



Electrochemical control of a biological process : glycerol electro-fermentation

Roman Moscoviz

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THÈSE

Pour obtenir le grade de
Docteur

Délivré par MONTPELLIER SUPAGRO

Préparée au sein de l'école doctorale GAIA
Et de l'unité de recherche Laboratoire de
Biotechnologie de l'Environnement (LBE, INRA
UR050)

**Spécialité : Agroressources, Procédés, Aliments,
Bioproduits (APAB)**

Présentée par Roman MOSCOVIZ

**Contrôle d'un bioprocédé par voie
électrochimique : électro-fermentation du
glycérol**

Soutenue le 28 février 2017 devant le jury composé de

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List of abbreviations

AEF	Anodic electro-fermentation
ATP/ADP	Adenosine tri/diphosphate
BES	Bioelectrochemical system
CE-SSCP	Capillary Electrophoresis Single-Strand Conformation
CEF	Cathodic electro-fermentation
CV	Cyclic voltammetry
DIET	Direct interspecies electron transfer
EET	Extracellular electron transfer
EF	Electro-fermentation
EFS	Electro-fermentation system
Fd_{red/ox}	Ferredoxin reduced/oxidized
HRT	Hydraulic retention time
IET	Interspecies electron transfer
MDC	Microbial desalination cell
MEC	Microbial electrolysis cell
MES	Microbial electrosynthesis
MFC	Microbial fuel cell
NADH₂/NAD⁺	Nicotinamide adenine dinucleotide reduced/oxidized
Ng	Number of population generation
ORP	Oxidation-reduction potential
OTU	Operational taxonomic unit
PCA	Principal component analysis
PDO	1,3-propanediol
PTT	Polytrimethylene terephthalate
qPCR	Quantitative real-time polymerase chain reaction
RMSE	Root mean squared error
RMSE_{cv}	Root mean squared error of cross validation
SCE	Saturated calomel electrode
SHE	Standard hydrogen electrode
TEE	Total electron equivalent
T_d	Doubling time
WE	Working electrode
Y_{NADH}	NADH production yield

Résumé

Introduction

Lors des deux dernières décennies, de nombreux pays de l'OCDE ont adopté des politiques en faveur des énergies renouvelables, ce qui a notamment conduit à l'augmentation de la production mondiale de biodiesel de 2,2 à 30,1 10^9 L en 2004 et 2015 respectivement. En conséquence le glycérol, coproduit à 10 %_{massique} du biodiesel de 1^{ère} génération, est devenu une molécule en surproduction dont l'utilisation et la valorisation est devenue nécessaire afin de rendre l'industrie du biodiesel durable et rentable. Le glycérol étant un précurseur polyvalent tant pour l'industrie chimique que biotechnologique, son utilisation en tant que substrat de fermentation constitue une opportunité pour la production de produits chimiques à valeur ajoutée tels que le 1,3-propanediol (PDO). A l'échelle industrielle, le PDO est actuellement produit à partir du glucose à l'aide de souches recombinantes. Celles-ci convertissent le glucose en glycérol avant de produire du PDO, ce qui ne permet pas d'atteindre de hauts rendements de production (0,30 %_{carbone}). Les résultats disponibles dans la littérature indiquent clairement qu'une production de PDO directement à partir de glycérol par fermentation serait à la fois pratiquement et économiquement faisable et pourrait concurrencer le procédé à base de glucose.

Les paramètres clés pour optimiser la production de PDO à partir de glycérol en fermentation peuvent se définir selon trois catégories : (1) La première concerne le génie des procédés appliqué à la fermentation du glycérol. En ce qui concerne la production de PDO, la fermentation « fed-batch » est préférable à la fermentation « batch » et continue. Une première raison réside dans le fait que le glycérol peut exercer une inhibition à des concentrations élevées (typiquement entre 60 et 110 g.L⁻¹), limitant ainsi l'efficacité des procédés « batch ». Une deuxième raison est qu'il est moins important d'optimiser les productivités que d'atteindre des titres en PDO élevés, ceci afin de réduire les coûts d'extraction et de purification. Cependant, à des fins de recherche, la fermentation « batch » peut être un moyen simple et peu coûteux de réaliser la fermentation du glycérol lorsque la concentration du substrat est maintenue basse. (2) Le deuxième paramètre clé est le choix d'un inoculum adapté. Les espèces bactériennes capables de fermenter le glycérol et produire du

Résumé

PDO sont majoritairement identifiées comme appartenant aux familles *Clostridiaceae*, *Enterobacteriaceae* et au genre *Lactobacillus*. Les meilleures souches sauvages utilisées dans la littérature sont *Clostridium butyricum* et *Klebsiella pneumoniae*. A ce jour, les meilleures performances de production de PDO à partir de glycérol ont été obtenues par des espèces recombinantes qui nécessitent des moyens de fermentation coûteux, des conditions stériles et des installations compatibles avec l'utilisation d'OGM. Une autre alternative consiste à utiliser des consortia bactériens pouvant fermenter le glycérol dans des conditions non stériles et sans ajout de vitamines coûteuses, tout en offrant des performances de production de PDO inférieures mais significatives. L'optimisation des procédures de sélection de populations lors de la fermentation du glycérol en cultures mixtes pourrait aider à structurer des consortiums microbiens efficaces qui pourraient rivaliser en termes de coûts de production avec les fermentations utilisant des souches recombinantes. L'efficacité de ces procédures de sélection dépend largement du choix des paramètres environnementaux pouvant conduire à l'émergence de producteurs de PDO efficaces et de leurs partenaires bactériens dans la communauté microbienne. (3) Les paramètres environnementaux constituent le troisième paramètre clé pour la production de PDO, mais restent cependant limités en nombre. Pour la production de PDO par fermentation du glycérol, les deux paramètres identifiés dans la littérature comme étant les plus influents en culture pure sont le pH et le redox. À cet égard, l'électro-fermentation, un nouveau procédé bio-électrochimique, pourrait être une nouvelle façon d'agir sur les balances redox en fermentation et être utilisée pour améliorer les performances de production de PDO, en particulier dans les procédés en cultures mixtes.

Lors de cette thèse, les fermentations seront conduites avec des concentrations en glycérol faibles ($< 20 \text{ g.L}^{-1}$), permettant l'utilisation de procédés « batch ». Ces procédés seront conduits en utilisant des cultures mixtes, environnementale dans le un premier temps, puis artificielle (co-culture). L'objectif de cette thèse est d'évaluer l'effet de paramètres abiotique (ex. pH et redox) et biotique (ex. ajout d'une espèce électro-active) sur la fermentation du glycérol, dans le but d'améliorer les rendements de production de 1,3-propanediol.

Résumé

Fermentation du glycérol pour la production de 1,3-propanediol en culture mixte sur une large gamme de pH

Actuellement, peu d'articles de la littérature traitent de l'utilisation de cultures mixtes pour la conversion du glycérol en PDO. Néanmoins, la meilleure performance a été atteinte par Dietz *et al.* [1], en utilisant des boues de station d'épuration municipale comme inoculum. Ainsi, des rendements allant de 0,56 à 0,76 mol_{PDO}.mol⁻¹_{glycérol} ont pu être obtenus en utilisant un milieu minimal non stérile. Selembo *et al.* [2] et Liu *et al.* [3] ont aussi obtenu des rendements de production de PDO proches du maximum théorique (respectivement 0,69 et 0,65 mol_{PDO}.mol⁻¹_{glycérol}) lors de l'utilisation de cultures mixtes. L'obtention de tels résultats dépendent grandement des procédures d'enrichissement pour conduire une culture mixte naturelle vers un consortium bien défini qui ne réalise que les fonctions biologiques visées. Les principaux paramètres environnementaux utilisés pour sélectionner un consortium microbien plus spécifique sont le pH, la température, le taux de charge organique, la composition du milieu et le temps de rétention hydraulique pour les processus en continu. Les résultats disponibles dans la littérature sur la fermentation du glycérol en cultures mixtes ont été obtenus dans différentes conditions expérimentales et, en particulier, avec des valeurs de pH allant de 5,5 à 8 mais avec différentes sources de glycérol [1–4], rendant difficiles à départager les effets des impureté du glycérol et les effets du pH. En culture pure, le pH a un effet significatif sur la production de PDO et pourrait être à ce titre un paramètre potentiellement important pour la fermentation en culture mixte. Ainsi, lors de ce travail de thèse, l'effet du pH initial sur la fermentation du glycérol par une culture mixte a été étudié pour une gamme allant de 4 à 10, en utilisant des réacteurs « batch » en triplicata, un milieu minimum contenant du glycérol raffiné (1,7 g.L⁻¹) et un inoculum mixte. Ainsi, le seul paramètre pouvant influencer les métabolites produits était le pH, rendant plus simple l'analyse des résultats.

Après 3 jours de fermentation, le glycérol a été consommé dans la plupart des réacteurs (voir Figure 1), sauf ceux fonctionnant à pH extrême 4, 5 et 10 avec une part de glycérol restante de 95,4%, 8,1% et 93,0% par rapport à l'initial, respectivement. Pour toutes les autres valeurs de pH, le principal métabolite produit était le PDO (60-74% de la DCO totale) avec de l'acétate comme sous-produit principal (11-17% de DCO totale). Les rendements de production de PDO ont varié de $0,52 \pm 0,01$ à $0,64 \pm 0,00$ mol_{PDO}.mol⁻¹_{glycérol}.

Résumé

Les meilleures valeurs ont été obtenues à pH 7 et 8 et correspondent à 90% du rendement théorique maximal de $0,72 \text{ mol}_{\text{PDO}} \cdot \text{mol}^{-1}_{\text{glycérol}}$ [1]. Ainsi, les rendements de PDO étaient élevés et comparables aux performances obtenues en procédé utilisant des cultures pures, et ce pour tout la gamme de pH considérée.

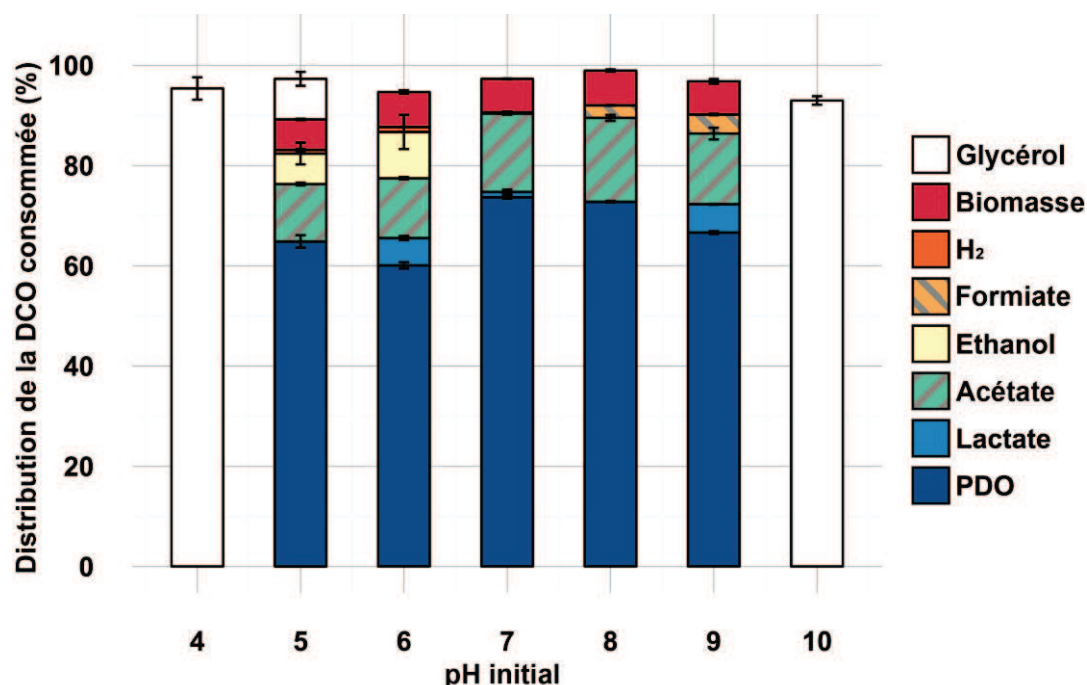


Figure 1. DCO calculés à partir des métabolites mesurés après 3 jours de fermentation en triple essai.

Les résultats sont normalisés sur la DCO initiale. La biomasse a été estimée à partir de la production d'ATP associée à la production de différents métabolites.

Concernant les structures de populations bactériennes, il est apparu dans cette étude que le pH a eu un impact significatif sur la croissance bactérienne et la composition de la communauté bactérienne finale. Les bactéries prédominantes présentes sur toute la gamme de pH, des familles *Clostridiaceae* et *Enterobacteriaceae*, n'ont pu expliquer à elles seuls les changements observés dans les profils métaboliques. Dans les espèces les moins dominantes, deux communautés différentes ont été observées, l'une à pH acide (*i.e.* < 7) et l'autre à valeurs de pH neutres à basiques (*i.e.* ≥ 7). Une étude des structures de corrélation a montré que cette dernière était favorable à la production de PDO même si aucune corrélation significative entre une famille bactérienne spécifique de cette communauté et un bon rendement en PDO n'a été trouvée. Ces résultats ont montré qu'il était probable qu'il y ait une forte redondance fonctionnelle au sein de la communauté microbienne finale. A partir de l'analyse théorique

Résumé

des voies métaboliques de la fermentation du glycérol et des corrélations avec les métabolites produits, il a été clairement démontré que la production de PDO était favorisée lorsqu'elle était produite conjointement avec l'acétate, ce qui a été majoritairement le cas dans cette étude. Même si des changements importants sont survenus dans la structure de la communauté microbienne sur la plage de pH étudiée, les rendements de production de PDO ont été assez peu dépendants du pH et étaient comparables au meilleur rendement obtenu dans des conditions similaires (culture mixte, glycérol pure et aucun additif tel que l'extrait de levure) de $0,69 \text{ mol}_{\text{PDO}} \cdot \text{mol}^{-1}_{\text{glycérol}}$ [2]. Les meilleurs résultats ayant été obtenus à pH 7, cette valeur sera retenue par la suite pour les expériences suivantes, utilisant le même inoculum.

Electro-fermentation du glycérol en culture mixte : effet sur la sélection de population

L'électro-fermentation (EF) est une approche récemment développée qui combine les systèmes de fermentation et de bio-électrochimie (BES) pour ajouter un contrôle supplémentaire sur le processus de fermentation [5,6]. L'EF consiste à conduire une fermentation spontanée en présence d'électrodes polarisées placées à l'intérieur du fermenteur. Cette source ou puit d'électrons supplémentaire offre de nombreux avantages aux micro-organismes, comme la possibilité d'effectuer des fermentations ayant un bilan électronique non équilibré (dissipation ou gain d'électrons à l'électrode) et peut aussi affecter directement les régulations métaboliques au travers de l'équilibre des transporteurs redox intracellulaires [5]. Contrairement à la plupart des autres BES, l'EF ne requiert pas nécessairement un apport énergétique élevé, et à l'inverse, même de faibles densités de courant peuvent avoir un effet important sur les performances globales de la fermentation [7]. Dans la présente étude, le potentiel de l'EF comme un outil pour rediriger les voies métaboliques et la sélection de population lors de la fermentation du glycérol par des cultures mixtes a été étudié.

Pour cela, deux conditions ont été considérées : un témoin fermentaire sans potentiel appliqué, et un réacteur contenant des électrodes, avec un potentiel de travail réducteur de -650 mV vs SHE. Ces deux conditions ont été conduites en utilisant des réacteurs « batch », un milieu minimum, du glycérol raffiné ($17 \text{ g} \cdot \text{L}^{-1}$) avec un pH contrôlé à 7. L'inoculum qui a été utilisé est identique à l'étude précédente. Dans une deuxième étape, des électrodes de travail

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pré-colonisées avec *Geobacter sulfurreducens* ont été utilisées afin d'évaluer le potentiel d'addition d'une bactérie électro-active comme biocatalyseur entre l'électrode de travail et les bactéries fermentatives lors d'une EF.

Les résultats obtenus ont montré que l'effet de la cathode sur les motifs de fermentation (*i.e.* sans *G. sulfurreducens*) était faible pour le potentiel de travail utilisé (-0,650 V *vs* SHE), mais était capable de modifier la production de métabolites secondaires. Lorsque *G. sulfurreducens* a été pré-cultivé sur l'électrode de travail, un enrichissement spécifique d'une espèce de la famille *Enterococcus* s'est produit (voir Figure 2). Le rendement de production de PDO, déjà élevé lors des fermentations témoins ($0,48 \pm 0,01 \text{ mol}_{\text{PDO}}.\text{mol}^{-1}_{\text{glycérol}}$), a alors été augmenté d'environ 10% pour atteindre la valeur de $0,54 \pm 0,02 \text{ mol}_{\text{PDO}}.\text{mol}^{-1}_{\text{glycérol}}$. Un modèle linéaire a montré que cette augmentation de performance était vraisemblablement due à un réarrangement de la communauté microbienne, suggérant que la présence de *G. sulfurreducens* sur la cathode a eu pour effet principal une sélection de la population. Cependant, les mécanismes sous-jacents à cette sélection de population et la nature des interactions spécifiques qui pourraient exister entre *G. sulfurreducens* et les espèces sélectionnées restent encore inconnus et nécessiteraient des études plus approfondies. Ainsi, une population microbienne simplifiée constituée uniquement de *G. sulfurreducens* et *C. pasteurianum*, une bactérie modèle pouvant fermenter le glycérol, sera ensuite étudiée afin d'améliorer la compréhension de l'effet de *G. sulfurreducens* sur les bactéries fermentaires.

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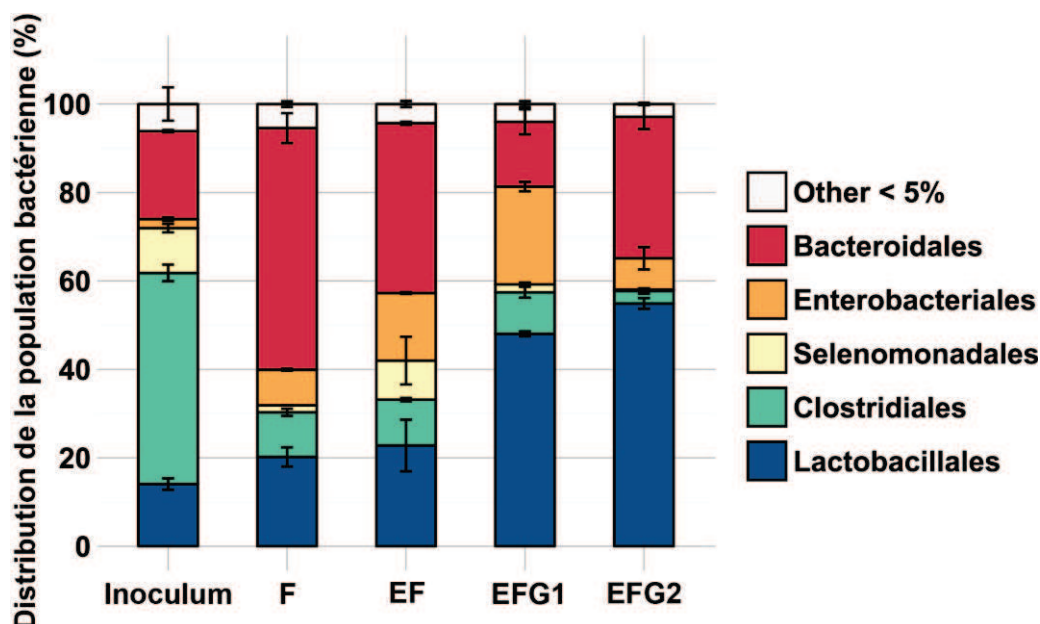


Figure 2. Distribution en classe taxonomique des bactéries issues du surnageant des fermenteurs

*Les barres d'erreur correspondent à l'écart-type des deux répétitions expérimentales pour chaque condition. F: fermentation classique. EF: Electro-fermentation. EFG1-2: Electro-fermentation en avec cathode pré-colonisée par *G. sulfurreducens* (batch successifs).*

Couplage électronique entre bactéries fermentaires et électro-actives: coculture de *C. pasteurianum* et *G. sulfurreducens*

Pour soutenir leur croissance et leur maintenance, les micro-organismes effectuent des réactions d'oxydoréduction à l'intérieur de leurs cellules. Ces réactions redox peuvent se résumer à des flux d'électrons provenant d'un donneur d'électrons qui sont transférés étapes par étapes à un accepteur final d'électrons (par exemple l' O_2 lors de la respiration aérobie) avec une libération globale d'énergie libre. Cependant, les micro-organismes individuels ne sont pas toujours capables d'effectuer seul toute la cascade de réactions. Dans ce cas, ils peuvent coupler leurs flux d'électrons avec d'autres espèces à travers des mécanismes de transfert d'électrons interspécifiques (IET) pour effectuer des réactions qui seraient éventuellement thermodynamiquement défavorables [8,9]. Le but de cette étude est de fournir une preuve de concept montrant que le couplage d'électrons entre bactéries fermentaires (accepteur d'électrons) et exo-électrogènes (donneur d'électrons) est possible. Ainsi, cette

Résumé

expérience a été conduite pour la fermentation du glycérol en utilisant une coculture de *G. sulfurreducens* et *C. pasteurianum* comme partenaires modèles.

Afin d'étudier les interactions possibles entre *G. sulfurreducens* et *C. pasteurianum*, les deux souches ont été inoculées dans un milieu contenant du glycérol (substrat de fermentation pour *C. pasteurianum*) et de l'acétate (donneur d'électrons pour *G. sulfurreducens*). Ces expériences ont été menées en réacteur « batch » sans système bio-électrochimique avec quatre répétitions expérimentales pour chaque condition.

La croissance de *G. sulfurreducens* et de *C. pasteurianum* a montré que *G. sulfurreducens* n'a été capable de croître qu'en présence de *C. pasteurianum* (voir Figure 3). Lorsqu'une croissance de *G. sulfurreducens* était détectée, les produits de fermentation de *C. pasteurianum* différaient du témoin de fermentation en culture pure. La production de PDO, butyrate et acétate était augmentée, tandis que la production de butanol et d'éthanol ainsi que la croissance bactérienne (pour *C. pasteurianum*) étaient toutes diminuées. Enfin, il a été montré que la croissance de *G. sulfurreducens* était dépendante de la présence de substrat de fermentation pour *C. pasteurianum*, soutenant l'hypothèse selon laquelle *G. sulfurreducens* dissipait l'excès d'électrons liés à sa propre croissance en utilisant *C. pasteurianum* comme accepteur d'électron, qui dissipait alors ces électrons en réduisant du glycérol.

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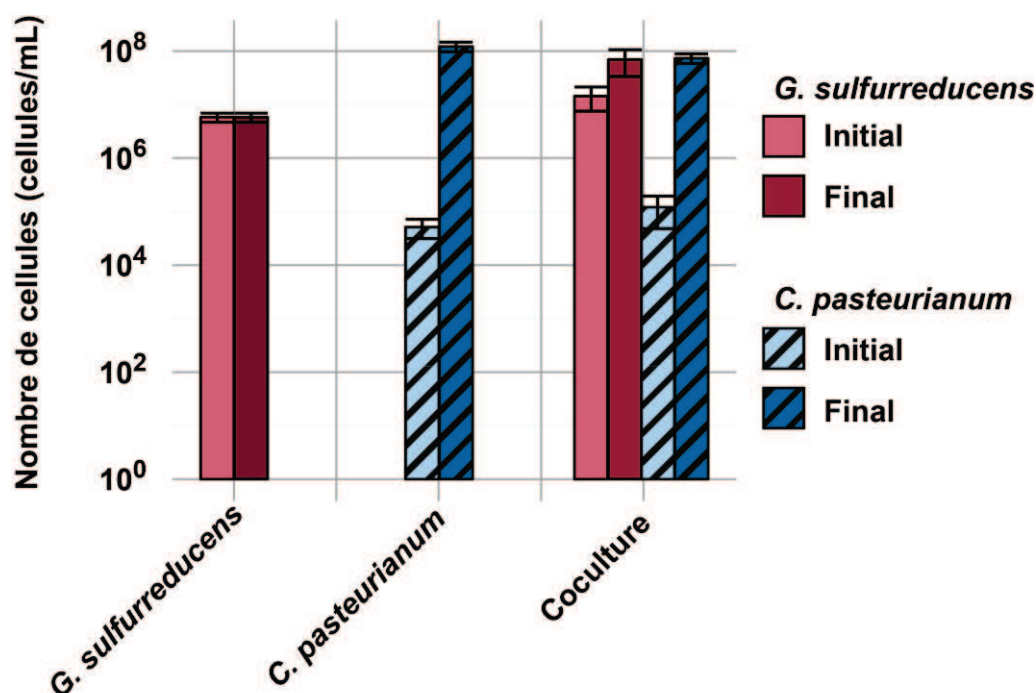


Figure 3. Suivi de la croissance de *G. sulfurreducens* et *C. pasteurianum*.

Les comptages de cellules sont basés sur les résultats de qPCR et corrigés par le nombre respectif de copies d'ARNr 16S pour chaque souche. Les barres d'erreur représentent l'écart-type des répétitions.

Ces résultats étendent le concept d'électro-fermentation au-delà de l'utilisation de systèmes BES: le rôle de *G. sulfurreducens* dans cette étude a été en effet très similaire à une cathode dans une électro-fermentation cathodique. Sous l'hypothèse que les interactions entre *G. sulfurreducens* et *C. pasteurianum* sont non spécifiques, des fermentations en réacteur continu en coculture avec des bactéries électro-actives et fermentaires apporteraient de nombreux avantages tels que: (i) recycler des électrons à partir de produits de fermentation indésirables pour promouvoir la production de métabolites plus réduits; (ii) éviter l'accumulation d'inhibiteurs tel que l'acide acétique; (iii) purifier le milieu de fermentation en éliminant les métabolites indésirables. Néanmoins, même si la présente étude est une preuve de concept très prometteuse, d'énormes efforts sur l'ingénierie des procédés et l'élucidation des principes fondamentaux d'interaction entre bactéries électro-actives / fermentaires restent nécessaires pour en tirer pleinement avantage.

Conclusions et perspectives

Les résultats rapportés dans cette thèse ont montré que la présence d'une électrode polarisée et / ou d'une espèce électro-active était capable de déclencher un changement de population dans une communauté bactérienne lors d'une fermentation, induisant en conséquence un changement de profil métabolique vers une molécule d'intérêt, ici le PDO. Néanmoins, les mécanismes sous-jacents à ce changement de population restent largement inconnus. La compréhension de la manière dont les consortiums microbiens sont affectés par l'électro-fermentation est une tâche particulièrement ambitieuse parce que (i) la connaissance liée à la réponse des souches pures à des conditions redox réductrices est encore très limitée; (ii) il en va de même pour les connaissances relatives à la consommation d'électrons à partir d'une électrode [10]; (iii) et les interactions biotiques entre les membres d'une communauté bactérienne sont par nature complexes et difficiles à identifier et quantifier. Une première étape vers une meilleure compréhension du couplage électronique entre microbes / microbes et microbes / électrodes a été initiée en étudiant une culture mixte simplifiée composée de seulement deux bactéries différentes. De manière similaire, les espèces d'intérêt identifiées dans des expériences de culture mixte peuvent être utilisées pour une série d'expériences de coculture en présence ou en absence d'électrodes. En mesurant les métabolites produits, la croissance bactérienne et les niveaux de transcription des gènes d'intérêt (par exemple impliqués dans le métabolisme central ou dans les interactions inter-espèces), ce type d'expérience permettrait d'améliorer la compréhension des mécanismes d'électro-fermentation et des interactions biotiques dans les consortiums microbiens. En identifiant les interactions favorables à la production de PDO, un meilleur contrôle des procédés en cultures mixtes pourrait être envisagé à l'avenir par l'ajout d'électrodes polarisées, d'espèces électro-actives ou d'autres espèces ayant des fonctions de support intéressantes. Cela pourrait constituer un pas en avant dans la mise en œuvre à plus grande échelle de procédés de cultures mixtes pour la production de métabolites à valeur ajoutée.

Lors de l'étude de cocultures de bactéries fermentaires/électro-actives, il a été montré qu'au lieu de fournir des électrons à une anode, *G. sulfurreducens* a pu transférer des électrons libérés par l'oxydation des sous-produits de fermentation à une espèce fermentaire, ici *C. pasteurianum*. Ce recyclage des métabolites est particulièrement intéressant car il pourrait améliorer la production de métabolites réduits d'intérêt (ex. le PDO à partir du glycérol, le butanol à partir du glucose) même en absence d'un système bio-électrochimique. Comme lors d'une électro-fermentation, les rendements de ces composés réduits peuvent dépasser les

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rendements théoriques maximums calculés pour la fermentation classique: dans le cas de la fermentation du glycérol, le rendement maximal de PDO serait augmenté de 0,72 à 0,85 $\text{mol}_{\text{PDO}} \cdot \text{mol}^{-1}_{\text{glycérol}}$. De plus, si tous les sous-produits de fermentation étaient recyclés, ce rendement maximal pourrait alors être approché sans même dépendre des voies métaboliques empruntées, car tous les sous-produits de fermentation ne seraient que des composés intermédiaires pour la production de CO_2 . En conséquence, ce processus de recyclage des métabolites pourrait théoriquement être une solution à l'absence de spécificité d'une fermentation. Cependant, l'application réelle de cette stratégie nécessiterait des études supplémentaire pour évaluer si (i) la fixation de biomasse électro-active sur un matériau conducteur pourrait être une solution aux différentes cinétiques de croissance observées pour les espèces fermentaires et électro-actives; (ii) des espèces fermentaires autres que *C. pasteurianum* peuvent recevoir des électrons provenant de bactéries électro-actives. Si l'on pouvait trouver de telles espèces "électro-fermentaires", les méthodes de recyclage des métabolites seraient un moyen permettant au moins de rediriger le métabolisme microbien vers une meilleure production de composés réduits, et au mieux une nouvelle façon de dépasser les rendements maximaux actuels.

Short introduction to the manuscript

In a context of environmental biorefinery, mixed-culture fermentation appears to be an ideal solution for single-step conversion of complex and heterogeneous organic waste into valuable chemicals that can be further used in green chemistry. In particular, it could be economically interesting to convert crude glycerol issued from the biodiesel industry into 1,3-propanediol, a valuable block chemical with a day-to-day expanding market. However, orienting mixed-culture fermentation end-products using the few usual environmental parameters (pH, temperature, organic loading rate...) is a very challenging issue. Electro-fermentation is a new approach that consists in using bioelectrochemical systems (*i.e.* polarized electrodes) as a way to stabilize and redirect fermentation pathways. As such, electro-fermentation is an additional tool to make more specific the conversion of organic waste by bacterial mixed consortia. In that sense, the main objectives of this thesis were:

- To assess the feasibility of efficient conversion of glycerol into 1,3-propanediol by mixed-consortia in a minimal fermentation medium.
- To evaluate the potential of electro-fermentation process for the improvement of product specificity in mixed-culture fermentation of glycerol.
- To provide better insight on the interactions that exist between electrodes, electro-active and fermentative bacterial species.

The manuscript is divided in four main parts: a literature review (Chapter 1), Material and methods (Chapter 2), Results and discussion (Chapters 3-5) followed by Conclusions and perspectives (Chapter 6).

The first chapter of this thesis presents the economic and societal aspects of biodiesel, glycerol and 1,3-propanediol production. An extensive review of glycerol fermentation for 1,3-propanediol production is then provided, where several key aspects for glycerol fermentation are explored: how to choose an efficient inoculum, the benefits and limitations of the different fermentation operational strategies (*i.e.* batch, fed-batch, continuous) and the effects of the most influent environmental parameters (*i.e.* temperature, pH, redox potential). In the last part of this chapter, some basics and principles better defining the electro-fermentation process are proposed. Hypothetical mechanisms that could explain the first electro-fermentation results reported in the literature are also proposed.

Short introduction to the manuscript

The second chapter presents the materials and methods used throughout this work, including fermentation media, culture conditions, analytical and molecular biology techniques, details of mass balance calculations and statistical analysis tools.

Chapter 3 is dedicated to the study of the impact of initial pH (from 4 to 10) on both bacterial population structure and metabolite production in glycerol mixed-culture fermentation. Results reported in this chapter showed that the bacterial community was remarkably functionally redundant whatever the pH ranging from 5 to 9 and was able to consistently produce 1,3-propanediol as main product at high yields (from 0.52 to 0.64 mol.mol⁻¹).

In chapter 4, glycerol mixed-culture electro-fermentation was carried out using the optimal pH assessed in chapter 3. The study demonstrates that both microbial community structure and metabolic patterns were altered in electro-fermentation as compared with classic fermentation. The possibility of increasing interactions between electrodes and fermentative bacteria was also explored by pre-colonizing *Geobacter sulfurreducens*, an electro-active species, directly on the electrode. The presence of this species did not increase current consumption from the electrochemical system. However, it triggered an important bacterial population shift that favored 1,3-propanediol production.

Chapter 5 focuses on investigating the possible direct electron transfer that could exist between fermentative and electro-active species. The study deals with the metabolic behavior of a co-culture of *G. sulfurreducens* and *Clostridium pasteurianum* during glycerol fermentation. Despite the absence of conventional electron acceptor, *G. sulfurreducens* was still able to grow in presence of *C. pasteurianum*. As a consequence, metabolic patterns of *C. pasteurianum* shifted toward better electron dissipation through an increase in 1,3-propanediol production.

Finally, the conclusive section of this manuscript draws possible outlooks concerning the use of mixed-culture and electro-fermentation for the production of value-added chemical.

Short introduction to the manuscript

The results of this thesis were valorized in the following publications:

- **Moscoviz R**, Trably E, Bernet N **(2016)** Consistent 1,3-propanediol production from glycerol in mixed culture fermentation over a wide range of pH. **Published in** *Biotechnology for Biofuels*. 9: 32. (IF 2015: 6.444)
- **Moscoviz R**, Toledo-Alarcón J, Trably E, Bernet N **(2016)** Electro-Fermentation: How To Drive Fermentation Using Electrochemical Systems. **Published in** *Trends in Biotechnology*. 34: 856–65. (IF 2015: 12.065)
- **Moscoviz R**, Trably E, Bernet N **(2017)** Electro-fermentation triggering population selection in mixed culture glycerol fermentation. **Under revision.** *Microbial Biotechnology*. (IF 2015: 3.991)
- **Moscoviz R**, de Fouchécour F, Santa-Catalina G, Bernet N, Trably E **(2017)** Cooperative growth of *Geobacter sulfurreducens* and *Clostridium pasteurianum* with subsequent metabolic shift in glycerol fermentation. **Published in** *Scientific Reports*. 7: 44334 (IF 2015: 5.228)

The results obtained in this thesis have also been presented in international conferences as listed below:

- **Moscoviz R**, Trably E, Bernet N **(2016)** Basics and principles of electro-fermentation “EU-ISMET 2016”, 3rd *European Meeting of the International Society for Microbial Electrochemistry and Technology*, Rome **(oral presentation)**
- **Moscoviz R**, Toledo-Alarcón J, Trably E, Bernet N **(2015)** Driving the fermentation patterns by redox potential control using BES. 14th *World Congress on Anaerobic Digestion* (AD14), Viña del Mar, Chile **(poster)**
- **Moscoviz R**, Trably E, Bernet N **(2015)** Electro-fermentation: a bio-electrochemical way to control glycerol fermentation. 5th *International Microbial Fuel Cell Conference* (MFC5), Tempe, USA **(poster)**

Chapter 1. Literature review

The aim of this chapter is to present the general context of glycerol fermentation and 1,3-propanediol production. Glycerol metabolism under anaerobiosis is detailed. The most influent fermentation parameters and modes of operation for glycerol fermentation are reviewed. Finally, the concept of electro-fermentation is presented along with experimental evidences from the literature. The part of this chapter dealing with electro-fermentation is based on an article published in Trends in Biotechnology:

Moscoviz R, Toledo-Alarcón J, Trably E, Bernet N (2016) Electro-Fermentation: How To Drive Fermentation Using Electrochemical Systems. *Trends Biotechnol.* 34: 856–65.

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1.1 Glycerol: an over-produced building block

1.1.1 The fast-growing market of biodiesel

Over the last decade, many countries as European Union (EU) member states or the United States of America (USA) have adopted policies in favor of renewable energies. Due to the high petroleum prices and to limit their environmental footprint related to its use as fuel, these countries have favored the development of alternatives fuels for transportation. Since the Energy Policy Act of 1992, the USA have promoted the production of biofuels through tax incentives [11], which have in turn developed the market of corn-based biofuels (*e.g.* ethanol or biodiesel). In the Energy Independence and Security Act of 2007, the USA have confirmed their will to promote bio-based fuels by setting a target volume of $140 \cdot 10^9$ L/year by 2022, including $80 \cdot 10^9$ L/year of non-corn-based biofuels [12]. Similarly, the EU has supported the biofuel industry since 2001 [13] and has now fixed a target value of 10 % for biofuels in the total fuel mix in the Member States by 2020 [14], with a maximum of 7 % of first generation biofuels (*i.e.* based on starch, sugar, animal fats and vegetable oil) [15]. As a result, the global biofuel production has boomed since the 2000's with a substantial increase of biodiesel production from 2.2 to $30.1 \cdot 10^9$ L/year in 2004 and 2015 respectively (see Figure 1-1) [16,17].

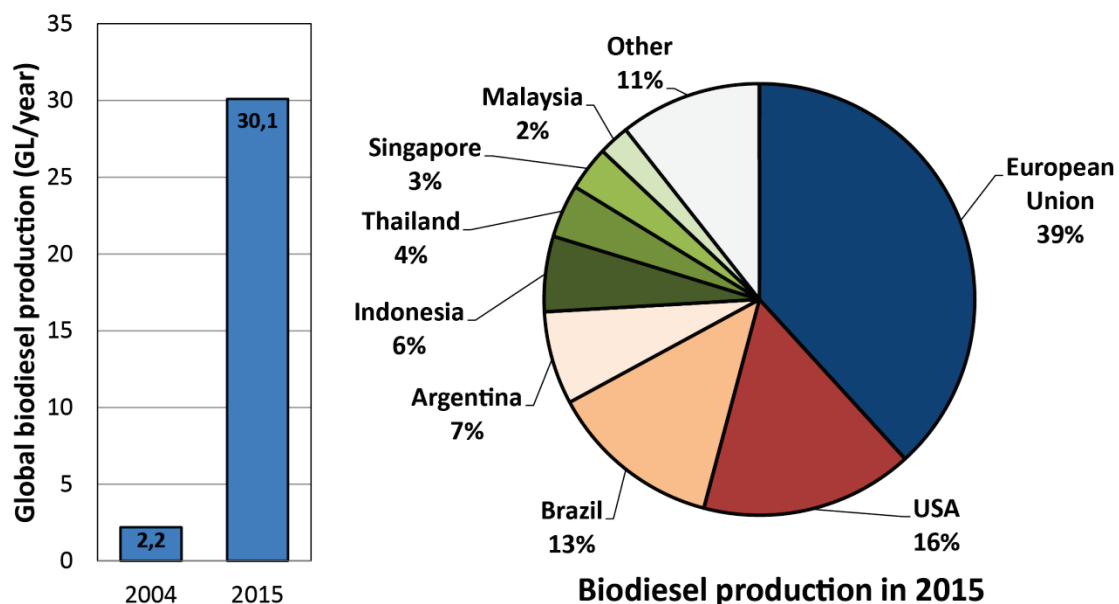


Figure 1-1. Global biodiesel production in 2004 and 2015 [16,17].

Chapter 1 - Literature review

Biodiesel is a first generation biofuel which is mainly produced from transesterification of triglycerides such as rapeseed, palm, soybean and sunflower oils [18]. This reaction consists in the esterification of triglycerides issued from vegetable oil or animal fats with an alcohol (*e.g.* methanol) in presence of alkali or acid catalysts [18–20]. When methanol is used, transesterification yields to methyl esters (*i.e.* biodiesel) and glycerol as major by-product (see Table 1-1 and Figure 1-2). In such reaction, about 100 kg of glycerol is produced per ton of biodiesel [18,21].

Table 1-1. Transesterification reactions in biodiesel production [18–20]

Triglycerides + CH ₃ OH	→ Diglycerides + R ₁ -COOCH ₃	Equation 1-1
Diglycerides + CH ₃ OH	→ Monoglyceride + R ₂ -COOCH ₃	Equation 1-2
Monoglyceride + CH ₃ OH	→ Glycerol + R ₃ -COOCH ₃	Equation 1-3
Triglycerides + 3 CH ₃ OH	→ Glycerol + Methyl esters (Biodiesel)	Equation 1-4

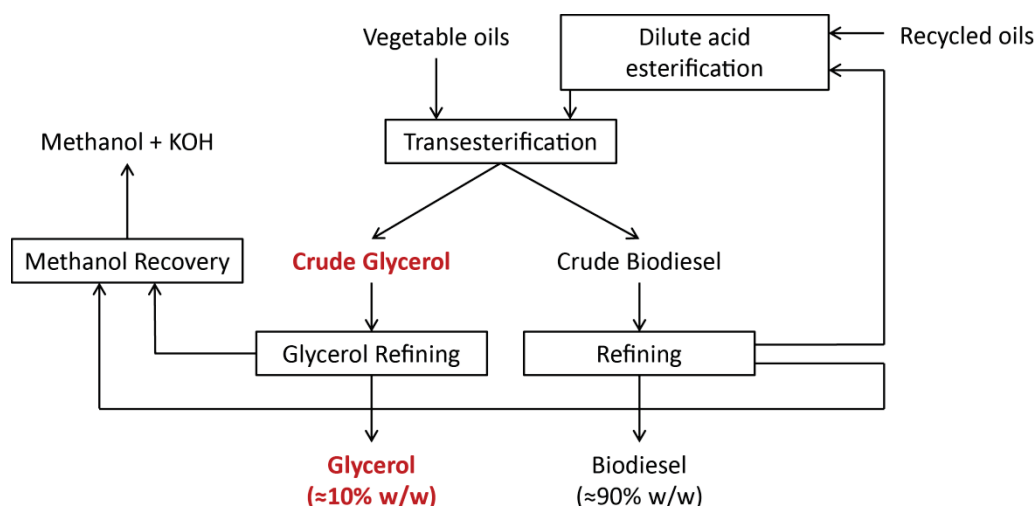


Figure 1-2. Transesterification of vegetable oils into biodiesel [21]

In addition, glycerol is obtained from four different processes: soap manufacture, fatty acid production, fatty ester production and microbial fermentation [18]. Production of glycerol from these four processes has been very stable since many decades and represents about

1.10⁶ t. Since the 2000's and the biodiesel production boom, crude glycerol production has exponentially increased (see Figure 1-3) and may reach up to 5.10⁶ t in 2020, with more than 70 % of its production originating from the biodiesel industry [22,23]. In response to this

Chapter 1 - Literature review

oversupply, crude glycerol price has dropped from about 0.45 to 0.10 US \$/kg in 2001 and 2011, respectively [22]. However, the glycerol market is in transition and the crude glycerol market price is subject to fluctuations [24]. The low crude glycerol prices open many opportunities for the use of this building block chemical for the production of value-added molecules. As the production costs are one of the main problems associated with biofuels production, finding new valorization way for crude glycerol is today required to make biodiesel production more sustainable [25].

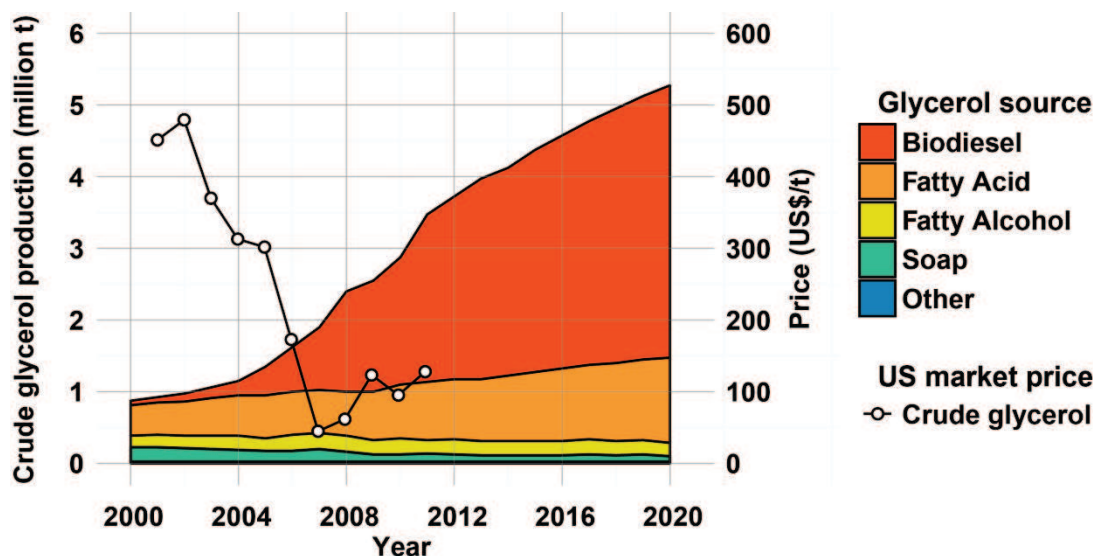


Figure 1-3. Evolution of raw glycerol production and price between 2000 and 2020 [22,23]

Values between 2012 and 2020 correspond to projections.

1.1.2 Existing strategies for glycerol utilization and conversion

1.1.2.1 Glycerol purification and utilization

A first way to valorize crude glycerol is through its purification to reach purities above 99 % for further utilization in the food, cosmetic or pharmaceutical industries [18]. The composition of the crude glycerol issued from biodiesel production can strongly vary depending on the biodiesel process. It typically contains 20-90 %w/w of glycerol and a large part of residual methanol that could represent up to 32 %w/w [26]. The transesterification catalysts (e.g. NaOH, KOH) can also be found in significant amounts (up to 5 %w/w). The other constituents of crude glycerol are mainly water, unreacted mono/di/triglycerides and methyl esters [22].

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Traditional purification of crude glycerol is composed of three steps (see Figure 1-4) [26]. In the first stage (neutralization), a strong acid is added to convert soaps into free fatty acids and neutralize the residual base catalyst. After this operation, the crude glycerol solution is constituted of three layers: (i) a top phase rich in fatty acids and methyl esters, which can be easily removed, (ii) a middle phase containing glycerol, water and methanol, and (iii) a bottom phase constituted of insoluble salts. In a second step, the remaining methanol is removed from the middle layer obtained in the first step using vacuum evaporation. Water is also evaporated during this step. After the second step, the glycerol solution typically contains about 85 % w/w of glycerol [27]. Different technologies have been developed to reach a glycerol purity higher than 95 % w/w: vacuum distillation, membrane separation and activated carbon adsorption [26]. Vacuum distillation is the most used method and can yield glycerol of high purity (about 95.5 % w/w [27]) but requires intensive energy supply as glycerol boiling point at atmospheric pressure is 290 °C [22]. Membrane separation techniques require much lower energy input and can be used to obtain also very high glycerol purity (up to 99 % w/w [26]). However, these techniques face severe limitations related to the fouling and the low durability of the membranes [26]. The activated carbon method is mainly used as a finalization step to adsorb the remaining dissolved small molecules and reduce both color and odors from the final glycerol solution [26]. Overall, the crude glycerol purification costs depend on the initial composition of the by-product solution issued from the biodiesel stream. A study from 2011 estimated that the cost of a glycerol purification to a minimum of 98 % w/w was at least 0.15 US \$/kg [22]. In the same year, high purity glycerol (>99.5 %) was sold at less than 0.50 US \$/kg [22]. Thus the valorization of crude glycerol into high purity glycerol is of limited relevance and may be economically viable only for biodiesel streams containing high glycerol content (*e.g.* from sunflower oil [26]).

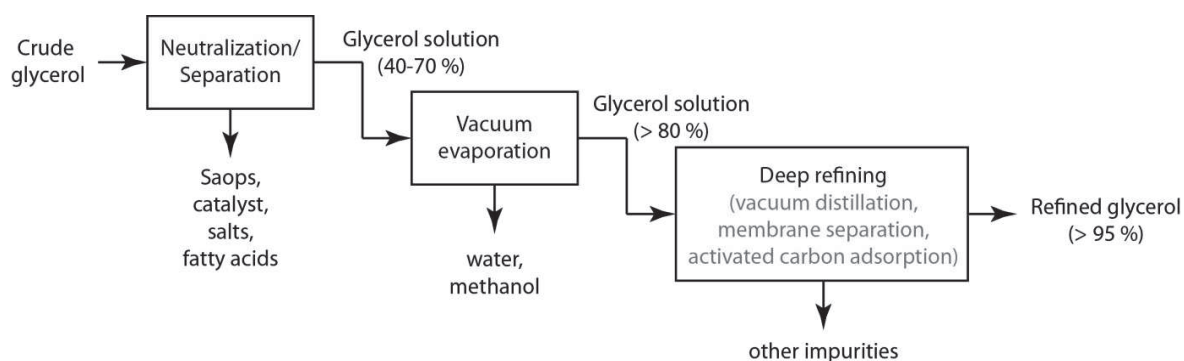


Figure 1-4. General refining process for crude glycerol [26]

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1.1.2.2 Glycerol conversion

Interestingly, glycerol represents also a feedstock for the generation of many chemicals with a wide range of applications [28]. Sofiprotéol, as a major actor of the biodiesel industry, provided in 2010 a list of glycerol derivatives having the highest economic interests (see Figure 1-5). Some of these value-added products are mainly obtained by chemical routes, such as glycerol carbonate (carboxylation) or acrylic acid (dehydration). Glycerol carbonate has wild applications such as the production of gas separation membrane or polymer synthesis [23]. Acrylic acid is also a building block that can be used in paints, coatings and plastics. The global demand for acrylic acid was about $1.1 \cdot 10^6$ t in 2010 with prices fluctuating between 1.1 and 2.5 US \$/kg [28].

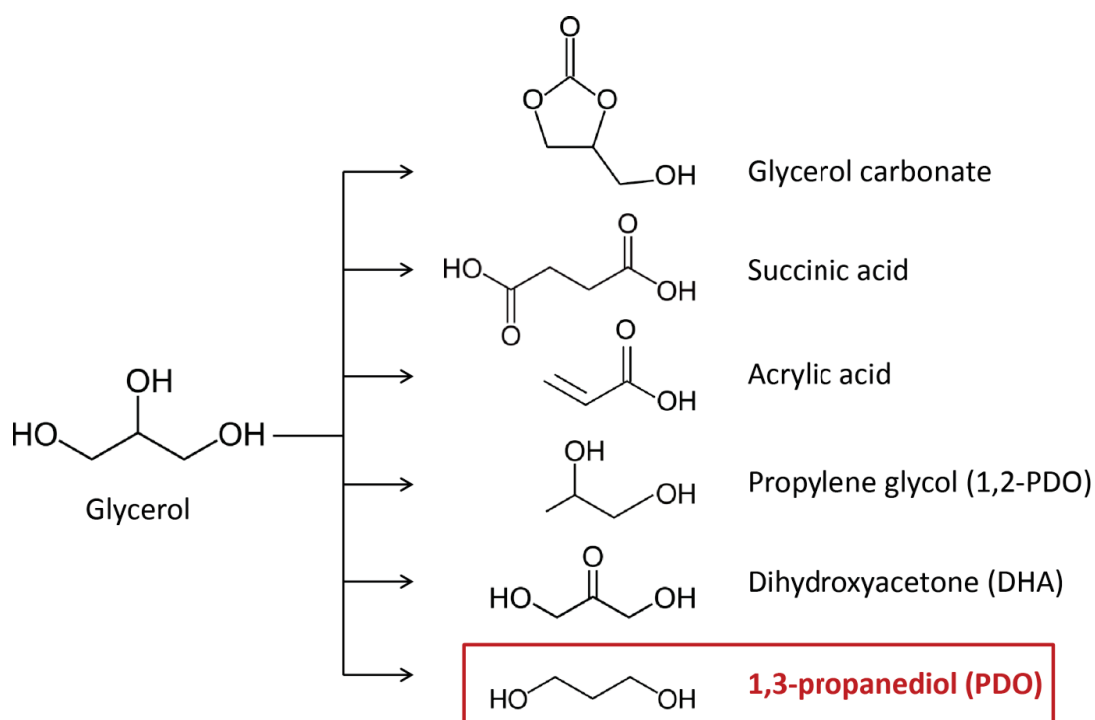


Figure 1-5. Selection of value-added chemicals possibly produced from glycerol
[19,23,25,28]

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Other compounds such as succinic acid, propylene glycol (1,2-propanediol), dihydroxyacetone (DHA) and 1,3-propanediol (PDO) can be obtained by glycerol fermentation and present major interest for their high value and wide application [28]. Within these compounds, PDO has gained a particular interest as it can be used for the production of polytrimethylene terephthalate (PTT) which is a polymer used in the textile industry [20]. PDO has also other utilities such as for the production of adhesives, solvents, and have recently been approved for food applications. In the following section, the PDO market will be presented along with the different existing methods used for its production.

1.1.3 1,3-propanediol market and means of production

1.1.3.1 PDO market

Historically, PDO production was fossil-based and its production requires costly processes. Before the 2000's, the price for PDO was about 30 US \$/kg [29]. In the early 2000's, Dupont Tate & Lyle Bio Products developed a glucose-based production of PDO by fermentation which reduced drastically the production costs. As a result, fermentation out-competed the petroleum-based processes and PDO price decreased to a value of 2.61 US \$/kg in 2012 (see Table 1-2) while being entirely bio-based [30,31]. The low cost of PDO have in turn favored the PTT production by DuPont (SORONA™) and Shell (CORTERRA™), used in the carpet industry, textile fibers, thermoplastics, films, automobiles, apparel and coatings [20,32]. Thus the demand for PDO had increased every year, from 60.2 to 128 kt/year in 2012 and 2015 respectively. A market study have predicted that the demand would reach up to 150 kt/year by 2019 with a PDO price slight increase to 3.73 US \$/kg [20].

Table 1-2. Price and production evolution of PDO

Year	Global demand (kt/year)	Price (US \$/kg)	Ref.
1993	N.A.	30	[29]
2012	60.2	2.61	[20]
2015	128	1.76	[30]
2019*	150	3.73	[20]

* Values for 2019 are projections made in a market study in 2012

1.1.3.2 Different processes for PDO production

Several PDO production processes already exist and are using not only glycerol as raw material but also glucose or petroleum derivatives (see Figure 1-6). Historically, PDO was synthesized from acrolein or ethylene oxide (petroleum derivatives). The ethylene oxide route was developed by Shell Chemical Company and consisted in a two-step reaction [20,33]. First, ethylene oxide reacts with carbon monoxide and a catalyst to produce a hydroxyl-aldehyde. The latter is then reduced to PDO by hydrogenation. Conversion yields of 0.90 mol/mol have been achieved via this hydroformylation route. However the final PDO solution contains many impurities (up to ten times the level of impurities than PDO derived from fermentation processes [20,33]), thus increasing the downstream process and overall production costs. PDO production from acrolein was proposed by Degussa and DuPont and consisted in the hydration of acrolein into 3-hydroxypropanal which is hydrogenolyzed in a second step to form PDO. Yields of such global reaction can reach up to 0.80 mol/mol [20,33]. Both of these routes have the disadvantage to consume petroleum-derived materials. Thus, other pathways using renewable materials as primary reactant have more recently been developed.

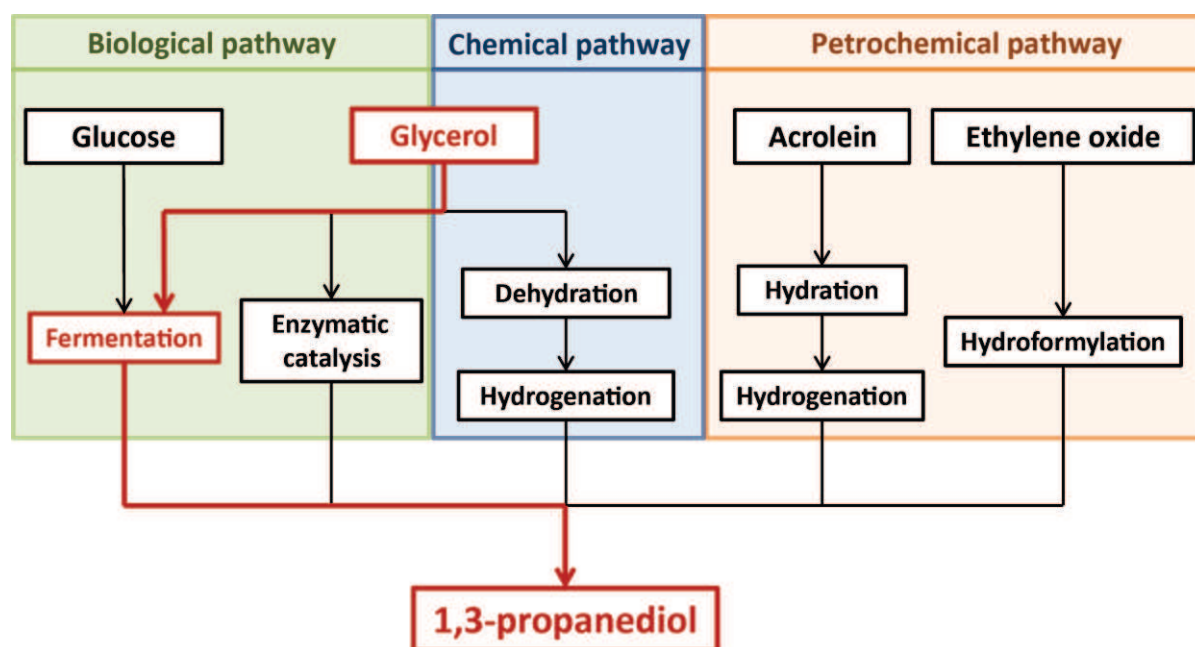


Figure 1-6. Different possible pathways for PDO production

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As reported in Section 1.1.1, glycerol has become an interesting low-cost and renewable building block that can be used for PDO synthesis. A chemical route consists in the hydrogenolysis of glycerol [20,34]. For that, glycerol is first dehydrated to form 3-hydroxypropanal which is subsequently hydrogenolyzed into PDO through the Degussa-Dupont route. The best yield obtained so far is 0.66 mol/mol with Pt-based catalyst [34]. In addition to the use of costly catalysts, this route requires high pressure (5.0–32.0MPa) and high temperature which limit the economic feasibility of the process [20]. Another catalytic pathway for the production of PDO from glycerol and H_2 has recently been proposed based on the use of enzymes [35]. The conversion was successfully implemented using two modules: a first module constituted of glycerol dehydratase and NADPH-dependent PDO dehydrogenase was able to convert glycerol into PDO by consuming one NADPH. In the second module, a hydrogenase regenerated the NADPH while consuming H_2 . Via this pathway, a very high 1,3-PDO yield of about 0.95 mol/mol was achieved [35]. However, severe limitations related to enzymes stability and production costs have to be addressed before any implementation at industrial scale. Although these two routes (*i.e.* hydrogenolysis and enzymatic conversion of glycerol) may become an economically viable alternative for PDO production in the future, fermentation is nowadays the cheapest way to produce PDO.

Fermentation presents several advantages, such as the possibility to be carried out at physiological temperature (37°C) and atmospheric pressure. When glycerol is used as substrate, yields between 0.50 and 0.72 mol/mol can be typically achieved. However, a large part of the actual PDO production is still based on glucose despite lower conversion yields (0.30 mol/mol) [31]. A life cycle analysis recently compared the glucose-based (corn) and fossil-based (ethylene oxide) production of PDO and concluded that the former consumes 38% less energy and emits 42% fewer greenhouse gases [36,37]. Switching from glucose to glycerol as fermentation substrate for PDO production could further decrease both production costs and environmental impacts and could help to make the biodiesel branch more sustainable. In the following sections, glycerol fermentation is presented in more details and placed in perspective with PDO production optimization.

1.2 Fermentative metabolism of glycerol

Glycerol fermentation pathways have been extensively studied in model microorganisms such as *Klebsiella pneumoniae* [38,39] or *Clostridium butyricum* [40]. For both microorganisms, similar pathways were found and can be summarized as in Figure 1-7 and Table 1-3. Glycerol can enter cells, either by diffusion or actively [41,42] to be used for both anabolism and catabolism. It is worth noticing that during glycerol fermentation, anabolism generates extra reducing equivalents as glycerol is more reduced than cell material, in average (see Equation 1-6). For this reason, all microorganisms fermenting glycerol possess at least one pathway for a net reducing equivalent dissipation: catabolism during glycerol fermentation consists in both a reductive (*i.e.* $Y_{\text{NADH}} < 0$) and an oxidative branch (*i.e.* $Y_{\text{NADH}} \geq 0$).

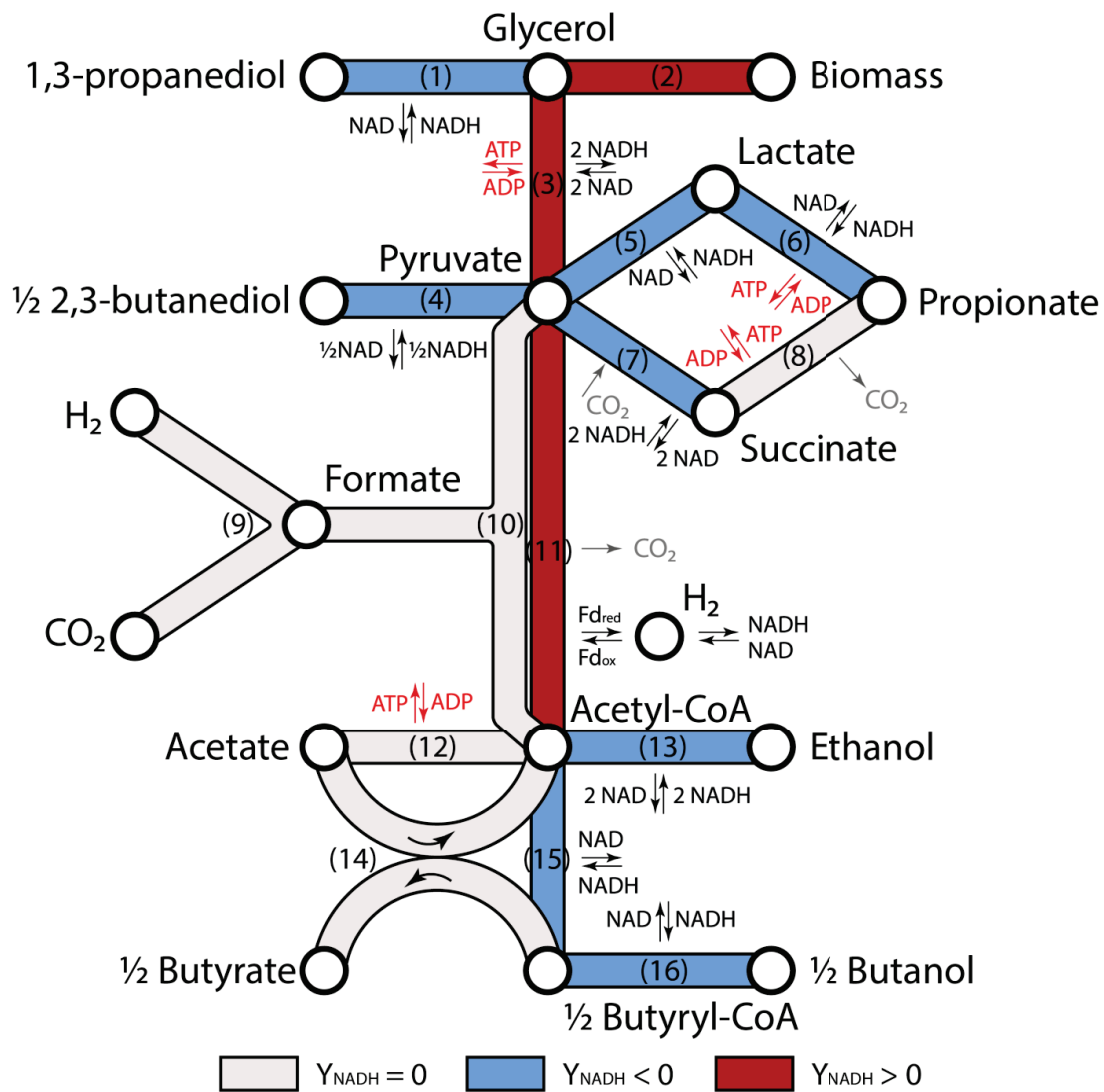


Figure 1-7. Simplified catabolic pathways of glycerol fermentation.

Fd_{ox} and Fd_{red} stand for the oxidized and reduced form of ferredoxin, respectively.

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In the oxidative branch, glycerol is oxidized into dihydroxyacetone and subsequently converted into pyruvate (glycolysis) while generating one mole of ATP and two moles of NADH per mole glycerol (see Equation 1-7). Then pyruvate reacts to produce various metabolites known from the fermentation of glucose. A first possibility is the lactic acid fermentation where pyruvate is converted to lactate by a lactate dehydrogenase while one NADH is consumed (Equation 1-9) [43]. One ATP can be generated by converting this lactate in propionate (acrylate pathway) with the consumption of one more NADH (Equation 1-10) [44]. Another way to produce propionate is through the succinic acid pathway in which pyruvate is converted to succinate with the consumption of one CO₂ and two NADH (Equation 1-11) [43]. This succinate can be then transformed in propionate with a release of one ATP and CO₂ (Equation 1-12) [45]. Some species of the *Klebsiella*, *Enterobacter* and *Bacillus* genera are also able to convert significant amount of pyruvate into 2,3-butanediol, a chemical of industrial interest (Equation 1-8) [46].

Table 1-3. Condensed metabolic pathways of glycerol fermentation

Reaction	Legend	Ref	
Glycerol + NADH → PDO + H ₂ O	(1)	[38,40]	Equation 1-5
Glycerol + ¾ NH ₃ + 7.5 ATP + 6 H ₂ O → ¾ C ₄ H ₇ O ₂ N + NADH	(2)	[38]	Equation 1-6
Glycerol → Pyruvate + ATP + H ₂ O + 2 NADH	(3)	[38,40]	Equation 1-7
Pyruvate + ½ NADH → ½ 2,3-Butanediol + CO ₂	(4)	[38,46]	Equation 1-8
Pyruvate + NADH → Lactate	(5)	[38,40,43]	Equation 1-9
Lactate + NADH → Propionate + ATP + 2 H ₂ O	(6)	[43,44,47]	Equation 1-10
Pyruvate + CO ₂ + 2 NADH → Succinate + 2 H ₂ O	(7)	[38,43]	Equation 1-11
Succinate → Propionate + ATP + CO ₂	(8)	[43,45]	Equation 1-12
Formate + H ₂ O → H ₂ + HCO ₃ ⁻	(9)	[38,43]	Equation 1-13
Pyruvate + CoA + H ₂ O → Acetyl-CoA + Formate	(10)	[38,43]	Equation 1-14
Pyruvate + CoA + H ₂ O → Acetyl-CoA + CO ₂ + Fd _{red}	(11)	[40,43]	Equation 1-15
Acetyl-CoA → Acetate + ATP + CoA + H ₂ O	(12)	[38,40,43]	Equation 1-16
Acetyl-CoA + 2 NADH → Ethanol + CoA	(13)	[38,40,43]	Equation 1-17
Butyryl-CoA + Acetate → Butyrate + Acetyl-CoA	(14)	[43,48,49]	Equation 1-18
2 Acetyl-CoA + 2 NADH → Butyryl-CoA + CoA + H ₂ O	(15)	[43,48,49]	Equation 1-19
Butyryl-CoA + 2 NADH → Butanol	(16)	[50,51]	Equation 1-20

For more readability, NAD⁺, H⁺ and ADP are omitted in the presented equations.

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Conversion of pyruvate into acetyl-coenzyme-A (acetyl-CoA) can be carried out through two pathways: the pyruvate formate lyase pathway [38,43] and the pyruvate dehydrogenase pathway [40,43]. The pyruvate formate lyase pathway (Equation 1-14) is a redox-neutral pathway that can occur without any involvement of reducing equivalents, where pyruvate and CoA are transformed in formate and acetyl-CoA. Formate can accumulate as fermentation product but can also be converted into carbonate and H_2 by a formate-hydrogen lyase complex (Equation 1-13) [52,53]. As the equilibrium ratio of the latter reaction mainly depends on the pH, conversion of formate into carbonate and H_2 is triggered by medium acidification and contributes to reduce acidity by removing formic acid [43,54]. The pyruvate dehydrogenase pathway consists in the conversion of pyruvate and CoA in acetyl-CoA, CO_2 , and reduced ferredoxin. This reduced ferredoxin can contribute to electron dissipation through the production of H_2 but can also be converted to NADH for further use of these electrons in other pathways (see Figure 1-8) [53].

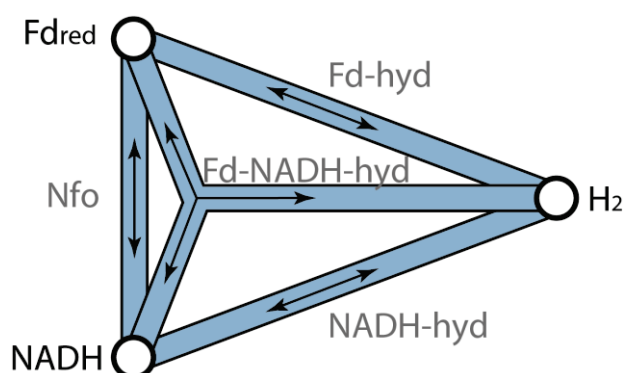


Figure 1-8. H_2 formation pathways from reducing equivalents.

Enzymes are displayed in grey. Fd_{red} : reduced ferredoxin; Nfo : NADH:ferredoxin oxidoreductase; $Fd-hyd$: Ferredoxin dependent hydrogenase; $Fd-NADH-hyd$: Ferredoxin and NADH dependent hydrogenase; $NADH-hyd$: NADH dependent hydrogenase.

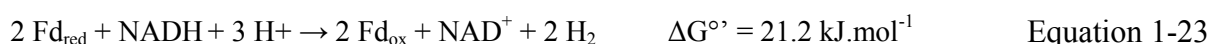
Acetyl-CoA is a precursor of many fermentation end products. It can be transformed in acetate while producing one ATP in a two-step reaction involving the phosphate acetyltransferase and the acetate kinase enzymes (Equation 1-16) [38,40,43]. Ethanol synthesis from acetyl-CoA also involves two enzymes, acetaldehyde dehydrogenase that converts acetyl-CoA in acetaldehyde, and ethanol dehydrogenase to transform the latter into

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ethanol [55]. The ethanol pathway dissipates two NADH but does not release any ATP (Equation 1-17).

Two acetyl-CoA can be condensed (2 NADH consumed) to form butyryl-CoA as precursor of several C4 metabolites (Equation 1-19) [43,48,49]. Butyrate synthesis from butyryl-CoA is a cycle which involves the consumption of a molecule of acetate along with butyryl-CoA and yields to one molecule of butyrate and acetyl-CoA (Equation 1-18). This acetyl-CoA can be then condensed with another acetyl-CoA to start a new cycle [43,48,49]. Butyryl-CoA can also be converted in butanol by some species such as *Clostridium pasteurianum* as a way to dissipate two more NADH (Equation 1-20) [50,51].

All these oxidative pathways are at best neutral in term of reducing equivalent production (e.g. NADH), but usually generates more NADH that must be dissipated through a reductive pathway. In addition, the NADH released from biomass synthesis has also to be dissipated to ensure the redox balance of the fermentation. For most microorganisms capable of glycerol fermentation, two pathways are possible to ensure NADH dissipation. The first one is the PDO pathway, in which glycerol is dehydrated to form 3-hydroxypropionaldehyde which is subsequently dehydrated to produce PDO (Equation 1-5) [38,40]. The second one is hydrogen formation (see Figure 1-8) from reducing equivalents, which depends on metabolic possibilities and is therefore strain-dependent. In particular, the following reactions are possible [43,56]:



These reductive pathways do not yield directly to any ATP production that could contribute to anabolism; they only consist in a net dissipation of reducing equivalents to ensure the redox balance of the fermentation. PDO pathway is the most common dissipation pathway used by glycerol fermenters [4,25,57]. As a consequence, PDO is found most of the time during glycerol fermentation, even though some specific species such as *Escherichia coli* were reported to ferment glycerol to form ethanol and H₂ only [58]. Thus, optimizing PDO production during glycerol fermentation is nearly equivalent to optimizing the production of oxidized metabolites such as acetate. In conclusion, the highest theoretical PDO yield is reached when acetate is the sole by-product, with a value of 0.72 mol_{PDO} mol⁻¹_{glycerol}.

1.3 Optimization of PDO production by glycerol fermentation

1.3.1 PDO-producing bacteria

1.3.1.1 *Clostridiaceae*

Many species from the *Clostridiaceae* family have been studied for their ability to convert glycerol into PDO [31]. Natural species that efficiently produce PDO are *Clostridium pasteurianum* [59,60], *Clostridium diolis* [61], *Clostridium bifermentans* [61] and *C. butyricum* [62], the latter being the most studied. All these species produce PDO as major metabolite during glycerol fermentation, along with a spectrum of by-products depending on the type of species and strain. For instance, *C. butyricum* generates mostly acetate, butyrate and lactate as by-products, whereas *C. pasteurianum* produces acetate, butyrate, ethanol and butanol [42,61]. These species are strict anaerobes and spore-forming, making them difficult to handle at industrial scale [63], also the PDO producers from this family have a biosafety level of 1 (non-pathogens) [64]. In *C. butyricum*, the glycerol dehydratase, converting glycerol into 3-hydroxypropionaldehyde (HPA, intermediate for PDO production), is extremely sensitive to oxygen and is inactivated even at very low levels[42]. As this enzyme is necessary for the reductive pathway of glycerol fermentation, its inactivation would result in a complete stop of the fermentation process. It is also noteworthy to mention that this enzyme is vitamin B₁₂ independent in *C. butyricum*, unlike glycerol dehydratases in *Enterobacteriaceae* and *Lactobacillus* species [42,65,66]. This means that no supplementation of expensive vitamin B₁₂ is required to sustain efficient production of PDO, resulting in reduced operating costs. *C. butyricum* has been reported as one of the most efficient bacterial species for the production of PDO, with both refined and crude glycerol (see Table 1-4 and Table 1-5). A high PDO titer of 93.7 g.L⁻¹ with a yield of 0.63 mol_{PDO} mol⁻¹_{glycerol} and a productivity of 3.35 g.L⁻¹.h⁻¹ was achieved in fed-batch using *C. butyricum* AKR 102a fed with refined glycerol [67]. This is the highest PDO concentration obtained so far using natural strains. A high PDO productivity of 10.3 g.L⁻¹.h⁻¹ was also reached by using continuous systems with *C. butyricum* VPI 3266 fed with refined glycerol, with a titer of 30 g.L⁻¹ and a yield of 0.60 mol_{PDO} mol⁻¹_{glycerol} [68].

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Table 1-4. Best PDO production performances reported in the literature

Organism	Mode of operation	Titer (g.L ⁻¹)	Yield (mol _C .mol _C ⁻¹)	Overall productivity (g.L ⁻¹ .h ⁻¹)	Glycerol source	Ref
<i>Clostridiaceae</i>						
<i>C. butyricum</i> CNCM1211	Batch	63.4	0.69	1.85	Crude	[69]
<i>C. butyricum</i> AKR 102a	Fed-batch	93.7	0.63	3.35	Refined	[67]
<i>C. butyricum</i> IK124	Fed-batch	87.0	0.65	1.9	Crude	[70]
<i>C. butyricum</i> VPI 3266	Continuous	30	0.60	10.3	Refined	[68]
<i>C. butyricum</i> F2b	Continuous	35-48	0.67	2.9-5.5	Crude	[62]
<i>C. butyricum</i> DSM 5431	Continuous	26.6	0.63	13.3	Refined	[71]
<i>Enterobacteriaceae</i>						
<i>K. pneumoniae</i> ZJU 5205	Batch	63.1	0.65	5.74	Refined	[72]
<i>K. pneumoniae</i> DSM 4799	Fed-batch	80.2	0.55	1.16	Crude	[73]
<i>K. pneumoniae</i> LX3	Fed-batch	68.2	0.62	3.43	Refined	[74]
<i>C. freundii</i> FMCC-B294	Fed-batch	68.1	0.48	0.79	Crude	[75]
<i>K. pneumoniae</i> DSM 2026	Continuous	35-48	0.61	4.9-8.8	Refined	[76]
<i>Lactobacillus</i>						
<i>L. diolivorans</i> DSM 14421	Fed-batch	85.4	0.56 ^a	0.46	Refined	[77]
<i>L. reuteri</i> ATCC 55730	Fed-batch	65.3	0.19 ^a	1.2	Refined	[63]
Genetically engineered strains						
<i>E. coli</i> K-12 ER2925	Fed-batch	104.4	- ^b	2.61	Refined	[78]
<i>E. coli</i> K-12	Fed-batch	135.0	0.3	3.5	- ^c	[31]
<i>K. pneumoniae</i> Cu ΔldhA	Fed-batch	102.7	0.50	1.53	Refined	[79]
<i>C. acetobutylicum</i> DG1 (pSPD5)	Fed-batch	84.0	0.65	1.70	Refined	[80]
Mixed cultures						
Biogas reactor sludge	Fed-batch	70.0	0.56	2.6	Crude	[1]

^a Fermentation with glucose as co-substrate

^b Fermentation was carried out with a glycerol / yeast extract mass ratio of 4. It was not possible to calculate an accurate carbon yield.

^c Glucose was used as substrate

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1.3.1.2 *Enterobacteriaceae*

Many species issued from the *Enterobacteriaceae* family and more specifically from the *Klebsiella*, *Citrobacter* and *Enterobacter* genera are known to have the ability to ferment glycerol [31]. Species from the *Enterobacteriaceae* family are easy-to-cultivate facultative anaerobes, but most species are considered as opportunistic pathogens [31,63] (biosafety level 2 [64]) which represents a significant constraint for their use at industrial scale. The most efficient natural PDO producers from this family include *Enterobacter agglomerans* [81], *Klebsiella oxytoca* [82], *Citrobacter freundii* [83,84] and *K. pneumoniae* [84], the latter two being the most studied species. For all these species, PDO and acetate are the major product from glycerol fermentation, but secondary products such as lactate, formate, succinate and ethanol can be found depending on the type of strain and culture conditions [42]. Within the *Enterobacteriaceae* family, *K. pneumoniae* is the species showing the best PDO production performances (see Table 1-4). However, lower yields were obtained with *K. pneumoniae* compared to *C. butyricum* due to ethanol production, which dissipates NADH and competes with PDO synthesis (see Section 1.2). Also, as the glycerol dehydratase of *K. pneumoniae* is vitamin B₁₂-dependent [65,66,85], yeast extract has to be supplemented in fermentation media because *Klebsiella spp.* are not able to synthesize this vitamin [65]. Despite these shortcomings, a maximum PDO titer of 80.2 g.L⁻¹ was achieved using *K. pneumoniae* DSM 4799 in a fed-batch fermentation of crude glycerol with a productivity and a yield of 1.16 g.L⁻¹.h⁻¹ and 0.55 mol_{PDO} mol⁻¹_{glycerol} respectively, [73]. This performance remains one of the best values ever reported for natural PDO producers.

1.3.1.3 *Lactobacillus*

The *Lactobacillus* genus is Generally Recognized As Safe (GRAS) [77,86] (biosafety level 1 [64]) and is composed of non-spore-forming facultative anaerobes [63]. Cultures are easy-to-handle and fermentation products can be used for food and cosmetics applications [63]. Some species from the *Lactobacillus* genus have been reported to produce PDO from glycerol [31], such as *Lactobacillus brevis* [87], *Lactobacillus buchneri* [87], *Lactobacillus panis* [86], *Lactobacillus diolivorans* [77,88] and *Lactobacillus reuteri* [63]. However, none of these species can grow using glycerol as sole substrate, because of the lack of certain enzymes from the glycerol oxidative pathway (see Section 1.2) [63,86–88]. For instance, *L. reuteri* lack the dihydroxyacetone kinase, an enzyme essential to connect glycerol to the glycolysis pathway. As a consequence, these *Lactobacillus* species are able to ferment glycerol only in presence of an additional substrate such as glucose. It is worth noticing that,

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similarly to *Enterobacteriaceae* species, the glycerol dehydratase (first enzyme of the glycerol reductive pathway) is vitamin B₁₂-dependent in *Lactobacillus* spp. [63,77,87], resulting in a lower glycerol utilization and PDO production when this vitamin is not sufficiently synthesized.

When glucose and glycerol are used as co-substrate, glycerol seems to be preferred for NADH dissipation through PDO production [63]. The glucose / glycerol ratio needs to be adjusted in order to maximize glycerol utilization and PDO carbon yield. However, optimization of this parameter seems species dependent, as increasing the glucose / glycerol ratio up to 1.5 resulted in an improvement of PDO productivity for *L. reuteri* [63], whereas PDO production was nearly stopped when the ratio was over 0.3 for *L. diolivorans* [77]. The best performance reached so far with *Lactobacillus* species was achieved by Pfügl *et al.* using *L. diolivorans* DSM 14421 in fed-batch fermentation with a glucose / glycerol ratio of 0.1 [77]. A final PDO titer of 85.4 g.L⁻¹ was attained with a yield and a productivity of 0.56 mol_C-PDO mol⁻¹_{C-substrate} and 0.46 g.L⁻¹.h⁻¹ respectively.

1.3.1.4 Metabolic engineering

Several strategies of genetic modifications have been investigated to improve fermentative PDO production. When looking at the glycerol oxidative metabolism, it is clear that PDO production is maximized during glycerol fermentation when acetate is the only other fermentation by-product (see Section 1.2). Therefore, a first idea was to reduce the by-products formation such as lactate and ethanol (see Figure 1-9). For instance, Zhang *et al.* (2006) inactivated the *aldA* gene encoding the acetaldehyde dehydrogenase in *K. pneumoniae* YMU2 [89]. As this enzyme is responsible for the conversion of acetyl-CoA in acetaldehyde (precursor of ethanol, see Section 1.2), ethanol production was reduced by a factor of 5 as compared to the wild-type strain. Simultaneously, PDO productivity and yield increased from 0.81 to 1.07 g.L⁻¹.h⁻¹ and from 0.36 to 0.70 mol_{PDO} mol⁻¹_{glycerol} respectively. A similar strategy was conducted by Yang *et al.* to produce a lactate deficient strain by knocking out the *ldhA* gene encoding lactate dehydrogenase in *K. oxytoca* M5a1 [90]. PDO productivity and yield increased from 0.63 to 0.83 g.L⁻¹.h⁻¹ and from 0.43 to 0.53 mol_{PDO} mol⁻¹_{glycerol} respectively as compared to the wild-type strain. Knocking out the same gene in *K. pneumoniae* Cu yielded to one of the best PDO production ever obtained. Indeed a final PDO titer of 102.7 g.L⁻¹ was achieved in a fed-batch fermentation using refined glycerol as substrate, with a productivity and a yield of 1.53 g.L⁻¹.h⁻¹ and 0.50 mol_{PDO} mol⁻¹_{glycerol} respectively [79]. Even though *C. butyricum* is the best natural PDO producer (see Table 1-4), this species has not been

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successfully genetically modified so far because no common genetic engineering tools for this species have been developed yet [61]. For a metabolic engineering approach, *C. butyricum* only serves as gene donor for genes encoding proteins involved in PDO synthesis.

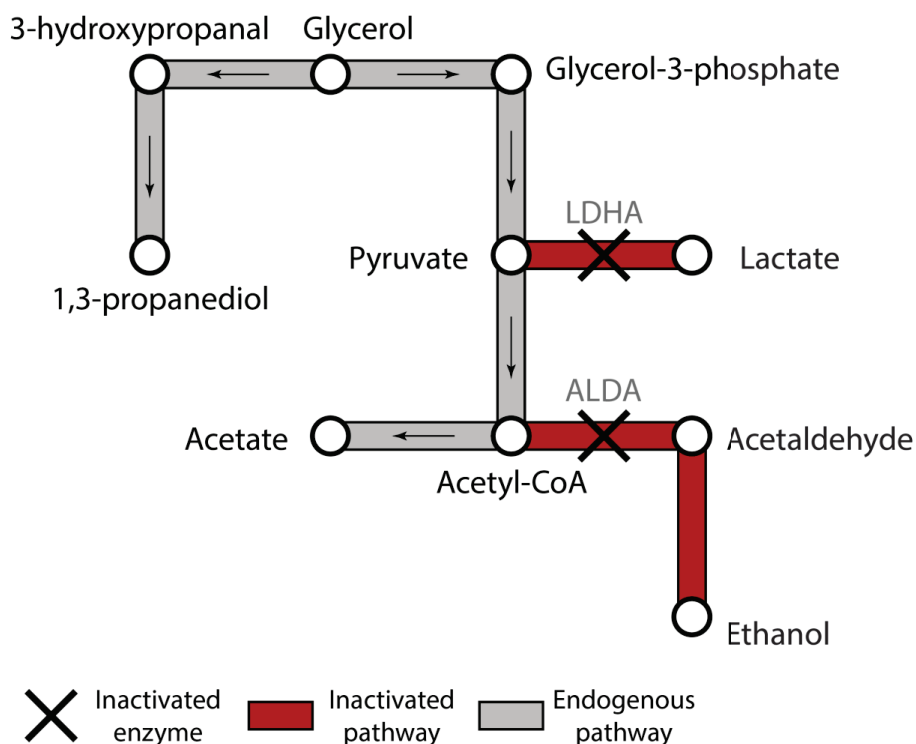


Figure 1-9. Genetic engineering strategies for microorganisms natively capable of glycerol fermentation (simplified catabolic pathways).

ALDA: aldehyde dehydrogenase; *LDHA*: lactate dehydrogenase *A*.

A second method consists in adding genes that encode enzymes required for PDO synthesis in organisms lacking the glycerol reductive pathway (see Figure 1-10). As such, a *C. acetobutylicum* recombinant was constructed by incorporating the pSPD5 plasmid containing the *dhaB1*, *dhaB2* and *dhaT* genes from *C. butyricum*, encoding for the B₁₂-independent glycerol dehydratase, its activating factor and the PDO dehydrogenase respectively [80]. As a result, a high PDO titer of 84.0 g.L⁻¹ was achieved while the wild-type *C. acetobutylicum* DG1 was not able to produce PDO. In a similar way, the *E. coli* K12 strain was modified by incorporating the pBV220 plasmid containing the *dhaB1* and *dhaB2* genes from *C. butyricum*, and the *yqhD* gene encoding PDO dehydrogenase from *E. coli* [78]. The *dhaT*-based PDO dehydrogenase can utilize NADH solely, while *yqhD*-based PDO dehydrogenase can utilize

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both NADH and NADPH [91]. Therefore, exploitation of *yqhD* gene instead of *dhaT* gene from *C. butyricum* enables greater provision of reducing equivalents for PDO production. The recombinant mutant was able to produce up to 104.4 g.L⁻¹ of PDO with a productivity of 2.61 g.L⁻¹.h⁻¹ in fed-batch using refined glycerol (purity > 95%).

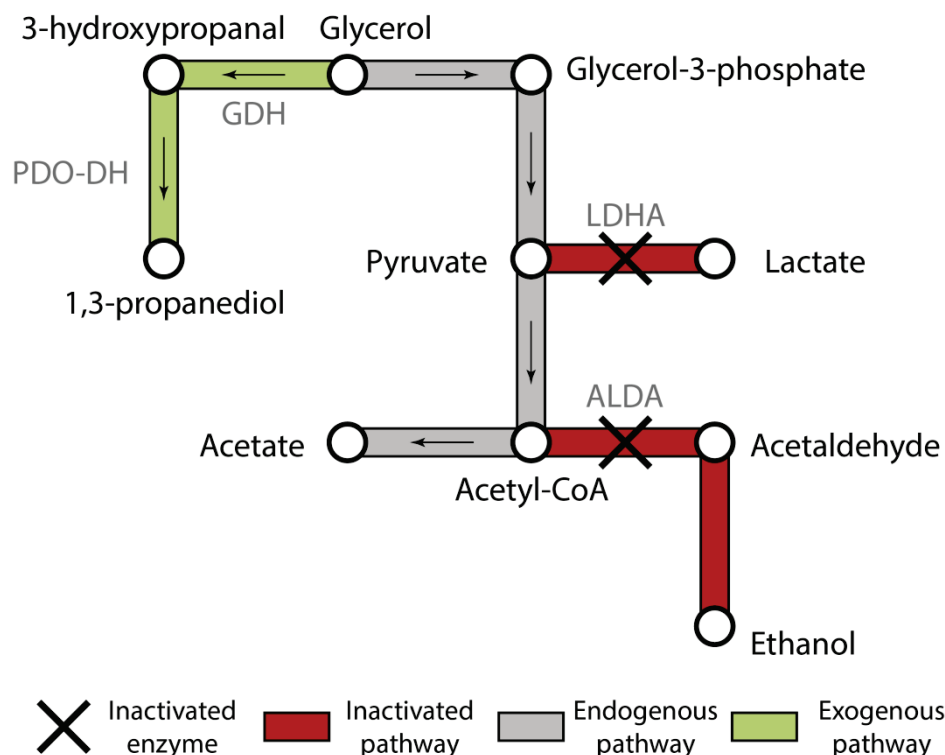


Figure 1-10. Genetic engineering strategies for microorganisms lacking glycerol reductive pathway (simplified catabolic pathways).

ALDA: aldehyde dehydrogenase; *GDH*: glycerol dehydratase; *LDHA*: lactate dehydrogenase A; *PDO-DH*: 1,3-propanediol dehydrogenase.

Another strategy is to connect glucose metabolism to the glycerol reductive pathway to produce PDO from glucose as alternative feedstock (see Figure 1-11) [92]. As an illustration, a recombinant *K. pneumoniae* expressing the gene encoding the glycerol-3-phosphatase from *Saccharomyces cerevisiae* was constructed by Laffend et al. from DuPont company [31]. This enzyme converts glycerol-3-phosphate, issued from glycolysis, in glycerol that can be further transformed in PDO. The same company applied a similar strategy in *E. coli* and reported a PDO titer of 135 g/L with a yield of 0.6 mol_{PDO}/mol_{Glucose}, which is the highest PDO titer obtained so far [31].

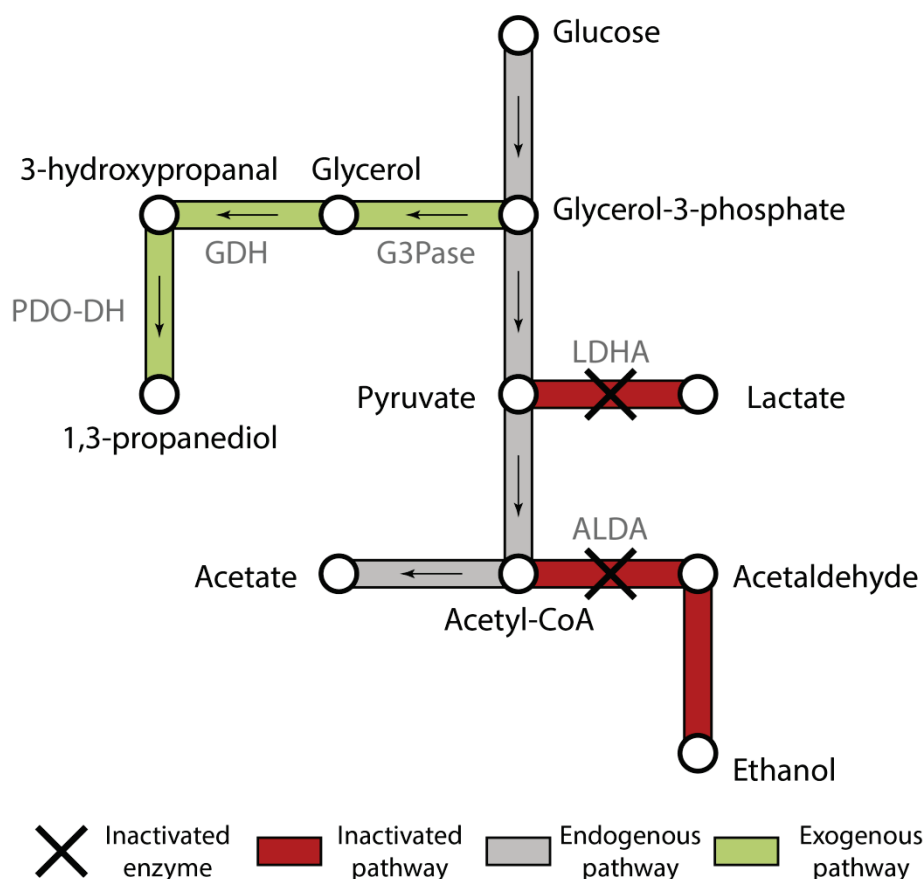


Figure 1-11. Genetic engineering strategies for producing PDO from glucose (simplified catabolic pathways).

ALDA: aldehyde dehydrogenase; G3Pase: glycerol-3-phosphatase; GDH: glycerol dehydratase; LDHA: lactate dehydrogenase A; PDO-DH: 1,3-propanediol dehydrogenase.

1.3.1.5 Open mixed cultures

So far, only few studies have reported that mixed cultures could be an interesting alternative to pure culture as an inoculum for PDO production [1–3,93,94]. An open mixed culture consists in a mixture of several different bacteria that are maintained in a reactor running under non-sterile conditions. They are usually derived from natural inocula that present high microbial diversity [95]. Drozdzyńska *et al.* succeeded in isolating bacteria able to produce PDO from diverse sources as groceries, soils, silages, composts, stagnant waters, sludges from municipal wastewater treatment plant, and biogas fermenters [83]. Microbial inocula from all these sources were suitable to carry out open mixed culture fermentations. As open mixed cultures fermentation can be carried out under unsterile conditions, their operational costs are drastically reduced when compared to pure culture fermentations. Other benefits of open mixed culture fermentations consist in a better substrate utilization, *in-situ*

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production of nutrients by symbiotic species (*e.g.* growth factors and vitamins), inhibitor removal and all kind of syntrophic interactions [96–99]. Hence, minimal cultivation medium that does not contain expensive additives such as yeast extract can be used in open mixed culture fermentations. However, the control of mixed-culture fermentation is a complicated task as interspecies interactions within a bacterial consortium are complex and difficult to predict and control. Also, when a population has been efficiently oriented towards an efficient production of a specific metabolite, there is no simple way to ensure its stability over time or to store it (*e.g.* lyophilization, freezing) without changing the population structure. As a result, mixed-culture processes often lack product specificity and are usually less reproducible.

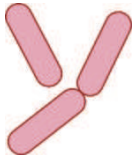
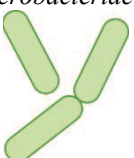
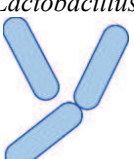

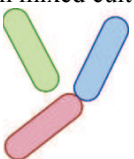
Regarding PDO yield, several authors have shown, despite their inherent drawbacks, mixed cultures can offer performances close to the best results obtained with pure cultures. Using organic soil as inoculum, Liu *et al.* obtained a PDO yield of $0.65 \text{ mol}_{\text{PDO}} \text{ mol}^{-1}_{\text{glycerol}}$ in batch mode which was close to the maximum theoretical yield of $0.72 \text{ mol}_{\text{PDO}} \text{ mol}^{-1}_{\text{glycerol}}$, although only 7 g.L^{-1} of refined glycerol was used [3]. With a similar inoculum and in batch fermentation at 35 g.L^{-1} of crude glycerol, Kanjilal *et al.* reached a final PDO titer of 19.4 g.L^{-1} , with a PDO yield of $0.67 \text{ mol}_{\text{PDO}} \text{ mol}^{-1}_{\text{glycerol}}$ [93]. So far only few studies have focused on optimizing the final PDO titer in open mixed cultures. Nonetheless, the best results were obtained by Dietz *et al.* with 70.0 g.L^{-1} of PDO at a yield and a productivity of $0.56 \text{ mol}_{\text{PDO}} \text{ mol}^{-1}_{\text{glycerol}}$ and $2.6 \text{ g.L}^{-1}.\text{h}^{-1}$ respectively, in a fed-batch fermentation fed with crude glycerol and using a biogas reactor sludge as inoculum (see Table 1-4) [1]. These results are particularly remarkable as they were obtained without any pretreatment of the crude glycerol or any addition of yeast extract (or equivalent).

It is noteworthy to mention that methane production could be avoided during glycerol fermentation with open mixed-cultures [1–3,93,94]. This is particularly important because PDO or glycerol can very probably be used in anaerobic mixed cultures to form methane [1], leading to a dramatic decrease of fermentation performances. Several strategies are possible to avoid methane production in mixed cultures: the inoculum can be heat-treated in order to remove non-spore-forming methanogens [100,101]; fermentations can be conducted at low pH [100] or with high carboxylic concentrations [102]; fermentations can be conducted in continuous reactor running at low hydraulic retention time [100]; or specific inhibitors such as 2-bromoethanesulfonate can be added in the fermentation broth [103]. High potassium or sodium concentrations such as those that can be found in crude glycerol [21] (used as alkali catalyst during transesterification, see section 1.1.1) are also not favorable to methanogenesis

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[102]. As all these techniques are compatible with PDO production from glycerol, methane production is not a major issue for future implementation of open mixed-culture processes for PDO production at larger scale.

Table 1-5. General features of PDO-producing bacteria

PDO producer	Oxygen tolerance	Sterile conditions	Biosafety level	Main by-products	Best performance (highest PDO titer)	Ref.
<i>Clostridiaceae</i> 	-	+	1	Acetic acid Butyric acid	<i>C. butyricum</i> AKR 102a C _f : 93.7 g.L ⁻¹ Y: 0.63 mol _C /mol _C Q: 3.35 g.L ⁻¹ .h ⁻¹	[67]
<i>Enterobacteriaceae</i> 	+	+	2	Acetic acid Lactic acid Ethanol 2,3-butanediol	<i>K. pneumoniae</i> DSM 4799 C _f : 80.2 g.L ⁻¹ Y: 0.55 mol _C /mol _C Q: 1.16 g.L ⁻¹ .h ⁻¹	[73]
<i>Lactobacillus</i> 	+	+	1 (+GRAS)	Acetic acid Lactic acid	<i>L. diolivorans</i> DSM 14421 C _f : 85.4 g.L ⁻¹ Y: 0.56 mol _C /mol _C Q: 0.54 g.L ⁻¹ .h ⁻¹	[77]
Engineered strains 	+ or -	+	2	NA	Recombinant <i>E. coli</i> K-12 C _f : 135 g.L ⁻¹ Y: 0.3 mol _C /mol _C Q: 3.5 g.L ⁻¹ .h ⁻¹	[31]
Open mixed cultures 	+	-	NA	Acetic acid Butyric acid Lactic acid Ethanol	Biogas reactor sludge C _f : 70.0 g.L ⁻¹ Y: 0.56 mol _C /mol _C Q: 2.6 g.L ⁻¹ .h ⁻¹	[1]

GRAS: Generally Recognized as Safe by the Food and Drug Administration (FDA, USA);
C_f: final PDO titer; Y: PDO yield; Q: PDO productivity

1.3.2 Mode of operation

As discussed in section 1.1.3, biotechnological technologies for PDO production are those showing less environmental impacts and possibly the lowest operating costs, as compared to chemical and petrochemical techniques. However, simple fermentation processes such as batch fermentation usually offer low reaction rates and relatively low product

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concentrations. As a consequence, huge volumes of fermentation broth have to be treated to extract and purify the product. In such fermentation, downstream processes can represent up to $\frac{2}{3}$ of the overall process costs [104]. Therefore, optimization of both product final concentration and purification/extraction procedures are the two key-elements to make cost-competitive the biotechnological production of PDO [30].

1.3.2.1 Batch mode

In batch reactors, fermentation is carried out at constant volume, without any feed inlet or outlet sampling. It is the most simple reactor operation and is often an effective and economic solution for slow processes. In order to achieve significant final PDO titers in batch fermentations, several authors have tried to use high initial glycerol concentrations. Unfortunately, glycerol concentrations ranging from 60 to 110 g.L⁻¹ were reported as inhibitory for both the bacterial growth and PDO production in model microorganisms such as *C. butyricum* [105–107] and *K. pneumoniae* [108]. This inhibitory effect was related to an osmotic stress caused by such high concentration of substrate [61]. Therefore, several approaches have been implemented to select PDO producers with a high resistance to osmotic stress. For instance, a chemical mutagenesis approach was used to select resistant *C. diolis* strains [109]. The wild-type strain DSM 15410 had a maximal glycerol concentration tolerance of 62 g.L⁻¹. This tolerance was improved by 77% to reach 109 g.L⁻¹ for a selected *C. diolis* mutant. Even with selective breeding, no strain has been reported to support more than 110 g.L⁻¹ without any inhibitory effect. Considering that the highest glycerol concentration that can be fed in batch mode is 110 g.L⁻¹ that can be further converted into PDO at a maximum theoretical yield of 0.72 mol_{PDO} mol⁻¹_{glycerol}, the maximum PDO titer that can be achieved in batch would be 65 g.L⁻¹. Such performance was attained by Barbirato *et al.* in a batch fed with 112 g.L⁻¹ of crude glycerol using a natural *C. butyricum* strain (CNCM1211) as bio-catalyst with a final titer of 63.4 g.L⁻¹, with a yield and productivity of 0.69 mol_{PDO} mol⁻¹_{glycerol} and 1.85 g.L⁻¹.h⁻¹ respectively (see Table 1-4) [69]. This result is the best performance achieved so far in batch mode without cell immobilization and probably represents the upper limit of this operation mode.

1.3.2.2 Fed-batch mode

In fed-batch fermentation, substrate is supplied to the bioreactor during cultivation and the products remain in the reactor until the end of the run (*i.e.* no outlet) [110]. Usually, the bioreactor is fed either continuously (*e.g.* constantly or exponentially) or intermittently in response to a control parameter (*e.g.* pH, pO₂ or other on-line measurements). Because

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substrates concentrations can be maintained at low levels, using fed-batch processes is an efficient way to avoid substrate inhibition [110,111]. It is also an effective process for accumulating desired products and achieving final titers higher than in batch mode. However, accumulation of fermentation products can induce several stresses to micro-organisms and eventually inhibit cell growth and product formation [61,112]. Regarding glycerol fermentation, it was hypothesized that PDO accumulation could inhibit cell growth by modifying membrane organization via an increased fluidity of the membrane [113,114]. Membrane ATPase and transport mechanisms could also be inhibited by PDO as reported for other alcohols [113,114]. PDO inhibition has been studied for several strains of *C. butyricum* and *K. pneumoniae* [74,107,113,115,116]. Similar results were reported for both species, and PDO was found to be inhibitory of the microbial growth in a range of 60-90 g.L⁻¹ for the wild-type strains [113,115,116]. Strong inhibition were also reported for by-products such as acetic acid and butyric acids, which were found to totally inhibit the microbial growth at concentrations of 27 g.L⁻¹ and 19 g.L⁻¹ respectively (as sum of both dissociated and undissociated form) [113]. Despite these limitations, Wilkens *et al.* attained a final PDO titer of 93.7 g.L⁻¹ with a yield and productivity of 0.63 mol_{PDO} mol⁻¹_{glycerol} and 3.35 g.L⁻¹.h⁻¹ respectively, using *C. butyricum* AKR 102a in fed-batch fermentation fed with refined glycerol [67]. This is the best performance achieved so far using natural PDO producers in fed-batch fermentation (see Table 1-4). The use of engineered strains allowed exceeding the limitations due to PDO inhibition, using strains more resistant to PDO than *C. butyricum* and *K. pneumoniae*. For instance, in a fed-batch using glucose as substrate and a *E. coli* K-12 recombinant, Laffend *et al.* from DuPont company were able to reach a maximum PDO concentration of 135 g.L⁻¹ [31]. As a consequence, fed-batch processes are those that are currently used for PDO production at industrial scale because of their highest final titers and the resulting lowest downstream process costs (see Section 1.1.3).

1.3.2.3 Continuous fermentation

Productivities in fed-batch processes can be substantially increased in continuous mode. Indeed, in fed-batch fermentations, initial PDO productivity is high (logarithmic growth phase) while it drops dramatically in the later period of fermentation due to product inhibition [111]. Continuous fermentation is a way to set substrate and product concentrations at a constant level by removing fermentation products continuously while providing nutrients at the same rate [117]. This mode of operation offers other advantages than batch and fed-batch fermentations such as a good control of the growth rate of the microorganisms [111] (by

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adjusting the dilution rate) and the possibility to reach steady states (*i.e.* variables become time-independent) which are convenient for process control and further downstream processes [118]. However, in such systems, final product titers are lower than those achieved in fed-batch: continuous fermentation is a trade-off between final concentration and productivity. Both *C. butyricum* and *K. pneumoniae* demonstrated good performances for continuous PDO production. At dilution rates between 0.1 and 0.25 h⁻¹, Menzel *et al.* reported that *K. pneumoniae* DSM 2026 was able to produce between 35.2 and 48.5 g_{PDO}.L⁻¹ at a yield and productivity of 0.61 mol_{PDO} mol⁻¹_{glycerol} and 4.9–8.9 g.L⁻¹.h⁻¹ respectively [76]. This productivity was approximately 2–3.5-fold higher than those obtained in batch and fed-batch cultures with the same *K. pneumoniae* strain. The best PDO productivities achieved so far in classic continuous fermentation (*i.e.* no cell recycling or immobilization) were obtained using *C. butyricum* VPI 3266 at a dilution rate of 0.30 h⁻¹. A final titer of 30 g_{PDO}.L⁻¹ was produced with a yield and productivity of 0.60 mol_{PDO} mol⁻¹_{glycerol} and 10.3 g.L⁻¹.h⁻¹ respectively [68].

1.3.2.4 Cell recycling and immobilization

Several strategies have been implemented to increase the cell densities in glycerol fermentation. Indeed, having high cell concentrations have advantages such as increased fermentation kinetics [32] and a better tolerance to high glycerol and fermentation by-products concentrations [32,71]. At some extent, achieving high cell concentration could be a solution to overcome substrate and product inhibitions as observed in classic suspended fermentation processes. Moreover, increasing cell concentration can improve process productivity. A first approach consists in recycling cells in continuous systems by passing the culture through a permeable membrane. The cell-free liquid can then be used for downstream processes while the concentrated cell suspension is reinjected into the reactor [71,119]. The best performances using this technology were reported by Reimann *et al.* using *C. butyricum* DSM 5431 at a dilution rate of 0.5 h⁻¹, with a final titer, yield and productivity of 26.6 g_{PDO}.L⁻¹, 0.63 mol_{PDO} mol⁻¹_{glycerol} and 13.3 g.L⁻¹.h⁻¹ respectively [71]. This productivity was about 30% higher than the best one achieved in classic continuous fermentation (10.3 g.L⁻¹.h⁻¹). In general, membrane clogging rapidly occurs in cell recycling systems, making difficult to maintain high and stable performances over time and limiting their implementation at industrial scale.

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Another approach to increase cell concentrations consists in immobilizing the microbial cells inside the reactor [32]. Several techniques have been explored, such as cell aggregation (*e.g.* self-immobilization as granules), cell attachment (*e.g.* biofilm formation on inert support) or cell entrapment (*e.g.* in porous materials). These techniques are used in continuous systems (*e.g.* packed-bed reactor, fluidized-bed reactor) as well as in -repeated- batch and fed-batch processes. In a repeated batch procedure, a final titer, yield and productivity of 63.1 g_{PDO}.L⁻¹, 0.65 mol_{PDO} mol⁻¹_{glycerol} and 5.74 g.L⁻¹.h⁻¹ respectively were achieved by encapsulating *K. pneumoniae* ZJU 5205 [72]. Although the final titer and yield obtained in this study were similar the best batch fermentation performances achieved with *C. butyricum* (see Table 1-6), the productivity was improved by a factor 3. Similar improvements were also reported for continuous fermentation using immobilized cultures. By immobilizing *K. pneumoniae* on ceramics balls, PDO productivity was improved from 4.9 g.L⁻¹.h⁻¹ to 9.8 g.L⁻¹.h⁻¹ for suspended and immobilized cultures respectively [120]. A comparable improvement (2.5-fold productivity increase) was reported for cultures of *Clostridium beijerinckii* immobilized on pumice stones [121]. These results show that cell immobilization is a very promising strategy for improving PDO productivity in glycerol fermentation. However, only few studies dealing with PDO production are currently available on cell immobilization and they all show that the choice of optimal material for cell immobilization is largely strain-dependent [32]. Therefore, there is no unique and optimal support material that could be used in all PDO fermentation processes. Further research on this matter could make this technology more suitable prior to application and improve PDO productivities in fermentation.

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Table 1-6. Comparison between suspended and immobilized / recirculated culture fermentation performances

Mode of operation		Best performance ^a				Ref.
		Final titer g _{PDO} .L ⁻¹	Yield mol _C /mol _C	Productivity g.L ⁻¹ .h ⁻¹	Microorganism	
Batch	Suspended	63.4	0.69	1.85	<i>C. butyricum</i> CNCM1211	[69]
	Immobilized	63.1	0.65	5.74	<i>K. pneumoniae</i> ZJU 5205	[72]
Fed-batch	Suspended	93.7	0.63	3.35	<i>C. butyricum</i> AKR 102a	[67]
	Immobilized ^b	80.2	0.55	1.16	<i>K. pneumoniae</i> DSM 4799	[73]
Continuous	suspended (w/o recirculation)	30	0.60	10.3	<i>C. butyricum</i> VPI 3266	[68]
	Immobilized / with recirculation	26.6	0.63	13.3	<i>C. butyricum</i> DSM 5431	[71]

^a Best performance reported correspond to the highest PDO titer reported for batch and fed-batch fermentation. For continuous fermentation it corresponds to the study showing the highest productivity with a final titer > 20g_{PDO}.L⁻¹.

^b Only one study was reported to use repeated fed-batch with immobilized cells.

1.3.3 Operating parameters

Once a mode of operation and an inoculum are selected, environmental parameters have to be adjusted in order to optimize the production of the molecule of interest. Apart from the parameters related to the mode of operation (*e.g.* dilution rate, substrate concentration and medium formulation), the most influent environmental parameters reported for fermentation are: availability of the trace elements and vitamins, temperature, pH and redox conditions (*e.g.* presence of oxygen or other electron acceptor) [122]. Trace elements and vitamins will not be discussed in this section as they are usually provided by complex additives such as yeast extract or tryptone prior to glycerol fermentation experiments, with no special focus on it in the published studies (apart from vitamin B₁₂, see Section 1.3.1). Influences of the other parameters over glycerol fermentation are reviewed in the following sections:

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1.3.3.1 Temperature

As described in Section 1.3.1, most efficient PDO-producing bacteria identified so far are mesophilic bacteria which grow typically between 20 and 45 °C [123]. Finding optimal environmental parameters for model organisms such as *C. butyricum* and *K. pneumoniae* has been the focus of numerous studies using statistical designs (e.g. Plackett-Burman, Taguchi) or kinetic models. For both species, temperature was reported to have only slight effect on PDO yield and titer, in a wide temperature range of 30°C - 39°C [105,124–126]. Nonetheless, temperature had an effect on the growth kinetics and PDO productivity, which were positively correlated for these two microorganisms. As 37 °C was the optimal temperature for both *C. butyricum* and *K. pneumoniae* growth and PDO productivity, such mesophilic temperature was described as optimal. No further significant effect of temperature on PDO titer and yield was observed as well for less-studied species such as *K. oxytoca* and *L. panis* in a similar range of temperature (30-39 °C) [127,128]. For these two species, 37 °C was also optimal for both the growth rate and PDO productivity. Overall, the only species able to produce PDO and affected by temperature was *C. freundii* for which the optimal temperature was found at 30 °C [75,83]. For all other mesophilic species, a temperature between 35 and 37 °C is commonly retained as optimal for PDO production [83,107,129].

It is noteworthy to mention that PDO production is also possible in thermophilic conditions. Wittlich *et al.* (2001) reported that among 60 thermophilic enrichment cultures, 16 were able to produce PDO from glycerol [130]. Isolates belonging to the *Caloramator* genus such as *Caloramator boliviensis* [131] and *Caloramator viterbensis* [132] were able to produce PDO at an optimal temperature of 60 °C. *C. viterbensis* was even able to produce PDO with acetate as sole by-product, reaching a PDO yield of $0.69 \text{ mol}_{\text{PDO}} \text{ mol}^{-1}_{\text{glycerol}}$ very close to the theoretical maximum of $0.72 \text{ mol}_{\text{PDO}} \text{ mol}^{-1}_{\text{glycerol}}$ [132]. Although thermophilic bacteria could be an interesting alternative for PDO production, almost no study has focused on their use in optimized glycerol fermentation processes and the best titer ever achieved so far was only $6.4 \text{ g}_{\text{PDO}} \cdot \text{L}^{-1}$ [130].

1.3.3.2 pH

The pH is usually described as a critical parameter affecting fermentation, because the catalytic activity of enzymes and therefore the metabolic activity of most microorganisms depend on extracellular pH [133]. In particular, highly acidic and alkaline environments (*i.e.* $\text{pH} < 4$ and $\text{pH} > 10$ respectively) can be extremely toxic to bacterial activity. Toxicity at low pH is often related to weak acids accumulation such as volatile fatty acids (*i.e.* acetic acid,

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propionic acid and butyric acid) and lactic acid that are produced in their dissociate form during fermentation. As the pKa values of these acids range from 4 to 5 (around 4.8 for volatile fatty acids, and 3.9 for lactic acid), an extracellular pH value below 4 would drastically increase the concentration of the undissociated form of these acids. Subsequently, these undissociated acids can freely diffuse inside the cells and cause proton imbalance [112]. To maintain an optimal intracellular pH, these extra protons have to be actively removed from the cell by proton pumps while consuming ATP. This mechanism is directly competing with bacterial growth that can even be totally inhibited. A similar mechanism was reported for ammonia (NH₃) inhibition at high pH. Ammonium (NH₄⁺) is a common source of nitrogen that can be found in most fermentations [102]. The concentration of ammonia, which is the undissociated form of ammonium, increases as pH become high (pKa = 9.2). Ammonia is also membrane-permeable and can cause proton imbalance as described for weak acids (although it increases intracellular pH) [102]. Except for specific extremophiles, the pH range compatible with bacterial growth is therefore usually comprised between 4 and 10.

Concerning the effect of extracellular pH on glycerol fermentation, no single and common behavior has been found for all PDO-producing bacteria (see Figure 1-12). As for temperature, pH has been the focus of optimization studies, especially for *C. butyricum* and *K. pneumoniae*. *C. butyricum* is able to ferment glycerol at large pH values between 5.4 and 8.2 [116], with an optimal growth and minimal inhibitions from both substrate and products between pH 6.5 and 7.0 [105,116]. *K. pneumoniae* was also capable of producing PDO from glycerol in a similar range of pH, *i.e.* from 6 to 8.5 [134,135] with an optimum in slightly alkaline conditions between 7.4 and 8 [126,135,136]. For *C. pasteurianum*, no clear dependence of the PDO production on pH was observed for values ranging from 5.0 to 7.5 [59,137]. *C. freundii* was able to grow on glycerol for a wide range of pH (4.6 to 8.2) with an optimal value for PDO production between 6.6 and 7.2 [138,139]. No optimization study of the pH was performed with *L. reuteri*. However this species was reported to ferment glycerol at pH between 4.7 and 6.5 and was more efficient for both PDO production and bacterial growth at pH 5.5 [129,140]. According to all these studies, it is clear that not all microorganisms have the same optimal pH range for PDO production. This is particularly important for the use of mixed consortia as inoculum prior to glycerol fermentation. Although no report is today available on the effect of pH on microbial population structures, the observations made on pure strains support that pH may be an important factor influencing population selection and consequently PDO production in glycerol fermentation.

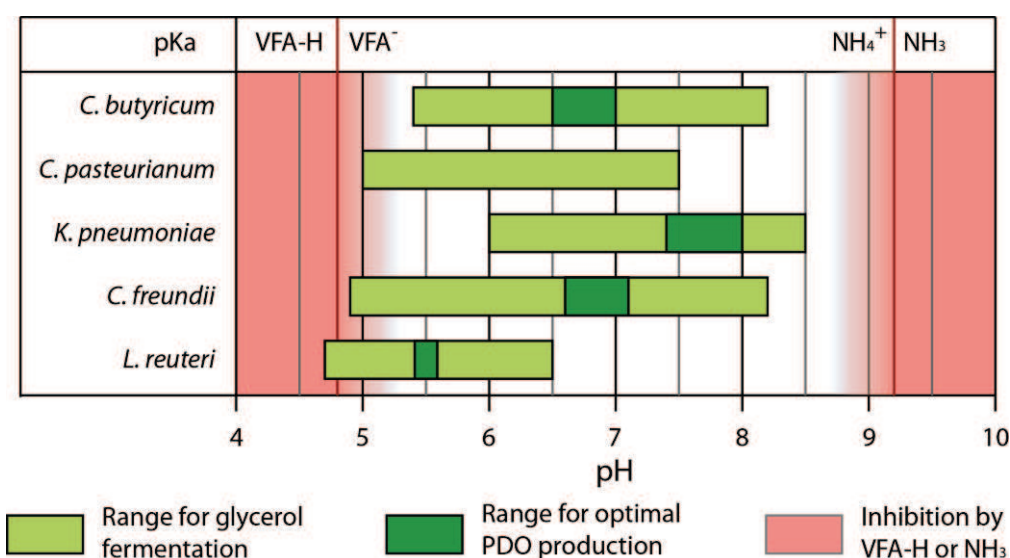


Figure 1-12. Diversity of pH ranges for glycerol fermentation and optimal PDO production for efficient PDO-producing bacteria.

VFA-H/VFA⁻: undissociated/dissociated volatile fatty acids.

1.3.3.3 Redox potential (ORP)

The oxidation-reduction potential (ORP) of the fermentation medium, also called extracellular ORP, appears to be a relevant parameter to control the microbial metabolism [10,141]. Indeed, a fermentation process corresponds to a cascade of oxidation and reduction reactions that must be kept in balance. These reactions are mostly thermodynamically favorable and spontaneous but they are also constrained by biological regulations within microorganisms and inter-species interactions in microbial communities. Similarly to pH as a measure of the protons activity, the extracellular ORP corresponds to the activity of the electrons present in the medium. It is mainly affected by temperature, chemical composition of the medium and the degree of reduction of the metabolites produced by fermentation. It can be easily measured with an ORP sensor located in the medium. The extracellular ORP is particularly important because it can subsequently affect the intracellular ORP through the NADH/NAD⁺ balance [142]. Intracellular ORP, representing the redox state inside a cell, can be estimated through the NADH/NAD⁺ ratio because of the intracellular redox homeostasis [142]. Intracellular ORP is known to influence gene expression and enzyme synthesis, that can further cause shifts in the metabolic pathways and impact the whole metabolism [142,143]. Chemical control (*e.g.* supply of chemical reductive or oxidative agents, bubbling

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gases [144]) of the extracellular ORP has already been successfully implemented to improve the production of metabolites such as succinate [145,146] or 1,3-PDO [147].

A first approach consists in providing oxygen (aeration or micro-aeration) to facultative anaerobes, such as *Enterobacteriaceae* or *Lactobacillus* species, in order to enhance their growth and subsequently improve PDO production rate. Oxygen is one of the terminal electron acceptor having the highest potential (see Figure 1-13). Thus microorganisms generates more energy (*i.e.* ATP) when using oxygen than under complete anaerobiosis. This strategy was applied for *K. pneumoniae* and successfully improved productivities in batch fermentation from 1.62 to 2.94 g.L⁻¹.h⁻¹ under anaerobic and micro-aerobic conditions respectively [148]. This improvement was correlated with higher bacterial biomass production but also anti-correlated with PDO yield (from 0.57 to 0.52 mol_{PDO} mol⁻¹_{glycerol}) as a result of the competition between O₂ and PDO as NADH sink. For lactic acid bacteria such as *L. diolivorans* [77] and *L. reuteri* [63], O₂ supply under both micro-aerobic and aerobic conditions successfully enhanced biomass production and kinetics. However PDO productivities and yield were always lower in presence of oxygen and PDO production was even completely stopped under aerobic conditions (*i.e.* pO₂ = 0.20). Thus, micro-aeration and aeration are efficient ways for accelerating bacterial biomass formation that also outcompete the PDO pathway. In fact, two-stage processes could be a way to take advantage of aeration: a first aerobic stage for increasing biomass production rate and a second anaerobic stage for producing PDO at higher yield and productivity [78,147].

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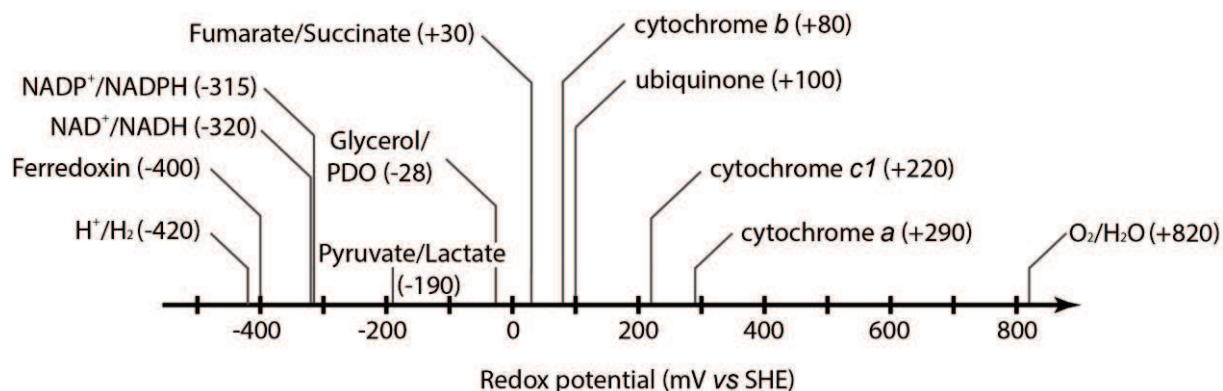


Figure 1-13. Biological standard potential of some intracellular redox pairs [142,149].

Values reported correspond to E° , the standard potential calculated in biological conditions (25°C, activities of 1 for all species except $[H_3O^+] = 10^{-7}$). Redox potential for cytochromes, ferredoxin and ubiquinone are strain-dependent and can differ from the reported value [150].

Another strategy consists in controlling the extracellular ORP at a specific value by a loop system with supply of both oxidant and reductant. As PDO production has for sole function to ensure redox homeostasis in glycerol fermentation [137] (see also Section 1.2), it is expected that its production might be tightly related to the environmental redox conditions. Du *et al.* (2006) investigated the response of *K. pneumoniae* M5aL under redox control environment at three levels (-140, -190 and -240 mV vs SHE) [147]. Interestingly, ORP changes resulted in a significant redistribution of the metabolic fluxes: rising ORP from -240 to -140 mV vs SHE increased the acetate production by 2.5-fold while decreasing lactate accumulation by 3-fold. As a result, PDO production was also affected by the extracellular ORP and an optimum for both its production and bacterial growth of this strain was found at -190 mV vs SHE. The same strain was then used in mutagenesis experiments to select mutants able to efficiently grow at low potential [151]. A mutant having a preferred ORP for growth of -280 mV vs SHE was able to produce 60% more PDO ($69.6 \text{ g}_{\text{PDO}} \cdot \text{L}^{-1}$) than its parent ($42.5 \text{ g}_{\text{PDO}} \cdot \text{L}^{-1}$) in fed-batch fermentation using their respective optimal ORP. It was also observed that the NADH/NAD⁺ ratio of this mutant was twice higher than in parent strain all along the fermentation time. This could have contributed to enhance the activity of the PDO dehydrogenase and consequently accelerate the PDO production. Similarly, Zhu *et al.* observed that *K. oxytoca* shifted its metabolism when the extracellular ORP decreased from -150 to -240 mV vs SHE [10]. Lower ORP accelerated glycerol consumption and enhanced

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PDO production while the bacterial growth was reduced. Proteomic analysis revealed that the abundance in PDO dehydrogenase encoded by the *yqhD* gene (see Section 1.3.11.3.1.4) increased by 7-fold when extracellular ORP decreased from -140 to -240 mV vs SHE. As PDO production was enhanced at lower potential, it is probable that this up-regulation was coupled to a higher availability in NADH, as reported with *K. pneumoniae* by Du *et al.* [151].

Overall, these studies show that extracellular ORP can influence the intracellular NADH/NAD⁺ ratio through metabolic regulations and subsequently redistribute the metabolic fluxes. For *K. pneumoniae* and *K. oxytoca*, low extracellular ORP was related with an enhancement of the PDO production as long as the strains were able to survive. If this behavior was to be generalized to other species, selecting mutants or microbial consortia able to grow at low extracellular ORP could be an efficient strategy for better PDO production from glycerol. Moreover, efficient strategies to control ORP would be necessary to benefit from such mutants. For this purpose, electrochemical tools could be of great interest for the possibility to influence ORP without adding any chemicals in the fermentation broth.

1.4 A novel tool for fermentation control: electro-fermentation

1.4.1 From microbial fuel cells to electro-fermentation

Bioelectrochemical systems (BESs) are bioreactors in which both biological and electrochemical processes can occur to generate electricity, hydrogen or other products of interest. To differentiate the various types of BESs, usually, a new name is given according to the product or service that is provided [152]. Initially, research on BESs mainly focused on the production of electricity in so-called microbial fuel cells (MFCs) [153–156]. Over the years, BESs have been used for many other applications, such as hydrogen production in microbial electrolysis cells (MECs) [157,158], chemical production from CO₂ reduction in microbial electrosynthesis processes (MES) [149,158,159] and water desalination in microbial desalination cells (MDCs) [160]. The main bottleneck of all these processes is the requirement of high current densities since electrons are either the desired product for MFCs, or the main driving force in MECs, MDCs and MES [161]. From the knowledge acquired by the use of these technologies, a new type of BES has been recently proposed to provide a novel mean to control and stabilize the fermentation process, with the possibility to exceed metabolic limitations of balanced reactions. Indeed, bioelectrochemical systems might be

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used to modify either extracellular ORP or directly intracellular ORP by supplying or collecting energy in the form of an electric current through the presence of electrodes, in a process so-called electro-fermentation (EF).

1.4.2 A novel type of BES: the electro-fermentation system (EFS)

1.4.2.1 *Electro-fermentation principles*

Electro-fermentation systems (EFS) could be defined as bioelectrochemical systems in which an electro-fermentation occurs to control self-driven fermentation. Electro-fermentation consists of operating the fermentation of an energy-rich substrate, such as a carbohydrate or an alcohol, in the presence of electrodes as supplementary electron source or sink. When the final product is more oxidized than the substrate (e.g. ethanol from glycerol), the working electrode (WE) works as an anode and is used to dissipate the excess of electrons in an anodic electro-fermentation (AEF). In contrast, for a reduced final product (e.g. butanol from glucose), the WE supplies electrons as a cathode in cathodic electro-fermentation (CEF). In this context, the electric current is not the product of interest nor the main energy source, but a trigger allowing the fermentation process to occur under unbalanced conditions. Moreover, in EF, the reaction is not only supported by the electronic current: even small current densities may affect both extracellular and intracellular ORP and thus the biological regulations through changes in the NADH/NAD⁺ ratio that can further impact the final fermentation product pattern [7,162–173]. The main difference between EF and other BESs is that EF does not require high current densities to occur. To discriminate these two processes, an electro-fermentation coefficient (η_{EF}) can be calculated (see Section 2.3.4.1). This parameter can also be used to estimate the energetic cost related to the production of a molecule of interest.

1.4.2.2 *Terminology*

As an emerging field of research, electro-fermentation has been investigated in only few studies and has not yet been well defined. Several terms have been used to describe this process, such as “unbalanced fermentation in microbial electrochemical cells” [162], “glycerol-fed bioelectrochemical system” [165], “bioelectrochemical fermentation” [166] or “electricity-driven biosynthesis” [167]. The concept and term of “electro-fermentation” was first proposed by Rabaey *et al.* [149] to designate this process. It was then used by several authors with the same meaning [7,166,174,175], but also to describe BES working as MFCs to produce H₂ and electricity from waste [176–179]. This lack of consensus may mislead the readers that are interested in this concept. To make more consistent this new way of using

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BES, we recommend the term “Electro-fermentation”. Conceptually, it is a clear way to designate a biological system that is mainly driven by the fermentative process, even though the metabolic pathways are influenced by the presence of electrodes.

1.4.3 Operational strategies for electro-fermentation

The effectiveness of the EFSs will mainly depend on (1) the interactions existing between microorganisms, (2) the dissolved redox couples of the medium, and (3) the interactions between microorganisms and the surface of the electrodes through cellular mechanisms of extracellular electron transfer (EET). Several strategies have been explored to ensure EET in electro-fermentation systems, as summarized in Table 1-7.

The use of pure cultures of electroactive microorganisms such as bacteria from the *Geobacteraceae* or *Shewanellaceae* families is of great interest because of their ability to perform direct electron transfer with the WE [180]. Such microorganisms are able to grow as an electroactive biofilm and thus directly interact with the WE. However, only few microorganisms, such as *Clostridium pasteurianum* [7], are known to be both electroactive and able to consume a large range of carbohydrates or alcohols [161,181]. To address this issue, co-cultures of electroactive and fermentative bacteria have been recently proposed to provide all the biological functions required for converting a substrate in electro-fermentation systems. As an illustration, such a strategy has been successfully applied with a co-culture of *Clostridium cellobioparum* and *Geobacter sulfurreducens* to produce ethanol from glycerol [163].

When none of the fermentative bacteria is electroactive, redox mediators such as neutral red [169] or methyl viologen [169,170] can be added to the fermentation medium and thus impact the extracellular ORP [162,169,172]. These chemicals can be oxidized or reduced by the fermentative bacteria and then recycled electrochemically at the electrode. They are here used as electron shuttles in a so-called mediated electron transfer [149,180]. Another way to add a redox mediator in the case of CEF is to produce H₂ at the cathode that could be further used as a one-way electron shuttle [164–167].

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Table 1-7. Electro-fermentation applications and operating parameters

Inoculum	Substrate	Aimed final product	Working potential (V vs. SHE)	Redox mediator	η_{EF}^*	Improvement vs. fermentation control	Ref.
Anodic electro-fermentation							
Engineered <i>Shewanella oneidensis</i>	Glycerol	Ethanol	0.40	No	0.25	No fermentative control	[168]
<i>Clostridium cellobioparum</i> <i>Geobacter sulfurreducens</i>	Glycerol	Ethanol	0.46	No	0.03	Acetate, H ₂ and formate removal Increased glycerol consumption	[163]
Engineered <i>Escherichia coli</i>	Glycerol	Ethanol Acetate	0.20	Methylene blue	0.02	Increased glycerol consumption rate	[162]
Cathodic electro-fermentation							
<i>Clostridium pasteurianum</i>	Glucose	Butanol	0.045	No	0.01	3-fold increase in butanol production yield	[7]
<i>Clostridium acetobutylicum</i>	Glucose	Butanol	NA	Methyl viologen	NA	26% increase in butanol production yield	[170]
<i>Clostridium tyrobutyricum</i>	Sucrose	Butyrate	-0.17	Neutral red	NA	30% increase in butyrate production yield	[169]
<i>Propionibacterium acidi-propionici</i>	Lactose	Propionate	-0.47	Cobalt sepulchrates	0.10	Propionate was the only product.	[171]
<i>Propionibacterium freudenreichii</i>	Glucose	Propionate	-0.39	Cobalt sepulchrates	0.15	No acetate production. Propionate was the only product.	[172]
<i>Clostridium pasteurianum</i>	Glycerol	1,3-propanediol	0.045	No	0.01	2-fold increase in 1,3-propanediol production yield	[7]
Mixed culture	Glycerol	1,3-propanediol	-0.90	No	0.34	2-fold increase in 1,3-propanediol production yield	[167]
Mixed culture	Glycerol	1,3-propanediol	$\sim -0.80^\dagger$	No	0.05	No fermentative control	[164]
Mixed culture	Glycerol	1,3-propanediol	$\sim -1.44^\dagger$	No	0.38	No fermentative control	[164]
Mixed culture	Glycerol	-	$\sim -1.28^\dagger$	No	NA	Increased glycerol consumption	[165]

* Electro-fermentation efficiency estimated from mass and electron balances available in the different studies.

† Bio-electrochemical reactors operated with an imposed electrical current

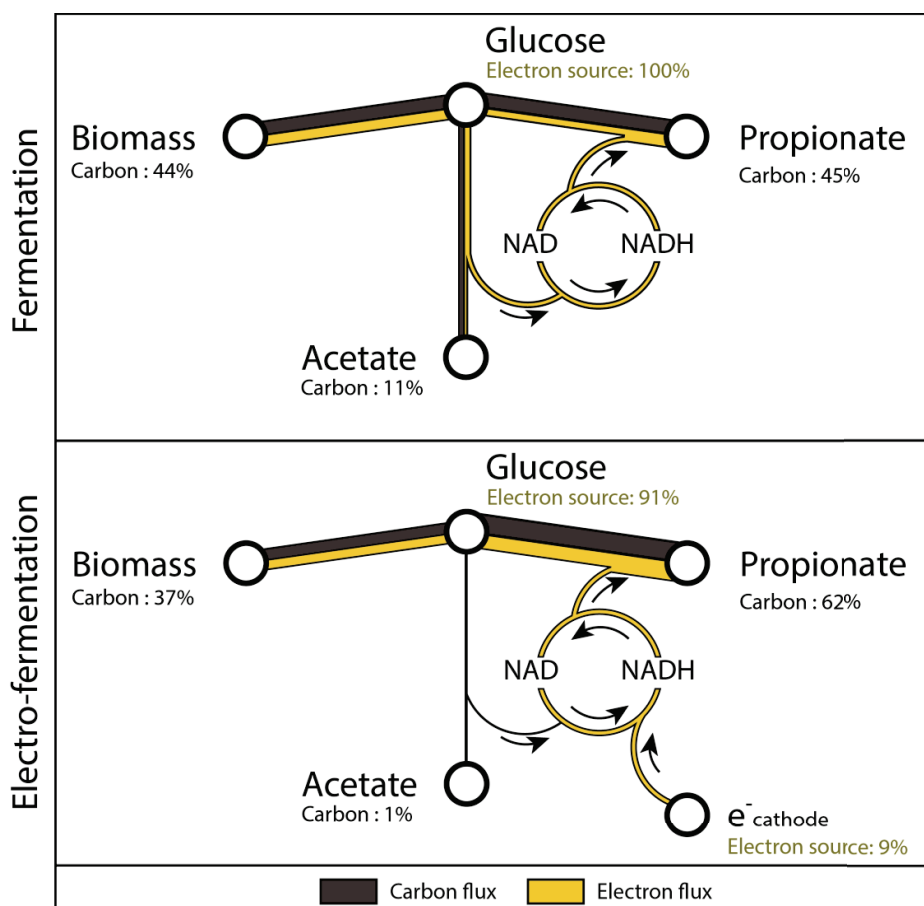


Figure 1-14. Comparison between a classical fermentation and an electro-fermentation with an electron mediator.

In addition, several authors proposed to metabolically engineer some fermentative bacterial strains of interest by adding the property of electro-activity. As an illustration, electron transfer in *Escherichia coli* was accelerated by 183% via a periplasmic heterologous expression of the c-type cytochromes CymA, MtrA and STC originated from *Shewanella oneidensis* [162]. However, in this case, the addition of methylene blue as electron shuttle was required. Reciprocally, electroactive bacterial species can also be engineered to uptake and grow on a broader range of substrates. This approach was performed on *S. oneidensis* to stoichiometrically convert glycerol to ethanol, a biotransformation that cannot occur unless two electrons are removed via an external reaction, here through electrode reduction [168].

Although research is emerging in this field, all of these methods are extendable to mixed culture fermentation processes, as long as the initial medium or microbial community contains components or bacteria able to interact directly or indirectly with the electrochemical system [164–167].

1.4.4 Hypothetical mechanisms of electro-fermentation

The mechanisms underlying the different observations in EF are not always well described. Likely, more than one basic mechanism is involved (see Figure 1-15).

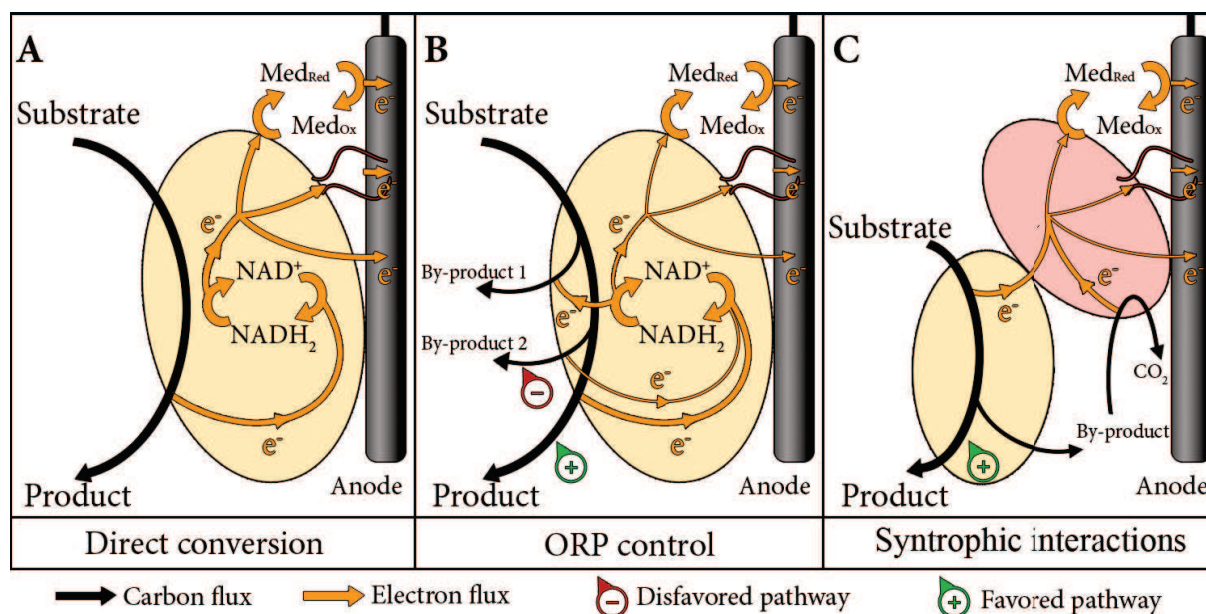


Figure 1-15. Hypothetical mechanisms that can occur during anodic electro-fermentation.

Mechanisms of cathodic electro-fermentation can be obtained by reversing all the electron fluxes. A: The substrate is directly converted into the product and the excess of electron is fully dissipated at the anode through mechanisms of extracellular electron transfer. B: The excess of electron generated during the oxidized products formation is not fully dissipated at the anode and part of the substrate is used for this purpose. Electron dissipation at the anode tends to decrease the $\text{NADH}_2/\text{NAD}^+$ ratio, resulting in regulations favoring one pathway to regenerate NADH_2 . C: The fermentative microorganism (yellow) consumes the substrate but is not able to interact with the anode. The electro-active microorganism (red) acts as a mediator between the fermentative microorganism and the anode through mechanisms of interspecies electron transfer. The electro-active microorganism also consumes by-products from the substrate fermentation, favoring the whole fermentation process.

1.4.4.1 Electron transfers and unbalanced fermentation

The electrodes present in the fermentation medium act like a non-soluble electron donor (cathode) or acceptor (anode) that is never limiting the reaction. Electron transfers between these electrodes and electro-active microorganisms can occur at the electrode surface through direct contacts or in presence of nanowires between the microorganisms and the electrode, or through extracellular polymeric substances produced by the microbial biofilms

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[149,150,180] (see Figure 1-16). Electron transfers can also be achieved without any biofilm formation through the presence of redox mediators either generated by fermentation, such as hydrogen, formate or acetate, or artificially added such as methyl viologen [149,150,180] or neutral red [169]. These EET mechanisms, well-described in the extensive literature dealing with the characterization of anodic reaction in MFCs, are likely to be also those that can occur during cathodic electron transfers [150].

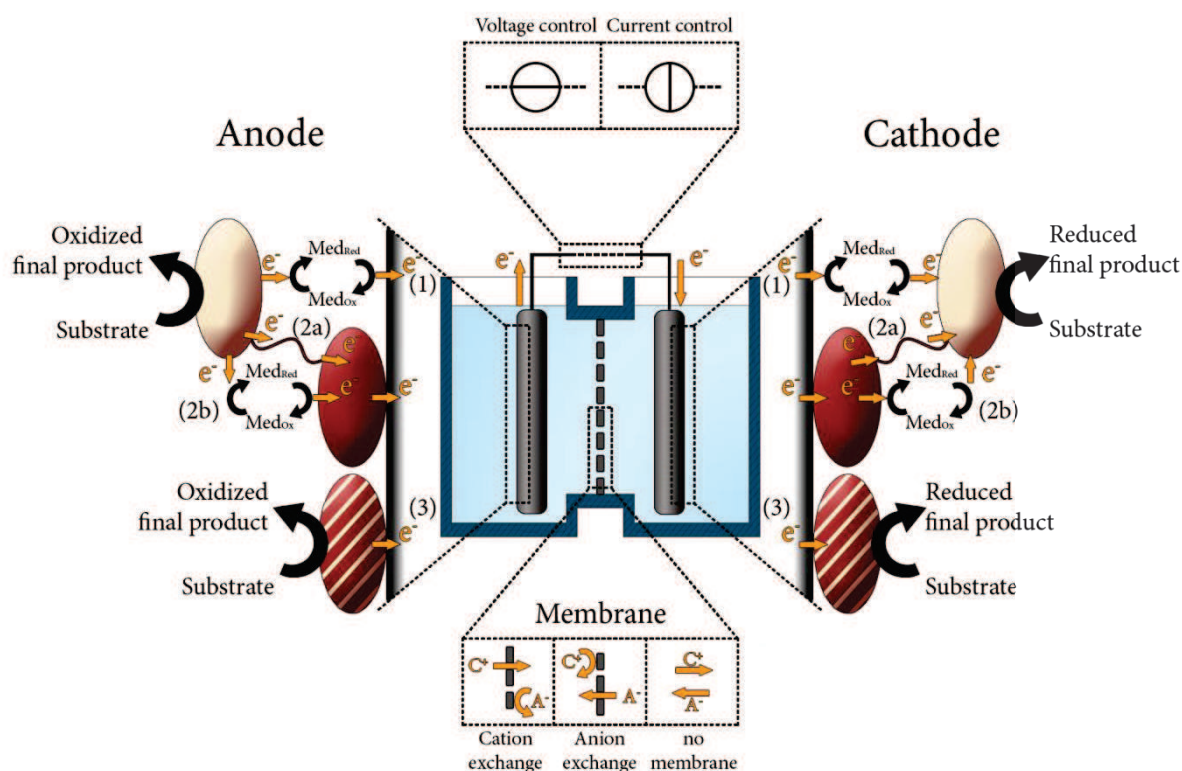


Figure 1-16. Different possible process configurations supporting the concept of electro-fermentation.

Interactions between electrodes and micro-organisms can be ensured using: (1) a redox mediator that can be oxidized (Med_{ox}) or reduced (Med_{red}) by an electrode and then used by fermentative micro-organisms (in light yellow); (2a) electro-active micro-organisms (in red) to perform a direct electron transfer to fermentative micro-organisms with nano-wires or (2b) to catalyze the regeneration of a redox mediator then used by fermentative micro-organisms; (3) electro-active micro-organisms that can also perform fermentation. The overall process can be voltage controlled to avoid electro-chemically produced by-products, or current controlled to ensure a high reaction rate. Different membranes can be used to separate the anodic and cathodic chamber depending on the compounds produced. Adapted from Rabaey and Rozendal (2010) [149].

In the context of EFSs, an immediate benefit of these EETs would be a direct dissipation of excess electrons in AEF [168], or a direct conversion of a substrate into a more reduced product in CEF [171–173] (see Figure 1-15A). Thus, CEFs is a kind of MES in which electrosynthesis starts from an electron-rich substrate instead of CO₂ (e.g. 1,3-propanediol from glycerol). Ideally, the substrate is stoichiometrically converted into the desired product.

1.4.4.2 Small current, high impact

Even though such a conversion has already been observed [168,171–173], electric current during EF is not always sufficient to explain the change in end products distribution [7,164,167]. The η_{EF} (see Section 2.3.4.1) were estimated from electron balances available in the different studies (see Table 1-7) and were often close to zero, indicating that significant impact on fermentation patterns was observed with only small current densities. For instance, Choi *et al.* [7] performed a CEF in which only 0.2% of the total electron input originated from the cathode. Considering a coulombic efficiency of 100% and that all these electrons were used to produce butanol from glucose, this would have led to a final butanol yield only 1.12-fold higher than the fermentation control (see Figure 1-17). The observed butanol yield increase was actually more than 3 times higher than the fermentation control, meaning that the electrons used for the extra butanol production were mainly diverted from other metabolic pathways.

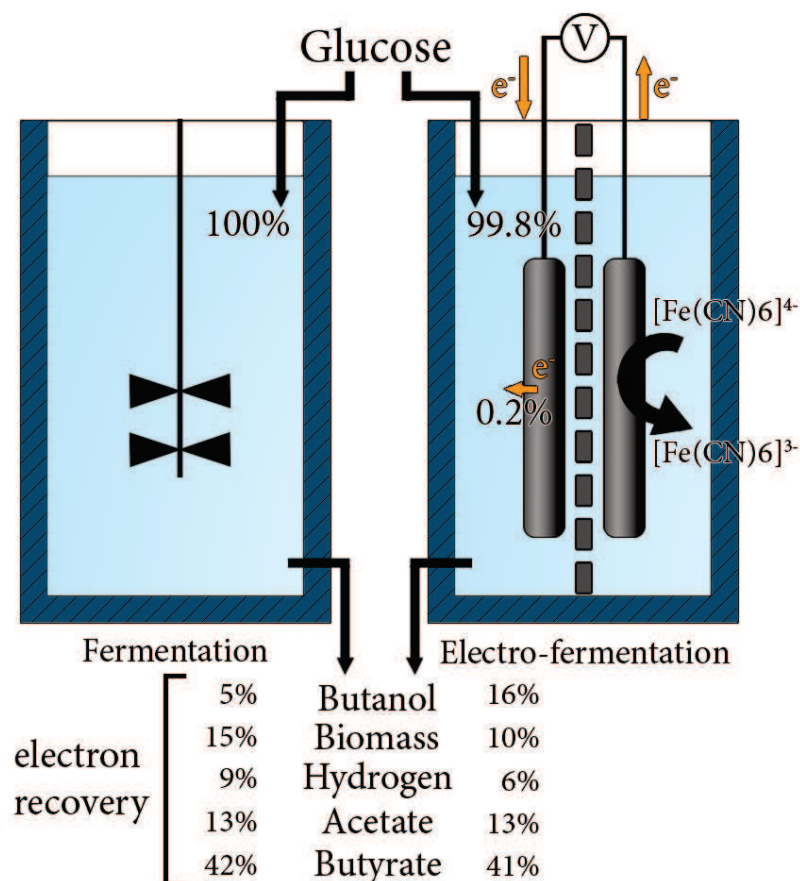


Figure 1-17. Comparison between a classical fermentation and an electro-fermentation with no electron mediator.

The values in percentage represent the initial electron contribution (substrates) or the electron recovery (products) obtained during experiments performed by Choi *et al.* [7]. Adapted from Harnisch *et al.* [175].

At a cellular level, the redox pairs homeostasis is crucial to ensure an optimal functioning of cellular metabolism [142,182]. Several metabolic regulatory enzymes are known to specifically detect changes in extracellular and intracellular ORP, and adjust electrons flow in the metabolism accordingly through NADH/NAD⁺ ratio stabilization [182,183]. It is expected that the NADH/NAD⁺ ratio might be affected by EETs with an electrode or soluble electron carriers as extra electron donor or acceptor [142]. In CEF operated with pure cultures, it was previously observed that more NADH was produced during EF when compared to the fermentation control. Choi *et al.* observed a NADH/NAD⁺ ratio at the beginning of EF that was 5 times higher than the one obtained in fermentation controls [7]. In response to such extra NADH, it was observed an increase of butanol

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production (net NADH-consuming) and a decrease of hydrogen and biomass production, with a final NADH/NAD⁺ ratio similar than the one obtained in the fermentation controls [7]. This would indicate that cellular regulations resulting from unbalanced NADH/NAD⁺ ratio have a stronger effect on metabolism than just a dissipation of the extra source of electrons (see Figure 1-15B) and, by extension, that other cellular mechanisms are involved. From a practical point of view, this would mean that EF can be performed with very low energy costs, resulting in a η_{EF} close to zero (see Table 1-7), albeit having high impact on the fermentation process. Also, in the cases EF was performed with redox mediators, a similar alteration of the NADH/NAD⁺ ratio was observed, meaning that an electro-active biofilm is not always essential for such a mechanism to occur [162,169,170].

1.4.4.3 *Syntrophic interactions*

Although the use of pure cultures is of great interest in EFSs, supplementary benefits can be obtained from the use of mixed cultures with both fermenters and electro-active bacteria. It was previously reported in MFCs that electro-active bacteria, able to perform anode respiration, are often associated in anodic biofilms with fermentative partners that can convert fermentable substrates into metabolites usable by the electro-active bacteria [9,184–187]. This relationship can be defined as syntrophic, as fermentative bacteria provide a substrate to electro-active bacteria that in return make the fermentation thermodynamically more favorable by removing the end-products [185]. The interactions between fermenters and electro-active bacteria rely on mechanisms of interspecies electron transfer (IET) either indirectly through the diffusion of electron carriers such as H₂, formate or other metabolites [9,188], or directly with the use of conductive pili [9,188–190], membrane to membrane contacts [9] or the presence of a conductive support on which a biofilm can attach [191–193]. These mechanisms usually occur in a biofilm in which contacts and interactions between microorganisms are favored. Such biofilms are spatially structured with electro-active bacteria being the most abundant organisms close to the electrode surface and fermenters dominating the top of the biofilm [163,194]. It is worth mentioning that the biofilm thickness can be a limitation for those interactions to occur. By increasing the biofilm thickness, the diffusivity in the biofilm decreases, resulting in gradients within the biofilm (e.g. pH, redox mediators) and a limitation of the IET [195].

Even though they occur at a limited rate, these interactions are of huge interest for EFSs as they can provide a substantial support to fermentative bacteria (see Figure 1-15C) [196]. Indeed, when co-metabolites such as organic acids or H₂ accumulate in too high

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concentrations in the fermentation bulk or headspace, they often strongly inhibit their own production and cell growth of fermentative bacteria, as observed in glycerol fermentation [163,166]. Their consumption by electro-active bacteria in the biofilm through IET mechanisms both stimulate the fermentation process and increase the purity of the final product by removing undesired by-products [163,197]. In this context, members of the *Geobacteraceae* family can be particularly preferred for their ability to consume several side-products of the fermentative pathways [181]. This mechanism is more likely to occur in an AEF because the electrons produced from the by-products oxidation can be transferred to the anode. However, it would also potentially exist in CEF if electrons are transferred from electro-active bacteria to fermenters through IET mechanism. Nonetheless the latter mechanism remains hypothetical and has never been proved in EF.

1.4.4.4 Mixed cultures

All of the mechanisms proposed above may also affect the selection of microbial populations when mixed cultures are used in EF. The addition of a driving force through a poised electrode creates a new ecological niche that may favor the growth of electroactive bacteria and their partners in a form of a mixed biofilm whose the microbial community is different from the planktonic community [164–167]. An indirect effect on population selection of planktonic bacteria would likely result in a significant effect on the final distribution of the fermentation products [166].

1.5 Conclusion

The increase of biodiesel production worldwide has led to an overproduction of glycerol. The use of this residual glycerol is necessary to make the biodiesel industry more sustainable. As glycerol is a well versatile precursor, its consumption is also an opportunity to produce useful value-added chemicals such as PDO. At industrial scale, the latter is currently produced from glucose by recombinant strains that convert glucose into glycerol before being able to produce PDO, thus achieving only low production yields. All the results reported in the present chapter clearly indicate that a glycerol-based production of PDO by fermentation is practically and economically feasible and could be an option to glucose-based processes.

The key parameters to optimize PDO production in glycerol fermentation fall into three different categories: (i) the first one concerns the process engineering of glycerol fermentation. Regarding PDO production, it is clear that fed-batch fermentation has to be preferred over batch and continuous fermentation systems. A first reason is that glycerol can be inhibitory at high concentrations and limit the efficiency of the process in batch. The second reason is that it is less important to optimize productivities than reaching high PDO titers in order to reduce downstream process costs. However, for research purpose, batch fermentation could be a simple and low-cost way to run glycerol fermentation when substrate concentration is kept low. (ii) The second key parameter is the choice of an appropriate inoculum. Nowadays, the best performances are achieved by recombinant species that require expensive fermentation media, sterile facilities and conditions that must be compatible with the use of GMOs. At the opposite, bacterial consortia can ferment glycerol under non-sterile conditions and without expensive vitamin addition while offering lower but yet significant performances for PDO production. Optimizing population selection procedures in mixed-culture glycerol fermentation could help structuring efficient microbial consortia that could be more competitive in term of production costs with GMO-based fermentations. Effectiveness of population selection procedures and maintenance depend on the choice of the environmental parameters that can drive the emergence of efficient PDO-producers and their bacterial partners in the microbial community. (iii) As third key parameter for optimizing PDO production, these environmental parameters are nevertheless limited in number. The two most influent parameters identified in pure culture fermentations are pH and ORP. In this respect, electro-fermentation could be a new way to act on redox balances in fermentation and be used to improve PDO production performances, in particular in mixed-culture processes.

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2.1 Overview of the materials and methods

This chapter gives a detailed account of the procedures that were followed in completing the experiments presented and discussed throughout the thesis. First, the specific materials and methods which were used in the different chapters are provided (one section for one chapter). Then the general techniques are detailed such a pure strain cultivation techniques or statistical analyses. A summary of the different experiments presented in the “Results and discussion” chapters is supplied as Table 2-1 and provides the main objectives of the studies along with important experimental parameters. An index presenting the different techniques employed for each study is also available as Table 2-2.

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Table 2-1. Summary of the main experimental parameters used in the “result & discussion” chapters

	Main objective	Inoculum	Type of reactor	Number of repetitions	C _{ini} * (g _{glycerol} .L ⁻¹)	pH	T (°C)	Potential (mV vs SHE)**
Chapter 3	- Assess effect of initial pH on PDO production by a chosen bacterial consortium	Mixed culture	500 mL glass bottle (200 mL working volume)	3	1.7	Buffered (4 to 10)	37	-
Chapter 4	- Evaluate the impact of an EF system on glycerol fermentation - Attempt to catalyze interactions between electrodes and bacteria by the addition of <i>G. sulfurreducens</i>	Mixed culture <i>G. sulfurreducens</i>	H-type reactors with dual chambers of 1L each (900 mL working volume)	2	17	Controlled at 7	37	-650
Chapter 5	- Proof-of-concept experiment to show that electro-active sp. can use fermentative sp. as electron acceptor	<i>C. pasteurianum</i> <i>G. sulfurreducens</i>	100 mL glass bottle (50 mL working volume)	4	10	Buffered At 6.5	37	-

* Initial concentration

** Potential applied to the working electrode of the electrochemical system.

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Table 2-2. Index of the different techniques employed for each study

Experiment	Techniques	Objective or measurement	Section
Wide range of pH (Chap 3)	HPLC	Metabolite concentrations	2.6.1
	GC	Gas composition	2.6.2
	MiSeq Sequencing	Bacterial population composition	2.7.3
	CE-SSCP	Bacterial population composition	2.7.4
	qPCR	Bacterial biomass quantification	2.7.2.1
	PCA	Bacterial population structure	2.2.4.1
	Correlation	Metabolite/Families correlation	2.9.1
	Mass balance	Pathway analysis	2.8
Electro-fermentation (Chap 4)	HPLC	Metabolite concentrations	2.6.1
	MiSeq Sequencing	Bacterial population composition	2.7.3
	Pure strain cultivation	<i>G. sulfurreducens</i> cultivation	2.5.3.1
		and pre-colonization on electrode	2.5.3.2
	Cyclic voltammetry	Electro-active bacteria detection	2.3.4.2
	Correlation	Metabolite/OTU correlation	2.9.1
	Flux-Balance analysis	Metabolic pattern for each OTU	2.3.4.3
	Mass balance		2.8
		Pathway analysis	2.3.4.1
Metabolite recycling (Chap 5)	HPLC	Metabolite concentrations	2.6.1
	GC	Gas composition	2.6.2
	CE-SSCP	Contaminant detection	2.7.4
	qPCR	Bacterial biomass quantification	2.7.2.2
	Pure strain cultivation	<i>G. sulfurreducens</i> cultivation	2.5.3.1
		<i>C. pasteurianum</i> cultivation	2.5.4
	Mass balance		2.8
		Pathway analysis	2.4.4.2

CE-SSCP: Capillary electrophoresis single-strand conformation polymorphism; GC: Gas chromatography; HPLC: High-performance liquid chromatography; OTU: Operational Taxonomic Unit; PCA: Principal Component Analysis; qPCR: Real-time polymerase chain reaction;

2.2 Mixed culture glycerol fermentation over a range of pH (Chapter 3)

2.2.1 Inoculum

The microbial inoculum used in this work was a mixed culture issued from a long-term continuous dark fermentation lab-scale reactor operated at pH 6.5 under micro-aerobic conditions for the production of H₂ from glycerol [198]. It was stored at 4°C for one month before use.

2.2.2 Fermentation medium

The composition of the fermentation medium (per liter of water) was modified from Dietz *et al.* [1] as follows: 1.66 g glycerol (Sigma-Aldrich, ≥99%), 1 g NH₄Cl and 0.5 g NaCl. In all experiments 20 mL/L of trace element solution (see Section 2.5.2) and 150mM phosphate buffer were added. The medium was not autoclaved.

2.2.3 Fermentation set-up

Batch experiments were performed in triplicates in glass bottles containing 200 mL of solution and around 300 mL of headspace. Bottles were sealed with butyl rubber septa and aluminum caps. Initial biomass was obtained after centrifugation of 33mL of the inoculum (Volatile Solids=0.40±0.01%total mass) at 12,000g for 15 min. The pellet was then suspended in the culture medium. Anoxic conditions were assured just after inoculation by flushing the media with high purity N₂ (>99.995%) for at least 30 min. The temperature was controlled at 37°C. Initial pH was adjusted at 4, 5, 6, 7, 8, 9 or 10 using 150mM phosphate buffer and NaOH (34%) or HCl (3M) solutions. After fermentation, final pH values were respectively 3.9±0.2, 4.2±0.2, 5.7±0.2, 6.9±0.1, 7.7±0.2, 8.0±0.2, 9.9±0.2.

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2.2.4 Specific techniques and calculations

2.2.4.1 Principal Component Analysis (PCA)

Principal component analysis (PCA) was used as multivariate analysis to explore bacterial population structures. The mathematical algorithm used in PCA creates a set of orthogonal basis vectors from a set of observations of possibly correlated variables. The new uncorrelated variables are called principal components. The first principal component is defined to explain the largest possible variance. Then, each succeeding component is defined to explain the highest possible remaining variance under the constraint that it is orthogonal to the preceding components. The PCA is so used to reduce the number of variables and reduce redundancy, which simplifies data interpretation. The principal component analysis (PCA) was performed on the microbial community compositions obtained from CE-SSCP with the R 2.12 software (R Development Core Team 2010), the vegan 2.12.2 package.

2.3 Mixed culture electro-fermentation of glycerol (Chapter 4)

2.3.1 Inoculum

The microbial inoculum used in this work was the same mixed culture used for pH experiments (see Section 2.3.1). It was stored at 4°C for two month before use.

G. sulfurreducens DSM 12127 was purchased from the DSMZ (Braunschweig, Germany) collection and grown as described in Section 2.5.3 prior to reactor inoculation.

2.3.2 Fermentation medium

Unsterile minimal medium with no vitamins or yeast extract was used for all experiments. The composition of the fermentation medium (per liter of water) was as follows: 17.5 g glycerol (Sigma-Aldrich, ≥ 99 %), 1.75 g NH₄Cl and 0.88 g NaCl. 10 mL of a trace element solution (see Section 2.5.2) and 100 mM phosphate buffer were added.

2.3.3 Fermentation and electro-fermentation set-up

Batch fermentation and electro-fermentation experiments were conducted in duplicates in potentiostat-controlled H-type reactors with dual chambers containing 900 mL of solution

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with 100 mL of headspace each. The two half-cells were separated with a cation exchange membrane (Fumasep® FAA-3-PK-130). The temperature was controlled at 37°C and pH was regulated at 7.0 by adding 2M NaOH (pH probe InPro 4260i, Mettler Toledo). Initial biomass was obtained after centrifugation of 140 mL of the mixed microbial inoculum (Volatile Solids = 0.40 ± 0.01 %_{total mass}) at 12,000g for 15 min. The pellet was then suspended in the culture medium. Anaerobic conditions were established just after inoculation by flushing the media with high purity N₂ (>99.995%) for at least 30 min. WE were 2.5 cm*2.5 cm*0.25 cm planar graphite plates and counter electrodes were 90% platinum–10% iridium grids. When a potential was applied, the WE was set at a fixed applied potential of -650 mV vs SHE to avoid electrochemical hydrogen production (see Figure 2-1), using a VMP3 potentiostat/galvanostat (BioLogic Science Instruments, France). For fermentation experiments, the electrical circuit was left open.

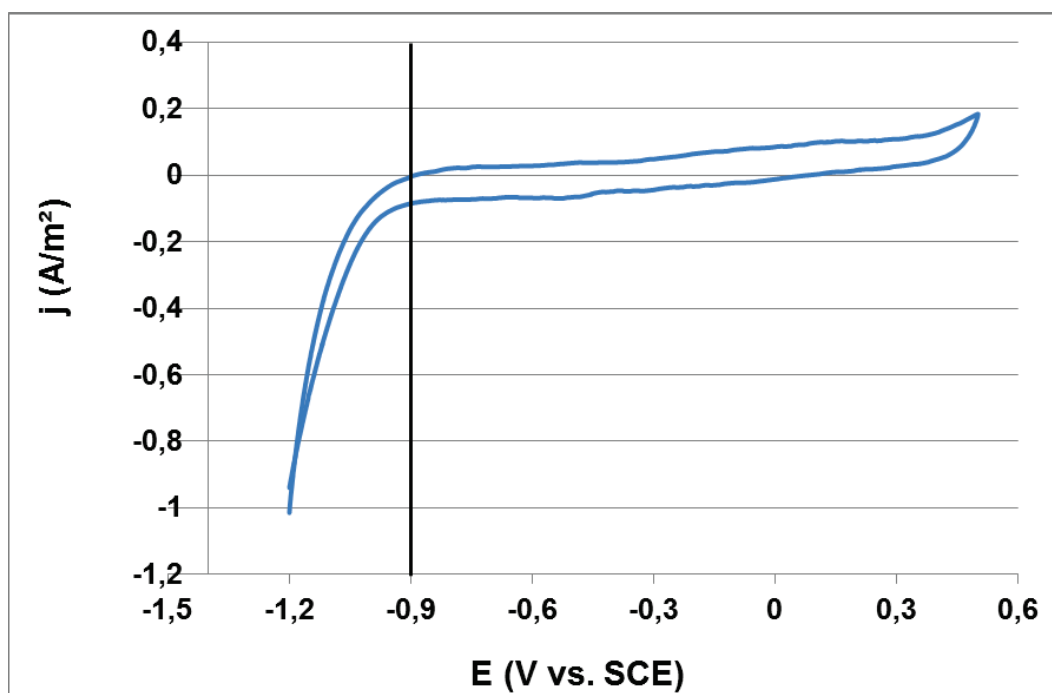


Figure 2-1. Abiotic CV control using the same reactor configuration and medium as during electro-fermentation experiments

The vertical black line corresponds to the potential chosen for electro-fermentation experiments.

For experiments with *G. sulfurreducens* pre-colonized electrodes, the protocol described in Section 2.5.3.1 was followed for the cultivation of *G. sulfurreducens* with an anode as sole electron acceptor (see Section 2.5.3.2). These colonized electrodes were then used for an electro-fermentation duplicate experiment labeled as “electro-fermentation with

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G. sulfurreducens pre-colonized electrodes” (EFG1). A second electro-fermentation batch series (EFG2) was also conducted using the biomass from the bulk of the two reactors with *G. sulfurreducens* pre-colonized electrodes as inoculum (10% w/w). The two working electrodes at the end of EFG1 were used as pre-colonized electrodes during EFG2. All the other parameters were the same as the first batch series.

2.3.4 Specific techniques and calculations

2.3.4.1 Electro-fermentation efficiency

The electro-fermentation efficiency is an index used to access the energy cost of an electro-fermentation. It corresponds to the ratio of the charge provided by the cathode over the charge recovered in the product. If it is close to 0, it means that the electrons provided by the cathode are negligible when compared to the product electron equivalent and that direct conversion was not the main mechanism (electrosynthesis). A higher value could indicate that: (i) direct conversion is occurring or (ii) electrons are dissipated through undesired side-reactions.

Electro-fermentation efficiencies were calculated as:

$$\eta_{EF} = \frac{Q_{e-}}{Q_{PDO}} \quad \text{Equation 2-1}$$

η_{EF} : electro-fermentation efficiency; Q_{e-} charge provided by the cathode; Q_{PDO} total charge in PDO i.e. the charge that would be produced by a total oxidation of the desired product. Q_{e-} was calculated from chronoamperometry, and Q_{PDO} by multiplying the molar amounts of PDO by its electron equivalent ($E_{eq} = 16$) and by the Faraday constant (96,485 C / mole-).

2.3.4.2 Cyclic voltammetry methods

In order to detect whether significant electro-active bacteria were attached to the working electrodes, cyclic voltammetry (CV) was performed at the end of all electro-fermentation experiments. The initial potential was -0.65V vs SHE and the maximum potential was +0.75V vs SHE. A slope of 1 mV.s⁻¹ was used and the CV was repeated 5 times. The last voltammogram of each experiment was then processed with QSOAS 1.0 [199] in order to remove the baseline and detect the different peaks.

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2.3.4.3 Estimation model of metabolic flux in mixed cultures

2.3.4.3.1 Model assumptions

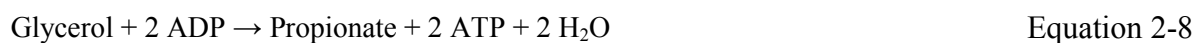
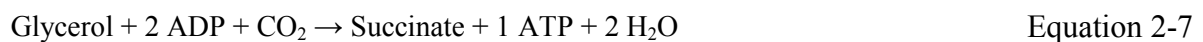
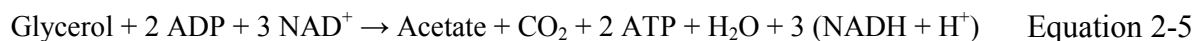
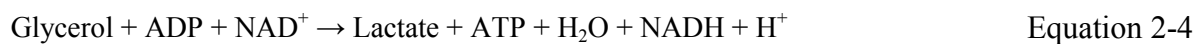
A linear inverse model was used to estimate the metabolite production of each OTU found in sequencing data of each mixed culture electro-fermentation experiment (see Chapter 4). Only the OTUs showing a relative abundancy higher than 1% in at least one reactor were selected in the model (15 OTUs).

The model assumptions were as follows: (ass.1) each OTU has the same metabolic profile in all reactors, (ass.2) each OTU contributes to the total metabolite production at a ratio equal to its abundancy (i.e. an OTU representing 30% of a reactor population will produce 30% of the metabolites of the reactor), (ass.3) each OTU has a balanced metabolism (the production and consumption of NADH and ATP are equals) and (ass.4) each OTU has closed electron and carbon mass balances.

In the model, six production yields were estimated: 1,3-propanediol (PDO), lactate, acetate, ethanol, succinate and propionate. The assumption (ass.2) was transformed into equations, by noting A_i the relative abundancy of the OTU $n^{\circ}i$, Y_j the experimental production yield of the product j and Y_{ij} the production yield of the product j by the OTU $n^{\circ}i$:

$$\frac{\sum A_i \cdot Y_{ij}}{\sum A_i} = Y_j \quad \text{Equation 2-2}$$

Because the sum of the A_i for the 15 OTUs considered in the model is not 1 (although it is always higher than 0.94), the left part of Equation 2-2 was normalized by the sum of the A_i in order to avoid an overestimation of the Y_{ij} . Equation 2-2 was thus written for the 6 estimated metabolite and the 8 reactors, leading to 48 equations. Then information about glycerol metabolism was added to constrain the model to produce only biologically compatible solutions. The following equations were used to describe the different metabolic pathways of the six products estimated (see Section 1.2):

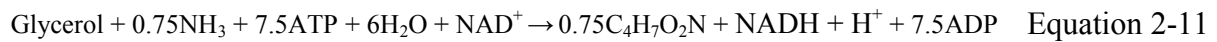


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The acetate and ethanol pathways (Equation 2-5 and Equation 2-6) were written as if only CO₂ and (NADH + H⁺) were produced during the conversion of pyruvate into Acetyl-CoA. However, H₂ and formate can also be produced during this conversion and were considered in the model with the following equations:



Biomass production was also taken into account using an elemental formula of C₄H₇O₂N a biomass production yield of 10.5 g/mol_{ATP} [38]:



Because of Equation 2-10, formate and H₂ are considered as equivalent for electron balances and were gathered in the model into the Y_i-(H₂+Formate) variable, as well as formate and CO₂ into the Y_i-(CO₂+Formate) variable for the carbon balance. Considering that only succinate production consumed CO₂ (15 inequations):

$$Y_{i\text{-Succinate}} + Y_{i\text{-(CO}_2\text{+Formate)}} \leq Y_{i\text{-Ethanol}} + Y_{i\text{-Acetate}} \quad \text{Inequation 2-12}$$

Considering that H₂ or formate was only produced during the conversion of pyruvate into acetyl-CoA (see Section 1.2), Y_i-(H₂+Formate) was also metabolically constrained for all OTU (15 inequations) by:

$$Y_{i\text{-(H}_2\text{+Formate)}} \leq Y_{i\text{-Ethanol}} + Y_{i\text{-Acetate}} \quad \text{Inequation 2-13}$$

Using Equation 2-3 to Inequation 2-13, the different assumptions of the model were converted into a set of equations and inequations. Assumption (ass.3) led to two equations for each OTU (*i.e.* 30 equations), traducing NADH (Equation 2-14) and ATP (Equation 2-15) mass balances:

$$-Y_{i\text{-PDO}} + Y_{i\text{-Ethanol}} + 3.Y_{i\text{-Acetate}} + Y_{i\text{-Lactate}} + \frac{4}{3}.Y_{i\text{-Biomass}} - Y_{i\text{-(H}_2\text{+Formate)}} = 0 \quad \text{Equation 2-14}$$

$$\frac{1}{10}Y_{i\text{-Ethanol}} + \frac{1}{5}Y_{i\text{-Acetate}} + \frac{1}{10}Y_{i\text{-Lactate}} + \frac{1}{10}Y_{i\text{-Succinate}} + \frac{1}{5}Y_{i\text{-Propionate}} - Y_{i\text{-Biomass}} = 0 \quad \text{Equation 2-15}$$

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Then, to ensure that the electron and carbon mass balances closed for each OTU, the number of electron and carbon equivalents of all metabolites were assessed (see Section 2.8) and led to the following equations (assumption (ass.4), 30 equations):

$$\begin{aligned} & \frac{16}{14} \cdot Y_{i\text{-PDO}} + \frac{12}{14} \cdot Y_{i\text{-Ethanol}} + \frac{8}{14} \cdot Y_{i\text{-Acetate}} + \frac{12}{14} \cdot Y_{i\text{-Lactate}} + Y_{i\text{-Succinate}} + Y_{i\text{-Propionate}} + \\ & \frac{16}{14} \cdot Y_{i\text{-Biomass}} + \frac{2}{14} \cdot Y_{i\text{-(H2+Formate)}} = 1 \end{aligned} \quad \text{Equation 2-16}$$

$$\begin{aligned} & Y_{i\text{-PDO}} + \frac{2}{3} \cdot Y_{i\text{-Ethanol}} + \frac{2}{3} \cdot Y_{i\text{-Acetate}} + Y_{i\text{-Lactate}} + \frac{4}{3} \cdot Y_{i\text{-Succinate}} + Y_{i\text{-Propionate}} + \\ & \frac{4}{3} \cdot Y_{i\text{-Biomass}} + \frac{1}{3} \cdot Y_{i\text{-(CO2+Formate)}} = 1 \end{aligned} \quad \text{Equation 2-17}$$

Finally, all the estimated yields were assumed to be positive (135 inequations):

$$0 \leq Y_{ij} \quad \text{Inequation 2-18}$$

In summary, a linear system of 108 equations and 165 inequations was obtained to estimate the 135 unknown parameters Y_{ij} . These unknown parameters were constrained as represented in Figure 2-2. This under-determined linear problem was solved using the function “lsei” of the package “limSolve” version 1.5.5.1 [200] on the R 3.2.3 software (R Development Core Team 2010). The function used pseudo-inverse matrices to solve the linear system and provided a unique solution that corresponded to the least square solution.

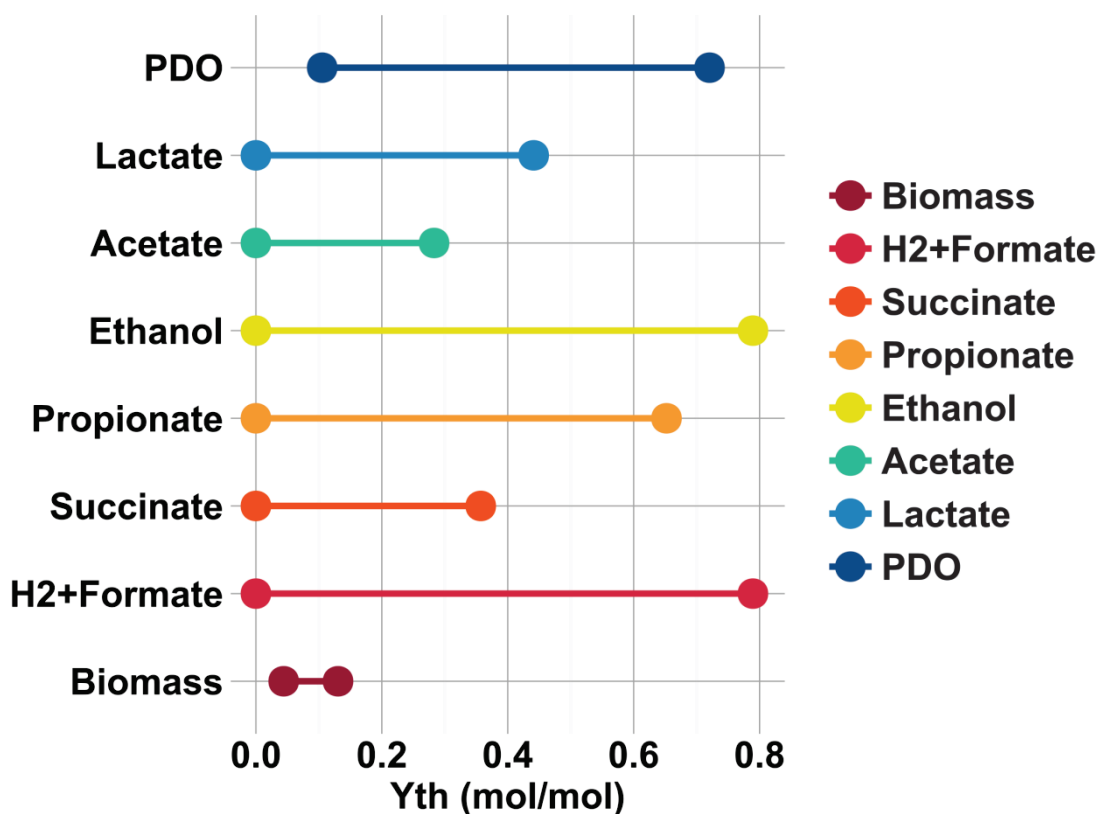


Figure 2-2. Theoretical range of production yield for the different metabolites considered in the linear model.

2.3.4.3.2 Model cross-validation

The model was validated using a k-fold cross-validation method. The model had 48 equations issued from the experimental observations, with only 8 independent reactors (6 equations per reactor). The cross validation was performed by removing six equations of one reactor and the model was calibrated on the 42 other equations. A root mean squared error of cross validation ($RMSE_{CV}$) was then calculated by comparing the predicted global production yields of the removed reactor with its experimental values. A standard deviation for each Y_{ij} was also extracted from the cross validation to assess the sensitivity of the model to one particular reactor.

2.4 Co-culture of *C. pasteurianum* and *G. sulfurreducens* (Chapter 5)

2.4.1 Inoculum

The co-culture was constituted of *G. sulfurreducens* DSM 12127 and *C. pasteurianum* DSM 525. They were both purchased from DSMZ (Braunschweig, Germany). These two microorganisms were cultivated according to Section 2.5.

2.4.2 Fermentation medium

A medium was designed for both co-culture experiments and control experiments with *G. sulfurreducens* and *C. pasteurianum* cultivated alone. The composition of the co-culture medium (per liter of water) was as follows: 10g glycerol ($\geq 99\%$), 0.82 g Na-Acetate, 2.00 g NH_4Cl , 0.75 g KCl , 2.45 g NaH_2PO_4 , 4.58 g Na_2HPO_4 , 0.28 g Na_2SO_4 , 0.26 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 2.90 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.50 g L-cysteine, 10 mL vitamin solution, 10 mL trace element solution (see Section 2.5.2), and 0.5 mL trace element solution SL-10 (DSMZ medium 320). The medium was then adjusted at pH 6.5.

2.4.3 Fermentation set-up

Experiments were conducted in quadruplicates using Hungate techniques and 100 mL serum bottles with 50mL of working volume and the fermentation medium was prepared as described in Section 2.5.1. *C. pasteurianum* was inoculated by adding 1 mL of *C. pasteurianum* full grown pre-culture (dilution 1/50). *G. sulfurreducens* pre-cultures were used after the cells naturally precipitated into red aggregates (~7 days). These pre-cultures were centrifuged at 3600 g for 10mn. Each pellet was then suspended in 2 mL of fresh co-culture medium (concentration x25). Finally, *G. sulfurreducens* was inoculated by adding 1 mL of this solution (final dilution 1/2). During experiments with different *G. sulfurreducens* inoculum concentrations, the concentrated inoculum solution was diluted using fresh co-culture medium prior to inoculation.

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2.4.4 Specific techniques and calculations

2.4.4.1 N_g calculations

To evaluate the growth of a population, the number of time a population doubles (N_g) can be used by calculating:

$$N_g = \frac{\ln(\frac{X_f}{X_i})}{\ln(2)} = \log_2(\frac{X_f}{X_i}) \quad \text{Equation 2-19}$$

Where X_i and X_f are the initial and final cell count respectively.

For each sample, 6 qPCR replicates were performed to assess the standard error of measurement of the technique. The raw qPCR results were \log_2 transformed before calculation of the variance between replicates. The standard error of measurement was calculated as:

$$\sigma = \sqrt{\frac{\sum Var}{n}} \quad \text{Equation 2-20}$$

with Var being the variance between the replicates of one sample, and n the number of samples. A value of $0.23 \log_2(\text{cell}).\text{mL}^{-1}$ was found. As a consequence, it was considered that there were no growth in samples with N_g inferior to twice σ ($\sim 0.5 \log_2(\text{cell}).\text{mL}^{-1}$).

2.4.4.2 Mass balance calculations for bacterial biomass

The molar amount of biomass was calculated by multiplying the cell counts obtained from qPCR by the respective mass of dried cell. For each strain, the later was determined using 12 samples from a unique pre-culture. From the samples taken, 6 were used for the qPCR analyses (cell quantification) and the other 6 were freeze-dried (measurement of mass of dried cells). Final values of $2.25 \cdot 10^{-13} \text{ g}_{\text{dried mass}}.\text{cell}^{-1}$ for *G. sulfurreducens*, and $5.86 \cdot 10^{-12} \text{ g}_{\text{dried mass}}.\text{cell}^{-1}$ for *C. pasteurianum* were obtained. Finally, the total mass was converted in mole using a molecular formula of $\text{C}_4\text{H}_7\text{O}_2\text{N}$ for the dry mass [54].

The acetate consumed by *G. sulfurreducens* was estimated using qPCR results and considering that 10% of the electron equivalents consumed by *G. sulfurreducens* was used for

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growth [201]. The remaining electron equivalents were considered to be transferred to *C. pasteurianum* as supplementary electron input (see Figure 5-4).

2.5 Pure strain cultivation

2.5.1 Anaerobic culture medium preparation for pure strains

Anaerobic strains were cultivated using 100 mL serum bottles sealed with butyl rubber stoppers and aluminum crimp caps, containing 50mL of cultivation medium. These bottles were prepared as follow:

- 1L of cultivation medium is prepared by dissolving all the ingredients corresponding to the optimal medium of a strain, except non-autoclavable compounds (*e.g.* vitamins, L-cysteine and compounds that could make the medium precipitate such as fumarate) and volatile compounds that could be stripped during the N₂ flush (*e.g.* sodium bicarbonate and ethanol). 1 mg/L of resazurin is added as a redox indicator.
- The medium is flushed with high purity N₂ (>99.995%) for at least 30 min.
- The volatile compounds are added and the pH is adjusted to the required value using concentrated NaOH or HCl solutions.
- The 100 mL serum bottles are flushed with high purity N₂ (>99.995%) for at least 10 min and filled with 50mL of cultivation medium. Then they are sealed with butyl rubber stoppers and aluminum crimp caps.
- All the bottles are autoclaved at 121°C during 20mn. They can be stored at room temperature afterwards.
- Prior to inoculation, all the non-autoclavable compounds are added to the medium using a sterile 0.2µm filter.

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2.5.2 Common vitamin and trace element solutions

Concentrated vitamin and trace element solutions were prepared in order to facilitate the preparation of cultivation media. They are used for pure culture experiments as well as for some mixed culture experiments (trace elements).

The trace element solution was composed of: 1.5 g/L Nitrilotriacetic acid; 3.0 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.50 g/L $\text{MnSO}_4 \cdot \text{H}_2\text{O}$; 1.0 g/L NaCl; 0.10 g/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; 0.18 g/L $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$; 0.10 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; 0.18 g/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$; 0.01 g/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$; 0.02 g/L $\text{KAl}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$; 0.01 g/L H_3BO_3 ; 0.01 g/L $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$; 0.03 g/L $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$; 0.30 mg/L $\text{Na}_2\text{SeO}_3 \cdot 5\text{H}_2\text{O}$; 0.40 mg/L $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$.

The vitamin solution was composed of: 2.0 mg/L Biotin; 2.0 mg/L Folic acid; 10.0 mg/L Pyridoxine-HCl; 5.0 mg/L Thiamine-HCl $\cdot 2\text{H}_2\text{O}$; 5.0 mg/L Riboflavin; 5.0 mg/L Nicotinic acid; 5.0 mg/L D-Ca-pantothenate; 0.1 mg/L Vitamin B₁₂; 5.0 mg/L p-Aminobenzoic acid; 5.0 mg/L Lipoic acid.

2.5.3 *Geobacter sulfurreducens*

Two methods were used to grow *G. sulfurreducens*: the first using 100 mL serum bottles and fumarate as final electron acceptor and the second using single cell bio-electrochemical reactor and an anode as final electron acceptor.

2.5.3.1 *With fumarate as electron acceptor*

The cultivation medium was prepared according to Section 2.5.1. The composition of the cultivation medium was as follows: 1.50 g/L NH_4Cl , 0.10 g/L KCl; 0.60 g/L Na_2HPO_4 ; 2.50 g/L NaHCO_3 ; 0.50 g/L L-cysteine; 0.82 g/L Na-acetate; 8.00 g/L Na_2 -Fumarate; 10mL/L trace element solution; 10mL/L vitamin solution (see Section 2.5.2) and pH was initially adjusted at 6.8.

The vitamin solution, Na_2 -Fumarate and L-cysteine were non autoclavable compounds and added prior to inoculation using a sterile 0.2 μm filter. The initial biomass inoculated corresponded to 5 mL of a grown *G. sulfurreducens* culture. Inoculated bottles were then stored and agitated in a room regulated at 35°C.

2.5.3.2 With an anode as electron acceptor

Electrodes were colonized with *G. sulfurreducens* using a single cell reactor containing 500 mL of medium and 300 mL of headspace. The temperature was controlled at 37°C and the medium was buffered at approximately pH 6.8. The inoculum was obtained after centrifugation of 50mL of pure *G. sulfurreducens* pre-culture at 12,000g for 15 min. The pellet was then suspended in the culture medium, composed as follows: 0.82 g/L Na-Acetate, 0.31 g/L NH₄Cl, 0.13 g/L KCl, 50mM phosphate buffer, 10mL/L trace element solution and 10 mL/L vitamin solution (see Section 2.5.2). Anaerobic conditions were established right after inoculation by flushing the media with high purity N₂ (>99.995%) for at least 30 min. Working electrodes (WE) were 2.5 cm*2.5 cm*0.25 cm planar graphite plates and the counter electrode was a 90% platinum–10% iridium grid. WE were set at a fixed potential of +450mV vs SHE in the same reactor, using a VMP3 potentiostat/galvanostat (BioLogic Science Instruments, France) in N-stat mode. The experiment was stopped when no more electric current was produced. A typical current production curve is provided in Figure 2-3.

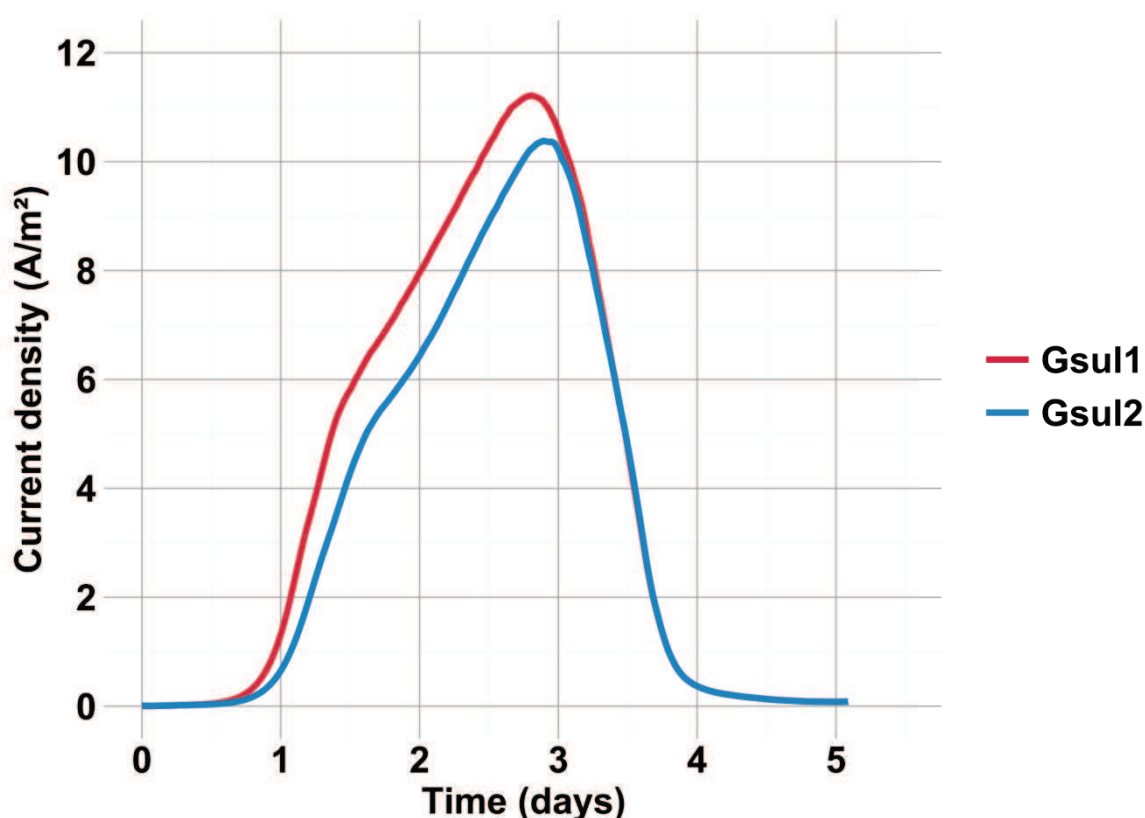


Figure 2-3. Current production during colonization of working electrodes by pure cultures of *Geobacter sulfurreducens*.

Gsul1 and *Gsul2* correspond to two working electrodes immersed in the same reactor (*Nstat* experiment).

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2.5.4 *Clostridium pasteurianum*

Clostridium pasteurianum was cultivated in 100 mL serum bottles with 50mL of working volume. The cultivation medium was prepared according to Section 2.5.1. The composition of the cultivation medium was as follows: 0.50 g/L NH₄Cl, 0.30 g/L KCl; 2.45 g/L NaH₂PO₄; 4.58 g/L Na₂HPO₄; 0.10 g/L Na₂SO₄; 0.15 MgCl₂·6H₂O; 10 g/L glycerol (Sigma-Aldrich, ≥99%); 0.50 g/L L-cysteine; 10mL/L trace element solution ; 10mL/L vitamin solution (see Section 2.5.2) and pH was initially adjusted at 6.5.

The vitamin solution and L-cysteine were non autoclavable compounds and added prior to inoculation using a sterile 0.2µm filter. The initial biomass inoculated corresponded to 5 mL of a grown *C. pasteurianum* culture. Inoculated bottles were then stored and agitated in a room regulated at 35°C.

2.6 Analytical chemistry methods

2.6.1 High-performance liquid chromatography (HPLC)

Concentrations of glycerol, PDO and organic acids were measured by HPLC with a refractive index detector (Waters R410). Samples were first centrifuged at 12,000g for 15 min and then supernatants were filtered with 0.2 µm syringe filters. HPLC analysis were performed at a flow rate of 0.4mL/min on an Aminex HPX-87H, 300 x 7.8 mm (Bio-Rad) column at a temperature of 35°C. H₂SO₄ at 4mM was used as mobile phase.

2.6.2 Gas chromatography (GC)

Biogas composition was determined using a gas chromatograph (Clarus 580, Perkin Elmer) equipped with a thermal conductivity detector. The columns used were a RtQbond column (for H₂, O₂, N₂ and CH₄) and a RtMolsieve column (for CO₂) and the gas vector was argon at a pressure of 3.5 bar.

2.7 Molecular biology techniques

2.7.1 DNA Extraction

During fermentation and electro-fermentation essays, DNA was extracted with the QIAamp fast DNA stool mini kit in accordance with manufacturer's instructions (Qiagen, Hilden, Germany). For pure strains or co-cultures of pure strains, DNA was extracted with the Wizard® Genomic DNA Purification Kit in accordance with manufacturer's instructions

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(Promega, Fitchburg, Wisconsin, United States of America). Extractions were verified using an Infinite 200 PRO NanoQuant (Tecan Group Ltd., Männedorf, Switzerland).

2.7.2 Real-time polymerase chain reaction (qPCR)

2.7.2.1 Open mixed culture assays

PCRs were prepared using 96-well real-time PCR plates (Eppendorf, Hamburg, Germany) and a Mastercycler ep gradient S (Eppendorf, Hamburg, Germany). Then, 6.5 µl of Express qPCR Supermix with premixed ROX (Invitrogen, France), 2 µl of DNA extract with three appropriate dilutions, 100 nM forward primer F338-354, 250nM reverse primers R805-785 (see Table 2-3), 50 nM TaqMan probe, and water were added to obtain a final volume of 12.5 µl for all analyses.

Table 2-3. Primers used during qPCRs

Specificity	Name	Sequence
<i>Bacteria</i>	F338-354	Forward 5'-ACTCC TACGG GAGGC AG-3'
	R805-785	Reverse 5'-GACTA CCAGG GTATC TAATC C-3'
<i>C. pasteurianum</i> DSM 525	W406	Forward 5'-GGAAT AGCCT CCCGA AAGGG-3'
	W407	Reverse 5'-TCCAA CTAGC TAATG CGCCG-3'
<i>G. sulfurreducens</i> DSM 12127	W410	Forward 5'-TGGGA AGTGC ATTGG AAAC T G-3'
	W409	Reverse 5'-GCGTC AGTAT CGGTC CAGAG-3'

An initial incubation of 2 min at 95°C and 40 cycles of denaturation (95°C, 7 s; 60°C, 25 s) were performed. One standard curve was generated from each assay by using 10-fold diluted solutions in sterile water (Aguettant Laboratory, Lyon, France) of a target plasmid (Eurofins Genomics, Germany). The initial DNA concentration was quantified using the Infinite 200 PRO NanoQuant (Tecan, France). The average number of bacterial cells was estimated by dividing the average number of 16S rRNA gene copies per cell by a factor of 4.1 [202].

2.7.2.2 Culture and co-culture of pure strains

PCRs were prepared using 96-well real-time PCR plates (Eppendorf, Hamburg, Germany) and Mastercycler ep gradient S (Eppendorf, Hamburg, Germany). Then, 12.5 µl of

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Express qPCR Supermix with premixed ROX (Invitrogen, France), 5 µl of DNA extract with three appropriate dilutions, 250 nM forward primer (W406 for *C. pasteurianum*, W410 for *G. sulfurreducens*, see Table 2-3), 250nM reverse primers (W407 for *C. pasteurianum*, W409 for *G. sulfurreducens*, see Table 2-3), and water were added to obtain a final volume of 25 µl for all analyses.

After an initial incubation of 2 min at 98°C, 40 cycles of denaturation (95°C, 15 s; 62°C, 30 s) were performed. One standard curve was generated from each assay by using 10-fold dilutions in sterilized water (Aguettant Laboratory, Lyon, France) of a target plasmid (Eurofins Genomics, Germany). The initial DNA concentration was quantified using the Infinite 200 PRO NanoQuant (Tecan, France). The average number of bacterial cells was estimated by dividing the average number of 16S rRNA gene copies per cell by a factor of 9 for *C. pasteurianum*, and by a factor of 2 for *G. sulfurreducens* [202].

2.7.3 Miseq Sequencing

The V4-V5 regions of the 16S rRNA genes were amplified using the primers 515F (5'-GTGYCAGCMGCCGCGGTA-3') and 928R (5'-CCCGYCAATTCMTTTRAGT-3'), which captures most of the bacterial and archaeal diversity [203]. The PCR mixtures (50 µl) contained 0.5 U of Pfu Turbo DNA polymerase (Stratagene) with its corresponding buffer, 200 mM of each dNTP, 0.5 mM of each primer, and 10 ng of genomic DNA. Reactions were performed in a Mastercycler thermal cycler (Eppendorf) as follows: 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 10 min. The amount and size of PCR products were determined using a Bioanalyzer 2100 (Agilent). The community composition was evaluated using MiSeq v3 (Illumina) with 2x300 bp paired-end reads at the GenoToul platform (<http://www.genotoul.fr>). Sequences were retrieved after demultiplexing, cleaning and affiliating sequences using Mothur [204].

2.7.4 Capillary electrophoresis single-strand conformation polymorphism (CE-SSCP)

DNA amplification for CE-SSCP was identical to the one performed for MiSeq sequencing. Then samples were heat-denatured at 95 °C for 5 min and re-cooled directly in ice for 5 min. CE-SSCP electrophoresis was performed in an ABI Prism 3130 genetic analyzer (Applied Biosystems) in 50 cm capillary tubes filled with 10% glycerol, conformation analysis polymer and corresponding buffer (Applied Biosystems). Samples were eluted at 12 kV and 32 °C for 30 min, as described elsewhere [205]. CE-SSCP profiles were aligned with an internal standard (ROX) to consider the inter-sample electrophoretic variability. CE-SSCP profiles were normalized using the StatFingerprints library [206] in R software version 2.9.2 (R. Development Core Team 2010).

2.8 Mass balance calculations

2.8.1 General calculations

Carbon and electron equivalent mass balances were calculated for all the experiments performed in this study to verify whether any major compounds was missing (non-closed balance) and compare the different fermentation products in similar units (*i.e.* Cmol for carbon balances or Emol for electron balances). The number of carbon equivalents (C_{eq}) of a compound corresponds to the number of atoms of carbon in one molecule of the compound (*e.g.* the number of carbon equivalents in glycerol - $C_3H_8O_3$ - is 3). The number of electron equivalents (E_{eq}) is comparable to the COD of a compound and must be calculated from the molecular formula as follows:

$$E_{eq} (C_w N_x O_y H_z^{n-}) = 4w - 3x - 2y + z + n \quad \text{Equation 2-21}$$

The Cmol and Emol of each compound were then calculated by multiplying the molar amount of this compound (obtained by HPLC or GC analysis) by the corresponding number of carbon and electron equivalents, respectively. Table 2-4 summarizes all the molar weights, carbon and electron equivalents of the compounds encountered during this study.

Carbon mass balances (resp. electron mass balances) were then calculated by dividing the final Cmol (resp. Emol) of each compound by the sum of the initial (or consumed) Cmol (resp. Emol) and by multiplying by 100 to obtain percentages. Illustratively, if the initial concentration of glycerol was 1 g/L, the initial Cmol content was equal to $1 \cdot 3/92 = 3.3 \cdot 10^{-2}$

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Cmol. At the end of the fermentation, if a concentration of 0.4 g/L of PDO was measured, this corresponded to $0.4 \times 3/76 = 1.6 \times 10^{-2}$ Cmol. Therefore the percentage of initial carbon content recovered as PDO was equal to $100 \times \frac{1.6 \times 10^{-2}}{3.3 \times 10^{-2}} = 48 \%$. A similar calculation for the percentage of initial electron content could have been made, and recovery as PDO would have been 55 %.

Table 2-4. Molar weight, carbon and electron equivalents of several compounds of glycerol metabolism.

Molecule	Formula	Molar weight (g/mol)	C _{eq}	E _{eq}
Dihydrogen	H ₂	2	0	2
Carbon dioxide	CO ₂	44	1	0
Formate	CHO ₂ ⁻	45	1	2
Acetate	C ₂ H ₃ O ₂ ⁻	59	2	8
Ethanol	C ₂ H ₆ O	46	2	12
Lactate	C ₃ H ₅ O ₃ ⁻	89	3	12
Propionate	C ₃ H ₅ O ₂ ⁻	73	3	14
Glycerol	C ₃ H ₈ O ₃	92	3	14
1,3-propanediol	C ₃ H ₈ O ₂	76	3	16
Succinate	C ₄ H ₅ O ₄ ⁻	117	4	14
Biomass	C ₄ H ₇ O ₂ N	101	4	16
Butyrate	C ₄ H ₇ O ₂ ⁻	87	4	20
Butanol	C ₄ H ₁₀ O	74	4	24

C_{eq} and E_{eq} stands for carbon equivalent and electron equivalent respectively.

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2.8.2 Biomass estimation

During fermentation and electro-fermentations assays, biomass was estimated to close the carbon and electron mass balances. The elemental constitution of biomass was assumed to be $C_4H_7O_2N$ with a biomass production yield of 10.5 g/mol_{ATP} [38]. ATP production was calculated from the metabolites measured at the end of fermentation, and was based on glycerol metabolism equations (see Section 1.2). The ATP production yields corresponding to each metabolite are provided in Table 2-5.

Table 2-5. Theoretical ATP production yields during glycerol fermentation

$Y_{ATP/PDO}$	0
$Y_{ATP/Ethanol}$	1
$Y_{ATP/Lactate}$	1
$Y_{ATP/Succinate}$	1
$Y_{ATP/Propionate}$	2
$Y_{ATP/Acetate}$	2
$Y_{ATP/Butanol}$	2
$Y_{ATP/Butyrate}$	3

2.9 Statistical analysis

2.9.1 Pearson correlations

Pearson correlations were used to assess statistical relationships between the variables encountered in the studies reported in this manuscript: bacterial population, environmental parameters and metabolic yields. The Pearson correlations and significance calculations were made with the R 3.1.3 software (R Development Core Team 2010). For correlation coefficient calculations, the function “rcorr” of the package Hmisc was used. Significance levels were assessed using random permutations with the function “sample” of the package combinat.

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2.9.2 Mean comparisons

In chapter 5, it was not possible to use parametric statistical tests such as t-test to compare the production yield means during co-culture experiments as the number of samples was too low. As a consequence, a two-sample Fisher-Pitman permutation test that does not require any distribution hypothesis was used. Two groups were compared ($n = 4$ and $n = 2$ resp.) with a total of 6 samples, meaning that 720 permutations could be generated. Therefore the p-values of the test were given at ± 0.0014 . The calculations were made using the “oneway_test” function of the package “coin” on R 3.1.3 software (R Development Core Team 2010).

Chapter 3. 1,3-propanediol production from glycerol in mixed culture fermentation over a wide range of pH

The aim of this chapter is to evaluate whether a previously selected mixed culture was suitable for efficient 1,3-propanediol production. Moreover, only few data is available in the literature on the effect of pH over both PDO production performances and microbial population structures. Therefore, pH values ranging from 4 to 10 were studied. The optimal pH value for this specific population was then further used with the same inoculum, as shown in the next chapter. The introduction, results and discussion of this chapter are issued from an article published in *Biotechnology for Biofuels*:

Moscoviz R, Trably E, Bernet N. Consistent 1,3-propanediol production from glycerol in mixed culture fermentation over a wide range of pH. *Biotechnol. Biofuels*. 2016;9.

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Chapter 3 - 1,3-propanediol production from glycerol in mixed culture fermentation over a wide range of pH

3.1 Introduction

Glycerol can be used as inexpensive carbon substrate to produce by fermentation many economically interesting chemicals including 1,3-propanediol (PDO). PDO is used for the production of solvents, cleaners, adhesives, resin and cosmetics. It can also be used as a monomer for the production of polytrimethylene terephthalate (PTT) in textile industry [207] (see Section 1.1.3). Many microorganisms from the *Enterobacteriaceae* and *Clostridiaceae* families are known as natural producers of 1,3-PDO from glycerol. So far, most studies about 1,3-PDO production from glycerol fermentation have focused on the use of pure cultures such as *Clostridium butyricum* [67] or *Klebsiella pneumoniae* [57] (see Section 1.3.1). High yields, productivities and final 1,3-PDO concentrations have been achieved with pure cultures which require sterile conditions and the use of yeast or meat extract in the culture medium. To reduce the costs, the use of mixed culture fermentations is of great interest because of their low operating costs. Indeed, cheap secondary products (*e.g.* molasses, crude glycerol) can be used as carbon sources during mixed culture fermentations without any sterilization or purification procedures [96–98]. Mixed consortia exhibit an improved resistance against contaminations [97] making possible their use in open fermentation systems (*i.e.* completely unsterile). When compared to pure culture processes, several other benefits can also be obtained from open fermentation such as better substrate utilization, *in-situ* production of nutrients by symbiotic species (*e.g.* growth factors and vitamins), inhibitors removal or all kind of syntrophic interactions [96–99].

Few articles have reported the use of mixed cultures to convert crude glycerol from biodiesel production into 1,3-PDO under non-sterile conditions. Dietz *et al.* [1] successfully used mixed cultures from municipal wastewater treatment plant and reached yields between 0.56 and 0.76 mol_{1,3-PDO} mol⁻¹_{glycerol} with a minimal culture medium containing crude glycerol. These production yields were slightly higher than the theoretical maximum yield of 0.72 mol_{1,3-PDO} mol⁻¹_{glycerol} (see Section 1.2) because of the impurities contained in crude glycerol that could be used as additional substrates. Selembo *et al.* [2] and Liu *et al.* [3] achieved 1,3-PDO production yields close to the theoretical maximum (resp. 0.69 and 0.65 mol_{1,3-PDO} mol⁻¹_{glycerol}) when using mixed culture on glycerol fermentation. However, efficient enrichment procedures are required to drive a natural mixed culture towards a well-defined consortium that performs only the aimed biological functions. Enrichment of a mixed consortium and maintenance of its specificity are probably the biggest challenges regarding mixed culture fermentation processes. The usual procedure for the selection of an efficient consortium is

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based on cultivation in sequencing batch or continuous reactors with a selection pressure imposed by the operating conditions [96,98]. The main environmental parameters used to select specific microbial consortium are pH, temperature, organic loading rate (OLR), medium composition and hydraulic retention time (HRT) in continuous processes. Previous reported results using mixed cultures were obtained in different experimental conditions and, in particular, with pH values ranging from 5.5 to 8 but with different sources of glycerol [1–4], making difficult to outline the effects of pH. Moreover, pH has been reported to have significant effect towards PDO production in pure culture fermentation and is expected to be an important parameter in mixed-culture as well (see Section 1.3.3.2).

The aim of this work was to investigate the effect of initial pH on batch production of 1,3-PDO under non-sterile conditions using a mixed culture as inoculum. Hence a minimal culture medium containing only pure glycerol with no additives such as yeast extract was used in order to reduce the sources of variability other than pH.

3.2 Experimental design

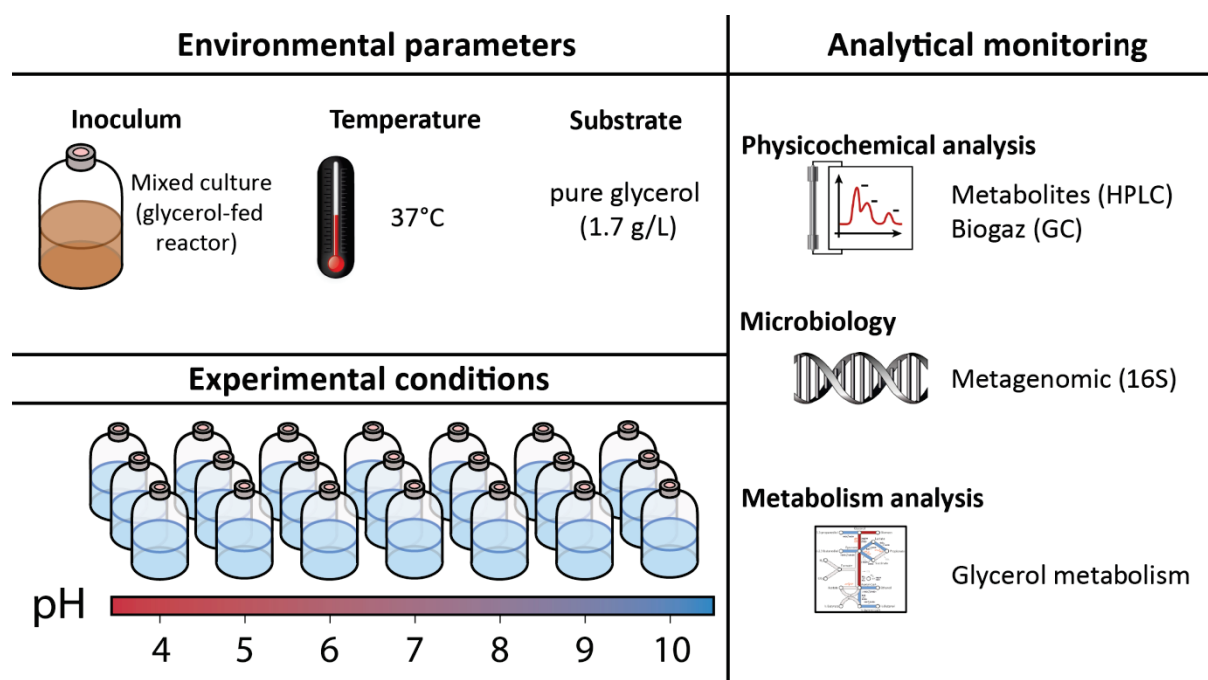


Figure 3-1. Experimental design for the study of 1,3-propanediol production from glycerol in mixed culture fermentation over a wide range of pH.

A simple experimental design plan was followed in this study: initial pH between 4 and 10 were studied in triplicate batch reactors all inoculated at the same time (see Figure 3-1). The inoculum used in this study was issued from a continuous reactor fed with glycerol

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under micro-aerophilic conditions. A constant temperature of 37°C was imposed by a heating chamber as this temperature was reported in the literature as optimal for PDO production in mesophilic conditions (see Section 1.3.3.1). There was no pH control system in this experiment. In order to avoid biases due the use of different pH buffers, pH was buffered using concentrated phosphate buffer (150mM) for all pH values. This buffer is efficient when pH is around 7.21 (value of pK_{a2}) and would not be suitable for extreme pH values such as 4 and 10. Thus a low initial glycerol concentration of 1.6 g.L⁻¹ was chosen in order to avoid excessive acidification of the fermentation broth. As reported by Samul et al. [208], the effects of crude glycerol impurities on the fermentation patterns can substantially vary, depending on their composition and the source of microorganisms. The additional effect of impurities would make it difficult to assess what is the effect of pH on microbial communities. Thus refined glycerol (Sigma-Aldrich, $\geq 99\%$) was used in this study. Methods employed during this study are more specifically described in Section 2.2.

3.3 Results

3.3.1 Effect of pH on fermentation products

To evaluate the effect of initial pH on glycerol fermentation by a mixed culture, a range of initial pH values between 4 and 10 was investigated in batch reactors. A low initial concentration of 1.66 g.L⁻¹ of glycerol was used to avoid a pH drop during the fermentation. COD mass balances are shown in Figure 3-2. COD mass balance closed between 93% and 102%, indicating that no major metabolic by-product was missed during the batch fermentation. After 3 days of fermentation, glycerol was depleted in most of the reactors except those running at the extreme pH 4, 5 and 10 with 95.4%, 8.1% and 93.0% of the initial glycerol remaining, respectively. It was assumed that no fermentation occurred at pH 4 and 10. For all other pH values, the main metabolite produced was PDO (60-74%_{total COD}) with acetate as major by-product (11-17%_{total COD}). The PDO production yields ranged from 0.52±0.01 to 0.64±0.00 mol_{PDO} mol⁻¹_{glycerol}. The best values were obtained at pH 7 and 8 and corresponded to 90% of the maximum theoretical yield of 0.72 mol_{PDO} mol⁻¹_{glycerol} [1] with a final concentration of 0.86±0.00 g.L⁻¹. Ethanol was only produced for pH values below 6 (6-9%_{total COD}) while acetate production was decreasing. At pH values over 7, formate production increased from 0 to 9%_{total COD}. H₂ was only detected for pH values below 7 and represented less than 1% of the total COD. Methane was not detected in any condition, which was not surprising since the initial inoculum originated from an output of a continuous reactor in

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which methanogenesis did not occur (low HRT). Although basic pH around 7-8 may favor the emergence of methanogens in long term operation of the reactor, several studies reported that high PDO final titers were obtained at pH between 5 and 6 [94], and pH 8 [2] without methane production.

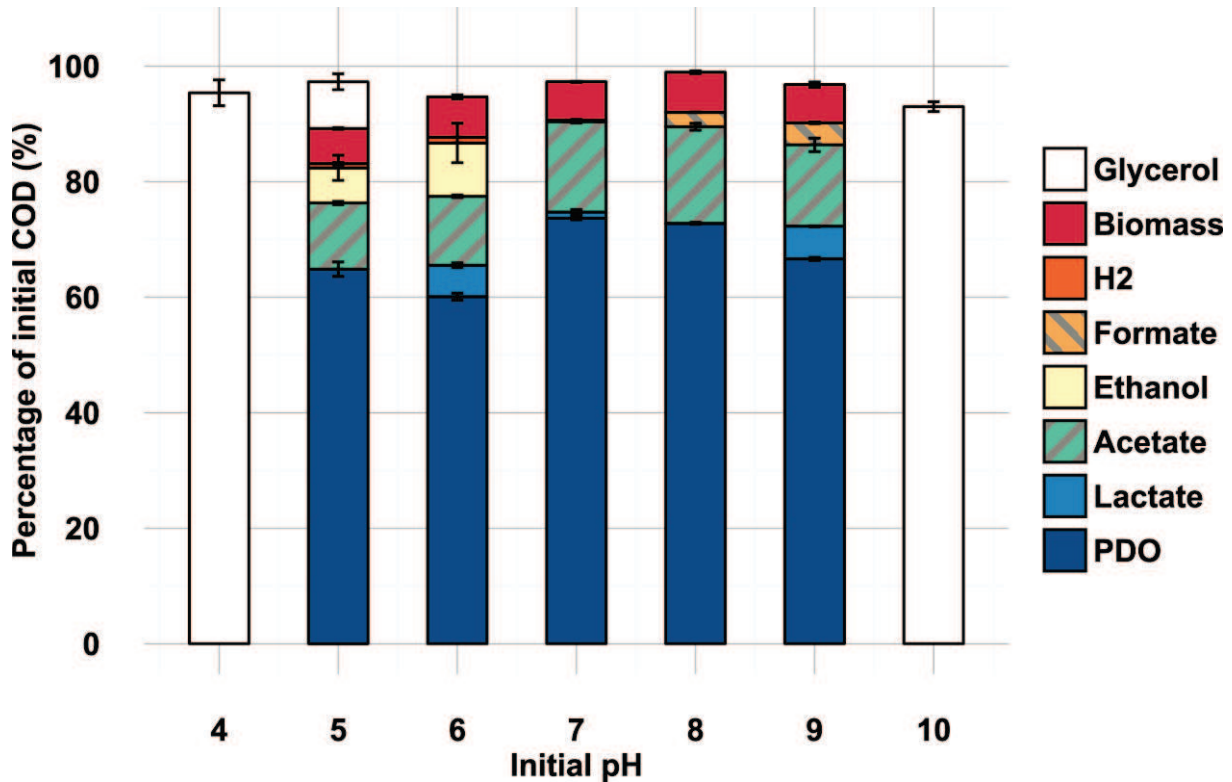


Figure 3-2. COD balances calculated from the metabolites measured after 3 days of fermentation in triplicate experiments.

Results are normalized on initial COD. The biomass was estimated from the ATP production associated to the different metabolites production.

3.3.2 Comparison with theoretical yields

Metabolic pathways of glycerol fermentation are well-known and have been described in Section 1.2. In order to find the global reactions leading to i) maximal PDO production, ii) maximal biomass growth and iii) minimal biomass growth, the following redox and ATP balanced reactions were calculated by aggregating the equations of glycerol metabolism as provided in Section 1.2 and presented in Figure 3-3 :

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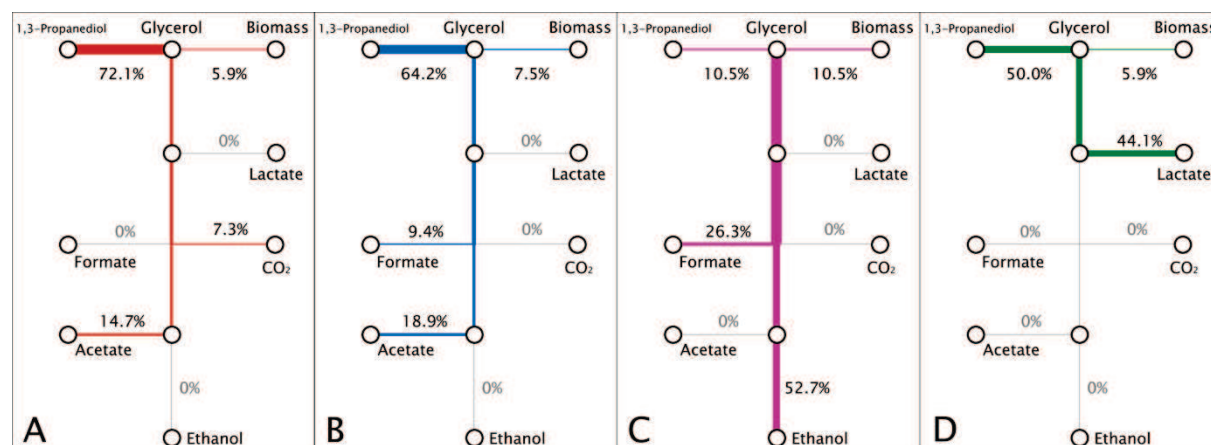
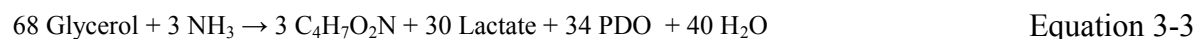
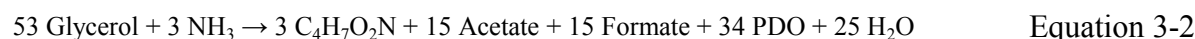
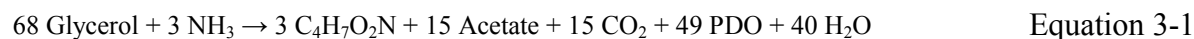


Figure 3-3. Carbon flux trees according to theoretical pathways.

A: Maximal 1,3-propanediol production; B: Acetate and Formate pathways; C: Maximal growth yield; D: Minimal growth yield. The values in percentage represent the proportion of initial carbon that is found in the final products.

The maximal theoretical production yield of PDO ($0.72 \text{ mol.mol}^{-1}$) could be obtained when only acetate was produced, according to Equation 3-1. The theoretical maximal growth was reached when ethanol was produced together with formate as in the Equation 3-4, leading to a minimal PDO yield of $0.11 \text{ mol.mol}^{-1}$. The theoretical biomass growth was minimal if only lactate and acetate were produced (Equation 3-1 and Equation 3-3). Lactate production was less favorable for PDO production than acetate production. The production of formate together with acetate had also a negative impact on PDO (Equation 3-2). These theoretical values have been compared to the actual values obtained at different pH values and are shown in Table 3-1. In this study, the best PDO production values were obtained at pH 7 and 8 and were close to those obtained with Equation 3-2 (i.e. $Y_{\text{Acetate/S}} = 0.28 \text{ mol.mol}^{-1}$ and $Y_{\text{PDO/S}} = 0.64 \text{ mol.mol}^{-1}$) but with much less formate or hydrogen produced, maybe due to measurement errors in hydrogen production.

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Table 3-1. Comparison of the experimental yields obtained in this study with theoretical yields calculated considering anabolism and catabolism.

	Theoretical values				Experimental values				
	Maximal PDO production	Minimal growth yield	Maximal growth yield	Acetate +formate pathway	pH 5	pH 6	pH 7	pH 8	pH 9
$Y_{X/S}$ (g/mol) ^a	4.45	4.45	7.97	5.72	5.39 ± 0.25	6.18 ± 0.62	5.90 ± 0.14	6.15 ± 0.41	5.87 ± 0.80
$Y_{ATP/S}$ (mol/mol) ^b	0.44	0.44	0.79	0.57	0.51 ± 0.02	0.59 ± 0.06	0.56 ± 0.01	0.59 ± 0.04	0.56 ± 0.07
$Y_{PDO/S}$ (mol/mol)	0.72	0.50	0.11	0.64	0.61 ± 0.04	0.52 ± 0.01	0.64 ± 0.00	0.63 ± 0.01	0.58 ± 0.01
$Y_{Acetate/S}$ (mol/mol)	0.22	0	0	0.28	0.21 ± 0.02	0.21 ± 0.01	0.27 ± 0.01	0.29 ± 0.02	0.25 ± 0.04
$Y_{Ethanol/S}$ (mol/mol)	0	0	0.79	0	0.07 ± 0.05	0.11 ± 0.07	0	0	0
$Y_{Lactate/S}$ (mol/mol)	0	0.44	0	0	0	0.06 ± 0.01	0.01 ± 0.01	0	0.07 ± 0.00
$Y_{(Formate+H_2)/S}$ (mol/mol)	0	0	0.79	0.28	0.06 ± 0.01	0.07 ± 0.00	0.01 ± 0.01	0.17 ± 0.01	0.26 ± 0.02

a The biomass yield was calculated assuming an elemental composition of $C_4H_7O_2N$ [38] and that all the ATP produced was used for biomass production.

b The ATP yield was calculated from the metabolites measured after three day of fermentation: $Y_{ATP/Acetate} = 2$; $Y_{ATP/Ethanol} = 1$; $Y_{ATP/Lactate} = 1$; $Y_{ATP/PDO} = 0$.

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3.3.3 Microbial communities and growth

Biomass was estimated after 3 days of fermentation from qPCR on total bacterial DNA. The low initial biomass concentration of $5.9 \pm 1.7 \cdot 10^5$ bact.mL⁻¹ after inoculation could explain the long lag phase observed at all pH values. The final biomass concentration ranged between 10^8 and 10^9 bact.mL⁻¹ in all reactors in which glycerol fermentation occurred, except for the reactors running at pH 9 ($7.4 \pm 1.3 \cdot 10^6$ bact.mL⁻¹). This value obtained at pH 9 is very low compared to the biomass estimated with ATP production. This could be due to ATP dissipation for maintaining intracellular pH at 7 (see Section 1.3.3.2). Therefore, it was clear that bacterial growth was strongly inhibited at extreme pH values lower than 5 and above 8.

To observe the effect of pH on microbial communities, MiSeq sequencing was performed on the inoculum and on samples after 3 days of fermentation (Figure 3-4). The inoculum was mainly composed of bacteria from the *Clostridiaceae* and *Enterococcaceae* families (resp. 50% and 18% of 82243 sequences). Two OTUs were dominant, one in each family, and represented 46% and 18% of the total bacterial community. Nucleotide sequence analyses of their 16S rRNA genes revealed resp. 99% and 100% of sequence homology with *Clostridium intestinale* and *Enterococcus cecorum*. *C. intestinale* is known to be an aerotolerant species, able to grow on glycerol and to produce H₂ [209–211], which is consistent with the inoculum origin. After three days of fermentation, the bacterial community observed at pH 9 was very close to the inoculum, probably because there was practically no bacterial growth. For every other pH condition, an *Enterobacteriaceae* species was enriched whose 16S rRNA gene had 100% of sequence homology with *Citrobacter freundii*, a species studied for PDO production from glycerol [20,83]. A *Brucellaceae* species which had 100% similarity with *Ochrobactrum anthropi* was also favored at pH 5.

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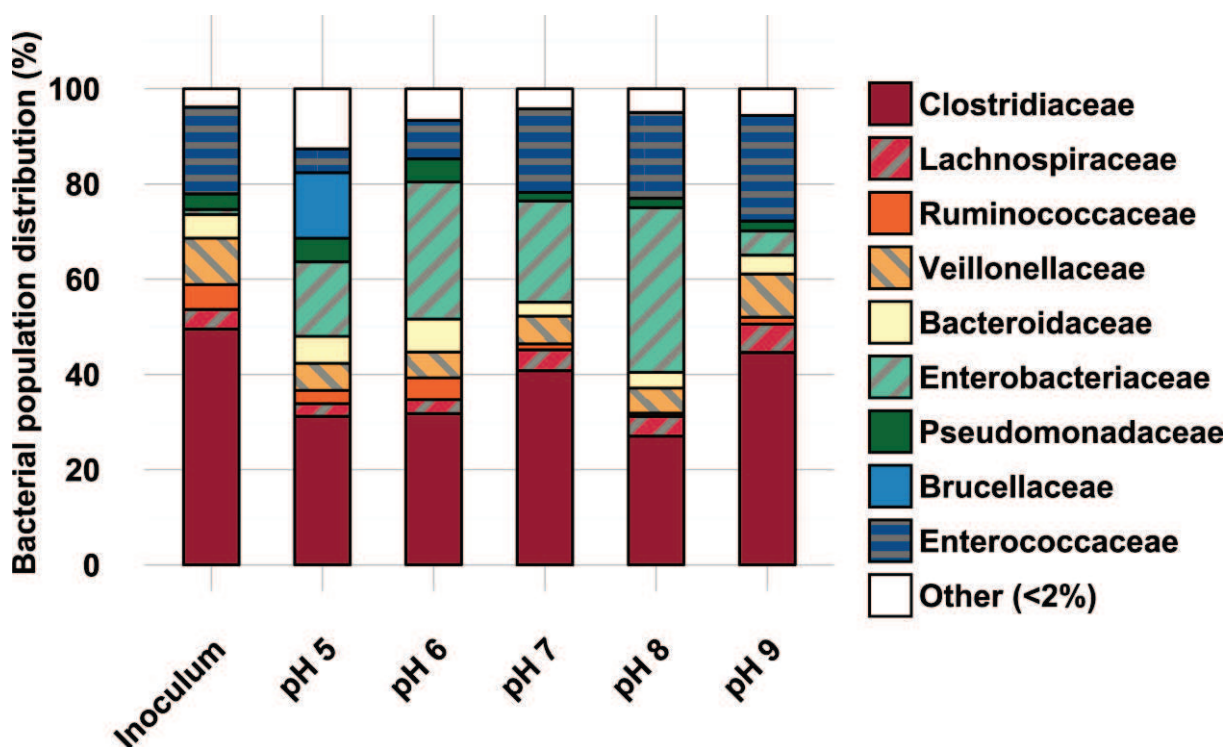


Figure 3-4. Bacterial population distribution within the taxonomic families of the inoculum and after 3 days of fermentation at different pH values.

This distribution is based on 16S rRNA genes identification retrieved from MiSeq sequencing.

“Other” stands for the families containing less than 2% of the total bacterial populations.

3.3.4 Correlations between microbial community and fermentation patterns

In order to highlight correlations between the composition of microbial communities and fermentation patterns, a Pearson correlation matrix was calculated with the bacterial families and metabolites produced as variables (Figure 3-5). PDO was found to be positively correlated to acetate ($r = .64$, $p \leq 0.01$) and negatively correlated to lactate ($r = -.78$, $p \leq 0.001$), ethanol ($r = -.65$, $p \leq 0.01$) and hydrogen ($r = -.60$, $p \leq 0.05$). It was also negatively correlated to the emergence of bacteria from the *Pseudomonadaceae* ($r = -.85$, $p \leq 0.05$), *Ruminococcaceae* ($r = -.92$, $p \leq 0.05$) and *Bacteroidaceae* ($r = -.96$, $p \leq 0.01$) families. A hierarchical cluster analysis on the Pearson correlation matrix also highlighted two groups of bacteria. The first one was composed of bacteria from *Veillonellaceae*, *Clostridiaceae*, *Lachnospiraceae* and *Enterococcaceae* families and was linked with formate production. The second one was composed of bacteria from *Pseudomonadaceae*, *Ruminococcaceae*,

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Bacteroidaceae and *Brucellaceae* and linked with ethanol and hydrogen production. There were high positive correlation between ethanol and the presence of *Brucellaceae* bacteria ($r = 0.99$, $p \leq 0.001$), and hydrogen production and the presence of *Pseudomonadaceae* bacteria ($r = 0.93$, $p \leq 0.05$). Lactate was not found to be correlated to a specific group of bacteria.

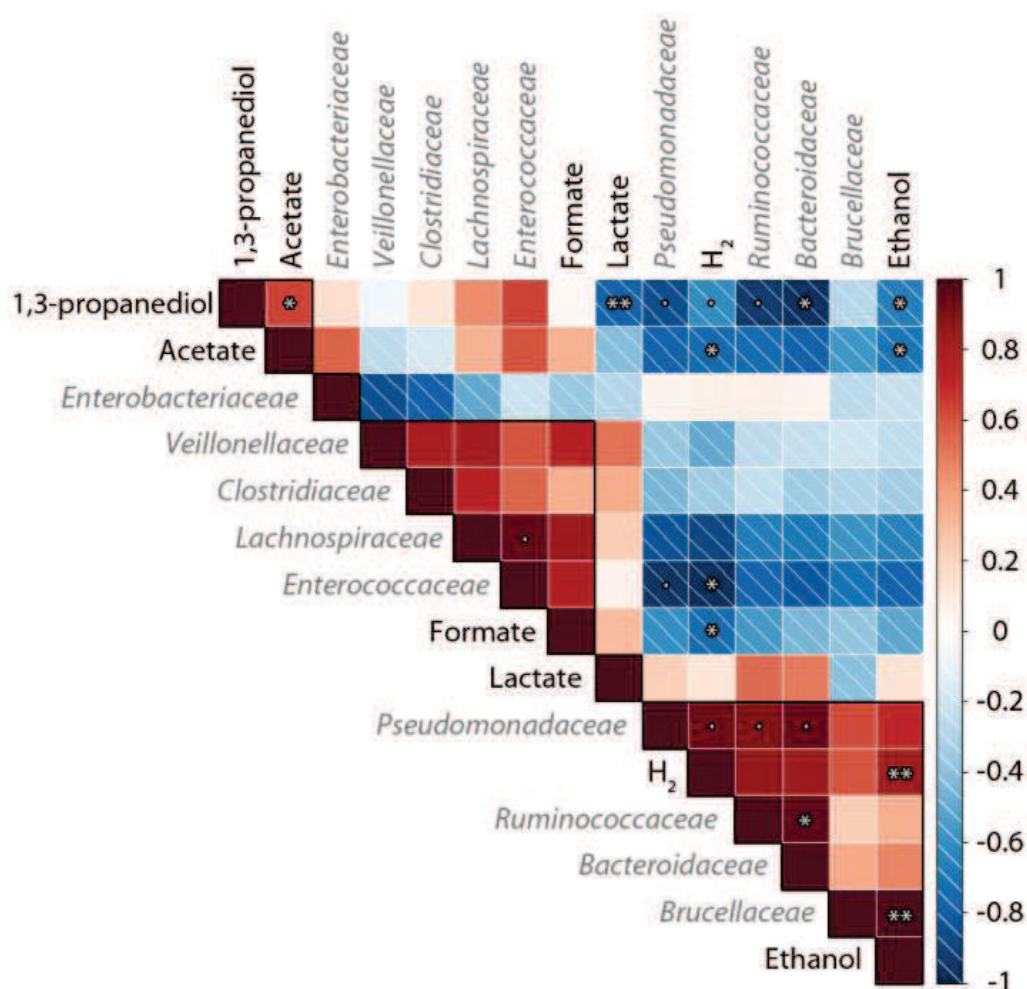


Figure 3-5. Pearson correlation matrix calculated from metabolite production profiles and sequencing results after 3 days of fermentation.

The hatched squares correspond to negative correlations and the full squares to positive correlations. The black outlines are the result of hierarchical clustering for $n=5$ groups.

P-values: ** ≤ 0.001 ; * ≤ 0.01 ; . ≤ 0.05

3.4 Discussion

3.4.1 Effect of pH on microbial population

In order to compare the bacterial populations obtained at the end of the fermentation with the different pH values, a PCA was performed (Figure 3-6). Most of the total variance (67.1%) was explained by the principal compound 1 (PC 1) that was able to discriminate samples between neutral pH from 6 to 8 and extreme pH values of 5 and 9. This PC was supported by the emergence of the *Enterobacteriaceae* species and the decrease of the *Clostridiaceae* species that were predominant in the inoculum. Surprisingly these two predominant families were found to have non-significant and low correlations with the metabolites produced suggesting that the differences found in the fermentation patterns were more related to less dominant species. It was shown that sub-dominant species in mixed culture fermentations can have significant effect on fermentation patterns and therefore have to be considered even at low abundance [212]. The PC 2 (16.4% of total variance) separated the bacterial population observed at low pH (≤ 6) and neutral to basic pH (≥ 7). This PC separated the two groups highlighted by the hierarchical clustering of the correlation matrix. The growth of *Pseudomonadaceae*, *Ruminococcaceae*, *Bacteroidaceae* and *Brucellaceae* species together with ethanol and H₂ production was then found to occur at low pH (< 6). On the other hand, the growth of the species from the *Enterococcaceae*, *Clostridiaceae*, *Lachnospiraceae* and *Veillonellaceae* families, associated to formate production, were favored at high pH (≥ 7). The high pH microbial community was more favorable for PDO than the one found for pH values below 6 in which many micro-organisms were strongly anti-correlated with PDO production. However, no significant and direct link between a specific bacterial family and a better PDO has been found. It was also found that lactate was neither correlated to a specific bacterial family nor to pH conditions.

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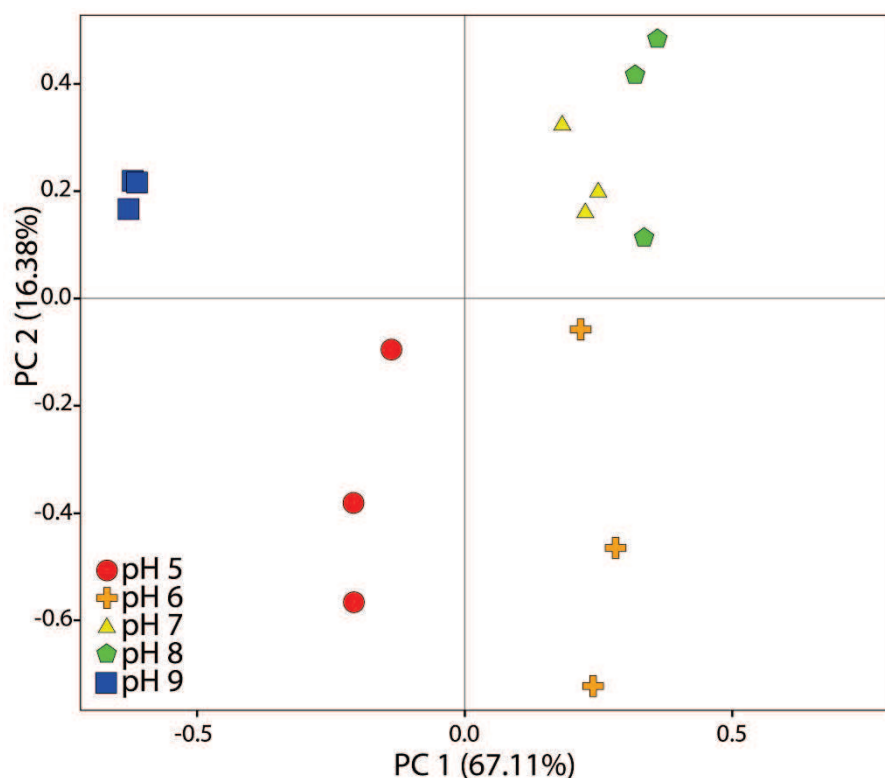
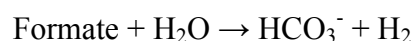


Figure 3-6. PCA performed on the composition of bacterial communities obtained with CE-SSCP after three days of fermentation.

3.4.2 pH-induced H₂/Formate shift

It is usual to observe H₂ production from glycerol or glucose fermentation depending strongly on the initial pH. The shift from formate to H₂ production observed in this study when pH decreased was previously described by Temudo *et al.* [43] who used a mixed culture for glucose fermentation. It was observed during this study that the hydrogen/formate molar ratio decreased concomitantly with the increase initial pH values. Considering the following equation and its Gibbs free energy [43]:



$$\Delta G^{\circ'} = 1.3 \text{ kJ.mol}^{-1} \quad \text{Equation 3-5}$$

The observed shift from formate to H₂ could be explained by thermodynamic considerations. This reaction is very close to the thermodynamic equilibrium and is catalyzed by the formate hydrogen lyase complex that is reversible. As the pK_a value of carbonate is 6.37 (at 25°C), a pH increase above this value would favor carbonate accumulation in the

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bulk and therefore inhibit formate splitting into carbonate and H₂. Considering that neither methanogenesis nor acetogenesis is occurring, a low H₂ production could mean that formate is produced and/or NADH₂ is formed from ferredoxin (see Section 1.2). However it is very likely that hydrogen was underestimated during this study when comparing the metabolic profiles obtained for pH values between 5 and 7 and theoretical values (see Table 3-1).

3.4.3 Ethanol production

From a theoretical analysis of all the possible glycerol fermentation pathways, it is clear that the acetate pathway leads to the highest PDO production. In this study, a shift in acetyl-CoA derived product was observed from acetate to ethanol at pH values below 6 with an expected decrease of the PDO production yields. From a thermodynamic point of view, Rodriguez *et al.* [213] showed in their metabolic-based model that for pH values below 5.6, ethanol is the metabolite that is generating the maximum energy for growth. Their calculation considers the energetic cost of acid transportation through the cellular membrane. At pH lower than 5.6, the energetic cost becomes more important than the energy supplied to the metabolism by the extra ATP produced during acetate production. Therefore, ethanol is energetically favored over acetate at low pH values. However, the ethanol shift cannot be only explained by energetic reasons and seems to be also strain-dependent. *Klebsiella variicola* has been reported to produce ethanol from glycerol with high yields at pH values ranging from 8 to 9 [214]. Temudo *et al.* [4] also showed ethanol production from glycerol at pH 8 from a mixed culture dominated by an *Enterobacteria* species close to *Klebsiella oxytoca*. On the contrary, *Clostridium acetobutylicum*, a bacterium used for acetone-butanol-ethanol production, is known for switching its metabolism from acidogenesis to solventogenesis when external pH drops under 5 [215]. In this study, ethanol production was highly correlated with Brucellaceae species and was only found when pH was below 6.

3.4.4 Towards high PDO concentrations

Initial high PDO production yields were obtained at low glycerol concentration with a low impact of the pH. To determine whether such performances could be reached at higher substrate concentration, an assay was performed in pH-regulated batch reactors with an initial glycerol concentration of 23.5 g.L⁻¹ at pH 7. In this experiment, a PDO yield of 0.53±0.02 mol_{PDO} mol⁻¹_{glycerol} was obtained, which is slightly lower but still consistent with the one obtained with the reactors buffered at pH 7 with an initial substrate concentration of 1.66 g.L⁻¹ (0.64±0.00 mol_{PDO} mol⁻¹_{glycerol}). Nevertheless, this yield is still high considering that a

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minimal medium with no vitamins or yeast extract was used. It is consistent with the results obtained by Dietz *et al.* in similar conditions with crude glycerol (yield of $\sim 0.60 \text{ mol}_{\text{PDO}} \text{ mol}^{-1} \text{ glycerol}$ and productivity of $\sim 1 \text{ g.L}^{-1}.\text{h}^{-1}$) and by Kanjilal *et al.* with pure glycerol ($0.52 \text{ mol}_{\text{PDO}} \text{ mol}^{-1} \text{ glycerol}$) [1,93]. These different results tend to show that mixed culture can be a viable option for PDO production from pure or crude glycerol, even though two major challenges remains to sustain an efficient production of high concentration of PDO. The first one is the use of crude glycerol issued from biodiesel production, which contains various impurities such as methanol and KOH at high concentrations [3,93,208,216] (see Section 1.1.2.1). These impurities may have positive effects through the addition of carbon sources and nutriment that can be used by the micro-organisms and thus increase the PDO production [1,93,208]. But methanol that is always present in these impurities can also inhibit the microbial growth, even at low concentration, and therefore decrease PDO productivity and glycerol consumption [3,216]. As crude glycerol composition may vary from a source to another, it is rather difficult to extend our conclusions when considering the combined effect of the impurities on glycerol fermentation. For that reason, mixed cultures fermentation has the advantage to be more robust to environmental changes. The second challenge is to increase final PDO concentration while keeping high productivities and production yields. A substrate inhibition has been reported at initial concentration higher than 70 g.L^{-1} of crude glycerol for *C. butyricum* [106,217]. This inhibition was also observed by Dietz *et al.* when mixed cultures were used [1]. Therefore, fed-batch process seems to be a good way to increase final PDO concentration while avoiding substrate inhibition. Using a fed-batch reactor with a continuous feed, mixed cultures and minimal medium, Dietz *et al.* obtained a final concentration of 70 g.L^{-1} of PDO with a yield of $0.56 \text{ mol}_{\text{PDO}} \text{ mol}^{-1} \text{ glycerol}$ and a productivity of $2.60 \text{ g.L}^{-1}.\text{h}^{-1}$ [1]. Another interesting process named electro-fermentation showed promising results by reaching a final PDO concentration of 42 g.L^{-1} [166]. These results are outstanding considering that non-sterile conditions and minimal medium were used and are comparable with the best performances obtained with pure culture [20].

3.5 Conclusion

When considering the Pearson correlation matrix (Figure 3-5) and the PCA results (Figure 3-6), it appeared in this study that pH had a significant impact on both bacterial growth, the composition of the bacterial community and metabolic profiles. The predominant bacteria from *Clostridiaceae* and *Enterobacteriaceae* families could not explain alone the changes in metabolic profiles. Within the less dominant species, two different communities were found, one at acid pH values and another at neutral to basic pH values. The latter one was favorable to PDO yield even if no significant correlation between a specific bacterial family of this community and a good PDO yield was found. Then, it was likely that there were a functional redundancy within this community. From the theoretical analysis of the metabolic pathways of glycerol fermentation (Table 3-1) and the correlation matrix (Figure 3-5), it was clear that PDO was favored when produced together with acetate, which was mostly the case in this study. Even if strong changes occurred in the microbial community structure over the pH range studied, high PDO production yields were obtained and were comparable to the best yield obtained in similar conditions (i.e. mixed culture, pure glycerin and no additive such as yeast extract) of $0.69 \text{ mol.mol}^{-1}$ [2]. As a pH of 7 led to the highest PDO production performances, this value was considered for the following studies when using the same inoculum.

Chapter 4. Electro-fermentation triggering population selection in mixed culture glycerol fermentation

The present chapter deals with mixed culture glycerol electro-fermentation consisting in a fermentation conducted in presence of polarized electrodes. Impact of the electrodes on glycerol fermentation was assessed by investigating the shifts of bacterial populations and metabolic patterns. Addition of *Geobacter sulfurreducens*, an electro-active species, as mediator between electrodes and fermentative bacteria was also studied. The introduction, results and discussion of this chapter are issued from an article currently under revision in *Microbial Biotechnology*:

Moscoviz R, Trably E, Bernet N. Electro-fermentation triggering population selection in mixed culture glycerol fermentation. Under revision. *Microbial Biotechnology*.

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Chapter 4 - Electro-fermentation triggering population selection in mixed culture glycerol fermentation

4.1 Introduction

Electro-fermentation (EF) is a recently developed approach that combines fermentation and bio-electrochemical systems (BESs) to add a supplementary way of control of fermentation patterns [5,6] (see Section 1.4). It consists in operating a self-driven fermentation in the presence of polarized electrodes inside the bulk phase. This additional electron sink or source provides many advantages to the micro-organisms, including the possibility to perform unbalanced fermentations (*e.g.* stoichiometric production of ethanol from glycerol [168]) and affect metabolic regulations directly through intracellular redox pair balance [5]. Contrary to most other BESs, EF does not necessarily require high energy input and even small current densities can have a great impact on the overall fermentation performances [7].

In this context, the fermentation of glycerol is of special interest because of its high electron content (4.7 moles of electrons per mole of carbon compared to 4 moles of electrons per mole of carbon for glucose). The substrate is so reduced that even biomass synthesis from glycerol generates an excess of intracellular electron carriers (*i.e.* NADH, NAPH). Most fermentation end-products (*e.g.* ethanol, acetate, butyrate, lactate, *etc.*) are also associated with a net NADH generation when produced from glycerol. On the other hand, only few pathways with a net NADH consumption are available in glycerol fermentation: either H₂ is produced or glycerol is converted into PDO (see Section 1.2). The latter is a chemical of industrial interest that can be used for the production of resins, cosmetics, solvents and polymers [207] and its production has been the focus of numerous scientific studies [20,25]. EF appears to be an appropriate tool to enhance PDO yield as its production is tightly related with intracellular electron balance and redox conditions. Adding a supplementary electron source (cathodic electro-fermentation) could provide several benefits such as: (i) a direct dissipation of these extra electrons into PDO (electrosynthesis); (ii) shifting metabolic patterns towards a more efficient electron dissipation (*i.e.* PDO production) caused by regulations ensuring NADH/NAD ratio homeostasis [5,7]; (iii) selection of microbial population that is more efficient for electron dissipation and more adapted to reduced conditions; (iv) disfavoring microbial H₂ production by electrochemically producing H₂ at the cathode, and therefore enhancing PDO production as sole NADH dissipating pathway.

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Up to date, only few studies have implemented an EF strategy to enhance PDO production in mixed culture glycerol fermentation [164–167]. In a study focusing on glycerol fermentation in batch tests, Zhou et al. observed that a strongly negative potential of -1.14 V vs Standard Calomel Electrode (SCE) applied at the cathode was able to improve PDO production from $0.25 \text{ mol}_{\text{PDO}} \text{ mol}^{-1}_{\text{glycerol}}$ in conventional fermentation to $0.50 \text{ mol}_{\text{PDO}} \text{ mol}^{-1}_{\text{glycerol}}$ in electro-fermentation [167]. In a second study, a cathodic current was applied (chronopotentiometry at -1 and -10 A.m⁻²) during continuous glycerol fermentation [164]. It was found that PDO production was positively correlated with the electron input, reaching an average yield of $0.51 \pm 0.07 \text{ mol}_{\text{PDO}} \text{ mol}^{-1}_{\text{glycerol}}$ during a three weeks operation time. In addition, Xafenias et al. [166] reached a maximum concentration of $42 \text{ g}_{\text{PDO}}.\text{L}^{-1}$ in fed-batch mode with a production yield of $0.46 \text{ mol}_{\text{PDO}} \text{ mol}^{-1}_{\text{glycerol}}$ while imposing a potential of -1.34 V vs SCE at the cathode. EF performances reported in this study were clearly higher than the fermentation controls, which reached only 18 g.L^{-1} of PDO in more than twice the experimental time. However, in these studies, the very low working potential applied led to hydrogen formation from water at the cathode and it was therefore difficult to assess if the predominant effect of EF was direct or indirect.

The aim of the present work was to investigate whether EF could be used as a tool to redirect mixed culture fermentation of glycerol without any electrochemical hydrogen production (see Figure 2-1) in order to minimize energy inputs. Impact on both fermentation patterns and bacterial community structure was studied. As a second step, *Geobacter sulfurreducens* pre-colonized working electrodes were used to evaluate the potential of adding an electro-active bacterium as biocatalyst between the working electrode and fermentative bacteria in EF.

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4.2 Experimental design

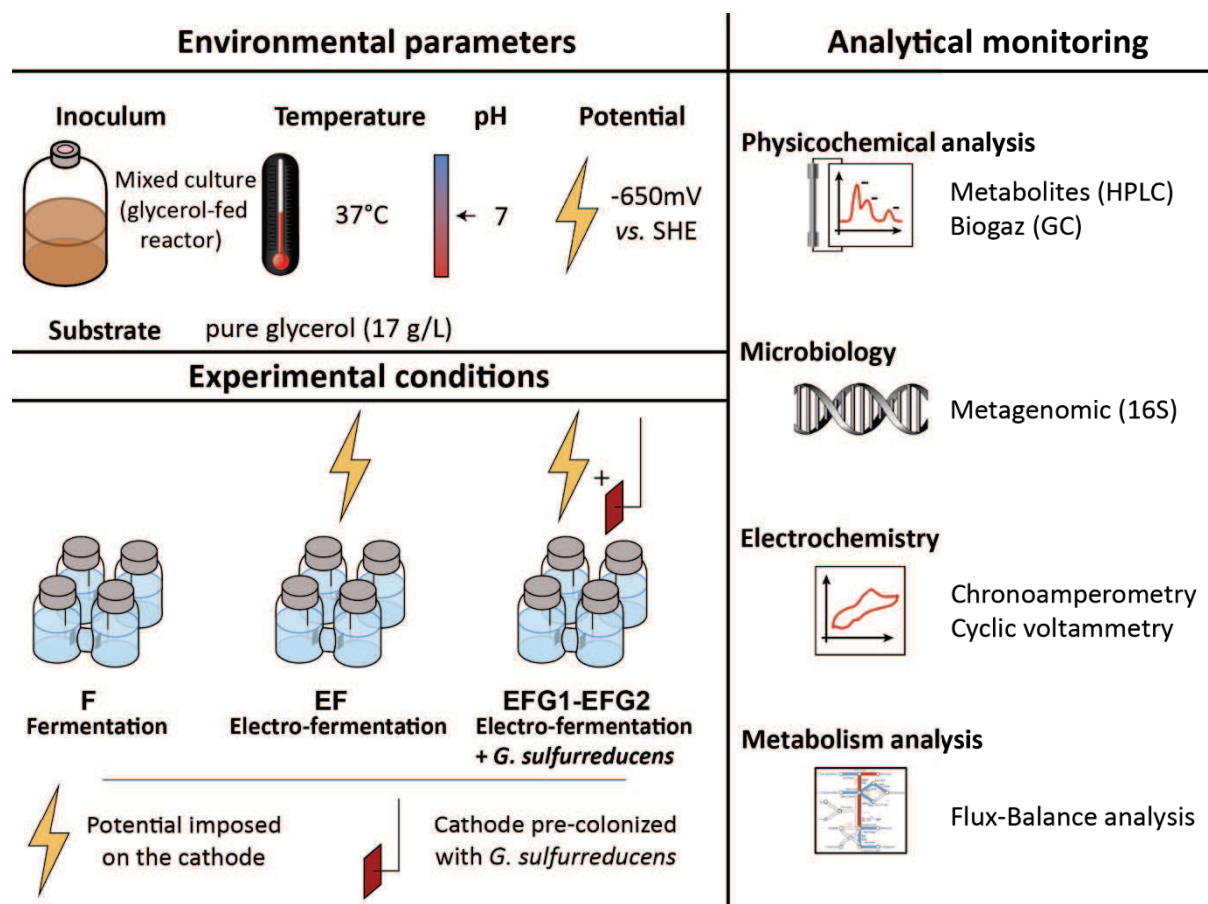


Figure 4-1. Experimental design for the study of electro-fermentation as a tool for control of mixed culture glycerol fermentation.

In the present study, three conditions were designed to observe the effect of polarized electrodes on glycerol fermentation: F for fermentation, EF for electro-fermentation, EFG1-2 for successive batches of electro-fermentation with *G. sulfurreducens* pre-colonized electrodes (see Figure 4-1). Fermentation conditions and medium were similar to those set in Chapter 3: 37°C, pH 7 (which was optimal for PDO production, see Figure 3-2), and an unsterile minimal medium containing no yeast extract or equivalent. As pH was controlled, initial glycerol concentration could be increased 10 times compared to Chapter 3 (from 1.7 to 17g.L⁻¹) without acidification of the fermentation broth. As in Chapter 3, refined glycerol was used (Sigma-Aldrich, ≥99%) to simplify the analysis of the results. The mixed consortium used as inoculum during this study was identical to the one used in Chapter 3. The three conditions were carried out in duplicate batches due the limited number of channels to control

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pH. Although electrodes were present in the three conditions, the potential of -650mV vs. SHE was only imposed in EF and EFG1-2. This potential was experimentally determined under abiotic conditions and chosen to be as low as possible while avoiding electrochemical production of hydrogen (see Figure 2-1). The pre-colonization of *G. sulfurreducens* on the cathode as mediator between the electrode and fermenters was studied in EFG1. EFG2 consisted in a batch conducted with fresh fermentation medium that was inoculated at 10% v/v with EFG1. The working electrodes (with their biofilms) from EFG1 were used in the corresponding successive batch in EFG2. These successive batches were conducted in order to observe if the population structure at the end of EFG1 was stable over time. Methods employed during this study are more specifically described in Section 2.3.

In particular, to estimate the metabolic fluxes of the different OTUs of the bacterial population, a model similar to the flux-balance analysis was developed for mixed cultures. The model assumptions were as follows: (ass.1) each OTU has the same metabolic profile in all reactors, (ass.2) each OTU contributes to the total metabolite production at a ratio equal to its abundance (i.e. an OTU representing 30% of a reactor population will produce 30% of the metabolites of the reactor), (ass.3) each OTU has a balanced metabolism (the production and consumption of NADH and ATP are equals) and (ass.4) each OTU has closed electron and carbon mass balances. Results of this model are presented and discussed in the following sections.

4.3 Results

4.3.1 Impact of electro-fermentation on metabolic patterns

Conventional fermentation (F) and electro-fermentation (EF) were compared in batch pH-controlled reactors with $17.5 \text{ g}_{\text{glycerol}}\cdot\text{L}^{-1}$. After about 35 h of lag-time, both F and EF started and glycerol was nearly depleted within 48 h. Electron mass balances closed between 90.5 and 92.8 % as shown in Figure 4-2. The missing part probably corresponds to measurement errors and unmeasured products such as hydrogen. During EF, the cathodic current was always more positive than $-0.35 \text{ A}\cdot\text{m}^{-2}$ and the electron input through the cathode represented only 0.2 % of the total electron input (*i.e.* glycerol plus electric current and initial biomass). The electro-fermentation efficiency η_{EF} was 0.004, meaning that the electric current could not contribute directly for more than 0.4 % of the PDO production and direct bioelectrosynthesis of PDO was therefore not the predominant reaction (see Section 2.3.4.1 for calculations). In both conditions, PDO was the main product and the other by-products were lactate, acetate and ethanol, being consistent with results reported in Chapter 3. Compared to F, EF had nearly no effect towards PDO production since in F and EF, similar yields of $0.48 \pm 0.01 \text{ mol}_{\text{PDO}} \text{ mol}^{-1}_{\text{glycerol}}$ and $0.46 \pm 0.01 \text{ mol}_{\text{PDO}} \text{ mol}^{-1}_{\text{glycerol}}$ corresponding to final PDO concentrations of $6.9 \text{ g}\cdot\text{L}^{-1}$ and $6.6 \text{ g}\cdot\text{L}^{-1}$ were respectively achieved. Comparable amounts of acetate (5.7 and 7.6 % Total Electron equivalent - TEE - during F and EF respectively), formate (1.6 and 2.7 % TEE resp.) and succinate (0.9 and 1.8 % TEE resp.) were found in both conditions. However, lactate production in F (16.9 % TEE) was higher than its production in EF (5.7 % TEE). In contrast, ethanol production was lower in F (5.5 % TEE) than EF (12.8 % TEE). Traces of propionate and butyrate were only found in EF.

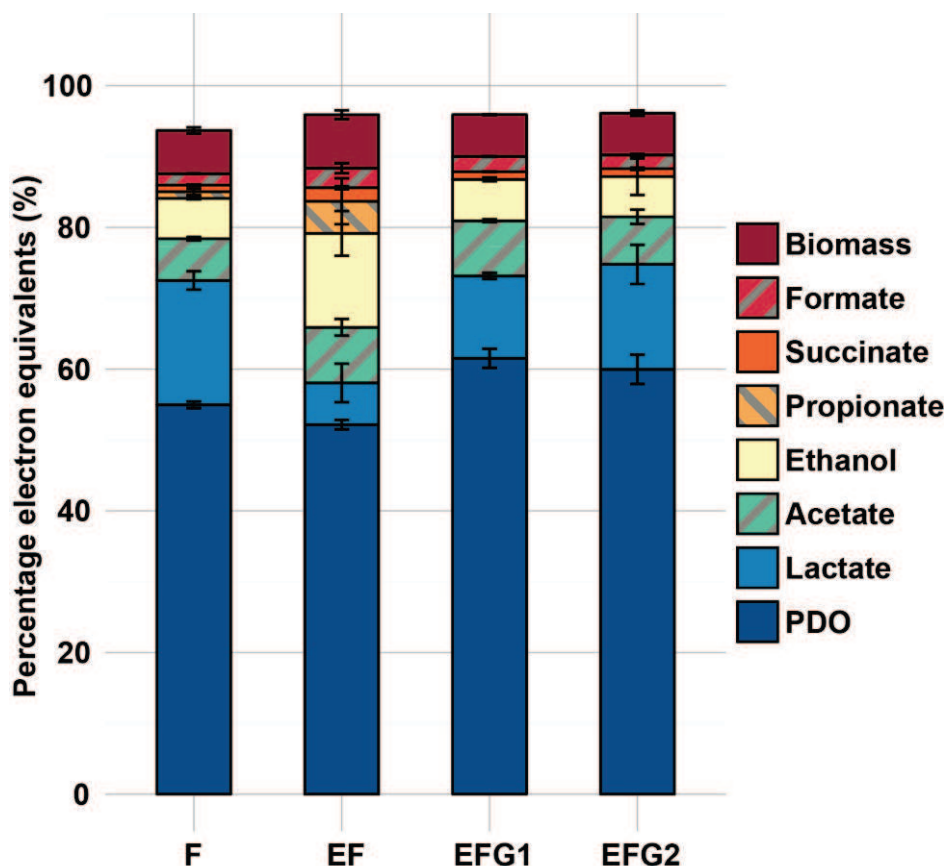


Figure 4-2. Electron mass balances calculated from the metabolites measured after glycerol depletion in duplicate experiments.

*Results are normalized on the sum of electron content from initial glycerol, initial biomass and cathodic current. The biomass was estimated from the ATP production associated to the different metabolites production. Error bars represent the minimum and maximum values of the replicates. F: Classic fermentation. EF: Electro-fermentation. EFG1-2: Successive batches of electro-fermentation with *G. sulfurreducens* pre-colonized cathode.*

4.3.2 The addition of *Geobacter sulfurreducens* increased PDO production

Two successive electro-fermentation series of batch experiment (EFG1 and EFG2) were carried out with *Geobacter sulfurreducens* pre-colonized WE. The lag-phase and fermentation time in EFG1 was less than 35h as observed in F and EF. However, the lag phase in EFG2 was reduced to less than 12h despite a similar fermentation. Electron mass balances are presented in Figure 4-2. and closed at about 93 % for both conditions. Similarly, the cathodic current was always more positive than -0.25 A.m^{-2} in EFG1 and was more positive than -0.05 A.m^{-2} in EFG2, representing 0.1 % and <0.1 % of the total electron input respectively (see Figure S6). In both conditions, η_{EF} was below 0.001, meaning that the

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electric current could not contribute directly for more than 0.1 % of the PDO production. PDO production increased by about 10 % when compared to conventional fermentation and electro-fermentation without *G. sulfurreducens*. PDO yield in EFG1 and EFG2 were 0.54 ± 0.02 and $0.53 \pm 0.02 \text{ mol}_{\text{PDO}} \text{ mol}_{\text{glycerol}}^{-1}$ (59.4 and 57.9 % TEE resp.), respectively. The final PDO concentrations were 7.8 g.L^{-1} and 7.7 g.L^{-1} for EFG1 and EFG2 respectively. In the two batch series, lactate was the main by-product (11.2-14.3 % TEE) along with acetate (6.5-7.5 % TEE) and ethanol (5.5-5.6 % TEE).

4.3.3 Microbial communities in the bulk

Microbial community compositions of the inoculum and bulk phase of all experimental conditions were measured using MiSeq sequencing. Relative abundancies and affiliations of each operational taxonomic unit (OTU) are provided in Table 4-1. The inoculum was mainly composed of bacteria from the *Clostridiales* (47.7 %), *Bacteroidales* (19.9 %) and *Lactobacillales* (14.1 %) orders and was dominated by OTUs 5 and 1 with 30.4 and 13.4% of the total bacterial community, respectively. These two OTUs were related to *Clostridium intestinale* (98 % 16S rRNA sequence similarity with OTU5) and *Enterococcus avium* (100 % 16S rRNA sequence similarity with OTU1). After substrate depletion, only 15 OTUs had a relative abundancy of more than 1 % in at least one reactor. OTU1, which was by far the most dominant species within the *Lactobacillales* order, was found to dominate the bacterial community of EFG1 and EFG2 (47.8 and 54.7 % of the total bacterial community resp.). OTU1 also represented a large part of the bacterial community of F and EF (20.1 and 22.7 % resp.). OTU1 was correlated to high PDO production yield ($R^2 = 0.66$, $p < 0.05$, $n = 8$). OTUs from the *Enterobacteriales* order were enriched in all the conditions, especially during EF and EFG1 with 15.3 and 22.1 % of the final bacterial community, respectively, while they represented only 2.0 % of the inoculum. The dominant OTU of the *Enterobacteriales* order was OTU2, which had 99 % 16S rRNA sequence similarity with *Citrobacter freundii*. OTU13 was the second dominant OTU of this taxonomic order that was only found during EFG1 and EFG2 (2.9 and 0.5 % of the total bacterial community resp.). OTU13 positively correlated with PDO production yield ($R^2 = 0.53$, $p < 0.01$, $n = 8$). This OTU had 100 % of 16s rRNA sequence similarity with species from *Escherichia-Shigella* genera such as *Shigella sonnei* and *Escherichia fergusonii*. Species from the *Bacteroidales* order were highly enriched in all conditions (54.6, 38.4 and 32.0 % of the total bacterial community for F, EF and EFG2 resp.) except in EFG1 where they represented only 14.6 % of the total bacterial community. OTU3 and OTU6 accounted for more than 91 % of the

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Bacteroidales species in all conditions. Nucleotide sequence analyses of the OTU3 16S rRNA genes revealed 98 % of sequence homology with *Dysgonomonas mossii* whereas OTU6 was not closely related to any cultured species.

Table 4-1. Clone abundancies and identification obtained after sequencing.

OTU n°*	Putative identification (% 16S rRNA sequence similarity)	Average abundance in the bulk (%)				
		Inoculum	F**	EF**	EFG1**	EFG2**
Firmicutes						
1	Enterococcus avium (100)	13.4± 2.5	20.1± 4.3	22.7± 11.6	47.8± 1.2	54.7± 2.2
5	Clostridium intestinale (98)	30.5± 3.1	1.0± 0.3	4.0± 1.2	1.2± 0.3	0.3± 0.0
9	Clostridium celerecrescens (100)	0.4± 0.4	2.2± 0.5	0.9± 0.1	5.0± 1.1	1.8± 1.1
12	Uncultured Lachnospiraceae sp.	5.4± 1.7	1.0± 0.2	1.7± 0.1	1.6± 0.5	0.3± 0.2
30	Clostridium oroticum (98)	0.4± 0.2	1.5± 1.5	1.3± 1.1	0.0	0.1± 0.1
31	Clostridium propionicum (99)	0.6± 0.4	3.0± 0.1	0.7± 0.2	0.0	0.0
34	Uncultured Veillonellaceae sp.	0.0	0.0	7.5± 10.6	0.0	0.0
Proteobacteria						
2	Citrobacter freundii (99)	2.0± 0.7	8.0± 0.4	15.1± 0.4	19.0± 4.2	6.4± 4.5
8	Stenotrophomonas pavanii (100)	0.4± 0.3	1.8± 0.5	1.3± 1.1	0.8± 0.6	1.7± 0.0
13	Escherichia fergusonii (100)	0.0	0.0	0.0	2.9± 2.1	0.5± 0.3
55	Telmatospirillum siberiense (98)	0.1± 0.1	0.6± 0.8	1.1± 1.6	0.0	0.0
61	Pseudomonas aeruginosa (100)	0.6± 0.9	0.0	0.8± 1.1	0.0	0.0
Bacteroidetes						
3	Dysgonomonas mossii (98)	2.0± 0.4	40.5± 23.6	29.0± 10.8	0.3± 0.0	28.0± 1.9
6	Uncultured Bacteroides sp.	5.4± 0.6	12.9± 17.0	8.4± 10.9	13.0± 5.9	3.7± 3.5
11	Bacteroides graminisolvens (99)	6.3± 1.1	0.8± 0.1	0.6± 0.4	0.8± 0.1	0.2± 0.1
Tenericutes						
33	Uncultured Mollicutes sp.	0.0	1.4± 1.6	0.2± 0.1	0.6± 0.3	0.3± 0.3

* Only the clones with a minimum of 1% abundancy in the bulk in at least one condition are reported.

** F: Classic fermentation (open-circuit). EF: Electro-fermentation (applied potential of -900 mV vs SCE). EFG1-2: Successive batches of electro-fermentation (applied potential of -900 mV vs SCE) with *G. sulfurreducens* pre-colonized cathode.

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4.3.4 Non-turnover cyclic voltammetry

Non-turnover cyclic voltammetry (CV) was performed after glycerol depletion on the cathode in EF and EFG1, to detect whether significant electro-active bacteria were attached to them [218]. The voltammograms are provided in Figure 4-3. A slow scan rate of 1 mV.s^{-1} in the range of -0.9 to $+0.5 \text{ V vs SCE}$ was used to detect as many redox peaks as possible. During the CV performed on EFG1 cathodes (Figure 4-3A), two anodic peaks (E_{pA1-2} at -0.254 and -0.086 V vs SCE resp.) and three cathodic peaks (E_{pC1-3} at -0.339 , -0.589 and -0.687 V vs SCE resp.) were detected. Only E_{pA1} and E_{pC1} seemed to be related to a reversible redox reaction, leading to a formal potential of $E^f = -0.297 \text{ V vs SCE}$. Similar formal potential (-0.286 V vs SCE) was previously observed during bio-electrosynthesis experiments using a homoacetogenic bacteria-enriched cathode [219]. E_{pA2} and E_{pC2} could be related to two redox complexes of *G. sulfurreducens* that were previously reported to play a minor role during anodic respiration [220] and that are not associated with cytochrome *c* electron transfers [150]. The last peak, E_{pC3} , could involve NAD^+ reduction into NADH (formal potential -0.564 V vs SCE) as hypothesized by Modestra *et al.* [219]. The non-turnover CV of the EF cathode (Figure 4-3B) revealed only three minor peaks, showing significantly less electro-activity on the electrode surface than in EFG1. Two peaks had a potential similar to the E_{pA1}/E_{pC1} redox system found on EFG1 cathodes ($E^f = -0.297 \text{ V vs SCE}$). The third peak, E_{pA3} , corresponded to an anodic peak with a potential of $+0.048 \text{ V vs SCE}$ that was not related to a known redox system.

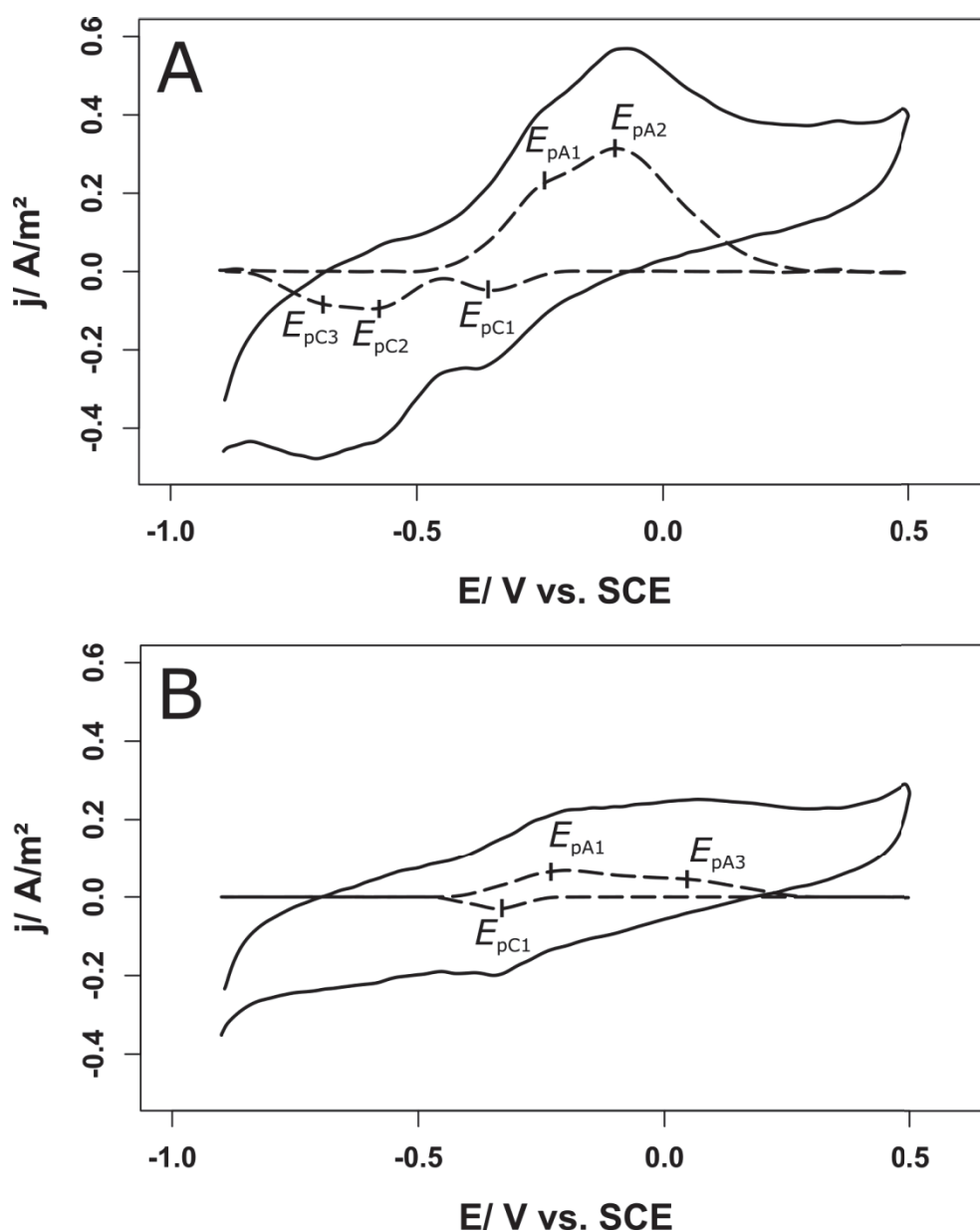


Figure 4-3. Representative non-turnover cyclic voltammogram of the cathode after substrate depletion.

(A) During electro-fermentation experiments with *G. sulfurreducens* pre-colonized cathodes (EFG1). (B) During electro-fermentation without *G. sulfurreducens* (EF). The dashed lines correspond to the baseline subtracted curves.

4.3.5 Population selection on *G. sulfurreducens* pre-colonized electrodes

To go further in the analysis of the cathodic electro-active biofilms, MiSeq sequencing was performed on cathode biofilm samples at the end of both EFG1 and EFG2. The bacterial population distribution within these biofilms is displayed in Figure 4-4. The microbial community of the EF cathodes was not sampled because of the weak signal found during CV (see Figure 4-3B). After the first batch series (EFG1), *G. sulfurreducens* was still the dominant species on the cathode (59% of the total abundancy) while representing only 0.2% of the populations in the bulk phase. The other dominant species attached to the cathode (>5% total abundancy) were OTUs 9, 14, 18 and 19 and were all affiliated to *Firmicutes*. OTU9 had 100% 16S rRNA sequence homology with *Clostridium celerecrescens* and represented 6.5% of the biofilm but was also present in significant amount in the bulk (5.0%). In contrast, OTUs 14, 18 and 19 (7.8, 6.5 and 5.0% of the total biofilm bacterial community resp.) were rare species in the bulk and accounted together for less than 0.03% of the total bacterial community. OTU14 was an uncultured *Firmicutes* species and OTUs 18 and 19 were found to be related to *Geosporobacter subterraneus* and *Lutispora thermophila* (100 and 98% 16S rRNA sequence similarity resp.). OTUs 1 and 2, which were two of the dominant OTUs in the bulk, were also attached to the cathode but only accounted for resp. 2.0 and 4.1% of the biofilm. At the end of the second batch series (EFG2), the biofilm bacterial community was more diversified than at the end of the first batch series (Simpson's index of diversity of 0.83 and 0.63 resp.). *G. sulfurreducens* was not the dominant species anymore (12.9% of the biofilm community) and was less abundant than OTUs 1 and 9 (25.6 and 27.9% of the biofilm community resp.). OTUs 14, 18 and 19 were still accounting for a similar percentage of the biofilm population as at the end of EFG1 (9.1, 3.5 and 4.0% of the total biofilm bacterial community resp.).

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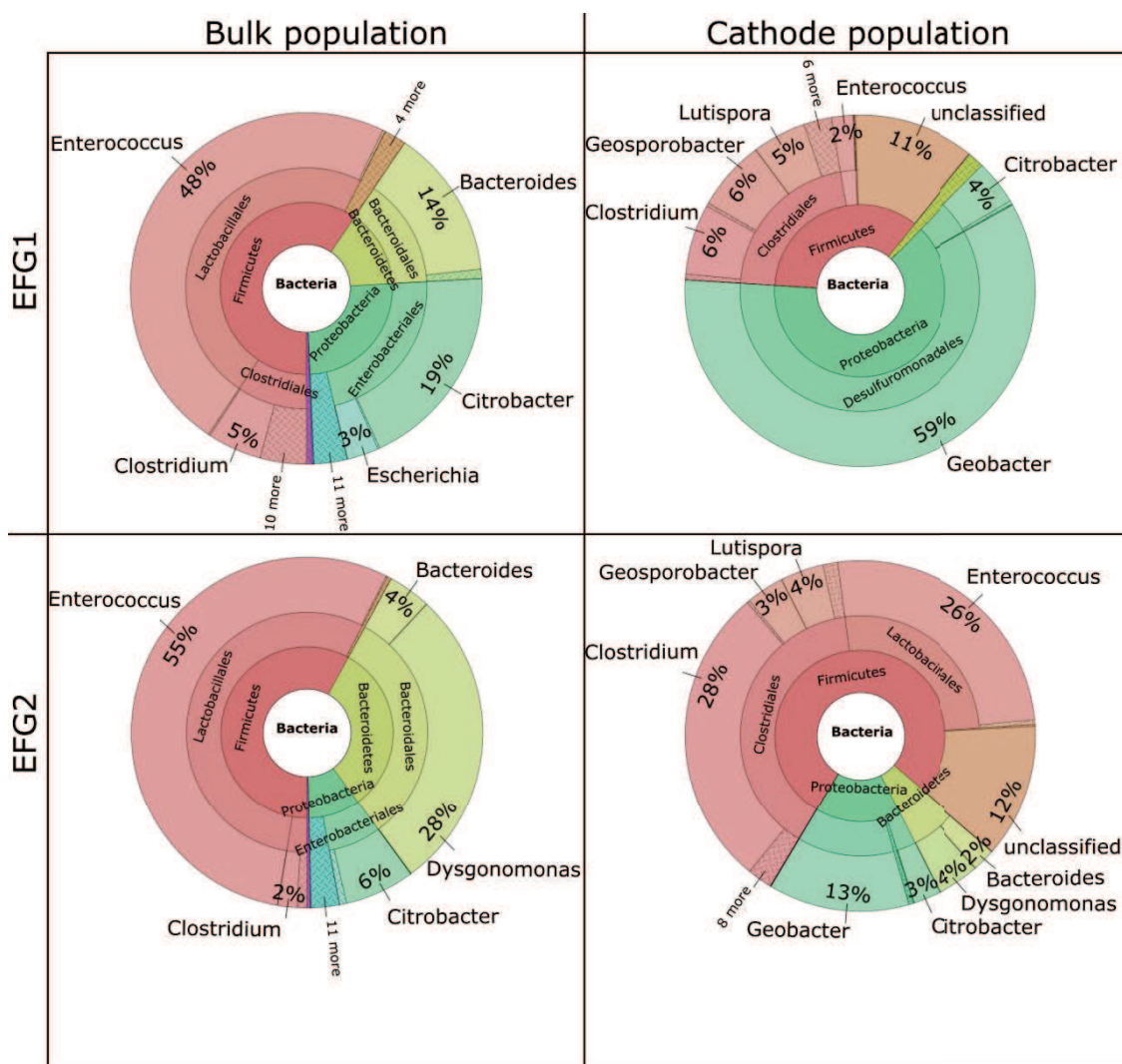


Figure 4-4. Pie charts representing the bacterial population composition in the bulk and on the cathode surface after substrate depletion during the two successive batches of electro-fermentation with *G. sulfurreducens* pre-colonized cathode (EFG1-2).

Only the taxonomic phyla, orders and genera are displayed.

4.3.6 Estimated metabolic patterns and clustering of the OTUs

As only 15 OTUs accounted for more than 94 % of the total bacterial population of the bulk in all conditions, a linear system could be written in order to estimate the metabolic profile of each of these OTUs. In order to simplify the analysis of the estimated metabolic patterns, the OTUs were clustered according to their normalized abundancy using k-means clustering ($k=4$) (Figure 4-5A). The metabolic profiles estimated by the model are displayed in Figure 4-5B. Cluster 1 was composed of OTUs 1, 2 and 9, which were over-represented in the bacterial population of EFG1 when compared to the mean abundancy in all conditions. They were all estimated to have a good PDO production yield (>0.50 mol/mol), especially OTU13 and OTU1 that showed the highest PDO theoretical production yield among all OTUs (0.64 and 0.55 mol/mol resp.). Interestingly, three out of four OTUs of the cluster 1 corresponded to the best acetate producers of all OTUs (OTU1, 2 and 13). Cluster 2 was more related to the OTUs that were over-represented during classic fermentation and was composed of OTUs 6, 31, and 33. These OTUs were all estimated to have a lactate orientated metabolism. Cluster 3 grouped OTUs 3, 8 and 30 that were mostly under-presented during EFG1. However the metabolic profiles proposed by the model were all different between these three OTUs. Finally, cluster 4 gathered OTUs 5, 12, 34, 55 and 61 that were over-represented during EF and estimated to have an ethanol or propionate metabolism. These OTUs (except for OTU 34) contained the worst PDO producers of the model.

Chapter 4 - Electro-fermentation triggering population selection in mixed culture glycerol fermentation

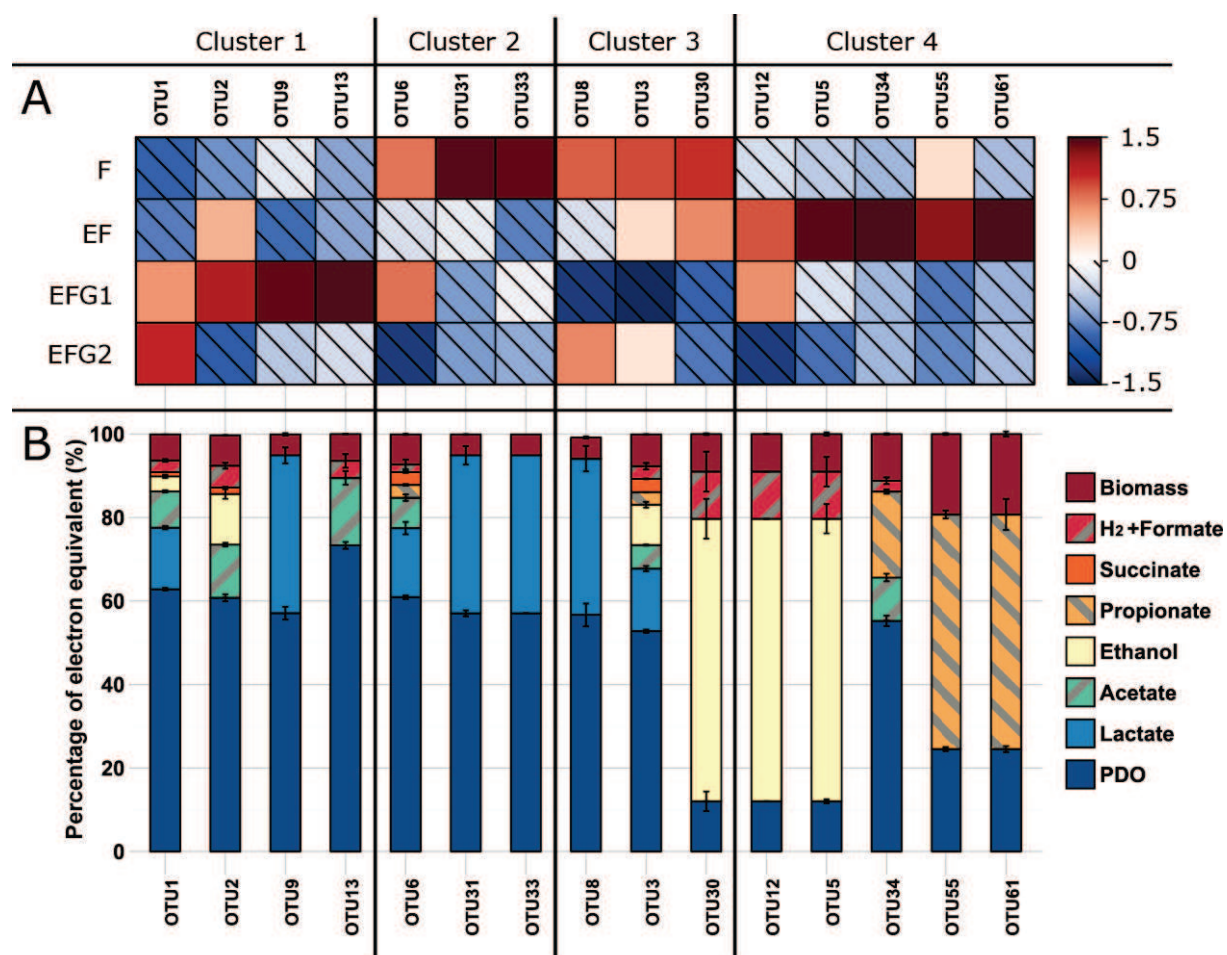


Figure 4-5. (A) Normalized abundancy of all OTUs representing more than 1% of the total bacterial community in at least one reactor (based bulk abundancies only). The hatched squares correspond to negative values of normalized abundancy. The clusters are the result of k-mean clustering for k=4 groups. (B) Electron mass balances estimated by the model and normalized on the electron equivalent of the glycerol consumed by each OTU. The error bars correspond to the standard deviation of the predicted values obtained by cross-validation.

4.3.7 Model accuracy and robustness

From the individually estimated metabolic pattern of each OTU, global yields could be assessed by the model and were compared with the actual yields (see Figure 4-6). The linear system was underdetermined, meaning that there were less independent equations (108) than unknowns (135), leading theoretically to an infinite amount of exact solutions. However, the range of the solutions was restrained by metabolic limitations in the form of an inequation system (see 0), by restricting the yields to only positive ones. No exact solution was found within these metabolically viable solutions, which was not surprising when considering that the experimental electron and carbon mass balances did not close perfectly. The solution provided by the model corresponded to the least square error solution and were used to calibrate the model. It was then validated using k-fold cross-validation ($k=8$) to assess the robustness of the solution. The different root square mean errors (RMSE) and RMSEs of the cross-validation ($RMSE_{CV}$) are provided in Table 4-2. The model was able to predict the global production yields satisfactorily ($RMSE=0.02$ mol/mol), especially for PDO ($RMSE=0.01$ mol/mol) and acetate ($RMSE=0.02$ mol/mol) production yields. Nonetheless, ethanol and lactate were less reliably predicted ($RMSE=0.03$ mol/mol). All the $RMSE_{CV}$ values were about twice the corresponding RMSE values, meaning that the model was highly robust. The OTUs with less stable metabolic profiles during cross-validation (Figure 4-5B) were mostly the less dominant OTUs. The most dominant OTUs (*i.e.* OTUs 1, 2, 3, 6 and 34) had their metabolic profile nearly unchanged during cross-validation.

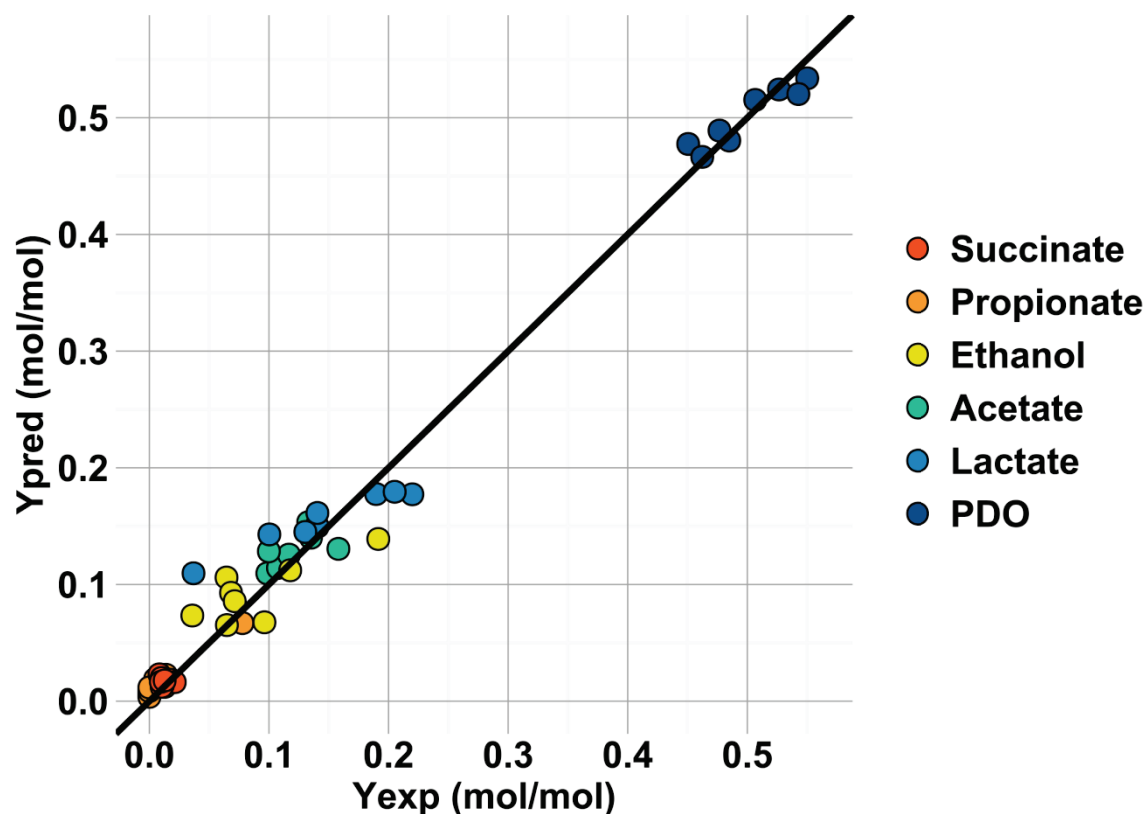


Figure 4-6. Global production yields predicted by the model in function of experimental production yields. Y_{exp} : experimental yields obtained in this study. Y_{pred} : yields predicted by the linear model.

Table 4-2. Inverse model errors of prediction.

	RMSE (mol/mol)	RMSE _{CV} (mol/mol)
Global	0.021	0.042
PDO	0.014	0.032
Lactate	0.034	0.063
Acetate	0.016	0.033
Ethanol	0.029	0.063
Propionate	0.009	0.014
Succinate	0.007	0.012

4.4 Discussion

4.4.1 *Clostridium celerecrescens* as a potential electro-fermentative bacteria

Without considering the presence of *G. sulfurreducens*, the biocathode biofilm composition was significantly different from the bacterial community in the bulk (see Figure 4-4). This different population composition was not due to contaminants that could have grown during pre-colonization procedures as all OTUs present on the cathode were also present in the fermentation inoculum. Therefore, the cathode acted as an ecological niche that selected species able to interact with either the electrode or *G. sulfurreducens* and could have been supplementary biocatalyst during glycerol EF. Some of the dominant OTUs (OTU 18 and 19) attached to the cathode at the end of EFG1 were related to species that have not been reported as glycerol consumers (*G. subterraneus* and *L. thermophila* resp.) [221,222]. Overall, little is known about the activity of these species which could be performing side-reactions such as homo-acetogenesis [223] as well as having specific interactions with fermenters in the bulk. More interestingly, OTU 9 (100% 16S rRNA sequence homology with *C. celerecrescens*) was highly selected on the cathode and was also found in significant amounts in the bulk in both EFG1 and EFG2. Consistently, *C. celerecrescens* has already been found on a cathode during bio-electrosynthesis experiments [224], was present in bacterial communities able to corrode steel pipes (*i.e.* the use of iron as electron donor) [225,226] and was able to grow using a mixture of CO₂ and H₂ as substrate [224], supporting a potential electro-activity of *C. celerecrescens* [227]. Considering that OTU 9 became the most dominant species at the cathode at the end of EFG2, it is probable that this OTU is electro-active and able to accept electrons from the cathode. Although *C. celerecrescens* was not reported as able to consume glycerol [228], OTU 9 represented 5% of the total bulk population at the end of EFG1. Either it was able to consume glycerol and would therefore be an electro-active fermenter, or it was growing by interacting with glycerol-fermenting bacteria (*e.g.* by-product consumption, direct electron transfer). In both cases *C. celerecrescens* might be interesting organisms for electro-fermentation of glycerol that requires further research.

4.4.2 Selection effect versus individual metabolic shift

In mixed culture fermentations, changing an environmental parameter can induce at least two effects: (i) a metabolic shift in a part or in all the bacteria composing the mixed

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culture, resulting in a global change of the metabolism but not impacting the composition of the microbial community and/or (ii) favoring or disfavoring the selection of specific populations, resulting in a change of the microbial community and a further change of the global metabolism. Direct effects of an electrode on glycerol metabolism have already been shown in pure culture experiments using *Clostridium pasteurianum*, even with small currents ($\eta_{EF} < 0.01$) [7]. Such a behavior could be possible for some species in our experiments. However, the significant changes in the composition of the bacterial community between the different experiments and the robustness of the model tend to support a selection as a dominant effect rather than a shift in individual metabolic behavior. Such strong changes in the composition of the bacterial community in electro-fermentation of glycerol were also reported by Xafenias et al. [166]. However, a highly negative potential was applied at the cathode in their experiments, resulting in the formation of significant amounts of H_2 on the electrode surface. As hydrogen is a common electron shuttle supporting interspecies electron transfers (IET) [9], it is not surprising that its continuous production at the cathode impacted the population composition. In the current study, electron inputs through the cathode were relatively low and the variations of microbial community composition in the bulk between fermentation controls (F) and electro-fermentation reactors (EF) were not so sharp. By contrast, a significant change of the microbial community occurred when *G. sulfurreducens* pre-colonized electrodes were used in electro-fermentation (EFG1). Some OTUs estimated as being good PDO producers by the model were specifically enriched in the bulk during EFG1. Some of these OTUs, such as OTU2 (99 % 16S rRNA sequence similarity with *Citrobacter freundii*), are related to species that are already known as efficient PDO producers [20,83]. Others, such as OTU1, which belongs to the *Enterococcus* family, became the most dominant OTU during EFG1 and counted for more than half of the microbial community. Species from the *Enterococcus* family were never reported to produce PDO, even though *E. avium* along with *Enterococcus faecalis*, *Enterococcus faecium* and *Enterococcus gallinarum* were described as glycerol consumers [229]. This family is closely related to the *Lactobacillaceae* family in which some species, such as *Lactobacillus reuteri*, have already been characterized as PDO producers [63,230]. It is then possible that these two families share the same ability to synthesize PDO from glycerol and further investigations on species belonging to the *Enterococcus* family could reveal new species of industrial interest.

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4.4.3 Electro-fermentation: towards a better PDO production

In the literature, the first electro-fermentation results showed significant changes in mixed culture glycerol fermentation after adding electrodes in the bulk phase [164–167]. The common strategy of these studies was to force an electron flow into the bioreactor by setting very low potentials at the WE (between -1.68 and -1.14 V vs SCE). This strategy led in some cases to an increase of PDO production yield [164,167], a higher final concentration of PDO [166] along with changes in microbial population structures [165,166]. The conversion of glycerol ($E_{eq} = 14$) into PDO ($E_{eq} = 16$) requires only two electrons, meaning that a direct bio-electrochemical conversion of glycerol into PDO would theoretically lead to a η_{EF} of 0.13 (2 electrons over the 16 electrons contained in PDO). Electro-fermentation efficiencies (η_{EF}) estimated from these studies ranged between 0.34 and 0.79, indicating that the large majority of the cathodic electrons were not used for PDO production but were probably consumed for electrochemical splitting of water into H_2 , making these processes highly electron consuming.

In the present study, glycerol electro-fermentation was conducted to avoid electrochemical H_2 production (see Figure 2-1). Nonetheless, a metabolic shift was observed but was not related to more efficient PDO production. The productions of lactate, ethanol and propionate were indeed affected even at very low cathodic current (always more positive than -0.35 A.m⁻²). As this low current density was likely due to a lack of electron mediators between the cathode and the bulk phase (e.g. H_2 , formate, metals, etc.), *G. sulfurreducens* was pre-colonized on the cathode prior to the experiment in an attempt to increase the bioavailability of cathodic electrons. *G. sulfurreducens* has already been described as being able to uptake electrons from a cathode [150] but also to transfer electron to other species through IET [201,231–233]. Then this species was a good candidate to facilitate electron transfers between the cathode and fermenters in the bulk. In the current study, *G. sulfurreducens* had a significant impact on population selection in the bulk but failed to improve cathodic electron densities as less cathodic electrons were transferred in EFG1 than in EF. Thus part of the population shift was likely due to specific interactions between some species from the bulk and *G. sulfurreducens* or other bacteria attached to the cathodes. The precise nature of these interactions is not yet elucidated.

4.5 Conclusions

Electro-fermentation is a promising concept that could help in specific mixed culture enrichments to improve metabolite production yields. The presence of an electrode inside a fermentation medium provides the opportunity to influence intracellular redox regulations without any addition of chemicals, but also to impact the microbial population structure during mixed culture processes. However, the main challenge regarding the future implementation of this concept is to find efficient catalysts able to ensure specific interactions between the electrochemical system and fermentative bacteria. Achieving high current densities is not relevant if this current is mainly used to sustain side-reactions such as water electrolysis. The present study suggests that high current densities are not necessary to influence metabolic patterns and/or microbial population structure during glycerol mixed-culture electro-fermentation. Moreover, the addition of *G. sulfurreducens* as mediator had a significant impact on population selection and seemed to trigger the emergence of good PDO producers. Whether this is due to a better utilization of the cathodic current or to specific interactions independent from the cathode remains unclear but could open new opportunities regarding the use of electro-active bacteria for a better control of fermentation processes.

Chapter 5. Electronic interactions between fermentative and electro-active bacteria: co-culture of *C. pasteurianum* and *G. sulfurreducens*

Experiments reported in this chapter are a proof-of-concept showing that despite the absence of electron acceptor, *G. sulfurreducens* is still able to grow in presence of *C. pasteurianum*. As a consequence, metabolic patterns of *C. pasteurianum* shift toward better electron dissipation (PDO production in glycerol fermentation). Possibility of direct interspecies electron transfer is discussed and put into perspective with electro-fermentation. This chapter is mainly based on an article currently under revision in Scientific Reports:

Moscoviz R, de Fouchécour F, Santa-Catalina G, Bernet N, Trably E. Cooperative growth of *Geobacter sulfurreducens* and *Clostridium pasteurianum* with subsequent metabolic shift in glycerol fermentation. Sci. Rep. 7: 44334

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5.1 Introduction

To sustain their growth and maintenance, microorganisms perform oxidative and reductive reactions inside their cells. These redox reactions consist in electron flows coming from an electron donor that are stepwise transferred to a terminal electron acceptor (*e.g.* O₂ in aerobic respiration) with an overall release of free energy. However, single microorganisms are not always able to perform the entire cascade of reactions. In this case, they can couple their electron flows with other species through mechanisms of interspecies electron transfer (IET) to carry out reactions that would otherwise be thermodynamically unfavourable [8,9]. A well described example is the IET existing between archaea and bacteria during methanogenesis through the diffusion of H₂ or formate [9,234]. More recently, direct interspecies electron transfer (DIET) that does not proceed through the diffusion of electron carriers has been discovered. During DIET, electrons are transferred via physical contacts between electron-donor (exoelectrogens) and electron-acceptor (electrotrophs) microorganisms [9,193,233]. Contacts between the two partners can be ensured by the establishment of a biofilm on a conductive material [8,193] (*e.g.* metals or carbon materials) or by connecting species with pili with metallic-like conductive properties [8,9,233]. These pili, named nanowires, can be produced by iron-reducing bacteria such as *Geobacter metallireducens* [235] or *Geobacter sulfurreducens* [236] that are even able to connect bacteria up to a centimetre scale [237]. Instead of using iron as electron sink, these species were reported to be able to transfer their electrons to other species, such as denitrifying bacteria [201,238] or hydrogenotrophic methanogens [190].

Interestingly, one of the two partners of a DIET can be replaced by an electrode that act as an artificial electron donor [150,239] (cathode) or acceptor [153] (anode). This is the basis of bio-electrochemical systems (BESs), processes that have been designed to take advantage of electro-active bacteria to produce electricity, chemicals or other services [161]. For instance, exoelectrogens (*e.g.* *Geobacter* species) can generate electrical power in microbial fuel cells while oxidizing organic matter from wastes [153]. Methanogens or denitrifying bacteria (electrotrophs) are also able to consume electrons from a cathode in microbial electrolysis cells to convert CO₂ into methane [240], or reduce nitrates, respectively [241,242]. The intensive research that has been conducted on BESs has revealed that besides the well-known *Geobacter* and *Shewanella* species, many other microorganisms are able to interact directly with electrodes [150,156,239]. In particular, metabolic patterns of

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fermentative bacteria could be affected by small input of electrons through a cathode during electro-fermentation experiments [6,243] (see Section 1.4). As an illustration, *Clostridium pasteurianum* was reported to be able to consume cathodic electrons during fermentation, and produced more butanol and 1,3-propanediol (PDO) from glucose and glycerol respectively than during classic fermentation [7]. As this fermenter was able to uptake extracellular electrons from a cathode, it is not excluded that electrons provided by DIET with an exoelectrogen organism could also be consumed and lead to a similar metabolic shift. To date, studies on electron flux between exoelectrogens such as *Geobacter* species and fermenters have always focused on the degradation of organic matters by the fermenters into simple carboxylic acids that could be readily converted by the exoelectrogens into electricity in microbial fuel cells, *i.e.* electron transfer from the fermenting to the electrogenic organism [185,244]. As highlighted in Chapter 4, the presence of *G. sulfurreducens* on cathodes in mixed-culture glycerol electro-fermentation had a significant impact on population selection. This population structure modification could not be solely due to electron transfer from fermenting (*i.e.* planktonic bacteria) to electrogenic organism (*G. sulfurreducens*) as the latter could not use the electrode as electron acceptor.

The aim of this study is to provide a proof-of-concept experiment showing that electron coupling between fermenters and exoelectrogens is also possible from exoelectrogens (electron donor) to fermentative species (electron acceptor). This could serve to trigger a metabolic shift towards the production of more reduced products such as PDO. This experiment was conducted for glycerol fermentation using a defined co-culture of *G. sulfurreducens* and *C. pasteurianum* as model partners for DIET.

5.2 Experimental design

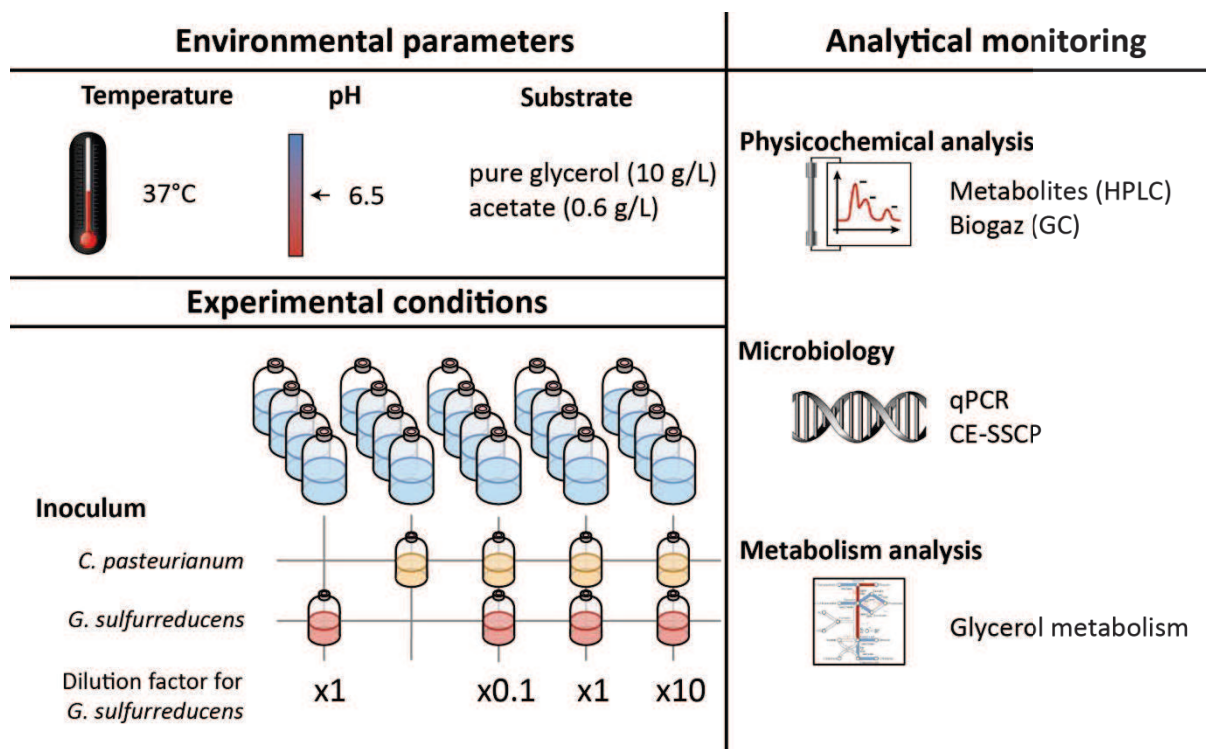


Figure 5-1. Experimental design for the study of a possible electronic interaction between *G. sulfurreducens* and *C. pasteurianum*.

The possible interactions between *G. sulfurreducens* (electro-active species) and *C. pasteurianum* (fermenter) were studied using 100mL flasks in quadruplicate co-cultures experiments. The fermentation medium used in this study was a compromise between the optimal media used for growing *G. sulfurreducens* (see Section 2.5.3.1) and *C. pasteurianum* (see Section 2.5.4). No fumarate (electron acceptor for *G. sulfurreducens*) was added to the medium in order to force *G. sulfurreducens* to interact with *C. pasteurianum*. Special attention was also paid to iron concentration in the fermentation medium to avoid any Fe^{3+} reduction by *G. sulfurreducens* that could interfere with the experiment. As a result, the total iron concentration was only $7.3 \mu\text{mol.L}^{-1}$ in the co-culture medium. Both 10 g.L^{-1} glycerol (fermentable by *C. pasteurianum*, but not by *G. sulfurreducens*) and 10mM acetate (electron donor for *G. sulfurreducens*, but non-usable by *C. pasteurianum*) were present in the fresh medium. An optimal temperature of 37°C and an initial pH value of 6.5 were set. For experiments with different *G. sulfurreducens* initial concentrations, *G. sulfurreducens* pre-cultures were concentrated and washed by centrifugation and resuspension in fresh co-culture medium. This concentrated medium (about 10 times compared to the pre-culture) was then

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diluted when needed. Methods employed during this study are more specifically described in Section 2.4.

5.3 Results

5.3.1 Growth of *G. sulfurreducens* and *C. pasteurianum* in co-cultures

To study the possible interactions that could exist between *G. sulfurreducens* and *C. pasteurianum*, the two strains were inoculated in a medium containing glycerol (fermentation substrate for *C. pasteurianum*) and acetate (electron donor for *G. sulfurreducens*). The growth of both *G. sulfurreducens* and *C. pasteurianum* were monitored using qPCR, as shown in Figure 5-2. To evaluate the growth of a population, the number of generation (N_g) can be used that corresponds to the \log_2 ratio of the final population count over the initial population count (see Section 2.4.4.1). During pure culture of *G. sulfurreducens*, no growth occurred as initial and final cell counts were strictly identical ($3.3 \pm 1.9 \cdot 10^6$ and $3.3 \pm 1.2 \cdot 10^6$ cells.mL⁻¹ respectively, $n = 8$ replicates), resulting in $N_g < 0.5$ (twice the standard error of qPCR measurements). This confirmed that no electron acceptor was available in the fresh medium to sustain the growth of *G. sulfurreducens*. Pure cultures of *C. pasteurianum* were inoculated at $5.2 \pm 2.0 \cdot 10^4$ cells.mL⁻¹ ($n = 4$). Growth was observed and stopped at $1.2 \pm 0.2 \cdot 10^8$ cells.mL⁻¹ after total substrate depletion ($N_g = 11.2 \pm 0.35$, $n = 4$).

Considering the co-culture experiments, *G. sulfurreducens* grew in only two of the four replicates. During these two replicates, a slightly lower growth of *C. pasteurianum* was observed when compared to the pure culture control ($N_g = 9.3 \pm 0.8$, $n = 2$), with an inoculation at $1.2 \pm 0.7 \cdot 10^5$ cells.mL⁻¹ and a final concentration of $7.4 \pm 1.5 \cdot 10^7$ cells.mL⁻¹ ($n = 2$). Interestingly, a significant growth of *G. sulfurreducens* was also observed, with $N_g = 2.2 \pm 0.1$ ($n = 2$). This means that *G. sulfurreducens* was able to use an electron acceptor that was not present in the fresh medium. The relatively low growth of *G. sulfurreducens* could be explained by its high doubling time (T_d) as previously reported in electron acceptor limiting conditions ($T_d = 6.93$ h) [201,245], especially when compared to *C. pasteurianum* doubling time in glycerol fermentation ($T_d = 1.87$ h) [59]. If *C. pasteurianum* accepts electrons from *G. sulfurreducens*, it can be hypothesized that *G. sulfurreducens* could only grow during *C. pasteurianum* growth phase. In this context, the ratio of generation numbers N_{gGsul} / N_{gCpast} ratio of *G. sulfurreducens* and *C. pasteurianum* respectively in co-culture should not exceed the ratio of the corresponding doubling times. During this experiment, the N_g ratio was $0.24 \pm$

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0.04 and was comparable to the T_d ratio of 0.27 calculated from values reported in the literature, supporting the synchronous growth of *G. sulfurreducens* and *C. pasteurianum*.

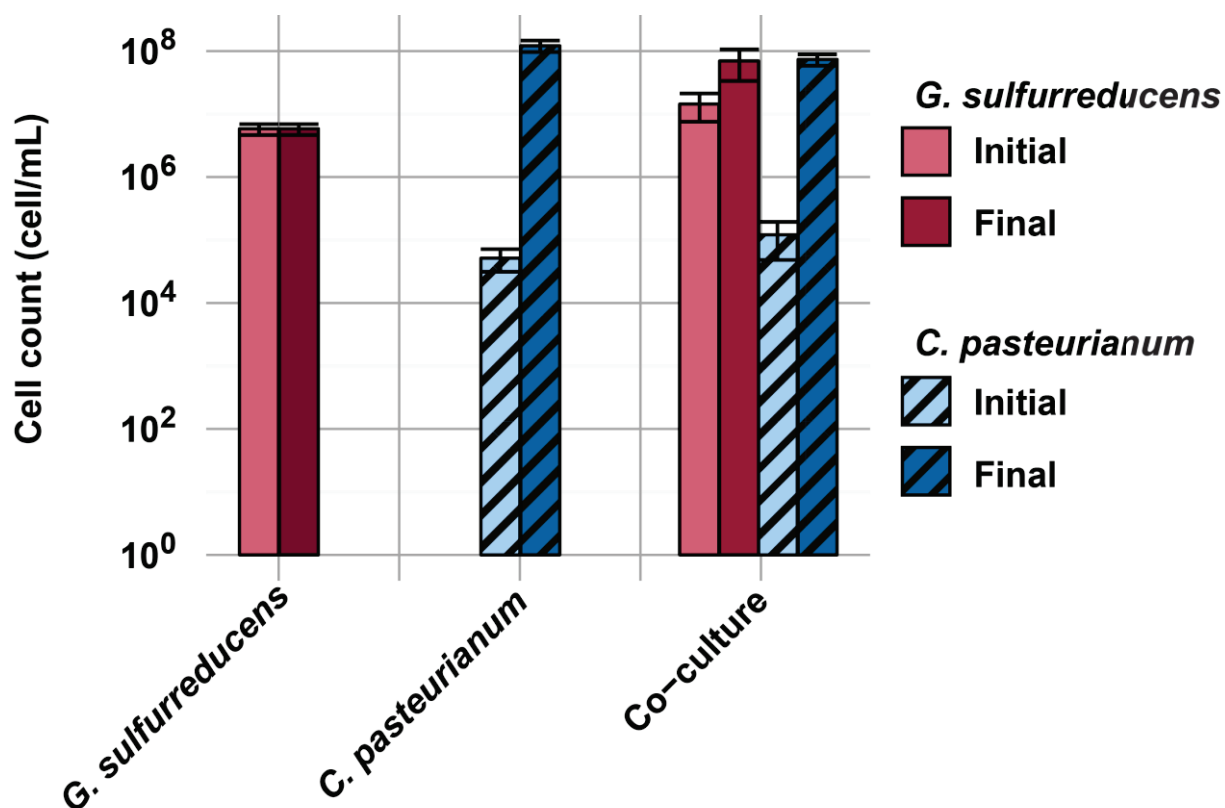


Figure 5-2. Growth monitoring of *G. sulfurreducens* and *C. pasteurianum*.

Cells counts are based on qPCR results and corrected by the respective number of copies of 16S rRNA for each strain. Error bars represent the standard deviation of the replicates.

5.3.2 Metabolic patterns shifted during co-cultures

After 72h of fermentation, metabolites in the liquid phase were measured to establish mass balances. Electron mass balances closed between 98.3 and 100.0 %, meaning that all major metabolic products were quantified (see Figure 5-3). When *G. sulfurreducens* was cultivated alone ($n=8$), no substrate was consumed. In contrast, all the glycerol was depleted after 72 h with the pure cultures of *C. pasteurianum* ($n = 4$). The main metabolite was butanol with a yield of $225 \pm 5 \text{ mmol.mol}_{\text{glycerol}}^{-1}$. Other major metabolic products were 1,3-propanediol (PDO, $176 \pm 13 \text{ mmol.mol}_{\text{glycerol}}^{-1}$), ethanol ($54 \pm 2 \text{ mmol.mol}_{\text{glycerol}}^{-1}$) and butyrate ($76 \pm 7 \text{ mmol.mol}_{\text{glycerol}}^{-1}$). Only the two replicates of *G. sulfurreducens* and *C. pasteurianum* co-cultures where *G. sulfurreducens* growth was observed had different metabolic patterns when compared to the pure cultures of *C. pasteurianum*. In these two

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replicates, PDO and butyrate yields significantly increased to $241 \pm 8 \text{ mmol.mol}_{\text{glycerol}}^{-1}$ ($p = 0.016$) and $105 \pm 6 \text{ mmol.mol}_{\text{glycerol}}^{-1}$ ($p = 0.018$) respectively, with regard to *C. pasteurianum* alone. On the opposite, butanol and ethanol yields significantly decreased to $188 \pm 9 \text{ mmol.mol}_{\text{glycerol}}^{-1}$ ($p = 0.016$) and $43 \pm 4 \text{ mmol.mol}_{\text{glycerol}}^{-1}$ ($p = 0.023$), respectively. In addition, samples were taken from the co-cultures after 240 h, to observe additional metabolite production or consumption following glycerol depletion. No significant changes were observed in comparison with the samples at 72h. As electron donors (*i.e.* acetate) were still present in the medium, this observation confirms that *G. sulfurreducens* was not able to directly use the end-products issued from *C. pasteurianum* fermentation as electron acceptors.

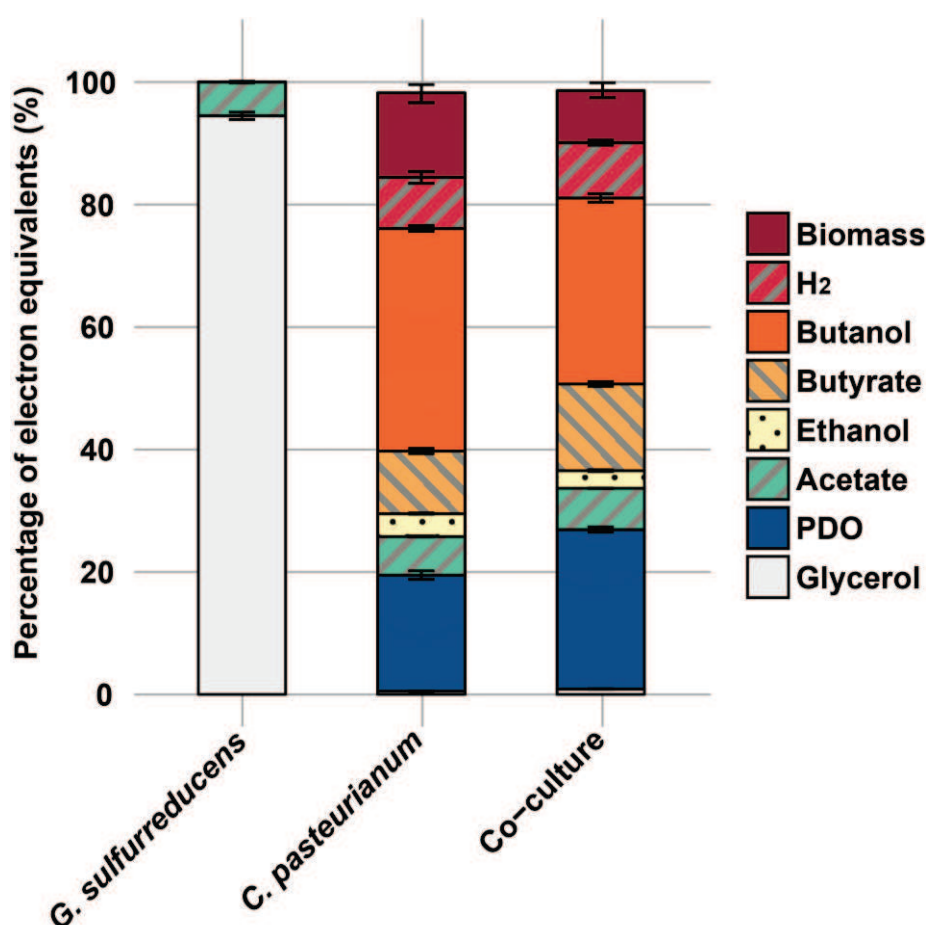


Figure 5-3. Electron balances calculated from the metabolites measured at the end of co-culture experiments.

Results are normalized on the sum of electron content from initial glycerol and acetate. The biomass was calculated from qPCR cell count results. Error bars represent the standard deviation of the replicates.

5.3.3 Metabolic shift of *C. pasteurianum*

Assuming that *G. sulfurreducens* transferred its electrons to *C. pasteurianum*, electron and carbon mass balances could be estimated for *C. pasteurianum* only (see Figure 5-4). Calculations were made under the following hypotheses: (i) acetate is the only carbon source and electron donor for *G. sulfurreducens* growth [181], (ii) this species uses only 10 % of the electron equivalents it consumed for its own growth [245] and (iii) all the remaining electrons are transferred to *C. pasteurianum*. In this context, the acetate consumed by *G. sulfurreducens* for its own growth could be assessed from qPCR data and was estimated around 1.1 ± 0.4 mM ($n = 2$). According to the different hypotheses, this acetate was used by *G. sulfurreducens* according to the following equation:



The electrons released from acetate consumption represented 0.6 % of the total electron equivalents consumed by *C. pasteurianum*. If this small amount of electrons was directly dissipated by *C. pasteurianum* by converting glycerol into PDO (2 moles electrons consumed per mole PDO produced), it would increase the PDO production from 17.6 %_{total carbon} (pure culture control) to only 21.5 %_{total carbon}. However, 24.1 ± 0.8 %_{total carbon} ($n = 2$) was recovered as PDO in the co-culture. It was therefore concluded that a direct dissipation of the electrons provided by *G. sulfurreducens* could not be the unique reason of the changes in metabolic patterns. In fact, carbon and electrons were also diverted from biomass synthesis and solventogenesis pathways (*i.e.* production of ethanol and butanol) to the production of carboxylic acids and PDO (see Figure 5-4). All these observations are consistent with previous electro-fermentation results reported by Choi et al. [7]: providing a small quantity of electrons from a cathode to the same strain of *C. pasteurianum* (2 % of the total electron input) resulted in a decrease of biomass and butanol production and an increase of PDO and butyrate production. Concerning biomass synthesis, this result is surprising since the increase and decrease in carboxylic acids and alcohols, respectively, should have theoretically led to an increase of the ATP production by 8.6 %. How this extra ATP was dissipated remains unknown.

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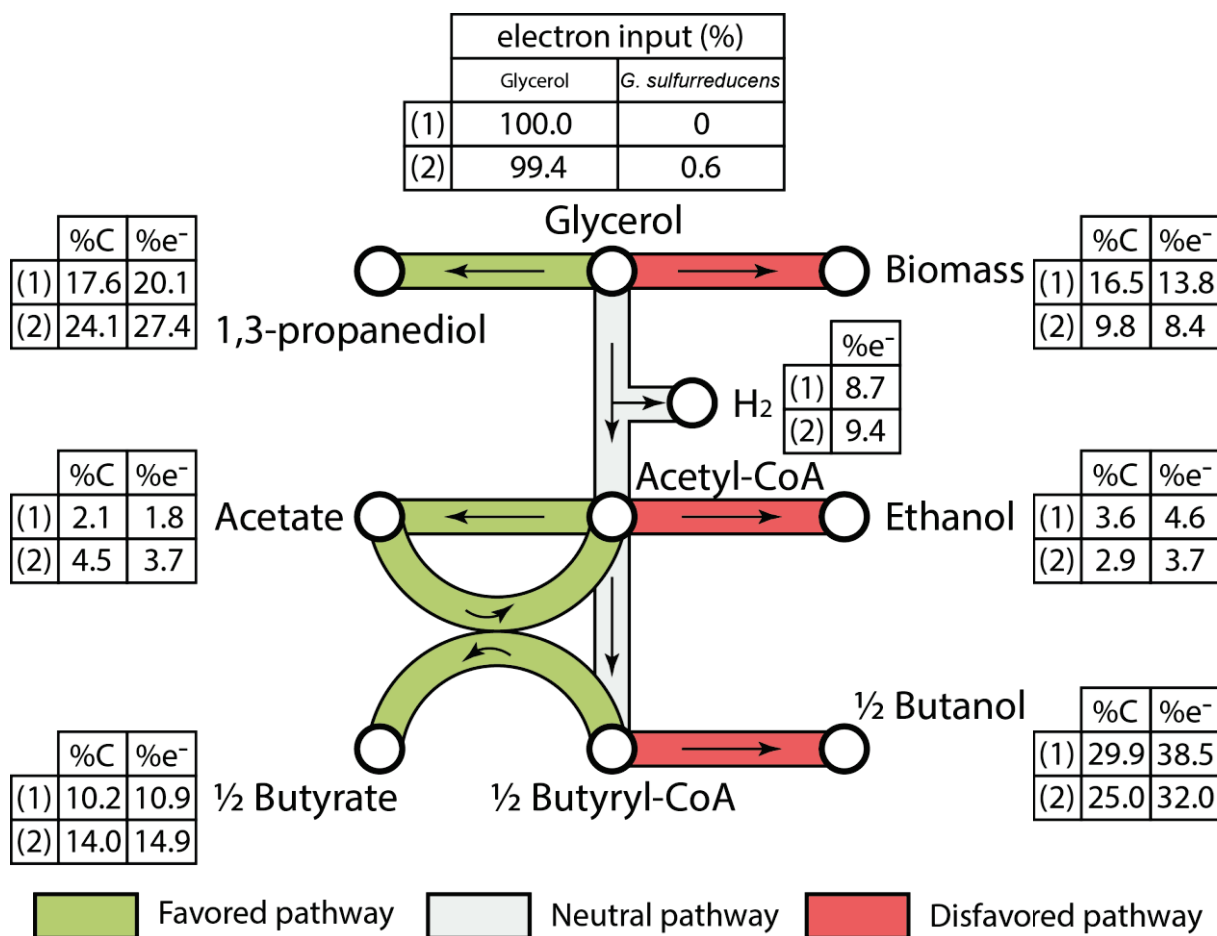


Figure 5-4. Average carbon and electron distributions of the products from glycerol fermentation during (1) pure cultures of *C. pasteurianum* and (2) co-cultures of *G. sulfurreducens* and *C. pasteurianum*.

Pathways are considered to be favored or disfavored if more than 10 % increase of decrease resp. was observed between (1) and (2).

5.3.4 Effect of *G. sulfurreducens* growth on *C. pasteurianum* production yields

In order to study the dependence of *C. pasteurianum* metabolic shift on biomass production of *G. sulfurreducens*, three quadruplicate experiments were carried out with distinct *G. sulfurreducens* initial concentrations of $7.4 \pm 2.8 \cdot 10^5$ (n = 4), $8.9 \pm 4.6 \cdot 10^6$ (n = 4) and $1.1 \pm 0.4 \cdot 10^8$ (n = 4) cells.mL⁻¹ respectively. In addition, four pure cultures of *C. pasteurianum* were performed as controls. All metabolite yields together with the growth yields of *C. pasteurianum* and *G. sulfurreducens* are provided in Table 5-1. Out of 12 co-cultures, only 8 experiments exhibited significant *G. sulfurreducens* growth ($N_g > 0.5 \log_2(\text{cells}).\text{mL}^{-1}$). The average numbers of generation $N_{g\text{Gsul}}$ of *G. sulfurreducens* ($N_{g\text{Gsul}} = 1.4$

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± 0.4 , $n = 8$) was lower than in the previous experiment ($N_{gGsul} = 2.2 \pm 0.1$). The N_{gCpast} of *C. pasteurianum* was also lower ($N_{gCpast} = 6.6 \pm 0.6$, $n = 8$) than the value of 9.3 ± 0.8 obtained in the previous experiment, as a consequence of a higher inoculum concentration. Nonetheless, this resulted in a N_{gGsul} / N_{gCpast} ratio of 0.21 ± 0.06 ($n = 8$) very comparable to the value of 0.24 ± 0.04 obtained previously. This supports again that *G. sulfurreducens* was only able to grow during the *C. pasteurianum* growth phase. For these 8 experiments, the metabolite yields as function of *G. sulfurreducens* growth are displayed in Figure 5-5. Significant correlations were found between the log-transformed *G. sulfurreducens* growth ($\log(\Delta\text{cells.mL}^{-1})$) and the metabolite yields. A high *G. sulfurreducens* growth positively correlated with an increase of PDO ($r = 0.95$, $p = 0.0002$, $n = 8$), butyrate ($r = 0.91$, $p = 0.0003$, $n = 8$) and acetate ($r = 0.93$, $p = 0.0069$, $n = 8$). In contrast, butanol ($r = -0.87$, $p = 0.0002$, $n = 8$) and ethanol ($r = -0.95$, $p = 0.0008$, $n = 8$) were disfavoured when *G. sulfurreducens* cells increased. Overall, between 76 and 90 % of total variance of the metabolite yields were explained by the growth of *G. sulfurreducens*.

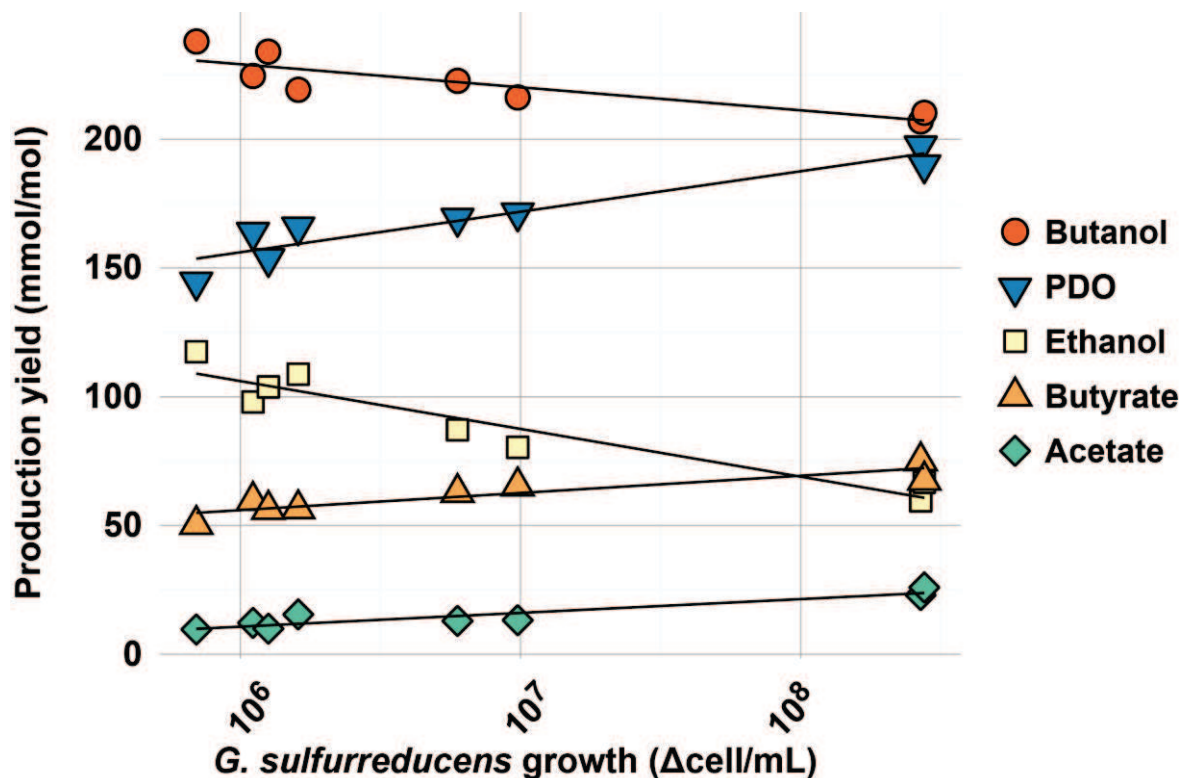


Figure 5-5. Production yields as a function of *G. sulfurreducens* growth during co-culture experiments with *C. pasteurianum*.

Only the results of experiments with a significant *G. sulfurreducens* growth are displayed ($N_g > 0.5 \log_2(\text{cell}).\text{mL}^{-1}$).

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Table 5-1. Metabolite production yields and growth of the co-culture.

<i>G. sulfurreducens</i> biomass		<i>C. pasteurianum</i> biomass	Production yield (mmol/mol _{Glycerol} ± std)					n
Initial (cell/mL)	N _{gGsul}	N _{gCpast}	PDO	Acetate	Butanol	Butyrate	Ethanol	
0	0	7.7 ± 0.3	159 ± 8	13 ± 2	233 ± 7	55 ± 3	106 ± 4	4
7.4 ± 2.8 10 ⁵	1.4 ± 0.3	7.1 ± 0.4	157 ± 10	12 ± 3	229 ± 8	56 ± 4	106 ± 8	4
8.5 ± 1.8 10 ⁶	1.0 ± 0.4	6.8 ± 0.3	170 ± 1	15 ± 3	220 ± 4	63 ± 2	85 ± 4	2*
1.2 ± 0.5 10 ⁸	1.7 ± 0.6	6.7 ± 0.4	194 ± 5	24 ± 2	209 ± 2	71 ± 5	63 ± 5	2*

* Only the results of experiments with a significant *G. sulfurreducens* growth are displayed ($N_g > 0.5 \log_2(\text{cell}).\text{mL}^{-1}$).

5.4 Discussion

5.4.1 *G. sulfurreducens* using *C. pasteurianum* as sole electron acceptor

G. sulfurreducens is a specialized microorganism with very limited metabolic capacities [181,246]. Amongst the metabolites found in fermentation and observed during this study, only acetate and H₂ could have been used by *G. sulfurreducens* as potential electron donors, and acetate was the only available carbon source. The range of known electron acceptors that can be used by *G. sulfurreducens* is also limited, and consists in some metal ions, elemental sulfur, malate and fumarate [181,246]. None of these electron acceptors was present in the fermentation medium to sustain *G. sulfurreducens* growth (see Figure 5-2). When growing together with *C. pasteurianum*, *G. sulfurreducens* could have also used glycerol fermentation end-products as sole electron acceptors. However, *G. sulfurreducens* was not able to grow after glycerol depletion, making the latter hypothesis invalid. The last

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alternative for *G. sulfurreducens* was to use *C. pasteurianum* as electron acceptor through mechanisms of interspecies electron transfers [8,9,188,233,247,248]. A similar behavior was previously reported in a co-culture of *Desulfovibrio vulgaris* and *Clostridium acetobutylicum* in which *D. vulgaris* was able to grow under nutritional stress using *C. acetobutylicum* as electron acceptor [249]. Metabolic pattern of *C. acetobutylicum* was in turn modified toward more electron dissipation via H₂ production. Since *C. pasteurianum* DSM 525 was reported to be able to uptake extracellular electrons from a cathode during glycerol electro-fermentation [7], it is highly probable that *G. sulfurreducens* transferred electrons to *C. pasteurianum*, either directly via interspecies wiring [8] or indirectly using soluble electron mediators present in the medium such as L-cysteine [250]. Through such interaction, *G. sulfurreducens* triggered a significant metabolic shift for *C. pasteurianum* that enhanced the production of PDO at the expense of butanol and ethanol.

This metabolic shift could not be only due to direct dissipation of the electrons released by the growth of *G. sulfurreducens*. Instead, a non-linear relationship was found between *G. sulfurreducens* growth (*i.e.* electrons transferred to *C. pasteurianum*) and the change of production yields (Figure 5-5). Extra electrons seemed to trigger metabolic regulations in favor of PDO pathway as main electron dissipation pathway. Once promoted, the PDO pathway could ensure intracellular redox balance by dissipating NADH, resulting in acetate and butyrate being produced from acetyl-CoA and butyryl-CoA instead of ethanol and butanol respectively. This would have theoretically led to more ATP production. Therefore a higher biomass production by *C. pasteurianum* could have been expected. In contrast, 39 % less biomass production was observed in co-cultures than in pure cultures of *C. pasteurianum*. Consistently, Choi et al. (2014) reported a very similar drop of biomass production in glucose electro-fermentation: *C. pasteurianum* grown with a cathode as electron donor produced 41 % less biomass than the open-circuit fermentation control [7]. The same phenomenon was also observed for other fermentative species growing in contact with a cathode [169,172]. If extracellular electrons uptake is an ATP-consuming process, then forcing electron consumption could be an interesting strategy to reduce biomass and by extension sludge formation during fermentation processes, leading to a better carbon and electron recovery. However, a better understanding of this decrease in biomass synthesis is necessary and should be the focus of further research.

5.4.2 Is nanowire expression the key for DIET?

In the present study, only 10 out of 16 co-culture experiments showed significant *G. sulfurreducens* growth, along with subsequent metabolic shift in glycerol fermentation. This observation could be related to the initial physiological state of *G. sulfurreducens* when inoculated. Indeed, a proteomic analysis previously revealed that under long-term terminal electron acceptor limiting conditions, *G. sulfurreducens* DSM 12127 enhanced the synthesis of some of its membrane-associated proteins [251]. In particular, this increased the synthesis of the pilA protein involved in nanowires conductivity [236]. The total heme content (e.g. cytochrome *c*) in the cells was also increased almost threefold compared with cells in midlog phase [251]. As a consequence, cells became poised and more susceptible to sense and use other electron acceptors that were encountered. In the present study, *G. sulfurreducens* pre-cultures were used as inoculum only after the cells precipitated as red aggregates (*i.e* with high heme content [251]). However the starvation time lasted only few days and the pre-cultures were probably heterogeneous and were constituted of both nanowire-rich aggregates and nanowire-poor planktonic cells. As the co-culture experiments duration was short, it is probable that only *G. sulfurreducens* cells that were already starved (*i.e* aggregates) at the inoculation time could have interacted with *C. pasteurianum*. That probably led to the heterogeneity in the observed results.

One option to ensure electrical connections during co-culture experiments could be to grow *G. sulfurreducens* as a biofilm on conductive materials. Studies focusing on electricity generation using *G. sulfurreducens* anodic biofilms have shown that such biofilms exhibit conductive properties through networks of nanowires [237,252]. Then, electron exchanges with a partner such as *C. pasteurianum* would be promoted as the two species would be physically connected through both pili networks and the conductive material [253]. This strategy has already been successfully applied to enhance DIET in methanogenesis [231,253–255]. For instance, inexpensive materials such as granular activated carbon were added into anaerobic digesters and resulted in an enrichment of *Geobacter*-like bacteria on the granules together with a significant increase of the methane production rate [231]. Moreover, attaching *G. sulfurreducens* on a carbon material could allow its use during continuous fermentation, thus overcoming the growth limitations observed during batch operations. In this case, the different growth rates of *G. sulfurreducens* and *C. pasteurianum* or other fermentative bacteria would not be an issue anymore and *G. sulfurreducens* wash-out would be avoided.

5.5 Conclusion

Results reported in this chapter extend the electro-fermentation concept beyond the use of BES: the role of *G. sulfurreducens* in the present study was very similar to the cathode in a cathodic electro-fermentation. Under the hypothesis that interactions between *G. sulfurreducens* and *C. pasteurianum* are non-specific, continuous co-culture fermentations with electro-active and fermentative bacteria would provide many benefits such as: (i) recycling electrons from undesired fermentation end-products to promote the production more reduced compounds; (ii) avoiding the accumulation of inhibitors such as acetic acid; (iii) purifying the fermentation medium by removing undesired metabolites. Nonetheless, even if the present study is a very promising proof-of-concept, huge efforts on both process engineering and fundamental principles elucidation are required to fully take advantage of electro-active/fermentative interactions.

Conclusions & perspectives

Mixed culture fermentation for the production of value-added chemicals

































As stressed by Kleerebezem *et al.* [96,256], open mixed-culture processes have the potential to become both economically and environmentally competitive to well-established pure strain processes when considering the production of certain bulk chemicals such as H₂ [257], 2nd generation bioethanol [258], short and medium chain fatty acids (*e.g.* acetate, *n*-caproate, *n*-caprylate) [259]. Glycerol-derived metabolites such as PDO are also good candidates for being produced by mixed-culture processes, especially if crude glycerol is directly used as substrate [1]. The aim of this thesis was not to develop a high-performance mixed-culture process for PDO production. Instead, the work focused on evaluating the response of a relatively complex bacterial community to influent environmental parameters previously identified from the literature. Several interesting features of mixed consortia usages can be highlighted by the results presented throughout this thesis (Chapters 3 and 4):



- The mixed community used in this work was able to ferment glycerol in a minimal medium containing no yeast extract or equivalent additive.
- The PDO yields reported in this work, although obtained in batch mode and without substrate or product inhibition, ranged from 0.46 to 0.64 mol_{PDO} mol⁻¹_{glycerol} and were comparable to the best yields reported in the literature for pure strains.
- The bacterial community was remarkably functionally redundant when exposed to perturbations such as a large range of pH.



However, the latter advantage can also be considered as a drawback because it means that changing the function of such mixed consortium (*e.g.* for better PDO production) is particularly challenging. Considering this, the use of electro-fermentation systems and the addition of electro-active species such as *G. sulfurreducens* could become additional tools for a better control of mixed culture processes. Results reported in Chapter 4 showed that the presence of a polarized electrode and/or an electro-active species was able to induce a population shift within the bacterial community, and the change of metabolic yields was directly linked to the new composition of the bacterial community. However, the mechanisms underlying this population shift remain largely unknown. Understanding how microbial

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consortia are affected by electro-fermentation is notably ambitious because (i) the knowledge related to the response of pure strains to low redox conditions is still very limited (see Section 1.3.3.3); (ii) the same goes for the knowledge related to electron consumption from an electrode [10]; (iii) the biotic interactions between the community members are by nature highly complex and difficult to measure. Nonetheless, a first step towards better comprehension of electron coupling between bacteria and bacteria or electrodes was initiated in Chapter 5 by studying a synthetic co-culture composed of two different well-known bacteria. To go beyond, species of interest previously identified in mixed culture experiments can be used for a series of co-cultures experiments in presence or absence of electrodes (see Figure P-1). By measuring metabolites, bacterial growth and transcript levels of genes of interest (*e.g.* involved in central metabolism, interspecies interactions), this kind of experiment would improve the understanding of both electro-fermentation mechanisms and biotic interactions in mixed consortia. By identifying the most favorable interactions for PDO production, better control of mixed-culture processes shall be considered in the future by the addition of polarized electrodes, electro-active species or other microorganisms with interesting key functions. This could be a step forward in the implementation at higher scale of mixed-culture processes for the production of value-added metabolites.

Number of species	1	2	3	4
	   	 +   +   +   +   +   + 	 +  +   +  +   +  +   +  + 	 +  +  + 
Number of experiments	4	6	4	1

G. sulfurreducens
E. avium

D. mossii
C. freundii

Figure P-1. Example of combinatory design for co-culture experiments based on species identified in Chapter 4.

In addition, same conditions can be carried out in presence of a polarized electrode to assess the influence of electrochemical systems towards biotic interactions.

Electromicrobiology as a new approach to conduct biotechnologies

Electromicrobiology was defined by Lovley as “the study of microbial electron exchange with electronic devices or the investigation of the electronic properties of microorganisms” [260]. Although this emerging field was initially dedicated to understanding mechanisms of electricity production in MFC, it is clear that its impact is now much wider and could change the way anaerobic digestion and fermentation are conducted [261]. In this thesis, it was shown that the presence of *G. sulfurreducens* could (i) induce a bacterial population shift in mixed culture fermentation of glycerol (Chapter 4) and (ii) trigger a metabolic shift of a single fermentative species as shown in Chapter 5. In both cases, PDO production was favored, supporting that the presence of this electro-active bacteria could promote better electron dissipation in the whole process. As *G. sulfurreducens* does not ferment glycerol, this species could only have a support function (*i.e.* indirect effect on metabolic patterns) that was very likely to be related with interspecies electron transfers. In the literature, DIET could be promoted by adding conductive support materials in digesters while enhancing methane production and avoiding inhibitor accumulation (*e.g.* propionate) [231,253–255]. A similar approach could be envisaged to ensure better contact between fermenters and electro-active bacteria and potentially catalyze interactions between these two groups. This could be relevant for both fermentation and electro-fermentation: *G. sulfurreducens* or other well-chosen electro-active bacteria could be added in the fermentation broth, along with a support material or an electrode that would give it a selective advantage. Hence, electro-active bacteria could grow along with fermentative bacteria while consuming non-desired fermentation by-products for their growth and providing beneficial functions to the process. For instance, such electro-active species could be chosen for their ability to release organic electron shuttles in the fermentation broth that would ensure interactions between fermenters and electrodes (see Figure P-2A), as demonstrated in a co-culture of *S. oneidensis* and *E. coli* [197]. Another strategy would consist in using *Geobacter* species [181] to specifically oxidize fermentation by-products while providing electrons to an anode (see Figure P-2B), thus purifying the product of interest and limiting eventual inhibitions that could result from the accumulation of these by-products [163].

Results reported in Chapter 5 prove that instead of providing electrons to an anode, *G. sulfurreducens* was able to give electrons released by the oxidation of fermentation by-products back to the fermentative species. Interestingly, this metabolite recycling (see Figure P-2C) would enhance the production of reduced metabolites of interest (*e.g.* PDO from

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glycerol, butanol from glucose) even without the presence of a bio-electrochemical system. As in electro-fermentation, the production yields of these reduced compounds could exceed the maximum theoretical yields calculated for classic fermentation: in the case of glycerol fermentation, maximum PDO yield would increase from 0.72 to 0.85 mol_{PDO} mol⁻¹_{glycerol} (see Appendix A). Furthermore, if all fermentation by-products would have been recycled, this maximum yield could be approached without any dependence on the nature of the by-products, as they would only be intermediary compounds for CO₂ production (see Appendix A for detailed calculation). As a result, metabolite recycling could theoretically be a solution to the lack of product specificity of such fermentations. However, actual application of this strategy would require dedicated studies to assess if (i) electro-active biomass fixation on conductive material could be a solution to the different growth kinetics between fermentative and electro-active species as observed in Chapter 5; (ii) fermentative species other than *C. pasteurianum* could receive electrons from electro-active bacteria. If such “electro-fermentative” species could be found, metabolite recycling approaches would be at least a way to shift microbial metabolism towards better reduced compound production, and at the best a new way to exceed current maximum yields.

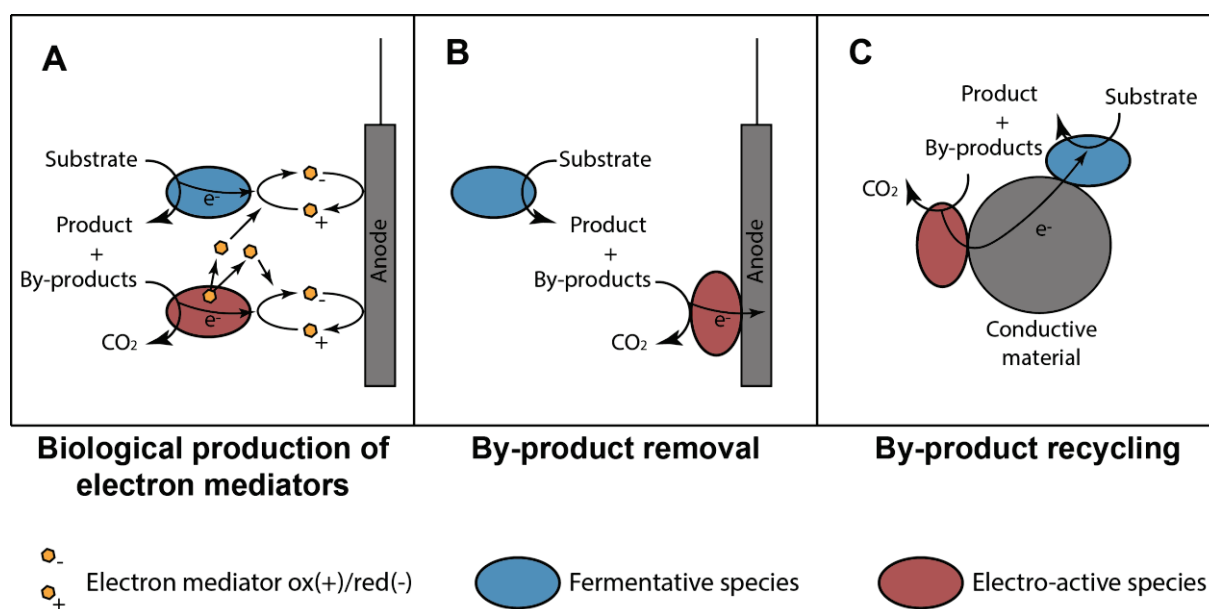


Figure P-2. Possible strategies for enhanced fermentation or electro-fermentation by the addition of electro-active species.

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Opportunities for electro-fermentation

Thermodynamics is not the sole limitation in fermentation production yields, as most of the overall reactions that occur during fermentation are spontaneous *i.e.* thermodynamically favorable. Other limitations are mostly due to biological regulations that keep the metabolism in a redox balanced environment. The presence of an electrode inside the fermentation medium is an opportunity to overcome these limitations at several levels.

First, EF makes possible the stoichiometric conversion of a substrate into a product of interest, by dissipating (resp. providing) all electrons released by (resp. needed for) the reaction. For instance, one mole of ethanol ($E_{eq} = 12\text{ e}^-$) can be produced from one mole of glycerol ($E_{eq} = 14\text{ e}^-$) if two moles of electrons are provided to an anode. Thus, EF presents the possibility of exceeding the theoretical maximum yields calculated for balanced fermentations, at even higher level than with metabolite recycling, as shown *in silico* by Kracke and Krömer (2014) (see Figure P-3) [174]. According to this simulation, many metabolites of economic interest, such as succinic acid or lysine, could be produced at significantly higher yields in EF when compared to classic fermentation, with very promising biotechnological outputs making EF a good candidate for full-scale application [174]. Such bioelectrochemical conversions will however require a relatively high current flow to ensure a good productivity, although lower than current consumed in MES, and therefore present similar limitations of most MFCs and MECs [175]. As stressed by Harnisch *et al.* [175], further fundamental research is needed and technological hurdles have to be taken.

Secondly, providing or consuming little amount of electrons through electrodes (*i.e.* η_{EF} close to zero) can be enough to affect the $NAD^+/NADH$ balance inside microorganisms, thus affecting whole metabolism. Since the amount of electrons passing through the electrochemical system is nearly negligible as compared with the electron flux from organic substrate oxidation, this approach does not allow exceeding theoretical yields. Nevertheless, it is an efficient way of controlling biological regulations that could lead to a more specific production of the desired end-product with low energy consumption. The use of redox mediators (*i.e.* artificial or from electro-active species) can make it even more attractive since no specific interactions between fermentative bacteria and the WE is required. Thus, EF could be potentially applied as an additional control tool for any fermentation process. More specifically, it could be a solution to the most challenging issue of mixed cultures processes, which is the increase of selectivity in fermentation patterns (*i.e.* production of a limited number of metabolites) and stability of this pattern.

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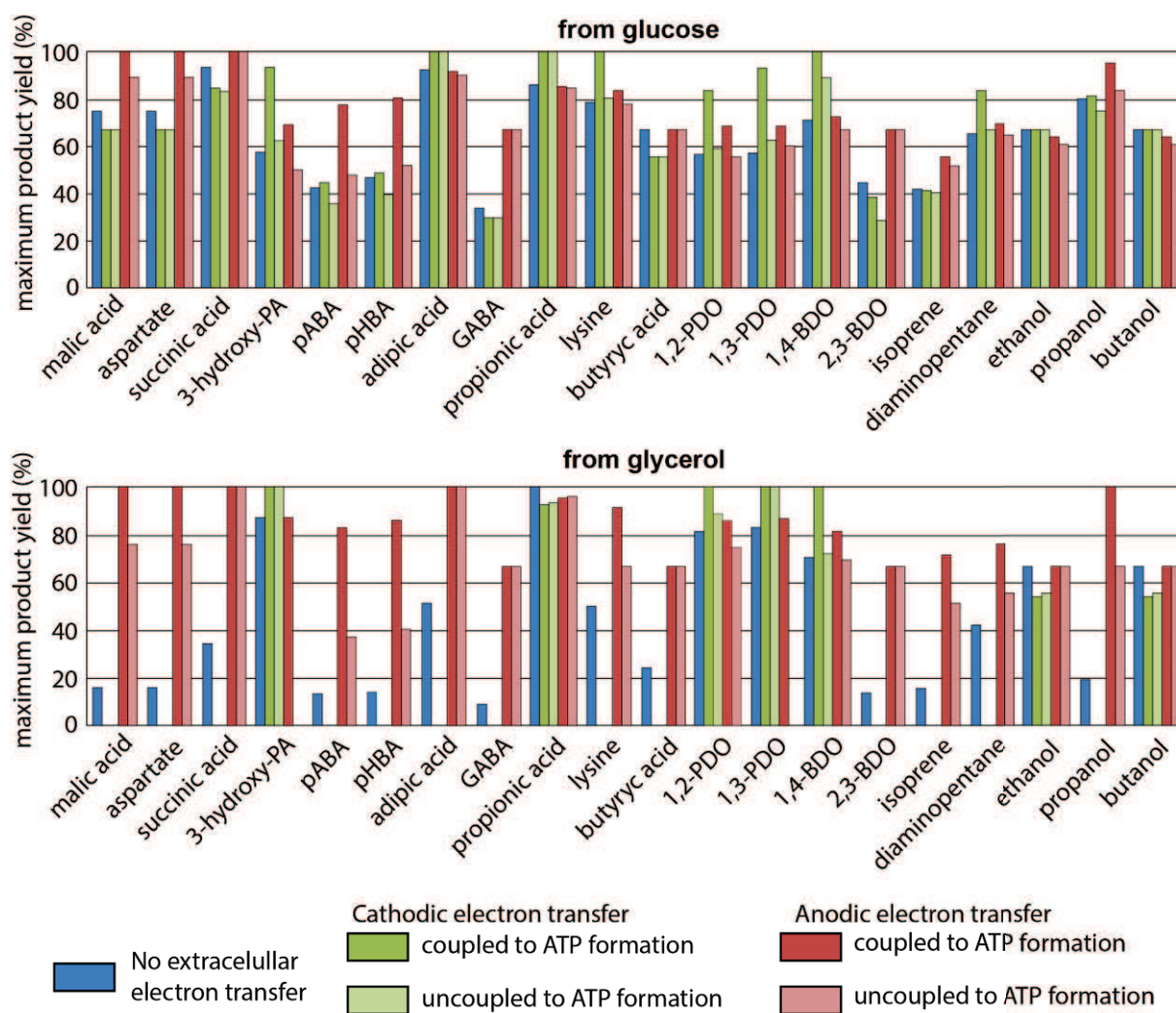


Figure P-3. Theoretical maximum carbon yields for different products in classic fermentation, cathodic and anodic electro-fermentation. (from Kracke and Krömer (2014) [174])

3-hydroxy-PA: 3-hydroxy-propionic acid; pABA: para-aminobenzoic acid; pHBA: para-hydroxybenzoic acid; GABA: γ -aminobutyric acid; BDO: butanediol;

Appendix A: Metabolite recycling

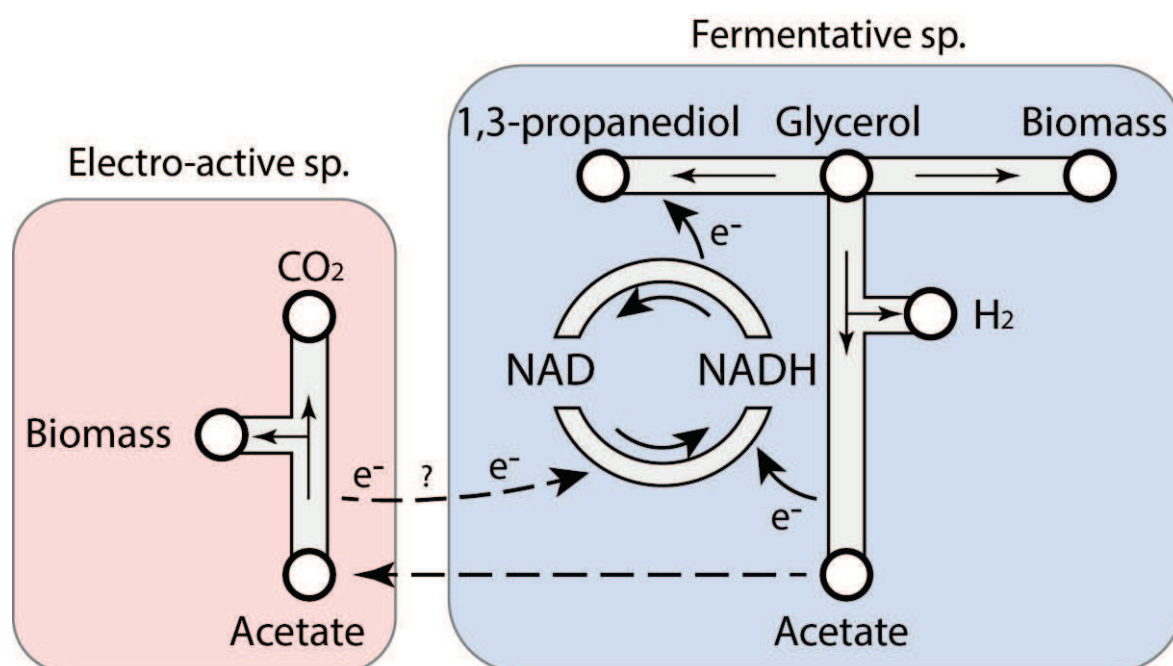


Figure A-1. Simplified metabolic pathways in co-culture fermentation with acetate recycling.

Table A-1. Glycerol fermentation pathways for acetate, lactate and ethanol production (no H₂/formate formation)

$68 \text{ Glycerol} + 3 \text{ NH}_3 \rightarrow 3 \text{ C}_4\text{H}_7\text{O}_2\text{N} + 15 \text{ Acetate} + 15 \text{ CO}_2 + 49 \text{ PDO} + 40 \text{ H}_2\text{O}$	Equation A1
$68 \text{ Glycerol} + 3 \text{ NH}_3 \rightarrow 3 \text{ C}_4\text{H}_7\text{O}_2\text{N} + 30 \text{ Lactate} + 34 \text{ PDO} + 40 \text{ H}_2\text{O}$	Equation A2
$68 \text{ Glycerol} + 3 \text{ NH}_3 \rightarrow 3 \text{ C}_4\text{H}_7\text{O}_2\text{N} + 30 \text{ Ethanol} + 30 \text{ CO}_2 + 34 \text{ PDO} + 40 \text{ H}_2\text{O}$	Equation A3

Table A-2. Redox reactions for metabolite recycling of acetate, lactate and ethanol

$\text{Glycerol} + 2 \text{ H}^+ + 2 \text{ e}^- \rightarrow \text{PDO} + \text{H}_2\text{O}$	Equation A4
$\text{Acetate}^- + 1.7 \text{ H}_2\text{O} + 0.05 \text{ NH}_3 \rightarrow 0.05 \text{ C}_4\text{H}_7\text{O}_2\text{N} + 1.8 \text{ CO}_2 + 7.2 \text{ e}^- + 6.2 \text{ H}^+$	Equation A5
$\text{Lactate}^- + 2.55 \text{ H}_2\text{O} + 0.075 \text{ NH}_3 \rightarrow 0.075 \text{ C}_4\text{H}_7\text{O}_2\text{N} + 2.7 \text{ CO}_2 + 10.8 \text{ e}^- + 9.8 \text{ H}^+$	Equation A6
$\text{Ethanol} + 2.55 \text{ H}_2\text{O} + 0.075 \text{ NH}_3 \rightarrow 0.075 \text{ C}_4\text{H}_7\text{O}_2\text{N} + 1.7 \text{ CO}_2 + 10.8 \text{ e}^- + 10.8 \text{ H}^+$	Equation A7

Table A-3. Glycerol fermentation pathways with metabolite recycling with acetate, lactate or ethanol as intermediary products

122 Glycerol + 3.75 NH ₃ → 3.75 C ₄ H ₇ O ₂ N + 42 CO ₂ + 103 PDO + 68.5 H ₂ O	Equation A8
230 Glycerol + 5.25 NH ₃ → 5.25 C ₄ H ₇ O ₂ N + 81 CO ₂ + 196 PDO + 125.5 H ₂ O	Equation A9
230 Glycerol + 5.25 NH ₃ → 5.25 C ₄ H ₇ O ₂ N + 81 CO ₂ + 196 PDO + 125.5 H ₂ O	Equation A10

Table A-4. PDO and biomass yields of the different glycerol fermentation pathways with and without metabolite recycling

Pathway	Y _{PDO/Gly} (mol/mol)	Y _{X/Gly (Ferm)} (g/mol)	Y _{X/Gly (Electro)} (g/mol)
Acetate (without H ₂)	0.72	4.46	0
Lactate	0.50	4.46	0
Ethanol (without H ₂)	0.50	4.46	0
Acetate + Recycling	0.84	2.48	0.62
Lactate + Recycling	0.85	1.32	0.99
Ethanol + Recycling	0.85	1.32	0.99

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Electrochemical control of a biological process: glycerol electro-fermentation

Electro-fermentation is a novel tool allowing to control classic fermentation through the use of polarized electrodes. Among all possible fermentation substrates, glycerol is a widely used by-product from the biodiesel industry that can be converted in value-added chemicals such as 1,3-propanediol. This PhD thesis aims at evaluating the potential of glycerol electro-fermentation for the improvement of product specificity in mixed-culture fermentation.

As a first step, classic fermentation of glycerol by mixed bacterial consortia was studied in order to characterize the main metabolic pathways according to the main influencing environmental parameter (pH). Then, the addition in fermentation broth of electrodes and electro-active bacteria, able to exchange electrons either with an electrode or other microorganisms has been investigated. This work was carried out in mixed-culture glycerol fermentation in order to optimize products selectivity and yields towards 1,3-propanediol. Finally, a model co-culture constituted of one fermentative and one electro-active species was used to elucidate part of the mechanisms underlying electro-fermentation.

This thesis opens a whole new range of possibility regarding the regulation of redox balances in fermentation. Hence electro-fermentation and the use of electro-active bacteria could become efficient tools for improving specificity and yield of 1,3-propanediol and other value-added products in fermentation.

KEYWORDS: Fermentation, Glycerol, 1,3-propanediol, Bio-electrochemistry, Electro-microbiology

Contrôle d'un bioprocédé par voie électrochimique : électro-fermentation du glycérol

L'électro-fermentation est un nouveau levier permettant le contrôle des procédés fermentaires à travers l'utilisation d'électrodes au potentiel contrôlé. Parmi de nombreux substrats fermentaires, le glycérol est une source de carbone largement utilisée issue de l'industrie du biodiesel, et permettant la production de molécules à valeur ajoutée comme le 1,3-propanediol. L'objectif de cette thèse est d'évaluer le potentiel de l'électro-fermentation du glycérol comme moyen de mieux maîtriser les spectres de produits fermentaires dans les procédés mettant en œuvre des cultures mixtes.

La thèse étudie dans un premier temps la fermentation du glycérol en cultures mixtes afin de caractériser les principales voies métaboliques d'intérêt en réponse au paramètre environnemental le plus influent pour la fermentation du glycérol (pH). L'effet de l'introduction d'électrodes colonisées par des bactéries électro-actives, capables d'échanger des électrons avec l'électrode et d'autres microorganismes, est ensuite étudié. Ce travail est réalisé en cultures mixtes dans l'objectif d'améliorer le procédé de fermentation en termes de spécificité des métabolites formés et de leur rendement de production. Enfin, un système modèle composé d'une souche fermentaire et une souche électro-active a ensuite été conçu afin de mieux comprendre les mécanismes mis en jeu lors de l'électro-fermentation.

Cette thèse ouvre de nouvelles possibilités quant à la régulation des balances redox lors de fermentation. L'électro-fermentation ainsi que l'utilisation de bactéries électro-actives ont le potentiel de devenir de puissants outils permettant d'améliorer les rendements et spécificité de production du 1,3-propanediol et d'autres molécules à valeur ajoutée.

MOTS-CLES: Fermentation, Glycérol, 1,3-propanediol, Bio-électrochimie, Electro-microbiologie.