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Study of aeration influence on the design of succinic acid production process using *Corynebacterium glutamicum* 2262

Abdoul Karim Kaboré

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UNIVERSITE DE LORRAINE (UL)

Ecole Nationale Supérieure d'Agronomie et des Industries Alimentaires (ENSAIA)

Ecole doctorale Ressources Procédés Produits Environnement (RP2E)

Laboratoire Réactions et Génie des Procédés (LRGP)

Centre National de la Recherche Scientifique (CNRS)

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Abdoul Karim KABORÉ

En vue de l'obtention du grade de

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Etude de l'influence de l'aération sur la mise en œuvre d'un procédé de production d'acide succinique par *Corynebacterium glutamicum* 2262

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« Nulle pierre ne peut être polie sans friction, nul homme ne peut parfaire son expérience sans épreuve. » Confucius

J'avais un peu étudié, étant plus jeune, entre les parties de la philosophie, à la logique, et entre les mathématiques, à l'analyse des géomètres et à l'algèbre, trois arts ou sciences qui semblaient devoir contribuer quelque chose à mon dessein. Mais, en les examinant, je pris garde que, pour la logique, ses syllogismes et la plupart de ses autres instructions servent plutôt à expliquer à autrui les choses qu'on sait ou même, comme l'art de Lulle, à parler, sans jugement, de celles qu'on ignore, qu'à les apprendre. Et bien qu'elle contienne, en effet, beaucoup de préceptes très vrais et très bons, il y en a toutefois tant d'autres, mêlés parmi, qui sont ou nuisibles ou superflus, qu'il est presque aussi malaisé de les en séparer, que de tirer une Diane ou une Minerve hors d'un bloc de marbre qui n'est point encore ébauché. Puis, pour l'analyse des anciens et l'algèbre des modernes, outre qu'elles ne s'étendent qu'à des matières fort abstraites, et qui ne semblent d'aucun usage, la première est toujours si astreinte à la considération des figures, qu'elle ne peut exercer l'entendement sans fatiguer beaucoup l'imagination ; et on s'est tellement assujetti, en la dernière, à certaines règles et à certains chiffres, qu'on en a fait un art confus et obscur, qui embarrasse l'esprit, au lieu d'une science qui le cultive. Ce qui fut cause que je pensai qu'il fallait chercher quelque autre méthode, qui, comprenant les avantages de ces trois, fût exempte de leurs défauts. Et comme la multitude des lois fournit souvent des excuses aux vices, en sorte qu'un État est bien mieux réglé lorsque, n'en ayant que fort peu, elles y sont fort étroitement observées ; ainsi, au lieu de ce grand nombre de préceptes dont la logique est composée, je crus que j'aurais assez des quatre suivants, pourvu que je prisse une ferme et constante résolution de ne manquer pas une seule fois à les observer.

Le premier était de ne recevoir jamais aucune chose pour vraie, que je ne la connusse évidemment être telle c'est-à-dire, d'éviter soigneusement la précipitation et la prévention ; et de ne comprendre rien de plus en mes jugements, que ce qui se présenterait si clairement et si distinctement à mon esprit, que je n'eusse aucune occasion de le mettre en doute.

Le second, de diviser chacune des difficultés que j'examinerais, en autant de pour celles qu'il se pourrait, et qu'il serait requis pour les mieux résoudre.

Le troisième, de conduire par ordre mes pensées, en commençant par les objets les plus simples et les plus aisés à connaître, pour monter peu à peu, comme par degrés, jusques à la connaissance des plus composés ; et supposant même de l'ordre entre ceux qui ne se précèdent point naturellement les uns les autres.

Et le dernier, de faire partout des dénombrements si entiers, et des revues si générales, que je fusse assuré de ne rien omettre.

*Discours de la méthode
René Descartes*

List of abbreviations and symbols

<i>[Ace], [Glu], [Lac], [Pyr], [Suc]</i>	Concentrations of acetate, glucose, lactate and pyruvate	g L ⁻¹ or mM
<i>ACK</i>	Acetate Kinase	
<i>ACN</i>	Aconitase	
<i>ADH</i>	Alcohol dehydrogenase	
<i>AMP</i>	Adenosine monophosphate	
<i>ATCC</i>	American type culture collection	
<i>ATP</i>	Adenosine triphosphate	
<i>ATP</i>	Adenosine Triphosphate	(mol)
<i>CAT</i>	CoA transferase	
<i>cdw</i>	Cell dry weight	(g)
<i>CS</i>	Citrate synthase	
<i>d</i>	Flask diameter	(m)
<i>d₀</i>	Shaking diameter	(m)
<i>dCO₂, dO₂</i>	Dissolved carbon dioxide, dissolved oxygen concentration	%
<i>Dct</i>	Dicarboxylate transport	
<i>Dcu</i>	Dicarboxylate uptake	
<i>DO</i>	Dissolved oxygen	
<i>DSP</i>	Downstream process	
<i>EMP</i>	Embden-Meyerhof-Parnas	
<i>FDH</i>	Formate dehydrogenase	
<i>FNR</i>	Fumarate and nitrate reductase	
<i>FUM</i>	Fumarase	
<i>g</i>	gram	
<i>gapA</i>	glyceraldehyde-3-phosphate dehydrogenase gene	
<i>GTP</i>	Guanosine triphosphate	
<i>GUR</i>	Glucose Uptake Rate	(mol L ⁻¹ h ⁻¹)
<i>ICD</i>	Isocitrate dehydrogenase	
<i>ICL</i>	Isocitrate lyase	

k_{La}	Gas-liquid mass transfer coefficient	(h^{-1})
L	litre	
LDH	Lactate dehydrogenase	
$MCGC$	Minimum <i>Corynebacterium glutamicum</i> Citrate	
MDH	Malate dehydrogenase	
ME	Malic enzyme	
min	minute	
mL	Milliliter	
mM	Millimolar	
MS	Malate synthase	
N	Shaking frequency	(s^{-1})
$NAD^{+}/NADH$	Nicotinamide Adenine Dinucleotide	
$NADP^{+}/NADPH$	Nicotinamide Adenine Dinucleotide Phosphate	
nm	Nanometer	
NRM	Nuclear magnetic resonance	
OD	Oxaloacetate decarboxylase	
$OD_{570\text{ nm}}$	Optical density measured at 570 nm	
$ODHC$	2-Oxoglutarate dehydrogenase complex	
OUR	Oxygen Uptake Rate	($mol\ L^{-1}\ h^{-1}$)
$[O_2]$	Dissolved oxygen concentration	($mol\ L^{-1}$)
$[O_2]^*$	Dissolved oxygen concentration at saturation	($mol\ L^{-1}$)
PC	Pyruvate carboxylase	
$PDHC$	Pyruvate dehydrogenase complex	
PEP	Phosphoenolpyruvate	
$PEPC$	Phosphoenolpyruvate carboxylase	

<i>PEPCK</i>	Phosphoenolpyruvate carboxykinase	
<i>PFR</i>	Plug flow reactor	
<i>PHB</i>	Polyhydroxybutyrate	
<i>PQO</i>	Pyruvate quinone oxidoreductase	
<i>PTA</i>	Phosphotransacetylase	
q_{max}^{OA}	Maximal specific organic acids production rate	$\text{g (g cdw)}^{-1} \text{ h}^{-1}$
q_{max}^{gluc}	Maximal specific consumption rate of glucose	$\text{g (g cdw)}^{-1} \text{ h}^{-1}$
$qPCR$	Quantitative polymerase chain reaction	
<i>rpm</i>	Rotation per minute	
<i>SCS</i>	succinyl-CoA synthetase	
<i>SDH</i>	Succinate dehydrogenase	
<i>STR</i>	Stirred tank reactor	
<i>TCA</i>	Tricarboxylic Acid	
μ	Specific growth rate	(h^{-1})
μg	microgram	
μL	microliter	
μm	Micrometer	
μ_{max}	Maximal specific growth rate	h^{-1}
<i>vvm</i>	gas volume flow per unit of liquid volume per minute	
<i>WT</i>	Wild type	
Y_{max}^{OA}	Maximal organic acid production yield from glucose	mol mol^{-1}

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Introduction

générale

Introduction générale et démarche adoptée

Les progrès de la biochimie, de la microbiologie et du génie génétique ont apporté des outils indispensables au développement des biotechnologies, en particulier des biotechnologies blanches qui visent l'application des procédés fermentaires et enzymatiques pour la valorisation industrielle des agroressources. Avec l'inquiétude grandissante liée aux procédés à base de ressources fossiles, aux politiques gouvernementales sur les pollutions environnementales, la dépendance du prix des ressources fossiles à la pression économique, les biotechnologies blanches pourraient être des alternatives technico-économiques et sociétales viables pour les industriels. C'est dans ce cadre que des rapports d'études sur la biomasse ont identifié des molécules biosourcées à fort potentiel. Parmi ces molécules, l'acide butanedioïque, aussi appelé acide succinique, a été cité comme une des plus importantes molécules pouvant servir de précurseur chimique (Werpy *et al.*, 2004b; Patel *et al.*, 2006; Bechthold *et al.*, 2008; Bozell and Petersen, 2010). L'acide succinique est un métabolite commun du métabolisme des plantes, des animaux et des microorganismes. C'est un acide dicarboxylique qui peut servir de précurseur pour de nombreux dérivés chimiques dont le 1,4-butanediol, le tétrahydrofurane, le γ -butyrolactone, le polybutylène de succinate etc. (Song and Lee, 2006; Cukalovic and Stevens, 2008). Même si la production industrielle d'acide succinique est, encore aujourd'hui, essentiellement réalisée par l'intermédiaire de procédés chimiques et pétrochimiques, depuis 2008, la production industrielle par voie biotechnologique commence à se développer de façon significative avec notamment des alliances stratégiques entre des groupes industriels. Ces principaux producteurs d'acide succinique bio-sourcé sont: Reverdia, Myriant, BASF-Purac, Bioamber, PTT-Mitsubishi chemical corporation (Cukalovic and Stevens, 2008; Cok *et al.*, 2014). La production de succinate par voie fermentaire, exploite les capacités et les voies métaboliques de microorganismes qui consomment du CO₂ pour pouvoir synthétiser l'acide succinique. En effet, le CO₂ est un co-substrat de la réaction de formation du succinate. Dans un contexte de valorisation du carbone, cette utilisation du CO₂ constitue un avantage de la production d'acide succinique biosourcé. Par ailleurs, Il existe une grande variété de microorganismes producteurs d'acide succinique. *Actinobacillus succinogenes*, *Anaerobiospirillum succiniciproducens* et *Mannheimia succiniciproducens*, sont cités comme étant les producteurs naturels d'acide succinique les plus intéressants (Cheng *et al.*, 2012a). Une des rares bactéries Gram positif produisant le succinate est *Corynebacterium glutamicum*, plus connue pour ses capacités de production d'acides aminés (Eggeling and Bott, 2005). C'est

une bactérie anaérobie facultative, qui ne se multiplie qu'en présence d'oxygène. En absence d'oxygène, elle n'est capable de se développer qu'en présence de nitrate au cours d'un processus respiratoire anaérobie (Takeno *et al.*, 2007). En condition anaérobie, sans nitrate, la croissance s'arrête mais les bactéries gardent la capacité métabolique de produire des acides organiques dont le succinate (Dominguez *et al.*, 1993). A ce jour, la majorité des travaux sur la production de succinate par *C. glutamicum*, est axée sur la modification génétique du microorganisme et l'utilisation d'un procédé en deux étapes physiquement séparées : une étape aérobie pour accumuler de la biomasse qui sera récoltée, rincée et utilisée dans la deuxième étape en condition anaérobie pour la production de succinate (Okino *et al.*, 2005, 2008a; Litsanov *et al.*, 2012b).

Les différents travaux présentés dans ce mémoire ont été réalisés dans le cadre d'un projet de thèse financé par l'Institut Carnot ICEEL et effectués au sein du Laboratoire Réactions et Génie des Procédés (LRGP). Ce laboratoire travaille depuis longtemps sur les procédés de mise en œuvre de *C. glutamicum*, et notamment la souche 2262, productrice d'acide glutamique. Dans la littérature sur la production d'acide succinique, il a été rapporté chez des microorganismes producteurs, anaérobies facultatifs comme *C. glutamicum*, qu'une phase de transition entre la phase aérobie et la phase anaérobie jouerait un rôle prépondérant dans l'induction des gènes impliqués dans les voies métaboliques de production de succinate et pourraient donc constituer une des cibles de l'amélioration des procédés de production (Vemuri *et al.*, 2002a; Martínez *et al.*, 2010; Zhu *et al.*, 2011). L'objectif global de cette thèse est donc d'explorer les capacités de production de succinate par *C. glutamicum* 2262 et d'élaborer un procédé de production, robuste et performant, ne nécessitant pas de récupération et de lavage des cellules entre la phase de croissance (aérobie) et la phase de production d'acide succinique (anaérobie). Cette contrainte impose une caractérisation de l'influence de l'aération pendant la phase de transition entre l'aérobiose et l'anaérobiose. Aussi, plusieurs questions se posent-elles pour lever les différents verrous scientifiques du projet.

Sachant que la croissance de *C. glutamicum* exige de l'oxygène et que la production d'acides organiques nécessite une limitation ou une absence en oxygène, existe-t-il des relations entre le transfert en oxygène imposé dans le système de culture utilisé et les performances du procédé (croissance et production d'acide succinique) ?

La phase de transition suppose une période de modification et d'adaptation du métabolisme des bactéries lors du passage de l'aérobiose à l'anaérobiose. Plusieurs questions se posent sur cette

transition. Doit-elle : être instantanée ? Progressive ? Echelonnée ? Comment opère-t-on cette transition, par la gestion du niveau d'oxygène dissous dans le milieu de culture ? Ou par la gestion des débits d'aération des cultures ?

Comment améliorer les performances de production de succinate par *C. glutamicum* 2262 sans avoir nécessairement recours à de nombreuses mutations génétiques ?

C. glutamicum réagissant différemment en présence et en absence d'oxygène, quelle est sa robustesse, sa capacité physiologique d'adaptation à une succession aléatoire d'environnements pauvre et riche en oxygène, comme les régions hétérogènes des grandes installations industrielles ?

Pour répondre à l'ensemble de ces interrogations, la démarche adoptée est schématisée sur la figure ci-dessous.

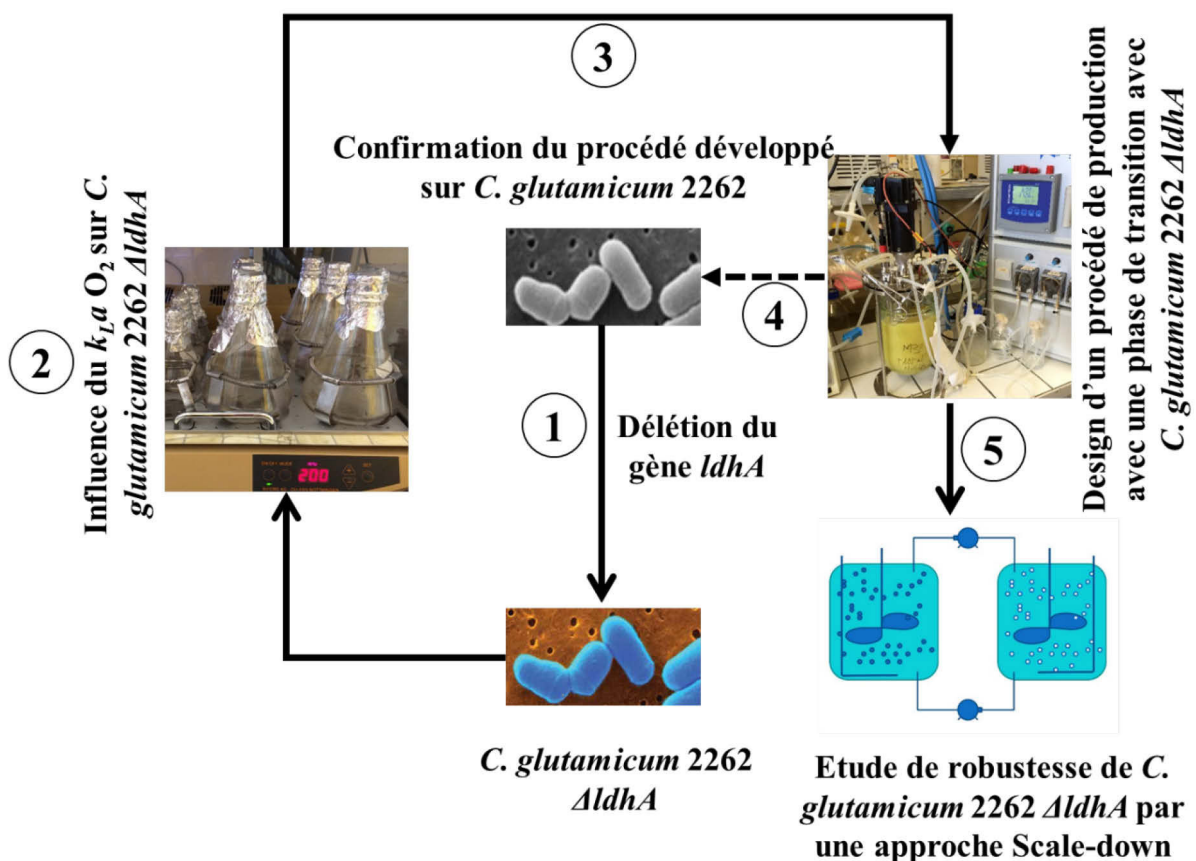


Figure A. Démarche suivie dans ce projet de thèse pour lever les différents verrous scientifiques

Ce mémoire de thèse est subdivisé en huit principales parties : une étude bibliographique, une partie matériel et méthodes, cinq parties de résultats et discussion et enfin, les conclusions et les perspectives de ce travail. Afin de valoriser le travail, chaque partie exceptée le « matériel et méthodes » est rédigée en anglais sous la forme de publication.

L'analyse bibliographique, qui constitue le chapitre I, fait un état de l'art et positionne la thématique de cette thèse. Cette partie traitera des propriétés de l'acide succinique, de sa production par voie chimique, de l'intérêt croissant pour une production biologique. Ainsi, les principaux microorganismes producteurs de succinate et en particulier la production par *C. glutamicum* seront étudiés. Enfin les capacités métaboliques de *C. glutamicum* à produire une large gamme de biocarburants et de molécules chimiques, dont la plus étudiée actuellement est le succinate, seront détaillées.

Le chapitre II est consacré à la description du matériel et des méthodes utilisés au cours de ce travail de thèse.

Dans la partie résultats et discussion :

Le chapitre III est axé sur la description de la construction d'un mutant de *C. glutamicum* chez lequel la lactate déshydrogénase codée par le gène *ldhA* a été supprimée (*C. glutamicum* 2262 Δ *ldhA*). Cette délétion est indispensable pour l'obtention d'une souche mutante stable produisant des quantités significatives d'acide succinique.

Le chapitre IV est orienté sur l'étude de l'influence des conditions d'oxygénation sur la production d'acides organiques par *C. glutamicum* 2262 Δ *ldhA*.

Le chapitre V est dédié à l'étude des différentes stratégies de transition entre la phase de croissance et la phase de production de succinate ainsi qu'au design d'un procédé tri-phasique de production d'acide succinique par *C. glutamicum* 2262 Δ *ldhA*.

Le chapitre VI est consacré à la confirmation de l'efficacité du procédé tri-phasique, avec une phase de transition, développé pour la production de succinate, en l'appliquant à la souche sauvage qui est hyperproductrice de lactate en conditions anaérobies.

Le chapitre VI présente des études menées sur la robustesse de *C. glutamicum* 2262 Δ *ldhA* en présence de concentrations en oxygène dissous hétérogènes dans le système de culture et les conséquences de cette hétérogénéité sur sa réponse physiologique ; par une approche scale-down.

Enfin, la dernière partie de ce manuscrit (chapitre VIII) est une synthèse des principaux résultats obtenus et des propositions de perspectives pour approfondir la thématique de recherche développée au cours de ce travail.

Chapitre I

Etude bibliographique

I. Biological production of the building block succinic acid: the biocatalyst *C. glutamicum* as cell factory

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Abstract

Succinic acid is a linear and bi-functional molecule that has several practical applications including food, chemical and pharmaceutical industries. It has been produced for a long time by chemical process using fossil resources. Faced to the limited availability of these resources and their indirect consequences in global warming, bioprocesses for the production of chemicals from renewable biomass have got an increased worldwide interest. Based on recent progress and succinic acid market size, bio-based succinate production could replace a part of the petroleum-based synthetic materials. Several promising anaerobic bacteria produce succinic acid naturally from renewable feedstock at elevated level. Thanks to increased knowledge on metabolism and pathway regulation of industrially relevant organisms, to the development of performant genetic tools and process engineering, recombinants strains (*Escherichia coli*, *Corynebacterium glutamicum*, *Saccharomyces cerevisiae* etc.) have been reported to be able to produce up to 100 g L⁻¹ with interesting yields (1.4 - 1.7 mole succinate per mole glucose). *C. glutamicum* is well known as one of the best industrial producer of numerous amino acids (glutamate, lysine, isoleucine etc.). However, recent extensive exploration of *C. glutamicum* revealed the capability of the strain to produce several organic acids (succinate, lactate, acetate, 2-ketoisovalerate etc.), biofuels (ethanol, butanol, isobutanol) and precursors for biodegradable polymers (putrescine, cadaverine). Actually, the researches on *C. glutamicum* are mainly focused on its improvement for efficient succinate production. This review summarizes the current knowledge on succinic acid producers, the markets, the different downstream process developed for succinate recovery and purification, the existing bioprocesses for succinate production. It also deals with the different pathways involved in *C. glutamicum* succinic acid production, its succinate production efficiency compared to natural and other recombinant producers.

Keywords: Succinic acid; Metabolic engineering; *Corynebacterium glutamicum*; Bioprocess engineering, Aerobic, Anaerobic.

I-1. Succinic acid: Origin and context

I-1.1. Origin

Succin or yellow amber is a fossil resinous matter. Indeed many studies reported that yellow amber resulted from the fossilized plant resins produced million years ago by conifers. The origin and the composition of succin have been the object of many speculations. These studies characterized succin as a mixture of, volatile oils, combined resins, succinic acid and residues that are insoluble in alcohol and ether. However, succinic acid was identified only in 1560, since George Agricola, while distilling the yellow amber, detected among the products obtained, a substance he described as " something of white and subtle which have some salt appearance, which is somewhat similar to salt. " Because of its subtlety, that substance was quickly classified as volatile alkali. In 1699, this succin salt was isolated and its acid character was characterized by experiments of Johann Conrad Barchusen and reported to French academy of science. In the treaty of chemistry of 1789, Antoine Laurent de Lavoisier, proposed some rehandlings to harmonize the nomenclature of the chemical species. Thus, "salt of succin" was replaced by succinic acid. On September 6th, 1834, Felix d' Arcet reported to the French academy of Science the properties of the succinic acid (Fievet, 1875).

Recently, several countries have evaluated the potential of biobased chemicals by governmentally (US Department of Energy and the European Commission) funded projects. The main aspects of these reports including national availability, integrated biorefinery concepts or white biotechnology led to the identification of several chemicals that can be biochemically produced and can be viable in an economic point of view. One of the chemical building blocks listed in the top 12 by the US Department of Energy as a promising platform chemical is succinic acid that is actually produced by petrochemical process. The suitability of succinic acid as a platform chemical is based on the renewable resources that can be used as raw materials for its production and the huge variety of chemicals that can be produced from it (Crank *et al.*, 2004; Werpy *et al.*, 2004a; Patel *et al.*, 2006). Considering the decrease in petroleum source availabilities and the increase in price of oil, development of systems for the production of succinic acid and its derivatives from renewable feedstock has become the necessity to cope up with the increasing demands for succinic acid (Adsul *et al.*, 2011).

I-1.2. Physico-chemical properties

Succinic acid, also known as 1,4-butanedioic acid, is a four-carbon dicarboxylic acid with molecular formula of $C_4H_6O_4$. In its natural state, it has a special flavor, slightly bitter. The pure substance is odorless, colorless but slightly yellow during simple sublimation. It is a colorless crystal soluble in water, ethanol, and acetone. Succinic acid is present either in a rhombic or hexagonal structure, or as fine translucent lanyards. Succinic acid is an optically inactive compound. It is volatilizable at very high temperatures ($> 235\text{ }^\circ\text{C}$) but is not volatile. This compound is difficult to oxidize, unalterable in the air and resistant to strong oxidizing agents such as chlorine, chromic acid, nitric acid (Fievet, 1875; Kaneuchi *et al.*, 1988).

I-1.3. Industrial relevance and market of succinic acid

The market of succinic acid and its derivatives is estimated to be more than 15 billion dollars. They have been widely used for many industrial applications as, precursors of biopolymers, pharmaceuticals, fibers, additives to food ingredients, acidulants, cosmetic products, and lubricants (Yi *et al.*, 2013). Furthermore, succinic acid also has a special chemical market since it is used for the production of coatings, surfactants, dyes, detergents, green solvents, biodegradable plastics, and ingredients for stimulation of animal and plant growth. Based upon the structure of linear and saturated dicarboxylic acid, it can be readily converted to other bulk chemicals (Cheng *et al.*, 2012a). The different products are grouped in four main markets which are the detergent/surfactant market, the ion chelator market, food market (acidulants, flavors or antimicrobials) and the pharmaceutical market (Zeikus *et al.*, 1999; Cukalovic and Stevens, 2008; Delhomme *et al.*, 2009; Beauprez *et al.*, 2010). In the agricultural area, succinic acid is known as growth regulator (Dougall and Weyrauch, 1980) which can be used for seed treatment and plant rooting. In the food industry, it is used for beverages flavoring, and food seasoning preparation (Chimirri *et al.*, 2010). Succinic acid is generally considered as safe and has been approved by the US Food and Drug Administration. Besides, its anticarcinogenic and insulinotropic properties have been also investigated in the pharmaceutical industry (Sener *et al.*, 1997). Due to the bi-functional structure, succinic acid is a precursor for the production of many high value chemicals including 1,4-butanediol, tetrahydrofuran, γ -butyrolactone, and 2-pyrrolidinone in the chemical industry (Landucci *et al.*, 1994; Zeikus *et al.*, 1999; Song and Lee, 2006; Sauer *et al.*, 2008; Yu *et al.*, 2011) (Figure I-1).

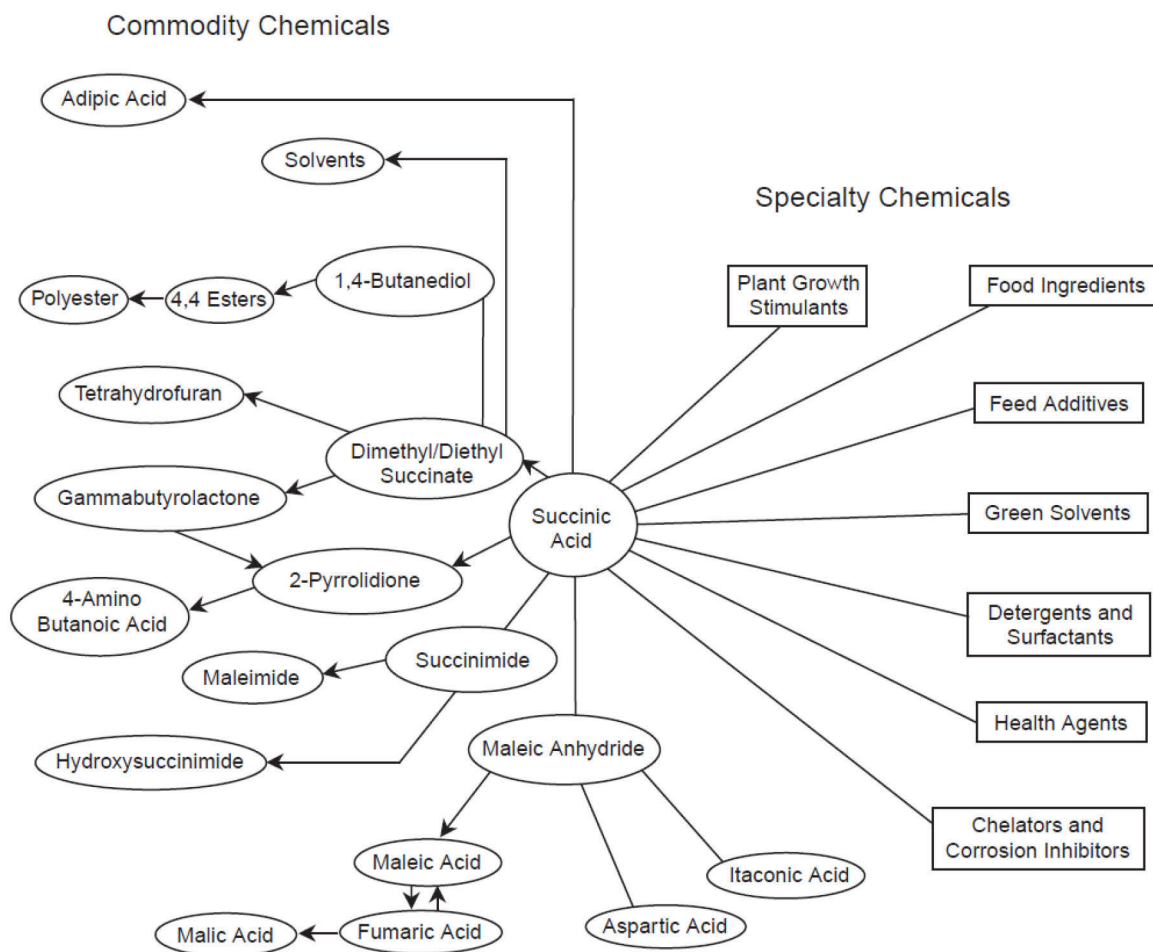


Figure I-1. Succinic acid and its various derivatives (Zeikus et al., 1999).

Finally, the most promising application area of succinic acid could be the specialized polyesters market, particularly, polybutylene succinate (Jun Xu, 2010; Xu and Guo, 2010). While the current global succinic acid production is 30,000 to 50,000 tons per year with the market price of 2,400 – 3,000 \$ per ton, the market is expected to reach 100,000 tons per year by 2015 (Beauprez et al., 2010). The global revenue for this market was estimated around 182.8 million \$ in 2010 and is expected to reach 496 million by 2016 (Succinic Acid Market by Source & Application - 2019 | MarketsandMarkets). Around the world, the largest succinic acid market demand was concentrated in Europe and North America in 2010, representing respectively 35 % and 31 % of the global succinate demand (Figure I-2). However, Asia-pacific is expected to be the fastest growing market in the near future due to the strong demand from key countries such as China and India (Cao et al., 2013).

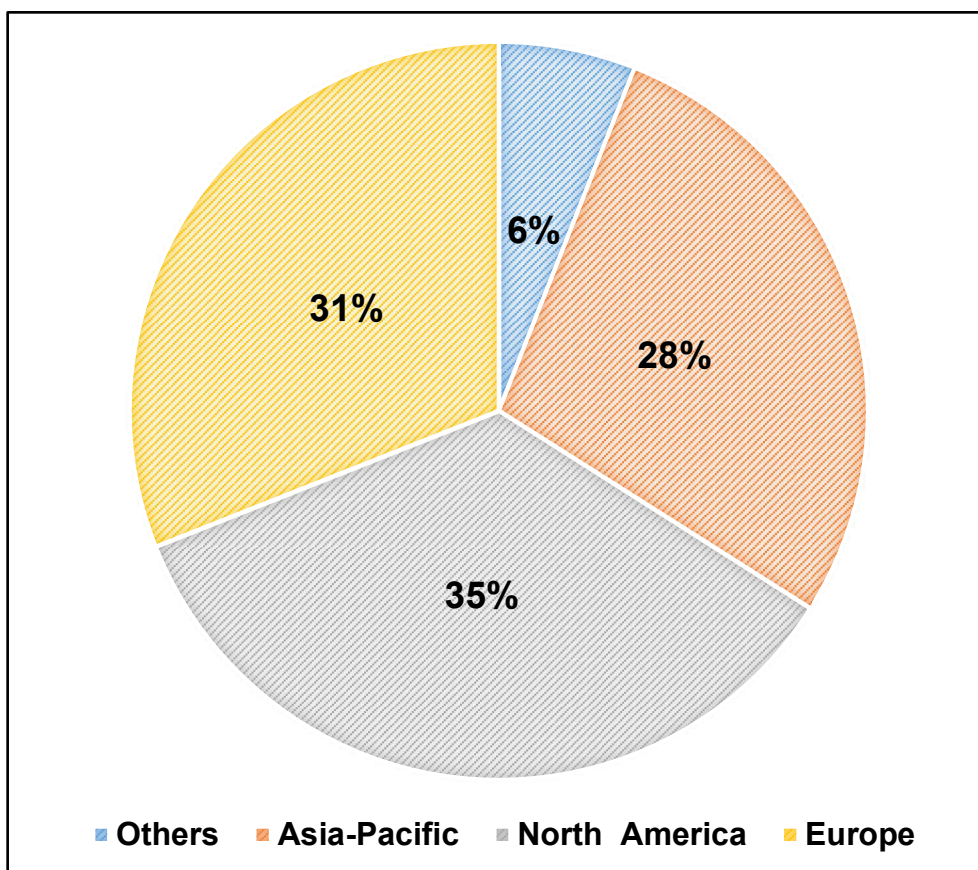


Figure I-2. Geographical succinic acid market repartition in 2010 (Cao *et al.*, 2013).

To face the increasing demand of succinate, one of the future challenge is the significant reduction of the production costs for the biological production routes in order to support its evergrowing markets. According to the US department of energy, in order to compete with petro-chemically produced succinate, bio-based production cost needs to be at or below \$ 0.55/kg. This is a significant technical challenge and should be undertaken with a long-term perspective (Werpy *et al.*, 2004c).

I-2. Main routes for succinic acid production

Currently, succinic acid is mainly produced by the chemical processes from *n*-butane or other petroleum derivatives. Such chemical processes involve fossil resources and are more and more regarded as having a heavy toll on the environment with atmospheric pollutions leading to global warning threats. In the past decades, efforts were focused on alternative solutions to replace part of the petroleum-based synthetic materials by natural products. In this context,

biotechnological strategies devoted to using plants, yeasts or bacteria and enzymes derived from them to produce succinic acid, could be an interesting solution. The industrial processes targeted through bioprocess researches, intend to decrease the use of fossil resources as raw material for succinate production and to make bio-products economically interesting and competitive in comparison to oil-derived processes.

I-2.1. Succinic acid production through chemical route

The manufacture of succinic acid is traditionally achieved through chemical routes. These processes include paraffin oxidation, catalytic hydrogenation, or electroreduction of maleic acid or maleic anhydride, acetylene, or acrylic acid as the starting materials (*Polly 1950; Cao et al. 2013*). In the paraffin oxidation process, catalysts such as calcium or manganese are used and several kinds of dicarboxylic acids are obtained at the same time. Succinic acid is then recovered and purified by distillation, crystallization, and drying. The yield and purity of succinic acid obtained by this process were relatively poor. Thus, process of maleic anhydride hydrogenation, industrialized as early as the 1930s, became the most direct and economical way to produce succinic acid. The hydrogenation reaction can be conducted homogeneously or heterogeneously by careful selection of the catalyst (*Jung et al., 2003; Pillai et al., 2003; Li et al., 2010b*). While high yield, purity, and selectivity of succinic acid can be obtained, the operation of this process is complicated, expensive, and might have environmental issues (*Vasudevan, 1995; Muzumdar et al., 2004; Cok et al., 2014*). The maleic anhydride, precursor of succinic acid, is produced by catalytic gas phase oxidation of suitable hydrocarbons. Today, maleic anhydride is still partly produced worldwide by the oxidation of benzene. In recent years, however, the rapid increase in benzene prices, the development of improved n-butane to maleic anhydride catalysts have boosted the attractiveness of n-butane in relation to benzene. The prices of petrochemical products such as benzene and butane are naturally strongly linked to the crude oil prices (*Emig and Martin, 1987*). Moreover, these chemical routes are almost carried out under high temperature and high pressure conditions, which are not environmental friendly. Even if, petrochemical processes are very competitive, with the decrease of global fossil fuel resources and the rise of crude oil price, these traditional succinate-producing methods deriving from petrochemicals are more and more costly and result in serious pollution problems. Therefore, new pathways for succinic acid production at industrial scale are required.

I-2.2. Biological process for succinic acid production

Since the industrial revolution, fossil fuels (petroleum, coal and natural gas) have been established as the main resources. Nowadays, the rising price of oil, the environmental impact of many petrochemical processes and political issues are imposing the reconsideration of many of the current chemical technologies. This trend channels resources towards the development of more economical as well as environmental sound routes. Furthermore, society is confronted today with the proliferation of wastes generated by municipalities, agriculture and all these considerations are the driving force for introduction of biochemical alternatives (Beauprez *et al.*, 2010). Thus, considerable effort is being invested in biotechnology to develop a chemical industry based on renewable resources, as a partial substitute for the decreasing fossil fuels. For this purpose, fermentation of biomass for the production of renewable building-block chemicals is gaining more and more interest (Delhomme *et al.*, 2009). The conversion of renewable resources or wastes to chemicals and fuels by microbial fermentations or enzymes represents a tremendous challenge for microbiologists in the future (Zeikus, 1980).

In the biological systems, succinic acid is an essential intermediate for cells metabolism. Moreover, it can also be one of the end-products of anaerobic fermentation. Since the succinate metabolic pathway has been resolved in different biological systems, it is possible to produce this valuable chemical through microbial fermentation. As microorganisms usually utilize renewable feedstocks and produce much fewer toxic by-products, biological processes are particularly attractive (Cao *et al.*, 2013). Up to now, a number of fermentative succinic acid-producing bacteria have been isolated and characterized. These microorganisms are generally classified into two categories: natural microbial producers and engineered microbial producers.

I-2.2.1. Natural succinic acid producing strains

Among the succinic acid producers, some bacteria, which produce succinate naturally in significant titers, have been isolated in the rumen of ruminants. Most of succinic acid producing microorganisms described in literature are capnophilic microorganisms. The most currently used microorganisms for succinic acid production are *Actinobacillus succinogenes*, *Anaerobiospirillum succiniciproducens*, *Mannheimia succiniciproducens* and *Escherichia coli*. The anaerobic bacterium, *A. succiniciproducens*, is considered as one of the best succinic acid producers. *Corynebacterium sp.* and *Bacteroides fragilis* have been introduced very recently as Gram positive succinic acid producers (Song and Lee, 2006; Beauprez *et al.*, 2010). *A. succinogenes*, *A. succiniciproducens*, *M. succiniciproducens* and *B. fragilis* produce a mixture

of volatile organic acids and as capnophiles, they can cope with high carbon dioxide (> to atmospheric CO₂ concentration, 0.04 %) concentration and use it as a carbon source together with sugars (Song and Lee, 2006). Optimized fungal succinate production systems are rarely described in literature, although *Fusarium spp.*, *Aspergillus spp.* and *Penicillium simplicissimum* are known to excrete few amounts of this acid (Magnuson and Lasure, 2004). The characteristics of some well-known succinic acid natural producers are summarized in the Table I-1.

Table I-1. Examples of natural succinic acid producers

Strains	Substrates	Mode of culture	Conc. (g L ⁻¹)	Productivity (g L ⁻¹ h ⁻¹)	References
<i>Actinobacillus succinogenes</i> 130z	Glucose	Anaerobic, batch	67.2	0.8	(Guettler <i>et al.</i> , 1998)
<i>Anaerobiospirillum succiniciproducens</i>	Glucose	Anaerobic, batch	50.3	2.1	(Glassner and Datta, 1992)
<i>Mannheimia succiniciproducens</i> MBEL55E	Glucose	Anaerobic, batch	14	1.8	(Lee <i>et al.</i> , 2002)
<i>Bacteroides fragilis</i>	Glucose	Anaerobic, batch	12.5	0.42	(Isar <i>et al.</i> , 2007)
<i>Prevotella ruminicola</i>	Glucose	Anaerobic, batch	18.9	0.52	(Guettler <i>et al.</i> , 1998)

I-2.2.2. Genetically modified strains

The accumulation of very high concentration of acid in fermentation broth is not a normal phenomenon for microorganisms. Therefore, for high succinic acid yields achievement with natural producers or non-natural producers, the development of commercial fermentation

process usually depends on the improvement of production strains (Guettler *et al.*, 1996). The strains are generally chosen on the basis of their genetic accessibility. Based on natural producers as mentioned above, a number of processes with metabolically engineered strains, selected variants, and optimized production conditions have been developed. For this purpose, one of the powerful technologies is the metabolic engineering which consists of the design, engineering and optimization of a microorganism to optimize a production process for the production of chemicals from renewable resources (Stephanopoulos *et al.*, 1998). However, to successfully perform metabolic engineering, sufficient genetic tools and information should be available for the targeted microorganism. Several microorganisms have been genetically modified in order to improve their succinic acid production ability (Table I-2).

I-3. *Corynebacterium glutamicum* as succinic acid producer

After the discovery of monosodium glutamate as a new taste in Japan, intensive researches for microorganism able to produce this compound resulted in the isolation of a bacterium initially named *Micrococcus glutamicus* by Kinoshita *et al.* (Kinoshita *et al.*, 1958, 2004). This soil bacterium is a Gram-positive bacterium that belongs to the Actinomycetes subdivision of the eubacteria, and is now known as *Corynebacterium glutamicum*. This bacterium is a non-sporulating, non-motile, with a straight or slightly curved rods, ovals, or clubs morphology and often have typical V-shaped arrangements of cell. Belonging to the *Actinomycetales* order, *C. glutamicum* is a member of the *Corynebacterineae* sub order, characterized by a distinctive cell wall architecture composed of mycolic acids. Widely used as a cost-effective industrial production of numerous metabolites including amino acids (Nakayama *et al.*, 1961; Kinoshita *et al.*, 2004), this species grows well at 30°C under aerobic conditions (Liebl, 2006). *C. glutamicum* is one of the most important species for industrial amino acid production, with L-glutamate (2.1 million tons per year) and L-lysine (1.4 million tons per year) as major products (Becker and Wittmann, 2012; Wieschalka *et al.*, 2013b). Due to this strong industrial interest, efforts have been made to investigate largely and in details, the whole metabolism of *C. glutamicum*. In order to decrypt the pathways for amino acid biosynthesis, their regulations, the reactions in the central metabolism, the genome of two representatives strains of the *Corynebacterium glutamicum* species, *C. glutamicum* ATCC13032 and *C. glutamicum* R have been sequenced (Ikeda and Nakagawa, 2003; Kalinowski *et al.*, 2003; Eggeling and Bott, 2005; Yukawa *et al.*, 2007) and numerous genetic tools allowing genetic engineering are available (Kirchner and Tauch, 2003a). Based on this extensive knowledge, metabolic engineering was

applied successfully to develop strains capable of producing a variety of other metabolites unlike amino acids.

Table I-2. Genetically modified strains for high yields succinic acid production

Strains	Substrate	Mode of culture	Conc (g L ⁻¹)	Productivity (g L ⁻¹ h ⁻¹)	Ref.
<i>A. succinogenes</i> FZ53	Glucose	Anaerobic, batch	105.8	1.36	(Guettler <i>et al.</i> , 1996)
<i>A. succinogenes</i> 130Z	Glucose	Anaerobic, batch	67.2	0.8	(Guettler <i>et al.</i> , 1998)
<i>A. succiniciproducens</i> FA-10	Glucose	Anaerobic, batch	34.1	0.77	(Guettler and Jain, 1996)
<i>M. succiniciproducens</i> LPK7	Glucose	Anaerobic, fed-batch	52.4	1.75	(Lee <i>et al.</i> , 2006)
<i>E. coli</i> NZN111	Glucose	Anaerobic, batch	12.8	0.29	(Stols and Donnelly, 1997)
<i>E. coli</i> AFP111/ pTrc99A- <i>pyc</i>	Glucose	Dual phase aeration, batch	99.2	1.31	(Vemuri <i>et al.</i> , 2002b)
<i>C. glutamicum</i> R <i>AldhA</i> -pCRA717	Glucose	Micro-aerobic, fed-batch with membrane for cell recycling	146	3.17	(Okino <i>et al.</i> , 2008a)

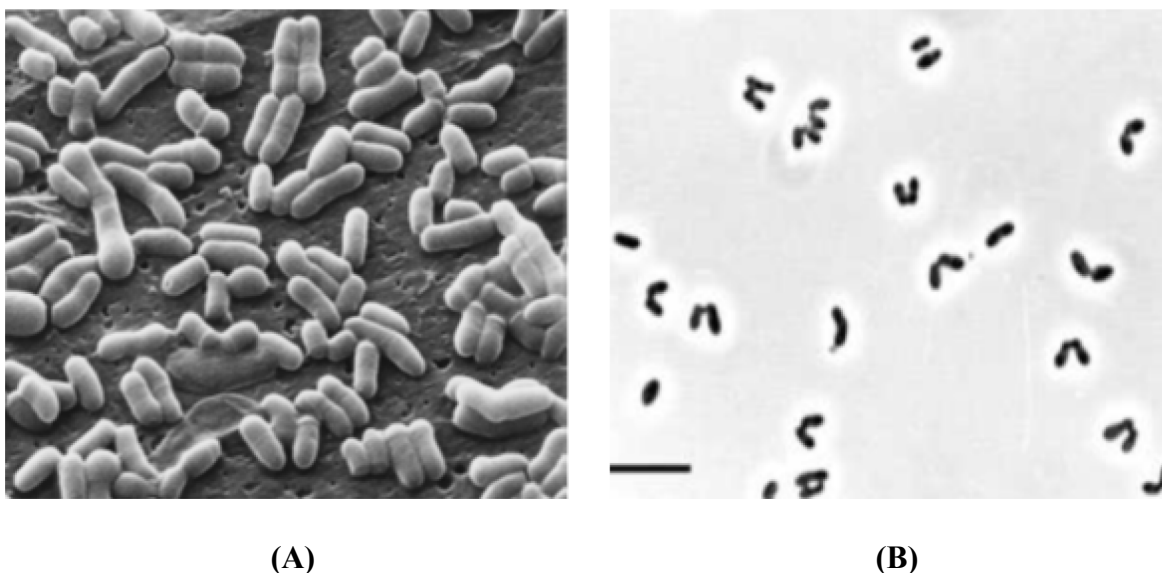


Figure I-3. Electron micrographs of wild-type strains of *C. glutamicum* (A), Phase contrast microphotograph (B) (Wehrmann *et al.*, 1998; Eggeling and Bott, 2005).

I-3.1. Relevant bio-based products of *C. glutamicum*

Biorefinery concept emerged recently and consists in performing industrial processes to manufacture various chemicals, fuels and energy from biobased feedstock. For this purpose, the main organisms used to date were *E. coli* and *S. cerevisiae* due to the extensive genetic tools and molecular biologic knowledge available for these species. However, with a long history of industrial amino acids production, a large progress in the understanding of its physiology and genome associated knowledge, *C. glutamicum* could be among the best platform for biorefinery organisms (Tatsumi and Inui, 2012). *C. glutamicum* has been for a long time used in aerobic processes for glutamate and other amino acids production and in the last decade novel bioprocesses have been developed for sugars conversion into chemicals and fuels (Inui *et al.*, 2004a, 2004b).

Aerobic processes production with C. glutamicum

The production of fuels such as propanol, isobutanol, 2-methyl-1-butanol, and 3-methyl-1-butanol have already been performed with *E. coli* (Atsumi and Liao, 2008; Atsumi *et al.*, 2008, 2009; Cann and Liao, 2008; Shen and Liao, 2008). Gram negative bacteria, *C. glutamicum* has also been modified genetically for fuels production. Smith *et al.*, engineered *C. glutamicum* to produce isobutanol by applying strategy similar to valine biosynthesis. In fact, valine and isobutanol share the same precursor, 2-ketoisovalerate. Isobutanol production was

accompanied by high chain alcohol byproducts formation such as propanol, butanol, 2-methyl-1-butanol, 3-methyl-1-butanol, and 2-phenylethanol. The authors also demonstrated that *C. glutamicum* exhibits better isobutanol tolerance than *E. coli* (Smith *et al.*, 2010).

Poly-hydroxybutyrate (PHB) is a biologically produced polyester that has received much attention as a biodegradable polymer. PHB and its derivatives have many attractive properties including biodegradability, biocompatibility and physical properties. *C. glutamicum* does not produce PHB naturally but the existing processes for amino acid production and the extensive genetic tools allowed the creation of a biosynthetic pathway for PHB production. Enzymes involved in PHB synthesis include beta-ketothiolase (PhbA), acetoacetyl-CoA reductase (PhbB) and PHB synthase (PhbC). Thus, this pathway was developed by introducing the *phbCAB* operon derived from *Ralstonia eutropha* (Jo *et al.*, 2006, 2007, 2009). Cells cultivated under aerobic conditions exhibited PHB synthase activity and intracellular PHB accumulated as inclusion granules up to 25 % (weight/weight) (Jo *et al.*, 2006).

Among the different classes of molecules, diamines such as cadaverine and putrescine are of particular interest. In fact, they are potential monomers for polyamide production. *C. glutamicum* has been enabled to produce putrescine by heterologous expression of genes encoding enzymes of the arginine and ornithine decarboxylase pathways from *Escherichia coli*. In the ornithine decarboxylase pathway, L-ornithine is directly converted to putrescine by ornithine decarboxylase while in the arginine decarboxylase pathway, L-arginine is first decarboxylated by arginine decarboxylase and then the resulting product was subsequently transformed into putrescine. The later pathway was shown to be 40 times less efficient than the former (Schneider and Wendisch, 2010; Schneider *et al.*, 2012). Furthermore, cadaverine (diaminopentane) is also a highly attractive building block for future production of bio-based polyamides. Cadaverine was produced by decarboxylation of lysine and since *C. glutamicum* is a strong lysine producer, this strain became an interesting candidate for cadaverine production (Mimitsuka *et al.*, 2007; Kind *et al.*, 2010a, 2010b, 2011).

C. glutamicum has also been tested as an alternative host for heterologous production of hyaluronic acid. For this purpose, the strain was transformed with vector containing the hyaluronic acid synthase from *Streptococcus equi*. Cultures performed in shake flask under aerobic conditions in minimal medium revealed, after purification, a concentration of 1.5 g L⁻¹ hyaluronic acid (Hoffmann and Altenbuchner, 2014).

Anaerobic processes performed with C. glutamicum

In anaerobiosis and in presence of nitrate ions, *C. glutamicum* is still able to grow thanks to the anaerobic respiration. This growth remains limited because of the accumulation of the nitrites which are not reduced in ammonium ions (Nishimura *et al.*, 2007; Takeno *et al.*, 2007). This metabolic capacity of reducing nitrate ions is due to the presence, in *C. glutamicum* genome, of the operon *narKGHJI* encoding a nitrate/nitrite transporter and a nitrate reductase. However, in deprived oxygen conditions, and in absence of nitrate ions, the growth of *C. glutamicum* is stopped but the cells retain a metabolic activity for chemicals and fuels production. It is noteworthy that this lack of growth can be considered as an advantage. Indeed, the accumulation in the culture medium of by-products, which are classically synthesized in anaerobiosis, remains highly limited. In anaerobiosis, high productivities were obtained for the production of lactate, succinate and also of other metabolites (Tatsumi and Inui, 2012). The arrest of growth combined with metabolic engineering approaches were used in order to explore the wide range of chemicals that can be produced by *C. glutamicum* as a robust and suitable host for industrial production. The extensive studies conducted, reported the ability of *C. glutamicum* to produce other commodity chemicals than amino acids under anaerobic conditions, including organic acids (pyruvate, lactate, acetate, succinate, etc.) and biofuels (ethanol, butanol, isobutanol, etc.)

Currently, most of studies are focused on bacterial strains that are able to synthesize succinate as a final product of catabolism, with the objective to establish competitive biotechnological processes for the industrial production of succinate that the demand and the use as a raw material for the manufacturing of biodegradable plastics is continuously increasing (Zeikus *et al.*, 1999; Flieger *et al.*, 2003). In this biological succinic acid production, the addition of bicarbonate has been demonstrated to promote an increase in the specific rate of glucose consumption and lactate production (2.5 times and 2.3 times, respectively) and a 3.6 times increase in the production of succinate. In this condition, the consumption of the bicarbonate and the production of succinate are almost equimolar. The stimulating effect of bicarbonate was explained by the fact that it promotes the increase of $\text{NAD}^+ / \text{NADH}, \text{H}^+$ ratio (2.9 vs 1.7 in the absence of bicarbonate) which is in favor of the glycolytic flux (Inui *et al.*, 2004a). Besides, the deletion of *ldhA*, encoding the L-lactate dehydrogenase, or the overexpression of the endogenous pyruvate carboxylase gene (*pyc*), has also led to a significant increase in succinate production (Inui *et al.*, 2004a; Okino *et al.*, 2008a). In these conditions with a deletion of *ldhA* and in presence of bicarbonate, the succinate production yield reached 1.4 mole per mole of glucose. This volumetric productivity, specific to this strain, were much higher than those

obtained during the production of succinate aerobically by *E. coli*. In addition succinate production processes involving *C. glutamicum* do not require complex media (Wendisch *et al.*, 2006). Therefore, *C. glutamicum* appears as a solid alternative to natural producers and *E. coli* strains for the biotechnological production of succinate. To date, studies dedicated to succinate production by *C. glutamicum* had to deal with two challenges: (i) the suppression or reduction of the acetate formation as a competing by-product and (ii) the improvement of succinate production yield from glucose which is limited by the availability of reducing equivalents that are needed for the conversion of oxaloacetate in succinate in the reductive branch of the cycle TCA. These issues have been recently resolved by the disruption of several genes, i.e. genes involved in acetic acid production (*cat*, *pqo*, *pta-ack*) and lactic acid productions (*ldhA*), in combination with the chromosomal insertion of the deregulated gene encoding the pyruvate carboxylase (*pycP458s*) of *C. glutamicum* DM1727. Additional integration of formate dehydrogenase gene (*fdh*) from *Mycobacterium vaccae* as well as the overexpression of *gapA* in *C. glutamicum* resulted in an increase in glycolytic flux, CO₂ and NADH,H⁺ availability (Litsanov *et al.*, 2012b). Under anaerobic conditions and in fed batch mode, this modified strain was able to use formate, and carbon dioxide as carbon sources and allowed to accumulate up to 1.13 M of succinate after 53 h of culture, with a yield of 1.67 mole of succinate produced per mole of glucose. In addition, other by products such as 2-ketoglutarate, malate, acetate, fumarate and pyruvate, accumulate in the fermentation broth but at very low concentrations (Litsanov *et al.*, 2012b). Furthermore, using ¹³C-NMR, a carbon flux analysis of succinate production by *C. glutamicum* under anaerobic condition indicated that succinate production was more influenced by CO₂ availability than NADH,H⁺ and that addition of CO₂ caused up to 2 and 3-fold increase in acetate and succinate production respectively, both at the expense of lactate production. In addition, flux analyses indicated that 97 % succinate was produced through the reductive TCA branch and a low but significant activity of the oxidative TCA cycle was observed and provided all the reducing equivalents needed for the redox balance (Rados *et al.*, 2014). Such study demonstrated clearly that natural cellular regulation could be overcome to obtain higher succinate yields by *C. glutamicum* without genetic manipulation.

Lactate, L and D isomers, is widely used for pharmaceutical, food industries, and biodegradable polymer production (Datta *et al.*, 1995; Hofvendahl and Hahn-Hägerdal, 2000; Wieschalka *et al.*, 2013a). Lactic acid bacteria which were mainly used as lactate producers need specific amino acids or complex nutrients for their growth. As complex media hampered the downstream processing, other biocatalysts such as *E. coli*, *S. cerevisiae* or *C. glutamicum* have

been genetically transformed to make them efficient D - and L-lactic acid producers (Okano *et al.*, 2010). For this purpose, *C. glutamicum* have been studied in a synthetic medium and under oxygen limitation. In this condition, it has been able to produce naturally up to 20 g L⁻¹ of L - lactic acid with a high productivity, generally in association with succinic acid and acetic acid (Inui *et al.*, 2004a). Influence of bicarbonate addition in lactate production by *C. glutamicum* has also been examined since it was shown that it resulted in an increase in glucose consumption and NADH,H⁺/NAD⁺ ratio (Inui *et al.*, 2004b). On the basis of these physiological effects, Okino *et al.* (2005) performed experiments with *C. glutamicum* R at density of 30 g cdw L⁻¹ under anaerobic conditions and in minimal medium containing 400 mM bicarbonate. The results showed a production of lactate with a concentration up to 97 g L⁻¹ with a production yield of 0.9 g g⁻¹ (Okino *et al.*, 2005). Taking advantage of this high lactate production capability, *C. glutamicum* R was also genetically modified for D - lactate production. It consisted in the introduction and the overexpression of the gene encoding the D - lactate dehydrogenase from *Lactobacillus delbrueckii*, associated to the suppression of the lactate dehydrogenase gene (*ldhA*). With high cell density of 60 g cdw L⁻¹ packed in anaerobic condition, the mutant produced up to 120 g L⁻¹ of D - lactic acid after 30 hours of fermentation, with an optical purity greater than 99.9 % (Okino *et al.*, 2008b).

Pyruvate is a key metabolite that supplies energy to living cells through the TCA cycle when oxygen is present. Alternatively, pyruvate is converted into fermentation products under anaerobiosis. Pyruvate, as a final fermentation product, is not generally considered because this molecule does not accumulate enough to be excreted. Therefore, production of pyruvate with *C. glutamicum* requires the knock out or the down-regulation of the different pathways that use pyruvate as precursor or intermediate. This can be achieved by inactivating the pyruvate dehydrogenase complex (PDHC) through the deletion of the *aceE* gene encoding the E1p subunit of PDHC. The resulting strain is unable to grow with glucose as the sole carbon source unless acetate or ethanol are added in the medium for complementation (Schreiner and Eikmanns, 2005; Blombach *et al.*, 2007, 2009). The $\Delta aceE$ mutant accumulated pyruvate with byproducts such as L - valine and L - alanine. To improve the production and reduce side products formation, additional deletions including (pyruvate:quinone oxydoreductase, lactate dehydrogenase, acetohydroxyacid synthase, alanine aminotransferase (*AlaT*) and valine-pyruvate aminotransferase (*AvtA*) genes) were introduced. The resulting strain, cultivated in fed-batch mode at high cell density and in limited oxygen supply conditions, produced more

than 500 mM pyruvate with a yield of 0.97 mole of pyruvate per mole of glucose (Wieschalka *et al.*, 2012a).

Finally, beside amino acids and organic acids, several research groups have intensively studied the ability of *C. glutamicum* to produce fuels by genetic modifications of its genome. Fuel like ethanol is widely used as gasoline additive in Europe and USA (Tatsumi and Inui, 2012). Ethanol is produced by *S. cerevisiae*, *E. coli*, and *Zymomonas mobilis*. Currently, the challenge for biofuels production is the use of lignocellulosic biomass which releases growth inhibitors such as phenols and furans during its hydrolysis. The biocatalysts cited above were seen to be less tolerant to the inhibitors and less efficient with sugar mixtures than *C. glutamicum*. (Dien *et al.*, 2003; Tatsumi and Inui, 2012). However, the central metabolism of *C. glutamicum* must be modified because enzyme activities involved in ethanol biosynthesis are not naturally present in this species. In *S. cerevisiae* and *Z. mobilis*, the metabolic pathway of ethanol biosynthesis from pyruvate is composed of two enzymes, pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH). These two enzymes are sufficient to convert the intracellular pyruvate and NADH,H⁺ to ethanol (Talarico *et al.*, 2005). Therefore, PDC and ADH II encoding genes from *Z. mobilis* were selected to introduce the ability to produce ethanol from pyruvate in *C. glutamicum* under the control of *ldhA* promoter which is induced under oxygen limitation conditions (Inui *et al.*, 2004b). In combination with the cloning of the *pdh* and *adhII* genes, Jojima *et al.* (2015), overexpressed four glycolytic genes encoding 6-phosphate isomerase (*pgi*), 6-phosphofruktokinase (*pfkA*), glyceraldehyde 3-phosphate dehydrogenase (*gapA*), and pyruvate kinase (*pyk*) in *C. glutamicum*. In fed-batch fermentation, the resulting strain, packed at a high density of 60 g cdw L⁻¹ produced up to 119 g L⁻¹ ethanol with a yield close to 95 % of the theoretical maximum value (Jojima *et al.*, 2015).

Isobutanol which have been described above during production under aerobic condition, has also been produced under anaerobic conditions (Blombach *et al.*, 2011). The productivity in growth-arrested process was reported to be more than twofold higher than the results of aerobic production (Tatsumi and Inui, 2012).

Among the different molecules that *C. glutamicum* have demonstrated interesting ability of production, succinic acid was the most regarded, these last years. The following part of this chapter will focused on the synthesis and production of succinic acid production by *C. glutamicum*.

I-3.2. Metabolic pathways for succinic acid biosynthesis

NMR isotopic filiation of ^{13}C -labelled carbon sources was used to investigate the tricarboxylic acid (TCA) pathway in *S. cerevisiae*. The ^{13}C distribution in the different intermediates suggested that the reductive branch of the TCA cycle was the main pathway for succinate production and that a surplus of succinate was produced by oxidative decarboxylation of 2-oxoglutarate (Camarasa *et al.*, 2003). In *E. coli*, the glyoxylate bypass was employed as a novel succinic acid pathway to sustain the main biosynthesis pathway of succinic acid (Vemuri *et al.*, 2002a; Hong, 2007). *C. glutamicum* shares these three biosynthesis routes with *S. cerevisiae*, *E. coli* and natural succinate producers. The fermentative pathway, activated under anaerobic conditions and corresponding to the reductive branch of the TCA cycle whereas the biosynthesis under aerobic conditions occurs through the oxidative branch of the TCA cycle, and through the glyoxylate pathway. The latter is essentially active in presence of acetate in the culture medium.

a. Glucose uptake system and glycolysis in *C. glutamicum*

C. glutamicum is able to uptake a variety of substrates. It possesses different phosphotransferase systems enabling for example, the uptake of glucose, fructose and sucrose (Mori and Shiio, 1987). The phosphoryl group of phosphoenolpyruvate is transferred to the imported sugar via several components of the PTS including the Enzyme E I (EI), Histidine Protein (HPr, Heat-stable Protein) and Enzyme E II (EII), a membrane permease, which phosphorylates and transports the corresponding substrate (Eggeling and Bott, 2005). *C. glutamicum* possesses a glucose EII with high affinity for glucose together with a non PTS permease with low affinity glucose uptake function (Cocaign-Bousquet *et al.*, 1996). A glucose kinase gene, for free intracellular glucose phosphorylating has been identified and could combine with non-pts permease for a non-pts glucose uptake system. Such system might contribute up to 25% of total glucose uptake under non-stress conditions (Moon *et al.*, 2007).

The substrate transported via pts will enter the central metabolic pathways including glycolysis and pentose phosphate pathway. The phosphorylated substrate in the glycolysis is metabolized to pyruvate through cascade of reactions catalyzed by several enzymes (Figure I-4). The first enzyme of the cascade, glucose phosphate isomerase was reported to be inhibited by erythrose-4-phosphate (Sugimoto and Shiio, 1989). The 6-phosphofructokinase catalyzes the conversion of fructose-6-phosphate to fructose-1,6-bisphosphate whereas the fructose-1,6-bisphosphatase

catalyzes the reverse reaction and is inhibited by AMP (Sugimoto and Shiio, 1989). The fructose-1,6-bisphosphate is reversibly transformed to dihydroxyacetone phosphate and glyceraldehyde-3-phosphate by the fructose-1,6-bisphosphate aldolase. The next reaction which yields 1,3-bisphosphoglycerate from glyceraldehyde-3-phosphate is catalyzed by glyceraldehyde-3-phosphate dehydrogenase (*gapA* gene). The former enzyme is strongly regulated by the ratio of $\text{NADH,H}^+/\text{NAD}^+$ (Dominguez *et al.*, 1998). The pyruvate kinase, that catalyzes the transformation of phosphoenolpyruvate in pyruvate coupled with the formation of an ATP, is considered as the most important control point of the glycolysis. It is activated by AMP and strongly inhibited by ATP (Eggeling and Bott, 2005). The reverse reaction from pyruvate to phosphoenolpyruvate is catalyzed by phosphoenolpyruvate synthetase.

The resulting metabolites of glycolysis (phosphoenolpyruvate, pyruvate), combined with oxaloacetate are involved in the phosphoenolpyruvate -pyruvate-oxaloacetate node that represents the switching point between the major pathways of carbon metabolism. This node insures the carbon flux repartition among catabolism, anabolism and energy supply of the cell. From this switching point, the pyruvate dehydrogenase decarboxylates pyruvate to acetyl-CoA which enters the oxidative branch of TCA cycle. Pyruvate can be also decarboxylated in non-oxidative condition to produce acetate directly or from acetyl-CoA. In addition, the anaplerotic pathways are replenished by phosphoenolpyruvate and/or pyruvate carboxylation or by decarboxylation of oxaloacetate- and/or malate to regenerate glycolysis metabolites (pyruvate and phosphoenolpyruvate) (Sauer and Eikmanns, 2005).

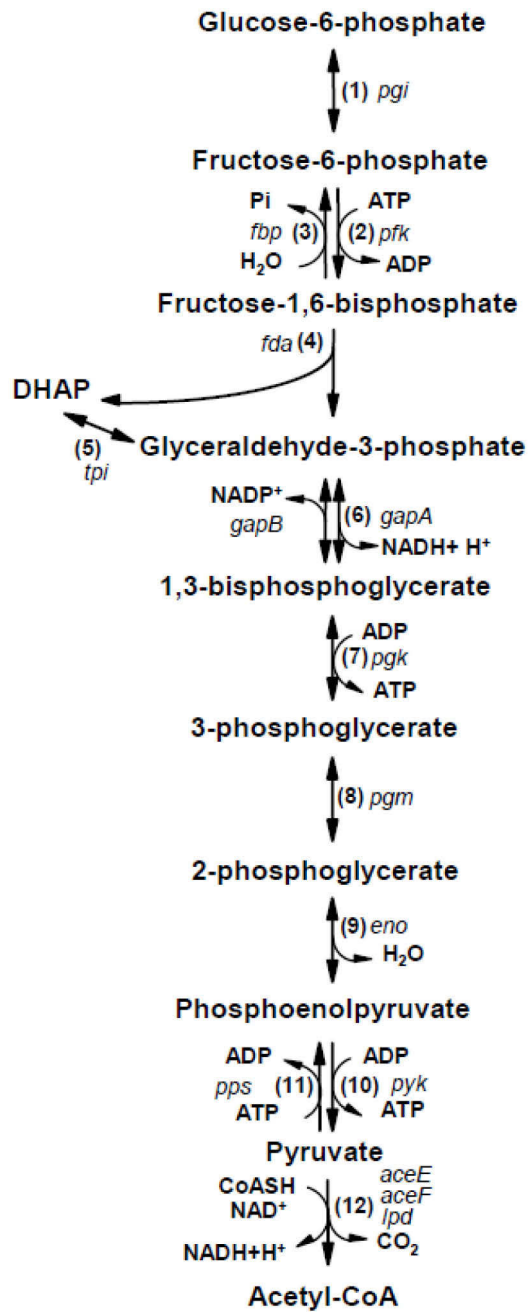


Figure I-4. Reactions involved in *C. glutamicum* glycolysis. Phosphate isomerase; (1). 6-phosphofructokinase; (2). Fructose-1,6-bisphosphatase; (3). Fructose-1,6-bisphosphate aldolase; (4). Glyceraldehyde-3-phosphate dehydrogenase; (5). Triose phosphate isomerase; (6). 3-phosphoglycerate kinase; (7). Phosphoglycerate mutase, (8), Enolase; (9). Pyruvate kinase; (10). Phosphoenolpyruvate synthetase; (11). Pyruvate dehydrogenase (12) (Botella, 2009).

b. Anaplerotic pathways

The TCA cycle of growing cells needs to be continuously replenished due to use of several intermediates in anabolism. Anaplerotic enzymes that catalyze such reactions fulfil this role. Unlike most of bacteria, *C. glutamicum* has a complex set of anaplerotic pathways for supplying oxaloacetate (Sahm *et al.*, 2000). The transformation of phosphoenolpyruvate to oxaloacetate by bicarbonate fixation is catalyzed by a phosphoenolpyruvate carboxylase which was reported to be activated by acetyl-CoA and fructose-1,6-bisphosphate and inhibited by aspartate and 2-oxoglutarate (Mori and Shiio, 1985a, 1985b). In *C. glutamicum*, PEP carboxylase was found to be the predominant anaplerotic enzyme for succinic acid production under oxygen deprived conditions (Inui *et al.*, 2004a). Besides, other studies with cell-free extracts of *C. glutamicum* revealed that it possesses phosphoenolpyruvate carboxykinase enzyme (Jetten and Sinskey, 1993; Peters-Wendisch *et al.*, 1993) but this activity, in the direction of oxaloacetate synthesis, is inhibited by low concentrations of ATP. Therefore, it is likely that the phosphoenolpyruvate carboxykinase of *C. glutamicum* is involved in gluconeogenesis rather than in anaplerosis (Jetten and Sinskey, 1993). In addition to phosphoenolpyruvate carboxylation, another anaplerotic enzyme, pyruvate carboxylase, participates to oxaloacetate replenishment. It catalyzes the irreversible carboxylation of pyruvate to oxaloacetate with concomitant ATP hydrolysis. Phosphoenolpyruvate carboxylase and pyruvate carboxylase genes deletions studies reported that the pyruvate carboxylase is essential for growth on lactate and pyruvate, and that the two enzymes can replace each other for the growth on glucose (Peters-Wendisch *et al.*, 1998). In succinic acid production with *C. glutamicum*, even if the predominant anaplerotic enzyme was reported to be phosphoenolpyruvate carboxylase, pyruvate carboxylase was shown to be rate limiting and its overexpression enhance the succinic acid production (Inui *et al.*, 2004a; Okino *et al.*, 2008a). Other anaplerotic enzymes described, act in the direction of reversible decarboxylation (Malic enzyme and oxaloacetate decarboxylase). Malic enzyme catalyzes the reversible decarboxylation of malate into pyruvate with simultaneous reduction of NADP⁺ (Eggeling and Bott, 2005). Gourdon *et al.* (2000), suggested that malic enzyme plays a role in NADPH generation on substrates other than glucose (Gourdon *et al.*, 2000). Oxaloacetate decarboxylase, is not strictly related to anaplerosis but is able to influence the flux of the anaplerotic reactions. It irreversibly converts oxaloacetate to pyruvate. The two main anaplerotic enzymes involved in the succinic acid production by *C. glutamicum* are then, phosphoenolpyruvate carboxylase and pyruvate carboxylase (Figure I-5).

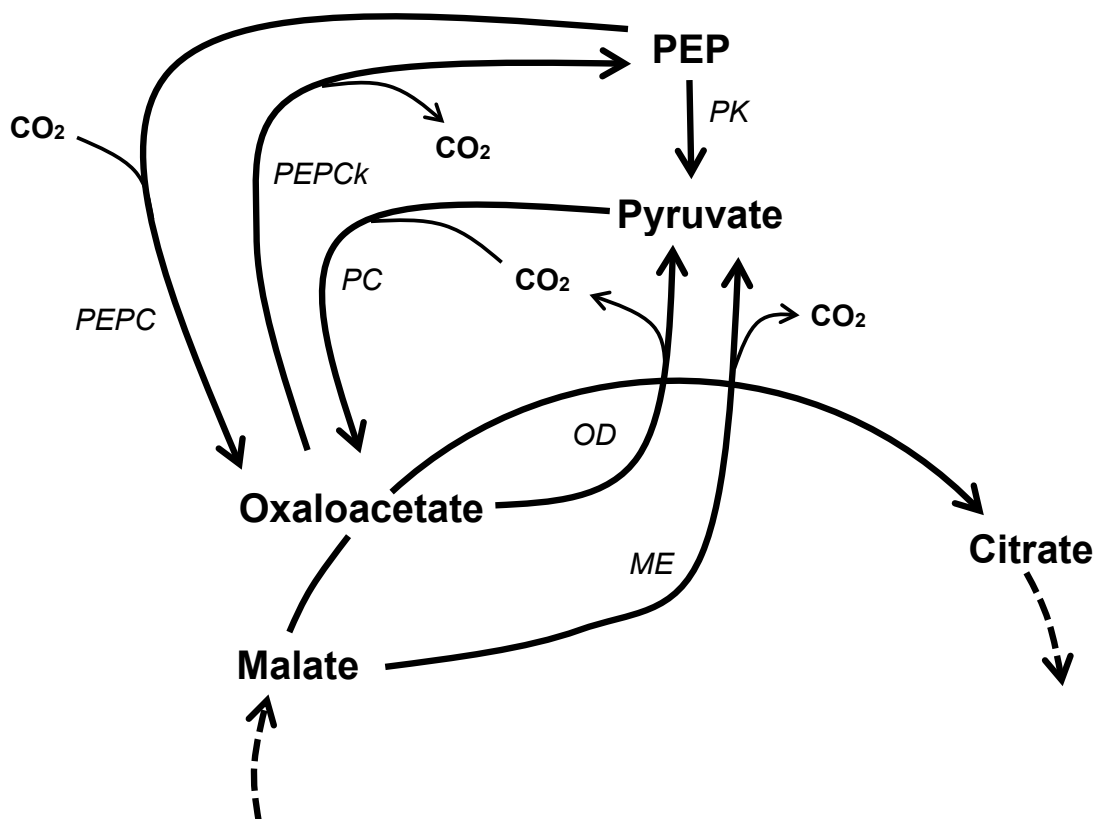


Figure I-5. *C. glutamicum* anaplerotic pathways involved in the PEP-pyruvate-oxaloacetate node. PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase; PEPCk, phosphoenolpyruvate carboxykinase; PK, pyruvate kinase; PC, pyruvate carboxylase; OD, oxaloacetate decarboxylase and ME, malic enzyme.

c. The oxidative branch of TCA cycle and the glyoxylate shunt

The citric acid cycle, also known as the tricarboxylic acid cycle (TCA) is involved in the breakdown of all the three major food groups; carbohydrates, lipids, and proteins. It plays an important anabolic and catabolic roles. It provides energy (ATP, GTP), reducing equivalents and precursor metabolites for biosynthetic processes. The most important intermediates for biosynthetic purpose are 2-oxoglutarate (precursor of glutamate and derivatives), and oxaloacetate (precursor of aspartate and derivatives) (Eggeling and Bott, 2005). After glycolysis, the pyruvate is transformed into Acetyl-CoA by the pyruvate dehydrogenase complex. Then Acetyl-CoA enters the TCA cycle by reacting with oxaloacetate to form citrate. This condensation is catalyzed by citrate synthase. In *C. glutamicum*, the specific activity of the citrate synthase was found to be independent of the growth substrate and of the growth phase. The enzyme was not affected by NADH or 2-oxoglutarate and was only weakly inhibited by

ATP and cis-aconitate activity. Inactivation of the citrate synthase encoding gene, *gltA*, leads to glutamate and citrate auxotrophy (Eikmanns *et al.*, 1994). Citrate is reversibly isomerized to isocitrate by aconitase with cis-aconitate as enzyme-bound intermediate. Isocitrate is decarboxylated to provide 2-oxoglutarate and release CO₂. This decarboxylation is catalyzed by a NADP-dependent isocitrate dehydrogenase. 2-oxoglutarate is an intermediate of TCA cycle but can also serve as a precursor for glutamate synthesis. This step of the TCA cycle gives important intermediates of the energy metabolism together with reducing equivalents to the cells. Isocitrate dehydrogenase of *C. glutamicum* is weakly inhibited by citrate, 2-oxoglutarate, and oxaloacetate and is found to be strongly inhibited by simultaneous addition of oxaloacetate and glyoxylate. The enzyme is formed constitutively (Shiio and Ozaki, 1968; Eikmanns *et al.*, 1995). The 2-oxoglutarate is further decarboxylated irreversibly to form succinyl-CoA by the 2-oxoglutarate dehydrogenase complex. The former reaction releases a reducing equivalent, NADH. The resulting Succinyl-CoA is transformed into succinate and coenzyme A. The reaction catalyzed by succinyl-CoA synthetase is accompanied by a concomitant production of ATP. It was reported that the genes *sucC* and *sucD* encode the succinyl-CoA synthetase subunits (Kalinowski *et al.*, 2003). Then, succinate is oxidized into fumarate by a membrane enzyme, succinate dehydrogenase (Hägerhäll, 1997). Fumarase or fumarate hydratase catalyzes the interconversion of fumarate and malate. The last reaction of the TCA cycle consists in the regeneration of oxaloacetate by malate oxidation. Two types of enzymes for oxidation of malate exist in *C. glutamicum*, a dehydrogenase which is a cytoplasmic NAD-dependent enzyme and a malate: quinone oxidoreductase, a membrane associated enzyme. The reaction catalyzed by the latter is more favorable than the first one under normal conditions (Molenaar *et al.*, 2000; Eggeling and Bott, 2005).

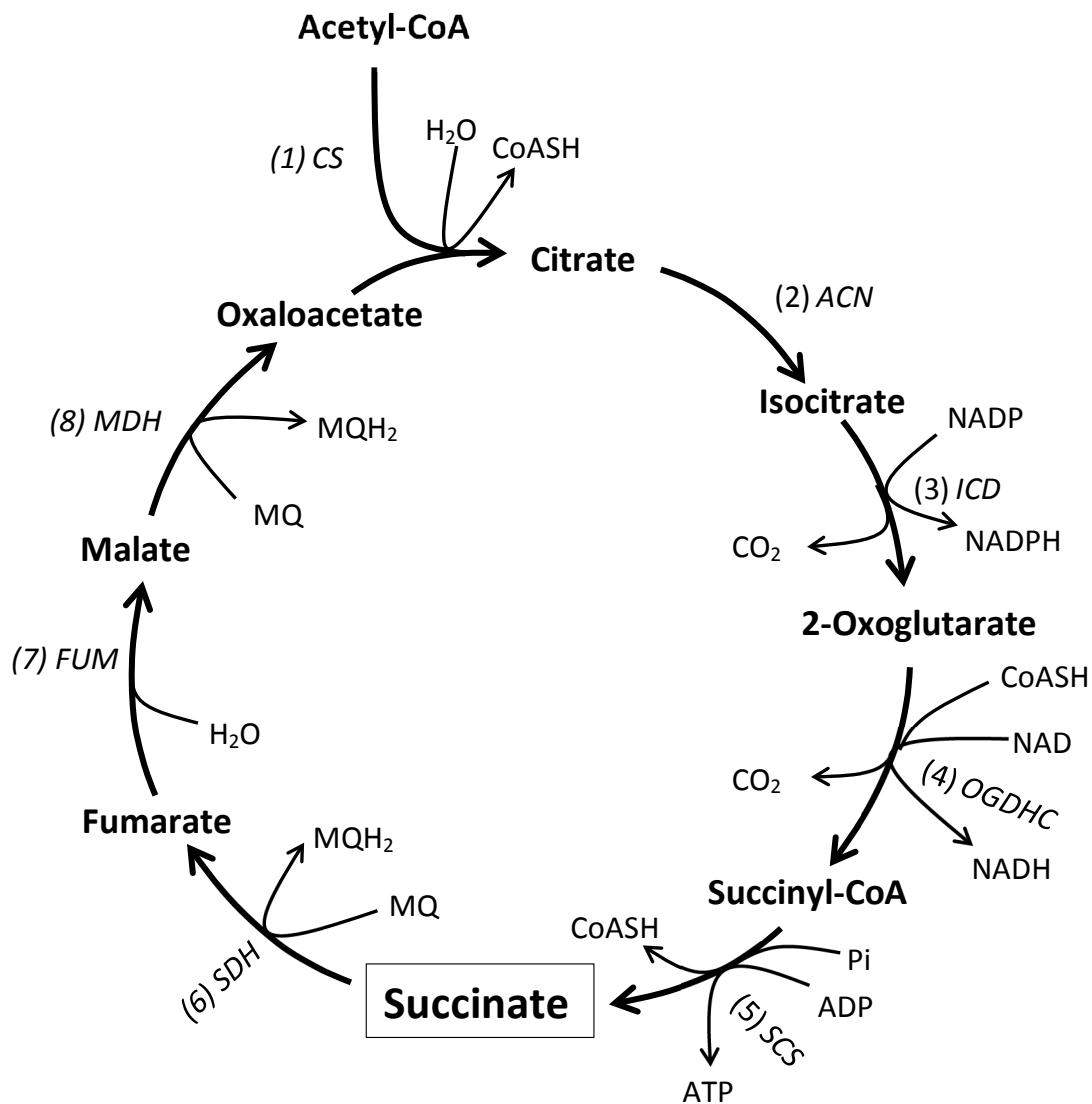


Figure I-6. The tricarboxylic acid cycle of *C. glutamicum*. (1) CS, citrate synthase ; (2) ACN, aconitase ; (3) ICD, isocitrate dehydrogenase; (4) OGDHC, 2-oxoglutarate dehydrogenase complex; (5) SCS, succinyl-CoA synthetase; (6) SDH, succinate dehydrogenase; (7) FUM, fumarase, and (8) MDH, malate dehydrogenase or malate: quinone oxidoreductase.

In fact, under aerobic conditions, succinic acid is only an intermediate of the Krebs cycle (TCA) (Figure I-6) and therefore can be hardly an end-product (Lin et al., 2005d). The capability to produce succinate under aerobic conditions would mean an active oxidative phosphorylation for generating energy with O_2 present as the electron acceptor. This would lead to higher biomass generation, faster carbon throughput and product formation. To produce succinate through the oxidative pathway, a key mutation corresponding to the deletion of the succinate dehydrogenase (SDH) genes is required. After this unavoidable mutation, subsequent deletions

of all known by-products producing pathways as well as overexpression of succinate biosynthesis genes could significantly increase succinic acid production. Recently, the central metabolism of *Yarrowia lipolytica* (Yuzbashev *et al.*, 2010), *C. glutamicum* (Litsanov *et al.*, 2012a) and *E. coli* (Lin *et al.*, 2005c) has been genetically modified by deleting *sdh*, the gene encoding the succinate dehydrogenase in the TCA cycle. In such conditions, the recycling of metabolite was made through the glyoxylate shunt (Figure I-7). The glyoxylate bypass is involved in aerobic metabolism of acetate, fatty acids or ethanol as an anaplerotic pathway to avoid the oxidative decarboxylation steps of the TCA cycle, and leads to the formation of malate and succinate as intermediates. It converts acetyl-CoA and isocitrate into malate and succinate from glyoxylate. The two enzymes involved in the glyoxylate shunt are isocitrate lyase and malate synthase enzymes (Figure I-6 and Figure I-7). The genes of the corresponding enzymes have been reported to be quite responsive to the availability of carbon sources (Wendisch *et al.*, 1997). Isocitrate lyase catalyzes the conversion of isocitrate, into glyoxylate and succinate (Reinscheid *et al.*, 1994). Malate synthase catalyzes the subsequent condensation of glyoxylate with acetyl-coenzymeA to produce malate. The glyoxylate pathway converts 2 moles of acetyl CoA and 1 mole of oxaloacetate to 1 mole of succinate and 1 mole of malate and contributes to avoid carbons lost as CO₂ by bypassing the two last decarboxylation steps in the TCA (Cok *et al.*, 2014). In *C. glutamicum*, isocitrate lyase and isocitrate dehydrogenase that share the same substrate, isocitrate, are subject to regulation. In fact, Isocitrate lyase activity is reduced when glucose is the substrate; meaning that the TCA cycle is the major pathway in such conditions. The isocitrate dehydrogenase activity is also reduced when glyoxylate cycle is processing (Reinscheid *et al.*, 1994; Han *et al.*, 2008).

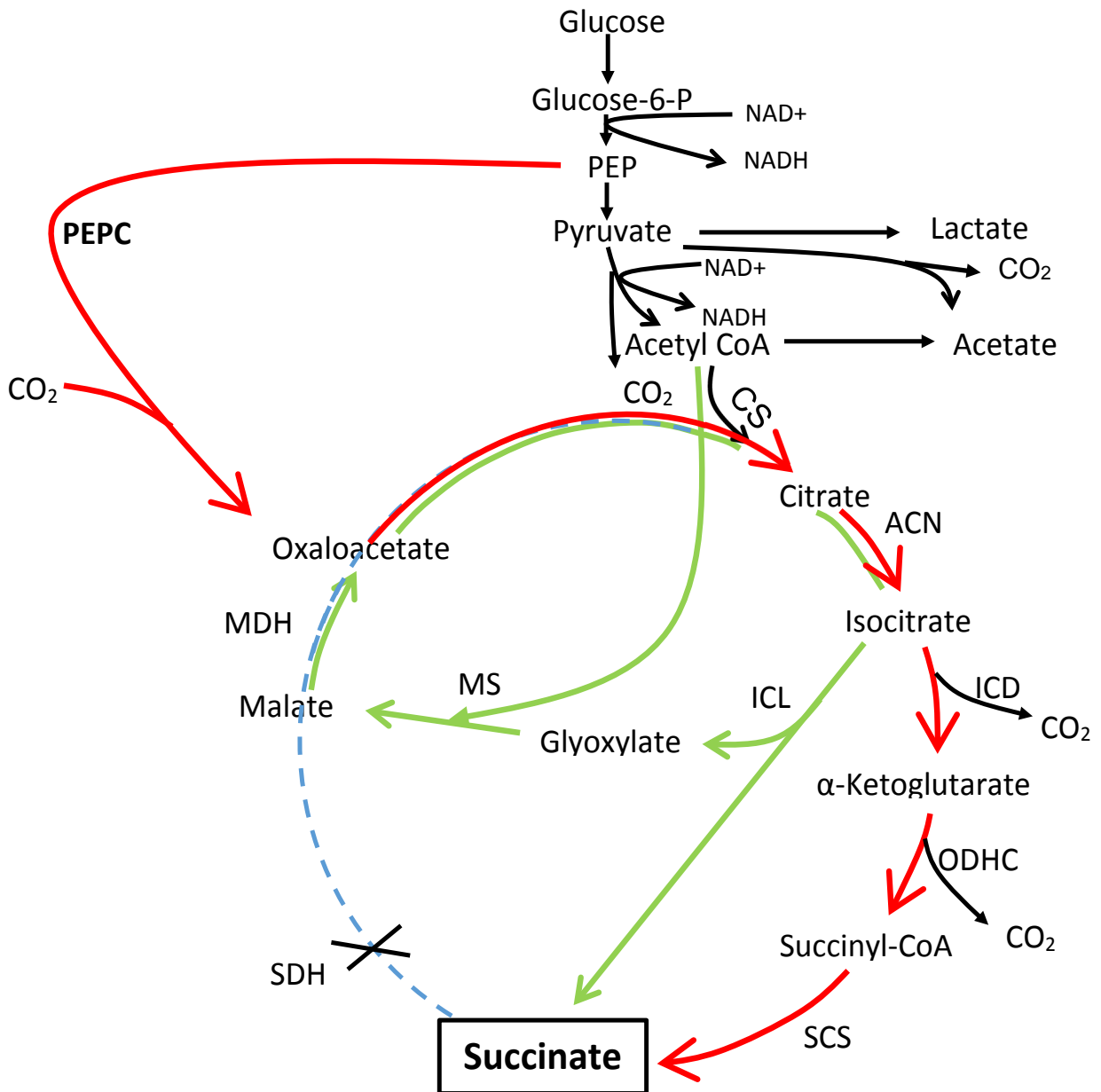


Figure I-7. Aerobic succinic acid production based on the two main routes, the glyoxylate shunt (green) and the oxidative arm of the TCA cycle (red). The relevant reactions leading to succinate are displayed in red, green and thick arrows. Abbreviations: Glyoxylate shunt (CS, citrate synthase; ACN, aconitase; ICL, isocitrate lyase; MS, malate synthase; MDH, malate dehydrogenase or malate: quinone oxidoreductase) and oxidative arm of TCA cycle (CS, citrate synthase; ACN, aconitase; ICD, isocitrate dehydrogenase; ODHC, 2-oxoglutarate dehydrogenase complex; SCS, succinyl-CoA synthetase). PEPC, phosphoenolpyruvate carboxylase. "X", indicated gene that should be deleted.

Taking advantage of this metabolic shunt, new succinate production pathway involving only the glyoxylate shunt could be investigated. For this purpose, two key regulatory genes, the succinate dehydrogenase (SDH) and isocitrate dehydrogenase (ICD) genes, have to be deleted. In such resulting mutant strain, the isocitrate is split into succinate and glyoxylate, which is

catalyzed by isocitrate lyase (Moat *et al.*, 2002; Meijer *et al.*, 2009). Then malate synthase condenses acetyl-CoA and glyoxylate to form malate which enters the residual TCA cycle to regenerate isocitrate (Figure I-8) (Lin *et al.*, 2005d; Litsanov *et al.*, 2012a; Cao *et al.*, 2013).

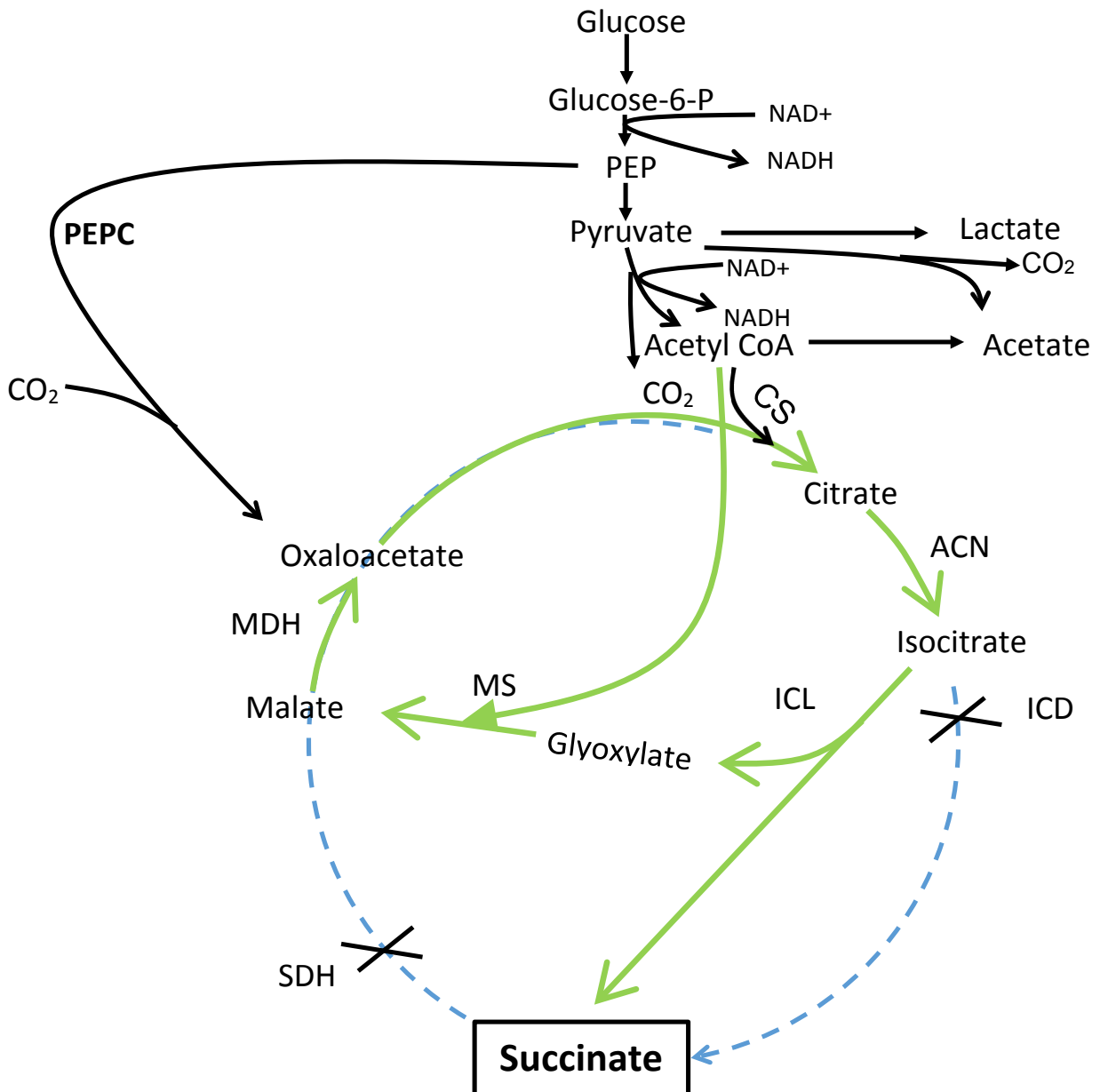


Figure I-8. Aerobic succinic acid production through the glyoxylate shunt. The relevant reactions leading to succinate are displayed in red, green thick arrows. Abbreviations: CS, citrate synthase; ACN, aconitase; ICL, isocitrate lyase; MS, malate synthase; MDH, malate dehydrogenase or malate: quinone oxidoreductase. "X", indicated genes that should be deleted for succinate production through only glyoxylate shunt; ICD, isocitrate dehydrogenase and SDH, succinate dehydrogenase.

d. *The reductive arm of tricarboxylic acid (TCA) cycle*

Some experiments performed with carbohydrates labeled with ^{14}C allowed the analysis of organic acids production pathways by anaerobe *Bacteroides fragilis*. The monitoring of fermentation products and the distribution of radioactivity were determined, and the labeling patterns in the products, as well as the enzyme analyses, reported that *B. fragilis* was able to ferment glucose via the Embden-Meyerhof-Parnas pathway and that CO_2 is needed for the formation of C_4 compounds from phosphoenolpyruvate. PEP condensates with CO_2 to form oxaloacetate. Oxaloacetate is then reduced to malate by malate dehydrogenase. Malate is dehydrated to fumarate and the latter is reduced to succinate by a membrane-bound fumarate reductase (Macy *et al.*, 1978). Besides *B. fragilis*, several enzymatic essays revealed that most succinic acid producing anaerobes use the Embden-Meyerhof-Parnas pathway for glycolysis and use pyruvate or phosphoenolpyruvate for anaplerotic replenishment of the reductive arm of the TCA cycle (Zeikus, 1980; Samuelov *et al.*, 1991; Litsanov *et al.*, 2014).

For examples, in *A. succinogenes*, enzyme assays and metabolic flux analyses were performed to characterize the succinic acid pathway and resulted in the following statements (Van der Werf *et al.*, 1997; McKinlay *et al.*, 2007b): Glucose is transported by a PEP-dependent phosphotransferase system as well as by a glucose permease. Glucose-6-phosphate is metabolized almost exclusively through the EMP pathway. Then, PEP formed in the EMP, is carboxylated to oxaloacetate by a PEP carboxykinase, the sole C_3 -carboxylating enzyme in *A. succinogenes*. Oxaloacetate is then converted to succinic acid via malate dehydrogenase, fumarase and fumarate reductase activity in the reductive branch of the TCA cycle. Thus, four key enzymes, responsible for succinic acid production, were identified to be PEP carboxykinase, malate dehydrogenase, fumarase and fumarate reductase (Song and Lee, 2006). Like *A. succinogenes*, *Mannheimia succiniciproducens*, mainly produce succinate through the carboxylation of PEP and use of the reductive TCA cycle (Hong *et al.*, 2004; Lee *et al.*, 2006). Additionally to anaerobes, some facultative aerobic bacteria have been proven to produce succinic acid through anaplerotic pathways and the TCA cycle. In fact, *E. coli*, possesses only a PEP carboxylase as anaplerotic enzyme for succinic acid synthesis through the reductive pathway (Vemuri *et al.*, 2002a; Wu *et al.*, 2007). In contrast to the microorganisms cited previously, *C. glutamicum* has many enzymes involved in the anaplerotic pathways (PEPC, PC, PEPCCK, OD and ME) and the reductive production of succinate (Sauer and Eikmanns, 2005). Studies with CO_2 donor such as bicarbonate in the anaerobic fermentation of *C. glutamicum*, led to a 3.6-folds increase in succinate production with consumed bicarbonate that is

proportional to succinate production. These observations led to the presumption that anaplerotic enzymes fixed bicarbonate to produce C₄ dicarboxylic acids through reductive TCA cycle (Inui *et al.*, 2004a; Song and Lee, 2006; Okino *et al.*, 2008a).

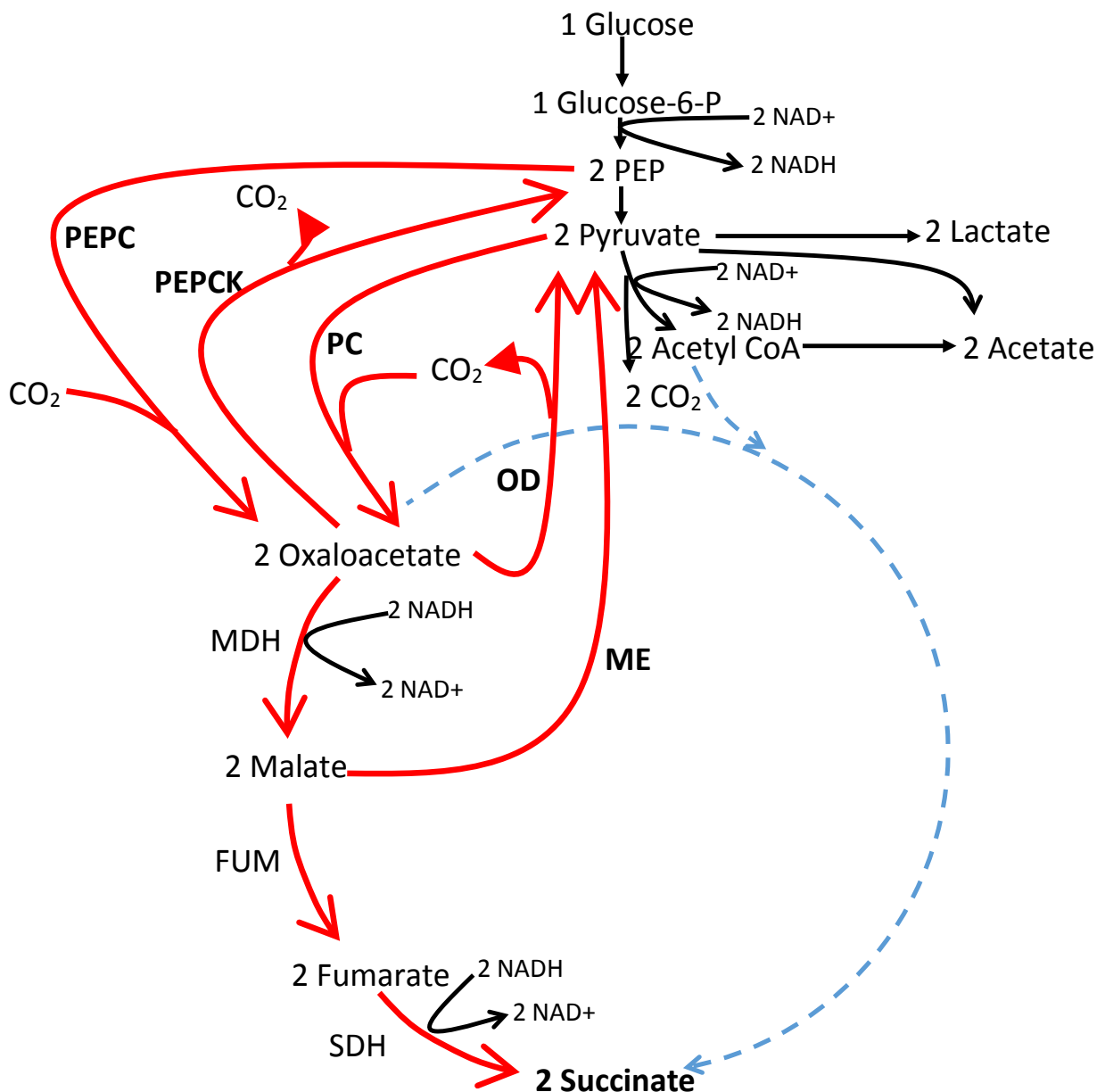


Figure I-9. Succinate biosynthesis pathway in *C. glutamicum* under anaerobic conditions. The relevant reactions leading to succinate are displayed in red and thick arrows. Abbreviations: Anaplerotic pathways (PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase; PEPCK, phosphoenolpyruvate carboxykinase; PC, pyruvate carboxylase; OD, oxaloacetate decarboxylase; ME, malic enzyme) and reductive arm of TCA cycle (MDH, malate dehydrogenase; FUM, fumarase; SDH, succinate dehydrogenase). LDH, lactate dehydrogenase.

e. Succinic acid membrane exporters

Bacteria contain secondary carriers for the uptake, exchange or efflux of C₄-dicarboxylates. In aerobic bacteria, dicarboxylate transport (Dct)A carriers catalyze the uptake of C₄-dicarboxylates in a H⁺- or - Na⁺ C₄-dicarboxylate symport. Carriers of the dicarboxylate uptake (Dcu)AB family are used for electro-neutral fumarate:succinate antiport which is required in anaerobic fumarate respiration. The DcuC carriers family apparently functions in succinate efflux during the fermentation. Some members of the DcuAB and DcuC families catalyze substrate antiport and function in succinate excretion direction (Janausch *et al.*, 2002). The synthesis of the transport system, in *E. coli*, requires the presence of FNR, the transcriptional regulator of the genes for anaerobic respiration. *E. coli* and other enteric bacteria excrete large amounts of succinate as the fermentation product in certain metabolic conditions. Under these conditions, succinic acid is synthesized in the cytosol and secreted via a symport system with protons (Engel *et al.*, 1994). Whereas the metabolic pathways for succinate formation are well described, transport of succinate across the cytoplasmic membrane is still under investigations for most of the succinic acid producers. In the genomes of *A. succinogenes* and *M. succiniciproducens*, genes encoding proteins similar to DcuA, DcuB and DcuC were found, but their function as carboxylic acid transporters were not confirmed (Huhn *et al.*, 2011). Besides, for *C. glutamicum*, Huhn *et al.* (2011), undertook extensive researches to identify putative succinate export systems by comparative genome analyses. Based on several screenings, a gene named *cg2425* was identified to encode a putative succinate export system. To analyze the role of this gene, a mutant was constructed. Cultivation of the *cg2425* mutant under appropriated conditions resulted in internal high amounts of succinic acid accumulated whereas its excretion was impaired. As the inactivation of *cg2425* caused a reduced succinate excretion, the authors concluded that the protein encoded by *cg2425* gene participates in succinate efflux. Then this gene was renamed *sucE*. Further studies demonstrated that *sucE* plays only a function as exporter but cannot import succinate from the culture broth (Huhn *et al.*, 2011).

I-4. The carbon dioxide role in succinic acid biosynthesis

Anaerobic succinic acid production is known to be a carbon dioxide fixing process. In fact, succinic acid is produced by carboxylation of C₃ compounds by most succinic acid producers under anaerobic conditions. In succinic acid production under anaerobic or oxygen deprived conditions, higher dissolved CO₂ availability was proven to increase the ratio of succinic acid concentration to the other acids production and the yield of succinic acid (Van der Werf et al., 1997; Lu et al., 2009; Zou et al., 2011). In *C. glutamicum* ATCC 13032, a characterization of glucose metabolism during anaerobic fermentation in the presence of CO₂ was performed with *In vivo* ¹³C-NMR analysis. The authors reported that, under pure gaseous CO₂, succinate was the major product whereas lactate was the main product in absence of CO₂ (Radoš et al., 2014). Thus, the availability of carbon dioxide is obviously an important aspect of the process. Such carboxylation is accomplished on pyruvate or phosphoenolpyruvate through anaplerotic enzymes which are pyruvate carboxylase, phosphoenolpyruvate carboxylase and/or phosphoenolpyruvate carboxykinase activities.

The theoretical fermentative stoichiometry of succinic acid formation from glucose can be written as follows:



From this equation, it appears that one mole of CO₂ is theoretically required for the synthesis of one mole of succinate. Studies have reported the effect of CO₂ level in culture medium on metabolic fluxes and the distribution of fermentation products (Samuelov *et al.*, 1991; Nghiem *et al.*, 1997; Lee *et al.*, 1999). As the level of CO₂ was found to promote the succinic acid production, CO₂ supply can be an important variable in succinate fermentation (Elsden, 1938). Carbon dioxide can be supplied to the fermentation medium in various ways: the medium can be sparged with gaseous CO₂, the CO₂ can be also mixed with other gases which do not interfere with the microorganism metabolism, and finally CO₂ can be supplied by the addition of carbonates or bicarbonates (Datta, 1992). When CO₂ or carbonates are dissolved in water, they produce HCO₃⁻ and CO₃²⁻ and an equilibrium between CO₂, HCO₃⁻ and CO₃²⁻ is obtained by the pH of the medium. Therefore, the pH of the culture medium is also an important parameter to be taken into account as it affects the solubility of CO₂ in the medium and obviously the availability of CO₂ for microorganisms. Although CO₂ exists in several different chemical

forms, in a CO₂ transport model proposed for *E. coli* (Figure I-10), it has been reported that the bicarbonate form (HCO₃⁻) is used for carboxylation reactions.

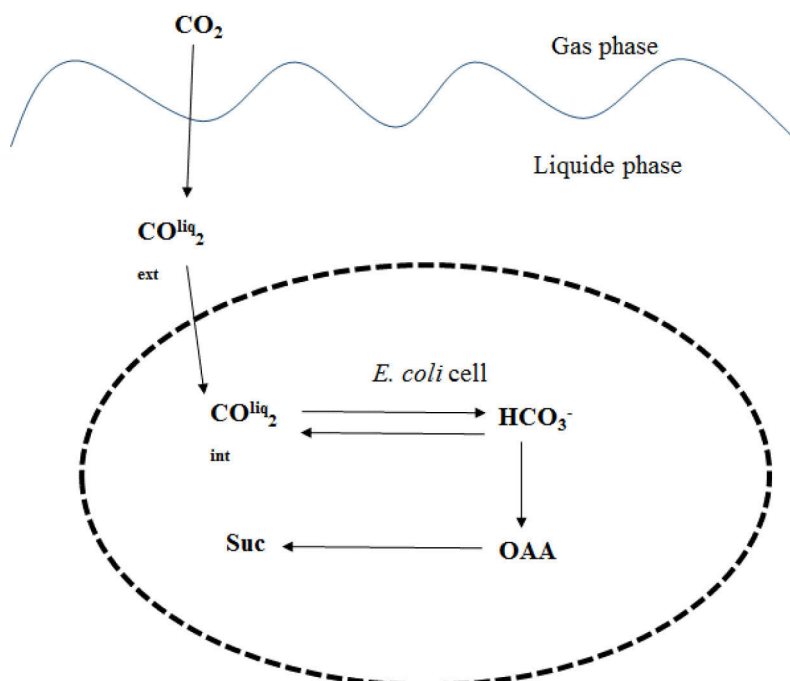


Figure I-10 Carbon dioxide transport from the gas phase to succinic acid pathway (Lu *et al.*, 2009).

I-5. Succinic acid production processes

I-5.1. Diversity of potential carbon sources

The choice of adequate carbon source for succinic acid production is an important point for improving yields and productivity. In fact, it was reported that the oxidation state of the carbon source affects the products formation as well as the accumulation of by-products which consequently affects the downstream process (Lin *et al.*, 2005a). Furthermore, feedstock represents the major cost for most bioprocesses for the production of bulk chemicals. Therefore, the identification of inexpensive feedstock devoted the production of succinate is of economic interest. Even if glucose is generally used as the carbon source for succinate production, its relative high price increases the production costs. In order to develop a more sustainable bio-based industrial production of succinate, low-cost substrates could be interesting alternative (Cao *et al.*, 2013). For example, glycerol which is an abundant waste product from the biodiesel industry with limited commercial uses, has been successfully employed for efficient succinate production with *A. succiniciproducens* (Lee *et al.*, 2001), *A. succinogenes* (Barros *et al.*, 2013), and metabolically engineered *E. coli* (Zhang *et al.*, 2010). Besides, many attempts targeted

various feedstocks. For this purpose, *A. succinogenes* has been studied for growth and succinic acid production based on various wastes used as the substrate: cane molasses (Liu *et al.*, 2008), corn stover (Li *et al.*, 2009b, 2011; Zheng *et al.*, 2010) wheat flour, corncob hydrolysates, corn stalk (Du *et al.*, 2008), sugarcane bagasse, hemicellulose hydrolyzate, and whey (Wan *et al.*, 2008, 130; Li *et al.*, 2010c; Borges and Pereira, 2011). Succinic acid production from various carbon sources is summarized in Table I-3.

Table I-3. The panel of substrates available for succinate bioproduction.

Strains	Substrates	Conc. (g L ⁻¹)	Productivity (g L ⁻¹ h ⁻¹)	Yield g g ⁻¹	References
<i>Actinobacillus succinogenes</i> 130z	Glucose	67.2	0.80	0.70	(Guettler <i>et al.</i> , 1998)
<i>Actinobacillus succinogenes</i> 130z	Sake lees	48	0.94	0.75	(Chen <i>et al.</i> , 2010)
<i>Actinobacillus succinogenes</i> CGMCC 1593	Cane molasses	50.6	0.84	0.80	(Liu <i>et al.</i> , 2008)
<i>Anaerobiospirillum succiniciproducens</i> ATCC 29305	Raw whey	34.7	1.02	0.91	(Samuelov <i>et al.</i> , 1999)
<i>E. coli</i> AFP184	Corn stalk enzymatic hydrolyzate	57.8	0.80	0.87	(Wang <i>et al.</i> , 2011a)
<i>Mannheimia succiniciproducens</i> MBEL55E	Wood hydrolyzate	11.7	1.17	0.56	(Kim <i>et al.</i> , 2004a)

I-5.2. The classical methods used for succinate biological production

Batch cultures are mostly employed as a production system even if higher titers and rates are obtained by fed-batch and continuous culture systems (Table I-4). One of the highest volumetric rate, $10.5 \text{ g L}^{-1} \text{ h}^{-1}$, was obtained under continuous culture with cell recycling using membrane at a dilution rate of 0.98 h^{-1} (Meynial-Salles *et al.*, 2008). In addition, a titer up to 146 g L^{-1} of succinic acid was obtained in a cell recycling fed batch culture with *C. glutamicum* R (Okino *et al.*, 2008a) under anaerobic conditions. Besides, most of the succinate-producing processes using facultative aerobic bacteria such as *E. coli* or *C. glutamicum*, are performed under anaerobic conditions. Unfortunately, long doubling time observed for several strains, slow carbon fluxes, low product formation rates and growth arrested (in the case of *C. glutamicum*) under anaerobic conditions constitute some disadvantages of anaerobic production systems. To overcome those constraints, strains are genetically modified in order to set up aerobic production processes (Lin *et al.*, 2005b, 2005d; Litsanov *et al.*, 2012a). However, even with such disadvantages, anaerobic production systems remain the most efficient. Thus, instead of aerobic production, a dual-phase fermentation process has been developed. This dual-phase consists in a first phase dedicated to the growth under aerobic conditions for biomass accumulation, followed by an anaerobic phase for succinate production (Vemuri *et al.*, 2002a, 2002b; Wu *et al.*, 2007; Lu *et al.*, 2009). The current dual-phase process is performed as follows: When the desired biomass is reached during the first phase, the air sparging is instantaneously replaced by a mixture of air CO_2/N_2 or CO_2 or N_2 or other gases except O_2 . However, studies have reported an important influence of the transition from aerobiosis to anaerobiosis on the process efficiencies. Indeed, enzymes that carry out the bio-transformations in the production phase, are largely expressed during the aerobic growth phase and the transition phase before entering the anaerobic phase. Several of them, particularly those involved in anaplerotic reactions were found to be upregulated during the transition from aerobiosis to anaerobiosis and remained active during the production phase (Vemuri *et al.*, 2002b; Inui *et al.*, 2007; Martínez *et al.*, 2010; Zhu *et al.*, 2011).

Table I-4. The different production strategies reported in literature for succinate production.

Strain	Process strategy	Titer (g L ⁻¹)	Yield (g g ⁻¹)	Productivity (g L ⁻¹ h ⁻¹)	Ref.
<i>E. coli</i> AFP111- <i>pyc</i>	Dual-phase fedbatch	99.2	1.10	1.30	(Vemuri <i>et al.</i> , 2002b)
<i>E. coli</i> HL27659k- <i>pepc</i>	Aerobic fed-batch	58.3	0.62	0.72	(Lin <i>et al.</i> , 2005b)
<i>C. glutamicum</i> Δ <i>ldhA</i> -pCRA717	Aerobic-microaerobic fed-batch	146	0.92	3.17	(Okino <i>et al.</i> , 2008a)
<i>C. glutamicum</i> BOL-3/pAN6- <i>gap</i>	Anaerobic fedbatch	134	1.10	2.5	(Litsanov <i>et al.</i> , 2012b)
<i>A. succiniciproducens</i> ATCC 53488	Anaerobic continuous culture with integrated membrane for cell recycling	83	0.88	10.4	(Meynial-Salles <i>et al.</i> , 2008)

I-5.3. The transition role in the process of succinate production

To understand the effect of transition between aerobic and anaerobic phases in succinic acid production process, Martinez *et al.* (2010) have performed experiments with *E. coli* SBS550MG in two different levels of aeration: a high aeration condition consisting in agitation rate of 800 rpm in combination with an aeration rate of 2.5 L min⁻¹ while the lower level consisted in an agitation of 500 rpm combined with an aeration rate of 1.5 L min⁻¹ of aeration rate. The cultures were carried out in aerobic conditions with a first batch of glucose until 2 g L⁻¹. When glucose was consumed, a second batch of glucose was fed until a final concentration of 2 g L⁻¹. When glucose was exhausted again, aeration was shut off and CO₂ was sparged at a

rate of 0.2 L min^{-1} then 20 g L^{-1} glucose were added for succinate production under anaerobic conditions. From this study, results showed a fast decrease of dissolved oxygen when aeration condition was lower, leading to a microaerobic condition that was maintained for about 3 h before the aeration was stopped. In the other side, the higher aeration experiment never reached microaerobic condition before switching to the anaerobic phase (Martínez *et al.*, 2010).

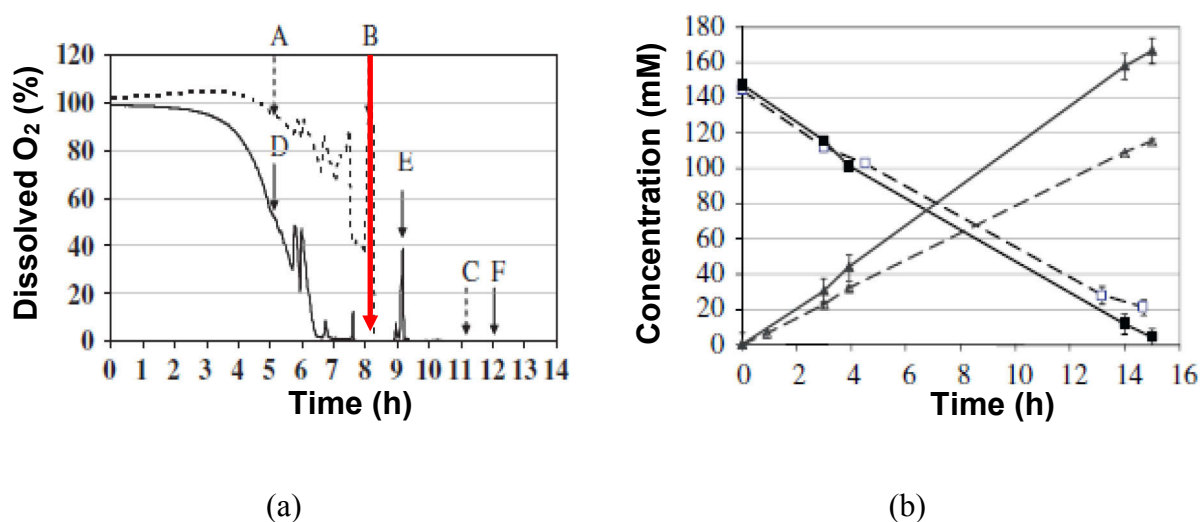


Figure I-11. Influence of aeration level during the initial phase of the process on succinic acid production during a fermentation process with *E. coli* SBS550MG. (a). Dissolved oxygen concentration along the fermentation time, (b). Glucose consumption and succinic acid production. Symbols: filled symbols and lines correspond to low aeration case while dotted line and open symbols represent high aeration conditions; (\square , \blacksquare) Glucose, (\triangle , \blacktriangle) Succinate. The different letters in figure (a) correspond to time of sampling for quantitative PCR analysis, red arrow corresponds to the time of aeration stop.

In fact, succinate concentration was 1.5 fold higher in the culture with low aeration level. To understand the influence of aeration levels on the metabolism of *E. coli*, samples were taken during the aerobic phase, the transition phase and the anaerobic phase for analysis of the expression level of the genes involved in the succinate production pathways. The results of the quantitative polymerase chain reaction (qPCR) revealed that the glyoxylate shunt genes *acnA* and *acnB* encoding aconitate hydratase, for the conversion of citrate into isocitrate, were up-regulated during the transition and anaerobic phases. Besides, the *mdh* gene, involved in the succinic acid production pathway, was strongly up-regulated during the anaerobic-production phase. The main anaplerotic genes *pyc* and *pepc* were also up-regulated, but only during the transition phase. From these different results, Martínez *et al.* (2010), stated that during the transition time the low aerated initial phase in the bioreactor generated microaerobic

environment for a few hours, and these conditions could have contributed to the up-regulation of fermentative genes and therefore to the preparation of the enzymatic machinery for the upcoming anaerobic environment.

I-5.4. Other factors than CO₂ affecting the succinic acid process

In the anaerobic succinic acid production pathways, many factors other than CO₂ availability have been reported to be rate-limiting. Succinic acid is produced as an intermediate of the tricarboxylic acid cycle and also as one of the fermentation products of anaerobic metabolism. As shown in the anaerobic biosynthesis pathway of succinic acid (Figure I-9), one mole of glucose can theoretically generate two moles of succinic acid via the C₄ pathway in excess of CO₂. Whereas the synthesis of two moles of succinic acid requires four moles of NADH, the glycolysis only provides two NADH reducing equivalents. NADH is not sufficient to reach the maximal theoretical yield of succinate. Moreover, several reports showed a relationship between redox potential and the physiological state of a microbial system and succinic acid production (Li *et al.*, 2010a). The intracellular NADH/NAD⁺ ratio was also found to influence succinic acid production by inhibiting the GAPDH for glycolysis (Inui *et al.*, 2004a; Chen *et al.*, 2012). To improve the availability of NADH, the NADH/NAD⁺ ratio, and the total NAD⁺, various strategies have been investigated, including low-oxidation-state carbon sources use, genes overexpression, reducing agents (Van der Werf *et al.*, 1997; Berríos-Rivera *et al.*, 2002a, 2002b; Lin *et al.*, 2005a; Chen *et al.*, 2012).

Finally, as pH affects the solubility of CO₂ in the medium and therefore influences the availability of CO₂ for micro-organisms, pH is also considered as a key factor in succinic acid production (Samuelov *et al.*, 1991; Cheng *et al.*, 2012a).

I-6. Downstream process: recovery and purification of succinate

Various downstream processes (DSP) for the recovery and purification of succinic acid have already been developed. In succinic acid recovery from culture broth, one of the difficulties of downstream process is the presence of other organic acids such as acetic, formic, and lactic acids generally found in mixed acid fermentation. These byproducts not only complicate the DSP but reduce the final succinic acid yield after recovery. As the pH of succinic acid production medium are generally around the neutrality, succinic acid is mainly present in the form of ionized succinate salt because the pK_a values of the two carboxyl functions which are

4.16 and 5.61 at 25°C are lower than the pH of the culture medium. According to the application, a conversion of succinate salt to free acid will be necessary. The downstream purification cost for fermentation-based processes normally amounts to more than 60 % of the total production costs. (Bechthold *et al.*, 2008; Lee *et al.*, 2008; Cheng *et al.*, 2012b; Yi *et al.*, 2013). The development of economically competitive separation technology is essential to reduce the production costs.

Several methods for purification of succinic acid, including electrodialysis, precipitation, and extraction have been developed (Table I-5). As an example, Datta *et al.*, 1992, precipitated succinic acid directly in the fermentation broth by addition of calcium dihydroxide, which simultaneously neutralized the fermentation broth. Calcium succinate was then recovered by filtration and converted to succinic acid by adding sulfuric acid followed by acidic and basic ion exchangers and resulted in product with a purity around 95% (Datta *et al.*, 1992a). In general, the different main steps of succinic acid DSP are:

- The first step consists of microbial cells separation from the fermentation broth by centrifugation or filtration
- This step is followed by ultrafiltration to eliminate proteins, polysaccharides, and other polymers from the supernatant
- Finally, one or combination of the following methods have been tested for the isolation of purified succinic acid isolation: crystallization, precipitation, extraction, electrodialysis, membrane separation, chromatography (Datta *et al.*, 1992a; Yedur *et al.*, 2001; Li *et al.*, 2009a; Nam *et al.*, 2011)

Table I-5. Downstream processes techniques comparison (Cheng et al., 2012b).

Process	Advantages	Disadvantages	Maturity of the technology/cost/ease of scale-up
Direct crystallization	Few unit operations	<p>Low yield and purity.</p> <p>Evaporation and distillation suffer from a large amount of energy consumption.</p> <p>Desalination and deproteinization are required</p>	Immature for commercial application
Precipitation	<p>The precipitation with Ca(OH)₂ or CaO can be a viable process for commercial biosuccinate production with very low technological barriers and risks.</p> <p>The process of precipitation with ammonia produces low amounts of by-products and the recycling used reagent is possible</p>	<p>These reagents cannot be used repeatedly.</p> <p>The substantial by-product calcium sulfate is low-value-added.</p> <p>The high energy consumption and equipment erosion due to high temperature in recycle of used reagent and low pH</p>	<p>Mature for commercial application.</p> <p>The operation cost is significant</p> <p>Needs to be verified in a pilot-scale facility before commercial application.</p> <p>The equipment cost is significant.</p> <p>Easy to scale up</p>
Membrane separation	A microfiltration, ultrafiltration, and nanofiltration combination can give high yield and purity	Membrane pollution can be very serious	<p>The equipment cost is the highest</p> <p>Mature for commercial application</p>

Extraction	High output and low energy consumption	No effective extractant has been so far found for liquid–liquid extraction of succinic acid. The reactive extraction is quite complicated. The removal of proteins as well as salts is necessary before reaction. The expensive extraction agent and diluent is an obstacle for reactive extraction application in a large scale	The operation cost is significant Not easy to scale up
Chromatography	Easy to scale up	The chromatographic medium or matrix has to be regenerated frequently. The regeneration of sorbent needs a large quantity of acid and alkali	Research at an initial stage. The performance of chromatography needs to be verified by using real fermentative broth
<i>In situ</i> separation	Improves the final succinic acid concentration. Reduces end-product inhibition	This process is complicated The regeneration of sorbent needs a large quantity of acid and alkali.	This process needs to be verified in a pilot-scale facility before commercial application

I-7. Conclusion

Succinic acid has been considered as one of the top value added chemicals from renewable feedstocks thanks to its potential to become a building block for various chemicals. However, even if biological production of succinate kept on improving, it is not competitive yet compared to petrochemical-derived succinate. The major drawbacks including, low product concentration in the fermentation broth, the accumulation of side-products like lactic and acetic acids, and the cost product recovery during the downstream process should be alleviated to increase the competitiveness of bio-succinate production. Thus, extensive studies have been focused on

succinate concentration and yield improvement through metabolic engineering of bacterial strains and utilization of low-cost renewable raw materials.

With a maximal succinic acid concentrations of 130 - 150 g.L⁻¹ and yields of 1.4 – 1.7 mole succinate per mole glucose and productivity of 1.6 mmol. (g cdw)⁻¹.h⁻¹, *C. glutamicum* is presented as one of the promising succinic acid producer (Okino et al., 2008a; Litsanov et al., 2012b). *C. glutamicum* main succinate biosynthesis pathway involved anaplerotic pathways (PEPC and PYC) and the reductive branch of the TCA cycle which is active under anaerobic conditions. Such reductive production requires the availability of carbon dioxide and reduced equivalents (NADH, NADPH). The major advantages of this strain were its ability, to metabolize substrate under growth arrested processes, to grow in synthetic medium which could reduce purification costs, to simultaneously catabolize various carbon sources including glucose, fructose, sucrose, xylose etc. Up to date, the production process is dual a phase processes with aerobic growth phase and anaerobic succinic acid production phase. Cells were generally harvested after aerobic growth and packed at high density in the anaerobic compartment. However several studies using facultative aerobic bacteria reported a primordial role of a transition phase on the induction of genes involved in succinate biosynthesis pathways, before starting the anaerobic phase (Vemuri et al., 2002b; Martínez et al., 2010; Zhu et al., 2011). Therefore, to improve the performances of succinic acid production process with *C. glutamicum*, future works should probably focused on:

- Characterization of the transition phase from aerobic to anaerobic condition,
- The availability of reduced equivalent and the maintenance of redox balance that governs the succinate synthesis reactions in the reductive pathway.

Finally, attention should also be given to relevant factors (pH, CO₂ concentration, management of O₂ concentration during aerobic and transition phase etc.) for process design and downstream processing for succinic acid recovery from mixed acids fermentation broth.

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Chapitre II

Matériel & Méthodes

II. Matériel et méthodes

II-1. Matériel

L'ensemble des réactifs utilisés et leur fournisseur sont donnés dans les tableaux suivants.

Table II-1. Ensemble des réactifs pour milieux de culture ainsi que leurs fournisseurs

BHI-Broth (Bouillon-cœur-cervelle)	Sigma Aldrich	Milwaukee, USA
LB	Sigma Aldrich	Milwaukee, USA
D(+) Glucose monohydraté	Sigma Aldrich	Milwaukee, USA
Saccharose	Fluka	Buchs, Suisse
Agar	BD	Le Pont de Claix, France
$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	Carlo Erba	Val de Reuil, France
KH_2PO_4	Carlo Erba	Val de Reuil, France
NaCl	Fluka	Buchs, Suisse
$(\text{NH}_4)_2\text{PO}_4$	Fluka	Buchs, Suisse
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	Fluka	Buchs, Suisse
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	Fluka	Buchs, Suisse
FeCl_3	Sigma Aldrich	Milwaukee, USA
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	Fluka	Buchs, Suisse
$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$	Fluka	Buchs, Suisse
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	Merck	Darmstadt, Allemagne
$(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$	Merck	Darmstadt, Allemagne
$\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$	Sigma Aldrich	Saint Louis, USA
CaCl_2	Fluka	Buchs, Suisse

D-biotine	Sigma Aldrich	Milwaukee, USA
Thiamine	Sigma Aldrich	Milwaukee, USA
Deferoxamine	Novartis	Stein, Suisse
Bétaïne	Sigma Aldrich	Saint Louis, USA
Urée	Prolabo	Paris, France
NaHCO ₃	Sigma Aldrich	Milwaukee, USA
Chloramphenicol	Fluka	Buchs, Suisse
Kanamycine	Sigma Aldrich	Saint Louis, USA

Table II-2. Réactifs pour analyses et dosages

Acide sulfurique	Carlo Erba	Val de Reuil, France
Acide acétique glacial	Sigma Aldrich	Saint Louis, USA
Acide lactique	Sigma Aldrich	Saint Louis, USA
Acide succinique	Sigma Aldrich	Saint Louis, USA
Acide fumarique	Sigma Aldrich	Saint Louis, USA
Acide pyruvique	Fluka	Buchs, Suisse

Table II-3. Autres réactifs

HCl	Carlo Erba	Val de Reuil, France
KOH	Carlo Erba	Val de Reuil, France
NaOH	Carlo Erba	Val de Reuil, France
Glycérol	Sigma Aldrich	Milwaukee, USA
PPG (Polypropylène glycol, 2000')	Sigma Aldrich	Milwaukee, USA

Réactifs de Gram	Sigma Aldrich	Milwaukee, USA
Agarose	Sigma Aldrich	Milwaukee, USA
Sybr safe	Invitrogen	New York, USA

Table II-4. Outil analytique : Chromatographie liquide haute performance

Chaîne LC20 avec injecteur automatique, détecteur à barrettes de diodes et réfractomètre différentiel	Shimadzu	Noisiel, France
Logiciel d'acquisition et traitement LC solution	Shimadzu	Noisiel, France
Colonne Aminex HPX-87H	Biorad	Marnes la Coquette, France
Analyseur multiparamétrique (Vitalab Selectra E)	Vital Scientific	Pays Bas

Table II-5. Matériel de microbiologie

Autoclave (100 L)	Science Médecine Industrie	Montpellier, France
Incubateur (table agitée)	Infors AG	Bottmingen, Suisse
PSM	ESI FluFrance	Val de Reuil, France
Fioles d'Erlenmeyer 250 -1000 mL	Thermo Fisher	Saint Herblain, France
Fioles en verre avec patch à oxygène dissous	System C	Saint Paul Trois Chateaux, France

Table II-6. Cultures en bioréacteurs de 2 - 5 L

5 L en verre et platine	Applikon	Schiedam, Pays-Bas
Manteau chauffant	Applikon	Schiedam, Pays-Bas
2 L en verre	Tryton-Pierre Guerrin	Niort, France
Automate de pilotage	Global Process Concept	Perigny, France
Electrode pH/rH (polarographique)	ISM Metler Toledo	Greifensee, Suisse
Sonde pO ₂ ISM (polarographique)	Metler Toledo	Greifensee, Suisse
Sonde pCO ₂ ISM (polarographique)	Metler Toledo	Greifensee, Suisse
Cryostat Lauda-régulation T°C	Global Process Concept	Perigny, France
Cryostat-système condensation	de LKB	Paris, France
Pompe péristaltique	Masterflex	Chicago, USA
Logiciel de supervision et pilotage des procédés	Global Process Concept	Perigny, France

Table II-7. Matériels divers

Filtres acétate de cellulose 0,22 µm	Millipore	Molsheim, France
Boîtes de Pétri polystyrène 90 mm	Sigma Aldrich	Milwaukee, USA
Microscope optique	Olympus	Paris, France
pH mètre (polarographique)	Aventis Pharma	Mumbai, Inde
Balance de précision AT200	Mettler	Greifensee, Suisse
Pipettes automatiques	Biohit, Sartorius	Dourdan, France
Vortex Top Mix	Bioblock Scientific	Strasbourg, France
Agitateur orbital Polymax 1040	Heidolph	Schwabach, Allemagne
Spectrophotomètre Hitachi 2000	Sciencetec	Les Ulis, France
Centrifugeuse (eppendorf)	Thermo Scientific	Waltham, USA
Centrifugeuse (Tube 250 mL)	Thermo Scientific	Waltham, USA
Electroporateur	Bio-Rad	Marnes-la-coquette, France
Shake Flask Reader	Precision Sensing GmbH	Regensburg, Germany

II-2. Les souches bactériennes utilisées et leurs milieux de culture

II-2.1. *Corynebacterium glutamicum* 2262

C. glutamicum 2262 est une souche industrielle qui a été utilisée pendant longtemps au sein du laboratoire pour étudier la production du glutamate. Durant cette thèse, la souche sauvage de *C. glutamicum* 2262 a été étalée sur boîtes de Pétri contenant du milieu riche comme le BHI-agar pour favoriser la prolifération bactérienne. A partir de ces étalements, des colonies ont été isolées sur de nouvelles boîtes de Pétri contenant du BHI-agar. A partir d'un clone isolé, une série de cultures en milieux liquides BHI (5 mL, 25 mL et 50 mL) a été réalisée jusqu'à obtenir une densité optique à 570 nm d'environ 20. A partir de cette dernière culture, une culture overnight est effectuée dans du MCGC (Minimum *Corynebacterium glutamicum* Citrate)

modifié. 3 mL de cette préculture ont servi d'inoculum pour ensemer 50 mL de MCGC modifié contenus dans une fiole chicanée de 500 mL de volume total et la culture est mise en incubation jusqu'à l'obtention d'une DO_{570nm} comprise entre 20 et 30. Dès que cette densité optique est atteinte, la croissance bactérienne est bloquée en plongeant la fiole dans de l'éthanol 70 % (vol/vol) à -20 °C jusqu'à l'observation d'une congélation sur les parois de la fiole puis elle est transférée dans de la glace. A ces cellules dont la croissance est bloquée, du glycérol stérile préalablement refroidi est rajouté à raison de 20 – 25 % en volume comme cryoprotecteur. Des aliquots de 1 mL de culture sont faits dans des microtubes de 2 mL et conservés à -80°C. Ces aliquots constituent le stock de travail.

Le milieu de culture de base utilisé dans ce travail est le milieu MCGC modifié dont la composition pour une culture en fiole ou en bioréacteur est indiquée dans la Table II-8. Par exemple pour une culture en fiole de 50 mL de MCGC, 40 mL de sels de base, 10 mL de supplément et 0,65 mL d'urée dans une fiole, sont mélangés après stérilisation.

II-2.2. *Escherichia coli* DH5 α et DH5 α mcr

La souche *E. coli* DH5 α mcr est utilisée dans ce travail pour la méthylation et la propagation de plasmides qui serviront à transformer *C. glutamicum* 2262 dans les expériences de construction de mutant déficient pour la lactate déshydrogénase. La souche DH5 α est utilisée uniquement pour la propagation plasmidique. Ces souches sont étalées sur du milieu LB-agar standard complété avec 40 $\mu\text{g}\cdot\text{mL}^{-1}$ de kanamycine et 35 $\mu\text{g}\cdot\text{mL}^{-1}$ de chloramphénicol (en fonction des besoins) pour cribler les cellules présentant les phénotypes conférés par les plasmides utilisés. Dans les expériences visant la prolifération des cellules afin d'augmenter la quantité de plasmides, les clones de la souche *E. coli* sélectionnés sur boîtes de Pétri sont ensemerés en milieu liquide LB contenant les mêmes quantités en antibiotiques. Les milieux LB sont stérilisés à l'autoclave à 121°C pendant 20 minutes, puis les antibiotiques sont rajoutés après refroidissement pour obtenir les concentrations finales souhaitées.

Table II-8. Composition du milieu MCGC modifié en remplaçant le citrate par la déféroxamine.

Composition	Culture en fioles	Volume Utilisé (mL)	Culture en Bioréacteur 2 L	Volume Utilisé (mL)
Sels de base	<u>Pour 1000 mL</u>	40	<u>Pour 1240 mL</u>	1240
	45,9 g Na ₂ HPO ₄ ·12H ₂ O 3,6 g KH ₂ PO ₄ 1,2 g NaCl 4,8 g (NH ₄) ₂ SO ₄ 900 mL H ₂ O pH ajusté à 7,6 H ₂ O qsp 1000 mL Autoclavé à 121°C, 20 min		11,35 g Na ₂ HPO ₄ ·12H ₂ O 9 g KH ₂ PO ₄ 3 g NaCl 12 g (NH ₄) ₂ SO ₄ 900 mL H ₂ O pH ajusté à 7,6 H ₂ O qsp 1240 mL Autoclavé à 121°C, 20 min	
Supplément	<u>Pour 175,5 mL</u>	10	<u>Pour 236,5 mL</u>	236,5
	100 mL de glucose à 300 g.L ⁻¹ 10 mL de sels A : 40 g MgSO ₄ ·7H ₂ O 4 g FeSO ₄ ·7H ₂ O 0,4 g FeCl ₃ H ₂ O qsp 1000 mL Autoclavé à 121°C, 20 min 1 mL de sels B: 1 g ZnSO ₄ ·7H ₂ O 0,4 g CuCl ₂ ·2H ₂ O 4 g MnSO ₄ ·H ₂ O 0,2 g (NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O		200 mL de glucose à 300 g.L ⁻¹ 15 mL de sels A : 40 g MgSO ₄ ·7H ₂ O 4 g FeSO ₄ ·7H ₂ O 0,4 g FeCl ₃ H ₂ O qsp 1000 mL Autoclavé à 121°C, 20 min 1 mL de sels B: 1 g ZnSO ₄ ·7H ₂ O 0,4 g CuCl ₂ ·2H ₂ O 4 g MnSO ₄ ·H ₂ O 0,2 g (NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O	

	0,4 g Na ₂ B ₄ O ₇ ·10H ₂ O H ₂ O qsp 1000 mL Autoclavé à 121°C, 20 min Vitamines : 0,5 mL CaCl ₂ 1,5 M 2 mL Biotine 1 g.L ⁻¹ 2 mL Thiamine 10 g.L ⁻¹ 30 µL Déféroxamine 100 g.L ⁻¹ 1,7 g Bétaïne 60 mL H ₂ O Stérilisées par filtration (0,22 µm)		0,4 g Na ₂ B ₄ O ₇ ·10H ₂ O H ₂ O qsp 1000 mL Autoclavé à 121°C, 20 min Vitamines : 0,75 mL CaCl ₂ 1,5 M 3 mL Biotine 1 g.L ⁻¹ 3 mL Thiamine 10 g.L ⁻¹ 45 µL Déféroxamine 100 g.L ⁻¹ 3 g Bétaïne 13,2 mL H ₂ O Stérilisées par filtration (0,22 µm)	
Urée	Solution à 300 g.L⁻¹	0,65		-

NB : En fonction des objectifs de l'étude, les concentrations en glucose pourront être modifiées. Dans ces cas de figures, ces concentrations spécifiques seront précisées dans la partie de l'étude en question. Par ailleurs, certaines études vont également nécessiter l'ajout supplémentaire de bicarbonate de sodium dont la quantité ajoutée sera mentionnée en temps opportun.

II-3. Les plasmides utilisés

II-3.1. pK19mobsacBAldhA

Pour l'obtention de la souche de *C. glutamicum* 2262 déficient pour le gène de la lactate déshydrogénase, nous avons procédé à une délétion totale du gène par génie génétique en utilisant le plasmide pK19mobsacBAldhA (Litsanov *et al.*, 2012b). Ce plasmide est un dérivé des plasmides suicides pK18mobsacB et pK19mobsacB décrits par Schafer *et al.* (Schäfer *et al.*, 1994). Le plasmide pK19mobsacBAldhA possède un site multiple de clonage au milieu du gène *lacZα*, une cassette de résistance à la kanamycine, le gène *SacB* codant la lévane sucrase de *Bacillus subtilis*, et une origine de répllication pour *E. coli*. Pour la délétion du gène de la lactate déshydrogénase, Litsanov *et al.* (2012b), ont cloné au niveau du site multiple de clonage, des fragments d'ADN qui sont homologues aux régions amont et aval flanquant le gène de la lactate déshydrogénase sur le génome de *C. glutamicum*. Ce plasmide pK19mobsacBAldhA est ainsi

utilisé pour transformer des cellules compétentes de *C. glutamicum* 2262. Après étalement sur milieu LB-agar contenant 40 $\mu\text{g.mL}^{-1}$ de kanamycine, seules les bactéries ayant intégré le plasmide dans leur génome par recombinaison homologue sont capables de pousser en présence de kanamycine. En effet, le plasmide ne possède pas d'origine de répllication pour *C. glutamicum*, il ne peut donc pas se répliquer sans s'intégrer au génome de *C. glutamicum*. Ces transformants sont à nouveau étalés sur boîte de Pétri contenant du LB-agar et 10% de saccharose. Le saccharose est transformé en levane par l'activité levane sucrase du gène *SacB* porté par le plasmide pK19mobsacB*AldhA* (Figure II-1). Les lévanes sont des exopolysaccharides intervenant généralement dans l'assemblage de la paroi bactérienne. Ces lévanes sont cités pour être toxiques chez *C. glutamicum* par perturbation des processus de transport à travers la membrane (Jäger *et al.*, 1992). La pression de sélection exercée par le saccharose, pousse les bactéries à un second évènement de recombinaison homologue pour exciser le plasmide précédemment intégré. Seules les bactéries ayant perdu le plasmide au cours de cette recombinaison sont capables de pousser sur LB-agar contenant 10% de saccharose. Vu que le plasmide contient des fragments homologues aux extrémités amont et aval de *ldhA* dans le génome, au moment de l'excision de ce dernier, dans 45 % des cas le plasmide est rejeté seul et un phénotype sauvage est retrouvé et dans le reste des cas, le plasmide est excisé avec le gène ciblé, le gène de la lactate déshydrogénase (Schäfer *et al.*, 1994); c'est ainsi que s'opère la construction du mutant.

II-3.2. pAN1

pAN1 est un plasmide utilisé pour la méthylation *in vivo* des plasmides de type ColE1 pour éviter leur dégradation par le système de restriction méthylcytosine de la souche transformée. Ce plasmide pAN1 possède une origine de répllication *Ori* et une cassette de résistance au chloramphénicol et le gène de la méthyltransférase du phage de *B. subtilis*($\Phi 3tI$) (Figure II-2) (Mermelstein and Papoutsakis, 1993). Le système de restriction de *C. glutamicum* est souvent une barrière majeure à son électrotransformation. Face à de très faibles taux d'efficacité d'électrotransformation lors d'expériences préliminaires, nous avons opté pour la méthylation du plasmide d'intérêt pK19mobsacB*AldhA*. Cette méthylation est effectuée en co-transformant *E. coli* DH5 α mcr avec le plasmide pAN1 et pK19mobsacB*AldhA* par électroporation. Les deux plasmides possédant respectivement une cassette de résistance au chloramphénicol et à la kanamycine, les transformants sont sélectionnés sur milieu LB-agar contenant 35 et 40 $\mu\text{g.mL}^{-1}$ d'antibiotiques.

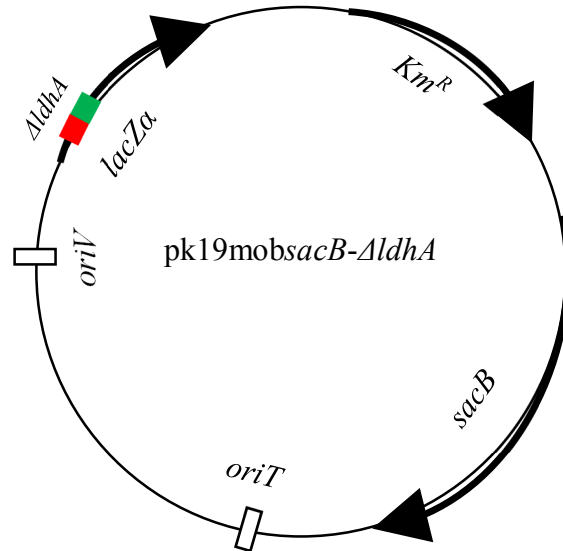


Figure II-1 Cartographie du plasmide *pk19mobsacB-ΔldhA*. *ΔldhA*: Construction correspondant aux bouts flanquants, homologues aux extrémités amont et aval du gène *ldhA* à déléter; *lacZα*: Complément du gène de la β -galactosidase *lacZ*; *Km^R*: Cassette de résistance à la kanamycine; *sacB*: Gène codant la lévansucrase de *B. subtilis*; *oriT/oriV*: Origines de réplication pour *E. coli*. Les dimensions des flèches sont juste schématiques.

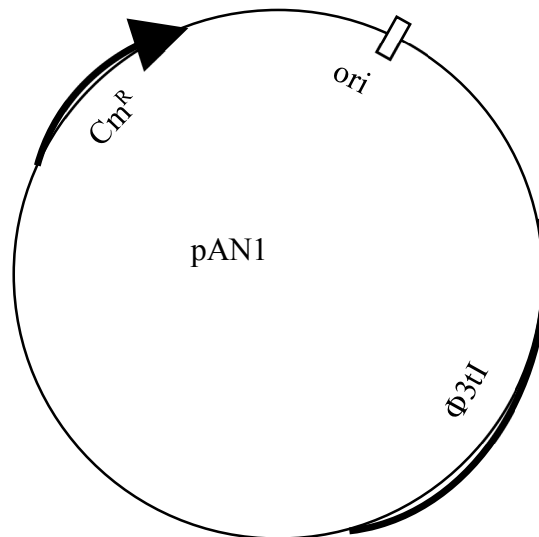


Figure II-2. Cartographie du plasmide *pAN1* utilisé pour la méthylation *in vivo* des plasmides *ColE1*. $\Phi 3tI$: Gène de la méthyltransférase du phage de *B. subtilis*; *ori*: Origine de réplication; *CmR*: Cassette de résistance au chloramphénicol. Les dimensions des flèches sont juste schématiques.

II-4. Conditions et modes opératoires des différentes cultures

II-4.1. Culture en boîtes de Pétri

Les cultures de *C. glutamicum* 2262 ensemencées sur boîtes de Pétri sont incubées dans une étuve humidifiée et maintenue à 33°C jusqu'à l'obtention des clones ou à l'arrêt de l'expérience. Pour les souches de *E. coli*, l'incubation se fait entre 33-37°C.

II-4.2. Pré-cultures

L'ensemble des précultures dans ce projet est préparé à partir d'un aliquot glycérolé, comme décrit plus haut. Toutes les cultures de *C. glutamicum* 2262 et 2262 *AldhA* sont effectuées dans le milieu MCGC modifié. A partir d'un stock glycérolé de 1 mL à -80°C, 50 mL de MCGC contenant environ 33 g.L⁻¹ de glucose et contenu dans une fiole chicanée stérile de 500 mL sont ensemencés. Cette culture est incubée sous agitation à 200 rpm et maintenue à 33°C pendant une nuit, et constitue une préculture A. 5 mL de cette préculture, servent d'inoculum pour une préculture B préparée comme la précédente et incubée dans les mêmes conditions jusqu'à l'obtention d'une densité optique à 570 nm d'environ 30. La croissance de la préculture B est alors stoppée par immersion du fond de la fiole dans de l'éthanol à 70% (vol/vol) à -20°C puis conservée dans de la glace pendant une dizaine de minutes avant d'être stockée à 4°C pour une durée d'environ 24 h.

II-4.3. Cultures en fioles d'Erlenmeyer

II-4.3.1. Conditions opératoires

Dans ce travail, un chapitre est consacré à l'étude de l'influence du coefficient de transfert en oxygène (k_{La}) dissous sur la production d'acides organiques par *C. glutamicum* 2262 *AldhA*. Dans cette partie, l'ensemble des expériences est effectué en fioles en verre non-chicanées. Pour ces expériences, le milieu MCGC est préparé en mélangeant les différents constituants dans les proportions adéquates avant de les distribuer dans les différentes fioles comme indiqué dans la Table II-9.

Table II-9 . Les différentes conditions de culture en fioles

Fréquence d'agitation (rpm)	Volume total de la fiole V_T (mL)	Volume du milieu V_L (mL)	Diamètre de la fiole (cm)	d
0 (statique)	250	100	8	
100	250	170	8	
170	500	250	8	
170	250	75	8	
350	500	200	10	
350	250	100	8	
350	1000	200	12	
350	250	40	8	
350	500	40	10	
350	1000	40	12	

Ces différentes cultures en fioles non-chicanées sontensemencées à partir de plusieurs fioles de préculture B. Un volume v de préculture B a été utilisé pour chaque culture afin d'obtenir une densité optique initiale à 570 nm comprise entre 1,5 – 2. Afin d'étudier l'influence du k_{LA} O_2 sur la réponse physiologique de *C. glutamicum*, le volume des fioles, le volume de remplissage des fioles, la fréquence d'agitation de l'incubateur ont été choisis dans le but d'imposer des k_{LA} allant de 0 à 170 h^{-1} . Pour ce faire, les fioles remplies selon la Table II-9, sont soumises à des fréquences d'agitation allant de 0 à 350 rpm sur une table d'agitation de 2,5 cm d'orbitale et la température d'incubation est maintenue à 33°C.

II-4.3.2. Détermination des coefficients de transfert d'oxygène (k_{LA})

Les différentes valeurs de k_{LA} ont été déterminées à partir de données expérimentales obtenues en appliquant les conditions de cultures de la Table II-9. Pour ce faire, des fioles en verre non-

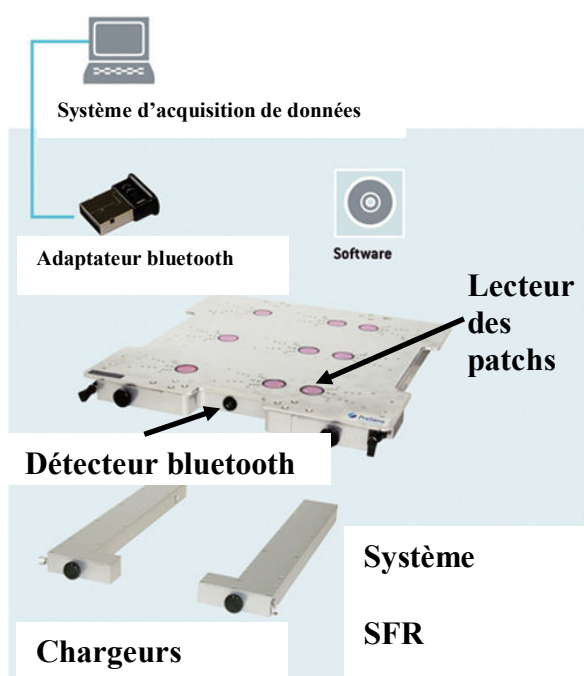
chicanées de 250 à 1000 mL, munies de patchs optiques pour la mesure de la concentration en oxygène dissous, sont utilisées. Le système PreSens utilisé pour la détermination des k_{La} est constitué (Figure II-3) :

- De fioles en plastiques ou en verre munies de patchs qui sont des colorants spécifiques pour l'oxygène dissous et pour le pH, basés sur un système de luminescence. Dans le cas des fioles en verres, seul le patch O_2 , est fixé. Leur temps de réponse est inférieur ou égal à 10 secondes correspondant au temps d'acquisition des données. Ces sondes sont précalibrées et les données de calibration sont fournies. Ces données sont enregistrées dans le logiciel qui permet la reconnaissance entre le plateau de lecture de patchs et l'ordinateur de contrôle, par bluetooth.
- D'un système SFR, qui est un système muni d'un ensemble de lecteurs qui permet la détection des différents patchs. Une fois le système bluetooth du SFR activé, il est reconnu par l'ordinateur de contrôle. Le lecteur émet à l'emplacement de chaque patch, une lumière excitatrice des molécules du colorant des patchs. La durée de vie de la luminescence générée est détectée de manière non invasive à travers les fioles transparentes. La durée de vie de la luminescence du colorant du capteur dépend de la pression partielle d'oxygène de l'échantillon. La luminescence émise est convertie en concentration d'oxygène dissous par le logiciel en utilisant les données d'étalonnage fournies, ce qui permet de pouvoir suivre au cours du temps l'évolution de la concentration en oxygène dissous.

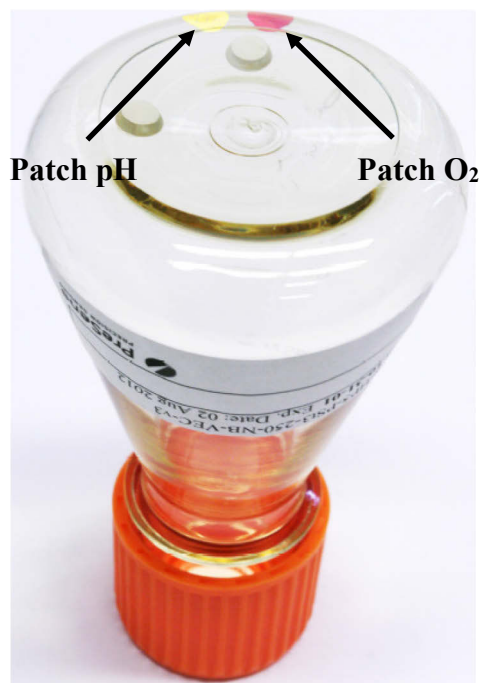
Dans ces travaux, le lecteur SFR est fixé sur une table d'agitation à orbitale de 2,5 cm. Au début des expériences, de l'azote est injecté dans les fioles remplies de milieu de culture avant que celles-ci ne soient placées sur le lecteur. La fréquence d'agitation est fixée à la valeur de travail et la température à 33°C. Les fioles sont laissées en agitation pendant un temps suffisamment long pour que les valeurs d'oxygène dissous affichées soient stables ; il a été ainsi considéré que la saturation du milieu en O_2 dissous était obtenue. Les patchs sont alors étalonnés. Enfin, de l'azote est de nouveau injecté dans le milieu de culture avant de replacer les fioles sur le plateau d'agitation et de commencer les mesures d' O_2 dissous. Les différentes conditions décrites dans la table Table II-9 sont imposées et l'évolution de la concentration en O_2 dissous est mesurée jusqu'à au moins 90 % de la saturation du milieu, au cours du temps. La valeur de k_{La} associée à chaque condition de culture est déterminée en utilisant la méthode statique selon l'équation ci-dessous :

$$\ln \left(\frac{[O_2]^*}{[O_2]^* - [O_2]} \right) = k_L a \cdot t \quad (\text{II-1})$$

Où $[O_2]^*$ correspond à la concentration d'O₂ dissous à la saturation, $[O_2]$ la concentration en O₂ dissous mesurée à chaque instant et t correspond au temps de mesure.



(A)



(B)

Figure II-3. Système PreSens. (A). système de détection et de lecture des données mesurées par les patches ; (B). Fioles en plastiques munies de 2 patches (les fioles en verres utilisées sont munies uniquement de patch O₂). (C). Plateau Presens fixé sur une table d'agitation.



(C)

II-5. Cultures en bioréacteurs

II-5.1. Cas des cultures pour l'élaboration d'un procédé de production de succinate

II-5.1.1. Montage du bioréacteur

Les cultures de *C. glutamicum* 2262 ou 2262 *AldhA* sont réalisées pendant 48 à 72 h dans des cuves en verre de 2 L (volume utile de 1,5 L). Ces cuves munies d'une double enveloppe sont couvertes d'une platine équipée :

- d'un axe d'agitation sur lequel sont fixés deux mobiles d'agitation de type Rushton comprenant 4 pales, dont l'agitation est fixée à 900 rpm pendant toute l'expérience,
- 2 contre-pales statiques permettant de limiter l'effet vortex lors des fortes agitations et d'améliorer les phénomènes de transfert d'oxygène,
- d'un condenseur permettant la condensation des vapeurs formées au cours de la culture. Celui-ci est enveloppé d'une double membrane où circule de l'eau à 10°C,
- d'une entrée d'air (de gaz) munie d'un diffuseur de gaz à 10 orifices. L'air comprimé est ajouté en continu pour maintenir la consigne d'O₂ dissous imposée au cours de la culture et dès l'instant où, à agitation fixe, l'augmentation du débit d'air n'est plus suffisante pour maintenir la consigne d'O₂ dissous demandée, le débit d'air est fixé à 60 L.h⁻¹ et le système de régulation est basculé sur l'enrichement de l'air par injection d'oxygène pur,
- d'une cane plongeante pour l'échantillonnage,
- d'une canule à remplir avec de l'eau pour la fixation de la sonde de température,
- d'emplacements pour les sondes pH/rH, pO₂, pCO₂ et un septum pour l'ajout d'antimousse ou d'autres suppléments injectables,
- d'entrée pour l'inoculum ou autre alimentation d'autres liquides,
- de 3 entrées supplémentaires dont deux utilisées pour la régulation acide/base.

Toutes les entrées utilisées pour une alimentation en gaz sont équipées de filtres PTFE de 0,22 µm. La température est régulée à 33°C à l'aide de la double enveloppe par une circulation d'eau froide ou chaude par un cryostat Lauda. Le pH est maintenu à 7,6 par ajout automatique de solutions de KOH 10 N ou HCl 2 N via des pompes péristaltiques reliées à un contrôleur de type on/off. Toutes les sondes, les moteurs, les débitmètres, les pompes ainsi que les cryostats

de régulation de température sont reliés à un automate de contrôle connecté à un ordinateur de pilotage utilisant le logiciel C-Bio développé par Global Process Concept (GPC). Le dispositif expérimental permet de gérer les consignes opératoires et de faire l'acquisition des données.

Avant chaque expérience, les différents éléments des bioréacteurs sont démontés, nettoyés et réinstallés de nouveau. L'électrolyte contenu dans la cellule de mesures des sonde O₂ et CO₂ dissous est changé et la réponse des sondes est testée. La sonde pH est calibrée à partir de solutions de références à pH 4, 7,02 et 9. Les différentes sondes (pO₂, pCO₂, pH) sont des sondes polarographiques. Une fois que le réacteur est prêt, il est rempli avec les sels de base (voir Table II-8) et autoclavé à 121°C pendant 20 minutes. Si la sonde CO₂ est utilisée, du fait de sa sensibilité aux fortes températures, l'ensemble est autoclavé à 105-110°C pendant 20 minutes. Les autres composants du milieu sont stérilisés séparément et rajoutés avant l'ensemencement. Une fois que le milieu est complet, que les différentes connections (sondes, solutions acide-base, circulation d'eau thermostatée) sont faites, les conditions de culture sont appliquées après la polarisation de la sonde à oxygène dissous. De l'air est alors injecté dans le milieu et maintenu jusqu'à l'obtention d'un signal constant de la sonde pO₂. A partir de cet instant l'étalonnage de cette sonde est effectué et les expériences, sont débutées.

II-5.1.2. Culture en phase aérobie pour l'accumulation de la biomasse microbienne

Le bioréacteur ainsi préparé estensemencé avec un volume donné de la préculture B afin d'avoir une densité optique initiale à 570 nm d'environ 1,5. Le volume final de la culture est de 1,5 L avec une température fixée à 33°C, une agitation fixée à 900 rpm et le pH à 7,6. Pendant la culture en phase aérobie, l'O₂ dissous est régulé à 50 % de la saturation. Dans le cas des expériences réalisées en bioréacteurs pour étudier la croissance et la production de CO₂, cette régulation est maintenue à 50 % jusqu'à la fin de l'expérience. Par contre, pour les expériences en bioréacteur visant la compréhension et la mise en œuvre d'un procédé de production d'acide succinique, l'O₂ dissous est régulé à 50 % de la saturation jusqu'à une DO_{570nm} de 30. Dès que cette DO est atteinte, plusieurs stratégies sont étudiées pour passer de la phase aérobie à la phase anaérobie pendant laquelle sont produits les acides organiques.

II-5.1.3. Stratégies de transition de l'aérobiose à l'anaérobiose

Deux sortes de stratégies de transitions ont été étudiées : Une transition basée sur la gestion de l'oxygène dissous en imposant des niveaux d'abaissement jusqu'à l'anaérobiose totale et un deuxième type de stratégies basé sur la gestion du débit d'aération en l'abaissant de palier en palier jusqu'à l'arrêt total de l'aération. La très grande différence entre ces deux stratégies est que dans le deuxième cas, le niveau de l'oxygène dissous, la création d'une phase de micro-aérobiose et d'anaérobiose sont induites par la prolifération bactérienne tandis que dans le premier cas, ils sont contrôlés et imposés. Pour la première catégorie de transitions, quatre transitions ont été retenues : i) dès que la DO_{570nm} de 30 est atteinte pendant la phase aérobie, la consigne d' O_2 dissous est passée brusquement à 0 % en stoppant l'aération, ii) La concentration en O_2 dissous est régulée à la saturation dès la phase aérobie et dès que la DO_{570nm} visée est obtenue, la consigne de régulation est abaissée à 50 % de la saturation et y est maintenue pendant 1 h, puis elle est abaissée à 20 % de la saturation. Après une 1 h de régulation à 20 % elle est réduite à 10 % et maintenue pendant 2 h avant de stopper totalement l'aération pour basculer en conditions anoxiques, iii) La concentration en O_2 dissous est maintenue à 20 % de la saturation pendant la phase aérobie et dès que la DO_{570nm} de 30 est obtenue, la consigne de régulation est abaissée à 10 % de la saturation. Après 4 h de régulation à 10 % de la saturation, les conditions anaérobies sont imposées en coupant l'aération, iv) dans cette transition, la concentration en O_2 dissous qui est régulée à 50 % de la saturation pendant la phase aérobie jusqu'à DO_{570nm} de 30 est abaissée à 20 % de la saturation et régulée autour de cette consigne pendant 2 h, elle est ensuite abaissée et maintenue à 10 % pendant 2 h et finalement l'aération est stoppée pour passer en anaérobiose. Pour la deuxième catégorie de stratégies de transitions par gestion du débit d'aération, trois exemples ont été retenus. Comme dans le cas précédent la concentration en O_2 dissous est régulée à 50 % de la saturation pendant la phase aérobie jusqu'à une DO_{570nm} de 30 puis les transitions sont appliquées comme suit : v) lorsque le système de régulation n'arrivait plus à réguler la concentration en O_2 dissous à 50 % de la saturation par simple injection d'air pendant la phase aérobie, le débit d'air est fixé à 100 $L.h^{-1}$ sans ajouter de l'oxygène pur et dès que la DO_{570nm} de 30 est atteinte, la transition est débutée en abaissant le débit d'aération à 60 $L.h^{-1}$ et en le maintenant à ce débit pendant 2 h puis il est abaissé à 25 $L.h^{-1}$ et après 2 h d'aération à 25 $L.h^{-1}$, l'aération est entièrement coupée, vi) lorsque le système de régulation de la concentration en O_2 dissous à 50 % par simple injection d'air devient difficile pendant la phase aérobie, le débit d'air est fixé à 60 $L.h^{-1}$ sans

ajouter d'oxygène pur et dès que la DO_{570nm} de 30 est atteinte, la transition est débutée en maintenant le débit d'aération à 60 L.h^{-1} . Après 4 de transition à 60 L.h^{-1} , l'aération est coupée pour passer en conditions anaérobies, vii) dans ce cas la concentration en O_2 dissous n'est pas régulée, du début à la fin. Pendant la phase aérobie, l'aération est fixée à 110 L.h^{-1} jusqu'à atteindre une DO_{570nm} de 30, puis la transition est débutée par une réduction de cette aération à 65 L.h^{-1} pendant 2 h puis à 10 L.h^{-1} pendant 2 h, avant l'arrêt total de l'aération.

II-5.1.4. Culture en phase anaérobie pour la production d'acide succinique

L'ensemble des différentes étapes du procédé est effectué dans le même bioréacteur sans étapes de récupération de biomasse ni de lavage. A la fin des différentes phases de transition, l'aération est totalement coupée et la culture est basculée en environnement dépourvu d'oxygène. En condition anaérobie, la croissance bactérienne cesse mais les bactéries gardent la capacité métabolique à produire des acides organiques dont le succinate. En condition anaérobie, l'agitation est maintenue à 900 rpm et la régulation de pH à 7,6.

II-5.2. Etude de l'influence de l'hétérogénéité en O₂ dissous du milieu

II-5.2.1. Montage expérimental

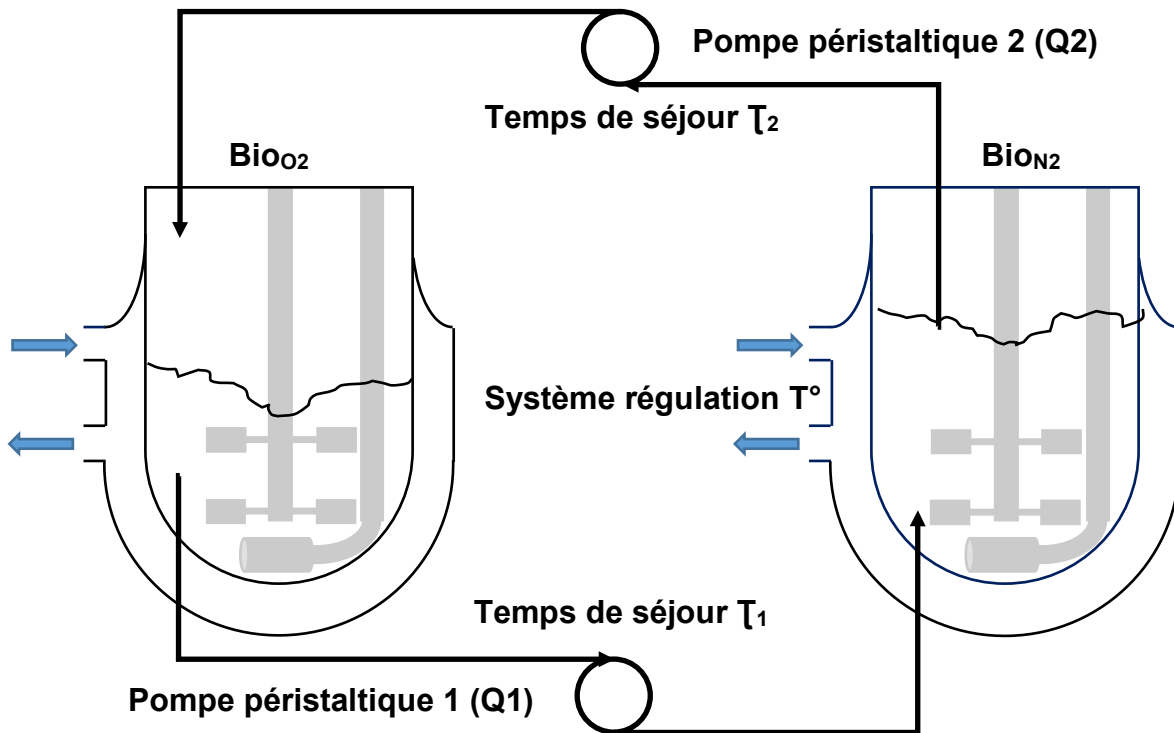


Figure II-4. Scale-down : système à deux compartiments RPA-RPA dont un est maintenu en condition aérobie et l'autre en condition anaérobie. La circulation du milieu entre les deux compartiments est assurée par deux pompes péristaltiques.

L'étude scale-down envisagée dans cette thèse consiste en un système bi-compartimenté avec deux réacteurs parfaitement agités (RPA) interconnectés (Figure II-4). Un des bioréacteurs est maintenu en condition aérobie en régulant l'oxygène dissous à 50 % de la saturation (BioO₂) et le second est maintenu en condition anaérobie par injection continue d'azote, l'objectif étant de mimer l'existence de zones aérobie et anaérobie, de gradients d'O₂ ; environnements typiquement rencontrés par les bactéries dans les grandes installations de production industrielle. Le milieu circule continuellement entre les deux compartiments. Afin d'augmenter ou réduire le temps de séjour des bactéries dans les différents compartiments, nous avons fait varier les volumes de culture ainsi que les débits de recirculation du milieu (Table II-10). Le milieu de culture est recirculé entre les deux bioréacteurs à travers des tubes de faible perméabilité aux gaz (Exacanal, 5 mm de diamètre interne et 2,5 m de long) grâce à deux

pompes péristaltiques (Masterflex, Cole-Parmer, Barrington IL, USA). La sortie du compartiment BioO₂ est placée en fond de cuve tandis que celle du compartiment BioN₂ est placée à la surface du liquide. Dans le bioréacteur (BioO₂), les conditions aérobies sont maintenues en injectant de l'air ou de l'oxygène pur à travers un diffuseur à 10 orifices à débit variable pour maintenir une pO₂ de 50 % de la saturation. Le bioréacteur (BioN₂) est maintenu en anaérobie en injectant de l'azote à travers un sparger de type fritté inox à des débits variant de 0,5 to 1,3 vvm, et équipé de 2 à 3 turbines Rushton à 4 pales. Pour les bioréacteurs de plus de 3 L, des bioréacteurs Applikon équipés de manteau chauffant pour la régulation de température sont utilisés (voir partie Matériel)

Table II-10. Les différentes conditions de temps de séjours dans l'étude scale-down

Ratio d'aérat.	BioO ₂		BioN ₂		Débit (mL/s)	Temps de séjour (s)	
	V ₁ (L)	[Ox. Diss] (% air sat.)	V ₂ (L)	[Ox. Diss] ⁽²⁾ (% air sat)	Q	τ ₁ (s)	τ ₂ (s)
0	1.5	0 ⁽¹⁾	1.5	0	7.3	205	205
0.2	1	50	4	0	7.3	137	548
0.25	1.5	50	4.5	0	7.3	205	616
0.33a	1	50	2	0	7.3	137	274
0.33b	1	50	2	0	1.8	556	1111
0.67a	2	50	1	0	7.3	274	137
0.67b	2	50	1	0	1.8	1111	556
1a ⁽³⁾	1.5	50	1.5	50	7.3	205	205
1b ⁽⁴⁾	1.5	50					

⁽¹⁾Dans ce cas, après l'étalonnage de la sonde pO₂, la culture est amorcée sans ajout ni d'air/oxygène pur ni d'azote tandis que dans le compartiment BioN₂, de l'azote est injecté continuellement. ⁽²⁾L'azote est injecté continuellement pour garantir des conditions anaérobies. ⁽³⁾Correspond à un cas de figure où l'O₂ dissous est régulé à 50% de la saturation dans les deux compartiments. ⁽⁴⁾Les ratios sont calculés suivant cette formule : ratio = Volume BioO₂/(Volume total circulant).

II-5.2.2. Ensemencement du système bi-compartmenté

Pour chaque condition de culture décrite dans la Table II-10, le volume de préculture B est préparé par rapport au volume total circulant afin d'avoir une DO_{570nm} initiale entre 1,5 et 2. L'ensemencement s'effectue dans le bioréacteur B-aérobie, et après un court temps de mélange, les échantillonnages se font dans ce même compartiment tout au long de l'expérience.

II-6. Détermination des paramètres cinétiques d'une culture

Pour quantifier les performances d'un procédé de culture en termes de croissance bactérienne, de consommation de substrats et de formation de produits, les grandeurs cinétiques sont calculées pour chaque expérience. Ainsi, à partir des données expérimentales de la biomasse (X), la vitesse spécifique de croissance maximale est calculée suivant :

$$\mu_{max} = \max_t \left(\frac{1}{[X]_f} \cdot \frac{d[X]_f}{dt} \right) \quad (\text{II-2})$$

Avec $[X]_f$ correspondant aux valeurs de lissage des données expérimentales en utilisant un modèle d'interpolation "smoothing splines".

La vitesse spécifique maximale de consommation du glucose q_{max}^{gluc} et les vitesses spécifiques maximales de production d'acides organiques q_{max}^{AO} sont déterminées respectivement par les formules suivantes :

$$q_{max}^{gluc} = - \max_t \left(\frac{1}{[X]_f} \cdot \frac{d[gluc]_f}{dt} \right) \quad (\text{II-3})$$

$$q_{max}^{gluc} = - \max_t \left(\frac{1}{[X]_f} \cdot \frac{d[gluc]_f}{dt} \right) \quad (\text{II-4})$$

$$q_{max}^{AO} = \max_t \left(\frac{1}{[X]_f} \cdot \frac{d[AO]_f}{dt} \right) \quad (\text{II-5})$$

Avec $[gluc]_f$ et $[AO]_f$, les valeurs lissées des données expérimentales de glucose et acides organiques en utilisant un modèle d'interpolation "smoothing splines".

Enfin, les rendements maximaux de conversion du glucose en biomasse et en acides organiques Y_{max}^{AO} et Y_{max}^X se calculent selon :

$$Y_{max}^{AO} = \left(\frac{AO_{max}^{exp} - AO_{t0}^{exp}}{Gluc_{t0}^{exp} - Gluc_{tAOmax}^{exp}} \right) ; Y_{max}^X = \left(\frac{X_{max}^{exp} - X_{t0}^{exp}}{Gluc_{t0}^{exp} - Gluc_{tXmax}^{exp}} \right) \quad (II-6)$$

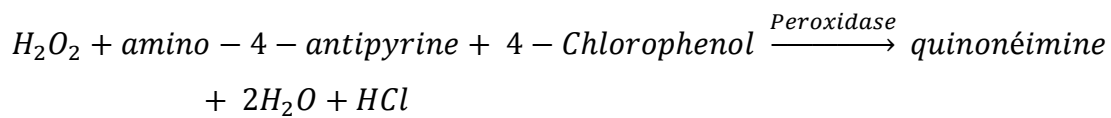
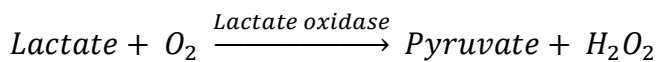
Où AO_{max}^{exp} et X_{max}^{exp} sont les concentrations expérimentales maximales en acides organiques et en biomasse, $Gluc_{t0}^{exp}$ et AO_{t0}^{exp} les concentrations initiales en glucose et en acides organiques et $Gluc_{tAOmax}^{exp}$ et $Gluc_{tXmax}^{exp}$ correspondent aux concentrations de glucose résiduelles lorsque la production d'acides ou de biomasse est maximale. Dans le cas d'une culture en mode fed-batch, $Gluc_{t0}^{exp}$ correspond à la valeur cumulée des concentrations de glucose ajoutées jusqu'à l'obtention de la concentration maximale en produits ou en biomasse.

II-7. Méthodes analytiques

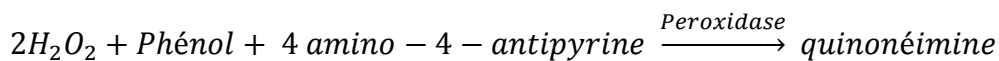
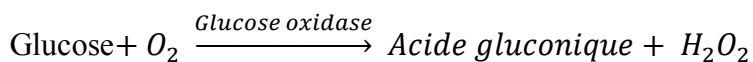
Pour chaque expérience de culture en fiole ou en bioréacteur, des prélèvements de 5 mL (bioréacteur) et 1-2 mL (fiole) d'échantillons sont effectués au cours du temps tout au long de la fermentation. A partir d'un échantillon de 5 mL, 1 mL est utilisé pour la détermination de la biomasse par turbidimétrie en mesurant une densité optique à 570 nm avec un spectrophotomètre Hitachi. Les valeurs de DO mesurées sont inférieures à 0,9 uDO ; au-delà de ce seuil, les échantillons, sont dilués avec de l'eau distillée. A partir de cette densité optique nous estimons la concentration de la biomasse en poids sec en utilisant un facteur de corrélation préalablement établi en faisant des courbes d'étalonnage DO-poids sec. Ainsi la concentration cellulaire (g poids sec.L⁻¹) correspond à la DO_{570nm} multipliée par le facteur de corrélation qui est de 0,27 g poids sec.L⁻¹.(unité DO)⁻¹. Le reste de l'échantillon est centrifugé à 5000 g pendant 10 minutes à 4°C. Après la centrifugation, les échantillons sont filtrés avec des filtres de porosité 0,22 µm et les concentrations résiduelles en glucose et en produits dans le surnageant sont analysées par Chromatographie Liquide Haute Performance (CLHP) ou par analyseur multiparamétrique. La concentration en glucose, lactate, succinate, acétate, pyruvate, fumarate est quantifiée par CLHP (10ADVP, Shimadzu) utilisant une colonne échangeuse d'ions (7.8 mm×300 mm, Bio-Rad Aminex HPX-87H) et une station SPD-M10A muni d'un détecteur à barrette de diodes DAD (Shimadzu) et un refractomètre RI 410 (Waters). L'analyse se fait par injection de 5 ou 10 µL d'échantillons et l'élution est faite en mode isocratique par une phase

mobile en acide sulfurique 5 mM pour l'analyse des sucres et une phase mobile 10 fois plus concentrée pour l'analyse des acides organiques pour bien prendre en compte des phénomènes de co-élutions. Le débit de la phase mobile est fixé à 0,7 mL.min⁻¹. La température de la colonne est maintenue à 50°C et chaque analyse dure 20 minutes. Les temps de rétention pour les différentes molécules dans ces conditions d'analyse sont de 7,7 - 9,2 - 10,2 - 10,9 - 13,1 et 13,5 minutes respectivement pour le glucose, le pyruvate, le succinate, le lactate, l'acétate et le fumarate. Pour faire une analyse croisée, le lactate et le glucose sont souvent dosés enzymatiquement en utilisant un analyseur automatique multiparamétrique utilisant des kits enzymatiques (Elitech-France pour le glucose et Biomerieux-France pour le lactate). Les principes des réactions colorimétriques sont les suivants :

- Pour le lactate,



- Pour le glucose,



L'apparition de la quinonéimine est détectée à 446 nm pour le lactate et 480 nm pour le glucose. L'intensité de coloration de la quinonéimine mesurée est proportionnelle à la quantité de lactate et de glucose dans l'échantillon.

II-8. Techniques de biologie moléculaire

II-8.1. Préparation de cellules compétentes

Le protocole de préparation de cellules compétentes est fourni sur la Figure II-5.

II-8.2. Propagation, méthylation de plasmides et électroporation

Les plasmides utilisés dans ce travail sont le plasmide pK19*mobsacB* Δ *ldhA* pour la création du mutant *ldhA* (-) de *C. glutamicum* 2262 et le plasmide pAN1 utilisé pour méthyler le plasmide pK19*mobsacB* Δ *ldhA* afin de favoriser son transfert et son maintien dans la bactérie receveuse. Afin d'avoir une quantité suffisante de plasmides, pK19*mobsacB* Δ *ldhA* a été propagé dans *E. coli* DH5 α selon le protocole de la Figure II-6.

Après cette étape de propagation, les plasmides sont purifiés par extraction à partir des culots cellulaires en utilisant un kit QIAprep Spin Miniprep (Qiagen, France).

Les plasmides ainsi purifiés sont mis à migrer sur gel d'agarose 1 % préparé dans du tampon tris borate EDTA (TBE) 0,5X. Après la migration, sur les différentes pistes de dépôt, les formes enroulées et relâchées du plasmide ont pu être observées sans aucune autre bande supplémentaire attestant de la pureté du plasmide extrait. La méthylation du plasmide pK19*mobsacB* Δ *ldhA* purifié est réalisée en co-transformant une souche *E. coli* DH5 α mcr avec le plasmide pK19*mobsacB* Δ *ldhA* et le plasmide de méthylation pAN1. Pour cela, la souche *E. coli* DH5 α mcr est transformée par électroporation en suivant le protocole de propagation de plasmide décrit ci-dessus. 2 μ L de chaque plasmide sont utilisés. Les étapes de criblage se font cette fois-ci dans du LB + 40 μ g.mL⁻¹ kanamycine + 35 μ g.mL⁻¹ chloramphénicol. Les plasmides sont purifiés comme précédemment. Le plasmide pK19*mobsacB* Δ *ldhA* ainsi méthylé est utilisé pour l'électrotransformation des cellules compétentes de *C. glutamicum* 2262 pour la construction du mutant *AldhA*.

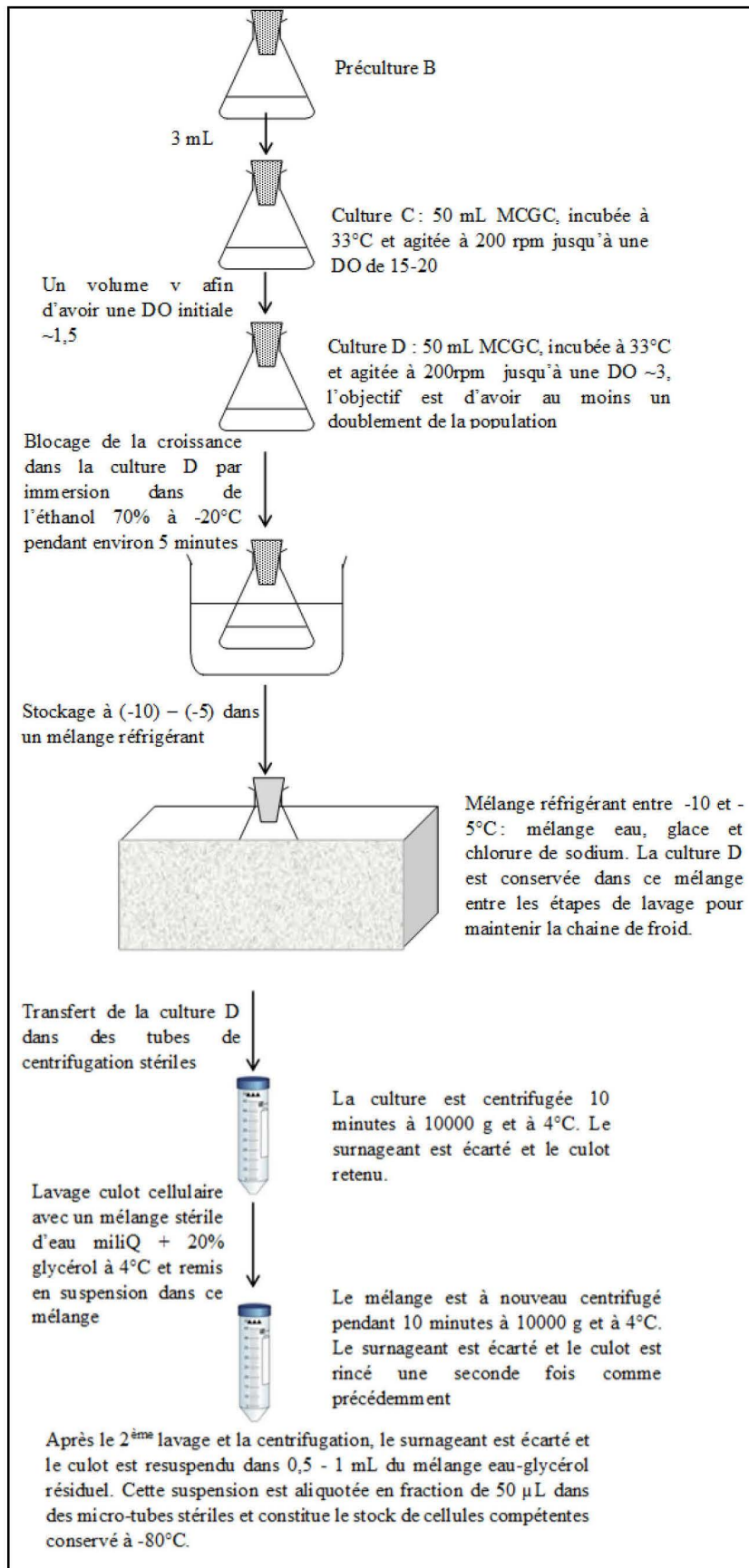


Figure II-5. Protocole de préparation des cellules compétentes.

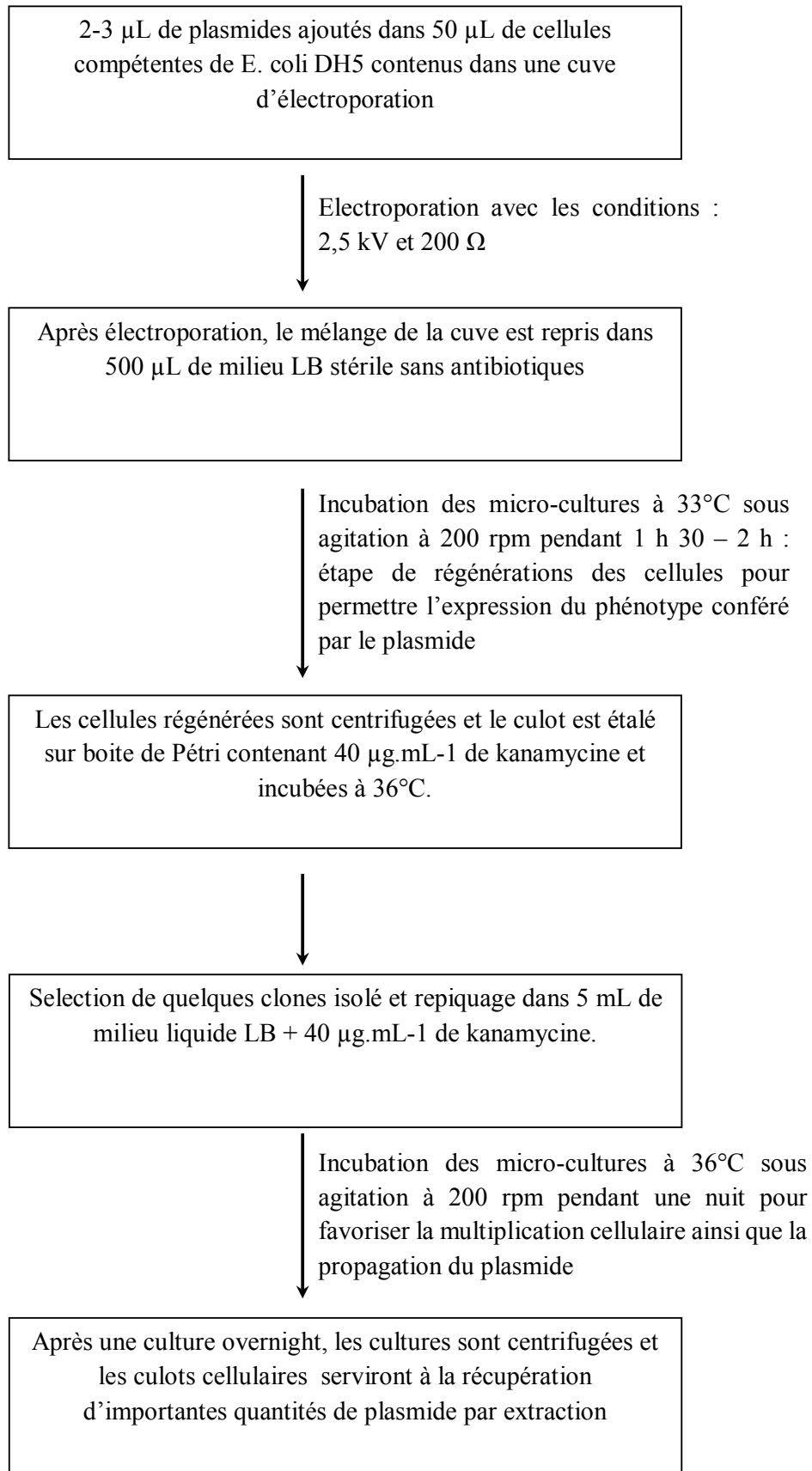


Figure II-6. Propagation, méthylation de plasmides et électroporation.

Chapitre III

**Construction et étude du
mutant, *Corynebacterium
glutamicum* 2262 Δ I_{dhA}**

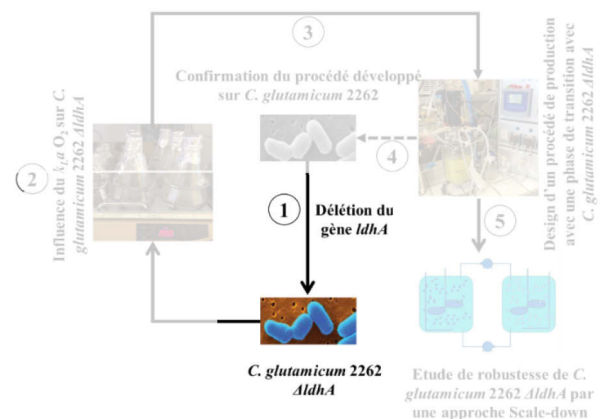
Chapitre III. Avant-propos

C. glutamicum, une bactérie aérobie facultative auxotrophe pour la biotine, a longtemps été utilisée uniquement pour la production d'acides aminés tels que le glutamate, la lysine, l'arginine, histidine et la valine (Eggeling and Sahm, 1999; Ikeda, 2003; Eggeling and Bott, 2005). Par ailleurs, Terasawa *et al.* (1990), ont montré qu'en conditions aérobies et en absence de biotine dans le milieu de culture, la croissance du microorganisme était arrêtée mais que ses capacités métaboliques de production d'isoleucine étaient maintenues (Terasawa *et al.*, 1990). Par ailleurs, il a été démontré qu'en conditions aérobies de culture mais lorsque l'oxygène devient limitant (soit des conditions dites de « microaérobie »), *C. glutamicum* pouvait excréter plusieurs acides organiques en faibles quantités et notamment l'acide succinique qui connaît un regain d'intérêt en tant que précurseur d'une large gamme de dérivés chimiques industriels. La production de succinate chez *C. glutamicum* utilise les voies anapérobiotiques pour fixer du CO₂ et alimenter la voie réductrice du cycle de Krebs (Inui *et al.*, 2004a). En conditions anaérobies, *C. glutamicum* produit des acides organiques même si sa croissance est stoppée. Cependant le lactate reste le produit majoritaire de cette fermentation avec une concentration d'environ 1,1 M contre 0,2 M de succinate (Okino *et al.*, 2005).

Afin de caractériser la capacité réelle de production de succinate d'une souche de *C. glutamicum* 2262, nous avons entrepris la construction d'un mutant déficient pour la lactate déshydrogénase, qui catalyse la dernière étape de la voie de biosynthèse du lactate. Les capacités de croissance et de production d'acides organiques

du mutant généré seront étudiées comparativement à la souche parentale. A noter aussi que, pendant la phase de croissance, la respiration bactérienne libère du CO₂ qui pourrait être avantageusement utilisé/recyclé pour la production d'acide succinique. Nous proposerons donc également une méthode de détermination de la production de CO₂ pendant la phase de croissance par simple utilisation d'une sonde CO₂ dédiée. Pour cela, un modèle simple de désorption du CO₂ produit pendant la phase de croissance sera proposé.

Les résultats de ces travaux sont présentés sous la forme d'un article.



III. *Corynebacterium glutamicum* L-lactate dehydrogenase gene deletion and evaluation of the CO₂ production during the oxidative metabolism

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Abstract

Corynebacterium glutamicum is widely used for industrial production of amino acids. Recently, its ability to produce organic acids in oxygen-deprived or anaerobic conditions has also been demonstrated. In such conditions, the bacterial growth was stopped. However, under anaerobic or oxygen deprived conditions, *C. glutamicum* wild type strain mainly produced lactate in comparison with other organic acids. Thus, to study the succinate production with *C. glutamicum*, the construction of lactate dehydrogenase deleted mutant strain seemed required. In this paper, the *ldhA* gene of *C. glutamicum* was deleted by using the plasmid vector *pk19mobsacBΔldhA*. The cultures in 2 L bioreactors of *ΔldhA* strain and parental strain presented similar growth kinetics with maximal growth rate of about 0.7 h⁻¹ with a maximal specific glucose uptake rate of 7.5 mmol (g cdw)⁻¹ h⁻¹ during aerobic culture. However, despite *C. glutamicum* 2262 *ΔldhA* did not present any lactate production, the quantities of succinate by *C. glutamicum* 2262 *ΔldhA* were only sensibly higher than those determined in the wild type strains. CO₂ desorption due to air bubbling was modelled and allowed the quantification of CO₂ produced during the aerobic phase with only a CO₂ probe measuring dissolved CO₂ concentrations. The results, in accordance with metabolic carbon balance, indicated that recycling the CO₂ produced during the aerobic phase would not bring sufficient CO₂ quantities to reach high concentrations of succinate during a dedicated anaerobic phase.

Keywords: Succinic acid, aerobic growth, *Corynebacterium glutamicum*, lactate dehydrogenase, CO₂ desorption model, *k_{LaCO2}*.

III-1. Introduction

Succinic acid is a C₄ dicarboxylic acid that has been identified by the U.S. Department of Energy as a top 12 target bio-based molecule with high-value added (Werpy *et al.*, 2004b) and that, thus, presents an increased interest. Mostly produced by chemical synthesis for industrial applications, succinic acid is an intermediate of the tricarboxylic acid cycle of almost all microbial, plant and animal cells. Succinic acid has a very broad spectrum of applications as precursor for various commodity chemicals such as 1,4-butanediol, tetrahydrofuran, γ -butyrolactone, adipic acid, maleic anhydride, various n -pyrrolidinones, aliphatic esters and biodegradable polymers such as polybutylene succinate and polybutylene succinate adipate (Zeikus *et al.*, 1999; McKinlay *et al.*, 2007b; Sauer *et al.*, 2008; Wieschalka *et al.*, 2013a). The markets of succinic acid and its derivatives include surfactants, ion chelators, and pharmaceuticals, and food industry (McKinlay *et al.*, 2007b). Several anaerobic bacteria, such as *Mannheimia succiniciproducens* (Lee *et al.*, 2003, 2006), *Actinobacillus succinogenes* (Van der Werf *et al.*, 1997; Guettler *et al.*, 1999), and *Anaerobiospirillum succiniciproducens* (Samuelov *et al.*, 1991; Lee *et al.*, 1999), efficiently produced succinic acid naturally with high productivity and tolerance to osmotic pressure. As succinic acid production is generally concomitant with other fermentation products, disruption of competitive pathways appears to be an obvious strategy for their production improvement. Aside of enhancing the performances of natural producers, alternative strategies consisted in the use of industrial micro-organisms which do not naturally produce significant amounts of succinic acid, namely *Escherichia coli*, *Corynebacterium glutamicum* or *Saccharomyces cerevisiae* and to metabolically engineer them for efficient succinic acid production yields (Chen and Nielsen, 2013; Jansen and van Gulik, 2014). The metabolic engineering included the directed modification of cellular metabolic properties through the amplification and/or the deletion of specific metabolic pathways or the introduction of new ones, using recombinant DNA technology. These non-natural producers are able to produce higher quantities of acetic acid, formic acid, lactic acid, and ethanol but not specifically of succinic acid during anaerobic fermentation process (Clark, 1989; Wieschalka *et al.*, 2013a; Khuat *et al.*, 2014). The redirection of metabolic fluxes and the reduction of other metabolites formation are therefore necessary. Towards this objective, a first general approach consisted in inactivating the lactate producing pathway by lactate dehydrogenase deletion (Sánchez *et al.*, 2005a) followed by subsequent deletions of other minor side-product pathways. For example, Sanchez *et al.* (2005b) created a recombinant *E. coli* strain by deleting the lactate dehydrogenase gene (*ldhA*), the alcohol dehydrogenase gene (*adhE*), the phosphate

acetyltransferase-acetate kinase (*ack-pta*) from the central metabolic pathway and by inactivating *iclR*, which encodes a transcriptional repressor protein of the glyoxylate bypass. The resulting mutant produced up to 40 g L⁻¹ of succinic acid with increased production yields up to 1.6 mole of succinate per mole of glucose under anaerobic conditions (Sánchez *et al.*, 2005b). On an other hand, a pyruvate formate lyase and lactate dehydrogenase double deleted mutant of *E. coli*, accumulated respectively succinic, pyruvic, and acetic acids at concentrations of 28.2, 2.31 and 2.17 g L⁻¹, respectively, with overall yield of 1.13 mole succinate per mole of glucose (Bunch *et al.*, 1997; Wu *et al.*, 2007). In addition to the gene knockout strategies, overexpression of the genes directly involved in the succinate biosynthesis pathways have also been investigated to enhance the succinic acid production. Since CO₂ is a co-substrate during succinic acid production under anaerobic conditions, these strategies consisted mainly in overexpression of endogenous or heterologous CO₂-fixing enzymes, such as pyruvate carboxylase, phosphoenolpyruvate carboxylase, pyruvate carboxykinase encoding genes (Vemuri *et al.*, 2002a; Kim *et al.*, 2004b; Lin *et al.*, 2004; Yu *et al.*, 2011). The combination of gene knockout and exogenous pyruvate carboxylase gene expression helped the creation of an *E. coli* strain AFP111 / pTrc99A – *pyc* which produced 99.2 g L⁻¹ of succinic acid with a production rate of 1.3 g L⁻¹ h⁻¹ in a dual phase fermentation (Vemuri *et al.*, 2002b). Furthermore, under anaerobic conditions, the reductive branch of the TCA cycle is used and succinate is produced from phosphoenolpyruvate and/or pyruvate via the fixation of CO₂. This reductive pathway converts oxaloacetate to malate, fumarate and succinate, which requires 2 moles of NADH per mole of succinate produced (Van der Werf *et al.*, 1997; McKinlay *et al.*, 2007a). In anaerobic production of succinate, CO₂ is a co-substrate generally provided by sparging the culture medium with gaseous CO₂ or by complementing the medium with bicarbonate salts (NaHCO₃, Ca(HCO₃)₂, Mg(HCO₃)₂ ...). For such reductive production of succinate, the theoretical maximal succinate production yield based only on carbon balance is 2 moles of succinate per mole of glucose. However, the limiting step of biochemical reactions in the anaerobic pathway is the reducing equivalents (NADH). In fact, 1 mole of glucose releases only 2 moles of NADH through the glycolytic pathway whereas one mole of succinic acid requires 2 moles of NADH. Therefore, during metabolic fluxes manipulation for the production of metabolites, an important attention should be paid to the redox balance. As succinate biosynthesis has been shown to be strongly related to the reducing equivalent availability, additional electron fluxes have been reported to improve the succinate production. Liang and coworker, developed *E. coli* NZN111/pTrc99a-*pncB* that overexpressed nicotinic acid phosphoribosyltransferase activity and reported an increase in succinate titer by a factor

3.7 (Liang *et al.*, 2012). *C. glutamicum* is another industrial bacterium reported as a good candidate for efficient succinic acid production. *C. glutamicum* is a Gram-positive, nonspore-forming and facultative anaerobic bacterium that has been widely used for the industrial production of various amino acids, nucleic acids and appears as a promising biocatalyst for succinic acid production (Terasawa and Yukawa, 1993; Kinoshita *et al.*, 2004; Nishimura *et al.*, 2007). Moreover, Dominguez *et al.* (1993), reported limited accumulation of organic acids during aerobic growth with oxygen limitation (Dominguez *et al.*, 1993). This observation was confirmed by Inui *et al.* (2014a), that showed that under oxygen deprived conditions, *C. glutamicum* growth was stopped but cells remained able to produce significant amount of lactate and trace amount of succinic acid (Inui *et al.*, 2004a). Besides, several *C. glutamicum* strains genome were sequenced and their central metabolisms were intensively described (Ikeda and Nakagawa, 2003; Kalinowski *et al.*, 2003; Yukawa *et al.*, 2007) and genetic tools for metabolic engineering of such strains are available (Kirchner and Tauch, 2003b; Burkovski, 2008). Thus, based on the different results reported, metabolic engineering has been successfully applied to *C. glutamicum* in order to develop a competitive succinic acid producer. In fact, by deleting lactate dehydrogenase and overexpressing pyruvate carboxylase in *C. glutamicum* R, Okino and coworkers produced 146 g L⁻¹ of succinic acid with a yield of 1.4 mole per mole of glucose and a productivity of 3.2 g L⁻¹ h⁻¹ in an anaerobic fed-batch process. This production involved mainly the reductive arm of the TCA cycle (Inui *et al.*, 2004a). In the same trend, a mutant strain of *C. glutamicum* ATCC 13032 was constructed by deleting all the main byproducts pathways, by overexpressing, the anaplerotic enzyme gene (*pyc*), the glycolytic glyceraldehyde-3-phosphate dehydrogenase (*gapA*) and by integrating genes to improve the supply of reducing equivalents. The resulting strain BOL- 3/pAN6-*gap* (*C. glutamicum* $\Delta cat \Delta p q o::Ptuf fdh \Delta p t a-ack::Ptuf pycP458S/pAN6-gap$) was able to produce up to 130 g L⁻¹ of succinate with a maximal production yield of 1.67 mol mol⁻¹ of glucose in an anaerobic fed-batch process with glucose and formate as substrates (Litsanov *et al.*, 2012b). Unlike the two previous metabolic engineering strategy, Zhu *et al.* (2014) overexpressed anaplerotic enzymes, deleted pathways of acetate and lactate synthesis, reconstructed the glyoxylate shunt and overexpressed the succinate exporter enzyme. In a dual production involving the reductive arm of TCA cycle and the glyoxylate shunt as succinate biosynthesis pathways, they obtained a concentration of 109 g L⁻¹ of succinic acid with a yield of 1.32 mole of succinate per mole of glucose (Zhu *et al.*, 2014). According to the literature, it appeared thus difficult to evaluate the succinic acid production potential of *C. glutamicum* without at least the

deletion of the lactate dehydrogenase gene¹. Thus, the objective of this study was the creation of *ldhA* mutant strain with unchanged growth performances and to study its physiological response in a mixed and sparged bioreactor, comparatively to the parental strain. As reported above, CO₂ was necessary for succinate production, and the oxidative metabolism release CO₂. We aimed also at the assessment of this CO₂ production during the aerobic culture conditions to evaluate the value of CO₂ recycling for succinate production in anaerobic culture conditions. In our conditions of cultures, the use of outer gas analyzer was not feasible. In fact, the gas analyzer requires a minimal threshold of a gas flow at the outer of the bioreactor which is not attained in the operating conditions. Thus, the CO₂ production was determined by establishing a mass balance taking into account CO₂ stripping by the air flow rate.

III-2. Material and methods

III-2.1. Bacterial strain, culture media and protocol

E. coli DH5 α mcr containing the plasmid pAN1 was used, for plasmid amplification and methylation. For culture of *E. coli* strain, LB or BHI medium was used with specific amount of antibiotics, if required. Batch cultures of *Corynebacterium glutamicum* 2262 and lactate dehydrogenase deleted mutant (*ΔldhA*) were performed in a modified MCGC mineral salt medium in which citrate was replaced by deferoxamine (Table III-1). Basal medium, salts A, salts B and urea were prepared separately and autoclaved during 20 minutes at 121°C. Vitamins solution was sterilized by filtration through 0.22 μm filter (Millex, Millipore, France). After sterilization, the different solutions were mixed together in accordance to the final concentrations mentioned in Table III-1. The culture medium was supplemented with glucose which was thus the sole carbon and energy source initially present in the medium. For specific study, 125 mM of sodium bicarbonate were added as exogenous CO₂ supply. From cell stocks maintained in 1.5 mL cryo-conservation tubes at -80 °C and containing 1 mL of MCGC with glycerol (20 % v/v), a first preculture (A) of *C. glutamicum* was prepared by transferring the total content of a tube to a baffled shake flask containing 50 mL of culture medium incubated at 33°C and shaken at frequency of 200 rpm. After 15 hours of culture, this preculture was used as inoculum for a second preculture (B) (inoculated at concentration of 10 % in MCGC

¹ This hypothesis will be questioned in chapter V of this PhD manuscript.

medium) incubated in the same conditions. When the optical density at of preculture (B) reached a value of 30 (570 nm), bacterial growth was stopped by cooling the flask in 70 % v/v ethanol at a temperature of -20°C; fermentation broth was then kept at a temperature of 4°C. Preculture (B) was used as the inoculum for the different bioreactors with a starting optical density of 1.5 (570 nm). Cultures were done in 2 L stirred tank bioreactors (Tryton, Pierre Guerin technologies, France) with 1.5 L working volume. The bioreactors were equipped with two four-blade Rushton turbines and maintained at a temperature of 33°C. During cultivation, dissolved oxygen was maintained at a constant concentration of 50 % of air saturation by sparging air or pure oxygen through a ten-orifice diffuser at varying gas flow rate. Dissolved oxygen and carbon dioxide concentrations were respectively measured using sterilizable oxygen probe (Mettler-toledo, ISM technology, Switzerland) and sterilizable CO₂ probe (Mettler-toledo, ISM technology, Switzerland). The pH was measured using sterilizable glass electrode (Mettler-toledo, ISM technology, InPro3253, Switzerland) and regulated at a set-point of 7.6 by automatic addition of 10 N KOH and 2 N HCl solutions using peristaltic pumps connected to an on/off pH controller. The foam formation was controlled by manual addition of drops of polypropylene glycol in culture broth with respect to a final concentration inferior to 1.2 mL ppg (L of culture)⁻¹. The temperature of cultures was maintained at 33 °C by automatic cool and hot water circulation in the bioreactor jacket.

III-2.2. Construction of a deleted *ldhA* mutant of *C. glutamicum* 2262

III-2.2.1. *C. glutamicum* 2262 competent cells preparation

From frozen cell stocks a preculture of *C. glutamicum* 2262 was prepared by transferring the total content of a tube (1 mL, glycerol 20 % (v/v)) to a baffled shake flask containing 50 mL of culture medium and incubated overnight at 33°C and shaken at 200 rpm frequency. This preculture was used as inoculum for 50 mL of MCGC culture medium with a starting optical density of 1.5 (570 nm). The culture was incubated at a temperature of 33 °C and also shaken at 200 rpm. When an optical density of 3 was reached, bacterial growth was stopped by cooling the flask in 70 % v/v ethanol maintained at a temperature of -20 °C, for 2 minutes and kept in a mixture of ice, salt and water at temperature of -5 to -10 °C. The cooled broth was centrifuged at 10,000 g for 10 minutes at 4 °C. After centrifugation, the supernatant was discarded and the pellet was washed with cooled milliQ water containing 20 % of glycerol. The resuspended cells were centrifuged, the pellet was washed again using the previous protocol and after a last

centrifugation, the supernatant was thrown and the pellet corresponding to competent cells was re-suspended in the small volume remaining. Aliquots of 50 μ L of competent cells were made and stocked at -80 °C. For *E. coli* DH5 α mcr/pAN1, 1 mL of glycerol stock was inoculated in 50 mL LB medium containing 35 μ g/mL of chloramphenicol. After an overnight culture at a temperature of 36 °C and shaken at a frequency of 200 rpm, this preculture was used to inoculate a culture with initial optical density of 0.3 (570 nm) and incubated in the same conditions as the preculture. When an optical density of 1.5 (570 nm), cell growth was stopped and competent cells were prepared following the method described above.

III-2.2.2. Plasmid propagation and transformation of *C. glutamicum* 2262

C. glutamicum mutant with in-frame deletion of *ldhA* was constructed via a two-step homologous recombination procedure as described by Schäfer *et al.* (Schäfer *et al.*, 1994), using the suicide vector pK19mobsacB. The plasmid pK19mobsacB*AldhA* constructed for precise deletion of lactate dehydrogenase gene was used in this study (Litsanov *et al.*, 2012b). The plasmid above bears the two main characteristics of plasmids pK18mobsacB namely 1) a selection marker represented by the resistance gene to kanamycin, 2) and a counter-selection marker, the *sacB* gene. It encodes levan sucrose, a transfructosylase which catalyzes the hydrolysis of sucrose and the synthesis of levan (fructan). The product synthesized from sucrose by the levan sucrose is lethal for cells. This lethality is then used as a counter selection marker for screening of mutants.

Table III-1. Composition of modified MCGC medium.

Compounds of the medium		Final concentration of the different compounds		
		For flask culture	For bioreactor culture	
Basal medium	Na ₂ HPO ₄ 12H ₂ O	36.72	7.44	g L ⁻¹
	KH ₂ PO ₄	2.88	5.90	g L ⁻¹
	NaCl	0.96	1.97	g L ⁻¹
	(NH ₄) ₂ SO ₄	3.84	7.86	g L ⁻¹
Salts A	MgSO ₄ 7H ₂ O	455.84	39.31	mg L ⁻¹
	FeSO ₄ 7H ₂ O	45.58	3.93	mg L ⁻¹
	FeCl ₃	4.56	0.39	mg L ⁻¹
Salts B	ZnSO ₄ 7H ₂ O	1.14	0.07	mg L ⁻¹
	CuCl ₂ 2H ₂ O	0.46	0.03	mg L ⁻¹
	MnSO ₄ H ₂ O	4.56	0.26	mg L ⁻¹
	(NH ₄) ₆ Mo ₇ O ₂₄ 4H ₂ O	0.23	0.01	mg L ⁻¹
	Na ₂ B ₄ O ₇ 10H ₂ O	0.46	0.03	mg L ⁻¹
Vitamins	CaCl ₂	94.85	8.18	mg L ⁻¹
	Biotin	2.28	0.20	mg L ⁻¹
	Thiamine	22.79	1.97	mg L ⁻¹
	Deferoxamine	3.42	0.29	mg L ⁻¹
	Betaïn	1937.32	0.20	mg L ⁻¹
	Urea	3.90		g L ⁻¹
	Glucose*	33	40	g L ⁻¹

*Concentration of glucose for bioreactor cultures could vary according to the aim of study. In this case, the dedicated concentration was specified.

To avoid the degradation of the exo plasmid pK19mobsacB*aldhA* by *C. glutamicum* methyl-cytosine restricting system, the plasmid was first methylated in *E. coli* DH5α mcr by using the plasmid pAN1 (Mermelstein and Papoutsakis, 1993). For this purpose, 2-5 μL of plasmid were used to transform 50 μL of electrocompetent DH5α mcr/pAN1 by electroporation (2.5 kVolts, 200 ohms). After electroporation, cells were regenerated in 0.5 mL of LB medium, incubated at a temperature of 36 °C for 1h30, and centrifuged at 5,000 g for 5 minutes. The supernatant was discarded and the pellet was plated on LB agar with 40 μg mL⁻¹ of kanamycin and 35 μg mL⁻¹ of chloramphenicol. These Petri dishes were incubated overnight at a temperature of 36

°C. An isolated colony from the plate was inoculated in 5 mL liquid LB containing 40 µg mL⁻¹ and 35 µg mL⁻¹ of chloramphenicol, incubated overnight at a temperature of 36 °C and shaken at a frequency of 200 rpm. The culture broth was centrifuged and the plasmids methylated using pAN1 were extracted and purified using a Qiagen kit (QIAprep Spin Miniprep Kit). The purified pK19mobsacBAldhA was then used for the deletion of LDH gene in *C. glutamicum* 2262. For this purpose, 3-5 µL of plasmid were mixed with 50 µL of competent cells and submitted to electroporation (1.8 kVolts, 200 ohms). After the electroporation, cells were regenerated in 0.5 mL of BHI medium incubated at a temperature of 33 °C and shaken at a frequency of 200 rpm for 1h30. The medium was then centrifuged and the pellet laid on plate of BHI plus 40 µg mL⁻¹ of kanamycin and incubated at a temperature of 33°C. After two days of incubation, less than 5 colonies were formed on every Petri dish. The colonies obtained were cells that integrated the plasmid in their genome by homologous recombination and that expressed the kanamycin resistance. The different colonies were transferred individually in 25 mL of liquid LB without antibiotic and were incubated at 33 °C and shaken at a constant frequency of 200 rpm. This step, was necessary to favor the cells proliferation and the occurrence of the second homologous recombination. Thereafter, culture broth was centrifuged in sterile microtubes and plated on Petri Dishes containing LB agar solid medium with 10 % sucrose. In such medium, cells holding the plasmid with *sacB* gene are not able to grow due to lethality resulting from SacB activity. After the second recombination, two kinds of events are possible: 1) the plasmid is excised alone, and the wild type phenotype is recovered, 2) the plasmid is excised with the targeted lactate dehydrogenase gene resulting in the creation of *C. glutamicum* 2262AldhA (details are available in (Schäfer *et al.*, 1994)). Thus, after 48 h incubation of Petri dishes at 33 °C, hundreds of colonies were formed on the plate and corresponded to the two kinds of transformants mentioned above. Finally, the screening for the right lactate dehydrogenase mutant of *C. glutamicum* included, macroscopic analysis, Gram coloration, lactate production ability.

III-2.3. Determination of CO₂ production during oxidative metabolism

The CO₂ production rate may be evaluated using two approaches. For the first one, a mass balance of dissolved carbon dioxide was made in the bioreactor considering CO₂ stripping due to air bubbling. For the second one, a metabolic mass balance was proposed. Both methods were finally compared and discussed.

III-2.3.1. CO₂ mass balance in the bioreactor

During the aerobic respiration of *C. glutamicum*, glucose and oxygen were consumed to release CO₂ in the culture broth. Due to constant agitation at 900 rpm associated to an increase in the sparging rate used to maintain constant the dissolved oxygen level, the dissolved CO₂ was stripped from the culture broth. In fact, Matsunaga et al., showed a linear increased of CO₂ stripping in relation with the aeration rate (Matsunaga *et al.*, 2009). Thus, during the culture, the CO₂ amount measured by probe corresponded to net accumulation of the CO₂ in culture broth. So, it could be assumed that the total CO₂ produced by the bacteria corresponded to the sum of the quantity of CO₂ stripped and of the time variation of the dissolved CO₂ concentration (dCO_2) measured by the dedicated probe. The net mass balance of CO₂ production could be written as following:

$$\frac{d(dCO_2)}{dt} = k_L a^{CO_2} (dCO_2^* - dCO_2) + R_{CO_2} \quad (\text{III-1})$$

Where $k_L a^{CO_2}$ (h⁻¹) corresponded to the liquid-gas mass transfer coefficient of CO₂ in the culture conditions, dCO_2^* (M) to the concentration of dissolved CO₂ at the bubble-liquid interface, dCO_2 (M) to the concentration measured along the experimentation time and R_{CO_2} (M.h⁻¹) corresponded to the CO₂ production rate. Some questions may arise from the determination of the concentration of dissolved CO₂ at the gas-liquid interface. Generally a thermodynamic equilibrium is assumed between the gas and the liquid phases and the concentration of dissolved gases is thus related to the partial pressure in the gas phase by the Henry's law.

$$p = P_{tot} \cdot y = He_{CO_2} \cdot dCO_2^* \quad (\text{III-2})$$

With P_{tot} the total pressure in the gas bubbles, He_{CO_2} (bar L mol⁻¹) the Henry constant and y the mole fraction of CO₂ in the gas phase. In our case, as the bioreactor was operating at atmospheric pressure, it could be supposed that $P_{tot} = 1$ bar. Equation (III-2) could be rewritten:

$$dCO_2^* = P_{tot} \cdot y / He_{CO_2} \quad (\text{III-3})$$

On the basis of equations (III-1) and (III-3), it could be stated that the CO₂ stripping rate was maximal ($dCO_2^* = 0$) when no CO₂ was present in the bubble. Commonly, the determination of dCO_2^* relies on the knowledge of the Henry constant and of the mole fraction of CO₂ in the gas phase. For CO₂/ water equilibrium at 25°C, the Henry constant was given equal to 29.41 bar L mol⁻¹ (Sander, 2014). It is interesting to note that this value was much lower than for O₂ and N₂ in equilibrium with water at 25°C with, respectively, He_{O_2} and He_{N_2} equal to 769 and 1640 (Sander, 2014). Thus, the molar fraction of oxygen in the gas phase should increase faster than the oxygen decrease. This meant that when the gas bubbles were ascending in the liquid phase, the molar fraction of CO₂ was increased continuously in the gas phase, reducing consequently the CO₂ stripping. To evaluate the relative importance of bubble enrichment in CO₂ on the stripping rate quantification, it was thus necessary to compare the characteristic residence time of bubbles in the bioreactor to the characteristic mass transfer time.

III-2.3.2. Characteristic mass transfer time

The characteristic mass transfer time was given by $t_{MT} = 1/k_L a^{CO_2}$. Despite the gas-liquid mass transfer coefficients $k_L a^{CO_2}$ were more precisely determined in paragraph III-3.2.4, its preliminary estimation should be made assuming a few hypothesis. $k_L a^{CO_2}$ is the product of the interfacial area a and of the liquid-side mass transfer coefficient k_L . It is reasonable to state that bubble shape, size and concentration do not depend on the gas composition and should be identical in the case of O₂ or CO₂ transfer. Consequently, the interfacial area a was not impacted by gas composition. For the k_L modelling, the theory of film renewal of Higbie (Higbie, 1935) should be advantageously used :

$$k_L = 2 \cdot \sqrt{\frac{D_{diff} \cdot u_B}{\pi \cdot d_B}} \quad (III-4)$$

With u_B (m s⁻¹) the bubble velocity, D_{diff,CO_2} the diffusivity (m² s⁻¹) of the dissolved gas in the culture medium and d_B (m) the bubble diameter. Similarly to previous hypothesis, it was reasonable to assume that gas composition had no impact on u_B . Finally, the general following equation, stating that the ratio of gas-liquid mass transfer coefficient was only related to the ratio of their mass diffusivities in the liquid phase, could be used:

$$\frac{k_L a^{CO_2}}{k_L a^{O_2}} = \frac{D_{iff}^{CO_2}}{D_{iff}^{O_2}} \approx 0.9 \quad (\text{III-5})$$

Thus, oxygen and carbon dioxide gas-liquid mass transfer were roughly determined by the same gas-liquid mass transfer coefficient. In the same bioreactor, the $k_L a$ value was given equal to approximately 50 h^{-1} in similar operating conditions (Haj-Husein, 2013), leading to a characteristic time of mass transfer of $t_{MT} = 72 \text{ s}$.

III-2.3.3. Characteristic bubble residence time

Supposing a vertical ascension of bubbles in the vessel and an average bubble vertical velocity of $u_B = 0.5 \text{ m s}^{-1}$, the residence time of bubbles t_B in a liquid height of approximately $H = 0.1 \text{ m}$ was given by:

$$t_B = \frac{H}{u_B} < 1 \text{ s} \quad (\text{III-6})$$

Thus, within the approximations made, it could be assumed that the bubble residence time is at least one order of magnitude shorter than the characteristic mass transfer time and that, the impact of CO_2 enrichment of gas bubbles on CO_2 stripping rate may be neglected. This was previously confirmed by Sieblist et al. who have intensively studied CO_2 stripping in large-scale culture bioreactors (Sieblist *et al.*, 2011).

III-2.3.4. Evaluation of $d\text{CO}_2$ stripping

The $k_L a$ of CO_2 stripping was determined in the culture medium containing 125 mM NaHCO_3 or not and without biomass inoculation. The bioreactor was filled up to working volume of 1.5 L with medium and pH was adjusted to 7.6 then pure CO_2 gas was sparged into the medium until $d\text{CO}_2$ reached the saturation value. Then, aeration was started with agitation under the normal culture conditions and $d\text{CO}_2$ desorption was measured continuously at the cultivation temperature of $33 \text{ }^\circ\text{C}$. The experimental results of $d\text{CO}_2$ (%) obtained under various air flow rates were given in Figure III-1A. The $k_L a$ of CO_2 stripping was evaluated as the slope of the following equation (Figure III-1B) (Matsunaga *et al.*, 2009):

$$\ln \frac{d\text{CO}_2(t)}{d\text{CO}_2(0)} = -k_L a^{CO_2} \cdot t \quad (\text{III-7})$$

Where $d\text{CO}_2(0)$ is the dissolved CO_2 concentration at the beginning of air sparging ($t = 0$)

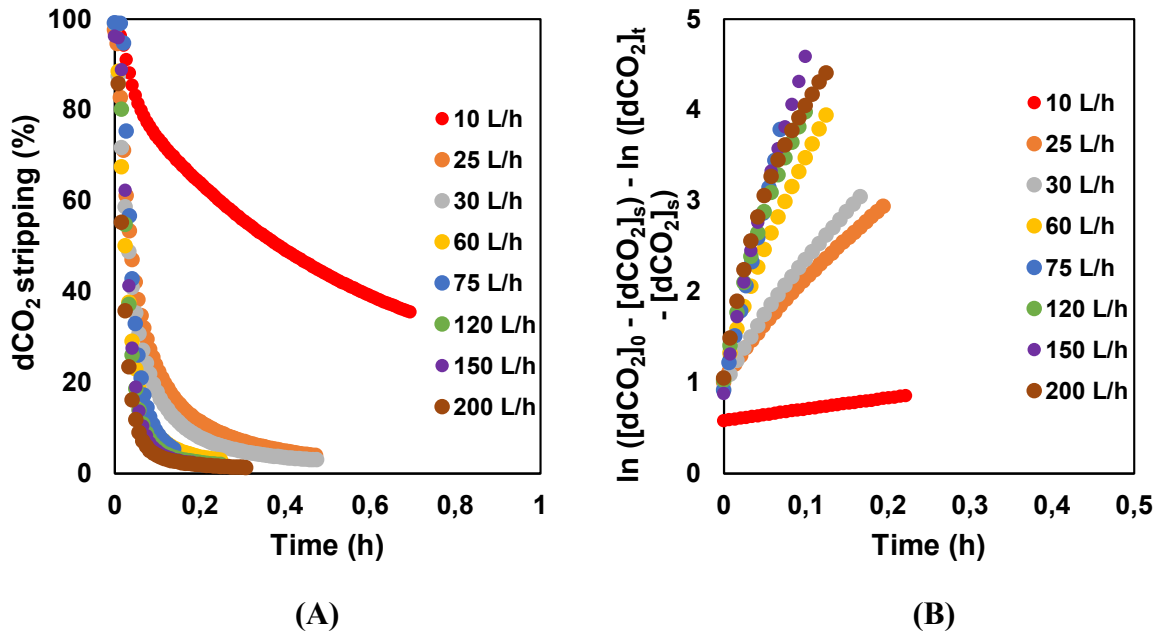


Figure III-1. Time course of dissolved CO_2 stripping at various air flow rates (A); determination of k_{La} of CO_2 stripping at different aeration rate using Equation (III-1) with no CO_2 production (B).

The results obtained for the various air flow rates were reported in Figure III-2 and were modelled for our aeration conditions to predict CO_2 stripping rate whatever the gas flow rate used (it is important to remember that gas flow rate was dynamically adjusted to maintain the set point of dissolved oxygen concentration). Therefore, the following equation could be used to approximately evaluate CO_2 stripping.

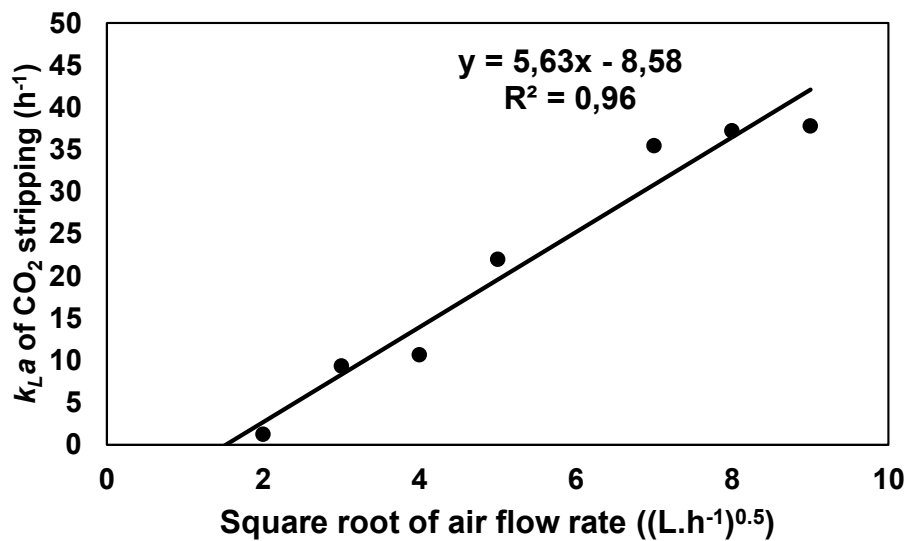


Figure III-2. Impact of the air flow rate on the $k_{La}^{\text{CO}_2}$ values determined experimentally.

$$R_{stripping}(t) = (5.23 * (Q_{air})^{0.5} - 8.58) * dCO_2(t) \quad (III-8)$$

Where $R_{stripping}$ represented the CO₂ removal rate related to broth aeration (% CO₂ h⁻¹), and dCO_2 was expressed in % of CO₂ saturation. It is worthwhile noticing that the stripping rate was related to $Q_{air}^{0.5}$, which was already demonstrated by Linek *et al.* (2004) for the oxygen transfer in a vessel mixed by a Rushton turbine.

III-2.3.5. Determination of CO₂ accumulated and CO₂ net production

Beside the stripped CO₂, a fraction of the CO₂ produced during aerobic growth of bacteria may accumulate in the culture broth and was measured with the dedicated CO₂ probe. The accumulation rate was given according to Equation (III-9):

$$R_{accum}(t) = \frac{d(dCO_2)}{dt} \quad (III-9)$$

The net CO₂ production rate during the respiratory phase of *C. glutamicum* culture, $R_{CO_2}(t)$ expressed in % CO₂ h⁻¹, could be then deduced from Equations (III-8) and (III-9):

$$R_{CO_2}(t) = R_{stripping}(t) + R_{accum}(t) \quad (III-10)$$

In order to compare the CO₂ production rate determined through experimental dissolved CO₂ measurements with its estimation via a metabolic mass balance, R_{CO_2} was converted from % CO₂ h⁻¹ to mM h⁻¹.

$$R_{CO_2}(t)(\text{mM h}^{-1}) = R_{CO_2}(t)(\% \text{ CO}_2 \text{ h}^{-1}) * \frac{17}{1.5 * 44} \quad (III-11)$$

Where 44 g mol⁻¹ corresponded to CO₂ molar weight and 17/1.5 corresponded to ratio obtained after calibration of CO₂ probe in our culture conditions (1.5 % of the saturation value corresponded to 17 mg L⁻¹ of dCO₂).

III-2.3.6. Evaluation of CO₂ production rate through a metabolic mass balance

During the microbial respiration, the CO₂ production rate could be estimated theoretically from substrate uptake rate. In calculation, it is admitted that, in aerobic culture conditions, when prokaryotic microorganism is grown in a defined medium of culture containing a defined substrate as carbon and energy source, half of the substrate was used for anabolism and the other part for catabolism. In fully aerobic conditions in the synthetic MCGC containing glucose as carbon and energy source, culture of *C. glutamicum* 2262 Δ ldh results in biomass accumulation and CO₂ release. Therefore, 50 % of glucose consumed were converted into biomass and the other 50 % were transformed into CO₂. The theoretical estimation of CO₂ production rate from glucose uptake rate is described below:

$$q_{glucose}(t) = -\frac{d[glucose]}{dt} = -\frac{[glucose]_{(t+1)} - [glucose]_{(t-1)}}{\Delta t} \quad (III-12)$$

Where $q_{glucose}(t)$ mM.h⁻¹ represented the glucose uptake rate, $[glucose]_{(t+1)}$ and $[glucose]_{(t-1)}$ (mM) represented the glucose concentrations obtained by fitting experimental data using interpolation model and Δt (h) corresponded to the interval of time.

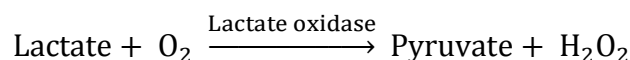
$$R_{CO_2}^{theo}(t) = 0.5 * q_{glucose}(t) \quad (III-13)$$

Where the coefficient 0.5 corresponded to the 50 % of total glucose used for CO₂ production and $R_{CO_2}^{theo}$ mM.h⁻¹ corresponded to theoretical CO₂ production rate.

III-2.4. Analytical methods

For each experiment, biomass concentration was determined by the measurement of absorbance at 570 nm with a Hitachi U2000 spectrophotometer (Sciencetec, les Ulis, France) and was transformed into dry cell weight (cdw) using the coefficient as: dry cell mass (g L⁻¹) = 0.27×OD₅₇₀. Samples of culture broth were first centrifuged at 5,000×g for 10 min and at 4°C. Glucose and lactate concentrations were analyzed in the supernatants by High Pressure Liquid Chromatography (10ADVP, Shimadzu) using an ion-exchange column (7.8 mm×300 mm, Bio-Rad Aminex HPX-87H) and SPD-M10A chromatography working station system equipped

with UV absorbance and refractive index detectors water 410 (Shimadzu). Each supernatant was injected with specific volume. The column was eluted isocratically at a rate of 0.7 mL min⁻¹ with 5 mM H₂SO₄ for sugar analysis and 50 mM H₂SO₄ for organic acids at a temperature of 50 °C. The approximate retention time for glucose, pyruvic, succinic, lactic and acetic acids were respectively 7.7, 9.2, 10.2, 10.9 and 13.1 minutes. Lactate was also quantified by enzymatic-colorimetric method using multiparametric automated analyzer (MPA) with enzymatic kits (Biomerieux, France). The principle of the method was described below:



Quinoneimine was detected at 446 nm and the color intensity of quinoneimine measured was proportional to the lactate amount in the sample.

III-3. Results and discussion

III-3.1. Screening for *C. glutamicum* 2262*AldhA* mutant strain identification

C. glutamicum 2262 was transformed with the plasmid pK19mobsac*BaldhA* methylated as described previously. Liquid culture broths were centrifuged and pellets were plated on LB agar containing 10 % sucrose. After 48 h of incubation at temperature of 33°C, hundreds of colonies were formed on the plate and corresponded to 2 kinds of transformants (see Material and methods). The different clones were morphologically identical, thus several clones were selected randomly for screening (Table III-2). The chosen clones were all composed of Gram positive bacteria presenting rod shape similar to *C. glutamicum* wild type strains. These clones were cultivated in 10 mL of MCGC containing glucose as the sole carbon source, in test tubes with poor conditions of agitations. These conditions should ensure oxygen-limited conditions which are necessary for lactate production by *C. glutamicum*. In some culture supernatants, the HPLC analytical analysis revealed a lactate production of 15 g L⁻¹ (Table III-2, samples 5, 13, 14, 16 and 17). This production level was comparable with that of wild type strain (17 g L⁻¹). The clones with such lactate production were those that lost the plasmid alone during the second homologous recombination and recovered the wild type phenotype. Other cultures produced lactate concentrations of 0-0.5 g L⁻¹, similar to negative control (0.3 - 0.4 g L⁻¹). These last clones, considered as lactate dehydrogenase deficient strains, were chosen for further study. The clones of samples 1, 2, 3, 12, 20, 21, 23 and 25 indicated in

Table III-2, were isolated on LB agar containing 10 % sucrose and a colony from each sample was transferred in 5 mL in MCGC containing 155 mM of glucose and incubated at a temperature of 33°C with an agitation of 200 rpm. The 5 mL culture was used as an inoculum for 25 mL MCGC culture medium with 155 mM of glucose as carbon and energy source. The cultures were run for 24 h at 33°C with an agitation of 200 rpm. After 8 h of culture, lactate concentration was measured enzymatically using MPA method. The results were summarized in Table III-2.

Table III-2. Results of screening for lactate producing and non-producing strains. Biomass concentration and HPLC quantification of lactate and glucose concentration after 8 hours of culture.

Sample	Biomass concentration (g L ⁻¹)	Gram coloration	Glucose concentration (g L ⁻¹)	Lactate concentration (g L ⁻¹)
1	3.8	Positive	8.51	nd
2	3.8	Positive	8.96	nd
3	3.9	Positive	8.45	nd
4	4.1	Positive	7.06	nd
5	3.8	Positive	5.03	16.1
6	3.9	Positive	7.89	nd
7	4.1	Positive	7.37	nd
8	3.9	Positive	8.37	nd
9	3.8	Positive	8.84	nd
10	4.1	Positive	9.15	nd
11	3.9	Positive	9.56	nd
12	4.2	Positive	8.69	nd
13	3.8	Positive	4.98	16.3
14	4.4	Positive	4.42	11.3
15	4.0	Positive	9.62	nd
16	4.0	Positive	5.13	16.3
17	3.7	Positive	5.12	15.9
18	3.6	Positive	8.75	nd
19	3.5	Positive	8.66	nd
NC 1	-	-	37.1	nd
NC 2	-	-	37.5	nd
WT 1	4.0	Positive	5.05	17.0
WT 2	4.2	Positive	4.88	17.1
20	11.7	Positive	4.27	nd
21	14.0	Positive	1.07	nd
22	13.0	Positive	1.78	nd
23	13.0	Positive	0.00	nd
24	14.6	Positive	0.00	nd
25	12.9	Positive	2.02	nd
WT3	15.2	Positive	0.00	5.08

NC, corresponded to the negative controls made with only culture medium without bacteria and incubated in the same conditions. WT, correspond to the positive controls and represent a culture with *C. glutamicum* 2262 and nd corresponded to samples where no lactate was detected.

Table III-3. Enzymatic analysis of lactate production for the selected *C. glutamicum* 2262 *AldhA* clones after 8 h of culture.

Sample	Biomass concentration (g L ⁻¹)	Lactate concentration (g L ⁻¹)
1	4.6	nd
2	5.3	nd
12	5.3	nd
20	5.4	nd
25	4.5	nd
¹ WT ¹	9.7	1.75
² NC ²	-	³ nd

¹WT, ²NC and ³nd represented the wild type strain assay, the negative control and no detected lactate samples, respectively

According to the different screening experiments, the sample 25 that showed no lactic acid production was chosen as the *C. glutamicum* 2262 *AldhA* mutant strain and was used for the next studies.

III-3.2. Comparison of growth and production kinetics of *C. glutamicum* 2262 *AldhA* mutant strain and wild type strain in aerobic conditions

Once the *C. glutamicum* 2262 *AldhA* constructed, the next steps consisted in studying its physiological response during aerobic growth compared to the wild type strain. The two strains were grown in a 2 L stirred tank bioreactor (pH 7.6, 33°C, and 900 rpm) with a dissolved oxygen concentration maintained around 50 % of air saturation. Kinetics of biomass growth, and glucose consumption were represented in Figure III-3, while trace amounts of metabolites (D-lactate and acetate) produced were noticed in Figure III-4. The deletion of *ldhA* gene did not affect the ability of growth in synthetic medium (MCGC) as growth profiles were similar between mutant and wild type strain with maximal biomass concentrations of 18.7 g L⁻¹ and 19 g L⁻¹, respectively (Figure III-3A). The specific growth rate remained also unchanged with maximal value of 0.7 h⁻¹ for both strains cultivated under aerobic conditions (Figure III-3B). During the culture, the glucose consumption was identical and a complete exhaustion of glucose was observed within 11 h for both cultures (Figure III-3C) with maximal specific consumption

rate of $7.5 \text{ mmol (g cdw)}^{-1} \text{ h}^{-1}$ (Figure III-3D). Therefore, it appeared that the deletion of *ldhA* gene that encodes L-lactate dehydrogenase, a key enzyme that allows L-lactate production as well as reoxidation of NADH formed during glycolysis, did not affect the growth and substrate uptake rates. In literature, *ldhA* gene deletion has been intensively used in *E. coli* and *C. glutamicum* to improve the ability of these strains to produce valuable metabolites, and no defect of growth and substrate uptake were also reported (Vemuri *et al.*, 2002b; Wendisch *et al.*, 2006). However, in lactic acid bacteria such as *Lactococcus lactis*, *Enterococcus faecalis*, and *Streptococcus pyogenes*, deletion of the *ldh* genes strongly affected the growth rate in chemically defined medium under microaerophilic conditions. The deletion effects depended also on the medium composition of cultures and pH (Fiedler *et al.*, 2011).

During the culture of *C. glutamicum* 2262 and 2262 *ΔldhA* mutant strains under aerobic conditions, a negligible amount of organic acids was detected ($< 0.4 \text{ g L}^{-1}$) (Figure III-4). Lactate and succinate productions by the wild type strain were sensibly higher than by 2262 *ΔldhA* mutant. Indeed in *C. glutamicum*, high organic acids production were proven to occur under oxygen deprived conditions only (Okino *et al.*, 2005; Inui *et al.*, 2007). Thus, as oxygen was controlled at a value of 50 % of air saturation, the low concentrations of organic acids observed was an expected result.

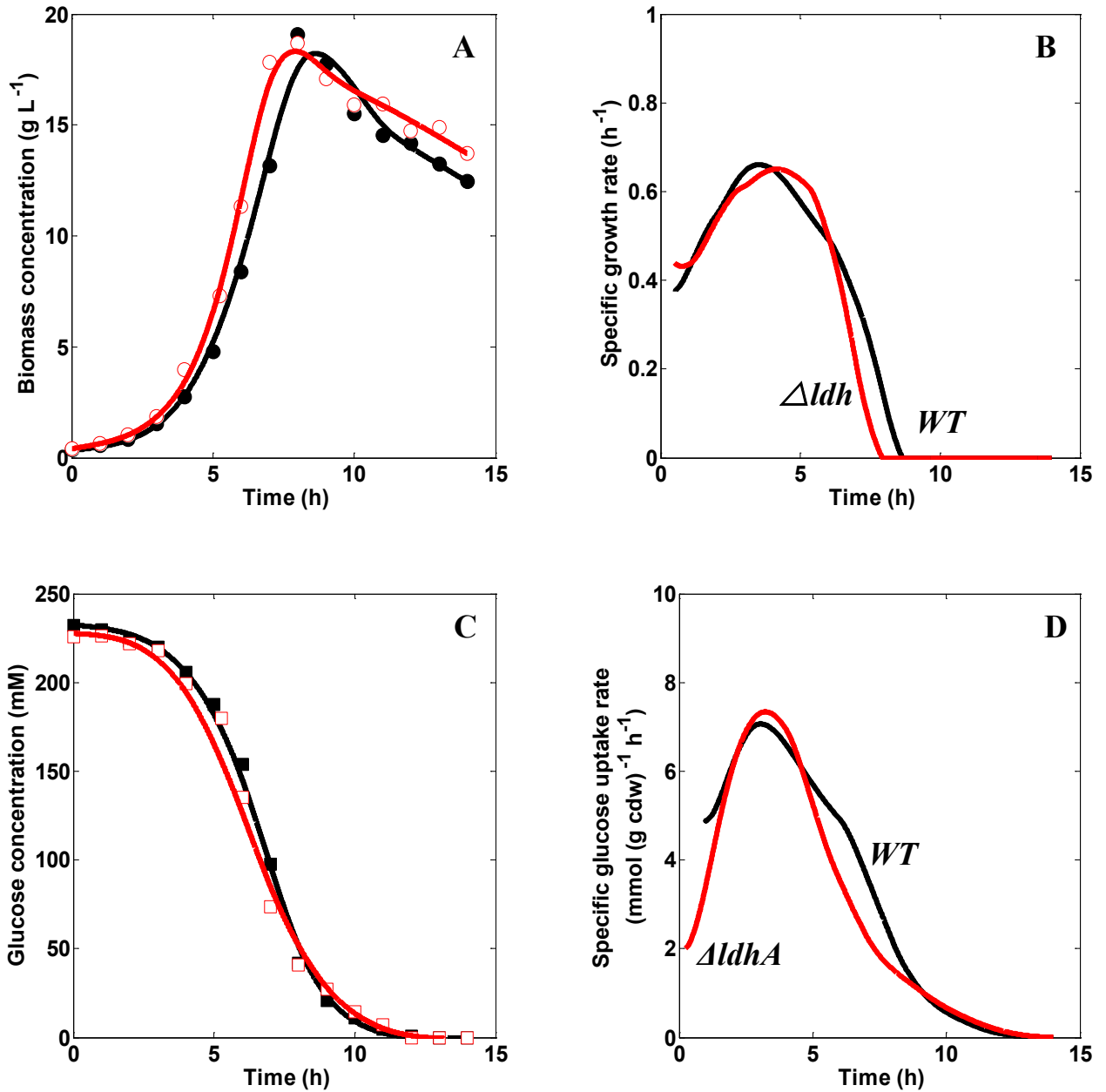


Figure III-3. Comparison of growth and glucose consumption kinetics of *C. glutamicum* 2262 (black) and *C. glutamicum* 2262 $\Delta ldhA$ (red) cultivated in aerobic growth conditions. The filled symbols represent wild type strain and open symbols correspond to mutant strain. A biomass concentration (\bullet , \circ); B. Specific growth rate of wild type strain (black) and $\Delta ldhA$ mutant (red); C. Glucose concentration (\blacksquare , \square); D. Specific glucose consumption rate of wild type strain (black) and $\Delta ldhA$ mutant (red).

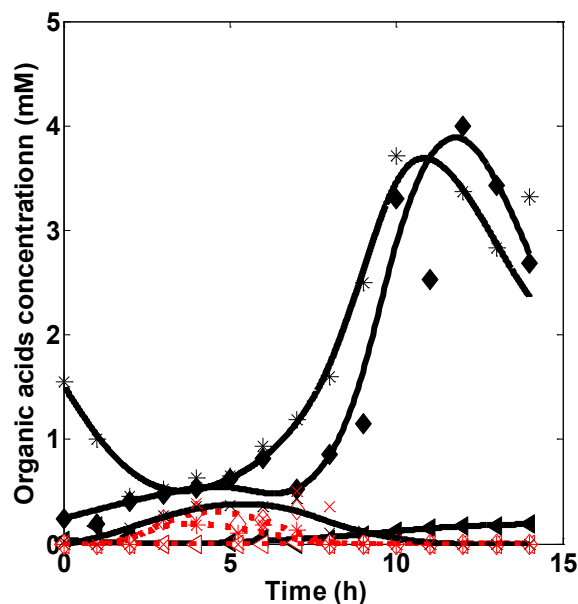


Figure III-4. Organic acids production by *C. glutamicum* 2262 (plain line) and *C. glutamicum* 2262 *Aldh* (dotted line) cultivated under aerobic conditions. The filled symbols represent the wild type strain whereas open symbols correspond to the mutant strain. Pyruvate (\blacktriangle , \triangle), succinate (\blacklozenge , \lozenge), lactate ($*$, $*$) and acetate (\times , \times) concentration.

III-3.3. Effects of bicarbonate addition on the growth of *C. glutamicum* 2262 *AldhA*

As mentioned in the introduction, bicarbonate salts like NaHCO_3 are frequently used as CO_2 donor during the succinate production under oxygen-limited or anaerobic conditions. Under reductive conditions, the production of one mole of succinic acid required the fixation of one mole of CO_2 (Inui *et al.*, 2004a; Okino *et al.*, 2005). Besides, according to the literature, the production of succinic acid by *C. glutamicum* involved two steps including a first initial aerobic phase for biomass accumulation, followed by anaerobic organic acids production phase. As NaHCO_3 is used as a co-substrate of succinic acid production during the anaerobic phase, it is worthwhile wondering if NaHCO_3 , considered as a CO_2 donor had any influence on bacterial growth during the aerobic phase. For this purpose, *C. glutamicum* 2262 *AldhA* was cultivated in a 2 L stirred bioreactor under aerobic conditions in MCGC medium supplemented with 125 mM of NaHCO_3 and 350 mM of glucose. The former experiments were compared to cultures performed in MCGC without NaHCO_3 . In the presence or absence of NaHCO_3 , the strain grew similarly during all the culture time with a maximal biomass concentration of 16 g cdw L^{-1} and specific growth rate of 0.65 h^{-1} (Figure III-5A and B). The kinetics of glucose consumption

were also comparable with a 9 % decrease observed for the maximal specific consumption rate when NaHCO₃ was added in the culture medium (Figure III-5C and D). According to these results, the addition of 125 mM of NaHCO₃ as CO₂ donor had no apparent effect on growth and substrate consumption of *C. glutamicum* 2262 *AldhA* during the aerobic phase.

III-3.4. Characterization of CO₂ production during aerobic growth of *C. glutamicum* 2262 *AldhA*

As exposed earlier, CO₂ is an indispensable co-substrate for succinate production through the reductive branch of TCA cycle. It is generally provided by external supply of the culture medium with NaHCO₃ or gaseous CO₂. However, the oxidative metabolism of *C. glutamicum* produces CO₂ that could be recycled during the succinate production. Therefore, the CO₂ production has been assessed. In order to analyze CO₂ production by *C. glutamicum* 2262 *AldhA*, the bacterium was cultivated in different culture conditions. In the first conditions, *C. glutamicum* 2262 *AldhA* was grown in a 2 L bioreactor with MCGC containing 225 mM of glucose (pH 7.6, 33°C and 900 rpm), with a dissolved oxygen concentration regulated at 50 % of air saturation by air or pure oxygen injection (Figure III-6). For this aerobic condition, glucose was entirely consumed within 12 hours with a maximal biomass concentration of 18.7 g L⁻¹ (Figure III-6A). Simultaneously to the bacterial growth, the carbon dioxide production rate estimated from glucose consumption rate using Equation (III-13), increased exponentially and reached the maximal rate of 26 mM h⁻¹. This maximal production rate was obtained when biomass was also maximal (7 h of culture). In addition, the CO₂ production profile determined from CO₂ stripping equations as described in the methods section was in good accordance with theoretical estimation (Figure III-6B). The maximal rate determined through CO₂ desorption equations was also reached when biomass was maximal. However this maximal value showed a 34 % increase in comparison to the theoretical estimation.

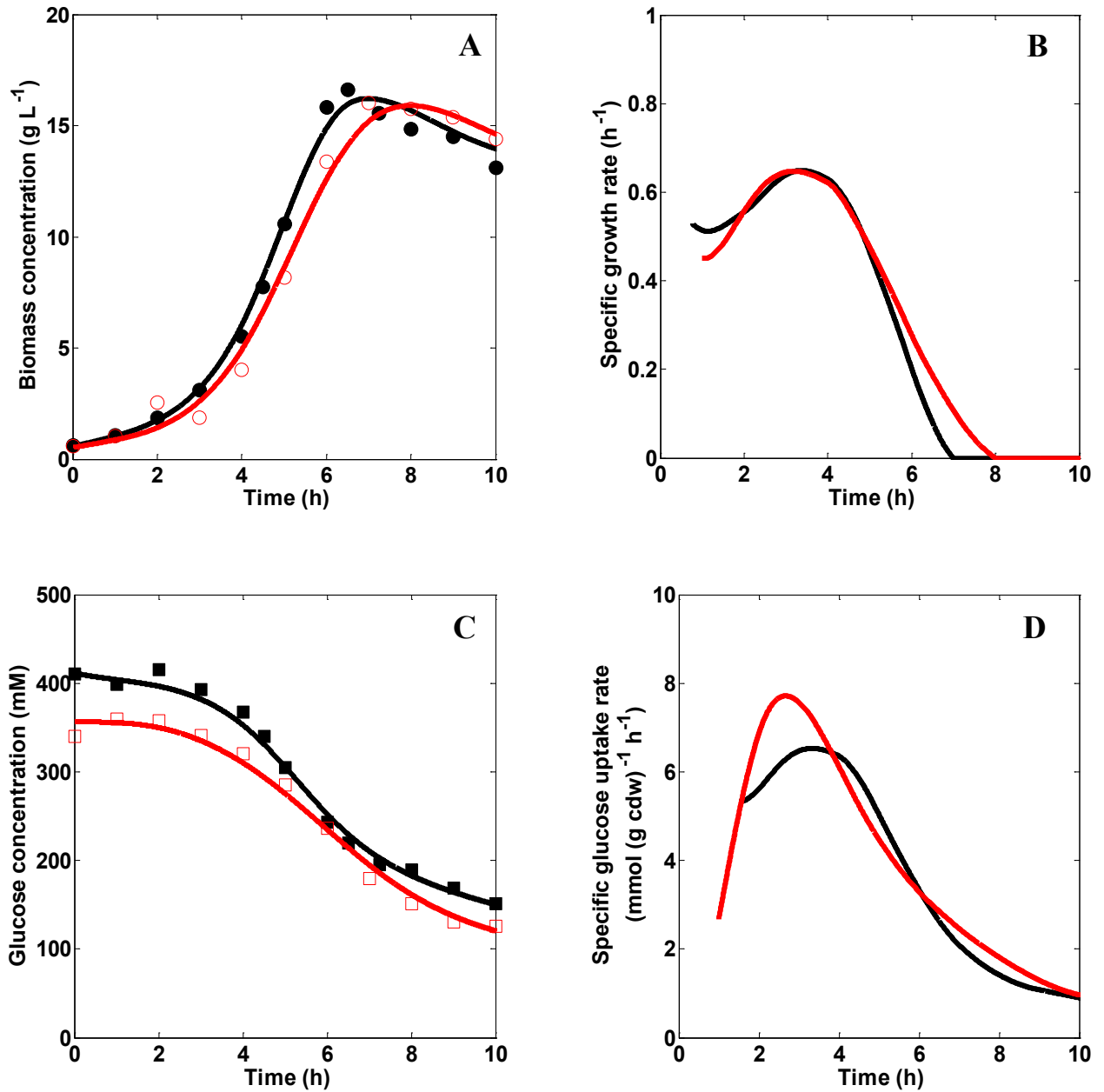


Figure III-5. Effects of NaHCO_3 addition on aerobic growth of *C. glutamicum* 2262 Δ ldhA. Kinetics of (A) growth, (B) specific growth rate, (C) glucose concentration and (D) specific glucose uptake rate. Open symbols/red color (without addition of NaHCO_3) and closed symbols/black color (with addition of 125 mM of NaHCO_3).

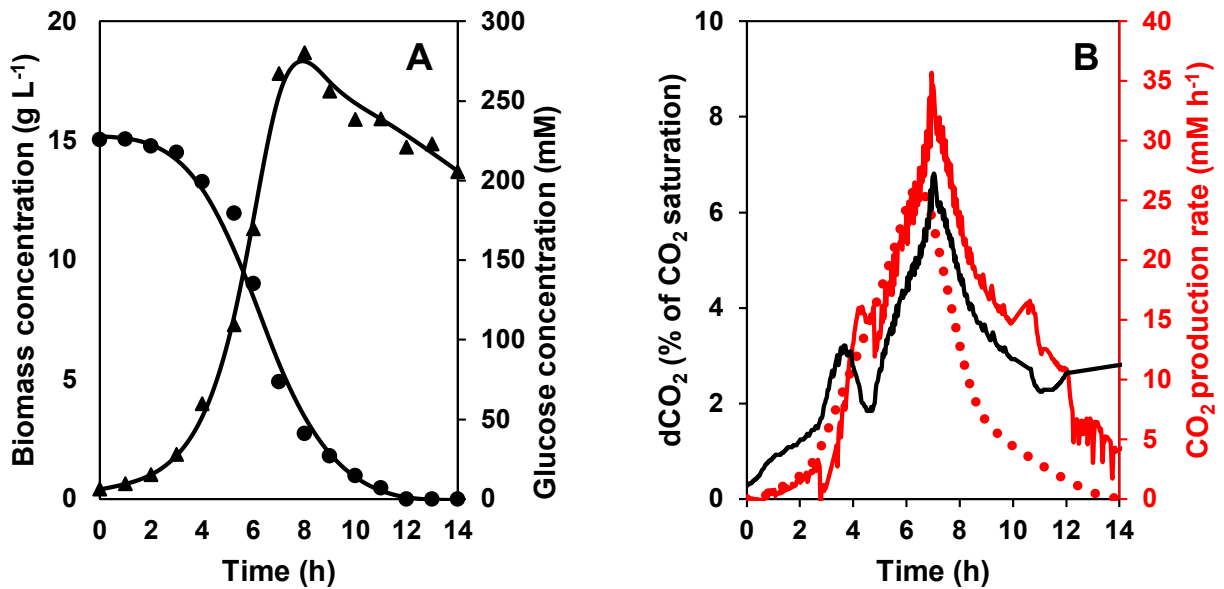


Figure III-6. (A) Time course of *C. glutamicum* 2262 *AldhA* growth and glucose consumption during aerobic culture in MCGC medium without addition of NaHCO₃. (B) Dissolved CO₂ concentration measured by probe (Black line) and carbon dioxide production rate calculated using CO₂ stripping rate (red line, Equation (III-10) and glucose consumption (red dotted line and Equation (III-13)).

The CO₂ production has been characterized in presence of sodium bicarbonate as the CO₂ donor for succinic acid production. *C. glutamicum* 2262 *AldhA* was cultivated in the same conditions as previously described but the MCGC medium was supplemented with 125 mM of NaHCO₃. Bacterial growth and glucose consumption were similar to those obtained in aerobic culture without addition of NaHCO₃, and maximal biomass concentration of 18.5 g L⁻¹ was obtained after 9 h of culture. The glucose has also been totally consumed within 10 hours (Figure III-7A). In Figure III-7B, the maximal CO₂ production rate calculated theoretically using Equation (III-13) or from CO₂ stripping quantification using Equation (III-10), was reached after 6-7 h of cultivation. Then, the CO₂ production decreased due to total exhaustion of residual glucose. The maximal rates of CO₂ production were about 60 and 32 mM h⁻¹, respectively calculated from Equations (III-10) and (III-13). Compared to culture without sodium bicarbonate, the CO₂ production was increased, mainly due to supplementation of the culture medium with NaHCO₃. Finally, in contrast to culture without NaHCO₃, Figure III-7B showed an increase in dissolved CO₂ concentration at 10 h of culture. At 10 h of culture, the growth was stopped due to glucose exhaustion and the rate of air injection, to maintain dissolved O₂ concentration at 50% of

saturation, was reduced drastically. Therefore, the CO₂ stripping was reduced resulting in the shift of equilibrium between bicarbonate ions and dissolved CO₂ causing the rise in dissolved CO₂ concentration observed in Figure III-7B.

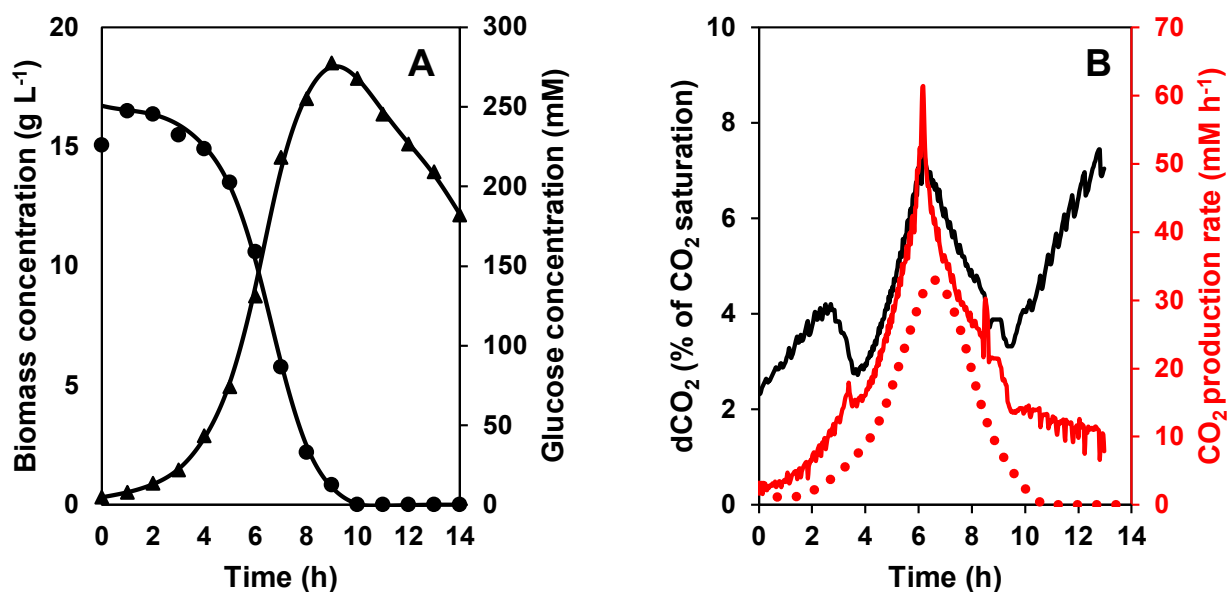


Figure III-7. (A) Time course of *C. glutamicum* 2262 *AldhA* growth and glucose consumption during aerobic culture in MCGC medium with addition of 125 mM of NaHCO₃. (B) Dissolved CO₂ concentration measured by probe (Black line) and carbon dioxide production rate calculated using CO₂ stripping rate (red line, Equation (III-10) and glucose consumption (red dotted line and Equation (III-13)).

III-3.5. Kinetics comparison of *C. glutamicum* 2262 *AldhA* and 2262 during a biphasic culture

It was previously demonstrated that *C. glutamicum* 2262 *AldhA* exhibited the same growth as the wild type strain under aerobic conditions. To analyze their organic acid production ability, the two strains were cultivated in a biphasic process consisting in a first aerobic growth phase followed by an anaerobic phase. *C. glutamicum* *AldhA* was cultivated in aerobic conditions in MCGC containing 125 mM NaHCO₃ until a biomass concentration ranging from 8 – 12 g cdw L⁻¹ was reached. Then, the aeration was stopped instantaneously and dissolved oxygen concentration dropped rapidly down to 0 % of air saturation. Bacterial growth was exponential until the aeration was stopped (Figure III-8A and C). The glucose was consumed at high rates of 6 and 7.5 mmol (g cdw)⁻¹ h⁻¹, respectively, for the mutant and the wild type strain during this exponential phase. When the culture was switched to anaerobic

conditions, growth ceased immediately and drastic reduction of glucose consumption was observed (Figure III-8A and C). The maximal CO₂ release rate was 10 mM h⁻¹ (Equation (III-13)) corresponding to 69 % decrease of the previous CO₂ production rate in presence of sodium bicarbonate. However, if the CO₂ production was reported to the amount of biomass, the decrease observed between the two cultures was only 28%. This observation was concordant since, the results displayed in Figure III-8A and C corresponded to cultures where the bacterial growth was stopped within 6 h.

The CO₂ production rate obtained from CO₂ stripping equations (Equation (III-10)), reached a maximum value of 15 mM h⁻¹ during the same time course. In experiments performed in presence of NaHCO₃, the difference observed between the two values of CO₂ production rates could be explained by bicarbonate supply; in fact, the calculation using metabolic mass balance took only in consideration the consumed glucose while CO₂ stripping model considered the dissolved CO₂ concentration which may be influenced by bicarbonate ions concentration depending also on the pH of the medium. When the aeration was stopped, the oxidative metabolism of bacteria reduced drastically, resulting in a sharp drop of the CO₂ production rate (Figure 6B). Finally, Figure III-8 B showed an increase in dissolved CO₂ concentration at 4-5 h of culture. At that moment, the shut-off of air bubbling resulted in CO₂ stripping stop; causing the shift of equilibrium between bicarbonate ions and dissolved CO₂ and the rise in CO₂ concentration observed (Figure III-8B).

During the aerobic growth phase, no organic acid production could be detected in the culture broth (Figure III-9). However, once the air bubbling was stopped, the residual dissolved oxygen has been consumed in less than 2 minutes, and the culture rapidly switched to anaerobic conditions. After 6-7 h of culture, organic acids production started for both strains. *C. glutamicum* 2262 produced up to 370 mM (33 g L⁻¹) of lactate while no lactate was detected for *AldhA* mutant strain (Figure III-9A and B). In these conditions 95 and 20 mM of succinate has been accumulated respectively by wild type and mutant strain (Figure III-9A and B). The cultures of wild type strain and mutant strain are not exactly similar. In fact at the switching point from aerobic to anaerobic conditions, the biomass concentration reached were different. Therefore, the specific succinate production was compared. After lactate dehydrogenase gene deletion, when cultivated under bi-phasic conditions, the mutant strain produced only 5.6 mmol of succinate per g cdw while the wild type strain produced 14.5 mmol per g cdw. From the results obtained, it is noteworthy that even with the lactate dehydrogenase gene deletion, the succinate production of the mutant strain remained fivefold lower than the wild type strain.

In the mutant strain, under the anaerobic conditions, lactate was not produced but the carbon flux was not redirected through succinate production pathway. In fact, under such condition, the glucose consumption by the mutant strain ceased few hours after aeration shut off (Figure III-9A).

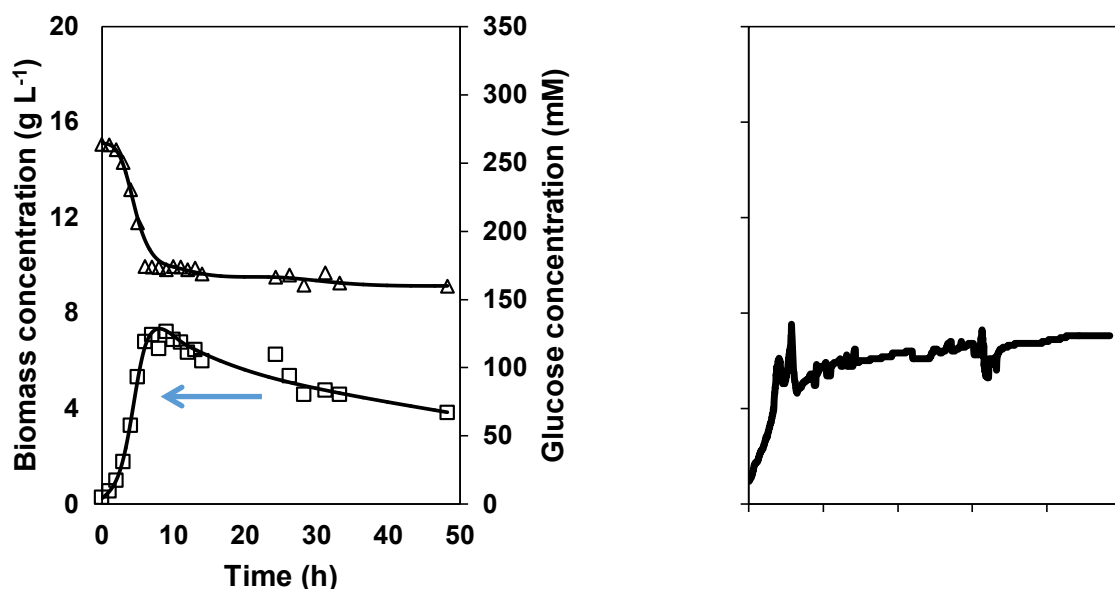


Figure III-8. (A) Kinetics of *C. glutamicum* 2262 Δ ldhA growth and glucose consumption. (B) Dissolved CO₂ concentration measured by probe during culture with Δ ldhA strain (black line) and carbon dioxide production rate calculated using CO₂ stripping model (red line and Equation (III-10)) or by metabolic mass balance (red dotted line and Equation (III-13)). (C) Kinetics of *C. glutamicum* 2262 growth and glucose consumption. The experiments have been performed in aerobic conditions (I) followed by anaerobic culture conditions (II) in MCGC medium complemented with 125 mM of NaHCO₃. Opened symbols (mutant strain), closed symbols (wild type strain).

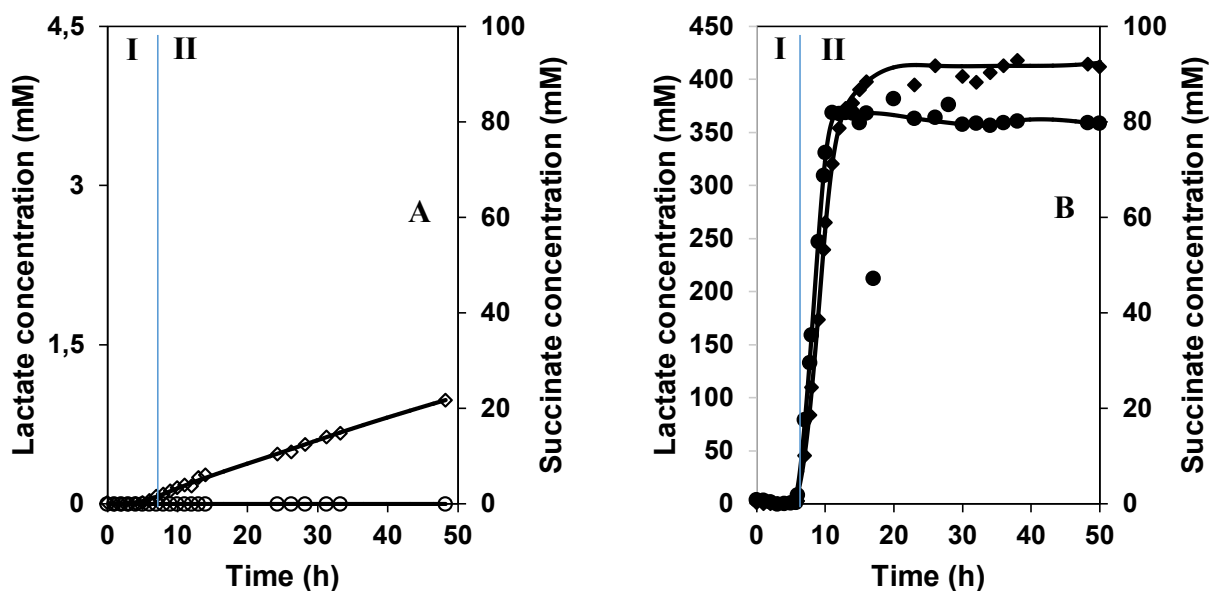


Figure III-9. (A) Lactate (circle) and succinate (diamond) production by *C. glutamicum* 2262 *AldhA*. (B) Lactate (circle) and succinate (diamond) production by *C. glutamicum* 2262. The experiments have been performed in aerobic conditions (I) followed by anaerobic culture conditions (II) in MCGC medium complemented with 125 mM of NaHCO_3 . Open symbols (*AldhA* mutant strain), closed symbols (wild type strain).

Table III-4. Summary of the CO_2 production in the different conditions performed

Cultures	Concentration (mM)		
	NaHCO_3	^(a) CO_2	^(b) Residual glucose
Figure 6A and B	0	178.2	-
Figure 7A and B	125	241.6	-
Figure 8A and B	125	28.4	174,3

^(a)Total CO_2 production estimated by determining the area under the curves obtained CO_2 stripping model using the method of trapezoids.

^(b) The amount of glucose at the moment when switching from growth to production phase.

From Table III-4, it appeared that during the aerobic growth, *C. glutamicum* 2262 *AldhA* produced 178 – 240 mM of CO_2 , meaning that equivalent amount of succinate could be produced by recycling the CO_2 . During the biphasic culture (Figure III-8), only 28 mM of CO_2 have been estimated through the stripping model. This amount has been produced during the

aerobic phase until the aeration was shut off. At this moment, the residual glucose concentration was 174 mM. According to stoichiometric production of succinic acid from glucose and CO₂, with 174 mM of residual glucose, at least 87 mM of CO₂ are required for optimal production. That means that in the biphasic process performed, the CO₂, from the aerobic phase is not enough. Thus, the addition of CO₂ donor such as bicarbonate is necessary. Finally, in the biphasic culture, there was 125 mM of NaHCO₃ combined with the CO₂ produced by cells but only 20 mM of succinate have been produced by *C. glutamicum* 2262 *AldhA* (Figure III-9A). In these conditions the CO₂ utilization seemed suboptimal.

In this chapter, lactate dehydrogenase deleted mutant was constructed and screened successfully. Through cultures in bioreactors, we have demonstrated that *ldhA* deletion did not affect growth performances. Indeed, *C. glutamicum* 2262 and *C. glutamicum* 2262 *AldhA* presented similar maximal growth rate of about 0.7 h⁻¹ with a maximal glucose uptake rate of 7.5 mmol (g cdw)⁻¹ h⁻¹ during aerobic culture. The *ldhA* deleted mutant has also been used to study and characterize the carbon dioxide production by *C. glutamicum* 2262 during aerobic conditions. For this purpose, CO₂ absorption and stripping were taken into account by establishing models for CO₂ stripping mass transfer coefficient calculations, related to aeration rates and the CO₂ production rates during a culture. In the three different culture conditions examined, the CO₂ production rate profiles estimated from equations were in satisfactory accordance with the theoretical profiles obtained through glucose uptake rates even if, in the different experiments, the maximum values of the former ones were higher than those obtained from equations of consumed glucose. From these equations for CO₂ production estimation in the given conditions, the potent amount of CO₂ that could be recycle have been estimated and could allow a succinate production up to 200 mM. Finally we evaluated the organic acids production ability of the *C. glutamicum* 2262 and *C. glutamicum* 2262 *AldhA* in a conventional biphasic process. It appeared that, even if lactate was not produced by the mutant strain, the glycolic flux was stopped instead of being redirected towards succinate production. Indeed, in the conditions of cultures, neither glucose, nor CO₂ was insufficient; the CO₂ utilization was not optimal according to calculations. It is noteworthy that, *C. glutamicum* 2262 compared to *C. glutamicum* R under similar anaerobic conditions, produces more succinate (Okino *et al.*, 2005); meaning that succinate production is specific to the strain 2262. Such observation could explain, in part, the relative low production obtain when *C. glutamicum* 2262 was deleted from *ldhA* gene. The further studies will thus focus on the development of enhanced process

operating conditions allowing the good utilization of CO₂ and the distribution of carbon flux towards the reductive pathways of the TCA cycle, with *C. glutamicum* 2262 *AldhA*.

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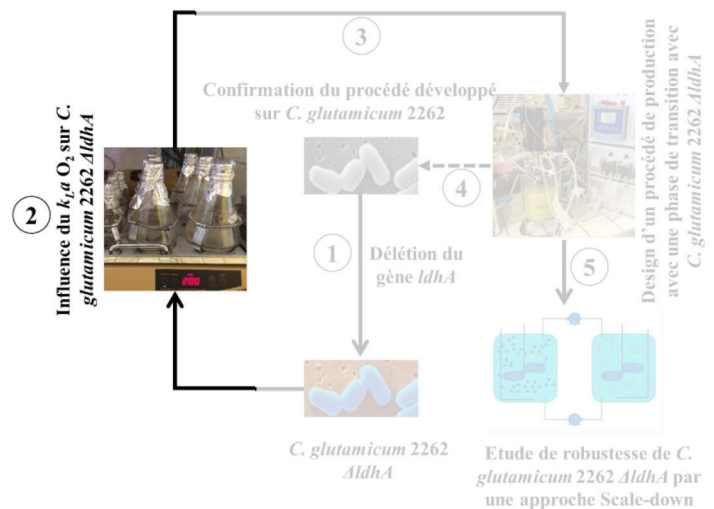
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Chapitre IV

Etudes cinétiques en fioles non-chicanées

Chapitre IV. Avant-propos

Chez *C. glutamicum*, la production d'acides organiques intervient en conditions de limitation en oxygène ou en conditions totalement anoxiques. Dans le chapitre III, une souche mutante *C. glutamicum* 2262 Δ *ldhA* a été construite. Lors d'un procédé classique de production d'acides organiques, la souche 2262 Δ *ldhA* a présenté de faibles performances de production de succinate par rapport à la souche parentale. Cependant la souche parentale produit principalement du lactate. Aussi, pour mieux comprendre l'influence des conditions d'oxygénation sur la production d'acides organiques (en particulier le succinate), le mutant *C. glutamicum* 2262 Δ *ldhA* est utilisé. Dans ce contexte, des cultures ont été réalisées en fioles d'Erlenmeyer lisses agitées sur table orbitale. En effet, ces fioles, qui sont largement utilisées dans le monde académique et industriel, sont un outil pratique pour un criblage rapide de plusieurs conditions opératoires (Garcia-Ochoa *et al.*, 2010). Dans ces fioles, l'oxygène est transféré par la surface libre du milieu de culture, sans bullage supplémentaire. Ce transfert est donc essentiellement conditionné par l'agitation imposée (fréquence d'agitation et diamètre orbital), le volume de remplissage et la taille de la fiole. En première approximation, il est possible de supposer que ce transfert reste relativement constant tout au long de la culture, ce qui, du fait de l'accroissement de la population bactérienne, induit généralement des limitations en oxygène dissous. Ainsi, des environnements dits de « micro-aérobiose » sont potentiellement observés, environnements que l'on pourrait définir par des concentrations nulles d'oxygène dissous mais sous un apport d'oxygène non-nul. Ces environnements pourraient être intéressants pour l'étude de la production d'acides organiques chez *C. glutamicum*, notamment l'identification des métabolismes attendus dans ces conditions de culture qui ne sont pas de l'anaérobiose *stricto sensu*. Pour relier la réponse physiologique et, notamment, la production d'acides organiques par *C. glutamicum* 2262 Δ *ldhA* aux capacités d'oxygénation des fioles, des systèmes non-invasifs pour la mesure en ligne de l'oxygène dissous et du pH dans ces fioles ont été utilisées. Ainsi, différentes conditions de k_{La} ont été imposées lors des cultures en fioles en verre lisses



et la réponse physiologique de *C. glutamicum* 2262 Δ *ldhA* a été analysée en termes de cinétiques de croissance, de consommation et de production.

Les résultats obtenus lors de ces études en fioles ont été présentés sous forme de publication et soumis dans le journal *Biochemical engineering Journal*. Cet article est actuellement au stade de revision.

IV. Impact of gas-liquid mass transfer on organic acids production by *Corynebacterium glutamicum* in unbaffled shake flasks.

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Abstract

Corynebacterium glutamicum is widely used for industrial production of amino acids. Recently, its ability to produce organic acids in oxygen-deprived or anaerobic conditions has also been demonstrated. In this study, the effects of oxygenation on *C. glutamicum* 2262 *AldhA* growth and organic acids production were investigated through cultures in shake flask with following gas-liquid mass transfer coefficients k_{La} conditions of 0.6, 3, 11, 19, 33, 36, 48, 77, 120 and 164 h⁻¹. These values were experimentally determined in unbaffled glass Erlenmeyer shake flasks equipped with Presens oxygen probes. The values were in very good agreement with similar values given in literature but using the sulfite chemical oxidation. Detailed growth, glucose consumption and organic acids production kinetics were presented and global kinetic parameters (growth and production rate or production yields) were related to the k_{La} values. The results showed that, increasing k_{La} values systematically increased growth performance, indicating growth-oxygen-limiting conditions. Our study also brought out that k_{La} values and thus oxygenation capacities of the flask were decisive regarding the fraction of carbon directed towards growth or organic acids production. Indeed, specific k_{La} ranges were observed for maximal (i) succinic acid concentration of 63 mM ($10 < k_{La} < 19$ h⁻¹), (ii) acetic acid concentration of 135 mM ($19 < k_{La} < 33$ h⁻¹) and (iii) biomass growth ($k_{La} > 164$ h⁻¹) were highlighted.

Keywords: *Corynebacterium glutamicum*, oxygen transfer, shake flask, organic acids production

List of symbols

ATP	Adenosine Triphosphate	(mol)
d	Flask diameter	(m)
d_0	Shaking diameter	(m)
cdw	Cell dry weight	(g)
$[Gluc]$	Glucose concentration	(M)
GUR	Glucose Uptake Rate	(mol L ⁻¹ h ⁻¹)
k_{La}	Gas-liquid mass transfer coefficient	(h ⁻¹)
μ	Specific growth rate	(h ⁻¹)
μ_{max}	Maximal specific growth rate	h ⁻¹
N	Shaking frequency	(s ⁻¹)
$[O_2]$	Dissolved oxygen concentration	(mol L ⁻¹)
$[O_2]^*$	Dissolved oxygen concentration at saturation	(mol L ⁻¹)
OUR	Oxygen Uptake Rate	(mol L ⁻¹ h ⁻¹)
q_{max}^{OA}	Maximal specific organic acids production rate	(g.(g cdw h) ⁻¹)
q_{max}^{gluc}	Maximal specific glucose uptake rate	(g.g cdw) ⁻¹ .h ⁻¹)
t	Time	(s)
V_L	Filling volume	(L)
V_T	Flask maximal volume	(L)
\underline{X}	Biomass concentration	g L ⁻¹
X_{max}	Maximal biomass concentration	g L ⁻¹
Y_{max}^{OA}	Maximal production yield of organic acids from substrate	(mol mol ⁻¹)
$Y_{ATP/gluc}$	Yield of ATP production from substrate	(mol mol ⁻¹)

IV-1. Introduction

Butanedioic acid, also known as succinic acid, is a dicarboxylic acid that is a common metabolite in microorganisms, plants and animals. It can be used as building-block for the production of molecules such as tetrahydrofuran, maleic anhydride and 1,4-butanediol, for its tensioactive properties or as additive in the food or chemical industries (Wieschalka *et al.*, 2013a). Due to the economic and ecological necessity of using renewable carbon sources and less polluting industrial processes, white biotechnologies are becoming valuable solutions for

the production of molecules which were traditionally synthesized using oil-derived resources (Zeikus *et al.*, 1999; Willke and Vorlop, 2004; Song and Lee, 2006). For instance, it was shown that the wild-type strain of *Corynebacterium glutamicum* R was able to synthesize succinate under oxygen-limited conditions (Inui *et al.*, 2004a; Okino *et al.*, 2005). However, to be competitive, the succinate production process using this microorganism has to reach the best compromise between an efficient and rapid growth, implying the use of fully aerobic culture conditions (meaning a sufficiently high concentration of dissolved oxygen) and succinate production in oxygen-limited culture conditions. Besides the use of more and more sophisticated genetically modified strains of *C. glutamicum* (Okino *et al.*, 2008a; Litsanov *et al.*, 2012b) the optimization of the production process, from a chemical engineering point of view, appears as a valuable complementary approach for the intensification of succinate production. For instance, to increase the production of succinate, Okino *et al.* (2008) used a two-step process: during the first step, a sufficient quantity of biomass was produced in fully aerobic conditions. The cell suspension was then centrifuged and the harvested biomass was placed in non-aerated conditions (Okino *et al.*, 2008a), promoting the production of organic acids and, notably, succinate (step 2). Another strategy consisted in a one-step process in which growth and production were simultaneously managed under aerobic conditions (Litsanov *et al.*, 2012a). For both culture strategies, the management of the transition from fully aerobic to anaerobic culture conditions was suggested to be a key step to optimize succinate production (Wieschalka *et al.*, 2013a). This was confirmed by Blombach and Eikmanns (Blombach and Eikmanns, 2011) and Blombach *et al.* (2011) during the iso-butanol production process by *C. glutamicum* (Blombach *et al.*, 2011) or during the succinate production process by *Escherichia coli* (Martínez *et al.*, 2010). In this last study, it was shown that the transition from fully aerobic to anaerobic phase had a strong impact on the ability of the microorganism to efficiently switch its enzymatic machinery from one metabolism to another. On the basis of these previous observations, it is thus worthwhile studying more intensively the impact of oxygenation conditions on the production of succinate by *C. glutamicum*.

For strict aerobic microorganisms, oxygen limitation and / or anaerobiosis may result in drastic effects on cell physiology and fermentation kinetics, similarly to nutrient limitation. When oxygen becomes limiting in the culture broth, microorganisms may respond by modifying their internal metabolism (Seletzky *et al.*, 2007; Suresh, 2009). In general, the central metabolism is known to display important differences under fully aerobic and oxygen-limited conditions. In fact, high activity in the respiratory chain is associated with high glucose uptake and utilization in aerobic bacteria. Besides, oxygen limitation results in cell respiration reduction and in

intracellular pyruvate accumulation which will be further converted to anaerobic fermentative byproducts (Mantzouridou *et al.*, 2005; Zimmermann *et al.*, 2006). As organic acids production by *C. glutamicum* was related to oxygen limited conditions in recent studies (Inui *et al.*, 2004a; Okino *et al.*, 2005), (Wendisch *et al.*, 2006; Khuat *et al.*, 2014), the physiological maximum oxygen consumption of the microorganisms should exceed the maximum amount of oxygen delivered by the gas–liquid mass transfer, promoting this production.

To get further insight in the impact of oxygenation conditions on succinate production by *C. glutamicum*, a one-step culture process, undertaken in shake flasks, was chosen in the present study. Indeed, shake flasks show numerous advantages for rapid screening of bioprocess conditions, specific activities of strains, mutant selection, medium characterization, metabolic pathway studies (Clark and Bushell, 1995; Büchs, 2001; John *et al.*, 2003; Garcia-Ochoa *et al.*, 2010). Interestingly, insufficient oxygen supply remains a frequent problem associated with the application of shake flasks (Büchs, 2001; Liu *et al.*, 2006); this drawback should be thus advantageously used in the present study. Indeed, during their culture in shake flask, microorganisms should switch progressively from an aerobic to an anaerobic metabolism, according to the oxygen transfer rate and to the oxygen uptake rate expected. This oxygen limited environment could lead to byproducts excretion which could, in return, affect the cells metabolism.

In shake flasks, the rotary and reciprocating action of the shaker apparatus achieve oxygen transfer through the gas-liquid free surface and then transport in the culture medium. The oxygen transfer process in the flask strongly depends on the liquid volume, flask size, shaking diameter and agitation rate of the table. As in mechanically stirred and sparged bioreactors, the quantification of oxygenation capacities relies on the determination of gas-liquid mass transfer coefficient (k_{La}), which relates the driving force ($[O_2]^* - [O_2]$) to the oxygen transfer rate (OTR) (Quesada-Chanto *et al.*, 1998, 12; Maier and Büchs, 2001). This coefficient has been measured in shake flasks using conventional systems equipped with sensor spots for online dissolved oxygen monitoring or using the sulfites reaction method (John *et al.*, 2003; Wittmann *et al.*, 2003; Schneider *et al.*, 2010; Schiefelbein *et al.*, 2013). Moreover, mathematical models and empirical correlations have also been used for gas-liquid mass transfer coefficient modeling (Henzler and Schedel, 1991; Maier and Büchs, 2001; Nikakhtari and Hill, 2005; Seletzky *et al.*, 2007). However, no literature data linking organic acids production by *C. glutamicum* and oxygen transfer in shake flasks could be found. Thus, in this study, the effects of oxygenation on *C. glutamicum* growth and organic acids production during cultures in unbaffled shake flasks were investigated by changing culture conditions (flasks size, filling volume, shaking rate).

Oxygenation was quantified by the determination of the gas-liquid mass transfer coefficient (k_{LA}) imposed to the shake flasks cultures while bioprocess efficiency was notably characterized in terms of growth rate, organic acids production yields and specific consumption rates. Original relationships coupling the physiological response of *C. glutamicum* and k_{LA} were then proposed and discussed in detail.

IV-2. Material and methods

IV-2.1. Bacterial strain, culture media and protocol.

Batch cultures of *Corynebacterium glutamicum* 2262 lactate dehydrogenase deleted mutant (*AldhA*) were performed in a modified MCGC mineral salt medium in which citrate was replaced by deferoxamine. Final concentrations of nutrients are indicated in Table IV-1. Basis salts A and B and urea were prepared separately and autoclaved for 20 min at 121°C. Vitamins solution was sterilized by filtration through 0.22 μm filter (Millex, Millipore, France). After sterilization, the different solutions were mixed together in accordance to the final concentrations mentioned in Table IV-1. The mutant strain was constructed via a two-step homologous recombination procedure using the vector pK19*obsacBAldh* (Litsanov *et al.*, 2012b). This plasmid was constructed using the suicide vector pK19*obsacB* as described by Schäfer *et al.* (Schäfer *et al.*, 1994). The culture medium was supplemented with 155 mM of glucose which was thus the sole carbon and energy source initially present in the medium. A first preculture (A) of *C. glutamicum* 2262 *AldhA* was performed in a 500 mL flask containing 50 mL of MCGC. Temperature and shaking rate of precultures and cultures were respectively 33°C and 200 rpm. When the optical density at 570 nm reached the value of 60 approximately, 5 mL of preculture (A) were used as inoculum for the second preculture (B) performed with the same culture medium than preculture A. When the optical density at 570 nm of preculture (B) reached a value of 30, bacterial growth was stopped by immersing the flask in 70 % v/v ethanol, at a temperature of -20°C; fermentation broth was then kept at a temperature of 4°C for 24 h maximum. Preculture B was used for the whole culture experiments. All the cultures were then performed in glass unbaffled shake flasks placed in a temperature-controlled ($T^\circ = 33^\circ\text{C}$) shaker during the cultures. A more efficient gas-liquid mass transfer should be expected in baffled shake flasks and thus should better prevent from oxygen limitations in the liquid phase. However, in baffled shake flasks, a more important variability of operating conditions may be encountered due to small variations of baffle geometry, to medium splashing on the flask wall

and on the cotton plug. Gas-liquid mass transfer modelling is also more complex than in un baffled shake (Büchs, 2001), thus justifying the use of un baffled shake flask in the present study. Shaking rate varied between 0 and 350 rpm and shaking diameter d_0 was equal to 2.5 cm. All culture conditions are given in Table IV-2.

Table IV-1. Composition of modified MCGC medium.

	Compounds	Final concentration (mg.L ⁻¹)
<i>Basis Salts</i>	Na ₂ HPO ₄ 12H ₂ O	36,720
	KH ₂ PO ₄	2,880
	NaCl	960
	(NH ₄) ₂ SO ₄	3,840
<i>Salts A</i>	MgSO ₄ 7H ₂ O	455.84
	FeSO ₄ 7H ₂ O	45.58
	FeCl ₃	4.56
<i>Salts B</i>	ZnSO ₄ 7H ₂ O	1.14
	CuCl ₂ 2H ₂ O	0.46
	MnSO ₄ H ₂ O	4.56
	(NH ₄) ₆ Mo ₇ O ₂₄ 4H ₂ O	0.23
	Na ₂ B ₄ O ₇ 10H ₂ O	0.46
<i>Vitamins</i>	CaCl ₂	94.85
	Biotin	2.28
	Thiamine	22.79
	Deferoxamine	3.42
	Betaïn	1,937.32
<i>Substrate</i>	Urea	3,900
	Glucose	27,900

IV-2.2. Determination of the gas-liquid mass transfer coefficient k_{LA}

In this paper, k_{LA} was determined in glass un baffled shake flasks equipped with non-invasive optical dissolved oxygen sensors and PreSens shake-flask reader (SFR) (Precision Sensing GmbH, Regensburg, Germany) described by Schneider et al. (Schneider *et al.*, 2010). The flask

was installed in an orbital shaker with 2.5 cm shaking diameter and the dissolved oxygen sensor was calibrated according to the SFR user manual. The determination of k_{LA} in the culture medium was performed by the gassing-out method. After degassing the culture medium with nitrogen, the shake flasks were placed in shaker and agitation was started according to the different values of k_{LA} desired. Dissolved oxygen level was recorded by SFR as a function of time. The values of k_{LA} were given by the slope of the logarithmic plot of $\left(\frac{[O_2]^*}{[O_2]^* - [O_2]}\right)$ versus time according to the equation IV-1

$$\ln\left(\frac{[O_2]^*}{[O_2]^* - [O_2]}\right) = k_{LA} \cdot t \quad \text{IV-1}$$

Where $[O_2]^*$ corresponds to value of dissolved oxygen at air saturation, $[O_2]$ to the value of dissolved oxygen measured at each sampling time and t to time course.

IV-2.3. Determination of global kinetic parameters

To quantify the culture performance in terms of growth, uptake and production, global kinetic parameters were calculated for each experiment. Given the biomass concentration X , the maximal specific growth rate was provided by:

$$\mu_{max} = \max_t \left(\frac{1}{[X]_f} \cdot \frac{d[X]_f}{dt} \right) \quad \text{IV-2}$$

With $[X]_f$ the biomass concentration obtained by fitting experimental data using smooth splines interpolation model. Maximal specific glucose q_{max}^{gluc} consumption and organic acids (OA) q_{max}^{OA} production rates were respectively calculated following:

$$q_{max}^{gluc} = - \max_t \left(\frac{1}{[X]_f} \cdot \frac{d[gluc]_f}{dt} \right) \quad \text{IV-3}$$

$$q_{max}^{OA} = \max_t \left(\frac{1}{[X]_f} \cdot \frac{d[OA]_f}{dt} \right) \quad \text{IV-4}$$

With $[gluc]_f$ and $[OA]_f$ respectively the fitted glucose and organic acids concentrations using smooth spline interpolation model. Finally, maximal yields of organic acids and biomass production on glucose concentration Y_{max}^{OA} were respectively calculated by the ratio of maximal, concentration of organic acids ($[OA]_{max}$) and biomass (X_{max}) to the quantity of glucose consumed to reach these maximal concentrations.

From the k_La determination, the oxygen uptake rate (OUR) could also be calculated using:

$$OUR = k_La([O_2]^* - [O_2]) - d[O_2]/dt \quad \text{IV-5}$$

The values of dissolved oxygen saturation was set to $[O_2]^* = 0.232$ mM (Schiefelbein *et al.*, 2013).

IV-2.4. Analytical methods

For each experiment, biomass concentration was determined by the measurement of absorbance at 570 nm with a Hitachi U2000 spectrophotometer (Sciencetec, les Ulis, France) and was transformed into cell dry weight (cdw) using the coefficient as: dry cell concentration (g L^{-1}) = $0.27 \times OD_{570}$. Before every sampling, the culture broth in the different shake flasks was mixed. Samples of culture broth were first centrifuged at $5,000 \times g$ for 10 min and at 4°C . Glucose and organic acids (succinate, lactate, acetate, pyruvate and fumarate) concentrations were analyzed in the supernatants by High Pressure Liquid Chromatography (10ADVP, Shimadzu) using an ion-exchange column (7.8 mm \times 300 mm, Bio-Rad Aminex HPX-87H) and SPD-M10A chromatography working station system equipped with UV absorbance and refractive index detectors water 410 (Shimadzu). The column was eluted isocratically at a rate of $0.7 \text{ mL} \cdot \text{min}^{-1}$ with 5 mM H_2SO_4 for sugar analysis and 50 mM H_2SO_4 for organic acids at a temperature of 50°C . The approximate retention time for glucose, pyruvic, succinic, lactic, acetic and fumaric acids were respectively 7.7, 9.2, 10.2, 10.9, 13.1 and 13.5 min.

IV-3. Results

IV-3.1. Gas-liquid mass transfer coefficients determination

The experimental values of gas-liquid mass transfer coefficients obtained using the static method for all operating conditions were reported in Table IV-2. A particular operating

condition (static flask i.e. $N=0$ rpm) was also added in the set of conditions and will be considered as representative of negligible oxygen transfer. For this condition, gas-liquid mass transfer was purely diffusive, explaining the close-to-zero value obtained (0.6 h^{-1}). Our results indicated that the operating conditions used allowed getting a broad range of k_{La} values from 0.6 to 164 h^{-1} .

Table IV-2. Gas-liquid mass transfer coefficient (k_{La}) determined from static method using shake flasks equipped with Presens sensor for all conditions used in the different experiments and in fresh culture medium. Shaking diameter was 2.5 cm for all conditions.

Shaking rate (rpm)	Flask maximal volume V_T (mL)	Filling volume V_L (mL)	Flask diameter d (cm)	Experimental k_{La} (h^{-1}) ^a
0 (static)	250	100	8	0.6 (0)
100	250	170	8	3 (5)
170	500	250	8	11 (11)
170	250	75	8	19 (20)
350	500	200	10	33 (31)
350	250	100	8	36 (36)
350	1000	200	12	48 (44)
350	250	40	8	77 (77)
350	500	40	10	120 (118)
350	1000	40	12	164 (168)

^a k_{La} values in parentheses were calculated with Eq. (IV-7).

IV-3.2. Effect of gas-liquid mass transfer coefficient on growth

To study the impact of gas-liquid mass transfer on biomass growth, cultures of *C. glutamicum* ATCC 2262 ΔdhA were performed in unbaffled shaking flasks under various values of k_{La} , as indicated in Table IV-2. During these cultures, it was noticed that microbial growth was significantly impacted by gas-liquid mass transfer as increasing k_{La} values systematically improved growth performance (quantified by specific growth rate and maximal biomass concentration). Indeed, as shown in Figure IV-1, maximal biomass concentration varied from 1.2 g cdw L^{-1} without flask shaking ($k_{La} \sim 0.6 \text{ h}^{-1}$) to $12.8 \text{ g cdw L}^{-1}$ for the highest gas-liquid mass transfer coefficient tested ($k_{La} = 164 \text{ h}^{-1}$). Moreover, the stationary phase was reached in a delayed manner for low k_{La} values. For instance, while biomass growth ended after 10 to 13 h of culture for a gas-liquid mass transfer coefficient higher than 44 h^{-1} , the growth arrest was

delayed to more than 20 h for k_{LA} inferior to 33 h^{-1} . The Figure IV-1B, provides time courses of dissolved oxygen concentrations recorded during the cultures for selected k_{LA} values (only three representative cases of dissolved oxygen profile were chosen). From this figure, it can be seen that, for all the cultures, dissolved oxygen concentrations reached zero values, which is commonly referred as oxygen limitation. This limitation appeared from the first minutes in culture with k_{LA} of 33 h^{-1} while for the highest k_{LA} condition (164 h^{-1}), the oxygen limitation appeared about 3.5 h after the culture started.

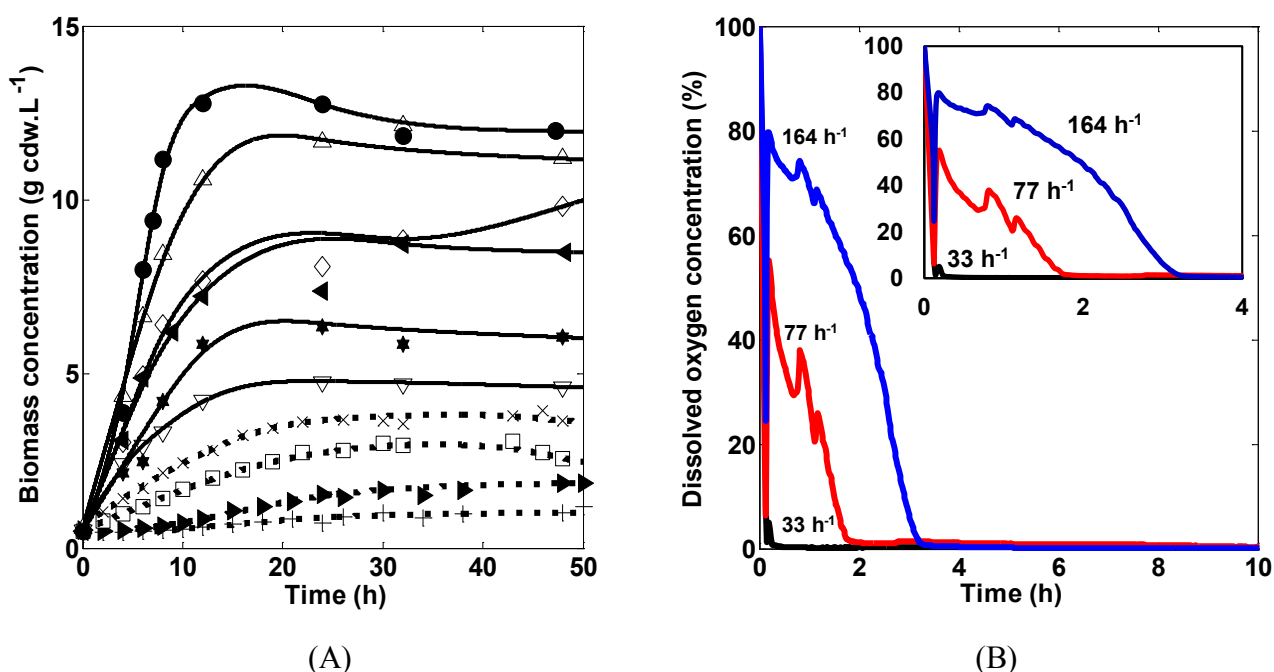


Figure IV-1. (A). Growth kinetics of *C. glutamicum* 2262 Δ ldhA. Values of k_{LA} are $0.6 (+)$, $3 (\blacktriangleright)$, $11 (\square)$, $19 (\times)$, $33 (\nabla)$, $36 (\star)$, $48 (\diamond)$, $77 (\blacktriangleleft)$, $120 (\Delta)$ and $164 \text{ h}^{-1} (\bullet)$. (B) Dissolved oxygen concentration profiles during cultivations for $k_{LA} = 33, 77$ and 164 h^{-1} .

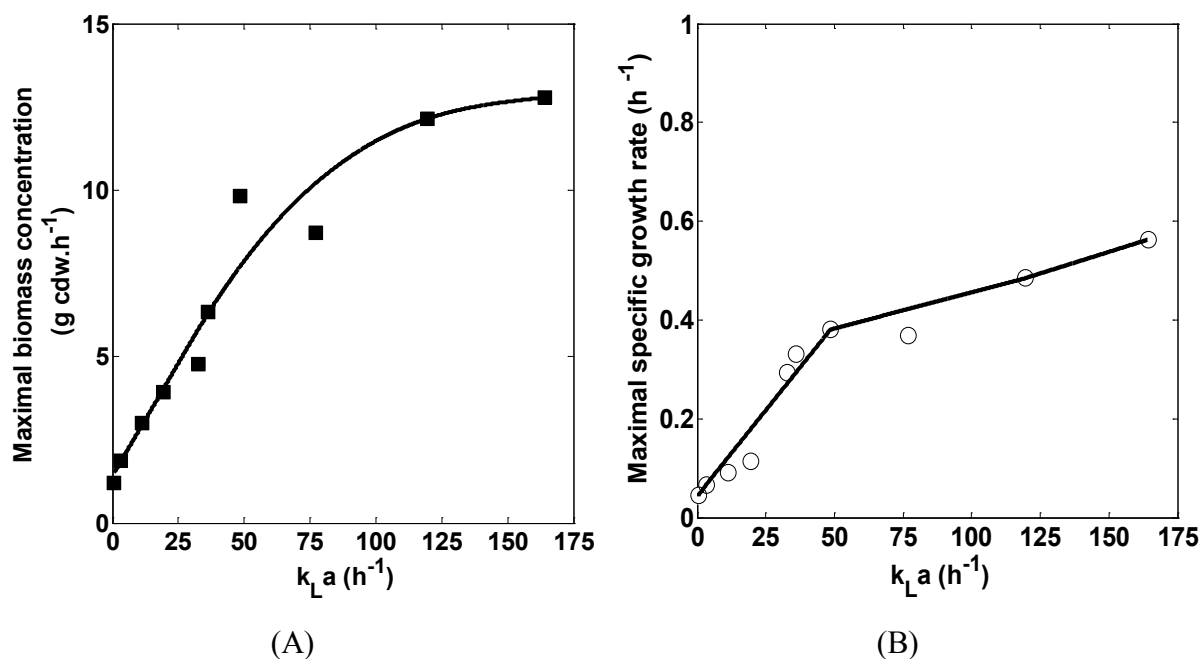


Figure IV-2. Relationships between gas-liquid mass transfer coefficient and maximal biomass concentration (A) and maximal specific growth rate (B) of *C. glutamicum* 2262 $\Delta ldhA$.

Relationships between the maximal biomass concentration X_{max} , maximal growth rate μ_{max} and k_{La} determined for each experiment were reported in Figure IV-2. As indicated in Figure IV-2A, a linear correlation was first observed between maximal biomass concentration and k_{La} from 0.6 to 36 h⁻¹. At higher k_{La} values (> 50 h⁻¹), the maximal biomass concentration was tending towards a plateau, suggesting that gas-liquid mass transfer became progressively sufficient to support maximal cell growth capacities but without reaching the asymptotic values in the range of operating conditions tested. This result was also shown in the case of the maximal specific growth rate (Figure IV-2B) with a strong impact of k_{La} on μ_{max} for k_{La} lower than 50 h⁻¹ whereas a slighter impact for higher oxygen transfer capacities was observed.

Combining oxygen profiles and k_{La} values (Equation IV-5), the maximal oxygen uptake rate OUR_{max} was found to vary from 7.4 to 38 mM h⁻¹ when k_{La} increased from 32 to 164 h⁻¹.

IV-3.3. Effect of gas-liquid mass transfer coefficient on glucose consumption

During the experiments, glucose was depleted before the end of the culture in the conditions of higher k_{La} (36, 48, 77, 120 and 164 h⁻¹) while residual glucose concentrations measured in supernatant of culture were 7, 14, 35, 85 and 124 mM for k_{La} values of 33, 19, 11, 3 and 0.6 h⁻¹, respectively (Figure IV-3A). Despite these differences, the maximal specific consumption

rate of glucose q_{max}^{gluc} was approximately maintained constant to $0.5 \text{ g (g cdw h)}^{-1}$ as far as k_{LA} values were non-zero (Figure IV-3B).

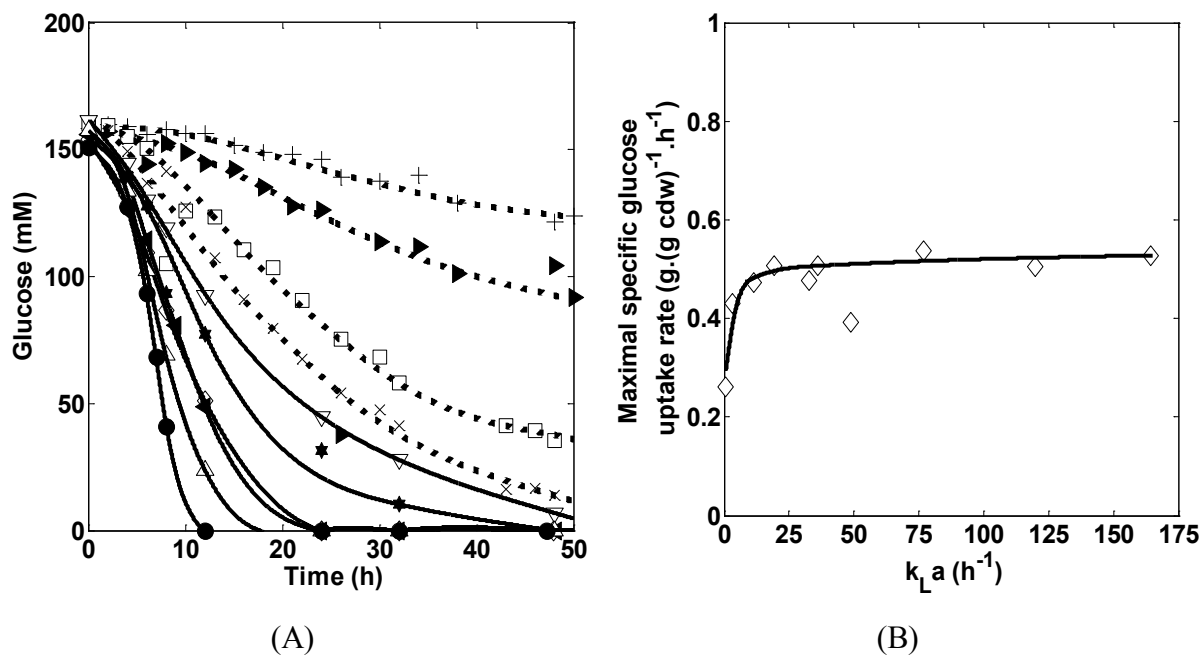


Figure IV-3. (A) Kinetics of glucose consumption and (B) maximal glucose uptake rate of *C. glutamicum* 2262 $\Delta ldhA$ for k_{LA} values of 0.6 (+), 3 (\blacktriangleright), 11 (\square), 19 (\times), 33 (∇), 36 (\star), 48 (\diamond), 77 (\blacktriangleleft), 120 (\triangle) and 164 h^{-1} (\bullet).

IV-3.4. Effect of gas-liquid mass transfer coefficient on organic acids production

Kinetics of production of succinate and acetate were reported in Figure IV-4. The concentrations of other organic acids (fumarate and pyruvate) were negligible ($< 4 \text{ mM}$) compared to succinate and acetate concentrations and were thus not discussed in the present paper. Furthermore, no lactate production was measured, confirming the deletion of the gene encoding the lactate dehydrogenase. All operating conditions led to succinic and acetic acid production, except for the static culture condition (no acetic acid production) and the highest value of k_{LA} (no succinic acid production). When it occurred, organic acids production started systematically during the first hours of cultures, independently of the gas-liquid mass transfer coefficient values. For the lowest k_{LA} values ($0.6, 3$ and 11 h^{-1}), the apparent succinic acid concentration kept increasing during all the culture while it decreased for $k_{LA} > 19 \text{ h}^{-1}$. The same

observation could be made with acetic acid concentration profiles which showed similar trends except that an acetate concentration decrease was observed only for $k_{La} > 33 \text{ h}^{-1}$. A decrease in organic acid concentrations corresponded to a consumption by *C. glutamicum* 2262 *AldhA* and was clearly related to the glucose depletion in the culture medium.

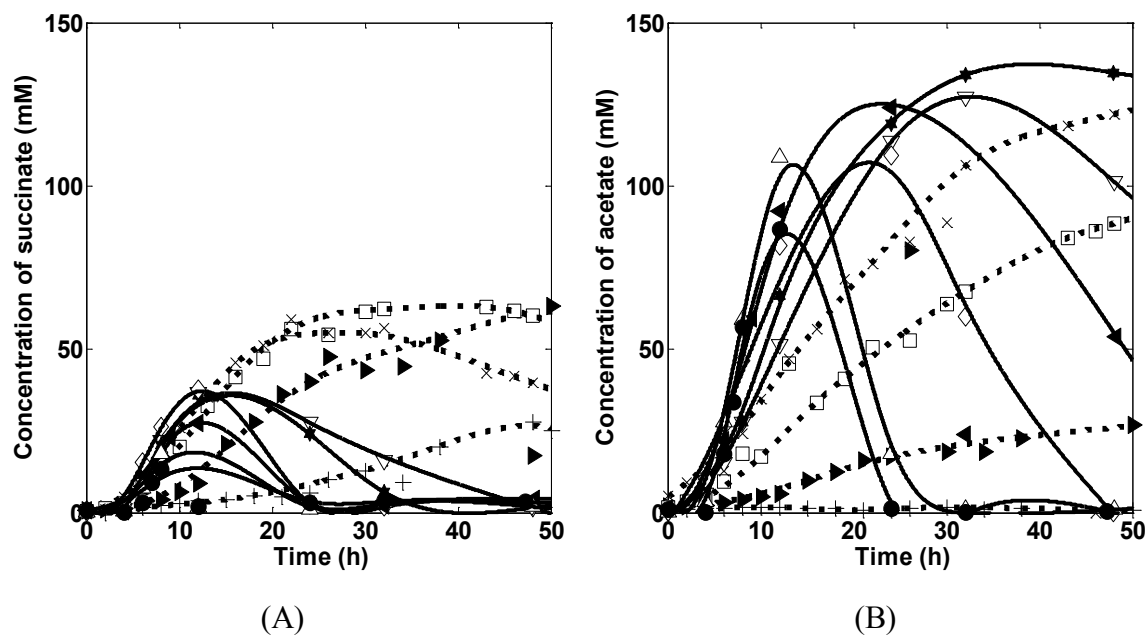


Figure IV-4. Kinetics of succinic acid (A) and acetic acid (B) production by *C. glutamicum* 2262 *AldhA* for k_{La} values of 0.6 (+), 3 (▶), 11 (□), 19 (×), 33 (∇), 36 (★), 48 (◇), 77 (◄), 120 (Δ) and 164 h^{-1} (●).

Consequently, oxygenation capacities within the shake flask had an impact on the maximal concentration of organic acids produced (Figure IV-5). Indeed, while experiments realized for k_{La} values increasing from 0 to 36 h^{-1} resulted in a maximal acetate concentration increase from 1.7 to 135 mM, a decrease in acetic acid concentrations from 135 to 87 mM was measured when k_{La} increased from 36 to 164 h^{-1} (Figure IV-5A). It is also important to note that acetic acid is the main fermentation product as the concentrations measured were 2 to 10 folds higher than succinic acid concentrations. In fact, the maximal concentrations of acetic and succinic acids were, respectively, 135.6 mM for a k_{La} of 36 h^{-1} and 62.9 mM for a k_{La} of 11 h^{-1} . It is noteworthy that the lowest succinic acid concentration was obtained with the highest gas-liquid mass transfer coefficient (164 h^{-1}) (Figure IV-5A). Indeed, excluding the culture without shaking ($k_{La} \sim 0.6 \text{ h}^{-1}$) the specific production rate of succinic acid seemed to be negatively influenced by a k_{La} value increase (Figure IV-6). The maximal specific production rate of acetate rose sharply

till k_{LA} of 33 h^{-1} , its value remained unchanged for higher gas-liquid mass transfer coefficients (Figure IV-6).

As expected, the conversion of glucose into biomass increased with the k_{LA} value. The highest biomass from glucose yield was calculated at the highest k_{LA} value (164 h^{-1}) (Figure IV-5B).

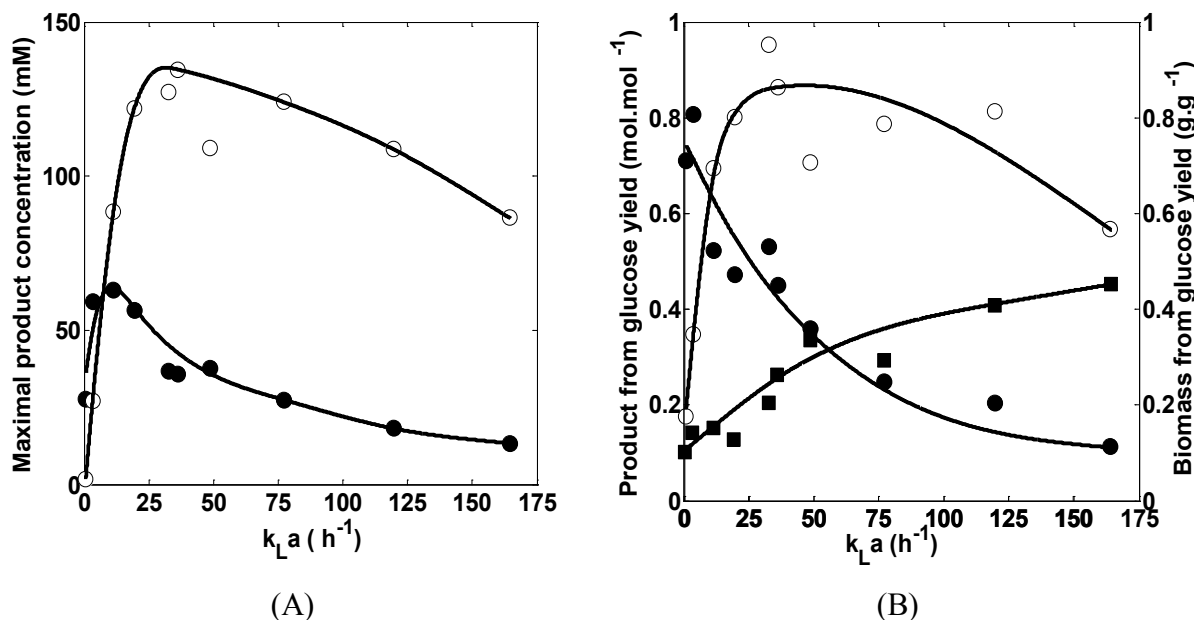
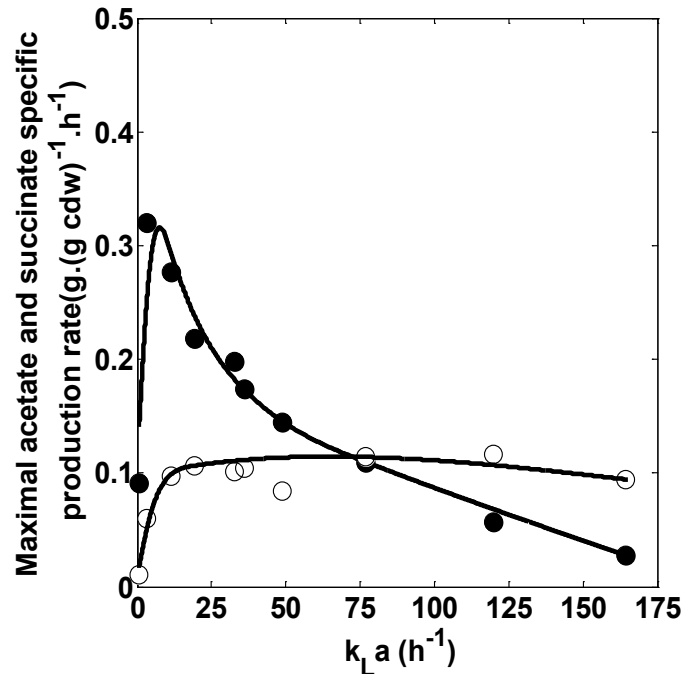


Figure IV-5. Relationship between oxygen mass transfer coefficient and organic acids production by *C. glutamicum* 2262 Δ ldhA. (A) Maximal products concentration. (B) Maximal production yields from glucose. Symbols: succinate (\bullet), acetate (\circ) and biomass (\blacksquare)

From the main products accumulated during cultivation and for all k_{LA} conditions, it appeared that the k_{LA} conditions ranging from 3 to 15 h^{-1} (in the case of succinic acid) and from 15 to 33 h^{-1} (in the case of acetic acid) also resulted in high product concentrations. Based on these observations, product to substrate yields were determined in order to analyze the differential use of the carbon source (Figure IV-5B). The highest yield of succinic acid production of $0.8 \text{ mol}_{\text{succ}}/\text{mol}_{\text{gluc}}$ was obtained when k_{LA} was low (3 h^{-1}). An increase in k_{LA} values resulted in a decrease in maximal succinate yields. In fact, for the highest k_{LA} studied, the lowest production yield of $0.1 \text{ mol}_{\text{succ}}/\text{mol}_{\text{gluc}}$ was obtained. The same result was observed with acetic acid production. Indeed, for less oxygenated cultures (k_{LA} between 0.6 and 33 h^{-1}), acetic acid production yield increased until the highest value of $0.95 \text{ mol}_{\text{acet}}/\text{mol}_{\text{gluc}}$ was reached. For k_{LA} higher than 33 h^{-1} , acetate yield decreased slowly. Consequently, the lowest yield was obtained at k_{LA} value of 0.6 h^{-1} .



(A)

Figure IV-6. Relationship between oxygen mass transfer coefficient and organic acids production by *C. glutamicum* 2262 Δ ldhA. Maximal specific succinic (●) and acetic (○) acid production rates.

Finally, our results demonstrated that, while the maximal specific production rates of acetate was not sensibly influenced by k_{La} values (for $k_{La} > 10 \text{ h}^{-1}$), the maximal specific production rates of succinate continuously decreased from 0.3 to 0.03 $\text{g} \cdot (\text{g}_{\text{CDW}} \text{h})^{-1}$ when k_{La} increased from 3 to 164 h^{-1} , respectively (Figure IV-6).

IV-4. Discussion

In shake flasks, the gas-liquid mass transfer coefficient k_{La} is generally determined either by the sulfites oxidation chemical reaction (Seletzky *et al.*, 2007) or by optical methods implying, among others, the Presens system (Wittmann *et al.*, 2003; Schneider *et al.*, 2010; Schiefelbein *et al.*, 2013). Both methods are considered robust allowing an accurate measurement of k_{La} in baffled and unbaffled glass or plastic shake flasks. In unbaffled plastic shake flasks, for instance, the following model was recently established by Schiefelbein *et al.* (Schiefelbein *et al.*, 2013):

$$k_{La} = y_0 + a \cdot N + b \cdot V_T + c \cdot N^2 + d \cdot V_T^2 \quad \text{IV-6}$$

In Equation IV-6, N was the shaking rate, V_T the flask volume and the parameters y_0 , a , b , c and d depended on flask filling ratio. These results brought interesting information on k_La values in shake flasks but, as they were validated for an orbital shaking diameter of 5 cm and plastic flasks only, they could not be used in the case of a 2.5 cm shaking table and glass flasks as used in this study.

Using unbaffled glass shake flasks and the chemical sulfites method, Seletzky *et al.* (Seletzky *et al.*, 2007) proposed a relationship allowing the determination of k_La in unbaffled flasks for a broad range of operating conditions ($50 < N < 500$ rpm, filling ratios between 4 and 20 % of total volume, $1.25 < d_0 < 10$ cm and $50 < V_T < 1000$ mL):

$$k_La = 0.024 \cdot N^{1.16} \cdot V^{-0.83} \cdot d_0^{0.38} \cdot d^{1.92} \quad \text{IV-7}$$

Where k_La is the volumetric gas-liquid mass transfer coefficient (h^{-1}), V (mL) the filling volume, d_0 (cm) the shaking diameter and d (cm) the maximal shake flask diameter. The values of the constant and of the exponents of Equation IV-7 are expected to be influenced by medium composition. However, it is worthwhile noticing that Büchs (Büchs, 2001) has shown that the gas-liquid mass transfer area a (which is equal to ratio between the free surface area and the liquid volume in the case of shake flasks) was a function of shaking operating conditions (flasks geometry, shaking rate, etc.) but not of medium composition. Moreover, Seletzky *et al.* (Seletzky *et al.*, 2007) have suggested that, due to the specific mechanisms of gas-liquid mass transfer in shake flasks, the influence of medium composition on the liquid-phase mass transfer coefficient k_L could be, as a first approximation, neglected. Thus, according to these authors, the accuracy of Equation IV-7 was 30 %. In previous works, Mehmood *et al.* used this correlation for calculation of the k_La value imposed to bacterial cultures (Mehmood *et al.*, 2010, 2011, 2012). However, these estimated values were not validated by experimental measurements. In the present work, the values obtained by this model were compared to experimental data reported in Table IV-2 and revealed that the relative differences were less than 10 % indicating a very good agreement between both determination methods. This also confirmed the low impact of medium composition on k_La values for the conditions considered in the present study. Furthermore, the range of OUR_{max} obtained in our study was also in good agreement with online respiration activity measurement with *C. glutamicum* ATCC 13032 by Seletzky *et al.* (Seletzky *et al.*, 2007) and Anderlei *et al.* (Anderlei *et al.*, 2004).

Gas-liquid mass transfer conditions, which depend on the shake flask size, the filling volume and the shaker agitation rate, greatly affects the bacterial physiological response during culture. Changing culture oxygenation conditions, biomass concentration and glucose consumption appeared to be strongly affected by volumetric oxygen mass transfer coefficient (k_{LA}). For instance, oxygen transfer had a clear effect on the maximal biomass accumulated. Within the k_{LA} range tested, oxygen transfer was limiting since the beginning of culture for lower k_{LA} (Figure IV-1). Using similar systematic approach and decoupling oxygen transfer and power dissipation, Mehmood et al. studied the effect of k_{LA} on the physiological response of filamentous bacterium *Streptomyces pristinaespiralis* during culture in unbaffled shake flasks. These authors reported a similar relationship linking k_{LA} to the maximal biomass obtained despite the occurrence of two-slope curve that they explained by the oxygen limitation inside the microbial pellets (Mehmood *et al.*, 2011). The effect of oxygen-supply rates on bacterial growth studied in unbaffled flasks with *Escherichia coli* also exhibited a dependence of maximal amount of biomass to oxygen supply rate even if, unlike in our study, a linear growth was observed for the different conditions in the two first hours (McDaniel *et al.*, 1965). Besides shake flask experiments, several studies reported such k_{LA} effects during enzymes and other metabolites production in stirred bioreactors (Demirtas *et al.*, 2003; Tang and Zhong, 2003; Souza *et al.*, 2009). Similarly to the maximal biomass accumulation, the maximal specific growth rate was affected by oxygen supply between 1 and 5 h of cultivation when glucose was not limiting. Furthermore, the duration of growth was also affected by oxygen and glucose availability, resulting in growth arrest within 10 h of cultivation. Combining results of Figure IV-2 and Figure IV-3A, it can be thus concluded that for k_{LA} values lower than 50 h^{-1} approximately, biomass final concentration was limited by gas-liquid mass transfer while for higher k_{LA} values, growth was rather limited by the initial quantity of glucose. This could explain the slighter influence of k_{LA} on microbial growth at the highest k_{LA} values. This conclusion is also supported by the fact that for k_{LA} lower than 50 h^{-1} , the dissolved oxygen concentration remained close to zero all over the culture (Figure IV-1B).

It was previously shown that in anaerobiosis and in absence of nitrate in the culture medium, *C. glutamicum* was not able to grow (Nishimura *et al.*, 2007; Takeno *et al.*, 2007). However, in each culture with *C. glutamicum* 2262 $\Delta ldhA$, except the static culture, a concomitant growth and production of fermentation end-products (acetate and/or succinate) was noticed. This suggests the simultaneous presence of both oxidative and fermentative metabolisms. This

coexistence could be the consequence of heterogeneity in the concentration of oxygen in the culture medium, resulting in the heterogeneity in cells behavior.

Indeed, despite the robustness of the experimental method developed in the present study, it should be kept in mind that dissolved oxygen concentrations were only measured at the sensor spot in the flask bottom vicinity. Thus, this measure was possibly not fully representative of the oxygen environment really encountered by the biomass inside the flask. One way to predict the occurrence of gradients inside a bioreactor is to compare the bioreactor mixing time to the characteristic consumption time. Tan *et al.* (2011) determined the mixing time in 250 mL unbaffled shake flask and reported values between 1 and 10 s depending of shaking rate (Tan *et al.*, 2011). Additionally, *C. glutamicum* 2262 was found to be able to consume dissolved oxygen from 10 to 0 % of air saturation within 5 seconds approximately for a biomass concentration of 12 g cdw L⁻¹ (data not shown). Thus, mixing time and consumption time were, at least, of the same order of magnitude for the conditions studied. In our work, the shaking conditions used to get low k_{La} values also corresponded to higher mixing times and thus to more probable occurrence of dissolved oxygen concentration gradients.

It is also interesting to reconsider the preceding conclusions regarding the physiological response of *C. glutamicum*. Indeed, from the growth and production kinetics, the simultaneous occurrence of both oxidative and fermentative metabolisms inside the flask was suggested. This statement was supported by the acetate production observed in the earlier stage of culture when bacteria were still growing (Figure IV-1A and Figure IV-4B). For instance, for the cultures at lower k_{La} (0.6 to 36 h⁻¹), the oxygen uptake is probably not sufficient to oxidize all the reduced coenzymes produced to balance the redox. Therefore most of the bacterial population should exhibit a fermentative metabolism. In the same way, Käß *et al.* (2014) studied the response of *C. glutamicum* in a stirred tank reactor connected to a plug flow reactor using a scale-down strategy (Käß *et al.*, 2014b). They reported a fast adaptation between low oxygen/ high substrate oscillation zones characterized by an ability to compensate concentration heterogeneities by a rapid, reversible switch from aerobic respiration to fermentative pathways entailing organic acids release and re-assimilation (Käß *et al.*, 2014b). According to these results, we could suggest that during cultures in shake flasks, temporal and/or spatial metabolism switches from aerobic to anaerobic and conversely could occur. In fact, on one hand, along the cultivation time, oxygen demand increased with biomass concentration increase, resulting in oxygen limitation and therefore a part of bacterial population switched from oxidative to fermentative metabolism. On the other hand, in the rotating bulk liquid, microorganisms in contact with the

headspace and those at the bottom side could encounter different oxygen levels and thus resulted in fermentative or oxidative metabolism. From this hypothesis, bacteria could be able to rapidly switch from respiration to fermentation leading to a concomitant formation of biomass and/or side-products.

The kinetics of biomass and organic acids production obtained during the study highlighted oxygen limitations for all oxygenation conditions with severe limitation for k_{LA} lower than 36 h^{-1} (Figure IV-5A). Oxygen limitation could physiologically result in an intracellular accumulation of pyruvate and its conversion to fermentation end-products (Zimmermann *et al.*, 2006). With a sufficient oxygenation of a culture of *C. glutamicum*, Dominguez *et al.*, noticed that reducing the gas flow rate and agitation rate in a stirred tank bioreactor, entailing oxygen limitations similar to the ones observed in our study, resulted in a shift in cell behavior with fermentation end-products formation (Dominguez *et al.*, 1993).

The ability of *C. glutamicum* to produce organic acids, under oxygen deprived conditions with growth arrested, has now been exploited to set up processes for succinic acid production. In fact, organic acids production by *C. glutamicum*, involved an aerobic phase for biomass accumulation followed by oxygen deprived conditions or fully anaerobic conditions to produce the organic acids (Inui *et al.*, 2004a; Okino *et al.*, 2005, 2008a; Litsanov *et al.*, 2012b). In this study, optimal values of k_{LA} for a maximal production of acetic and succinic acids were put into evidence in unbaffled shake flasks. When k_{LA} was between 10 and 19 h^{-1} , the concentration of succinic acid was the highest and the maximal yield of succinate to substrate was higher for k_{LA} lower than 19 h^{-1} (Figure IV-5). The maximal production of acetic acid occurred when k_{LA} values were ranging from 19 to 33 h^{-1} . The maximal production yields also showed a maximal peak for acetic acid for k_{LA} between 19 and 33 h^{-1} (Figure IV-5B). Meaning that, according to oxygen supply limitation degree, a specific sequence of fermentation end-products formation was thus observed. Similar production sequences have been reported using metabolic flux simulations by Shinfuku *et al.* (Shinfuku *et al.*, 2009). These authors reconstructed a genome-scale metabolic model of a strain of *C. glutamicum* and simulated the changes that could occur in the metabolic flux profiles when changing the oxygen uptake rate by using a flux balance analysis (FBA). After verifying the agreement between experiments and FBA simulations, they analyzed the changes in the intracellular metabolic profiles by changing the oxygen supply. From the simulations results, the metabolic profiles have revealed a specific sequence of organic acids production. The results showed that in conditions with sufficient oxygenation, the tricarboxylic acid cycle (TCA) was activated for biomass production while when oxygen supply

decreased (according to given ratio of OUR/GUR), cells started to produce acetate for ATP generation. Further decrease in the oxygen supply led to lactate production, instead of acetate, to oxidize the NADH produced in order to maintain the intracellular redox balance (Shinfuku *et al.*, 2009).

Lastly, comparing the culture performances in terms of yields (Figure IV-5), a growth decoupled phenomenon was observed. Indeed, a reduction of the oxygen mass transfer coefficient resulted in a reduction of biomass yield while an increase of $Y_{OA/S}$ in the very low k_{La} range was observed. Above $k_{La} = 50 \text{ h}^{-1}$, despite $Y_{X/S}$ seemed weakly impacted by oxygen transfer, organic acids production were drastically reduced. This result is a strong physiological characteristic of *C. glutamicum* and was thus used in succinic acid production processes to reach higher productivities (Okino *et al.*, 2008a; Litsanov *et al.*, 2012b). In the same way, Käb *et al.* (2014) studied the impact of various levels of gas-liquid mass transfer limitation within a microtiter plate based cultivation with *C. glutamicum* on physiological response (Käb *et al.*, 2014c). Working in maximum oxygen transfer rate ranging from $0.8 \text{ mM}\cdot\text{h}^{-1}$ to $22.8 \text{ mM}\cdot\text{h}^{-1}$, they noticed a growth alteration associated to more side-products accumulation in very low oxygen transfer range. Despite these authors emphasized lysine production whereas the focus was put on organic acids production in our study, similar conclusions between both studies could be underlined. For instance, $Y_{X/S}$ was negatively affected at reduced oxygen supply while substrate to lysine or substrate to organic acids production yields were improved (Käb *et al.*, 2014c). Therefore, according to the process target, oxygen transfer limitation level appears as a critical process parameter to reach high productivities.

In conclusion, for the first time, unbaffled glass shake flasks with oxygen patched probe and PreSens system were used for oxygen transfer assessment showing that they could be useful complementary tools to the establishment of correlations. The effects of oxygen supply on *C. glutamicum* were investigated and it was shown that there was a critical level of oxygenation at which cell growth was altered and organic acid production was reduced. Specific k_{La} ranges for (i) optimal succinic acid, (ii) acetic acid production and (iii) biomass accumulation were highlighted and respectively corresponded (i) $10 < k_{La} < 19 \text{ h}^{-1}$, (ii) $19 < k_{La} < 33 \text{ h}^{-1}$ and (iii) $k_{La} > 164 \text{ h}^{-1}$.

IV-5. References

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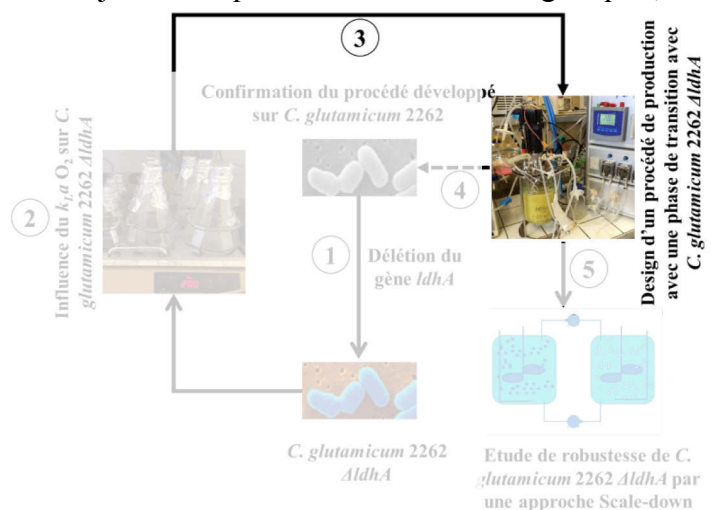
Chapitre V

**Mise en œuvre d'un procédé de
production d'acide succinique
par *Corynebacterium*
glutamicum 2262 Δ ldhA**

Chapitre V. Avant-propos

La plupart des études de production d'acide succinique par *C. glutamicum* est basée sur la modification génétique de la souche et l'utilisation d'un procédé commun en deux phases. Dans une première phase, la bactérie est mise en culture en condition aérobie jusqu'à une densité bactérienne très élevée puis, les cellules sont récoltées, rincées et remises en culture en conditions anaérobies pour la production de succinate (Okino et al., 2008a; Litsanov et al., 2012b; Zhu et al., 2014). Parallèlement au procédé en deux phases séparées, des travaux chez *E. coli*, une bactérie aérobie facultative à l'instar de *C. glutamicum*, ont utilisé un procédé appelé « dual-phase process ». Un des résultats majeurs de ces études a été de démontrer que les enzymes nécessaires à la bioconversion du substrat en succinate sont essentiellement produites avant l'anaérobiose. En effet, une stratégie mettant en œuvre une première phase de croissance directement suivie d'une phase de production de succinate, et sans séparation physique des deux phases, permettraient l'induction des gènes impliqués dans la biosynthèse du succinate (Martínez et al., 2010; Zhu et al., 2011). Par ailleurs, les résultats du chapitre IV ont montré que chez *C. glutamicum AldhA*, cultivée en fiole d'Erlenmeyer non chicanée, le coefficient volumique de transfert d'oxygène avait un effet majeur sur la production des acides organiques, dont le succinate. En effet, dans ces cultures en fioles, l'occurrence de conditions limitantes en oxygène (nécessaires à la production de succinate) est liée à l'accroissement de la densité bactérienne. Ce passage progressif vers un environnement de limitations pourrait favoriser l'adaptation des bactéries, et améliorer les productions en succinate.

En considérant ces résultats et les données de la littérature, nous avons focalisé nos travaux sur la stratégie de désoxygénation du milieu de culture entre la phase de croissance et la phase de production. Nous avons ainsi développé plusieurs stratégies de transition aérobie-anaérobie qui ont permis d'élaborer un procédé de production de succinate en utilisant *C. glutamicum 2262 AldhA*. Celles-ci ont été basées, soit sur le contrôle de la concentration en oxygène dissous, soit sur celui du débit d'air appliqué. Les différentes expériences réalisées sont présentées sous la forme d'un projet de publication dans le chapitre V.



V.Design of a one-step process for succinic acid production by *Corynebacterium glutamicum* 2262 Δ *ldhA*: Influence of the aerobic – anaerobic transition strategy on the process performance

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Abstract

Corynebacterium glutamicum have been engineered for the production of succinic acid. The commonly processes used are composed of aerobic phase for biomass accumulation followed by anaerobic phase for succinate production. The two phases have been performed either in the same bioreactor or physically separated with steps of centrifugation and washing. In this work, we have developed a new tri-phasic process consisting in an aerobic growth at dissolved oxygen regulated at 50 % of air saturation until OD_{570nm} of 30; when the desired biomass was reached, the DO was reduced to 20 % of air saturation and was maintained for 2 h before switching to 10% and maintained for 2 h and finally the aeration was stopped to switch into anaerobic conditions. The application of this tri-phasic process during the fermentation of *C. glutamicum* 2262 Δ *ldhA* in a 2 L bioreactor containing sodium bicarbonate resulted in the production of 327 mM of succinic acid with a yield of 0.94 mole per mole glucose while a similar process without transition produced 127 mM of succinate with a yield of 0.46 mole per mole glucose. Unlike the transition based on dissolved oxygen regulation, other transitions were developed through the regulation of aeration rates. The aerobic and anaerobic phases were run like previously. One of those transitions, consisting in regulation of the aeration rate at 60 L h⁻¹ (after the OD_{570nm} of 30 was reached) for 2 h followed by a reduction to 25 L h⁻¹ for 2 h before aeration shut off, produced 290 mM of succinate with a yield of 0.81 mole per mole glucose in a medium containing sodium bicarbonate. All the fermentations performed with transition resulted in higher titer and yield than process without transition phase and acetate was produced with yields

ranging from 0.06 to 0.2 mole per mole glucose. Finally, using the transition with dissolved oxygen regulation in a culture started with a large inoculum (OD_{570nm} of 50), with a feeding of glucose and sodium bicarbonate, 424 mM of succinate were produced with a yield of 1 mole per mole of glucose.

Keywords: Succinic acid, *Corynebacterium glutamicum* 2262 Δ *ldhA*, bioprocess, aerobiosis, anaerobiosis, aerobic-anaerobic transition.

V-1. Introduction

Succinic acid, also named butanedioic acid, is a four-carbon dicarboxylic acid which is a common metabolic intermediate of the tricarboxylic acid cycle (TCA) in plants, animals and microorganisms. In 2004, it has been cited as one of the top 12 molecules with multiple functional groups that possess the potential to be transformed into new families of useful material via biological conversion (Werpy *et al.*, 2004b). With bi-functional and linear structure, succinic acid could be used as precursor for industrial production of large spectrum of bulk chemicals such as N-methylpyrrolidone, 1,4-butanediol, tetrahydrofuran, γ - butyrolactone, adipic acid, dimethyl succinate (Tsao *et al.*, 1999; Zeikus *et al.*, 1999; McKinlay *et al.*, 2007b; Bozell and Petersen, 2010). Succinic acid production from renewable resources by microbial fermentation got increased interest because of its broad applications. Thus, these molecules find applications in various domains such as green solvents, biodegradable plastics, for plasticizers, as surfactant, detergent or foaming agent, as ion chelator or anti-microbial agent in the food industry (McKinlay *et al.*, 2007b; Sauer *et al.*, 2008; Beauprez *et al.*, 2010).

With efforts dedicated to develop biological processes for succinic acid production, numerous succinic acid producers have been isolated and described in rumen such as *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, *Actinobacillus succinogenes*, *Bacteroides amylophilus*, *Prevotella ruminicola*, *Succinimonas amylolytica*, *Succinivibrio dextrinisolvans*, *Wolinella succinogenes*, *Cytophaga succinicans* *Actinobacillus succinogenes* and *Mannheimia succiniciproducens* (Gokarn *et al.*, 1997). A non-rumen anaerobic bacterium, *Anaerobiospirillum succiniciproducens* (Lee *et al.*, 1999; Samuelov *et al.*, 1991) associated to *Actinobacillus succinogenes* and *Mannheimia succiniciproducens*, were reported as the best anaerobic microorganisms that naturally produce succinic acid as a major fermentation product (Zeikus *et al.*, 1999; Lee *et al.*, 2004; Song and Lee, 2006).

In addition to the natural producers, many microorganisms have been engineered to produce succinate as a fermentative end-product. *Escherichia coli*, *Corynebacterium glutamicum* whose wild type strains produce minor amount of succinic acid under anaerobic or oxygen deprived conditions (Clark, 1989; Dominguez *et al.*, 1993) are among the most representatives of engineered producers. Several metabolic engineering strategies have been developed to improve succinic acid production by *E. coli*. Those strategies included the inactivation of genes of enzymes which compete with succinic acid pathways, the amplification of enzymes involved in succinic acid pathways and the overexpression of exogenous enzymes resulting in the increase in the succinic acid production. A concluding example is *E. coli* AFP111/pTrc99A-pyc that has been able to achieve a final succinic acid concentration of 99.2 g L⁻¹ with an overall yield of 1.1 g g⁻¹ glucose and a productivity of about 1.3 g L⁻¹ h⁻¹ (Vemuri *et al.*, 2002b). The working strain of the following study, *C. glutamicum* has a so called GRAS-status. It is used industrially to produce L-glutamate (2.2·10⁶ t year⁻¹) and L-lysine (1.4·10⁶ t year⁻¹) (Wieschalka, 2012). In the past decade, extensive studies on organic acids production by *C. glutamicum* have been undertaken and production of acids like 2-ketoisovaleric acid, pyruvic acid, lactic acid and succinic acid under anaerobic culture conditions were reported (Okino *et al.*, 2005; Krause *et al.*, 2010; Wieschalka *et al.*, 2012a; Khuat *et al.*, 2014). However, currently, the investigations are mainly focused on the improvement of succinic acid production by *C. glutamicum* (Inui *et al.*, 2004a; Okino *et al.*, 2008a, 2008b; Litsanov *et al.*, 2012b). Under oxygen deprivation or anaerobic conditions, even if growth is stopped, wild type strains of *C. glutamicum* produces succinic acid via glycolysis, carboxylation of phosphoenolpyruvate or pyruvate to oxaloacetate by PEP carboxylase and/or pyruvate carboxylase, which is subsequently converted by malate dehydrogenase, fumarase, and succinate dehydrogenase (Figure V-1) (Inui *et al.*, 2004a; Wieschalka *et al.*, 2013a). According to Inui *et al.* (2004), the ability of Corynebacteria to produce organic acids with growth stopped under oxygen-deprived conditions presents two advantages: i) most of energy is dedicated to acids production and ii) the use of microorganisms at high density (Inui *et al.*, 2004a). Exploiting these properties, a two-step process of succinate production has been established with genetically modified strains of *C. glutamicum* R and ATCC 13032. These strains contained several deleted genes, overexpressed endogenous and exogenous genes, new introduced pathways (Okino *et al.*, 2008a; Litsanov *et al.*, 2012b; Zhu *et al.*, 2014). The two steps of the production process, physically separated, consisted of a first stage with a fully aerobic growth; then, the cells were harvested, washed and transferred in a bioreactor with anaerobic conditions for succinic acid production. With such a process, 146 g L⁻¹ of succinate were produced with a yield of 1.4 mole

per mole of glucose in fed-batch mode. However, a significant amount of acetate was also observed in the culture medium at the end of this process. With a similar strategy, Litsanov et al. (2012), improved the succinate yield (1.67 mole per mole of glucose) with a reduced acetate production by using an engineered *C. glutamicum* ATCC 13032 (Litsanov *et al.*, 2012b).

However, it has been reported that the aerobically induced enzymes can maintain their activity during the anaerobic phase and significantly influence the succinate production (Vemuri *et al.*, 2002a, 2002b; Wu *et al.*, 2007). Therefore using an adequate transition strategy of deoxygenation from aerobic to anaerobic phases based on the activities of the key enzymes and physiological states could improve the process performances. Similarly, Martinez *et al.* (2010), reported that an introduction of microaerobic phase at the end of the aerobic growth phase of an *E. coli* resulted in an enzymatic machinery mobilization and an enhanced succinate production during the anaerobic phase (Martínez *et al.*, 2010). Recently, Wieschalka (2012), conducted a one-step fed-batch fermentation process with *C. glutamicum* ELB-P by combining an aerobic growth phase on glucose plus acetate followed by a microaerobic phase at the end of the exponential growth before the anaerobic production phase. This process produced 39 g L⁻¹ of succinate with a yield of 1.02 mole succinate per mole of glucose (Wieschalka, 2012).

As the operation conditions of a process could greatly affect the behavior and the enzymatic machinery of the production strain, the genetic manipulation has to be combined to process engineering to improve the strain's performances. In this study, a lactate dehydrogenase deleted mutant of *C. glutamicum* 2262 was used to design a succinic acid process. According to the literature, the transition from aerobic to anaerobic phase has an important impact on the succinic acid production. The physiological changes establishing during a fastest or slowest transition to anaerobic conditions could be some key parameters in anaerobic succinic acid production process. Thus, the following study is mainly focused on the understanding of the influences of deoxygenation transition phase on succinate production. Several strategies of switching based on dissolved oxygen level or aeration rates management are investigated in a one-step process with *C. glutamicum* 2262 *AldhA* in minimal medium (MCGC).

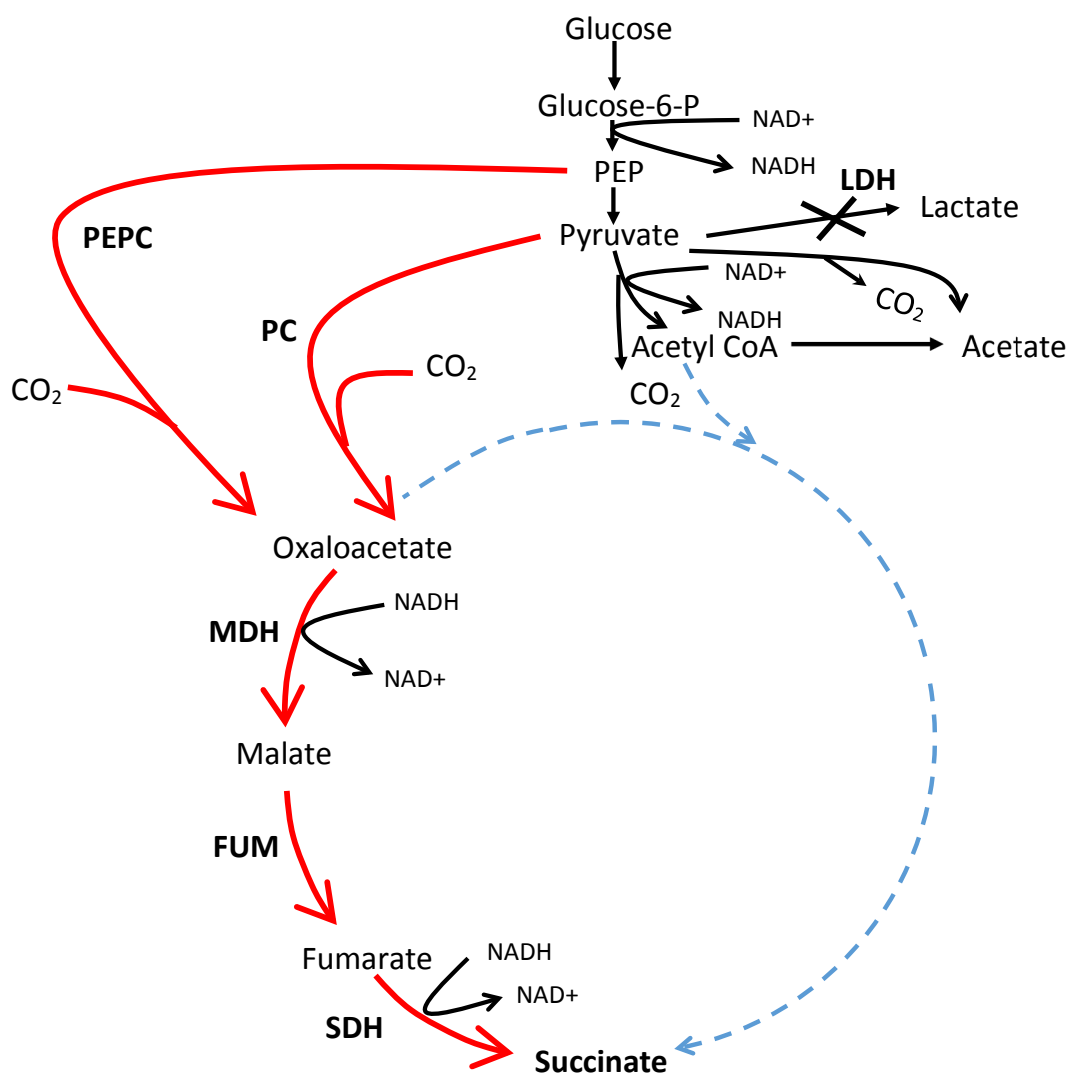


Figure V-1. Succinate biosynthesis pathway in *C. glutamicum* under anaerobic conditions. The relevant reactions leading to succinate are displayed in red and thick arrows. Abbreviations: PEPC, phosphoenolpyruvate carboxylase; PC, pyruvate carboxylase; MDH, malate dehydrogenase; FUM, fumarase SDH, succinate dehydrogenase; LDH, lactate dehydrogenase. The lactate dehydrogenase, whose encoding gene was deleted, is indicated by “X”. The stoichiometric coefficients were indicated only for the main pathways.

V-2. Material and methods

V-2.1. Bacterial strain, culture media and protocol

Batch cultures of a mutant strain of *Corynebacterium glutamicum* 2262, deleted for lactate dehydrogenase gene (*AldhA*) were performed in a modified MCGC mineral salt medium in which citrate was replaced by deferoxamine. Final concentrations of nutrients were indicated

in Table V-1. Basal medium, salts A and B and urea were prepared separately and autoclaved for 20 min at 121°C. Vitamins solution was sterilized by filtration through a 0.22 µm filter (Millex, Millipore, France). After sterilization, the different solutions were mixed in accordance to the final concentrations mentioned in Table V-1. The mutant strain was constructed via a two-step homologous recombination procedure using the vector pK19*mobsacBAldhA* (Litsanov et al., 2012b). This plasmid was constructed using the suicide vector pK19*mobsacB* as previously described (Schäfer *et al.*, 1994). The culture medium was supplemented with glucose as carbon and energy source at the desired concentration and with 125 mM of NaHCO₃ as CO₂ donor for the succinic acid production. A first pre-culture (A) of *C. glutamicum* 2262 *AldhA* was performed in a 500 mL baffled flask containing 50 mL of MCGC. Temperature and shaking rate of pre-cultures and cultures were respectively 33°C and 200 rpm. When the optical density at 570 nm reached approximately the value of 60, 5 mL of pre-culture (A) were used as inoculum for the second pre-culture (B) performed with the same culture conditions than pre-culture A. When the optical density at 570 nm of pre-culture (B) reached the value 30, bacterial growth was stopped by immersing the flask in 70 % v/v ethanol, at a temperature of -20°C; culture broth was then kept at 4°C for 12 – 24 h.

V-2.2. Aerobic production of biomass for anaerobic succinate production

Pre-culture (B) was used to inoculate different bioreactors with a starting optical density at 570 nm of 1.5. Cultures were performed in 2 L stirred tank bioreactors (Tryton, Pierre Guerin technologies, France) with a 1.5 L working volume. The bioreactors were equipped with two four-blade Rushton turbines. During cultivation, dissolved oxygen concentration (DOC) was maintained at a constant concentration of 50% of air saturation by sparging air or pure oxygen through a ten-orifice diffuser at varying gas flow rates and by using a sterilizable oxygen probe (Mettler-toledo, ISM technology, Switzerland). The pH was measured using sterilizable glass electrode (Mettler-toledo, ISM technology, InPro3253, Switzerland) and regulated at a set-point of 7.6 by automatic addition of a 10 N KOH solution or 2 N HCl using a peristaltic pump. The foam formation was controlled by manual addition of drops of polypropylene glycol (ppg) with the respect of a maximal final concentration of 1.2 mL ppg (L of broth)⁻¹. The temperature of cultures was maintained at 33°C by automatic cool and hot water circulation in the bioreactor jacket. For experiments with different sizes of inoculum, pre-cultures were prepared in one or two bioreactors as described above. In these conditions, cells were harvested in sterile tubes

and centrifuged at 5000 g, 4°C, for 10 minutes. Cells were re-suspended in MCGC and used as inoculum for experiments performed to study the influence of initial inoculum size.

Table V-1. Composition of modified MCGC medium.

Compounds of the medium		Final concentration		
		For flask culture	For bioreactor culture	
<i>Basal medium</i>	Na ₂ HPO ₄ 12H ₂ O	36.72	7.44	g L ⁻¹
	KH ₂ PO ₄	2.88	5.90	g L ⁻¹
	NaCl	0.96	1.97	g L ⁻¹
	(NH ₄) ₂ SO ₄	3.84	7.86	g L ⁻¹
<i>Salts A</i>	MgSO ₄ 7H ₂ O	455.84	39.31	mg L ⁻¹
	FeSO ₄ 7H ₂ O	45.58	3.93	mg L ⁻¹
	FeCl ₃	4.56	0.39	mg L ⁻¹
<i>Salts B</i>	ZnSO ₄ 7H ₂ O	1.14	0.07	mg L ⁻¹
	CuCl ₂ 2H ₂ O	0.46	0.03	mg L ⁻¹
	MnSO ₄ H ₂ O	4.56	0.26	mg L ⁻¹
	(NH ₄) ₆ Mo ₇ O ₂₄ 4H ₂ O	0.23	0.01	mg L ⁻¹
	Na ₂ B ₄ O ₇ 10H ₂ O	0.46	0.03	mg L ⁻¹
<i>Vitamins</i>	CaCl ₂	94.85	8.18	mg L ⁻¹
	Biotin	2.28	0.20	mg L ⁻¹
	Thiamine	22.79	1.97	mg L ⁻¹
	Deferoxamine	3.42	0.29	mg L ⁻¹
	Betaïn	1937.32	0.20	mg L ⁻¹
	Urea	3.90		g L ⁻¹
	Glucose*	33	40	g L ⁻¹

*Concentration of glucose for bioreactor cultures could vary according to the aim. In this case, the dedicated concentration was specified.

V-2.3. Transition from aerobic growth to anaerobic conditions

Two kinds of deoxygenation strategies were applied to *C. glutamicum* 2262 *AldhA* cultures: Either a transition based on the control of the dissolved oxygen concentration or through the management of the aeration flow rate. For the first one, *C. glutamicum* 2262 *AldhA* was first cultivated in fully aerobic conditions as described previously until a desired optical density at 570 nm was reached (10, 30 or 50). Then, step decrease from aerobic to anaerobic conditions was applied. For this kind of transition, the DOC was maintained constant at a targeted value (50 %, 20 % and 10 %) for at least 2 hours before decreasing to a lower level which was maintained also for at least 2 hours. Lastly the oxygen sparging was stopped to create anoxic conditions. The second type of transition strategy, with a regulation of the aeration flow rate

instead of the DOC, was similarly made but the reduction in the DOC was the consequence of the bacterial oxygen uptake. After the aerobic growth, the aeration flow rate was reduced from 100 L h⁻¹ to 60 L h⁻¹, maintained for 2 h at this value, then reduced, once again, to 25 L h⁻¹ for 2 h, and, lastly, to 0 to get anaerobic conditions. Furthermore, a similar transition was conducted with the following steps: 110 L h⁻¹ to 65 L h⁻¹ which was maintained for 2 h, 65 L h⁻¹ to 10 L h⁻¹, for 2 h, and finally 10 L h⁻¹ to 0 till the end of the culture. For the third and last transition, after the aerobic growth phase, the aeration rate was set to 60 L h⁻¹ for 4 h before the total shut down of the aeration.

Table V-2. Summary of the different conditions of transition

Transitions	Strategy of transition	
	Control of the dissolved oxygen (% of air saturation)	Control of the aeration rates (L h ⁻¹ of air)
<i>Control</i>	50 to 0 % promptly	Not controlled
<i>Tr1</i>	100 → 50 % (1h) → 20 % (1 h) → 20 % (1 h) → 10 % (2 h) → 0 %.	Not controlled
<i>Tr2</i>	20 % → 10 % (4 h) → 0 %.	Not controlled
<i>Tr3</i>	50 % → 20 % (2 h) → 10 % (2 h) → 0 %.	Not controlled
<i>Tr4</i>	Not controlled	100 → 60 (2 h) → 25 (2 h) → 0 L h ⁻¹
<i>Tr5</i>	Not controlled	60 (4 h) → 0 L h ⁻¹
<i>Tr6</i>	Not controlled	110 (5 h) → 65 (2 h) → 10 (2 h) → 0 L h ⁻¹

(→) Corresponded to quick shift from one level to another. The time indicated in parentheses corresponded to the duration of the regulation at the desired level of dissolved oxygen or aeration.

V-2.4. Anaerobic succinate production in batch mode

As the different steps of the process were conducted in the same bioreactor, at the end of the transition stage, aeration was totally stopped and culture broth was changed into anaerobic conditions. The agitation rate was maintained at a value 900 rpm and the pH was always

controlled at a set-point of 7.6 by automatic addition of a 10 N KOH solution or 2 N HCl with a peristaltic pump connected to an on/off pH controller.

V-2.5. Anaerobic succinate production in fed-batch mode

Fed-batch cultures were performed as shown for batch cultures. Three additions of 50 mL of a solution of 3.5 M glucose combined with 50 mL of 1.5 M NaHCO₃ solution were performed during the succinic acid production phase. The pH was kept at 7.6 by automatic addition of a 10 N KOH solution or 2 N HCl with a peristaltic pump connected to an on/off pH controller.

V-2.6. Determination of global kinetic parameters

To evaluate the culture performances on growth, substrates uptake and organic acids production, global kinetic parameters were calculated for each experiment. The maximal specific growth rate was provided by:

$$\mu_{max} = \max_t \left(\frac{1}{[X]_f} \cdot \frac{d[X]_f}{dt} \right) \quad (\text{V-1})$$

With $[X]_f$ the biomass concentration obtained by fitting experimental data using smooth splines interpolation model. Maximal specific glucose q_{max}^{gluc} consumption and organic acids (OA) q_{max}^{OA} production rates were respectively calculated as follows:

$$q_{max}^{gluc} = - \max_t \left(\frac{1}{[X]_f} \cdot \frac{d[gluc]_f}{dt} \right) \quad (\text{V-2})$$

$$q_{max}^{OA} = \max_t \left(\frac{1}{[X]_f} \cdot \frac{d[OA]_f}{dt} \right) \quad (\text{V-3})$$

With $[gluc]_f$ and $[OA]_f$ the fitted glucose and organic acids concentrations respectively using smooth spline interpolation model. Maximal yields of organic acids and biomass production on glucose concentration Y_{max}^{OA} were respectively calculated by the ratio of maximal concentration of organic acids ($[OA]_{max}$) and biomass (X_{max}) on the concentration of glucose consumed.

V-2.7. Analytical methods

For each experiment, biomass concentration was determined by the measurement of absorbance at 570 nm with a Hitachi U2000 spectrophotometer (Sciencetec, les Ulis, France) and was transformed into cell dry weight (cdw) using the relation: dry cell concentration (g L^{-1}) = $0.27 \times \text{OD}_{570\text{nm}}$. For the determination of glucose residual concentration and organic acids concentration, samples of culture broth were first centrifuged at $5,000 \times g$ for 10 minutes and at 4°C . Glucose and organic acids (succinate, lactate, acetate, pyruvate and fumarate) concentrations were measured in the supernatants by High Pressure Liquid Chromatography (10ADVP, Shimadzu) using an ion-exchange column ($7.8 \text{ mm} \times 300 \text{ mm}$, Bio-Rad Aminex HPX-87H) and SPD-M10A chromatography working station system equipped with UV absorbance and refractive index detectors water 410 (Shimadzu). The column was eluted isocratically at a rate of 0.7 mL min^{-1} with $5 \text{ mM H}_2\text{SO}_4$ for sugar analysis and $50 \text{ mM H}_2\text{SO}_4$ for organic acids, at a temperature 50°C . The approximate retention time for glucose, pyruvic, succinic, lactic, acetic and fumaric acids were respectively 7.7, 9.2, 10.2, 10.9, 13.1 and 13.5 min.

V-3. Results

V-3.1. Production of succinate by *C. glutamicum* 2262 *AldhA* after a sudden stop of the aeration, during a batch culture

In the literature, succinate production by facultative aerobic bacteria was performed through a process comprising an aerobic growth phase followed by an anaerobic production phase. The two phases were done either in the same bioreactor (dual-phase process) or separately. In this last case, cells were harvested by centrifugation and were introduced into the acid production medium. The culture broth was then sparged with CO_2 or N_2 to strip oxygen before the inoculation (Vemuri *et al.*, 2002a, 2002b; Chen *et al.*, 2012; Wieschalka, 2012). The protocol of the dual phase process was applied to *C. glutamicum* 2262 *AldhA*.

This strain was cultivated in bioreactors, containing MCGC and 125 mM of NaHCO_3 . In a first aerobic growth phase, *C. glutamicum* 2262 *AldhA* was grown at constant DOC of 50 % of air saturation. It was previously shown with *C. glutamicum* 2262 that the transition time from aerobic to anaerobic conditions influences greatly the performance of growth and organic acids production (Khuat *et al.*, 2014). For this reason, two transition OD were tested with *C. glutamicum* 2262 *AldhA*. The first was at OD_{570} of 10 (2.7 g cdw L^{-1}) and the second of 50 (13.5

g cdw L⁻¹) (Figure V-2A). During this growth phase, the maximal specific growth rates were 0.56 h⁻¹ and 0.62 h⁻¹, respectively (Figure V-2C). When the desired OD₅₇₀ was reached, the aeration was stopped to promote anaerobic conditions. During the anaerobic culture, growth ceased in both cases with an important cell lysis observed in the case of an OD_{570nm} of 50 (Figure V-2A). *C. glutamicum* 2262 *AldhA* consumed glucose with a maximum specific rate of 9.6 mmol (g cdw)⁻¹ h⁻¹ (Figure V-2B and D). During anaerobic conditions, glucose consumption was dramatically reduced. The residual glucose concentrations were 280 and 40.5 mM for biomass concentrations of 2.7 g cdw L⁻¹ and 13.5 g cdw L⁻¹, when the transition was performed at OD₅₇₀ of 10 and 50, respectively (Figure V-2B). With instantaneous deoxygenation to reach anaerobic conditions, *C. glutamicum* 2262 *AldhA* showed only a weak organic acids production. 127 mM of succinic acid and 47 mM of acetic acid were produced when the transition was applied at an OD₅₇₀ of 50 (Figure V-3A and B). When aeration was stopped at an OD₅₇₀ of 10, succinic and acetic acid concentrations were also low with only 23 and 4 mM, respectively (Figure V-3A and B).

V-3.2. Influence of sodium bicarbonate supplementation the growth and organic acids production by *C. glutamicum* 2262 *AldhA*

Succinate production in *C. glutamicum* requires CO₂ as substrate (Figure V-1). For this reason, a supplementation of the culture medium with 125 mM of NaHCO₃ was performed. As previously, a sudden shut down of aeration was performed when these OD values were reached:

- 2.7 g cdw L⁻¹ (OD_{570nm} of 10) (Figure V-4A and C).
- 13.7 g cdw L⁻¹ (OD_{570nm} of 50) (Figure V-4B and D).

Very similar pattern were observed with or without sodium bicarbonate concerning the growth indicating that no impact of NaHCO₃ on microbial growth in the range of values tested (Figure V-4).

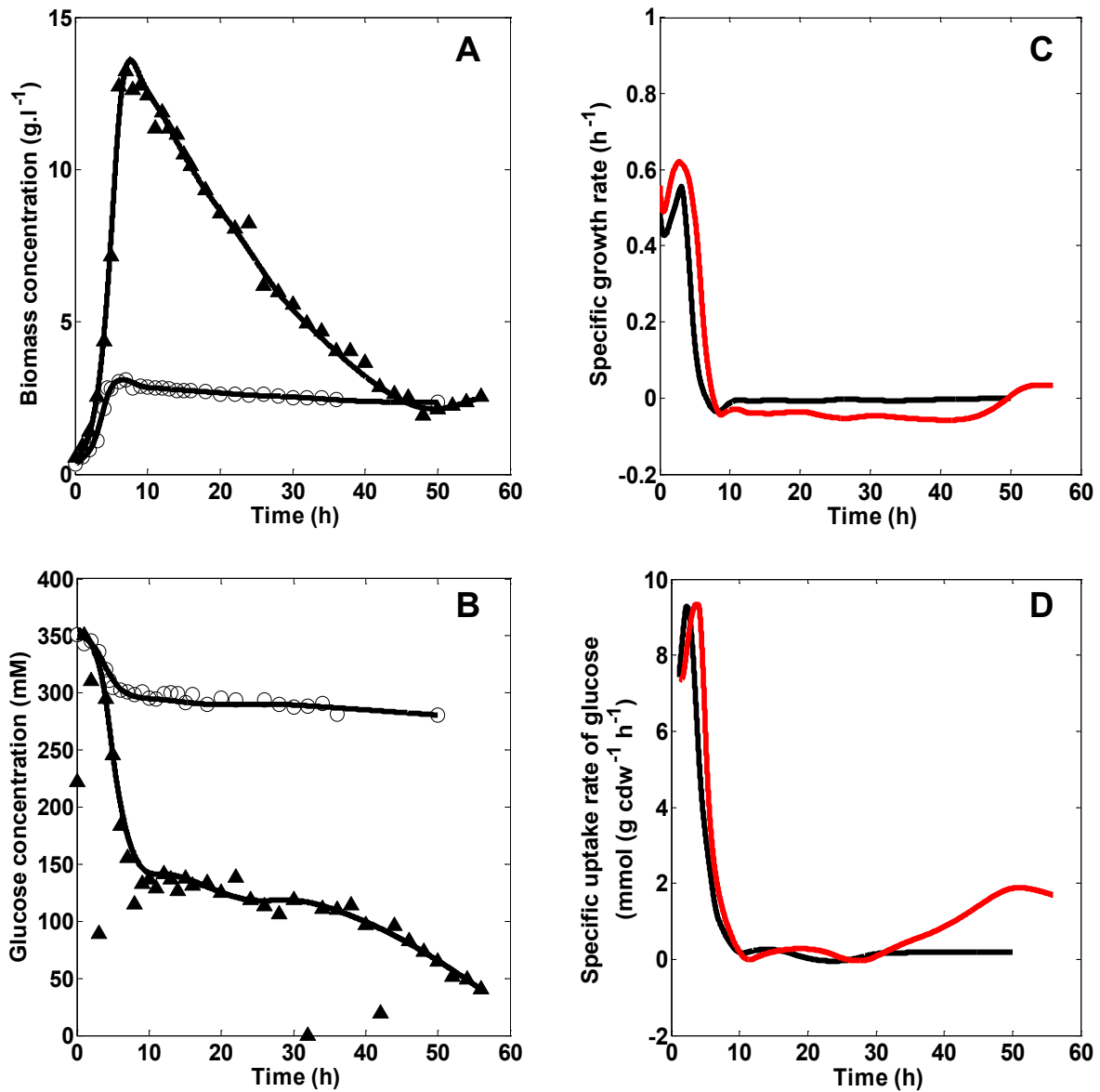


Figure V-2. Kinetics of *C. glutamicum* 2262 AldhA culture during a process with an instantaneous deoxygenation from aerobiosis to anaerobiosis, applied at OD_{570} of 10 (O, —) or 50 (▲, —). A Biomass concentration, B Residual glucose concentration, C Specific growth rates, D Specific consumption of glucose.

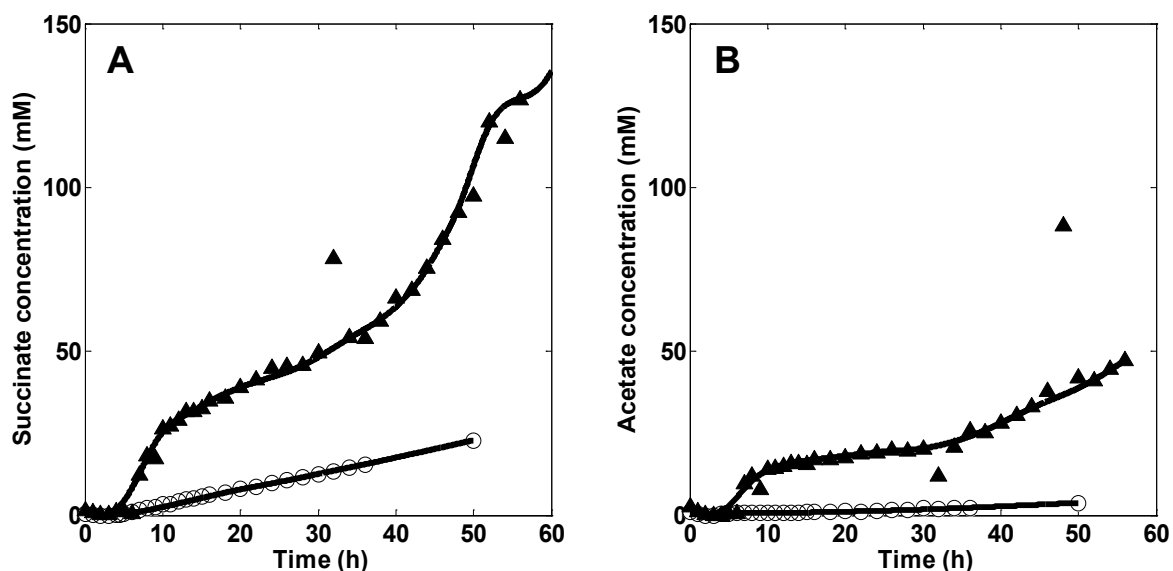


Figure V-3. Organic acids production of *C. glutamicum* 2262 Δ ldhA during a dual-phase culture with an instantaneous transition from aerobiosis to anaerobiosis, applied at OD₅₇₀ of 10 (○) or 50 (▲). A Succinic acid concentration, B Acetic acid concentration.

The glucose consumption is shown in Figure V-5. A slight difference was observed between cultures performed with or without NaHCO₃ (Figure V-5A and B). In both cases, glucose was consumed with maximal specific rates during the aerobic growth: 6.7 and 8 mmol (g cdw)⁻¹ L⁻¹ in absence and in presence of 125 mM of bicarbonate respectively for cultures with a transition at biomass concentration of 2.7 g cdw L⁻¹ (Figure V-5C). The addition of 125 mM bicarbonate resulted in a 19 % increase in the glucose maximal uptake rate. When aeration was performed up to a biomass concentration of 13.7 g cdw L⁻¹, the maximal rates were 7.8 and 9.3 mmol (g cdw)⁻¹ L⁻¹, respectively, corresponding to a 20 % increase (Figure V-5D).

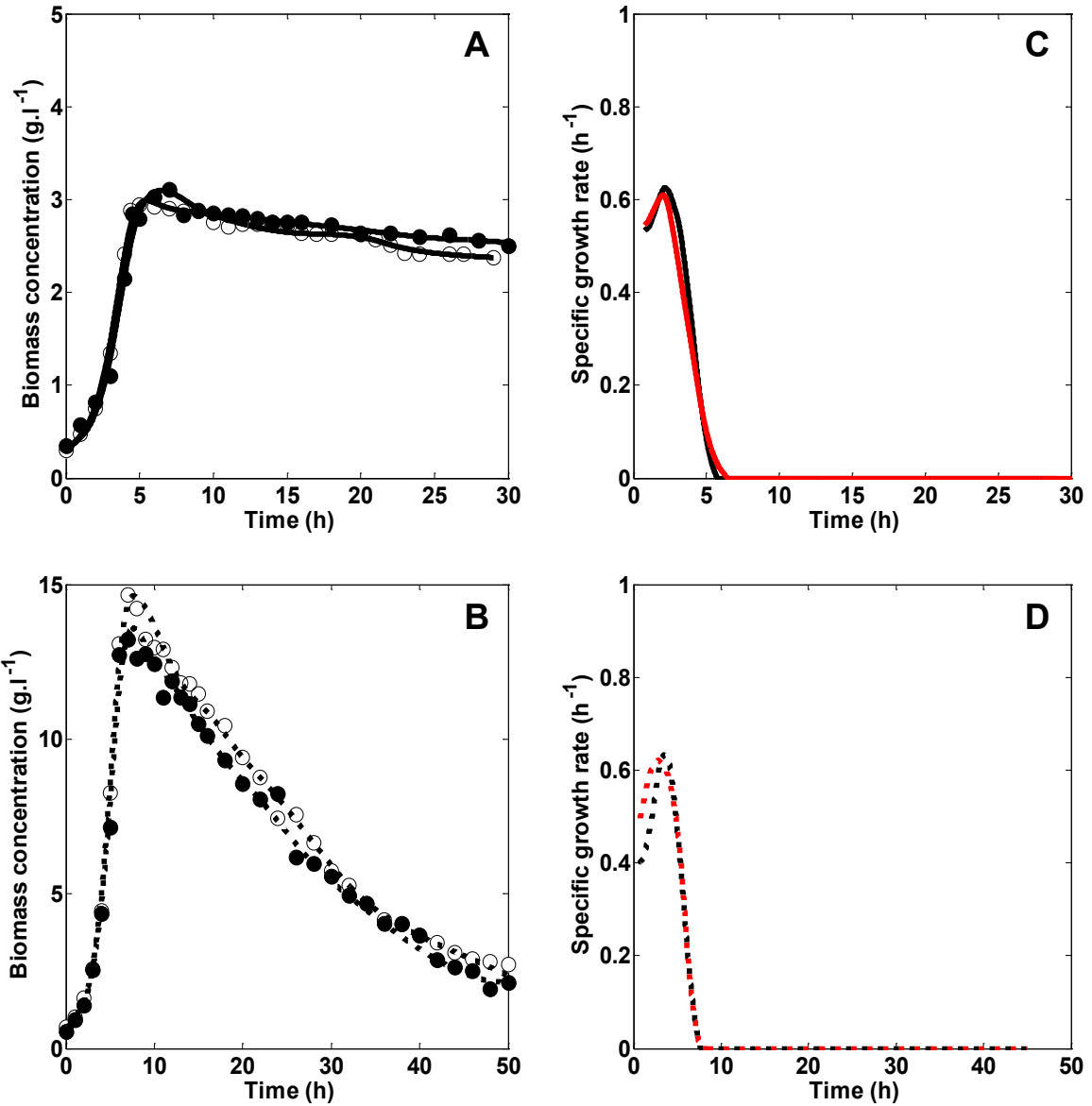


Figure V-4. Influence of NaHCO_3 addition on *C. glutamicum* 2262 ΔldhA growth during cultures with an instantaneous deoxygenation from aerobiosis to anaerobiosis, applied at OD_{570} of 10 (A and C) or 50 (B and D). The cultures were performed without NaHCO_3 (O, black) and with 125 mM NaHCO_3 (●, red). (A - B) Biomass concentration, (C - D) Specific growth rates.

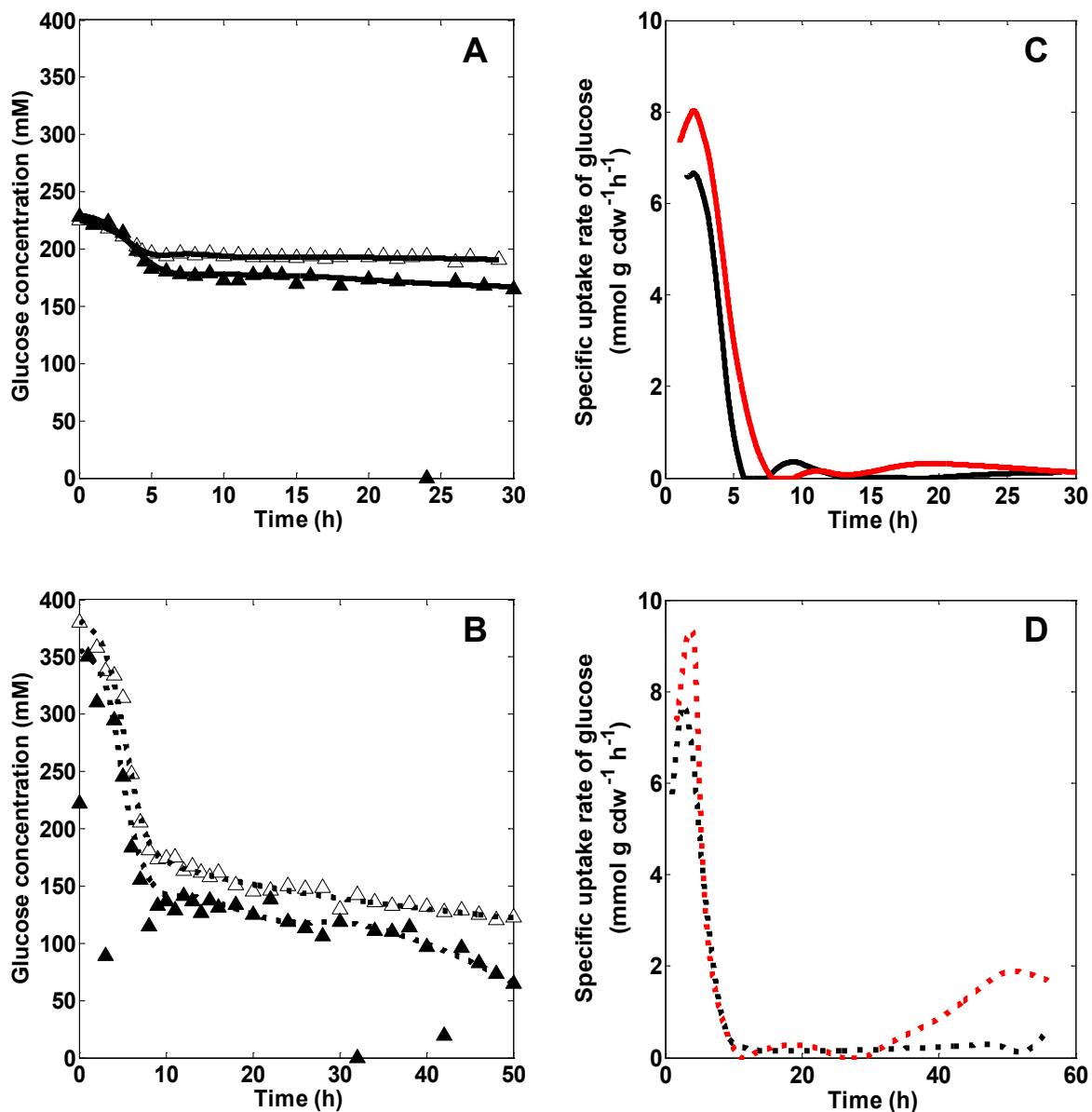


Figure V-5. . Influence of NaHCO_3 addition on *C. glutamicum* 2262 *AldhA* glucose consumption during cultures with an instantaneous deoxygenation from aerobiosis to anaerobiosis, applied at OD_{570} of 10 (A and C) or 50 (B and D). The cultures were performed without NaHCO_3 (Δ , black) and with 125 mM NaHCO_3 (\blacktriangle , red). (A - B) Residual glucose concentration, (C - D) Specific glucose consumption rates.

Then, the addition of bicarbonate in the culture medium led to an increase of glucose consumption rates (Figure 5). However, according to the succinic acid production pathway, the anaplerotic enzymes phosphoenolpyruvate carboxylase (PEPC) and/or pyruvate carboxylase (PC), consumed CO_2 to produce C_4 dicarboxylic acids from phosphoenolpyruvate (PEP) or pyruvate (Figure 1). Thus, succinic acid production was expected to be increased in presence

of bicarbonate. In the one-step process performed with dual phases, for aerobic growth until a biomass concentration of 2.7 g cdw L^{-1} , the amounts of succinate without and with NaHCO_3 were 9.7 and 13.6 mM, respectively (Figure 6A). Concerning the cultures performed with an aerobic phase until a biomass concentration of $13.7 \text{ g cdw L}^{-1}$, the maximal concentrations of succinate were close with 109 and 126 mM respectively without and with bicarbonate (Figure 6B) even though the production profiles were quite different during the first 35 h of culture. Less acetate was produced in the presence of 125 mM bicarbonate (Figure 6C and D). When the aeration was maintained up to a biomass concentration of $13.7 \text{ g cdw L}^{-1}$, an increase in acetate concentration was determined in presence of NaHCO_3 , from 32 to 42 mM (Figure 6D).

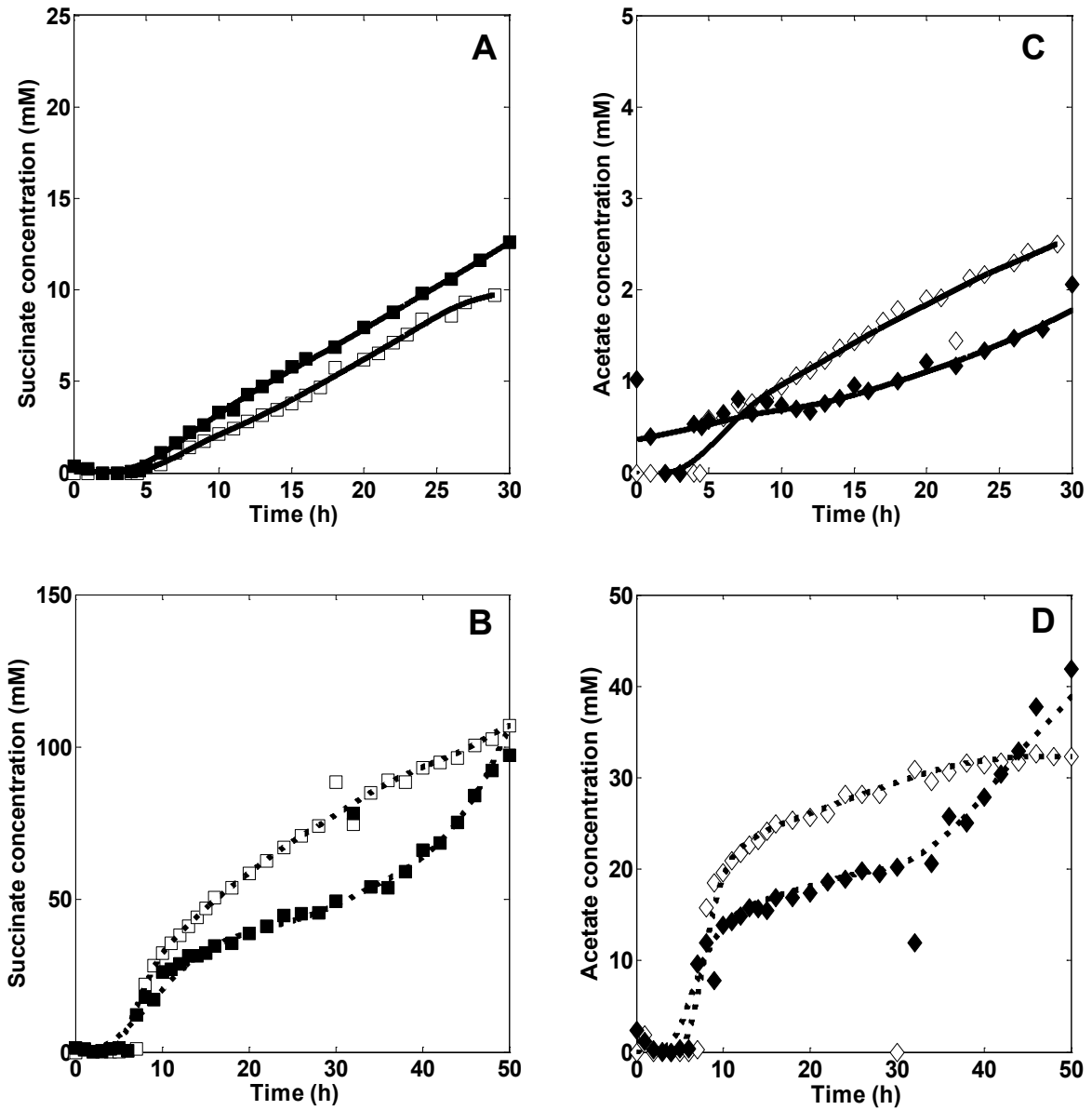


Figure V-6. Influence of $NaHCO_3$ addition on *C. glutamicum* 2262 Δ ldhA organic acids production during cultures with an instantaneous deoxygenation from aerobiosis to anaerobiosis, applied at OD_{570} of 10 (A and C) or 50 (B and D). The cultures were performed without $NaHCO_3$ (\square , \diamond) and with 125 mM $NaHCO_3$ (\blacksquare , \blacklozenge). (A - B) Succinate concentration, (C - D) Acetate concentration.

V-3.3. Design of a one-step process with progressive deoxygenation phases between aerobiosis and anaerobiosis

V-3.3.1. Management of deoxygenation via the regulation of DOC

Recently, Martinez *et al.* (2010) have reported the metabolic effect of aeration conditions for *E. coli* on metabolite and gene expression profiles during the succinic acid production phase. The results showed that, with a high aeration flow rate, which induced high DOC (> 40 % of air saturation) before starting the anaerobic phase promoted low succinate production. Lower aeration flow rates during the first phase of process resulted in lower DOC (near 0 %) before anaerobic phase which promoted higher succinate concentrations than previously (Martínez *et al.*, 2010). Furthermore, during experiments in shake flasks with application of different k_{La} values, it was shown the weakest k_{La} values allowed the direction of carbon flux toward the succinate synthesis (Chapter IV). These different observations, suggested a relationship between the DOC applied to the culture during aerobic and transition phases and the succinate production during the anaerobic phase. Therefore, several experiments, in which the deoxygenation sequence was precisely controlled between the two phases of the process, were performed.

In the different trials, the first phase was systematically an aerobic phase in which the DOC was maintained between 20 and 100 % of air saturation according to the cultures. In this phase, bacteria were cultivated until an optical density at 570 nm of 30 (8.1 g cdw L⁻¹) was reached. This optical density was chosen because it was previously reported that this optical density was suitable for organic acids production (Khuat *et al.*, 2014). Three different strategies of this deoxygenation phase were in described in Figure V-7 and compared to a control culture (*Control*) which deoxygenation corresponded to the conventional process of a succinate production. In this case, it was noteworthy that, once the aeration was stopped, the dissolved oxygen was rapidly consumed within less than 2 minutes. After the transition phase, the last phase was the shut off of aeration to impose anaerobic conditions to the cells.

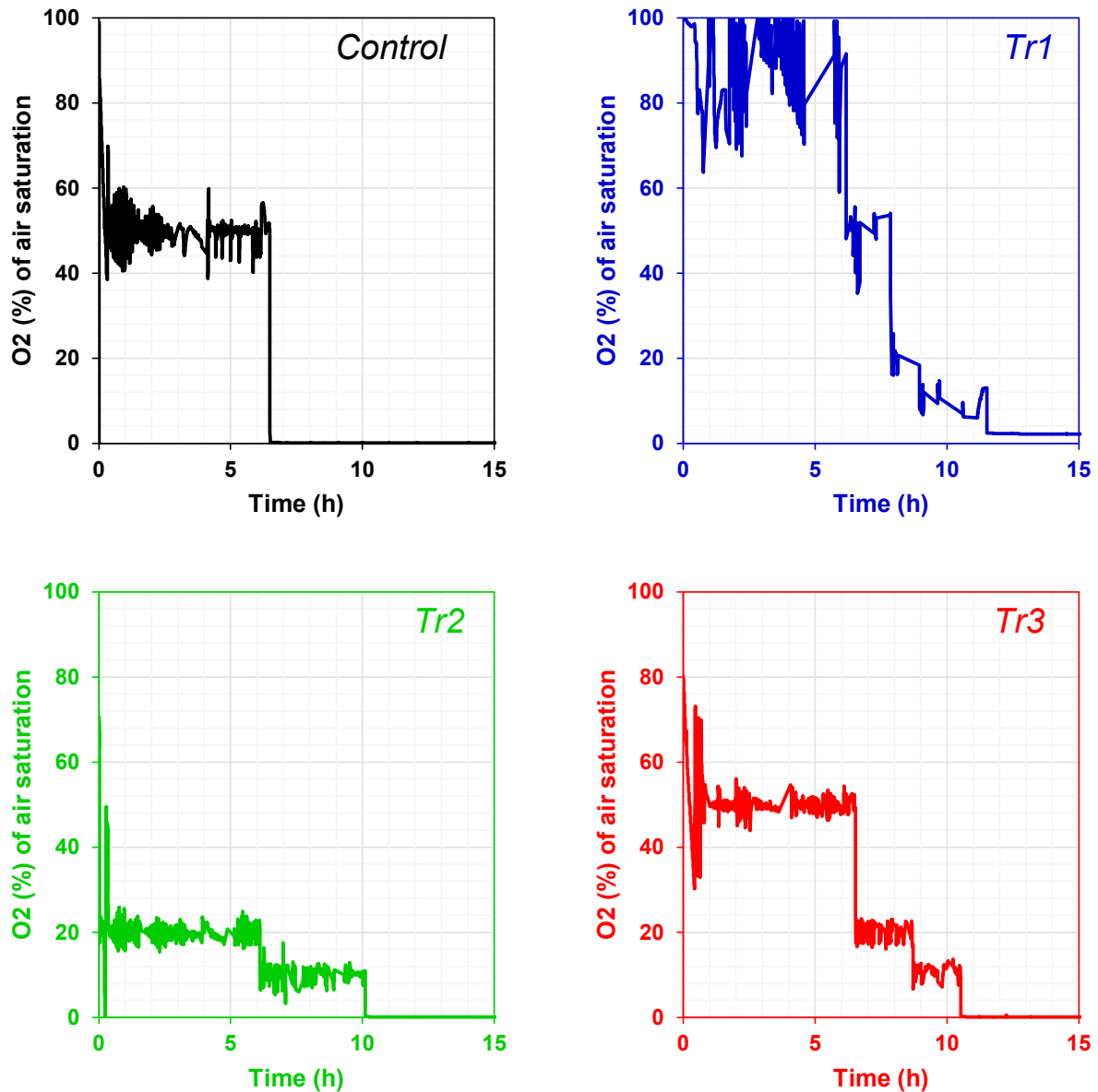


Figure V-7. Time course of dissolved oxygen concentrations recorded during succinic acid production process involving a transition phase between aerobic and anaerobic phase using *C. glutamicum* 2262 Δ ldhA. The experiments were performed with fully aerobic conditions until a biomass concentration of 8.1 g cdw L^{-1} . Four strategies of transitions were applied. Control: Bacteria were grown with DOC controlled at 50 % of air saturation until OD_{570} of 30, then aeration was suddenly stopped. Tr1: During the growth phase, DOC was maintained around the saturation until the targeted biomass was reached before switching to 50 % (1 h) then to 20 % (1 h) and finally, aeration was turned off. Tr2: Culture was conducted under DOC controlled at 20 % of air saturation until an OD_{570} of 30, then, the DOC was reduced at 10 % for 4 h before the aeration was stopped. Tr3: First aerobic growth with dissolved O₂ concentration at 50 % of air saturation until an OD_{570} of 30, then the DOC was reduced to 20 % (for 2 h) and to 10 % for 2 h and finally the aeration was shut off.

V-3.3.2. Consequences of deoxygenation strategies on succinic acid production by *C. glutamicum* 2262 *AldhA*

To determine the influence of deoxygenation strategy on the the performance of the process, *C. glutamicum* 2262 *AldhA*, cultivated under aerobic conditions, was shifted into anaerobic conditions after different transition phases. Cultures were performed in MCGC containing 350 mM of glucose and 125 mM NaHCO₃, in batch mode. The cultures were performed in a 2-L bioreactor (pH 7.6, agitation rate of 900 rpm and temperature of 33°C).

During the first phase of the process, until the biomass concentration of 8.1 g cdw L⁻¹, similar growth profiles were determined in the four cultures with a maximal specific growth rate of 0.6 h⁻¹ after 2 h of culture (Figure V-8A and C, Table V-3). During the different transition phases, *C. glutamicum* 2262 *AldhA* kept on growing up to OD₅₇₀ of about 55 (15 g cdw L⁻¹) (Figure V-8A, Table V-3). Once the aeration was stopped at the end of the transition, bacterial growth ceased for all the cultures and during the anaerobic phase, the bacterial amount progressively decreased to a lowest values of 2.5, 4.9, 1.1, 3.2 g cdw L⁻¹ for *Control*, *Tr1*, *Tr2* and *Tr3*, respectively at the end of the experiment (Figure V-8A). Glucose was entirely consumed within 20 – 30 h for the experiments with transitions *Tr1*, *Tr2* and *Tr3* whereas a residual glucose concentration of 50 mM was measured in the *Control* culture (Figure V-8B). The highest specific glucose consumption rates were obtained when *C. glutamicum* 2262 *AldhA* was during the aerobic phase and were 9.3, 7, 9.1 and 7.4 mmol (g cdw)⁻¹ h⁻¹, in *Control*, *Tr1*, *Tr2* and *Tr3* cultures, respectively (Figure V-8D, Table V-3).

As shown in Figure V-9A, the succinate concentrations increased continuously since the beginning of the anaerobic phase with differences in the concentrations of succinate between the different transitions phases. The maximal titer of succinate produced were 118.2 (13.6 g L⁻¹), 183 (21.6 g L⁻¹) and 326.9 (36.9 g L⁻¹) mM for cultures performed with *Tr1*, *Tr2* and *Tr3*, respectively (Table V-3). The yields of succinate reached the highest value of 0.94 mole per mole of glucose in the *Tr3* experiment (Table V-3). Whatever the transition strategy, succinate was the main product with productions 5 and 7 times higher than acetate and pyruvate, respectively. According to the transitions applied, unlike the effects observed on succinate production, the influence on acetate production was less pronounced (Figure V-9B and C). The maximal titer of acetate produced was 68 mM with a yield of 0.19 mole per mole of glucose (Table V-3). The pyruvate concentration reached 48.7 mM with a conversion yield of 0.14 mole

per mole of glucose (Table V-3). The accumulated pyruvate was re-consumed along the culture time.

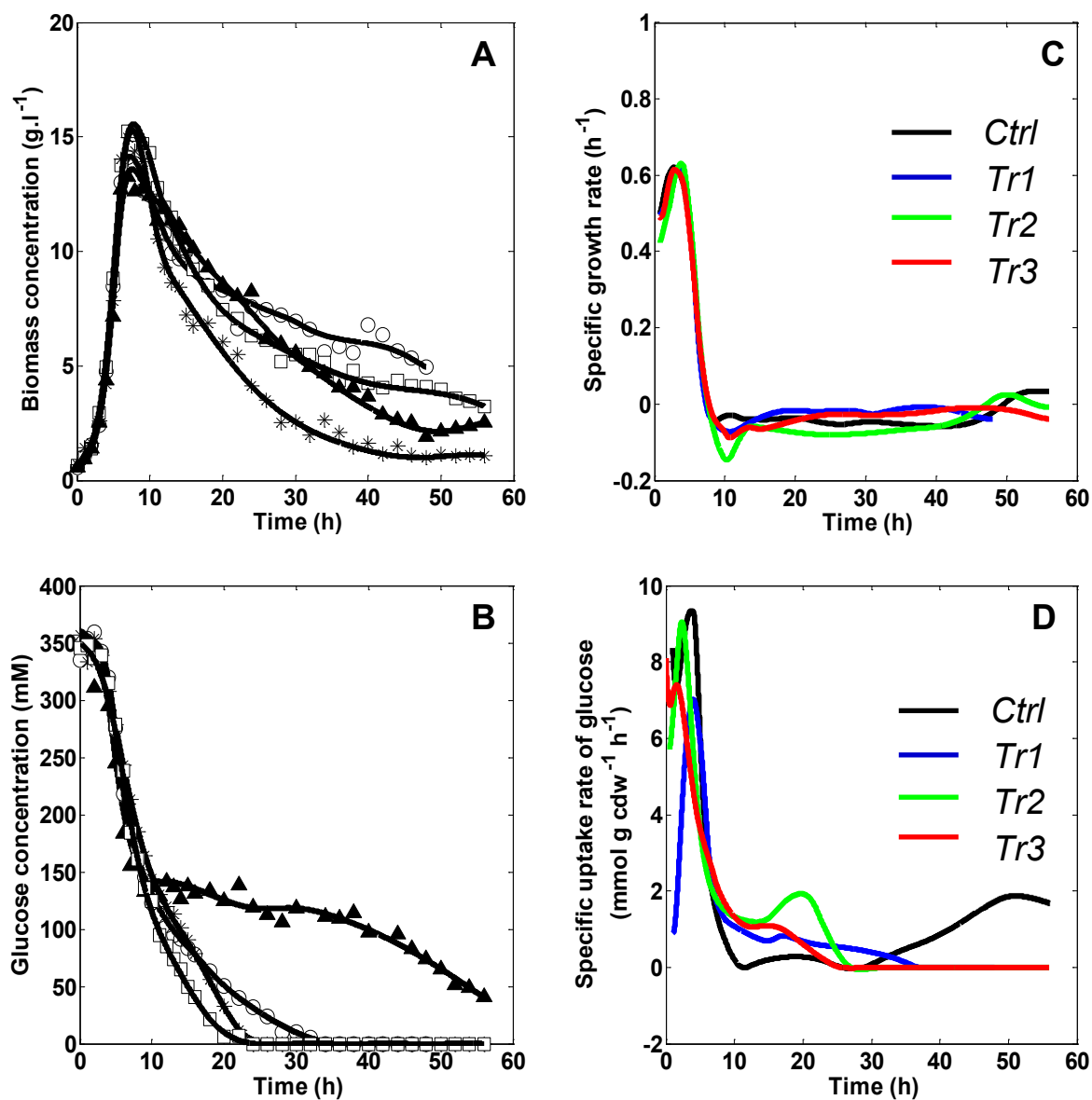


Figure V-8. Influence of transition strategies on kinetics of *C. glutamicum* 2262 AldhA. A. Profiles of biomass concentrations, B. Residual glucose concentration, C. Specific growth rate, D. Glucose specific consumption rate. The different symbols correspond to: (\blacktriangle) Control, (\circ) Tr1, ($*$) Tr2 and (\square) Tr3.

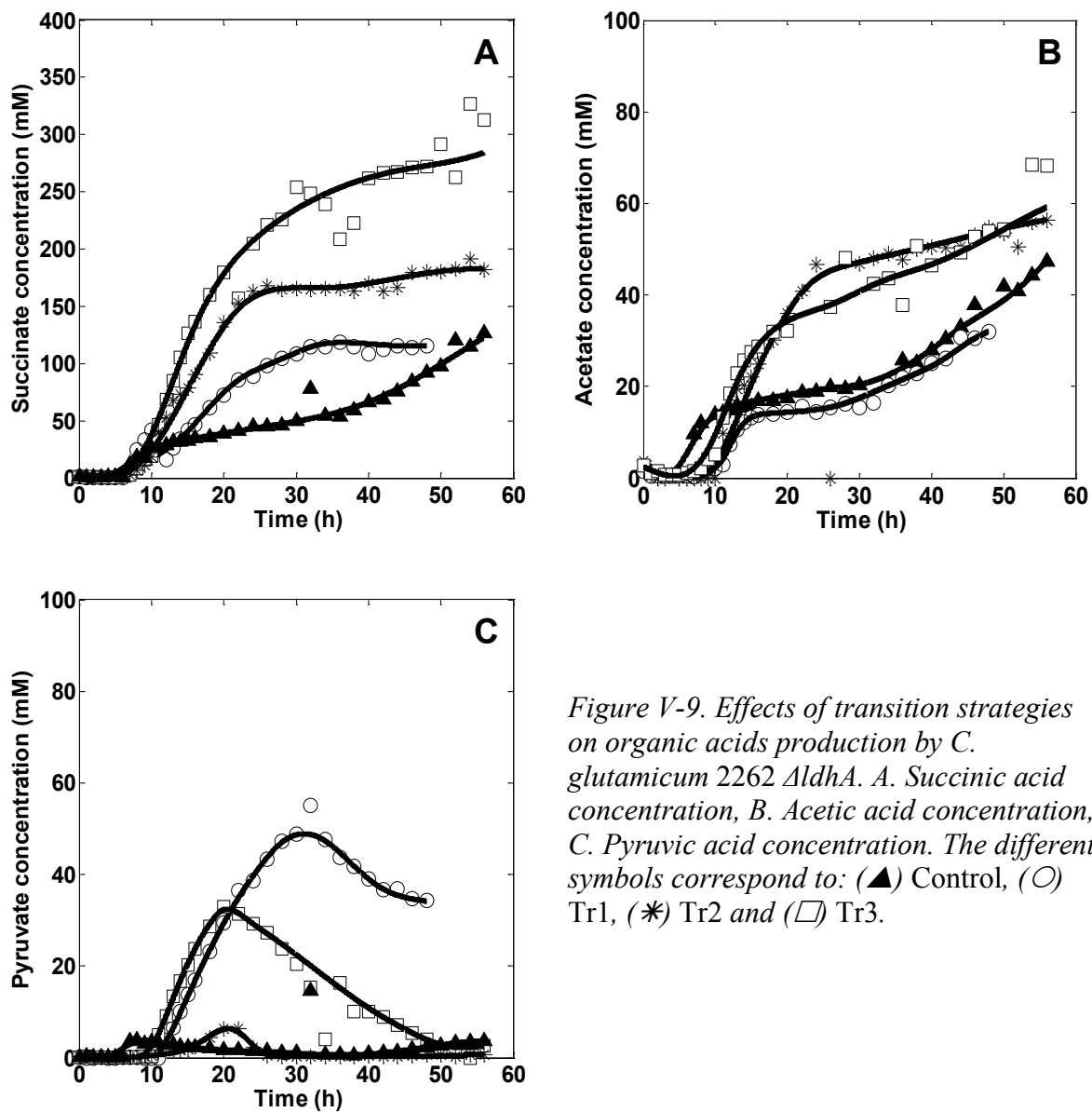


Figure V-9. Effects of transition strategies on organic acids production by *C. glutamicum* 2262 *AldhA*. A. Succinic acid concentration, B. Acetic acid concentration, C. Pyruvic acid concentration. The different symbols correspond to: (▲) Control, (○) Tr1, (*) Tr2 and (□) Tr3.

V-3.4. Management of deoxygenation phase via the control of air flow rates

Studies on the influence of operating parameters reported that changes in the level of aeration and agitation during the cell growth phase significantly influenced the succinate production by *E. coli*. Indeed, during experiments at constant aeration flow rates till the beginning of the anaerobic phase and according to the level of aeration applied, self-induced microaerobic conditions could occur resulting in different succinate production levels (Martínez *et al.*, 2010; Zhu *et al.*, 2011, Chapter IV). For this reason, the succinate production by *C. glutamicum* 2262 *AldhA* was studied by varying the aeration flow rates during the transition phase before.

V-3.4.1. Description of the various transitions applied and of the resulting DOC

In this section, contrary to the cultures previously performed with *C. glutamicum* 2262 *AldhA*, the transition phase was managed through the control of aeration flow rates. In such conditions, microaerobic and oxygen deprived conditions were induced by bacterial oxygen consumption and could occur before the 'true' anaerobic phase of culture. As previously, three phases were successively applied to *C. glutamicum* 2262 *AldhA*. These three phases of the process were all done in the same bioreactor, containing MCGC and 125 mM of bicarbonate. The first phase was an aerobic phase of growth in which the DOC was maintained at 50 % of air saturation. In this phase, bacteria were cultivated until an optical density at 570 nm of 30 (8.1 g cdw L⁻¹). When the targeted biomass concentration was reached, cells were submitted to transition phase. For this transition, three different deoxygenation strategies were set-up as:

- *Tr4*, DOC was controlled at 50 % of air saturation until an OD₅₇₀ reached the value of 30. At that time, aeration flow rate was 100 L h⁻¹. Then, the aeration rate was shifted to 60 L h⁻¹ and maintained for 2 h. It was reduced again to 25 L h⁻¹ for 2 h before aeration was stopped;
- *Tr5*, During the growth phase, the DOC was maintained at 50 % of air saturation by air injection only in order to reach an optical density at 570 nm of 30. Once air was no longer sufficient to maintain the DOC to 50 %, the aeration flow rate was set to 60 L h⁻¹ and maintained for 4 h before aeration was shut off;
- *Tr6*, Culture was performed under constant aeration flow rate of 110 L h⁻¹ until an OD of 30. When this OD was reached, the aeration was decreased to 65 L h⁻¹ and maintained for 2 h, the aeration rate was shifted to 10 L h⁻¹ for 2 h before the aeration was stopped (Figure V-10).

After the transition phase, during the last phase of the process the aeration was arrested to induce anaerobic conditions.

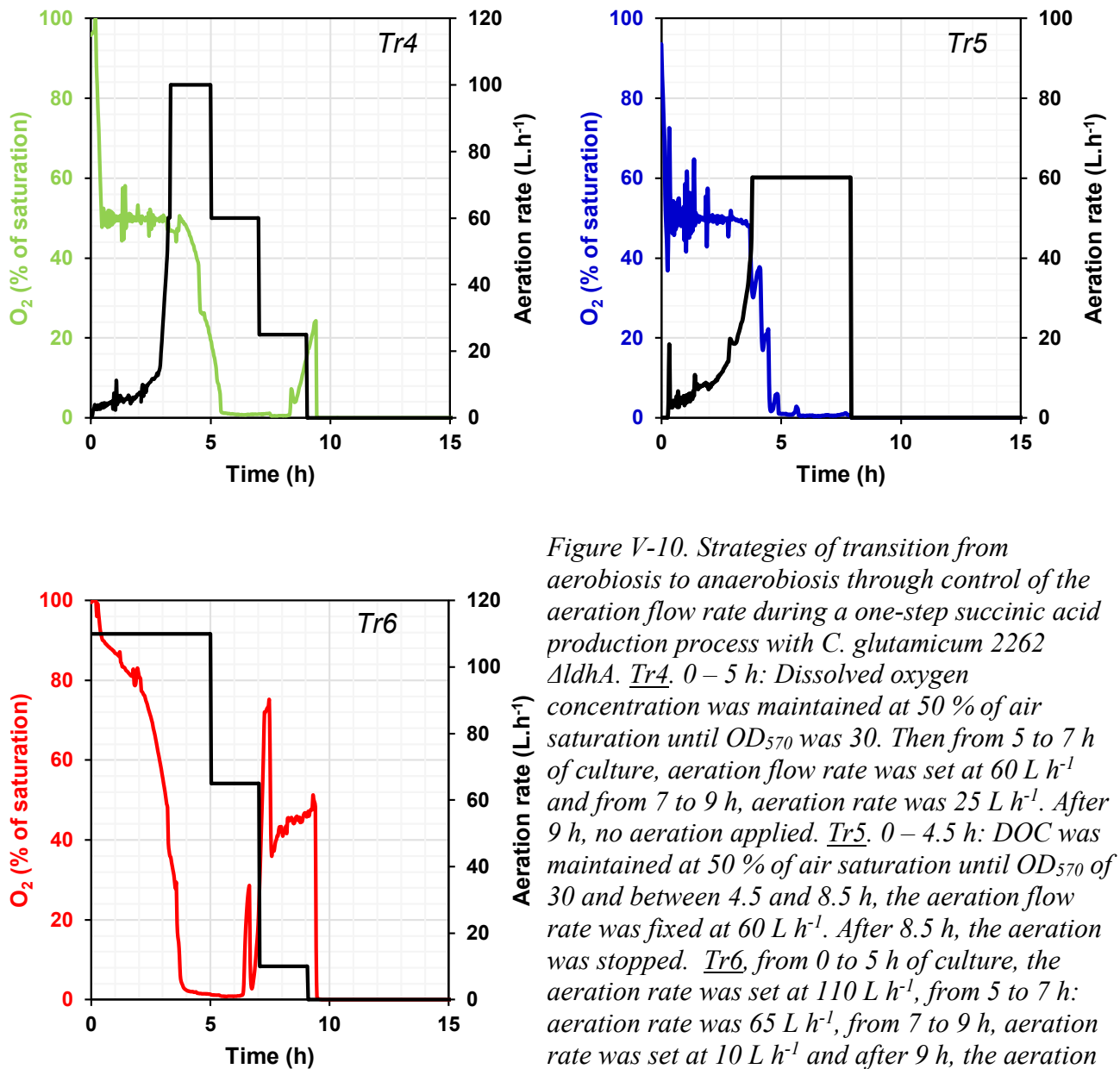


Figure V-10. Strategies of transition from aerobiosis to anaerobiosis through control of the aeration flow rate during a one-step succinic acid production process with *C. glutamicum* 2262 *AldhA*. *Tr4*. 0 – 5 h: Dissolved oxygen concentration was maintained at 50 % of air saturation until OD_{570} was 30. Then from 5 to 7 h of culture, aeration flow rate was set at 60 L h^{-1} and from 7 to 9 h, aeration rate was 25 L h^{-1} . After 9 h, no aeration applied. *Tr5*. 0 – 4.5 h: DOC was maintained at 50 % of air saturation until OD_{570} of 30 and between 4.5 and 8.5 h, the aeration flow rate was fixed at 60 L h^{-1} . After 8.5 h, the aeration was stopped. *Tr6*, from 0 to 5 h of culture, the aeration rate was set at 110 L h^{-1} , from 5 to 7 h: aeration rate was 65 L h^{-1} , from 7 to 9 h, aeration rate was set at 10 L h^{-1} and after 9 h, the aeration was shut off. Colors: black (aeration rate); blue, green and red (DOC).

V-3.4.2. Succinic acid production by *C. glutamicum* 2262 *AldhA*: effects of the strategies of the control of aeration rate during the deoxygenation transition phase from aerobiosis to anaerobiosis

The growth was perfectly similar between the three conditions during the first aerobic phase (Figure V-11A) with a maximal specific growth rate of 0.6 h^{-1} (Figure V-11C). For the *Tr4* and *Tr5* experiments (Figure V-10), during the first phase, the dissolved oxygen level was

regulated at 50 %, thus during the transition phases, *C. glutamicum* 2262 *AldhA*, kept on growing until a biomass concentration of about 13.7 g cdw L⁻¹. For *Tr6* experiment which was a culture with a constant air flow rate during the first aerobic phase instead of a DOC control, the culture showed a DOC decline that contributed to lower the maximal biomass obtained (10 g cdw L⁻¹) during the transition phase (Figure V-11A). For the experiments performed with transitions *Tr4* and *Tr5*, the glucose consumptions were similar until the beginning of anaerobic phase with a maximal specific uptake rate of 6.8 mmol (g cdw)⁻¹ h⁻¹ (Figure V-11B and D, Table V-3). When the anaerobic phase was set, the glucose was slightly consumed and a residual amount of 77 mM remained in *Tr5* while glucose was entirely consumed in *Tr4* experiment. In the case of *Tr6*, the maximum specific consumption rate was reduced to 4.5 mmol (g cdw)⁻¹ h⁻¹ (Figure V-11B and D).

Other consequences of the transition phase performed by the control aeration flow rate, was a clear difference on organic acids production. Succinate remained the main fermentation end-product for all cultures conditions (Figure V-12). The maximal succinate titer obtained was 290 mM (34 g L⁻¹) (Figure V-12A) when transition *Tr4* was applied (Figure V-10). 81 % of glucose consumed were converted into succinic acid while only 58 and 44 % were used for succinate production during the cultures with transitions *Tr5* and *Tr6*, respectively (Table V-3). The maximal acetate production of 35 mM, with a yield of 0.12 mole per mole of glucose, was obtained during *Tr5* culture and 48 mM of pyruvate, with a yield of 0.16 mole per mole of glucose, was reached when experiment was conducted with *Tr6* transition (Figure V-12B and C, Table V-3).

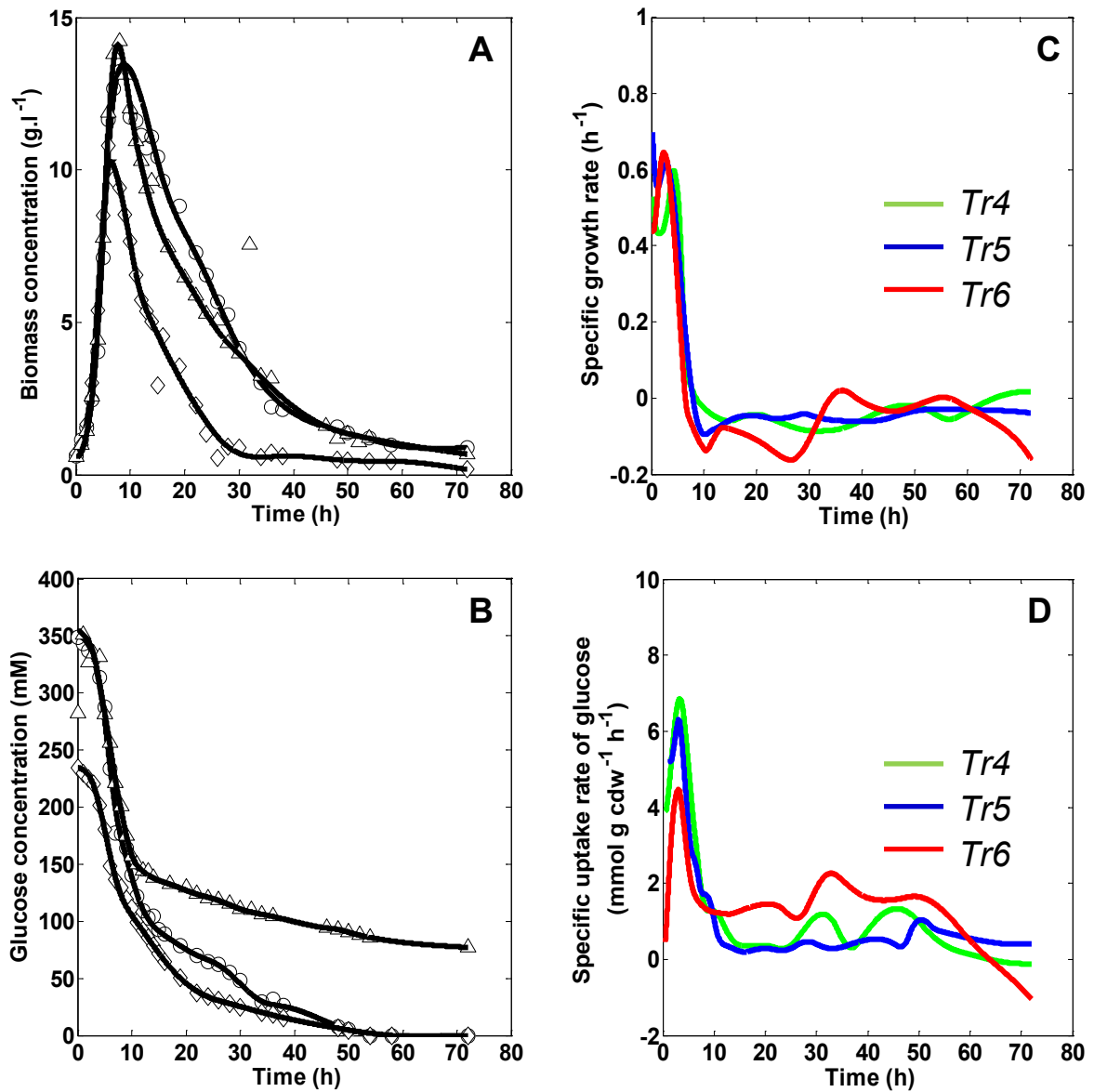


Figure V-11. Consequences of the regulation of the aeration rate during the transition phase during the succinate production process with *C. glutamicum* 2262 Δ ldhA. A time course of biomass concentration, B residual glucose concentration, C specific growth rate, D glucose specific consumption rate. The different symbols correspond to transitions (○) Tr4, (△) Tr5 and (◇) Tr6.

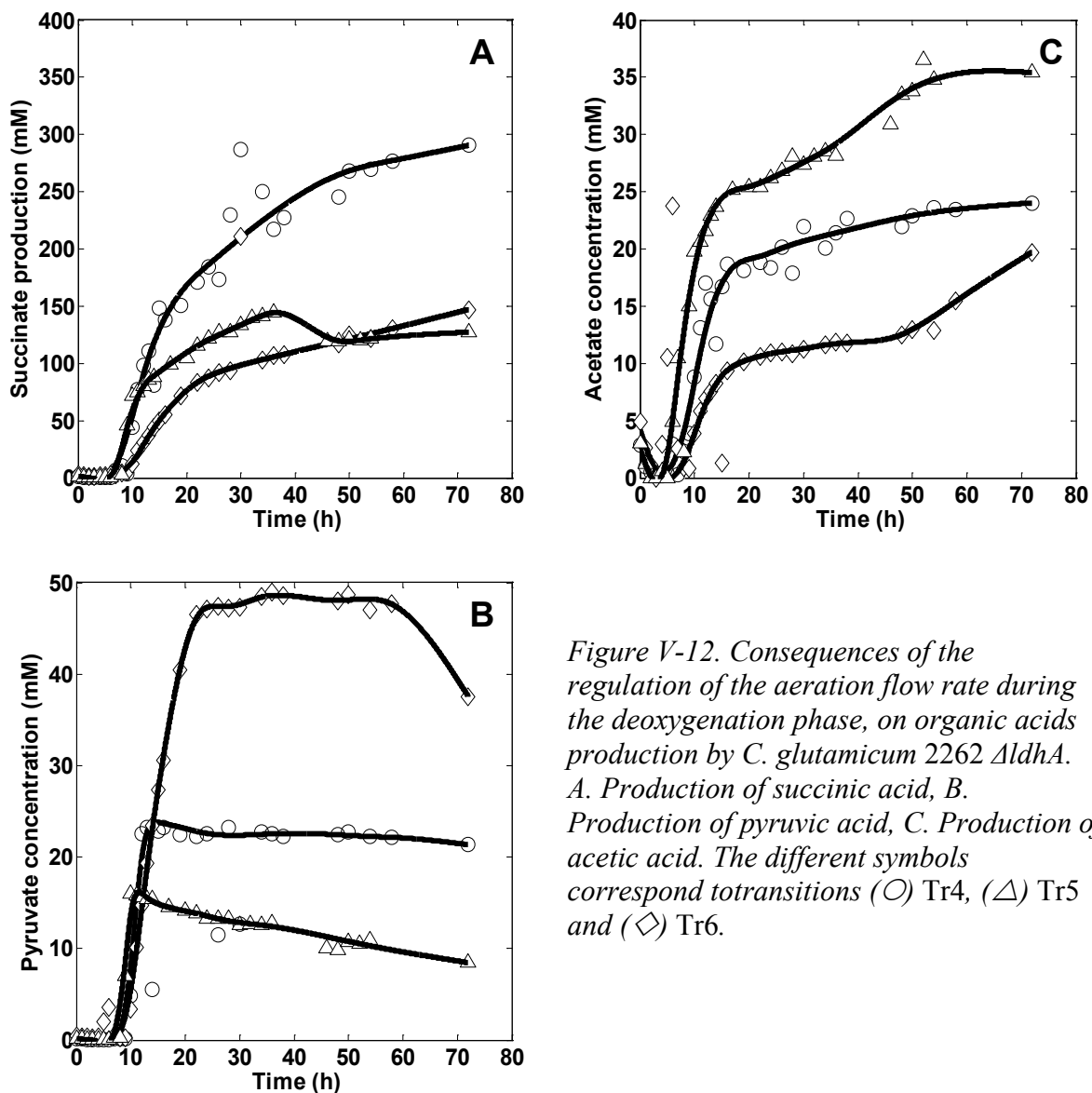


Figure V-12. Consequences of the regulation of the aeration flow rate during the deoxygenation phase, on organic acids production by *C. glutamicum* 2262 Δ ldhA. A. Production of succinic acid, B. Production of pyruvic acid, C. Production of acetic acid. The different symbols correspond to transitions (○) Tr4, (△) Tr5 and (◇) Tr6.

V-3.5. Influence of inoculum sizes on succinate production during a process with transition *Tr3*

Taking advantage of *C. glutamicum* ability to excrete in significant amounts of several metabolites including organic acids, production processes based on high cell density were developed in the literature. Using such process, *C. glutamicum* was cultured at high cell-density under anaerobic conditions, and produced lactate and succinate at high levels (Okino *et al.*, 2005, 2008a, 2008b; Litsanov *et al.*, 2012b; Tsuge *et al.*, 2013). However, in the present work, the onset of transition and the strategy of deoxygenation were reported to influence the succinate production in the anaerobic. Thus, dedicated cultures were also performed to analyze the influence of the starting concentration of biomass.

Table V-3. Influence of different deoxygenation strategies on the performance of succinate production process.

Transition	Biomass		Maximal products conc. (mM)			*Maximal yields (mol.mol ⁻¹)			Maximal rates (mmol (g cdw) ⁻¹ h ⁻¹)			
	g L ⁻¹	h ⁻¹	[Suc]	[Ace]	[Pyr]	Ysuc	Yace	Ypyr	qsuc	qace	qpyr	qglc
<i>Control</i>	13.2	0.62	126.7	47.2	3.83	0.46	0.17	0.02	0.4	0.3	0.1	9.3
<i>Tr1</i>	14.1	0.62	118.2	32.0	48.7	0.33	0.08	0.1	0.8	0.3	0.4	7.0
<i>Tr2</i>	15.0	0.63	183.0	56.4	6.45	0.51	0.15	0.02	1.7	0.5	0.2	9.1
<i>Tr3</i>	15.2	0.61	327.0	68.4	33.0	0.94	0.19	0.10	1.6	0.3	0.4	7.4
<i>Tr4</i>	13.2	0.60	290.5	24.0	23.3	0.81	0.06	0.10	1.4	0.2	0.5	6.9
<i>Tr5</i>	14.2	0.62	144.6	35.4	16.0	0.58	0.12	0.10	1.3	0.3	0.4	6.7
<i>Tr6</i>	10.8	0.65	147.1	19.7	48.9	0.44	0.04	0.20	1.1	0.2	0.9	4.5

*The yields were calculated using the quantity of glucose consumed from the beginning of culture to the moment where the maximal concentration of product was measured.

Among the different strategies of transitions studied, the results of experiments performed with transition *Tr3* (Figure V-7) showed the highest titer and yield of succinate produced. Therefore, the transition *Tr3* has been used to study the impact of inoculum size effects on succinic acid production. Two different inoculum sizes of strain *C. glutamicum* 2262 *AldhA* were studied:

- ‘Small’ inoculum corresponding to approximately 8 % of inoculation.
- ‘Large’ inoculum corresponding to approximately 17 % of inoculation.

The experiments were carried out in a fed-batch bioreactor containing MCGC medium with 125 mM of NaHCO₃. The initial glucose concentration was about 380 mM. When the residual glucose concentration was lower than 50 mM, a pulse of 50 mL of glucose at a concentration of 3.5 M combined with 50 mL of NaHCO₃ solution at concentration of 1.5 M was added to the culture broth. The small inoculum resulted in an initial cell concentration of 6.3 g cdw L⁻¹ (OD₅₇₀ of 23) whereas the large inoculum resulted in an initial biomass concentration of 13.9 g cdw L⁻¹ (OD₅₇₀ of 51). The culture started at an OD_{570nm} of 23, generated a succinate yield of 1.06 mole per mole of glucose. Even if the succinate production kinetics showed important differences during the first 30 h, the highest titers reached within 50 h of fermentation, were 404 mM (48 g L⁻¹) and 412 mM (49 g L⁻¹) during cultures inoculated with small inoculum or large inoculum concentration, respectively (Figure V-13C). At the end of the

cultures, elevated residual glucose concentrations were measured in both cultures (Figure V-13B). Results thus showed a significant reduction in succinate yield when a large inoculum was used, indicating that glucose was less metabolized through the succinate synthesis pathways. The culture at the starting OD₅₇₀ of 23 achieved a global succinate productivity of 0.9 g L⁻¹ h⁻¹ until the higher titer was obtained. This succinate productivity was substantially higher than that of the culture inoculated with the large inoculum which was 0.7 g L⁻¹ h⁻¹. The final biomass concentrations for the small and the large inoculum cultures were 18 and 26 g cdw L⁻¹, respectively (Figure V-13A). Acetate was also produced with a final molar ratio of succinate to acetate for the small inoculum and large inoculum cultures of 13.1 mol mol⁻¹, 10.7 mol mol⁻¹, respectively. This implies that more carbon flux was directed towards succinate synthesis when starting the culture with a smaller biomass concentration (Figure V-13D). In addition, pyruvate was produced in higher concentration than acetate (Figure V-13D and E). The final molar ratios of succinate to pyruvate were 11.6 mol mol⁻¹ and 5.4 mol mol⁻¹ for cultures inoculated with small or large inoculum, respectively.

V-4. Discussion

To verify its ability to produce organic acids under oxygen deprived conditions, batch cultures of *C. glutamicum* 2262 *AldhA* were established in a one-stage process in bioreactor containing a minimal medium, with or without bicarbonate. During a first aerobic growth, bacteria were cultivated until two different concentrations of biomass (OD_{570nm} of 10 and 50). Once the targeted biomass concentration was reached, the cultures were switched instantaneously under anaerobic conditions by stopping the aeration. In such conditions, up to 127 mM succinate were produced in presence of bicarbonate and when the biomass concentration was around 14 g L⁻¹ (OD_{570nm}=50). This suggested that, the higher the biomass concentration was during the process, the higher the final concentration of succinate also was. This observation corroborated the classic process of succinic acid production based on high cell density in the succinic acid production phase (Inui *et al.*, 2004a; Okino *et al.*, 2005; Wang *et al.*, 2011b). In a similar process, Wieschalka (2012) cultivated *C. glutamicum* ELB-P in a dual-phase culture with an aerobic growth phase under oxygen excess, followed by an anaerobic production phase during which CO₂ was bubbled when the bacterial growth ended at OD_{600nm} of 55 (Wieschalka, 2012). In this condition, 80 mM of succinate were produced which was lower than the 127 mM obtained in our study. However, the succinic acid yield was identical and was about 0.46 mole succinate per mole of glucose (Wieschalka, 2012) (Figure V-3).

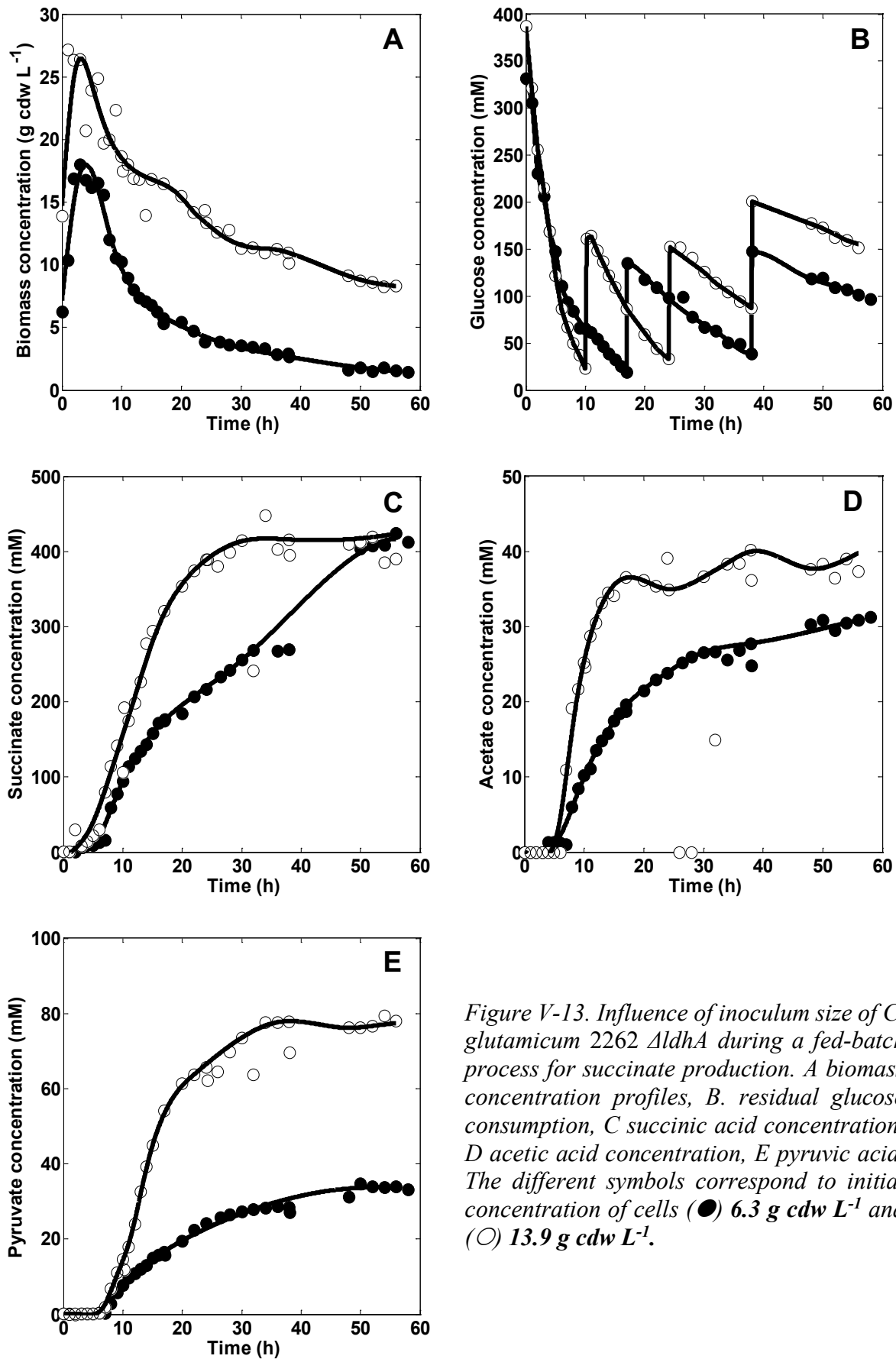


Figure V-13. Influence of inoculum size of *C. glutamicum* 2262 AldhA during a fed-batch process for succinate production. A biomass concentration profiles, B. residual glucose consumption, C succinic acid concentration, D acetic acid concentration, E pyruvic acid. The different symbols correspond to initial concentration of cells (●) 6.3 g cdw L⁻¹ and (○) 13.9 g cdw L⁻¹.

Succinic acid production pathway in *C. glutamicum AldhA* or wild type strain (Figure V-1), involved the reductive branch of the TCA cycle under anaerobic conditions. Under these conditions, succinate is produced via anaplerotic enzymes consisting in carboxylation of PEP or pyruvate to OAA by PEPC and/or PC with fixation of CO₂. OAA is subsequently transformed by MDH, FUM and SDH to succinate (Inui *et al.*, 2004a). Thus, CO₂ is necessary for reductive production of succinate. As a CO₂ donor, sodium bicarbonate effects have been investigated with *C. glutamicum AldhA-pCRA717* and revealed that, without bicarbonate, succinate production from glucose was very low. Increasing bicarbonate concentration up to 100 mM during different individual cultures, resulted in increased yields from 0.5 to 1.4 mole per mole of glucose (Okino *et al.*, 2008a). In this study, a one-step process was done in medium containing 125 mM of NaHCO₃. When aerobic growth was conducted until OD_{570nm} of 10, the presence of bicarbonate resulted in a 48 % increase in the maximal glucose uptake rate and a 40 % increase in succinic titer. When aerobic phase was conducted until OD_{570nm} of 50, only a 20 % increase in glucose specific uptake rate and a 16 % increase in succinate amount were noticed. Bicarbonate added in this study appeared to exhibit lower influence on succinate production at high optical density culture compared to literature studies. In fact, cultures with *C. glutamicum* R reported an increase of glucose specific consumption rate from 3.3 to 6.8 mmol (g cdw h)⁻¹ when 100 mM of NaHCO₃ were added to the culture medium. In the same time the succinate concentration increased from 15 to 32 mM (Okino *et al.*, 2005, 2008a). Furthermore, in contrast to the results observed by Wieschalka for which addition of bicarbonate increased the succinate to substrate yield from 0.78 to 0.91 mole succinate per mole of glucose (Wieschalka, 2012) in the present study, the yield was decreased from 0.46 to 0.35 mol mol⁻¹. The difference observed could be due to the fact that with *C. glutamicum* 2262 *AldhA*, CO₂ was probably not the limiting substrate as the CO₂ produced during the first phase of the process remained in the culture medium, on contrary to the processes including cells harvest and wash. In these cases, bicarbonate addition was essential. However, addition of various concentrations of bicarbonate did not show any change in biomass concentrations (Okino *et al.*, 2005) as it was observed with cultures grown until OD_{570nm} of 10 and 50 (Figure V-4).

In the literature, efficient fermentation processes to produce succinate with *C. glutamicum* have been reported but they were mainly based on strains improvement. Indeed, impressive and interesting results have been obtained using metabolic engineering of *C. glutamicum* strains. Okino *et al.* (2008) constructed *C. glutamicum AldhA-pCRA717* from *C. glutamicum* R. In a

two step-process, this mutant strain was grown aerobically and the cell mass was harvested, washed and incubated at a biomass concentration of 50 g cdw L⁻¹, in anaerobic conditions. In this process and with a strain lacking *ldhA* gene and overexpressing *pyc* gene, 1.2 M of succinate with yields of 1.4 and 0.3 mole per mole glucose of succinate and acetate, respectively (Okino *et al.*, 2008a). Based on a similar strategy, Litsanov *et al.* (2012) designed *C. glutamicum* BOL-3/pAN6-*gap* with deletions of all known genes involved in acetate and lactate synthesis. The *fdh* gene, encoding formate dehydrogenase, was integrated into the genome of the resulting mutant in order to provide more reduced equivalents and CO₂ from formate. By over expressing the *gapA* gene, encoding glyceraldehyde phosphate dehydrogenase, for glycolytic flux increase, the final mutant strain produced up to 1.1 M of succinate with the highest yield of 1.67 mole succinate per mole glucose during a fed-batch culture on a substrate mixture of glucose and formate, complemented with bicarbonate (Litsanov *et al.*, 2012b). Compared to the above mentioned metabolic strategies, this work was focused on the process engineering as a complementary strategy for succinate production improvement. In previous experiments in un baffled shake flasks (Chapter IV), with a biomass concentration of ~2.8 g cdw L⁻¹, 63 mM succinate were measured whereas a controlled culture in bioreactor resulted in only 10 mM of succinate with a similar biomass concentration (Table V-2). In flask cultures, bacteria self-induced the shift from aerobic to anaerobic with transition while the shift in bioreactor was imposed by a sudden interruption of aeration. From this observation, the strategy to switch from aerobic to anaerobic conditions appears as a probable key parameter in succinate production. Thus, the influence of the different transitions from aerobic phase to an anaerobic succinic acid production phase was studied. By switching instantaneously from aerobic to anaerobic, a succinate concentration of 127 mM with a yield of 0.46 mol mol⁻¹ was reached. When a transition phase was introduced before starting the anaerobic conditions (Figure V-7, Figure V-8 and Figure V-9), the maximal succinic acid concentration of 327 mM with the highest yield of 0.94 mol mol⁻¹ was obtained. This yield was obtained when *C. glutamicum* 2262 *ΔldhA* was cultivated in aerobic phase with controlled DOC at 50 % of air saturation until a biomass of 8.7 g cdw L⁻¹ was reached followed by a 2 h period with a dissolved oxygen level at 20 % of air saturation, then 2 h at 10 % and finally the stop of the aeration to begin the anaerobic phase. Different transition durations were tested and it appeared that the transition with more progressive lowering of DOC resulted in improved succinate production in comparison to instantaneous switching from aerobiosis to anaerobiosis (Table V-3). Besides, Zhu *et al.* (2011) designed a one-step triphasic process with *E. coli* SBS550MG, with non-controlled agitation and pH during growth, followed by a transition phase in which DOC was always at a relatively

low level (0–20 %), induced by bacterial oxygen uptake. The duration of this phase was controlled by glucose feeding before switching to the anaerobic phase. It was observed that the air supply conditions in the aerobic and micro-aerobic transition phases, affected the cell density and cell viability at the switch to anaerobic phase, which affected the overall production rate and the final succinate titer. A transition duration of 2-5 hours was shown to be optimal for the pathway fine-tuning in the cells which could have resulted in improved succinate production process. Indeed, Zhu *et al.* (2011) performed tri-phasic process for succinate production with *E. coli* SBS550MG and showed that transition duration lower than 2 h resulted in succinate titer of 300 mM. When this duration was between 2 – 5 h, the titer was between 380 and 450 mM. For a duration of transition phase higher than 5 h, the succinate concentration dropped to around 100 mM (Zhu *et al.*, 2011). In the present study, it is important to note that, the different types of transitions clearly influenced the succinate production while the by-products synthesis like acetate and pyruvate were not really impacted. 68 mM of acetate and 48 mM of pyruvate with production yields of 0.19 and 0.16 mole per mole of glucose, respectively, were produced.

In *C. glutamicum* 2262 Δ *ldhA* cultures in shake flasks at different oxygen transfer rates (Chapter IV, Figure IV-1B), a progressive reduction of DOC was measured. This was the consequence of the cells growth and resulted in oxygen-limited conditions and micro-aerobiosis. The ‘self-induced’ micro-aerobic phase before anaerobic has already been reported to have a beneficial effect on succinate production. Martinez *et al.* (2010), applied two different aeration and agitation conditions (low aeration and high aeration) during the aerobic-growth phase of *E. coli* SBS550MG before switching the cells in oxygen deprived conditions. The different aeration conditions have led to different dissolved oxygen profiles. The dissolved oxygen level decreased quickly with the lower aeration, leading to a micro-aerobic condition that was maintained for about 3 h before fully anaerobic conditions. With the higher aeration, the micro-aerobic condition was never obtained before reaching the anaerobic phase. The consequences of these two conditions were succinate concentrations of 167 mM with a yield of 1.6 mol mol⁻¹ and 115 mM with a yield of 1.3 mol mol⁻¹ for low and high aeration rates, respectively. It was shown that the consequence of the micro-aerobiosis was an up-regulation of the genes encoding anaplerotic enzymes and enzymes involved in the glyoxylate pathway (Martínez *et al.*, 2010). Considering the potential impact of some of these genes in the succinate biosynthetic pathway, transitions based on the control of aeration flow rate instead of the DOC regulation were performed. It appeared that, with constant air flow rates, and according to the different steps applied, cells growth introduced different profiles of oxygen concentrations

resulting in micro-aerobic environments. The occurrence of such micro-aerobiosis might enable the cells to adjust their enzymatic machinery by expressing the proteins that were required in the anaerobic production phase. Thus, cells that have experienced micro-aerobiosis through self-induced oxygen limitations could be the best biocatalysts for succinate production. During this study, in a dual phase, when cells, cultivated at DOC of 50 %, were promptly shifted to DOC = 0 %, only 15 mM of succinate were produced when biomass concentration was 3 g cdw L⁻¹. When the shift was operated at a biomass concentration of 14 g cdw L⁻¹, about 100 mM were produced. In the same time, cultures were performed during 50 h at constant agitation of 200 rpm and constant air flow rate of 120 L h⁻¹; the DOC was 100 % of air saturation at the beginning of culture and dropped to approximately 0 within 2 h until the end of the culture. In these conditions, cells were exposed to microaerobiosis. The maximal biomass concentration reached was 4.5 g cdw L⁻¹, and the succinate produced was 144 mM; meaning that even with low biomass concentration, the cells that have been cultivated in such conditions were more efficient than those cultivated until a biomass concentration of 14 g cdw L⁻¹, with instantaneous shift of DOC from 50 to 0 %. In addition, the culture performed with transitions *Tr4* to *Tr6*, presented a progressive decrease in DOC, promoting microaerobiosis and showed better succinate production, in comparison with the *Control* (rapid and sudden deoxygenation).

A similar one-step process involving *C. glutamicum* ELB-P have been developed by Wieschalka (Wieschalka, 2012). In this study, the agitation rate varied from 400 to 750 rpm with a constant aeration of 1 vvm during the aerobic phase and 400 to 1000 rpm, 1.25 vvm all over the micro-aerobiosis phase. CO₂ has also been sparged during the whole anaerobic phase. With this process, *C. glutamicum* ELB-P produced up to 350 mM succinate with a yield of 1 mole succinate per mole of glucose compared to 291 mM with yield of 0.8 mol mol⁻¹ obtained in this study. Similar performances were then obtained with both strains although their genetic background was really different. Whereas *C. glutamicum* 2262 was deleted only for *ldhA* gene, the strain constructed by Wieschalka (2012) was modified for several genes (*C. glutamicum* $\Delta aceE \Delta pqq \Delta ldhA \Delta C-TilvN \Delta alaT \Delta avtA$) (Wieschalka, 2012). The other major difference between the two processes is the transition time. With *C. glutamicum* 2262 $\Delta ldhA$, the transition was started when bacteria were in active growth phase whereas with *C. glutamicum* ELB-1, the anaerobic conditions were imposed during the stationary growth phase. The physiological states obtained at the switching time could have influenced bacteria ability to adapt to a new environment. In fact, Vemuri *et al.* (2002b) set up six transition times based on physiological states of bacteria to study dual -phase succinate production with *E. coli* AFP111 and *E. coli*

AFP111/pTrc99A-*pyc*. The authors performed full aerobic cultures and according to the respiratory quotient and DOC, physiological milestones were determined to be useful mark for transition point. Three milestones corresponding to physiological moment where cells growth rate was linear, with decreasing DOC, associated to increased activity of fumarate reductase, were used as transition time in dual-phase culture. The dual-phase resulted in significantly greater volumetric productivities. Fed-batch dual phase culture of *E. coli* AFP111 / pTrc99A-*pyc* using such milestones for transition time determination, resulted in succinic acid concentration of 99 g L⁻¹ with an overall volumetric productivity of 1.3 g (L h)⁻¹. Thus, the ideal time of transition should probably be selected based on physiological conditions (Vemuri *et al.*, 2002b).

Finally, *Tr3* transition was used to study the influence of the initial biomass concentration on the succinate production. Surprisingly, this parameter had only a slight influence on succinate production. Indeed, beginning the culture at a biomass concentration of 6.3 or 13.9 g cdw L⁻¹ resulted in similar concentrations, 424 mM succinate with a yield of 1.1 mol mol⁻¹ or 453 mM succinate with a yield of 0.86 mol mol⁻¹, respectively. However, some modifications in the transition time, according to the biomass concentration and the cellular physiological state could probably allow a further enhancement of these performances.

V-5. Conclusion

With *C. glutamicum* 2262 Δ *ldhA*, most of the deoxygenation strategies that were based on a gradual transition to anaerobic conditions results in higher concentrations of succinate and conversion yields than the processes in which cells were submitted to an instantaneous transition from aerobic to anaerobic conditions. This confirmed the importance of the transition phase in the succinate producing process using mutant strains of *C. glutamicum*. Moreover, it appeared that the addition of bicarbonate had no impact on *C. glutamicum* 2262 Δ *ldhA* growth. The influence of the transitions could be associated with the occurrence of a micro-aerobiosis period. With the most efficient process developed in the present study, 327 mM and 424 mM succinate were produced in batch and fed-batch process, respectively. These final titers were comparable with the performance of some natural producers such as *M. succiniciproducens*, *A. succinogenes*, *A. succiniciproducens* (Lee *et al.*, 2001, 2006; Urbance *et al.*, 2004) and non-natural producers with multiple mutations like *E. coli* (Sánchez *et al.*, 2005b; Wu *et al.*, 2007). Thus, the present study underlined the key parameters influencing the microorganism metabolic behavior. Therefore, the performances of the succinate producing process resulted of a complex

interaction between the physiology of the producing cells and the process parameters. So, the next step would consist in studies at the transcriptional, translational and enzymatic levels to understand the physiological responses to DOC changes, particularly during the transition from aerobic to anaerobic conditions.

V-6. References

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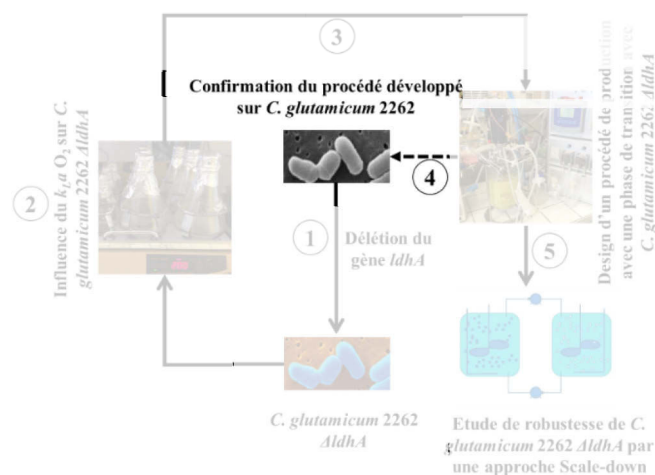
Chapitre VI

Application du procédé de
production d'acide succinique
à la souche sauvage,
C. glutamicum 2262

Chapitre VI : Avant-propos

Dans le chapitre précédent, plusieurs stratégies de désoxygénation ont été appliquées pendant la phase de transition entre la phase aérobie et la phase anaérobie. L'étude du choix de ces stratégies sur les performances de production a abouti à la mise en œuvre d'un procédé efficace présentant des rendements intéressants par rapport aux données de la littérature. Ce procédé a été développé en utilisant une souche mutante *C. glutamicum* 2262 *AldhA* afin de limiter la production de lactate, considéré ici comme un sous-produit du procédé. Par ailleurs, la souche sauvage de *C. glutamicum* R est citée dans la littérature comme une souche surproductrice de lactate en condition anaérobie avec des coproduits tels que le succinate et l'acétate en faibles quantités (Okino et al., 2005). Nous avons également démontré que les quantités de succinate produites par la souche mutante restaient sensiblement inférieures à celles produites par la souche sauvage (Chapitre III).

Dans le présent chapitre, nous avons évalué l'efficacité du procédé de production basé sur une désoxygénation progressive du milieu de culture, lors de cultures mettant en œuvre la souche sauvage, *C. glutamicum* 2262. Pour cela, cette souche sauvage a été cultivée en bioréacteurs de 2 L en appliquant soit le procédé avec une désoxygénation progressive soit une désoxygénation brusque. L'impact du mode de culture (discontinu fermé ou discontinu alimenté) sur la réponse physiologique a été également étudié et les performances de production de succinate comparées à celles reportées dans la littérature.



VI. Application of a one-step process with a designed aerobic-anaerobic transition phase to *Corynebacterium glutamicum* 2262

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Abstract

Corynebacterium glutamicum has been widely used for the industrial production of amino acids (glutamate, lysine, isoleucine, etc.). Recent studies have also extensively demonstrated its ability to produce organic acids in oxygen-deprived or anaerobic conditions, in the absence of growth. However, under such conditions, *C. glutamicum* produced mainly lactate at the expense of succinate. In this work, the new tri-phasic process previously developed on *C. glutamicum AldhA* was applied to the parent wild type strain, *C. glutamicum* 2262, in both batch and fed-batch modes. This tri-phasic process consisted successively of an aerobic, a deoxygenation and an anaerobic phase. A particular attention was paid to the design of culture feed composition. Our results showed that batch culture of *C. glutamicum* 2262, in a 2 L bioreactor containing basic culture medium MCGC supplemented with bicarbonate, using designed tri-phasic process, resulted in 280 mM of succinate production, a yield of 0.78 mole per mole of glucose and succinate to lactate ratio of 1.1 mole of succinate per mole of lactate. A similar fermentation performed without transition phase resulted only in a succinate concentration of 164 mM, a yield of 0.5 mole per mole of glucose and succinate to lactate ratio of 0.45 mole of succinate per mole of lactate. Among the four feed compositions studied, the addition of 8 pulses of 50 mL of glucose (3.5 M) and 50 mL of NaHCO₃ (1 M), in a 2 L bioreactor led to 793 mM (94 g L⁻¹) of succinate and 785 mM (71 g L⁻¹) of lactate. The succinate production yield was 1.1 mole of succinate per mole of glucose and acetate production was negligible. Using the fed-batch tri-phasic production process, the highest concentration of succinate reported, so far, for a wild type strain of *C. glutamicum* was thus obtained.

Keywords: Succinic acid, *Corynebacterium glutamicum* 2262, bioprocess, aerobiosis, anaerobiosis, aerobic-anaerobic transition.

VI-1. Introduction

Due to its potential as an important building block from bio-based substrates, the investigations of fermentative processes for succinic acid production (Jansen and van Gulik, 2014) have emerged these last years. In the literature, the studies on fermentative production of succinate were mostly focused on enzyme activities characterization through deletion of genes involved in the competing pathways, overexpression of genes encoding enzymes in the pathways towards succinate formation (Jost *et al.*, 2014; Tsuge *et al.*, 2014; Zhu *et al.*, 2014). However, the studies dedicated to processes involving the modified succinate producers, particularly *Corynebacterium glutamicum*, have to be deeper investigated. In fact, the process strategies used for succinic acid production with genetically improved *C. glutamicum* mainly considered a two-step production. In the first step, the strains were grown under fully aerobic conditions, then cells were harvested, washed and incubated under anaerobic conditions at high cell density (Okino *et al.*, 2005, 2008a; Litsanov *et al.*, 2012b). Recently, with the reported importance of the transition phase between aerobic and anaerobic conditions on succinic acid production performances, one step processes were investigated (Martínez *et al.*, 2010; Zhu *et al.*, 2011; Wieschalka *et al.*, 2012b). For this purpose, we have previously developed a one-step process with three phases including the commonly used aerobic and anaerobic phases between which a deoxygenation transition phase was introduced. This transition phase was studied in details in Chapter V and it was shown that the process yield of succinic acid production by *C. glutamicum* 2262 *AldhA* was improved when this phase was carefully designed.

In the present study, a one-step process of succinic acid production developed in this previous work was applied to *C. glutamicum* 2262, wild type strain. *C. glutamicum* wild types have been reported to produce only few amount of succinic acid whereas lactic acid was predominant under anaerobic conditions unlike natural succinate producers. The objectives of the study were:

- To demonstrate the real potential of succinate production by *C. glutamicum* wild type strains under suitable process conditions without metabolic engineering
- To confirm the importance of dissolved oxygen management during the process of succinate production with *C. glutamicum*.

- To validate the efficiency of the process previously developed with *C. glutamicum* 2262 *AldhA*.

VI-2. Material and methods

VI-2.1. Bacterial strain, pre-cultures media and protocol

Batch cultures of *C. glutamicum* 2262 were performed in a modified MCGC mineral salt medium in which citrate was replaced by deferoxamine. Final concentrations of nutrients were indicated in Table VI-1. Basis salts A and B and urea were prepared separately and autoclaved for 20 min at 121°C. Vitamins solution was sterilized by filtration through 0.22 µm filter (Millex, Millipore, France). After sterilization, the different solutions were mixed in accordance to the final concentrations mentioned in Table VI-1. The culture medium was supplemented with glucose as the carbon and energy source at the desired concentration. A first pre-culture (A) of *C. glutamicum* 2262 was performed in a 500 mL baffled flask containing 50 mL of MCGC medium. Temperature and shaking rate of pre-cultures were respectively 33°C and 200 rpm. After overnight culture, 5 mL of pre-culture (A) were used as the inoculum for the second pre-culture (B) performed with the same culture medium as pre-culture (A). When the optical density at 570 nm of pre-culture (B) reached 30, bacterial growth was stopped by immersing the flask in 70 % v/v ethanol, at a temperature of -20°C; culture broth was then kept at a temperature of 4°C for 12 – 24 h.

VI-2.2. Protocols and cultures in bioreactors

Each culture in bioreactor was inoculated with two flasks of pre-culture (B) which were previously pooled. Cultures were done in 2 L stirred tank bioreactors (Tryton, Pierre Guerin technologies, France). The bioreactors filled with 1.5 L of MCGC medium (Table VI-1) containing 125-200 mM of NaHCO₃ as the CO₂ donor for succinic acid production and glucose as the carbon and energy source at the desired concentration. The bioreactors were equipped with two four-blade Rushton turbines. During cultivation, dissolved oxygen was maintained at a constant concentration of 50 % of air saturation. This was performed by sparging air or pure oxygen through a ten-orifice diffuser at varying gas flow rate using a sterilizable oxygen probe (Mettler-toledo, ISM technology, Switzerland). The pH was measured using a sterilizable glass electrode (Mettler-toledo, ISM technology, InPro3253, Switzerland) and regulated at a set-point of 7.6 by automatic addition of a 10 N KOH solution or 2 N HCl with a peristaltic pump

connected to an on/off pH controller. The foam formation was controlled by manual addition of drops of pure polypropylene glycol (ppg) with respect to a final concentration inferior to 1.2 mL ppg (L of broth)⁻¹. The temperature of the medium was maintained at 33°C by automatic cool and hot water circulation in the bioreactor jacket. For experiments with different sizes of inoculum, pre-cultures were prepared in one or two bioreactors as described above. In these conditions, cells were cultivated until the OD_{570nm} reached 30.

Table VI-1. Composition of modified MCGC medium.

Compounds of the medium		Final concentration		
		For flask culture	For bioreactor culture	
<i>Basal medium</i>	Na ₂ HPO ₄ ·12H ₂ O	36.72	7.44	g L ⁻¹
	KH ₂ PO ₄	2.88	5.90	g L ⁻¹
	NaCl	0.96	1.97	g L ⁻¹
	(NH ₄) ₂ SO ₄	3.84	7.86	g L ⁻¹
<i>Salts A</i>	MgSO ₄ ·7H ₂ O	455.8	39.31	mg L ⁻¹
	FeSO ₄ ·7H ₂ O	45.58	3.93	mg L ⁻¹
	FeCl ₃	4.56	0.39	mg L ⁻¹
<i>Salts B</i>	ZnSO ₄ ·7H ₂ O	1.14	0.07	mg L ⁻¹
	CuCl ₂ ·2H ₂ O	0.46	0.03	mg L ⁻¹
	MnSO ₄ ·H ₂ O	4.56	0.26	mg L ⁻¹
	(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O	0.23	0.01	mg L ⁻¹
	Na ₂ B ₄ O ₇ ·10H ₂ O	0.46	0.03	mg L ⁻¹
<i>Vitamins</i>	CaCl ₂	94.85	8.18	mg L ⁻¹
	Biotin	2.28	0.20	mg L ⁻¹
	Thiamine	22.79	1.97	mg L ⁻¹
	Deferoxamine	3.42	0.29	mg L ⁻¹
	Betaïn	1937	0.20	mg L ⁻¹
	Urea	3.90		g L ⁻¹
	Glucose*	33	40	g L ⁻¹

*Initial concentration of glucose could vary according to the aim of study. In such a case, the dedicated concentration was specified.

VI-2.3. Transition from aerobic growth to anaerobic conditions

Once an OD_{570nm} value of 30 was obtained in the first aerobic phase, a transition strategy developed in Chapter V named *Tr3* was applied in comparison to a *Control* transition. The *Control* transition corresponded to an instantaneous deoxygenation including the switch from aerobiosis to anaerobiosis. For this *Control*, when the desired optical density was reached, the aeration was instantaneously stopped. Transition *Tr3* corresponded to a switch in the dissolved O₂ level from 50 % to 20 % of air saturation. After 2 h of regulation at 20 %, the set point was

switched to 10 % and maintained at 10 % of air saturation for 2 h before the aeration was arrested and as a consequence, culture switched under anaerobic conditions.

VI-2.4. Anaerobic succinate production in batch mode

The different steps of the process were carried out in the same bioreactor. When the transition phase was finished, aeration was totally stopped and culture broth was switched into anaerobic conditions. The agitation was maintained at 900 rpm and the pH was always controlled at a set-point of 7.6 by automatic addition of a 10 N KOH solution or 2 N HCl with a peristaltic pump connected to an on/off pH controller.

VI-2.5. Anaerobic succinate production in fed-batch mode

Except the initial volume in the reactor which was 0.9 - 1.1 L, fed-batch experiments were performed using the same operating conditions as batch experiments but with an additional feeding of glucose and bicarbonate/or basis salts. Feeding included pulses of 50 mL of glucose at a concentration of 3.5 M combined with 50 mL of NaHCO₃ solution at a concentration of 1 M. In one experiment, NaHCO₃ solution was replaced by basis salts solution in order to verify the importance of NaHCO₃ addition during the fed-batch processes. The pH was controlled at 7.6 by automatic addition of a 10 N KOH solution or 2 N HCl with a peristaltic pump connected to an on/off pH controller.

VI-2.6. Determination of global kinetic parameters

Performance of microbial growth, substrate uptake and organic acids production were determined by the calculation of global kinetic parameters for each experiment. The maximal specific growth rate was provided by:

$$\mu_{max} = \max_t \left(\frac{1}{[X]_f} \cdot \frac{d[X]_f}{dt} \right) \quad (\text{VI-1})$$

With $[X]_f$ the biomass concentration obtained by fitting experimental data using smooth splines interpolation model. Maximal specific glucose q_{max}^{gluc} consumption and organic acids (OA) q_{max}^{OA} production rates were respectively calculated as follows:

$$q_{max}^{gluc} = -\max_t \left(\frac{1}{[X]_f} \cdot \frac{d[gluc]_f}{dt} \right) \quad (\text{VI-2})$$

$$q_{max}^{OA} = \max_t \left(\frac{1}{[X]_f} \cdot \frac{d[OA]_f}{dt} \right) \quad (\text{VI-3})$$

With $[gluc]_f$ and $[OA]_f$ the fitted glucose and organic acids concentrations respectively using smooth spline interpolation model. Maximal yields of organic acids and biomass production on glucose concentration Y_{max}^{OA} were respectively calculated by the ratio of maximal concentration of organic acids $[OA]_{max}$ and biomass concentrations $[X]_{max}$ on the concentration of glucose consumed.

VI-2.7. Analytical methods

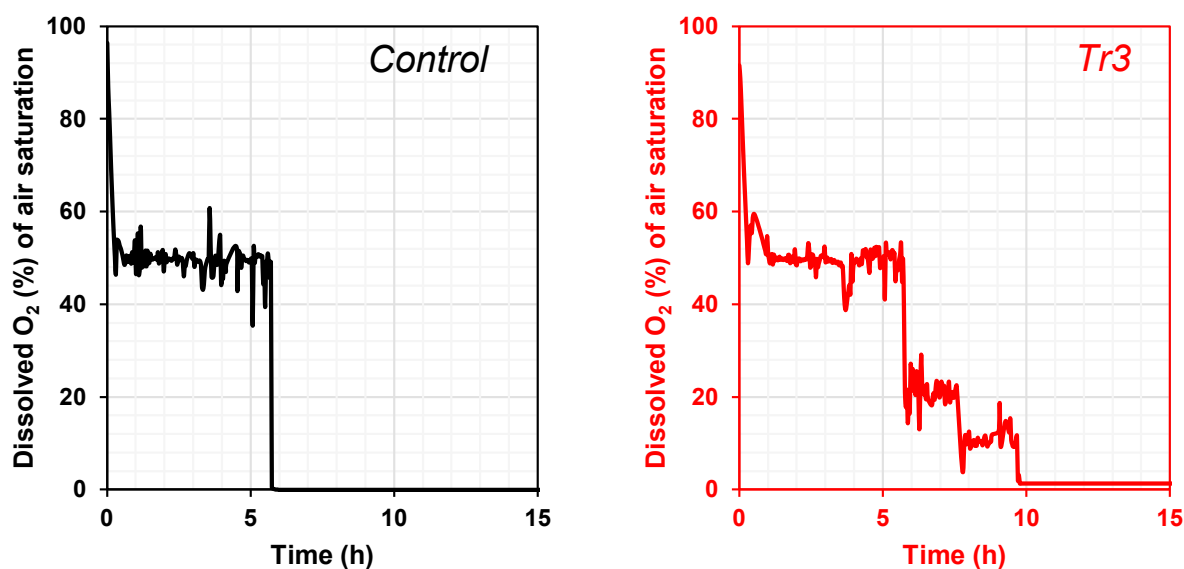
For each experiment, biomass concentration was determined by the measurement of absorbance at 570 nm with a Hitachi U2000 spectrophotometer (Sciencetec, les Ulis, France) and was transformed into cell dry weight (cdw) using the relation dry cell concentration (g L^{-1}) = $0.27 \times \text{OD}_{570\text{nm}}$. For the determination of glucose and organic acids concentrations, samples of culture broth were first centrifuged at $5,000 \times g$ for 10 minutes and at 4°C . Glucose and organic acids (succinate, lactate, acetate, pyruvate and fumarate) concentrations were measured in the supernatants by High Pressure Liquid Chromatography (10ADVP, Shimadzu) using an ion-exchange column (7.8 mm \times 300 mm, Bio-Rad Aminex HPX-87H) and SPD-M10A chromatography working station system equipped with UV absorbance and refractive index detectors water 410 (Shimadzu). The column was eluted isocratically at a rate of 0.7 mL min^{-1} with 5 mM H_2SO_4 for sugar analysis and 50 mM H_2SO_4 for organic acids, at 50°C . The approximate retention time for glucose, pyruvic, succinic, lactic, acetic and fumaric acids were respectively 7.7, 9.2, 10.2, 10.9, 13.1 and 13.5 min.

VI-3. Results

VI-3.1. Dissolved oxygen profiles during the cultivation of *C. glutamicum* 2262

In Chapter V, an efficient process for succinate production has been designed with *C. glutamicum* 2262 *AldhA*. This process, realized in a single step, was characterized by a first aerobic phase for bacteria proliferation, followed by the *Tr3* deoxygenation transition before

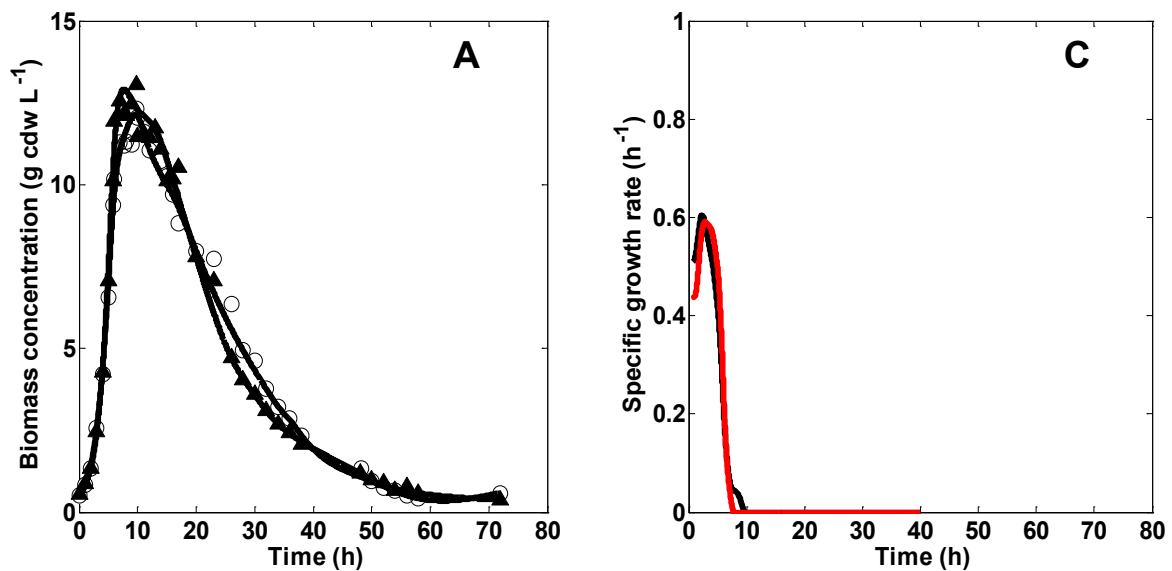
the anaerobic succinate production phase took place. To study the influence of the process with such transition on succinic acid production with *C. glutamicum* 2262, cultures were performed in a 2-L bioreactor containing MCGC medium (Table VI-1) with initially 300 - 350 mM of glucose and 125 mM of bicarbonate. The *Control* and the process with the transition *Tr3* were applied and compared. The different dissolved oxygen level recorded were plotted in Figure VI-1. In *Control* experiment, the first phase of the process is an aerobic phase where the dissolved oxygen was maintained at 50 % of air saturation (by air injection or pure oxygen if needed). When the desired biomass was reached, the aeration was instantaneously stopped promoting anaerobic conditions (Figure VI-1). Cultures performed with transition *Tr3* started with similar aerobic phase until optical density of 30 (570 nm) was obtained, then the dissolved oxygen regulation was reduced to 20 % and was maintained for 2 h. The set point was lowered again to 10 % of air saturation. Finally, after 2 h of regulation at 10 %, anaerobic conditions were obtained by aeration shut off (Figure VI-1).



*Figure VI-1. Time course of dissolved oxygen concentrations during succinic acid production process involving a transition phase between aerobic and anaerobic by using *C. glutamicum* 2262. The cultures were performed under fully aerobic conditions until an optical density at 570 nm of 30 (8.1 g cdw L^{-1}) then two different transitions phases were applied. Control. The aeration was stopped promptly and culture was turned into anaerobic conditions, Tr3. The dissolved O_2 level was lowered step by step before aeration shut off promoting the anaerobic conditions.*

VI-3.2. Impact of deoxygenation strategy on succinic acid production by *C. glutamicum* 2262 in batch mode

When the dissolved oxygen level was regulated at 50 % of air saturation during the aerobic phase, the exponential phase was reached quickly within 2 hours with a maximal growth rate of 0.6 h^{-1} (Figure VI-2C). In this phase, bacteria were cultivated until an optical density at 570 nm of 30 was reached because at this optical density, *C. glutamicum* 2262 cells were in active growth phase. It has been reported previously that this OD was suitable for organic acids production (Khuat *et al.*, 2014). The maximal specific rate of glucose consumption was reached during this phase with values comprised between $5.8 - 6.8 \text{ mol (g cdw)}^{-1} \text{ h}^{-1}$ (Figure VI-2B and D). During the anaerobic phase, growth was totally stopped and a lysis phenomenon was observed in the control and the culture with transition phase; the final biomass concentration was only 0.6 g cdw L^{-1} in both cultures (Figure VI-2A).



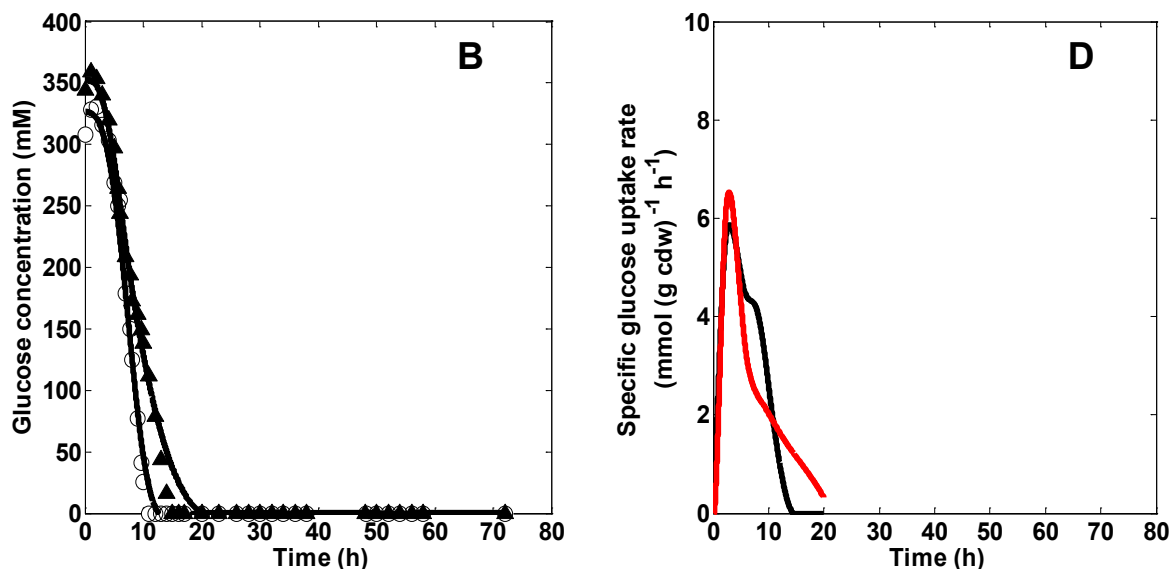


Figure VI-2. Comparison of growth and substrate consumption kinetics of *C. glutamicum* 2262 in a one step process Control (O, —) and Tr3 (▲, —) deoxygenation strategies, in batch mode. (A) bacterial growth, (B) glucose consumption, (C) specific growth rates, (D) specific uptake rate of glucose.

During the transition and anaerobic phases, the organic acids production increased steeply and reached their maximal values after 15 - 20 h of culture for the main organic acids, lactate and succinate (Figure VI-3A and B). Cultures performed with instantaneous switching from aerobiosis to anaerobiosis, resulted in maximal concentrations of 104 and 369 mM of succinate and lactate respectively with a ratio of succinate to lactate of 0.28 mol mol⁻¹ for the *control* culture (Table VI-2), whereas organic acids produced during processes with transition *Tr3* were respectively 280 and 261 mM of succinate and lactate with a ratio of 1.1 mol mol⁻¹. Thus, the deoxygenation strategy *Tr3* clearly promoted a redirection of the carbon flux towards succinate pathways at the expense of lactate (Table VI-2). The maximal lactate production rate of 7.8 mmol (g cdw)⁻¹ L⁻¹ was reached during the *control* culture. This rate was 50 % reduced when a step by step transition was applied (*Tr3*) (Figure VI-3E). The highest specific succinate production rate of 3 mmol (g cdw)⁻¹ L⁻¹ was obtained with transition *Tr3* (Figure VI-3D). In either *Control* culture or process with transition *Tr3*, acetate production was not really affected and acetate remained a minor product with a final concentration of about 35 mM (Figure VI-3C and F).

VI-3.3. Impact of deoxygenation strategy on succinic acid production by *C. glutamicum* 2262 in fed-batch mode

In the preceding batch experiments, glucose which represented the carbon and energy source, was entirely exhausted within 15 h of cultivation. This substrate depletion might have hampered the succinic acid production. To confirm this hypothesis, fed-batch experiments were undertaken. The starting volume was 1.2 L of MCGC medium supplemented with glucose and 142 mM of NaHCO₃. During the culture, three pulses of 50 mL of solution of 3.5 M glucose and 50 mL of 2 M NaHCO₃ were added in the culture broth after 10, 24 and 48 h, respectively.

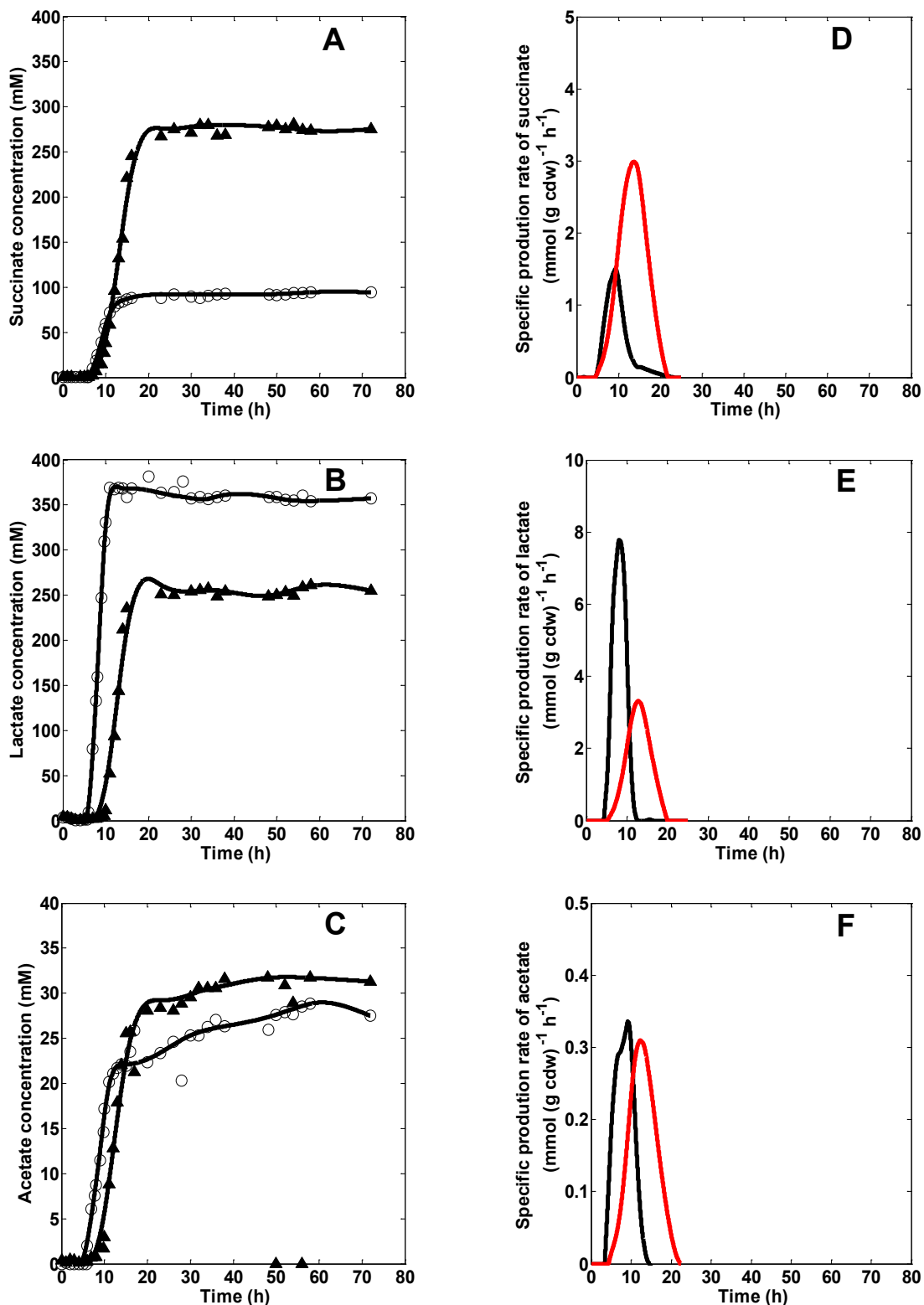


Figure VI-3. Comparison of organic acids production kinetics of *C. glutamicum* 2262 in a one-step process with Control (O, —) and Tr3 (▲, —) deoxygenation strategies, in batch mode. (A) succinate production; (B) lactate production, (C) acetate production, (D) specific succinate production rate, (E) specific lactate production rate, (F) specific acetate production rate.

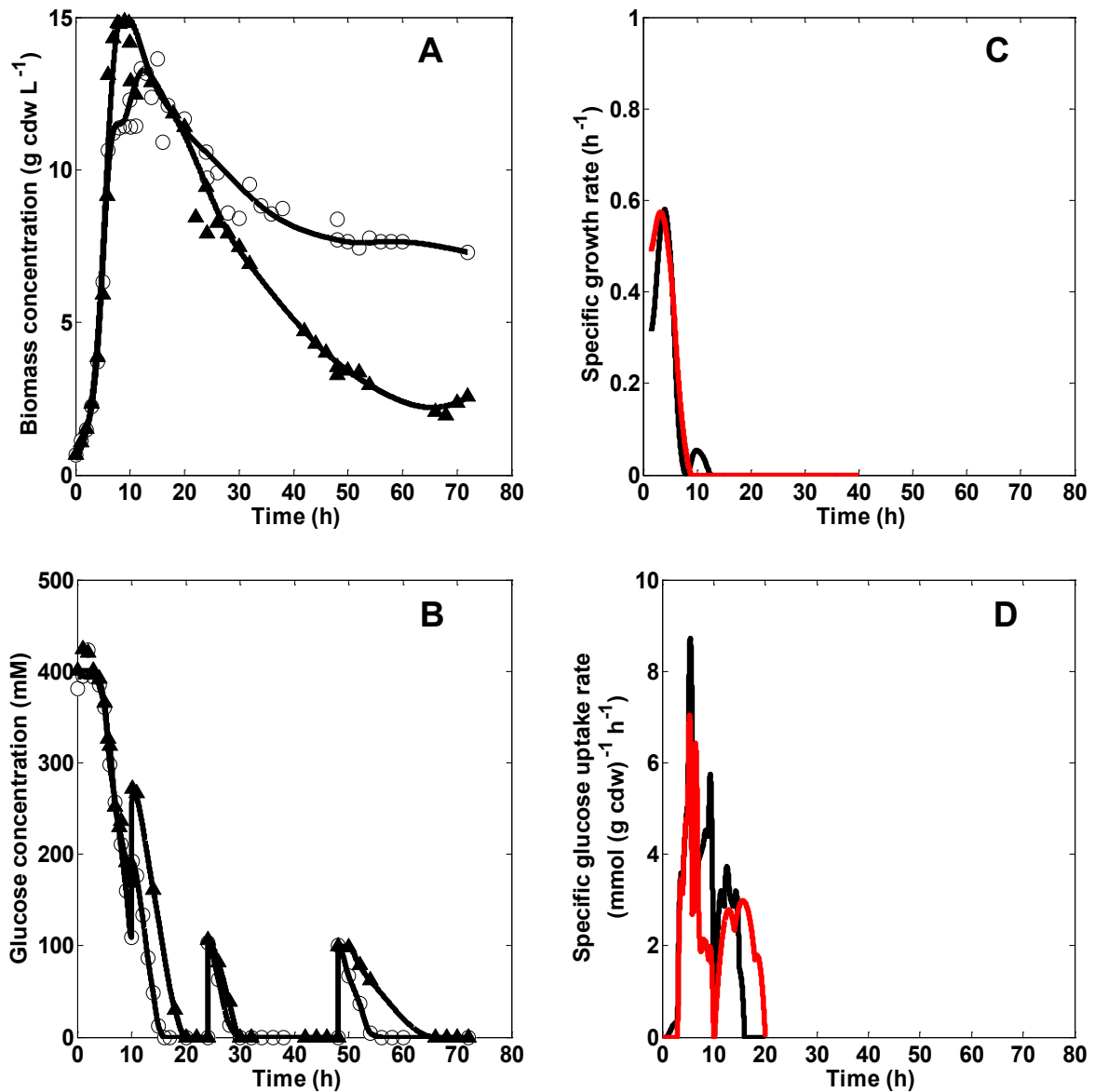


Figure VI-4. Comparison of growth and substrate consumption kinetics of *C. glutamicum* 2262 in a one step process Control (O, —) and Tr3 (▲, —) deoxygenation strategies, in fed-batch mode. (A) bacterial growth, (B) glucose consumption, (C) specific growth, (D) specific uptake rate of glucose.

As it was previously observed, with the *Control* or the *Tr3* deoxygenation strategies, the growth of cells remained similar during the first aerobic phase with a maximal growth rate of 0.6 h⁻¹ (Figure VI-4A and C). The lysis of cells was more pronounced for the culture with transition strategy *Tr3* and a final biomass concentration of 2.5 g cdw L⁻¹ was obtained (Figure VI-4A). After each pulse of glucose and bicarbonate, glucose was quickly consumed after 20 hours starvation between the second and the third pulse. At the end of the fermentation, about 700

mM of glucose were consumed (Figure VI-4B and D). As a consequence to glucose starvation periods, some plateaus were observed on the organic acids concentrations. The production of these organic acids followed the glucose addition and was stopped when glucose was exhausted (Figure VI-5). The products accumulation observed after feeding corresponded also to the increase of the specific production rates (Figure VI-5D – F). The peaks of the different production rate indicated that cells remained still able to convert substrate properly. In the different fed-batch cultures, when the process was conducted without transition (*Control*), the production of lactate (697 mM) was higher than succinate (328 mM) with maximal specific production rates of 5.4 and 1.2 mmol (g cdw)⁻¹ L⁻¹, respectively. A succinate to lactate ratio of 0.47 mol mol⁻¹ similar to those in batch culture was obtained. The glucose and NaHCO₃ feeding in the culture with *Tr3* transition resulted in an enhancement of the succinate to the detriment of lactate. In fact, the use of *Tr3* transition resulted in a succinate concentration increase from 328 to 575 mM (76 % increase) while the lactate produced was 549 mM (21 % decrease) (Figure VI-5A). The succinate to lactate ratio of 1.1 mol mol⁻¹ was also similar to that obtained during batch culture using *Tr3* transition. Acetate production was still lower compared to succinate and lactate (Figure VI-5C). It appeared that the addition of glucose and bicarbonate improved mainly the maximal titer of succinate produced without affecting the ratio between succinate and lactate.

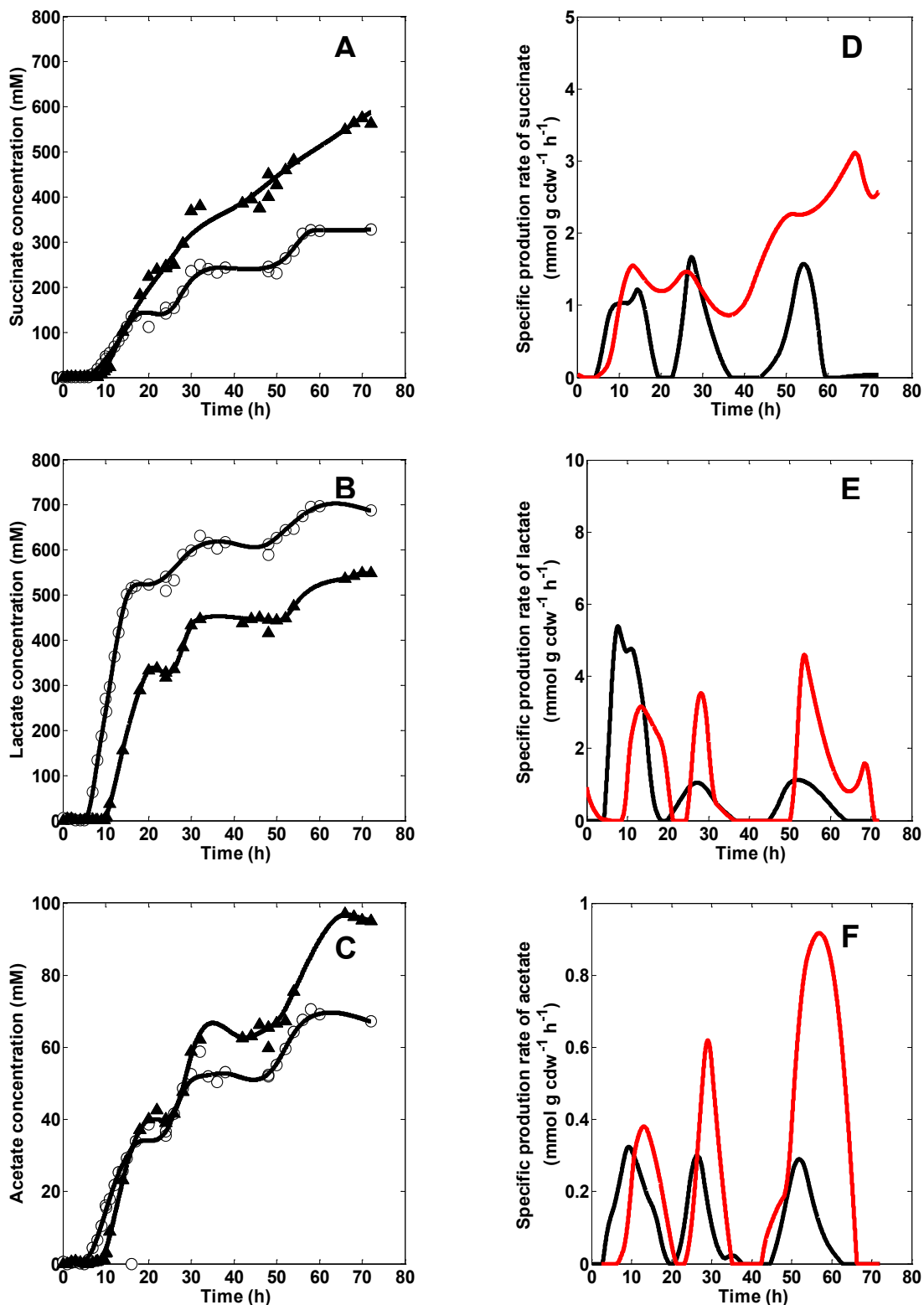


Figure VI-5. Comparison of organic acids production kinetics of *C. glutamicum* 2262 in a one-step process with Control (O, —) and Tr3 (▲, —) deoxygenation strategies, in fed-batch mode. (A) succinate production, (B) lactate production, (C) acetate production, (D) specific succinate production rate, (E) specific lactate production rate, (F) specific acetate production rate.

VI-3.4. Impact of feeding strategies during one-step fed-batch cultures of *C. glutamicum* 2262 with *Tr3* deoxygenation

Application of *Tr3* deoxygenation improved the succinate production by *C. glutamicum* 2262 in both batch and fed-batch modes. Therefore, this transition was used for further fed-batch cultures of *C. glutamicum* 2262 to enhance the titer of succinate, without metabolic engineering. Considering the osmotic pressure exerted by the additional feedings of glucose and NaHCO_3 , various feeding strategies of fed-batch cultures were studied. The first feeding (E1), corresponded to 8 pulses of 50 mL of glucose (3.5 M) and 50 mL of NaHCO_3 (1 M) starting with an initial volume of 1.2 L of MCGC medium containing 475 mM glucose and 168 mM NaHCO_3 . For the second feeding (E2), the bicarbonate was replaced by basis salts medium (see Table VI-1). The initial conditions were identical to the previous one but 7 pulses of 50 mL of glucose (3.5 M) and 50 mL of basis salts medium were fed along the culture. The last experiment (E3) started with an initial volume of 0.95 L of MCGC medium containing approximately 600 mM of glucose and 200 mM of bicarbonate. During the culture, 8 additions of 50 mL of glucose (3.5 M) and 50 mL of NaHCO_3 (1 M) were done. Growth was identical in the three cultures with only slight differences in the maximal biomass concentration (from 16 to 18 g cwt L⁻¹). The biomass was accumulated with similar maximal specific growth rate of about 0.6 h⁻¹ (Figure VI-6A and B). The first addition was performed after 10 h of culture whatever the culture conditions. After the fifth pulse, all the glucose added in the culture medium was not consumed. As a consequence, glucose accumulated in the culture medium and reached between 250 and 300 mM at the end of culture according to the process (Figure VI-6B).

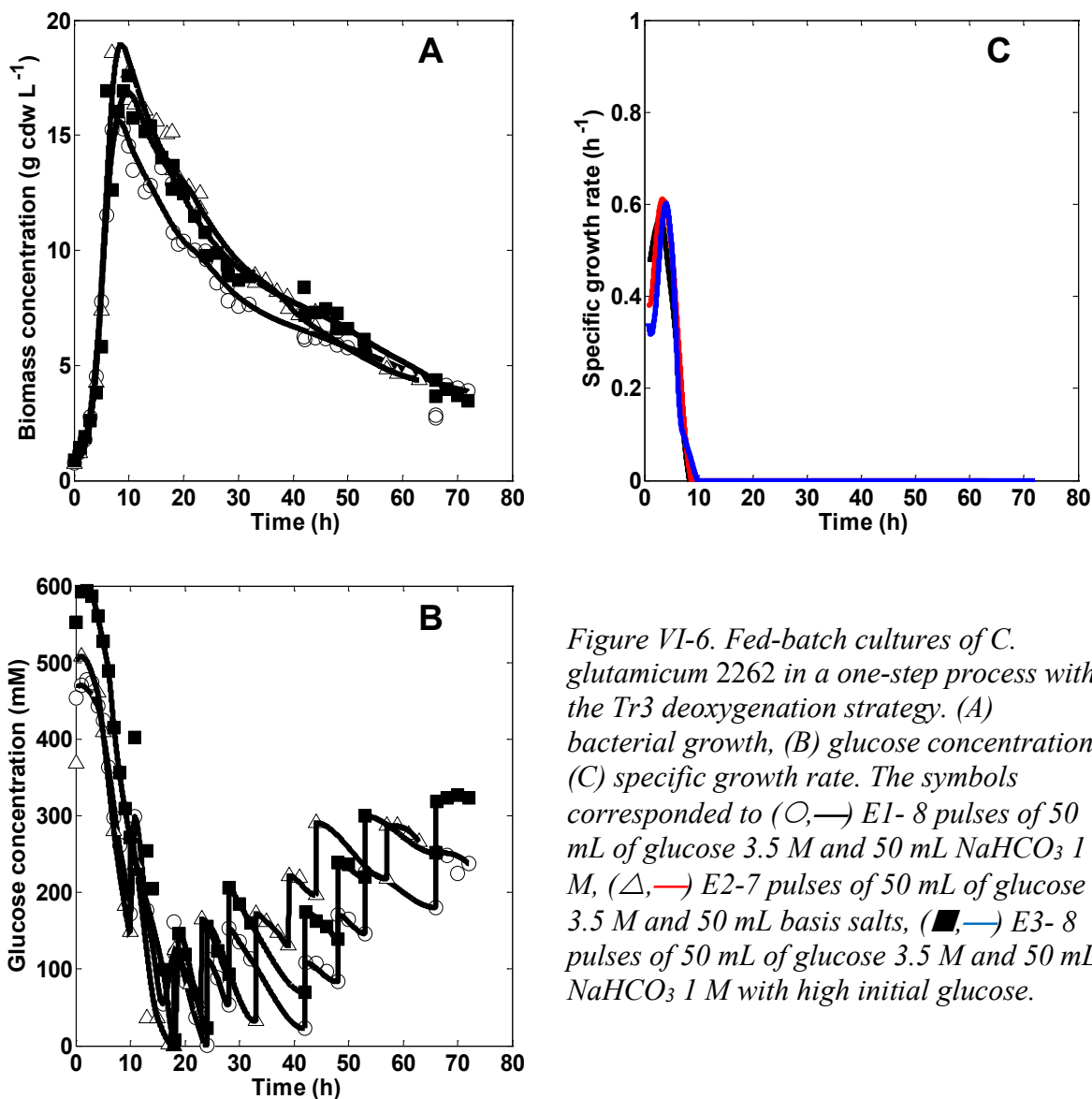


Figure VI-6. Fed-batch cultures of *C. glutamicum* 2262 in a one-step process with the Tr3 deoxygenation strategy. (A) bacterial growth, (B) glucose concentration, (C) specific growth rate. The symbols corresponded to (O, —) E1- 8 pulses of 50 mL of glucose 3.5 M and 50 mL NaHCO₃ 1 M, (Δ, —) E2-7 pulses of 50 mL of glucose 3.5 M and 50 mL basis salts, (■, —) E3- 8 pulses of 50 mL of glucose 3.5 M and 50 mL NaHCO₃ 1 M with high initial glucose.

Concerning the organic acids production, the three cultures resulted in similar kinetics of lactate formation with a maximal lactate concentration of 800 mM and a lactate yield of 1 mole per mole of glucose (Figure VI-7A and Table 2). In fed-batch culture, with bicarbonate replaced by basis salts medium (E2), the succinate production was dramatically reduced and the absence of bicarbonate hampered the succinate production with a decreased titer of 309 mM and a succinate yield of 0.56 mole per mole of glucose. The maximal specific production rate of succinate was 2.1 mmol (g cdw)⁻¹ h⁻¹ (Figure VI-7B and D). The experiment (E1) demonstrated that *C. glutamicum* 2262 was able to produce up to 800 mM (95 g L⁻¹) of succinate with a yield of 1 mole per mole of glucose and a succinate to lactate ratio of 1 mol.mol⁻¹ (Figure VI-7B and Table VI-2). When the culture was started with 600 mM of glucose (E3), only 710 mM of succinate were accumulated and the residual glucose concentration at the end of culture was

325 mM. The succinate yield in this condition was 0.8 mole per mole of glucose (Figure VI-7B, D and Table VI-2).

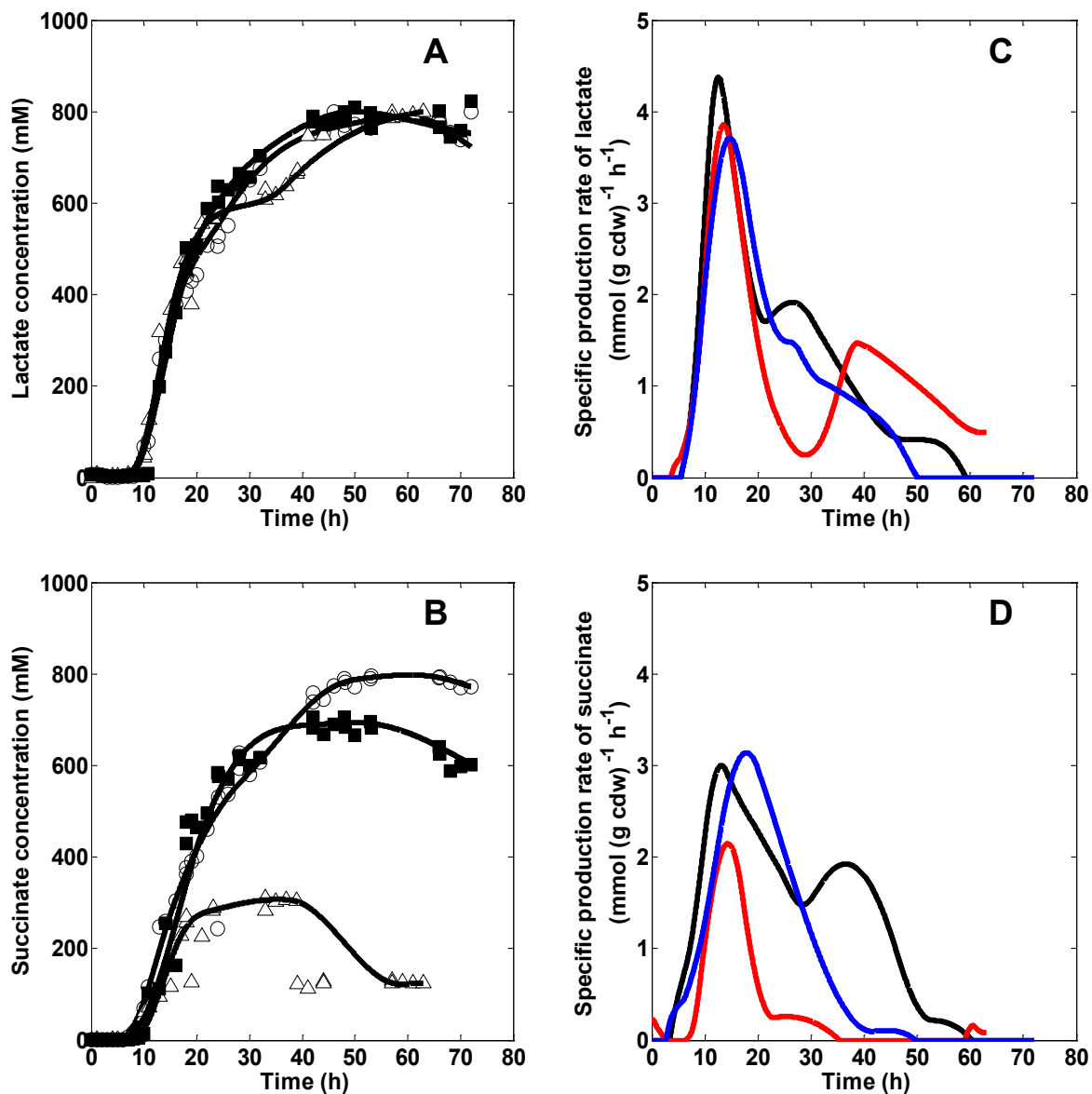


Figure VI-7. Organic acids production kinetics of *C. glutamicum* 2262 in a one-step process with deoxygenation transition Tr3 during fed-batch cultures. (A) Lactate production, (B) succinate production, (C) specific production rate of lactate, (D) specific production rate of succinate. The symbols corresponded to (O, —) E1- 8 pulses of 50 mL of glucose 3.5 M and 50 mL NaHCO₃ 1 M, (Δ, —) E2-7 pulses of 50 mL of glucose 3.5 M and 50 mL basis salts, (■, —) E3- 8 pulses of 50 mL of glucose 3.5 M and 50 mL NaHCO₃ 1 M with high initial glucose.

Table VI-2. Summary of the performances of the different experiments in one-step process performed with *C. glutamicum* 2262.

	Transi- tion	Biomass		Maximal product concentrations. (mM)		Maximal production to substrate yields (mol mol ⁻¹) ⁽⁴⁾		Ratio of maximal concentrations (mol mol ⁻¹)
		[X] _{max} g L ⁻¹	μ_{max} h ⁻¹	[Suc] _{max}	[Lac] _{max}	$Y_{suc/gluc}$	$Y_{lac/gluc}$	[Suc] _{max} / [Lac] _{max}
Batch	<i>Control</i>	12.3	0.60	104	369	0.32	1.1	0.28
Batch	<i>Tr3</i>	12.6	0.59	280	261	0.78	0.71	1.1
Fed-batch	<i>Control</i>	13.3	0.58	327	697	0.51	1.1	0.47
Fed-batch	<i>Tr3</i>	14.9	0.57	575	549	0.86	0.83	1.0
Fed-batch ⁽¹⁾	<i>Tr3</i>	15.7	0.57	793	785	1.0	0.99	1.0
Fed-batch ⁽²⁾	<i>Tr3</i>	18.6	0.61	309	800	0.52	1.1	0.39
Fed-batch ⁽³⁾	<i>Tr3</i>	17.6	0.60	706	822	0.82	1.0	0.86

⁽¹⁾ (E1)-8 pulses of 50 mL of glucose 3.5 M and 50 mL NaHCO₃ 1 M.

⁽²⁾ (E2)-7 pulses of 50 mL of glucose 3.5 M and 50 mL basis salts.

⁽³⁾ (E3)-8 pulses of 50 mL of glucose 3.5 M and 50 mL NaHCO₃ 1 M with high initial glucose.

⁽⁴⁾ Yields were calculated by considering the product accumulation from the beginning of the culture to moment when the maximum amount was reached.

VI-4. Discussion

C. glutamicum is a facultative anaerobic bacterium which can grow aerobically with oxygen as the final electron acceptor or anaerobically in the presence of nitrate (Takeno *et al.*, 2007). In anaerobic conditions without nitrate, *C. glutamicum* cannot grow but cells keep their capability to metabolize sugars to organic acids. Dominguez *et al.* (1993) reported that *C. glutamicum* is able to excrete several organic acids at low level when oxygen is limited during aerobic growth (Dominguez *et al.*, 1993). In this study, to produce succinate, *C. glutamicum* 2262 was cultured in a one-step process with successively aerobic, transition and anaerobic phases. In the batch process performed with an instantaneous switching from aerobic to anaerobic conditions (*Control*), only 164 mM of succinate with a yield of 0.5 mol mol⁻¹ were produced. The lactate concentration produced in the same time was 2.25 fold higher than the succinate one with a production yield of 1.1 mole lactate per mole of glucose. A similar approach was applied by Okino *et al.* (2005) with *C. glutamicum* R. However, their production was based on a two-step process with physical separation between biomass growth and organic

acid production. Even though the specific rates of glucose consumption and organic acid were comparable, the final succinate concentration was lower in batch mode (Okino *et al.*, 2005). In the same study, a fed-batch culture in presence of 400 mM NaHCO₃, resulted in a concentration of 1.1 M of lactate and of 192 mM, respectively. In similar process but without harvest and washing steps, the fed-batch culture of *C. glutamicum* 2262 allowed the production of 697 and 328 mM of lactate and succinate, respectively. As it has been reported in literature and developed in Chapter V, the strategy of deoxygenation influenced the induction of the genes involved in the succinic acid production pathway mainly (*pepc*, *pc*, *mdh* etc.) (Martínez *et al.*, 2010). Thus, the best transition strategy developed in Chapter V was applied to *C. glutamicum* 2262. The application of the one-step process with *Tr3* transition to *C. glutamicum* 2262 allowed the production of 280 mM of succinate and 260 mM of lactate in batch mode culture. These data corresponded to more than 70 % increase in succinate production and 30 % decrease in the lactate accumulation. Compared to the two-step process performed by Okino *et al.* (2005) with the wild type strain, *C. glutamicum* R, the management of the transition phase improved by more than 100 % the succinate production at the expense of lactate with the wild type strain, *C. glutamicum* 2262 (Okino *et al.*, 2005). Therefore, the increase in the succinic acid titer and yield with an adequate transition could be partially attributed to the enhancement of the PEPC activity. In fact, as PEPC has been reported to be the main anaplerotic pathway under oxygen deprivation conditions (Inui *et al.*, 2004a), the application of the transition *Tr3* which is progressive could have induced PEPC expression and as a consequence, could have reduced the pool of PEP that could be further converted by pyruvate kinase and by lactate dehydrogenase. The role of PEPC in *C. glutamicum* anaplerosis under anaerobic conditions have been demonstrated. Inui *et al.*, investigated the genetic expression pattern of *C. glutamicum* using DNA microarrays and RT-qPCR when cells were shifted from aerobic to oxygen deprived conditions. They reported that PEPC, encoded by *ppc*, was threefold upregulated in *C. glutamicum* R, under oxygen deprivation conditions. Under such conditions, the oxaloacetate pool was replenished via conversion of phosphoenolpyruvate rather than via conversion of pyruvate (Inui *et al.*, 2004a, 2007).

After the observation that *C. glutamicum* 2262 was able to produce up to 280 mM (33 g L⁻¹) by process engineering only, feeding strategy was enhanced to increase the maximal concentration of succinic acid that could be produced by this strain. When glucose and bicarbonate were fed during the fermentation phase, the highest concentration of 793 mM (94 g L⁻¹) of succinic acid was obtained with a yield of 1.1 mol mol⁻¹. Lactate was also produced to

the same extend. This concentration is the highest value of succinate reported to date for a wild type strain of non-natural succinate producer.

For cultures supplied with glucose and basis salts medium instead of bicarbonate, succinate production never exceeded 310 mM but the lactate production remained at its highest level (800 mM). Thus, bicarbonate appeared to be rate limiting in this fed-batch culture. Such influence of NaHCO₃ or CO₂, on succinate production have been extensively studied. In fact, CO₂ is a co-substrate of PEP or pyruvate for tricarboxylic acid cycle replenishment and elevated carbon dioxide concentrations have been shown to trigger enhanced activities of anaplerotic enzymes, leading to elevated succinate production (Samuelov *et al.*, 1991; Inui *et al.*, 2004a; Okino *et al.*, 2008a). As mentioned above, even if the process with transition could trigger the induction of PEPC, the decrease of the succinate to lactate ratio from 1 to 0.39 mol mol⁻¹ (Table VI-2) in the absence of bicarbonate, indicated that NaHCO₃ was limiting factor, resulting in a reduced flux from PEP to succinic acid via the TCA reductive arm. The ability of *C. glutamicum* 2262 to produce succinate, as demonstrated in this study, has been compared to some of the most efficient succinate producer strains in oxygen deprived conditions from glucose (Table VI-3). The one-step process with the *Tr3* transition developed in this project compete not only with the natural producer, but also with metabolically engineered strains regarding the final concentration, the volumetric productivity and the yields even if lactate is accumulated at the same level than succinate.

Except the known combinations of glucose plus glutamate, acetate plus ethanol and glucose plus ethanol for whom a diauxic growth was observed (Krämer *et al.*, 1990; Arndt and Eikmanns, 2007; Arndt *et al.*, 2008), *C. glutamicum* possess the ability to catabolize different sugars simultaneously without a diauxic phenomenon (Dominguez *et al.*, 1997; Wendisch *et al.*, 2000; Blombach and Seibold, 2010) unlike the other succinate producers. Furthermore *C. glutamicum* possesses several anaplerotic enzymes at the PEP-pyruvate-oxaloacetate node that govern the succinate production pathways (Sauer and Eikmanns, 2005) in contrast to *E. coli*. Besides, both *E. coli* and *C. glutamicum* are able to use mineral media which allow the reduction of the downstream process costs in comparison to natural producers which require complex media (Datta *et al.*, 1992b; Guettler *et al.*, 1996; Scholten and Dägele, 2008). Finally, the ability to produce organic acid in growth arrested conditions might confer an advantage to *C. glutamicum* during cultures on lignocellulosic biomass hydrolysates in which growths inhibitors are often abundant. However *C. glutamicum* compared to yeasts, needs a near-neutral cultivation pH which requires the addition of alkali during fermentation and a more complex process recovery (Jansen and van Gulik, 2014).

Table VI-3. Summary of process performances described for anaerobic succinate production in literature.

Producing microorganisms	$[Suc]_{max}$ (mM)	Volumetric productivity (mM h ⁻¹)	$Y_{suc/gluc}$ (mol mol ⁻¹)	Applied process	References
<i>M. succiniciproducens</i> LPK7	444	15 ^a	1.16	One step anaerobic fed-batch process in complex medium	(Lee <i>et al.</i> , 2006)
<i>A. succiniciproducens</i> ATCC 53488	180	9 ^a	1.37	One step-anaerobic batch process in complex medium	(Datta <i>et al.</i> , 1992b)
<i>E. coli</i> NZN111	237	5.9 ^a	1.13	One-step, aerobic growth in complex Medium followed anaerobic fed-batch production in complex medium	(Wu <i>et al.</i> , 2007)
<i>E. coli</i> AFP111/pTrc99A- <i>pyc</i>	839	11 ^a	1.7	One-step, dual phase culture in complex medium	(Vemuri <i>et al.</i> , 2002b)
<i>C. glutamicum</i> R <i>AldhA</i> -pCRA717	1,240	21 ^a	0.85 (1.40 ^c)	Two steps ^(d) , aerobic growth in complex medium, anaerobic fed-batch production in minimal medium	(Okino <i>et al.</i> , 2008a)
<i>C. glutamicum</i> BOL-3/pAN6- <i>gap</i>	1,134	16 ^a	1.36 (1.67 ^c)	Two steps, aerobic growth in minimal medium, anaerobic fed-batch production in saline solution	(Litsanov <i>et al.</i> , 2012b)
<i>C. glutamicum</i> ELB-P	330	5.6 ^a	1.02	One-step , aerobic, microaerobic and anaerobic culture in minimal medium	(Wieschalka, 2012)
<i>C. glutamicum</i> SA5	926	9.4 ^a	1.32	Two-steps, aerobic, followed anaerobic fed-batch culture in complex medium	(Zhu <i>et al.</i> , 2014)
<i>C. glutamicum</i> 2262 <i>AldhA</i>	452.8	6.3	0.86 (1.2 ^c)	One step, aerobic, transition Control anaerobic batch culture in minimal medium	Chapter V
<i>C. glutamicum</i> 2262	164.4	8.2 ^b	0.5 (0.65 ^c)	One step, aerobic, transition Control anaerobic batch culture in minimal medium	This work
<i>C. glutamicum</i> 2262	280	8.74 ^b	0.78 (1.7 ^c)	One step, aerobic, transition Tr3 anaerobic batch culture in minimal medium	This work
<i>C. glutamicum</i> 2262	793	12 ^b	1.05 (1.7 ^c)	One step, aerobic, transition Tr3 anaerobic fed-batch culture in minimal medium	This work

^a Volumetric productivity calculated for the overall process time (aerobic + anaerobic phases).

^b Volumetric productivity calculated for the overall process until the maximal succinate titer was reached; sometime experiments were ended when titer was the highest.

^c In one-step or two-step processes, approximate values of yields calculated by taking into account only the anaerobic phase of production were displayed in parentheses.

^dTwo-step processes correspond to processes where the biomass production phase is physically separated from the production phase. After growth, cells were harvested, washed and re-suspended in anaerobic conditions.

VI-5. Conclusion

In summary, the present study demonstrated that the process designed in Chapter V and that consisted in a one-step process with a deoxygenation transition strategy *Tr3* promoted succinate production by *C. glutamicum* at the expense of other products (lactate, acetate). The application of such transition to a lactate dehydrogenase deleted mutant or to the wild type strain, *C. glutamicum* 2262, resulted in an increase in 100 % in succinate titer with yields of 1.1 mol mol⁻¹. The application of the designed process to *C. glutamicum* 2262 in a fed-batch culture resulted in the highest titer of 94 g L⁻¹ of succinate with 71 g L⁻¹ of lactate. This titer of 94 g L⁻¹ of succinate is the highest titer reported for a wild type, whereas other studies reported only minor production. Furthermore, the results of the study demonstrated that using the facultative anaerobic properties of *C. glutamicum* and the indirect influences of key operating conditions on *C. glutamicum* physiological response, it was possible to create and consolidate a new process of succinate production in minimal medium that hold several advantages over existing processes, such as high titer and volumetric productivity. Unlike the present work, most of studies involved mutant strains, however only the deletion of undesired genes or pathways in several cases, didn't improve the desired product formation due to unreasonable metabolic flux redistribution and new undesired metabolite's accumulation (Chapter III). In contrast to mutants where the elimination of some by-products could break the original intracellular redox balance, wild type strains could adapt more easily to intracellular and extracellular changes.

Finally, the study highlighted larger production capacity for two bio-based precursors for biodegradable plastics with great interest as cited by US department of energy (Industrial bioproducts: today and tomorrow). Next steps could be: (i) the improvement of the fed-batch process through the control of glucose and bicarbonate supply to prevent their accumulation in the culture broth and (ii) the elaboration of convenient purification methods for an efficient separation of succinic and lactic acids.

VI-6. References

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Chapitre VII

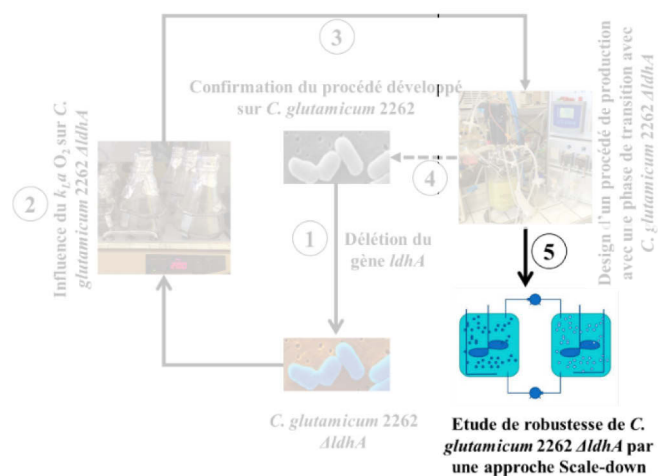
**Etude de la réponse physiologique
de *C. glutamicum* 2262 Δ ldhA à
des oscillations de concentration
en oxygène dissous par une
approche 'scale-down'**

Chapitre VII. Avant-propos

Dans une hypothèse d'industrialisation du procédé de production de succinate par *C. glutamicum*, une problématique de montée en échelle du bioréacteur de culture devra être posée. A l'échelle industrielle, les volumes de culture escomptés étant de l'ordre de la centaine de mètres cubes, une augmentation des temps de mélange dans ces grandes installations est attendue (Lara *et al.*, 2006), ce qui peut induire des gradients de concentration non-négligeables, en particulier en oxygène dissous dans le cas de cultures aérobies à fortes concentrations de biomasse. Ces hétérogénéités impliquent des oscillations de concentration le long de la trajectoire des bactéries, oscillations qui peuvent potentiellement influencer, en retour, les performances des procédés. Dans le cadre de nos travaux, nous avons montré que, dans des conditions de micro-aérobiose, les métabolismes oxydatifs et fermentaires pouvaient être conjointement observés (chapitre IV). Par ailleurs, nous avons développé un procédé de production avec une phase de transition aérobie-anaérobie qui nous a permis une nette progression de la production de succinate (Chapitres V et VI). Sur la base de ces résultats, il semble très intéressant d'évaluer la robustesse de *C. glutamicum* vis-à-vis d'oscillations périodiques de la concentration en oxygène dissous et des éventuelles conséquences sur le procédé de production de succinate. Cette étude devrait permettre, en effet, de prédire à terme la réponse physiologique de *C. glutamicum* lors d'une culture à l'échelle industrielle dans des environnements hétérogènes d'oxygène dissous.

Pour cela, des oscillations en oxygène dissous ont été reproduites à l'échelle du laboratoire en utilisant un système « scale-down » constitué de deux bioréacteurs parfaitement agités et interconnectés grâce à deux pompes péristaltiques. Un des bioréacteurs est maintenu en aérobie par régulation de l'oxygène dissous à 50 % de la saturation en air tandis que le second est maintenu en anaérobie par bullage

continu d'azote. Pour tester la robustesse de *C. glutamicum* 2262 *AldhA* face aux oscillations d'oxygène dissous, nous avons fait varier les temps de séjour des bactéries dans les deux compartiments par modification des volumes utiles de chaque bioréacteur et des débits de



circulation des pompes. Nous avons défini des ratios de temps d'aération correspondant au rapport du temps d'exposition à des conditions d'aérobiose sur le temps total de culture. Nous avons ainsi étudié l'influence de ratios d'aération variant de 0 (les deux bioréacteurs en anaérobiose) à 1 (les deux bioréacteurs en aérobiose) sur la réponse physiologique de *C. glutamicum* 2262 *AldhA* lors des cultures. Les différents résultats issus de ces expériences en bioréacteurs bi-compartmentés sont présentés sous la forme d'un projet de publication (Chapitre VII).

VII. Characterization of *Corynebacterium glutamicum* 2262 *AldhA* physiological response to dissolved oxygen concentration oscillations using a scale-down approach

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Abstract

Corynebacterium glutamicum is a facultative aerobic bacterium that may be potentially used for the industrial production of organic acids (succinate, acetate, pyruvate, etc.). At the industrial scale, the culture volumes may exceed hundreds of cubic meters resulting in increased mixing times and, consequently, to spatial heterogeneities of substrate, dissolved oxygen or dissolved CO₂ concentrations, or pH. The chronic subjection of cells to concentration fluctuations could negatively influence the carbon metabolism, among others. Thus, the results obtained in the preceding chapters should be questioned when biochemical environments are not homogeneous. Following the main conclusions of previous chapters and especially the impact of deoxygenation on organic acids production, the focus was thus put on the study of the robustness of *C. glutamicum* 2262 *AldhA* against dissolved oxygen oscillations. Aiming at this, a dedicated two compartment scale down system composed of two stirred tank reactors interconnected with two peristaltic pumps for medium recirculation was set-up. One bioreactor was maintained in aerobic condition while the second one was flushed by a constant N₂ flow rate. The residence time of bacteria in each compartment was modified by adapting circulation rates and culture volumes in each bioreactor. A mean aeration fraction α experienced by the cells was defined and it was showed that the global physiological response was clearly linked to this variable. The results of cultures demonstrated that, when the mean aeration fraction were higher than 0.67, thus corresponding to longer residence times in the aerobic bioreactor, no detrimental consequences of dissolved oxygen oscillation on *C. glutamicum* 2262 *AldhA* growth performance were observed. However, when α was below 0.33, maximal substrate uptake rate decreased from 1.3 g (g cdw)⁻¹ h⁻¹ to 0.2 g (g cdw)⁻¹ h⁻¹ associated to bacterial growth performance loss and overflow of organic acids increase. Despite this increase, organic acids

production remained anecdotic because (i) organic acids were re-consumed in the aerobic bioreactor and (ii) the instantaneous transitions of dissolved oxygen concentrations experienced by the cells did not promote adequate deoxygenation conditions for optimal organic acids productions. Lastly, an original lagrangian model coupling kinetic modelling and Residence Time Distribution showed that on the contrary to the fermentative metabolism, the oxidative metabolism could rapidly be effective in the bacteria after an anaerobic sequence, with no consequences on real growth performance.

Keywords: Bacterial growth, *Corynebacterium glutamicum* 2262 *AldhA*, Scale-down STR-STR, DOC fluctuations, aerobiosis, anaerobiosis, kinetic modelling, RTD.

Nomenclature

Variables

DOC	Dissolved Oxygen Concentration	(% air saturation)
k_d	Death rate	(s^{-1})
M	Number of circulations	()
N	Agitation rate or number	(s^{-1}) or ()
Np	Power number	()
\underline{Q}	Circulation flow rate	($m^3 s^{-1}$)
P	Power dissipation	(W)
Re	Reynolds number	()
V_1	Volume of aerobic bioreactor	(m^3)
V_2	Volume of anaerobic bioreactor	(m^3)
V_{tube}	Volume connection tube	(m^3)
vvm	Volume of gas per volume of liquid and per minute	($L L^{-1} h^{-1}$)
X_f	Fitted biomass concentration	(g cdw L^{-1})
X	Biomass concentration	(g cdw L^{-1})

Greek letters

α	Aeration ratio	()
β	Dynamic fraction of aeration	()
η	Dynamic viscosity	(Pa.s)
ρ	Density	($kg.m^{-3}$)
μ	Specific growth rate	(h^{-1})
τ_1	Mean residence time in aerobic bioreactor	(s)
τ_2	Mean residence time in anaerobic bioreactor	(s)

VII-1. Introduction

Whereas the first step of process development consists in the research of value-added products production at the laboratory scale by selecting productive microbial strains for instance, scale-up of bioreactor is the second paramount step to allow the large-scale synthesis of the target molecule. Indeed, process scale-up has been many times cited as the bottleneck in fermentation technology due to difficulties arising from incomplete similitude between lab and industrial scales. In particular, using a constant power dissipation per unit of volume as criterion for bioreactor scale-up entails an increase of mixing times (Nienow, 1997) that may promote concentration heterogeneities. As cell physiology, maximum productivity, product concentration, product yields and product quality strongly depend on environmental conditions, prediction of concentration gradients and bioreactor heterogeneities could be of great interest before scaling-up. To predict concentration heterogeneities, regime analysis, consisting in the identification of the limiting step of the bioprocess by comparing mixing time, that corresponds to the time required to attain reactor homogeneity after a step change in an arbitrary variable, to the characteristic consumption time of a given substrate, is generally undertaken (Lara *et al.*, 2006a). Indeed, when mixing time is expected longer than a relevant cellular consumption, concentration gradients are likely to occur (Sweere *et al.*, 1987; Paul *et al.*, 2004; Lara *et al.*, 2006a). Another way to predict bioreactor heterogeneities is the use of computational fluid dynamics (Hristov *et al.*, 2001; Morchain *et al.*, 2014). Using this approach, possibly combined with experimental studies, it has been demonstrated that, in industrial-scale bioreactors, cells may be exposed to unsteady biochemical and hydro-mechanical micro-environments that could impact their metabolic and physiologic behavior (Namdev *et al.*, 1993; Bylund *et al.*, 1998, 1999, 2000; Hewitt *et al.*, 2000). Concentration gradients may concern dissolved oxygen concentrations (Oosterhuis and Kossen, 1984), pH (Reuss *et al.*, 1994; Langheinrich and Nienow, 1999; Onyeaka *et al.*, 2003), nutrients such as glucose (Larsson *et al.*, 1996; Bylund *et al.*, 1998), temperature, and dissolved CO₂. Among the previous parameters identified, dissolved oxygen is one of the most critical due to inhomogeneity of the oxygen injection, to spatial variation of hydrostatic pressure and thus of oxygen transfer capacities and to high oxygen consumption rates when high biomass concentrations are encountered (Bryant, 1977). For instance, in a 25 m³-bioreactor equipped with two Rushton turbines, Oosterhuis and Kossen (1984) measured variations of dissolved oxygen concentrations (DOC) from 8 - 15 % of air saturation in the turbine region to dramatically low values of 0-6 % away from the turbine region (Oosterhuis, 1984). To investigate the physiological effects of chronic exposure of

microorganisms to the heterogeneous distribution of micro-environmental conditions, scale-down models were introduced since 1980's as a rational and practical approach to simulate the micro-environment experienced by cells at large scale using small-scale set-ups (Oosterhuis, 1984). In most studies, fluctuating environments are controlled by combining two or three small-scale bioreactors. These set-ups can either be plug-flow (PF) or continuous stirred tank (CST) bioreactors which are connected using a recirculation loop. These systems, intensively reviewed in Lara *et al.* (2006) have already been applied for various models of microorganisms (Lara *et al.*, 2006a). For instance, DOC oscillations imposed by a scale-down set-up using *Escherichia coli* promoted an increase in byproducts accumulation and a reduction in production yield (loss of up to 87 %), specific growth rate, and recombinant protein production (Namdev *et al.*, 1993; Sandoval-Basurto *et al.*, 2005; Delvigne *et al.*, 2006a, 2006b; Lara *et al.*, 2006b). Another industrial enzymes and antibiotic producer, *Bacillus subtilis* has been used for mixing and mass transfer studies in bioreactors by scale-down strategy (Moes *et al.*, 1985; Suphantharika *et al.*, 1995). The effects of fluctuating environmental conditions on yeast metabolism were also investigated through scale-down studies (Sweere *et al.*, 1988a, 1988b; Cortés *et al.*, 2005).

Besides these industrial strains, *Corynebacterium glutamicum*, a promising candidate for bio-based chemicals production, is a gram-positive bacterium isolated in 1950s (Kinoshita *et al.*, 2004) which is used for the production of millions of tons of amino acid per year including, glutamate, lysine, valine and alanine (Eggeling and Sahm, 1999; Radmacher *et al.*, 2002; Hermann, 2003; Hasegawa *et al.*, 2012). Currently, the interest for *C. glutamicum* as an efficient organic acids producer under deprived oxygen conditions is increasing (Inui *et al.*, 2004a; Okino *et al.*, 2005; Litsanov *et al.*, 2012b; Khuat *et al.*, 2014). To meet the demand of bio-based products, optimized large scale bioreactors will be looked for, implying the characterization of the possible spatial gradients expected. Till now, only few scale-down approaches have been carried out with *C. glutamicum*. Schilling *et al.* (1999) investigated the performance of L-lysine production by *C. glutamicum* under substrate gradients (Schilling *et al.*, 1999). Furthermore, glucose and oxygen oscillations impact on *C. glutamicum* metabolism have been very recently analyzed in a CST bioreactor connected to PF bioreactor (Käß *et al.*, 2014a, 2014b). Finally, the metabolic and transcriptional response in *C. glutamicum* exposed to carbon dioxide gradients in a three stirred tank compartment bioreactors were recently analyzed. Indeed Buchholz *et al.* (2014) reported that the imposed pCO₂ gradients at industry-relevant residence times of about 3.6 min did not hamper the process performances in comparison to a control.

However, transcriptional analysis have revealed up to 66 differentially expressed genes already after 3.6 min under pCO₂ gradient exposure and the genes expression was correlated to gradient intensity and the residence time of the cells (Buchholz *et al.*, 2014).

In previous work (Chapters V and VI), the regulation of dissolved oxygen was reported to greatly influence the succinic acid production process. Moreover, the availability of dissolved oxygen could have an impact on the physiological state of cells which could subsequently affect negatively the metabolite production. In fact, the physiological state of cells was shown to play an important role during the succinic acid production process particularly at the transition time from growth to succinate production phase. Therefore, as cells physiological state could change along the culture time due to the growth environment changes (dissolved oxygen, dissolved carbon dioxide concentration etc.), it was of first importance to increase the knowledge concerning the physiological response of *C. glutamicum* when exposed to DOC fluctuations. The present work was dedicated to the study of this response when this microorganism was subjected to DOC oscillations, using a two compartment scale-down set-up approach. An original lagrangian kinetic model coupled to Residence Time Studies was also proposed.

VII-2. Materials and methods

VII-2.1. Bacterial strain, culture media

Batch cultures of a mutant strain of *C. glutamicum* 2262, deleted for lactate dehydrogenase gene (*AldhA*) were performed in a modified MCGC mineral salt medium in which citrate was replaced by deferoxamine. Final concentrations of nutrients are indicated in Table VII-1. Basal medium, salts A and B and urea were prepared separately and autoclaved for 20 min at 121°C. Vitamins solution was sterilized by filtration through a 0.22 µm filter (Millex, Millipore, France). After sterilization, the different solutions were mixed together in accordance to the final concentrations mentioned in Table VII-1. The mutant strain was constructed via a two-step homologous recombination procedure using the vector pK19*mobsacBAldh* (Litsanov *et al.*, 2012b). This plasmid was constructed using the suicide vector pK19*mobsacB* as previously described (Schäfer *et al.*, 1994). The culture medium was supplemented with glucose as carbon and energy source at the desired concentration.

Table VII-1. Composition of modified MCGC medium.

Compounds of the medium		Final concentration		
		For flask culture	For bioreactor culture	
<i>Basal medium</i>	Na ₂ HPO ₄ ·12H ₂ O	36.72	7.44	g L ⁻¹
	KH ₂ PO ₄	2.88	5.90	g L ⁻¹
	NaCl	0.96	1.97	g L ⁻¹
	(NH ₄) ₂ SO ₄	3.84	7.86	g L ⁻¹
<i>Salts A</i>	MgSO ₄ ·7H ₂ O	455.8	39.31	mg L ⁻¹
	FeSO ₄ ·7H ₂ O	45.58	3.93	mg L ⁻¹
	FeCl ₃	4.56	0.39	mg L ⁻¹
<i>Salts B</i>	ZnSO ₄ ·7H ₂ O	1.14	0.07	mg L ⁻¹
	CuCl ₂ ·2H ₂ O	0.46	0.03	mg L ⁻¹
	MnSO ₄ ·H ₂ O	4.56	0.26	mg L ⁻¹
	(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O	0.23	0.01	mg L ⁻¹
	Na ₂ B ₄ O ₇ ·10H ₂ O	0.46	0.03	mg L ⁻¹
<i>Vitamins</i>	CaCl ₂	94.85	8.18	mg L ⁻¹
	Biotin	2.28	0.20	mg L ⁻¹
	Thiamine	22.79	1.97	mg L ⁻¹
	Deferoxamine	3.42	0.29	mg L ⁻¹
	Betaïn	1937	0.20	mg L ⁻¹
	Urea	3.90		g L ⁻¹
	Glucose*	33	40	g L ⁻¹

*Initial concentration of glucose could vary according to the aim of study. In such a case, the dedicated concentration was specified.

VII-2.2. Inoculum preparation

The cell stocks were maintained in 1.5 mL cryo-conservation tubes at -80 °C. Each tube contained 1 mL of MCGC with glycerol (20 %). A first preculture (A) of *C. glutamicum* 2262 *Al dhA* was prepared by transferring the total content of a tube to a baffled shake flask containing 50 mL of culture medium, incubated at 33°C and mixed at a 200 rpm shaking frequency. After 15 hours cultivation, this preculture was used as inoculum for a second preculture (B) (10 % inoculation in MCGC medium) incubated in the same conditions. When the optical density at 570 nm of pre-culture (B) reached a value of 30, bacterial growth was stopped by immersing the flask in 70 % v/v ethanol, at a temperature of -20°C; culture broth was then kept at 4°C for 12 – 24 h. This pre-culture was used as inoculum for the different bioreactors with an initial optical density of 1.5 (570 nm).

VII-2.3. Experimental set-up and culture conditions

To impose oscillating dissolved oxygen concentration (DOC) to cultures of *C. glutamicum* 2262 *AldhA*, a scale-down bioreactor system described in the literature was used (Oosterhuis et al., 1983; Sandoval-Basurto et al., 2005; Lara et al., 2006b). This system (Figure VII-1) consisted of two interconnected stirred tank bioreactors (Tryton, Pierre Guerin technologies, France). One bioreactor was maintained at a constant DOC of 50 % of air saturation (Bio_{O_2}) while the second was set at $DOC = 0\%$ of air saturation (Bio_{N_2}) to mimic transition from aerobic to anaerobic regions encountered by cells in large-scale production.

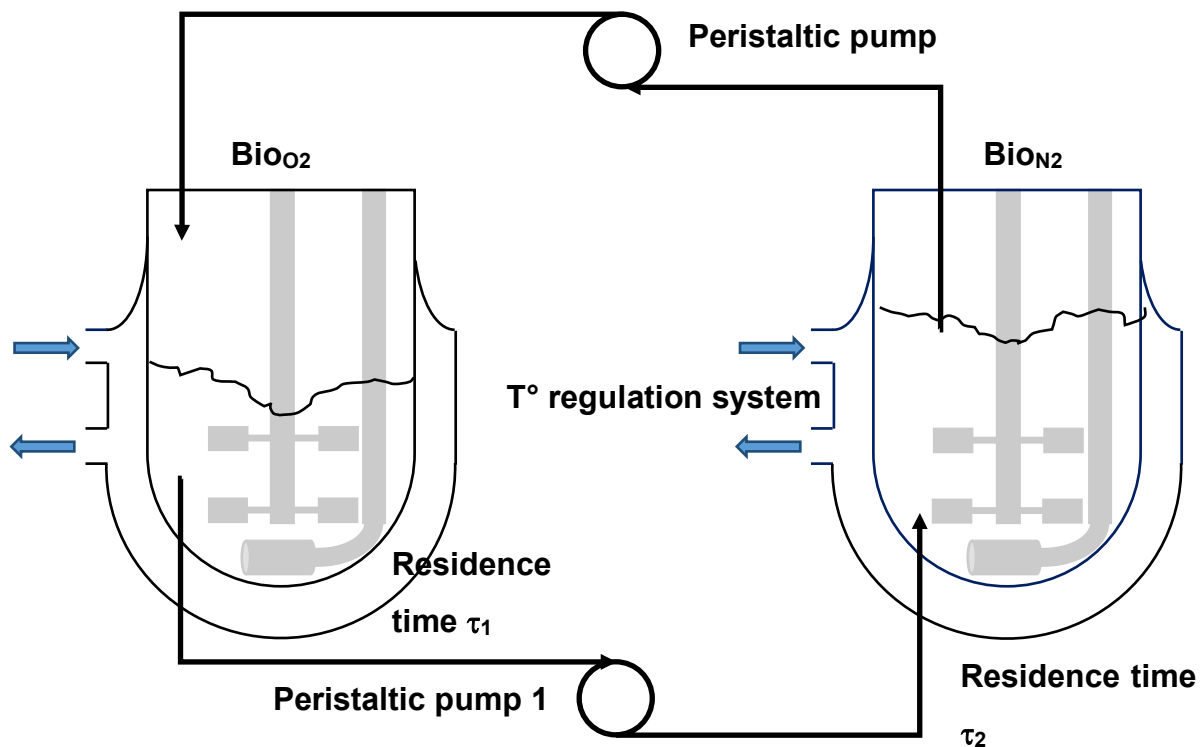


Figure VII-1. Two-compartment scale-down bioreactors system with aerobic (Bio_{O_2}) and anaerobic (Bio_{N_2}) bioreactors. The culture broth was recirculated between the two bioreactors by using peristaltic pumps.

The culture broth was continuously pumped between both vessels at various circulation flow rates through a low gas permeability tubing (Exacanal, 5 mm internal diameter and 2.5 m long) using two peristaltic pumps (Masterflex, Cole-Parmer, Barrington IL, USA). Depending on liquid volume, each bioreactor was equipped with two or three four-blade Rushton turbines (diameter $D = 0.06$ m). In the aerobic bioreactor, DOC was maintained constant by sparging air or pure oxygen through a ten-orifice diffuser and by varying gas flow rate. Anaerobic conditions were ensured in Bio_{N_2} bioreactor by sparging pure nitrogen through a porous diffuser at a flow

rate of 0.5 to 1.3 vvm. Liquid volumes in both aerobic and anaerobic bioreactors were modified according to Table VII-2. The total volume in the circulation loop was approximately 100 mL which is small compared to mixed vessel volumes. The mean circulation times calculated as the ratio between total culture volume and broth circulation flow rate ranged from 400 s to 1,600 s. However, in large scale cultures, the circulation time was estimated lower, around 50-100 s (Amanullah *et al.*, 2003; Paul *et al.*, 2004) even if higher circulation times from 600 – 1,200 s have been reported in fluidized-bed reactors with immobilized bacterial cells (Sweere *et al.*, 1987). Obtaining very short circulation times is very challenging as it either implies to (i) use very small liquid volumes (which makes kinetic study more difficult) or (ii) high recirculation flow rates. Moreover, recirculation times from an aerobic to an anaerobic bioreactor is also limited to approximately 100 s by the gas-liquid mass transfer coefficients achievable in each system (Sandoval-Basurto *et al.*, 2005). Thus, our study mainly focused on relative residence time in each bioreactor by varying the ratios between the two bioreactor volumes.

DOC was measured in both bioreactors using sterilizable polarographic probes (Mettler-toledo, ISM technology, Switzerland). In Bio_{O₂} the dissolved oxygen concentration was controlled at a constant value by a proportional-integral-derivative (PID) algorithm that automatically varied the flow rate of the individual gases (air or pure oxygen) through two mass flow controllers (Brooks, France). The DOC set point for Bio_{O₂} was 50 % of air saturation for all cultures except for the anaerobic control fermentation (Table VII-2). Agitation rates were set to constant values of 900 rpm and 500 rpm respectively for Bio_{O₂} and Bio_{N₂}. pH was measured using sterilizable glass electrode (Mettler-toledo, ISM technology, InPro3253, Switzerland) and regulated in the two compartments at a set-point of 7.6 by automatic addition of a 10 N KOH solution or 2 N HCl with a peristaltic pump connected to an on/off pH controller. The foam formation was controlled by manual addition of drops of pure polypropylene glycol (ppg) in both vessels with respect to final concentration less than 1.2 mL ppg/ L of broth. The temperature of cultures was maintained at 33°C by automatic cool and hot water circulation in the jacket of the bioreactors.

Table VII-2. Scaled-down culture conditions.

Mean aeration ratio α	Bio _{O2}		Bio _{N2}		Flow rate (mL/s)	Residence time (s)	
	V_1 (L)	DOC (% air sat.)	V_2 (L)	DOC ⁽²⁾ (% air sat)	Q	τ_1 (s)	τ_2 (s)
0	1.5	0 ⁽¹⁾	1.5	0	7.3	205	205
0.2	1	50	4	0	7.3	137	548
0.25	1.5	50	4.5	0	7.3	205	616
0.33a	1	50	2	0	7.3	137	274
0.33b	1	50	2	0	1.8	556	1111
0.67a	2	50	1	0	7.3	274	137
0.67b	2	50	1	0	1.8	1111	556
1a ⁽³⁾	1.5	50	1.5	50	7.3	205	205
1b ⁽⁴⁾	1.5	50					

⁽¹⁾In this case, once the oxygen probe was calibrated, the culture was started without air/oxygen sparging nor nitrogen sparging whereas the Bio_{N2} compartment was sparged continuously with nitrogen. ⁽²⁾Nitrogen was sparged continuously to warrant anaerobic conditions. ⁽³⁾Corresponded to two aerobic bioreactors interconnected, ⁽⁴⁾ corresponded to a single anaerobic bioreactor.

VII-2.4. Determination of global kinetic parameters

To quantify the culture performance in terms of growth, uptake and production, global kinetic parameters were calculated for each experiment. Given the biomass concentration X , the maximal specific growth rate was provided by:

$$\mu_{max} = \max_t \left(\frac{1}{X_f} \cdot \frac{dX_f}{dt} \right) \quad (\text{VII-1})$$

With X_f the experimental biomass concentration fitted using smooth splines interpolation model and the Matlab® software. Maximal specific glucose q_{max}^{gluc} consumption rates were respectively calculated following:

$$q_{max}^{gluc} = - \max_t \left(\frac{1}{[X]_f} \cdot \frac{d[gluc]_f}{dt} \right) \quad (VII-2)$$

With $[gluc]_f$ the fitted glucose concentrations using smooth spline interpolation model. Finally, maximal yields of biomass on glucose $Y_{max}^{X/S}$ were calculated by Equation (VII-3).

$$Y_{max}^{X/S} = \max_t \left(\frac{X_{exp}}{[Gluc]_{exp}} \right) \quad (VII-3)$$

X_{exp} was the experimental concentration of biomass.

VII-2.5. Analytical methods

For each experiment, biomass concentration was determined by the measurement of absorbance at 570 nm with a Hitachi U2000 spectrophotometer (Sciencetec, les Ulis, France) and was transformed into cell dry weight (cdw) using the relation: dry cell concentration (g.L^{-1}) = $0.27 \times \text{OD}_{570\text{nm}}$. For the determination of glucose residual concentration and organic acids concentration, samples of culture broth were first centrifuged at $5,000 \times g$ for 10 minutes and at 4°C . Glucose and organic acids (succinate, lactate, acetate, pyruvate and fumarate) concentrations were measured in the supernatants by High Pressure Liquid Chromatography (10ADVP, Shimadzu) using an ion-exchange column (7.8 mm \times 300 mm, Bio-Rad Aminex HPX-87H) and SPD-M10A chromatography working station system equipped with UV absorbance and refractive index detectors water 410 (Shimadzu). The column was eluted isocratically at a rate of 0.7 mL min^{-1} with 5 mM H_2SO_4 for sugar analysis and 50 mM H_2SO_4 for organic acids, at 50°C . The approximate retention time for glucose, pyruvic, succinic, lactic, acetic and fumaric acids were respectively 7.7, 9.2, 10.2, 10.9, 13.1 and 13.5 min.

VII-3. Results

VII-3.1. Control cultures at constant dissolved oxygen concentration

To analyze the physiological responses of *C. glutamicum* 2262 *AldhA* used in the present study, three different control cultures were first performed at constant DOC, without concentrations fluctuations. The first one, Bio_{Ref1}, consisted of a culture performed using a single aerobic bioreactor at a constant DOC of 50 % air saturation and thus without medium circulation. This experiment was identified as the reference culture conditions for biomass growth promotion (Figure VII-2A and C). The second reference culture Bio_{Ref2} consisted of two identical CST bioreactors controlled at a DOC of 50 % of air saturation and connected by a continuous recirculation (Table VII-2). This control culture condition was used to assess the impact of two-compartment experimental set-up on microbial response (Figure VII-2C and E). For the third reference culture Bio_{Ref3}, two CST bioreactors operating in anaerobic conditions were interconnected and were considered as reference cultures of *C. glutamicum* 2262 *AldhA* for organic acids production (Figure VII-2E-G).

Kinetics of growth, consumption of glucose, production of organic acids for the anaerobic culture, pH and DOC profiles are shown in Figure VII-2. It can be noticed that the regulation of these parameters was very satisfactory whatever the culture conditions. Biomass concentration increased exponentially until exhaustion of glucose with maximal biomass concentrations of 18.2 and 19.8 g cdw L⁻¹ respectively obtained in one aerobic and two aerobic interconnected bioreactors (Figure VII-2B and E). Only minor amounts of organic acids were detected in the aerobic control cultures including pyruvate, acetate with maximal concentrations of 0.4 g L⁻¹ approximately and succinate with a maximal concentration 1 g L⁻¹ for both aerobic bioreactors. For the anaerobic control culture Bio_{Ref3}, a negligible growth with maximal biomass concentration of 0.7 g L⁻¹ was formed after 50 h of culture (Figure VII-2F). Whereas for the aerobic cultures the initial amount of glucose was completely exhausted, only 29 mM (4.3 g L⁻¹) were consumed for the anaerobic culture (Figure VII-2F). In these anaerobic conditions, the glucose consumed was mainly converted into organic acids. After 50 h of culture also, the maximal concentrations of pyruvate, acetate and succinate were respectively 4.6 mM (0.4 g L⁻¹), 10.9 mM (0.7 g L⁻¹) and 14.6 mM (1.7 g L⁻¹) (Figure VII-2G). The linearity of the organic acids production kinetics seemed to indicate that this production was mainly related to biomass concentration and not to specific growth rate. From the results obtained, it could be concluded that (*i*) similar results were obtained in the two-compartment and single compartment

aerobic bioreactors, (ii) in fully-anaerobic cultures, no growth but organic acids production were observed, (iii) in fully-aerobic culture conditions, exponential growth but no organic acids production were measured.

VII-3.2. Oxygen oscillations imposed to *C. glutamicum* 2262 *AldhA* cells

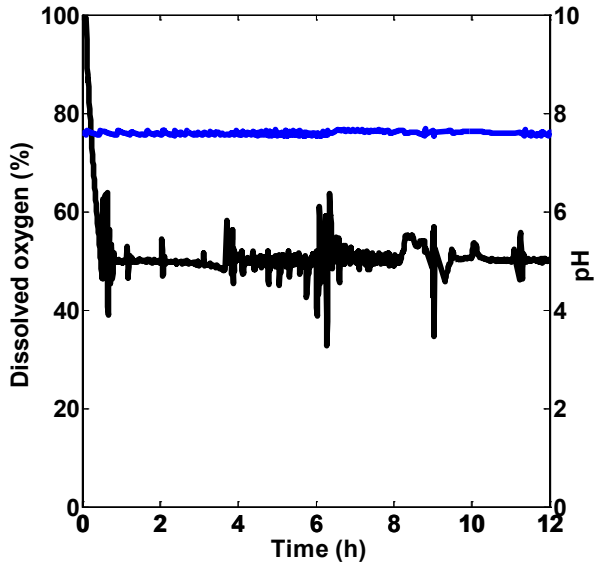
Oscillations of dissolved oxygen were imposed by continuously circulating cells from the aerobic bioreactor operating at a DOC of 50 % of air saturation to the anaerobic bioreactor (DOC = 0 % and N₂ sparging). Regarding bioreactors geometry, agitation and circulation conditions, it could be assumed as a first approximation that the two mixing vessels used were similar to homogeneous Continuous Stirred-Tank Reactor (CSTR) vessels while connection tubes could be assumed to heterogeneous plug-flow bioreactors. To confirm this, the mixing time of the bioreactors were compared to mean residence times of the liquid. For a four-blade Rushton turbine, it could be assumed (Roustan, 2005) that the impeller number was:

$$Np = \frac{P}{\rho \cdot N^3 \cdot D^5} = 4 \quad (VII-4)$$

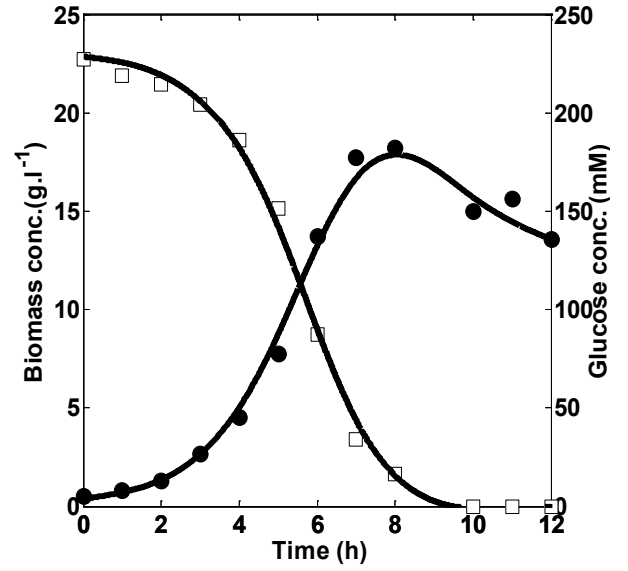
With P (W) the power dissipation, N (s⁻¹) the agitation rate and D (m) the impeller diameter. The flow regime was systematically turbulent as the Reynolds number $Re = \frac{\rho \cdot N \cdot D^2}{\mu}$ was comprised between 40,000 and 72,000 approximately, supposing that the culture broth viscosity and density were similar to water properties at a temperature of 33°C. Thus, the mixing times obtained for the various culture conditions could be given by Nienow (1997):

$$t_M = 5.9 \cdot T^{2/3} \cdot \left(\frac{n_R \cdot P}{\rho \cdot V} \cdot \gamma \right)^{-1/3} (D/T)^{-1/3} \quad (VII-5)$$

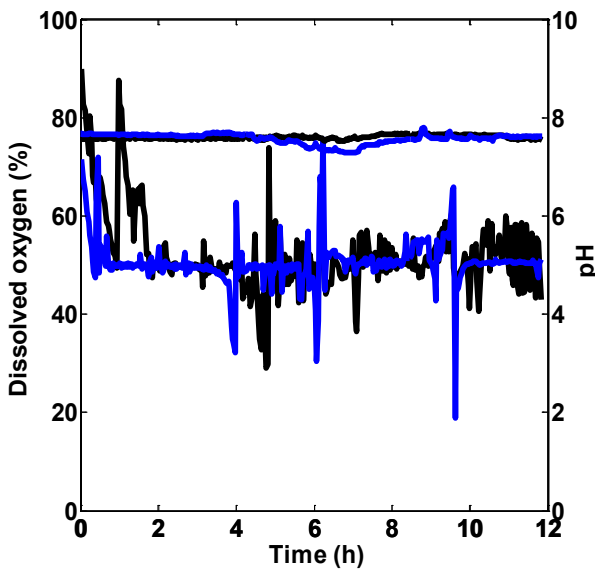
$T = 0.117$ m was the vessel diameter, n_R was the number of Rushton turbines. The coefficient γ was added in the preceding equation to take into account the impact of gas bubbling on the loss of mechanical power. In our case, as the bubbling flow rate changed over time to allow the dissolved gas concentration control, it was chosen to make the dramatic hypothesis of impeller flooding ($\gamma = 0.5$). Using equation (VII-5), the calculation of mixing times provided values of approximately 1 and 2 s in the BioO₂ and BioN₂ bioreactors respectively.



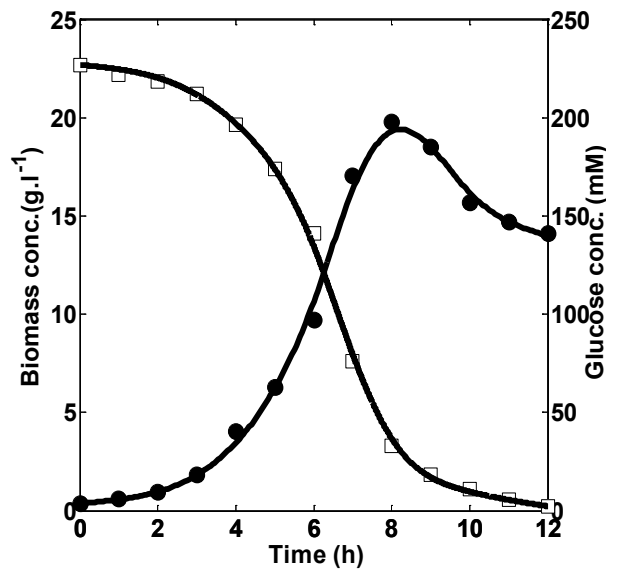
(A)



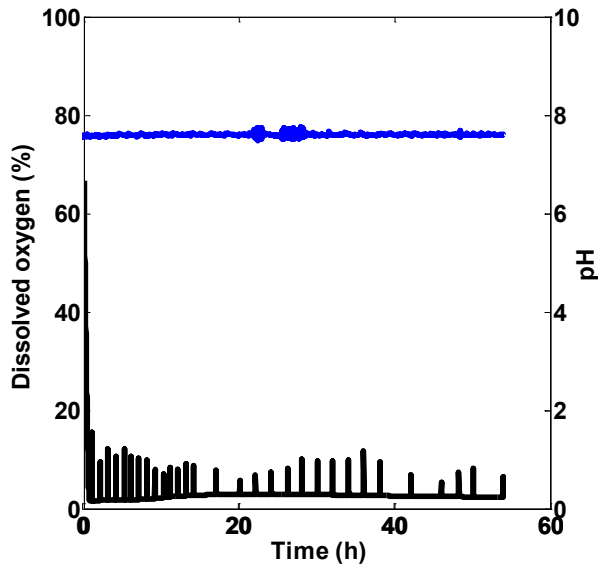
(B)



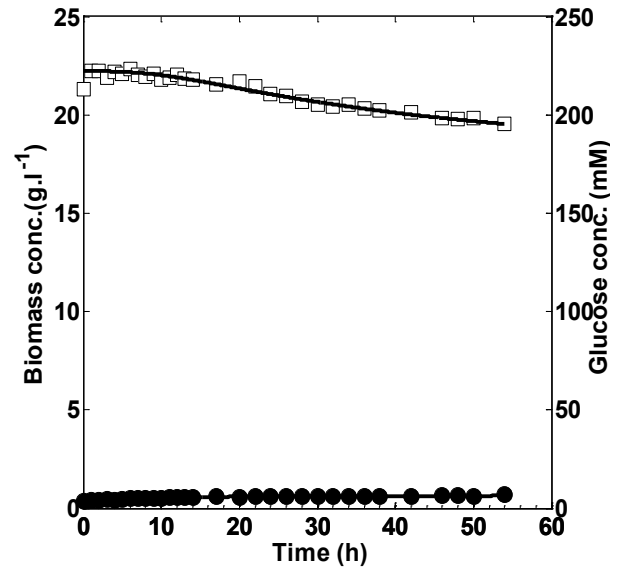
(C)



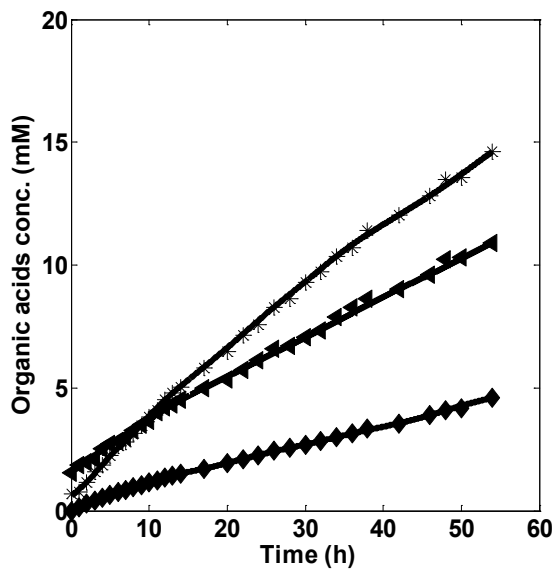
(D)



(E)



(F)



(G)

Figure VII-2. Reference culture kinetics of *C. glutamicum* 2262 *AldhA* under constant dissolved oxygen concentration. Dissolved oxygen concentrations profiles (A, C, E), biomass concentration (B, D, F) and organic acid concentration (G) kinetics obtained in a single bioreactor with constant dissolved oxygen concentration of 50 % of air saturation (A, B), in two interconnected stirred tank bioreactors with constant dissolved oxygen concentration of 50% of air saturation (C, D) or in two interconnected stirred anaerobic bioreactors (E, F, G). The different symbols represent: glucose (\square), biomass (\bullet), succinate ($*$), acetate (\blacktriangleleft), pyruvate (\blacklozenge).

Thus, these values were two orders of magnitude lower than the mean residence time (Table VII-2), which was satisfying regarding the assumption of CSTR for both bioreactors.

For these oscillating conditions, it could be possible to determine the mean fraction of time α spent by the biomass in aerobic conditions:

$$\alpha = \frac{\tau_1 + (V_{tube}/2)/Q}{\tau_1 + \tau_2 + V_{tube}/Q} \quad (VII-6)$$

With Q ($\text{m}^3 \text{s}^{-1}$) the circulation flow rate, τ_1 and τ_2 (s) respectively the mean residence time in both bioreactors of volume V_1 and V_2 :

$$\tau_1 = \frac{V_1}{Q} \text{ and } \tau_2 = \frac{V_2}{Q} \quad (\text{VII-7})$$

Indeed, usually, the mean residence time is used in scale-down studies to characterize the set-up and the period of oscillations of dissolved oxygen (or other substrates) concentrations. However, it should be noted that mixed bioreactors also perform a mixing of the microbial populations through the residence time distributions (RTD) imposed. While in the case of PF bioreactors, it could be supposed that RTD only consists in a delay function, in the case of CSTR, for a mean residence time τ , the RTD $E(t)$ takes the well-known following form:

$$E(t) = \frac{1}{\tau} e^{-t/\tau} \quad (\text{VII-8})$$

Supposing that the bacteria perfectly followed the liquid flow, the succession of DOC oscillations encountered was specific to each bacterium and related to the RTD distributions in each CSTR, which represented the probability for a given entity to spend a time t in the bioreactor. It is thus necessary to validate first the robustness of the set-up by quantifying the dispersion of the aeration ratio α around its mean value given by equation (VII-6). Aiming at this, the oscillations encountered by a statistically representative population of 1,000 microbial sub-populations were modeled using a dedicated Matlab script. From the oscillations profiles obtained, it was then possible to calculate the dynamic fraction of time spent in aerobic conditions $\beta(j, M)$ for each sub-population j after M circulations inside the set-up.

$$\beta(j, M) = \frac{\sum_{i=1}^M t_1(i, j) + M \times \left(\frac{V_{tube}}{2 \times Q}\right)}{\sum_{i=1}^M [t_1(i, j) + t_2(i, j)] + M \times \left(\frac{V_{tube}}{Q}\right)} \quad j \in \{1 \dots 1,000\} \quad (\text{VII-9})$$

With $t_1(i, j)$ and $t_2(i, j)$ the time spent by microbial sub-populations in aerobic and anaerobic conditions. The value of M was chosen to match the simulation time to the experimental culture duration.

The variation of β results obtained for $\alpha = 0.33$ and $\alpha = 0.67$ are given in Figure VII-3. It can be seen that each dynamic profile of aerobic fractions are converging to the mean value α

(equation (VII-6)) but also that some subpopulations may exhibit sensibly higher or lower values of aeration fraction, especially during the first hours of culture.

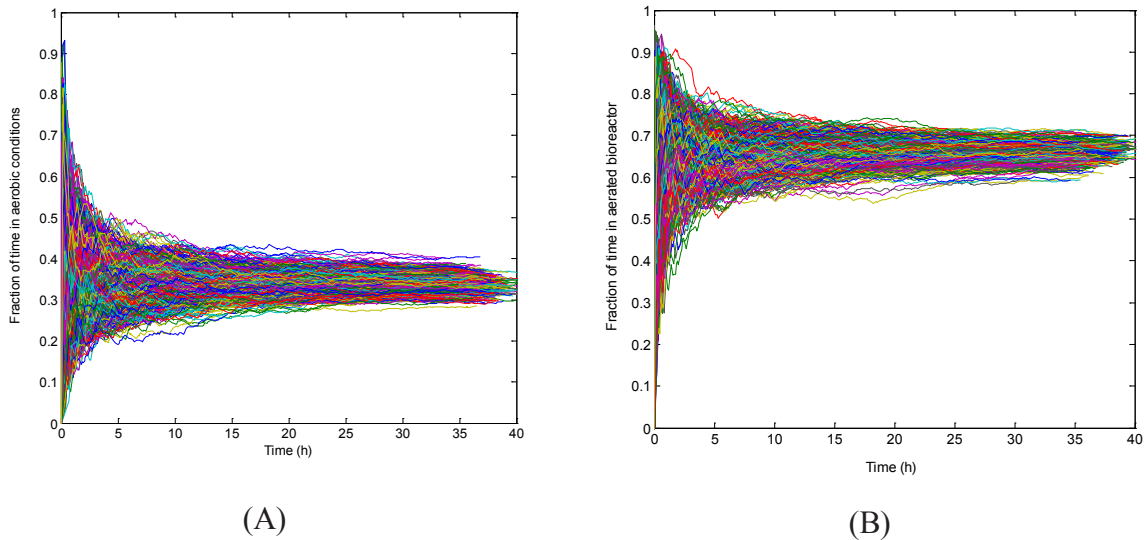


Figure VII-3. Variation of the dynamic fraction of aeration β for 10,000 sub-populations in function of time for mean aeration fractions of $\alpha = 0.33$ (A) and $\alpha = 0.67$ (B).

To quantify the dispersion of oxygenation conditions imposed to the microorganisms, mean and standard deviations of the dynamic fraction of aeration β were calculated within the microbial sub-populations. The results were depicted in Figure VII-4.

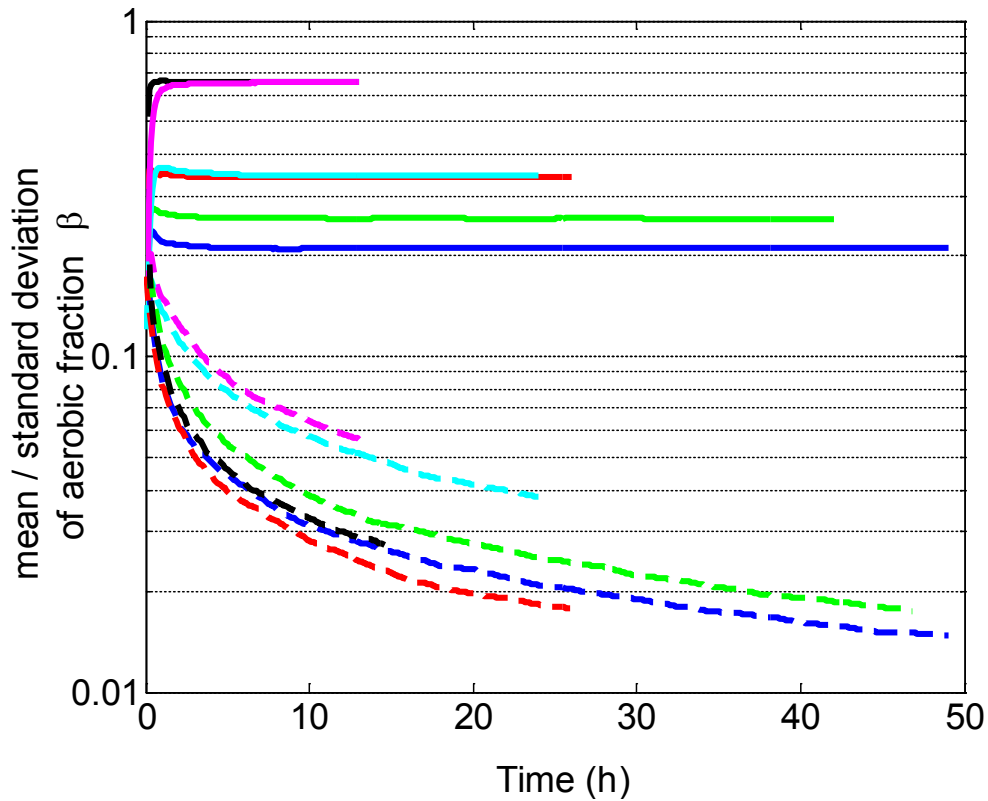


Figure VII-4. Mean and standard deviation values of dynamic aerobic fraction β in course of time. Mean aerobic fractions α are 0.2 (—), 0.25 (—), 0.33a (—), 0.33b (—), 0.67a (—), 0.67b (—). Continuous and dotted lines respectively depict mean and standard deviations of dynamic aerobic fractions β .

It could be noted that mean values of aerobic fractions β were rapidly converging to the value of the corresponding mean aeration fractions α . Moreover, a moderate dispersion around the mean value could be observed, especially during the first hours of culture.

Lastly, it was necessary to ensure the reliability of the scale-down set-up in terms of dissolved oxygen concentration control. Indeed, the control of DOC may become problematic if the characteristic time of liquid circulation between the two tanks becomes similar to the characteristic time of oxygen transfer or stripping in each vessel, thus limiting the liquid flow rate used for the experiments. To verify the ability of the set-up to provide well-controlled oxygenation conditions, DOC temporal profiles have been reported in Figure VII-5 for two operating conditions. Except some minor fluctuations of DOC in the aerobic vessel due to switching between air and pure oxygen injection loop during the regulation, DOC set points were clearly respected during the cultures.

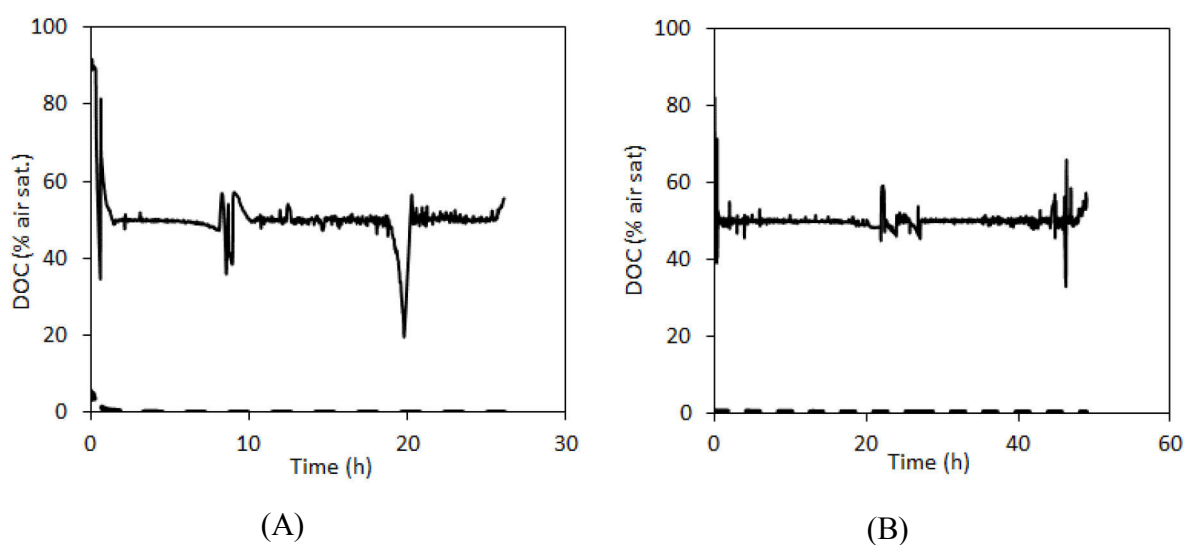


Figure VII-5. Dynamic profiles of DOC in the aerobic (continuous line) and in the anaerobic (dashed line) bioreactor. Mean aerobic fractions were 0.33a (A) and 0.2 (B).

VII-3.3. Cultures under oscillating DOC conditions

In order to mimic the effect of dissolved oxygen gradients encountered by cells in large-scale bioreactors, *C. glutamicum* 2262 ΔdhA was continuously circulated between the aerobic and anaerobic compartments of the scale-down set-up. DOC set points for aerobic and anaerobic bioreactors were 50 (except case of 0 ratio) and 0 % of air saturation, respectively. The impact of DOC oscillations on microbial growth kinetics and glucose consumption were depicted in Figure VII-6. When the cells were cultivated in fully aerobic conditions (reference culture), the maximal biomass concentration reached a value of 19 g cdw L⁻¹ (Figure VII-6A). For a mean aeration fraction $\alpha = 0.67$, no sensible difference was observed between $\alpha = 0.67a$ and $\alpha = 0.67b$ and the maximal biomass accumulated was 16.3 g cdw L⁻¹. Compared to the reference culture, circulation between aerobic and anaerobic environment with this mean aeration fraction resulted in a biomass concentration decrease of approximately 14 %. Similar growth were also obtained comparing mean aeration fractions of $\alpha = 0.33a$ and 0.33b, meaning that increasing the residence time at same mean aeration fraction had no sensible influence on bacterial growth. However, comparatively to reference culture, a decrease of 31 % in maximal biomass concentration was noticed. For lowest mean aeration fractions of 0.25 and 0.2, an important lag phase and the decrease of maximal biomass concentration were more pronounced with 37 % and 51 % decrease in comparison with reference culture, respectively. Lastly, when

C. glutamicum 2262 ΔdhA was cultured in two anaerobic bioreactors ($\alpha = 0$), no sensible growth was observed, resulting in a loss of 96 % of the maximal biomass concentration compared to the reference culture (Figure VII-6A).

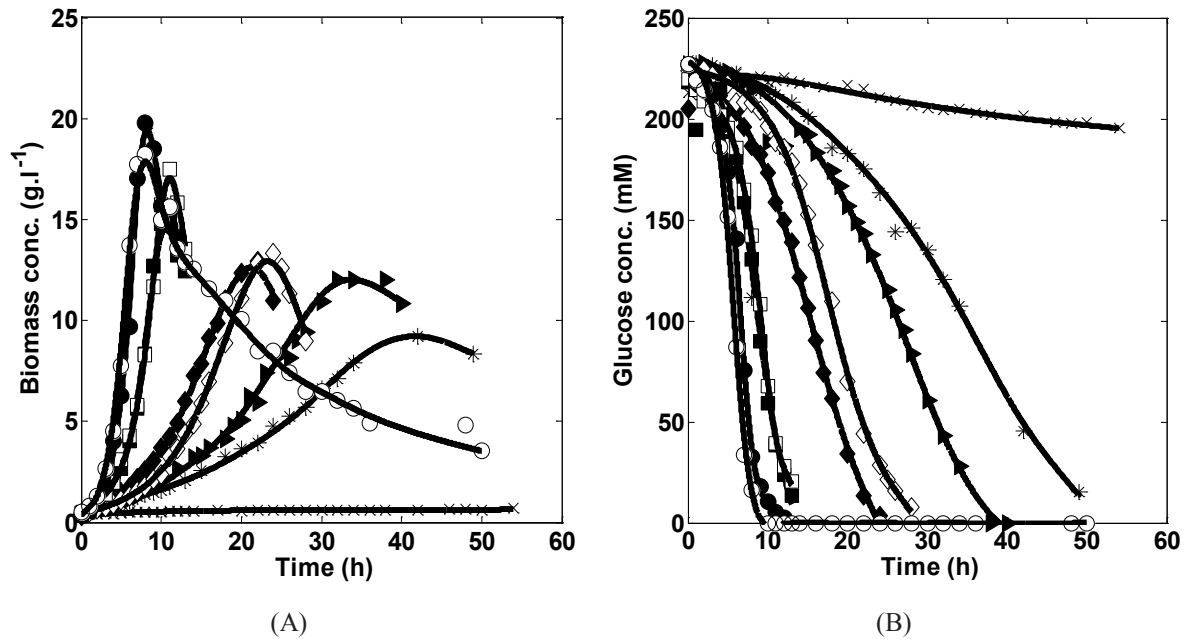


Figure VII-6. Influence of dissolved oxygen oscillation on *C. glutamicum* 2262 ΔdhA growth and substrate consumption during a two compartment scale-down system. (A). Growth kinetic; (B). Glucose uptake profile. The mean aerobic fractions α are: 0 (\times), 0.2 (*), 0.25 (▶), 0.33a (◆), 0.33b (◇), 0.67a (■), 0.67b (□), 1a (●), 1b (○).

For each experiment, the maximal specific growth rate μ_{max} was determined by Equation (VII-1). Interestingly, Figure VII-7 showed that μ_{max} increased linearly with the mean aeration fraction α and varied between 0.02 h⁻¹ and 0.6 h⁻¹ for $\alpha = 0$ and $\alpha = 1$, respectively. This would indicate that the apparent growth rate of the bacteria were strongly related to mean fraction of aeration and would thus also impact the time necessary to reach the maximal biomass concentration $t(X_{max})$.

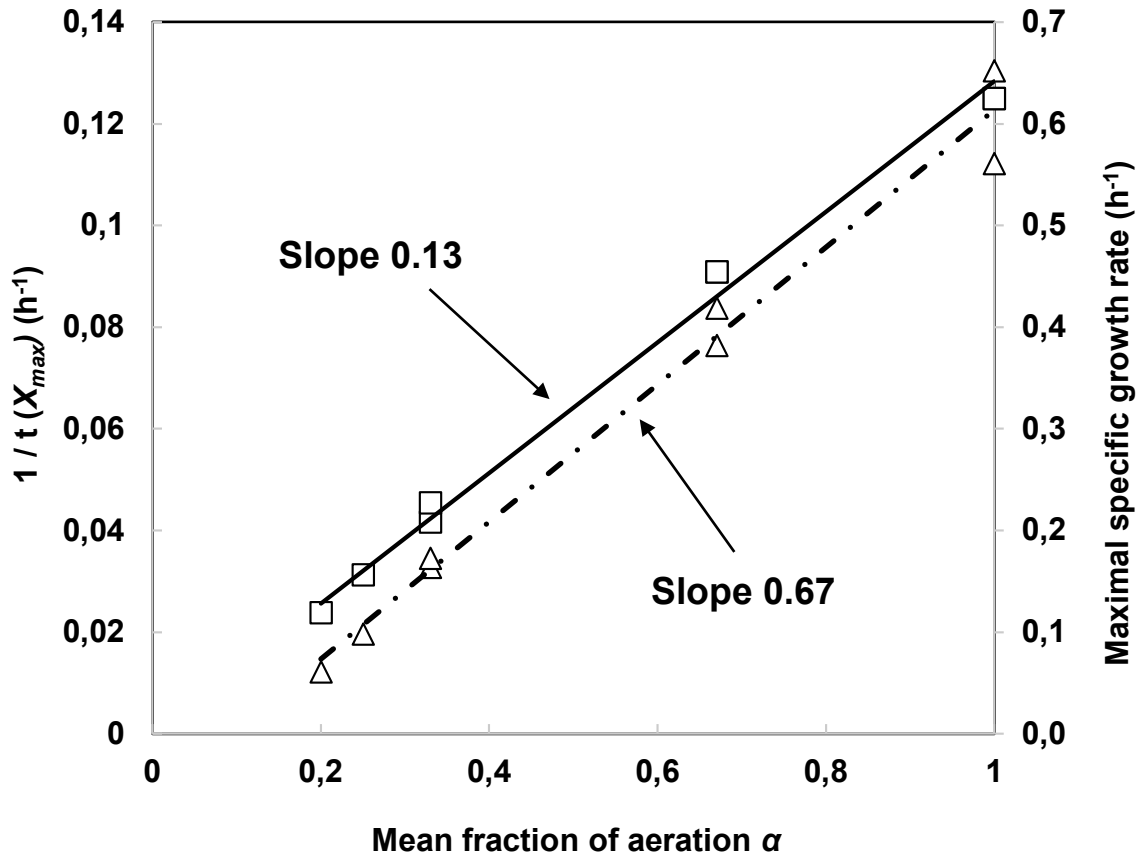


Figure VII-7. Relationship between mean fraction of aeration α , $1/t(X_{max})$ (□) and maximal specific growth rate μ_{max} (Δ).

Indeed, the report of $1/t(X_{max})$, which has the same dimension (h^{-1}) as the specific growth rate, as a function of α , also indicated a linear relationship between both variables (Figure VII-7). It could be thus suggested that the oxidative metabolism of the bacteria could sequentially operate, without lag phase but with a loss of productivity, as indicated by the decrease of the maximal biomass concentration (Figure VII-6). This hypothesis of “on/off” metabolism will be further discussed in the paragraph dedicated to kinetics modelling (paragraph VII-3.4).

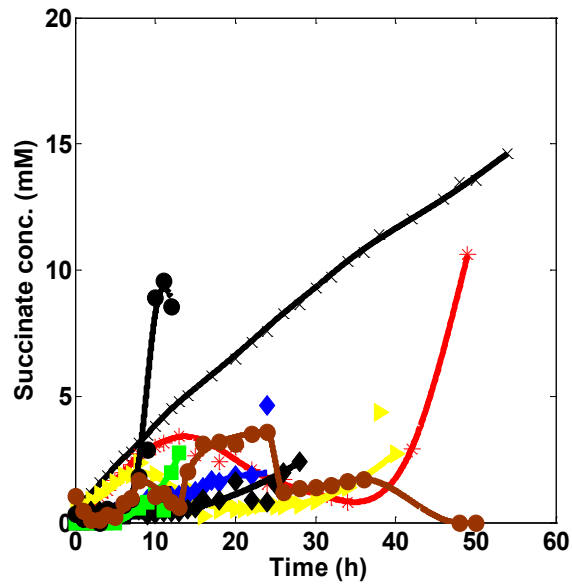
VII-3.4. Effect of DOC oscillations on organic acids production and glucose consumption

Influence of DOC oscillations on the organic acids production by *C. glutamicum* 2262 $\Delta ldhA$ was also investigated. Decreasing mean aeration fraction resulted in transitory production of organic acids (Figure VII-8). The data obtained were compared with the production obtained during cultures at constant DOC of 50 % and 0 % of air saturation. As shown in Figure VII-8,

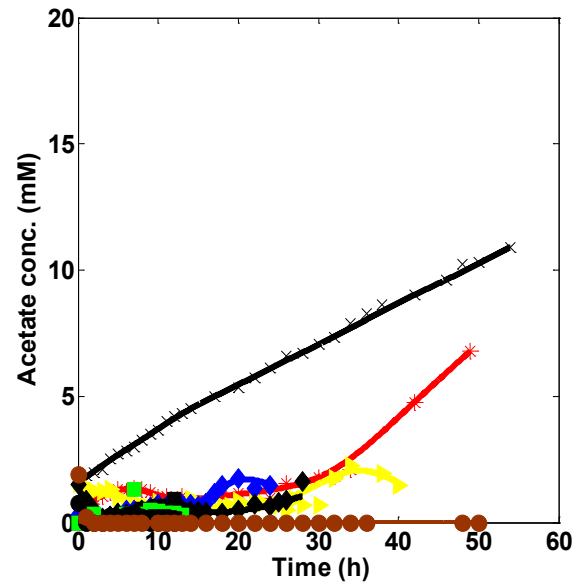
succinic, acetic and pyruvic acids produced in the scale down system were re-consumed along the culture time for mean aeration fractions from 0.25 to 1. This observation made hard the analysis of the results, especially in terms of specific production rates or maximal quantities produced. For the two compartment cultivation of *C. glutamicum* 2262 *AldhA* under anaerobic conditions ($\alpha = 0$), the production of organic acids was maximal (0.4 g L⁻¹ of pyruvate, 1.7 g L⁻¹ of succinate and 0.7 g L⁻¹ of acetate) despite the weak value of biomass concentration and, more interestingly, these acids were not re-consumed over the time. It should be thus mainly noted that the concentrations of organic acids were dramatically lower than the ones obtained during the one-step and biphasic process of production (see chapter V, first paragraph of results).

Concerning glucose consumption and according to Figure VII-6B, the fastest glucose consumption was obtained for the reference culture ($\alpha = 1a$ and $1b$) with a total glucose exhaustion which was observed within 10 hours. For the lower mean aeration ratios, glucose was also totally consumed later than for the reference cultures. In the absence of aeration ($\alpha = 0$), 90 % of glucose remained in the culture (Figure VII-6B).

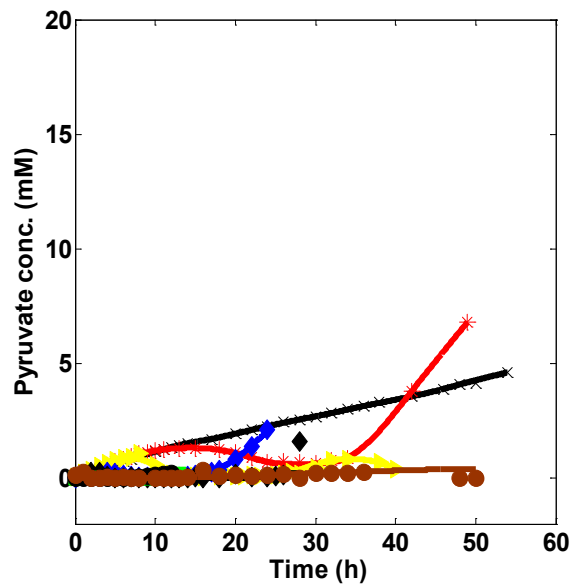
According to substrate flux distribution, when cultivated under one aerobic or two interconnected aerobic bioreactors, the carbon was thus mainly converted into biomass with maximal biomass on glucose yield, Y_{XS} , of 0.5 g cdw g⁻¹ glucose (Figure VII-9). This yield decreased progressively when the mean fraction of aeration also decreased. Thus, in comparison with the fully aerobic reference culture, maximal yield showed a 56 % decrease in the scale-down experiment with mean aeration fraction of 0.2. Indeed, this yield dropped to 0.07 g cdw g⁻¹ glucose for the fully-anaerobic culture. It could be also noted that maximal biomass concentration and maximal specific glucose consumption rate exhibited similar non-linear trends while, similarly to maximal specific growth rate, maximal biomass to glucose yield was linearly linked to the mean fraction of aeration. This would thus further suggest the hypothesis of “on/off” oxidative metabolism of *C. glutamicum* during their culture in the scale-down set-up.



(A)



(B)



(C)

Figure VII-8. Kinetics of organic acids production during an oscillating scale-down process using *C. glutamicum* 2262 Δ ldhA. (A). Succinate concentration; (B). Acetate concentration; (C). Pyruvate production. The mean aeration fraction α are 0 (\times), 0.2 ($*$), 0.25 (\blacktriangleright), 0.33a (\blacklozenge), 0.33b (\blacklozenge), 0.67a (\blacksquare), 0.67b (\blacksquare), 1a (\bullet), 1b (\bullet).

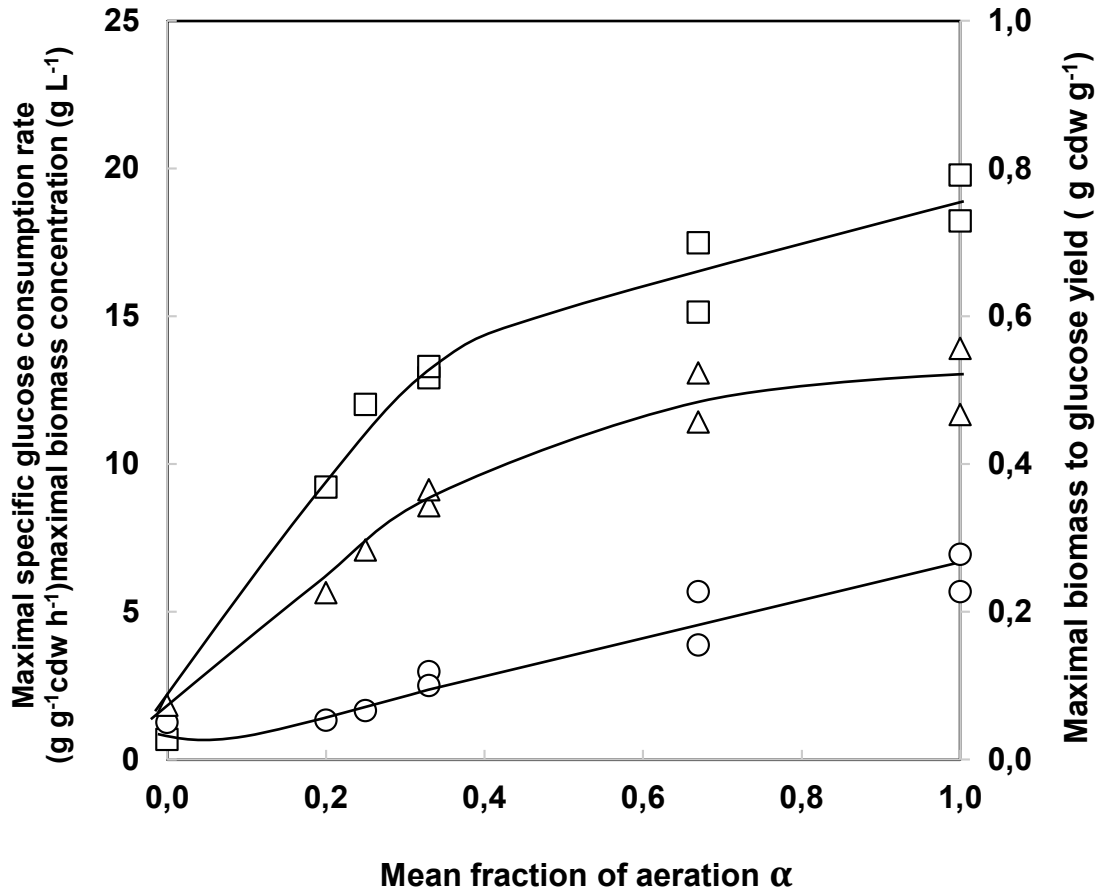


Figure VII-9. Relationship between mean fraction of aeration α maximal biomass concentration X_{max} (□), maximal specific glucose consumption rate (○) and maximal biomass on glucose yield Y_{XS} (Δ).

VII-3.5. Growth kinetics modelling using a coupled RTD-Monod model

To get further insight in the microbial physiological response, it was proposed to couple the results of RTD modelling (paragraph VII-3.1) with a Monod growth kinetic model. The model presented, based on Monod's classic law of growth must be considered as a first attempt of growth modelling. Further developments should be probably necessary to increase the robustness of its prediction capacity. First, Monod law was applied to model for each group of bacteria i bacterial growth (Monod, 1949):

$$\frac{dX_i}{dt} = \mu_{max} \frac{[Gluc]}{[Gluc] + K_s} \cdot X_i - k_d \cdot X_i, \quad i \in \{1 \dots 1,000\} \quad (VII-10)$$

A cell death rate was added in the growth kinetics to model the decrease of biomass concentration observed experimentally (Figure VII-6A) and the shape of growth kinetics for each mean fraction of aeration. Death kinetics was modelled using a first-order kinetic equation:

$$\frac{dX_{d,i}}{dt} = k_d \cdot X_i, i \in \{1 \dots 1000\} \quad (VII-11)$$

Total biomass concentration was: $X = \sum_i^N X_i$

For glucose consumption, a constant biomass to glucose yield was supposed:

$$\frac{d[Gluc]}{dt} = -\frac{\mu}{Y_{X/S}} \cdot X \quad (VII-12)$$

The kinetic modelling was based on several hypotheses

- i. Only $N = 1000$ groups of bacteria were tracked inside the two bioreactors to decrease calculation time. Each group thus represented an initial biomass concentration of X_0/N ; Following Monod kinetic model, the mass of each bio-entity increased following equation (VII-10).
- ii. Kinetic parameters, namely maximal specific growth rate μ_{max} , biomass on substrate yield $Y_{X/S}$, death rate k_d and half-velocity constant K_s were maintained constant whatever the culture modelled. Whereas μ_{max} , $Y_{X/S}$ and k_d were obtained using the reference fully-aerobic culture, K_s was given by Seletzky *et al.* (2007). The values of the kinetic parameters were given in Table VII-3.
- iii. In the aerobic bioreactor, bacteria grew and consumed glucose, whatever the DOC oscillations imposed before (no history effect). It was also assumed that transition from anaerobic to aerobic metabolism was immediate.
- iv. In the anaerobic bioreactor, bacteria neither grew nor produced organic acids nor consumed glucose. In fact, at every run, the bacteria switched promptly from aerated to non-aerated zones, whereas a suitable induction of anaerobic metabolism requires a progressive transition from aerobic to anaerobic (see chapter V). Growth and glucose consumption stopped immediately when the microorganisms entered the anaerobic bioreactor.

Table VII-3. Values of the optimized parameters for kinetic modelling.

Parameter	μ_{max} (h^{-1})	$Y_{X/S}$ ($g\ g_{CDW}^{-1}$)	K_s ($g\ L^{-1}$)	k_d (h^{-1})
Value	0.55*	0.4*	0.0045**	0.0005*

* Parameters determined using the reference fully aerobic culture.

** Value given by Seletzky *et al.* (2007)

The results obtained by the RTD-kinetic model were given in Figure VII-10. It can be noted that the results were in correct agreement with the experimental measurement of biomass concentrations. In particular, the coupled RTD-kinetic model was able to satisfactory predict the decrease of maximal biomass concentration X_{max} and the increase of the time necessary to reach X_{max} when the mean fraction of aeration was increased. This result confirmed the fact that, if a “on/off” metabolism as supposed for the oxidative metabolism of each bacterium, the global physiological response of the whole microbial population was matching the experimental observations.

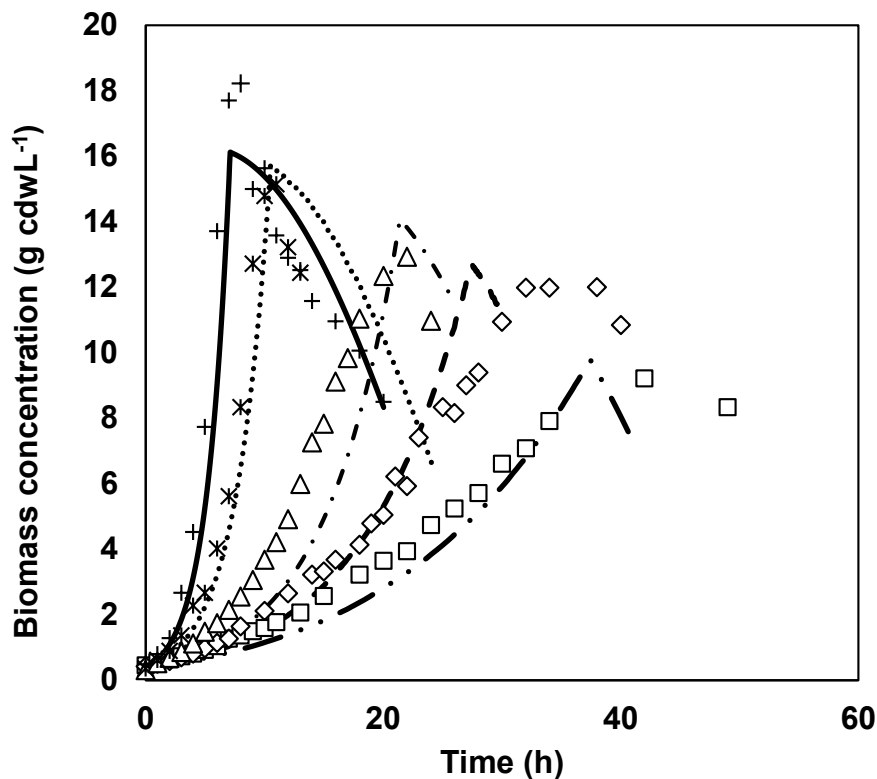


Figure VII-10. Comparison of experimental and modelled growth kinetics and impact of DOC oscillations. Dots are experimental values while continuous lines are modelled kinetics. Mean aerobic fractions α are 0.2 (\square , —●●), 0.25 (\diamond , ---), 0.33a (Δ , -●-), 0.67a ($*$, ●●●), 1 ($+$, —).

VII-4. Discussion

This study provided new insights into metabolic and physiological responses of *C. glutamicum* 2262 *AldhA* when subjected to oscillating DOC. The comparison of reference fully-aerobic cultures, either performed in a single aerobic compartment or two interconnected aerobic bioreactors revealed unchanged growth and metabolic responses (Figure VII-2A-F). Thus, the scale-down set-up had, in itself, no influence on the microbial physiological response as far as the DOC was maintained at the set point value of 50 % of air saturation. However, subjecting *C. glutamicum* 2262 *AldhA* to various mean aeration fractions α by continuously circulating bacterial cells between an aerobic and an anaerobic bioreactors had relative impacts on cell growth and organic acids production. For instance, for $\alpha = 0.67a$ and $0.67b$, DOC oscillations with residence times in the anaerobic bioreactor of 138 s and 551 s respectively (Table VII-2 and Figure VII-3) resulted in a small decrease (14 %) of maximal biomass concentration and glucose uptake rate despite the bacteria were oxygenated only 67 % of total culture time. Scale-down experiments using stirred tank bioreactor (STR) and plug flow reactor (PFR) for the culture of *C. glutamicum* ATCC 13032 under DOC oscillations with residence times in anaerobic vessel of 45 and 87 s with total oxygenation ratio of 82 %, have also reported unchanged growth yields (Käb *et al.*, 2014b). In our case, the time spent in anaerobic bioreactor was sensibly higher, which could explain the decrease observed. It is also interesting to notice that biomass concentration decrease was also observed in scale-down systems, with anaerobic residence times of the same order of magnitude, but for other microorganisms such as *Saccharomyces cerevisiae* (George *et al.*, 1993). When the anaerobic residence time was increased to 551 s while aerobic residence time was 138 s (see condition $\alpha = 0.2$ in Table VII-2) or anaerobic residence of 616 s associated to aerobic residence time of 206 s (see condition $\alpha = 0.25$ in Table VII-2), detrimental consequences on microbial growth and glucose consumption were observed (Figure VII-6). Lastly, our study proposed an original coupled RTD-Monod growth model that considered independently 1000 microbial sub-populations on the basis of their respective residence time in each vessel. Our model showed a good agreement with experimental measurements of biomass concentrations. In particular, it revealed that the global physiological response of *C. glutamicum* 2262 *AldhA* observed in the scale-down set-up could be modelled by considering that the metabolic parameters (maximal specific growth rate, biomass on glucose yield) were not impacted by the mean fraction of aeration.

By simulating DOC oscillations effects on *B. subtilis*, using a scale-down approach, Amanullah and Buckland reported a similar decrease in maximal biomass concentration and specific

growth rate when the circulation time was increased at a constant ratio of volume aerobic reactor/volume anaerobic reactor (Amanullah *et al.*, 2003).

The ability of *C. glutamicum* for amino acids production has been intensively underlined in literature (Hermann, 2003; Ikeda and Takeno, 2013). Recently its suitability for large scale industrial applications for bulk chemical as organic acids has shown a growing interest. In order to investigate the large scale applicability of such processes, experiments were made taking into consideration the possible occurrence of gradients in dissolved oxygen in industrial scale reactors, as *C. glutamicum* is a high oxygen requiring organism. In our scale-down study, minor quantities of organic acids were obtained (Figure VII-8). In *C. glutamicum*, it is well known that the lack of oxygen and nitrate as external electron acceptors in such conditions resulted in cell growth stop but still allowing the fermentation of carbohydrates into organic acids (Dominguez *et al.*, 1993; Inui *et al.*, 2004a; Khuat *et al.*, 2014). When *C. glutamicum* 2262 *AldhA* was cultured in two interconnected anaerobic bioreactors ($\alpha = 0$), the concentration of organic acids was maximal although growth was almost undetectable. For the other experiments ($\alpha > 0$), organic acids were produced and re-consumed as a second substrate; bacteria travelled from aerobic to anaerobic bioreactors without decrease of growth capacities, as far as DOC could feed their oxidative metabolism. Decreasing the mean fraction of aeration resulted in lengthening the culture time. Besides the prolonged lag phase observed when *C. glutamicum* 2262 *AldhA* was faced to low oxygenation ratio (< 0.2), Oosterhuis *et al.* (1985), reported detrimental influence of oscillating DOC not only on gluconic acid productivity but also on the rate of growth of *Gluconobacter oxydans* (Oosterhuis *et al.*, 1985). Metabolization of produced organic acids have also been reported in STR - PFR scale down of *C. glutamicum*. In fact, Käß *et al.* (2014) showed in their scale down system that the majority of glucose entering the PFR was transformed into lactate during the passage through the PFR and was quickly metabolized once the bacteria were in the aerated STR again (Käß *et al.*, 2014b). Through the different scale down experiments, decreasing the volume ratio resulted in a negative impact on growth performance while fermentation products accumulation were slightly enhanced but still anecdotic (Figure VII-8). Such observation underlined the interest of well characterizing the process conditions according to a given goal. For aerobic process, occurrence of important anaerobic zones could result on carbon overflow. A rapid redirection of substrate carbon flow was observed directly by the increase in extracellular organic acids in the lowest ratios experiments (Figure VII-8). *C. glutamicum* has been demonstrated to be able to switch rapidly from aerobic respiration to fermentative pathways (Okino *et al.*, 2005; Yamamoto *et al.*, 2011).

Recent scale-down studies involving different strains of *C. glutamicum* performed in a two compartment STR-PFR system, revealed no loss of process performance with DOC oscillation frequencies in the minute range. Indeed, analysis of proteome, metabolome, transcriptome indicated that no change was noted in the response to DOC oscillations. Therefore, they emphasized the robustness of *C. glutamicum* in comparison to other industrial microorganisms (Käb *et al.*, 2014a, 2014b). This conclusion was verified in our study when experiments were performed with mean aeration fractions between 0.67 and 1. For these conditions, the physiological response of *C. glutamicum* was not significantly different from those of fully-aerobic reference culture (Figure VII-6). However when mean aeration fraction were below 0.33, maximal substrate uptake rate decreased from $1.3 \text{ g g}^{-1} \text{ cdw h}^{-1}$ to $0.2 \text{ g g}^{-1} \text{ cdw h}^{-1}$ (Figure VII-9) associated to bacterial growth performance loss and overflow of side product increase (Figure VII-8).

VII-5. Conclusion

Using a scale-down set-up, it was shown that when the mean fraction of aeration was lower than 0.33, carbon flux was diverted to side-products accumulation, which was also concomitant with growth stop. But, when this fraction was higher than 0.67, no detrimental consequences of dissolved oxygen oscillation on *C. glutamicum* 2262 *AldhA* performance was observed. A negligible accumulation of organic acids was detected as these acids were re-consumed leading to a kind of futile cycle of extracellular side products production and consumption. In such conditions, which may be observed at the industrial scale, *C. glutamicum* thus exhibits a remarkable robustness towards DOC oscillations. The results of this study indicated that *C. glutamicum* 2262 *AldhA* is robust against oscillating oxygen supply limitation environments within some boundaries of heterogeneities. The presented scale-down approach for this phenomenon investigation helped identifying physiological properties and responses to process inhomogeneity. Finally, the data of our study have shown that the effect of gradients, particularly oxygen gradients in production scale bioreactors should be studied before the process is scaled up. In this regard, it is important to have some knowledge concerning the influence of local dissolved oxygen concentrations and the sweep of these oxygen gradients in order to achieve an efficient reactor design.

VII-6. References

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Conclusion générale

et

Perspectives

VIII. Conclusion et perspectives

VIII-1. Conclusion

Dans un contexte sociétal et industriel, plus général, de recherche de nouvelles voies de synthèse d'intrants chimiques utilisant des biocatalyseurs et des réactifs biosourcés, les procédés de production d'acides organiques par voie microbienne semblent aujourd'hui très prometteurs de par leur productivité et leur compétitivité. Ainsi, ces travaux de doctorat ont été dédiés à l'étude de l'influence de l'oxygénation sur la réponse physiologique de deux souches de *C. glutamicum*, la souche sauvage, *C. glutamicum* 2262, et une souche génétiquement modifiée, *C. glutamicum* 2262 Δ *ldhA*, qui sont décrites comme excellentes candidates pour la production d'acides organiques en conditions d'anaérobiose. Notre attention s'est plus particulièrement portée sur les verrous scientifiques suivants :

- Impact de la délétion d'un gène sur les capacités de croissance et de production de *C. glutamicum*.
- Réponse physiologique microbienne dans des conditions micro-aérobies de culture.
- Impact de la stratégie de désoxygénation lors de la phase de transition entre aérobiose et anaérobiose.
- Dynamique du métabolisme oxydatif face à des fluctuations de concentrations en oxygène dissous.

Dans un premier temps, nous avons construit un mutant de *C. glutamicum* 2262 par délétion du gène *ldhA* afin de limiter la production de co-produits, notamment du lactate. A travers des expériences de cultures en bioréacteur, nous avons montré que la délétion du gène n'avait aucune incidence sur la croissance de la bactérie en condition aérobie ; *C. glutamicum* 2262 et *C. glutamicum* 2262 Δ *ldhA* présentaient, en effet, des vitesses spécifiques maximales de croissance similaires et comprises entre 0,6 et 0,7 h⁻¹ avec une vitesse spécifique de consommation de glucose d'environ 7,5 mmol (g poids sec)⁻¹.h⁻¹. Ceci s'explique probablement par une activité réduite de la lactate déshydrogénase pendant la phase de croissance (Inui et al., 2004a). Nous avons ensuite évalué la production de CO₂ observée pendant la croissance en établissant un modèle désorption du CO₂ sous l'effet du débit d'air. Néanmoins, nos estimations ont montré que les productions de CO₂ ne seraient pas suffisantes à alimenter, à elles seules, un second bioréacteur de production qui fonctionnerait en anaérobiose sans apport supplémentaire de CO₂.

Sachant que la croissance de *C. glutamicum* exige de l'oxygène et que la production d'acides organiques nécessite une limitation ou une absence en oxygène, nous avons étudié l'influence du coefficient de transfert en oxygène (k_{La}) sur sa croissance et sa production d'acides organiques dans des fioles en verre non-chicanées. Pour cela, nous avons imposé les k_{La} suivants : 0,6 ; 3 ; 11 ; 19 ; 33 ; 36 ; 48 ; 77 ; 120 et 164 h⁻¹ qui ont été déterminés expérimentalement dans des fioles en verres munies de patches à oxygène en utilisant un lecteur PreSens. Les résultats obtenus montrent qu'une augmentation du k_{La} se traduit par une amélioration de la croissance tandis qu'une diminution du k_{La} , favorise la production d'acides organiques. Cette étude a démontré qu'en fioles : (i) pour des k_{La} entre 10 et 19 h⁻¹, la production de succinate était maximale (63 mM), (ii) pour des valeurs de k_{La} comprises entre 19 et 33 h⁻¹, c'est la production d'acétate qui est maximale (135 mM) et enfin (iii) pour orienter le métabolisme vers la formation de biomasse, les k_{La} devraient être supérieurs à 164 h⁻¹.

Afin d'améliorer les performances de production de succinate, nous avons entrepris des cultures de la souche mutante en bioréacteur de 2 L en présence ou non de bicarbonate de sodium comme donneur de CO₂ pour la réaction de formation du succinate. Toutes les cultures ont été réalisées dans des procédés mono-étape où les phases de croissance et de production d'acides organiques sont effectuées dans le même bioréacteur, sans étapes supplémentaires de récolte ni de lavage de la biomasse. Nos résultats ont également montré que l'ajout du bicarbonate n'avait pas d'incidence sur la phase de croissance.

Lors des cultures en procédés mono-étape, nous avons développé deux types de stratégies de désoxygénation lors de la transition de la phase de croissance à la phase de production : une première basée sur le contrôle du profil dynamique de l'oxygène dissous et une seconde basée sur celui du débit d'air. Nous avons ainsi développé un procédé mono-étape et tri-phasique composé (voir Figure B) :

- d'une phase de croissance en aérobiose,
- d'une phase de transition permettant la désoxygénation,
- d'une phase de production de succinate en anaérobiose.

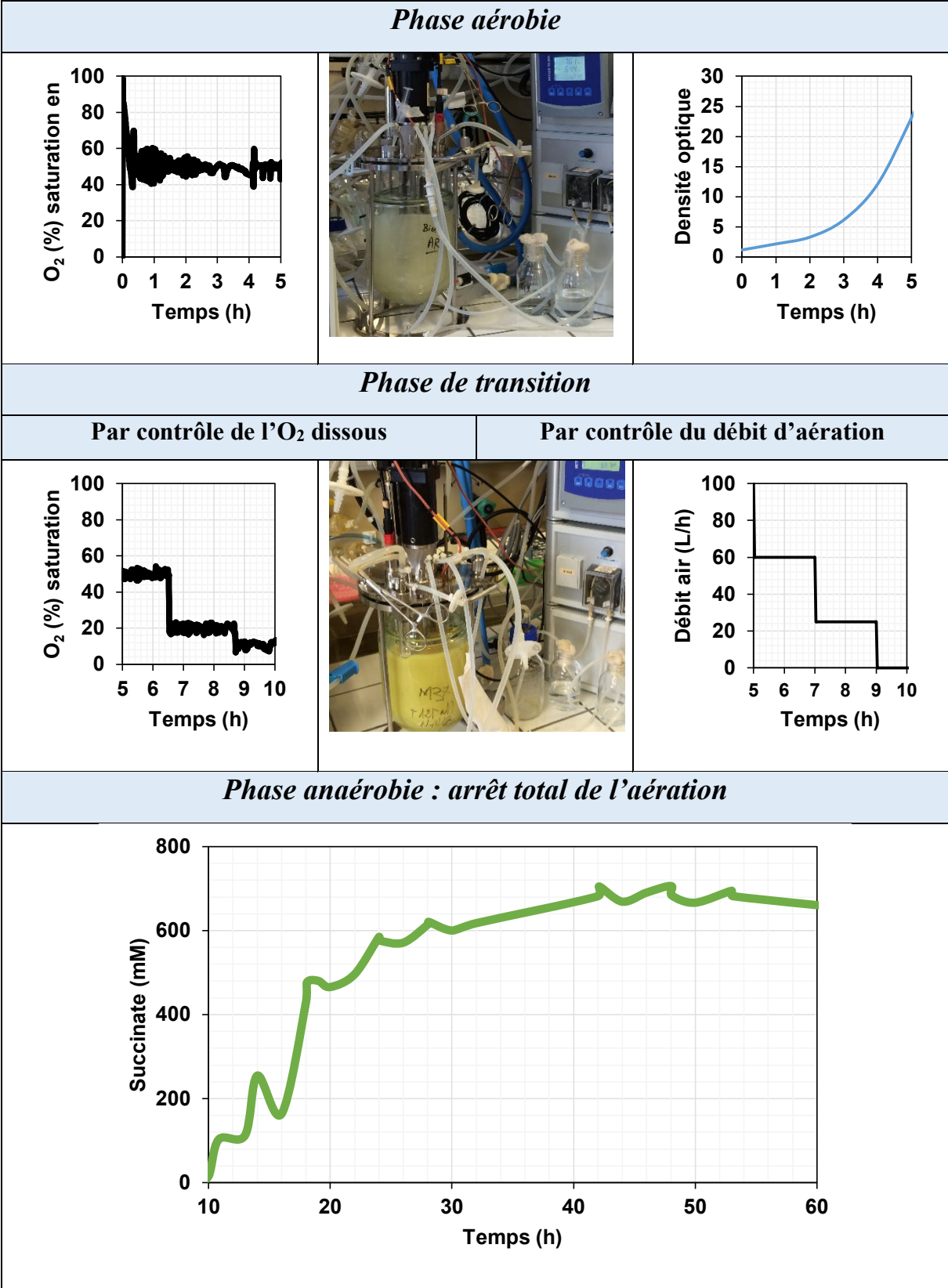


Figure B. Enchaînement des différentes étapes du procédé tri-phasique de production

Cette étude nous a permis de montrer que la réduction par paliers successifs de la concentration en oxygène dissous, ou du débit d'air, jusqu'à l'anaérobiose, améliorerait nettement la production de succinate par rapport à un passage instantané (en moins d'une minute) de l'aérobiose à l'anaérobiose. L'influence de ces transitions serait associée à l'apparition d'un environnement de micro-aérobiose lors de l'abaissement de l'oxygène dissous à de faibles niveaux ou à une auto-induction de la micro-aérobiose lorsqu'on gère la transition par le débit d'air. Cette micro-aérobiose favoriserait, probablement, la régulation de la machinerie enzymatique (les enzymes anapérotyques, celles du shunt de glyoxylate, ou les régulateurs de l'aérobiose/anaérobiose comme les systèmes Arc et FNR présents chez *E. coli*) et l'adaptation des cellules à l'appauvrissement de leur environnement en oxygène.

Parmi les différentes transitions testées, les expériences réalisées avec la transition *Tr3*, qui consistent en une phase de croissance aérobie jusqu'à une DO_{570nm} de 30 avec une régulation de l'oxygène dissous à 50% de la saturation suivie de la transition *Tr3* (réduction du niveau d' O_2 dissous à 20% de la saturation et maintien à 20% pendant 2 h, abaissement de cette consigne à 10% et après 2 h de régulation à 10% de la saturation, arrêt total de l'aération) et enfin la phase de production en anaérobiose, ont révélé que l'application de cette transition a permis une meilleure performance de production de succinate en terme de concentration (327 mM), de rendement (0,94 mol par mol de glucose), et de productivité ($6 \text{ mM}\cdot\text{h}^{-1}$). La transition par la gestion de l' O_2 dissous est aussi meilleure par rapport à celle basée sur la gestion du débit car elle permet une maîtrise plus fine du temps de transition vers l'anaérobiose au contraire d'une stratégie basée sur le contrôle du débit d'air, qui implique un temps de transition dépendant de la consommation d'oxygène bactérienne. Avec le procédé tri-phasique intégrant la transition *Tr3*, la souche *C. glutamicum* 2262 ΔldhA , a produit jusqu'à 424 mM avec un rendement de $1 \text{ mol}\cdot\text{mol}^{-1}$ de glucose.

En adaptant le procédé, nous avons obtenu des performances comparables à celles des microorganismes capables de produire naturellement du succinate comme *M. succiniciproducens*, *A. succinogenes*, *A. succiniciproducens* ou celles d'autres organismes ayant subi de multiples mutations génétiques comme *E. coli*.

Afin de vérifier la transposabilité de ce procédé ainsi que son efficacité sur la souche parentale qui, naturellement, a une production ultra-majoritaire de lactate mais aussi une production significative de succinate. Les cultures de *C. glutamicum* 2262 mettant en œuvre la désoxygénation progressive *Tr3*, ont abouti à une augmentation de la concentration produite de plus de 100 % avec un rendement de $1,1 \text{ mol}\cdot\text{mol}^{-1}$ par rapport à un procédé avec une transition

rapide. Par ailleurs, la production du lactate qui était majoritaire est devenue équimolaire à celle du succinate. En effet, avec le procédé à transition instantanée (*Control*), la production n'a été que de 164 mM avec un ratio succinate/lactate de 0,45 mol.mol⁻¹ en culture en mode batch tandis qu'avec la transition *Tr3*, on obtient respectivement 280 mM et 1 mol.mol⁻¹ de lactate. L'application du procédé avec la transition *Tr3* à une culture de *C. glutamicum* 2262 lors d'une fermentation en fed-batch avec ajout supplémentaire d'une série de pulses de glucose et de bicarbonate de sodium a abouti à une concentration maximale de 94 g.L⁻¹ (793 mM) de succinate avec 71 g.L⁻¹ (785 mM) de lactate. Cette concentration en succinate de 94 g.L⁻¹ est la concentration la plus élevée jamais rapportée dans la littérature pour une souche sauvage référencée comme non productrice naturelle de succinate. Ces résultats démontrent ainsi qu'avec une souche sauvage, en l'occurrence *C. glutamicum* 2262, et avec l'étude de paramètres opératoires clés, il est possible d'exploiter le maximum des capacités métaboliques de la souche.

Ces différents résultats très intéressants témoignent de la capacité de production d'acide succinique par *C. glutamicum* 2262, ainsi que l'influence de l'aération et de l'oxygène dissous sur le procédé fermentaire. Par ailleurs, différentes études sur la réponse physiologique de la bactérie en milieux hétérogènes en oxygène dissous ont été effectuées. En bioréacteur industriel, ces différentes hétérogénéités sont souvent inévitables du fait de l'augmentation des temps de mélange. Dans le cas du procédé de production de succinate avec une phase de transition en micro-aérobiose, l'amplitude des zones non aérées par rapport à celles aérées peut avoir un effet drastique sur la maîtrise de cette transition et donc modifier les performances de production d'acide succinique. A ce propos, une étude en scale-down utilisant des bioréacteurs parfaitement agités et interconnectés a été mise en œuvre. Les expériences en scale-down ont montré que, lorsque les bactéries oscillent fréquemment entre des zones parfaitement aérées et des zones non aérées, la croissance bactérienne est apparemment fortement impactée si les ratios volumiques de la zone aérée par rapport au volume total des deux zones sont inférieurs à 0,67. Si les ratios sont compris entre 0,67 et 1 les résultats montrent seulement une diminution de moins de 14 % de la biomasse maximale tandis que pour des ratios inférieurs à 0,33 une perte en biomasse de plus de 50 % est observée. De plus, pour des ratios inférieurs à 0,33 la consommation spécifique en substrat chute de 1,3 g.(g poids sec)⁻¹.h⁻¹ à 0,2 g.(g poids sec)⁻¹.h⁻¹. En établissant un modèle lagrangien couplant distribution des temps de séjour et modèle simple de croissance, nous avons montré que *C. glutamicum* 2262 Δ *ldhA* était capable d'activer très rapidement son métabolisme oxydatif, avec une efficacité maximale, même lorsque les

fractions temporelles d'aération n'étaient au global que de 20 %. Néanmoins, et en accord avec les résultats déjà établis dans nos études, la production d'acides organiques est restée marginale du fait des transitions rapides entre les deux environnements aérés, non-aérés.

En définitive, de la construction du mutant à l'élaboration d'un procédé de production, en passant par l'étude des influences du transfert d'oxygène en fiole, les performances de production de *C. glutamicum* 2262, ont été considérablement augmentées comme l'atteste l'évolution de la production au cours de la thèse (voir Figure C).

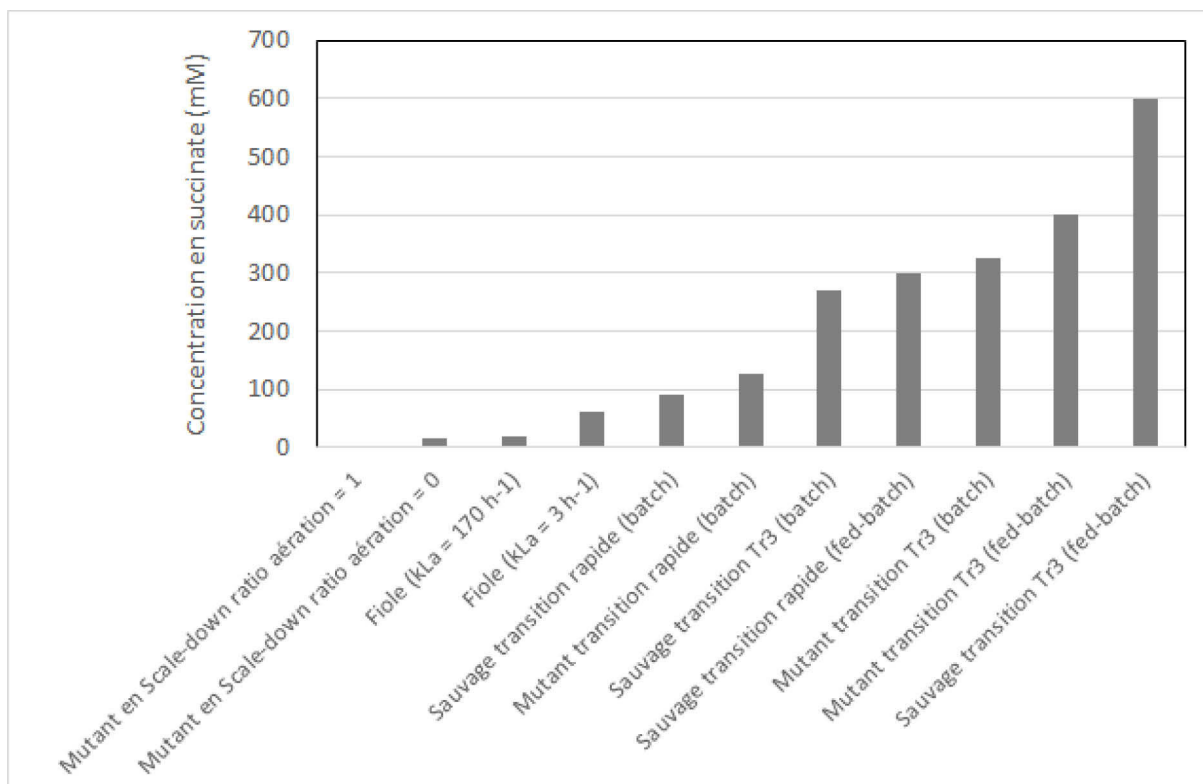


Figure C. Evolution de la production de succinate au cours de la thèse.

VIII-2. Perspectives

Au vu des résultats obtenus lors de ces travaux de doctorat, nous sommes en mesure de proposer des perspectives intéressantes pour améliorer la compréhension de la réponse métabolique de *C. glutamicum* face à la dynamique des microenvironnements biochimiques et *in fine*, optimiser la production de succinate.

Les résultats du chapitre IV ont clairement démontré que pour la gamme de valeurs de k_{La} allant de 0 à 170 h⁻¹, les cultures de *C. glutamicum* 2262 ΔdhA pouvaient présenter simultanément

un métabolisme respiratoire et fermentaire. Pour approfondir ces observations de métabolisme mixte ou de double population, il serait intéressant d'envisager un suivi par cytométrie de flux combinée avec des protéines fluorescentes afin de mieux caractériser l'hétérogénéité de la population bactérienne (Delvigne *et al.*, 2011)². Par ailleurs, pour compléter l'étude, des expériences à des k_{La} plus élevés que 170 h^{-1} pourraient être effectuées afin de déterminer les conditions favorisant uniquement la respiration, sans accumulation de produits de fin de fermentation. Ainsi, avec les résultats déjà obtenus, la production de succinate en fioles pourrait être améliorée et davantage caractérisée. En effet, on pourrait envisager des cultures en fioles avec des conditions initiales de k_{La} favorisant la respiration pour atteindre un certain seuil de biomasse suivie d'une deuxième partie de culture où les conditions de k_{La} seraient favorables à la production de succinate.

Les différents travaux sur l'élaboration du procédé de production en bioréacteur ont démontré que la transition jouait indubitablement un rôle primordial dans le procédé de production du succinate. Même si des études similaires sur *E. coli* ont montré qu'une phase de micro-aérobiose avant la phase anaérobie destinée à la production de succinate (Vemuri *et al.*, 2002b; Martínez *et al.*, 2010; Zhu *et al.*, 2011) améliorent les rendements et les concentrations de production, les connaissances sur les relations entre inductions, transcriptions et traductions des principales enzymes impliquées restent à explorer afin de mieux comprendre l'adaptation des bactéries. Aussi, des analyses enzymatiques et génomiques sont indispensables pour apporter des éclaircissements aux observations macroscopiques faites lors des cultures. Nous proposons donc de réaliser lors des procédés de production :

- *Au niveau enzymatique.* Par des prélèvements d'échantillons, pendant la phase de croissance, pendant la transition et pendant la phase anaérobie permettant des extractions et des mesures *ex-vivo* de l'activité enzymatique des enzymes anaplérotiques (PYC, PEP), qui catalysent les réactions alimentant la voie de production du succinate, de la MDH, de la FUM et de la SDH. Ces mesures donneront une idée de l'évolution de ces différentes activités enzymatiques selon les conditions opératoires.

² Delvigne, F., Brognaux, A., Francis, F., Twizere, J. C., Gorret, N., Sorensen, S. J., & Thonart, P. (2011). Green fluorescent protein (GFP) leakage from microbial biosensors provides useful information for the evaluation of the scale-down effect. *Biotechnology journal*, 6(8), 968-978

- *Au niveau génomique.* Comme les activités enzymatiques correspondent à une mesure nette des gènes traduits, il est indispensable de cibler ces gènes au niveau transcriptionnel. Pour ce faire, il serait utile de procéder à des mesures de l'expression relative des gènes correspondant aux enzymes citées ci-dessus par PCR quantitative. L'expression de ces gènes pourraient également être suivie lors des différentes phases du procédé afin de déterminer, avec plus de certitude, le moment/période d'induction des gènes et ceux qui sont réellement induits.

Quant au procédé en lui-même, des améliorations sont encore possibles et souhaitables. En effet, sur l'ensemble des profils cinétiques de croissance, dès la mise en place des conditions anaérobies, on constate un arrêt total de la croissance mais surtout une mort cellulaire très importante qui jouent incontestablement sur la baisse de production et de productivité en succinate. Aussi, pour pallier ce problème, un procédé discontinu alimenté, non seulement en substrats, mais également en bactéries pré-conditionnées, pourrait être envisagé. Pour cela, parallèlement à la culture principale, des cultures secondaires pourraient être effectuées pour pouvoir, à la fin de la phase de transition, récolter les cellules produites et alimenter le bioréacteur principal de production. En outre, l'alimentation en glucose peut être surement optimisée, en reliant ses ajouts à l'état physiologique cellulaire. Par exemple, il est possible de trouver une relation entre le profil d'ajout de base pour réguler le pH dont la baisse est occasionnée par l'acidification du milieu et la consommation en glucose.

Enfin, les études sur le k_{LA} réalisées en fioles pourraient être extrapolées aux cultures en bioréacteur. En effet, il pourrait être intéressant d'appliquer différents k_{LA} aux cultures en bioréacteur afin de voir les niveaux de production en biomasse et en acides organiques (succinate, acétate). A l'instar du procédé tri-phasique élaboré pour la production de succinate et selon les résultats obtenus avec les études de k_{LA} en bioréacteurs, un procédé avec une combinaison de différentes conditions de k_{LA} peut être imaginé et comparé aux résultats avec les transitions basées sur la gestion de l'oxygène dissous et celles basées sur la gestion du débit d'aération.

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Résumé

L'acide succinique est un diacide linéaire qui possède de nombreuses applications notamment alimentaires, chimiques et pharmaceutiques. Dans ce travail de thèse, un procédé de production de succinate mettant en œuvre *Corynebacterium glutamicum* a été étudié avec les outils du génie métabolique et du génie des bioprocédés. Dans un *premier chapitre*, nous avons supprimé le gène *ldhA* de *C. glutamicum* en utilisant un vecteur plasmidique *pk19mobsacBΔldhA* afin de rediriger le flux de carbone de la production de lactate vers la production de succinate. Le mutant *ΔldhA* et la souche parentale cultivés en bioréacteurs de 2 L ont montré des cinétiques de croissance similaires avec un taux de croissance maximal d'environ $0,7 \text{ h}^{-1}$ et une vitesse spécifique maximale de consommation de glucose de $7,5 \text{ mmol} \cdot (\text{g poids sec})^{-1} \cdot \text{h}^{-1}$. De plus, tandis que *C. glutamicum ΔldhA* ne produit pas de lactate, la souche sauvage a présenté des niveaux de production de succinate supérieurs. Par la modélisation de la désorption du CO_2 lors de cultures aérobies, nous avons également montré que la simple mesure du CO_2 dissous par une sonde dédiée permettait une très bonne estimation du CO_2 produit lors de la culture. Dans un *second chapitre*, nous avons étudié les effets de l'oxygénation sur la réponse physiologique de *C. glutamicum 2262 ΔldhA* à travers des expériences de cultures en fioles en verre lisses en imposant différentes conditions de k_{LA} déterminés expérimentalement grâce à un système PreSens. Les résultats ont montré que (i) des k_{LA} entre 10 et 19 h^{-1} sont optimaux pour la production d'acide succinique (63 mM) tandis que des k_{LA} entre (ii) 19 et 33 h^{-1} favorisaient des concentrations maximales d'acétate (135 mM) et que pour favoriser la production de biomasse, les k_{LA} doivent être au moins supérieurs à 164 h^{-1} (iii). Sur la base de ces observations, nous avons développé, lors d'un *troisième chapitre*, un procédé consistant en trois phases successives (aérobie, désoxygénation, anaérobie). *La phase de désoxygénation s'est révélée particulièrement critique vis-à-vis des quantités de succinate produite.* Ainsi, lors de cultures de *C. glutamicum 2262 ΔldhA* en mode discontinu pour les nutriments et en bioréacteur de 2 L, 327 mM de succinate ont été produits avec un rendement de 0,94 mole par mole de glucose tandis qu'en utilisant un procédé similaire sans une phase de transition, seulement 127 mM de succinate sont produits avec un rendement de 0,46 mole par mole de glucose. D'autres stratégies de transition, basées sur la régulation du débit d'aération ont également donné de meilleurs rendements par rapport aux procédés sans une phase de transition. Pour confirmer l'efficacité du procédé tri-phasique, celui-ci a été appliqué à *C. glutamicum 2262* sauvage (*chapitre 4*). Lors de cultures en mode discontinu alimenté par des ajouts supplémentaires des pulses de glucose et de NaHCO_3 , *C. glutamicum 2262* sauvage a produit jusqu'à 793 mM ($94 \text{ g} \cdot \text{L}^{-1}$) de succinate et 785 mM ($71 \text{ g} \cdot \text{L}^{-1}$) de lactate. Le rendement de la production du succinate est de 1,1 mole par mole de glucose et la production d'acétate n'était pas mesurable. *Ces productions sont sensiblement plus grandes que celles reportées dans la littérature pour une souche sauvage.* Enfin, la robustesse de *C. glutamicum 2262* face à une oscillation de la concentration en oxygène dissous a été évaluée par une étude scale-down en système bi-étagé (*Chapitre 5*). Sur la base des résultats expérimentaux et de ceux fournis par un modèle couplant DTS et cinétiques cellulaires, il a été montré que le métabolisme oxydatif de *C. glutamicum 2262 ΔldhA* était insensible aux fluctuations d'oxygène dissous, même pour des fractions moyennes d'aération très faibles, de l'ordre de 0,2. Par contre, la production d'acides organiques n'était alors pas observée du fait d'une reconsommation continue des produits et des séquences rapides de désoxygénation imposées.

Keywords: *Corynebacterium glutamicum*, acide succinique, génie génétique; modes d'alimentation, k_{LA} , aérobiose, anaérobiose, transition aérobiose-anaérobiose, génie des procédés, scale-down RPA-RPA, modélisation cinétique.