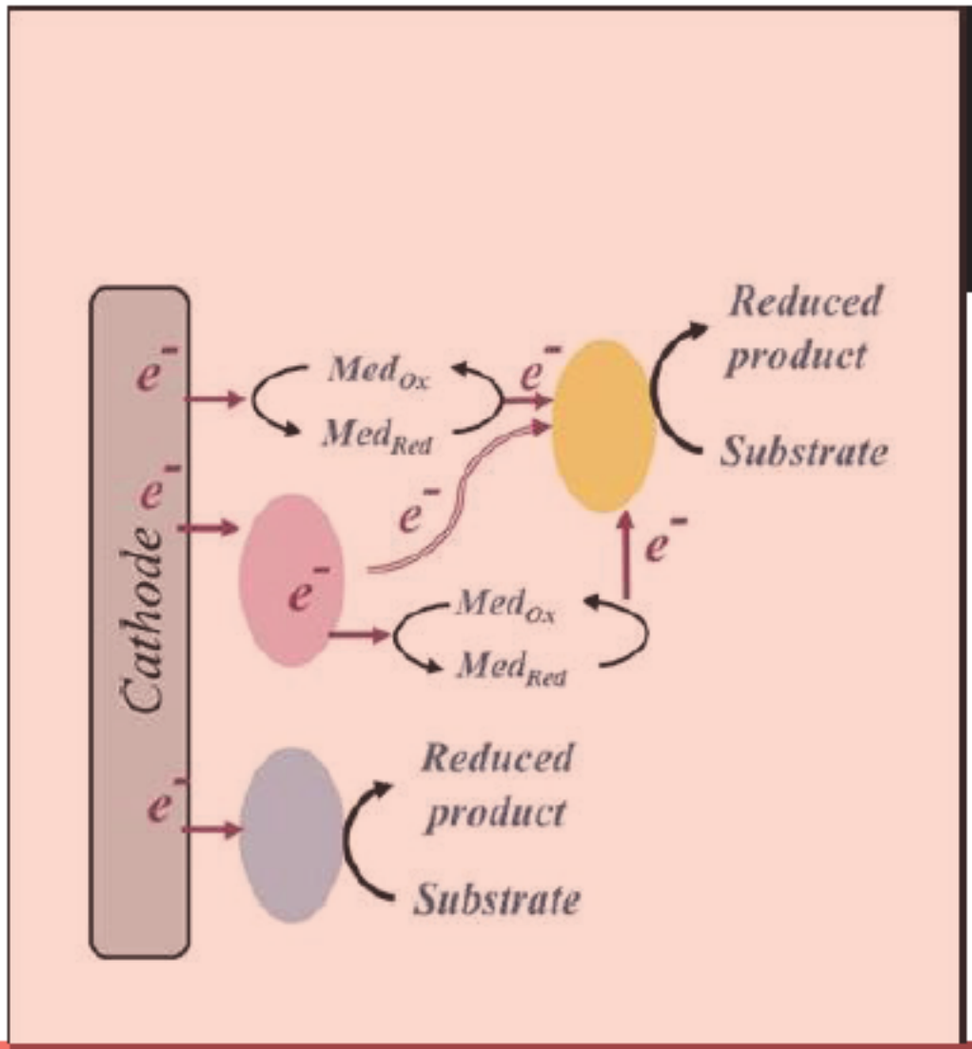
Etude et contrôle de la fermentation électro-assistée en cultures mixtes : rôle et ingénierie des interactions microbiennes

Javiera Belen Toledo Alarcon

Javiera TOLEDO ALARCÓN



PhD thesis 2018

STUDY AND CONTROL OF ELECTRO-ASSISTED FERMENTATION IN MIXED CULTURES

THE ROLE OF ENGINEERING OF MICROBIAL INTERACTIONS

ETUDE ET CONTRÔLE DE LA FERMENTATION ÉLECTRO-ASSISTÉE EN CULTURES MIXTES: RÔLE ET INGÉNIERIE DES INTERACTIONS MICROBIENNES

INRA SCIENCE & IMPACT

Membre fondateur de agreenium

THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE MONTPELLIER SUPAGRO

Biotechnologie et Microbiologie

École doctorale GAIA
Portée par l'Université de Montpellier

Laboratoire de Biotechnologie de l'Environnement
(LBE, INRA UR050)

Etude et contrôle de la fermentation électro-assistée en cultures mixtes : rôle et ingénierie des interactions microbiennes

Présentée par Javiera TOLEDO ALARCÓN

Le 28 septembre 2018

Sous la direction de Nicolas BERNET
et Eric TRABLY

Devant le jury composé de

Pierre FONTANILLE, Maître de conférences, Polytech Clermont-Ferrand	Rapporteur
Benjamin ERABLE, Chargé de recherche, CNRS Toulouse	Rapporteur
Eric DUBREUCQ, Professeur, Montpellier SupAgro	Président du jury
Nicolas BERNET, Directeur de recherche, INRA Narbonne	Directeur de thèse
Eric TRABLY, Ingénieur de recherche, INRA Narbonne	Co-directeur de thèse
Antonella MARONE, Chercheuse contractuelle, UAB Barcelone	Invitée

UNIVERSITÉ DE MONTPELLIER

Montpellier SupAgro

Abstract

Dark fermentation (DF) is a biological process used to produce hydrogen (H_2). In this process a wide variety of substrates could be used including simple substrates such as glucose or more complex substrates such as industrial wastewaters and waste. Mixed cultures can be used as inoculum that are more robust than pure cultures. However, H_2 producers and consumers can coexist in such mixed cultures, so that they often have to be pre-treated to inhibit H_2-consumers. Moreover, operational parameters such as pH and temperature play a key role in the selection of H_2-producing bacterial community. Up to date, great efforts have been made to optimise the operational parameters, but only few controllers are available to maintain the DF process stable. In this context, an electro-fermentation (EF) process is proposed as a new tool to control bioprocesses through polarised electrodes. Depending on the applied potential, EF can occur at the anode or cathode, acting either as electron sink or additional source of energy, respectively. High current densities are not necessary to have a significant impact on cell metabolism since the electrical current is not the main electrons source, nor the product of interest. The mechanisms behind EF are still unknown, but microbial interactions between fermentative and electroactive bacteria may be the key factor of the process. The objective of this thesis was: "Better understanding of the EF mechanisms through the characterization of microbial interspecies-interactions as well as interactions with the polarized electrode". Our results show that the presence of polarized electrodes led to the selection of H_2-producing bacteria, and more particularly from Enterobacteriaceae and *Clostridiaceae* families. Such microbial selection was concomitant with a significant increase in H_2 and butyrate production, at the expense of lactate production. However, when different inoculum were used, different behaviours were observed with an increase, a decreased or no effect on H_2 production. This observation evidences that the inoculum microbial community composition, and more particularly the relative abundance of the *Clostridiaceae* family, can significantly affect the microbial community behaviour in EF, i.e. microbial community trajectories and the related metabolic patterns. Finally, the microbial interactions were further investigated with a mixed inoculum enriched in *G. sulfurreducens*, as well-known electroactive bacteria. Here, a substantial change in the metabolic pathways towards higher H_2 and butyrate production was observed, at the expense of 2,3-butanediol production. This change was associated with an increase in relative abundance of the *Clostridiaceae* family at the end of fermentation, probably due to a cooperative growth that G. sulfurreducens occurring with the members of the *Clostridiaceae* family. Overall, although the mechanisms behind the microbial interactions are not yet well know, the EF process showed a great potential as a new type of control for mixed-culture bioprocesses with significant effects of the polarized electrodes on glucose fermentation.

Keywords: Electro-Fermentation; Dark fermentation; Microbial Community Interactions; Clostridia species.

Résumé

La fermentation sombre (DF) est un procédé biologique utilisé pour la production d'hydrogène (H_2). Dans ce processus, une grande variété de substrats peut être utilisée, des substrats simples comme le glucose ou plus complexes comme les effluents industriels. De plus, des cultures pures et mixtes peuvent être utilisées comme inoculum, ces dernières étant très attrayantes car conduisent à des systèmes plus robustes. Toutefois, des bactéries productrices et consommatrices d'H_2 coexistent dans ces systèmes, de sorte que l'inoculum mixte nécessite d'être pré-traité pour inhiber l'activité des micro-organismes consommateurs d'H_2. Les paramètres opérationnels tels que le pH et la température jouent également un rôle clé dans la sélection de la communauté bactérienne productrice de H_2. Jusqu'à présent, de grands efforts ont été faits pour optimiser les paramètres de prétraitement et opérationnels, mais seulement peu de leviers sont disponibles pour maintenir la stabilité des bioréacteurs (pH, température, TSH). Dans ce contexte, l'électrofermentation (EF) est proposée comme un nouvel outil de contrôle des bioprocédés par le biais d'électrodes polarisées. Selon le potentiel appliqué, l'EF peut se réaliser au niveau de l'anode ou de la cathode, agissant respectivement comme dissipateur d'électrons ou comme source d'énergie supplémentaire. Des densités de courant élevées ne sont pas nécessaires pour avoir un impact significatif sur le métabolisme cellulaire car le courant électrique n'est pas la principale source d'électrons, ni le produit d'intérêt. Les mécanismes d'action derrière l'EF restent encore inconnus, mais les interactions microbiennes entre les bactéries fermentaires et électroactives peuvent en être la clé. Ainsi, l'objectif de cette thèse est : "Meilleure compréhension des mécanismes EF par la caractérisation des interactions microbiennes inter-espèces ainsi que des interactions avec l'électrode polarisée". Nos principaux résultats montrent que des électrodes polarisées permettent de sélectionner les bactéries productrices de H_2, en particulier des entérobactéries et des clostridies. Cette sélection a conduit à une augmentation significative de la production de H_2 et de butyrate, au détriment de la production de lactate. Toutefois lorsque différents inocula sont utilisés, 3 comportements différents sont observés : une augmentation, une diminution ou aucun effet sur la production de H_2, par rapport à une fermentation conventionnelle. Ceci montre que la composition de la communauté microbienne de l'inoculum, et en particulier l'abondance relative de la famille des *Clostridiaceae*, affecte significativement le comportement de l'EF, c'est-à-dire la communauté microbienne finale et les voies métaboliques. Enfin, afin d'étudier le processus d'interaction, un inoculum mixte enrichi en G. sulfurreducens (bactéries électroactives connues) a été étudié. Un changement dans les voies métaboliques vers une production plus élevée de H_2 et de butyrate a alors été observé, au détriment de la production de 2,3-butanediol. Ce changement a été associé à une augmentation de l'abondance relative de la famille des *Clostridiaceae* à la fin de la fermentation, probablement en raison d'une croissance coopérative que *G. sulfurreducens* avec les membres de la famille des *Clostridiaceae*. Globalement, et même si les mécanismes d'interactions microbiennes restent non élucidés, le procédé EF a montré un certain potentiel en tant que nouveau moyen de contrôle de bioprocédés opérés en cultures mixtes.

Mots-clés : Électro-fermentation ; Fermentation sombre ; Interactions entre les communautés microbiennes ; Clostridia species.

Résumé étendu

A ce jour, environ 85% de toute l'énergie consommée dans le monde provient du pétrole (35%), du charbon (28%) et du gaz naturel (22%). Au cours des 40 dernières années, son utilisation a été grandement remise en question, principalement pour trois raisons : i) une forte dépendance énergétique à l'égard des pays disposant de ressources fossiles ; (ii) il s'agit d'une ressource limitée ; (iii) les dommages importants causés à l'environnement par son utilisation excessive. Dans ce contexte, il est désormais urgent de trouver une source d'énergie renouvelable qui soit respectueuse de l'environnement et qui génère la stabilité énergétique par l'utilisation des ressources propres à chaque pays.

L'hydrogène (H_2) est considéré comme le combustible du futur et peut être considéré comme renouvelable s'il est produit à partir de sources renouvelables. Actuellement, environ 96% du H_2 produit provient de combustibles fossiles, principalement par reformage à la vapeur, un procédé très énergivore et polluant. H_2 est également produit par l'électrolyse de l'eau en utilisant de l'énergie électrique provenant de combustibles fossiles, mais ce procédé ne libère pas de CO_2 dans l'atmosphère.

Cependant, pour être une alternative respectueuse de l'environnement, et renouvelable, l'H_2 doit être produit à l'aide de procédés écologiques tels que les méthodes biologiques, comme la fermentation sombre. Ici, les micro-organismes dégradent la matière organique complexe en molécules plus simples et génèrent simultanément de l'H_2. La communauté microbienne impliquée dans ce processus se trouve facilement dans la nature, avec la coexistence d'espèces productrices et consommatrices d'H_2, grâce à l'établissement de différentes interactions microbiennes. Les principaux facteurs affectant la production d'H_2 par fermentation sombre comprennent : la communauté microbienne de l'inoculum de départ, le

type de substrat, la température, le pH, le mode de fonctionnement et le temps de séjour hydraulique. Cependant, seuls ces quelques moyens de contrôle sont disponibles pour maintenir stable le processus de fermentation sombre, c'est-à-dire les profils métaboliques et les performances de l'H_2.

Récemment, l'électro-fermentation (EF) a été proposée comme nouveau type de contrôle des bioprocédés en présence d'électrodes polarisées. L'EF est un système bio-électrochimique, dans lequel une densité de courant élevée n'est pas nécessaire pour obtenir un effet important sur le métabolisme cellulaire. Ainsi, une petite quantité d'électrons peut avoir un impact significatif sur les schémas métaboliques. L'EF peut se produire à l'anode ou à la cathode, agissant soit comme puits d'électrons, soit comme source d'énergie supplémentaire, respectivement, mais pas comme source d'énergie majoritaire. En effet, la source principale d'électrons pour générer le produit attendu est le substrat organique comme dans un processus de fermentation conventionnel.

Dans ce contexte, l'objectif général de cette thèse a été d'avoir une "Meilleure compréhension des mécanismes de EF par la caractérisation des interactions microbiennes interespèces ainsi que des interactions avec des électrodes polarisées". Pour répondre à cet objectif général, quatre objectifs plus spécifiques ont été proposés pour :

i. Déterminer les principaux paramètres affectant l'EF du glucose.
ii. Identifier l'influence des électrodes polarisées sur la communauté microbienne et les profils métaboliques pendant l'EF du glucose.
iii. Étudier l'influence de la source d'inoculum sur les performances de l'EF du glucose.
iv. Étudier les interactions microbiennes entre les bactéries électroactives et fermentaires

Démarrage de l'électro-fermentation et détermination des paramètres d'EF

Comme l'EF est un concept récent, peu de travaux sont documentés et il n'y a pas assez de connaissances sur les conditions de fonctionnement optimales et les matériaux les plus appropriés pour réaliser une EF efficace. La littérature montre que le matériau de l'électrode et sa taille, ainsi que la tension appliquée sur l'électrode de travail, peuvent altérer la production de courant et générer différentes interactions avec les communautés microbiennes. En outre, la configuration des réacteurs (c'est-à-dire les réacteurs à un ou deux compartiments) est importante lorsqu'on considère l'efficacité des systèmes électrochimiques.

Dans ce Chapitre, différentes expériences d'EF ont été réalisées sur glucose afin de déterminer les meilleurs paramètres opératoires et de sélectionner les matériaux adéquats. Après avoir comparé différents matériaux comme électrodes de travail, des grilles en platine ont été sélectionnées sur la base des performances de production d'H_2 pour réaliser les expériences suivantes d'EF. De plus, un changement significatif sur les profils métaboliques a été observé lorsqu'une électrode polarisée a été placée dans le milieu de fermentation. Dans ce cas, la production de H_2 et de butyrate a augmenté alors que le lactate n'était pas favorisé par rapport au contrôle. L'analyse de la communauté microbienne a montré que les familles dominantes présentes à la fin de l'opération par lots ne pouvaient pas expliquer ces changements. Néanmoins, et malgré une faible abondance relative, la présence de membres de la famille des *Aeromonadaceae* a montré une corrélation positive et significative avec l'indice d'efficacité de EF (η_{EF}). Ceci suggère l'importance des espèces sous-dominantes dans les interactions microbiennes ayant un impact subséquent sur les patterns métaboliques.

Par la suite, différentes tensions ont été appliquées sur l'électrode de travail, mais aucune différence n'a été observée entre les conditions, y compris le contrôle. Dans ce cas, une voltampérométrie cyclique (CV) initiale a été effectuée et on a émis l'hypothèse que la CV

était peut-être la cause de ces performances similaires. En outre, des interférences électriques ont été observées entre le contrôle et les tests EF, en raison de la voltampérométrie cyclique initiale effectuée lors des tests de EF. Bien que le phénomène à l'origine de ce comportement soit inconnu et n'ait pas été étudié plus avant, les réacteurs témoins ont ensuite été placés dans un bain-marie distinct de celui des réacteurs d'électrofermentation et aucune interférence électrique n'a dans ce cas été observée.

Enfin, deux tailles d'électrodes de grilles en platine ont été testées dans des réacteurs à dual compartiment. Les grandes électrodes de 3,5 x 3,5 cm^2 ont été choisies car elles permettent un plus grand échange d'électrons avec le milieu de fermentation et un effet maximal sur la production de H_2 a été observé.

Effet de l'électro-fermentation sur la sélection des bactéries productrices de H_2

Les cultures mixtes ont été largement utilisées pour la production de H_2 par fermentation sombre. Cependant, de nombreux micro-organismes différents peuvent se développer dans les milieux de fermentation et aucune pression de sélection directe ne peut être appliquée pour la sélection de bactéries portant des voies efficaces de production de H_2, telles que *Clostridium* sp. Jusqu'à présent, beaucoup d'efforts ont été faits pour optimiser les paramètres d'exploitation. Cependant, seuls quelques contrôleurs sont disponibles pour maintenir des performances stables de fermentation sombre. Dans ce contexte, l'électro-fermentation a été proposée comme un nouveau type de bioprocédé contrôlé par des électrodes polarisées.

Ce chapitre met en évidence un effet clair des électrodes polarisées sur les voies métaboliques et la structure de la communauté microbienne lors de la fermentation sombre. Une forte corrélation a été observée entre les communautés microbiennes sélectionnées et les

métabolites produits, y compris H_2. Deux comportements métaboliques différents pour la production de H_2 ont été observés dans l'EF. Le premier a conduit à une production de H_2 plus élevée par rapport au témoin avec une forte sélection de *Clostridium* sp. Le second comportement a conduit à une production de H_2 plus faible avec la production d'éthanol et était fortement corrélé avec la sélection des genres *Escherichia* et *Enterobacter*. De tels mécanismes d'interaction entre les électrodes polarisées et la communauté microbienne restent cependant peu clairs mais un nouveau champ d'investigation dans la fermentation de cultures mixtes a été ouvert.

Les effets de l'électro-fermentation sont influencés par la composition microbienne initiale

Lors de la production de H_2 par fermentation sombre, la source d'inoculum et leur composition microbienne sont cruciales pour définir les performances des réacteurs finaux. Pour cela, de nombreux pré-traitements ont été employés pour obtenir un inoculum adéquat capable de produire des rendements élevés en H_2 sans production de méthane, comme les chocs thermiques. De plus, certaines conditions opératoires peuvent influencer directement la sélection de la communauté microbienne, notamment le pH, la température, le temps de séjour hydraulique et le type de substrat. En général, ces pré-traitements et conditions opérationnelles sont principalement focalisés sur l'élimination de l'activité méthanogène et non sur la sélection des bactéries productrices de H_2.

Selon les résultats observés au chapitre précédent, l'EF est également un moyen de sélectionner les bactéries productrices de H_2. Par conséquent, la sélection microbienne peut d'abord dépendre de la structure initiale de la communauté microbienne. Comme l'EF est

potentiellement utilisable pour contrôler les bioprocédés, il serait intéressant d'évaluer l'effet des électrodes polarisées lorsque les communautés microbiennes changent au fil du temps.

De ce chapitre, il a été conclu que EF n'avait qu'un impact sur la sélection initiale de la communauté bactérienne en mode de fonctionnement continu de réacteur. Lorsque la communauté microbienne change en raison de la pression de sélection imposée par le temps de séjour hydraulique, l'effet des électrodes polarisées n'est pas observable. Cela nous permet d'émettre l'hypothèse que l'effet de l'EF dépend de la communauté microbienne et donc de l'inoculum initial.

Par la suite, il a été montré que la source d'inoculum avait en effet un impact significatif sur la communauté microbienne finale et la production de métabolites. Sur la base des résultats de production de H_2, 3 groupes peuvent être constitués en fonction de l'effet observé lors de l'EF : effet positif, effet négatif et effet neutre. L'abondance relative des bactéries productrices de H_2, en particulier de la famille des *Clostridiaceae*, dans l'inoculum initial semble être un paramètre clé déterminant l'effet de l'EF.

Investigation des mécanismes d'électro-fermentation par l'étude des interactions microbiennes

Comme indiqué dans les chapitres précédents, l'EF est un outil de contrôle prometteur pour les bioprocédés qui dépend de la composition et de la structure de la communauté bactérienne. Le mécanisme d'action étant derrière la sélection des bactéries productrices de H_2 et qui contribue aux changements dans les voies métaboliques reste encore inconnu. Une hypothèse serait que les bactéries électroactives, telles que *G. sulfurreducens*, joueraient un rôle clé dans le transfert d'électrons, même s'il s'agit d'espèces sous dominantes. Les bactéries

électroactives peuvent prendre/donner des électrons directement à des électrodes polarisées, tout en établissant différents types d'interactions avec la communauté bactérienne.

Dans ce contexte, ce chapitre a pour objectif d'étudier les changements du profil métabolique en présence de *G. sulfurreducens,* pendant la fermentation sombre conventionnelle et l'EF. Des changements significatifs dans les voies métaboliques liés à la sélection des communautés bactériennes lorsque l'inoculum a été enrichi avec *G. sulfurreducens* ont été observés. Le premier inoculum, composé principalement d'*Enterobacteriaceae* et de *Clostridiaceae,* a montré une augmentation du rendement en H_2 et de la production de butyrate, associée à une augmentation de l'abondance relative des *Clostridiaceae*. Ce changement a été supposé résulter d'une croissance coopérative entre *Clostridiaceae* et *G. sulfurreducens*. Le deuxième inoculum, composé principalement de *Saprospiraceae* et de *Rhodocyclaceae,* a montré une diminution de la production de H_2, associée à une augmentation de la production de lactate et à l'émergence de membres de la famille des *Streptococcaceae*. Cependant, dans ce cas, l'action de *G. sulfurreducens* n'était pas claire parce que toutes les interactions possibles avec les différentes bactéries fermentaires restent encore inconnues.

Enfin, aucun effet sur les voies métaboliques n'a été observé lorsqu'une électrode précolonisée avec *G. sulfurreducens* a été utilisée pendant l'EF du glucose. Ces résultats étaient probablement dus à l'impossibilité pour *G. sulfurreducens* de trouver un accepteur d'électrons (non ajouté dans le milieu de fermentation) et son impossibilité d'interagir avec les bactéries en solution pour établir une interaction coopérative.

Perspectives

En général, les changements observés pendant l'EF ne sont pas associés au courant qui passe à travers les électrodes polarisées, il serait donc intéressant d'évaluer les changements physiques possibles que les cellules subiraient pendant l'EF et, ensuite, comment ces changements peuvent modifier les interactions microbiennes en culture mixte. Dans ce contexte, il serait intéressant d'étudier l'EF du glucose en utilisant des mono et co-cultures de bactéries clés observées au cours de cette thèse, en particulier *C. butyricum*, *E. fergusonii*, *E. cloacae* et *S. equinus*. en se concentrant sur l'étude des changements du potentiel zêta et de la morphologie cellulaire.

D'autre part, nos résultats montrent que l'abondance relative de *Clostridia spp*. dans l'inoculum semble être la clé du type d'effet qui sera observé pendant l'EF. Dans ce contexte, il serait intéressant d'utiliser des *Clostridia spp*. comme *C. pasteurianum* (bactérie électroactive et producteur de H_2) et *C. butyricum* (espèce sélectionnée lors de cette thèse), pour bioaugmenter des cultures mixtes avec différentes communautés microbiennes. Ces inocula bioaugmentés pourraient alors être testés en EF. De plus, différents pourcentages de *Clostridia spp*. pourraient être testés pour étudier l'influence de leur abondance relative sur le comportement final de EF. Cette étude pourrait fournir des informations cruciales sur les interactions microbiennes et la façon dont la communauté microbienne interagit avec l'électrode polarisée.

Enfin, l'une des hypothèses sur les mécanismes d'action de l'EF suggère que les bactéries électroactives comme *G. sulfurreducens*, peuvent agir comme médiateur redox entre l'électrode polarisée et les bactéries de fermentation dans la communauté microbienne. Ainsi, la croissance coopérative entre *G. sulfurreducens* et *C. pasteurianum* a été montrée précédemment au LBE. Dans ce contexte, il est proposé d'étudier l'interaction dans les co-

cultures de *G. sulfurreducens* et de bactéries fermentaires clés telles que rapportées lors de cette thèse. Ces espèces comprennent *C. butyricum*, *E. fergusonii*, *E. cloacae* et *S. equinus*. En outre, il serait intéressant d'évaluer les changements sur les régulateurs d'oxydoréduction cellulaire tels que NAD^+/NADH, ainsi que l'expression des gènes des hydrogénases. Ce dernier pourrait mettre en évidence la consommation de H_2 comme source d'électrons par *G. sulfurreducens*.

List of Communications

Papers in Peer Reviewed International Journals

i. R. Moscoviz, **J. Toledo-Alarcón**, E. Trably, and N. Bernet, "Electro-Fermentation: How to Drive Fermentation Using Electrochemical Systems," Trends Biotechnol., vol. 34, no. 11, pp. 856–865, 2016.

ii. **J. Toledo-Alarcón**, G. Capson-Tojo, A. Marone, F. Paillet, A. D. N. Ferraz Júnior, L. Chatellard, N. Bernet, and E. Trably, "Basics of bio-hydrogen production by dark fermentation", no. 9789811076763. 2018.

iii. **Toledo-Alarcón J**., Moscoviz R., Trably E. and Bernet N. Glucose electro-fermentation as main driver for efficient H_2-producing bacteria selection in mixed cultures. International Journal of Hydrogen Energy. Accepted

International conferences

i. **Toledo-Alarcón J**, Moscoviz R, 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). Award: Best poster presentation.**

ii. **J. Toledo-Alarcon**, N. Bernet, E. Trably (2017). Selection of H_2 producing bacteria during glucose electro-fermentation. 2nd International Conference on Alternative Fuels and Energy: Future and Challenges – ICAFE 2017, Daegu, South Korea. **(Oral)**

Other presentations

i. **Toledo-Alarcón J**, Trably E, Bernet N (2016). Study and Control of Electro-Assisted Fermentation in Mixed Cultures: the Role of Engineering of Microbial Interactions. Journée de l'Ecole Doctorale GAIA, Montpellier Supagro, 14 Juin 2016, Montpellier, France. **(Poster). Award: Best poster presentation.**

ii. **Toledo-Alarcón J**, Trably E, Bernet N (2017). Study and Control of Electro-Fermentation in Mixed Cultures. Journée de l'Ecole Doctorale GAIA, Montpellier Supagro, 23 Juin 2017, Montpellier, France. **(Oral)**

Acknowledgements

With courage and perseverance dreams come true and for me, my PhD in France has been the realization of my greatest dream. When we dare to dream and work to fulfill them, the whole universe conspires in our favor and God takes care of what is not in our hands. All along the way there were very important people who unknowingly blessed my life and my family.

Living abroad made me grow enormously as a person, valuing what I took for obvious, valuing family and my roots. France taught me how to respect personal life outside of work and the LBE showed me the friendly side of research. I learned a lot from many in the laboratory, especially a little English and French. Many thanks to all, there will always be a beautiful memory of you in my heart.

Especially, I want to thank Nicolas B and Eric T for directing my thesis work, thank you very much for the opportunity to grow professionally with great people. Thank you for guiding me and motivating me to be the best version of myself. It is difficult to separate the work from the personal life, but I cannot fail to mention the enormous gratitude I feel for the great support given during my pregnancy and also during the first years of my babies' lives. I really believe that you did much more than I expected from my thesis supervisors.

Diana, thank you so much for being our angel. Thank you Gaelle and Helene C for your constant concern and closeness. To my office partners, sorry to speak so loudly on the phone. Aurelie already missed you next to my desk. Iza, I will miss talking about maternity and research. It's hard to list everybody and I don't want to forget anyone, but know that I will remember everyone I met and shared during my stay at the LBE.

Especially, I want to dedicate this thesis to Gonzalo Ruiz, my dear professor. A very special person, always ready to listen to crazy ideas and support them. Thanks so much.

I want to thank my parents for their unconditional support despite the distance. You have been fundamental during my life, being an example of perseverance and resilience for me.

Jhony, thanks for venturing with me, thanks for making my dreams yours. Thanks for accompanying me with love, patience and motivation, always encouraging me to follow. Thanks my love! To my Tomy and Lucky, my lovely children, the joy and the motor of my life.

Contents

List of tables

List of figures

List of abbreviations

Hydrogen	H_2
Dark fermentation	DF
Electro-fermentation	EF
Hydrogen-consuming bacteria	HCB
Hydrogen-producing bacteria	HPB
Volatile fatty acids	VFA
Maximum growth rate	μ_{max}
Adenosine triphosphate	ATP
Nicotinamide adenine dinucleotide oxidized/reduced	NAD^+/NADH
Saturated calomel reference electrode	SCE
Electro-fermentation efficiency coefficient	η_{EF}
Ferredoxin oxidized/reduced	Fd, Fd_{ox}/Fd_{red}
Pyruvate-ferredoxin oxidoreductase	PFOR
Pyruvate formate lyase	PFL
Formate hydrogen lyase	FHL
Hydraulic retention time	HRT
Oxidation-reduction potential	ORP
Bioelectrochemical reactors	BES
Microbial fuel cells	MFC
Microbial electrolysis cells	MEC
Microbial electrosynthesis	MES
Microbial desalination cells	MDC
Extracellular electron transfer	EET
Indirect extracellular electron transfer	MIET
Direct extracellular electron transfer	DIET
Chemical oxygen demand	COD
Paleontological statistics	PAST
Analysis of variance	ANOVA
Principal component analysis	PCA
Non-metric multidimensional scaling	NMDS
Analysis of similarities	ANOSIM
Similarity percentage	SIMPER
Chronoamperometry	CA
Cyclic Voltammetry	CV

Introduction

Industrial Revolution was the most important process of economic, social and technological transformation over the human history. From that moment, a transition was initiated that would end centuries of manual labour and the use of animal traction, being replaced by machinery for the industrial manufacture and goods and transportation of passengers. During this period the wood was replaced by coal, as main energy source. Coal led the industrial growth until the mid-twentieth century, when oil and natural gas emerged as alternative energy sources (Fig. 1-1-A). These provoked new inventions, such as internal combustion engines, that propelled the cars and, later, the planes. The appearance of electricity was also decisive, since it constituted a way for storing and transporting energy in a refined and standardized way, greatly facilitating its consumption and the usages [1], [2].

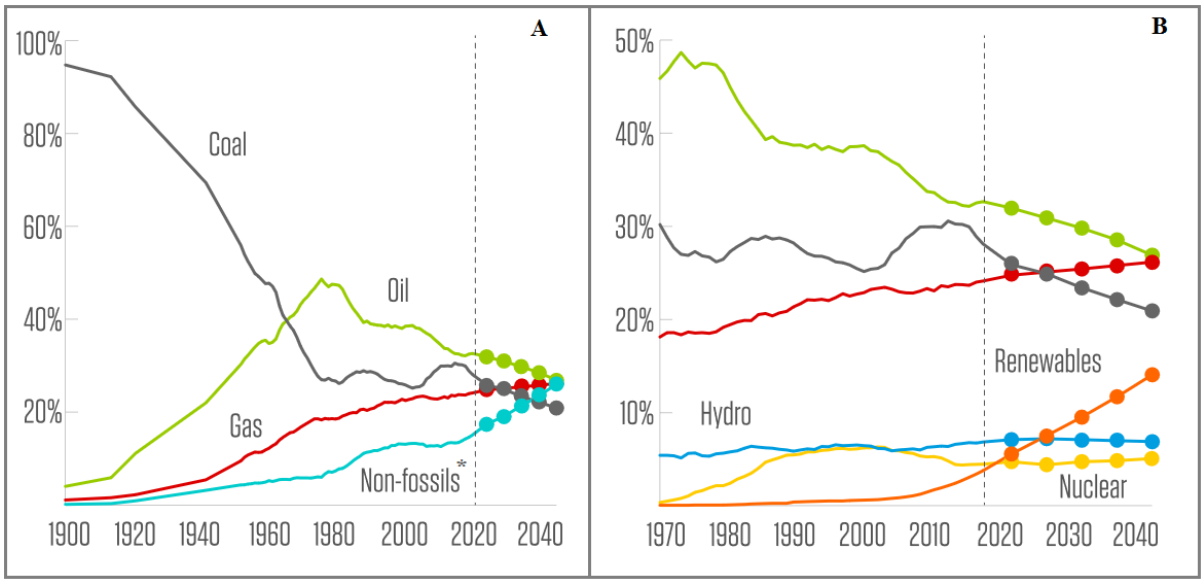


Fig. 1-1: Shares of primary energy since 1900 and its projection until 2040

**Non-fossils includes renewable, nuclear and hydro. Source: BP energy Outlook 2018 (https://www.bp.com)*

Since then, oil consumption has been increasing faster than natural gas, becoming the most used energy source until today, as shown in Fig. 1-1-A. However, a decrease in oil

consumption has been observed since 1973 because of the Oil Crisis, when the OPEC (Organization of the Petroleum Exporting Countries) countries refused to export their fossil fuels for politic reasons. Then, a successive crisis in 1979, because of Islamic Revolution in Iran and the confrontation of this county with Iraq, delayed the recovery of supplies. This problem opened the debate on energy dependencies and consequently the security of fossil fuels supplies [1], [2]. Besides, the abusive use of fossil fuels has led to serious environmental problems including ecological damage, water and air pollution, alteration by greenhouse effect and public health problems. To count on clean energies from their production to their final use is urgent. Third, fossil fuel reserves are limited and are currently being faster consumed than they are produced. As an example, in one year the human being consumes what nature has taken one million years to produce [3]–[5].

In this context, finding new sources of renewable energy is necessary for clean, unlimited production that is adapted to the natural resources of each country. Renewables energies include solar, wind, geothermal, hydraulic, nuclear, electric and biomass. Today renewable energy grows strongly mainly by the increasing of wind and solar power energy. Projected exponential growth predict to reach 20% of total energy consumption by 2040 (Fig. 1-1-B), with China and India as main clean energy producers [1], [2].

Hydrogen (H_2) is considered as the fuel of the future and can be considered renewable if it is produced from renewable sources. H_2 can be used in internal combustion or jet engines, gas power turbines but also in fuel cells. The main advantage of H_2, as a fuel, is attributed to its high net calorific value of 120 $kJ.g^{-1}$ compared to methane (50 $kJ.g^{-1}$), ethanol (26.8 $kJ.g^{-1}$) and gasoline (46.7 $kJ.g^{-1}$) [4]. Besides, it is an ecologically clean energy source because its combustion is CO_2 free. However, an important limiting factor in the use of H_2 as an energy source is its explosivity in a mixture with oxygen and its storage is more difficult compared

other fuels [5], [6]. Although H_2 is slowly beginning to be used as transportation fuel, its main applications include chemical, refinery and pharmaceutical industries. In addition, it is particularly used for ammonia and methanol synthesis and for hydrogenation of liquid oils [4], [6]. At present, about 96% of H_2 produced come from fossil fuels mainly by steam reforming, a process very energy-intensive and polluting. H_2 can also be produced through water electrolysis using electrical energy [4], [7].

To stand as an environmental-friendly and renewable alternative, H_2 must be produced using sustainable processes such as biological methods. These methods are based on the biological capability of some microorganisms to produce H_2 by degradation of the organic matter, thus making it possible to efficiently combine the organic waste treatment and the energy production. This is the case of dark fermentation (DF), a fermentation process in which microorganisms degrade complex organic matter to simpler molecules and simultaneously generate H_2. The main co-products are acetate, butyrate, lactate and ethanol. All of them are valuable chemicals that are also used in the chemical industry. Therefore, DF appears as a promising technology that can be included in a concept of environmental biorefinery towards circular economy, where organic residues are not anymore considered as a waste but as a resource of multiple products [8].

Microbial communities involved in H_2 production by dark fermentation can be easily found in Nature, mainly in anaerobic environments, such as in lakes, guts, sludge from wastewater treatment plant or manure digestate [9]. Many of these communities have been extensively studied, revealing how different microbial populations can coexist under complex interactions and efficient cooperative relationships. These microbial interactions make mixed cultures more attractive than pure cultures for biotechnological purposes. Mixed culture-based bioprocesses are generally more robust, overcoming sudden environmental changes

and carrying out more complex activities [10], [11]. However, many different microorganisms can grow during dark fermentation including those than can consume H_2. Thus, inocula pre-treatment is generally required to prevent methanogenic activity during dark fermentation. Heat shock is the most applied pre-treatment, aiming to eliminate the non-spore-forming microorganisms (*e.g.* methanogenic archaea) and favour species from *Clostridium* genus, well known as the most efficient H_2 producers [11], [12]. To date, a lot of efforts have been made on optimizing the different operating parameters, including: carbon sources, macro-micro nutrients, temperature, pH, HRT, organic loading rates, H_2 partial pressure [13]–[15]. However, only few controllers (pH, OLR, HRT) are available to maintain stable the dark fermentation process, *i.e.* metabolic patterns and H_2 performances [16], [17].

Electro-fermentation has been proposed as a new type of bioprocess control in presence of polarized electrodes [18]. Electro-fermentation can occur at the anode or cathode, acting either as electron sink (*i.e.* passing the electrons excess from fermentation medium towards electric circuit) or additional energy source (*i.e.* passing electrons towards fermentation medium from the circuit), respectively [17], [18]. Electro-fermentation is a bioelectrochemical system, in which a high current density is not necessary to have a strong effect on cellular metabolism, and only a small amount of electrons involved has a significant impact on the metabolic patterns [16]–[19]. Indeed, electro-fermentation relies on the modification of cellular metabolism with a low amount of electrons. Thus, the main source of electrons to generate the expected product comes from the organic substrate, as found in conventional fermentation process [18]. That differentiates electro-fermentation from microbial electrosynthesis where the main source of electrons is the electrode. As an illustration, electro-fermentation could be considered as a control tool of H_2-producing dark

fermentation process, while H_2 is chemically produced at the cathode in microbial electrolysis cells [20].

In this context, the general objective of this thesis is to provide a "*Better understanding of the electro-fermentation process in microbial communities fermenting glucose*". To fulfil this general objective, four specific objectives were investigated and aimed to:

i. Determine the main parameters affecting the glucose electro-fermentation.
ii. Identify the influence of polarized electrodes on microbial community and metabolic patterns during glucose electro-fermentation.
iii. Evaluate the influence of inoculum source on glucose electro-fermentation performances.
iv. Characterize the microbial interactions between electroactive and fermentative bacteria.

This document includes six chapters, and is structured as follows:

Chapter 1 corresponds to the literature review including the metabolic pathways for H_2 production by dark fermentation, its microbiology, and the main operational parameters that can affect it. Basis of electro-fermentation are presented as well as the hypothetical mechanisms of EF action and the possible interactions existing between electrodes and fermentative bacteria.

Chapter 2 describes the materials and methods used in this thesis. That includes biochemical analysis, molecular biology techniques and statistical data analytical tools. In addition, the experimental methodology used for each test is presented.

Chapter 3 aims to determine the main parameters affecting the glucose EF. While, the specific objectives are as follows: (i) determine how the electrode material can affect the H_2

production during EF; (ii) study different potential applied on the working electrode using a single – chamber reactor; (iii) determine how the electrode size can affect the H_2 production during EF.

Chapter 4 aims to identify the influence of polarized electrodes on microbial community and metabolic patterns during glucose EF through different applied potential.

Chapter 5 aims to evaluate the influence of inoculum source on glucose EF performances. While, the specific objectives are as follows: (i) study the effect of EF on continuous H_2 production in chemostat; (ii) study the influence of the initial inoculum on EF in batch mode.

Chapter 6 aims to characterize the microbial interactions between electroactive and fermentative bacteria. While, the specific objectives are as follows: (i) study the changes in metabolic pathways during glucose dark fermentation when a mixed culture is enriched in *G. sulfurreducens*; (ii) study the influence on metabolic pattern during glucose EF when an electrode is precolonized with *G. sulfurreducens*.

Finally, a section of general conclusion from this thesis and prospects are proposed for future research to be carried out beyond this thesis.

CHAPTER 1
Literature review

1.1 Synopsis

This chapter aims to put in context the research questions addressed by this thesis. The main aspects of hydrogen production by dark fermentation are presented, including the different metabolic routes, the main bacterial species involved in the fermentative process and their known interactions in mixed cultures. Typical operational parameters such as substrate, pH, inoculum source is also reviewed, as well as how they affect the microbial communities and subsequent hydrogen performances. Finally, basics of electro-fermentation are presented together with the operational parameters that can affect it. So far not well documented, the possible mechanisms behind electro-fermentation and the use of polarized electrodes are discussed.

This chapter has been written based on the following scientific publications:

i. **J. Toledo-Alarcón**, G. Capson-Tojo, A. Marone, F. Paillet, A. D. N. Ferraz Júnior, L. Chatellard, N. Bernet, and E. Trably, Basics of bio-hydrogen production by dark fermentation, no. 9789811076763. 2018.
ii. R. Moscoviz, **J. Toledo-Alarcón**, E. Trably, and N. Bernet, "Electro-Fermentation: how to drive fermentation using electrochemical systems" Trends Biotechnol., vol. 34, no. 11, pp. 856–865, 2016.

1.2 Context

Nowadays, fossil fuels are extensively used in transport, agriculture, domestic and industrial sectors to generate power. By consequence, the interests in finding alternative energy source have gained more and more followers around the word over the past decades. That aims to decrease the energy dependence and diversify the energy resources by specially focussing on clean, renewable and environment-friendly energies. In this context, hydrogen (H_2) is considered as one of the most interesting future fuels because H_2 has numerous advantages compared to current fossil fuel-based energy sources. H_2 has the main advantage of being cleanly used in both combustion motors and electrical vehicles using fuels cell, reaching an efficiency 30% higher with this last one [21]–[23]. Additionally, H_2 has the highest specific energy (141.9 $J.kg^{-1}$) among known fuels, *e.g.* methane (55.7 $J.kg^{-1}$), natural gas (50.0 $J.kg^{-1}$), biodiesel (37.0 $J.kg^{-1}$) and ethanol (29.9 $J.kg^{-1}$) [21]. Despite these advantages, H_2 is not yet commercialized as a fuel mainly due to high production cost, the important technical requirements for storage and distribution methods. However, it is widely used as a reactant for fertilizers production, diesel refining and ammonium synthesis. Only about 4% of all H_2 in the world (about 0.1 GT per year [24]) is produced by water electrolysis, while about 96% by steam reformer, thermochemical process that use fossil sources [22], [23], [25]. These techniques consume a lot of energy (about of 63.3 $kJ.mol_{H2}^{-1}$ [24]) and has a high environmental impact (10kg of CO_2 by kg of H_2 are released [26]). Thus, a massive production of H_2 for transportation purposes would not be sustainable with that way. Alternatively, some promising methods for H_2 production are emerging and more particularly by using autotrophic or heterotrophic microorganisms through photobiological and dark fermentation processes. In autotrophic conversions (*i.e.* direct or indirect biophotolysis), solar energy is converted into H_2 via photosynthetic reactions mediated by photosynthetic

microorganisms such as microalgae or photosynthetic bacteria. Under heterotrophic conversions, photo-fermentation carried out by photosynthetic bacteria and dark fermentation carried out by anaerobic bacteria [15], [27]. Dark fermentation is the most studied and promising technology for H_2 production at larger scale with regards to its high production rates and its capacity for treating complex organic waste [15], [23], [27].

1.3 Hydrogen production by dark fermentation

Dark Fermentation (DF) is a biological process that is commonly used to produce H_2. DF does not require solar energy to occur and hence the configuration of the bioreactor is simpler and cheaper than photo-biological processes. Most importantly, this technology has attracted attention because it can use a wide range of substrates, particularly organically rich residues. This technology is particularly suitable to be integrated into wastewater or waste treatment systems to produce H_2 [21], [23].

However, the development of dark fermentation processes at industrial scale is still limited since lower H_2 yields have been reported when compared to the theoretical maximum. Thus, different ways to increase H_2 yields have been tested including the optimization of process design and operation parameters of dark fermentation reactors. Pre-treatments and inoculum enrichment methods can also increase the H_2 yields. Recently, to overcome the issue of low H_2 yields, several solutions have been proposed by coupling the dark fermentation process with photofermentation or bioelectrochemical systems, making thus the global energetic balance both positive and competitive [15].

1.3.1 H_2 producing enzymes

Molecular H_2 is produced when the cells must release their excess of electrons through H_2-producing enzymes. These enzymes under anaerobic conditions catalyse the simple redox

reaction: $2H^+ + 2e^- \leftrightarrow H_2$ [15], [28]. In order to perform this reaction, most of these enzymes have complex metalloclusters as active sites and require special maturation proteins [28]. The three main H_2-producing enzymes used by most microorganism are nitrogenases, [NiFe]-hydrogenases and [FeFe]-hydrogenases [15], [28].

Nitrogenases catalyse the nitrogen fixation reaction to ammonium, which requires the protons reduction to H_2. The nitrogenase is a protein complex composed for two subunits encoded by three structural *nif* genes. The first subunit is MoFe-protein, encoded by *nif*D and *nif*K, and the second subunit is Fe-protein, encoded by *nif*H. Nitrogenase catalysis reaction requires adenosine triphosphate (ATP) and proceeds at a slow turnover rate of about 6.4 s^{-1} [28]–[30].

[NiFe]-hydrogenases can work producing H_2 but also as uptake hydrogenases, using the electrons from H_2 to reduce intracellular NAD^+. The catalytic core of the [NiFe]-hydrogenases is composed of a heterodimeric protein. The large subunit contains the Ni-Fe active site and the small one contains several Fe-S clusters which serve as electron transfer points [28], [30], [31]. [NiFe]-hydrogenases work to turnover rate of 98 s^{-1}, about 15 times faster than nitrogenases for producing H_2 and do not require ATP to function as nitrogenases [30].

[FeFe]-hydrogenases enzyme is the most efficient hydrogenase and H_2 producing enzyme [32], which can have an activity 1000 times higher than nitrogenases and about 10-100 times than [NiFe]-hydrogenases. Structurally this enzyme consists of a protein with an Fe-Fe catalytic core that can accept a large variety of electron donors and acceptors. Depending of the environment conditions this enzyme can either produce or consume H_2 [28], [31].

1.3.2 Metabolic pathways to H_2 production

In dark fermentation process, a substrate rich in carbohydrates is anaerobically consumed by different fermentative microorganisms including strict and facultative anaerobes. Fig. 1-1 shows the main metabolic pathways observed during glucose fermentation when mixed microbial cultures are used. First, 1 mole of glucose is converted into 2 moles of pyruvate through the glycolytic pathway, producing 2 moles of ATP and 2 moles of reduced nicotinamide adenine dinucleotide (NADH). Pyruvate is further oxidized to acetyl-CoA, and different intermediates and by-products are produced depending on the microbial community composition and the environmental conditions [15]. Strict anaerobic bacteria commonly use the pyruvate ferredoxin oxidoreductase pathway (Fig. 1-1, right side). In this pathway, the oxidation of pyruvate into acetyl-CoA requires the reduction of one ferredoxin (Fd) supported by a pyruvate-ferredoxin oxidoreductase (PFOR), reduced Fd (Fd_{red}) is then oxidized by a hydrogenase that regenerates the Fd_{ox} and releases H_2. Additional H_2 can be produced from the NADH that is generated during glycolysis. The NADH is oxidized by Fd_{red} and a NADH-[FeFe] hydrogenase , but only at very low partial pressures of H_2 (<60 Pa) (Fig. 1-3) [23], [28]. Finally, 2 or 4 mol of H_2 per mole of glucose can be obtained, depending on the metabolic pathway, which in turn is directly related to the H_2 partial pressure inside the reactor [23].

In facultative anaerobes the pyruvate formate lyase (PFL) pathway is preferred (Fig. 1-1, left side). In this pathway, the pyruvate is oxidized into acetyl-CoA and formate. The acetyl-CoA is then broken down into acetate and ethanol, while formate is directly converted into H_2 and CO_2 by a formate hydrogen lyase (FHL) [23], [28]. With a maximum yield of 2 mol of H_2 per mole of glucose [33].

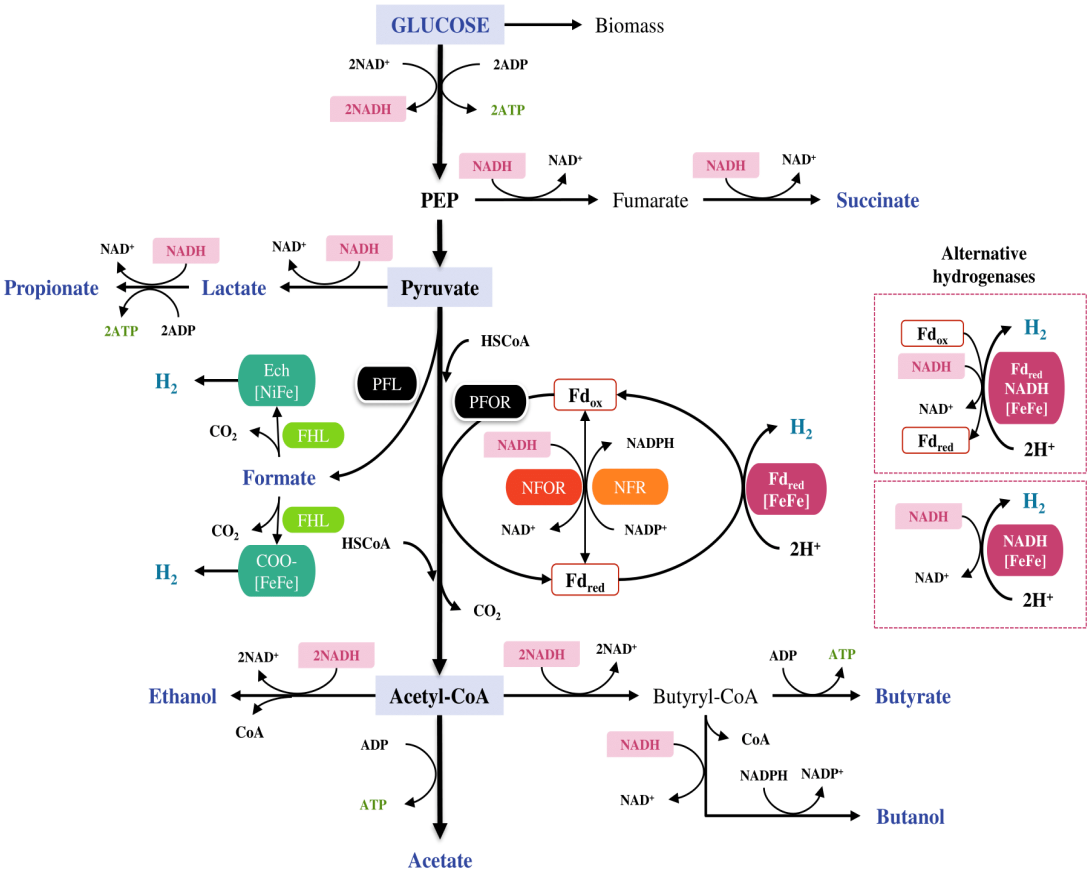


Fig. 1-1: Metabolic pathways during dark fermentation when mixed cultures are used. (Adapted from [23], [34], [35]**)**

Theoretically, a maximum of 12 mol of H_2 per mol of glucose can be produced. However, this reaction does not consider biological redox balances and regulations, and on average the highest reported H_2 yield that can be expected through fermentative pathways is only about 20% of this maximum, *i.e.* about 2.4 mol of H_2 per mol of glucose [21]. Dark fermentation is less efficient in terms of converting substrates to H_2 because about of 70% of carbon flux goes mainly into the formation of volatile fatty acids (VFA) and/or alcohols. In conclusion, the maximum energy conversion from glucose to H_2 is only 33% via the acetate pathway (*i.e.* 4 $molH_2.mol_{glucose}^{-1}$, Table 1-1, Eq.1), 17% via the butyrate pathway (*i.e.* 2 $molH_2.mol_{glucose}^{-1}$,

Table 1-1, Eq.2) and only 17% via the acetate and ethanol pathway (*i.e.* 2 molH_2.mol$_{glucose}^{-1}$, Table 1-1, Eq.3) [15], [21].

Table 1-1: Main reactions during H_2 production by dark fermentation (from [35]**)**

	ΔG°' (kJ/mol)	N° Eq.
Hydrogen production		
$\boldsymbol{Glucose + 2H_2O \rightarrow 2Acetate + 2CO_2 + 4H_2}$	– 215	Eq. 1
$\boldsymbol{Glucose \rightarrow Butyrate + 2CO_2 + 2H_2}$	– 264	Eq. 2
$\boldsymbol{Glucose + H_2O \rightarrow Ethanol + Acetate + 2CO_2 + 2H_2}$	– 225	Eq. 3
Acetogenic reactions		
$\boldsymbol{Propionate + 2H_2O \rightarrow Acetate + 3H_2 + CO_2}$	+ 76.2	Eq. 4
$\boldsymbol{Butyrate + 2H_2O \rightarrow 2Acetate + 2H_2}$	+ 48.4	Eq. 5
$\boldsymbol{Lactate + 2H_2O \rightarrow Acetate + HCO_3^- + 2H_2}$	– 4.2	Eq. 6
$\boldsymbol{Ethanol + H_2O \rightarrow Acetate + H_2}$	+ 9.6	Eq. 7
Hydrogen consumption		
$\boldsymbol{Glucose + 2H_2 \rightarrow 2Propionate + 2H_2O}$	– 279	Eq. 8
$\boldsymbol{4H_2 + 2CO_2 \rightarrow Acetate + 4H_2O}$	– 136	Eq. 9
$\boldsymbol{Butyrate + Acetate + 2H_2 \rightarrow Caproate + 4H_2O}$	– 359	Eq. 10
$\boldsymbol{Butyrate + 2CO_2 + 6H_2 \rightarrow Caproate + 4H_2O}$	– 143	Eq. 11
$\boldsymbol{4H_2 + CO_2 \rightarrow CH_4 + 2H_2O}$	– 135	Eq. 12
$\boldsymbol{SO_4^{2-} + 4H_2 + H^+ \rightarrow HS^- + 4H_2O}$	– 152.2	Eq. 13
$\boldsymbol{4S^0 + 4H_2 + H^+ \rightarrow 4HS^- + 4H^+}$	– 112	Eq. 14

Besides some acetogenic bacteria can synthesis acetate and H_2 during dark fermentation, particularly from propionate (Eq.4), butyrate (Eq.5), lactate (Eq.6) and ethanol (Eq.7) (see Table 1-1). However, except from lactate, the other reactions are thermodynamically

unfavourable (positive ΔG) particularly under high partial pressures of H_2, promoting the VFAs accumulation [35].

In Nature, microorganisms seek to maximize their biomass production and not H_2. In this context, there are many metabolic pathways that allow the cell to function in equilibrium including pathways that are not producing H_2 and consume reducing equivalents (NADH), and others that directly consume its molecular form (H_2). Consumption of NADH prevents Fd_{red} and formate formation, which both lead to H_2 production [35]. Such NADH balance without H_2 generation is observed in lactate, propionate, succinate, butyrate, ethanol and butanol pathways (Fig. 1-1).

Molecular H_2 consumption is thermodynamically very favourable, and has been evidenced by homoacetogenic bacteria to produce acetate (Table 1-1, Eq.9), as well as metabolic pathways for propionate (Table 1-1, Eq.8) and caproate production (Table 1-1, Eq.10 & Eq.11) [35].

1.3.3 Microbiology of mixed cultures

Dark fermentation can be carried out by pure or mixed cultures of microorganisms. According studies using pure cultures important knowledge for H_2 production could be provided [36]. Although higher H_2 yields have been reported with pure cultures, mixed cultures are preferred due to the wide variety of substrates that can be potentially used, cheaper systems and easier controls of the bioprocesses [22]. Mixed cultures are more flexible and resist to environmental stresses including limited availability of the substrates or changes in pH and temperature. Moreover, the diverse microflora present in the mixed culture might provide synergistic interactions that can improve H_2 production such as consumption of O_2 traces by facultative anaerobes that allows strict anaerobes to emerge safely [21], [23], [37], [38]. Mixed cultures capable of producing H_2 are widely present in

environment such as anaerobic and aerobic sludge, compost, soil, sediments, leachate and organic wastes etc. and microbial diversity differs with each inoculum source [22]. In a H_2 producing community coexist microorganisms with different functions, grouped as H_2 producers, H_2 consumers, as well auxiliary bacteria which are not able to produce H_2. But all them are key supporting the microbial community activity according thermodynamic possibilities of the system.

1.3.3.1 H_2 producing bacteria

H_2 producing bacteria (HPB) are commonly classified as strict and facultative anaerobes. *Clostridia* species and *Enterobacter* species are the most abundant in H_2-producing reactors. This is why those species are the most studied reported as pure culture to inoculate or bioaugment reactors [11], [36], [37]. *Clostridia* species are spore-forming bacteria and have a great potential to produce H_2 by dark fermentation. They are considered as the most efficient HPB since they are usually predominant in reactors with high H_2 yields *e.g.*, from 1.5 to 3.0 $mol_{H2}.mol_{glucose}^{-1}$ [21], [37]. With strict anaerobes, the most common fermentative pathways correspond to two main routes: acidogenesis and solventogenesis. Acidogenesis refers to organic acid production such as acetate and butyrate, while solventogenesis corresponds to solvent production such as ethanol and butanol [12], [37].

The most found strict anaerobes species include *C. butyricum*, *C. pasteurianum*, *C. beijerinckii* and *C. acetobutylicum*. These species exhibit different H_2 production pathways depending mainly on the type of substrate, the operating conditions and the reactors configuration [37]. For example, *C. acetobutylicum* can switch its metabolism from acidogenesis to solventogenesis under conditions of low growth rate, low pH and high concentrations of carbohydrates [39]. However, under optimal conditions, this specie provided the highest H_2 production rate from starch-containing waste in pure culture [40].

Besides, during glucose-fed CSTR, *C. histolyticum* was dominant during the periods of solventogenesis pathways at pH 4.0–4.5, with highest H_2 yields, while *C. lituseburense* was dominant during periods of acidogenesis pathways at pH 6.0–6.5, with lower H_2 yield [41].

Others strict anaerobes species reported are *Ethanoligenens harbinens* and *Acetanaerobacterium elongatum* that produce ethanol and H_2 simultaneously. These HPB have highly resistant against the bactericidal effect of ethanol during H_2 production, probably due to their specific [FeFe] hydrogenase [37], [42]–[46].

Facultative HPB correspond mainly to members of the *Enterobacteriaceae* family, such as *Enterobacter*, *Escherichia*, *Citrobacter*, and *Klebsiella* species. The most important characteristic of this family is the high 'tolerance' of the cells to oxygen. This allows to protect the enzymes not oxygen-tolerant like hydrogenases, as for example, when a reactor is accidentally exposed to oxygen, the presence of facultative anaerobes favours the immediate oxygen consumption to rapidly recover the hydrogenase activity [21]. The most studied model species include *Enterobacter aerogenes*, *Enterobacter cloacae*, *Citrobacter amalonaticus*, *Escherichia coli* and *Klebsiella pneumoniae*. Similarly to *Clostridium* species, members from this family could use different H_2 production pathways, but commonly are linked with poor H_2 production compared to strict anaerobes, because additionally H_2 production from NADH is not possible (Section 1.3.2) [12], [37]. For example, high H_2 yields are usually reported for *E. aerogenes* compared with other species from *Enterobacteriaceae* family [47], [48], while *Klebsiella* spp. is known to produce H_2 and alcohols such as ethanol and 2.3-butanediol from a variety of substrates [49].

Bacillales members are other facultative anaerobes found in H_2-producing reactors, mainly when operated with complex substrates, maybe due to their capacity to excrete hydrolytic enzymes [50], [51]. Particularly, *Bacillus megaterium* withstand high salinity levels (up to

15%) making possible the H_2 production from high salt-content wastewaters or polluted sea waters [21], [52]. Studies with this species have not been deepened, so the reasons for its salt tolerance are unknown.

1.3.3.2 H_2 consuming bacteria

Probably, the most famous H_2-consuming microorganism are methanogenic archaea (Table 1-1, Eq.12) and many efficient methods to eliminate or inhibit them have been developed. However, other H_2 consuming bacteria (HCB) can also be present in mixed cultures such as sulfate-reducing bacteria, lactic acid bacteria and homoacetogens.

Sulfate-reducing bacteria are lithotrophic and use H_2 as electron donor to reduce sulfate to hydrogen sulfide (Table 1-1, Eq.13 & Eq.14) [37]. Thermodynamically, sulfate reduction is the most efficient H_2-consuming reaction. Besides, is highly competitive for H_2, even at very low sulfate concentration because they can grow through an interspecies H_2 transfer by cooperating with methanogens and syntrophic bacteria [53]. Some genera include *Desulfovibrio*, *Desulfomicrobium*, *Desulfobulbus*, *Desulfobotulus* and *Desulfococcus* [35], [37]. For example, a high abundance of sulfate-reducing bacteria has been reported in an anaerobic CSTR under sulfate-limiting condition [54]. This is likely because many of these species can grow using a variety of fermentative metabolites as electron acceptor. However, generally short HRT as 2 h is not enough to inhibit the sulfate-reducing bacteria, but pH values lower than 6 significantly inhibit their activity [55], [56].

Several lactic acid bacteria such as *Lactobacillus paracasei*, *Lactobacillus ferintoshensis* and *Enterococcus sp* . have already been reported in H_2-producing reactors, especially in reactors operated at low pH and at high organic loads [35], [37], [57]. Some studies reported that the presence of acid lactic bacteria acted as an inhibitor of H_2 producers due to (i) substrate competition and (ii) excretion of bacteriocins [35], [57]. This toxin inhibit the growth of other

bacteria and more particularly clostridial species [58]. Contradictorily, many reports have suggested that these lactic acid bacteria could contribute to H_2 production. Indeed, lactate is a rapid way for the cells to release the excess of electrons, but in mixed cultures lactate could be consumed by some *Clostridia spp*. such as *C. diolis* to produce butyrate, CO_2 and H_2 [37].

Another type of H_2 consumers are fermentative homoacetogens that are strict anaerobes using H_2 as electron donor to autotrophically reduce CO_2 into acetate, through the acetyl-CoA pathway (Table 1-1, Eq.9). Because some homoacetogens are spore-forming, they difficult to remove them from the anaerobic reactors because they are resistant to pre-treatment. Besides, they are facultatively hydrogenotrophic, *i.e.* can grow autotrophically on H_2/CO_2 or heterotrophically on organic compounds such as sugars. Their presence results in lower H_2 production because can consume about 11% and 43% of H_2 produced accumulating the acetate [35], [37]. Homoacetogenic microorganisms include several clostridial species that exhibit a reverse metabolism of either production or uptake of H_2 through bidirectional hydrogenases [35], [37]. Some bacteria belonging to *Acetobacterium*, *Butyribacterium*, *Clostridium*, *Eubacterium*, *Peptostreptococcus* and *Sporomusa* have been classified as homoacetogens [35].

1.3.3.3 Contribution of auxiliary non H_2-producing bacteria in microbial community behaviour

As reported in many cases, the presence of auxiliary bacteria which are not able (or less efficient) to produce H_2 could be playing an important role supporting the microbial community with activities that include: (i) oxygen consumption often attributed to *Bacillus* and *Klebsiella*; (ii) pH regulation by lactic acid bacteria; (iii) hydrolysis of complex substrates attributed to lactic acid bacteria, *Bacillus sp.* and *Pseudomonas sp.* and

Ruminococcus; (iv) formation of cellular aggregates attributed to lactic acid bacteria, *Bacillus racemilacticus, Paenibacillus polymyxa* and *Prevotella sp.* [37], [59].

1.3.4 Main operating conditions for H_2 production

1.3.4.1 Mixed cultures: inoculum pre-treatments

Mixed cultures are composed of not only HPB, but also HCB that redirect the electron fluxes towards methane production or VFA (lactic, acetic, butyric acid etc.) accumulation , thus decreasing the total amount of accumulated H_2 [12], [22]. Therefore, it is essential to initially eliminate/inhibit the activity of HCB and enrich the inoculum in native HPB by applying physical or chemical pre-treatment methods [21]. Compared to untreated mixed cultures (<1.0 $molH_2.mol_{glucose}^{-1}$), generally successful improvement of the H_2 yields has been reported when pre-treated mixed cultures are used. This effect was confirmed by the level of hydrogenase activity in the pre-treated sludge that was three fold higher when compared to untreated sludge [60]. Fig. 1-2 summarizes the main pre-treatment methods reported in the literature, as discussed here below. Besides Table 1-2 shows the H_2 yields obtained with different pre-treatments when glucose was used as substrate [22].

i. Heat pre-treatment

Heat pre-treatment is a physical treatment, considered as the most effective one. For that reason, it is the most reported inoculum treatment in literature. Heat shock pre-treatment aims to eliminate non-spore forming microorganisms such as methanogens. This is considered as a simple and efficient method to eliminate HCB with a high potential for industrialization. However, some non-spore forming HPB could also be eliminated such as *Enterobacter* spp. and *Bacillus spp.*, Both are well-known HPB and particularly *Bacillus spp* could participate to the efficient degradation of complex substrates [39], [61]–[63].

Temperatures and exposition duration time are ranging from 65 °C – 100 °C and 10 min – 10 h, respectively [21], [22]. Intuitively, it is believed that higher temperatures require shorter exposure times, however the best combination to improve H_2 yields is strongly dependent on the inoculum source, *i.e.* native microbial diversity. Nonetheless, 2.49 $molH_2mol^{-1}_{hexose}$ is the highest H_2 yield obtained using heat pre-treatment, in this case from anaerobic sludge treated at 100 °C for 60 min, using sucrose as substrate [22], [64]–[66].

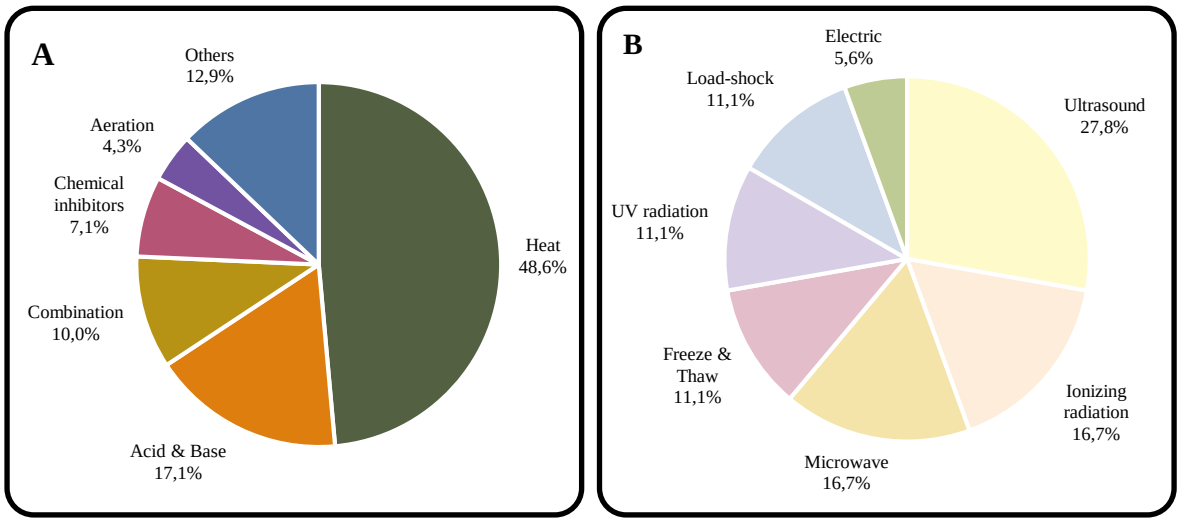


Fig. 1-2: Principals pretreatments reported using on mixed cultures to increase the H_2 yields. A: Main pretreatments used. B: Details of "Others" pretreatments shown in A. (Adapted from [22]**)**

ii. Acid-base pre-treatment

Acid-base inoculum pre-treatment is a chemical method causing changes in the extracellular pH that affect the net charge on the cell surface and consequently the enzyme activity and microbial nutrient absorption [22]. In principle, extreme pH induces spores formation, or lysis of not sporulating cells [21]. This method has been used to reduce the methanogenic activity in mixed cultures. The pH for acid pre-treatment commonly ranges from 2 to 4 with an exposition time from 30 min to 24h. The most common combination seems to be pH=4 for 24h. While the pH of basic pre-treatment is usually ranging from 10 to 12 with an exposition

time from 30 min to 24h, the most frequently found combination seems to be pH=10 for 24h [21], [22]. Although a higher H_2 yield has been reported using a basic pretreatment (3.1 $molH_2mol^{-1}_{hexose}$), from sucrose as substrate [67], no conclusive evidence to define on whether acidic or basic pre-treatment is better to enrich HPB and increase H_2 production. Because different studies comparing both treatment differ when concluding which was the best [21], [22], [63], [67].

iii. Chemical inhibitors

Some chemical inhibitors can selectively inhibit methanogenic activity without impacting H_2 production, including chloroform, bromoethanesulphonic acid (BESA), iodopropane and unsaturated fatty acids. Particularly, Chloroform and BESA can inhibit the Methyl-coenzyme M reductase (MCR), the key enzyme responsible for methane production. However, these inhibitors are often also lethal to the HPB and highly toxic and harmful to humans and the environment, which restricts their wide application [21], [22], [67].

Table 1-2: H_2 yields reported in literature when different inoculum pre-treatments where used during glucose dark fermentation (Adapted from [22]**)**

Inoculum Source	Pre-treatment	Glucose ($g.l^{-1}$)	Operational conditions	H_2 yields ($molH_2.mol_{glucose}^{-1}$)	Ref.
Anaerobic sludge	Heat (65 – 121 °C 10 – 90 min)	2 – 25	30 – 40 °C pH 5.5 – 7.5	0.9 – 2.41	[64], [68]–[76]
Compost	Heat (105 °C, 2 h)	13.1	33.5 °C, pH 5.0	2.15	[77]
Anaerobic sludge	Acid (pH 3, 24 h)	8 – 20	35 – 37 °C pH 6.5 – 7.0	0.27 – 1.51	[42], [63], [69], [71]–[73]
Anaerobic sludge	Base (pH 10, 24 h)	8 – 20	35 – 37 °C pH 6.5 – 7.0	0.25 – 1.72	[63], [69], [71]–[73]
Anaerobic sludge	Aeration (DO < 0.5 $mg.l^{-1}$, 12 h)	10	35 °C, pH 6.8	1.96	[42]
Anaerobic sludge	Aeration (Continuous 24 h)	10	35 °C, pH 7.0	0.86	[69]
Anaerobic sludge	Chloroform (2%, 24 h)	10	37 °C, pH 7.0	0.66	[69]
Anaerobic sludge	Ultrasonication (20 – 79 $Kj.gTS^{-1}$, 24 h)	8 – 8.5	37 °C, pH 6.5 – 6.8	1.03 – 1.70	[72], [78]
Anaerobic sludge	Freeze & thaw (–25 °C, 24 h and room temperature, 5h)	20	37 °C, pH 7.2	0.17	[79]
Anaerobic sludge	Electric current (10V, 10 min)	20	37 °C, pH 7.0	1.43	[80]
Anaerobic sludge	Ionizing radiation (0.5 – 10 kGy)	10	36 °C, pH 7.0	1.41 – 2.14	[71], [81], [82]
Anaerobic sludge	Heat (100°C, 30 min) & Base (pH 10, 24 h)	10	35 °C, pH 6.5	2.45	[83]

iv. Aeration

Aeration pre-treatment applies an oxidative stress aiming to deactivate strict anaerobic HCB such as methanogens, but also to enrich the inoculum in facultative HPB such as *Enterobacter* sp. However, the oxidative stress also severely damages strict anaerobic HPB like *Clostridium* spp. For that reason, aeration pre-treatment has been shown as inefficient by lowering the H_2 yields, compared with heat pre-treatment [21], [67]. Commonly, aeration conditions correspond to continuous aeration during dark fermentation or intermittent aeration and aeration times before to inoculate the reactor from 30 min to 4 d [22], [42], [67].

v. Other pre-treatments

Other physical and chemical pretreatments have been reported to a lower extent in the literature as shown in Fig. 1-2B.

Ultrasonication is a physical-chemical pre-treatment that uses sound waves to eliminate HCB and keeping the spore-former HPB. Micro bubbles are formed and collapse, generating shear forces, high localized temperature (5000 K) and pressure (180 MPa), and highly active radicals [22]. Although same damages can be caused on HPB, they can be prevented by controlling the pre-treatment duration and energy input [21], [22], [72], [84], [85].

Freezing and thawing implies the use of extreme and abrupt changes of temperature for several cycles leading to ice crystal formation inside the cells and thus making irreversible damages in the microbial structures. In general, this method causes cell lysis of HCB but also HPB, and very low H_2 yield were reported compared to heat pre-treatment (0.04 - 0.17 $molH_2.mol^{-1}_{hexose}$). Thus it can be considered as inefficient [21], [22], [79], [86].

Ultraviolet radiation (UV) can denature the intracellular DNA, leading to the death or cell inactivation. The recommended pre-treatment condition is 15 min of UV irradiation. Such

conditions successfully eliminate methanogens increasing the H_2 yield of 1.39-fold respect to untreated mixed cultures. However, HCB inside dark coloured sludge particles are protected from UV irradiation and only bacteria present on the surface are eliminated [21], [87], [88].

Electric current was also found to have the capacity of destructing cell membranes while preserving the microorganisms capable of forming spores. However, the mechanism of action of the electric current is not totally clear. Different values of electrical field have been applied within a range of 3.0 - 12 V, but using 10V the highest H_2 yield was observed (1.43 $molH_2mol^{-1}_{hexose}$) [21], [80], [89].

Microwaves are another kind of electromagnetic radiation with frequencies ranging from 0.3 GHz to 300 GHz and wave lengths in air from 1 m to 0.0001 m. In general, the effectiveness of microwaves in destructing microorganisms depends on the time of exposure and the power of the electromagnetic field used. The specific frequency of the microwares for enriching HPB was found at 2450 MHz Although the effectiveness of pre-treatment depends on the inoculum microbial community, *Clostridia spp*. is generally the most favoured [22], [90], [91].

Ionizing radiation comprises gamma rays (0.5 – 10.0 KGy), X-rays and the higher UV part of the electromagnetic spectrum. When aqueous solutions are irradiated by such ionizing radiations, highly reactive chemical species are formed that can react with other substances existing in the solutions. Due to the low water content of the spores, less hydroxyl radicals are generated inside microbes in the form of spores during ionizing irradiation process. Thus, spore-forming bacteria are expected to be more likely to survive under such ionizing radiation, increasing H_2 production [22], [71], [92].

In addition, pre-treatments are sometimes combined to ensure the efficient HCB elimination, while seeking to enrich the inoculum with HPB. In this context has been reported a high H_2 yields of 2.45 $molH_2.mol_{glucose}^{-1}$ by combining heat (100°C, 30 min) and base (pH 10, 24h) pre-treatment [83].

1.3.4.2 Simple and complex substrate

For proper bacterial growth, appropriate sources of carbon, nitrogen, phosphorus and other micronutrients must be available in the fermentation medium and in correct proportions. Generally, carbon source is considered as the most important element and is commonly referred as THE substrate. Glucose has been the most used substrate and is considered as model because is consumed by most of the microorganisms. In the literature, substrates are distinguished as simple or complex. Monomeric or pure polymeric sugars such as sucrose, lactose, fructose, xylose, starch, cellulose and pure glycerol are considered as simple substrates, while industrial wastewaters or waste including rice winery, food waste, dairy wastewaters, beer lees and crude glycerol are considered as complex substrates [11], [21].

Organic substrates found in wastewaters are moreover cheap and easily available. However, industrial wastewaters may not be sufficiently nutritious to support H_2 production and it is not practical to continuously supply the fermentation process with costly supplements such as peptone or yeast extract. One of the solutions is to improve the nutrient content using a combination of different types of wastewaters, for instance by mixing different waste streams rich in carbohydrate or nitrogen [21]. For example, when Wang *et al*. (2011) used as substrate a combination of 75% food wastewater (carbohydrate rich) and 25% sewage sludge (nitrogen rich), a higher H_2 yield was obtained, compared with the yield observed with each substrate alone [93].

Besides, a balanced concentration of substrate plays an important role in H_2 production. Increasing the substrate concentration may increase H_2 production but it must be appropriate as it has also been evidenced that beyond certain substrates concentrations the H_2 yields decreaseq [11], [94], [95]. Furthermore, excess substrate decreases H_2 production by shifting fermentation pathways to produce alcohol or lactic acid [11], [21]. For example, Levin *et al.* (2006) reported during *Clostridium thermocellum* dark fermentation, that lactate and H_2 production were increased and decreased, respectively, when using high levels of cellulosic biomass as substrate (4.5 $g.l^{-1}$) [96].

1.3.4.3 Macro and micro-nutrients requirements

As already mentioned in the previous section, the carbon source in not the only requirement to support proper bacterial growth. Microorganisms need other elements for their growth, such as nitrogen, phosphorus and other important micronutrients. That is why the nutritional requirements and the culture medium composition are important variables that directly affect the microbial metabolism in dark fermentation and therefore are critical for H_2 production [14], [97], [98].

Nitrogen is an important component of the cells to synthetize proteins, nucleic acids and enzymes such as hydrogenases. However, there are still disagreements with respect to the optimum concentration. But a nitrogen excess could be detrimental to the intracellular pH and can eventually inhibit the bacterial growth and enzymatic activity [11], [14]. High nitrogen concentrations can induce ammonification, which is not favorable for H_2 production [11], [14]. Phosphate is also required to synthetize ATP and other nucleic acids, as well as to provide a buffering capacity [11], [14]. But high phosphorus concentration can also stimulate excessive VFAs production, which leads to significant decrease in H_2 production [14]. On top of the optimal concentrations to be adjusted prior to fermentation, appropriate C/N and C/P

ratios are fundamental to balance the carbon fluxes between biomass production and H_2-producing pathways. Among the studies that dealt with this aspect, there is no common agreement on the optimal values and the ratios to be respected, with a wide range from 47 to 200 and 559 to 1000 for C/N and C/P, respectively [98]–[100]. Such substantial differences in optimal values of these ratios are probably due to different carbon source, inoculum source and operational conditions were used.

In addition, metal ions are necessary to activate enzymes and co-enzymes related to microbial metabolism and consequently are essential for cell growth and any fermentation processes [14]. The most studied metal in H_2 production is iron (Fe^{2+}) since the most efficient hydrogenases have bimetallic Fe-Fe active sites surrounded by Fe-S protein clusters [11], [101]. For example, has been reported an increase in the H_2 production by 1.5-fold when Fe concentration in the fermentation medium increased from 15 to 55 $mg.l^{-1}$ [102].

Light metal ions have also been studied including magnesium (Mg^{2+}), sodium (Na^{+}) and calcium (Ca^{2+}). Magnesium ions are present in cellular walls and membranes and are required for building cellular proteins and they are also serving as activators and cofactors of many enzymes such as kinases and synthetases, mainly useful during glycolysis [7]. For example, Alshiyab *et al.* (2008) reported an increase in the H_2 yields when was added $MgSO_4.7H_2O$ in range of 50-500 $mg.l^{-1}$ [103].

Sodium ions have been reported as a micronutrient for bacterial growth [104]. For example, a maximum H_2 production was obtained with 1 $gNa^{+}.l^{-1}$, while using 2 $gNa^{+}.l^{-1}$ caused a decrease due to Na^{+} inhibition. Probably, more ATP was used to maintain the cellular viability than for growth under high Na^{+} concentration conditions [105].

Calcium (Ca^{2+}) has been reported to enhance bacterial growth and mechanical strength of granular sludge while also promoting cell retention [103], [105], [106]. Besides, some studies report increase of H_2 production associated to addition of Ca^{2+} [102], [105], [107].

1.3.4.4 pH and Temperature

The most crucial operational parameters that affect the H_2 production pathways are pH and the temperature. pH is an important parameter during de H_2 production impacting: (i) the enzymatic activities and more particularly the hydrogenases that present an optimal pH between 6.0 and 6.5 [108], [109]; (ii) the microbial community selection through *e.g.* methanogen inhibition at pH lower than 6; (iii) the metabolic pathways and consequently the H_2 yields. In the last case, acetate and butyrate pathways are mostly favoured at neutral (pH 6.5–7.0) and acid (pH 5.0–5.5) pH, respectively; (iv) the hydrolysis of complex substrates [15], [27]. In general, the operational pH is set within a range that supports the growth of many HPB , *i.e.* between 4.5 and 8.0 [14], [15], [27]. An optimum pH helps to maintain the surface change on the cell membrane which facilitates nutrient uptake and sustains bacterial growth [21]. Indeed, for simple substrates such as glucose, the highest H_2 yields were reported at pH of 6.0 (1.83 $molH_2.mol_{glucose}^{-1}$) in batch experiments [110]. When fermenting a complex substrate (as food waste), maximum H_2 yields were reported at pH 8.0 (1.92 $molH_2.mol_{hexose}^{-1}$) [111]. However, there is an agreement of the negative effect of pH values below 4.5 – 5.5, generally caused by the accumulation of VFAs, which can reduce H_2 production due to shifts in the metabolite production pathways towards solventogenesis (acetone, butanol, ethanol) [112].

Temperature is also a key parameter determining the physiological activities of the microbial community and similarly to pH, temperature can affect substrate hydrolysis, in particular for lignocellulosic compounds. Operational temperatures are classified as ambient (15 – 27 ºC),

mesophilic (30 – 45 ºC), thermophilic (50 – 60 ºC) or extreme thermophilic (>60 ºC) [11], [14], [15]. Varying the temperature affects greatly the Microbial community structure. Lazaro *et al.* (2014) explained that significant differences between the microbial communities at 37 °C and 55 °C exist. A shift from *Clostridium* at mesophilic conditions to *Thermoanaerobacterium* when thermophilic conditions were applied was shown. However, the H_2 yield was not significantly impacted by the temperature regime (2.31 and 2.23 $mmolH_2.gCOD_{influent}^{-1}$ at mesophilic and thermophilic, respectively [113]. As reported by Ghimire *et al.* (2015), temperature also affects the metabolic pathways, thus modifying the by-products produced during DF [15]. Consistently, the study of Valdez-Vasquez *et al.* (2005) showed a significant difference in the average distribution of metabolites between thermophilic and mesophilic conditions. The predominant metabolite produced under mesophilic temperatures was butyrate, while in thermophilic conditions acetate was the main metabolite [114].

1.3.4.5 H_2 partial pressure

The main objective for optimizing H_2 production is to maximize the H_2 yields with subsequent accumulation in reactors headspace. However, such H_2 over-accumulation can lead to a strong inhibition further resulting in lower H_2 yields than expected [23]. In general, H_2 production is thermodynamically limited by the enzymatic reaction of the [Fe Fe] hydrogenases involved in the PFOR pathway (Fig. 1-1). When the H_2 partial pressure is less than 0.3 atm (3×10^4 Pa), H_2 could be produced via Fd_{red} producing a maximum of 2 moles of H_2 per mole of glucose with butyrate as final product (Fig. 1-3, a). When the H_2 partial pressure is < 60 Pa, additional H_2 could be produced from NADH excess by NADH-[FeFe] hydrogenase to produce an additional 2 moles of H_2 *i.e.* a maximum of 4 moles of H_2 per mole of glucose, with acetate as final product (Fig. 1-3, b) [23], [115]. Several strategies have

been proposed to decrease the H_2 partial pressure in dark fermentation including a continuous gas release, larger headspace volume in reactor, vacuum stripping or sparging with inert gas like N_2 or CO_2 [116]–[122]. In general, all these strategies evidence an efficient increase in the H_2 production. For example, Hussy *et al.* (2005) reported an increase in the H_2 yield from 1.0 to 1.9 $molH_2.mol_{hexoxe}^{-1}$ by continuous N_2 sparging at 40 $ml.min^{-1}$ [116]. Similarly, Bastidas-Oyanedel *et al.* (2012) reported an H_2 yield increase from 1.0 to 3.25 $molH_2.mol_{glucose}^{-1}$ by continuous N_2 flushing at 40.5 $ml.min^{-1}$ [122].

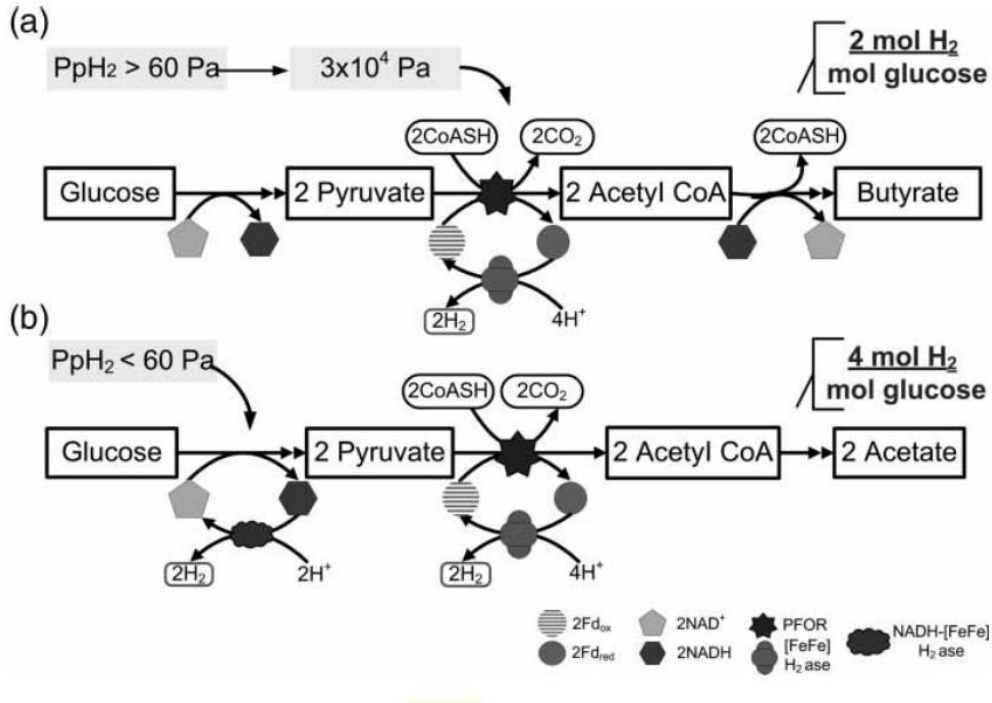


Fig. 1-3: H_2 partial pressure effect on dark fermentation pathway

H_2 partial pressure (PpH_2) >60 Pa produce different reduced metabolites with a maximum H_2 yield of 2 $molH_2.mol_{glucose}^{-1}$ (a); NADH oxidation by NADH-[FeFe] hydrogenase is possible only when the PpH_2 is <60 Pa, with a maximum H_2 yield of 4 $molH_2.mol_{glucose}^{-1}$ (b). (Figure source: [23])

1.3.4.6 Hydraulic retention time

The hydraulic retention time (HRT) is an important parameter also impacting H_2 production in continuous systems. HRT in suspended-cell systems *e.g.* CSTR, is the inverse of the dilution rate (D, Eq. 1). When the system reaches the steady-state *i.e.* equilibrium, D is equal to the microbial growth rate (μ). Thus, manipulating D makes possible the selection of growing microorganisms that become rapidly dominant in the system. When using mixed cultures, two situations may occur in the system: (i) $D > \mu_{max}$, all the microorganisms under this condition are washed-out from the reactor; (ii) $D \leq \mu_{max}$, all the microorganisms under this condition are kept in the reactor. To modify the D value, the input flow of the reactor can be changed since the reactor volume remains generally constant.

Eq. 1

$$D[h^{-1}] = \frac{Flow_{input}[l \cdot h^{-1}]}{Volume_{reactor}[l]}$$

Since methanogens (HRT ≥ 1 d) are growing more slowly than HPB, HRT values around 4 to 24h are usually preferred to favour H_2 production. In general, this value depends on the type of substrate and which HPB type is desired in the reactor [22]. Typically, short HRTs (< 24h) and low pHs (pH 5.5 – 6.0) are coupled to decrease the methanogenic activity and reach efficient H_2 production [15], [22], [23], [123].

1.3.5 Oxidation-reduction potential and fermentation

The fermentation process is defined as a cascade of oxidation-reduction reactions occurring in equilibrium to provide enough energy to the cells for their growth and maintenance. Under fermentative conditions, the substrate is only partially oxidized and the energy yield is lower than in respiration, *i.e.* when using oxygen as final electron acceptor (Fig. 1-4) [124].

The importance of the "redox" potential, also called Oxidation-Reduction Potential (ORP), on the microbial activity has been well addressed and anaerobes during any fermentation are particularly sensitive to its changes [125], [126]. ORP reflects the overall electrons transfer and redox balance involved in extracellular metabolism. Two compounds are required for an oxidation-reduction reaction to occur, during which the reducing compound donates electrons to an oxidizing compound. Each redox pair has its intrinsic ORP value. The higher is the affinity to the electrons, the higher the ORP of a redox pair would be [127]. Table 1-3 shows the reduction potential of some redox pairs of microbiological importance [124].

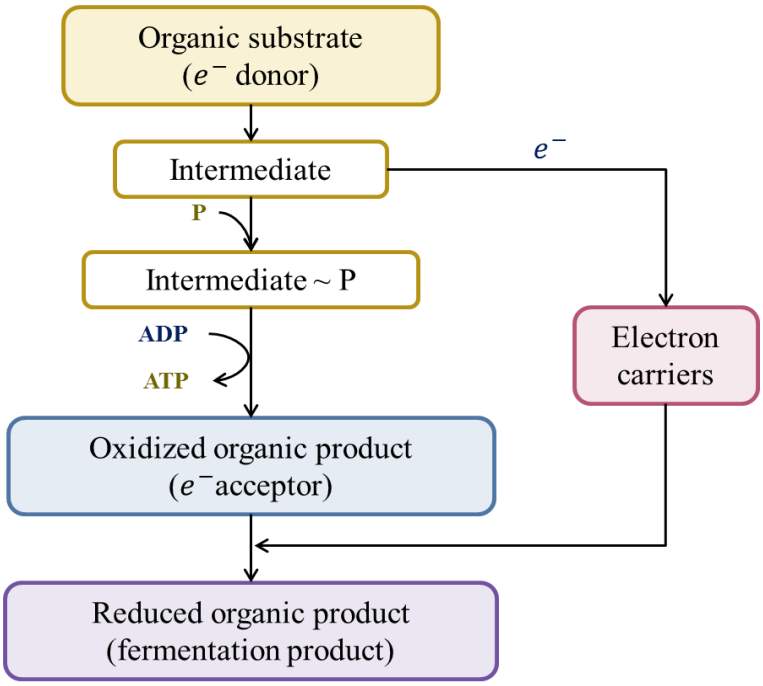


Fig. 1-4: Simplified diagram of a fermentation process (Adapted from [124]**)**

By measuring ORP in the extracellular medium is possible to know what takes place inside the cell [127]. Key factors modulating ORP in fermentation medium include: temperature, traces of dissolved oxygen, reduction degree of the substrate and the metabolic products released during fermentation [127]–[129]. Fig. 1-5 summarize the main factors affecting both extracellular and intracellular redox and how they are interacting.

Table 1-3: Reduction potential of important redox pairs involved in biological process

Redox pairs	E^0 (V)	Redox pairs	E^0 (V)
$2H^+/H_2$	– 0.41	**$Pyruvate^-/lactate^-$**	– 0.19
Ferredoxin ox/red	– 0.38	**Fumarate/Succinate**	+ 0.03
$NAD^+/NADH$	– 0.32	**Cytochrome *b* ox/red**	+ 0.03
Cytochrome c_3 ox/red	– 0.29	**NO_2^-/NO**	+ 0.36
$CO_2/Acetate^-$	– 0.29	**NO_3^-/NO_2^-**	+ 0.43
CO_2/CH_4	– 0.24	**O_2/H_2O**	+ 0.82

The conjugated redox pairs and their homeostasis are fundamental in the intracellular metabolism, since many biological functions are sensitive to ORP changes including genetic expression, enzymatic synthesis and metabolic patterns [127], [130]–[133]. NAD^+/NADH cofactor is the most important redox pairs of catabolism. NADH is formed from NAD^+ during the oxidation of molecules such as glucose. However, the cell seeks to regenerate the NAD^+ and release the electrons excess by transferring these electrons to intermediary metabolites such as ethanol, butyrate and H_2 [127], [133]. Particularly, in clostridia, H_2 is produced to regenerate the NADH through a ferredoxin oxidoreductase and a hydrogenase [134], [135]. The activity of these H_2 evolving enzymes is regulated by the intracellular NADH/NAD^+ and acetyl-CoA/CoA ratios and all excess of reducing power is disposed as H_2 [136]. Meanwhile, in facultative anaerobes, NADH is usually regenerated through the 2,3-butanediol, ethanol and lactate pathways with no H_2 generation [128]. Nonetheless, some studies operated with *E. aerogenes* reported that H_2 production was highly dependent on the NADH/NAD^+ ratio, suggesting that NADH accumulation is a critical factor for H_2 production [137].

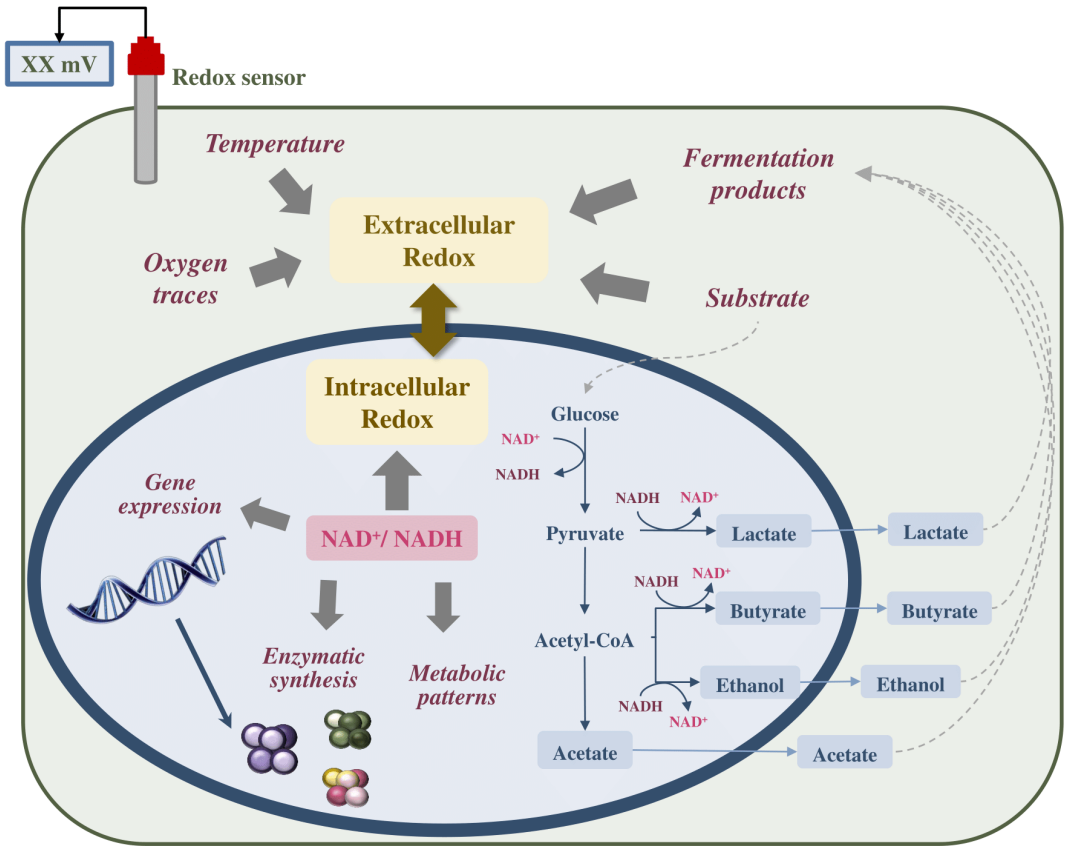


Fig. 1-5: Main factors affecting extracellular and intracellular redox

Extracellular Redox and Intracellular redox are linked, thus, the extracellular redox is the reflection of the intracellular redox. Extracellular redox is mainly affected by temperature, oxygen traces and by reduction degree of organic molecules in solution as fermentation products and substrate. Intracellular redox is mainly affected by ratio NAD^+/NADH, which in turn affects the gene expression, the enzymatic synthesis and metabolic patterns. (adapted from [127]*)*

Different strategies have been tested and implemented to better control the cellular ORP including metabolic engineering, chemical addition, gas bubbling and supply of external energy by bioelectrochemical reactors. For instance, redox reactants such as potassium ferricyanide, methyl viologen and neutral red can act as electron transporters affecting the redox balance and thus the cellular metabolism [126], [127], [138]. Active redox gases such as oxygen, H_2 and CO can also be bubbled into the fermentation medium to control the ORP. Oxygen increases the ORP while H_2 decreases it. Other inert gases such as nitrogen or helium can be bubbled, eliminating oxygen and H_2 and changing indirectly the ORP of the medium

[127]. Finally, energy can also be supplied in the form of an electric current in bioelectrochemical reactors (BES). BES are equipped with electrodes to control the ORP during fermentation. The efficiency of these systems mainly depends on the interactions existing between the microorganisms and the surface of the working electrode [127], [139]. In the next section, more details about BES are given and more particularly about electro-fermentation.

1.4 Electro-fermentation: how to drive fermentation using electrochemical systems?

As described in previous Section 1.3, many parameters have been used to control bioprocesses. However, only few controllers are available to maintain stable the metabolic patterns *i.e.* H_2 and metabolite production. In this context, electro-fermentation (EF) has been proposed as a new type of bioprocess control in presence of polarized electrodes. EF aims to maximize the performances of production in a high valuable end-products through a better control of the metabolic patterns. Besides, depending on specific operational conditions could allow to decrease purification and separation cost, face to possible industrial applications. Despite EF is a new technology and the action mechanisms are not totally understood, EF is a promising technology.

1.4.1 Basics of Electro-fermentation

EF is a BES that couples fermentation and electrochemical processes to better control the microbial metabolism through extracellular ORP regulation [18], [127]. Typically, a BES is composed of an anode, a cathode and sometimes a reference electrode. This last one has a stable and known equilibrium potential and is placed in the working electrode chamber (anode or cathode depending on working potential) to facilitate the control of the system.

Then, a potential is applied at the working electrode by changing the potential on the counter electrode [19]. Single or dual-chamber reactors can be used, although dual chamber BES requires the presence of a membrane to separate each chamber. Classically, an ion exchange membrane is used, selective either for cations or anions (*a.* and *b.*, Fig. 1-6) [19]. Fig. 1-6 shows a classical EF reactor and three possible types of interaction between the electrode and the fermentation medium to support this system.

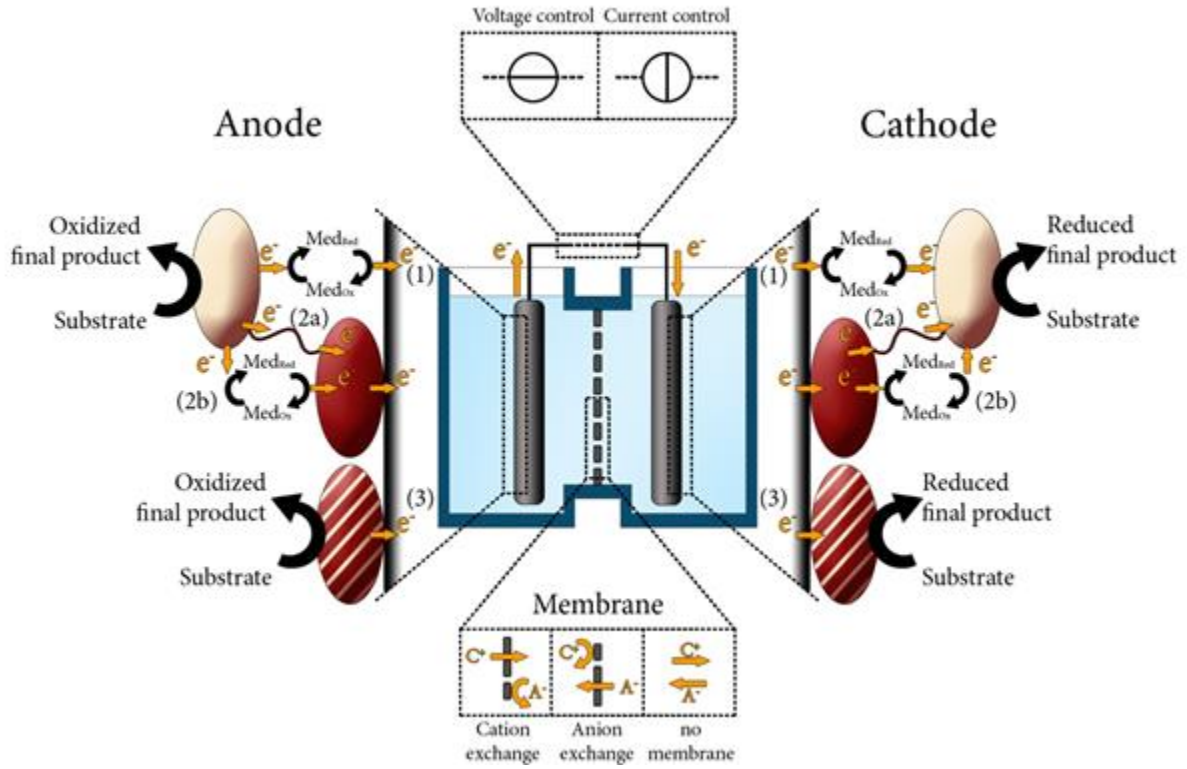


Fig. 1-6: Process configurations of electro-fermentation system including possible interactions between electrode and fermentation medium

Three possible interaction between electrode and fermentation medium could be supporting this system: (1) a redox mediator that can be oxidized (Med_{ox}) or reduced (Med_{red}) by an electrode and then used by fermentative bacteria (in light yellow); (2a) electroactive bacteria (in red) to perform a direct electron transfer to fermentative bacteria with nanowires or (2b) to catalyse the regeneration of a redox mediator then used by fermentative bacteria; (3) electroactive bacteria that can also perform fermentation. The overall process can be voltage controlled to avoid electrochemically 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 (Figure source [140]*)*

Different types of BES exist including microbial fuel cells (MFC) to produce electricity, microbial electrolysis cells (MEC) to produce hydrogen, microbial electrosynthesis (MES) to produce chemical products from CO_2 reduction and microbial desalination cells (MDC) to desalinate saline waters [18]. The common denominator of all these processes is the requirement of large current density because electrons are either the final product of interest (in MFC) or the main driving force [18], [141].

EF implies the fermentation of an organic substrate in presence of polarized electrodes. Depending on the potential applied, the system can provide a supplementary source of electrons, or act as an electron acceptor. When electrodes provide more electrons to the fermentation medium the operation can be called cathodic electro-fermentation. Meanwhile if the system is acting as electron sink the operation is called anodic electro-fermentation [18]. The function of polarized electrodes during EF is only to assist the microbial metabolism and is not the main energy source supporting the microbial growth and activity because the main energy source during fermentation comes from the organic substrate and the main electron acceptors are intermediate products of fermentation (Fig. 1-4). Consequently, a high current density is not involved in the process [18], [142], [143]. This point is the main difference between EF and others BES. To illustrate this, a coefficient of electro-fermentation efficiency, η_{EF}, can be estimated, as detailed in Section 2.4.1.

How polarized electrodes are affecting the metabolic patterns depends on many factors, including (i) the type of inoculum: pure or mixed cultures; (ii) the composition of the microbial community with complex interactions; (iii) the presence in the fermentation medium of redox mediators, naturally produced such as H_2 and formate, or artificially added such as methyl viologen and neutral red; (iv) the microbial interactions with the polarized electrode surface.

1.4.2 First evidence of metabolic pattern changes and microbial community selection in electro-fermentation

The first research works in EF have been performed with both pure and mixed cultures. In both cases, a clear effect of polarized electrodes was shown on the fermentation processes. In pure cultures, a substantial change of the metabolic pathways was observed [142], while in mixed culture, the metabolic patterns changes were probably due to the selection of specific microbial community [143].

When using pure cultures different strategies were employed to ensure the extracellular electron transfer (EET) between the fermentative microorganisms and the polarized electrode. Choi *et al.*, 2014 showed that *Clostridium pasteurianum*, a fermentative bacteria, was capable to receive electrons directly from a cathode poised +0.045V *vs* SHE, suggesting some electroactive properties of this microorganism. Butanol and 1,3-propanediol production were then enhanced 3 and 2 folds using glucose and glycerol as substrates, respectively [142].

Metabolic engineering was also used aiming to use electroactive bacteria such as *Shewanella Oneidensis* to ferment a wider range of substrates. Flynn *et al.*, (2010) worked with a genetically modified strain of *S. Oneidensis* to produce ethanol from glycerol. This pathway is only possible by removing two electrons through an external reaction. In this case an anode poised at +0.4V *vs* SHE acting as electron sink, was enough to target the metabolic flow towards the ethanol production [144]. Also, *Escherichia coli* was genetically modified to give its electroactive properties, allowing to accelerate the electron transfer by 183% via periplasmic heterologous expression of the c-type cytochromes CymA, MtrA, and STC. However, methylene blue as electron shuttle was required [145].

Another strategy consists in the addition in the fermentation medium of redox mediators such as methylene blue [145], neutral red [146], methyl viologen [147] and cobalt sepulchrate

[148]. These redox mediators are not degraded and act as electron shuttles since they can be oxidized or reduced by fermentative bacteria and then be recycled at the electrode surface [18], [139]. An interesting study reported propionate as unique fermentation product from glucose after addition of 0.4mM cobalt sepulchrate and using *Propionibacterium freudenreichii*. In this study, a three-electrode amperometry culture system was poised at –0.39V *vs* SHE [148].

The syntrophic relationship between electroactive and fermentative bacteria can be harnessed and replicated to ensure the transfer of electrons. *S. Oneidensis* and *Geobacter sulfurreducens* are famous for interacting directly with the electrode surface (more detail in Section 1.4.3). For example, ethanol production and glycerol consumption were increased when using *C. cellobioparum* and polarized electrodes previously colonized with *G. sulfurreducens* during MEC system poised at +0.46V *vs* SHE [149].

The few research made using mixed cultures have shown changes in microbial communities with a consequence on the metabolic patterns. Xue *et al.*, 2018 reported an increase of 4.7 times in lactate production from organic wastes when working with a cathodic EF poised at –0.1V *vs* SHE. This change was attributed to a strong selection of *Lactobacillus* and *Caldicoprobacter* genera [150]. In contrast, Xafenias *et al.*, 2015 reported an increase of 1.3 – propanediol production related to *Clostridium sp.* selection during glycerol EF, while in the control test (without electrode) lactate production related to the presence of *Lactobacillaceae* was shown [151]. Consistently, Dennis *et al.*, 2013 showed that an electrical current of –240 mA impacted the microbial community leading to the production of different molecules, depending the microbial community selected during 9 weeks of operation. Thus, during the first week *Citrobacter* population was dominant, with 1,3-propanediol and ethanol as main metabolites. Then, *Pectinatus* population was increased at expenses of *Citrobacter*

population, and propionate was the main metabolite. After about 6 weeks of operation, *Clostridium* population was dominant and valerate production was also increased [152]. Moscoviz *et al.*, 2017 studied the influence of polarized electrodes, previously colonized with *Geobacter sulfurreducens*, in glycerol EF by mixed cultures. This study showed an increase in 1,3 – propanediol production supported by the selection of a specific microbial community in a system poised at – 0.9V *vs* SCE [143]. Unfortunately, molecular biology analyses have not been performed in all mixed cultures studies making difficult the comparison of the microbial communities between these systems operated with polarized electrodes [153], [154].

1.4.3 Possible microbial interactions with the polarized electrodes

When considering the microbial interactions with the electrodes, the microbial community can take/release electrons from/towards electrode surface through indirect and direct EET, also called MIET and DIET respectively. MIET involves the production or use of electron shuttles to transport the electrons, while DIET not. Fig. 1-7 summarize the main EET mechanisms reported in literature [19], [155].

The idea of an electrically connected microbial community through DIET has begun to be recognized as key to diverse environments. Three potential strategies for DIET have been reported to date as evidence the Fig. 1-8 [156]. These strategies include electron transfer through electrically conductive pili, conductive materials and electron transfer between proteins associated to outer membrane as cytochromes [156].

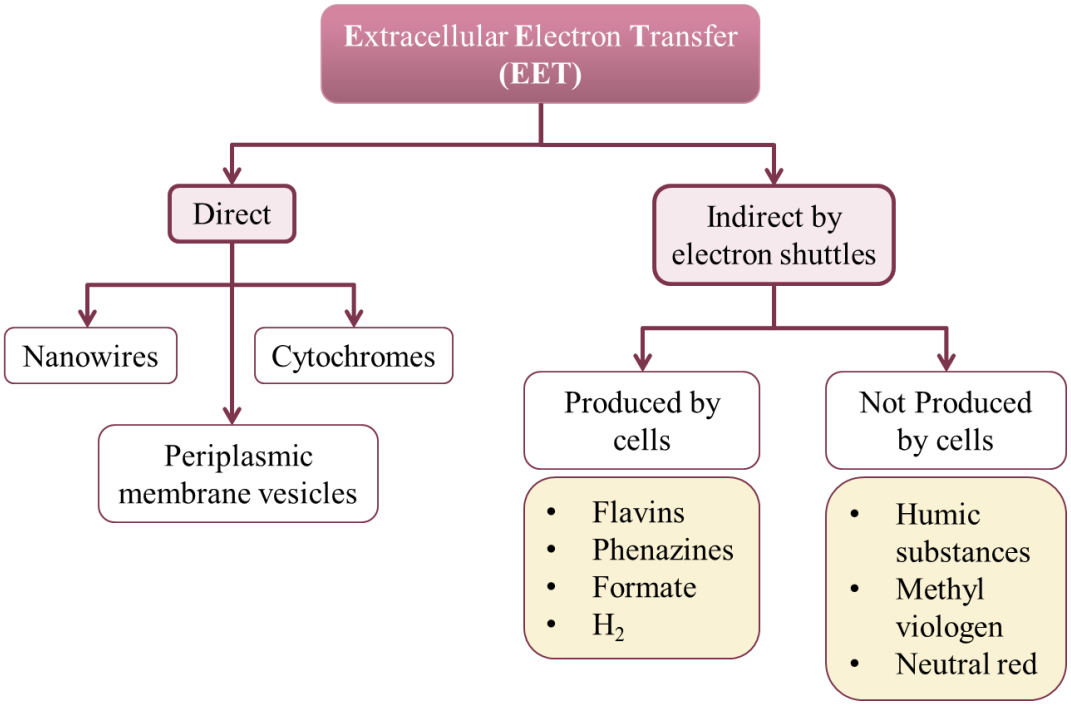


Fig. 1-7: Main extracellular electron transfer mechanism reported on literature

1.4.3.1 Direct interspecies electron transfer (DIET)

Particularly, DIET has been studied in pure cultures of *G. sulfurreducens* and *S. Oneidensis* [19]. *S. Oneidensis* is a fermentative bacteria exhibiting electroactive properties [157]. This bacteria uses metal reducing (Mtr) pathway, which includes a series of protein components (*i.e.*, CymA, MtrB, MtrC and OmcA) that transfer electrons from the quinone (soluble electron shuttles) pool embedded in the cytoplasmic membrane (*i.e.* inner membrane) to outer membrane and finally outside the cell towards insoluble materials [144], [155], [158]. Besides, *S. Oneidensis* can release flavins to transfer electrons, but also the flavins can bind to Mtr proteins and enhance extracellular electron transfer rates [144], [155], [159]. Periplasmic membrane vesicles excreted by this bacteria have also been documented as extracellular electron carrier [155]. Similarly, *G. sulfurreducens* uses a trans-outer membrane porin-cytochrome complex containing OmcB and porin-like outer membrane proteins along

with a periplasmic c-type cytochrome to transfer electrons across the outer membrane [155]. This bacteria use also nanofilaments or bacterial nanowires to establish electron transport with the electrodes surface but also with other microorganisms even a long-range (50 µm or more when assembled to form biofilm) [19], [155]. These nanowires have been comparable with doped silicon nanowires or conductive polymers [155].

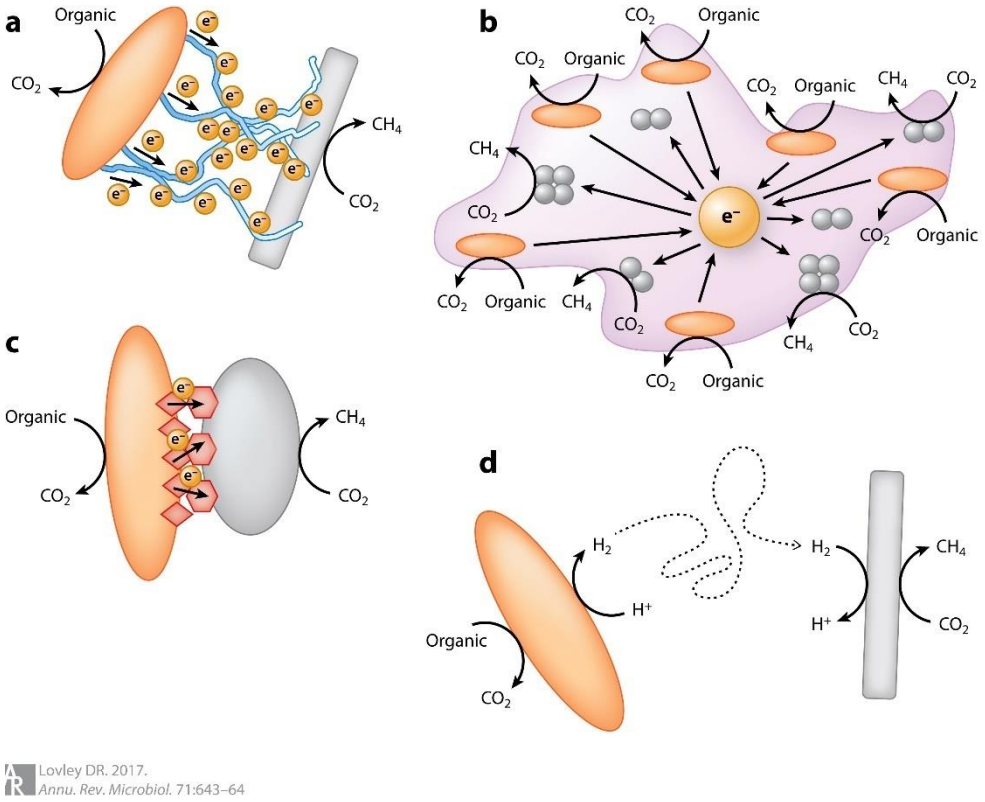


Fig. 1-8: Proposed mechanisms for interspecies electron transfer through Direct interspecies electron transfer (DIET)

(a) electron transfer through electrically conductive pili (blue); (b) electron transfer through electrically conductive materials (purple); (c) electron transfer between electron transport proteins (red) associated with outer cell surfaces. (d) DIET contrasts with the diffusive exchange of electrons between species through soluble electron shuttles such as H_2. In these examples the electron-accepting microbe is a methanogen (Figure source [156]*)*

1.4.3.2 Mediated interspecies electron transfer (MIET)

MIET implies the use of electroactive substances so-called electron shuttles. Examples of electron shuttles produced by microorganisms are phenazines, flavins, H_2 and formate [19], [155]. External electron shuttles that can be added to the system include humic substances, methyl viologen, and neutral red [19], [139]. Microorganisms can selectively oxidize or reduce these species without consuming them, leaving them free for recycling at the electrode [139]. The best-known form of MIET is interspecies hydrogen transfer, a key-concept that revolutionized the understanding of how methanogenic microbial community worked during anaerobic digestion [156].

1.4.4 Action mechanisms of electro-fermentation

The action mechanisms in EF are still not well understood and resolved. Moscoviz *et al.*, 2016 proposed three hypothetical mechanisms. Fig. 1-9 shows the hypothetical mechanisms that could be occurring the anode, however, the same mechanisms are expected in the cathode. The first one considers a direct conversion of the substrate to a product of interest. Here the electrodes act as an unlimited source or sink of electrons, depending on the working potential [18].

The second one considers a modification of the oxidation-reduction potential through (i) a partial dissipation of the electrons in excess (*i.e.* towards polarized electrode) produced by substrate fermentation or (ii) a small addition of extra electrons (*i.e.* from polarized electrode) to the fermentation medium. In both cases, a change in the $NADH/NAD^+$ ratio is promoted, contributing to metabolic regulation of many important cellular functions, including genetic expression and enzymatic synthesis [17], [127], [139]. This second hypothesis is probably responsible for why the electric current during EF is not always sufficient to explain the

change in metabolic patterns [142], [153], [154]. However, this small current densities that are passing through polarized electrodes is enoguh to observe a significative impact [18].

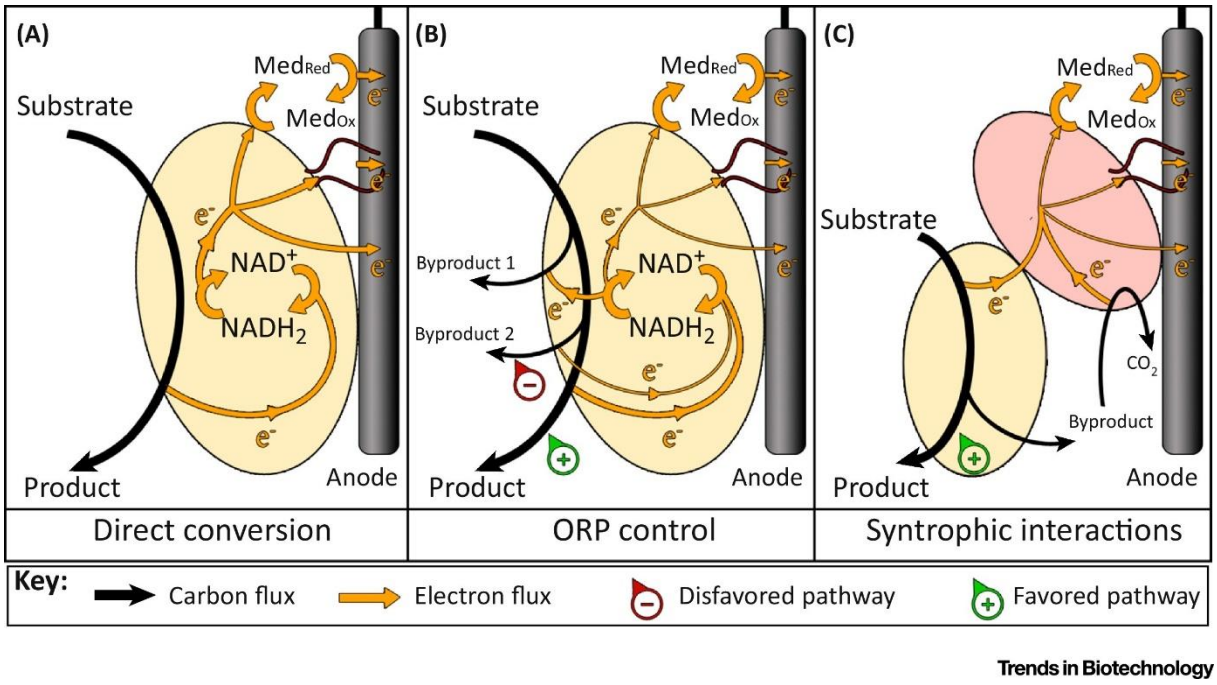


Fig. 1-9: Hypothetical mechanisms that could be occurring during anodic electro-fermentation

Mechanisms of cathodic EF can be obtained by reversing all the electron fluxes. (A) The substrate is directly converted into the product, and the electron excess is fully dissipated at the anode through mechanisms of extracellular electron transfer. (B) The electron excess generated during the formation of oxidized products 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 NADH/NAD$^+$ ratio, resulting in compensatory cellular regulation favoring pathways to regenerate NADH. (C) The fermentative bacteria (yellow) consumes the substrate but is not able to interact with the anode. The electroactive microorganism (red) acts as a mediator between the fermentative bacteria and the anode through mechanisms of interspecies electron transfer. The electroactive microorganism also consumes by-products from substrate fermentation, favouring the overall fermentation process. Abbreviations: Med$_{Ox}$, oxidized mediator; Med$_{Red}$, reduced mediator; ORP, oxidation–reduction potential. (Figure source: [18])

The third mechanism consists in a syntrophic interaction between fermentative (*e.g. Clostridia* species) and electroactive bacteria (*e.g. Geobacter* species) [18], [142], [143], [160]. This is considered as syntrophic because the fermentative partner provides a substrate to the electroactive bacteria that in return makes the fermentation thermodynamically more

favourable by removing the by-products [18]. Interactions between fermenters and electroactive bacteria rely on the mechanisms of interspecies electron transfer, either indirectly through the diffusion of electron shuttles such as H_2, formate, or other metabolites or directly through the use of conductive pili, membrane to membrane contacts, or via a conductive support onto which a biofilm can attach (See section 1.4.3) [18], [160].

In general, all the mechanisms proposed above (Fig. 1-9) may affect the selection of microbial communities when mixed cultures are used. Through the use of polarized electrodes, electroactive species can be favoured, leading to the formation of mixed biofilm with a different microbial community than the fermentation bulk [151], [152], [154]. Likewise, the microbial community selection in the fermentation bulk could be influenced by establishing cooperative partnerships with bacteria attached to the biofilm. Then, significant changes in metabolism patterns could be observed [18].

On the other hand, the polarized electrode could affect the microbial community not only by taking or releasing electrons from the fermentation medium [89]. Illustratively, the presence of a polarized electrode can lead to changes in the zeta potential of cell membranes. This could favour biofilm formation or make some bacteria more (or less) competitive [142], [161]. A study run with *Clostridium pasteurianum* showed that the presence of a polarized electrode of graphite felt caused large changes in cell structure and, more particularly, the formation of extracellular appendages. Polarized electrodes can also affect electrostatic interactions between the cells and the polarized electrode, making the cells less electronegative and thus facilitating the biofilm formation on a poised electrode (0.045V *vs* SHE) [142].

CHAPTER 2
Materials and methods

2.1 Synopsis

This chapter summarizes the experimental and analytical methodology used during this thesis. Information on the sampling procedures and storage is detailed. The different techniques to analyse liquid and gaseous samples, as well as molecular biology samples are presented. Statistical tools for data analysis are described, together with the calculations made to compare the experiments. Experimental design is detailed per chapter including the specific materials used, microbial inoculum and the modes of operation.

2.2 Biomass determination and chemical analysis

2.2.1 Liquid samples collection and biogas quantification

Liquid samples were collected from the reactor to evaluate the chemical composition. 2 ml of liquid were collected in a screw-top tube and were then centrifuged at 13400 rpm for 15 min. Supernatants were filtered with 0.2µm syringe filters and then stored in a clean safe-lock tube at 4 ºC until HPLC analysis. Additionally, pellets were stored at -20 ºC in the initial tubes for molecular biology analysis.

Biogas production was quantified by two different ways depending on the test. First, when used 1L reactor, the biogas was measured by liquid displacement using an inverted graduated cylinder. Second, when the tests were performed in 100 mL bottles, the biogas production was estimated by measuring the gas pressure in the bottle head space according Eq. 2-1. Where P_f and P_i are final and initial gas pressure. V correspond to volume of bottles head space. T is the measuring temperature.

Eq. 2-1

$$Biogas\ [mmol] = \frac{(P_f - P_i)[bar] \times V_{HeadSpace}[mL]}{T[K] \times 0.083[mL.bar.K^{-1}.mmol^{-1}]}$$

2.2.2 Volatile suspended solids and volatile solids

Volatile suspended solids (VSS) corresponds to the biomass concentration measured by dry weight technique. During this thesis, VSS were measured at the end of the tests at least in duplicates. First, a known volume was centrifuged at 7 830 rpm for 15 min. The supernatant was then discarded, and the pellet fraction was resuspended with a small amount of distilled water and placed into an aluminum dish. Samples were dried in an oven at 105 ºC until constant weight (about 8 h). Before weighing (A), the sample was placed in a desiccator to balance temperature. Then, samples were ignited to constant weight (about 3 h) in a muffle at 550 ºC. Before weighing (B), it was placed in desiccator to balance temperature. Finally, the biomass concentration is estimated according the Eq. 2-2 [162].

Eq. 2-2

$$VSS\ [g.L^{-1}] = \frac{(A-B)[g] \times 1000[mL.L^{-1}]}{Volume[mL]}$$

Besides, volatile solids (VS) determination was performed in some cases to characterize the inoculum. VS is performed following the same protocol described above for VSS. As a single exception, in VS the centrifugation is not necessary because VS consider all volatile mass present in the sample. When the sample has a low water content, VS could be a useful estimate of VSS.

2.2.3 High-performance liquid chromatography

Principle: High-performance liquid chromatography (HPLC) is an analytical technique in which a liquid mobile phase transports a sample through a column containing a solid stationary phase. The interaction of the sample with the stationary phase selectively retains individual compounds and permits the separation of the sample components. Detection of the

separated sample compounds is achieved using different detectors types including conductivity, electro-chemical, fluorescence, ultraviolet, refractive index and others [162].

Concentration of glucose, succinate, lactate, formate, acetate, propionate, ethanol, butyrate and 2.3-butanediol were measured by HPLC coupled to a refractive index (RI) detector (Waters R410). HPLC analysis were performed using an Aminex HPX-87H, 300 x 7.8 mm (Bio-Rad) column at 35 ºC. H_2SO_4 at 4mM was used as mobile phase. The firsts tests were carried out at a flow rate of 0.4 $mL.min^{-1}$, then the method was changed to 0.3 $mL.min^{-1}$ to shorten the analysis.

2.2.4 Gas chromatography

Principle: Gas chromatography (GC) uses a mobile phase (a carrier gas) and a stationary phase (column packing or capillary column coating) to separate individual compounds. The carrier gas is nitrogen, argon-methane, helium or hydrogen. For packed columns, the stationary phase is a liquid that has been coated on an inert granular solid, called the column packing that is held in borosilicate glass tubing. The column is installed in an oven with the inlet attached to a heated injector block and the outlet attached to a detector. Precise temperature of the injector block, oven, and detector is maintained constant [162].

Biogas composition was determined using a GC Clarus 580, Perkin Elmer equipped with a thermal conductivity detector (TCD) and argon as carrier gas at 3.5 bar. The columns used were RtMolsieve for CO_2 determination and RtQbond for H_2, O_2, N_2 and CH_4 determination.

2.3 Molecular biology techniques

2.3.1 DNA extraction

In the experiments described in Chapter 4, DNA was extracted with the QIAamp fast DNA stool mini kit in accordance with manufacturer's instructions (Qiagen, Hilden – Germany). For all other experiments, DNA was extracted with FastDNA^{TM} SPIN Kit in accordance with manufacturer's instructions (MP Biomedical; Santa Ana, California – USA). Extractions were confirmed, and DNA concentration was obtained using Infinite 200 PRO NanoQuant (Tecan Group Ltd., France).

2.3.2 MiSeq sequencing

The V3-V4 regions of the 16S rRNA genes were amplified using the primers W338F (CTTTCCCTACACGACGCTCTTCCGATCTACGGRAGGCAGCAG) and W339R (GGAGTTCAGACGTGTGCTCTTCCGATCTTACCAGGGTATCTAATCCT). The PCR mixtures (50µL) contained 0.5 U of Pfu Turbo DNA polymerase, Pfu Turbo buffer, 200 mM of each dNTP, 500nM of each primer and 10 ng of genomic DNA. Reactions were performed in a Mastercycler thermal cycler (Eppendorf, Hamburg, Germany) as follows: denaturing at 94 ºC for 2 min, 35 cycles of 94 ºC for 1 min, 65 ºC for 1 min and 72 ºC for 1 min, with a final elongation at 72 ºC 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 [163]. Sequences reported in Chapter 4 have been submitted to GenBank, under the accession numbers from KX632761 to KX632946.

2.3.3 Real-times polymerase chain reaction (qPCR)

PCRs were prepared using 96-well real-time PCR plates (Eppendorf, Hamburg, Germany) and 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 (5'-ACTCC TACGG GAGGC AG-3'), 250 nM reverse primer R805-785 (5'-GACTA CCAGG GTATC TAATC C-3'), 50 nM TaqMan probe, and water were added to obtain a final volume of 12.5 µL for all analyses.

An initial incubation of 20 min at 95 ºC and 40 cycles of denaturation (95 ºC, 7 s; 60 ºC, 25 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 concentrations were quantified using the Infinite 200 PRO NanoQuant (Tecan Group Ltd., France). The average number of bacterial cells were estimated by dividing the average number of 16S rRNA gene copies per cell by factor of 4.1 [164].

2.4 Calculations and statistical analysis

2.4.1 Electro-fermentation efficiency

The electro-fermentation (EF) efficiency coefficient, η_{EF}, was used to compare the EF with other bioelectrochemical systems. It corresponds to the ratio of electrons passing through for the electrical circuit on the number of electrons recovered in the product of interest, *i.e.* hydrogen (Eq. 2-3). For EF systems, this value should be between 0 and 1 showing that electricity production or consumption was not predominating H_2 production. A value higher than 1 means electricity production, direct electrosynthesis or electrolysis.

Eq. 2-3

$$\eta_{EF} = \frac{Q_{e-}}{Q_{H_2}} = \frac{\int Idt}{n_{H_2} \times N_{H_2} \times F}$$

Eq. 2-4

$$N\left(C_w N_x O_y H_z\right) = 4w - 3x - 2y + z$$

Q_{e-} is estimated from chronoamperometry by integrating the electric current over the operation time. Q_{H_2} is calculate from $n_{H_2} \times N_{H_2} \times F$. Where n_{H_2} is moles of H_2 produced, N_{H_2} is 2 mol H_2/ mol e^- (Eq. 2-4) and F is Faraday constant (96,485 C/mol e^-).

2.4.2 Estimation of the theoretical chemical oxygen demand

Mass balances were calculated after all experiments to verify whether most of the metabolic products were detected and quantified. In environmental area, mass balances based on the chemical oxygen demand (COD) is often used. In principle, the COD is defined as the amount of a specified oxidant that reacts with the samples under controlled conditions. The quantity of oxidant consumed is expressed in terms of oxygen equivalents. COD could be calculated by experimental methods (including open reflux, titrimetric and colorimetric methods), generally used when complex samples need to be analysed such as wastewaters and natural waters [162]. However, it is also possible to estimate the theoretical COD of each individual compound knowing the chemical composition of the sample (Eq. 2-5).

Eq. 2-5

$$C_n H_a O_b N_d + xO_2 \rightarrow yCO_2 + wH_2O + zNH_3$$

According to Eq. 2-5 , it is just necessary to balance this equation based on the empirical formula of each compound and then to calculate the ratio $gO_2.g^{-1}_{compound}$. Table 2-1 summarize the theoretical COD ratio for each compound measured in this thesis. As an

example, considering glucose the balanced equation is $C_6H_{12}O_6 + \mathbf{6}O_2 \rightarrow 6CO_2 + 6H_2O$. Then, the theoretical $COD_{glucose}$ is estimated as follows:

$$\frac{6mol_{O2}}{1mol_{Glucose}} \times \frac{32g_{O2}}{mol_{O2}} \times \frac{mol_{Glucose}}{180g_{Glucose}} = \mathbf{1.07}\ [\boldsymbol{g_{O2}/g_{Glucose}}]$$

Table 2-1: Theoretical COD ratio calculated for each compound measured in this thesis.

Compound	Empirical formula	Molar weight [g.mol^{-1}]	Theoretical COD [go$_2$.g$^{-1}_{compound}$]
Glucose	$C_6H_{12}O_6$	180.2	1.07
Dihydrogen	H_2	2.0	8.00
Formic Ac.	CH_2O_2	46.0	0.35
Succinic Ac.	$C_4H_6O_4$	118.1	0.95
Lactic Ac.	$C_3H_6O_3$	90.1	1.07
Ethanol	C_2H_6O	46.1	2.09
Acetic Ac.	$C_2H_4O_2$	60.1	1.07
Propionic Ac.	$C_3H_6O_2$	74.1	1.51
Butyric Ac.	$C_4H_8O_2$	88.1	1.82
2,3-Butanediol	$C_4H_{10}O_2$	90.1	1.95
Biomass	$C_5H_7O_2N$	113.1	1.41

COD Mass balance is performed by comparing the COD of each final fermentation products and COD of substrate consumed through multiplication of final concentration (obtained from HPLC or GC analysis) and theoretical COD ratio for each compound. Table 2-1 shows the theoretical COD ratio ($go_2.g_{compound}^{-1}$) calculated for each compound measured in this thesis. As an example, considering that 5 g.l^{-1} of initial glucose is totally consumed to produce acetate (1.5 g.l^{-1}), butyrate (1.2 g.l^{-1}) and ethanol (0.5 g.l^{-1}). COD is 5.35, 1.61. 2.18 and 1.05

$gO_2.l^{-1}$ for the glucose consumed, acetate, butyrate and ethanol, respectively. Then, by dividing each fermentation products by the glucose consumed and multiplying per 100, estimation of the mass balance is obtained. In this example the balance is 30.1%, 40.7% and 19.6% for acetate, butyrate and ethanol respectively.

2.4.3 Pearson correlations

Principle: The Pearson correlation index is used to calculate the linear correlation between quantitative variables no mattering the scale of measurement. Index value (r) ranges between -1 and 1, both included. When $0< r <1$ a positive correlation is observed, while $r =0$ means no correlation and $-1< r <0$ represents a negative correlation. Pearson correlation was calculated using PAST (PAleontological STatistics) version 3.20.

2.4.4 Analysis of Variance (ANOVA)

Principle: Analysis of variance (ANOVA) allows to compare the variability existing between several data groups with the average data variability of each group. A statistical value is used to accept or reject the null hypothesis. If null hypothesis is accepted, it is concluded in no difference between the data, while if it is rejected, that means that at least one group is different. ANOVA test was calculated using PAST (PAleontological STatistics) version 3.20.

2.4.5 Principal component analysis

Principle: Principal component analysis (PCA) is a statistical technique used to reduce the dimensionality of a data set. Independent factors are obtained, that are a linear combination of the original variables. PCA allow identifying patterns in data and expressing the data in such a way as to highlight their similarities and differences. PCA tests were calculated using PAST (PAleontological STatistics) version 3.20.

2.4.6 Mantel test and partial Mantel test

The Mantel test is a permutation test for correlation between two distance or similarity matrices derived from either multi or univariate data. The test compares the matrix in pairs under the null hypotheses: 'the distances among objects in the first matrix are not linearly correlated with the second matrix'.

The Mantel statistic, Z_M, is computed adding the cross products of the two similarity matrices being tested. As similarity matrices are symmetrical about their diagonals, half of each matrix (excluding values on the diagonal) is unfolded *i.e.* vectorized by stacking the values in each row below those of the previous the row, to compute their cross products. The r_M value is simply the Pearson's correlation coefficient between all the entries in the two matrices. It ranges from – 1.0 to + 1.0. The permutation test compares the original r_M to r_M computed in 9999 random permutations. Depending of *p-value* obtained the null hypothesis will be accepted or refused. In this thesis a *p-value* <0.05 was considered statically significant leading to refuse the null hypothesis, accepting the alternative hypothesis: 'the distances among objects in the first matrix are linearly correlated with the second matrix' [165]–[167].

Partial Mantel test allows to include the influence of a third data matrix. Only have sense to perform Partial-Mantel test when significative correlations were found between the two first data matrices tested. Thus, partial Mantel test is performed for the correlation of two first matrices, controlling for similarities given in the third data matrix [165], [168]. Mantel test and partial-Mantel test were calculated using PAST (PAleontological STatistics) version 3.20.

2.4.7 Non-metric multidimensional scaling

Non-metric multidimensional scaling (NMDS) is an indirect gradient analysis approach which produces an ordination based on a distance or similarity matrix. In this thesis were used matrix based on Bray-Curtis similarity index. Contrary to methods which attempt to maximize the variance as PCA, NMDS attempts to represent, as closely as possible, the pairwise similarity between objects in a low-dimensional space. NMDS is a rank-based approach meaning that the original distance data is substituted with ranks [169]. NMDS test was performed using PAST (PAleontological STatistics) version 3.20.

2.4.8 ANOSIM & SIMPER

ANOSIM (Analysis of similarities) is a non-parametric test of significant difference between two o more groups, based on any distance measure. This test has some similarity with ANOVA however, distances between groups with distances within groups are compared. For that, ANOSIM to evaluate a similarity matrix. Large positive R (up to 1) signifies dissimilarity between groups. The significance of R statistic is determined by permuting group membership.

SIMPER (Similarity percentage) is a simple method attempts assessing which average percent contribution of individual variable to the dissimilarity between objects in a Bray-Curtis similarity matrix. This method is useful to identify variables that are likely to be the major contributors to difference between groups detected by using *e.g.* ANOSIM [166], [170].

ANOSIM & SIMPER tests were performed using PAST (PAleontological STatistics) version 3.20.

Table 2-2: Summary of the statistical analyses performed during this thesis

Type of analysis	Objective of analysis	Chapter
Pearson correlation	Correlation between metabolite production and final microbial community	Chapter 3, 4, 5 & 6
Pearson correlation	Correlation between members of final microbial community	Chapter 6
ANOVA	Determine whether H_2 yields have statistically significant differences between the conditions studied	Chapter 4
PCA	Represent the relationships between final microbial communities and reactor performances	Chapter 4 & 5
Mantel test	Evaluate the correlation existing between the inoculum microbial community, the metabolites produced and the final microbial community	Chapter 5
Partial Mantel test	Know whether the inoculum microbial community is affecting the correlation between metabolites produced and the final microbial community	Chapter 5
NMDS	Represent the gradient of inoculum microbial community from different sources	Chapter 5
ANOSIM	Determine whether the differences between the inoculum microbial community are statistically significant	Chapter 5
SIMPER	Determine which families of inoculum microbial community were contributing in a major percentage to the differences between different inoculum sources.	Chapter 5

2.5 Electro-fermentation systems

2.5.1 Fermentation medium composition

During all EFs tests, only glucose was used as substrate along with others macro and micro nutrients as is detailed in Table 2-3. When dual-chamber reactors were used the fermentation medium and inoculum were added in the working electrode chamber, while in the counter electrode chamber only fermentation medium free of glucose was added.

Table 2-3: Composition of fermentation medium used during electro-fermentation tests during this thesis

Compound	Concentration ($g.l^{-1}$)	Solution A (Oligoelements)	Concentration ($g.l^{-1}$)
Glucose	5.0	HCl 37%	46.0 mL
NH_4Cl	2.0	$MgCl_2 \times 6H_2O$	55.0
K_2HPO_4	0.5	$FeSO_4(NH_4)_2SO_4 \times 6H_2O$	7.0
$FeCl_2 \times 4H_2O$	8.6 mg	$CoSO_4 \times 7H_2O$	1.3
Solution A	1 mL	$MnCl_2 \times 4H_2O$	1.2
MES (100 mM)	19.5	$ZnCl_2 \times 2H_2O$	1.0
		$Mo_7O_{24}(NH_4)_6 \times 4H_2O$	1.0
		$CuSO_4 \times 5H_2O$	0.4
		BO_3H_3	0.1
		$NiCl_2 \times 6H_2O$	0.05
		$Na_2SeO_3 \times 5H_2O$	0.01
		$CaCl_2 \times 2H_2O$	60.0

2.5.2 Electro-fermentation reactors set-up

All EFs tests were performed using dual-chamber reactors (Fig. 2-1A), except in 3 of 4 tests showed in Chapter 3 (See section 2.6.1, 2.6.2 and 2.6.3), that were performed using single chamber reactors (Fig. 2-1B). Conventional fermentation as control tests were performed also using single chamber reactors. When used dual-chamber reactor a membrane of cation exchange (FKE-50, FuMA-Tech GmbH, Germany) was placed between both chambers (Fig. 2-2D).

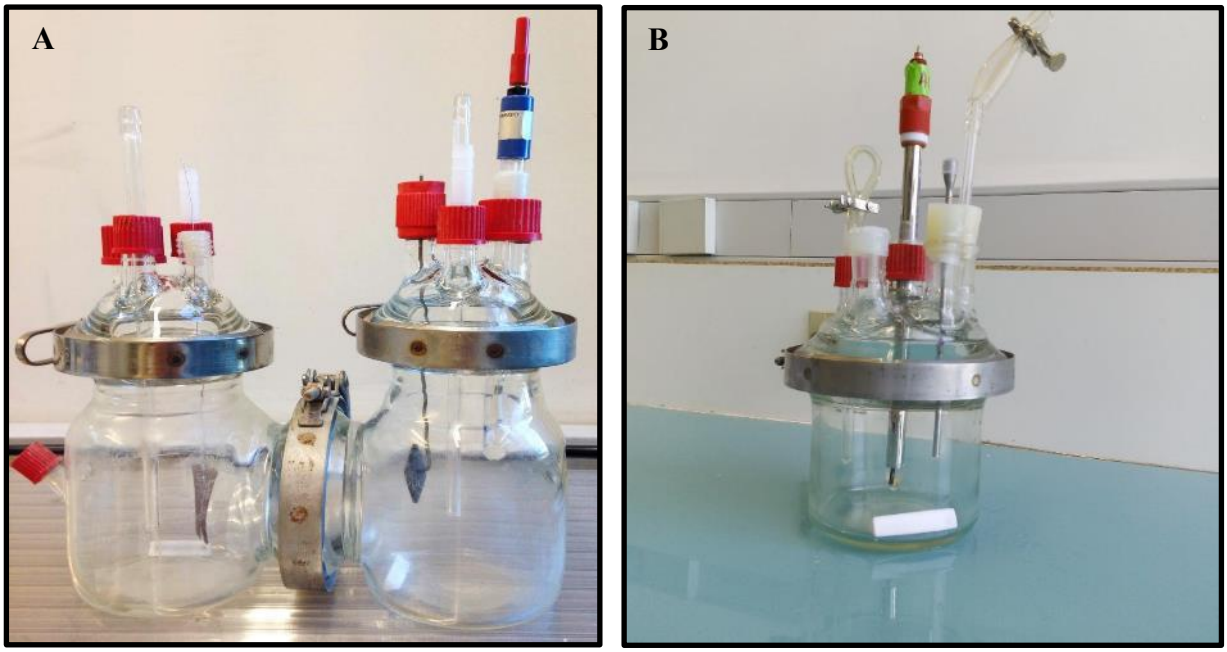


Fig. 2-1: Referential image showing reactors design used during this thesis

(a) *Dual-chamber reactor with 1L of volume util. (b) single chamber reactor with 1L of volume util.*

The EF systems were composed of three electrodes: working, counter and reference electrodes. These electrodes were connected to a VSP Potentiostat/Galvanostat interfaced to a VMP3B-80 Current Booster unit (BioLogic Science Instruments, France) to maintain a constant the applied potential at the working electrode. For all EFs tests as reference electrode was used calomel (SCE) (Fig. 2-2E). While, as counter electrodes were used grids

of 90% platinum – 10% iridium (Fig. 2-2C). Working electrode used in Chapter 3 were planar graphite plate (Fig. 2-2A), graphite felt (Fig. 2-2B) and grids of 90% platinum – 10% iridium (Fig. 2-2C), as is detailed in Section 2.6. While that in EF test performed in Chapter 4 and Chapter 5, the working electrodes were grids of 90% platinum – 10% iridium of 3.5 x 3.5 cm^2 (Fig. 2-2C). In the Chapter 6 as working electrode were used planar graphite plate (Fig. 2-2A) as is detailed in Section 2.9.2.

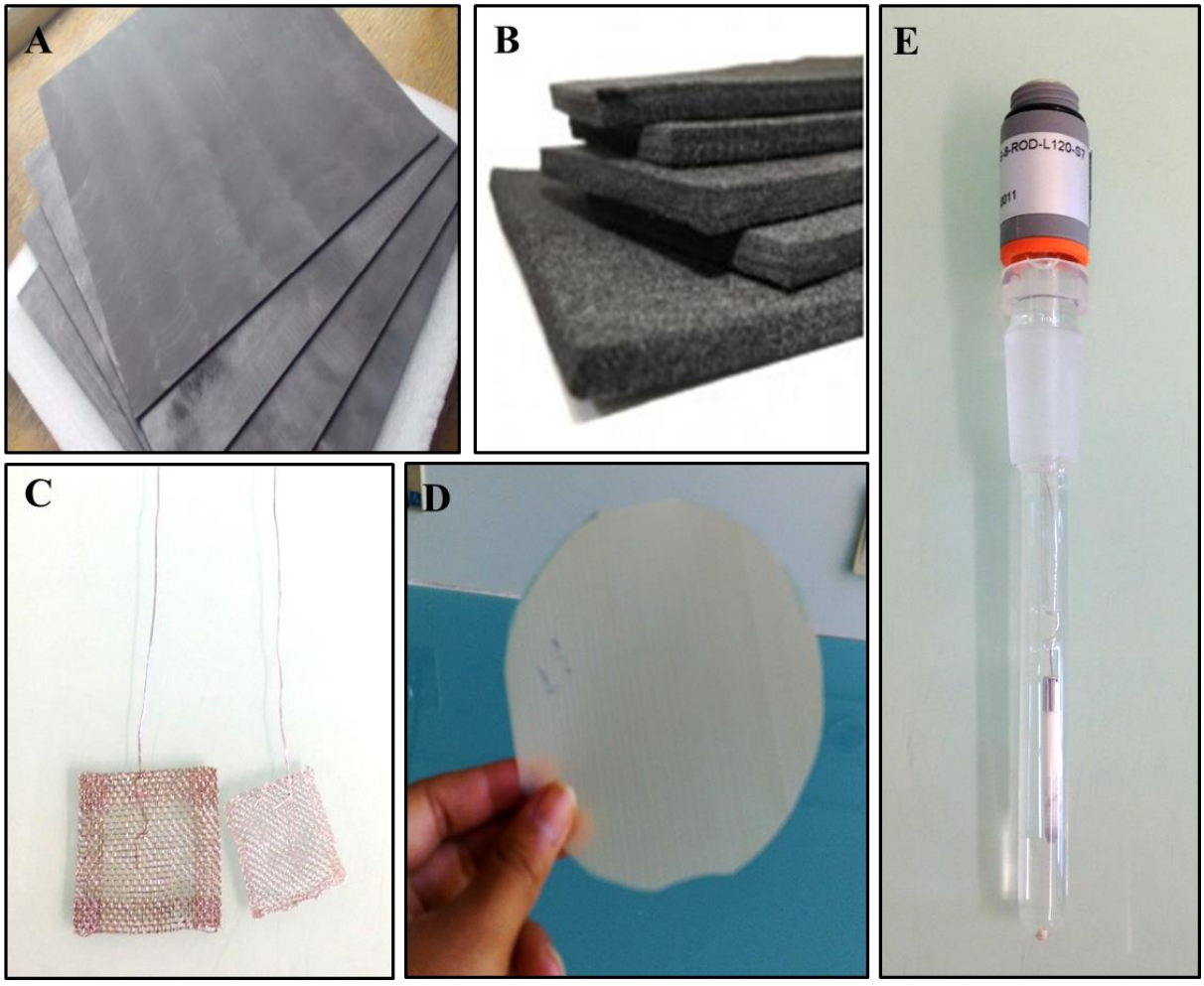


Fig. 2-2: Materials used to equip electro-fermentation reactors in this thesis

(a) *Planar graphite plate electrodes. (b) Graphite felt electrodes. (c) Grids of 90% platinum – 10% iridium with two sizes 2.5 cm x 2.5 cm and 3.5 cm x 3.5 cm. (d) Cation exchange membrane. (e) Reference electrode of saturated calomel (SCE).*

In all EF tests, the temperature was maintained at 37 °C using a water bath equipped with a thermocirculator. The stirring was set at 250 rpm using a magnetic stirrer and the initial pH was adjusted at 6.0 with 2 M NaOH during batch EF. When the operation was performed in continuous mode in Chapter 5 (Section 2.8.1), the pH was continuously adjusted at 6.0 with 2M NaOH using a peristaltic pump connected to a pH sensor.

The Fig. 2-3 shows a schematic diagram of experimental start-up for EF tests performed in dual-chamber reactors.

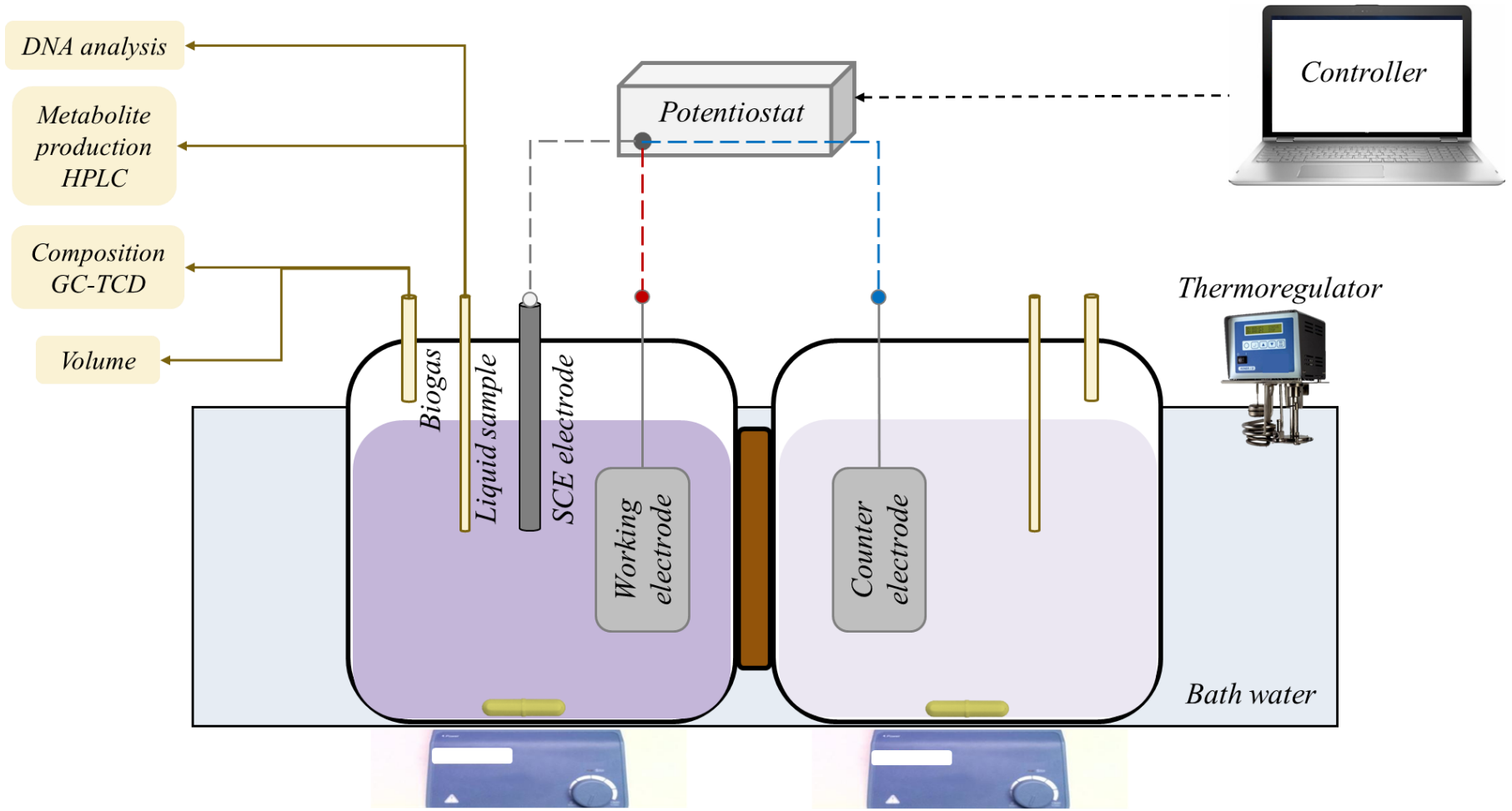


Fig. 2-3: Schematic diagram of experimental start-up for electro-fermentation tests performed in dual-chamber reactors

2.5.3 Chronoamperometry and cyclic voltammetry

Chronoamperometry (CA) is a controlled-potential technique and was used during all EF test performed during this thesis. With this technique a constant potential is applied in the working electrode for a fixed time duration and the current is measured. The current-time response reflects the change of the concentration gradient near the electrode surface. Particularly, in this thesis CA was used to stimulate the microbial metabolism by fixing the potential applied [171].

Cyclic Voltammetry (CV) is the most widely used technique to acquire quantitative information about electrochemical reactions. CV provides information on redox processes, heterogeneous electron transfer reactions and adsorption processes. It offers a rapid location of redox potentials of the electroactive species. The CV technique consists in scanning the potential of a stationary working electrode using a triangular potential waveform. During the potential sweep, the potentiostat measures the current resulting from electrochemical reactions occurring at the electrode interface and consecutive to the applied potential. CV was performed only in the initial EF tests in Chapter 3, especially to study the effect of different voltage applied on the working electrode in Section 2.6.3 [171]. CA and CV were controlled using EC-Lab® software [171].

2.6 Experimental design in Chapter 3

The inoculum used was anaerobic sludge sampled from a sewage treatment plant in Narbonne (3.5 $gVS.l^{-1}$). Tests were inoculated at a ratio of S/X = 10, where S is substrate ($g.l^{-1}$) and X is initial biomass ($gVS.l^{-1}$) calculated from Eq. 2-2. Glucose was used as substrate along with others macro and micro nutrients (Table 2-3).

To remove oxygen traces, the reactor medium and headspace were bubbled with N_2 gas (purity ≥ 99.9%) for 30 minutes before experiments, using a commercial aquarium air stone.

The Table 2-4 summarize the experiments show in the Chapter 3.

2.6.1 Study on 'the selection of the Electrode material'

Batch EF experiences were performed for 24 hours using a single chamber reactor with 0.5 L of fermentation volume (50% of total volume). Three different materials were used as working electrodes: Graphite felt with a size of 5.2 cm x 3.5 cm x 3.5 cm, planar graphite plate with a size of 2.5 cm x 2.5 cm and grids of 90% platinum – 10% iridium with a size of 2.5 cm x 2.5 cm. In all cases, grids of 90% platinum – 10% iridium were used as counter electrodes. The applied potential at working electrode was –0.9V *vs* SCE. Control tests were also performed using a single-chamber reactor, in absence of polarized electrodes.

All the experiments were carried out in duplicates

2.6.2 Study on the 'effect of the Platinum electrode on metabolic patterns'

Batch EF experiences were performed for 24 hours using a single chamber reactor with 0.5 L of fermentation volume (50% of total volume). Grids of 90% platinum – 10% iridium with a size of 2.5 cm x 2.5 cm were used as working and counter electrodes. The applied potential at working electrode was –0.9V *vs* SCE.

Two control tests were performed. On the first one the electrodes were placed on fermentation medium but not connected to circuit electric, *i.e.* not polarized electrodes. This is to evaluate whether the electrode material has any effect on metabolism patterns. On the second control polarized electrodes were placed in the fermentation medium free of inoculum

i.e. abiotic control. This to evaluate if is possible the chemical H_2 production by water hydrolysis under conditions used. All experiments were carried out in duplicates.

2.6.3 Study on 'the effect of different potential applied on the working electrode'

Batch EF experiences were performed for 24 hours using a single chamber reactor with 0.5 L of working volume (50% of total volume). Grids of 90% platinum – 10% iridium with a size of 2.5 cm x 2.5 cm were used as working and counter electrodes. Four potential values were applied at the working electrode: –0.9, –0.7, –0.5 and –0.3 V *vs* SCE. Control tests were also performed using a single-chamber reactor, in absence of polarized electrodes. All the experiments were carried out in duplicates.

Table 2-4: Summary of electro-fermentation tests show in the Chapter 3

Study	Material of working electrode	Potential applied (V *vs* SCE)	Reactor type
Selection of the electrode material	i. Graphite felt (5.2x3.5x3.5 cm^3) ii. Planar graphite plate (2.5x2.5 cm^2) iii. Grids of 90% platinum–10% iridium (2.5x2.5 cm^2)	– 0.9	Single chamber
Effect of the platinum electrode on metabolic patterns	Grids of 90% platinum–10% iridium (2.5x2.5 cm^2)	– 0.9	Single chamber
Effect of different potential applied on the working electrode	Grids of 90% platinum–10% iridium (2.5x2.5 cm^2)	–0.9, –0.7, –0.5 & –0.3	Single chamber
Effect of the electrode size	Grids of 90% platinum–10% iridium of two sizes: i. 2.5x2.5 cm^2 ii. 3.5x3.5 cm^2	– 0.9	Dual chamber

2.6.4 Study on the effect of the electrode size

Batch EF experiences were performed for 24 hours using a dual-chamber reactor with 0.5 L of working volume (50% of total volume) in each chamber. Two grids of 90% platinum – 10% iridium were used: 2.5 cm x 2.5 cm and 3.5 cm x 3.5 cm. The applied potential at working electrode was –0.9V *vs* SCE. Control test were also performed in absence of polarized electrodes using a single-chamber reactor. The experiments were carried out in duplicates.

2.7 Experimental design in Chapter 4

The inoculum used in this chapter corresponded to an anaerobic sludge sampled from a lab-scale anaerobic digester treating sewage sludge (37.7 $gVS.l^{-1}$). The sludge was heat-treated at 90°C for 30 minutes using a water bath before inoculation at ratio S/X = 10, where S is substrate ($g.l^{-1}$) and X is initial biomass ($gVS.l^{-1}$) calculated from Eq. 2-2. Glucose was used as substrate along with others macro and micro nutrients (Table 2-3).

Batch EF experiences were performed for 20 hours using a dual-chamber reactor with 0.5 L of working volume and 0.5 L of headspace in each cell. The values of applied potentials at working electrode were –0.9, –0.4, +0.4 and +0.9 V *vs* SCE. To remove oxygen traces, the reactor medium and headspace were bubbled with N_2 gas (purity ≥ 99.9%) for 30 minutes before experiments, using a commercial aquarium air stone. Batch control experiments were also performed using a single-chamber reactor, in absence of polarized electrodes under similar operating conditions. The experiments were carried out in duplicates (–0.9V and –0.4V), triplicates (+0.4V and +0.9V) and quintuplicates (controls).

2.8 Experimental design in Chapter 5

2.8.1 Electro-fermentation in continuous operation mode

The inoculum used was an acidogenic sludge sampled from H_2-producing reactor fed with molasses (9.9±0.3 $gVS.l^{-1}$). Fresh sludge was added corresponding to 14.3% of fermentation volume (100 mL). Glucose was used as substrate along with others macro and micro nutrients (Table 2-3).

EF test in continuous operation mode was performed for 40 days (143.3 HRTs) in dual-chamber reactors with 0.7 L of fermentation volume and 0.3 L of headspace in each cell. The applied potential at working electrode was –0.4V *vs* SCE. Before starting continuous operation, a batch was performed for 24 hours to grow biomass and for its acclimatization. The input/output of working electrode chamber were continuously supplied/retired by peristaltic pump. The pump flows were adjusted to maintain the HRT at 6.7 hours.

Control tests were also performed using a single-chamber reactor, in absence of polarized electrodes under similar operating conditions.

2.8.2 Electro-fermentation in batch operation mode: Comparison of different inoculum source

Four types of inoculum were used to compare the batch EF: (i) acidogenic sludge from H_2-producer reactor fed with glucose (1.5 $gVS.l^{-1}$); (ii) anaerobic sludge from a lab-scale anaerobic digester treating food waste (7.1 $gVS.l^{-1}$). The sludge was heat-treated at 90°C for 30 minutes using water bath before inoculation; (iii) anaerobic sludge from a lab-scale anaerobic digester treating volatile fatty acids (9.5 $gVS.l^{-1}$) and (iv) activate sludge from sewage treatment plant at Narbonne (10.0 $gVS.l^{-1}$). Glucose was used as substrate along with others macro and micro nutrients (Table 2-3).

Batch EF tests were performed for 24 hours using a dual-chamber reactor with 0.9 L of working volume. The applied potential at working electrode was –0.4V *vs* SCE. Batch control tests were also performed using a single-chamber reactor, in absence of polarized electrodes and operated under similar conditions.

2.9 Experimental design in Chapter 6

2.9.1 Microbial interaction between different mixed cultures and electroactive bacteria

2.9.1.1 Inoculum and fermentation medium

Two different sludge were used as inoculum: (i) acidogenic sludge from H_2-producer reactor fed with molasses (9.9±0.3 $gVS.l^{-1}$). The sludge was stored in a freezer at –20°C and defrost at room temperature and (ii) activate sludge from sewage treatment plant at Narbonne (10.0 $gVS.l^{-1}$). In all cases, the sludge was enriched with *Geobacter sulfurreducens* DSMZ 12127, which was precultured as shown in Section 2.9.1.2. Fermentation medium was composed for 5 $g.l^{-1}$ glucose and 1.65 $g.l^{-1}$ acetate as carbon sources along with others macro and micro nutrients (Table 2-3).

2.9.1.2 Electroactive bacteria growth: *G. sulfurreducens* DSMZ 12127

Geobacter sulfurreducens DSMZ 12127 was cultivated in 100 mL bottles with 50 mL of culture medium. This was composed as follow: 0.82 $g.l^{-1}$ Na – acetate, 1.5 $g.l^{-1}$ NH_4Cl, 0.1 $g.l^{-1}$ KCl, 0.6 $g.l^{-1}$ Na_2HPO_4, 10 $mL.l^{-1}$ trace element solution, 10 $mL.l^{-1}$ vitamin solution (Table 2-5), 8.0 $g.l^{-1}$ Na_2 – fumarate and 2.5 $g.l^{-1}$ $NaHCO_3$. 1 L of culture medium was prepared without vitamin solution and Na_2 – fumarate. 1 $mg.l^{-1}$ of resazurin was added as redox indicator. pH was adjusted at 6.8 and then 50 mL were distributed in each 100 mL bottles. The bottles were closed with butyl rubber stoppers and aluminium crimp caps.

Anaerobic conditions were established bubbling N_2 gas (purity ≥ 99.9%) for at least 30 min. Then bottles were autoclaved at 121°C for 20 min. Before inoculation vitamin solution, Na_2 – fumarate and 0.5 $g.l^{-1}$ L-cysteine were added using a sterile 0.2µm filter. Inoculated bottles were cultivated in a room acclimatized at 35°C under agitation.

Table 2-5: Composition of trace element solution and vitamin solution

Trace element solution	Concentration ($g.l^{-1}$)	Vitamin solution	Concentration ($mg.l^{-1}$)
Nitrilotriacetic acid	1.50	Biotin	2.0
$\mathbf{MgSO_4 \times 7H_2O}$	3.00	Folic acid	2.0
$\mathbf{MnSO_4 \times H_2O}$	0.50	Pyridoxine – HCl	10.0
NaCl	1.00	Thiamine – HCL $\times 2H_2O$	5.0
$\mathbf{FeSO_4 \times 7H_2O}$	0.10	Riboflavin	5.0
$\mathbf{CoSO_4 \times 7H_2O}$	0.18	Nicotinic acid	5.0
$\mathbf{CaCl_2 \times 2H_2O}$	0.10	D – Ca – pantothenate	5.0
$\mathbf{ZnSO_4 \times 7H_2O}$	0.18	Vitamin B_{12}	0.10
$\mathbf{CuSO_4 \times 5H_2O}$	0.01	p – Aminobenzoic acid	5.0
$\mathbf{KAI(SO_4)_2 \times 12H_2O}$	0.02	Lipoic acid	5.0
$\mathbf{H_3BO_3}$	0.01		
$\mathbf{Na_2MoO_4 \times 2H_2O}$	0.01		
$\mathbf{NiCl_2 \times 6H_2O}$	0.03		
$\mathbf{Na_2SeO_3 \times 5H_2O}$	0.30 mg		
$\mathbf{Na_2WO_4 \times 2H_2O}$	0.40 mg		

2.9.1.3 Fermentation bottles and start-up

Batch fermentation experiences were performed using 100 mL bottles with 50 mL of working volume. The experiments were carried out until glucose was totally consumed between 24 and 72 hours in a room acclimatized at 35°C under agitation. Initial pH was adjusted at 6.0 with 2 M NaOH. To remove oxygen traces, bottles were bubbled with N_2 gas (purity ≥ 99.9%) for 30 min before experiments, using a commercial aquarium air stone. Each bottle was inoculated with 2.5 mL of sludge and 2.5 of *G. sulfurreducens* DSMZ 12127. Control experiences were performed by inoculating only 5.0 mL of sludge and were operated under similar conditions.

The experiments were carried out in triplicates and quadruplicates when used activate sludge from sewage treatment plant at Narbonne and acidogenic sludge from H_2-producer reactor fed with molasses, respectively.

2.9.2 Mixed culture electro-fermentation using electrodes colonized by *Geobacter sulfurreducens*

2.9.2.1 Inoculum and fermentation medium

The inoculum used was acidogenic sludge from a H_2-producing reactor fed with molasses (9.9±0.3 $gVS.l^{-1}$). The sludge was stored in a freezer at –20°C and defrost at room temperature. Glucose was used as substrate along with others macro and micro nutrients (Table 2-3)

2.9.2.2 Pre-colonization of the electrode with *G. sulfurreducens* DSMZ 12127

Geobacter sulfurreducens DSMZ 12127 pre-cultivated as showed in the Section 2.9.1.2, was used to pre-colonize electrode in a single-chamber reactor with 0.8 L of medium. The fermentation medium was composed as follows: 1.65 $g.l^{-1}$ Na – acetate, 0.63 $g.l^{-1}$ NH_4Cl,

0.26 $g.l^{-1}$ KCl, 50 mM MES Buffer (9.75 $g.l^{-1}$), 20 $mL.l^{-1}$ trace element solution and 20 $mL.l^{-1}$ vitamin solution (Table 2-5). The temperature was maintained at 37 °C using a water bath equipped with a thermocirculator. The stirring was set at 250 rpm using a magnetic stirrer and initial pH was adjusted at 6.8 with 2 M NaOH. Anaerobic conditions were established bubbling N_2 gas (purity ≥ 99.9%) after inoculation for 30 min.

Planar graphite plate with a size of 5.0 cm x 5.0 cm x 0.25 cm and grids of 90% platinum – 10% iridium with a size of 3.5 cm x 3.5 cm were used as working and counter electrode, respectively. The applied potential at working electrode was +0.2V *vs* SCE. The working electrode, counter electrode and calomel reference electrode (SCE) were connected to a VSP Potentiostat/Galvanostat interfaced to a VMP3B-80 Current Booster unit (BioLogic Science Instruments, France) to maintain a constant the applied potential at the working electrode. The experiment was stopped when no more electric current was produced. The Fig. 2-4 show a typical curve of current production during electrode colonization.

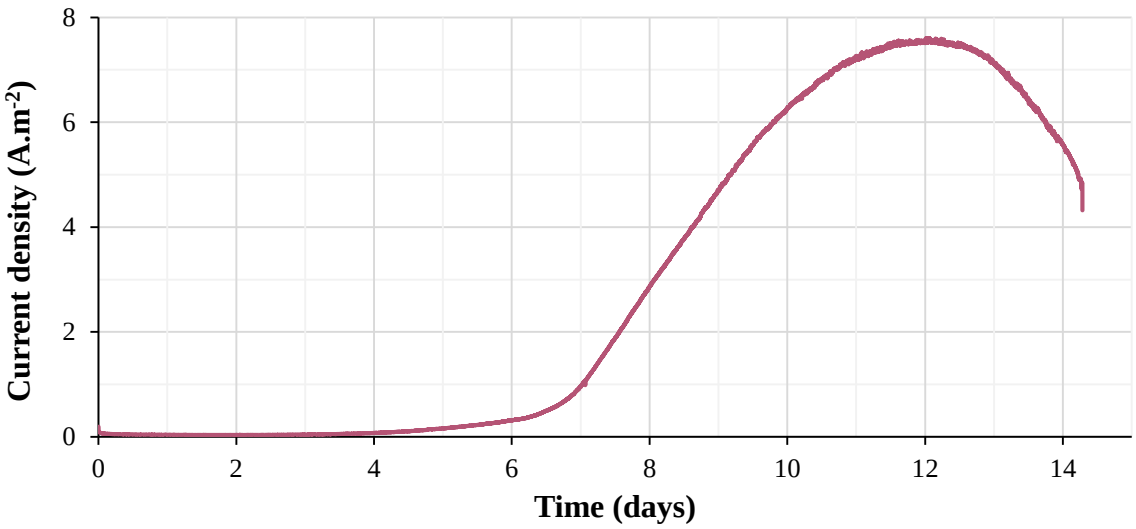


Fig. 2-4: Typical curve of current production during electrode colonization by *G. sulfurreducens*

2.9.2.3 Electro-fermentation reactors and start-up

Batch EF experiences were performed for 18 hours using a dual-chamber reactor with 0.9 L of working volume and 0.1 L of headspace in each chamber. Precolonized electrode with *G. sulfurreducens* (Section 2.9.2.2) and 90% platinum – 10% iridium grids with a size of 3.5 cm x 3.5 cm were used as working and counter electrodes, respectively. The values of applied potentials at working electrode were –0.4 V *vs* SCE. Batch control experiments were also performed using a single-chamber reactor, in absence of polarized electrodes under similar operating conditions.

The EF experiments were carried out in two batch series keeping only the precolonized electrode. While controls were performed in duplicates.

CHAPTER 3
Starting with electro-fermentation and determination of parameters

3.1 Introduction

EF is not only a new operating configuration for BES, but it also introduces a new type of control for anaerobic bioprocesses. As EF is a recent concept, only few works are documented and there is not enough knowledge about the optimal operating conditions and which are the most suitable materials for performing efficient EF. To date EF is carried out under the same laws that dominate in traditional BES. However, it is well admitted and obvious that it is not the same to work with a MEC or an MFC, although both configurations are BES, each configuration having its own particularities, as well as EF.

The literature evidences that electrode material and size, as well as the potential applied at the working electrode, can alter the current production and generate different interactions with the microbial community. Besides, reactor configuration (*i.e.* single or dual-chamber reactors) is important when considering the efficiency of electrochemical systems. Then, this first chapter aims to determine the main parameters affecting EF. The specific objectives are as follows:

i. Determine how the electrode material can affect H_2 production during EF.
ii. Study different potential applied at the working electrode using a single – chamber reactor.
iii. Determine how the electrode size can affect H_2 production during EF.

3.2 Selection of electrode material

This section aims to select the better electrode material according to H_2 production for the next experiments. For that, three types of electrode material were tested as working electrode in single chamber reactors: graphite felt (**Felt – Pt**), planar graphite plate (**Graf – Pt**) and grids of 90% platinum – 10% iridium (**Pt – Pt**). In all cases, grids of 90% platinum – 10%

iridium were used as counter electrodes. The applied potential at working electrode was –0.9V *vs* SCE. Besides, conventional fermentation as control was performed without electrodes in the fermentation medium.

After 24 hours of batch operation, the glucose was totally consumed in all conditions (3.8±0.6 $gCOD.l^{-1}$). Total COD mass balance measured as soluble and H_2 gas, was between 74.2 and 88.6%. It is assumed that 10-15% of the missing COD was corresponding to biomass growth. The rest COD could correspond to unknown metabolites.

Fig. 3-1 shows the H_2 yield obtained for each electrode. A significant increase of the H_2 yield by a factor of 1.36 was observed using Pt – Pt (1.76±0.08 $mol_{H2}.mol^{-1}_{glucose}$), when compared to control (1.29±0.05 $mol_{H2}.mol^{-1}_{glucose}$). Using Graf – Pt (1.26±0.02 $mol_{H2}.mol^{-1}_{glucose}$), the H_2 yield was almost same than in the control, while a lower H_2 yield was obtained using Felt – Pt (0.98±0.003 $mol_{H2}.mol^{-1}_{glucose}$). The metabolites were measured but no important difference among the conditions was observed.

The electrons transferred as electric charge in Coulombs (C) from the polarized electrode to the fermentation medium during batch operation time were of –424.7±27.8 C, –69.2±96.0 C and –115.2±43.3 C for Pt – Pt, Graf – Pt and Felt – Pt, respectively. While, EF efficiency (ηEF) (Section 2.4.1 – Materials and methods) respect to H_2 production were of 0.12±0.01, 0.04±0.06 and 0.06±0.02, respectively. This means that as maximum only 12% of the H_2 produced came from the electrons supplied by the electrodes. Particularly, in the case of Pt – Pt, the electrons supply was more stable between the replicates, as shown by the low standard deviation value observed in the average of electrical charge. Contrary, using Graf – Pt and Felt – Pt, large differences between the duplicates were observed, evidencing an unstable supply of electrons under the operational conditions used here.

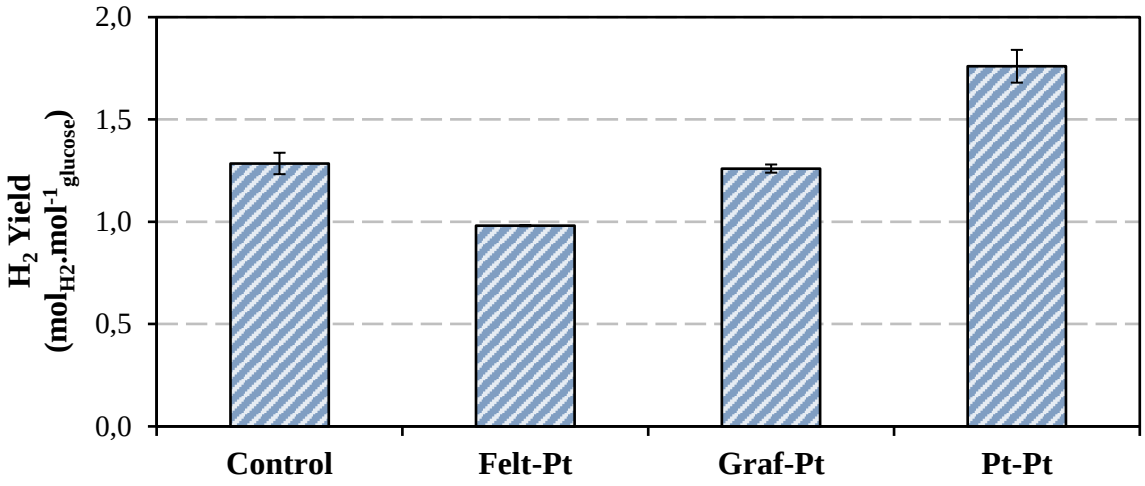


Fig. 3-1: Hydrogen yield observed during Electro-fermentation tests using single chamber reactors and different electrode materials

Control: *without electrode on fermentation medium;* ***Felt:*** *graphite felt;* ***Graf:*** *planar graphite plate;* ***Pt:*** *grids of 90% platinum – 10% iridium. As convention, first is mentioned the working electrode and then the counter electrode. Error bars represent the standard deviation calculated from replicates.*

The critical parameters governing any bioelectrochemical system include the composition of the electrolyte (in this thesis, it is the same as the fermentation medium) [172], pH [173], electrode materials [174], applied potential [175] and composition of the microbial community [176]. Except the composition of the microbial community, these parameters determine which abiotic reactions (*i.e.* not catalysed by microorganism or biological enzymes) could take place on electrode surface, consequently affecting microbial community behaviour [139], [177]. Particularly, electrodes based on metallic materials as platinum are highly reactive due to a good electrical conductivity, thus transporting electrons more rapidly than others with low conductivity capacity [139], [177], [178]. Consistently, ours results evidence a major electric charge transferred when Pt – Pt was used compared to carbon-based electrodes tested.

Besides, H_2 can likely be produced from water electrolysis, when highly negative potential are applied, *i.e.* –0.9 V *vs* SCE, especially when metallic-based electrodes are used [139],

[178]. This could explain the increase of the H_2 yield when Pt – Pt electrodes were used. However, an abiotic control (*i.e.* EF tests without inoculum) was not performed to corroborate or discard this hypothesis. This point will be discussed in more detail in the following section.

As a conclusion, it was decided to use Pt – Pt electrodes for the next experiments because higher H_2 yields were obtained and the electron supply was more stable compared with the other materials tested. Besides, interestingly Pt electrodes do not facilitate biofilm formation due to the smooth surface [179]. This property can be interesting to study the interaction between polarized electrodes and microbial community without biofilm attached.

3.3 Effect of platinum electrode on fermentation patterns

This section seeks to deepen the results observed in previous section. For that, EF experiences were carried out under similar conditions with grids made of 90% platinum – 10% iridium as working and counter electrodes (Pt – Pt). The applied potential at working electrode was –0.9V *vs* SCE. Besides, two control tests were performed: (i) in the first one the electrodes were placed on fermentation medium but not connected to circuit electric, *i.e.* not polarized electrodes. This is to evaluate whether the electrode material has any effect on metabolism patterns; and (ii) in the second control polarized electrodes were placed in the fermentation medium free of inoculum *i.e.* abiotic control. This to evaluate if is possible the chemical H_2 production by water hydrolysis under conditions used. All experiments were carried out in duplicates.

After 24 hours of batch operation, the glucose was totally consumed in all reactors (4.5±0.7 $gCOD.l^{-1}$), except in the abiotic control. Total COD mass balance measured as soluble and H_2

gas, was 82.5±1.1%. It is assumed that the 10-15% of the missing COD was corresponding to biomass growth.

3.3.1 Hydrogen production and metabolite distribution

During EF, the H_2 yield was 70% higher (0.97±0.09 $mol_{H2}.mol^{-1}_{glucose}$) than in conventional fermentation or control (0.57±0.09 $mol_{H2}.mol^{-1}_{glucose}$, Fig. 3-2). Besides, not H_2 production was observed in the abiotic control (*i.e.* EF tests without inoculum). Despite, literature evidence of electrochemical H_2 production at potential applied of –0.9 V *vs* SCE, our results corroborate that electrolyte or fermentation medium in our case (because single chamber reactors are used), are affecting the abiotic reactions occurring in the electrode surface [139], [178], [180]. Thus, even by applying a so low potential, the chemical H_2 production by water electrolysis is not assured. Consequently, the H_2 produced is the result of biological microbial community activity.

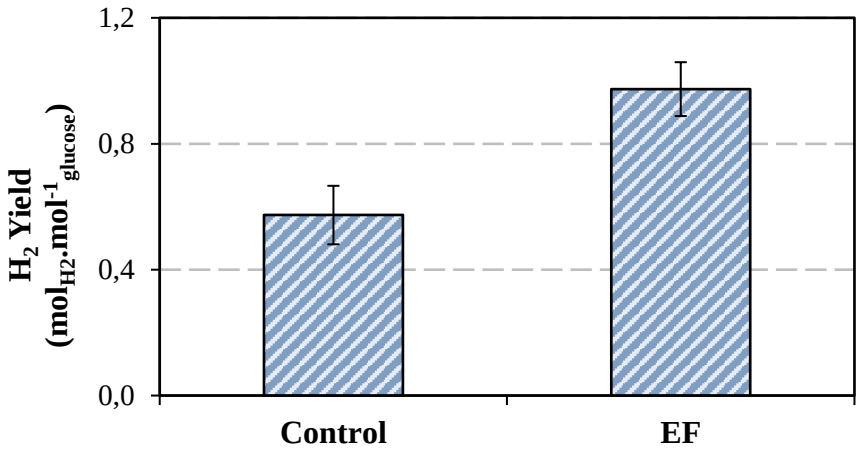


Fig. 3-2: Hydrogen yield observed in fermentation control and electro-fermentation tests using single chamber reactors and Pt-Pt electrodes.

Fig. 3-3 shows the metabolites distribution according to the COD mass balance. In the control, the main metabolite was lactate that represented 32.5±6.0%$_{COD}$. Ethanol, acetate and butyrate were also observed, representing 20.6±6.0%$_{COD}$, 11.8±2.7%$_{COD}$ and 8.6±5.4%$_{COD}$,

respectively. Less amounts of propionate and succinate accumulated, representing only 3.0 ±2.7%$_{COD}$ and 2.1 ±0.6%$_{COD}$, respectively.

Interestingly, during EF significant changes were observed on lactate and butyrate pathways. Lactate decreased down to 3.4±0.7%$_{COD}$ and butyrate increased up to 34.4±8.2%$_{COD}$. Such shifts of the metabolic pattern can explain the higher H_2 production in EF. This is consistent with literature since lower H_2 yields are related to lactate accumulation [37], [181], while higher H_2 production is often associated with butyrate [15], [34]. Other metabolites were not affected by polarized electrodes. Thus, ethanol, acetate and succinate also accumulated in EF, ranging 20.6±1.0%$_{COD}$, 13.1±4.6%$_{COD}$ and 2.5±0.2%$_{COD}$, respectively.

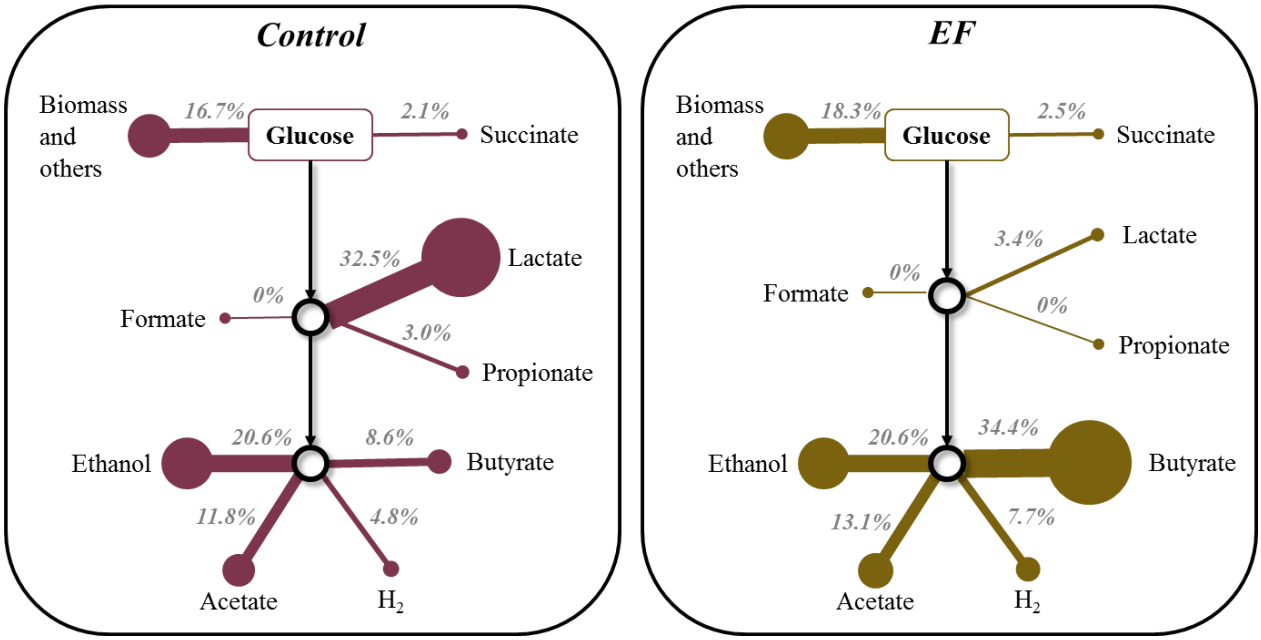


Fig. 3-3: Metabolite distribution observed during electro-fermentation tests using single chamber reactors and Pt-Pt electrodes

Since electrodes were not polarized (*i.e.* not connected to circuit electric) in the control tests, it can be assumed that changes on metabolic pathways as observed in EF were due to polarization of electrodes and not only to the presence of the Pt conductive material. Only a small quantity of electrons transferred from the polarized electrode to the fermentation

medium during the first 24 hours of batch operation (– 322.5±229.8 C). That was confirmed by a low EF efficiency of η_{EF} = 0.18±0.12, with respect to H_2 production. This value means that a maximum of 18% of the H_2 produced came from the electrons supplied by the electrodes [18]. Besides, a global coefficient of η_{EF} = 0.010±0.007, respect to all fermentation product was obtained. This value means that only a maximum of 1.0% of all fermentation product are affected for the electrons supplied by the electrodes, evidencing that only few extra electrons can substantially impact the metabolic patterns by dark fermentation.

3.3.2 Microbial community analysis and link with metabolic patterns

Microbial community analyses were performed with MiSeq sequencing. The samples were taken from the inoculum and at the end of the batch operation in the control and electro-fermentation experiments. The main phyla found in the inoculum (Fig. 3-4) were *Proteobacteria, Bacteroidetes* and *Actinobacteria,* representing 56.3±1.7%, 26.0±0.8% and 4.0±0.8% of the total abundance, respectively. No OTU reached a relative abundance higher than 4.0%, that is consistent with a high diversity Simpson index of 0.992±0.001. In control tests, the main phyla were *Proteobacteria* and *Firmicutes* representing 62.4±6.5% and 33.9±6.0 of the abundance, respectively. Meanwhile, in EF tests, the main phyla were *Proteobacteria, Firmicutes* and *Bacteroidetes* representing 59.4±5.8%, 18.8±8.1% and 14.0±2.1, respectively. In both cases the Simpson diversity indexes decreased to 0.855±0.028 and 0.840±0.016 for Control and EF, respectively. As is expected, the microbial community after a fermentation process is less diverse compared with the inoculum. This because the operating conditions applied allow to this selecting of species.

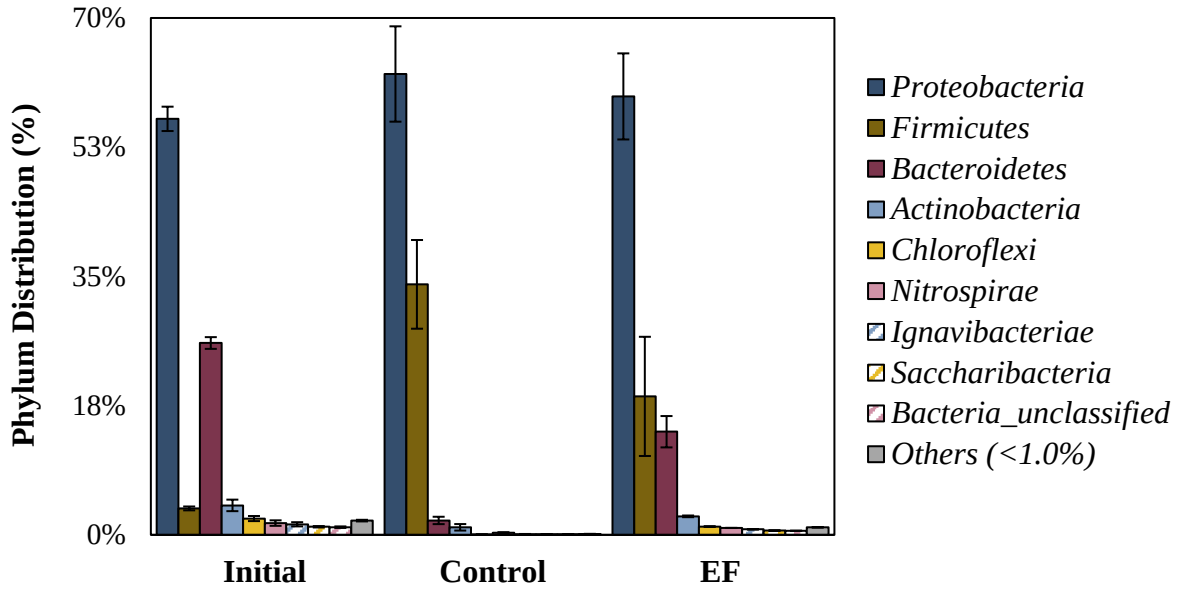


Fig. 3-4: Phylum distribution of microbial community observed in inoculum and at the end of both control and electro-fermentation tests

In the inoculum, *Saprospiraceae* and *Rhodocyclaceae* were the dominant representing 14.8±0.5% and 11.6±3.0%, respectively. About 70% of the microbial community are represented for families with a relative abundance <6.0%. Fig. 3-5 shows further analysis on the family distribution of the final microbial communities of the control and EF operation. No significant difference was observed between the control and EF, with respect to the most abundant families constituting the microbial communities. The dominant families included *Enterobacteriaceae*, *Clostridiaceae*, *Streptococcaceae* and *Aeromonadaceae* that represented 52.9±6.2% / 51.5±3.3%, 11.6±4.1% / 14.4±9.2%, 17.1±3.0% / 19.5±1.4% and 3.9±1.4% / 6.9±2.2% in Control / EF, respectively. Only small differences between the control and EF within the low abundant families were observed. In particular, the control test had more families lower than 1.0% than the EF test. Probably, theses sub-dominants families generate a different support for dark fermentation in each case, changing the microbial interactions and consequently the metabolic patterns. Consistently, the key role of sub-dominant species was

previously reported during H_2 production by dark fermentation [59] but also during 1.3 – propanediol production in mixed cultures fermentation [182].

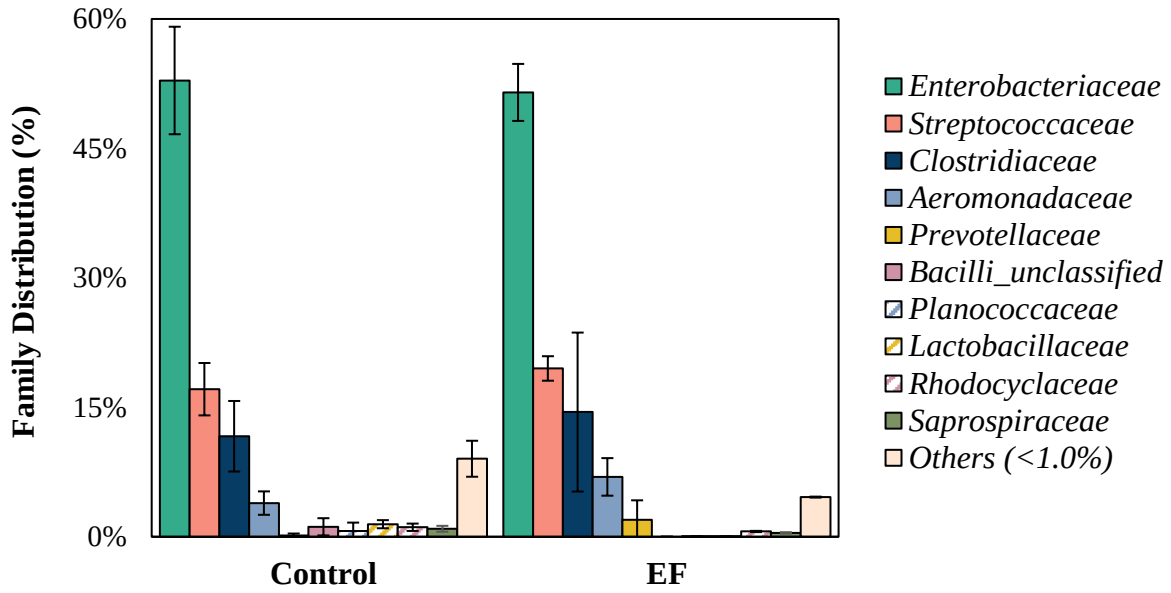


Fig. 3-5: Family distribution of microbial community observed during control and electro-fermentation tests using single chamber reactors and Pt-Pt electrodes

In addition, Table 3-1 shows the Pearson correlation matrix that was performed to evaluate the influence of microbial family abundance on metabolic patterns. Lactate production negatively correlated with H_2 production (R^2 = –0.93, p < 0.05) and positively with the following families: *Lactobacillaceae* (R^2 = 0.95, p < 0.05), *Saprospiraceae* (R^2 = 0.88, p < 0.05) and other bacteria <1.0% (R^2 = 0.96, p < 0.05). Many bacteria can produce lactate, either to rapidly release their excess of electrons or because they are natural lactate producers. In both cases, H_2 production is affected and lactate and H_2 are mostly negatively correlated [37], [181], [183]. However, a positive correlation could occur by using lactate to produce H_2 along with butyrate [184]. In this case, lactate production during dark fermentation could act as an "electron reservoir" for H_2 production when the substrate (*e.g.* glucose) is totally depleted [185]. Besides, our results are consistent with the literature, and species belonging to the family *Lactobacillaceae* are well known as lactate producers [37]. *Lactobacillaceae* were

present in a low relative abundance, but their contribution in lactate production seems to be significant, evidencing once again the importance of subdominant bacteria in the main microbial community activities.

Despite the low amount of propionate in the control tests, two correlations were observed with microbial family abundance including a *Bacilli_unclassified* (R^2 = 0.93, $p < 0.05$) and *Streptococcaceae* (R^2 = –0.95, $p < 0.05$). Similarly and consistently with literature, ethanol production shows a positive correlation with *Enterobacteriaceae* family (R^2 = 0.95, $p < 0.05$) [28].

Table 3-1: Pearson correlation matrix based on metabolite production and microbial family abundance

	Enterobacteriaceae	*Clostridiaceae*	*Streptococcaceae*	*Aeromonadaceae*	*Prevotellaceae*	*Bacilli_unclassified*	*Planococcaceae*	*Lactobacillaceae*	*Rhodocyclaceae*	*Saprospiraceae*	*Others (<1.0%)*	H2
Succinate	*0.64*	*0.04*	*-0.45*	*0.51*	*0.20*	*-0.01*	*-0.71*	*-0.74*	*-0.78*	*-0.67*	*-0.63*	*0.23*
Lactate	*0.00*	*-0.27*	*-0.29*	*-0.85*	*-0.67*	*0.46*	*0.68*	***0.95***	*0.83*	***0.88***	***0.96***	***-0.93***
Acetate	*0.78*	*-0.68*	*-0.17*	*0.79*	*0.77*	*0.14*	*-0.56*	*-0.45*	*-0.50*	*-0.60*	*-0.44*	*0.30*
Propionate	*0.66*	*-0.21*	***-0.95***	*-0.17*	*-0.37*	***0.93***	*-0.34*	*0.37*	*-0.12*	*-0.01*	*0.20*	*-0.61*
Butyrate	*-0.47*	*0.55*	*0.57*	*0.48*	*0.39*	*-0.70*	*-0.30*	*-0.76*	*-0.50*	*-0.54*	*-0.73*	*0.86*
Ethanol	***0.95***	*-0.40*	*-0.68*	*0.42*	*0.20*	*0.36*	*-0.61*	*-0.39*	*-0.56*	*-0.48*	*-0.31*	*-0.12*
ηEF	*0.05*	*-0.21*	*0.57*	***0.90***	***0.96***	*-0.56*	*-0.39*	*-0.78*	*-0.51*	*-0.68*	*-0.71*	***0.89***
H2	*-0.25*	*0.25*	*0.54*	*0.81*	*0.74*	*-0.52*	*-0.50*	*-0.80*	*-0.64*	*-0.76*	*-0.86*	

The data used include all replicates from electro-fermentation and control reactors. In bold were marked the significant correlations with a p-values ≤0.05. Positive (■ for 1.0), negative (■ for –1.0) and null (□ for zero) correlations were marked with gradient colour depending on value.

Table 3-1 shows also the correlation existing between the EF efficiency (η_{EF}), metabolic patterns and family distribution of the microbial communities. The η_{EF} shows a positive correlation with the H_2 yields ($R^2 = 0.89$, $p < 0.05$), and the presence of *Aeromonadaceae* ($R^2 = 0.90$, $p < 0.05$) and *Prevotellaceae* ($R^2 = 0.96$, $p < 0.05$). Interestingly, some members of the *Aeromonadaceae* family were previously reported in anodic (*i.e.* being part of the transfer electrons toward electrode) biofilms in MFCs, including anodes made with metallic materials such as titanium [186]–[189]. This electrochemical activity could also occur in the opposite direction, *i.e.* taking electrons from the cathodic surface, thus, being able to actively participate in the electrons transfer from the electrode to the fermentation medium. In addition, members of the *Prevotellaceae* family are associated to the production of extracellular polymeric substances for cellular aggregation and possibly could favour biofilm formation [37], [190]. The positive correlation between η_{EF} and the selection of both *Aeromonadaceae* and *Prevotellaceae* families, could explain the increase in electron transfer from the electrode surface toward the fermentation medium. Even they were not dominant in the soluble microbial community, their contribution to metabolic pattern changes should not be neglected.

3.4 Effect of the applied potential on metabolic patterns

In this section, the influence of the applied potential on metabolic patterns was investigated. For that, four potential values were applied at the working electrode: –0.9, –0.7, –0.5 and –0.3 V *vs* SCE. Batch EF tests were performed using a single chamber reactor using as working and counter electrodes grids of 90% platinum – 10% iridium. Besides, conventional fermentation as control tests were also performed using a single chamber reactor, in absence of polarized electrodes. All the experiments were carried out in duplicates.

After 24 hours of batch operation, the glucose was totally consumed in all reactors (4.9±0.3 $gCOD.l^{-1}$). Total COD mass balance measured as soluble and H_2 gas, was 85.3±6.6%. It was assumed that the 10-15% of the missing COD corresponded to biomass growth.

3.4.1 Hydrogen production and metabolite distribution

The maximum H_2 yield was reached at –0.7V and –0.5V, with 1.09±0.07 $mol_{H2}.mol^{-1}_{glucose}$ (Fig. 3-6) that represented an increase of 42% with respect to the control (0.77±0.002 $mol_{H2}.mol^{-1}_{glucose}$). A lower H_2 yield was obtained at –0.9V and –0.3V, with only 0.83±0.02 $mol_{H2}.mol^{-1}_{glucose}$ and 0.85±0.05 $mol_{H2}.mol^{-1}_{glucose}$, respectively. Only the tests made at –0.7V and –0.5V were statistically different (ANOVA: F=10.89, *p*=0.011) from the control. The tests at –0.9V and –0.3V did not show significant differences with the control (ANOVA: F=3.48, *p*=0.1656).

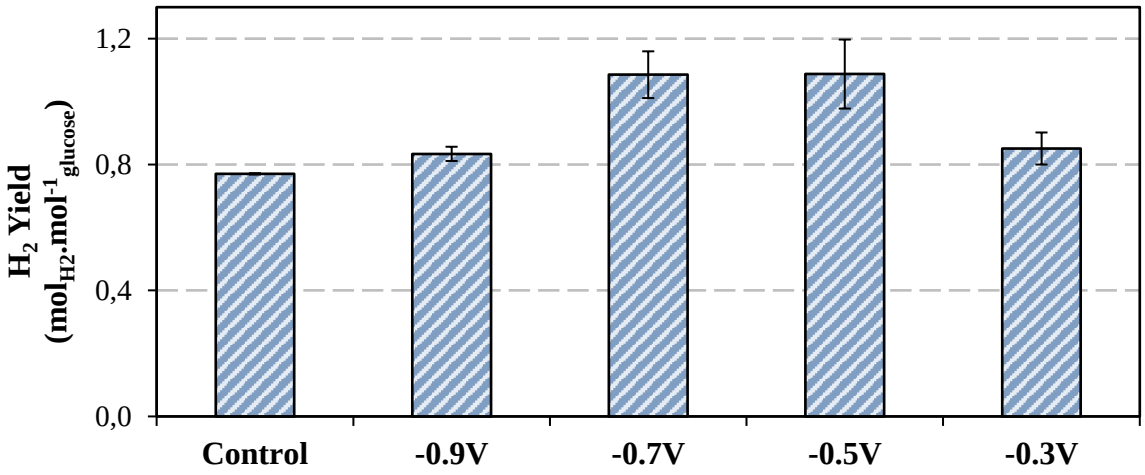


Fig. 3-6: Hydrogen yield observed during electro-fermentation tests at different potential applied on the working electrode using single chamber reactors

Regarding the metabolites produced, no significant difference was observed in all conditions including the control. The ANOVA analysis performed did not show a significant *p-value* to reject the null hypothesis *i.e.* group means are all equal. The F / *p-value* for acetate,

propionate, butyrate and ethanol were 1.171 / 0.423, 0.454 / 0.782, 0.956 / 0.503 and 0.472 / 0.757, respectively.

Illustratively, Fig. 3-7 shows the metabolic distribution based on COD mass balance in the control and at –0.7V. A focus on this particular condition was made since the H_2 yield was significantly different from the control. Butyrate was the main metabolite in all the cases, ranging between 36.8±1.0%$_{COD}$ and 52.8±6.3%$_{COD}$. Acetate, propionate and ethanol were also produced, in a range between 10.4±0.3%$_{COD}$ and 12.5±1.9%$_{COD}$, 4.0±3.2%$_{COD}$ and 12.2±7.9%$_{COD}$ and 8.7±3.4%$_{COD}$ and 11.7±2.0%$_{COD}$, respectively. Low quantities of lactate were also detected, *i.e.* between only 1.9±0.3%$_{COD}$ and 8.4±11.9%$_{COD}$.

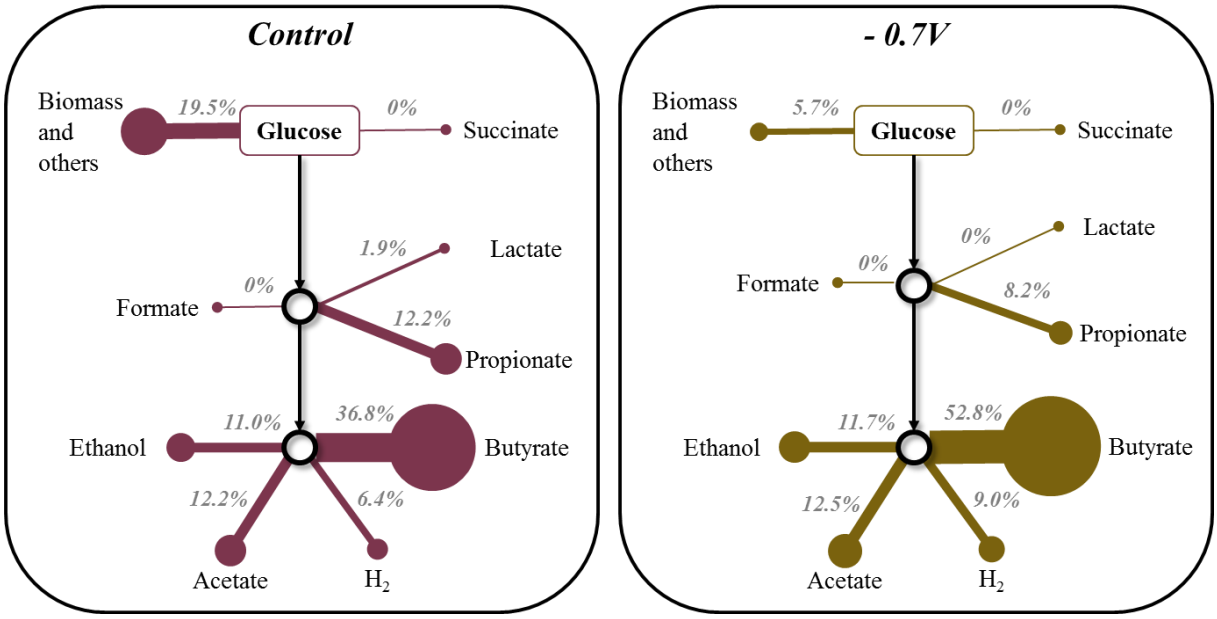


Fig. 3-7: Metabolite distribution observed during electro-fermentation tests at different potential applied on the working electrode using single chamber reactors

As not significant differences were found on metabolic pattern during any EF conditions and control, illustratively only the control and – 0.7V are shown.

3.4.2 Analysis of bio-electrochemical parameters

In EF, the electric charges transferred from (*i.e.* negative sign) or to (*i.e.* positive sign) the electrodes were –304.8±98.1 C, –26.1±20.2 C, +0.09±0.07 C and +1.0±1.2 C for –0.9V, –0.7V, –0.5V and –0.3V, respectively. The η_{EF}, considering H_2 as interest product was 0.093±0.027 for –0.9V, while that for the others conditions the values were <0.003. According to the η_{EF}, a maximum between 0.3% and 9.3% of the total electrons that have passed by the electrical circuit could have contributed to H_2 production.

During the experiments showed in this section, a cyclic voltammetry (CV) was performed during 3.3 hours at the beginning and at the end of the batch operation. The CV was performed 10 times from – 1.0 V to 1.0 V with a scan rate of 1 $mV.s^{-1}$. CV is a standard tool in electrochemistry and has regularly been exploited to study and characterize electron transfer interactions between microorganisms or microbial biofilms and electrodes [191], [192]. However, after EF tests no conditions studied *i.e.* potential applied, allowed to observe any active site in the CV profile. This means that there was no electroactive biofilm formation on the electrode surface.

Fig. 3-8 shows the ORP measurements during batch operation time. Initially, a similar ORP profile was observed in EF tests due to CV performed at the beginning of batch operation. During the CV, the potential swapped and the electrode polarization changes over a short period of time. Since EF systems need only small amount of electron supplied (or harvest) to have a high impact [18], probably this initial CV performed was enough to cause a change in the microbial community. Besides, as CV was performed under the same parameters in all EF reactors, it could be responsible for the similar performances observed. Surprisingly, the ORP profile of the control showed similar disturbances, coinciding with the CV performed in EF reactors. Probably for cable ORP system electrical insulation reasons, the CV seemed to have

also affected the control and for that not significative differences were observed with the EF tests.

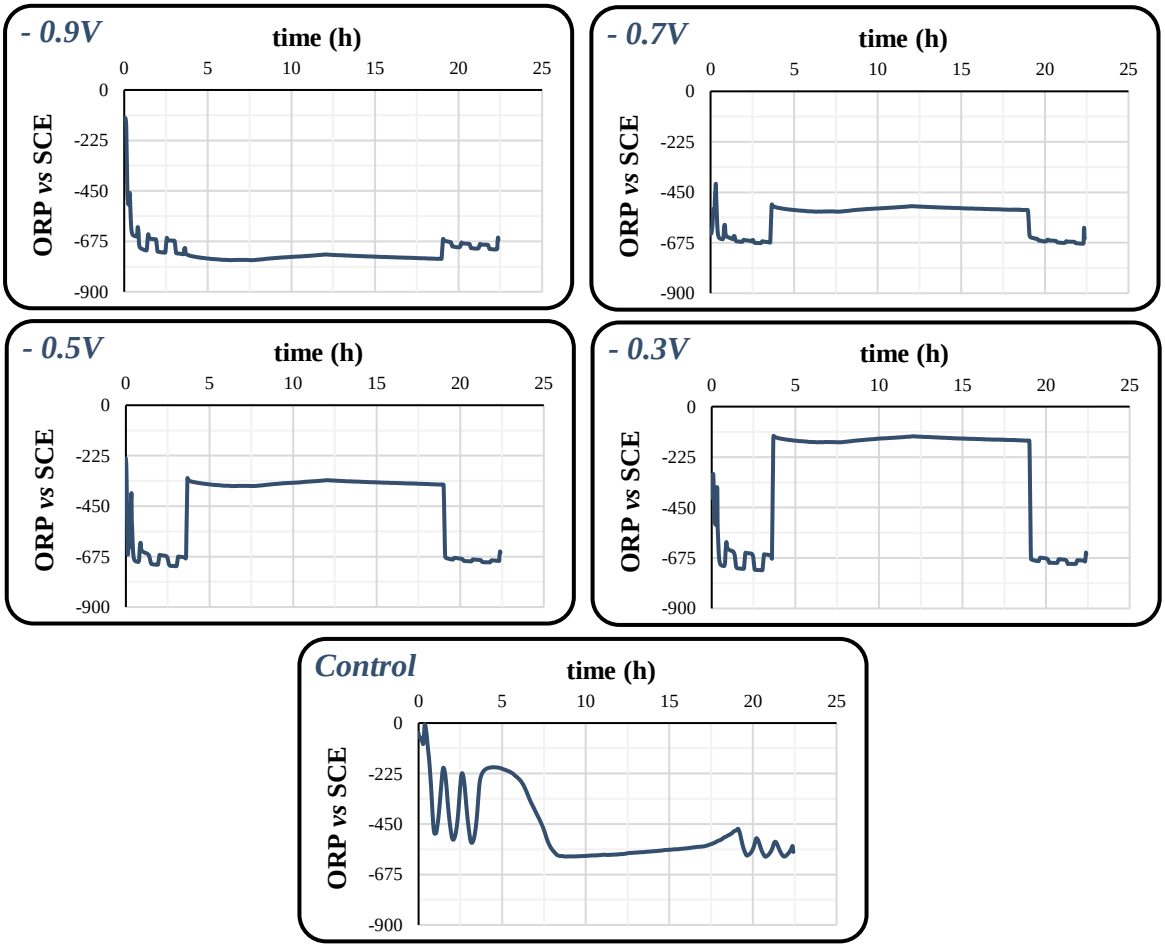


Fig. 3-8: ORP measurement in the fermentation bulk during electro-fermentation tests at different potential applied on the working electrode using single chamber reactors

The ORP measured (Fig. 3-8) in the control had a typical shape for biological kinetics, where fermentation products accumulate and stabilize the ORP, here around a negative value of – 0.57±0.03V.

In EF reactors, after the initial CV, a constant ORP value of – 0.75±0.01 V, – 0.52±0.01 V, – 0.35±0.01 V and – 0.15±0.01 V was reached when the potential applied at the working electrode was – 0.9 V, – 0.7 V, – 0.5 V and – 0.3 V, respectively (Fig. 3-8). A linear correlation between ORP and applied potential was shown (Fig. 3-9). However, ORP values

and applied potential are not equal and ORP measured probably reflects the joint effect of the working and counter electrodes because both are in the same reactor, *i.e.* single chamber reactor.

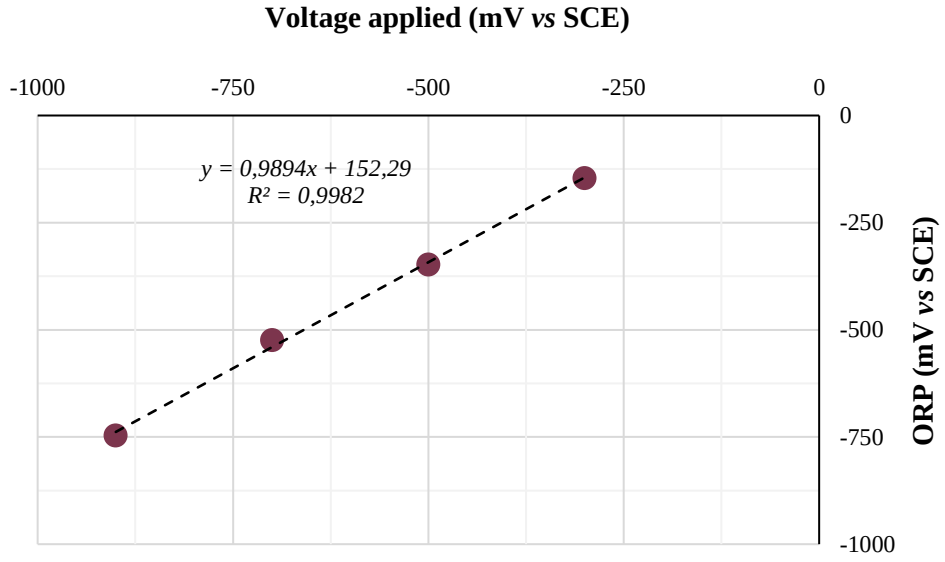


Fig. 3-9: Linear correlation between ORP measured in fermentation bulk and potential applied at working electrode

3.5 Size electrode selection

In this last section, the influence of electrode size on H_2 production and metabolic pathways during glucose EF was evaluated. For that, two electrode sizes of working and counter electrode of 2.5x2.5 cm^2 (**SE**) and 3.5x3.5 cm^2 (**BE**) were tested. It should be noted that working and counter electrodes always have the same size. So when BE (or SE) was used, the same size was used for both electrodes. Dual-chamber reactors were used to avoid low reproducibility as observed in simple-chamber reactors (data not shown). Besides, control test were performed in absence of polarized electrodes using a single-chamber reactor and all the experiments were carried out in duplicates.

After 24 hours of batch operation, glucose was totally consumed in all reactors (5.3±0.1 $gCOD.l^{-1}$). Total COD mass balance measured as soluble and H_2 gas, was 76.5±9.1%. 10-15% of missing COD was likely corresponding to biomass growth. The rest of missing COD probably correspond to unknown metabolites.

A maximum H_2 yield of 1.39±0.17 $mol_{H2}.mol^{-1}_{glucose}$ (Fig. 3-10) was reached when BE was used, but no significant difference (ANOVA: F=4.73, p=0.118) was observed when compared to the control (1.26±0.09 $mol_{H2}.mol^{-1}_{glucose}$) and when SE (1.04±0.05 $mol_{H2}.mol^{-1}_{glucose}$) was used.

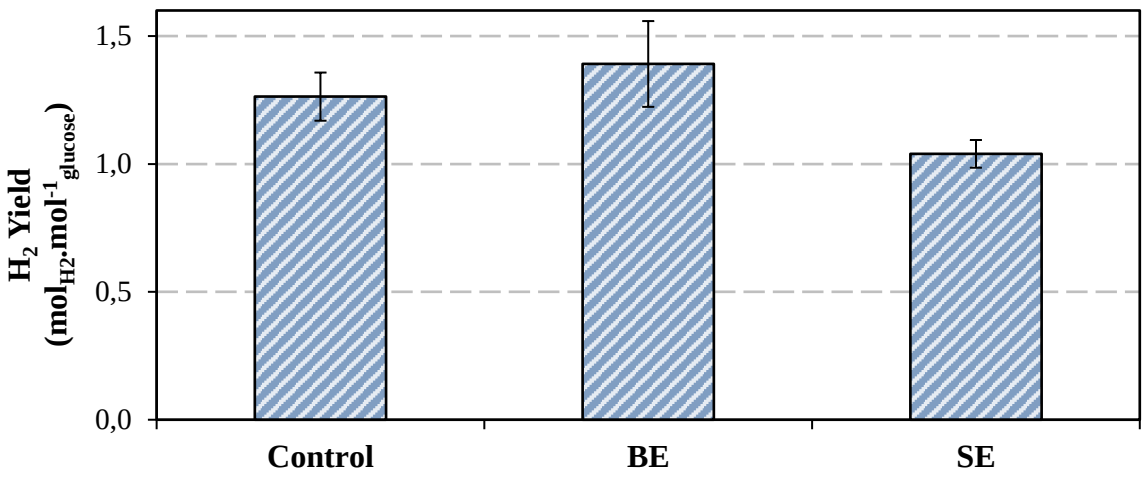


Fig. 3-10: Hydrogen yield observed during electro-fermentation tests comparing two electrodes sizes using dual chamber reactors

*Two electrodes size were compared of 2.5x2.5 cm^2 (**SE**) and 3.5x3.5 cm^2 (**BE**).*

With respect to metabolites distribution (Fig. 3-11), ethanol was the main metabolite in the control by reaching 31.7±0.3 $\%_{COD}$. Butyrate and acetate were also produced at lower extents, representing 12.6±4.3$\%_{COD}$ and 8.5±0.7$\%_{COD}$, respectively. Low quantity of succinate and lactate were also detected, representing only 2.2±0.1$\%_{COD}$ and 1.1±1.6$\%_{COD}$, respectively.

During EF, ethanol production decreased to 19.0±6.3%$_{COD}$ and 12.1±2.3%$_{COD}$ for BE and SE, respectively. Meanwhile, lactate production increased and represented 29.9±9.3%$_{COD}$ and 47.8±3.8%$_{COD}$ for BE and SE, respectively. Acetate, butyrate and succinate were also produced, representing 9.2±1.2%$_{COD}$ – 8.3±0.5%$_{COD}$, 7.2±3.4%$_{COD}$ – 6.2±0.02%$_{COD}$ and 1.5±0.3%$_{COD}$ – 1.0±0.01%$_{COD}$ for BE and SE, respectively. Despite that in all experiments performed in this chapter the same inoculum was used under similar operational conditions, the metabolite distributions observed show great differences compared to results from previous sections. Probably, this is due to an effect of inoculum age it has been stored at –4°C for around 4 months before the experiment observed in this section. However, as no microbial analysis was performed, it is not possible to compare the differences in the microbial community composition.

In EF tests, the electric charges transferred from the electrodes surface to the fermentation medium were –747.9±126.0 C and –244.1±61.9 C for BE and SE, respectively. The η_{EF} indices calculated on H_2 production were 0.20±0.01 and 0.09±0.02 respectively. That suggests that a maximum of 19.8% and 9.0% of the electrons passing through the electrical circuit could have contributed to H_2 production. Besides, respect to all fermentation product a global coefficient of η_{EF} = 0.031±0.002 and 0.009±0.002 for BE and SE, respectively, was obtained. This value means that only a maximum of 3.1% of all fermentation product are affected for the electrons supplied by the electrodes.

As already shown no significant differences on the H_2 production was observed here. Moreover, a direct relationship between the electrode size and the electric charge was observed, as already reported in the literature [157], [193], [194]. Indeed, Oh et al (2004) reported an increase of 22% in the power density during electricity generation in microbial fuel cells by tripling the cathode surface area [195].

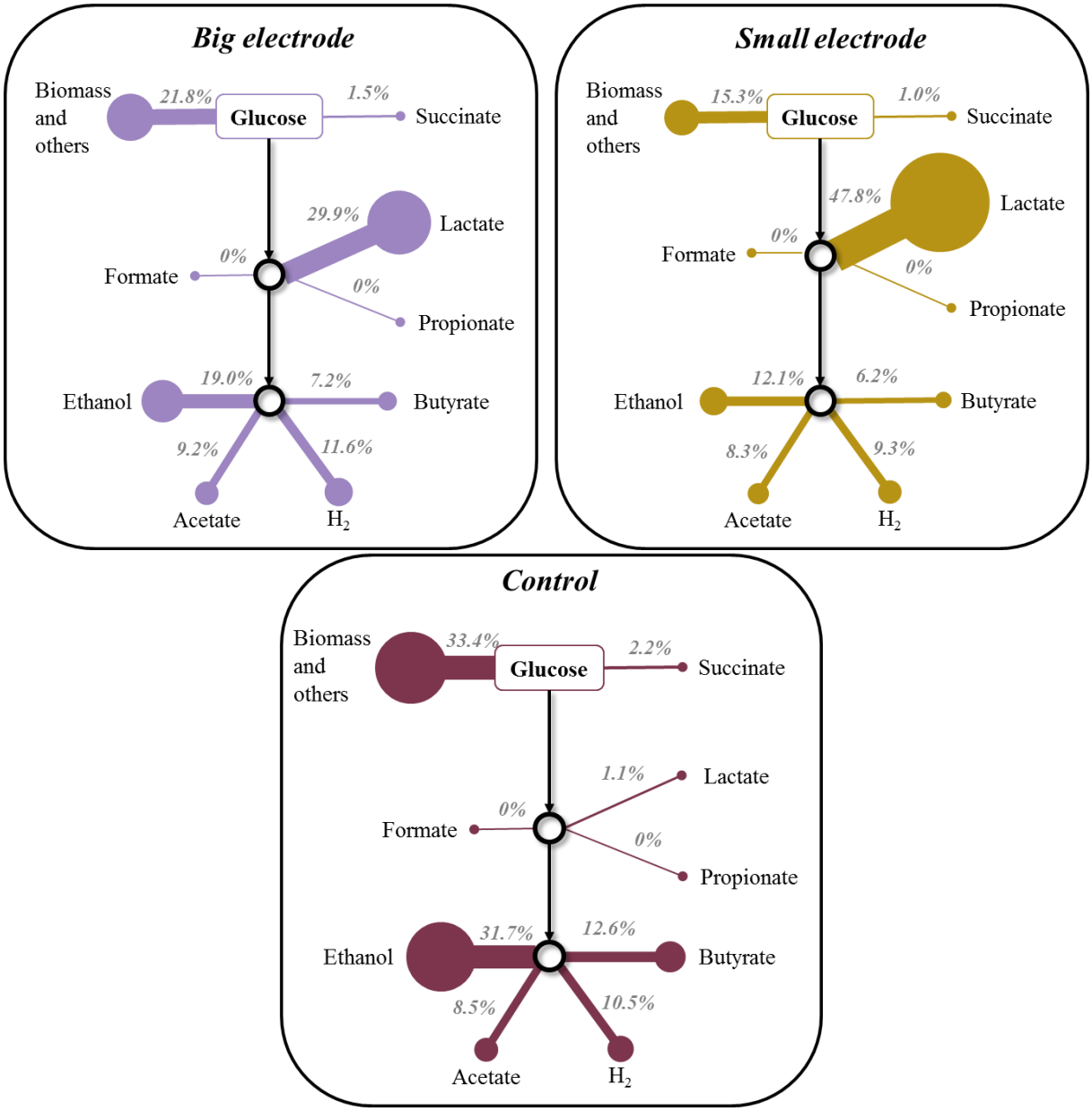


Fig. 3-11: Metabolite distribution observed during electro-fermentation tests comparing two electrodes sizes using dual-chamber reactors

3.6 Conclusion

In this chapter, different electro-fermentation experiments using glucose were performed to determinate the best operational parameters and select adequate materials. After comparing different materials as working electrode, platinum grids were selected based on H_2 production

performances to carry out the next electro-fermentation experiences. Besides, a significant change on the metabolic patterns was observed when a polarized electrode was placed in the fermentation medium. In this case, H_2 and butyrate production were both increased while lactate was unfavoured when compared to the control. Microbial community analysis showed that the dominant families present at the end of the batch operation could not explaining these changes. Nonetheless, and despite a low relative abundance, the presence of members of the *Aeromonadaceae* family showed a positive and significant correlation with the electro-fermentation efficiency index. This suggests the importance of subdominants species in the microbial interactions having a subsequent impact on metabolic patterns.

Afterwards, different potential were applied on the working electrode, but not differences were observed between the conditions including the control. In this case, an initial cyclic voltammetry was performed, and it was hypothesized that the CV was possibly the cause of such similar performances. Besides, some electrical interferences were observed between the control and the EF tests, due to the initial cyclic voltammetry performed in electro-fermentation tests. Although the phenomenon behind this behaviour is unknown, and was not further investigated, the control reactors were then placed in another water bath that electro-fermentation reactors and no longer electrical interference was observed.

Finally, two sizes of platinum grids electrodes were tested using dual-chamber reactors. The big electrodes of 3.5 x 3.5 cm^2 were selected because more electrons, *i.e.* Coulombs, were supplied to the fermentation medium.

CHAPTER 4

Electro-fermentation triggering H_2-producing bacteria

4.1 Introduction

Mixed cultures have been widely used for H_2 production by dark fermentation. However, many different microorganisms can grow in fermentation media and no selection pressure can be directly applied for the selection of bacteria carrying efficient H_2-producing pathways, such as *Clostridium* sp. Besides, not only the action of dominant species is crucial, subdominant species also play a key role supporting the microbial community [59]. Consistently, in the Chapter 3 the results evidence that only members of the subdominant family *Aeromonadaceae* showed a positive and significant correlation with the EF efficiency index. This suggests the importance of subdominants species in the microbial community interactions. However, there is not much research focused on the microbial interactions studies.

To date, lot of efforts have been made on optimizing operating parameters, including: carbon sources, macro-micro nutrients, temperature, pH, HRT, organic loading rates, H_2 partial pressure. However only few controllers (pH, OLR, HRT) are available to maintain stable dark fermentation performances, *i.e.* metabolic patterns and H_2 production. In this context, the electro-fermentation process has been proposed as a new type of bioprocess controlled by polarized electrodes.

The objective of this chapter is to use polarized electrodes in glucose fermentation to evaluate their influence in the fermentation medium on metabolic pathways and H_2 production, with a special focus on how the bacterial populations could be affected.

This chapter has been written based on the article accepted in International Journal of Hydrogen Energy: **Toledo-Alarcón J**., Moscoviz R., Trably E. and Bernet N. Glucose

electro-fermentation as main driver for efficient H_2-producing bacteria selection in mixed cultures.

4.1.1 Context and specific experimental methodology

In this Chapter the effect of polarized electrodes was evaluated by testing four different values of applied potential at working electrode *i.e.* –0.9, –0.4, +0.4 & +0.9 V *vs* SCE. Batch operation was performed using dual-chamber reactor and as working and counter electrode were used grids of 90% platinum – 10% iridium of 3.5 x 3.5 cm^2. Conventional fermentation *i.e.* without electrodes, was performed as control in single chamber reactor, under similar conditions than electro-fermentation test. More details are provided in Chapter 2 (Materials and methods).

4.2 Increase in H_2 production and changes in metabolite distribution during glucose electro-fermentation

After 20 hours of batch operation, glucose was totally consumed in all conditions (5.2±0.2 $g_{COD}.l^{-1}$). Total COD mass balance, calculated from soluble products and H_2 gas accumulation, ranged between 73.2% and 80.6%. Approximately 10-15% of the missing COD was likely corresponding to the biomass growth. Whatever the conditions, no methane was detected in headspace.

First, a low H_2 yield of 0.74±0.09 $mol_{H2}.mol^{-1}_{glucose}$ was observed in the control (conventional fermentation). H_2 production was significantly enhanced by a factor between 1.8 and 2.5 in presence of the polarized electrodes. The highest H_2-yield was 1.81±0.32 $mol_{H2}.mol^{-1}_{glucose}$ and was reached at the applied potentials of –0.4V and +0.9V. Lower H_2 yields were observed at –0.9V and +0.4V, *i.e.* 1.49±0.06 and 1.34±0.12 $mol_{H2}.mol^{-1}_{glucose}$, respectively. The H_2 yields were statistically different only between the control and the EF experiments

(ANOVA: F=20.68, *p*=0.0001), but not between EF tests. This result suggests that placing a polarized electrode, whatever the applied potential, in the fermentation medium is enough to observe a clear effect on H_2 production. The maximum H_2 yield obtained in this study is comparable to reported for glucose dark fermentation in batch operation using mixed cultures as inoculum (2.5 $mol_{H2}.mol^{-1}_{glucose}$) [21]. However, depending on inoculum source and the pre-treatment employed, the H_2 yields could be between 0.1 and 3.0 $mol_{H2}.mol^{-1}_{glucose}$ [15], [37], [69], [79], [196].

Fig. 4-1 shows the metabolites distribution according to the COD mass balance. In the control, lactate was the main metabolite reaching to 66.9±4.9%$_{COD}$. Ethanol and acetate were also observed representing 14.0±2.5%$_{COD}$ and 9.5±1.0%$_{COD}$, respectively. In less quantity butyrate and propionate were produced, representing 1.6±3.1%$_{COD}$ and 0.4±0.9%$_{COD}$, respectively.

In EFs tests, lactate production was significantly decreased representing between 1.1 – 32.1%$_{COD}$. Regardless of the applied potential, ethanol and acetate productions were increased representing between 24.7 – 38.6%$_{COD}$ and 14.0 – 18.5%$_{COD}$, respectively. Particularly, in –0.4V and +0.9V experiments, butyrate production increased by 14.6 and 16.8-fold, when compared to the control. While it represented about 24.6 and 29.3%$_{COD}$ at –0.4V and +0.9V, respectively. As minority compounds, succinate production (not detected in the control) also increased to a lesser extent, representing 1.0 – 6.9%$_{COD}$. Propionate was produced at only very low concentrations (<1.6%$_{COD}$).

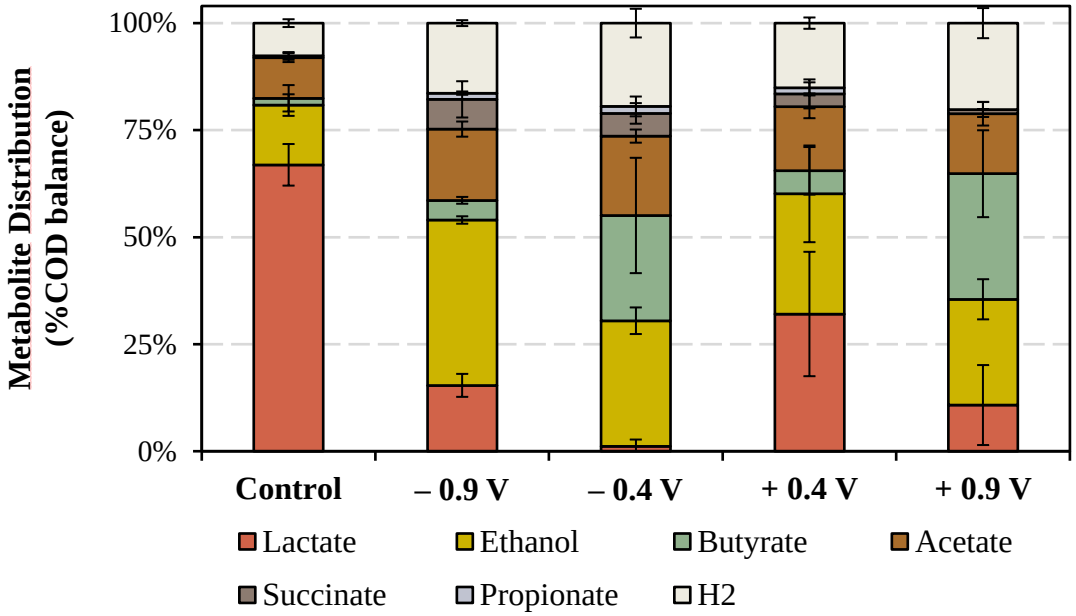


Fig. 4-1: Metabolite distribution based on COD mass balance in final samples of glucose electro-fermentation

Values were calculated based on total glucose consumed. Values represent the average from quintuplicates (Control), triplicates (+0.4V and +0.9V) or duplicates (–0.9V and –0.4V). Error bars represent the standard deviation of the data.

In general, high lactate production is consistent with low H_2 performances like in our control tests. This, because lactate is directly produced from pyruvate (not by the acetyl-coA pathway) by consuming NADH, allowing the cell to quickly get rid of the excess in electrons through NAD^+ regeneration without H_2 production [37]. Increasing H_2 yields were observed in EF tests and correlated with ethanol and acetate accumulation. Acetate is a key molecule for H_2 production due to the high energy gain of this pathway through ATP production [15], [197] Although ethanol is a more reduced molecule than glucose and its production allows to release the excess of electrons by direct NAD+ regeneration, ethanol is also associated with H_2 production [18]. Finally, this study show the maximum H_2 was linked with the butyrate production. This metabolite has been often associated with additional ATP production and butyrate production was suggested as the most thermodynamically favourable reaction during

dark fermentative H_2 production [34]. As a consequence, butyrate production is often associated with high H_2 producing reactors [15].

4.3 Microbial community analysis

DNA samples were taken from inoculum and after 20 hours of batch operation to analyse the effect of the polarized electrodes on the microbial communities. A total of 589 operational taxonomic units (OTUs) were found after MiSeq sequencing in all samples. The dominant family found in the inoculum was *Clostridiaceae* representing 17.6±1.6% of the microbial community (Fig. 4-2). Families with an abundance relative lower than 5.0% represented 46.9±3.1% of the microbial community in the inoculum, evidencing a high diversity (Simpson index of 0.955±0.003) at start of the experiments.

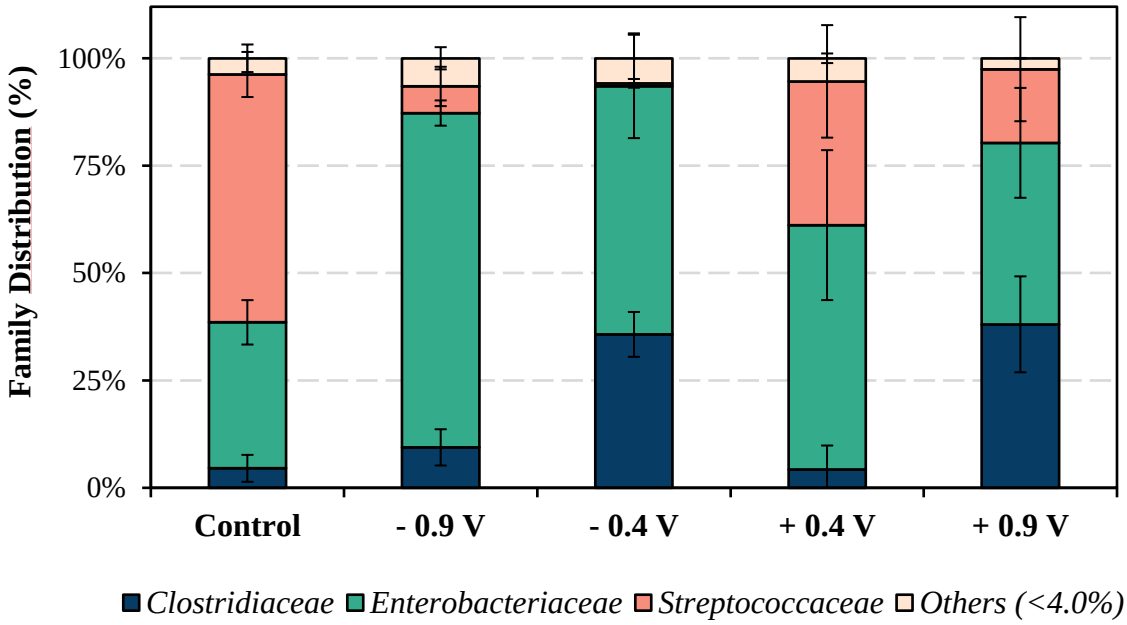


Fig. 4-2: Family distribution of the microbial communities found in final samples of glucose electro-fermentations and fermentation controls

Values represent the average of triplicates (+0.4V and +0.9V) or duplicates (Control, –0.9V and –0.4V). Error bars represent the standard deviation of the data.

After batch operation, the Simpson diversity index decreased about 26.1 – 39.4% and only three families (*Streptococcaceae, Enterobacteriaceae* and *Clostridiaceae)* dominated the microbial community, representing about 93.8 – 97.8% of the total abundance (Fig. 4-2). Only 8 OTUs showed a relative abundance higher than 1.0% in at least one sample (Table 4-1). In the control, *Streptococcaceae* (57.9±5.1%) and *Enterobacteriaceae* (34.0±5.2%) families were the most representative families, and OTU2 and OTU1 were dominant with 57.7±5.2 and 28.9±4.9% of the total bacterial community, respectively. These two OTUs were related to *Escherichia fergusonii* (99% 16S rRNA sequence similarity with OTU1) and *Streptococcus equinus* (99% 16S rRNA sequence similarity with OTU2).

The *Clostridiaceae* family was mainly represented by OTU3 and was enriched at –0.4V (35.7±4.2%) and +0.9V (38.1±11.2%). OTU3 had 99% of 16S rRNA sequence similarity with *Clostridium butyricum.* The *Enterobacteriaceae* family was the most abundant in all EF reactors, reaching 77.8±2.9, 57.7±12.0, 56.9±17.5 and 42.2±12.8% of total microbial community at –0.9V, –0.4V, +0.4V and +0.9V, respectively. Two main OTUs represented this family, *i.e.* OTU1 and OTU4. OTU1 was already described at the beginning of this section and OTU4 had 99% of 16S rRNA sequence similarity with *Enterobacter cloacae.*

Table 4-1: Relative abundance (%) of microbial community after 20 hours of batch operation based on MiSeq sequencing of 16S rRNA gene

OTU	Putative identification (16S rRNA sequence similarity)	**Control**		**–0.9V**		**–0.4V**		**0.4V**			**0.9V**		
		n=1	*n=2*	*n=1*	*n=2*	*n=1*	*n=2*	*n=1*	*n=2*	*n=3*	*n=1*	*n=2*	*n=3*
Enterobacteriaceae													
1	*Escherichia fergusonii* (99)	25.4	32.3	70.8	32.4	64.7	45.7	40.6	18.6	29.2	38.5	9.6	15.0
4	*Enterobacter cloacae* (99)	4.9	5.3	9.1	43.4	1.5	3.5	26.6	48.1	7.5	18.4	27.5	17.9
Total[a]		*30.8*	*38.6*	*80.7*	*76.5*	*66.5*	*49.9*	*68.0*	*67.3*	*36.9*	*57.2*	*38.5*	*33.6*
Streptococcaceae													
2	*Streptococcus equinus* (99)	61.4	54.0	3.0	9.4	0.0	1.4	24.6	27.3	48.5	10.7	9.6	31.1
Clostridiaceae													
3	*Clostridium butyricum* (99)	6.7	2.3	10.8	6.4	29.8	37.8	1.8	0.3	10.6	29.9	49.3	33.3
7	*Clostridium intestinale* (97)	0.0	0.0	1.6	0.1	2.2	1.7	0.1	0.0	0.0	0.1	1.5	0.1
Enterococcaceae													
8	*Enterococcus casseliflavus* (100)	0.0	2.0	0.0	0.0	0.0	0.8	0.6	0.0	0.1	0.0	0.0	0.0
Total[a]		*68.5*	*60.3*	*16.9*	*16.6*	*32.8*	*43.5*	*30.1*	*27.8*	*59.6*	*41.0*	*60.7*	*65.2*
Corynebacteriaceae													
5	*Corynebacterium vitaeruminis* (99)	0.1	0.7	1.8	1.5	0.4	0.8	1.2	4.1	3.1	1.0	0.1	0.7
Total[a]		*0.2*	*0.8*	*2.0*	*1.7*	*0.5*	*0.9*	*1.4*	*4.2*	*3.2*	*1.2*	*0.2*	*0.8*
Prevotellaceae													
6	*Prevotella paludivivens* (99)	0.0	0.0	0.0	5.0	0.0	5.1	0.2	0.4	0.1	0.2	0.5	0.2
Total[a]		*0.3*	*0.2*	*0.2*	*5.1*	*0.1*	*5.7*	*0.4*	*0.6*	*0.3*	*0.5*	*0.5*	*0.3*
Others		0.2	0.2	0.3	0.1	0.1	0.1	0.1	0.0	0.0	0.1	0.1	0.0

[a] *including OTUs with <1.0% relative abundance.*

In all the samples, H_2-producing bacteria were selected, and their relative abundances increased, and more specially members of the *Enterobacteriaceae* and *Clostridiaceae* families. Despite the inoculum was heat-treated before reactor inoculation, none-spore forming bacteria of the *Enterobacteriaceae* family such as OTU1 and OTU4 were selected and dominated at the end of operation. Probably these species can survive due to limitations in heat transfer depending on the system used for pre-treatment. Consistently, it has been reported in literature that even methanogens can survive after some operation days after heat shock pre-treatment, and the type of inoculum source plays a key role in its effectiveness [21], [198].

4.4 The three mains metabolic pathways for H_2 production in electro-fermentation resulted from microbial community selection

To represent the relationships between microbial communities and reactor performances, a principal component analysis (PCA) was performed. Fig. 4-3 shows the PCA based on bacterial population in EF and control reactors evidencing the categorical differences between using or not polarized electrodes. Three main H_2 production pathways correlated well with the selection of specific microbial communities. In the first pathway (Fig. 4-3 on the right side) observed in control reactors, *Streptococcaceae* family abundance was correlated with high lactate production ($R^2 = 0.92$, $p<0.01$) and with a low H_2 production ($R^2 = –0.73$, $p<0.01$).

Details of the correlation matrix are presented Fig. 4-4. *Streptococcus equinus* belongs to a known lactic acid bacteria group [199], that was previously found in low H_2-producing reactors [37], [200]–[202]. Overall, lactic acid bacteria act as a suppressor of H_2 production through substrate competition (*i.e.* pyruvate) and produce lactate at the expense of H_2,

resulting in lower yields or the release of bactericides inhibiting the growth of H_2 producing bacteria [12], [199].

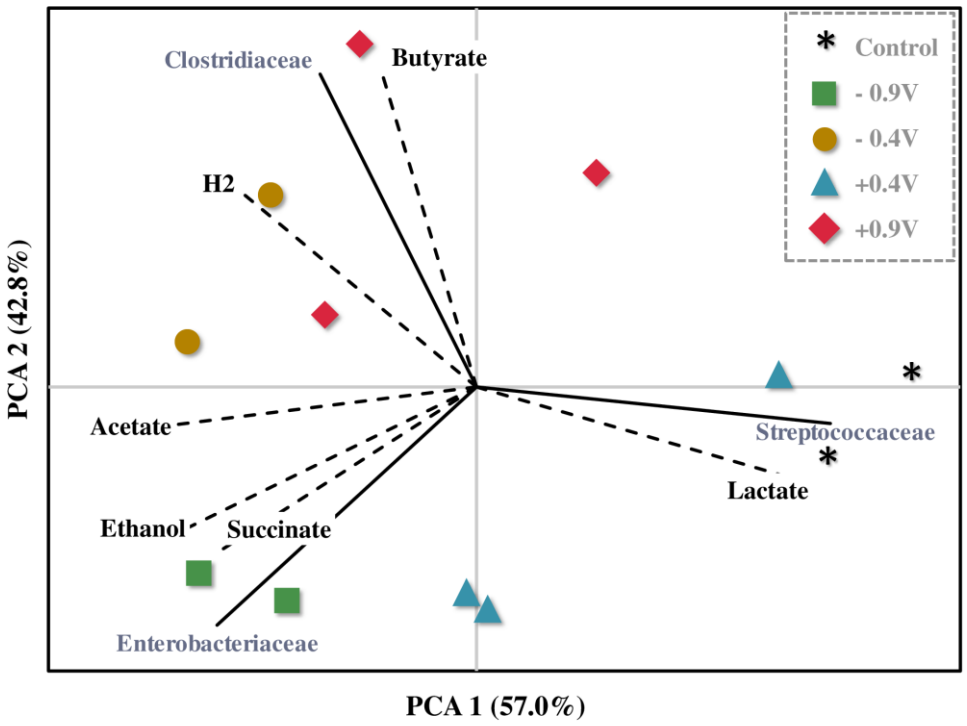


Fig. 4-3: Principal component analysis (PCA) based on microbial population distribution

Black plain lines and dotted lines represent correlations between PCA axes and taxonomic families and metabolic yields, respectively. Stars, squares, triangles, circles and diamonds represent control (n=2), –0.9V, +0.4V, –0.4V and +0.9V reactors, respectively.

In the second pathway (Fig. 4-3 on the left upper side), as observed at –0.4V and +0.9V, *Clostridiaceae* abundance correlated well with high H_2 yields (R^2 = 0.79, p<0.01, Fig. 4-4) and high butyrate production (R^2 = 0.94, p<0.01, Fig. 4-4). Additionally, a negative correlation was evidenced with lactate production (R^2 = –0.63, p<0.05, Fig. 4-4). By selecting *Clostridium* species, up to 45% of the theoretical H_2 – yield (4 $mol_{H2}.mol_{glucose}^{-1}$) was reached [15], [34], along with an important increase in the butyrate production. In this case, OTU3 (related to *Clostridium butyricum*) was greatly favoured when compared to the control. This

species was the most abundant in only one condition (+0.9V) but, at the family level, the *Enterobacteriaceae* dominated in all other EF experiences. *Clostridium butyricum* is a well-known efficient H_2 producer producing butyrate as main metabolite [15], [203], [204] which is consistent with the present findings. More generally, species belonging to the *Clostridia* genus are considered as efficient H_2 producers [37] and they have been found in most of the mixed culture-based H_2 producing fermentative systems. [15].

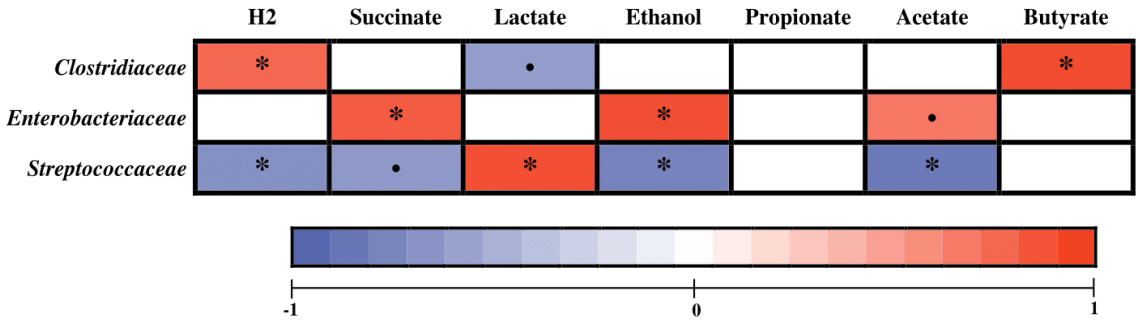


Fig. 4-4: Pearson correlation matrix based on metabolite production and microbial family abundance

The data used include all data from electro-fermentation and control reactors. • = $p_{value}<0.05$; * = $p_{value}<0.01$

A third pathway (Fig. 4-3 on the left bottom) was observed at –0.9V and +0.4V and was related to the *Enterobacteriaceae* abundance which positively correlated with succinate (R^2 = 0.86, p <0.01, Fig. 4-4), ethanol (R^2 = 0.93, p <0.01, Fig. 4-4) and acetate (R^2 = 0.71, p <0.05, Fig. 4-4). The *Enterobacteriaceae* selection in EF tests led to a lower H_2 production than when the second pathway was promoted, but H_2 yields were still higher than in the control. In these reactors the OTU3 related to *Clostridium butyricum* was not favoured and the *Enterobacteriaceae* family was largely dominating. OTUs 1 and 4, related to *Escherichia fergusonii* and *Enterobacter cloacae*, respectively, were the main species and both belong to genera that have been widely used in pure cultures for H_2 production [36], [205]–[210].

However, species from the *Enterobacteriaceae* family were already found during periods of poor H_2 production [211]. Consistently, our results show that this family is positively correlated with succinate and ethanol accumulation [28], [37].

4.5 Electro-fermentation patterns: low current is enough to trigger high changes in fermentation patterns

During EF, the electric charges transferred from (*i.e.* negative sign) / to (*i.e.* positive sign) the electrodes were –615.4 ±378.6 C, –0.17 ±0.11 C, +2.85 ±1.75 C and +1.89 ±0.83 C at an applied potential of –0.9V, –0.4V, +0.4V and +0.9V, respectively. Although the measured current was significant, the quantity of electrons at –0.9V represented only 1.9% of the total electrons, *i.e.* the electrons issued from glucose or from and the electric current. Current was close to zero in all other EF conditions (Table 4-2). To differentiate EF from other bioelectrochemical systems, an efficiency coefficient of EF (η_{EF}), analogous to the coulombic efficiency in conventional BES, could be estimated, as previously proposed by Moscoviz et al. (2016). η_{EF} values range between 0 and 1 in EF systems and values higher than 1 indicate the possible occurrence of direct bio-electrosynthesis. When considering H_2 as targeted product, the η_{EF} value at –0.9V was 0.16, meaning that the electric current could not directly contribute to more than 16.0% of the total accumulated H_2. Thus, hydrogen production mainly resulted from glucose catabolism. In other EF conditions, the η_{EF} was <0.001, meaning that the electric current did not significantly contribute to the whole metabolic reaction (<0.1% of H_2 production). Consequently, only a small amount of energy was sufficient to generate a great impact, as evidenced by the low values of η_{EF} [18]. This is consistent with EF articles already published, where current was not the main source of energy for microbial metabolism [18], [142]. Overall, such low amount of energy could explain the relative independence of the H_2 yields to the applied potential since electrons are

not directly converted to H_2 and polarized electrodes rather contribute to change the local environment around the electrode leading to microbial selection and subsequently different H_2 yields.

Table 4-2: Summary of ηEF calculations for all replicates from each condition evaluated

Test		Q_{e-} (C)	$mmol_{Qe-}/mol_{e-total}$	ηEF
–0.9V	*(n=1)*	-347.71	-11.309	8.93E-02
	(n=2)	-883.17	-26.516	2.26E-01
–0.4V	*(n=1)*	-0.10	-0.003	2.22E-05
	(n=2)	-0.25	-0.008	4.59E-05
+0.4V	*(n=1)*	4.30	0.135	1.13E-03
	(n=2)	3.35	0.105	1.04E-03
	(n=3)	0.91	0.028	2.50E-04
+0.9V	*(n=1)*	1.25	0.039	3.22E-04
	(n=2)	2.83	0.087	5.06E-04
	(n=3)	1.60	0.049	3.10E-04

Electric charge (Qe-, Coulomb) transferred from/toward circuit electric through working electrode placed in fermentation medium. Molar ratio (mmolQe-/mole-total) of electrons transferred through circuit electric per total electrons feed on the system (electrons from glucose + or – current electric). The electrofermentation efficiency (ηEF) was calculated considering hydrogen as metabolite of interest.

4.6 Hypothetical action mechanisms during glucose electro-fermentation

To better understand the mechanisms that drive the EF process, Arunasri et al. (2016) reported that the microbial community could be affected by exposure to different applied potentials during the H_2 production in a single chamber microbial electrolysis cell (MEC). These authors showed that members of the *Firmicutes* phylum were favored by increasing the applied potentials [177]. In contrast, in the present study, no significant linear relationship

between the applied potential and population selection was observed. The only OTU which had an abundance that linearly correlated to the applied potential was OTU 1 (*Escherichia fergusonii*) which was selected at low applied potential (R^2 = –0.71, p<0.05). However, substantial changes in the microbial community were triggered by small amounts of current that could not sustain an electrochemical H_2 production that would explain the difference between controls and EF conditions.

One hypothetical action in EF is related to the changes that could occur on the net charge of the cell surface. Choi et al. (2014) reported that the zeta potential of *C. pasteurianum* DSM 525 cells grown with electricity was near to zero, whereas cells were electronegative in open circuit [142]. The selection of OTU1 could have resulted from a change in the net charge of the cell surface in response to the potential applied on the working electrode. This would have likely caused physiological changes on growth rate and cell division and would consequently disadvantage it in substrate competition while giving the opportunity to other species to emerge [142], [212]. In addition, changes in cell surface net charge towards values close to zero in microorganisms such as *Clostridium* could have made them more resistant to bactericide produced by OTU 2 (related to *Streptococcus equinus*). As an illustration, nisin is a known toxin released by lactic acid bacteria causing a depolarization of energized bacterial membranes, especially affecting *Clostridia* species. This toxin stimulates the formation of potential-dependent multi-state pores when membrane potential is negative (–80 to –100 mV vs SHE). Through these pores, vital gradients equilibrate with the extracellular medium and metabolites and salts can be lost causing cell deaths [57], [58], [213], [214]. By modifying the cell surface net charge, the EF process could help sensitive bacterial species to counter this effect.

A second hypothetical mechanism is related to small changes in the extracellular redox potential caused by the polarized electrodes. That would affect the regulation of key membrane-bound enzymes involved in H_2 production (hydrogenases), due to their high sensitivity to redox potential variations [28], [44], [177]. However, such mechanisms are still difficult to clearly identify in mixed cultures. In all cases, interactions among species within microbial community are crucial to structure this community and the subsequent metabolism.

4.7 Conclusion

This chapter evidences a clear effect of polarized electrodes on both metabolic pathways and microbial community structure in dark fermentation. A strong correlation was observed between the selected microbial communities and the metabolites produced including H_2. In all EF conditions, an increase in the H_2-yields was shown, independently on the applied potential. The effects on microbial community were mostly non-linear except for specific OTUs related to *Escherichia fergusonii* and *Streptococcus equinus*. Such interaction mechanisms between polarized electrodes and microbial community remain however unclear but a new field of investigation in mixed cultures fermentation has been opened.

CHAPTER 5

Electro-fermentation is affected by initial microbial composition

5.1 Introduction

During H_2 production by dark fermentation, the inoculum source and their microbial composition are crucial for defining final reactor performances. However, different scenarios could lead to a specific selection of the microbial community with consequent changes in the H_2 production and in the metabolic pathways. For that, many strategies have been employed to obtain an adequate inoculum capable to produce H_2 high yields without methane production. These strategies include inoculum pre-treatment and setting of important operational parameters as pH, temperature and hydraulic retention time for continuous systems. Recently, it has been reported that the sugar type and the polymerization degree of complex sugars are also determine a specific microbial community, so it could be considered as a new strategy [9].

Particularly, the results shown in Chapter 4 evidence that an efficient tool for selecting H_2-producing bacteria is proposed through polarized electrodes. Therefore, microbial selection may first depend on the initial structure of the microbial community, so-called inoculum. Second, since electro-fermentation seeks to control bioprocesses, it is interesting to evaluate the effect of polarized electrodes when microbial communities change over time. For that continuous reactors that allow to study dynamic behaviors of ecosystems were used.

In this context the objectives of this chapter are the following:

i. Study the effect of electro-fermentation on continuous H_2 production in chemostat.
ii. Study the influence of the initial inoculum on electro-fermentation in batch mode

5.2 Glucose electro-fermentation during reactor operation in continuous mode

5.2.1 Context and specific experimental methodology

Continuous operation was performed for 40 days at a hydraulic retention time (HRT) of 6.7 h. Electro-fermentation (**CEF**) operation was performed in dual-chamber reactor, while the control, as conventional fermentation test (**CF**), was performed in single chamber reactor. In CEF only the working electrode chamber was operated in continuous. Electrodes present in CEF were connected to the potentiostat only the first 23 operation days (CEF-A). Then, electrodes were disconnected but left in the reactor until the end of operation (CEF-B).

During steady state operation, the glucose feed concentration was 5.2±0.2 $g_{COD}.l^{-1}$, 5.0±0.3 $g_{COD}.l^{-1}$ and 5.3±0.2 $g_{COD}.l^{-1}$, while glucose consumption was 87.8±7.3%, 82.7±10% and 86.5±5.3%, for CF, CEF-A and CEF-B, respectively. Total COD mass balance, calculated from soluble products and H_2 gas, ranged between 72.0±4.2 and 74.1±6.2%. Approximately 10-15% of the missing COD was likely corresponding to biomass growth, while the rest (10-15%) was attributed to unknown metabolites.

5.2.2 Hydrogen and metabolite production

Fig. 5-1 shows the H_2 productivity during all time of reactors operation. At the beginning of CF (d9-d10) and CEF-A (d9-d10 and d14-d15) operation, H_2 production was unstable due to operational problems related with pH control. The software had a limit in the data acquisition and when the system was overcharged it lost its functionality, leading to uncontrolled pH in the reactors. Thus, due to volatile fatty acids production, the pH decreased at values so low that H_2 production was inhibited [197]. To avoid future setbacks the software was restarted every four days of operation.

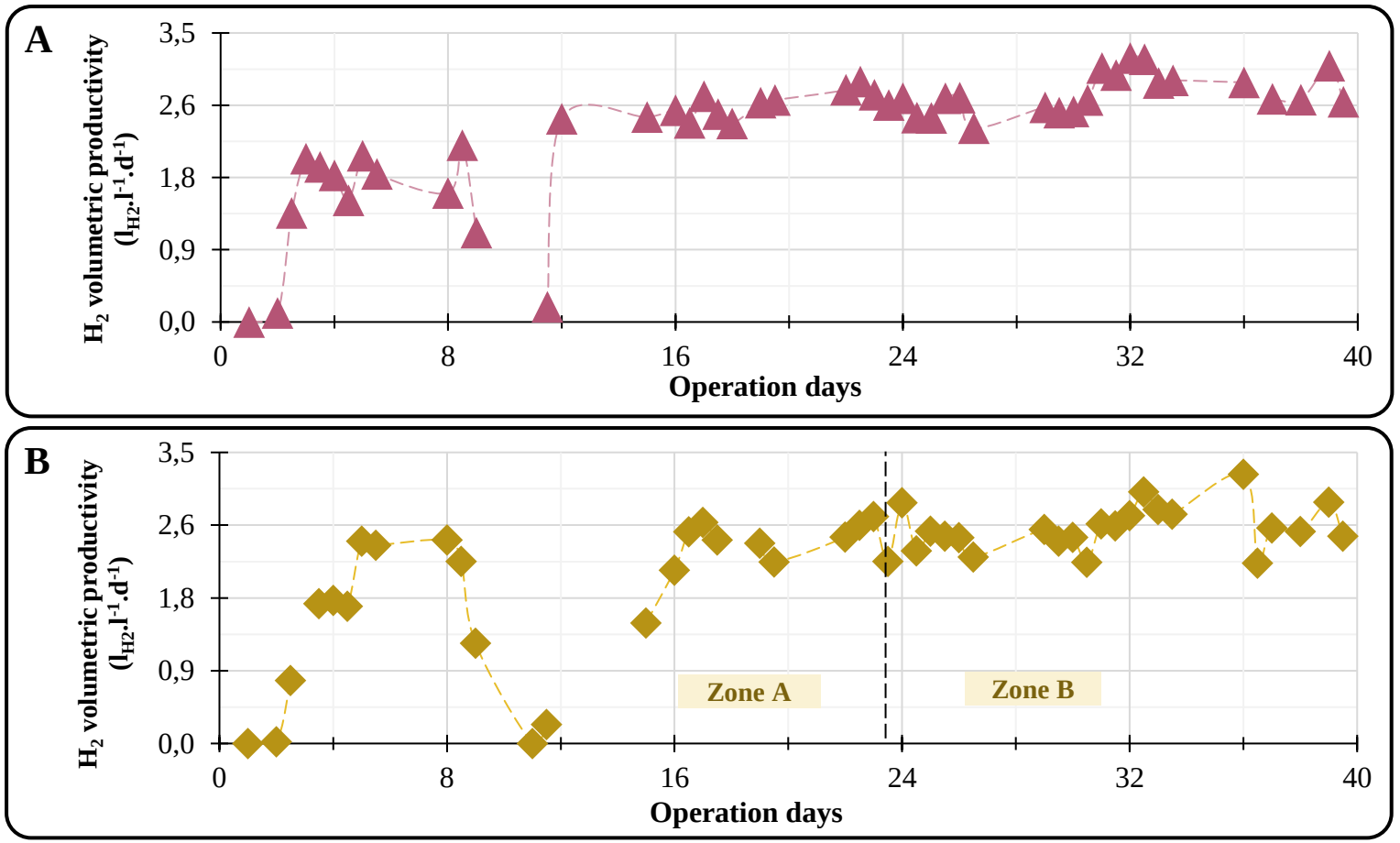


Fig. 5-1: Hydrogen volumetric productivity during all time of continuous operation

Figure A *shows the conventional fermentation (CF) operation i.e. without electrodes.* ***Figure B*** *shows the electro-fermentation operation where two zones are differentiating. Zone A (CEF-A) correspond to operation with polarized electrodes i.e. connected at potentiostat. Zone B (CEF-B) correspond to operation with electrodes but not connected at potentiostat.*

Steady-state was considered after 16 days of operation for CF and CEF-A, 25 days for CEF-B. The H_2 volumetric productivity was 2.7±0.2, 2.4±0.2 and 2.6±0.3 $l_{H2}.d^{-1}.l^{-1}$ for CF, CEF-A and CEF-B, respectively. According to the ANOVA test, significant differences were observed within the group of CF, CEF-A and CEF-B (F=6.084 and *p-value*= 0.004). Furthermore, and according to Mann-Whitney pairwise test, a significant difference was found between CF and CEF-A (*p-value*= 0.003). The high H_2 volumetric productivity reached in CF, CEF-A and CEF-B is comparable to those obtained with a pure culture of *Clostridium bifermentans* (2.65 $l_{H2}.d^{-1}.l^{-1}$) under similar operational conditions *i.e.* pH 5.5, HRT 6 h and 35°C [215]. Therefore, our results show a high productivity compared the study performed by Wu *et al* (2008) where anaerobic sludge was used as inoculum (0.6 $l_{H2}.d^{-1}.l^{-1}$) [76].

Moreover, H_2 yields were 1.54±0.18, 1.48±0.07 and 1.40±0.10 $mol_{H2}.mol_{glucose}^{-1}$ for CF, CEF-A and CEF-B, respectively. The ANOVA test showed significant differences between CF, CEF-A and CEF-B (F=3.594 and *p-value*= 0.039). According to Mann-Whitney pairwise test the only significant difference was found between CF and CEF-B (*p-value*= 0.017). Overall, the H_2 yields obtained in CF, CEF-A and CEF-B reached between 70 and 77% of the theoretical maximum when butyrate is the main metabolite. These values are comparable with the reported values found in literature [76], [215]–[217].

Regarding the metabolite production, no significant difference was observed between CF, CEF-A and CEF-B. Illustratively, Fig. 5-2 shows the metabolite distribution at steady state of the CF reactor. The main metabolite was butyrate that represented between 37.4±2.0 and 38.6±4.6 $\%_{COD}$. An important percentage of the COD, between 25.9±6.2 and 28.0±4.2 $\%_{COD}$, was associated to the production of biomass and other unknown metabolites. Acetate and

lactate represented only between 13.8±0.8 – 15.1±1.6 $\%_{COD}$ and between 6.8±1.3 – 8.2±0.9 $\%_{COD}$, respectively.

No difference was observed in metabolite patterns between CF, CEF-A and CEF-B. This fact is evidencing that, in continuous operation, the polarized electrodes in CEF-A were not affecting the metabolite production compared to conventional fermentation (CF). Then, same trend was observed when the electrodes were disconnected from the potentiostat (CEF-B).

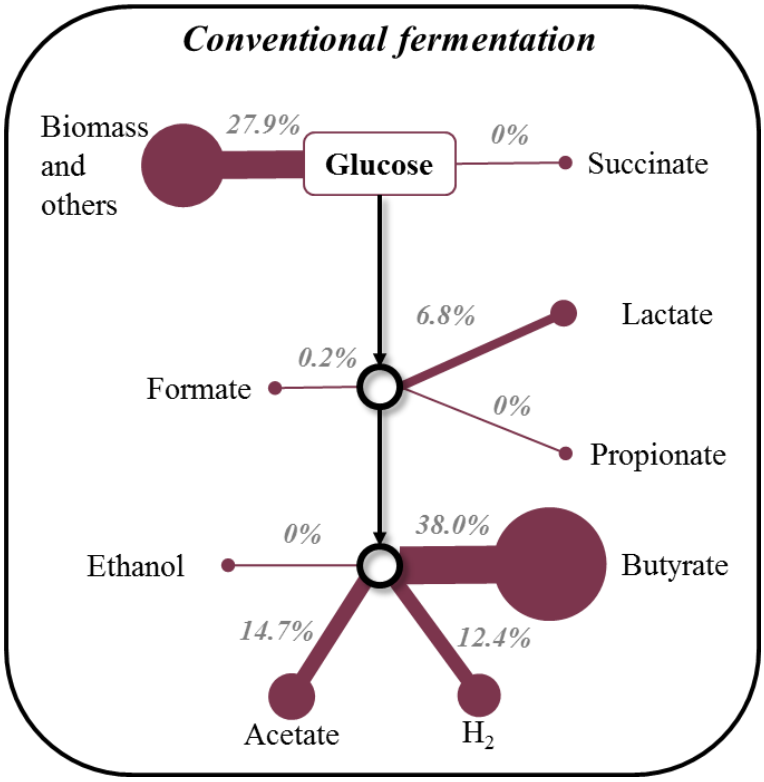


Fig. 5-2: Metabolite distribution observed during steady state of continuous operation when conventional fermentation (CF) was performed

5.2.3 Microbial community analysis and link with metabolic patterns

Microbial community analyses were performed with MiSeq sequencing. The samples were taken at different operational days. Fig. 5-3 shows the relative distribution of the major

families in the microbial communities. Before starting the continuous operation, a batch was performed for 24 hours to acclimatize the inoculum to glucose substrate and medium. In the case of CEF the electrodes were placed in the fermentation medium but was not connected to the potentiostat. In Fig. 5-3A, data from d2 until d17 correspond to CEF-A while data d25 and d26 to CEF-B. The microbial community after the batch period (d1) was more diverse in CEF than in CF, with a Simpson diversity index of 0.80 and 0.65 respectively. CF was dominated by *Clostridiaceae* and *Lactobacillaceae* families, representing 78.5 and 15.9% respectively. Meanwhile, CEF was dominated by *Clostridiaceae*, *Sporolactobacillaceae*, *Pseudomonadaceae* and *Streptococcaceae* families, representing 63.7, 11.8, 6.4 and 5.0% respectively. *Clostridiaceae* family has several well-known H_2 producing species [36], [37]. Species from *Lactobacillaceae*, *Sporolactobacillaceae* and *Streptococcaceae* family are lactate producers widely reported in literature and found in reactors during H_2 production by dark fermentation [10], [37], [181], [218]. More scarcely, species from the *Pseudomonadaceae* family have been reported in H_2-producing reactors mainly associated with the degradation of complex substrates [10], [37]. Interestingly, they have also been reported in electroactive biofilms [219], [220]. Probably species belonging to this family benefited from the presence of a conductive material in the fermentation medium, despite electrodes were not connected to the potentiostat. The literature reports that when conductive materials carbon-based are placed in anaerobic reactors, the microbial community composition and their interactions changes leading to increase the methane production, as well as the kinetic of degradation of volatile fatty acids [221], [222]. This process is known as direct interspecies electron transfer and has been widely documented and recently reviewed in Lovley (2017) [156]. Thus, conductive materials would act as efficient redox mediators [223]. However, here, only small differences were observed in initial batch between CF and CEF likely because of the initial microbial community structure.

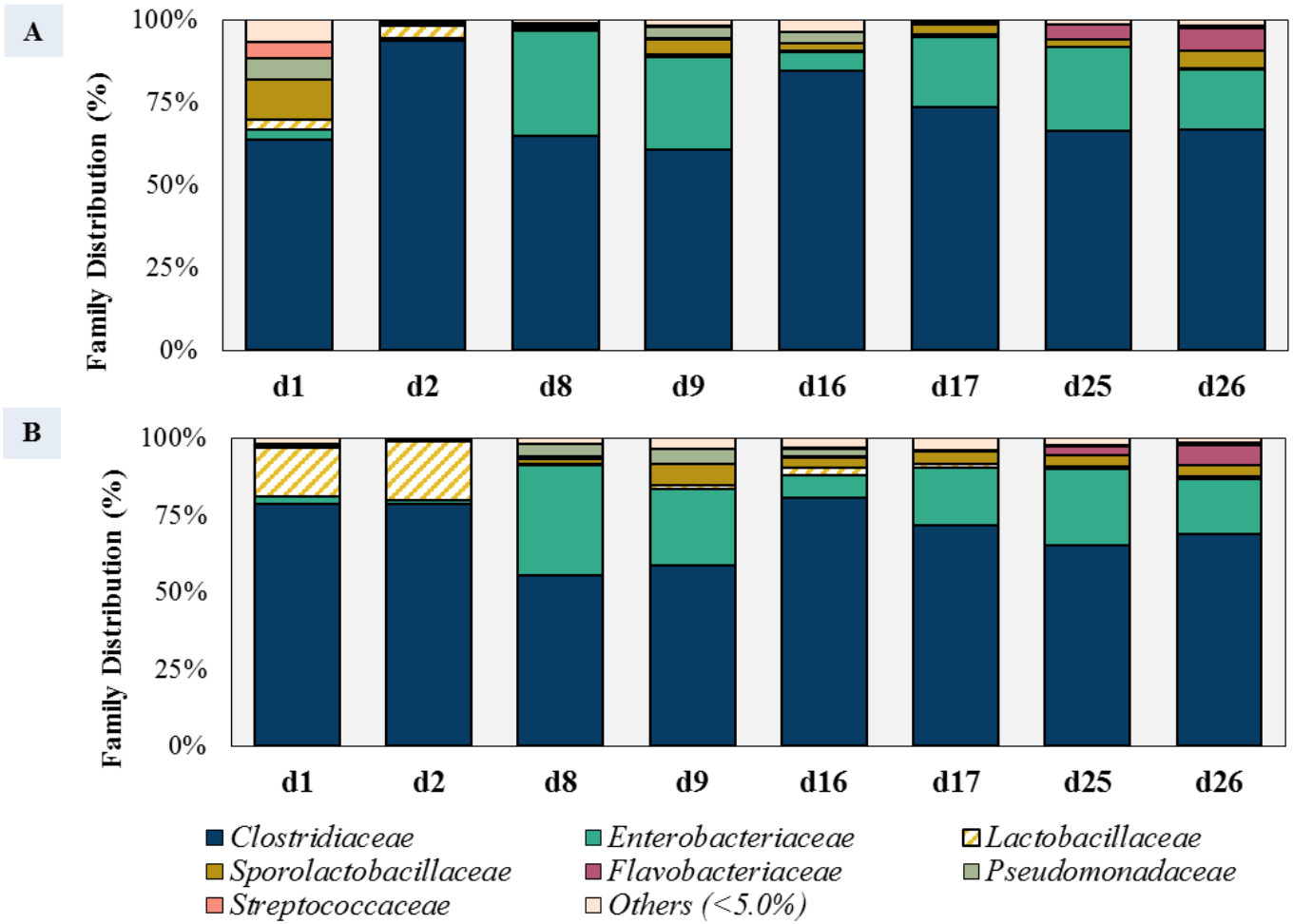


Fig. 5-3: Family distribution of microbial community observed during continuous operation of electro-fermentation and conventional fermentation.

Figure A *shows the electro-fermentation operation (CEF).* ***Figure B*** *Shows the conventional fermentation (CF) i.e. without electrodes.*

After the batch period, continuous operation was started, and the electrodes were connected to the potentiostat (CEF-A). After 24 hours of operation (d2, Fig. 5-3), *Clostridiaceae* family remained dominant in CF (78.5%) and CEF-A (93.8%). Particularly, *Clostridiaceae* was favored with the electrode polarization in CEF-A, displacing *Sporolactobacillaceae*, *Pseudomonadaceae* and *Streptococcaceae* families. This is consistent with the result observed in Chapter 4, where H_2-producing bacteria were favoured during the electro-fermentation tests, while that *Streptococcaceae* family was disadvantaged.

After 8 days of operation, *Enterobacteriaceae* family was favoured in CF, CEF-A and CEF-B, representing between 18.3% and 35.8% of microbial community, respectively. The only exception was day 16 where *Enterobacteriaceae* represented only between 5.4 and 7.0%. In fact, at day 9 a problem with the pH control led to system instability decreasing the H_2 production, with an increase in *Sporolactobacillaceae* was observed, when compared with d8.

PCA-right in Fig. 5-4, shows the relation between microbial community and metabolic patterns. Consistently, H_2 production was related to acetate and ethanol production, while Others+biomass (*i.e.* biomass production and unknown metabolite) and *Lactobacillaceae* family were opposite in the PCA, evidencing a negative correlation. Butyrate production was correlated with the *Clostridiaceae* family and anticorrelated with *Enterobacteriaceae* family, which is consistent with the literature because high butyrate production is often associated to members of *Clostridiaceae* as *C. tyrobutyricum* [10], [37], [146], [224].

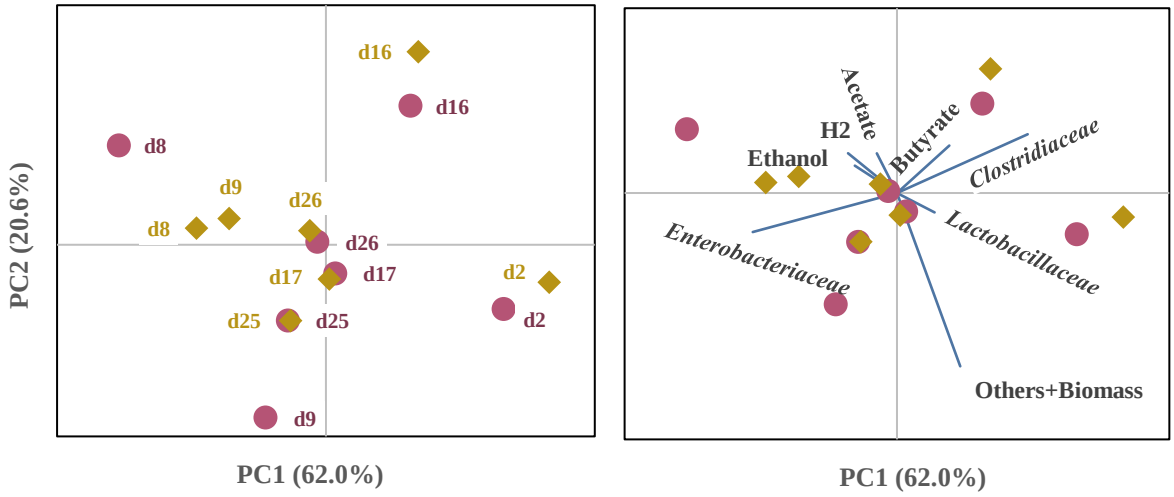


Fig. 5-4: Principal component analysis (PCA) based on microbial population distribution and metabolite production.

Left PCA *shows the operation days distribution for continuous conventional fermentation (CF) and electro-fermentation (CEF-A and CEF-B).* ***Right figure*** *shows a biplot representing correlations between PCA axes, taxonomic families and metabolic yields. Diamonds and circles represent operation of CF and CEF-A&CEF-B, respectively. Only are presented the families and metabolites that which showed stronger contributions to PCA axes.*

In general, no substantial difference was observed in the microbial community from d2 to d26 in all CF, CEF-A and CEF-B tests. This point is shown in the left PCA in Fig. 5-4, where operation days are independently grouped whatever if it is CF, CEF-A or CEF-B. Apparently, differences between electro-fermentation and *conventional* fermentation can only be evidenced during a batch operation, as shown in this section and observed in Chapter 4. Probably, the microbial selection pressure imposed by the HRT was pre-dominant over the selection pressure made by the polarized electrodes. Indeed, HRT is directly related with maximum growth rate (μ_{max}) allowing to maintain in the reactor the microorganisms that can grow faster than the rate fixed by the HRT value. The HRT used here (*i.e.* 6.7h) is in the range of typically applied values for H_2 production in suspend biomass systems as CSTRs [11], being considered efficient to select H_2 producing bacteria from glucose, while the methanogenic activity is often negligible. However, possible selection of electroactive

bacteria due to polarized electrode seems to be complicated, maybe because electroactive bacteria grow slower than fermentative bacteria in these conditions and higher HRT values would be necessary [149], [225], [226].

5.3 Glucose electro-fermentation effect is influenced by inoculum microbial community

5.3.1 Context and specific experimental methodology

In this section, the inoculum source was evaluated for its influence on the electro-fermentation. For that, 5 different inocula (Table 5-1) were tested at –0.4 V *vs* SCE using as working and counter electrodes grids of 90% platinum – 10% iridium with a size of 3.5 x 3.5 cm^2. Particularly, the experiments performed with inoculum InA correspond to results showed in Chapter 4 (EF, –0.4V). However, they are shown here for a better comparison with the results obtained using the others inoculum source.

Table 5-1: Inoculum from different sources used during glucose electro-fermentation

Inoculum	Source	Inoculum biomass ($g_{VS}.l^{-1}$)
InA	Heat-treated anaerobic sludge sampled from a lab-scale anaerobic digester treating sewage sludge	37.7
InB	Activated sludge sampled from sewage treatment plant in Narbonne	10.0
InC	Acidogenic sludge sampled from H_2-producing reactor fed with glucose	1.5
InD	Heat-treated anaerobic sludge sampled from a lab-scale anaerobic digester treating food waste.	7.1
InE	Anaerobic sludge sampled from a lab-scale anaerobic digester treating volatile fatty acids	9.5

After batch operation, the glucose was totally consumed in all reactors (5.3±0.2 $gCOD.l^{-1}$). Total COD mass balance calculated from soluble products and H_2 gas ranged between 72.5% and 87.7%, except for Inoculum D that was only 42.1±1.2% where probably the most metabolites were unknown. Approximately 10-15% of the missing COD was likely corresponding to biomass growth. While the rest of the COD correspond to unknown metabolites.

5.3.2 Hydrogen and metabolite production

Fig. 5-5 shows the H_2 yields obtained during conventional fermentation (F) and electro-fermentation (EF) tests for each inoculum. When InA was used, H_2 production was favoured in EF (1.80±0.31 $molH_2.mol^{-1}_{glucose}$) with respect to F (0.70±0.12 $molH_2.mol^{-1}_{glucose}$). In contrast, when InB was used, H_2 production was disfavoured in EF (0.57±0.17 $molH_2.mol^{-1}_{glucose}$) compared to F (1.14±0.09 $molH_2.mol^{-1}_{glucose}$). Interestingly, no difference was observed in H_2 production between EF and F when InC, InD and InE were used as inoculum. The H_2 yields obtained were of 0.88±0.02 $molH_2.mol^{-1}_{glucose}$, 0.49±0.01 $molH_2.mol^{-1}_{glucose}$ and 0.73±0.004 $molH_2.mol^{-1}_{glucose}$ respectively.

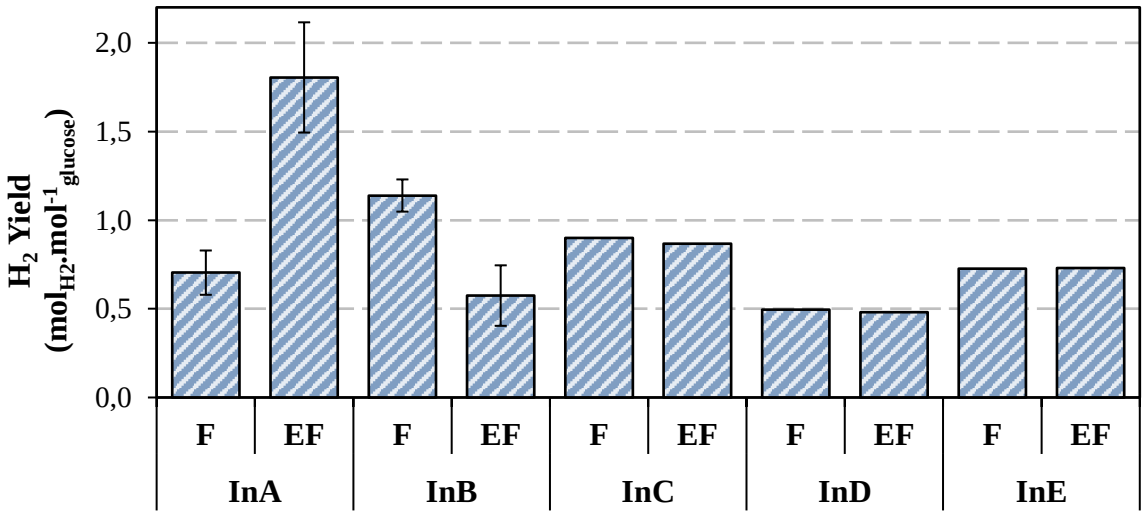


Fig. 5-5: Hydrogen yields during glucose electro-fermentation using different inoculum

In addition, Fig. 5-6 shows the metabolic distribution for each inoculum used. When InA was used, butyrate (19.0±10.4%$_{COD}$), acetate (14.3±1.2%$_{COD}$) and ethanol (22.7±2.4%$_{COD}$) increased during EF, with regards to F where the main metabolite was lactate (56.9±2.1%$_{COD}$). When InB was used, the main metabolites during EF were butyrate (26.8±9.2%$_{COD}$) and lactate (25.7±33.7%$_{COD}$), while in F, butyrate reached 57.5±0.1%$_{COD}$. In this case, a large difference was observed in lactate and propionate production between the EF duplicates. However, there are species that can consume lactate to produce propionate, which could explain the great variability of these two metabolites in the duplicates [16].

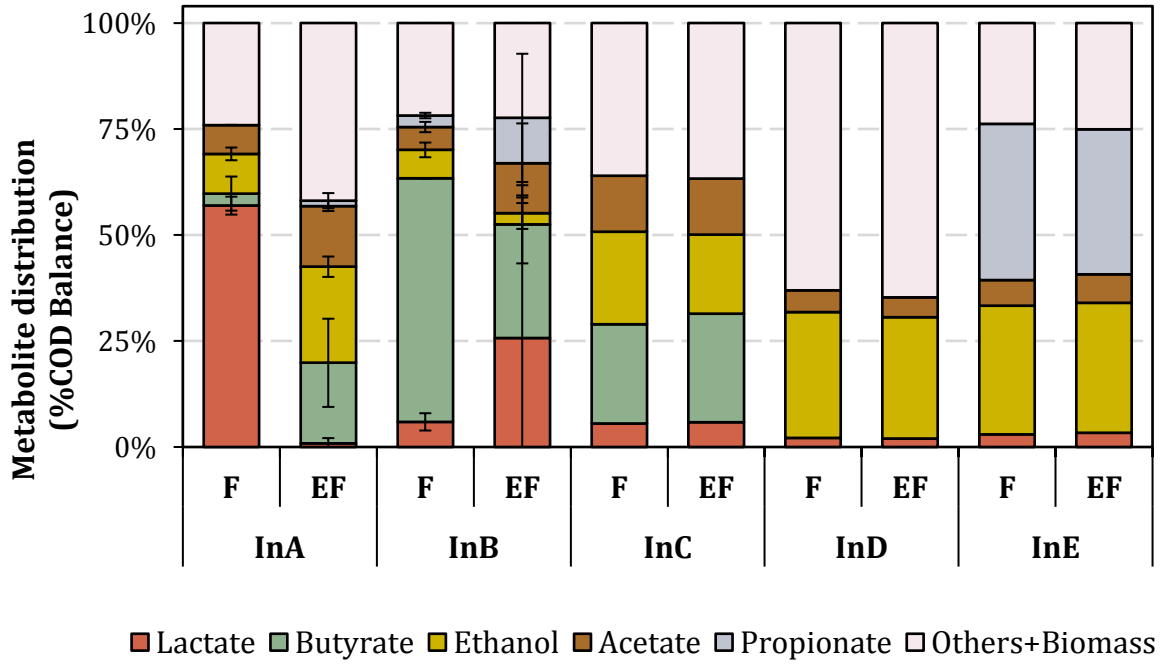


Fig. 5-6: Metabolite distribution during glucose electro-fermentation using different inoculum

When InC, InD and InE were used, not difference between EF and F was observed with respect to the metabolic patterns. In InC, the main metabolites were butyrate (24.5±1.6%$_{COD}$) and ethanol (20.2±2.2%$_{COD}$). While, ethanol (29.2±0.8%$_{COD}$) was predominant in InD, an

important part of the COD was attributed to Others+Biomass (57.9±1.2%$_{COD}$). In InE, the main metabolites were propionate (35.5±1.8%$_{COD}$) and ethanol (30.5±0.2%$_{COD}$).

In general, maximal H_2 yields yield were associated with butyrate production (InA-EF and InB-F), as well as decreased in lactate. Consistently, the literature often refers to high H_2 yields when butyrate and acetate are the most important metabolites [9], [34], [224]. While lactate production is commonly associated with lower H_2 yields because its production leads to the consumption NADH *i.e.* allows the cells to release electron excess [9]. Thus, the conversion of NADH into additional H_2 production by *Clostridia* species is constrained [28].

5.3.3 Link between final microbial community and metabolite patterns

Microbial communities present at the end of the batch were analysed. Fig. 5-7 shows the distribution of microbial families, evidencing that in all cases the main families were *Enterobacteriaceae*, *Streptococcaceae* and *Clostridiaceae*. In InA *Streptococcaceae* family was dominating during F (57.9±5.1%). However, during EF was replaced by *Enterobacteriaceae* (57.7±12.1%) and *Clostridiaceae* (36.5±5.5%). In InB, the *Streptococcaceae* family was dominant in both F (42.1±3.2%) and EF (56.2±23.7%). Despite the differences observed in the duplicates in EF, a lower relative abundance of *Enterobacteriaceae* (10.6±4.7%) and *Clostridiaceae* (23.7±19.9%) than in F was observed. In InC, InD and InE the main family was *Enterobacteriaceae* for EF and F, representing 86.0±5.6%, 95.3±0.3% and 97.9±0.9%, respectively.

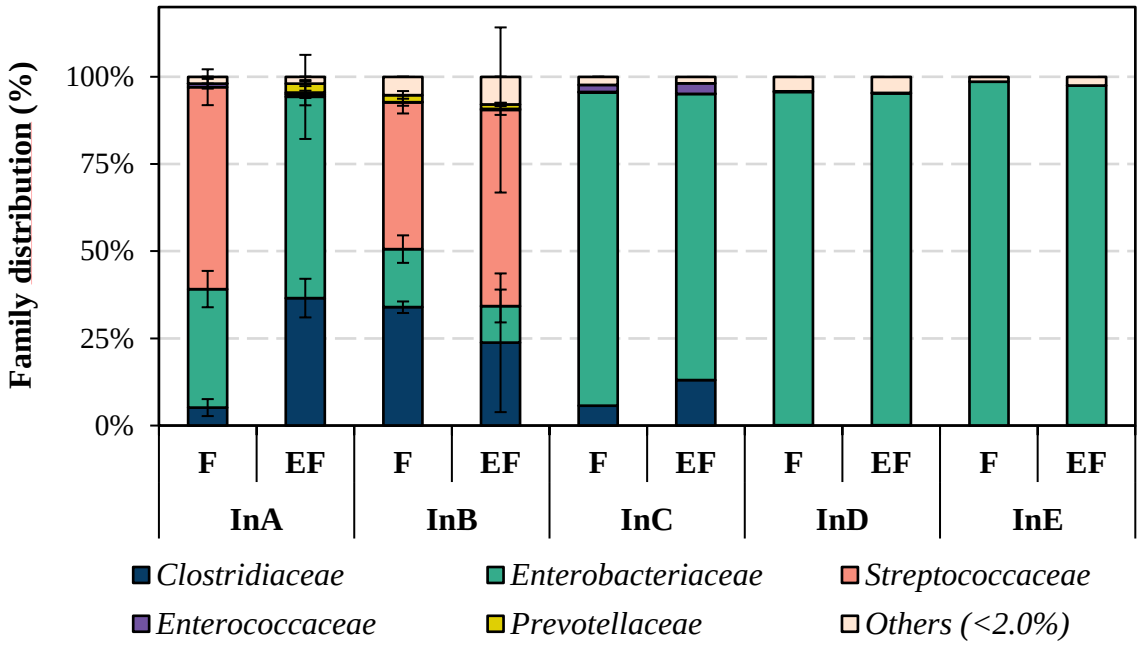


Fig. 5-7: Family distribution of final microbial community from glucose electro-fermentation using different inoculum

*Five inoculum from different sources were compared during Electro-fermentation **(EF)** under the same operational conditions. Besides conventional fermentation **(F)** was performed as control without electrodes.*

For better visualization and comparison of all reactors, a PCA analysis was performed using variance-covariance matrices from both metabolic patterns and final family distribution (Fig. 5-8). All data from F and EF were used in the analysis, however, only the biplot representation shows the most important variables in the components determination. The PCA shows that tests performed using InC, InD and InD are grouped in the left side. Consistently, none of these inoculums showed significant differences between F and EF respect to metabolic patterns. Besides, these reactors were related to the emergence of members of the *Enterobacteriaceae* family and with the production of ethanol and others+biomass.

At the right side of the PCA, the reactors showed some effect during EF. On the right above is InA-EF and along the horizontal axis is InB-F. Both are related to H_2 yield, *Clostridiaceae*

family and butyrate production. On the right below are InA-F and InB-EF related to lactate production and *Streptococcaceae*.

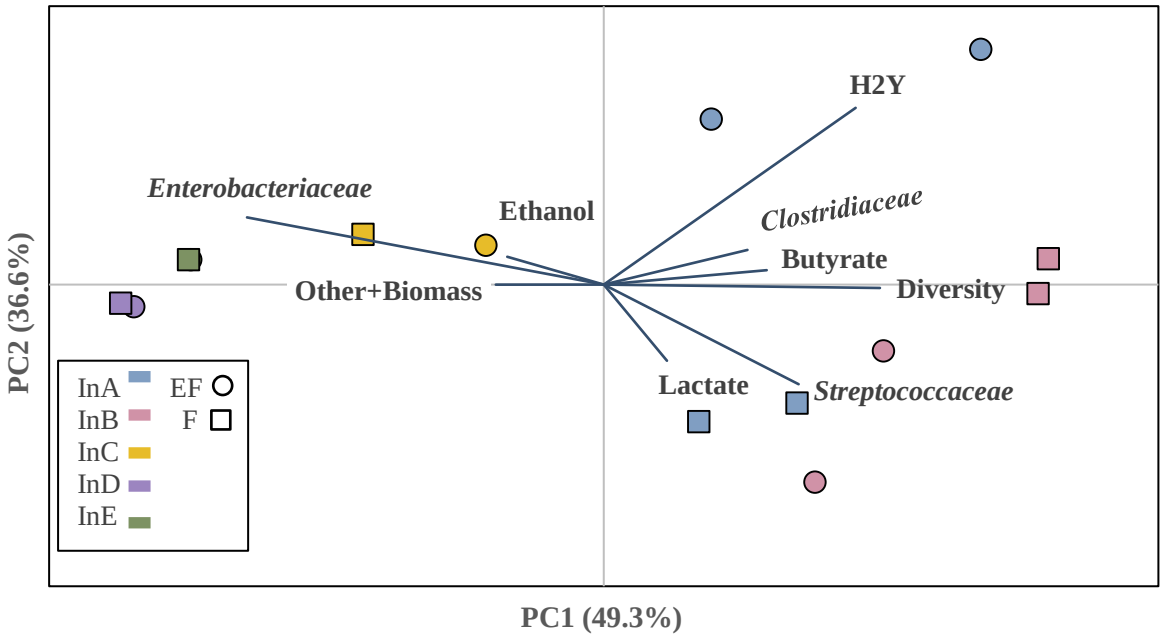


Fig. 5-8: Principal component analysis (PCA) performed with variance-covariance matrix from microbial population distribution after batch operation and metabolic patterns

Pearson test was performed to evaluate the significant correlations existing between metabolic patterns and final microbial community structures (Table 5-2Table 5-2). H_2 yield positively correlated with *Clostridiaceae* and *Prevotellaceae* families. As it has already been discussed, high H_2 yields are commonly associated with members of the *Clostridiaceae* family [37], [227]. *Prevotellaceae* has been widely reported as a subdominant species in H_2 production reactors, and its function is not entirely clear. *Prevotellaceae* could contribute to the decomposition of complex substrates, but they may also be competing for the substrate [37], [228]. Butyrate production is positively correlated with microbial diversity of *Clostridiaceae* and *Prevotellaceae*, and negatively correlated with *Enterobacteriaceae*. This is consistent with literature, since butyrate production is a typical metabolic product of

Clostridia spp. but not of *Enterobacter spp.* [15]. Lactate production is positively correlated with *Streptococcaceae* and negatively with ethanol production. *Streptococcaceae* family is known as lactate producers commonly reported in H_2 production reactors [37], [199], [229]. Particularly, lactate production could be negatively correlated with ethanol production because they are produced by bacteria that commonly outcompete for the substrate [37].

Finally, ethanol production was positively correlated with *Enterobacteriaceae* and negatively with butyrate, microbial diversity and *Streptococcaceae* family. Ethanol production could be linked to solventogenic fermentation by *Clostridia spp.* Some species from the *Enterobacteriaceae* family can produce it during the H_2 production, as evidenced by our results [128], [181], [230]. On the other hand, ethanol was an important metabolite when InC, InD and InE were used, which at the end of operation had a low microbial diversity because *Enterobacteriaceae* family was widely dominating.

Table 5-2: Pearson correlation matrix from microbial population distribution after batch operation and metabolic pattern using different inoculum

	H2Y	Succinate	Lactate	Ethanol	Acetate	Butyrate	Other+Biomass	Diversity	*Enterobacteriaceae*	*Streptococcaceae*	*Clostridiaceae*	*Prevotellaceae*
H2Y		0.41	-0.35	-0.05	0.49	0.40	-0.35	0.44	-0.14	-0.26	***0.74***	***0.67***
Succinate	0.41		-0.49	***0.73***	0.29	-0.39	0.29	-0.49	***0.66***	***-0.77***	-0.05	-0.14
Lactate	-0.35	-0.49		***-0.57***	-0.33	-0.24	-0.22	0.23	-0.48	***0.79***	-0.31	-0.30
Ethanol	-0.05	***0.73***	***-0.57***		-0.03	***-0.60***	0.53	***-0.80***	***0.95***	***-0.91***	-0.44	-0.25
Acetate	0.49	0.29	-0.33	-0.03		0.20	-0.23	0.32	-0.05	-0.27	***0.54***	0.42
Butyrate	0.40	-0.39	-0.24	***-0.60***	0.20		-0.48	***0.73***	***-0.62***	0.30	***0.76***	***0.56***
Other+Biomass	-0.35	0.29	-0.22	0.53	-0.23	-0.48		***-0.54***	***0.56***	-0.44	-0.45	-0.38
Diversity	0.44	-0.49	0.23	***-0.80***	0.32	***0.73***	***-0.54***		***-0.86***	***0.59***	***0.80***	***0.57***
Enterobacteriaceae	-0.14	***0.66***	-0.48	***0.95***	-0.05	***-0.62***	***0.56***	***-0.86***		***-0.87***	***-0.60***	-0.43
Streptococcaceae	-0.26	***-0.77***	***0.79***	***-0.91***	-0.27	0.30	-0.44	***0.59***	***-0.87***		0.14	0.03
Clostridiaceae	***0.74***	-0.05	-0.31	-0.44	***0.54***	***0.76***	-0.45	***0.80***	***-0.60***	0.14		***0.77***
Prevotellaceae	***0.67***	-0.14	-0.30	-0.25	0.42	***0.56***	-0.38	***0.57***	-0.43	0.03	***0.77***	

All data were used including F and EF in duplicates when correspond. In bold were marked the significant correlations with p-values ≤0.05 (*) and p-values ≤0.01 (**). Positive (■ for 1.0), negative (■ for –1.0) and null (□ for zero) correlations were marked with gradient colour depending on value

Table 5-2 also provides interesting information about ecological interactions occurring between species. Thus, *Enterobacteriaceae* is negatively correlated with *Clostridiaceae* and *Streptococcaceae*, evidencing a possible competitive interaction. Besides, *Clostridiaceae* is positively correlated with *Prevotellaceae* suggesting a cooperation interaction between both families.

5.3.4 Inoculum source is influencing the glucose electro-fermentation effect

Bacterial communities of the inoculum were also studied with the objective to study how the microbial distribution was affecting the EF. Fig. 5-9 shows the phylum distribution of the microbial communities of all inocula. The dominant phylum in InA, InC and InD was Firmicutes, representing 45.6±2.6%, 96.5±2.3% and 56.2±0.9%, respectively. Meanwhile InB and InE were dominated by Proteobacteria (48.4±3.8%) and Bacteroidetes (40.5±0.3%), respectively. Firmicutes are commonly linked to H_2 production systems mainly for *Clostridia spp.* and *Bacillus spp*. [123]. Besides, Firmicutes play a very important role in anaerobic environments such as in the lakes depths and landfill sediments, associated with the degradation of organic matter [231]. Species belonging to Proteobacteria have also been widely studied in the H_2 production, thanks to their ability to maintain anaerobic conditions in the reactors (*e.g.* Enterobacter spp.) [217], [231], [232]. Proteobacteria are widely reported in anaerobic sludge, aerobic sludge and food waste [231]. Bacteroidetes have also been reported in H_2-producing reactors, but since their function is not clearly elucidated since it has not received a great attention [37].

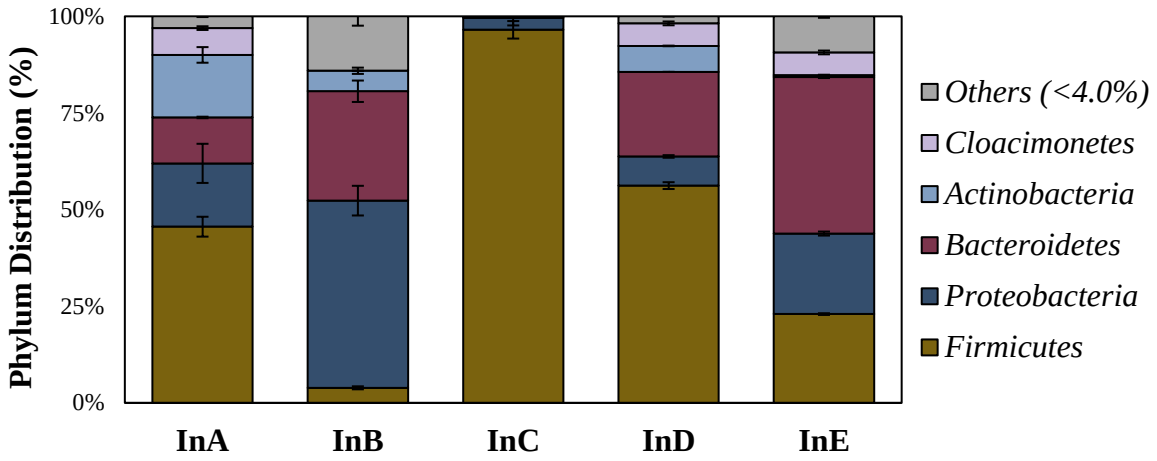


Fig. 5-9: Phylum distribution of microbial community in the inoculum from different sources

Family distribution of the microbial community from the different inoculum is shown in Fig. 5-10. Except for InC, most of the families represented less than 10% of the microbial community. Dominant family in InA was *Clostridiaceae* representing 17.6±1.6%. As this inoculum was heat pre-treated, it is expected that spore-forming bacteria were preferentially selected [39], [233], [234]. In InB the dominant families were *Saprospiraceae* and *Rhodocyclaceae*, representing 14.6±1.4% and 11.3±0.6%, respectively. Both families have been reported in wastewater treatment systems, performing important functions such as the degradation of complex organic matter and in denitrification processes, respectively [235]–[237]. In InC, *Sporolactobacillaceae* was dominant, representing 83.8±2.4%. Generally, this lactate-producing bacteria is not dominant in H_2-producing reactors. However, since the inoculum was taken from the reactor outlet storage, probably uncontrolled pH conditions favoured its abundance. In InD, the dominant families were *group_MBA03* (Clostridia), *Bacteroidaceae* and *Ruminococcaceae*, representing 15.8±0.3%, 13.9±0.1% and 13.3±0.2%, respectively. Particularly uncultured *group_MBA03* (Clostridia), has recently been reported in an anaerobic reactor processing wastewater with high oil content [238]. Although the

Bacteroidaceae family is not spore-forming, it survived the heat pre-treatment performed in InD, as already been reported in literature [61]. In the case of *Ruminococcaceae* family, this family has been reported with an important hydrolytic activity when complex substrates are used during H_2 production [9], [234]. Finally, uncultured *group_vadinHA17* (Bacteroidetes) family was dominant in InE, representing 19.5±0.4%. Particularly, this family has been reported as dominant in a UASB reactor treating poultry slaughterhouse wastewater [239].

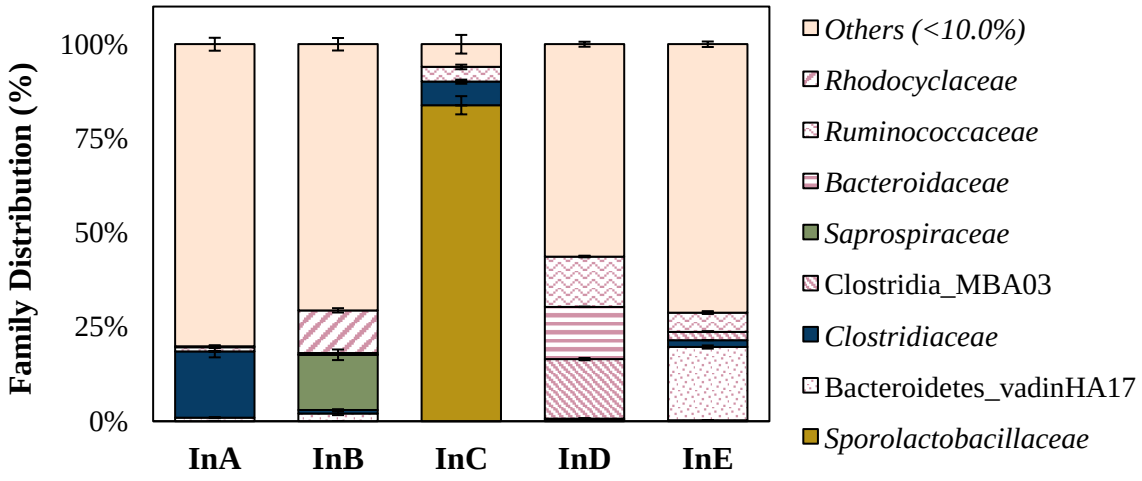


Fig. 5-10: Family distribution of microbial community in the inoculum from different sources

Below are some statistical analyses to determine significantly how the inoculum source is affecting the metabolic patterns and the final microbial community, as well as which members of the inoculum microbial community are key in determining the final EF behaviour.

First, a Mantel test was performed to evaluate the correlation existing between the initial inoculum microbial community, the final metabolites produced and the final microbial community. For that, three matrices with Euclidean distance data were prepared, including *conventional* fermentation (F) and electro-fermentation (EF) data. As observed in Table 5-3, the only positive lineal correlation of r_M= 0.283 (Pearson correlation) with a significance of

p_{value}= 0.004, was found between metabolites produced matrix and final microbial community matrix.

Table 5-3: Mantel tests performed with Euclidean distance matrix from inoculum microbial composition, metabolite production and final microbial composition

	Inoculum microbial community	**Metabolic patterns**	**Final microbial community**
Inoculum microbial community		Z_M=56.3 r_M= –0.179 p_{value}=0.300	Z_M=46.9 r_M= 0.070 p_{value}=0.458
Metabolic patterns			**Z_M=89.0** **r_M=0.283** **p_{value}=0.004**
Final microbial community			

***Z_M** is the Mantel statistic; **r_M** value is simply the Pearson's correlation coefficient and ranges from – 1.0 to + 1.0. Significance of the test was calculated from 9999 permutation. In this thesis a **p-value** <0.05 was considered statically significant to refuse the null hypothesis.*

Then, to know whether the inoculum microbial community distance matrix was affecting the correlation between the other two matrices *i.e.* metabolites produced and final microbial community, a Partial Mantel test was performed. Interestingly, this new test shows that the inoculum source affected the correlation between final microbial community and the metabolites produced (Z_M= 6.33, r_M= 0.301 and p_{value}= 0.003).

As the inoculum source influences the effect of electro-fermentation, it is interesting to find which are the bacterial families that mainly contribute to the differences and similarities between inocula. To start, a Non-metric multidimensional scaling (NMDS) was performed with Bray Curtis similarity index matrix from inoculum data (Fig. 5-11). This figure shows that inocula are separated depending on the source, through their microbial community distances.

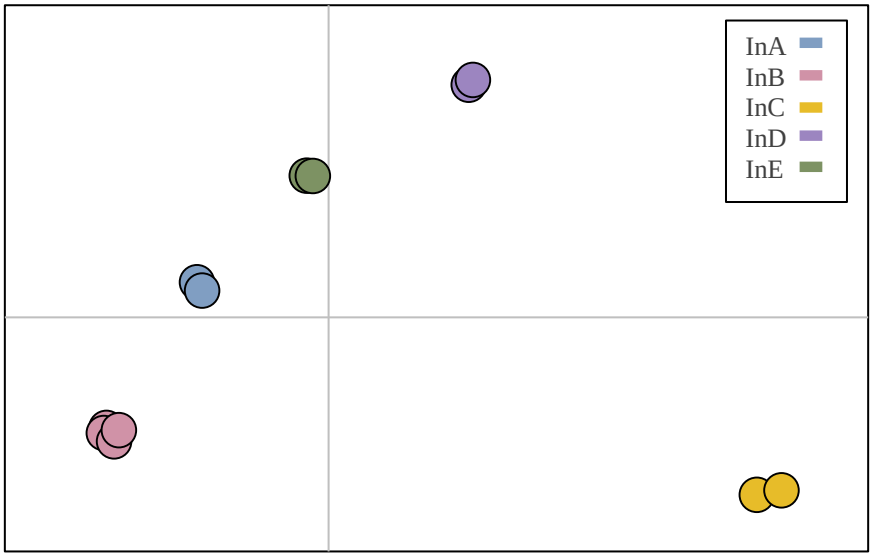


Fig. 5-11: Non-metric multidimensional scaling (NMDS) performed with Bray Curtis Similarity index from inoculum data.

Different points for the same inoculum correspond to the number of replicates analysed. All inocula were tested in duplicate, except for InB which was quadruplicate.

Then, to determine whether the differences between the different inoculum sources (Fig. 5-11) are statistically significant, an analysis of similarities was made, ANOSIM. This analysis was performed with 9999 permutations using Bray Curtis similarity index, reporting a mean rank within inoculum of 5.5, mean rank between inoculum of 38.5, R=1 and p_{value}=0.0001. According to this analysis, when R is close to 1, more differences exist between the analysed data, *i.e.* in our case the inocula. Thus, it is possible to conclude that the differences observed in Fig. 5-11 are statistically significative, *i.e.* all inocula used are significative different.

Based on H_2 production results, 3 groups can be made according to the effect observed during EF: positive effect (E_P; InA), negative effect (E_N; InB) and neutral effect (E_0; InC, InD and InE). In this context, studying the similarities and differences between bacterial communities could reveal what are the key bacteria determining the effect of electro-fermentation. For

that, a similarity of percentages (SIMPER) test was performed to determine which families was mainly contributing to the differences between inoculum and in what percentage (Table 5-4).

Table 5-4: Similarity of percentage analysis (SIMPER) performed to compare the family microbial composition of all inoculum data.

	E_N & E_0		E_P & E_0		E_N & E_P	
Family	**Dissim. contrib. %**	Total %	**Dissim. contrib. %**	Total %	**Dissim. contrib. %**	Total %
Sporolactobacillaceae	**22.3**	22.3	**23.4**	23.4	0.0	0.0
Clostridiaceae	1.8	24.1	**7.3**	30.7	**12.2**	12.2
Saprospiraceae	**6.3**	30.4	0.0	30.7	**10.7**	22.9
Rhodocyclaceae	4.9	35.3	0.1	30.8	**8.1**	31.0
Bacteroidetes vadinHA17	**5.6**	40.9	**5.7**	36.5	0.8	31.8
Family_XI	1.5	42.4	3.2	39.7	**5.6**	37.4
MBA03	4.6	47.0	4.8	44.5	0.0	37.4
Unknown	1.9	48.9	3.0	47.4	4.3	41.7
Ruminococcaceae	4.2	53.1	4.1	51.5	0.5	42.2
Draconibacteriaceae	1.1	54.2	2.5	54.0	4.1	46.2
Planococcaceae	0.1	54.3	2.4	56.4	4.1	50.3
Bacteroidaceae	3.8	58.1	3.9	60.4	0.0	50.3
Christensenellaceae	0.1	58.2	1.8	62.2	3.0	53.3
Enterobacteriaceae	0.6	58.7	2.1	64.3	3.0	56.3
Peptostreptococcaceae	1.0	59.7	2.0	66.3	2.9	59.2
uncultured	1.8	61.5	0.4	66.7	2.8	62.1
*Acidimicrobiales_I.S.**	0.3	61.8	1.8	68.5	2.6	64.6
Rikenellaceae	2.3	64.1	2.6	71.1	1.2	65.9
Rhodospirillaceae	1.5	65.5	0.0	71.1	2.5	68.4
Porphyromonadaceae	2.4	68.0	2.3	73.4	1.2	69.6
Intrasporangiaceae	0.2	68.1	1.5	74.8	2.2	71.8
Desulfuromonadaceae	1.9	70.1	2.0	76.9	0.0	71.8
*Xanthomonadales_I.S.**	1.2	71.3	0.0	76.9	2.0	73.8

I.S: *abreviation of Incertae Sedis.* **E_N:** *negative effect.* **E_P:** *positive effect.* **E_0:** *neutral effect.* **Dissim. contrib. %** *correspond to percentage that each family is contributing to dissimilarity between the groups compared.* **Total %** *correspond to accumulative contribution of each family to dissimilarity percentage.*

Table 5-4 summarizes the results obtained in the SIMPER test, showing only families with a contribution to dissimilarity ≥2.0%. Comparing E_N and E_0, a dissimilarity of 92.7% was

observed, mainly due to *Sporolactobacillaceae* (22.3%) and *Saprospiraceae* (6.3%). *Sporolactobacillaceae* was present in E_0 and abundantly in InC but was absent in E_N. Well-known families producing H_2 such as *Enterobacteriaceae* and *Clostridiaceae* are found in very low concentrations in both inoculum groups and contributing less than 2.0% in the dissimilarity of these groups.

By comparing E_P and E_0 a dissimilarity of 84.3% was observed, determined mainly by *Sporolactobacillaceae* (23.4%) and *Clostridiaceae* (7.3%) families. *Clostridiaceae* family is present in E_P with a greater abundance than E_0, while *Sporolactobacillaceae* is only present in E_0. Besides, *Enterobacteriaceae* family is contributing with 2.1% in dissimilarity between these two groups. By comparing E_P y E_N a dissimilarity of 85.6% was observed, determined mainly by *Clostridiaceae* (12.2%) and *Saprospiraceae* (10.7%) families. Besides *Enterobacteriaceae* family is contributing with 3.0% in dissimilarity between these two inoculum groups. In particular, *Clostridiaceae* family was more abundant in E_p inoculum.

From the statistical analyses it can be concluded that the members of the *Clostridiaceae* family are key. Because the *Clostridiaceae* family was negligible in the InC, InD and InE inocula, no effect of the polarized electrodes was observed. However, members of this family were significantly present in InA and InB, but with a higher relative abundance in InA leading to this inoculum causing a positive effect due to polarized electrodes, increasing the production of H_2.

In short, our findings are insufficient to ensure that polarized electrodes have a special effect on *Clostridia* species, but their relative abundance in the inoculum plays a crucial role.

5.4 Conclusion

In this Chapter was studied the influence of initial microbial community during the electro-fermentation operated in both continuous and batch mode.

Our results evidence that electro-fermentation had only an impact on the initial bacterial community selection that is lost in continuous operation. The effect of selection of H_2-producing bacteria in presence of polarized electrodes observed in Chapter 4 was not predominant, maybe because the selection pressure was more important by imposing the hydraulic retention time. However, different inoculum source were used in both cases, which could explain different EF behaviour.

Thereafter, inocula from different sources showed a significant impact on the existing correlations between final microbial community and metabolic patterns. The relative abundance of H_2-producing bacteria, especially *Clostridiaceae* family, in the initial inoculum seems to be a determining parameter affecting the final electro-fermentation behaviour.

CHAPTER 6
Investigating the electro-fermentation mechanisms by studying microbial interactions

6.1 Introduction

As seen in the previous chapters, electro-fermentation is a promising control tool for bioprocesses that depends on the bacterial community composition and structure. However, the mechanism of actions behind the selection of hydrogen-producing bacteria that contribute to the changes in metabolic pathways are still unknown. One hypothesis could be that electroactive bacteria, such as *G. sulfurreducens*, play a key role in transferring electrons, even though they were subdominant species. Electroactive bacteria can take/release electrons directly from/toward the polarized electrodes, while at the same time they establish different types of interactions within the bacterial community in the bulk. But, many of the possible interactions that can occur are unknown to date [18].

In this context, this chapter aims to study the metabolic pattern changes in presence of *G. sulfurreducens*, during conventional dark fermentation and electro-fermentation. Specific objectives are:

i. Study the changes in metabolic pathways during glucose dark fermentation when a mixed culture is enriched in *G. sulfurreducens*.
ii. Study the influence on metabolic pattern during glucose electro-fermentation when a electrode is precolonized with *G. sulfurreducens*.

6.2 Increased fermentative hydrogen production using acidogenic sludge as inoculum enriched with *G. sulfurreducens*

6.2.1 Context and specific experimental methodology

In this section, the effect on metabolic patterns, mainly focused on H_2 production by dark fermentation, of an inoculum enriched in electroactive bacteria was investigated. In this case acidogenic sludge was used as inoculum and was previously enriched with *G. sulfurreducens* (**MG**) at the ratio 1:1. As control, non-enriched inoculum from the same source, was used (**M**). All tests were performed in 100 ml bottles in quadruplicate using glucose and acetate as carbon source.

6.2.2 Hydrogen production and metabolite distribution

After 24 hours of batch operation, the glucose was totally consumed in all reactors (4.6±0.03 $gCOD.l^{-1}$). Total COD mass balance calculated from soluble products and H_2 gas ranged between 81.7 and 88.8%. The biomass concentration at the end of operation was 0.54±0.05 $gVSS.l^{-1}$ and 0.40±0.03 $gVSS.l^{-1}$ for MG and M, respectively.

H_2 production increased by 55.5% in MG (0.28±0.04 $molH_2.mol^{-1}_{glucose}$) with respect to M (0.18±0.01 $molH_2.mol^{-1}_{glucose}$). Despite the increase, only 7.0% of the theoretical maximum yield was reached [15]. The low H_2-yields observed are related with the high alcohol production, including 2,3-butanediol (2,3-BDO) and ethanol (Fig. 6-1). In M tests 2,3-BDO and ethanol represented 38.4±4.3%$_{COD}$ and 23.9±1.7%$_{COD}$, respectively. However, during MG tests metabolic flux changed toward a higher butyrate production (16.7±0.9%$_{COD}$), decreasing the proportion of 2,3-BDO (23.1±0.5%$_{COD}$). Acetate was also produced,

representing 17.1±0.5%$_{COD}$ for M and MG. Lactate, succinate and propionate were detected, but at only very low concentrations (<3.5%$_{COD}$).

Ethanol and 2.3-BDO are more reduced than glucose, so their production is an alternative pathway to release the excess electrons from the cell catabolism. Consequently, H_2 yields are lowered because acetate was not the main pathway for equilibrating the intracellular redox [16], [49].

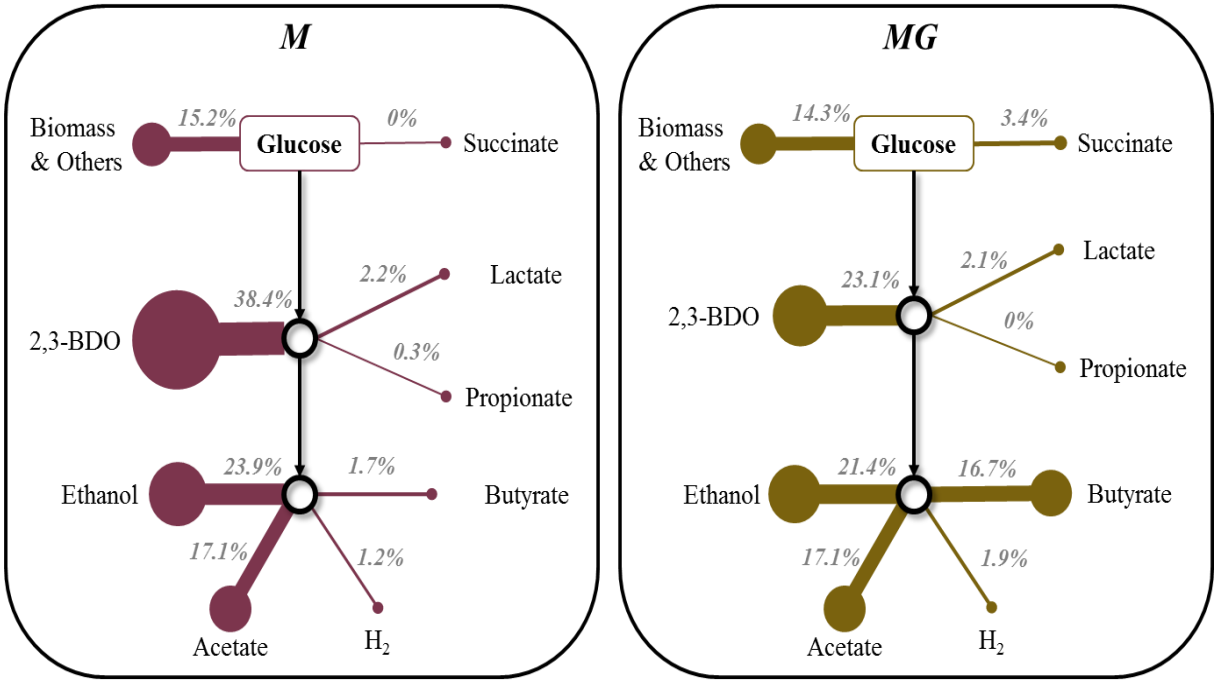


Fig. 6-1: Metabolite distribution observed during dark fermentation using acidogenic sludge as inoculum enriched with *G. sulfurreducens*

***M**: wild inoculum. **MG**: inoculum enriched with G. sulfurreducens. The figures represent the average of all replicates for each test. The lines size is proportional to %$_{COD}$ represented by each metabolite.*

6.2.3 Microbial community analysis and its link with the metabolic patterns

Microbial community analysis of the inoculum and after batch operation were performed. Fig. 6-2 shows the family distribution of the microbial communities. In the inoculum (M.i)

Enterobacteriaceae and *Clostridiaceae* were dominant, representing 73.8±2.1% and 14.9±1.6%, respectively. After inoculum was enriched with *G. sulfurreducens* (MG.i), the microbial community distribution was as follows: *Enterobacteriaceae* (54.8±1.3%), *Geobacteraceae* (31.0±1.6%) and *Clostridiaceae* (8.5±1.3%).

After 24 hours of operation, the microbial community in M (M.f) tests was similar to the inoculum and *Enterobacteriaceae* (69.3±5.7%) and *Clostridiaceae* (23.9±5.8%) remained dominants. However, in MG tests *Clostridiaceae* became dominant, representing 52.7±0.9%. While *Enterobacteriaceae* (38.7±0.8%) and *Geobacteraceae* (1.9±0.1%) decreased in terms of relative abundance.

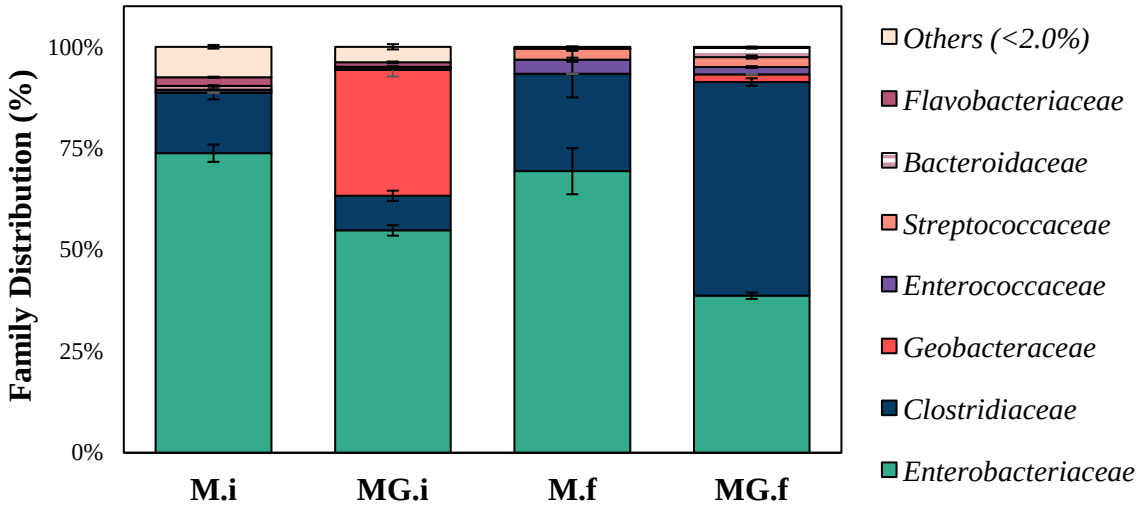


Fig. 6-2: Family distribution of microbial community during dark fermentation using acidogenic sludge as inoculum enriched with *G. sulfurreducens*.

***M**: wild inoculum. **MG**: inoculum enriched with G. sulfurreducens. The samples taken before and after fermentation are marked with "i" and "f", respectively. The figures represent the average of all replicates for each test. Error bars are representing the standard deviation.*

Significative correlations between metabolic patterns and final microbial community were statistically analysed with a Pearson correlation test (Table 6-1). H_2 production positively correlated with *Geobacteraceae* and *Clostridiaceae* abundances and negatively with

Enterobacteriaceae. Butyrate production was positively correlated with H_2 production, *Geobacteraceae* and *Clostridiaceae,* while negatively with *Enterobacteriaceae*. As observed in previous chapters and consistently with the literature, high productions of H_2 along with butyrate, are associated to an increase in the relative abundance of *Clostridiaceae* [224], [227].

Moreover, 2,3-BDO production was negatively correlated with H_2 production, *Geobacteraceae* and *Clostridiaceae*, and positively correlated with abundance of *Enterobacteriaceae*. This is consistent with the literature since 2,3-BDO production is often associated to the presence of species from the *Enterobacteriacea* family, such as *Enterobacter aerogenes* [240] and *Klebsiella pneumoniae* [49]. With respect to H_2 and 2,3-BDO production, as mentioned above, they are two pathways available to control the intracellular redox potential, so that a high production of one leads to a decrease of the others [49].

In particular, members of the *Geobacteraceae* family strongly correlated with the main metabolites, *i.e.* H_2, butyrate and 2,3-BDO, evidencing the same tendency than *Clostridiaceae*. This suggests that *Geobacteraceae* promoted changes in microbial interactions leading to an increase in H_2 and butyrate production while 2,3-BDO production decreased. This point will be further investigated in the next section (Section 6.2.4).

Table 6-1: Pearson correlation matrix from family distribution of final microbial community and metabolic patterns

	H2Y	Succinate	Lactate	Ethanol	Acetate	Propionate	Butyrate	2,3-BDO	Others+biomass
H2Y		***0.90****	*-0.06*	*-0.61*	*-0.08*	***-0.92****	***0.93****	***-0.89****	*-0.24*
Enterobacteriaceae	***-0.90****	***-0.97****	*0.11*	*0.61*	*-0.13*	***0.97****	***-0.98****	***0.99****	*0.08*
Clostridiaceae	***0.90****	***0.97****	*-0.10*	*-0.61*	*0.15*	***-0.97****	***0.97****	***-0.99****	*-0.05*
Geobacteraceae	***0.93****	***1.00****	*-0.23*	***-0.71****	*0.00*	***-1.00****	***1.00****	***-0.95****	*-0.24*
Enterococcaceae	***-0.81****	***-0.93****	*0.00*	*0.53*	*-0.25*	***0.93****	***-0.92****	***0.97****	*-0.01*
Streptococcaceae	*-0.38*	*-0.31*	*0.68*	***0.79****	*-0.03*	*0.33*	*-0.29*	*0.17*	*-0.03*
Bacteroidaceae	***0.89****	***1.00****	*-0.26*	***-0.74****	*0.03*	***-1.00****	***0.99****	***-0.94****	*-0.21*
Flavobacteriaceae	***-0.72****	*-0.69*	*-0.43*	*0.12*	*0.05*	***0.71****	***-0.71****	***0.75****	*0.29*
Others (<2.0%)	***-0.75****	***-0.79****	*-0.08*	*0.47*	*-0.16*	***0.81****	***-0.78****	***0.78****	*0.18*

The data used include all replicates from M and MG. In bold were marked the significant correlations with p-values ≤0.05 (*) and p-values ≤0.01 (**). Positive (■ for 1.0), negative (■ for –1.0) and null (□ for zero) correlations were marked with gradient colour depending on value.

6.2.4 Electroactive bacteria are promoting novel microbial interaction to increase hydrogen production

Table 6-2 shows the correlation matrix constructed by family distribution of the microbial communities sampled at the end of batch operation. *Enterobacteriaceae* family negatively correlated with *Clostridiaceae* and *Geobacteraceae*, while *Clostridiaceae* and *Geobacteraceae* positively correlated.

Enterobacteriaceae and *Clostridiaceae* families are composed of numerous well-known H_2-producing species. Commonly, in dark fermentation systems, many species from both families are interacting and contributing together for H_2 production [37]. Unlike *Enterobacter spp.*, *Clostridia spp.* are linked to higher H_2 yields. Nonetheless, *Enterobacter spp.* are also considered as key species to maintain the anaerobic conditions that are strictly necessary for the growth of *Clostridia spp.* [37]. Our results are evidencing that high both H_2 yields and butyrate production are linked with an increase in the relative abundance of *Clostridiaceae*.

In addition, a positive correlation between *Clostridiaceae* and *Geobacteraceae*, was found to be responsible for changes in the carbon flux from more reduced metabolites (*i.e.* 2,3-BDO) toward the H_2 and butyrate production. Consistently, it was previously reported in the literature that *C. pasteurianum* and *G. sulfurreducens* can cooperatively grow through direct interspecies electron transfer [225]. In other words, *G. sulfurreducens* could oxidize the acetate from fermentation medium as sole electron source using *C. pasteurianum* as sole electron acceptor. This electron transfer would be favorable for both species, causing changes in metabolic pathways, as already reported in literature [225].

Table 6-2: Pearson correlation matrix from family distribution of final microbial community

	Enterobacteriaceae	*Clostridiaceae*	*Geobacteraceae*	*Enterococcaceae*	*Streptococcaceae*	*Bacteroidaceae*	*Flavobacteriaceae*	*Others (<2.0%)*
Enterobacteriaceae		***-1.00*****	***-0.98*****	***0.95*****	*0.25*	***-0.97*****	*0.69*	***0.74****
Clostridiaceae	***-1.00*****		***0.97*****	***-0.95*****	*-0.26*	***0.96*****	*-0.68*	***-0.74****
Geobacteraceae	***-0.98*****	***0.97*****		***-0.91*****	*-0.33*	***0.99*****	*-0.68*	***-0.77****
Enterococcaceae	***0.95*****	***-0.95*****	***-0.91*****		*0.19*	***-0.93*****	***0.74****	***0.87*****
Streptococcaceae	*0.25*	*-0.26*	*-0.33*	*0.19*		*-0.34*	*-0.25*	*0.24*
Bacteroidaceae	***-0.97*****	***0.96*****	***0.99*****	***-0.93*****	*-0.34*		*-0.68*	***-0.79****
Flavobacteriaceae	*0.69*	*-0.68*	*-0.68*	***0.74****	*-0.25*	*-0.68*		***0.80****
Others (<2.0%)	***0.74****	***-0.74****	***-0.77****	***0.87*****	*0.24*	***-0.79****	***0.80****	

The data used include all replicates from M and MG. In bold were marked the significant correlations with p-values ≤0.05 (*) and p-values ≤0.01 (**). Positive (■ for 1.0), negative (■ for –1.0) and null (□ for zero) correlations were marked with gradient colour depending on value.

6.3 Decrease of fermentative hydrogen production using anaerobic sludge as inoculum artificially enriched with *G. sulfurreducens*

6.3.1 Context and specific experimental methodology

In this section, anaerobic sludge was used as inoculum and was enriched with *G. sulfurreducens* (**MC+G**) at the ratio 1:1. As control, a non-enriched inoculum was used (**MC**). Tests were performed in 100 ml bottles in triplicate using glucose and acetate as carbon sources.

6.3.2 Hydrogen production and metabolite distribution

After 48 hours of batch operation, glucose was totally consumed in all reactors (5.6±0.2 $gCOD.l^{-1}$). Total COD mass balance calculated on the basis of soluble products and H_2 gas ranged between 72.1% and 86.9%. The biomass concentration at the end of operation was 1.45±0.03 $gVSS.l^{-1}$ and 1.06±0.06 $gVSS.l^{-1}$ for MC and MC+G, respectively. Contrary results were observed in the Section 6.2, where higher biomass concentration was obtained after fermentation in the inoculum enriched with *G. sulfurreducens* (MG) compared to wild inoculum (M).

Weirdly, the H_2 yields decreased by 93% during MC+G tests (0.05±0.02 $molH_2.mol^{-1}_{glucose}$) with respect to MC tests (0.73±0.15 $molH_2.mol^{-1}_{glucose}$). Low H_2 yield observed in MC+G test was linked to high lactate production (Fig. 6-4). Main metabolites in MC tests were butyrate and ethanol, representing 34.5±2.4%$_{COD}$ and 25.4±2.0%$_{COD}$, respectively. However, in MC+G tests these metabolites decreased, increasing lactate production at 43.4±1.3%$_{COD}$. Acetate was also produced, representing 18.0±1.0%$_{COD}$ and 21.5±2.7%$_{COD}$ for MC+G and

MC, respectively. Succinate, formate and propionate were detected in both tests, at very low concentrations (<3.4%$_{COD}$).

In this case, the changes in the microbial interactions motivated by the inoculum enrichment with *G. sulfurreducens*, was detrimental to H_2 production. Unlike to results observed in Section 6.2, neither H_2 nor butyrate production were increased. Instead, lactate production was increased.

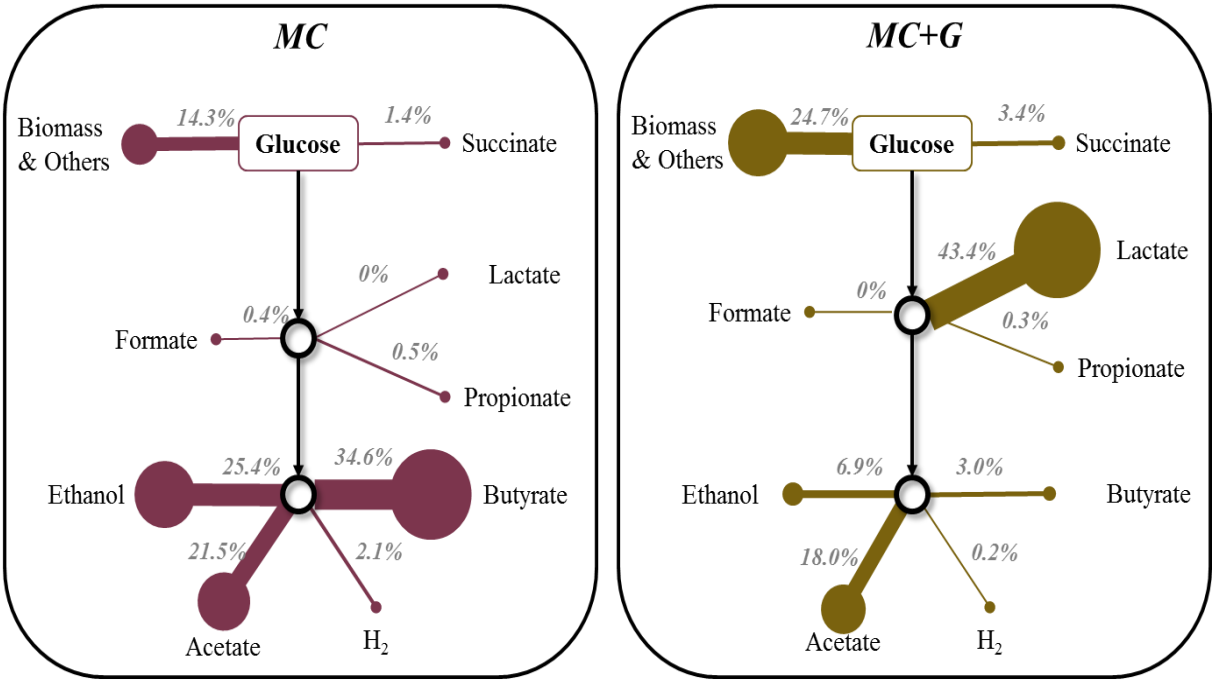


Fig. 6-3: Metabolite distribution observed during dark fermentation using anaerobic sludge as inoculum enriched with *G. sulfurreducens*

***MC**: wild inoculum. **MC+G**: inoculum enriched with G. sulfurreducens. The figures represent the average of all replicates for each test. The lines size is proportional to %COD represented by each metabolite.*

6.3.3 Microbial community analysis and its link with the metabolic patterns

Microbial community analysis of the inoculum and after batch operation were performed. Fig. 6-4 shows the family distribution of the microbial communities. The dominant families in the inoculum (MC.i) were *Saprospiraceae* and *Rhodocyclaceae* representing 13.7±0.5% and 13.4±0.1%, respectively. When the inoculum was artificially enriched with *Geobacter sp.* (MC+G.i), the microbial composition was as follows: *Geobacteraceae* (13.9±1.5%), *Saprospiraceae* (12.8±0.05%) and *Rhodocyclaceae* (11.3±0.02%).

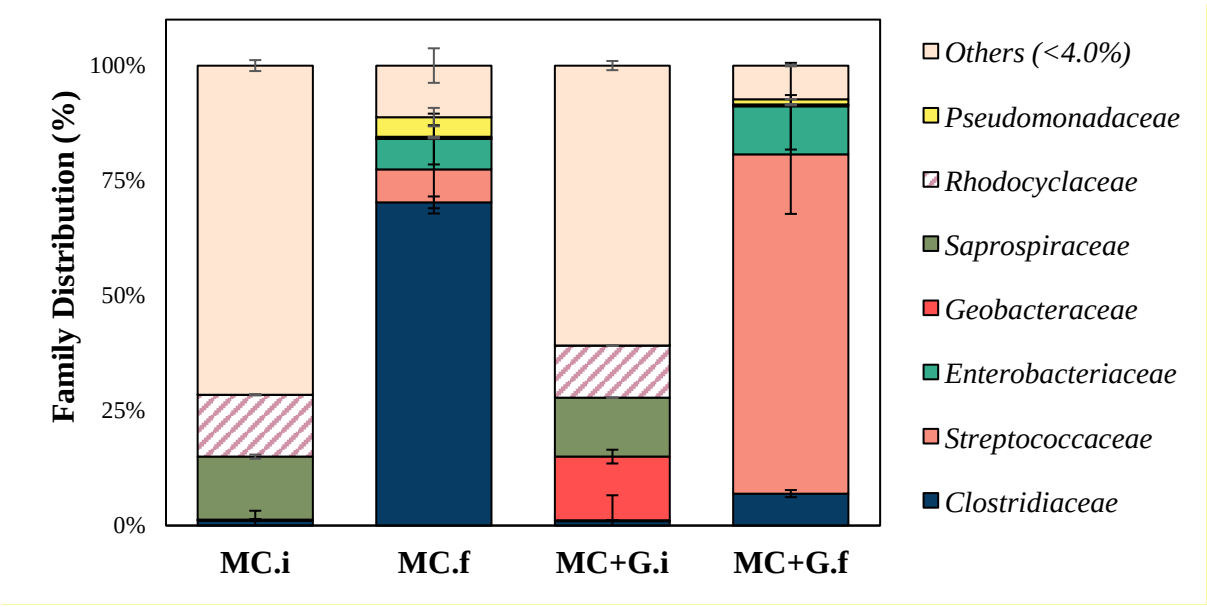


Fig. 6-4: Family distribution of microbial community during dark fermentation using anaerobic sludge as inoculum enriched with *G. sulfurreducens*

***MC**: wild inoculum. **MC+G**: inoculum enriched with G. sulfurreducens. The samples taken before and after fermentation are marked with "i" and "f", respectively. The figures represent the average of all replicates for each test. Error bars are representing the standard deviation*

After 48 hours of operation, in MC tests the microbial community was dominated by *Clostridiaceae*, followed by *Streptococcaceae*, representing 70.3±27.4% and 7.2±9.6%,

respectively. In MC+G tests *Streptococcaceae* was widely dominant, followed by *Enterobacteriaceae* representing 73.7±12.9% and 10.5±9.4%, respectively.

Correlations existing between metabolic patterns and microbial communities were analysed by a Pearson correlation test (Table 6-3). H_2 production positively correlated with the abundance of *Clostridiaceae*, but negatively with *Streptococcaceae* and *Geobacteraceae*. Lactate production positively correlated with *Streptococcaceae* and negatively with H_2 production. While, butyrate production negatively correlated with *Streptococcaceae.* The results are consistent with literature where *Streptococcaceae* family is a known lactate producer and often is linked to low H_2 yields [37], [58], [229].

Table 6-3: Pearson correlation matrix from family distribution of final microbial community and metabolic patterns

	H2Y	Succinate	Lactate	Formate	Acetate	Propionate	Ethanol	Butyrate	Others+Biomass
H2Y		***-0.98****	***-0.96****	*0.91*	*0.84*	*0.76*	*0.89*	*0.95*	*-0.79*
Clostridiaceae	***0.99***	***-0.99***	*-0.92*	*0.86*	*0.89*	*0.69*	*0.84*	*0.90*	*-0.73*
Streptococcaceae	***-0.98****	*0.92*	***0.97****	*-0.94*	*-0.78*	*-0.84*	*-0.92*	***-0.96****	*0.81*
Enterobacteriaceae	*-0.43*	*0.59*	*0.33*	*-0.27*	*-0.36*	*-0.12*	*-0.34*	*-0.37*	*0.38*
Geobacteraceae	***-0.98****	***0.99***	*0.88*	*-0.80*	*-0.91*	*-0.61*	*-0.79*	*-0.86*	*0.68*
Saprospiraceae	*0.26*	*-0.15*	*-0.53*	*0.64*	*-0.28*	*0.82*	*0.66*	*0.56*	*-0.75*
Rhodocyclaceae	*0.87*	*-0.80*	***-0.97****	***1.00***	*0.47*	***0.98****	***0.99***	***0.98****	***-0.96****
Aeromonadaceae	*0.43*	*-0.29*	*-0.66*	*0.76*	*-0.07*	*0.90*	*0.75*	*0.67*	*-0.79*
Bacillaceae	*0.33*	*-0.20*	*-0.59*	*0.69*	*-0.20*	*0.86*	*0.69*	*0.60*	*-0.76*
Pseudomonadaceae	*0.65*	*-0.55*	*-0.84*	*0.91*	*0.16*	***0.99****	*0.91*	*0.86*	*-0.94*
Bacillales_unclassified	*-0.67*	*0.78*	*0.63*	*-0.58*	*-0.46*	*-0.46*	*-0.65*	*-0.66*	*0.68*

The data used include all replicates from MC and MC+G. In bold were marked the significant correlations with p-values ≤0.05 (*) and p-values ≤0.01 (**). Positive (■ for 1.0), negative (■ for –1.0) and null (□ for zero) correlations were marked with gradient colour depending on value.

6.3.4 Influence of electroactive bacteria on fermentative hydrogen production

On the other hand, and contrary to the observations made in the past section (Section 6.2) *Clostridiaceae* and *Geobacteraceae* families negatively correlated, so a cooperative growth did not occur in that case. Possibly, *G. sulfurreducens* was oxidizing the H_2 produced by *Clostridia spp.* evidencing a extremely low H_2 yield as observed in MC+G tests [241], [242]. However, a final electron acceptor such as Fe(III), fumarate and quinines are required by *G. sulfurreducens*, but any of them were supplied in the fermentation medium [241], [242]. An alternative hypothesis would be that *G. sulfurreducens* was donating its electrons to some unknown species, analogously to what has been evidenced with *C. pasteurianum* [9]. However, it is not possible to affirm it since more research is necessary.

Table 6-4 shows the correlation matrix from family distribution of microbial communities at final time of batch operation. Significative correlations were observed between several families during this experiment. *Clostridiaceae* family negatively correlated with *Streptococcaceae* and *Geobacteraceae*. The influence of lactic acid bacteria such as members from the *Streptococcaceae* family, in H_2-producing reactors, has been in debate because they are not always harmful [37], [229]. However, they have a toxic effect by bactericide synthesis, especially on *Clostridia spp*., mostly leading to a decrease of H_2 yields [58], [59], [183].

On the other hand, and contrary to the observations made in the past section (Section 6.2) *Clostridiaceae* and *Geobacteraceae* families negatively correlated, so a cooperative growth did not occur in that case. Possibly, *G. sulfurreducens* was oxidizing the H_2 produced by *Clostridia spp.* evidencing a extremely low H_2 yield as observed in MC+G tests [241], [242]. However, a final electron acceptor such as Fe(III), fumarate and quinines are required by *G. sulfurreducens*, but any of them were supplied in the fermentation medium [241], [242]. An alternative hypothesis would be that *G. sulfurreducens* was donating its electrons to some unknown species, analogously to what has been evidenced with *C. pasteurianum* [9]. However, it is not possible to affirm it since more research is necessary.

Table 6-4: Pearson correlation matrix from family distribution of final microbial community

	Clostridiaceae	*Streptococcaceae*	*Enterobacteriaceae*	*Geobacteraceae*	*Saprospiraceae*	*Rhodocyclaceae*	*Aeromonadaceae*	*Bacillaceae*	*Pseudomonadaceae*	*Bacillales_unclassified*
Clostridiaceae		***-0.95****	-0.48	***-0.99****	0.15	0.81	0.32	0.22	0.56	-0.68
Streptococcaceae	***-0.95****		0.24	0.91	-0.38	-0.92	-0.57	-0.46	-0.74	0.52
Enterobacteriaceae	-0.48	0.24		0.54	0.16	-0.22	0.24	0.22	-0.04	0.94
Geobacteraceae	***-0.99****	0.91	0.54		-0.06	-0.75	-0.22	-0.12	-0.48	0.72
Saprospiraceae	0.15	-0.38	0.16	-0.06		0.71	***0.96****	***0.99****	0.90	-0.13
Rhodocyclaceae	0.81	-0.92	-0.22	-0.75	0.71		0.81	0.75	0.94	-0.55
Aeromonadaceae	0.32	-0.57	0.24	-0.22	***0.96****	0.81		***0.99****	***0.95****	-0.09
Bacillaceae	0.22	-0.46	0.22	-0.12	***0.99****	0.75	***0.99****		0.93	-0.10
Pseudomonadaceae	0.56	-0.74	-0.04	-0.48	0.90	0.94	***0.95****	0.93		-0.38
Bacillales_unclassified	-0.68	0.52	0.94	0.72	-0.13	-0.55	-0.09	-0.10	-0.38	

The data used include all replicates from MC and MC+G. In bold were marked the significant correlations with p-values ≤0.05 (*) and p-values ≤0.01 (**). Positive (■ for 1.0), negative (■ for –1.0) and null (□ for zero) correlations were marked with gradient colour depending on value.

6.4 Mixed culture electro-fermentation using electrodes precolonized by *G. sulfurreducens*

6.4.1 Context and specific experimental methodology

In the previous sections it was seen how electroactive bacteria could influence the metabolic patterns during conventional dark fermentation. In this section, the effect on metabolic patterns during glucose electro-fermentation by using an electrode precolonized of planar graphite plate with *G. sulfurreducens* was investigated. For that, two sequential batchs were performed, **GEF1** and **GEF2** at –0.4 V *vs* SCE. Precolonized electrodes were used, while fresh inoculum + fermentation medium was added each time. Besides, conventional fermentation (**F**) was performed in duplicate as control.

6.4.2 Hydrogen production and metabolite distribution

After 24 hours of batch operation, the glucose was totally consumed in all reactors (5.3±0.1 $gCOD.l^{-1}$). Total COD mass balance calculated from soluble products and H_2 gas ranged between 60.7 and 70.5%.

H_2 yields and metabolic patterns (Fig. 6-5) showed no significant difference between F, GEF1 and GEF2. The H_2 yields were 0.34±0.07 $molH_2.mol^{-1}_{glucose}$, 0.30 $molH_2.mol^{-1}_{glucose}$ and 0.31 $molH_2.mol^{-1}_{glucose}$ respectively. In all the cases, the main metabolites were ethanol and 2,3-BDO, representing between 33.4 – 34.4 $\%_{COD}$ and 33.4 – 34.4 $\%_{COD}$, respectively. However, a large amount of COD was associated to "Others+Biomass", representing between 31.1 – 34.4$\%_{COD}$. Lactate, acetate and butyrate were also detected, but in lower concentrations (<6.4$\%_{COD}$).

The electrons passing from fermentation medium to circuit electric was 43.9 C and 2.0 C for GEF1 and GEF2, respectively. Besides, considering H_2 as the interest product, ηEF

calculated was low, representing 0.031 and 0.001, respectively. So, the biological H_2 production was influenced at maximum 3.1% of the electrons passing through the polarized electrodes. These low ηEF values are consistent with the literature for electro-fermentation process [142], [143].

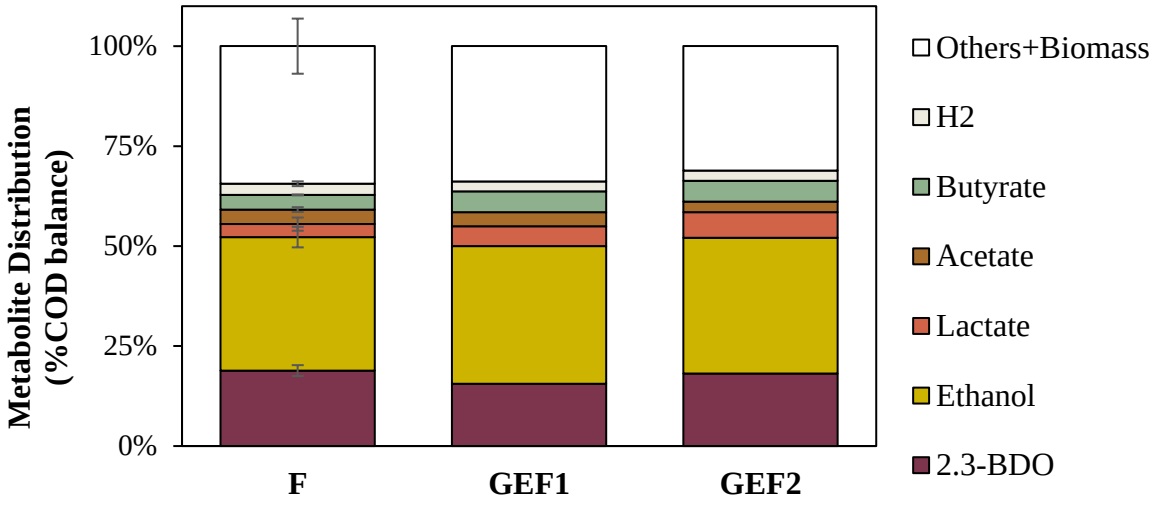


Fig. 6-5: Metabolite distribution observed during electro-fermentation using precolonized electrodes with *G. sulfurreducens*

***F**: conventional fermentation. **GEF1**: First electro-fermentation batch using precolonized electrodes with G. sulfurreducens. **GEF2**: Second electro-fermentation batch using precolonized electroes with G. sulfurreducens.*

6.4.3 Microbial community analysis

Initial and final microbial communities were analysed for samples taken in the fermentation bulk and electroactive biofilm (Fig. 6-6). Dominant families in the inoculum were *Enterobacteriaceae* and *Clostridiaceae*, representing 64.5±2.3% and 23.6±0.6%, respectively. Initial biofilm in the precolonized electrode (We.i) was mainly composed by *Geobacteraceae* (94.9%), as expected because *G. sulfurreducens* was used to form the biofilm.

After batch fermentation, the dominants families in the inoculum remained dominant in F and GEF1, evidencing only small differences in their relative abundances. *Enterobacteriaceae* and *Clostridiaceae* families represent 76.4±12.5% and 18.1±11.7% respectively in F, while that in GEF1 55.0% and 35.0%, respectively. Besides, the microbial composition of the biofilm (We.1) changed and *Geobacteraceae* (28.2%) was no longer dominant and species from fermentation bulk, such as *Enterobacteriaceae* (31.6%) and *Clostridiaceae* (31.1%), were also attached to biofilm.

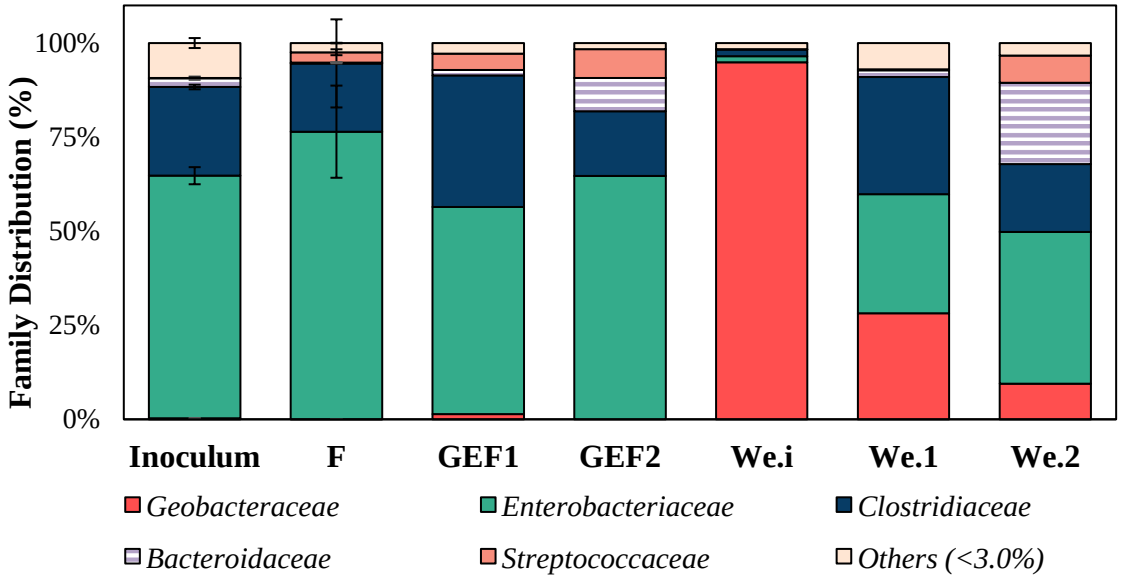


Fig. 6-6: Family distribution of microbial community observed during electro-fermentation using precolonized electrodes with *G. sulfurreducens*

***Inoculum**: initial sample from bulk fermentation. **F**:final conventional fermentation. **GEF1**: final of first electro-fermentation batch performed using precolonized electrode with G. sulfurreducens. **GEF2**: final of second electro-fermentation batch performed using precolonized electrode with G. sulfurreducens. **We.i**: initial sample from electrode biofilm. **We.1**: electrode biofilm after first batch. **We.2**: electrode biofilm after second batch. Inoculum and F represent the average of all replicates. Error bars are representing the standard deviation.*

After second batch (CEF2) the microbial composition in the fermentation bulk was still dominant for *Enterobacteriaceae* and *Clostridiaceae*, representing 64.7% and 17.2%, respectively. Besides, an increase in relative abundance of *Bacteroidaceae* (8.9%) family was

observed. With respect to the biofilm (We.2), *Geobacteraceae* (9.4%) decreased in relative abundance, while *Enterobacteriaceae* (40.4%) and *Bacteroidaceae* (21.7%) became dominant. Additionally, our results show that ηEF is positively linked to the relative abundance of *Geobacteraceae* in the biofilm.

The results show that using an electrode pre-colonized by *G. sulfurreducens* had no effect on metabolic pathways during glucose electro-fermentation. Contrary results have been reported in literature, where important changes in both metabolic pathways and microbial community, have been linked to the interaction between *G. sulfurreducens* attached as biofilm and fermentative bacteria in the bulk [143]. Probably, the applied potential during the electro-fermentation (*i.e.* –0.4 V *vs* SCE) was not adequate to promote interactions between *G. sulfurreducens* and fermentative bacteria and to favour its growth in the biofilm because its relative abundance decreased considerably between GEF1 and GEF2.

More probably, the inoculum microbial community structure and its composition are key factors in electro-fermentation process as it been evidenced in Chapter 5. Literature evidence that *G. sulfurreducens* can take electrons from electrode and release them to the fermentation medium through reduction reactions using redox mediators or with some bacteria such as *Clostridia* species by establishing a syntrophic relationship. In all cases, a better understanding of the microbial interactions occurring between electroactive and fermentative bacteria are essential for better controlling the process, but to date these interactions have not been intensively investigated. So, possibly the inoculum used was not adequate due to the absence of bacteria that could establish a cooperative growth with *G. sulfurreducens*

6.5 Conclusion

In this chapter, we investigated the microbial interactions using two different mixed cultures enriched with *G. sulfurreducens* during conventional H_2 production by dark fermentation as well as the interaction between a mixed culture and *G. sulfurreducens* attached on the electrode during glucose electro-fermentation.

Significant changes in metabolic pathways related to bacterial communities selection when the inoculum was enriched with *G. sulfurreducens* were observed. The first inoculum, mainly composed of *Enterobacteriaceae* and *Clostridiaceae*, showed an increase in both H_2 yield and butyrate production, associated with an increase in relative abundance of *Clostridiaceae*. This change was assumed to result from a cooperative growth between *Clostridiaceae* and *G. sulfurreducens*. The second inoculum, mainly composed of *Saprospiraceae* and *Rhodocyclaceae*, showed a decrease in H_2 production, associated with increased in lactate production and the emergence of members of the *Streptococcaceae* family. However, in this case *G. sulfurreducens* action was not clear because of all the possible interactions that could have been established with the different fermentative bacteria, are still unknown.

Finally, no effect on the metabolic pathways was observed when a pre-colonized electrode with *G. sulfurreducens* was used during glucose electro-fermentation. These results were probably due to the impossibility of *G. sulfurreducens* to find an electron acceptor (not added in the fermentation medium) and therefore did not interact with bulk bacteria to establish a cooperative interaction. In this context, once again it is evident that initial microbial community is playing a crucial role in all electro-fermentation system.

Conclusion & Perspectives

Polarized electrodes physically affect the microbial community

An efficient selection of H_2-producing bacteria and consequent changes in metabolism patterns was observed in Chapter 4. Two main metabolic behaviors were observed in EF. The first one was associated with high H_2 yields and butyrate production and related to *Clostridium butyricum* selection. The second H_2 producing pathway was associated to ethanol and acetate production related to *Escherichia fergusonii* and *Enterobacter cloacae* selection. During conventional fermentation, a third metabolic pathway was observed and was associated to low H_2 yields and lactate production due to *Streptococcus equinus* selection. Our results are not fully explained for the electric current passing through the electrodes, which is consistent with the literature [142], [143], [153], [154].

In particular, Choi et al. (2014) reported that the zeta potential of *C. pasteurianum* can be more positive in the presence of a polarized electrode, while the cells are more electronegative with an open circuit [142]. Polarized electrodes could cause physiological changes in the surface cell affecting growth rates, which would disadvantage some species and thus giving others species the opportunity to emerge [212]. However, this physiological change could be positive by providing an advantage over the action of an inhibitor such as bacteriocin nisin (produced by lactate-producing bacteria), as proposed in Chapter 4. On the other hand, Jeong *et al.* 2013 efficiently applied an electric field as a inoculum pre-treatment for H_2 production by dark fermentation, by selecting mainly *Clostridia* species [80].

In this context, it is interesting to study the glucose EF using keys species found in Chapter 4, through mono and co-cultures, as proposed in Table C&P.1. Studies with mono-cultures can show how each species is affected by the presence of polarized electrode with a special focus

on how the zeta potential and cell morphology are changed. Then, co-culture studies could provide valuable information about changes in microbial interactions between two species due to polarized electrode influence. Finally, the EF mecanisms can be studied by simulating a mixed culture using a mixture of all these key species, as synthetic microbial consortium. Regarding the operational parameters, the same ones used in the Chapter 4 experiments should be tested.

Table C&P. 1: Experimental design proposed to study electro-fermentation using mono and co-cultures of main OTU selected in Chapter 4

	C. butyricum	*E. fergusonii*	*E. cloacae*	*S. equinus*
C. butyricum	*Mono-culture*	*Co-culture*	*Co-culture*	*Co-culture*
E. fergusonii		*Mono-culture*	*Co-culture*	*Co-culture*
E. cloacae			*Mono-culture*	*Co-culture*
S. equinus				*Mono-culture*

Importance of *Clostridia* spp. abundance in the inoculum

Our results evidence that the inoculum source has a significative impact on final microbial community and metabolite production (See Chapter 5). By using five different inoculum sources, three different EF behaviours were observed with respect to H_2 production: positive, negative and neutral effect. *Clostridiaceae* family and their relative abundance seem to be critical in determining the final EF behaviours. Although *Clostridia spp*., specially *C. pasteurianum* is a known species collecting electrons directly from an electrode, the mechanism behind the EF on this genus selection remains unclear [142].

Influence of inoculum source on H_2 production by dark fermentation is often not considered and only few research studies are available [234], [243]. Depending of the microbial

community composition in the inoculum, different metabolic patterns can be observed during dark fermentation [243]. In addition, along with dominant bacteria, subdominant bacteria are known to play a key role during H_2 production [59]. This evidence the importance of microbial interactions that govern the microbial community, which could be changed in the presence of polarized electrodes [18]. On the other hand, bioaugmentation is a strategy used to increase the relative abundance of key H_2-producing species, in a mixed cultures already containing a high microbial diversity [37]. This strategy has been reported as efficient to increase H_2 production between 1.5 – 4 times, because the whole microbial community is supporting the H_2 producing bacteria performances [37], [244], [245].

In this context, *Clostridia spp.* such as *C. pasteurianum* (known as electroactive and H_2-producing bacteria) and *C. butyricum* (species selected in Chapter 4) could be used to bioaugment mixed cultures with different microbial communities. Then, these bioaugmented inocula could be tested in glucose –fed EF. Additionally, different percentages of *Clostridia spp.* could be tested to study the influence of their relative abundance on the final EF behaviour. Overall, this study could provide key information about microbial interactions and how the microbial community interacts with the polarized electrode.

Interaction between *G. sulfurreducens* and fermentative bacteria

To date, EF mechanisms are not clearly elucidated. One hypothesis is that electroactive bacteria may be interacting with the polarized electrode, but also with fermentative bacteria [18]. *G. sulfurreducens* is a known electroactive bacteria, which has evidenced a cooperatively growth with *C. pasteurianum*, a famous H_2 producing bacteria [225]. Based on this cooperative growth, *G. sulfurreducens* was used to enrich two inocula with different microbial community compositions. The first inoculum, mainly composed of *Enterobacteriaceae* and *Clostridiaceae*, showed an increase in both H_2 yield and butyrate

production, associated with an increase in relative abundance of *Clostridiaceae*. While the second inoculum mainly composed of *Saprospiraceae* and *Rhodocyclaceae* showed a decrease in H_2 production, associated with an increase in lactate production and the emergence of members of the *Streptococcaceae* family.

Our results evidence that depending on the inoculum and probably the presence of *Clostridia spp.*, the interactions between *G. sulfurreducens* and the fermentation bacterial community change. However, *G. sulfurreducens* has limited metabolic capacity and can only use acetate and H_2 as electron donors, and only acetate as carbon source [242], [246]. The number of electron acceptors that can be used by *G. sulfurreducens* is also limited, including metal ions such as Fe^{+3}, elemental sulphur, malate and fumarate. But none of them was added to the fermentation medium. The only option was that *G. sulfurreducens* releases its electrons by direct electron transfer toward fermentative bacteria as *Clostridia spp.* or other undetermined species.

In this context, it is proposed to study the interactions in co-cultures of *G. sulfurreducens* and fermentative bacteria keys reported in the Chapter 4. These species include *C. butyricum*, *E. fergusonii*, *E. cloacae* and *S. equinus*. This study would reveal whether *G. sulfurreducens* can use other bacteria than *C. pasteurianum* as electron acceptors, providing new insights into microbial interactions. On the other hand, in addition to monitoring metabolism patterns during H_2 production. It would also be interesting to evaluate changes on important redox regulators such as NAD^+/NADH, as well as hydrogenases gene expression. The latter could evidence the H_2 consumption as electron source by *G. sulfurreducens*.

References

[1] J. Devries, "The industrial revolution and the industrious revolution," *J. Econ. Hist.*, vol. 54, no. 2, pp. 249–270, 1994.

[2] BP Energy Economics, "BP Energy Outlook," p. 125, 2018.

[3] S. A. Endesa energia, "TWENERGY." [Online]. Available: https://twenergy.com/. [Accessed: 20-Aug-2006].

[4] R. Łukajtis *et al.*, "Hydrogen production from biomass using dark fermentation," *Renew. Sustain. Energy Rev.*, vol. 91, no. April 2017, pp. 665–694, 2018.

[5] I. Sreedhar, K. M. Kamani, B. M. Kamani, B. M. Reddy, and A. Venugopal, "A Bird's Eye view on process and engineering aspects of hydrogen storage," *Renew. Sustain. Energy Rev.*, vol. 91, no. November 2017, pp. 838–860, 2018.

[6] C. Grimes, O. Varghese, and S. Ranjan, "Light, Water, Hydrogen," in *The Solar Generation of Hydrogen by Water Photoelectrolysis*, Springer, Boston, MA, 2008.

[7] P. Sinha and A. Pandey, "An evaluative report and challenges for fermentative biohydrogen production," *Int. J. Hydrogen Energy*, vol. 36, no. 13, pp. 7460–7478, Jul. 2011.

[8] J. Toledo-Alarcón *et al.*, *Basics of bio-hydrogen production by dark fermentation*, no. 9789811076763. 2018.

[9] L. Chatellard, E. Trably, and H. Carrere, "The type of carbohydrates specifically selects microbial community structures and fermentation patterns," *Bioresour. Technol.*, vol. 221, pp. 541–549, 2016.

[10] C.-H. Hung, Y.-T. Chang, and Y.-J. Chang, "Roles of microorganisms other than Clostridium and Enterobacter in anaerobic fermentative biohydrogen production systems--a review.," *Bioresour. Technol.*, vol. 102, no. 18, pp. 8437–44, Sep. 2011.

[11] J. Wang and W. Wan, "Factors influencing fermentative hydrogen production: A review," *Int. J. Hydrogen Energy*, vol. 34, no. 2, pp. 799–811, Jan. 2009.

[12] M. A. Z. Bundhoo and R. Mohee, "Inhibition of dark fermentative bio-hydrogen production : A review," *Int. J. Hydrogen Energy*, vol. 41, pp. 6713–6733, 2016.

[13] G. Kumar *et al.*, "Enhancement of biofuel production via microbial augmentation : The case of dark fermentative hydrogen," *Renew. Sustain. Energy Rev.*, vol. 57, pp. 879–891, 2016.

[14] K. Chandrasekhar, Y. Lee, and D.-W. Lee, "Biohydrogen Production : Strategies to Improve Process Efficiency through Microbial Routes," *Int. J. Mol. Sci.*, vol. 16, no. 4, pp. 8266–8293, 2015.

[15] A. Ghimire *et al.*, "A review on dark fermentative biohydrogen production from organic biomass : Process parameters and use of by-products," *Appl. Energy*, vol. 144, pp. 73–95, 2015.

[16] R. D. Hoelzle, B. Virdis, and D. J. Batstone, "Regulation mechanisms in mixed and pure culture microbial fermentation.," *Biotechnol. Bioeng.*, vol. 111, no. 11, pp. 2139–54, Nov. 2014.

[17] A. Schievano *et al.*, "Electro-Fermentation - Merging Electrochemistry with Fermentation in Industrial Applications," *Trends Biotechnol.*, vol. xx, pp. 1–13, 2016.

[18] R. Moscoviz, J. Toledo-Alarcón, E. Trably, and N. Bernet, "Electro-Fermentation: How To Drive Fermentation Using Electrochemical Systems," *Trends Biotechnol.*, vol. 34, no. 11, pp.

856–865, 2016.

[19] K. Rabaey and R. A. Rozendal, “Microbial electrosynthesis - revisiting the electrical route for microbial production.,” *Nat. Rev. Microbiol.*, vol. 8, no. 10, pp. 706–16, Oct. 2010.

[20] P. Parkhey and P. Gupta, “Improvisations in structural features of microbial electrolytic cell and process parameters of electrohydrogenesis for efficient biohydrogen production: a review,” *Renew. Sustain. Energy Rev.*, vol. 69, no. September 2016, pp. 1085–1099, 2017.

[21] Y. M. Wong, T. Y. Wu, and J. C. Juan, “A review of sustainable hydrogen production using seed sludge via dark fermentation,” *Renew. Sustain. Energy Rev.*, vol. 34, pp. 471–482, Jun. 2014.

[22] J. Wang and Y. Yin, “Principle and application of different pretreatment methods for enriching hydrogen-producing bacteria from mixed cultures,” *Int. J. Hydrogen Energy*, vol. 42, no. 8, pp. 4804–4823, 2017.

[23] J. E. Ramírez-Morales, E. Tapia-Venegas, J. Toledo-Alarcón, and G. Ruiz-Filippi, “Simultaneous production and separation of biohydrogen in mixed culture systems by continuous dark fermentation,” *Water Sci. Technol.*, vol. 71, no. 9, pp. 1271–1285, 2015.

[24] P. Nikolaidis and A. Poullikkas, “A comparative overview of hydrogen production processes,” *Renew. Sustain. Energy Rev.*, vol. 67, pp. 597–611, 2017.

[25] D. Das, “Hydrogen production by biological processes: a survey of literature,” *Int. J. Hydrogen Energy*, vol. 26, no. 1, pp. 13–28, 2001.

[26] N. Muradov, “Low to near-zero CO2production of hydrogen from fossil fuels: Status and perspectives,” *Int. J. Hydrogen Energy*, vol. 42, no. 20, pp. 14058–14088, 2017.

[27] M. Y. Azwar, M. a. Hussain, and a. K. Abdul-Wahab, “Development of biohydrogen production by photobiological, fermentation and electrochemical processes: A review,” *Renew. Sustain. Energy Rev.*, vol. 31, pp. 158–173, 2014.

[28] J. Mathews and G. Wang, “Metabolic pathway engineering for enhanced biohydrogen production,” *Int. J. Hydrogen Energy*, vol. 34, no. 17, pp. 7404–7416, Sep. 2009.

[29] P. Tamagnini *et al.*, “Cyanobacterial hydrogenases : diversity , regulation and applications,” *FEMS Microbiol. Rev.*, vol. 31, no. July, pp. 692–720, 2018.

[30] P. C. Hallenbeck and J. R. Benemann, “Biological hydrogen production; Fundamentals and limiting processes,” *Int. J. Hydrogen Energy*, vol. 27, no. 11–12, pp. 1185–1193, 2002.

[31] P. Vignais, B. Billoud, and J. Meyer, “Classification and phylogeny of hydrogenases,” *FEMS Microbiol. Rev.*, vol. 25, pp. 455–501, 2001.

[32] S. Morra *et al.*, “Expression of different types of [FeFe]-hydrogenase genes in bacteria isolated from a population of a bio-hydrogen pilot-scale plant,” *Int. J. Hydrogen Energy*, vol. 39, no. 17, pp. 9018–9027, 2014.

[33] P. C. Hallenbeck, M. Abo-Hashesh, and D. Ghosh, “Strategies for improving biological hydrogen production.,” *Bioresour. Technol.*, vol. 110, pp. 1–9, Apr. 2012.

[34] H. Lee, M. B. Salerno, and B. E. Rittmann, “Thermodynamic Evaluation on H2 Production in Glucose Fermentation,” *Environ. Sci. Technol.*, vol. 42, no. 7, pp. 2401–2407, 2008.

[35] N. M. C. Saady, “Homoacetogenesis during hydrogen production by mixed cultures dark fermentation: Unresolved challenge,” *Int. J. Hydrogen Energy*, vol. 38, no. 30, pp. 13172–13191, 2013.

[36] O. Elsharnouby, H. Hafez, G. Nakhla, and M. H. El Naggar, “A critical literature review on biohydrogen production by pure cultures,” *Int. J. Hydrogen Energy*, vol. 38, no. 12, pp. 4945–4966, 2013.

[37] L. Cabrol, A. Marone, E. Tapia-Venegas, J. P. Steyer, G. Ruiz-Filippi, and E. Trably, "Microbial ecology of fermentative hydrogen producing bioprocesses: Useful insights for driving the ecosystem function," *FEMS Microbiol. Rev.*, vol. 41, no. 2, pp. 158–181, 2017.

[38] M. M. Arimi, J. Knodel, A. Kiprop, S. S. Namango, Y. Zhang, and S. U. Geißen, "Strategies for improvement of biohydrogen production from organic-rich wastewater: A review," *Biomass and Bioenergy*, vol. 75, no. 0, pp. 101–118, 2015.

[39] B. Baghchehsaraee, G. Nakhla, D. Karamanev, A. Margaritis, and G. Reid, "The effect of heat pretreatment temperature on fermentative hydrogen production using mixed cultures," *Int. J. Hydrogen Energy*, vol. 33, no. 15, pp. 4064–4073, 2008.

[40] H. Argun, F. Kargi, and I. K. Kapdan, "Microbial culture selection for bio-hydrogen production from waste ground wheat by dark fermentation," *Int. J. Hydrogen Energy*, vol. 34, no. 5, pp. 2195–2200, 2009.

[41] J. Song, D. An, N. Ren, Y. Zhang, and Y. Chen, "Effects of pH and ORP on microbial ecology and kinetics for hydrogen production in continuously dark fermentation," *Bioresour. Technol.*, vol. 102, no. 23, pp. 10875–10880, 2011.

[42] N. Ren *et al.*, "Effects of different pretreatment methods on fermentation types and dominant bacteria for hydrogen production," *Int. J. Hydrogen Energy*, vol. 33, no. 16, pp. 4318–4324, Aug. 2008.

[43] S. Chen and X. Dong, "Acetanaerobacterium elongatum gen. nov., sp. nov., from paper mill waste water," *Int. J. Syst. Evol. Microbiol.*, vol. 54, no. 6, pp. 2257–2262, 2004.

[44] D. Xing, N. Ren, and B. E. Rittmann, "Genetic diversity of hydrogen-producing bacteria in an acidophilic ethanol-H2-coproducing system, analyzed using the [Fe]-hydrogenase gene," *Appl. Environ. Microbiol.*, vol. 74, no. 4, pp. 1232–1239, 2008.

[45] W. Q. Guo, Z. H. Meng, N. Q. Ren, Z. P. Zhang, and F. Y. Cui, "Optimization of key variables for the enhanced production of hydrogen by Ethanoligenens harbinense W1 using response surface methodology," *Int. J. Hydrogen Energy*, vol. 36, no. 10, pp. 5843–5848, 2011.

[46] X. Zhao, D. Xing, L. Zhang, and N. Ren, "Characterization and overexpression of a [FeFe] -hydrogenase gene of a novel hydrogen-producing bacterium Ethanoligenens harbinense," *Int. J. Hydrogen Energy*, vol. 35, no. 18, pp. 9598–9602, 2010.

[47] E. Seol, S. Kim, S. M. Raj, and S. Park, "Comparison of hydrogen-production capability of four different Enterobacteriaceae strains under growing and non-growing conditions," *Int. J. Hydrogen Energy*, vol. 33, no. 19, pp. 5169–5175, 2008.

[48] H. S. Jayasinghearachchi, P. M. Sarma, S. Singh, A. Aginihotri, A. K. Mandal, and B. Lal, "Fermentative hydrogen production by two novel strains of Enterobacter aerogenes HGN-2 and HT 34 isolated from sea buried crude oil pipelines," *Int. J. Hydrogen Energy*, vol. 34, no. 17, pp. 7197–7207, 2009.

[49] K. Niu, X. Zhang, W. S. Tan, and M. L. Zhu, "Characteristics of fermentative hydrogen production with Klebsiella pneumoniae ECU-15 isolated from anaerobic sewage sludge," *Int. J. Hydrogen Energy*, vol. 35, no. 1, pp. 71–80, 2010.

[50] P. Kumar, S. K. S. Patel, J. K. Lee, and V. C. Kalia, "Extending the limits of Bacillus for novel biotechnological applications," *Biotechnol. Adv.*, vol. 31, no. 8, pp. 1543–1561, 2013.

[51] S. K. S. Patel, H. J. Purohit, and V. C. Kalia, "Dark fermentative hydrogen production by defined mixed microbial cultures immobilized on ligno-cellulosic waste materials," *Int. J. Hydrogen Energy*, vol. 35, no. 19, pp. 10674–10681, 2010.

[52] H. Liu and G. Wang, "Hydrogen production of a salt tolerant strain Bacillus sp. B2 from marine intertidal sludge," *World J. Microbiol. Biotechnol.*, vol. 28, no. 1, pp. 31–37, Jan. 2012.

[53] L. Raskin, B. E. Rittmann, and D. A. Stahl, "Competition and coexistence of sulfate-reducing and methanogenic populations in anaerobic biofilms," *Appl. Environ. Microbiol.*, vol. 62, no. 10, pp. 3847–3857, 1996.

[54] Tian Cheng Zhang and T. Noike, "Influence of retention time on reactor performance and bacterial trophic populations in anaerobic digestion processes," *Water Res.*, vol. 28, no. 1, pp. 27–36, 1994.

[55] O. Mizuno, Y. Y. Li, and T. Noike, "The behavior of sulfate-reducing bacteria in acidogenic phase of anaerobic digestion," *Water Res.*, vol. 32, no. 5, pp. 1626–1634, 1998.

[56] C. Y. Lin and H. P. Chen, "Sulfate effect on fermentative hydrogen production using anaerobic mixed microflora," *Int. J. Hydrogen Energy*, vol. 31, no. 7, pp. 953–960, 2006.

[57] A. Sikora, M. Błaszczyk, M. Jurkowski, and U. Zielenkiewicz, "Lactic Acid Bacteria in Hydrogen-Producing Consortia: On Purpose or by Coincidence?," in *Lactic Acid Bacteria - R & D for Food, Health and Livestock Purposes*, M. Kongo, Ed. Rijeka: InTech, 2013.

[58] T. Noike, H. Takabatake, O. Mizuno, and M. Ohba, "Inhibition of hydrogen fermentation of organic wastes by lactic acid bacteria," *Int. J. Hydrogen Energy*, vol. 27, no. 11–12, pp. 1367–1371, 2002.

[59] Y. Rafrafi *et al.*, "Sub-dominant bacteria as keystone species in microbial communities producing bio-hydrogen," *Int. J. Hydrogen Energy*, vol. 38, no. 12, pp. 4975–4985, Apr. 2013.

[60] L. R. V. De Sá *et al.*, "Hydrogenase activity monitoring in the fermentative hydrogen production using heat pretreated sludge: A useful approach to evaluate bacterial communities performance," *Int. J. Hydrogen Energy*, vol. 36, no. 13, pp. 7543–7549, 2011.

[61] M. A. Z. Bundhoo, R. Mohee, and M. A. Hassan, "Effects of pre-treatment technologies on dark fermentative biohydrogen production: A review," *J. Environ. Manage.*, vol. 157, pp. 20–48, 2015.

[62] I. Valdez-Vazquez and H. M. Poggi-Varaldo, "Hydrogen production by fermentative consortia," *Renew. Sustain. Energy Rev.*, vol. 13, no. 5, pp. 1000–1013, 2009.

[63] S. Chang, J. Z. Li, and F. Liu, "Evaluation of different pretreatment methods for preparing hydrogen-producing seed inocula from waste activated sludge," *Renew. Energy*, vol. 36, no. 5, pp. 1517–1522, 2011.

[64] L. R. V. De Sá, M. C. Cammarota, T. C. De Oliveira, E. M. M. Oliveira, A. Matos, and V. S. Ferreira-Leitão, "Pentoses, hexoses and glycerin as substrates for biohydrogen production: An approach for Brazilian biofuel integration," *Int. J. Hydrogen Energy*, vol. 38, no. 7, pp. 2986–2997, 2013.

[65] C. N. Lin, S. Y. Wu, and J. S. Chang, "Fermentative hydrogen production with a draft tube fluidized bed reactor containing silicone-gel-immobilized anaerobic sludge," *Int. J. Hydrogen Energy*, vol. 31, no. 15, pp. 2200–2210, 2006.

[66] S. O-Thong, P. Prasertsan, and N. K. Birkeland, "Evaluation of methods for preparing hydrogen-producing seed inocula under thermophilic condition by process performance and microbial community analysis," *Bioresour. Technol.*, vol. 100, no. 2, pp. 909–918, 2009.

[67] H. Zhu and M. Beland, "Evaluation of alternative methods of preparing hydrogen producing seeds from digested wastewater sludge," *Int. J. Hydrogen Energy*, vol. 31, no. 14, pp. 1980–1988, 2006.

[68] R. Abdallah *et al.*, "Dark fermentative hydrogen production by anaerobic sludge growing on glucose and ammonium resulting from nitrate electroreduction," *Int. J. Hydrogen Energy*, vol. 41, no. 12, pp. 5445–5455, 2016.

[69] J. L. Wang and W. Wan, "Comparison of different pretreatment methods for enriching

hydrogen-producing bacteria from digested sludge," *Int. J. Hydrogen Energy*, vol. 33, no. 12, pp. 2934–2941, 2008.

[70] B. Baghchehsaraee, G. Nakhla, D. Karamanev, and A. Margaritis, "Revivability of fermentative hydrogen producing bioreactors," *Int. J. Hydrogen Energy*, vol. 36, no. 3, pp. 2086–2092, 2011.

[71] Y. Yin, J. Hu, and J. Wang, "Enriching hydrogen-producing bacteria from digested sludge by different pretreatment methods," *Int. J. Hydrogen Energy*, vol. 39, no. 25, pp. 13550–13556, 2014.

[72] E. Elbeshbishy, H. Hafez, and G. Nakhla, "Enhancement of biohydrogen producing using ultrasonication," *Renew. Energy*, vol. 35, no. 12, pp. 6184–6193, 2010.

[73] E. Kan, "Effects of pretreatments of anaerobic sludge and culture conditions on hydrogen productivity in dark anaerobic fermentation," *Renew. Energy*, vol. 49, pp. 227–231, 2013.

[74] E. L. Cavalcante de Amorim, A. R. Barros, M. H. Rissato Zamariolli Damianovic, and E. L. Silva, "Anaerobic fluidized bed reactor with expanded clay as support for hydrogen production through dark fermentation of glucose," *Int. J. Hydrogen Energy*, vol. 34, no. 2, pp. 783–790, 2009.

[75] G. Davila-Vazquez, F. Alatriste-Mondragón, A. de León-Rodríguez, and E. Razo-Flores, "Fermentative hydrogen production in batch experiments using lactose, cheese whey and glucose: Influence of initial substrate concentration and pH," *Int. J. Hydrogen Energy*, vol. 33, no. 19, pp. 4989–4997, 2008.

[76] S. Wu *et al.*, "HRT-dependent hydrogen production and bacterial community structure of mixed anaerobic microflora in suspended, granular and immobilized sludge systems using glucose as the carbon substrate," *Int. J. Hydrogen Energy*, vol. 33, no. 5, pp. 1542–1549, Mar. 2008.

[77] X. Wu, H. Yang, and L. Guo, "Effect of operation parameters on anaerobic fermentation using cow dung as a source of microorganisms," *Int. J. Hydrogen Energy*, vol. 35, no. 1, pp. 46–51, 2010.

[78] B. R. Dhar, E. Elbeshbishy, and G. Nakhla, "Influence of iron on sulfide inhibition in dark biohydrogen fermentation," *Bioresour. Technol.*, vol. 126, pp. 123–130, 2012.

[79] H. Liu, G. Wang, D. Zhu, and G. Pan, "Enrichment of the hydrogen-producing microbial community from marine intertidal sludge by different pretreatment methods," *Int. J. Hydrogen Energy*, vol. 34, no. 24, pp. 9696–9701, 2009.

[80] D. Y. Jeong, S. K. Cho, H. S. Shin, and K. W. Jung, "Application of an electric field for pretreatment of a seeding source for dark fermentative hydrogen production," *Bioresour. Technol.*, vol. 139, pp. 393–396, 2013.

[81] J. Yin *et al.*, "Enhancement of acidogenic fermentation for volatile fatty acid production from food waste: Effect of redox potential and inoculum," *Bioresour. Technol.*, vol. 216, pp. 996–1003, 2016.

[82] Y. Yin, J. Hu, and J. Wang, "Gamma irradiation as a pretreatment method for enriching hydrogen-producing bacteria from digested sludge," *Int. J. Hydrogen Energy*, vol. 39, no. 25, pp. 13550–13556, 2014.

[83] Z. Liu, F. Lv, H. Zheng, C. Zhang, F. Wei, and X. H. Xing, "Enhanced hydrogen production in a UASB reactor by retaining microbial consortium onto carbon nanotubes (CNTs)," *Int. J. Hydrogen Energy*, vol. 37, no. 14, pp. 10619–10626, 2012.

[84] L. Guo *et al.*, "Impacts of sterilization, microwave and ultrasonication pretreatment on hydrogen producing using waste sludge," *Bioresour. Technol.*, vol. 99, no. 9, pp. 3651–3658, 2008.

[85] J. Kim *et al.*, "Effects of Various Pretreatments for Enhanced Anaerobic Digestion with Waste Activated Sludge," *J. Biosci. Bioeng.*, vol. 95, no. 3, pp. 271–275, 2003.

[86] P. Mohammadi, S. Ibrahim, M. S. Mohamad Annuar, and S. Law, "Effects of different pretreatment methods on anaerobic mixed microflora for hydrogen production and COD reduction from palm oil mill effluent," *J. Clean. Prod.*, vol. 19, no. 14, pp. 1654–1658, 2011.

[87] H. Wang, M. Fang, Z. Fang, and H. Bu, "Effects of sludge pretreatments and organic acids on hydrogen production by anaerobic fermentation," *Bioresour. Technol.*, vol. 101, no. 22, pp. 8731–8735, 2010.

[88] Y. B. Wang, R. J. Li, W. W. Li, Y. T. Fan, and H. W. Hou, "Effects of pretreatment of natural bacterial source and raw material on fermentative biohydrogen production," *Int. J. Hydrogen Energy*, vol. 37, no. 1, pp. 831–836, 2012.

[89] J. C. Park *et al.*, "Inactivation of bacteria in seawater by low-amperage electric current," *Appl. Environ. Microbiol.*, vol. 69, no. 4, pp. 2405–2408, 2003.

[90] M. Kuglarz, D. Karakashev, and I. Angelidaki, "Microwave and thermal pretreatment as methods for increasing the biogas potential of secondary sludge from municipal wastewater treatment plants," *Bioresour. Technol.*, vol. 134, pp. 290–297, 2013.

[91] Y. Singhal and R. Singh, "Effect of microwave pretreatment of mixed culture on biohydrogen production from waste of sweet produced from Benincasa hispida," *Int. J. Hydrogen Energy*, vol. 39, no. 14, pp. 7534–7540, 2014.

[92] Y. Yin and J. Wang, "Optimization of Hydrogen Production by Response Surface Methodology Using γ-Irradiated Sludge as Inoculum," *Energy & Fuels*, vol. 30, no. 5, pp. 4096–4103, 2016.

[93] W. Wang, L. Xie, J. Chen, G. Luo, and Q. Zhou, "Biohydrogen and methane production by co-digestion of cassava stillage and excess sludge under thermophilic condition," *Bioresour. Technol.*, vol. 102, no. 4, pp. 3833–3839, 2011.

[94] Y. C. Lo, W. M. Chen, C. H. Hung, S. Der Chen, and J. S. Chang, "Dark H2 fermentation from sucrose and xylose using H2-producing indigenous bacteria: Feasibility and kinetic studies," *Water Res.*, vol. 42, no. 4–5, pp. 827–842, 2008.

[95] S. Van Ginkel, S. Sung, and J.-J. Lay, "Biohydrogen Production as a Function of pH and Substrate Concentration," *Environ. Sci. Technol.*, vol. 35, no. 24, pp. 4726–4730, 2001.

[96] D. B. Levin, R. Islam, N. Cicek, and R. Sparling, "Hydrogen production by Clostridium thermocellum 27405 from cellulosic biomass substrates," *Int. J. Hydrogen Energy*, vol. 31, no. 11, pp. 1496–1503, 2006.

[97] C. Y. Lin and C. H. Lay, "A nutrient formulation for fermentative hydrogen production using anaerobic sewage sludge microflora," *Int. J. Hydrogen Energy*, vol. 30, no. 3, pp. 285–292, 2005.

[98] C. Y. Lin and C. H. Lay, "Carbon/nitrogen-ratio effect on fermentative hydrogen production by mixed microflora," *Int. J. Hydrogen Energy*, vol. 29, no. 1, pp. 41–45, 2004.

[99] H. Argun, F. Kargi, I. Kapdan, and R. Oztekin, "Biohydrogen production by dark fermentation of wheat powder solution: Effects of C/N and C/P ratio on hydrogen yield and formation rate," *Int. J. Hydrogen Energy*, vol. 33, no. 7, pp. 1813–1819, Apr. 2008.

[100] S. O-Thong, P. Prasertsan, N. Intrasungkha, S. Dhamwichukorn, and N.-K. Birkeland, "Optimization of simultaneous thermophilic fermentative hydrogen production and COD reduction from palm oil mill effluent by Thermoanaerobacterium-rich sludge," *Int. J. Hydrogen Energy*, vol. 33, no. 4, pp. 1221–1231, Feb. 2008.

[101] A. D. Tsaousis, E. Nývltová, R. Šuták, I. Hrdý, and J. Tachezy, "A Nonmitochondrial

hydrogen production in naegleria gruberi," *Genome Biol. Evol.*, vol. 6, no. 4, pp. 792–799, 2014.

[102] D. H. Kim, S. H. Kim, H. W. Kim, M. S. Kim, and H. S. Shin, "Sewage sludge addition to food waste synergistically enhances hydrogen fermentation performance," *Bioresour. Technol.*, vol. 102, no. 18, pp. 8501–8506, 2011.

[103] H. Alshiyab, M. S. Kalil, A. A. Hamid, and W. M. Wan Yusoff, "Effect of salts addition on hydrogen production by C. acetobutylicum," *Pakistan Journal of Biological Sciences*, vol. 11, no. 18. pp. 2193–2200, 2008.

[104] X. Hao, M. Zhou, H. Yu, Q. Shen, and L. Lei, "Effect of sodium ion concentration on hydrogen production from sucrose by anaerobic hydrogen-producing granular sludge," *Chinese J. Chem. Eng.*, vol. 14, no. 4, pp. 511–517, 2006.

[105] M. J. Lee, T. H. Kim, B. Min, and S. J. Hwang, "Sodium (Na+) concentration effects on metabolic pathway and estimation of ATP use in dark fermentation hydrogen production through stoichiometric analysis," *J. Environ. Manage.*, vol. 108, pp. 22–26, 2012.

[106] Z. Yuan, H. Yang, X. Zhi, and J. Shen, "Increased performance of continuous stirred tank reactor with calcium supplementation," *Int. J. Hydrogen Energy*, vol. 35, no. 7, pp. 2622–2626, 2010.

[107] S. Y. Wu, C. Y. Chu, and Y. C. Shen, "Effect of calcium ions on biohydrogen production performance in a fluidized bed bioreactor with activated carbon-immobilized cells," *Int. J. Hydrogen Energy*, vol. 37, no. 20, pp. 15496–15502, 2012.

[108] N. Mnatsakanyan, K. Bagramyan, and A. Trchounian, "Hydrogenase 3 but not hydrogenase 4 is major in hydrogen gas production by Escherichia coli formate Hydrogenlyase at acidic pH and in tyhe presence of external formate Acidic pH and in the Presence of External Formate," *Cell Biochem. Biophys.*, vol. 41, no. 3, pp. 357–366, 2004.

[109] A. A. Tsygankov, E. A. Minakov, N. A. Zorin, K. S. Gosteva, O. G. Voronin, and A. A. Karyakin, "Measuring the pH dependence of hydrogenase activities," *Biochemistry. (Mosc).*, vol. 72, no. 9, pp. 968–73, Sep. 2007.

[110] Z. Li, H. Wang, Z. Tang, X. Wang, and J. Bai, "Effects of pH value and substrate concentration on hydrogen production from the anaerobic fermentation of glucose," *Int. J. Hydrogen Energy*, vol. 33, no. 24, pp. 7413–7418, 2008.

[111] G. De Gioannis *et al.*, "Biohydrogen production from dark fermentation of cheese whey: Influence of pH," *Int. J. Hydrogen Energy*, vol. 39, no. 36, pp. 20930–20941, 2014.

[112] G. J. Xie *et al.*, "Control strategies for hydrogen production through co-culture of Ethanoligenens harbinense B49 and immobilized Rhodopseudomonas faecalis RLD-53," *Int. J. Hydrogen Energy*, vol. 35, no. 5, pp. 1929–1935, 2010.

[113] C. Z. Lazaro, V. Perna, C. Etchebehere, and M. B. a Varesche, "Sugarcane vinasse as substrate for fermentative hydrogen production: The effects of temperature and substrate concentration," *Int. J. Hydrogen Energy*, vol. 39, no. 12, pp. 6407–6418, 2014.

[114] I. Valdez-Vazquez, E. Ríos-Leal, F. Esparza-García, F. Cecchi, and H. M. Poggi-Varaldo, "Semi-continuous solid substrate anaerobic reactors for H2 production from organic waste: Mesophilic versus thermophilic regime," *Int. J. Hydrogen Energy*, vol. 30, no. 13–14, pp. 1383–1391, 2005.

[115] L. T. Angenent, K. Karim, M. H. Al-Dahhan, B. A. Wrenn, and R. Domíguez-Espinosa, "Production of bioenergy and biochemicals from industrial and agricultural wastewater," *Trends Biotechnol.*, vol. 22, no. 9, pp. 477–485, 2004.

[116] I. Hussy, F. Hawkes, R. Dinsdale, and D. Hawkes, "Continuous fermentative hydrogen production from sucrose and sugarbeet," *Int. J. Hydrogen Energy*, vol. 30, no. 5, pp. 471–483,

Apr. 2005.

[117] D. Kim, S. Han, S. Kim, and H. Shin, "Effect of gas sparging on continuous fermentative hydrogen production," vol. 31, pp. 2158–2169, 2006.

[118] O. Mizuno, R. Dinsdale, F. R. Hawkes, and D. L. Hawkes, "Enhancement of hydrogen production from glucose by nitrogen gas sparging," vol. 73, pp. 59–65, 2000.

[119] T. D. Nguyen, S. Jong, J. Pyo, M. Sun, and S. Jun, "Hydrogen production of the hyperthermophilic eubacterium , Thermotoga neapolitana under N 2 sparging condition," *Bioresour. Technol.*, vol. 101, no. 1, pp. S38–S41, 2010.

[120] B. Mandal, K. Nath, and D. Das, "Improvement of Biohydrogen Production Under Decreased Partial Pressure of H2 by Enterobacter cloacae," *Biotechnol. Lett.*, vol. 28, no. 11, pp. 831–835, 2006.

[121] J. T. Kraemer and Æ. D. M. Bagley, "Supersaturation of dissolved H 2 and CO 2 during fermentative hydrogen production with N 2 sparging," pp. 1485–1491, 2006.

[122] J. Bastidas-oyanedel *et al.*, "Gas controlled hydrogen fermentation," *Bioresour. Technol.*, vol. 110, pp. 503–509, 2012.

[123] B. Si *et al.*, "The role of hydraulic retention time on controlling methanogenesis and homoacetogenesis in biohydrogen production using upflow anaerobic sludge blanket (UASB) reactor and packed bed reactor (PBR)," *Int. J. Hydrogen Energy*, vol. 40, no. 35, pp. 11414–11421, 2015.

[124] T. D. Brock and M. T. Madigan, *Biology of microorganisms*, 6th ed. Englewood Cliffs, New Jersey: Prentice Hall, 1991.

[125] S. Hirano, N. Matsumoto, M. Morita, K. Sasaki, and N. Ohmura, "Electrochemical control of redox potential affects methanogenesis of the hydrogenotrophic methanogen Methanothermobacter thermautotrophicus.," *Lett. Appl. Microbiol.*, vol. 56, no. 5, pp. 315–21, May 2013.

[126] K. Bagramyan, A. Galstyan, and A. Trchounian, "Redox potential is a determinant in the Escherichia coli anaerobic fermentative growth and survival: effects of impermeable oxidant," *Bioelectrochemistry*, vol. 51, no. 2, pp. 151–156, Jun. 2000.

[127] C. G. Liu, C. Xue, Y. H. Lin, and F. W. Bai, "Redox potential control and applications in microaerobic and anaerobic fermentations.," *Biotechnol. Adv.*, vol. 31, no. 2, pp. 257–65, 2013.

[128] Y. Nakashimada, M. A. Rachman, T. Kakizono, and N. Nishio, "Hydrogen production of Enterobacter aerogenes altered by extracellular and intracellular redox states," *Int. J. Hydrogen Energy*, vol. 27, pp. 1399–1405, 2002.

[129] X. Chen, S. Jiang, Z. Zheng, L. Pan, and S. Luo, "Effects of culture redox potential on succinic acid production by Corynebacterium crenatum under anaerobic conditions," *Process Biochem.*, vol. 47, no. 8, pp. 1250–1255, 2012.

[130] D. B. Murray, K. Haynes, and M. Tomita, "Redox regulation in respiring Saccharomyces cerevisiae," *Biochim. Biophys. Acta - Gen. Subj.*, vol. 1810, no. 10, pp. 945–958, 2011.

[131] G. N. Vemuri, E. Altman, D. P. Sangurdekar, a B. Khodursky, and M. a Eiteman, "Overflow Metabolism in Escherichia coli during Steady-State Growth : Transcriptional Regulation and Effect of the Redox Ratio Overflow Metabolism in Escherichia coli during Steady-State Growth : Transcriptional Regulation and Effect of the Redox Ratio †," *Appl. Environ. Microbiol.*, vol. 72, no. 5, pp. 3653–3661, 2006.

[132] C. Riondet, R. Cachon, Y. Waché, G. Alcaraz, and C. Diviès, "Extracellular oxidoreduction potential modifies carbon and electron flow in Escherichia coli," *J. Bacteriol.*, vol. 182, no. 3,

pp. 620–626, 2000.

[133] D. Kültz, "Molecular and Evolutionary Basis of the Cellular Stress Response," *Annu. Rev. Physiol.*, vol. 67, no. 1, pp. 225–257, 2005.

[134] L. Girbal, C. Croux, I. Vasconcelos, and P. Soucaille, "Regulation of metabolic shifts in Clostridium acetobutylicum ATCC 824," *FEMS Microbiol. Rev.*, vol. 17, no. 3, pp. 287–297, 1995.

[135] D. T. Jones and D. R. Woods, "Acetone-Butanol Fermentation Revisited," *Microbiol. Rev.*, vol. 50, no. 4, pp. 484–524, 1986.

[136] M. W. Adams, L. E. Mortenson, and J.-S. Chen, "Hydrogenase," *Biochim. Biophys. Acta*, vol. 594, pp. 105–176, 1981.

[137] S. Tanisho and N. Kadokurat, "Effect of CO2 removal on hydrogen production by fermentation," *Int. J. Hydrogen Energy*, vol. 23, no. 7, pp. 559–563, 1998.

[138] S. Peguin and P. Soucaille, "Modulation of metabolism of Clostridium acetobutylicum grown in chemostat culture in a three-electrode potentiostatic system with methyl viologen as electron carrier," *Biotechnol. Bioeng.*, vol. 51, no. 3, pp. 342–348, 1996.

[139] J. C. Thrash and J. D. Coates, "Review : Direct and Indirect Electrical Stimulation of Microbial Metabolism," *Environ. Sci. Technol.*, vol. 42, no. 11, pp. 3921–3931, 2008.

[140] R. Moscoviz, "Electrochemical control of a biological process : glycerol electro-fermentation," Montpellier Supagro, 2017.

[141] J. B. A. Arends and W. Verstraete, "100 years of microbial electricity production: Three concepts for the future," *Microb. Biotechnol.*, vol. 5, no. 3, pp. 333–346, 2012.

[142] O. Choi, T. Kim, H. M. Woo, and Y. Um, "Electricity-driven metabolic shift through direct electron uptake by electroactive heterotroph Clostridium pasteurianum," *Sci. Rep.*, vol. 4, no. 6961, 2014.

[143] R. Moscoviz, E. Trably, and N. Bernet, "Electro-fermentation triggering population selection in mixed-culture glycerol fermentation," *Microb. Biotechnol.*, vol. 0, pp. 000–000, 2017.

[144] J. M. Flynn, D. E. Ross, and K. A. Hunt, "Enabling Unbalanced Fermentations by Using Engineered Electrode-," vol. 1, no. 5, pp. 1–8, 2010.

[145] K. Sturm-Richter *et al.*, "Unbalanced fermentation of glycerol in Escherichia coli via heterologous production of an electron transport chain and electrode interaction in microbial electrochemical cells," *Bioresour. Technol.*, vol. 186, pp. 89–96, 2015.

[146] O. Choi, Y. Um, and B. I. Sang, "Butyrate production enhancement by clostridium tyrobutyricum using electron mediators and a cathodic electron donor," *Biotechnol. Bioeng.*, vol. 109, no. 10, pp. 2494–2502, 2012.

[147] T. S. Kim and B. H. Kim, "Electron flow shift in Clostridium acetobutylicum fermentation by electrochemically introduced reducing equivalent," *Biotechnol. Lett.*, vol. 10, no. 2, pp. 123–128, 1988.

[148] R. Emde and B. Schink, "Enhanced propionate formation by Propionibacterium freudenreichii subsp. freudenreichii in a three-electrode amperometric culture system," *Appl. Environ. Microbiol.*, vol. 56, no. 9, pp. 2771–2776, 1990.

[149] A. M. Speers, J. M. Young, and G. Reguera, "Fermentation of glycerol into ethanol in a microbial electrolysis cell driven by a customized consortium.," *Environ. Sci. Technol.*, vol. 48, no. 11, pp. 6350–8, Jun. 2014.

[150] G. Xue *et al.*, "Efficient bioconversion of organic wastes to high optical activity of L-lactic acid stimulated by cathode in mixed microbial consortium," *Water Res.*, vol. 131, pp. 1–10,

2018.

[151] N. Xafenias, M. Oluchi, and V. Mapelli, "Electrochemical startup increases 1 , 3-propanediol titers in mixed-culture glycerol fermentations," *Process Biochem.*, vol. 50, no. 10, pp. 1499–1508, 2015.

[152] P. G. Dennis, F. Harnisch, Y. K. Yeoh, G. W. Tyson, and K. Rabaey, "Dynamics of cathode-associated microbial communities and metabolite profiles in a glycerol-fed bioelectrochemical system.," *Appl. Environ. Microbiol.*, vol. 79, no. 13, pp. 4008–14, Jul. 2013.

[153] M. Zhou, J. Chen, S. Freguia, K. Rabaey, and J. Keller, "Carbon and electron fluxes during the electricity driven 1,3-propanediol biosynthesis from glycerol.," *Environ. Sci. Technol.*, vol. 47, no. 19, pp. 11199–205, Oct. 2013.

[154] M. Zhou, S. Freguia, P. G. Dennis, J. Keller, and K. Rabaey, "Development of bioelectrocatalytic activity stimulates mixed-culture reduction of glycerol in a bioelectrochemical system," *Microb. Biotechnol.*, vol. 8, no. 3, pp. 483–489, 2015.

[155] R. C. G. Creasey, A. B. Mostert, T. A. H. Nguyen, B. Virdis, S. Freguia, and B. Laycock, "Microbial nanowires – Electron transport and the role of synthetic analogues," *Acta Biomater.*, vol. 69, pp. 1–30, 2018.

[156] D. R. Lovley, "Syntrophy Goes Electric: Direct Interspecies Electron Transfer," *Annu. Rev. Microbiol.*, vol. 71, no. 1, p. annurev-micro-030117-020420, 2017.

[157] L. E. Doyle and E. Marsili, "Weak electricigens: A new avenue for bioelectrochemical research," *Bioresour. Technol.*, vol. 258, no. December 2017, pp. 354–364, 2018.

[158] Q. Cheng and D. F. Call, "Hardwiring microbes: Via direct interspecies electron transfer: Mechanisms and applications," *Environ. Sci. Process. Impacts*, vol. 18, no. 8, pp. 968–980, 2016.

[159] M. J. Edwards *et al.*, "Redox Linked Flavin Sites in Extracellular Decaheme Proteins Involved in Microbe-Mineral Electron Transfer.," *Sci. Rep.*, vol. 5, no. December 2014, pp. 1–11, 2015.

[160] A. Kouzuma, S. Kato, and K. Watanabe, "Microbial interspecies interactions : recent findings in syntrophic consortia," *Front. Microbiol.*, vol. 6, no. May, pp. 1–8, 2015.

[161] P. Schär-zammaretti *et al.*, "Influence of Fermentation Medium Composition on Physicochemical Surface Properties of Lactobacillus acidophilus Influence of Fermentation Medium Composition on Physicochemical Surface Properties of Lactobacillus acidophilus †," *Appl. Enviromental Microbiol.*, vol. 71, no. 12, pp. 8165–8173, 2005.

[162] E. W. Rice, R. B. Baird, A. D. Eaton, and L. S. Clesceri, *Standard Methods for the Examination of Water and Wastewaster*, 22nd ed. Washington DC: American Public Health Association, 2012.

[163] P. D. Schloss *et al.*, "Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities," *Appl. Environ. Microbiol.*, vol. 75, no. 23, pp. 7537–7541, Dec. 2009.

[164] F. Braun, J. Hamelin, G. Gèvaudan, and D. Patureau, "Development and application of an enzymatic and cell flotation treatment for the recovery of viable microbial cells from environmental matrices such as anaerobic sludge," *Appl. Environ. Microbiol.*, vol. 77, no. 24, pp. 8487–8493, 2011.

[165] J. A. F. Diniz-Filho *et al.*, "Mantel test in population genetics," *Genet. Mol. Biol.*, vol. 36, no. 4, pp. 475–485, 2013.

[166] K. R. Clarke, "Non-parametric multivariate analyses of changes in community structure," *Aust. J. Ecol.*, vol. 18, no. 1988, pp. 117–143, 1993.

[167] N. Mantel, "The detection of disease clustering and a generalized regression approach,"

Cancer Res., vol. 27, no. 4, pp. 1183–1185, 1967.

[168] P. Legendre and L. F. J. Legendre, *Numerical Ecology*. Elsevier Science, 1998.

[169] Y. H. Taguchi and Y. Oono, "Relational patterns of gene expression via non-metric multidimensional scaling analysis," *Bioinformatics*, vol. 21, no. 6, pp. 730–740, 2005.

[170] D. I. Warton, S. T. Wright, and Y. Wang, "Distance-based multivariate analyses confound location and dispersion effects," *Methods Ecol. Evol.*, vol. 3, no. 1, pp. 89–101, 2012.

[171] BioLogic science instruments, "EC-Lab Software: Techniques and Applications," no. August. 2014.

[172] M. D. Merrill and B. E. Logan, "Electrolyte effects on hydrogen evolution and solution resistance in microbial electrolysis cells," *J. Power Sources*, vol. 191, no. 2, pp. 203–208, 2009.

[173] M. Lenin Babu, G. Venkata Subhash, P. N. Sarma, and S. Venkata Mohan, "Bio-electrolytic conversion of acidogenic effluents to biohydrogen: An integration strategy for higher substrate conversion and product recovery," *Bioresour. Technol.*, vol. 133, pp. 322–331, 2013.

[174] S. Cheng and B. E. Logan, "High hydrogen production rate of microbial electrolysis cell (MEC) with reduced electrode spacing," *Bioresour. Technol.*, vol. 102, no. 3, pp. 3571–3574, 2011.

[175] S. Venkata Mohan and M. Lenin Babu, "Dehydrogenase activity in association with poised potential during biohydrogen production in single chamber microbial electrolysis cell," *Bioresour. Technol.*, vol. 102, no. 18, pp. 8457–8465, 2011.

[176] J. M. Pisciotta, Z. Zaybak, D. F. Call, J. Y. Nam, and B. E. Logan, "Enrichment of microbial electrolysis cell biocathodes from sediment microbial fuel cell bioanodes," *Appl. Environ. Microbiol.*, vol. 78, no. 15, pp. 5212–5219, 2012.

[177] K. Arunasri, J. Annie Modestra, D. K. Yeruva, K. Vamshi Krishna, and S. Venkata Mohan, "Polarized potential and electrode materials implication on electro-fermentative di-hydrogen production: Microbial assemblages and hydrogenase gene copy variation," *Bioresour. Technol.*, vol. 200, pp. 691–698, 2016.

[178] M. Zhou, M. Chi, J. Luo, H. He, and T. Jin, "An overview of electrode materials in microbial fuel cells," *J. Power Sources*, vol. 196, no. 10, pp. 4427–4435, 2011.

[179] J. Wei, P. Liang, and X. Huang, "Recent progress in electrodes for microbial fuel cells," *Bioresour. Technol.*, vol. 102, no. 20, pp. 9335–9344, 2011.

[180] L. Lu and Z. J. Ren, "Microbial electrolysis cells for waste biorefinery: A state of the art review," *Bioresour. Technol.*, vol. 215, pp. 254–264, 2016.

[181] R. Palomo-Briones, E. Razo-Flores, N. Bernet, and E. Trably, "Dark-fermentative biohydrogen pathways and microbial networks in continuous stirred tank reactors: Novel insights on their control," *Appl. Energy*, vol. 198, no. April, pp. 77–87, 2017.

[182] R. Moscoviz, E. Trably, and N. Bernet, "Consistent 1,3-propanediol production from glycerol in mixed culture fermentation over a wide range of pH," *Biotechnol. Biofuels*, vol. 9, no. 1, p. 32, 2016.

[183] J. H. Jo, C. O. Jeon, D. S. Lee, and J. M. Park, "Process stability and microbial community structure in anaerobic hydrogen-producing microflora from food waste containing kimchi," *J. Biotechnol.*, vol. 131, no. 3, pp. 300–308, 2007.

[184] A. Noblecourt, G. Christophe, C. Larroche, and P. Fontanille, "Hydrogen production by dark fermentation from pre-fermented depackaging food wastes," *Bioresour. Technol.*, vol. 247, no. July 2017, pp. 864–870, 2018.

[185] S. H. Duncan, P. Louis, and H. J. Flint, "Lactate-Utilizing Bacteria , Isolated from Human Feces , That Produce Butyrate as a Major Fermentation Product Lactate-Utilizing Bacteria , Isolated from Human Feces , That Produce Butyrate as a Major Fermentation Product," *Appl. Environ. Microbiol.*, vol. 70, no. 10, pp. 5810–5817, 2004.

[186] C. A. Pham *et al.*, "A novel electrochemically active and Fe(III)-reducing bacterium phylogenetically related to Aeromonas hydrophila, isolated from a microbial fuel cell," *FEMS Microbiol. Lett.*, vol. 223, no. 1, pp. 129–134, 2003.

[187] U. Michaelidou, A. Ter Heijne, G. J. W. Euverink, H. V. M. Hamelers, A. J. M. Stams, and J. S. Geelhoed, "Microbial communities and electrochemical performance of titanium-based anodic electrodes in a microbial Fuel Cell," *Appl. Environ. Microbiol.*, vol. 77, no. 3, pp. 1069–1075, 2011.

[188] S. Ishii *et al.*, "Microbial population and functional dynamics associated with surface potential and carbon metabolism," *ISME J.*, vol. 8, no. 5, pp. 963–978, 2014.

[189] K. Chung and S. Okabe, "Characterization of electrochemical activity of a strain ISO2-3 phylogenetically related to Aeromonas sp. isolated from a glucose-fed microbial fuel cell," *Biotechnol. Bioeng.*, vol. 104, no. 5, pp. 901–910, 2009.

[190] C. Li, T. Zhang, and H. H. P. Fang, "Fermentative hydrogen production in packed-bed and packaging-free upflow reactors," *Water Sci. Technol.*, vol. 54, no. 9, pp. 95–103, 2006.

[191] K. Fricke, F. Harnisch, and U. Schröder, "On the use of cyclic voltammetry for the study of anodic electron transfer in microbial fuel cells," *Energy Environ. Sci.*, vol. 1, no. 1, p. 144, 2008.

[192] F. Harnisch and S. Freguia, "A basic tutorial on cyclic voltammetry for the investigation of electroactive microbial biofilms," *Chem. - An Asian J.*, vol. 7, no. 3, pp. 466–475, 2012.

[193] C. Santoro, C. Arbizzani, B. Erable, and I. Ieropoulos, "Microbial fuel cells: From fundamentals to applications. A review," *J. Power Sources*, vol. 356, pp. 225–244, 2017.

[194] B. E. Logan, "Exoelectrogenic bacteria that power microbial fuel cells," *Nat. Rev. Microbiol.*, vol. 7, no. 5, pp. 375–381, 2009.

[195] S. Oh, B. Min, and B. E. Logan, "Cathode performance as a factor in electricity generation in microbial fuel cells," *Environ. Sci. Technol.*, vol. 38, no. 18, pp. 4900–4904, 2004.

[196] Y.-Y. Wang, P. Ai, C.-X. Hu, and Y.-L. Zhang, "Effects of various pretreatment methods of anaerobic mixed microflora on biohydrogen production and the fermentation pathway of glucose," *Int. J. Hydrogen Energy*, vol. 36, no. 1, pp. 390–396, Jan. 2011.

[197] S. K. Khanal, W. H. Chen, L. Li, and S. Sung, "Biological hydrogen production: Effects of pH and intermediate products," *Int. J. Hydrogen Energy*, vol. 29, no. 11, pp. 1123–1131, 2004.

[198] G. Kumar, G. Zhen, T. Kobayashi, P. Sivagurunathan, S. H. Kim, and K. Q. Xu, "Impact of pH control and heat pre-treatment of seed inoculum in dark H2 fermentation: A feasibility report using mixed microalgae biomass as feedstock," *Int. J. Hydrogen Energy*, vol. 41, no. 7, pp. 4382–4392, 2016.

[199] J. Ferrer-Valenzuela, L. A. Pinuer, A. García-Cancino, and R. Bórquez-Yáñez, "Metabolic Fluxes in Lactic Acid Bacteria — A Review," *Food Biotechnol.*, vol. 29, no. 2, pp. 185–217, 2016.

[200] C. Hung, C. Cheng, D. Guan, and S. Wang, "Interactions between Clostridium sp . and other facultative anaerobes in a self-formed granular sludge hydrogen-producing bioreactor," *Int. J. Hydrogen Energy*, vol. 36, no. 14, pp. 8704–8711, 2010.

[201] Z. Song, Y. Dai, Q. Fan, X. Li, Y. Fan, and H. Hou, "Effects of pretreatment method of natural bacteria source on microbial community and bio-hydrogen production by dark fermentation,"

Int. J. Hydrogen Energy, vol. 37, no. 7, pp. 5631–5636, 2012.

[202] C. Chu, S. Wu, Y. Wu, B. Sen, and C. Hung, "Phase holdups and microbial community in high-rate fermentative hydrogen bioreactors," *Int. J. Hydrogen Energy*, vol. 36, no. 1, pp. 364–373, 2011.

[203] P. Sivagurunathan, B. Sen, and C. Lin, "Batch fermentative hydrogen production by enriched mixed culture : Combination strategy and their microbial composition," *J. Biosci. Bioeng.*, vol. 117, no. 2, pp. 222–228, 2014.

[204] H. H. P. Fang, T. Zhang, and H. Liu, "Microbial diversity of a mesophilic hydrogen-producing sludge," *Appl. mi*, vol. 58, pp. 112–118, 2002.

[205] J. J. Seppala, J. A. Puhakka, O. Yli-Harja, M. T. Karp, and V. Santala, "Fermentative hydrogen production by Clostridium butyricum and Escherichia coli in pure and co-cultures," *Int. J. Hydrogen Energy*, vol. 36, pp. 10701–10708, 2011.

[206] D. Ghosh and P. C. Hallenbeck, "Fermentative hydrogen yields from different sugars by batch cultures of metabolically engineered Escherichia coli DJT135," *Int. J. Hydrogen Energy*, vol. 34, no. 19, pp. 7979–7982, Oct. 2009.

[207] L. M. Rosales-Colunga, E. Razo-Flores, and A. De León Rodríguez, "Fermentation of lactose and its constituent sugars by Escherichia coli WDHL: Impact on hydrogen production," *Bioresour. Technol.*, vol. 111, pp. 180–184, May 2012.

[208] N. Khanna, S. M. Kotay, J. J. Gilbert, and D. Das, "Improvement of biohydrogen production by Enterobacter cloacae IIT-BT 08 under regulated pH," *J. Biotechnol.*, vol. 152, no. 1–2, pp. 9–15, Mar. 2011.

[209] N. Kumar and D. Das, "Enhancement of hydrogen production by Enterobacter cloacae IIT-BT 08," *Process Biochem.*, vol. 35, no. 6, pp. 589–593, Jan. 2000.

[210] K. Nath, A. Kumar, and D. Das, "Effect of some environmental parameters on fermentative hydrogen production by *Enterobacter cloacae* DM11," *Can. J. Microbiol.*, vol. 52, no. 6, pp. 525–532, Jun. 2006.

[211] Y. Cho and T. Lee, "Variations of hydrogen production and microbial community with heavy metals during fermentative hydrogen production," *J. Ind. Eng. Chem.*, vol. 17, no. 2, pp. 340–345, 2011.

[212] W. W. Wilson, M. M. Wade, S. C. Holman, and F. R. Champlin, "Status of methods for assessing bacterial cell surface charge properties based on zeta potentil measurements," *J. Microbiol. Methods*, vol. 43, pp. 153–164, 2001.

[213] M. A. Riley and J. E. Wertz, "Bacteriocins: Evolution, Ecology, and Application," *Annu. Rev. Microbiol.*, vol. 56, no. 1, pp. 117–137, 2002.

[214] F. Schuller, R. Benz, and H. Sahl, "The peptide antibiotic subtilin acts by formation of voltage-dependent multi-state pore in bacterial and artificial membanes," *Eur. J. Biochem.*, vol. 186, pp. 181–186, 1989.

[215] S. Zhang, Y. Lee, T. H. Kim, and S. J. Hwang, "Effects of OLRs and HRTs on hydrogen production from high salinity substrate by halophilic hydrogen producing bacterium (HHPB)," *Bioresour. Technol.*, vol. 141, pp. 227–232, 2013.

[216] E. Tapia-Venegas, J. E. Ramirez, A. Donoso-Bravo, L. Jorquera, J.-P. Steyer, and G. Ruiz-Filippi, "Bio-hydrogen production during acidogenic fermentation in a multistage stirred tank reactor," *Int. J. Hydrogen Energy*, vol. 38, no. 5, pp. 2185–2190, Feb. 2013.

[217] H. H. P. Fang and H. Liu, "Effect of pH on hydrogen production from glucose by a mixed culture," *Bioresour. Technol.*, vol. 82, no. 1, pp. 87–93, 2002.

[218] F. Silva-Illanes, E. Tapia-venegas, M. C. Schiappacasse, E. Trably, and G. Ruiz-filippi,

"Impact of hydraulic retention time (HRT) and pH on dark fermentative hydrogen production from glycerol," *Energy*, 2017.

[219] G. D. Saratale *et al.*, "A comprehensive overview on electro-active biofilms, role of exo-electrogens and their microbial niches in microbial fuel cells (MFCs)," *Chemosphere*, vol. 178, no. March, pp. 534–547, 2017.

[220] R. G. Saratale *et al.*, "Microbiome involved in microbial electrochemical systems (MESs): A review," *Chemosphere*, vol. 177, no. June, pp. 176–188, 2017.

[221] Y. Dang *et al.*, "Enhancing anaerobic digestion of complex organic waste with carbon-based conductive materials," *Bioresour. Technol.*, vol. 220, pp. 516–522, 2016.

[222] G. Capson-Tojo *et al.*, "Addition of granular activated carbon and trace elements to favor volatile fatty acid consumption during anaerobic digestion of food waste," *Bioresour. Technol.*, vol. 260, no. March, pp. 157–168, 2018.

[223] F. Liu, A.-E. Rotaru, P. M. Shrestha, N. S. Malvankar, K. P. Nevin, and D. R. Lovley, "Promoting direct interspecies electron transfer with activated carbon," *Energy Environ. Sci.*, vol. 5, no. 10, pp. 8982–8989, 2012.

[224] R. Palomo-Briones *et al.*, "Hydrogen metabolic patterns driven by Clostridium-Streptococcus community shifts in a continuous stirred tank reactor," *Appl. Microbiol. Biotechnol.*, vol. 102, no. 5, pp. 2465–2475, 2018.

[225] R. Moscoviz, F. De Fouchécour, G. Santa-Catalina, N. Bernet, and E. Trably, "Cooperative growth of Geobacter sulfurreducens and Clostridium pasteurianum with subsequent metabolic shift in glycerol fermentation," *Sci. Rep.*, vol. 7, no. March, 2017.

[226] H.-S. Lee, C. I. Torres, and B. E. Rittmann, "Effects of Substrate Diffusion and Anode Potential on Kinetic Parameters for Anode-Respiring Bacteria," *Environ. Sci. Technol.*, vol. 43, no. 19, pp. 7571–7577, Oct. 2009.

[227] H.-S. Lee, M. B. Salerno, and B. E. Rittmann, "Thermodynamic evaluation on H2 production in glucose fermentation.," *Environ. Sci. Technol.*, vol. 42, no. 7, pp. 2401–7, 2008.

[228] E. Tapia-Venegas *et al.*, "Adaptation of acidogenic sludge to increasing glycerol concentrations for biohydrogen production," *Appl. Microbiol. Biotechnol.*, vol. 99, no. 19, pp. 8295–8308, 2015.

[229] E. Castelló, L. Braga, L. Fuentes, and C. Etchebehere, "Possible causes for the instability in the H2production from cheese whey in a CSTR," *Int. J. Hydrogen Energy*, vol. 43, no. 5, pp. 2654–2665, 2018.

[230] M. Wietzke and H. Bahl, "The redox-sensing protein Rex, a transcriptional regulator of solventogenesis in Clostridium acetobutylicum," *Appl. Microbiol. Biotechnol.*, vol. 96, no. 3, pp. 749–761, 2012.

[231] S. I. Maintinguer, I. K. Sakamoto, M. A. T. Adorno, and M. B. A. Varesche, "Bacterial diversity from environmental sample applied to bio-hydrogen production," *Int. J. Hydrogen Energy*, vol. 40, no. 8, pp. 3180–3190, 2015.

[232] S. K. S. Patel, P. Kumar, and V. C. Kalia, "Enhancing biological hydrogen production through complementary microbial metabolisms," *Int. J. Hydrogen Energy*, vol. 37, no. 14, pp. 10590–10603, Jul. 2012.

[233] X. M. Guo, E. Trably, E. Latrille, H. Carrère, and J.-P. Steyer, "Hydrogen production from agricultural waste by dark fermentation: A review," *Int. J. Hydrogen Energy*, vol. 35, no. 19, pp. 10660–10673, Oct. 2010.

[234] M. Navarro-Díaz, I. Valdez-Vazquez, and A. E. Escalante, "Ecological perspectives of hydrogen fermentation by microbial consortia: What we have learned and the way forward,"

Int. J. Hydrogen Energy, vol. 41, no. 39, pp. 17297–17308, 2016.

[235] E. Kim, S. G. Shin, M. A. H. Jannat, J. V. Tongco, and S. Hwang, "Use of food waste-recycling wastewater as an alternative carbon source for denitrification process: A full-scale study," *Bioresour. Technol.*, vol. 245, no. June, pp. 1016–1021, 2017.

[236] N. K. Kim, S. Oh, and W. T. Liu, "Enrichment and characterization of microbial consortia degrading soluble microbial products discharged from anaerobic methanogenic bioreactors," *Water Res.*, vol. 90, pp. 395–404, 2016.

[237] D. Xu, S. Liu, Q. Chen, and J. Ni, "Microbial community compositions in different functional zones of Carrousel oxidation ditch system for domestic wastewater treatment," *AMB Express*, vol. 7, no. 1, 2017.

[238] T. N. Nazina *et al.*, "Phylogenetic Diversity of Microorganisms from the Sludge of a Biogas Reactor Processing Oil-Containing and Municipal Waste," *Microbiology*, vol. 87, no. 3, pp. 416–424, May 2018.

[239] V. T. Mota, A. D. N. Ferraz Júnior, E. Trably, and M. Zaiat, "Biohydrogen production at pH below 3.0: Is it possible?," *Water Res.*, vol. 128, pp. 350–361, 2018.

[240] C. Zhang, K. Ma, and X. Xing, "Regulation of hydrogen production by Enterobacter aerogenes by external NADH and NAD+," *Int. J. Hydrogen Energy*, vol. 34, no. 3, pp. 1226–1232, Feb. 2009.

[241] M. V Coppi, R. A. O. Neil, and D. R. Lovley, "Identification of an Uptake Hydrogenase Required for Hydrogen-Dependent Reduction of Fe (III) and Other Electron Acceptors by Geobacter sulfurreducens Identification of an Uptake Hydrogenase Required for Hydrogen-Dependent Reduction of Fe (III) and Ot," vol. 186, no. Iii, pp. 3022–3028, 2004.

[242] F. Caccavo, D. J. Lonergan, D. R. Lovley, and M. Davis, "Geobacter sulfurreducens sp. nov., a Hydrogen- and Acetate- Oxidizing Dissimilatory Metal-Reducing Microorganism," *Microbiology*, vol. 60, no. 10, pp. 3752–3759, 1994.

[243] Y. Akutsu, D.-Y. Lee, Y.-Y. Li, and T. Noike, "Hydrogen production potentials and fermentative characteristics of various substrates with different heat-pretreated natural microflora," *Int. J. Hydrogen Energy*, vol. 34, no. 13, pp. 5365–5372, Jul. 2009.

[244] S. M. Kotay and D. Das, "Novel dark fermentation involving bioaugmentation with constructed bacterial consortium for enhanced biohydrogen production from pretreated sewage sludge," *Int. J. Hydrogen Energy*, vol. 34, no. 17, pp. 7489–7496, 2009.

[245] A. Marone, G. Massini, C. Patriarca, A. Signorini, C. Varrone, and G. Izzo, "Hydrogen production from vegetable waste by bioaugmentation of indigenous fermentative communities," *Int. J. Hydrogen Energy*, vol. 37, no. 7, pp. 5612–5622, 2012.

[246] D. R. Lovley, "Reach out and touch someone: Potential impact of DIET (direct interspecies energy transfer) on anaerobic biogeochemistry, bioremediation, and bioenergy," *Rev. Environ. Sci. Biotechnol.*, vol. 10, no. 2, pp. 101–105, 2011.

STUDY AND CONTROL OF ELECTRO-ASSISTED FERMENTATION IN MIXED CULTURES: THE ROLE OF ENGINEERING OF MICROBIAL INTERACTIONS

Dark fermentation (DF) is a biological process commonly used to produce hydrogen (H_2). Up to date, great efforts have been made to optimise the operational parameters, but only few controllers are available to maintain the DF process stable. In this context, an electro-fermentation (EF) process is proposed as a new tool to control bioprocesses through polarised electrodes. The mechanisms behind EF are still unknown, but microbial interactions between fermentative and electroactive bacteria may be the key factor of the process. The objective of this thesis was: «Better understanding of the EF mechanisms through the characterization of microbial interspecies-interactions as well as interactions with the polarized electrode». Our results show that the presence of polarized electrodes led to the selection of H_2-producing bacteria, and more particularly from *Enterobacteriaceae* and *Clostridiaceae* families. Such microbial selection was concomitant with a significant increase in H_2 and butyrate production, at the expense of lactate production. However, when different inoculum were used, different behaviours were observed with an increase, a decreased or no effect on H_2 production. This observation evidences that the inoculum microbial community composition, and more particularly the relative abundance of the *Clostridiaceae* family, can significantly affect the microbial community behaviour in EF, i.e. microbial community trajectories and the related metabolic patterns. Finally, the microbial interactions were further investigated with a mixed inoculum enriched in *G. sulfurreducens*, as well-known electroactive bacteria. Here, a substantial change in the metabolic pathways towards higher H_2 and butyrate production was observed, at the expense of 2,3-butanediol production. This change was associated with an increase in relative abundance of the Clostridiaceae family at the end of fermentation, probably due to a cooperative growth that *G. sulfurreducens* occurring with the members of the *Clostridiaceae* family. Overall, although the mechanisms behind the microbial interactions are not yet well know, the EF process showed a great potential as a new type of control for mixed-culture bioprocesses with significant effects of the polarized electrodes on glucose fermentation.

DEFENDED ON 28TH SEPTEMBER 2018 AT :

WITH THE FINANCIAL SUPPORT OF :

Becas CONICYT
Programa de Formación de Capital Humano Avanzado

INRA
SCIENCE & IMPACT

INSTITUT NATIONAL DE LA RECHERCHE AGRONOMIQUE
Unité de recherche (UR0050) - Laboratoire de Biotechnologie de l'Environnement

102, avenue des Etangs
F-11100 Narbonne
France

Tél. : +33 4 68 42 51 51
Courriel: lbe-contact@supagro.inra.fr
www.montpellier.inra.fr/narbonne