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FERMENTATION DU GLYCÉROL BRUT EN CO-CULTURE POUR LA PRODUCTION D'HYDROGÈNE

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DÉDICACE

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RÉSUMÉ

L’hydrogène est un combustible à haute teneur en énergie et peut garantir un transport propre pour l'environnement dans les prochaines années. Compte tenu de ces perspectives, de nombreux groupes de recherche travaillent au développement de bio-procédes rentables de production d’hydrogène (H_2) comme source d'énergie.

Les substrats, tels que le glucose pur et le saccharose, ne sont pas économiquement viables pour la fermentation. Les déchets riches en carbone ont donc été explorés en tant que substrats d’intérêts. Le marché mondial du biodiesel, une énergie renouvelable, durable et plus respectueuse de l’environnement connaît une croissance annuelle à la hausse. La production atteindrait 30,28 milliards de litres en 2020. Avec chaque tonne de biodiesel produit, 100 kg de glycérol brut (GB) est généré en tant que sous-produit (déchets). Ce GB est une bonne source de carbone. La valorisation de ce sous-produit dans la production de bio H_2 est donc une stratégie très prometteuse.

Pour commencer, le calcul du bilan énergétique a été réalisé en comparant l'utilisation du GB dans le procéde de fermentation sombre et la purification industrielle du GB en glycérol pur. L'apport total d'énergie pour la purification du glycérol (872,4 MJ) était de 2,5 fois plus élevée par rapport à l'énergie totale maximale nécessaire pour un procédé de production de H_2 à partir de GB (344,3 MJ). La purification industrielle du glycérol à partir du GB coûtera quelque part autour de 0,66 $/L avec la diminution de la valeur du marché. Alternativement, la fermentation sombre du glycérol brut avec une production de 1 kg de H_2 peut remplacer 2,47 L de diesel conventionnel, ce qui représente une valeur de 3,40 $ sur le marché. Pour une utilisation efficace du GB, la bioconversion et la production H_2 par fermentation peuvent être considérées comme une option de génération de carburant durable complémentaire pour l’industrie du biodiesel.

De nombreuses espèces de microorganismes coexistent dans la nature en interaction les uns avec les autres et sont plus efficaces lorsqu'ils sont associés entre eux. En utilisant des microorganismes en culture pure, le procéde de fermentation fournit un faible rendement en H_2. En comparaison avec les mono-cultures, les co-cultures offrent des effets synergiques avec un rendement de production de H_2 supérieur. Cette

approche génère également de meilleures performances que l'utilisation des cultures mixtes et permet de surmonter les limitations et les contraintes de l'utilisation des cultures pures, nécessitant moins de temps de fermentation et de meilleurs rendements de production de H_2.

La production d'hydrogène par fermentation sombre (non-photonique) du GB se révèle être plus appropriée que les autres produits à valeur ajoutée, tels que le 1,3-propanediol (1,3-PD) et l'éthanol entre autres. L'objectif principal est d'augmenter la production d'hydrogène lors de la fermentation du glycérol brut en utilisant différentes stratégies de co-culture. *E. aerogenes* et *C. butyricum* dans un système de co-culture sans pré-traitement du substrat et sans addition d'agent réducteur coûteux laissent entrevoir un procéde rentable pour la production de H_2. L'originalité de la thèse est la production améliorée d'hydrogène et de produits à valeur ajoutée à partir de glycérol brut en utilisant le système de co-culture. La fermentation du GB génère des rendements de 15,64 et 17,44 mmol-H_2/L de milieu pour un système en mono-culture en comparaison avec des rendements aussi élevés que 19,46 mmol-H_2/L de milieu avec le système proposé de co-culture. Le rendement de H_2 est d'environ 0,95 mol-H_2/mol de GB, ce qui est comparable à d'autres études de co-culture utilisant la même combinaison de cultures pures, mais avec des substrats différents, tels que l'amidon et l'hydrolysat de pulpe de manioc.

L'utilisation de co-substrat de différents déchets avec des caractéristiques complémentaires peuvent aider à maintenir des niveaux de pH recommandés, et agit comme supplément pour éviter l'ajout de sources d'azote coûteuses. Également, en utilisant des co-substrats, une gestion efficace du procédé peut être réalisée pour améliorer le rendement de production de H_2. L'optimisation des concentrations des substrats et co-substrats, ainsi que de la taille de l'inoculum est de première importance pour l'industrie des bioprocédés. Le modèle a permis de déterminer une réponse de rendement supérieur avec 15 g/L de GB, 5 g/L d'APH (d'hydrolysat de marc de pomme) et 15% (*v/v*) d'inoculum. Les rendements ont été de 26,07 mmol de H_2/L avec une faible production de 1-3-propanediol. Le concept d'addition de co-substrat permet de limiter également l'inhibition du substrat, ce qui entraîne une augmentation de la production de H_2 à une concentration plus élevée de GB.

Également, l'utilisation de la co-culture permet d'améliorer le procédé en éliminant l'étape de barbotage à l'azote pour la production de H_2. En utilisant 1% du glycérol brut sans barbotage à l'azote un rendement de production de H_2 de 1,5 mmol-H_2/mol de glycérol a été obtenu par rapport à 1,2 pour les systèmes utilisant le barbotage à l'azote. Le rendement de 1,3-PD au cours du barbottage au N_2 était d'environ 0,43 et un changement soudain vers les procédé aérobies a entraîné une diminution de rendement de 0,04. L'élimination de l'étape fastidieuse du barbotage à l'azote peut offrir une nouvelle stratégie améliorée pour la production de H_2 à moindre coût, de limiter les risques de contamination, de réduire la production de métabolites et de faire baisser le coût de récupération du produit pour simplifier le procédé de fermentation. La stratégie a démontré la capacité de changer de voie métabolique de la voie réductrice à la voie oxydative poussant le flux de carbone vers une augmentation de la production de H_2 ce qui rend la production de H_2 à partir du GB plus efficace et économiquement plus intéressante.

L'étape initiale de pré-traitement a consisté à diminuer la viscosité du GB par mélange avec de l'eau distillée, suivi par l'ajustement du pH et l'étape de centrifugation pour éliminer les précipités de lipides ou de gras libres qui ont un effet néfaste sur la production de H_2. Dans cette étude, en même temps que le mélange de GB et de l'eau distillée pour diminuer la viscosité, l'addition d'un agent tensio-actif afin d'améliorer l'utilisation du glycérol a été étudiée. Une modélisation statistique a été réalisée afin de cibler les meilleures conditions optimales. Les conditions optimales sont: une concentration de GB de 17,5 g/L et de Tween 80 à 15 mg/L. Cette combinaison a entraîné une augmentation de la production de H_2 atteignant un maximum de 32,1 ± 0,03 mmol/L avec 87,7% de l'utilisation du glycérol. L'utilisation de Tween 80 à une concentration minimale et à faible coût offre une meilleure stratégie pour stimuler l'industrie du biodiesel grâce à une production accrue de H_2 à partir du GB.

Il est bien connu que les procédés de fermentation sombre (non-photonique) génèrent des rendements de H_2 modérés, ce qui nécessite l'ajout de composantes supplémentaires, telles que les acides et les bases pour maintenir le pH, l'utilisation d'un co-substrat et les techniques d'immobilisation pour accroître la production de H_2. Le coût supplémentaire des composantes du milieu de culture, des agents tampons

externes et le coût des matériaux pour l'immobilisation peuvent être éliminés en utilisant une approche rentable et environnementale en utilisant des résidus de coquilles d'oeufs. La production maximale de H_2 a été obtenue avec des coquilles de taille x_5 (33 µm $<x_5<$ 75 µm) avec 36,53 ± 0,53 mmol de H_2/L pendant une première fermentation, suivie par 41,16 ± 0,95 mmol/L pendant une seconde fermentation discontinue répétée. Dans l'étude de mise à l'échelle en bioréacteur (7,5 L), des rendements de près de 1,5 fois plus élevés (en comparaison de mono-culture) ont été obtenus à savoir 312,12 mmol ou 7,69 L H_2/L de milieu avec 86,65% d'utilisation du glycérol. La valorisation des résidus de coquilles d'œufs comme agent neutralisant et d'immobilisation permet de réduire de manière significative les coûts de production du H_2.

Les médias usés post-fermentation contenant des composés organiques et des composantes inutilisées sont d'un grand intérêt comme additif ou supplément pour refaire une deuxième fermentation. Il s'agit d'une approche intéressante de réutilisation d'une partie des déchets de fermentation. Le système de culture mixte a été étudié pour la première fois pour la production de H_2 avec des boues de l'industrie du biodiesel. Un inoculum à partir de boues à une proportion de 20% *v/v*, du GB à 20 g/L, ainsi que les milieux usés ont permis d'obtenir une production accrue de H_2 d'environ 38,12 ± 0,84 mmol/L. Dans une autre approche, le milieu usé de fermentation du GB a remplacé le milieu frais pour la croissance des algues. Cette approche a permis de faire croître des algues pour la production de lipides. Le volume du mélange de milieu usé (30 mL) et du milieu frais (20 mL) a donné lieu à un rendement de 0,098 ± 0,007 g/L de production de lipides. L'approche global d'un système efficace pour utiliser le GB et les milieux usés confirme la valorisation prometteuse du GB et élève également le potentiel l'industrie du biodiesel afin de la rendre plus compétitive sur le marché.

ABSTRACT

Hydrogen with high energy content can guarantee environmentally clean transport in coming years. Given these perspectives, scientists are developing cost-effective processes by pursuing biological hydrogen production to aid the ongoing research on H_2 as energy source.

Pure glucose and sucrose as substrate of dark fermentation are not economically feasible and hence, currently carbon rich wastes have been explored as suitable substrates. Global biodiesel market is renewable, healthier, environment friendly and sustainable alternative for fossil fuels and with increasing annual growth, production would reach 30.28 billion liters by 2020. With each ton of biodiesel produced, 100 kg of crude glycerol (CG) is generated as a by-product. Alternatively, CG is a good source of carbon; therefore valorization of this by-product into fermentative H_2 production seems to be a very promising strategy.

To begin with, energy balance calculation was carried out between CG utilization by dark fermentation process and industrial enrichment to pure glycerol. The total energy input for glycerol purification (872.39 MJ) was 2.5-fold higher in comparison to maximum total energy input of vegetable feedstock derived CG (344.25 MJ). Industrial enrichment with the purified glycerol will cost somewhere around $0.66/L with decreasing market value. Alternatively, the dark fermentation of crude glycerol with 1 Kg of H_2 production can replace 2.47 L of conventional diesel at $3.40/L market value. For efficient utilization of CG, bioconversion to H_2 production by dark fermentation can be considered as energy-efficient and sustainable fuel generation option for biodiesel industries.

Many species of microorganisms coexist in nature by interacting with each other and are most effective when associated with other group of microorganisms. By using single microorganism, the fermentation process delivers low H_2 content and low yield resulting in limited efficiency for H_2 production. In comparison to mono-culture, co-culture offers synergistic effects with higher H_2 yield, better performance than mixed-cultures,

overcomes the limitations of pure cultures, requiring less fermentation time and producing more H_2.

Hydrogen production by dark fermentation using CG is found to be more suitable than any other valuable products, such as 1,3-propanediol (1,3-PD), ethanol, among others. Main objective of the thesis is increasing the hydrogen production during the fermentation of CG using different strategies of co-culture. The studies dealing with *E. aerogenes* and *C. butyricum* in a co-culture system without pre-treatment of substrate and no addition of expensive reducing agent represented a novel process using CG for H_2 production. Thesis originality is improved production of hydrogen and value-added products from crude glycerol using the co-culture system.With CG fermentation, compared to 15.64 and 17.44 mmol-H_2/L of medium for a mono-culture system; as high as 19.46 mmol-H_2/L of medium has been obtained by proposed co-culture system. The H_2 yield was around 0.95 mol-H_2/mol of crude glycerol, which was comparable to other co-culture studies using the same combination of pure cultures over starch and cassava pulp hydrolysate.

Co-substrate utilization of different wastes with complementary characteristics can help in maintaining recommended pH levels, act as supplementary to avoid expensive nitrogen source and with co-substrate effective management of the process can be achieved to improve the H_2 yield. The optimization of substrate and co-substrate concentration along with inoculum size is of primary importance for bioprocess industry. The model helped in determining the higher response at 15 g/L CG, with 5 g/L of APH (apple pomace hydrolysate) and 15% (*v/v*) inoculum resulting in H_2 production around 26.07 mmol/L with trace amount of 1,3-PD production. The concept of co-substrate addition also deals with substrate inhibition, resulting in increased H_2 production at higher concentration of CG.

Improvement in the process by eliminating nitrogen sparging step was investigated for H_2 production using the co-culture system. Using 1% of crude glycerol without nitrogen sparging resulted in H_2 yield of 1.5 mmol-H_2/mol of glycerol in comparison to 1.2 for nitrogen sparged media. The yield of 1,3-PD during N_2 sparging was around 0.43 and sudden shift towards aerobic process resulted in decreased yield of 0.04. Decision on

tedious nitrogen sparging step can offer a new improved strategy for H_2 production at lower cost, avoid risk of contamination, reduce metabolite production and bring down the product recovery cost to streamline the fermentation process. The strategy demonstrated the ability to shift metabolic pathway from reductive to oxidative pushing the carbon flux towards increase in H_2 production and making H_2 production from CG more efficient and economical.

Initial pretreatment step of decreasing viscosity of CG by mixing with distilled water, followed by pH adjustment and centrifugation step to remove precipitated free fatty resulted in decreased H_2 production. In this study, along with mixing of CG and distilled water to decrease the viscosity, addition of the surfactant to further improve the glycerol utilization was investigated along with H_2 production using statistical modeling. The optimized conditions of CG: 17.5 g/L and Tween 80: 15 mg/L resulted in increased H_2 production reaching a maximum of 32.1 ± 0.03 mmol/L with 87.7% of glycerol utilization. The utilization of Tween 80 at minimum concentration offered low-cost improved strategy to boost biodiesel industry through increased H_2 production from CG.

Dark fermentation often end ups in moderate H_2 yield, requiring additional media components, addition of exogenous acids/bases to maintain pH, co-substrate utilization and immobilization techniques for increased H_2 production. The added cost of media components, external buffering agents and material cost for immobilization can be eliminated by using a cost-effective and environmental approach of recycling eggshell. The maximum H_2 production resulted with eggshell size of x_5 (33 µm $<x_5<$ 75 µm) with 36.53 ±0.53 mmol/L during batch followed by 41.16 ± 0.95 mmol/L during repeated-batch fermentation. In the scale-up study with bioreactor (7.5 L), almost 1.5-fold increase (in comparison to mono-culture) i.e. 312.12 mmol or 7.69 L H_2/ L of medium with 86.65% glycerol utilization was obtained. Valorization of eggshells biowaste as a neutralizing, and immobilizing agents can significantly reduce production cost of H_2 production.

The spent media containing organic compounds and unutilized substrate with media components is of high interest as promising choice for waste utilization. The mixed-culture system was studied for the first time during H_2 production with biodiesel sludge

at 20% inoculum, CG at 20 g/L along with spent media resulted in increased H_2 production of around 38.12 ± 0.84 mmol/L. In another approach, the fresh media was replaced with spent media and used for algal growth for lipid production. The mixture volume of spent media (30 mL) and fresh media (20 mL) resulted in 0.098 ± 0.007 g/L of lipid production. The efficient closed system approach of utilizing CG and spent media confirms valorization of CG and also uplifts the biodiesel industry to be competitive in the market.

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TABLE DES MATIÈRES

LISTE DES TABLEAUX

LISTE DES FIGURES

LISTE DES ÉQUATIONS

LISTE DES ABRÉVIATIONS

APH	apple pomace hydrolysate
AS	apple pomace sludge
ANOVA	analysis of variance
BLAST	Basic Local Alignment Search Tool
BOD	biological oxygen demand
BPS	biodiesel primary sludge
CG	crude glycerol, GB= glycérol brut
CFR	code of federal regulations
COD	chemical oxygen demand, DCO= la demande chimique en oxygène
CCD	central composite design
DNA	deoxyribonucleic acid
DNS	dinitrosalicylic acid
DCW	dry cell weight
DGGE	denaturing gradient gel electrophoresis
FID	flame ionization detector
FISH	fluorescence in situ hybridization
GHG	greenhouse gas
InS	inoculum size
LOF	lack of fit
mRNA	messenger ribonucleic acid
RT-PCR	reverse trascriptase- polymerase chain reaction
RSM	response surface methodology
SEM	scanning electron microscopy
TCD	thermal conductivity detector
TRS	total reducing sugar
1,3-PD	1,3-propanediol

CHAPITRE 1

SYNTHÈSE

PARTIE 1. LA PRODUCTION D'HYDROGÈNE PAR UN SYSTÈME DE CO-CULTURE: REVUE DE LA LITTÉRATURE

1.1. Introduction

Le monde sera confronté à une crise énergétique car les réserves de pétrole fossiles seront épuisées dans moins de 45 ans (Ahmad *et al*., 2011). Les sources renouvelables, telles que le biodiesel et le biohydrogène sont des alternatives prometteuses aux combustibles pétroliers et gagnent en importance pour le futur (Sarma *et al*., 2015b; Varrone *et al*., 2013). L'enjeux limitant pour la production commerciale d'hydrogène (H_2) repose principalement sur une consommation d'énergie onéreuse couplée avec l'augmentation du coût de production. Environ 95% de l'hydrogène produit vient principalement des énergies fossiles libérant du dioxyde de carbone à l'atmosphère affectant ainsi le réchauffement climatique (Zeidan *et al*., 2009). Cependant, dans un proche avenir, la production biologique d'hydrogène pourrait offrir une alternative économique et favorable à l'environnement (Zeidan *et al*., 2009).

Pendant les 25 dernières années, des carburants de types biodiesel produits à partir des graisses ou d'huile animales ou végétales ont été employés dans les moteurs diesel et les systèmes de chauffage à travers le monde. Les avantages du biodiesel resident dans le fait qu'il s'agit d'un carburant renouvelable non-toxique, ne produisant aucun sulfate, ni composé aromatique, avec un impact de production de CO_2 moindre et une combustion génèront moins de fumée et de particules. Le biodiesel est une alternative concurentielle au combustible pétrolier (Atabani *et al*., 2012; Varrone *et al*, 2012). Récemment, la production de biodiesel à travers le monde s'est développée exponentiellement, attirant l'attention des chercheurs et des scientifiques pour les aspects économique et durable pour un remplacement des carburants fossiles (Ahmadet *et al*, 2011; Ayoub *et al*, 2012; Xiao *et al*, 2013). Pour satisfaire la demande croissante mondiale, la production projetée de biodiesel en 2020 sera environ 30,28 milliards de litres (Ayoubet *et al*, 2012). Le sous-produit principal de la production du biodiesel est le glycérol brut (GB); un litre de GB est généré pour 10 L de biodiesel produits (Astals *et al* 2012; Sarma *et al*, 2013a). Les données projetées suggèrent que

la production globale de glycérol atteigne ~5,8 milliards de litres d'ici 2020 (Ayoub *et al*. 2012).

En 2012 le Canada a produit 190 millions de litres de biodiesel pour remplacer 5% du diesel, qui génère plus de 1,1 milliard $ de revenus agricoles supplémentaires. Cette production génère également environ 19 millions de litres de GB en tant que déchets (www.eia.gov, 2015; www.greenfuels.org, 2015). Le GB est classé comme déchet dangereux dans l'UE et le raffinage du glycérol n'est pas un procédé rentable (Pott *et al*., 2014).

La gestion alternative de GB couramment utilisée par les industries est la combustion, cependant, de nombreuses industries trouvent qu'il est difficile en raison de propriétés dangereuses d'ignition du méthanol dans le GB (Johnson *et al*., 2007). En cas de disposition du GB, les normes de traitement conventionnelles doivent être appliquées. Dans certaines situations, le GB peut-être est disposé ene site d'enfouissement, ce qui peut causer un ruissellement et rejoindre des cours d'eau intoxiquant les plantes et la faune aquatique. L'utilisation du GB pour l'alimentation animale n'est plus recommandée en raison d'impuretés potentiellement dangereuses (DeFrain *et al*., 2004; Yang *et al*., 2012). Le procédé de purification de la glycérine à partir du GB en utilisant la distillation, l'évaporation ou des techniques combinées de séparation est très énergivore et coûteux (Hunsom *et al*., 2013).

Théoriquement, le GB est un substrat intéressant pour la production de H_2 (Heyndrickx *et al*., 1991). Toutefois, depuis les 50 dernières années, la conversion du glycérol en divers composés, tels que l' H_2 (Ito *et al*., 2005b; Jitrwung *et al*., 2011; Marques *et al*., 2009; Sarma *et al*., 2013b), l'éthanol (Choi *et al*., 2011; Ito *et al*., 2005b) et le 1,3-propanediol (Barbirato *et al*., 1995; Afroditi Chatzifragkou *et al*., 2011c) a été étudiée en utilisant des espèces microbiennes appartenant aux genres *Enterobacter*, *Klebsiella*, *Citrobacter* et *Clostridium* (Ito *et al*., 2005b; Sarma *et al*., 2012). L'état de réduction chimique élevé du glycérol nécessite un excès d'équivalents réducteur, ce qui peut être accompli par la voie de consommation du NADH vers la production de H_2 ou via des produits finaux réduits (tels que l'éthanol ou du 1,3-PD) (Heyndrickx *et al*., 1991).

La production de H_2 à partir du GB est une approche intéressante où le H_2 produit peut être facilement séparé du milieu de fermentation et ne nécessite aucun coût supplémentaire de purification par rapport à l'éthanol et le 1,3-PD (Ito *et al*., 2005b; Sarma *et al*., 2013a). La hausse de la production du biodiesel contribue à la disponibilité du GB en tant que source de carbone abondante, à bon marché et de qualité (Chatzifragkou *et al*., 2011a; Wilkens *et al*., 2012). En donnant ces perspectives, les scientifiques mettent en évidence des procédés rentables pour produire le bio-hydrogène et ainsi lancer de nouveaux travaux de recherche sur l'utilisation du bio-hydrogène comme source d'énergie (Vatsala *et al*., 2008a). La production d'hydrogène biologique utilisant le glycérol brut est moins énergivore, plus économique et constitue une méthode durable de couplage de production d'énergie et de traitement des déchets.

Une recherche approfondie sur un grands groupes de bactéries produisant de l'hydrogène a été effectuée et depuis le H_2 est considéré comme une source future d'énergie propre. Les organismes les plus couramment utilisés sont les genres *Enterobacter* et *Clostridium* avec un rendement en hydrogène de 1 à 2 mol H_2/mol de glucose dans les systèmes en mono-culture (Yokoi *et al*., 1998). Les méthodes de production d'hydrogène réalisées à l'aide de co-culture avec des bactéries spécifiques sont plus efficaces et plus robustes que celles utilisant une culture mixte (bactéries non définies) ou en mono-culture. L'approche de construire de microorganismes spécifiques producteurs de H_2 offrent de meilleures performances, des effets synergiques avec un rendement de H_2 supérieur (Patel *et al*., 2014; Zeidan *et al*., 2010), ce qui nécessite moins de temps de fermentation pour produire plus de H_2 (Kao *et al*., 2014). Quelques études ont été répertoriées portant sur *E. aerogenes* et *C. butyricum* dans un système de co-culture utilisant comme substrat des résidus amidonés tels que l'amidon des patates douces (Yokoi *et al*., 2001; Yokoi *et al*., 1998) et de la pâte de manioc hydrolysée (Phowan *et al*., 2010). Cependant, malgré un rendement en H_2 élevée en utilisant une source de carbone pur, la production de H_2 en utilisant du glycérol brut comporte de nombreuses questions qui doivent être abordées pour faciliter une application à l'échelle industrielle.

La section de revue de la littérature présente une étude détaillée et comparative des différents documents actuellement disponibles sur la production de H_2 à l'aide de co-culture et introduit de nouvelles stratégies qui sont abordées dans **la partie II du chapitre 2**. Tout d'abord, la composition du glycérol brut généré lors de la production de biodiesel est décrite, suivie par l'évaluation du potentiel du glycérol brut comme source de carbone pour la production de H_2. La quantité du bio-hydrogène produite par le système de co-culture est très avantageuse par rapport au système de monoculture. En effet, le système de co-culture a été proposé comme approche expérimentale pour augmenter la production du bio-hydrogène. L'approche de l'utilisation du système en co-culture sans ajout d'agent réducteur coûteux pour augmenter le taux d'utilisation du substrat avec un co-substrat, l'élimination de l'étape de barbotage à l'azote pour limiter les changements métaboliques causés par l'oxygène, l'absence de pré-traitement du substrat avec addition d'agents tensioactifs et l'utilisation de la coquille d'œuf comme médias alternatifs sont tous des aspects qui seront étudiés. Toutes ces approches représentent, dans l'ensemble, une stratégie pour améliorer la rentabilité du procédé tout en étant axées sur les procédés d'augmentation de la production d'hydrogène.

Chapter 2 Part I: Pachapur VL, Sarma SJ, Brar SK, Le Bihan Y, Buelna G & Verma M (2015c) Biological hydrogen production using co-culture versus mono-culture system. *Environmental Technology Reviews* 4(1):55-70.

Chapter 2 Part II: Pachapur VL, Sarma SJ, Brar S K, Le Bihan Y, Soccol CR, Buelna G & Verma M (2015) Co-culture strategies for increased biohydrogen production. *International Journal of Energy Research* 39(11):1479-1504.

1.2. Glycérol brut: sous-produits dérivés du biodiesel

1.2.1. Biodiesel: le marché mondial

Le monde sera confronté à une crise énergétique et les réserves de pétrole seront épuisées dans moins de 45 ans (Ahmad *et al*., 2011). Le biodiesel est renouvelable, plus sain et c'est une alternative durable pour le respect de l'environnement en remplacement des carburants petroliers (Sarma *et al*., 2013a). La production de biodiesel à travers les continents en 2005 et 2011 (Ayoub *et al*., 2012) ainsi que la demande croissante de la production à travers le monde. Le gouvernement américain et l'Union européenne ont fait la promotion de diverses subventions et de nouvelles politiques en matière de carburants renouvelables qui sont favorables à la production de biodiesel (Sarma *et al*., 2013a).

Le Canada étant l'un des principaux producteurs de biodiesel aux États-Unis, la production estimée est susceptible d'atteindre 3,75 milliers barils/jour. Le Canada a mis 500 millions de litres/an de production de biodiesel en accord avec le protocole de Kyoto en 2010 et également mis en place l'exemption de 14,3% de taxe provinciale sur l'utilisation du biodiesel dans les provinces (Canada, 2014). Le tableau 1.1 montre les usines de biodiesel au Canada fonctionnant sur différentes matières premières avec la capacité et les détails des statuts des usines.

Table 1.1 Détails des usines de biodiesel à travers le Canada, avec les détails de la charge d'alimentation, la capacité avec l'état actuel (Biodiesel, 2014, Industrie).

Nom de l'usine	Province	Feedstock	Capacité*	statut
Archer Daniels Midland	Alberta	Source végétale	265	En construction
BIOX Corporation	Ontario	Multi-alimentation	66	Opérationnel
City-Farm Biofuel Ltd.	British Columbia	Déchets d'animaux	10	Opérationnel
Consolidated Biofuels Ltd.	British Columbia	Déchets d'animaux	11	Opérationnel
FAME Biorefinery	Alberta	Source végétale	1	Installation de démonstration
Great Lakes Biodiesel	Ontario	Multi-alimentation	170	Opérationnel
Kyoto Fuels Corp	Alberta	Multi-alimentation	66	En construction
Methes Energies Canada	Ontario	Déchets d'animaux	5	Opérationnel
Methes Energies Canada	Ontario	Multi-alimentation	50	En construction
Milligan Bio-Tech Inc.	Saskatchewan	Source végétale	20	Opérationnel
Noroxel Energy Ltd.	Ontario	Déchets d'animaux	5	Opérationnel
QFI Biodiesel Inc.	Quebec	Multi-alimentation	5	Opérationnel
Rothsay Biodiesel	Quebec	Multi-alimentation	55	Opérationnel
Cowichan Biofuel Facility	BC	Source végétale	0.36	Opérationnel
Innoltek	QC	Multi-alimentation	3	Opérationnel

*Mmly= Millions de litres par an.

1.2.2. Transestérification de la matière de première génération

Le biodiesel est dérivé de la transestérification de matières premières renouvelables biologiques, tels que des graisses d'huiles végétales ou animales, ainsi que d'alcool en présence d'un catalyseur (Ahmad *et al*., 2011). Les produits de réaction sont des esters monoalkyliques d'acides gras à longue chaîne (biodiesel) ainsi que le glycérol brut (Ayoub *et al*., 2012) tels que représentés sur la figure 1.1. Pour la production du biodiesel, les triglycérides provenant des matières premières de première, deuxième et troisième générations sont transestérifiées avec un alcool en présence d'un catalyseur alcalin ou acide (Astals *et al*., 2012; Ayoub *et al*., 2012). Au cours de la réaction, la structure du glycerol retrouvée dans le triacylglycérol est substituée par du méthanol (couramment utilisé pour son faible coût) en présence d'hydroxyde de sodium (communément utilisé pour un court temps de réaction) (Atabani *et al*., 2012; Li Cheng *et al*. 2013a).

Les paramètres tels que la nature de la teneur en acides gras libre du matériau, du rapport huile-alcool, le type de catalyseur et la température réactionnelle de départ ont une influence sur le procédé de transestérification (Chatzifragkou *et al*., 2012). Le mélange réactionnel est agité et chauffé à la température d'ébullition du méthanol pour produire deux phases non miscibles distinctes. Les esters méthyliques (biodiesel brut) se séparent dans la phase supérieure avec la phase inférieure contenant le glycérol et le méthanol brut en tant que sous-produits (Ayoub *et al*., 2012; Sarma *et al*., 2013a). Peu après la phase de séparation le biodiesel compte pour 30-50%, avec d'autres impuretés, telles que des savons, l'alcool, l'eau, les sels, les mono- et tri-diglycérides qui n'ont pas réagi et la composition du glycérol en quantités variables (Amaral *et al*., 2009; Astals *et al*., 2012; Ito *et al*., 2005a). Pour augmenter la pureté, le biodiesel brut est en outre soumis à une étape de purification pour éliminer toutes les contaminations restantes suivies avec l'analyse de contrôle de qualité pour répondre aux spécifications des normes.

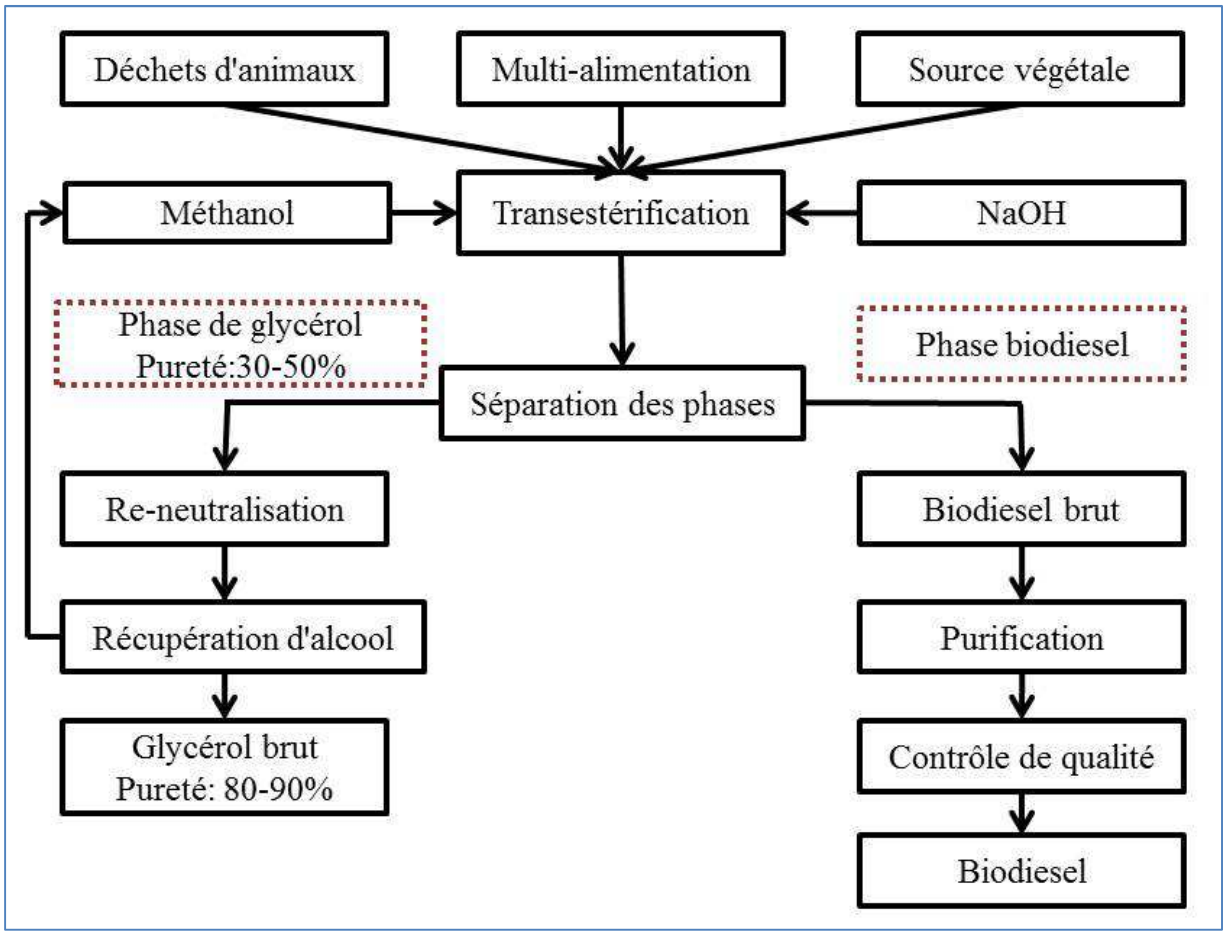

Figure 1.1: Utilisation de la charge de génération avec de l'alcool dans la réaction de transestérification pour la production de biodiesel et de glycérol brut. Détails du processus de production de biodiesel à partir de différentes matières premières dans les industries du biodiesel.

1.2.3. Glycérol brut: valeur du marché

La teneur en glycérol dans le glycérol brut (GB) est d'environ 30 à 50% dans la phase inférieure et celui-ci comprend de nombreuses impuretés. Pour augmenter la pureté et le raffinage du glycérol, des étapes supplémentaires de distillation sous vide/d'échange d'ions, re-neutralisation et la récupération de l'alcool sont des étapes nécessaires pour atteindre une pureté d'environ 99% pour un potentiel d'application pharmaceutique

(Chatzifragkou *et al*, 2012, www.rothsaybiodiesel.ca/our_biodiesel.html). La matière première contribue à environ 75-95% des coûts industriels; la purification supplémentaire du GB se traduira par des coûts de traitement inévitables pour l'industrie du biodiesel (Da Silva *et al*., 2009; Cheng Li *et al*., 2013a). Avec des teneurs de 55-75% en glycérol et diverses impuretés présentes, l'approche de purification est non compétitive pour les usines de biodiesel à petite et moyenne échelle (Chatzifragkou *et al*., 2012). Sur le marché, il existe deux types de glycérol: le glycérol raffiné et le GB. Avec l'industrie du biodiesel, la fourniture en glycérol est passée de 9% à 64%, devenant un des principaux moteurs d'approvisionnement du glycérol sur le marché (Ayoub *et al*., 2012). Finalement, l'augmentation significative de la demande de biodiesel a donné lieu à un grand excédent de glycérol diminuant ainsi le prix du marché (Da Silva *et al*., 2009).

Le prix de GB a diminué de (0,44$ à 0,11$ par kg) en raison de la grande disponibilité sur le marché et avec un effet direct sur le prix du glycérol raffiné provoquant une chute de 1,15$ à 0,66$ par kg (Cheng Li *et al*., 2013a). Le coût du biodiesel est ~1,5 fois plus élevé que celui des combustibles à base de pétrole et les facteurs importants sont le coût des matières premières, la dimension des usines de transformation et la valeur du GB. À l'avenir avec la valorisation et la valeur des sous-produits générés à partir du GB, les coûts totaux de production de carburants biodiesel pourront être réduits de 13-14%. Par conséquent, le coût total de la production de biodiesel est inversement proportionnelle au crédit sur le GB (Ayoub *et al*., 2012). Le GB généré est considéré comme un flux de déchets et il est nécessaire de trouver une destination économique et respectueuse de l'environnement (Cheng Li *et al*., 2013a). La disposition du GB a conduit à la diminution de la viabilité et de l'économie de l'industrie du biodiesel (Ayoub *et al*., 2012). Par conséquent, la nécessité de trouver une stratégie efficace pour transformer le GB en produits à valeur ajoutée est nécessaire pour les usines de biodiesel et pourrait être une solution au problème du coût actuel de valorisation du GB.

1.2.4. Gestion industrielle du glycérol brut

Dans cette section, la gestion alternative du GB couramment utilisé par les industries peut être accomplie par une transformation chimique ou une application directe. Les modes de valorisation actuels par transformation chimique sont la combustion et le prétraitement par compostage. Pour les applications directes, l'enfouissement et l'alimentation animale sont les principales alternatives (Li *et al*., 2013a). Certaines méthodes, telles que la combustion, le compostage et l'alimentation animale, (Ayoub *et al*., 2012) ont des normes strictes et nécessitent des traitements supplémentaires (Sarma *et al*., 2013a), ce qui augmente les coûts de valorisation (Johnson *et al*., 2007). En outre, la décharge potentiellement dangereuse et l'écoulement des eaux de ruissellement causent des perturbations dans l'écosystème aquatique. (DeFrain *et al*., 2004; Yang *et al*., 2012). Avec la récente progression des méthodes de purification du GB dans les industries du biodiesel des techniques ont été développées telles que la distillation par lots simple, l'évaporation ou la séparation membranaire ou des techniques combinées. Le procédé de purification de la glycérine provenant du GB en utilisant la distillation, l'évaporation et d'autres techniques de séparation sont très énergivores (Hunsom *et al*., 2013). La transformation du GB en produit à haute valeur ajoutée avec une consommation énergétique plus faible en comparaison avec la transformation directe et chimique peut être réalisée par conversion microbienne.

1.2.5. Valorisation du glycérol brut

La conversion microbienne du GB surmonte les limites reliées à la transformation chimique et directe, comme une faible spécificité du produit en raison des différents contaminations et le peu d'énergie nécessaire pour la réaction contrairement à la combustion qui nécessite un apport énergétique important (pression/température) (Cheng Li *et al*., 2013a). La méthode industrielle possible pour la bioconversion de GB offre plusieurs avantages, tels qu'un prétraitement ou une purification limitée, un faible apport d'éléments nutritifs et un procédé très efficace pour la production de biogaz ou de biocarburants. Avec la fermentation microbienne, la conversion directe du GB en produits à forte valeur ajoutée semble être économiquement viable pour les industries du biodiesel (Varrone *et al*., 2013).

La fermentation microbienne a la capacité de convertir le GB en tant que seule source de carbone pour la synthèse de plusieurs produits tels que des alcools (éthanol, butanol), des diols (1,3-propanediols, 2,3-butanediol) et les acides (acide lactique, l'acide succinique acide), entre autres. Cependant, la biosynthèse de la majorité de ces produits a des besoins en milieusynthétiques plus complexes (Gonzalez-Pajuelo *et al*., 2004), nécessite des suppléments en vitamines (Jitrwung *et al*., 2011) pour générer des produits à une concentration plus élevée (Chatzifragkou *et al*., 2011b) et les effets des impuretés du GB sur la fermentation doivent être surmontés à des coûts minimes (Ito *et al*., 2005b; Jitrwung *et al*., 2011). La technologie pour la production de ces alcools, diols et des acides est déjà bien connue et l'exigence de pureté pour les applications industrielles est sont harmonisées avec les exigences de la synthèse chimique (Almeida *et al*., 2012). Une option possible serait d'utiliser le GB pour la production d'hydrogène (H_2). Enfin, la production de H_2 par fermentation sombre (non-photonic) en utilisant le GB est plus appropriée que tous les autres produits de valeur (alcools, diols et acides). La présence de diverses impuretés dans le GB rend l'étape de récupération des produits à valeur ajoutée coûteuse. Cependant, le H_2 produit avec 80-99% de pureté peut être facilement séparé des médias et peut être utilisé directement comme combustible (Sarma *et al*., 2013a; Sarma *et al*., 2012). Les méthodes industrielles classiques utilisées pour 95% de la production de H_2 sont coûteuses et utilisent des combustibles fossiles (Costa *et al*., 2011; Sarma *et al*., 2012).

Par conséquent, il existe un besoin d'une technique moins coûteuse de production de H_2 à partir de sources renouvelables en utilisant des espèces microbiennes comme méthode alternative aux méthodes industrielles actuelles (Markov *et al*., 2011).

1.3. Glycérol brut: une source de carbone pour la production d'hydrogène

1.3.1. Types de sources de carbone utilisées pour la production d'hydrogène

L'abondance des déchets organiques déversés à partir de sources municipales, agricoles et industriels causent des préoccupations environnementales graves, tels que les odeurs les émanations de gaz toxiques, la contamination des eaux souterraines au site d'enfouissement (Kim *et al*., 2013; Kim, 2004). La quantité de déchets organiques est estimée entre 2,75 à 4 kg/habitant/jour dans les pays développés et 0,5 kg/habitant/jour dans les pays à faible revenu à travers le monde (Kim *et al*., 2013). La plupart des déchets organiques solides sont facilement disponibles et contiennent des compositions diverses en source de carbone et d'azote nécessaire pour la production microbienne d'hydrogène (Chong *et al*., 2009, Kim *et al*., 2013). Les critères principaux pour la sélection des matériaux organiques afin de produire du H_2 sont la disponibilité des ressources, le coût, le rapport d'hydrate de carbone et l'azote, le besoin de pré-traitement et le taux de biodégradabilité (Kim, 2004). Le potentiel de production de H_2 a été étudié à l'aide de divers déchets organiques et celui-ci est résumé ci-dessous.

Matières lignocellulosiques

Un autre résidu organique facilement disponible et abondant qui peut être utilisé pour la production de H_2 microbien est le déchet lignocellulosique, avec une production annuel de plus de 220 milliards de tonnes dans le monde entier (Kim *et al*., 2013). La matière cellulosique est le biopolymère le plus abondant obtenu à partir des rebuts de la sylviculture, les résidus de bois, le déchets de papier, et les déchets agricoles (Kim *et al*., 2013). Les matières lignocellulosiques contiennent de la cellulose (32-47%), de l'hémicellulose (19-27%) et de la lignine (5-24%) avec les plus grandes sources renouvelables des sucres de type hexoses et pentoses (Carere *et al*., 2008; Kim *et al*. , 2013). Pour un procédé de production de H_2 à partir d'un résidu lignocellulosique il est nécessaire d'utiliser des microorganismes cellulolytique ou une étape de prétraitement supplémentaire pour dégrader la cellulose en sucre fermentescible simples (Chong *et al*., 2009). L'hydrolyse de l'hémicellulose de la bagasse de canne à sucre a été

effectuée en présence d'acide sulfurique (0,5% v/v) à 121 °C avec un temps de réaction de 60 min pour obtenir des sucres pour la production de H_2 (Pattra *et al*., 2008). Les déchets lignocellulosiques nécessitent un prétraitement alcalin doux avec du NaOH à 3% en utilisant un rapport liquide solide de 25:1 à 80 °C pendant 3 heures pour hydrolyser les structures complexes en sucres fermentescibles pour avoir un rendement final de 50,05 ± 1,51 mmol H_2/L (Cheng *et al*., 2013).

Déchets alimentaires

La plupart des déchets organiques générés par les municipalité sont formés de déchets alimentaires qui représente environ 1,3 milliard de tonnes per an, soit un tiers des aliments produits dans le monde entier (www.unep.org/wed/2013/quickfacts/, 2013). Les déchets alimentaires retrouvés dans les déchets solides municipaux, comprennent les résidus de cuisine, les déchets municipaux et les co-produits alimentaires. Ils contiennent des niveaux élevés de constituants organiques volatils variant de 85-95%, qui agissent en tant que substrat possible pour la production anaérobique de H_2 (Kim *et al*., 2013).

La pulpe de manioc, un substrat complexe contenant 66% (p/p) d'amidon nécessite une hydrolyse acide en utilisant 0,5% (v/v) de H_2SO_4 1:15 (rapport liquide solide) à 121 °C pendant 30 minutes pour générer un rendement élevé de 345,8 mL H_2/g DCO (la demande chimique en oxygène) (Phowan *et al*., 2010). Selon certaines études, un prétraitement avec une hydrolyse chimique suivie d'une hydrolyse enzymatique supplémentaire de l'amidon du blé en glucose et maltose a donné lieu à un plus fort taux de production de H_2 à raison de 280 mL/jour (Ozmihci *et al*., 2011). La conversion des déchets organiques pour une utilisation durable afin de produire du H_2, sans étape de prétraitement ni d'ajout de nutriments supplémentaires, conduira à une utilisation du substrat à un faible coût résultant d'un avantage économique pour la production biologique de H_2.

1.3.2. Glycérol brut pour la production d'hydrogène

Le glycérol brut représente un substrat peu coûteux, durable et approprié pour la bioconversion microbienne par rapport aux substrats de bioraffineries classiques, tels

que le glucose et le saccharose provenant de déchets organiques (Li Cheng *et al*., 2013a). Le haut degré de l'état de réduction du glycérol génère une augmentation accrue de NADH, ce qui en fait une matière première facilement assimilable pour la biomasse microbienne. En outre, la base à 3 carbone du glycérol génère deux moles de NADH par rapport à la demi-mole de glucose qui génère seulement une mole de NADH (Li Cheng *et al*., 2013a). Le rôle du NADH est très important dans le métabolisme du glycérol, et le rapport de $NADH/NAD^+$ définit le métabolisme oxydatif du glycérol vers la voie réductrice pendant la production de H_2 (Nakashimada *et al*., 2002). Compte tenu de la nature hautement réductrice du glycérol, la production de H_2 à des rendements très élevés à partir de GB est possible par rapport aux sucres communs, tels que le glucose et le xylose en utilisant des bactéries anaérobies ou facultatives (Murarka *et al*., 2008).

Bactéries anaérobies

Les bactéries anaérobies sont surtout recherchées pour leur taux élevé de production de H_2 et leur capacité à utiliser une large gamme de substrats. Les bactéries anaérobies appartenant à *Clostridium* sont généralement utilisées pour la bioconversion des hydrates de carbone en H_2, acétate, butyrate, CO_2 et les solvants organiques (Ito *et al*., 2005). La production d'hydrogène suit la voie du butyrate et de l'acétate, en présence d'une enzyme hydrogénase et un rapport approprié en butyrate/acétate pour produire de l'H_2 à partir du glucose (Chong *et al*., 2009). Les expériences de traitement par lots pour une plus grande production de H_2 peuvent être réglées de manière à accélérer la phase acidogène ou exponentielle pour plus de temps d'exécution en réduisant la consommation de glucose et en limitant la co-production d'éthanol (Chong *et al*., 2009).

Bactéries anaérobies facultatives

Ces bactéries possèdent la double propriété de produire de l'ATP en présence d'oxygène, ainsi que la production de H_2 par fermentation anaérobie. Les bactéries anaérobies facultatives ont des avantages sur les bactéries anaérobies strictes (Chong *et al*., 2009). Beaucoup de recherches pour l'amélioration des bactéries anaérobies

facultatives par mutagenèse chimique, par effets simples et réciproques de substrat avec la température et le pH ont été expérimentées (Sarma *et al*., 2012). La bactérie *Enterobacter sp*. est active, même dans des conditions acides (pH 3,9) (Sarma *et al*., 2013c) et peut également remplacer les agents réducteurs par sa capacité à consommer de l'oxygène (Phowan *et al*., 2010; Yokoi *et al*., 1998b).

1.3.3. Voie de la production d'hydrogène à partir du glycérol

Le métabolisme de conversion du glycérol et la production d'hydrogène pour les bactéries anaérobies facultatives et strictes suit la voie oxydative et réductrice. Dans la voie oxydative, le NAD^+ se réduit en NADH avec le glycérol et entre dans la voie de la glycolyse pour produire le pyruvate (Sarma *et al*., 2012). Le pyruvate, en fonction du microorganisme, sera décomposé en divers produits (éthanol, le lactate, l'acétate, le butyrate, entre autres) ainsi que la production de H_2. Au cours de la voie réductrice, le NADH est réoxydé en NAD^+ avec le glycérol qui se fait déshydrater en 1,3-propanediol (1,3-PD) (Sarma *et al*., 2012). Lors de la production de H_2 à partir du GB, l'éthanol, l'acétate, le butyrate et le 1,3-PD sont communs aux sous-produits générés par *Enterobacter* (Ito *et al*., 2005; Jitrwung *et al*., 2011) et *Clostridium* (Heyndrickx *et al*., 1991).

La diversité des produits de fermentation à partir du glycérol dépend du ratio entre $NADH/NAD^+$. Le rapport supérieur favorise la voie réductrice dans les deux souches avec une production du 1,3-PD seulement. Lors d'un passage d'un métabolisme anaérobie à aérobie, le ratio diminue de manière significative et favorise la voie oxydative. Dans le cas de *Clostridium*, le NADH se réoxyde avec la production de H_2 avec la ferrédoxine oxydoréductase (FD_{ox}) et des enzymes de types hydrogénases sont synthétisés. L'enzyme FD_{ox} régule le rapport de $NADH/NAD^+$ et l'acétyl-CoA/CoA avec des pouvoirs réducteurs supplémentaires pour synthétiser le H_2. Dans le cas *d'Enterobacter*, l'hydrogénase produit du H_2 à partir d'un dérivé de formiate en divisant la pyruvate-formate lyase (Nakashimada *et al*., 2002). La fermentation du glycérol avec une éventuelle voie de libération de H_2 en même temps que la production de

métabolites grâce à l'équilibre du carbone est indiquée ci-dessous dans les équations 1-6 (Barbirato *et al*., 1995; Biebl *et al*., 1992).

1,3-Propanediol: $C_3H_8O_3 + NADH + H^+ \rightarrow C_3H_8O_2 + H_2O + NAD^+$ (1)

Acetate: $C_3H_8O_3+H_2O+2NAD++2ADP \rightarrow C_2H_4O_2+HCOOH+H_2+2NADH+2H^++2ATP$ (2)

Butyrate: $2C_3H_8O_3 \rightarrow C_4H_8O_2+2CO_2 + 2H_2 + 2NADH +2 H^+$ (3)

Ethanol: $C_3H_8O_3 + ADP \rightarrow C_2H_6O + HCOOH + ATP$ (4)

Formic acid: $C_3H_8O_3+H_2O+2Co\text{-}A \rightarrow 2(CH_2O_2) \rightarrow 2CO_2 +2H_2 + 2CH_3\text{-}CoA$ (5)

Lactate: $C_3H_8O_3 + NAD + + ADP \rightarrow C_3H_6O_3 + NADH + H^+ + ATP$ (6)

1.3.4. Raison de bioconversion du glycérol brut en hydrogène

Le glycérol brut est une bonne source de carbone, son utilisation comme matière première pour la production H_2 a été proposée pour compenser le crédit sur le GB pour l'industrie du biodiesel (Sarma *et al*., 2012). La bioconversion du glycérol (pur et brut) en H_2 peut atteindre 75-80% de la production de H_2 théorique (Sabourin-Provost *et al*., 2009). Le GB est renouvelable, économique et est une source efficace de substrat. Son utilisation durable pour la production H_2, sans ajout de nutriment additionnel, serait une utilisation industrielle à valeur ajoutée avec des avantages économiques de valoriser le GB pour l'industrie du biodiesel.

Avec la conversion de GB en H_2, d'autres avantages environnementaux en termes d'équivalent d'émission de CO_2 (CO_2-éq) et de réduction des GES (gaz à effet de serre) peuvent être obtenus. Le montant total de la réduction des GES, compte tenu que 2,56 L de diesel fossile est remplacé par du carburant H_2 à partir du GB a donné lieu à une réduction de 7,66 kg d'équivalent CO_2 (Sarma *et al*., 2013a). Sur les mêmes bases, les détails de calcul des avantages environnementaux qui découlent de la production de GB à travers le Canada et dans le monde sont présentés du tableau 1.2. La réduction des GES calculée par la production H_2 à partir du GB aiderait le Canada à réduire et maintenir les émissions de 17% de GES fixées d'ici 2020.

Table 1.2 Estimation des avantages environnementaux de la bioconversion de 1 kg de glycérol brut en utilisant la fermentation sombre et estimation des résultats pour la production de glycérol brut par jour en 2020 à travers le Canada et dans le monde.

Processus de bioconversion	Avantages de la production d'hydrogène	Avantages estimatifs pour la CG produite/ jour en 2020 dans l'ensemble du Canada	Estimation des avantages pour le GB produit/jour en 2020 dans le monde entier
Glycérol brut	1 kg	44715 kg	594600 kg
Hydrogène	20,75 g	927 kg	12338 kg
Remplacement du combustible fossile	2,5 L	110446 L	1468662 L
Réduction des GES (CO_2 eq)	7,33 kg	0,3 millions kg	4.3 millions kg

Dans l'ensemble, avec l'utilisation des déchets tel que le GB, la production de H_2 réduit les émissions de CO_2 conduisant à une plus grande réduction et un avantage potentiel sur les émissions de GES.

1.4. Système de co-culture de production d'hydrogène

1.4.1. Système de mono-culture de production d'hydrogène

Le procédé de fermentation en présence de culture pure spécifique est connu en tant que système mono-culture. L'utilisation des déchets organiques complexes par le système en mono-culture nécessite des étapes de prétraitement et l'ajout d'enzymes externes pour hydrolyser la cellulose et l'amidon contenus dans les déchets organiques en hydrates de carbone simples pour la production de H_2 (Capdan *et al*., 2006). La production de l'hydrogène par fermentation peut s'effectuer selon deux modes.

Fermentation sombre (non-photonique)

La production de H_2 microbienne en l'absence de lumière, mais en présence de bactéries soient anaérobies ou facultatives et en utilisant des déchets organiques est communément appelé fermentation sombre (Hema *et al*., 2012). Pendant la fermentation sombre, le pool d'électrons et de protons sont les paramètres les plus

précieux en utilisant des formes complexes de substrat organique nécessitant de faible demande en énergie, de conception simple, sous des conditions ambiantes avec un mélange afin d'obtenir un taux de production en H_2 supérieur (équation 7) (Lee *et al*., 2010). Cependant, la dégradation complète du substrat avec une diminution de rendement semblait un obstacle majeur dans le procédé de fermentation sombre pour la production de H_2 (Hema *et al*., 2012). Le taux de conversion du substrat diminue avec la variation du pH due à l'accumulation de sous-produits, tels que l'acide acétique, l'acide butyrique et de l'acide lactique (Yokoi *et al*., 1998a). L'inhibition par les acides organiques peut être minimisée par l'échange de sous-produits par le système à deux phases (Sarma *et al*., 2015) ou en effectuant la fermentation sombre et la photo-fermentation combinée pour augmenter le rendement en H_2.

$$C_6H_{12}O_6 + 2\ H_2O \text{ ---- > } 2\ CH_3COOH + 2\ CO_2 + 4\ H_2 \quad (7)$$

Photofermentation

La production microbienne de H_2 en présence de lumière est appelée photofermentation. Au détriment de l'énergie lumineuse, des petites chaînes d'acides organiques qui agissent comme donneurs d'électrons sont utilisées par des bactéries photosynthétiques pour la production de H_2 (équation 8). Les bactéries phototrophes peuvent travailler sous de large éventail de spectres d'absorption pour atteindre un rendement de H_2 théorique. Les bactéries photosynthétiques sont capables d'utiliser les acides accumulés, ralentir la baisse du pH, augmenter la dégradation du substrat et maximiser le rendement de H_2 (Hema *et al*., 2012). La combinaison de deux fermentations a un potentiel pour atteindre un rendement de H_2 théorique (équations 7 et 8) (Chen *et al*., 2008). Certaines études indiquent que la co-culture de *C. butyricum* et *E. aerogenes* cultivés sur de l'amidon en l'absence d'agents réducteurs, en éliminant les traces d'oxygène a permis de produire du H_2 à environ 2 moles/mole de glucose. La combinaison de *C. butyricum* et *Rhodobacter* sp. M-19 a abouti à environ 6,6 moles de H_2/mole de glucose (Yokoi *et al*., 2001).

$$2\ CH_3COOH + 4\ H_2O \text{ ----> } 4\ CO_2 + 8\ H_2 \quad (8)$$

Dans certains cas, les médias usés contenant des acides organiques après la fermentation sombre ont été centrifugés et supplémentés avec des composants de médias pour réaliser la photo-fermentation. Le rendement de production de H_2 en deux phases a été plus élevé en comparaison avec la fermentation sombre et la photo-fermentation séparées.

1.4.2. Système séquentiel en deux étapes de production d'hydrogène

Afin d'augmenter la production de H_2, une approche intégrée de combinaison de la fermentation sombre avec la photofermentation pour les déchets organiques est présenté aux équations 7 et 8. Dans un système séquentiel en deux étapes, les bactéries fermentaires sombres vont agir sur les déchets organiques complexes pour produire des acides organiques de faibles poids moléculaires (équation 7), qui sont facilement dégradés par les bactéries photosynthétiques pour la production de H_2 (équation 8) (Hema *et al*., 2012).

Ce faisant, le H_2 produit par le procéde en deux étapes séquentiel peut générer un niveau de H_2 près du taux théorique de 12 moles de H_2 en utilisant le glucose (Hema *et al*., 2012). Ce taux ne peut être atteint qu'en combinant la fermentation sombre et la photofermentation dans un bioréacteur (Chen *et al*., 2008). L'adaptation du procéde en deux étapes séquentielles à l'échelle industrielle est moins efficace et nécessite du temps et des efforts supplémentaires afin d'optimiser les conditions de production H_2.

En utilisant le système de co-culture le rendement de production en H_2 a été augmenté. Également, une meilleure récupération d'énergie à partir des déchets de la biomasse et une faible teneur en DCO dans le produit final peuvent être obtenues. En outre, un système en co-culture pour la conversion de la biomasse nécessaire pour la production de H_2 peut être plus efficace par rapport au système à deux étapes dans deux réacteurs différents (Geng *et al*., 2010).

1.4.3. Système de co-culture: Avantages par rapport à la mono-culture

Un consortium est rien d'autres que des microorganismes qui vivent dans une communauté très diverse et complexe, capable d'effectuer des tâches complexes, des

interactions diverses en passant par la coopération à la réalisation directe (Zuroff *et al*., 2013). Une étape raisonnable simple à concevoir au sein de chaque souche est d'obtenir un phénotype complémentaire, ce qui est bénéfique pour la communauté afin que le consortium demeure stable (Weibel, 2008). Les microbiologistes et les ingénieurs ont exploité les communautés microbiennes pour concevoir des consortiums synthétiques pour la production de biocarburants. Les interactions syntrophiques entre les organismes aident à la production de H_2 à partir des déchets organiques (Fang *et al*., 2006). Les rendements de H_2 à travers différents procédé (mono-, co- et mixtes culture).

La création d'une population syntrophique en mélangeant deux ou plusieurs microorganismes fonctionnels spécifiques pour améliorer les propriétés individuelles qui sont défaillante n'est rien d'autre que le système de co-culture. Le système de co-culture possède des avantages économiques et techniques sur le système mono- et en culture mixte. En effet, ce système permet d'éviter le prétraitement/l'hydrolyse enzymatique (Elsharnouby *et al*., 2013), élimine l'utilisation d'agents réducteurs (Yokoi *et al*., 1998a) et peut produire du H_2 avec une pureté de 58% (Bao *et al*., 2012) à 99,99% (Singh *et al*., 2008). Les avantages de la co-culture sur les mono- et cultures mixtes pour l'utilisation des déchets organiques sont présentés. La figure montre les différentes étapes à travers plusieurs systèmes pour la conversion des déchets organiques en H_2.

Avant la fermentation pour produire du H_2, le système en mono-culture nécessite l'étape de prétraitement et l'ajout d'enzymes externes pour l'hydrolyse des déchets organiques (Capdan *et al*., 2006). Dans le cas des cultures mixtes, un traitement supplémentaire en terme de chaleur, pH, aération, produits chimiques et traitements de gel et de dégel, suivi avec l'ajout des nutriments complémentaires pour enrichir les bactéries productrices de H_2 sont nécessaires (Kim *et al*., 2013).

Dans un système de co-culture, les microorganismes ne présentent pas de dominance entre eux, chacun métabolise son substrat spécifique et les microorganismes sont peu affectés par la présence d'autres microbes ce qui facilite la neutralisation de l'inhibition

des produits finaux de la réaction (Park *et al*., 2012). Les mélanges de substrats complexes sont simultanément convertis en produits plus simples de façon séquentielle en H_2 afin d'amortir les fluctuations du système à travers le flux d'alimentation du procédé (Eiteman *et al*., 2008; Quéméneur *et al*., 2011).

Contrairement à la monoculture, la co-culture possède une plus grande résistance aux fluctuations de l'environnement et assure la stabilité pendant la période de traitement (Brenner *et al*., 2008). La combinaison de bactéries facultatives avec des bactéries anaérobies strictes, permet de consommer l' O_2 et d'éliminer l'étape de barbotage à l'azote et, finalement, permet de déplacer les voies métaboliques vers une augmentation de la production de H_2 avec une diminution de la formation de sous-produits (Yokoi *et al*., 1998).

Un autre avantage de la co-culture est de réaliser deux procédé ou plus en une seule étape par culture de deux ou plusieurs microorganismes complémentaires. La co-culture avec deux ou plusieurs microorganismes compatibles pour la dégradation cellulolytiques permet d'hydrolyser la cellulose et d'utiliser les sucres dissous pour produire efficacement du H_2 (Harish *et al*., 2010). L'avantage d'utiliser le système de co-culture permet non seulement d'améliorer le rendement en H_2, mais d'augmenter la formation de biofilm en tant que caractéristique potentielle dans les systèmes de fermentation industrielle (Zeidan *et al*., 2010). La technique de co-culture à l'aide de la fermentation sombre et de la photofermentation permet de définir une voie pour une plus grande production de H_2 par rapport au procédé en une seule étape (Afsar *et al*., 2011).

Le liquide fermenté généré par la combinaison de système en co-culture répond à la demande d'élimination de la DCO par rapport à la fermentation unique et en deux étapes (Lee, 2012). L'utilisation d'acides organiques par les photo-bactéries dans un système de co-culture fournit une meilleure qualité de traitement des effluents en termes de réduction de la DCO. L'étude de Vatsala *et al*., (2008) avec un système en co-culture a montré 60% de réduction de la DCO. Une extrapolation de leur résultats permettrait d'obtenir une réduction des coûts de traitement conventionnel de ~

271000$/an. Le développement du système de co-culture est capable de faire évoluer l'économie des combustibles fossiles basés sur des biocarburants en améliorant le rendement en H_2 par l'ingénierie des microorganismes producteurs de H_2 à l'échelle industrielle (Pachapur *et al*., 2015).

1.4.4. Défis dans le système de co-culture

Les principaux défis à relever pour une production efficace de H_2 à l'échelle commerciale peuvent être décrits de la façon suivante. Dans un système de co-culture, la sélection de la microflore productrice de H_2 joue un rôle très important. La sélection de la microflore de diverses sources est une tâche difficile, en raison de la co-existence d'autres bactéries consommant le H_2.

Une fois que les microorganismes sont choisis pour le système en co-culture, des études ont démontré que l'analyse des métabolites lors de la fermentation a indiqué que *C. freundii* s'est développée plus rapidement que *C. butyricum*, ce qui a entraîné la diminution du rendement final de production en H_2. Le rapport de l'inoculum optimal joue un rôle clé dans le système en co-culture pour la consommation anaérobie de substrat et pour la création d'un environnement anaérobie par les bactéries facultatives (Thonart *et al*., 2010). Le microorganisme dominant en compétition pour le substrat génère des sous-produits ou métabolites qui ont pour effet de diminuer le pH et d'inhiber l'activité hydrogénase qui produit l' H_2 des autres microorganismes (Li *et al*., 2013b). Dans le cas du système combiné en co-culture, le pH acide à 5 ou 6 est optimal pour les bactéries de la fermentation sombre par rapport à un pH neutre de 6,5 à 7,0 qui est préféré par les bactéries photo-fermentaires. Ces conditions sont nécessaires pour obtenir un rendement élevé en production de H_2 (Lee *et al*., 2012). Les bactéries de la fermentation sombre ont une croissance rapide et produisent rapidement les acides volatils provoquant une diminution du pH. Au contraire, les bactéries photo-fermentaires ont une croissance lente, ce qui provoque un déséquilibre du taux de consommation des acides volatils limitant la production H_2 (Liu *et al*., 2010). Dans le cas du système combiné de co-culture, l'augmentation de la concentration de l'inoculum

en bactéries anaérobies va diminuer le taux d'utilisation de la lumière par les bactéries photo-fermentataires (Wu *et al*., 2012).

Les problèmes de co-culture mentionnés ci-dessus peuvent être minimisés en réalisant des outils statistiques comme discuté pour la production de H_2 (**chapitre 2, Partie I**) et en intégrant des stratégies de co-culture (**Chapitre 2, Partie II**) ainsi que des améliorations dans la conception du réacteur. Le coût du procédé global de l'utilisation des déchets organiques avec la production d'énergie simultanée et le traitement des effluents peut être diminué en réduisant le coût de la récolte, le transport et le traitement des déchets de la biomasse par la réalisation du procédé de production de H_2 à la source des résidus (Sarma *et al*., 2013a).

1.4.5. Approche de co-culture pour la production d'hydrogène

La production de H_2 per fermentation comprend les étapes typiques suivantes: le dépistage des bactéries productrices de H_2; l'hydrolyse acide ou enzymatique d'un substrat complexe en sucres fermentescibles; fermentation anaérobie des sucres pour produire du H_2 et des acides organiques volatils (métabolites finaux) et; l'utilisation de ces acides organiques volatils dans la production de H_2 en présence de bactéries photofermentaires. Il est impossible de réaliser toutes ces étapes en utilisant qu'une seule mono-culture pure unique. Cette approche est possible dans un système en co-culture avec l'utilisation de deux ou plusieurs cultures pures définies. Les différentes stratégies de co-culture pour accroître la production H_2 sont présentées dans le **Chapitre 2 Partie II**.

La sélection de microorganisme dépend des tests biochimiques. Néanmoins, dans le cas d'une production maximale de H_2 en utilisant le système en co-culture, le meilleur choix sera l'utilisation de *Clostridium* et *Enterobacter*, l'un agit comme dégradeur et l'autre intervient comme agent réducteur, suivi avec utilisation des métabolites finaux à l'aide des bactéries photo-fermentaires (*Rhodobacter* ou *Rhodopseudomonas*) pour la production de l'H_2. La fréquence d'utilisation de ces quatre microorganismes a compté pour environ 50-64% de tous les microorganismes répertoriés à travers toutes les études portant sur la co-culture.

En fonction du substrat, l'étape de prétraitement peut être sélectionnée. Par contre, si les microorganismes de la co-culture sont capables de dégrader le substrat complexe en sucre fermentescible simple, l'étape de pré-traitement peut être évitée. Ensuite, le taux d'inoculation, le pH initial, la vitesse d'agitation, la concentration en substrat et la température optimale de croissance des microorganismes ont besoin d'être optimisés pour la production de H_2 en utilisant des outils statistiques. Un accroissement supplémentaire de la production de H_2 peut être obtenu grâce à l'optimisation des paramètres extérieurs, tels que l'utilisation de plusieurs substrats complémentaires, l'immobilisation des microorganismes et des techniques de criblage biochimiques.

La teneur en H_2 produite peut varier de 58% (Bao *et al*., 2012) à 99,99% de pureté (Singh *et al*., 2008). En utilisant des microorganismes purs et spécifiques, le H_2 peut être exempt de CO_2 et, dans certains cas, la teneur en CO_2 peut varier de 10 à 20% (Reith *et al*., 2003). Même en présence de quantités traces de CO_2, la pureté de H_2 peut être augmentée en utilisant du NaOH, un procédé d'absorption du CO_2 dans une solution basique (Junyapoon *et al*., 2011). Cette approche permet de générer un gaz répondant aux exigences commerciales actuelle en terme de pureté du H_2 pureté (99,9%) (Vatsala *et al* ., 2008b).

Les projets du 21^{ième} siècle utilisent des "énergies renouvelables" et des "énergies vertes" pour répondre à la demande croissante et assurer une moindre dépendance envers les combustibles fossiles. Les scientifiques du monde entier participent à la production commerciale de H_2 comme une source viable d'énergie. Les investissements dans le développement de la filiale H_2 renforceront la nation à travers le marché mondial (Jain, 2009). Les nations dont les entreprises sont expérimentées dans le développement spatiale sont prédestinées à gérer la production, le stockage et le transport de grandes quantités de H_2. Les principales applications de H_2 sont répertoriées dans l'industrie de l'équipement électrique et électronique, dans le verre et les fabricants d'aliments et l'utilisation importante et inévitable dans le transport aérien et spatial (Winter, 2009).

Pour augmenter la production dans les systèmes d'énergie renouvelable, le mélange de H_2 à environ 5-15% dans les gazoducs existants a été proposé (Melaina *et al*., 2013). Pour répondre à la demande croissante, le système énergétique actuel pour la production de l'H_2 peut être amélioré en utilisant des ressources renouvelables, tels que le glycérol brut avec la stratégie susmentionnée en utilisant un système de fermentation en co-culture.

PARTIE II. PROBLÉMATIQUE

À travers la revue de la littérature (Pachapur *et al*., 2015) et les stratégies de système de co-culture (Pachapur *et al*., 2015) présentées au chapitre 2, les problématiques suivantes ont été soulevées au cours de la production d'hydrogène.

2.1. Gestion industrielle du glycérol brut

Les industries font face à une problématique d'élimination et de valorisation du GB. En cas de combustion du GB, les propriétées dangereuses et inflammables du méthanol contenu dans le GB peuvent provoquer une combustion spontanée et nécessite un coût supplémentaire pour la disposition de ces déchets (Johnson *et al*., 2007). Pour l'élimination, des normes de traitement universelles doivent être appliquées et pour le compostage, une source supplémentaire de nutriments est nécessaire (Sarma *et al*., 2013a). Dans certaines situations, où le GB a été mis à l'enfouissement, du ruissellement a été observé causant un effet toxique pour les plantes. Si le GB est utilisé comme engrais, il diminue l'absorption d'azote du sol lors de sa dégradation. Les niveaux toxiques du méthanol et les impuretés potentiellement dangereuses dans le GB rendent défavorable son application en alimentation animale (DeFrain *et al*., 2004, Yang *et al*., 2012). Ce résidu de l'industrie du biodiesel est constitué d'un pourcentage variant de glycérol ainsi que diverses impuretés telles que des sels organiques et inorganiques, les savons, les alcools, et quelques traces de glycérides et des pigments d'origine végétale. Le procédé de purification du glycérol provenant du GB en utilisant la distillation, l'évaporation ou d'autres techniques de séparation sont à forte consommation énergétique (Hunsom *et al*., 2013). La valorisation du GB est réalisable

soit par fermentation sombre ou par purification du glycérol en tenant compte de l'analyse du bilan énergétique.

2.2. Composants de milieu de culture coûteux pour la production d'hydrogène

Les microorganismes les plus couramment utilisés pour la production de H_2 par fermentation sont du genre *Enterobacter* et *Clostridium* avec un rendement de H_2 variant entre 1-2 moles H_2/mole de glucose dans le système de type mono-culture (Yokoi *et al*., 1998). En général, les bactéries anaérobies strictes, telles que *Clostridium* peuvent théoriquement avoir plus de rendement de H_2 que les bactéries anaérobies facultatives. Cependant, des conditions anaérobies devraient être maintenues pour assurer la croissance microbienne soit par le barbotage du milieu à l'azote/hélium ou par addition d'agents réducteurs coûteux (Phowan *et al*., 2010; Yokoi *et al*., 1998).

2.3. Inhibition du substrat à une concentration plus élevée lors de la production d'hydrogène

L'état de réduction élevé du glycérol favorise les souches *Enterobacter* et *Clostridium* vers la voie réductrice avec une production de 1,3-PD (Barbirato *et al*., 1995, Papanikolaou *et al*., 2004). Le GB était responsable de 66% de l'inhibition par le substrat de la production de H_2 et favorise la voie réductrice du métabolisme du glycérol entraînant la formation de 1,3-PD au lieu de H_2 (Szymanowska-Powałowska *et al*., 2014; Viana *et al*., 2012). Afin de déplacer le métabolisme de la voie réductrice vers la voie oxydative, l'acétate en tant que co-substrat agi comme un accepteur d'électrons augmentant ainsi la production de H_2 (Heyndrickx *et al*., 1991).

2.4. Effet du barbotage à l'azote sur la production d'hydrogène

L'utilisation de l'azote lors de l'étape initiale de barbotage des milieux de cultures est un procédé fastidieux et est effectuée pour créer des conditions anaérobies (Morsy, 2014). Un générateur d'azote doit être installé pour obtenir du N_2 pur (Sarma *et al*., 2013a) ou il est possible d'utiliser un cylindre de N_2 avec 99,99% de pureté au coût de 63$ environ (en dollars américains). Cependant, l'utilisation d'un coût élevé pour le barbotage au N_2

entraverait l'application à l'échelle industrielle avec l'augmentation des coûts de production (Kim *et al*., 2012)..

2.5. Étape de pré-traitement supplémentaire pour réduire la viscosité du GB

La production d'hydrogène dépend des caractéristiques du GB et la composition des principales impuretés, tels que le méthanol et du savon (Ito *et al*., 2005b; Ngo *et al*., 2011, Nwachukwu *et al*., 2013). L'inhibition par le méthanol est pris en charge lors de la stérilisation des médias (Ito *et al*., 2005b; Sarma *et al*., 2012). L'effet du savon est atténué par l'étape de prétraitement initiale qui consiste à diminuer la viscosité du GB en mélangeant avec de l'eau distillée (1:4 v/v), suivi par l'ajustement du pH et l'étape de centrifugation pour éliminer les précipités d'acides gras libres (Athalye *et al*, 2009; Chi *et al*., 2007; Ethier *et al*., 2011; Ngo *et al*., 2011). L'élimination du savon conduit à une baisse de production de H_2, ce qui suggère que la présence de savon joue un rôle d'agent tampon avec des effets bénéfiques sur l'utilisation du glycérol (Sarma *et al*., 2014).

2.6. Production d'acides organiques lors de la production d'hydrogène

Pendant la fermentation sombre il y a une production d'acides organiques, une forte baisse du pH du milieu de culture avec une diminution de la production en H_2. Cette réaction nécessite un ajout d'agents tampons externes pour maintenir le pH (Sarma *et al*., 2015a; Tenca *et al*., 2011). La production de H_2 est seulement capable d'utiliser 30-40% du substrat et le reste 60-70% est utilisé dans la production de métabolites. La bioconversion du GB pour produire 1 kg de H_2 produit 8700 litres de milieux usés contenant du carbone organique, de l'azote, de la biomasse ainsi que des métabolites et des nutriments résiduels (Sarma *et al*., 2015b).

PARTIE III. HYPOTHÈSE

Ce travail de recherche comprend les hypothèses suivantes:

1. Le GB dérivé du biodiesel est produit par le procédé de transestérification de différentes matières lipidiques. Les déchets générés par les restaurants et l'industrie de transformation de la viande contenant de l'huile, les graisses animales et huiles de cuisson sont utilisés comme matière première pour la production de biodiesel. Par conséquent, la composition de GB peut être variée de sorte que se caractérisation est essentielle avant son utilisation pour la production de H_2.

2. Afin de soutenir l'industrie du biodiesel, le GB peut être utilisé pour d'autres produits à valeur ajoutée, tels que la purification en glycérol pur ou la fermentation sombre pour la production de H_2. L'apport d'énergie pour chacune des étapes du procédé dans les deux cas, déterminera la dépense énergétique totale avec la valeur marchande du produit final.

3. Parmi les bactéries productrices de H_2, *Clostridium* a un rendement plus élevé de H_2 par rapport à *Enterobacter* sp. Par conséquent, la combinaison d'*Enterobacter* et de *Clostridium* dans un système de co-culture dans des conditions optimales peut fournir un haut rendement de production de H_2.

4. Dans le système de production de H_2 par co-culture, l'équilibre du profil métabolique détermine le comportement de la voie des cultures pendant la fermentation du glycérol. Chaque microorganisme suit une voie spécifique avec une production de métabolites distincts selon l'absorption d'une source de carbone. L'analyse des métabolites pourrait permettre de distinguer le rôle des cultures pures dans la fermentation du glycérol.

5. L'utilisation de co-substrat à partir des différents déchets présentant des caractéristiques complémentaires peut fournir un support complet pour augmenter la production de H_2. Le marc de pomme peut être hydrolysé et utilisé comme une alternative de source de carbone dans les systèmes de mono- et de co-culture pour la production de H_2.

6. Les études portant sur le co-substrat en utilisant le GB sont rares et sans aucune optimisation statistique des paramètres de fermentation. Les optimisations de la concentration en substrat et en co-substrat, ainsi que la volume de l'inoculum sont de première importance pour l'amélioration des bioprocédés pendant la production de H_2.

7. L'étape de barbotage à l'azote est mise en oeuvre pour créer des conditions anaérobies pendant la production de H_2. Sous des conditions anaérobies, la fermentation du glycérol favorise la voie réductrice avec une production de 1,3-propanediol entraînant une diminution de la production de H_2. En éliminant l'étape de barbotage à l'azote en utilisant le système de co-culture avec *E. aerogenes et C. butyricum* la modification de la voie métabolique peut être évaluée pendant la production de H_2.

8. Le GB possède une viscosité plus élevée en raison de la présence de diverses impuretés. La diminution de la viscosité est effectuée avec de l'eau distillée lors de la production de H_2. Des surfactants supplémentaires peuvent réduire la viscosité et augmenter la disponibilité du glycérol pour accroître la production de H_2.

9. La fermentation sombre nécessite des composantes de milieux supplémentaires, des acides et des bases pour maintenir le pH, ainsi que des techniques d'immobilisation pour augmenter la production de H_2. Les propriétés de la coquille d'œuf comme agent neutralisant et comme support peu coûteux seront explorés pour accroître la production de H_2.

10. Afin de faire correspondre le niveau de production et la valeur du marché du biohydrogène avec d'autres combustibles commerciaux, il est important de valoriser les milieux utilisés. Par conséquent, la culture mixte et les bactéries photosynthétiques possèdent la capacité de convertir les acides gras en H_2 et les milieux usées peuvent servir à la croissance des microalgues. Les milieux usés peuvent être réutilisés en remplacement de l'eau distillée lors de la préparation des médias.

PARTIE IV. OBJECTIFS

L'objectif principal de cette étude est d'augmenter la production d'hydrogène par fermentation du glycérol brut en utilisant diverses stratégies de co-culture et la conversion des milieux de culture usés pour la production d'hydrogène.

1. Caractérisation du glycérol brut

La composition du glycérol brut étant variable, il est essentiel de le caractériser adéquatement en tant que substrat de fermentation. La teneur en cendres, l'humidité et les MONG (matière non-glycérol organique), mesurées en termes de contaminants du GB seront analysées selon la méthode standard (ISO 2098-1972).

2. Équilibre énergétique du glycérol brut pour la production d'hydrogène

Afin de déterminer l'apport d'énergie efficace de l'utilisation de GB, des comparaisons seront faites entre la production de H_2 par fermentation sombre et la gestion industrielle du GB via sa purification en glycérol pur. Cet objectif apportera une contribution significative telle que des informations sur les dépenses d'énergie pour chacun des matériaux utilisés lors de l'inoculum, les étapes de préparation des médias et de l'électricité consommée au cours du procédé de fermentation sombre pour la production d'hydrogène. La meilleure gestion industrielle actuelle serait la méthode de purification du glycérol. Toutefois, l'apport en énergie déterminera le procédé le plus efficace de valorisation du GB en termes de glycérol raffiné ou de H_2 purifié produit par fermentation sombre.

3. Évaluation de la capacité de *E. aerogenes* comme agent de remplacement de l'agent réducteur dans le système en co-culture pour la production d'hydrogène

En général, les bactéries anaérobies strictes, telles que *Clostridium* peuvent théoriquement avoir plus de rendement de H_2 que *E. aerogenes*. Cependant, l'état anaérobie doit être maintenue pour assurer leur croissance par addition d'agents réducteurs coûteux. Alternativement, les bactéries anaérobies facultatives peuvent consommer l'oxygène de l'espace libre et peuvent être utilisées pour éviter l'utilisation des agents chimiques réducteurs coûteux.

4. Développement des méthodes par GC et LC-MS pour l'analyse des métabolites finaux lors de la production d'hydrogène

Les métabolites finaux de la fermentation du glycérol, tels que le 1,3-DP, l'éthanol et l'acide butyrique, entre autres, ont une importance commerciale. L'analyse des métabolites finaux de fermentation représente une composante essentielle de cette étude. Celle-ci permettra d'avoir une connaissance approfondie sur la voie d'utilisation du glycérol par *E. aerogenes* et *C. butyricum* et d'effectuer le calcul du bilan de carbone.

5. Stratégie de co-culture avec l'addition de co-substrat pour augmenter la productivité en H_2.

L'utilisation du glucose et du saccharose pur comme substrats ne sont pas économiquement viables et actuellement les déchets riches en carbone sont explorés en tant que substrats appropriés. Le but de cette étude sera d'évaluer le potentiel de production de H_2 à partir du GB en cas de co-fermentation avec l'hydrolysat de marc de pomme pour produire le 1,3-PD (<3 g/L) et de l'éthanol (<3 g/L). La production de H_2 en présence et absence de co-substrat sous les systèmes mono- et co-culture sera également testée.

6. Étude de l'effet du glycérol brut et de la concentration de co-substrat sur la production d'hydrogène

Les effets individuels et interactif de chacun des principaux paramètres (concentration de GB (5-15 g/L), concentration d'hydrolysat de marc de pomme (5-15 g/L) et taille d'inoculum (5-15%)) seront étudiés par une méthodologie de plan de surface de réponse. Le GB utilisé et la quantité de co-substrat seront analysés.

7. Élimination de l'étape de barbotage à l'azote pendant la production d'hydrogène par l'utilisation d'un système en co-culture

L'utilisation de l'azote lors de l'étape initiale de barbotage des médias est un procédé fastidieux et le barbotage au N_2 après l'étape de l'inoculum pose un risque élevé de contamination. Par conséquent, pour minimiser les coûts de production, sans co-

substrat, l'étape de barbotage au N_2 sera éliminée et des conditions microaérobies seront créées pour étudier la production de H_2 en utilisant le système de co-culture.

8. Augmentation de la disponibilité du glycérol brut pour la production d'hydrogène

L'optimisation de la concentration du GB (10-30 g/L) et de la concentration en surfactant (1-25 mg/L) sera réalisée par la méthodologie de réponse de surface pour évaluer l'effet individuel et interactif sur la production de l'H_2 et de l'éthanol avec une concentration de glycérol résiduelle. La variation de la viscosité pendant la préparation du milieu et à la fin de la fermentation sera analysée.

9. Coquilles d'œuf comme remplaçant de composants de milieu de culture en utilisant un bioreacteur de 7,5 L en système de co-culture

Différentes tailles de coquilles (1,7 mm à 75 µm) à plusieurs concentrations (0,5 à 4% w/v) en mode de fermentation en lot seront utilisées pour la production de H_2. Le dispositif expérimental en l'absence et en remplacement de chaque composante en présence de coquille d'œuf sera effectuée. Les résultats obtenus en bouteille de sérum de 125 mL de volume seront mis à l'échelle, jusqu'en bioréacteur de 7,5 litres de volume.

10. Photo-fermentation du milieu de culture riche en acides organiques provenant des milieux de culture usés utilisés lors de la production d'hydrogène

Les milieux de culture usés contiennent de la biomasse morte, des nutriments résiduels, des biomolécules et du glycérol inutilisé qui peuvent agir tous comme une source supplémentaire de substrat. Le milieu usé sera utilisé comme co-complément à différentes concentrations (10 à 50 mL) au cours de la production de H_2 et des lipides.

PARTIE V. ORIGINALITÉ

Sur la base des hypothèses et des objectifs proposés, les composantes de l'originalité du projet de Ph.D. sont les suivantes:

1. Jusqu'à ce jour, le GB est surtout utilisé pour la production de 1,3-propanediol et de l'éthanol au détriment des composantes du milieu de culture. Toutefois, aucune tentative n'a été faite en utilisant une co-culture de *E. aerogenes* et *C. butyricum*, sans aucun prétraitement du GB. Pour réduire le coût du procédé, *E. aerogenes* doit être utilisé comme une alternative aux agents chimiques réducteurs coûteux.

2. Le GB est essentiellement une source de carbone et son utilisation par *E. aerogenes* et *C. butyricum* peut facilement être évaluée en effectuant un bilan carbone. L'analyse des métabolites des systèmes de mono- et de co-culture dans différentes conditions de fermentation doit être étudiée.

3. La production de H_2 peut être améliorée en présence d'un milieu complet. Cependant, aucune tentative n'a été faite pour évaluer l'hydrolysat de marc de pomme (déchets de l'industrie de la pomme) en tant que co-substrat avec le GB pour la production de H_2 et l'analyse des métabolites générés par un système en co-culture. Cette approche sera évaluée.

4. Aucune tentative n'a été faite à ce jour pour évaluer les interactions de différentes concentrations de GB et d'hydrolysat de marc de pomme (co-substrat) ainsi que la taille de l'inoculum (InS) sur la production de H_2 en utilisant un système en co-culture. Dans ce contexte, la méthodologie de plan de surface de réponse pourrait être adoptée pour étudier l'interaction entre les GB, APH et InS, avec les réponses de la production de H_2, du 1,3-propanediol et de la production d'éthanol.

5. Classiquement, l'azote est utilisé pour faire un barbotage et créer des conditions anaérobies pendant la production de H_2 en utilisant du GB. Ainsi, en présence de *E. aerogenes* (une bactérie anaérobie facultative) et *C. butyricum* (une bactérie anaérobie stricte), l'étape du barbotage au N_2 sera éliminée et le profil métabolique et la production de H_2 seront évalués dans des systèmes en mono et en co-culture.

6. Le GB ainsi que les composantes des milieux de culture sont mélangés avec de l'eau distillée lors de l'étape de préparation des médias pour diminuer la viscosité facilitant ainsi la production H_2. Ainsi, la présence d'un agent tensio-actif et l'absence d'un prétraitement seront évaluées à différentes concentrations initiales de GB et des concentrations variables en agent tensio-actif.

7. Les exigences du développement durable pour le recyclage des déchets solides tels que les coquilles d'œufs mélangées avec le GB auront un effet d'assurer une production de H_2 plus verte. Le procédé d'utilisation des coquilles d'œufs lors de la production de H_2 à partir du GB par le système de co-culture est une approche novatrice.

8. En vue d'élever l'industrie du biodiesel en tant que vecteur énergétique majeur, de nouvelles approches pour l'utilisation des déchets générés doivent être explorées. Un modèle de fermentation sombre et en photo-fermentation sera évalué pour le recyclage des milieux de culture usés comme intrants supplémentaires lors de la production de H_2 et la production d'algues.

Dans l'ensemble, l'originalité de la recherche proposée se définie par ***La production améliorée d'hydrogène et de produits à valeur ajoutée à partir de glycérol brut en utilisant un système de co-culture.***

PARTIE VI. SOMMAIRE DES DIFFÉRENTS VOLETS DE RECHERCHE EFFECTUÉS DANS CETTE ÉTUDE

6.1. La production d'hydrogène par un système de co-culture: Différents types et stratégies (deux articles publiés)

6.2. La production d'hydrogène en utilisant une co-culture sans prétraitement du substrat (deux articles publiés)

6.3. La production d'hydrogène en utilisant une co-culture pour plusieurs substrats (un article publié)

6.4. La production d'hydrogène en utilisant une co-culture pour optimiser les procédé (deux articles publiés)

6.5. La production d'hydrogène à l'aide de co-culture avec des cellules immobilisées (un article soumis)

6.6. La production d'hydrogène en utilisant une co-culture avec une fermentation sombre et une photofermentation (un article soumis)

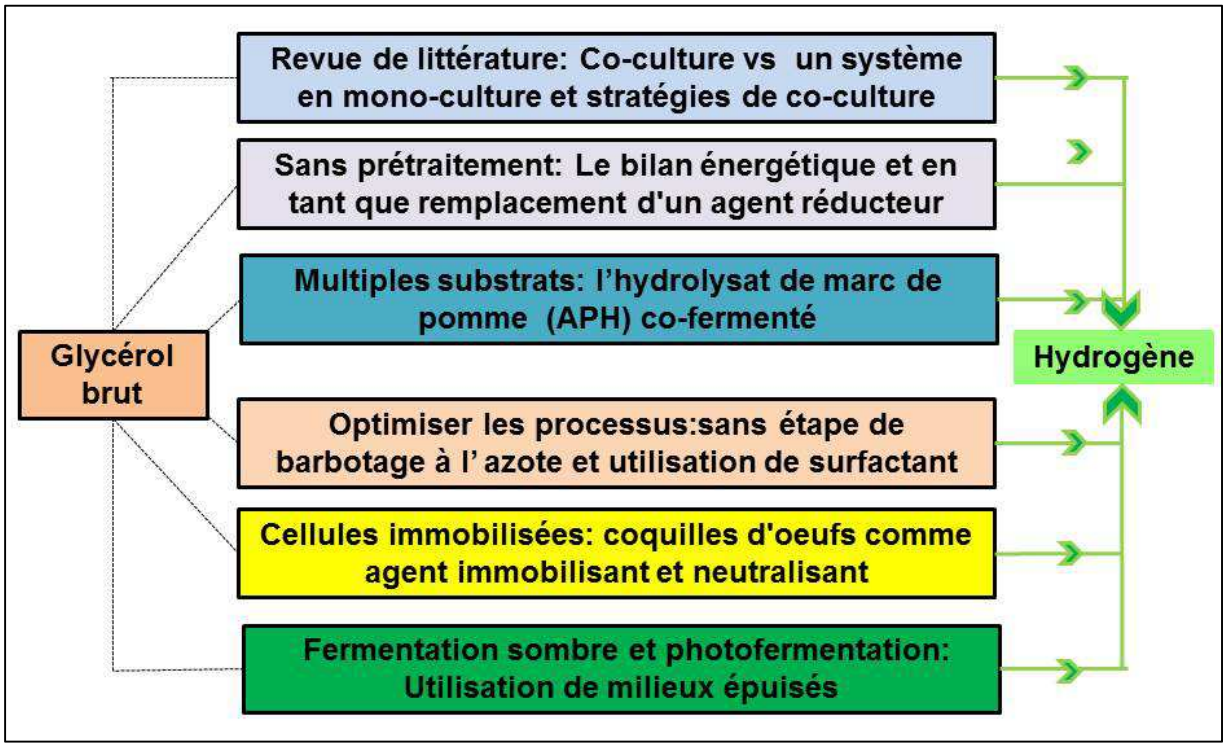

Figure 1.1: Présentation de la stratégie d'étude de la thèse portant sur la co-culture pour accroître la production de biohydrogène.

6.1. Production d'hydrogène par un système de co-culture: Différents types et stratégies (Chapitre 2)

6.1.1. Production biologique d'hydrogène en utilisant un système de co-culture vs un système en mono-culture (Chapitre 2, Partie I, un article de publié)

Une approche durable pour la production de H_2 à partir de diverses méthodes, telles que photo-fermentation, la fermentation sombre et séquentielle à deux étapes présente des avantages importants pour compléter le procédé traditionnel de production. Pendant la fermentation, des microorganismes consortium spécifiques sont caractérisés et leur métabolisme est bien étudié. Des efforts de recherche importants ont été réalisés pour augmenter la production de H_2 en utilisant le système de co-culture, qui

offre l'avantage d'un rendement accru en H_2 et un taux de production supérieur par rapport aux mono-culture. Le concept du système de co-culture est une approche simple qui consiste à mélanger ensemble différentes souches microbiennes pour améliorer les propriétés manquantes des souches individuelles. Le système en co-culture est un système rentable, qui élimine éventuellement l'étape de prétraitement et évite l'utilisation d'un agent chimique réducteur coûteux. En éliminant ces deux étapes, le coût global du procédé peut être réduit sans affecter le rendement de production en H_2. Le système en co-culture hydrolyse directement les substrats organiques complexes en sucres fermentescibles avec 94,1% d'amélioration de rendement par rapport au système en mono-culture. La co-culture offre divers avantages, par exemple, la réduction de la phase de latence, la résistance aux fluctuations de l'environnement et une stabilité des taux de production de H_2 sans interruption, et selon certaines études le taux de production de H_2 serait 8 fois plus élevé par rapport aux mono-culture. Les systèmes de production de H_2 en co-culture sont aussi une méthode de traitement des effluents avec une réduction de 60% de la DCO et peut être facilement intégré dans un procédé à l'échelle pilote pour réaliser une production continue de H_2. L'élaboration d'un système en co-culture suggère un potentiel énorme pour la production de H_2 en utilisant des déchets organiques complexes avec une application viable et applicable à l'échelle industrielle.

6.1.2. Stratégies de co-culture pour accroître la production de biohydrogène (Chapitre 2, Partie II, un article de revue publié)

La production biologique de H_2 à partir de déchets organiques est moins coûteuse, exige moins d'énergie et est un procédé respectueux de l'environnement. L'approche en mono-culture pure fournit un contenu en H_2 et un rendement faible; ces limitations sont surmontées par l'utilisation d'un système en co-culture, qui surpasse les cultures mixtes avec un rendement accru de production en H_2. Les stratégies utilisées dans le système de co-culture pour augmenter la production de H_2 ont été discutés dans cette revue. Les stratégies comprennent l'hydrolyse de différents substrats complexes, tels que la cellulose, la mélasse, le glycérol brut, et la biomasse algale en sucres fermentescibles simples en éliminant l'utilisation d'enzymes exogènes. Les stratégies

peuvent mettre en contact des microorganismes qui se retrouvent dans des environnements différents et distants pour une utilisation simultanée du substrat et de métabolites finaux afin de produire de H_2 à 99,99% pure, sans les coûts d'agent réducteurs. Il est possible d'obtenir une production maximale d'hydrogène en utilisant des stratégies de co-culture avec *Clostridium*, *Enterobacter* et des bactéries photo-fermentaires dans un système de bioprocédés consolidé. Le système en co-culture permet d'obtenir un rendement près de la valeur théorique de H_2 avec une haute efficacité de conversion des déchets organiques. Cette approche permet également d'améliorer la viabilité économique de la production H_2, de fournir une meilleure qualité de traitement des effluents et en même temps répondre aux problématiques de production en H_2.

6.2. Production d'hydrogène en utilisant une co-culture sans prétraitement du substrat (Chapitre 3)

6.2.1. Bilan énergétique pour l'utilisation du glycérol brut pendant la production d'hydrogène et pendant la purification du glycérol (Chapitre 3, Partie I, un article de revue publié)

La production de biodiesel à travers le monde a entraîné une augmentation proportionnelle du glycérol brut (GB) comme déchets. La valorisation du GB augmentera la viabilité économique de l'industrie du biodiesel à l'avenir. Cette étude compare le bilan énergétique pour une utilisation du GB pour la production de H_2 par fermentation sombre avec la purification du glycérol brut pour en faire du glycérol pur. Le bilan énergétique pour chacun des matériaux utilisés lors de l'inoculum, les étapes de préparation des médias et de l'électricité consommée dans les deux méthodes ont été calculées. L'entrée d'énergie totale pour la purification du glycérol (872,39 MJ) était 2,5 fois plus élevé par rapport à l'apport d'énergie totale maximale de la matière première du GB (344,25 MJ). La valeur de marché du glycérol pur diminue par rapport à l'augmentation de la valeur de marché de H_2 comme combustible. Pour une utilisation efficace du GB, la bioconversion pour la production H_2 par fermentation sombre peut être considérée comme l'option favorable et durable pour la production de carburant à

haut rendement énergétique. L'énergie nette (MJ) pour différentes matières premières (source végétale -158,90, multi-matières premières -113,23 et les déchets animaux -83,14) pendant la production de H_2 varie avec la teneur en glycérol. Il est possible de faire la fermentation sombre sous des conditions ambiantes et d'utiliser de l'électricité produite à partir du biogaz pour réduire l'apport énergétique total. Ce faisant, l'énergie nette pour différentes matières premières aura une valeur positive.

6.2.2. Production d'hydrogène à partir de déchets de l'industrie du biodiesel en utilisant une co-culture de *Enterobacter aerogenes* et *Clostridium butyricum* (Chapitre 3, Partie II, un article de revue publié)

La valorisation du glycérol brut (GB), une perte dans le procédé de production du biodiesel a été étudiée pour augmenter la production d'hydrogène par un système en co-culture en utilisant *Enterobacter aerogenes* NRRL B-407 et *Clostridium butyricum* NRRL B-41122. La capacité de *E. aerogenes* a été évaluée en tant que remplacement d'un agent réducteur coûteux afin de maintenir des conditions anaérobies pour la croissance de *C. butyricum*. La co-culture a donné lieu à une production accrue de H_2, atteignant un maximum de 19,46 ± 0,95 mmol-H_2/L-de milieu de culture par rapport aux mono-culture de *E. aerogenes* (15,64 ± 0,47 mmol/L) et *C. butyricum* (17,44 ± 0,38 mmol/L) avec la présence d'un agent réducteur. L'effet du rapport de l'inoculum a été étudié et le rendement en H_2 était de 0,95 mol-H_2/mol-glycérol pour un rapport de l'inoculum de 1:11 avec plus de 85% de l'utilisation du substrat. Ces résultats étaient comparables à ce que d'autres études répertorient pour des cultures mixtes et co-culture. En plus du H_2 comme produit à valeur ajoutée, des sous-produits tels que le 1,3-propanediol, l'acide acétique, l'acide butyrique et l'éthanol ont également été produits. La co-culture a démontré une capacité accrue de valorisation du GB, en tant que consortium fonctionnel stable avec une plus grande efficacité de conversion pour augmenter l'hydrogène ainsi que générer des sous produits à hautes valeurs ajoutées. En outre, l'étude a fourni la preuve que la composition du milieu contrôle les changements de voie métabolique lors de la bioconversion du GB. La valorisation du GB à faible coût avec une co-culture efficace pour la production dH_2 peut aider l'industrie du biodiesel en générant une source d'énergie interne.

6.3. Production d'hydrogène en utilisant une co-culture pour plusieurs substrats (Chapitre 4)

6.3.1. Production d'hydrogène par co-fermentation du glycérol brut et de l'hydrolysat du marc de pomme en utilisant la co-culture de *Enterobacter aerogenes* et *Clostridium butyricum* (Chapitre 4, un article de revue publié)

L'utilisation de co-substrat provenant de divers déchets présentant des caractéristiques complémentaires peut fournir un support complet pour une plus grande production d'hydrogène. Cette étude a permis d'évaluer le potentiel de l'hydrolysat de marc de pomme (APH) co-fermenté avec du glycérol brut (GB) pour augmenter la production de H_2 et une diminution des sous-produits formés. La conception de la composante centrale (CCD) a été utilisée comme outil d'optimisation et 15 g/L de GB, 5 g/L d'APH et 15% (v/v) d'inoculum ont été identifiées comme conditions optimales. Dans ces conditions, la production en H_2 a été aussi haute que 26,07 ± 1,57 mmol H_2/L de milieu. La valeur p de 0,0017 a indiqué que l'APH à faible concentration a eu un effet significatif sur la production de H_2. Le GB utilisé comme unique source de carbone, favorise le métabolisme de la voie réductrice du glycérol et permet une production de 19,46 mmol H_2/L. Cependant, avec l'APH, la voie oxydative a été favorisée par une plus grande production de H_2 (26,01 mmol/L) et la diminution de la formation des sous-produits réduits tels que (1,3-propanediol et de l'éthanol). L'APH augmente la production de H_2, et diminue l'inhibition du substrat.

6.4. Production d'hydrogène en utilisant une co-culture pour optimiser les procédé (Chapitre 5)

6.4.1. Preuve des changements métaboliques sur la production d'hydrogène, l'éthanol et le 1,3-propanediol à partir de glycérol brut par barbotage de l'azote sous des conditions micro-aérobies en utilisant une co-culture de *Enterobacter aerogenes* et de *Clostridium butyricum* (Chapitre 5, Partie I, un article de revue publié)

L'hydrogène (H_2), un éventuel futur vecteur d'énergie propre, exige des voies d'amélioration basés sur les procédé pour couper le coût de production. L'impact du

barbotage à l'azote (N_2) sur la production de H_2 à partir du GB en système de co-culture de *E. aerogenes* et *C. butyricum* a été étudié pour réduire le coût global du procédé. La production de H_2 en utilisant 1% GB sous des conditions de barbotage du milieu à l'azote avant l'autoclavage a abouti à une production de 1,2 mol-H_2/mol de glycérol par rapport à 1,5 mol-H_2/mol de glycérol sans barbotage. En présence d'air variant de 5 à 75 mL dans le volume d'espace de tête, la production de H_2 a augmenté jusqu'à un maximum de 26,14 mmol/L et 1,4 g/L pour la production d'éthanol. La concentration en 1,3-propanediol lors du barbotage avec le N_2 était d'environ 3,0 g/L et a diminué à 0,5 g/L en raison de la présence de 75 mL d'air dans l'espace de tête. Cette observation peut être attribuée à un changement du métabolisme réducteur et oxydant pour le glycérol. Une stratégie d'amélioration basée sur les procédé pour optimiser la formation H_2 a entraîné un changement de voie métabolique de réducteur à oxydant avec l'augmentation de la production de H_2. L'influence synergique du système de co-culture en l'absence d'agent réducteur coûteux et sans étape de barbotage à l'azote peut offrir une meilleure stratégie économique. Cette approche permet de minimiser la production de métabolites et accroît l'application sur le terrain à l'échelle industrielle.

6.4.2. Utilisation de surfactant améliorant l'absorption du glycérol et la production d'hydrogène à partir des déchets de l'industrie du biodiesel à l'aide de co-culture de *Enterobacter aerogenes* et *Clostridium butyricum* (Chapitre 5, Partie II, un article de revue publié)

Dans la présente étude, le Tween 80, un agent tensioactif non ionique, a été utilisé pour améliorer la production d'hydrogène à partir du GB en utilisant la bioconversion par une co-culture de *Enterobacter aerogenes* et *Clostridium butyricum*. Le but de l'introduction de l'agent tensio-actif est de diminuer la viscosité du glycérol brut, de sorte que la solubilité et la biodisponibilité apparente du glycérol peuvent être améliorées au détriment des étapes de prétraitement. Des expériences ont été planifiées en utilisant le concept de composite central (CCD); les concentrations de GB et Tween 80 ont été optimisées alors que la production de H_2, l'utilisation du glycérol et de la viscosité des médias ont été considérés comme des réponses. La surface de réponse pour le modèle quadratique montre que la concentration de Tween 80 a un effet significatif ($p < 0,05$)

sur les trois réponses. En utilisant les conditions optimisées à 17,5 g/L de GB et 15 mg/L de Tween 80, la production de H_2 a atteint un maximum de 32,1 ± 0,1 mmol/L de milieu. L'augmentation de la production de H_2 est d'environ 1,25 fois, en présence de Tween 80 par rapport à son absence, avec 25,56 ± 0,91 mmol/L de production. Des conditions optimales sélectionnées ont également été validées par rapport à l'absence de GB (4,69 ± 0,76 mmol/L), l'utilisation de GB prétraitée (20.06 ± 0.51 mmol/L) et à travers le système de mono-culture (15,43 ± 0,79 à 22,14 ± 0,94 mmol/L). L'introduction du Tween 80 dans le milieu de fermentation améliore le taux d'utilisation du glycérol, ce qui entraîne une augmentation de la production de H_2 et permet d'éliminer les étapes de prétraitement.

6.5. Production d'hydrogène à l'aide de co-culture avec des cellules immobilisées (Chapitre 6)

6.5.1. Bio-valorisation du glycérol brut avec des coquilles d'oeufs en remplacement des composants de support au cours de la production d'hydrogène par des études d'augmentation d'échelle en utilisant un système de co-culture (Chapitre 6, un article soumis)

Les propriétés des coquilles d'oeufs comme agent immobilisant et neutralisant ont été examinées lors de la production de H_2 en utilisant le GB par co-culture de *Enterobacter aerogenes* et *Clostridium butyricum*. Différentes tailles de coquilles d'œufs à plusieurs concentrations ont été testées lors de la réalisation des lots de fermentation discontinue répétée. La production de H_2 maximum selon la taille des coquilles d'œufs de 33 µm < x_5 < 75 µm avec 36,53 ± 0,53 mmol/L pendant le lot suivie par 41,16 ± 0,95 mmol/L pendant la seconde fermentation répétée. La production de H_2 a proportionellement augmenté avec la diminution de la taille de la coquille d'œuf qui maintient aussi le pH de fermentation (6,0-6,3). De plus, la propriété d'immobilisation a été vérifiée par des images de microscopie électronique à balayage. La concentration en coquille d'oeuf de 0,25% (v/v) a été jugée optimale et il a complété le rôle de support microbien et de substance de remplacement à des composés chimiques du milieu de culture avec 31,66 ± 0,55 mmol/L par rapport à la présence de composantes du milieu

avec 32,07 ± 0,92 mmol/L lors de la production de H_2. L'étude de mise à l'échelle avec des conditions optimisées à l'aide d'un bioréacteur de 7,5 L a entraîné près de 1,5 fois plus de H_2 par rapport à la mono-culture. Une production de 312,12 mmol ou 7,69 L H_2/L de milieu avec 86,65% de glycérol utilisé a été mesurée. L'utilisation des coquilles d'œufs en remplacement des composants des milieux de culture peut faire baisser le coût des milieux de 85-90% pour la production de H_2. La valorisation des coquilles d'œufs comme un agent neutralisant, agent immobilisant et comme source d'éléments nutritifs peut réduire de façon significative le coût de production du H_2. En outre, cette valorisation réduit les effets des biodéchets sur l'environnement faisant ainsi l'industrie du biodiesel et de H_2 plus compétitive.

6.6. Production d'hydrogène en utilisant une co-culture avec une fermentation sombre et une photofermentation (Chapitre 7)

6.6.1. Approche alternative de la co-culture dans les milieux de culture utilisés à travers la culture mixte et photo-fermentation (Chapitre 7, un article soumis)

En vue d'élever l'industrie du biodiesel en tant que vecteur énergétique majeur, une nouvelle approche pour minimiser les déchets et utiliser ces déchets générés a été explorée. Les milieux usés générés par le système en co-culture sont rentables et peuvent servir comme supplément renouvelable pour les culture mixtes afin de produire du H_2. Ce milieu épuisé peut également remplacer les milieux utilisés en photo fermentation pour produire des lipides. La conversion directe des milieux usés avec le glycérol brut à 20 g/L à l'aide de boues de station d'épuration traitées par choc thermique a donné lieu à 38,12 ± 0,84 mmol/L de H_2. Dans une autre approche, les milieux usés ont été utilisés comme co-supplément avec un milieu frais à un rapport de 3:2 pour la croissance des algues, ce qui entraîne un rendement de 0,098 ± 0,007 g/L de production de lipides. Les milieux usés contiendraient de la biomasse morte, des nutriments résiduels, des biomolécules et du glycérol inutilisé. Cette matrice pourrait agir comme source supplémentaire au cours de la production de l'hydrogène et des lipides. L'hydrogène produit peut être utilisé comme source d'énergie interne dans l'industrie et les lipides peuvent être utilisés comme matière de première génération.

L'étude explore l'utilisation du glycérol brut, des milieux usés et des boues primaires vers une approche en système fermé efficace pour l'industrie du biodiesel afin de minimiser la production de déchets et augmenter la valeur commerciale du GB.

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CHAPTER 2

HYDROGEN PRODUCTION BY CO-CULTURE SYSTEM: COMPARISON AND STRATEGIES

PART1

BIOLOGICAL HYDROGEN PRODUCTION USING CO-CULTURE VERSUS MONO-CULTURE SYSTEM

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Résumé

La produire l'hydrogène à partir de diverses méthodes, telles que la photo-fermentation, la fermentation sombre et séquentielles en deux étapes, afin d'améliorer le processus traditionnel. Pendant la fermentation, des microorganismes consortium spécifiques sont caractérisés et leur métabolisme est bien étudié. Des efforts de recherche importants ont été réalisés pour augmenter la production de H_2 en utilisant le système de co-culture, qui offre l'avantage d'un rendement accru en H_2 et un taux de production supérieur par rapport aux mono-culture. Le concept du système de co-culture est une approche simple qui consiste à mélanger ensemble différentes souches microbiennes pour améliorer les propriétés manquantes des souches individuelles. Le système en co-culture est un système rentable, qui élimine éventuellement l'étape de prétraitement et évite l'utilisation d'un agent chimique réducteur coûteux. En éliminant ces deux étapes, le coût global du procédé peut être réduit sans affecter le rendement de production en H_2. Le système en co-culture hydrolyse directement les substrats organiques complexes en sucres fermentescibles avec 94,1% d'amélioration de rendement par rapport au système en mono-culture. La co-culture offre divers avantages, par exemple, la réduction de la phase de latence, la résistance aux fluctuations de l'environnement et une stabilité des taux de production de H_2 sans interruption, et selon certaines études le taux de production de H_2 serait 8 fois plus élevé par rapport aux mono-cultures. Les systèmes de production de H_2 en co-culture sont aussi une méthode de traitement des effluents avec une réduction de 60% de la DCO et peut être facilement intégré dans un procédé à l'échelle pilote pour réaliser une production continue de H_2. L'élaboration d'un système en co-culture suggère un potentiel énorme pour la production de H_2 en utilisant des déchets organiques complexes avec une application viable et applicable à l'échelle industrielle.

Mots clés: Co-culture, fermentation sombre, hydrogène, monocultures, déchets organiques, photo fermentation

Abstract

A sustainable approach for hydrogen production from various methods, such as photo-, dark fermentation and sequential two-stage has significant advantages to complement traditional process. During hydrogen fermentation, defined, well characterized and composite microorganisms are studied. Substantial research efforts have been carried out to increase hydrogen production by using co-culture system, which offers advantage of increased H_2 yield and production rate in comparison to mono-culture. Concept of co-culture system is a simple step of mixing together different microbial strains for improving the individual properties that other strain lacks. Co-culture system is a cost-effective, which potentially eliminates pre-treatment step and avoids use of expensive reducing agent. By eliminating these two steps, the overall process cost can be reduced without negotiating the hydrogen yield. Co-culture system directly hydrolyzes complex organic substrates into fermentable sugar with 94.1% improved yield in comparison to mono-culture. Co-culture offers various advantages, example, reduction in lag phase, resistance to environmental fluctuations and provides stability with uninterrupted hydrogen production rate, which is 8 times in comparison to mono-culture. Co-culture system of hydrogen production is also an alternative effluent treatment method with 60% reduction in COD level and can be easily integrated into pilot-scale to achieve continuous H_2 production. The elaboration on co-culture system suggests a huge potential of hydrogen production using complex organic wastes with viable application towards industrialization.

Keywords: Co-culture, dark fermentation, hydrogen, mono-culture, organic wastes, photo fermentation

Introduction

Hydrogen is a clean energy carrier; however presently used commercial methods for its production is not environmentally friendly. Around 95% of hydrogen produced comes primarily from fossil releasing carbon dioxide to atmosphere with effect of greenhouse gas on global climate change (Zeidan *et al.*, 2009). Future hydrogen economy will not be sustainable, due to depletion of fossils in near future (Zeidan *et al.*, 2009). In addition, H_2 production requires expensive energy input with increasing process cost being the largest impediment. Given these perspectives, scientists are developing cost-effective processes by pursuing biological hydrogen production to aid the ongoing research on H_2 as energy source (Vatsala *et al.*, 2008). Biological hydrogen production offers an environmentally friendly alternative and less energy input in comparison to thermochemical and electrolysis processes (as in Table 2.1.1) and represents a promising route with utilization of organic wastes for hydrogen production. Despite these advantages, the yield of biological hydrogen production using mono-culture system is lower and the process is less economical. Substantial improvements have been made to increase hydrogen yield by using co-culture system, which has many advantages in comparison to mono-culture system of hydrogen production.

In nature, many microorganisms coexist with proper interaction, assisting each other to perform effectively amongst the specific groups (Chang *et al.*, 2008). In the case of co-culture system, microorganisms communicate with each other with exchange of metabolites and perform division of tasks for degrading complex substances (Balachandar *et al.*, 2013). Technically, incubation of different microbial strains under sterile conditions defines a co-culture. The technique of co-culture with pre-defined microbial characteristics to perform complex functions, simultaneous consumption, exchange of metabolites, working with different substrates has several advantages over mono-culture system (Balachandar *et al.*, 2013; Chang *et al.*, 2008; Das *et al.*, 2008; Hema *et al.*, 2012; Masset *et al.*, 2012; Zeidan *et al.*, 2010).

The combination with pure cultures of both aerobic and anaerobic microorganisms plays an important role in developing industrialized hydrogen process from solid organic wastes (Chang *et al.*, 2008). To generate hydrogen using mono-culture system requires

expensive pre-treatment to hydrolyse the complex organic materials into soluble fermentable sugars and needs expensive reducing agent for the optimal growth of microorganisms to transform hydrolysed matter into hydrogen (A. Wang, 2008). Combination of mesophilic and thermophilic anaerobic bacteria proved great potential for hydrogen production with an effective utilization of cellulase without addition of exogenous cellulase for cellulose hydrolysis in an interactive cooperation during co-culture system (Y. Liu *et al.*, 2008) and use of facultative bacteria as reducing agent reduced increased cumulative hydrogen production by 14.50% in comparison to mono-culture (Phowan *et al.*, 2010). In order to reduce the expensive polypeptone usage, Yokoi *et al*. (Thonart *et al.*, 2010) used corn steep liquor as a nitrogen source in the co-culture media for effective and economical hydrogen production. The co-culture system can increase hydrogen production rate in comparison to mono-culture system (Thonart *et al.*, 2010) and also has demonstrated hydrogen yield near to theoretical maximum hydrogen yield (Ghosh *et al.*, 2012; Sun *et al.*, 2010).

The access to co-culture studies is very limited, in particular details on the fermentation, culturing condition, pre-treatment of the biomass, bioreactor design and optimization of dark and photo-fermentation (Ding *et al.*, 2009). Researchers have defined different approaches for various substrates and accordingly used co-culture techniques to increase hydrogen yield and production rate. Therefore, this review provides useful information and the challenges of co-culture for hydrogen production. The article reviews co-culture fermentation for its process improvements, advantages, and challenges and highlights the concepts behind co-culture technique for hydrogen production.

Types of waste materials

Organic waste is estimated to range from 2.75 to 4 kg/capita/day in developed countries and 0.5 kg/capita/day in low-income countries across the globe (Kim *et al.*, 2013). The massive organic wastes discharged from municipal, agricultural and industrial sources cause serious environmental contamination, such as odor emanation, toxic gas emission, groundwater contamination at collection and landfill source (Kim *et al.*, 2013;

Kim, 2004). The sustainable waste management strategies are in progress for the conversion of organic wastes into energy. Most of the organic solid wastes are readily available and contain varied composition of carbon and nitrogen source necessary for microbial hydrogen production (Chong *et al.*, 2009; Kim *et al.*, 2013). The major criteria in hydrogen production for the selection of the organic waste materials are the resource availability, cost, carbohydrate to nitrogen ratio, pre-treatment requirement and rate of biodegradability (Kim, 2004). The hydrogen production potential has been investigated using various organic wastes and these are summarized below.

Lignocellulosic materials: Another readily available and abundant organic waste that can be used for microbial hydrogen production is lignocellulosic waste, with annual yield exceeding 220 billion tonnes worldwide (Kim *et al.*, 2013). Cellulosic waste is the abundant biopolymer obtained from the discarded forestry, wood residues, paper wastes, and agriculture wastes (Kim *et al.*, 2013). Lignocellulosic materials contain cellulose (32–47%), hemicelluloses (19–27%) and lignin (5–24%) with largest renewable sources of hexose and pentose sugars (Carere *et al.*, 2008; Kim *et al.*, 2013). A potential hydrogen production process either needs cellulolytic degrading microorganism or additional pretreatment step to degrade cellulose into simple fermentable sugar (Chong *et al.*, 2009). Higher hydrogen production was obtained using pretreatment steps, which helped in bioconversion of cellulose to hydrogen at faster rate. Acidification with diluted HCl treatment of lignocellulosic waste materials compared to raw waste resulted in 10 to 136-fold increase in hydrogen production (Chong *et al.*, 2009). The hydrolysis of sugar bagasse hemicellulose was carried out in presence of sulfuric acid (0.5 % v/v) at 121 ºC with a reaction time of 60 min to obtain sugars for hydrogen production (Pattra *et al.*, 2008). The lignocellulosic wastes require mild alkali pretreatment with 3% NaOH using a liquid to solid ratio of 25:1 at 80 °C for 3 h to hydrolyse complex structure into fermentable sugar to have a final yield of 50.05 ± 1.51 mmol H_2/L (Cheng *et al.*, 2013). Taguchi *et al*. (Taguchi *et al.*, 1995) used *Clostridium* sp. strain no. 2, a cellulose degrading bacteria for enzymatic hydrolysis of xylan for hydrogen production with a yield of 18.6 mmol/g substrate.

Food wastes: Most common organic wastes are food waste amounting to roughly 1.3 billion tonnes which is one third of produced food across the world. The amount of food wasted every year is equivalent to net food production of Africa and almost equals to half of worldwide annual cereal production (2.3 billion tonnes) (www.unep.org/wed/2013/quickfacts/, 2013). The food wastes account for municipal solid wastes, which include kitchen refuse, municipal sludge and food co-products. They contain high levels of organic volatile constituents at 85–95%, which act as a possible substrate for anaerobic hydrogen production (Kim *et al.*, 2013).

Cassava pulp, a complex substrate containing 66% (w/w) of starch requires acid hydrolysis using 0.5% (v/v) H_2SO_4 with 1:15 (solid to liquid ratio) at 121 ºC for 30 min resulting in highest yield of 345.8 mL H_2/g COD suggesting that the cassava pulp hydrolysate could be used for H_2 production (Phowan *et al.*, 2010). Waste wheat was also considered as reliable resources for H_2 production, contains around 97% (w/w) starch and needed boiling for 1.5 h for partial hydrolysis of starch. Pretreatment of hydrolysis followed by additional enzymatic hydrolysis of wheat starch into glucose and maltose resulted in highest H_2 production rate (280 mL/day) (Ozmihci *et al.*, 2011).

Glycerol waste: In recent years, biodiesel has gained importance as promising alternative for future fossil fuels with exponential production across the world. To meet the depleting fossil oil reserves and to avoid energy crisis in future, the projected biodiesel production in 2020 will be around 30.28 billion L, which will eventually generate around 3 billion L of crude glycerol as by-product (pachapur *et al.*, 2015c). Crude glycerol is generated during the production of biodiesel at around 10 kg of crude glycerol for every 100 kg of biodiesel production. Crude glycerol is a good source of carbon, its use as feedstock for hydrogen production as alternative for CG treatment has been proposed to compensate the decreasing cost of CG in the market (Sarma *et al.*, 2012). Crude glycerol contains major impurities, such as methanol and soap, which affect the microbial growth during H_2 production (Sarma *et al.*, 2012). Rotary evaporation step to remove alcoholic compounds from crude glycerol resulted in H_2 production, which was 1.55-fold higher in pre-treated glycerol in comparison to untreated glycerol (Ngo *et al.*, 2011). Additional precipitation steps involve mixing of

crude glycerol with distilled water, adjusting the pH and carry out centrifugation to separate fatty acid from crude glycerol (Sarma *et al.*, 2012). With anaerobic treatment, at mesophilic and thermophilic temperature, in the presence of indigenous hydrogentrophic bacteria, glycerol waste was used for hydrogen production. The production rate increased to higher rate by using yeast extracts along with NH_4Cl, KCl and $CaCl_2$ (Chong *et al.*, 2009). CG being good source of carbon, two different strains with a co-culture technique can be evaluated for bioconversion of fermentable end products (acetic and butyric acid) into hydrogen (Sarma *et al.*, 2012).

Organic waste conversion into sustainable use for hydrogen production, with no pretreatment step or additional media supplements, will lead to a value added utilization of low cost substrate resulting in economic benefits for hydrogen production. Researchers have used cellulose degrading bacteria to convert raw lignocellulosic waste into a fermentable substrate and exploited the property of facultative anaerobe as a replacement for expensive media components and used aciduric tolerant microorganisms to minimize unwanted substrate inhibition during hydrogen production. The benefits of using two or more functional microorganisms in a co-culture system on these organic wastes, offers greater selectivity towards the substrate degradation, utilization and conversion rate to obtain relatively high hydrogen yield in comparison to mono-culture system.

Hydrogen producing microorganisms

Depending upon the availability of substrate, the selection of functional microorganisms necessary for hydrogen production becomes an important step. In nature, numerous hydrogen producing microorganisms have been reported; the common ones are the strict anaerobes, followed by mesophiles, phototrophic and lastly thermophiles with optimum working pH range within 4-7. The working temperature for hydrogen production ranges from 30 to 40 ºC (anaerobes, mesophiles and phototrophic) or 60 ºC (moderate thermophiles) or 70 to 80 ºC (thermophiles).

Anaerobic bacteria: Anaerobic bacteria are mostly researched for its increased hydrogen production rate and ability to work on a wide range of substrates. The anaerobic bacteria belonging to *Clostridium* species are typically used for bioconversion

of carbohydrate to hydrogen, acetate, butyrate, CO_2 and organic solvents (Ito *et al.*, 2005). Hydrogen production follows the butyrate and acetate pathway, in the presence of hydrogenase enzyme at suitable butyrate/acetate ratio to produce 6 moles of hydrogen from two moles of glucose. The anaerobic bacteria with hydrogen production, tend to shift the metabolism to solvent production during the stationary phase causing decrease in hydrogen production (Chong *et al.*, 2009). The batch experiments for higher hydrogen production can be set in such a way to accelerate the acidogenic or exponential phase for longer run time by reducing the glucose input and by restricting co-production of ethanol (Chong *et al.*, 2009). Few *Clostridium* species possess hydrogenase genes and amylase hydrolytic activity to degrade complex starch into simple sugar (Yokoi *et al.*, 2001).

Clostridium species require complete anaerobic environment and usually an expensive reducing agent is used to maintain this condition, which imposes additional process cost for hydrogen production.

Facultative anaerobic bacteria: With dual property of producing ATP in the presence of oxygen and also switching to hydrogen production through anaerobic fermentation, the facultative anaerobic bacteria have advantages over the anaerobic bacteria (Chong *et al.*, 2009). The facultative anaerobic bacterium, *Enterobacter aerogenes* is active even under acidic condition (pH 3.9) (Sarma *et al.*, 2013b) and also used as reducing agent for its ability to consume oxygen (Phowan *et al.*, 2010; Yokoi *et al.*, 1998b). By using *E. aerogenes* strain HO-39, without pH control, a continuous hydrogen production process employing glucose and polypeptone as substrate has been reported to achieve hydrogen productivity as high as 120 mL/L/h (Chong *et al.*, 2009). *Bacillus* sp. are the newest entrants into the co-culture system, with quite a few features with the presence of hydrogenase genes, implying major role by producing hydrolytic enzymes, efficiently consuming the oxygen and a better contributor in improving H_2 production (Patel *et al.*, 2014). A lot of research for the improvement of facultative anaerobic bacteria through chemical mutagenesis, single and mutual effects of substrate with temperature, pH is carried out. A continuous mode operation using immobilized cells and repeated batch processing have been tested to increase hydrogen yield (Sarma *et al.*, 2012). Using

facultative anaerobe along with strict anaerobic bacteria in a co-culture system resulted in improved hydrogen production.

Photosynthetic bacteria: Photosynthetic bacteria are studied for their capacity to produce hydrogen due to their versatile enzyme property. Nitrogenase enzyme catalyses the reduction of H^+ to molecular hydrogen and hydrogenase is necessary for photoautotrophic growth and also for recycling of H_2 production by nitrogenase (Odom *et al.*, 1983). They require small-chain organic acids as electron donors to drive the hydrogen production (Chang *et al.*, 2008). The photosynthetic bacterium, *Rhodopseudomonas palustris* converts glycerol (pure and crude) to hydrogen at 6 mol/mol of glycerol to achieve 75% of theoretical hydrogen production with no effect of inhibition or toxicity (Sabourin-Provost *et al.*, 2009). In another example of *R. palustris* using crude glycerol yielded hydrogen at 6.69 mol/mol of glycerol. The results near to theoretical maximum hydrogen yield (8 mol/mol of glycerol) were achieved by studying the interactive effects among important process parameters using response surface methodology (Ghosh *et al.*, 2012). In a two-stage system by using the spent biomass from the dark fermentation, the photosynthetic bacteria *Rhodopseudomonas sphaeroides* O.U. 001 yielded hydrogen at 1.7 mol/mol of acetic acid (Das, 2009). Photosynthetic bacteria can work across batch processes, through continuous and two-stage system; however hydrogen production rates, stabilities and process efficiency are far below to result in industrial commercialization. In order to minimize process cost and fermentation time, in a combined dark and photo-fermentation involving a co-culture system, phototrophic microorganisms can utilize the organic acids produced from dark fermentation into hydrogen production.

Thermophilic bacteria: The thermophilic bacteria operate at temperature greater than 60 ºC. This potential property favors the reaction kinetics by avoiding contamination during the process. These species show oxygen tolerance and maximum hydrogen production during their exponential phase and possess protease, lipase and amylase activities. Many thermophilic bacteria utilize a wide range of substrates from simple to complex carbohydrates, are superior in hydrogen yield and possess favorable thermodynamic conditions with reduced by-product formation (Pattra *et al.*, 2008).

Thermoanaerobacterium neapolitana and *Thermoanaerobacterium maritime* are active at 75 to 80 ºC with (6.5 to 7.0) pH range using carbohydrate substrate and hydrogen yield around 172 mL/mL of culture in the presence of carboxymethycellulose (Chong *et al.*, 2009). Use of thermophilic bacteria in a co-culture system for the hydrogen production from biomass will contribute significantly to an energy efficient bioprocess system (de Vrije *et al.*, 2009). Extreme thermophiles used for H_2 production had superior production rate, better profile of fermentation by-products making their application economically and technically more interesting in reaching 95% theoretical stoichiometry (4 mol H_2/mol glucose) with H_2 yield of 3.8 (Zeidan *et al.*, 2009).

These microorganisms possess the functional property to utilize various organic wastes either in dark (dark fermentation) or in the presence of light (photo fermentation) for hydrogen production. Exploiting the functional property of these microorganisms by using a co-culture system will result in consortium of microorganisms leading to efficient process for hydrogen production.

Co-culture system

A consortium is nothing but microbes which are living in a highly diverse and complex community. They are capable of performing complex tasks, interacting in numerous ways with cooperation to direct completion (Zuroff *et al.*, 2013). Creation of a syntrophic population by mixing different functional microorganisms for improving the individual properties that other strain lacks. A simple reasonable step to engineer within each strain encodes a required phenotype, which is beneficial for consortium to reach stable population (Weibel, 2008). Microbiologists and engineers have exploited the microbial communities to engineer synthetic consortia for biofuel production. The best way to increase the hydrogen production could be achieved by using either anaerobic or photosynthetic microorganism (Chong *et al.*, 2009).

Syntrophic interactions between the organisms help in the enhanced hydrogen production from the biomass or wastewater (Fang *et al.*, 2006). The hydrogen yields across different processes (mono-, co- and mixed-culture) are presented in Table 2.1.2.

Co-culture in comparison to mono-culture offers syntrophic effects with increased H_2 yield (Patel *et al.*, 2014), at reduced fermentation time and producing more amount of

H_2 (Kao *et al.*, 2014). Co-culture system operates in harmony with greater process stability (Patel *et al.*, 2010), out performs mixed-cultures and overcomes the limitations of mono-culture (Zeidan *et al.*, 2010). Co-culture system possesses economical and technical advantages over mono- and mixed-culture system, in terms of pretreatment/enzymatically hydrolysed cellulose (Elsharnouby *et al.*, 2013) and also by eliminating use of reducing agents during H_2 production (Yokoi *et al.*, 1998a) to produce hydrogen with purity of 58% (Bao *et al.*, 2012) to 99.99% pure (Singh *et al.*, 2008). The advantages of co-culture over mono- and mixed-cultures for utilization of organic wastes during hydrogen production are presented in the Figure 2.1.1. The figure demonstrates the various steps across different systems for conversion of organic wastes into hydrogen. Utilization of organic wastes by mono-culture system requires pretreatment step and addition of enzymes for hydrolysis of cellulose and starch containing organic wastes into simple carbohydrates for hydrogen production (Kapdan *et al.*, 2006). In the case of mixed-cultures, additional treatment in terms of heat, pH, aeration, chemical and freeze and thawing treatments to enrich hydrogen producing bacteria is required (Kim *et al.*, 2013). However, anaerobic *Clostridia* and aerobic *Bacillus* are often detected at various sites, where cellulose degradation occurs. Incorporating *Bacillus* possess increased growth rate with ability to create anaerobic condition and secrete extracellular enzymes for hydrolysis of complex insoluble cellulose compounds to soluble monomers with *Clostridium* utilizing soluble monomers for H_2 production. Coexistence of these two bacteria utilized brewery yeast waste with effective cellulose degradation by eliminating expensive hydrolysis step and in absence of reducing agent for increased hydrogen production (Chang *et al.*, 2008). Mesophilic bacteria need exogenous cellulase enzymes for cellulose hydrolysis to produce H_2. Nevertheless, co-culturing with thermophilic bacteria effectively helped cellulose utilization and possessed potential for H_2 production. The co-culture system resulted in increased H_2 yield from 0.8 to 1.8 mol H_2/mol glucose and the system could utilize corn-cob powder and corn-stalk powder in the absence of exogenous cellulase (Liu *et al.*, 2008). Likewise, bioagumentation of *Clostridium acetobutylicum* X_9 with *E. harbinense* B_{49} effectively hydrolysed partially delignified lignocellulose complex (carboxymethyl cellulose and cornstalk), significantly

improved cellulose hydrolysis to decrease pretreatment cost and resulted in 2.4 times hydrogen yield in comparison to mono-culture (Ren *et al.*, 2006; A. Wang, 2008). A combination of *Enterococcus gallinarum* G1 (highly-active cellulose-hydrolyzing) with *Ethanoigenens harbinense* B49 (H_2-producing) bacteria, increased cell growth, shortened H_2 production peak period by 10 h and 31% increase in H_2 production yield indicated the requirement of co-culture system in degrading organic wastes (Aijie Wang *et al.*, 2009). Co-culturing of *Clostridium* sp. resulted in tolerance of presence of phenols (<1000 mg/L), degraded cellobiose and achieved H_2 yield of 2.4-2.6 mol H_2/mol cellobiose from wastewater (Ho *et al.*, 2010). Direct microbial conversion of lignocellulosic wastes showed 94.1% higher hydrogen yield using the co-culture of *Clostridium thermocellum* and *Clostridium thermosaccharolyticum* (Qian Li *et al.*, 2012). Using *Clostridium* sp. ability to produce 2 mol H_2/mol of glucose required addition of expensive reducing agent for stable H_2 production (Yokoi *et al.*, 1998b). Yokoi *et al*. (Yokoi *et al.*, 1998b) used *Enterobacter aerogenes* as reducing agent to create anaerobic conditions for *Clostridium butyricum* growth. The co-culture system consumed oxygen, rapidly replaced the gaseous phase of reactors and eliminated argon/nitrogen sparging step (pachapur *et al.*, 2015a; Phowan *et al.*, 2010; Yokoi *et al.*, 1998b). The concentration of facultative anaerobic bacteria at the time of inoculation is very important to create anaerobic condition in the absence of reducing agent (Pachapur *et al.*, 2015b; Qian *et al.*, 2011; Seppälä *et al.*, 2011; Thonart *et al.*, 2010) with the potential strict anaerobes to reach high experimental H_2 yield, which promotes higher production rates for facultative anaerobes. This co-culture system degrades more substrate at faster rate and outperforms the mono-cultures in hydrogen production volumes (Seppälä *et al.*, 2011).

Co-culture system is an alternative to mono-culture and mixed-culture with process strategy involving hydrolysis, fermentation and production steps in a single process by providing syntrophy among two species (Carere *et al.*, 2008; Lynd *et al.*, 2005; Salimi *et al.*, 2010). On the same principle, several reports using co-culture system in a single step hydrogen production demonstrated a perfect cooperation between the two microorganisms, with decrease in lag time, lowered total process time, increase in

hydrogen yield and no pH adjustment due to automatic stabilization between the microorganisms (Zuroff *et al.*, 2013).

The conceptual model of the co-culture system is depicted in Figure 2.1.2. The co-culture system is not only related to decreasing process cost, but also possesses potential ability to combine dark- and photo-fermentation. Use of a co-culture system of dark fermentative and photosynthetic bacteria together will help in increasing hydrogen yield. Application of the co-culture system for reducing accumulated fermented end products during dark fermentation with suitable photosynthetic microorganisms possessing metabolizing property will help in improved hydrogen production.

Co-culture system with combined dark- and photo-fermentation

Dark fermentation: The microbial hydrogen production in the absence of the light but in the presence of either anaerobic or facultative bacteria utilizing organic waste is commonly known as dark fermentation (Hema *et al.*, 2012). During dark fermentation, pool of electrons and protons are most valuable parameters utilizing complex forms of organic substrate, requiring low energy demand, simple design with predominant mixing for hydrogen production (Equation 7) (Lee *et al.*, 2010). The hydrogen production rate was higher in comparison to photo-fermentation but complete degradation of substrate to hydrogen production seemed a major bottleneck in the dark fermentation process (Hema *et al.*, 2012). To address this issue, Yokoi *et al*. (Yokoi *et al.*, 2001) used *E. aerogenes and C. butyricum* to degrade sweet potato residue, carried out repeated batch cultures and in presence of polypeptone (0.1%), the hydrogen yield 2.4 mol/mol of glucose was higher than those for mono-culture. Co-immobilization of these two strains over glass beads helped to achieve hydrogen yield of around 2.6 mol/mol of glucose with higher substrate consumption rate in comparison to mono-culture (Yokoi *et al.*, 1998b). With the same set of co-culture system, Phowan *et al*. (Phowan *et al.*, 2010) demonstrated reduction in lag phase during hydrogen production using cassava pulp with production rate being 14.50 % higher in comparison to mono culture of *C. butyricum*. A study on proteomic and genetic approach was carried out using various carbohydrates and different *Clostridium* species in a co-culture system for hydrogen production. The characteristics of carbohydrates, hydrolysis rate, glycosidic chain

length, and difference in metabolic routes of *Clostridium* species influenced the performance of dark fermentation (Quéméneur *et al.*, 2011).

Substrate conversion rate in dark fermentation decreases with change in pH caused due to accumulation of by-products, such as acetic acid, butyric acid and lactic acid (Yokoi *et al.*, 1998a). The inhibition of organic acids can be minimized through exchange of organic acids by two-phase system (Sarma *et al.*, 2015) or by carrying out combined dark and photo-fermentation for increased hydrogen yield.

Photofermentation: The microbial hydrogen production in the presence of light is referred to as photofermentation; at the expense of light energy, for hydrogen production small-chain organic acids are used by photosynthetic bacteria acting as electron donors (Equation 8). The phototropic bacteria can work under wide range of absorption spectra to reach theoretical hydrogen yield. Photosynthetic bacteria are capable of utilizing the accumulated acids, slow down the drop in pH, increase the degradation of substrate and maximize the hydrogen yield (Hema *et al.*, 2012). Combinations of both dark- and photo-fermentation have potential to reach theoretical hydrogen yield. In the combined system, the acidogenic bacteria will produce hydrogen by degrading organic substrate into end metabolites and photosynthetic bacteria will act on these metabolites in the presence of light to produce hydrogen (Equation 7 and 8) (Chen *et al.*, 2008). The co-culture of *C. butyricum* and *E. aerogenes* was grown on starch biomass in the absence of reducing agents by removing trace amount of oxygen producing hydrogen at around 2 mol/mol of glucose and a combination of *C. butyricum* and *Rhodobacter sp*. M-19 yielded hydrogen of around 6.6 mol/mol of glucose (Yokoi *et al.*, 2001).

A combination of both the processes was carried out on algal biomass by using anaerobic lactic acid bacterium *Lactobacillus amylovorus* for conversion of algal starch to lactic acid. Later, lactic acid was used by photosynthetic *Rhodium marinum* bacterium for the production of hydrogen. The combination of *L. amylovorus* and *R. marinum* A-501 resulted in 1.5 fold increase in production rate in comparison to that of *Vibrio fluvialis* T-522 and *R. marinum* A-501 (Kawaguchi *et al.*, 2001). In a typical co-culture system, even though pure culture of *Rhodobacter* species utilizes glucose, but it never

competes with *Clostridium* species for glucose and only grows syntrophically by utilizing the fatty acids for hydrogen production. Even in the case of cell number for both the microorganisms, a higher cell number is seen much earlier in a co-culture system in comparison to its pure culture system (Fang *et al.*, 2006). In some cases, the spent media containing organic acids after dark fermentation was centrifuged and supplied along with fresh media components to carry out photo-fermentation. The hydrogen yield of two-stage was higher in comparison to combined dark- and photo-fermentation.

Sequential two- stage dark and photofermentation: In order to increase hydrogen production, an integrated approach of combining dark with photofermentation or fermentative bacteria with photosynthetic bacteria for biomass wastes is presented in Equation 7 and 8. In a two-stage sequential system, dark fermentative bacteria will act on solid organic wastes to produce low-molecular weight organic acids, which are easily degraded by photosynthetic bacteria for hydrogen production (Hema *et al.*, 2012).

Stage I. Dark fermentation (facultative anaerobes).

$C_6H_{12}O_6 + 2\ H_2O$ ---- > $2\ CH_3COOH + 2\ CO_2 + 4\ H_2$……Equation: (7)

Stage II. Photo-fermentation (photosynthetic bacteria).

$2\ CH_3COOH + 4\ H_2O$ ----> $8\ H_2 + 4\ CO_2$……..Equation: (8)

An effective system with increased yield of hydrogen production, better energy recovery from biomass waste, with a low COD in the final product can only be achieved by combining dark and photo-hydrogen fermentation in a two-stage bio-reactor (Chen *et al.*, 2008). By doing so, the hydrogen produced through the sequential two stage process can yield hydrogen level that can match theoretical rate of 12 moles of hydrogen using glucose in a combined process (Hema *et al.*, 2012). However, a single cultivation system for the desired biomass conversion for hydrogen production can be more effective when compared to two-step system running on two different reactors (Geng *et al.*, 2010). The adaptation of the sequential two-step process at industrial scale is less efficient and requires further efforts to optimize the conditions of sequential two-step fermentation process for hydrogen production.

Co-culture as an alternative to sequential two-stage

Using a co-culture system of anaerobic and photosynthetic bacteria will be more efficient and cost-effective in comparison to sequential two-step process of dark- and photo-fermentation. With both fermentations working simultaneously in the same bioreactor will save time, cost and result in increased hydrogen production. To investigate different parameters on combined system using *C. butyricum* and *Rhodopseudomonas faecalis* RLD-53 over glucose for hydrogen production, immobilization of *R*. *faecalis* RLD-53, with 1:10 (ratio of dark to photo bacteria) at light intensity of 8000 lux, resulted in hydrogen yield of 4.13 mol/mol of glucose (Ding *et al.*, 2009). Yokoi *et al*. (Yokoi *et al.*, 1998b) studied the combined system of *C. butyricum* IFO13949 and *Rhodobacter* sp. M-19 in presence of starch at 30 ºC under illumination at 5,000 lux. A maximum hydrogen yield of 6.6 mol/mol of glucose was obtained using a combined system in comparison to 3.6 mol/mol of glucose for two-step process. A combined system using *Lactobacillus delbrueckii* and *Rhodobacter sphaeroides RV* resulted in maximum hydrogen yield of 7.1 mol/mol of glucose. The technique of co-immobilization of both the bacteria gave the highest results accompanied by molasses treatment with simultaneous energy production (Asada *et al.*, 2006). In order to develop a cost effective process, a combined co-culture system for hydrogen production using sugarcane distillery effluent was evaluated. The co-culture of *Citrobacter freundii* 01, *E. aerogenes* E10 and *R. palustris* P2 resulted in 21.38 kg of H_2 at 0.53 kg/100 m^3/h production rate (Vatsala *et al.*, 2008). To increase the hydrogen production, each of the key factors needs to be characterized to determine its optimal conditions for improved sustainable hydrogen production.

Effect of key factors on a co-culture system

Hydrogen being the cleanest energy carrier, its production from various others sources has been studied using co-culture system. The hydrogen production is limited by some of the key factors, such as substrate concentration, working pH, inoculation ratio, and temperature with varying co-culturing conditions. Some key factors have a very strong effect, each parameters needs to be evaluated individually and in combination for hydrogen production.

Several studies reported a favorable hydrogen production using initial substrate concentration of 1% under dark fermentation (Yokoi *et al.*, 1998a; Yokoi *et al.*, 1998b). To determine the effect of substrate concentration, Ding *et al*. (Ding *et al.*, 2009) utilized varying concentrations of glucose from (3, 6, 9, 12 and 15) g/L and hydrogen yield increased with glucose concentration from 3 to 6 g/L. Hydrogen yield and fatty acid concentration decreased with increase in concentration from 9 to 15 g/L. At 6 g/L, hydrogen yield was highest with complete consumption of glucose.

The key factors, such as inoculation ratios of *C. thermopalmarium* to *C. thermocellum* at volume fraction of 0.0005:1, 0.05:1 and 0.17:1 was used for hydrogen production until 60 h after inoculation, giving the best results at the volume fraction of 0.05:1 in comparison to other ratios. At this particular volume fraction (0.05:1), the relative abundance of both the microorganisms was at highest point, making a "cellulase-enzyme-microbe" complex for efficient utilization of cellulose and resulted in the cumulative hydrogen production near to 400 mL/L culture (Geng *et al.*, 2010).

Subsequently, to find out the reason for the decrease in cellulose utilization (0.11 g/L), Geng *et al*. (Geng *et al.*, 2010) observed that the lowering of pH below 6.0 decimated growth and caused inhibition of cellulose-degradation ability of *C. thermocellum*. To increase the alkalinity, autoclaved $KHCO_3$ was added in increasing concentration from 0 to 60 mM, resulting in utilization of cellulose from 14 to 94 % along with increase in pH from 5.53 to 6.26 with highest hydrogen production of 1387 mL/L of culture saturated with 40 mM $KHCO_3$. These data indicated maintenance of the pH of the system is an important parameter to avoid the inhibition activity of the microorganisms in a co-culture system (Geng *et al.*, 2010). To investigate optimal working pH, co-culture system of *C. butyricum* TISTR 1032 and *E. aerogenes* TISTR 1468 was used. Too low (≤ 5.0) or too high (≥ 6.5) pH resulted in decrease in hydrogen production due to inhibition of *C. butyricum* hydrogenase activity with similar results even at (7.0 to 8.0) pH. The maximum hydrogen production rate of 3580 mL/L per day was achieved at pH 5.5 (Phowan *et al.*, 2010).

Consideration all these factors, hydrogen produced must be continuously removed from the system along with the end products. The produced hydrogen in the reactor system

gets accumulated in the headspace making it difficult for the mixing and distribution of gases across the system (Lee, 2012). With increase in hydrogen production, there is a possibility of increase in partial pressure. In such cases, the hydrogen production will be switched to solvent production later inhibiting the hydrogen production (Phowan *et al.*, 2010).

Further understanding of synergic relationship and physiological mechanisms involved in co-culture technique will definitely help in delivering higher hydrogen yield. In order to attain the theoretical value, conventional techniques require statistical based experimental design for effective, time-saving co-culture system experiments to attain highest hydrogen yield.

Co-culture model for hydrogen production

A common defined strategy in co-culture model allows isolation of the dominant members among the mixed culture in order to maintain the stability and also the original function of the mocroflora (Zeidan *et al.*, 2010). Such modeling helps in attaining faster substrate consumption at higher concentration for the dominant organism, diluting its activity to pure culture will improve metabolic reconstructions for the second microorganism (Hanly *et al.*, 2013). A co-culture mathematical model was designed on this principle to determine the stable co-existence among the microbes using maximum specific growth rate and Monod equations (Zeidan *et al.*, 2010). The co-culture model helped in better understanding, predicting high community mass, highlighting energy balanced reaction and using ecological theory to predict fate of population, fitness of resistance, productivity rate in overall consortium community (Zuroff *et al.*, 2013). In general, the co-culture model helped in the selection of the best performing microbes. With high activity in the overall conversion process, improved metabolic pathways and offered higher resistance to environmental stress (Yokoi *et al.*, 2001). A central composite design (CCD) and Placket-Burman design was applied on co-culture system of *C. acidisoli* and *R. sphaeroides*. The design model identified substrate concentration, initial pH and inoculum ratio as simultaneous and interdependent factors on co-culture system. The model analysis helped to obtain hydrogen yield of around 10.16 mol/mol of

sucrose, which was near to expected value (10.70 mol/mol of sucrose) (Sun *et al.*, 2010).

To understand the complexity and population dynamics of the co-culture communities, dynamic changes between substrate and products, it is necessary to reveal each of the key factors. Constructing defined co-culture model using hydrogen producers will offer better performance and better approach to understand the ecological relationship between distant microorganisms for continuous hydrogen production.

Co-culture system for large scale hydrogen production

A novel approach of co-culture system adapted by Integrated Project "HYVOLUTION" funded in EU Framework was to provide highest hydrogen production efficiency for small scale and cost-effective industries (Zeidan *et al.*, 2009). In a continuous commercial hydrogen production process, obtaining a stable microbial consortium with continuous enrichment technique is especially valuable (Zeidan *et al.*, 2010). A stable coexistence must last for longer period to consistently grow and produce hydrogen at commercial scale for its future industrial application (Masset *et al.*, 2012). A stable existence of two closely related but geographically-distant habitat organisms exhibited a remarkable stability over a period of 70 days under carbon-sufficient conditions with a maximum H_2 yield of 3.7 mol/mol of glucose (Zeidan *et al.*, 2010). With co-culture, production cycle was shortened with enhanced average rate and increased hydrogen production suggesting comparatively high production rates using 6 L reactor under continuous fermentation. The continuous fermentation represented a logarithmic phenomenon with gas accumulation, acquired better substrate utilization rate, indicated excellent gas production stability and was beneficial for long time hydrogen production (Qian *et al.*, 2011). The co-culture ability to simultaneously consume pentose or hexose with consistent growth and H_2 production was evaluated using *Clostridium* sp., during 13-day fermentation in a 20 L sequencing batch reactor (SBR). The hydrogen yield of 2.3 mol/mol of glucose was highest reported in a 20 L large lab-scale bioreactor in a starch-containing medium, without any need of pretreatment, efficiently consumed the lactate present, re-oxidizing produced formate with higher biogas production rates (Masset *et al.*, 2012). To investigate the economically viable process for H_2 production,

a combined co-culture system from lab-scale was scaled-up to 100,000 L using distillery effluent. Sustainable way of H_2 production along with treatment of effluent produced 21.38 kg of H_2 in less than 40 h (Vatsala *et al.*, 2008). Decrease in production time increases production rate and benefits overall production cost of hydrogen.

Hydrogen production rate during co-culture system

In a co-culture system, even the residual substrate present in minimal quantity is converted to final products and substrate utilization is increased resulting in increased hydrogen yield per mole of the substrate. The hydrogen production rates across the studies are compared between mono- and co-culture systems and presented in Table 2.1.3.

Hydrogen production, regardless of the type of reactor used in both carbon and non-carbon limiting conditions showed higher production rate using a co-culture in comparison to mono-culture strains (Zeidan *et al.*, 2010). Co-culturing of two extreme thermophilies resulted in faster consumption of multiple substrates with relatively higher production rate of 38 mmol/g/h in comparison to mono-culture of 21 mmol/g/h. The synergy between both the microorganisms during co-culture system helped to reach significant H_2 yield approaching theoretical stoichiometry (Zeidan *et al.*, 2009). Bioaugmentation of *C. acetobutylicum* X_9 and *E. harbinense* B_{49}, completed the fermentation with doubling time of 6 h in comparison to mono-culture of 7.2 h and H_2 production rate was 8.6 times in comparison to mono-culture (Wang, 2008). Combination of cellulose-hydrolysis with H_2-producing bacteria presented a potential route of cellulose conversion to H_2 by decreasing the production time by 10 h in comparison to mono-culture. The decrease in production period increased the production rate by 1.5 times in comparison to mono-culture (Wang *et al.*, 2009). The co-culture system decreased the production time to almost 2 days from 4 days (Patel *et al.*, 2010) and helped to increase number of batch runs per year. This benefited overall cost of hydrogen production and achieved net gain of $37,070/year (Vatsala *et al.*, 2008). Hydrogen production utilizing organic wastes indicates a viable process alternative as energy source and for effluent treatment.

Advantages of co-culture system

A co-culture system can be a potential bioprocess system with effective system for industrial use. A schematic diagram of co-culture approach for overall process improvement during hydrogen production utilizing organic wastes is presented in Figure 2.1.3.

The microbes show no cross-interactions among each other, each metabolizing its own substrate and is least affected by the presence of other microbe thereby neutralizing the inhibition of reaction end products (Park *et al.*, 2012). In a co-culture system, each of the complex substrate mixtures is simultaneously converted to simpler products through sequential manner. The co-culture system is potentially more suitable for transformation of a non-sterile substrate into hydrogen by cushioning the system fluctuations across the process feed stream (Eiteman *et al.*, 2008; Quéméneur *et al.*, 2011).

The co-culture can perform multiple functions, a defined communication, division of tasks, completion of combined tasks in a balanced way and performing multiple steps which otherwise is impossible for a single microorganism (Brenner *et al.*, 2008). Co-culture technique during dark fermentation and photofermentation can define a route for higher hydrogen production in comparison to single step process (Afsar *et al.*, 2011). The fermented liquid from combined co-culture system satisfies the demand of COD removal in comparison to single and two-stage fermentation (Lee, 2012).

In comparison to monoculture, co-culture possesses higher resistance to foreign invasion, environmental fluctuations and provides stability through the process period (Brenner *et al.*, 2008). The pretreatment methods, such as hydrolysis and acid treatment for the reduction of complex substrate, which are used for mono-culture, can be avoided in co-culture system (Pattra *et al.*, 2008). Isolation with characterization of the major contributors from the original mixed microflora and the beneficial effects of associated microbes can be engineered for efficient hydrogen production (Chang *et al.*, 2008). Another advantage is combination of two processes into a single step process by co-culture of two or more compatible microorganisms for cellulolytic hydrolysis and utilization of dissolved sugars for production of renewable energy for efficient processes

(Harish *et al.*, 2010). The combination of facultative and anaerobic bacteria helped in eliminating the nitrogen sparging step and shifted the metabolic pathways towards increased hydrogen production with decrease in by-product formation (Pachapur *et al.*, 2015a). The advantage of using co-culture system not only improved the product yield, but increased biofilm formation as a potential feature in industrial fermentation system (Zeidan *et al.*, 2010).

The successful development of co-culture system is capable of transitioning the economy from fossil fuel based to biofuel-based with improvement in hydrogen yield by engineering geographically distant microorganism possessing higher tolerance and displaying hydrogen production ability at industrial scale.

Challenges in co-culture system

Major challenges to be overcome for an efficient production of hydrogen at commercial scale are outlined as follows. In a co-culture system, selection of hydrogen producing microflora plays a very important role. The selection of microflora from various sources is a difficult task, due to co-existence of other hydrogen consuming bacteria. In some cases, additional analysis using molecular tools will help in screening and selection of microbial communities that produce only hydrogen. Using genetically modified microorganisms, controlling the photosynthetic protein expression and altering the light harvesting ratio in mutant bacteria will result in increased hydrogen production (Das, 2009). Large scale profiling techniques using OMICS (transcriptomics, proteomics, and metabolomics) can guide experimental investigation, define gene regulatory networks, triggering pathways with optimal conditions for microbes best suited for hydrogen production at the expenses of additional process cost (Das, 2009; Das *et al.*, 2008).

Once the microorganisms are selected for the co-culture system, metabolite analysis during the fermentation indicated that *C. freundii* grew faster than *C. butyricum*, which resulted in decrease in final hydrogen yield. The optimum inoculum ratio pays a key role in co-culture system for substrate consumption by anaerobes and creation of anaerobic environment by facultative bacteria (Thonart *et al.*, 2010). Similarly, if one microorganism dominates over the other, the H_2 yield decreases. The dominating microorganism competes over substrate with production of end-metabolites, which can

be toxic and inhibit the H_2 metabolism of other (Li *et al.*, 2013). In the case of combined co-culture system, the increase in inoculum concentration of mesophilic bacteria will block the light utilization rate of photo-fermenting bacteria (Wu *et al.*, 2012). It was necessary to control the pH during hydrogen production as its direct effect was seen on the hydrogenase activity. In the case of combined co-culture system, acidic pH of 5 to 6 is optimum for dark-fermentative bacteria in comparison to neutral pH of 6.5 to 7.0 preference for photo-fermentative bacteria for increased hydrogen yield (Lee *et al.*, 2012). Dark-fermentative bacteria are fast growing and produce volatile acids at faster rate. On the contrary, photo-fermentative bacteria are slow growing, which causes an imbalance in utilization rate of volatile acids limiting H_2 production (Liu *et al.*, 2010).

The above stated co-culture issues can be minimized by carrying out design of experiment as discussed in the co-culture model of hydrogen production and by incorporating improvements in reactor design. In terms of bioreactor design, using rhomboidal reactor for issues of hydrogen gas-hold up and multi-layer photo-bioreactor for proper light distribution in photofermentation reaction can resolve many problems (Das, 2009). A possible two-phase system needs to be designed to prevent interspecies hydrogen transfer or use of reverse micelles systems for significant improvement in H_2 production (Sarma *et al.*, 2015; Singh *et al.*, 2008). The overall process cost of utilization of organic wastes with simultaneous energy production and effluent treatment can be further decreased by reducing the cost of growing, harvesting, transporting and treatment of biomass waste by carrying out hydrogen production process at the source (Sarma *et al.*, 2013a).

These concerns can be overcome with scientific advancement and technical breakthrough with effective co-culture system of hydrogen production for application at industrial scale.

Conclusion

Primary advantage of co-culture system is a cost-effective process, which potentially avoids costly enzymatic hydrolysis pretreatment steps and eliminates use of expensive reducing agents. The co-culture system display the interspecies well-organization of two dissimilar microorganisms, which helped in eliminating addition of expensive polypeptone nitrogen sources and used discharged organic waste as supplement which otherwise is impossible for a mono-culture. Co-culture system effectively hydrolyzed lignocellulosic complex into fermentable sugar to decrease pretreatment cost of additional hydrolysis step and improved hydrogen yield by 94.1% in comparison to mono-culture. A co-culture system offers an efficient process, which is capable of consuming headspace oxygen, eliminate use of expensive reducing agent and additional sparging step, and also possess the ability to recover H_2 production during accidental process snag. The reduction in lag phase and decrease in H_2 production period by 10 h during co-culture system benefits production rate by 14.50% and 8.6 times in comparison to mono-culture. Decrease in production time indirectly increases batch runs and directly profits overall H_2 production cost. Co-culture system systematically channels the dark-fermentation by-products into H_2 production by photo-fermentation using combined system in a single reactor. Co-culture system demonstrates better energy recovery during organic waste utilization by simultaneously achieving theoretical hydrogen yield and reaching the specified limit of COD removal of around 60 %. The diversity of co-culture substrate ranges from organic wastes, such as cassava pulp, brewery waste, starch residue, sugar cane distillery effluent to synthetic substrates, such as glucose and xylose. The simultaneous consumption of substrate with consistent growth during hydrogen production over 13 days of fermentation period, demonstrated the viability for industrial scale production. Integration of co-culture results at lab-scale helped to design an economically viable process at 100,000 L to achieve a sustained state of H_2 production.

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Table 2.1.1: Advantages and disadvantages of different hydrogen production process.

Production process	**Advantages**	**Disadvantages**	**Ref.**
Steam reforming of hydrocarbons	Feedstock for fuel cell and other hydrocarbon products	CO_2 released into the atmosphere	(Kapdan *et al.*, 2006)
Thermal processing	Maximum conversion	Significant gas conditioning is required.	(Kapdan *et al.*, 2006)
Electrolysis of water	H_2 used in fuel cell, O_2 generated used for space.	80 % operating cost going to electricity consumption	(Masset *et al.*, 2012)
Mono-culture fermentation	At ambient temperature and atmospheric pressure	Low rate of H_2 production and yield	(Das *et al.*, 2008)
Co-culture fermentation	Cost effective system, increased H_2 production, resistance to foreign invasion, resistance to process fluctuation, efficient recovery of biomass waste and low COD in final product.	Engineering applications, better bioreactor design and continuous process requirements.	(Hema *et al.*, 2012)

Table 2.1.2: Microorganisms used across different processes using diverse sources for total hydrogen yield.

Microorganism	Co-culture approach	Source	Source composition	Operating conditions	Process	H_2 yield (mol/mol of glucose)	Ref.
Clostridium butyricum (CWBI952)[a] , *Citrobacter freundii* (CWBI1009)	Avoid addition of expensive reducing agents	Glucose monohydrate, maltose, sucrose, lactose or starch	-	200 mL working volume, pH 7.3, inoculation with 5/3+3 mL (pure/ co-culture) culture at 30°C ± 0.5°C with 150 rpm	Mono-culture[a]	0.69[a]	(Thonart *et al.*, 2010)
					Co-culture	0.73	
Clostridium thermocellum JN4, Thermoanaerobacterium thermosaccharolyticum GD17[a]	Complete utilization of cellobiose and glucose	Microcrystalline cellulose, filter paper or cellobiose	-	10 mL working volume, pH 6.8, at 60 °C with 2-10 % inoculation volume in hungate tubes	Mono-culture[a]	0.8[a]	(Liu *et al.*, 2008)
					Co-culture	1.8	
Enterobacter aerogenes HO-39[a] , *Clostridium butyricum* IFO13949	Remove O_2 and avoid addition of expensive reducing agents	Starch	-	200 mL working volume, pH 5.2, inoculation with 8/8+4 mL (pure/co-culture) cultures at 36 °C with 150 rpm	Mono-culture[a]	1.0[a]	(Yokoi et *al.*, 1998b)
					Co-immobilized cells	2.6	
E. aerogenes NRRL B-407[a], *C. butyricum* NRRL B-41122	Remove O_2 and avoid addition of expensive reducing	Crude Glycerol	23.63 % glycerol, 3.06 % ash, 5.75 % moisture	50 mL working volume, pH 6.5, inoculation with 1.25+1.25 mL cultures at 36 °C with 150 rpm	Mono-culture[a]	0.82[b]	(Pachapur *et al.*, 2015)
					Co-culture	0.95[b]	

Microorganism	**Co-culture approach**	**Source**	**Source composition**	**Operating conditions**	**Process**	**H_2 yield (mol/mol of glucose)**	**Ref.**
	agents without pretreatment						
Clostridium butyricum IFO13949[a], *Rhodobacter sphaeroides* M-19	Utilization of by-products in H_2 production	Starch	-	50 mL working volume, pH 7.0, inoculation with 2/2+5/2+3 mL (pure/two-stage/co-culture) culture at 30 °C with stirring under illumination at 5,000 lux	Mono-culture[a]	1.9[a]	(Yokoi et *al.*, 1998a)
					Two-stage	3.6	
					Co-culture	6.6	
Enterobacter aerogenes HO-39 [a], *Clostridium butyricum*[a], *Rhodobacter sphaeroides* M-19	Use of organic waste rich in nitrogen source for effective and economical H_2 production	Sweet potato starch residue	50% starch, 14% moisture, 5% ash	200 mL working volume, pH 5.25 at 37 °C, inoculation with 11+1 mL (co-culture) and 2+3 mL (two-stage) culture at 35 °C with stirring under illumination at 5,000 lux	Co-culture[a]	2.7[a]	(H Yokoi *et al.*, 2002)
					Two-stage	7.2	
Clostridium	Utilization of	Sweet	50%	200 mL working volume, pH	Co-culture[a]	2.4[a]	(Yokoi et

Microorganism	**Co-culture approach**	**Source**	**Source composition**	**Operating conditions**	**Process**	**H_2 yield (mol/mol of glucose)**	**Ref.**
butyricum IFO13949[a], *Enterobacter aerogenes* HO-39 [a], *Rhodobacter sphaeroides* M-19	starch and avoid addition of reducing agents	potato starch residue	starch, 14% moisture, 5% ash	5.25 at 37 °C, inoculation with 11+1 mL (co-culture) and 2+3 mL (two-stage) culture at 35 °C with stirring under illumination at 5,000 lux	Two-stage	7.0	*al.*, 2001)
Caldicellulosiruptor saccharolytics DSM 8903, *Caldicellulosiruptor kristjanssonill* DSM 12137	Rapid and efficient utilization of lignocellulosic sugars	Glucose and xylose	-	1 L working volume, pH 6.7, with inoculation of both cultures at 15 % (v/v) stirred at 70 °C 300 rpm	Co-culture	3.7	(Zeidan *et al.*, 2010)
Clostridium acidisoli DSM12555, *Rhodobacter sphaeroides* ZX-5	To improve efficiency of sucrose conversion to H_2	Sucrose	-	30 mL working volume, pH 7.0, at 30 °C, 1.2 mL mixed culture inoculation, with illumination at 4000 lux	Co-culture	5.08	(Sun *et al.*, 2010)
Clostridium butyricum, Rhodopseudomonas faecalis RLD-53	Effect of different process parameters	Glucose	-	80 mL working volume, pH 7.5, 35 °C with 1:10 inoculation ratio at 120 rpm under illumination at 4000 lux	Co-culture	4.13	(Ding *et al.*, 2009)
Clostridium butyricum DSM 10702, *Rhodobacter*	Utilization of glucose and by-products in	Glucose	-	200 mL working volume, pH 7.8, inoculation with 1:5.9 culture at 30 °C with stirring	Co-culture	3.7	(Fang *et al.*, 2006)

Microorganism	**Co-culture approach**	**Source**	**Source composition**	**Operating conditions**	**Process**	**H_2 yield (mol/mol of glucose)**	**Ref.**
sphaeroides DSM 158	H_2 production			under illumination at 135 Wm-2			
Clostridium thermocellum, Clostridium thermopalmarium	Effect of different process parameters	Whatman filter paper	98% cellulose	30 mL working volume, pH 7.0 , with inoculation of both cultures at 0.05-0.17:1 ratio at 55 °C	Co-culture	1.36	(Geng *et al.*, 2010)
Enterobacter aerogenes TISTR 1468, *Clostridium butyricum* TISTR 1032	Removal of O_2 by using *Enterobacter* as reducing agents	Cassava pulp hydrolysate	66.4% starch, 2.1% protein, 28.8% fiber, 0.2% fat and 2.5% ash	100 mL working volume, pH 5.5, inoculation with 2+4 mL cultures at 36 °C with 150 rpm	Co-culture	1.9	(Phowan *et al.*, 2010)
Enterobacter aerogenes E10, *Citrobacter freundii* 01 and *Rhodopseudomonas palustris* P2	Improve H_2 production from sugar cane distillery effluent	Sugar cane distillery effluent	6.4% reducing sugar, 3.5% fatty acids, 12.3% total nitrogen, 78.2% total protein	100 m^3 working volume, pH 5.25 at 37 °C, inoculation with 10 % (mixed) cultures at 37 °C under illumination at 7,000 lux	Co-culture	2.76	(Vatsala *et al.*, 2008)
Mixed Culture	To efficiently convert crude glycerol into H_2	Crude glycerol	90% glycerol, 7% salts, 2% ash, 1% methanol	45 mL working volume, pH 7.9, 5 mL inoculation of mixed cultures at 37 °C with 120 rpm	Mixed-culture	0.96[b]	(Varrone *et al.*, 2012)
Mixed Culture	The effect of pH on the	Glucose	-	1.7 L working volume, pH 5.5, 50 mL inoculation with	Mixed-culture	2.1	(Fang *et al.*, 2002)

Microorganism	**Co-culture approach**	**Source**	**Source composition**	**Operating conditions**	**Process**	**H_2 yield (mol/mol of glucose)**	**Ref.**
	conversion of glucose to H_2			mixed cultures at 36 °C with 200 rpm			

[a] The microorganisms used for the process with its respective hydrogen yield.

[b] H_2 yield (mol/mol of crude glycerol)

Table 2.1.3: Comparison of H_2 production rate among mono- and co-culture systems.

Microorganism	Substrate	Process	H_2 production rate (mmol/g of cell dry weight/h)	Ref.
Clostridium acetobutylicum X9[a] and *Ethanoigenens harbinense* B49	Microcrystalline cellulose	Mono-culture[a]	6.4	(Wang, 2008)
		Co-culture	55.4	
Citrobacter freundii 01 [a], *Enterobacter aerogenes* E10 and *Rhodopseudomonas palustris* P2	Distillery effluent	Mono-culture[a]	8.8	(Vatsala *et al.*, 2008)
		Co-culture	10.6	
Caldicellulosiruptor owensensis[a] *and Caldicellulosiruptor saccharolyticus*	Glucose and xylose	Mono-culture[a]	21	(Zeidan *et al.*, 2009)
		Co-culture	38	

[a] The microorganisms used for the study with its respective hydrogen yield.

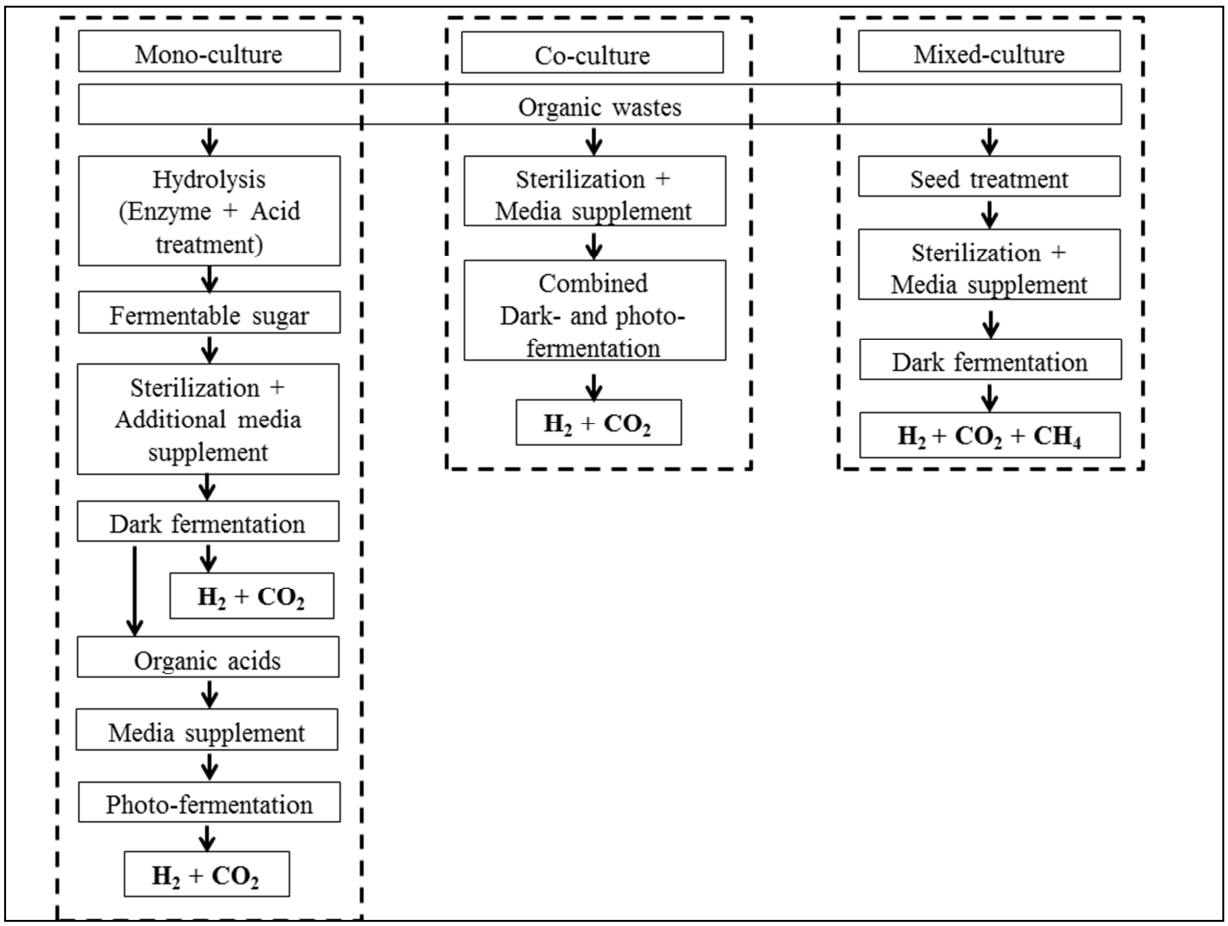

Figure 2.1.1: A schematic diagram for hydrogen production from organic wastes across different systems.

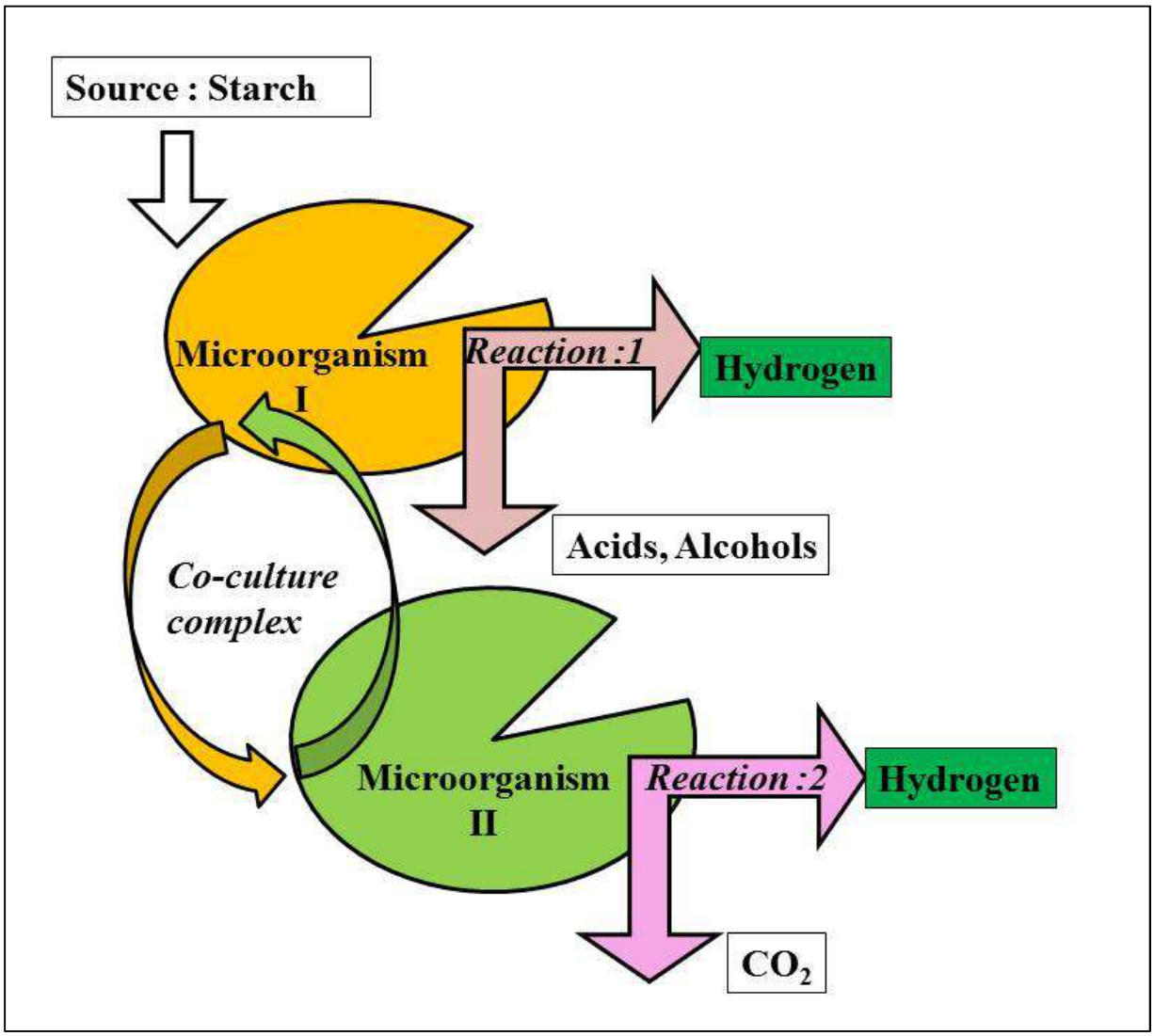

Figure 2.1.2. The conceptual model of the co-culture system in comparison to mono-culture system of fermentation. In Reaction: 1, starch is converted to H_2 and end metabolites such as acids and alcohols in the presence of *C. butyricum* in a mono-culture system. In a co-culture system, by using *Rhodobacter* strain the end metabolites are converted into additional H_2 (Reaction: 2).

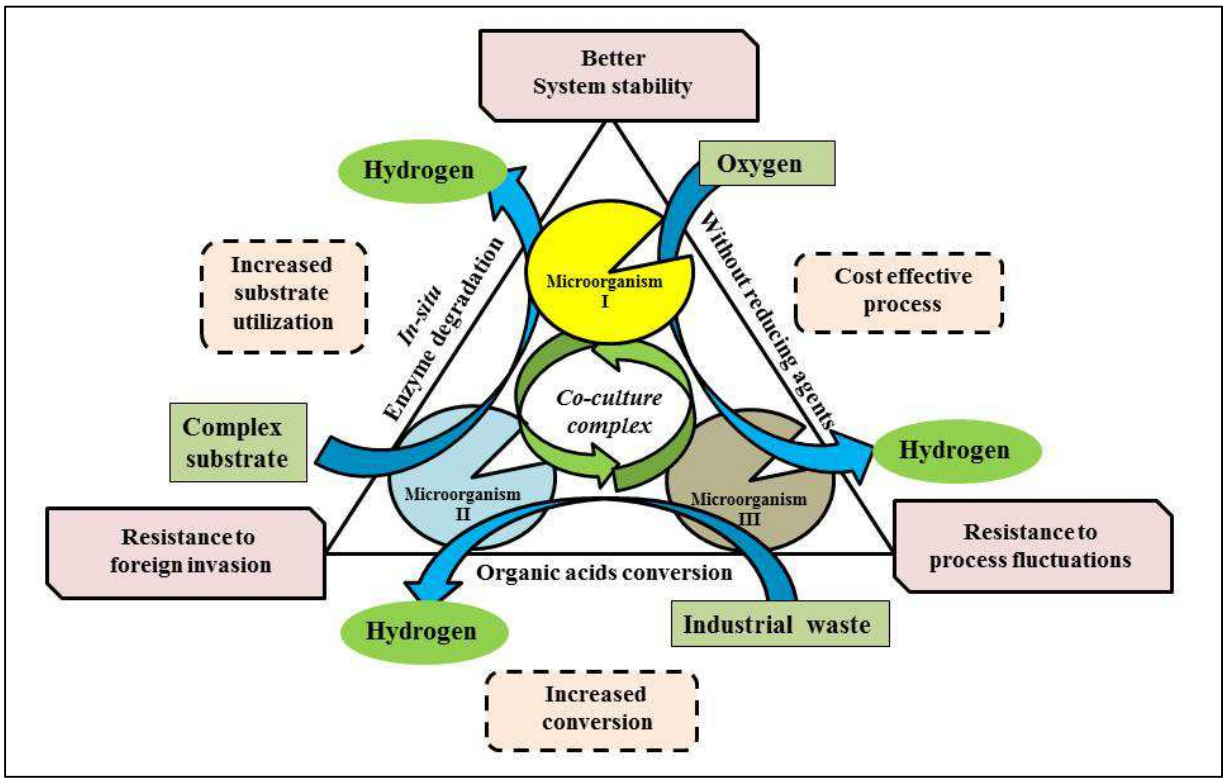

Figure 2.1.3: A schematic representation of co-culture approach for overall process improvement during hydrogen production.

PART 2

CO-CULTURE STRATEGIES FOR INCREASED BIOHYDROGEN PRODUCTION

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CHAPTER 3

HYDROGEN PRODUCTION BY CO-CULTURE SYSTEM WITH NO PRE-TREATMENT OF SUBSTRATE

PART 1

ENERGY BALANCE OF HYDROGEN PRODUCTION FROM WASTES OF BIODIESEL PRODUCTION

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Résumé

La production de biodiesel à travers le monde a entraîné une augmentation proportionnelle du glycérol brut (GB) comme déchets. La valorisation du GB augmentera la viabilité économique de l'industrie du biodiesel à l'avenir. Cette étude compare le bilan énergétique pour une utilisation du GB pour la production de H_2 par fermentation sombre avec la purification du glycérol brut pour en faire du glycérol pur. Le bilan énergétique pour chacun des matériaux utilisés lors de l'inoculum, les étapes de préparation des médias et de l'électricité consommée dans les deux méthodes ont été calculés. L'entrée d'énergie totale pour la purification du glycérol (872,39 MJ) était 2,5 fois plus élevé par rapport à l'apport d'énergie totale maximale de la matière première du GB (344,25 MJ). La valeur de marché du glycérol pur diminue par rapport à l'augmentation de la valeur de marché de H_2 comme combustible. Pour une utilisation efficace du GB, la bioconversion pour la production H_2 par fermentation sombre peut être considérée comme l'option favorable et durable pour la production de carburant à haut rendement énergétique. L'énergie nette (MJ) pour différentes matières premières (source végétale -158,90, multi-matières premières -113,23 et les déchets animaux -83,14) pendant la production de H_2 varie avec la teneur en glycérol. Il est possible de faire la fermentation sombre sous des conditions ambiantes et d'utiliser de l'électricité produite à partir du biogaz pour réduire l'apport énergétique total. Ce faisant, l'énergie nette pour différentes matières premières aura une valeur positive.

Mots clés: Glycérol brut; fermentation sombre; bilan énergétique; gaz a effet de serre; hydrogène

Abstract

Biodiesel production across the globe has resulted in proportional increase of crude-glycerol (CG) as waste by-product. Utilization of CG will increase the economic viability of biodiesel industry in future. This study compared energy balance for CG utilization during dark fermentation for hydrogen production with glycerol purification for glycerol. The energy balance for each of the materials used during inoculum, media preparation steps and electricity consumed across both the methods was calculated. The total energy input for glycerol purification (872.39 MJ) was 2.5 fold times higher in comparison to maximum total energy input of vegetable feedstock derived CG (344.25 MJ). The market value of pure glycerol is decreasing in comparison to increasing market value of hydrogen as fuel. For efficient utilization of CG, bioconversion to hydrogen production by dark fermentation can be considered as energy-efficient and sustainable fuel generation option. The net energy (MJ) for different feedstock (vegetable source -158.90, multi-feedstock -113.23 and animal waste -83.14) during hydrogen production varied with glycerol content. Alternatively, performing dark fermentation at ambient condition and utilization of electricity generated from biogas capture will surely reduce the total energy input. By doing so, the net energy for different feedstock will have a positive value.

Key words: crude glycerol; dark fermentation; energy balance; greenhouse gas; hydrogen

Introduction

The world will be confronted with an energy crisis as the fossil oil reserves will be exhausted in fewer than 45 years (Ahmad *et al.*, 2011). In the past 25 years, biodiesel fuels produced from vegetable oils or animal fats has been used in diesel engines and heating systems across the world. Due to various advantages of being non-toxic renewable fuel, produces no sulfates, aromatic compounds, small net contribution of CO_2, very low CO, less smoke and particulate matter, it is actually competing as an alternative to petroleum fuels (Atabani *et al.*, 2012; Pachapur *et al.*, 2016; Varrone *et al.*, 2012). In the recent years, biodiesel production across the world has been growing exponentially, attracting attention of researchers and practitioners for economical, environmental and sustainable future fuel energy (Ahmad *et al.*, 2011; Ayoub *et al.*, 2012; Xiao *et al.*, 2013). Renewable sources, such as biodiesel, biofuels and biohydrogen are gaining importance as promising future alternative for fossil fuels (Varrone *et al.*, 2013). To meet the increasing world demand the projected biodiesel production (L/day) in 2020 will be around 30.28 billion liters (Ayoub *et al.*, 2012). The main by-product of biodiesel production generates crude glycerol (CG), one volume of crude glycerol is generated for every 10 volume of biodiesel produced (Astals *et al.*, 2012; Sarma *et al.*, 2013a). The projected data suggests that the global crude glycerol production will attain 3 billion liters after the year 2020 (Ayoub *et al.*, 2012). Biodiesel production across major continents during 2005 and 2011 is illustrated in Figure 3.1.1 (Data generated from (US, 2014)), along with the estimation for the biodiesel and crude glycerol production for the year 2020.

Consequent increase in demand of biodiesel has resulted in large surplus of CG which may be treated as a waste or turned into useful purpose (Li *et al.*, 2013). Some of the methods for disposal and utilization of crude glycerol, such as combustion, composting, animal feeds, distillation/evaporation (Ayoub *et al.*, 2012) among others have additional treatment standard (Sarma *et al.*, 2013a), with increasing cost (Johnson *et al.*, 2007), potential hazardous results (DeFrain *et al.*, 2004; Yang *et al.*, 2012) and requiring high energy intense separation techniques (Hunsom *et al.*, 2013).

In order to support the biodiesel industry, a comparative and combinative approach for crude glycerol purification resulted in highest glycerol purity in comparison to other high-energy input techniques (Ayoub *et al.*, 2012; Hunsom *et al.*, 2013). Pure glycerol can be utilized for value-added products which opens a new market of crude glycerol in near future and this could credit crude glycerol with reduction of 13-14% in the total manufacturing cost of biodiesel fuel (Ayoub *et al.*, 2012).

Another best possible industrial method with bioconversion of CG delivers several advantages, such as no need of pretreatment or purification method, low nutrient addition and highly efficient process with production of biohydrogen seems to be economically viable for biodiesel industries (Varrone *et al.*, 2013).

Anaerobic (dark) fermentation of the crude glycerol for hydrogen production holds better promise as compared to aerobic (photo) fermentation (Fukushima *et al.*, 2011; Jitrwung *et al.*, 2011; Sarma *et al.*, 2013a). Hydrogen is considered as potential efficient carriers of future energy due to its high energy content (120-142.9 MJ/kg), higher conversion efficiency, recyclability and reduction in GHG (greenhouse gas) (Pachapur *et al.*, 2015; Pachapur *et al.*, 2015c; Sarma *et al.*, 2013a). Hydrogen production is more suitable than any other valuable products (1,3-propanediol, ethanol etc) with no expensive step for product recovery and can be used directly as fuel (Sarma *et al.*, 2013a; Sarma *et al.*, 2012).

To test the best approach of crude glycerol credit for biodiesel industry either by dark fermentation or glycerol purification, calculation of energy balance was employed as platform to determine the efficient and feasibility of the two methods. The concept behind the proposed study is thus to evaluate: (i) energy balance during hydrogen production by dark fermentation from crude glycerol; (ii) energy balance during glycerol purification via combination of solvent extraction and adsorption from crude glycerol; (iii) comparison and evaluation of dark fermentation and glycerol purification options for crude glycerol. This study also attempted to provide an insight into hydrogen production from crude glycerol and revealed energy-efficient approach for hydrogen production.

Material and methods

The articles were screened with literature on utilization of CG by dark fermentation for hydrogen production and excluding topics related to production of other products,

such as 1,3-propanediol, ethanol etc. The energy balance depending on the literature framework during dark fermentation, such as pretreatment of CG, microorganism selection, inoculum development, media preparation, hydrogen production and utilization of spent media are considered. In case of glycerol purification, energy balance on pretreatment of CG and combined purification treatment (chemical and physical) is considered. In this study, the energy balance is calculated based on 1 kg of crude glycerol utilization either from dark fermentation or glycerol purification process. The electricity used for sterilization, incubation, fermentation and shaking, drying, evaporation and adsorption steps was considered as direct energy and their energy values with power consumption were used in the calculation. Likewise, other media components, such as yeast extract, peptone, tryptone, cysteine, supplementary nutrients, buffering agents, butanol, iso-propanol etc have been also considered as source of indirect energy for which energy consummation during production of these materials per kg of crude glycerol was used in the calculation (Batan *et al.*, 2010; Ruggeri *et al.*, 2010; Zhang *et al.*, 2013). The energy terms are defined as follows and represented as equations (1-4): *Total energy input of process (TEI):* the sum of each process energy input; *Energy credit (EC)*: the energy contained in produced hydrogen and by-products/dry biomass; *Net energy balance (NEB):* the difference between the energy contained in generated hydrogen or by-products/dry biomass produced, and the total energy spent to obtain and maintain the hydrogen production process*; Theoretical Energy output (TEO):* theoretical energy contained in 1 mole of glycerol i.e. 4 moles of hydrogen, on conversion to energy equals to 8.44 MJ [8] and *Net Energy Ratio(NER):* theoretical energy output to net energy balance (*TEO* / *NEB*) (Ruggeri *et al.*, 2010).

The different equations can be represented as follows:

Total Energy Input: TEI = ∑ Energy input (Pretreatment+ Inoculation+ Media preparation+ Dark fermentation) (1)

Energy Credit: EC = ∑ Energy credit (Hydrogen + by-products/ dry biomass) (2)

Net Energy Balance: *NEB= EC – TEI* (3)

Theoretical Energy Output: TEO= 8.44 MJ (4 moles of hydrogen/mole of glycerol)

Net Energy Ratio: NER= TEO / *NEB* (4)

Table 3.1.1, lists the literature findings on dark fermentation for hydrogen production from pure glycerol (as positive control) and crude glycerol. Different steps in the literature have been considered for energy balance calculation.

Results and Discussion

Energy balance of dark fermentation: Microbial strains

The glycerol uptake in many of the microorganisms is through passive diffusion, channel protein or by using an active uptake mechanism. Many of the microorganisms (Table 3.1.1) use CG as sole carbon and energy source during dark fermentation. The strains, such as *Enterobacter, Citrobacter, Clostridium and Klebsiella* metabolize glycerol using oxidative and reductive pathway (Seifert *et al.*, 2009).

Along with hydrogen, other reduced products, such as butyrate, butanol, ethanol, 1, 3-propanediol and acetone are also produced depending upon the microorganisms used (Pachapur *et al.*, 2016, Pachapur *et al.*, 2015a, Pachapur *et al.*, 2015b). Studies in Table 3.1.1, have reported single strains, and mixed cultures of more than two microorganisms for hydrogen production. The microbial strains are collected from sludge (Seifert *et al.*, 2009; Sittijunda *et al.*, 2012; Varrone *et al.*, 2012), from bottom portion of up-flow anaerobic blanket reactor of a soyebean processing plant (Costa *et al.*, 2011), few from wheat/tomato soil samples (Selembo *et al.*, 2009) and engineered microorganisms (Yazdani *et al.*, 2008) for hydrogen production. In this study, the energy balance on collection, screening and isolation of microorganisms is not considered. The energy expenditure step during microbial growth, with the culture preparation is considered in the inoculum development step.

Energy balance of dark fermentation: Pretreatment of crude glycerol

Crude glycerol released from biodiesel plant contains different impurities at variable percentage contribution. Methanol and soap are the two major contributors, which have inhibitory effect on the growth of microorganisms. In most dark fermentation studies listed in Table 3.1.1, the sterilization of CG media evaporates methanol, hence additional pretreatment step for methanol removal is not carried out. In the case of soaps, dilution with distilled water and treatment with HCl, converts insoluble free fatty acids and the resulting precipitated solid can be removed after centrifugation (Sarma *et al.*, 2013b). Ngo *et al*. (Ngo *et al.*, 2011) carried out

methanol removal by rotary evaporation at 45 ^{o}C and settling of solids by centrifugation at 15,000 rpm for 15 min and demonstrated that pretreatment plays a key role in conversion of CG into hydrogen at a rate of 1.55-fold higher than the untreated one. In other studies, different concentrations of the CG without pretreatment, from 0.25% (w/v), to 15% (w/v) g/L (Sittijunda *et al.*, 2012), was investigated to study its effect on hydrogen production. Higher concentration of glycerol resulted in decreased yields of hydrogen (Ito *et al.*, 2005). In this study, 1 kg of crude glycerol will be diluted to 400 L at 0.25% (w/v) concentration in a 1000 L reactor for hydrogen production. The energy balance during methanol usage and electricity used for centrifugation step is calculated in the pretreatment step of crude glycerol. Table 3.1.2 features the energy balance calculations across different feedstock (4(a) vegetable source (Ngo *et al.*, 2011) , 4(b) multi-feedstock (Yazdani *et al.*, 2008) and 4(c). animal waste (Sarma *et al.*, 2013b)) shortlisted for this study.

Energy balance of dark fermentation: Inoculation preparation

The mixed culture collected from varied sources was subjected to different treatments prior to the inoculation step (Costa *et al.*, 2011, Seifert *et al.*, 2009, Selembo *et al.*, 2009). The microbial strains need to be enriched with nutritional medium, which helps in the cultivation of the glycerol fermenting microorganisms. The medium contained carbohydrate source (glycerol), nitrogen source (peptone/tryptone and yeast/urea), buffering agents or salts along with micro-nutrients. Inoculum percentage across studies varied from 5-30% (v/v) (Sarma *et al.*, 2013b; Sittijunda *et al.*, 2012). Inoculum with 5% (v/v) was considered for the proposed dark fermentation requiring media components for 20 L inoculum preparation. The increase in the media components for inoculum step will increase the energy input (a).Table 3.1.3 features the summary of the energy balance across different feedstock. From Table 3.1.3, least and highest energy contribution for the inoculum step was seen for multi-feedstock (Jitrwung *et al.*, 2013) with 0.46% contribution and vegetable source (Varrone *et al.*, 2012) with 28.78% contribution of total energy input.

Energy balance of dark fermentation: Media preparation

The success of the fermentation technology depends on the statistical optimization of media composition and process parameters, which improves the process

efficiency, stability and is very important to boost fermentation process (Selembo *et al.*, 2009; Varrone *et al.*, 2012). Varrone *et al*. (Varrone *et al.*, 2012) used Plackett-Burman screening design, Box-Behnken design and Response Surface Method (RSM) to obtain high yield of hydrogen, using a very simple synthetic medium, without nutrients, tryptone or yeast extract. The model used by Sittijunda *et al*. (Sittijunda *et al.*, 2012) suggested that parameter optimization of glycerol concentration, nitrogen source, buffer concentration and amount of nutrients improved hydrogen yield. Preparation of the media by diluting the CG with suitable buffer salts and supplementary nutrients increased cell growth and hydrogen yield. However, addition of any supplements will increase the fermentation cost, disposal treatment and it also adds on to the total energy input (Ito *et al.*, 2005). In the absence of any supplementary media component, 92% reduction in process cost was possible (Sarma *et al.*, 2013a). The energy consumption during production of these media components was higher (Zhang *et al.*, 2013) and there utilization for instance, in Table 3.1.3, contributes to the increase in total energy input (a). From the Table 3.1.3, the least energy contribution for the media preparation step was seen in animal waste (Sarma *et al.*, 2013b) with 0.001% contribution and the highest was in vegetable feedstock (Ngo *et al.*, 2011) with 46.42% contribution of total energy input.

Energy balance of dark fermentation: Hydrogen production

Hydrogen production using dark fermentation involves three types of biochemical reactions. The first reaction is typically found in *Escherichia coli* and *Enterobacteriacease*, where pyruvate is spilt into acetyl-CoA and formate by pyruvate formate lyase (PFL) enzyme. Later, in the presence of formate hydrogen lyase (FHL) enzyme, formate is broken to molecular hydrogen and carbon dioxide release. Second type of reaction involves *Clostridium* species, whereby pyruvate oxidation into acetyl-CoA with reduction of ferredoxin (Fd) is carried out by pyruvate ferredoxin oxidoreductase (PFOR). The reduced Fd is oxidized in the presence of Fd-dependent hydrogenase (HydA) to release molecular hydrogen. In the third type of reaction, several thermophilic bacteria and many *Clostridium* species carry out the reaction by NADH: ferredoxinoxidoreductase (NFOR) and HydA. In this reaction, the NADH reduced oxidized Fd in the presence of NFOR, and the Fd (red) was

transferred to molecular hydrogen by HydA (Hay *et al.*, 2013). During bioconversion of CG, after 30 h, along with hydrogen production, acids are also generated. The increase in acids level causes reduction in optimal pH, which inhibits microorganisms growth and hydrogen production (Ngo *et al.*, 2011). Varrone *et al*. (Varrone *et al.*, 2012) carried out fermentation for 6 days, only to observe the best results reached during the second day of experiments. The hydrogen production reached a stationery phase at 48 h and later started to decrease with no hydrogen production after 60 h (Sarma *et al.*, 2013b). Markov *et al*. (Markov *et al.*, 2011) concluded that hydrogen production in the bioreactor starts after 24 h and reaches the maximum production rate on the second day of operation. In the energy balance calculation, fermentation of 48 h is assumed for the production of hydrogen from crude glycerol. From Table 3.1.3, the least and highest energy consumption for the total electricity was seen in vegetable source (Ngo *et al.*, 2011) with 51.14% contribution and animal waste (Sarma *et al.*, 2013b) with 98% contribution of total energy input.

Energy balance of glycerol purification: Pre-treatment of CG

The constituents of crude glycerol, such as soap, fatty acid, esters of glycerol, salts, methanol and matter organic non-glycerol (MONG), are present at varying concentrations. Hunsom *et al*. (Hunsom *et al.*, 2013) carried out initial and most important step in glycerol purification with pretreatment step to concentrate glycerol from other impurities. The most effective treatment step is acidification, with addition of phosphoric acid (H_3PO_4) at pH 2.5 in constant rpm of 200 for 1 h. Later after 12 h, the glycerol-rich layer is separated and neutralized with NaOH at pH 7.0 and drying step at 110 ºC for 15 h to obtain pre-treated crude glycerol. The pretreatment steps in case of dark fermentation with energy expenditure of 0.18 MJ is efficient in comparison to the treatment steps of glycerol purification process with 35.41 MJ. The energy balance across the pretreatment accounts for the acid, base utilization along with electricity used for shaking and drying is calculated and presented in Table 3.1.4.

Energy balance of glycerol purification: Combined purification treatment

Purification of pre-treated crude glycerol was tested by Hunsom *et al*. (Hunsom *et al.*, 2013) with chemical (solvent extraction) and physical (adsorption) treatment. Initial chemical extraction using polar solvents: propanol (C_3H_7OH) at volume ratio of

2 (solvent to pre-treated glycerol) under constant shaking at 200 rpm for 4 h, separation of layer with decantation followed with evaporation of solvent at 95 ºC for 12 h to obtain glycerol with purity of 97.9%. In physical treatment, using heat dried (at 105 ºC for 12 h) pre-activated carbon at ratio 67 g/L with pre-treated crude glycerol under constant shaking at 200 rpm followed with vaccum filtration resulted in 68.9% glycerol purity. To increase the purity, Hunson *et al*. (Hunsom *et al.*, 2013) carried out combined solvent extraction followed with activated carbon adsorption to end-up with glycerol purity of 99.0%. The purity obtained by simple solvent extraction and activated carbon adsorption was better across glycerol purification studies using high-energy input techniques such as vacuum distillation (96%), electrochemical technique (95%), physicochemical approach (93%) and saponification-acidification-neutralization (96%). The energy balance considering the combined chemical and physical treatment is calculated and presented in Table 3.1.4.

Energy balance for dark fermentation

As seen in Table 3.1.1 (average value of CG: ~70% across all the studies) and in general CG from biodiesel contains approximately 70% (w/w) glycerol (Sarma *et al.*, 2013a) was considered and used for theoretical calculation of hydrogen production from CG. From the Table 3.1.3, the key contributors of the total energy input are the electricity used, inoculation and media preparation steps. The inoculation and media preparation step have varying percentage contribution to the total energy input, but the major contribution is from the electrical energy used for the dark fermentation. In the minimum energy contribution with 0.46% during inoculum step for multi-feedstock (Jitrwung *et al.*, 2013) used minimum supplement in terms of beef extract and peptone for the microorganism growth. However, with 28.78% of inoculum energy contribution for vegetable source (Varrone *et al.*, 2012) used variety of costly buffer resulting in highest energy input across the studies. Considering the media preparation, the lowest energy contribution by animal waste feedstock used by Sarma *et al*. (Sarma *et al.*, 2013b) was 0.002 MJ of energy. The reason being the fermentation media with only CG and no additional media supplement required minimal energy input just to maintain initial pH. The highest contribution was seen for the multi-feedstock carried out by Ngo *et al*. (Ngo *et al.*, 2011) with 159.80 MJ for using most of the media supplements such as buffering salts, yeast extract, mineral

solution and cysteine. In most of the references, minimal electricity consumption remained almost same for 48 h fermentation, unless additional step of pre-treatment was carried out.

The only steps which can be controlled by minimizing the total energy input can be use of CG as a carbon source and avoiding use of additional supplements. In Varrone *et al*. (2) study, the fermentation process along with inoculum development and media preparation was carried out using CG. Similarly, Sarma *et al*. (Sarma *et al.*, 2013b) used the minimal media components after carrying out a techno-economic analysis (Sarma *et al.*, 2013a), which helped in designing low cost experiments with lowest *Total Energy Input* of 179.45 MJ, with *Net Energy Balance* of around -83.14 MJ followed by lowest *Net Energy Ratio* of -9.85 using equations (1-4).

Net Energy Balance in case of animal waste can be given by Equation 5:

NEB = ∑ Energy credit [0.42+95.89) - ∑ Total Energy Input (0+6.66+0.002+172.80*)*

NEB = -83.14

Figure 3.1.2 represents the overall energy balance calculation for animal feedstock (Sarma *et al.*, 2013b), considering each step of dark fermentation for bioconversion of CG to hydrogen production. For feedstock, animal waste (Sarma *et al.*, 2013b) resulted in the lowest net energy followed by the multi-feedstock and vegetable source. Minimizing the inoculum and media components and utilizing CG as the only carbon source will reduce the total energy input. To further reduce the total energy input, electricity consumption during fermentation need to be minimized. Increase in electricity input during sterilization and fermentation steps reduces the efficiency of dark fermentation process (Manish *et al.*, 2008). The electricity contribution for animal feedstock (Sarma *et al.*, 2013b) is around 98% (175.14 MJ) of total energy input 179.45 MJ (as seen from Table 3.1.3 and Figure 3.1.2). So in case of countries with ambient climate conditions in case of Asia and South America (Brazil and Mexico) the fermentation can be carried out ambient condition, eliminating the electricity input for a net positive energy gain during fermentation. In another approach, the produced biogas from the dry biomass (5.5 kg/kg glycerol = 95.89 MJ/kg produced) can be captured for electricity generation (Themelis *et al.*, 2007) and utilized for fermentation. By doing so, the net energy for different feedstock used

for dark fermentation will have a near positive value. The conclusions are only made on the calculated results considering the experimental results from various references. More experiments with CG from different feedstock using a common dark fermentation platform will help in designing an optimal process for higher hydrogen yield.

Energy balance for glycerol purification

Hunsom *et al*. (Hunsom *et al.*, 2013) carried out a best possible parameters across chemical extraction and physical adsorption to determine combination effect on the property of purified glycerol. The percentage of glycerol content used in the purification method matched with the average value of glycerol content used in hydrogen production experiment, which helped to make a comparable energy balance calculation across the studies. Calculating the energy balance on glycerol purification study for the initial pre-treatment of CG, followed by combined chemical and physical adsorption step along with total electricity used resulted in a *Total Energy Input* (*TEI*) of 872.39 MJ using equation (1). Table 3.1.4 shows the energy balance for each of the steps involved during glycerol purification process.

Comparison of energy balance between two methods

The comparison of energy balance for glycerol purification was made across the highest and lowest energy input for dark fermentation. From the Table 3.1.4, the *Total Energy Input* (*TEI*) of the glycerol purification process (872.39 MJ) was found to be 2.5 fold times higher in comparison to maximum total energy input of vegetable feedstock (Ngo *et al.*, 2011) derived crude glycerol with 344.25 MJ and almost 4.9 times in comparison to animal waste feedstock used by Sarma *et al*. (Sarma *et al.*, 2013b) with minimum total energy input 179.45 MJ. With high energy intensity for purification, industries use combustion for CG disposal instead of purification, as purification is costly and exceeds the range of economic feasibility for small scale industries (Pachauri *et al.*, 2006; Sarma *et al.*, 2013a). In final comparison, the final product obtained across the methods in terms of market values is calculated. Considering the conversion of 1 kg of crude glycerol by glycerol purification resulted in 99% pure glycerol with market value of around $0.66/L with decreasing market value (Li *et al.*, 2013). Alternatively the dark fermentation with bioconversion of 1 Kg crude glycerol with H_2 and biogas production can replace 2.47 L of conventional

diesel (Sarma *et al.*, 2013a) at $3.40/L market value. The Figure 3.1.3, compiles the comparison of energy balance for utilization of crude glycerol across hydrogen production and glycerol purification along with market value of the final products. Thus, dark fermentation of CG utilization offered advantages in comparison to the industrial purification of CG in terms of energy input and with increasing market value of H_2.

Conclusions

Biodiesel industries are looking for alternative approach to decrease disposal cost of crude glycerol and increase crude glycerol utilization to credit manufacture cost of biodiesel fuel. The energy balance study for utilization of crude glycerol concluded 2.5-4.9 fold energy input in glycerol purification along with decreasing market value of purified glycerol in comparison to dark fermentation with increased market value of hydrogen as future fuel. Energy balance of hydrogen production from CG was impacted by electricity (around 68%), inoculum (7%) and media components (26%) used during dark fermentation. The net energy (MJ) for vegetable source -158.90, multi-feedstock -113.23 and animal waste -83.14 during hydrogen production varied with glycerol content. Minimizing the media, inoculum components and electricity consumption will reduce the energy input during dark fermentation, albeit with low hydrogen yield. To increase the hydrogen yield, alternative and low cost inoculum and media components can be used with improvement in reducing the electricity consumption during dark fermentation. Thus, for efficient utilization of CG, bioconversion of CG to hydrogen production by dark fermentation can be considered as energy-efficient and feasible for sustainable fuel generation option.

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Table 3.1.1: Hydrogen productions at lab scale by dark fermentation process using crude glycerol derived from biodiesel production plants which used different feedstock as its raw material

Feedstock	**Composition of CG**	**Microorganisms**	**Temp/pH**	**H_2 yield (mol/mol glycerol)**	**Ref.**
Commercial	Pure	Anaerobic digested sludge	37 ºC/6	0.71	(Seifert *et al.*, 2009)
Commercial	Pure	*Enterobacter aerogenes*	37 ºC/6.8	0.89	(Markov *et al.*, 2011)
Vegetable source (soybean oil)	glycerol: 70%,	Microbial mixed culture	30 ºC/6.2	0.31 ± 0.01	(Selembo *et al.*, 2009)
Vegetable source (soybean, rapeseed, used vegetable oil)	glycerol: 80%	*Thermotoga neapolitana*	75 ºC/7-7.5	2.73 ± 0.14	(Ngo *et al.*, 2011)
Vegetable source (waste vegetable oil)	glycerol 41%, TOC:540g/l, ash:8%, methanol:25%, acylglycerol:0.05%	*Enterobacter aerogenes HU-101*	37 ºC/ 6.8	1.12	(Ito *et al.*, 2005)
Vegetable source (rapeseed, sunflower and soybean)	glycerol:90%, salts:7%, ashes:2%, methanol:1% and moisture:<0.4%	Microbial mixed culture	37 ºC/ 7.9	0.96, 0.94	(Varrone *et al.*, 2011; Varrone *et al.*, 2012)
Vegetable source	glycerol:86%, MONG:6.2%, ash:4.6% and methanol:0.03%	*Enterobacter aerogenes*	37 ºC/ 6.5	1.05	(Marques *et al.*, 2009)
Vegetable source (soybean oil)	glycerol:82.82%, ash:5.5%, NaCl:5.91%, mositure:11.2%, monoacylglycerols:0.48%	*Klebsiella pneumoniae* BLb01	35 ºC/ 7.0	0.45	(Costa *et al.*, 2011)
Animal waste (fried chicken oil)	glycerol: 441.3g/l, methanol:230g/l, NaCl:10g/l, nitrigen:0.5g/l and phosphrous:0.05g/l	*Thermoanaerobacterium*	55 ºC/5.5	0.30	(Sittijunda *et al.*, 2012)

Feedstock	**Composition of CG**	**Microorganisms**	**Temp/pH**	**H_2 yield (mol/mol glycerol)**	**Ref.**
Animal waste (meat processing and restaurant waste)	glycerol: 23.63 + 2.5%, carbon:35.9%, nitrogen:3.25% and	*Enterobacter aerogenes*	37 ºC/6	0.31 ± 0.01	(Sarma *et al.*, 2013b)
Multi-feedstock (soybean oil, beef tallow, pork lard)	glycerol 84%, salts 5%and methanol 0.02%	Engineered	37 ºC/6.3	1.02	(Yazdani *et al.*, 2008)
Multi-feedstock (animal fats and recycled cooking oil)	glycerol 82%, ash 2.6%, moisture12.3%, fatty acid1.7%, MONG: 3.4%	*E. aerogenes (ATCC 35029)*	37 ºC/6.8	0.85, 0.84	(Jitrwung *et al.*, 2013; Jitrwung *et al.*, 2011)

Table 3.1.2: Energy balance for per kg crude glycerol bioconversion to hydrogen production from dark fermentation for different feedstock (4(a): vegetable source (Ngo *et al.*, 2011) 4(b): multi-feedstock (Yazdani *et al.*, 2008), and 4(c): animal waste (Sarma *et al.*, 2013b)).

Energy balance for vegetable source (Ngo *et al.*, 2011).	**Item required**	**Quantity required (kg)**	**Energy contained in chemical used/produced (MJ/ kg of crude glycerol)**
Pretreatment of CG	Evaporation and centrifugation (1 h)		0.18
Inoculum development + Media preparation (20 L +380 L)	KH_2PO_4	0.60	7.84
	Na_2HPO_4	1.68	20.69
	NH_4Cl	0.20	1.18
	$MgCl_2$	0.08	0.25
	NaCl	10.80	75.98
	Yeast extract	1.60	21.76
	NaOH	3.20	34.00
	$Na2S.9H_2O$	1.00	1.52
	Cysteine	1.00	4.75
	HEPES buffer	0.05	0.25
	Sterilization and incubation (17 h)		3.06
Dark fermentation	Fermentation (48 h)		172.80
Total Energy Input (TEI)			344.25
Hydrogen	2.73 mol/mol		2.88
Dry Biomass	5.5 kg/kg glycerol		95.89

Energy balance for multi-feedstock (Yazdani *et al.*, 2008)	**Item required**	**Quantity required (kg)**	**Energy contained in chemical used/produced (MJ/ kg of crude glycerol)**
Pre-treatment of CG	No		
Inoculum development (20 L)	Agar	0.30	0.33
	Na_2HPO_4	0.00	0.06
	NH_4Cl	0.01	0.06
	$MgCl_2$	0.00	0.01
	NaCl	0.06	0.41
	KCl	0.07	0.43
	Tricine	0.01	0.12
	Sterilization and incubation (17 h)		3.06
Media preparation	Yeast extract	1.90	25.84
	Tryptone	3.80	54.22
	Centrifugation (1 h) for pre-culture pellet		0.18
Dark fermentation	Fermentation 48 h		172.80
Total Energy Input (TEI)			257.51

Hydrogen	1.02 mol/mol		1.08
Ethanol	1.01 mol/mol		0.46

Energy balance for animal waste (Sarma *et al.*, 2013b)	**Item required**	**Quantity required (kg)**	**Energy contained in chemical used/produced (MJ/ kg of crude glycerol)**
Pre-treatment of CG	No		
Inoculum development (20 L)	Glucose	0.10	1.56
	Peptone	0.10	1.43
	Yeast extract	0.01	0.14
	KH_2PO_4	0.04	0.36
	$MgSO_4.7H_2O$	0.01	0.11
	Sterilization and incubation (17 h)		3.06
Media preparation (380 L)	HCl	0.001	0.002
Dark fermentation	Fermentation 2 days at 30 ºC 150 rpm		172.80
Total Energy Input (TEI)			179.45
Hydrogen	0.40 mol/mol		0.42

Table 3.1.3: Energy balance (%) summary across each step during dark fermentation with values of total energy input (MJ), energy credit (MJ) and net energy (MJ) details of different feedstock per kg of crude glycerol conversion.

Feedstock	Energy Required								Ref.
	Inoculum Step (%)	Media preparation Step (%)	Electricity consumed (%)	Total Energy Input (MJ)	Energy Credit (MJ)	Net Energy Balance (MJ)	Theoretical Energy Output (MJ)	Net Energy Ratio	
Pure glycerol	0.00	36.68	63.32	278.32	118.90	-159.42	8.44	-18.89	(Seifert *et al.*, 2009)
Pure glycerol	1.37	42.86	55.77	315.33	96.83	-218.50	8.44	-25.89	(Markov *et al.*, 2011)
Vegetable source	3.58	0.89	95.53	180.88	95.89	-84.67	8.44	-10.03	(Selembo *et al.*, 2009)
Vegetable source	2.44	46.42	51.14	344.25	95.89	-245.48	8.44	-29.09	(Ngo *et al.*, 2011)
Vegetable source	27.44	16.79	55.77	315.33	97.18	-218.15	8.44	-25.85	(Ito *et al.*, 2005)
Vegetable source	28.78	1.57	68.65	256.16	97.26	-158.90	8.44	-18.83	(Varrone *et al.*, 2012)
Vegetable source	2.20	41.80	56.00	314.01	95.89	-218.12	8.44	-25.85	(Marques *et al.*,

									2009)
Vegetable source	1.92	39.85	58.23	302.01	96.36	-205.65	8.44	-24.37	(Costa *et al.*, 2011)
Animal waste	4.39	9.93	85.68	205.25	96.21	-109.04	8.44	-12.92	(Sittijunda *et al.*, 2012)
Animal waste	2.00	0.001	98.00	179.45	96.31	-83.14	8.44	-9.85	(Sarma *et al.*, 2013b)
Multi-feedstock	0.55	31.09	68.36	257.51	97.42	-160.08	8.44	-18.97	(Yazdani *et al.*, 2008)
Multi-feedstock	0.46	14.49	85.05	210.36	97.13	-113.23	8.44	-13.42	(Jitrwung *et al.*, 2013)

Table 3.1.4: Energy balance calculation for glycerol purification process.

Energy balance	Item required	Quantity required (kg)	Energy contained in chemical used/produced (MJ/ kg of crude glycerol)
Pre-treatment of CG	H_3PO_4	0.20	2.63
	NaOH	0.38	4.04
	Shaking and Drying (1+15 h)		28.80
Combined Enrichment treatment (Chemical and Physical)	Extraction with C_3H_7OH	1.30	43.69
	Shaking and Evaporation (4+12 h)		28.80
	Adsorption with activated carbon	20.60	735.63
	Adsorption step (drying + shaking + vaccum filtration) (12+2+2 h)		28.80
Total Energy Input			872.39

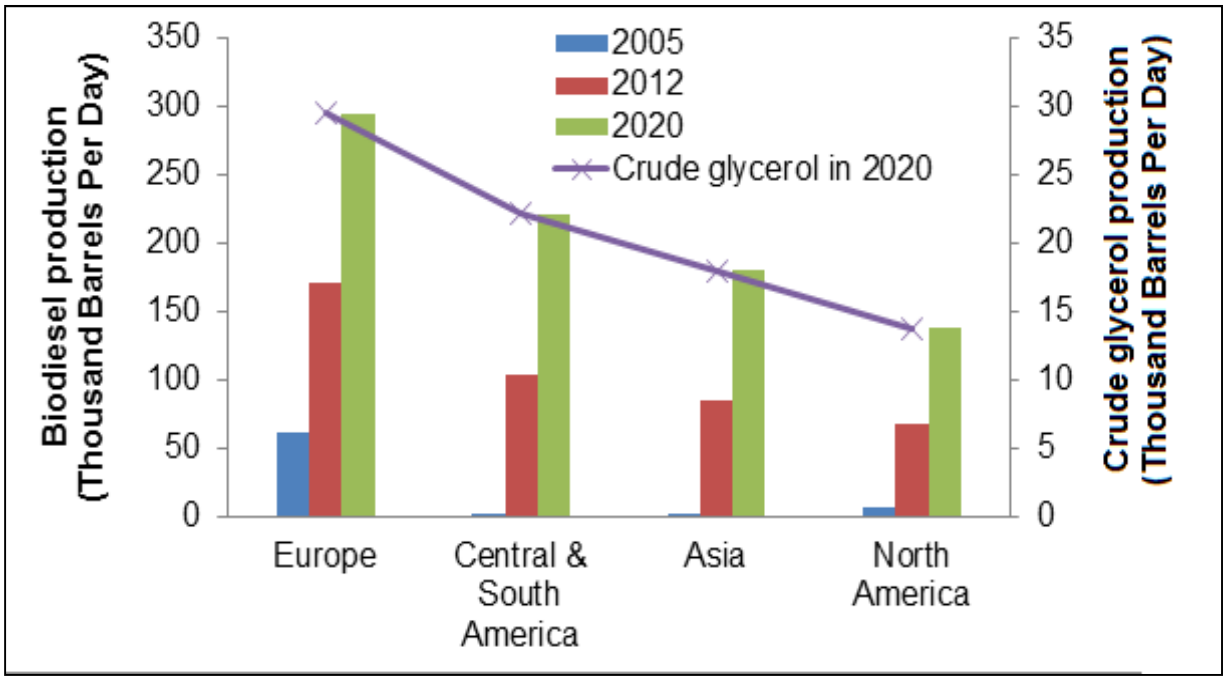

Figure 3.1.1: Biodiesel production (L/day) across major continents during 2005 and 2011 with estimated biodiesel and crude glycerol production for the year 2020.

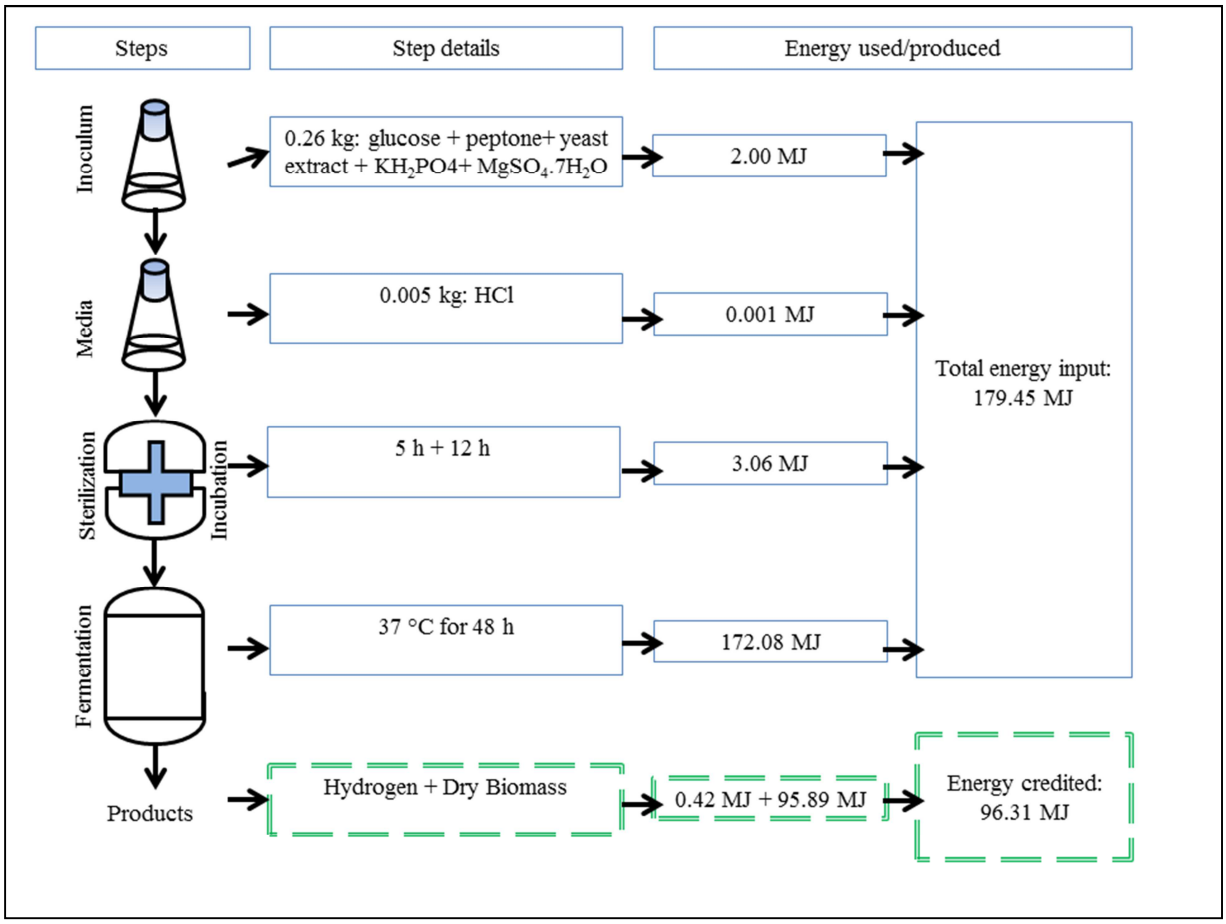

Figure 3.1.2: Schematic representation of the dark fermentation with energy balance calculation for the animal waste feedstock derived crude glycerol used in (Sarma *et al.*, 2013).

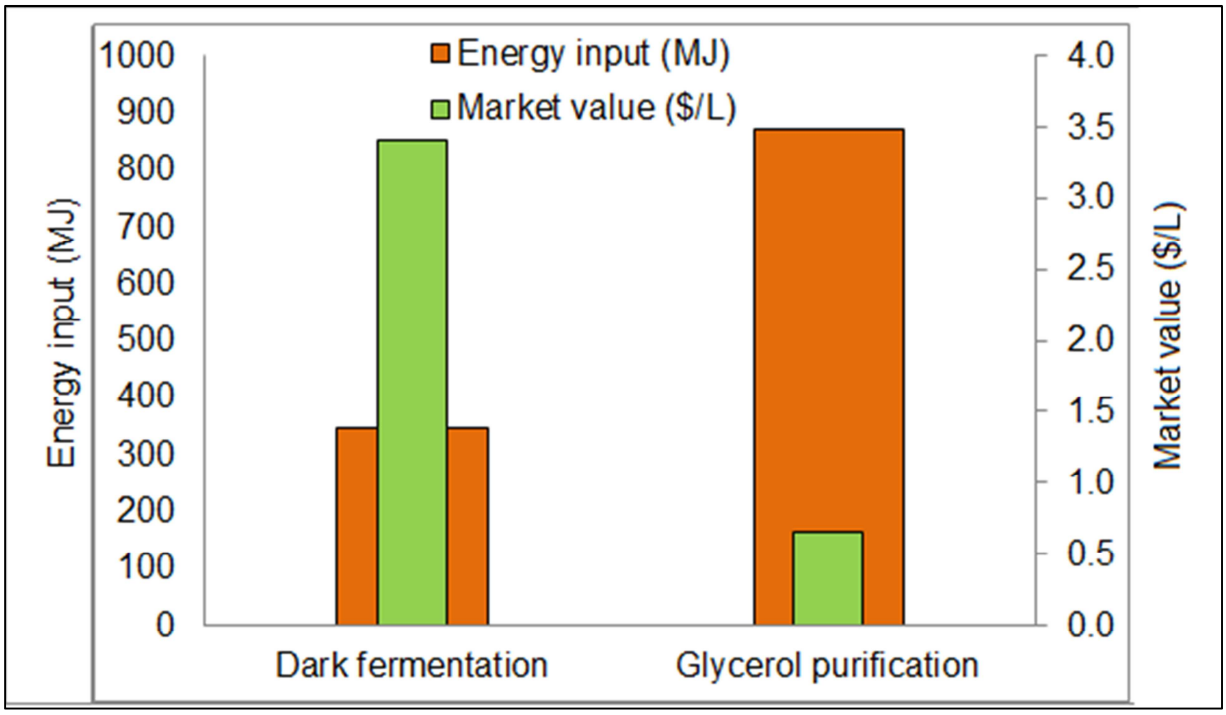

Figure 3.1.3: Comparison of crude glycerol utilization across dark fermentation and glycerol purification process with respect to total energy input and market value of the end product (H_2 and purified glycerol).

PART 2

HYDROGEN PRODUCTION FROM BIODIESEL INDUSTRY WASTE BY USING A CO-CULTURE OF *ENTEROBACTER AEROGENES* AND *CLOSTRIDIUM BUTYRICUM*

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Résumé

La valorisation du glycérol brut (GB), un sous-produit de la production du biodiesel a été étudiée pour augmenter la production d'hydrogène par un système en co-culture en utilisant *Enterobacter aerogenes* NRRL B-407 et *Clostridium butyricum* NRRL B-41122. La capacité de *E. aerogenes* a été évaluée en tant que remplacement d'un agent réducteur coûteux afin de maintenir des conditions anaérobies pour la croissance de *C. butyricum*. La co-culture a donné lieu à une production accrue de H_2, atteignant un maximum de 19,46 ± 0,95 mmol-H_2/L-de milieu de culture par rapport aux mono-culture de *E. aerogenes* (15,64 ± 0,47) et *C. butyricum* (17,44 ± 0,38) avec la présence d'un agent réducteur. L'effet du rapport de l'inoculum a été étudié et le rendement en H_2 était de 0,95 mol-H_2/mol-glycérol pour un rapport de l'inoculum de 1:11 avec plus de 85% de l'utilisation du substrat. Ces résultats étaient comparables à ce que d'autres études répertorient pour des cultures mixtes et co-culture. En plus du H_2 comme produit à valeur ajoutée, des sous-produits tels que le 1,3-propanediol, l'acide acétique, l'acide butyrique et l'éthanol ont également été produits. La co-culture a démontré une capacité accrue de valorisation du GB, en tant que consortium fonctionnel stable avec une plus grande efficacité de conversion pour augmenter l'hydrogène ainsi que générer des sous produits à hautes valeurs ajoutées. En outre, l'étude a fourni la preuve que la composition du milieu contrôle les changements de voie métabolique lors de la bioconversion du GB. La valorisation du GB à faible coût avec une co-culture efficace pour la production de H_2 peut aider l'industrie du biodiesel en générant une source d'énergie interne.

Mots clés: Biodiesel glycérol brut, Co-culture, Hydrogène, *Enterobacter aerogenes*, *Clostridium butyricum*

Abstract

Valorization of crude glycerol (CG), a waste of biodiesel production process was investigated for increased hydrogen production by co-culture system using *Enterobacter aerogenes* NRRL B-407 and *Clostridium butyricum* NRRL B-41122. The ability of *E. aerogenes* was evaluated as a replacement of expensive reducing agent to maintain anaerobic conditions for the growth of *C. butyricum*. The co-culture resulted in increased hydrogen production, reaching a maximum of 19.46 ± 0.95 mmol-H_2/L-medium in comparison to mono-culture of *E. aerogenes* (15.64 ± 0.47) and C. butyricum (17.44 ± 0.38) in the presence of reducing agent. The effect of inoculum ratio was investigated and hydrogen yield was 0.95 mol-H_2/mol-glycerol at 1:11 inoculum ratio with more than 85% of substrate utilization was comparable to other mixed- and co-culture studies. In addition to hydrogen value-added by-products such as 1,3-propandiol, acetic acid, butyric acid and ethanol have also been produced. Co-culture indicated the possibility of valorization of CG, as a stable functional consortium with higher conversion efficiency for increased hydrogen along with high-value by-products production. Additionally, the study provided the evidence of medium composition controlled metabolic pathway shift during CG bioconversion. Valorization of low cost CG with efficient co-culture of hydrogen production can aid biodiesel in-house energy source.

Keywords: Biodiesel crude glycerol, Co-culture, Hydrogen, *Enterobacter aerogenes*, *Clostridium butyricum*

Introduction

Biodiesel is renewable, healthier, environmental friendly and sustainable alternative for fossil fuels (Sarma *et al.*, 2013a). Global biodiesel market is estimated to reach 140 billion liters by 2016 and with increasing annual growth, production would reach 159 billion liters by 2020 (Nwachukwu *et al.*, 2013). Biodiesel is produced by transesterification of feedstock, such as, vegetable oil and animal fats, causing an increase in the cost of these raw materials (Ito *et al.*, 2005). To meet the increasing demand, waste generated from restaurants/meat processing industry containing cooking oil and animal fats are considered as low cost materials for biodiesel production (Sarma *et al.*, 2013b). For each ton of biodiesel produced, 100 kg of crude glycerol (CG) is generated as a by-product. CG contains large amounts of impurities at varying percentage requiring higher purification process costs and often renders unprofitable situation for small- and medium-scale biodiesel plants (Chatzifragkou *et al.*, 2012; Varrone *et al.*, 2012). CG being a good source of carbon valorization of this by-product into fermentative hydrogen production seems to be a very promising alternative strategy (Varrone *et al.*, 2012).

Commercial hydrogen production through electrochemical and thermochemical are dependent on fossil fuels, emitting carbon dioxide, causing severe ecological and environmental effects globally (Bundhoo *et al.*, 2015, Pachapur *et al.*, 2015c, Sekoai *et al.*, 2015). Renewable biohydrogen production has attracted global attention as stated by International Energy Agency (Sekoai *et al.*, 2015). Hydrogen production by dark fermentation uses simple set-up, diverse substrates, requires lower energy with higher production rate in comparison to complex set-up, light consuming photo-fermentation requiring organic acids (Gundogdu *et al.*, 2013; Pachapur *et al.*, 2015a; Sekoai *et al.*, 2015). The process cost of dark fermentation is minimum by using abundant, low-cost and renewable organic wastes (Sekoai *et al.*, 2015)

Hydrogen production by dark fermentation using crude glycerol is found to be more suitable than any other valuable products, such as 1,3-propanediol (1,3-PD), ethanol, among others (Sarma *et al.*, 2012). Hydrogen gets easily separated from the media but

recovery of other valuable products will be an expensive step due to the presence of various impurities in CG (Sarma *et al.*, 2013b). Hydrogen can be used as fuel with higher energy content (142.3 kJ/g) with no harmful emissions during combustion and reduction in GHG (greenhouse gas) emissions (Sarma *et al.*, 2013a; Seifert *et al.*, 2009).

Extensive investigation on large group of pure hydrogen-producing bacteria has been carried out since hydrogen is considered as future clean source of energy. The most commonly used organisms were of genus *Enterobacter* and *Clostridium* sp. with hydrogen yield of 1 and 2 mol H_2/mol of glucose in mono-culture system (Yokoi et *al.*, 1998). In general, strict anaerobic bacteria, such as *Clostridium* can theoretically have higher hydrogen yield than facultative anaerobes. However, anaerobic conditions should be maintained to grow them either by nitrogen sparging of the medium or by addition of expensive reducing agents (Yokoi et *al.*, 1998). Alternatively, hydrogen production by co-culture of strict and facultative anaerobic bacteria can be considered to avoid the addition of the reducing agents (Phowan *et al.*, 2010).

Hydrogen production carried out using co-culture with defined microorganisms are more practical and robust than those using mono- and mixed-culture (undefined microorganisms) system (Pachapur *et al.*, 2015c). Constructing co-culture of defined H_2 producers offer better performance, utilize the synergy among the microorganisms involved and overcome poor H_2 yield (Zeidan *et al.*, 2010). Co-culture system of defined microorganisms minimizes the additional pre-treatment steps, which are necessary for substrate hydrolysis in case of mono-culture and also suppress hydrogen consumers in case of mixed culture system (Bundhoo *et al.*, 2015; Pachapur *et al.*, 2015c).

The studies dealing with *E. aerogenes* and *C. butyricum* in a co-culture system have been very scarce using starch, sweet potato starch residue (Yokoi et *al.*, 2002; Yokoi *et al.*, 2001; Yokoi *et al.*, 1998) and hydrolyzed cassava pulp as substrate (Phowan *et al.*, 2010). According to Sarma *et al*, *E. aerogenes* performed hydrogen production ability using crude glycerol as sole substrate in the absence of additional media supplements (Sarma *et al.*, 2013b). This approach of hydrogen production using co-culture system

without pre-treatment of substrate and with no addition of expensive media supplements or reducing agent could represent a cost-effective process. In this study, the *in-situ* operating parameters (media components, buffering agent, reducing agent and different inoculum ratio) were tested for increased hydrogen production using co-culture system with crude glycerol as the sole carbon source.

Materials and Methods

Microorganisms, pre-culture media and inoculum development

Enterobacter aerogenes NRRL B-407 (facultative anaerobe) and *Clostridium butyricum* NRRL B-41122 (strict anaerobe) considered in this study are collected from ARS, USDA, USA. *E. aerogenes* was precultured at 30 ºC in a basal synthetic medium consisting (w/v) of 1% glucose, 2.0% casein polypeptone, 0.2% KH_2PO_4, 0.05% yeast extract and 0.05% $MgSO_4.7H_2O$ maintained anaerobically at pH 6.5. *C. butyricum* was precultured at 36 ºC in a modified basal medium supplemented with 0.1% L-cysteine-$HCl.H_2O$ as a reducing agent (Yokoi et *al.*, 1998). Each of the media components was mixed in distilled water; initial pH was adjusted to 6.5 by NaOH and final volume of 47.5 mL was transferred to 125 mL serum bottles. The basal medium was used to grow and maintain the microorganisms in serum bottles with working volume of 50 mL. Prior to sterilization by autoclave (Tuttnauer 3870-Heidolph), the medium was sparged with pure N_2 gas for 3 min to create anaerobic conditions. The log phase culture broth was used as inoculum (5% *v/v*) throughout the study and cultures were incubated in orbital incubator shaker (INFORS HT–multitron standard) maintained at 150 rpm (Sarma *et al.*, 2013b).

Source of substrate, chemicals and reagents

Crude glycerol was supplied by Rothsay®, Canada, recycles inedible by-products (fat-containing waste) from meat processing plants and used grease from restaurants for biodiesel production (www.rothsaybiodiesel.ca). It contained up to 23.6% of glycerol, with carbon and nitrogen content around 35.9% and 3.2% (Sarma *et al.*, 2013b). Chemicals, such as glucose, $MgSO_4.7H_2O$, NaOH, casein peptone, L-cysteine-$HCl.H_2O$ and KH_2PO_4 were purchased from Fisher scientific, Canada and VWR, Canada along with yeast extract being a gift from Lallemand, Canada.

Characterization of crude glycerol

The ash content was analyzed according to the standard method (ISO 2098-1972) as described by Hunsom *et al* (Hunsom *et al.*, 2013). The sum of moisture and MONG (matter organic non-glycerol) measured in terms of contaminants was calculated by the difference in weights from Eq. (1) as reported by (Hansen *et al.*, 2009).

$$Contaminant(\%\, moisture + \%\, MONG)(wt.\%) = 100 - [Glycerol\ content\ (wt.\%) + Ash\ content\ (wt.\%)] \quad (1)$$

Hydrogen production in batch cultures

For hydrogen production by pure culture of *E. aerogenes* and *C. butyricum*, 2.5 mL of precultured broth was added to 47.5 mL of pre-sterilized culture media (1% (*w/v*) of crude glycerol and distilled water) in a 125 mL serum bottle for anaerobic fermentation at 36 ºC without pH control. In the case of co-culture experiments, 1:2 ratio of precultured broth of *E. aerogenes* and *C. butyricum* was used as inoculum. The experiments were carried out in triplicates. During the incubation, 1 mL of gas sample was collected every 24 h till 168 h for hydrogen analysis. At the end of fermentation 1 mL of aqueous sample was collected for glycerol and end metabolite analysis.

Effect of buffering agents on hydrogen production

To evaluate the effect of buffering agents on CG bioconversion for hydrogen production, separate set of experiments were carried out. In order to try other buffering agents, salts of succinic acid at 0.05 M was used in culture media containing only 10 g/L of crude glycerol with distilled water. Similarly, the effect of two other buffering agents (*w/v*), at 0.2 % KH_2PO_4 with 0.05 % $MgSO_4.7H_2O$ along with crude glycerol was also investigated.

Hydrogen production using modified basal media

In the present case, hydrogen production by co-culture was investigated with modified basal media, containing (*w/v*) crude glycerol (1%), casein polypeptone (2%), KH_2PO_4 (0.2%), yeast extract (0.05%) and $MgSO_4.7H_2O$ (0.05%). The media preparation, nitrogen sparging and autoclave step was carried out similar to that of inoculum

development. Co-culture at the inoculum ratio of 5% (*v/v*) at 1:2 ratio of precultured broth of *E. aerogenes* and *C. butyricum* was used.

Hydrogen production at varying inoculum ratio of co-culture

The inoculum percentage of both the cultures at varying concentrations was carried out. To determine the exact inoculum ratio for increased hydrogen production, ratios from co-culture experiments (Phowan *et al.*, 2010; Yokoi *et al.*, 1998) were considered. The modified basal media was used with ratios of *E. aerogenes* and *C. butyricum* ranging from (0.09, 0.14, 0.33, 0.50, 1.00, 2.00 and 3.00) at 5% (*v/v*) inoculum size. All the experiments across the study were carried out in triplicates, presented vales are the average of triplicates and error bars in the figures represent the standard deviation (±) values.

Analysis: Hydrogen

The gas sample composition was analyzed by gas chromatography (Varian 3800, USA) equipped with a thermal conductivity detector (TCD) fitted with a 3 m PoraPLOT Q® column (Agilent technology, USA). The carrier gas, nitrogen was used at a flow rate of 3.5 mL/min, with injector, column and detector temperature set at 100 ºC. The volume of gas produced was converted to mmol, considering the temperature and atmospheric pressure (Sarma *et al.*, 2013b).

Analysis of end-metabolites/by-products

GC-FID

The aqueous samples at the end of fermentation were centrifuged (Minispin plus, Eppendorf) at 6708 x *g* for 10 min and filtered through 0.2 µm syringe filters prior to analysis. The methanol and ethanol concentrations were verified by gas chromatography (GC) (7890B GC-Agilent, Santa Clara, CA) with a flow of 1 mL/min over the ZB-WAXplus column (30 m x 0.25 mm, 0.25 µm film thickness) using flame ionization detector (FID) detector with a helium carrier gas and a temperature profile of 150– 250 ºC at 16 min run time. Using the same sample preparation method, the concentrations of glycerol and by-products (acetic acid, butyric acid, 1,3-propanediol) were measured over GC with slight modification to original method, at a flow rate of 2

mL/min using FID detector temperature profile of 80– 240 ºC at 8.4 min run time (Heyndrickx *et al.*, 1991).

LC-MS/MS

Akin to GC sample preparation, the concentration of lactate and succinate was measured using LC-MS/MS. The instrument comprised a Thermo TSQ Quantum (International Equipment Trading Ltd, USA) equipped with Surveyor autosampler and Surveyor LC pump with an Electrospray Ionization (ESI) detector, using C18 Hypersil Gold column (100 mm x 2.1 mm, 3 µm film thickness). Operating conditions were: 20 µL injection volume, 0.3 mL/min flow rate over a runtime of 8 min using gradient of formic acid and acetonitrile buffers.

Results and Discussion

Crude glycerol characterization

The glycerol content of the CG sample was found to be around 23.63% (*w/w*), with total organic carbon content of around 35.9% (*w/w*) suggesting considerable amount of carbonaceous material other than glycerol (Sarma *et al.*, 2013b). In the present study, the ash (3.06%) and moisture content (5.75%) was relatively higher in comparison to 2% and 0.4% of vegetable feedstock derived CG (Varrone *et al.*, 2012). The amount of MONG determined using Eq. (1) was about 67.56%, suggesting the presence of varying concentration of methanol, free fatty acids, soap, fatty acid methyl esters, and glycerides altogether. The reason for increased percentage of MONG is due to the absence of costly purification step for the crude glycerol purity and recovery of methanol during biodiesel production (www.rothsaybiodiesel.ca). The final purity of glycerol depends on the feedstock used during the biodiesel production and also additional glycerol purification steps undertaken by biodiesel plants. Soon after phase separation from crude biodiesel, the glycerol purity in crude glycerol will be around 30-50%. Later after the re-neutralization and alcohol recovery step the purity of glycerol reaches 80-88%. Further, with additional vacuum distillation/ion-exchange step, the purity will reach 99% amendable for pharmaceutical application (Chatzifragkou *et al.*, 2012). The glycerol content (23.63%) in this study was lower in comparison to the ones reported in

other studies: 45% (Morsy, 2014), 69.5% (Selembo *et al.*, 2009) and 90% (Varrone *et al.*, 2012), respectively. Hence, the initial concentration of crude glycerol was set at 10 g/L in order to minimize the inhibitor effect of crude glycerol impurities at higher concentration during hydrogen production.

Hydrogen production in batch cultures using crude glycerol as only substrate

The studies carried out using co-culture and mixed culture system, along with the purpose of the study, the substrate used, and experimental details with hydrogen yield are presented in Table 3.1.1. Across the study the main purpose of co-culture system was to efficiently convert crude glycerol into value-added products, avoid use of reducing agents, increase utilization of substrate and economically produce hydrogen. In order to further reduce the cost of hydrogen production, co-culture system using crude glycerol as sole substrate in absence of media components was investigated.

The purpose of avoiding media components resulted in less hydrogen production across the co-culture system. The hydrogen production for mono-culture *E. aerogenes*, *C. butyricum* and co-culture using CG as the stand alone substrate is presented in Figure 3.2.1. The maximum hydrogen production from *E. aerogenes* was around 15.77 ± 0.24 mmol/L of medium, which was higher in comparison to *C. butyricum* (13.84 ± 0.74 mmol/L) and co-culture system (12.12 ± 0.85 mmol/L) as seen in Figure 3.2.1. In the presence of only crude glycerol, *E. aerogenes* was able to produce hydrogen reaching the maximum production. However, the same condition was not favorable for the growth of *C. butyricum* resulting in decreased hydrogen production in comparison to *E. aerogenes.* The ability of hydrogen production for mono-cultures of *C. butyricum* also reflected during co-culture system with decreased production. A possible reason could be unfavorable growth condition for the growth of *C. butyricum.* It was observed soon after the sterilization step, the media pH dropped from 6.5 to below 5.0 causing a drastic change due to absence of additional buffering agents.

The drop in pH at the beginning of the inoculum for *C. butyricum* created difficult conditions for its optimal growth, due to which there was neither growth nor increase in hydrogen production in comparison to mono-culture. *E. aerogenes* was surviving in the co-culture as *C. butyricum* hardly showed growth with H_2 production at medium pH

lower than 5.5. The ability of substrate consumption and H_2 production by *C. butyricum* decreases with low medium pH due to accumulation of organic acids (Yokoi et *al.*, 1997a). In a co-culture system of *E. aerogenes* and *C. butyricum*, the dominance of *C. butyricum* plays an important role in the utilization of substrate and induce facultative anaerobes to achieve higher production rates (Thonart *et al.*, 2010, Yokoi *et al.*, 1998). However, the fast growing *E. aerogenes* with utilization of 20-30% glycerol in the first 24 h created an acidic condition (pH 3.6-4.2) (Sarma *et al.*, 2013b) which resulted in unfavorable growth condition for *C. butyricum*. H_2 evolution rate of the co-culture was higher than that of the pure cultures at controlled pH 5.0-5.5, where *C. butyricum* played an major contribution in the hydrogen production (Yokoi et *al.*, 1998). The hydrogen produced in the co-culture system (12.12 ± 0.85 mmol/L) as represented in Figure 3.2.1, is contributed by the *E. aerogenes*. *E. aerogenes* possesses the property to grow in absence of media components and produce hydrogen at acidic pH 3.9 (Sarma *et al.*, 2013b). However, the acidic pH conditions resulted in decreased hydrogen production for *C. butyricum.* During H_2-production, organic acids produced decrease the culture pH to inhibit the bacterial H_2 production process (Yokoi et *al.*, 1997b). The end-metabolite production is accompanied by bacterial fermentation and analysis of these metabolites could be useful indicators for monitoring hydrogen production (Phowan *et al.*, 2010). The end metabolites analysis at the end of fermentation by using crude glycerol as the sole substrate is presented in Table 3.2.2.

Glycerol fermentation follows two possible pathways depending upon the bacterial type and fermentation conditions (Pachapur *et al.*, 2015a). In oxidative pathway glycerol is converted to pyruvate entering glycolysis to produce various organic acids, hydrogen, and ethanol. Across reductive pathway, glycerol is reduced by $NADH_2$-linked oxidoreductase into 1,3-PD production (Pachapur *et al.*, 2015a; Wilkens *et al.*, 2012). When crude glycerol was used as stand-alone substrate, *E. aerogenes* followed oxidative production pathway, which produced acetate around (360 ± 84 mg/L) with very low concentration ethanol (150 ± 70 mg/L). Even *C. butyricum* followed oxidative pathway, produced more ethanol (1050 ± 43 mg/L) in comparison to acetate (620 ± 42 mg/L). This was also true in case of co-culture system, followed oxidative pathway with

higher amount of ethanol (1400 ± 35 mg/L) in comparison to acetate production (360 ± 88 mg/L) as seen in Table 3.2.2. Theoretically, hydrogen yield will be maximum around (3 mol/mol of glycerol) with production of acetate (Sarma *et al.*, 2012) and ethanol formations do not yield hydrogen (Argun *et al.*, 2008). Increased ethanol formation reduces the media pH, utilize the $NADH_2$-reducing equivalents and favors solvent production affecting H_2 production (Mangayil *et al.*, 2012). In the case of crude glycerol as stand-alone substrate, ethanol production was higher (1400 mg/L), which caused decrease in the pH and lowered the hydrogen production in co-culture system. The results were in accordance with Phowan *et al*., observed reduced hydrogen yield with increased ethanol concentration at a final pH of around 4.21.

The only possibility of using CG as sole carbon source will be for *E. aerogenes* and in case of co-culture using *C. butyricum*, addition of buffering agent needs to be incorporated. Increase to higher pH before sterilization alters crude glycerol characteristics with formation of soap and decrease in hydrogen production (Sarma *et al.*, 2012). The investigation of using CG as the only carbon source along with distilled water was unfavorable due to the absence of any buffering agents in the fermentation media. Different buffering agents are commonly used in CG based fermentation medium during hydrogen production to prevent drastic change in medium pH (Sarma *et al.*, 2013b). Addition of different buffering agent was evaluated in further experiments.

Effect of buffering agents on hydrogen production

The optimal growth condition for highest hydrogen production for *E. aerogenes* and *C. butyricum* was around pH (5.5–6.5) (Yokoi et *al.*, 1998), thus salts of 0.05 M succinic acid (pK_a=5.64) having effective pH range around (5.5–6.5) was selected to maintain the culture pH during the batch fermentation. In a similar way, HEPES buffer (pK_a=7.5) was used to maintain the pH (6.8–7.5) for optimum growth of *Thermotoga neapolitana* during hydrogen production from crude glycerol (Ngo *et al.*, 2011). Hydrogen production profiles using the salts of succinic acid for both mono and co-culture experiments are presented in Figure 3.2.2.

The hydrogen production for *E. aerogenes* showed maximum production around 20.11 ± 0.54 mmol of H_2/L. The maximum production was reached at 96 h and maintained a

constant level until 144 h with no further increase. In the case of *C. butyricum*, there was lag-time of around 48 h and within the next 24 h, it reached a maximum production of 18.43 ± 0.91 mmol/L. Using a co-culture system there was decrease in the lag-time for *C. butyricum* and hydrogen production was observed within 48 h and reaching maximum of 15.68 ± 0.99 mmol/L as seen in Figure 3.2.2. The purpose of co-culture to reduce the lag-time matched the results of (Yokoi et *al.*, 1998) and (Phowan *et al.*, 2010). Nevertheless, there was no increase in hydrogen production with co-culture system in comparison to mono-culture. Use of salts of succinic acid helped in achieving the maximum hydrogen production for *E. aerogenes,* but there was no increase in the hydrogen production for co-culture system. The final pH for all the three set-ups was within (4.2– 4.7) and there was sufficient growth in the medium at the end of the fermentation (data not shown). The role of succinic acid addition either helped for the growth of biomass or deviated glycerol fermentation pathway for hydrogen production. In presence of succinic acid as buffer, microorganisms can assimilate succinic acid as carbon source, which result in increased cell growth and succinic acid production (Lee *et al.*, 2001; Soares *et al.*, 2000). The metabolite analysis in presence of succinic acid as buffer indicated both oxidative and reductive pathway for formation of metabolites (as seen in Table 3.2.2).

In case of *E. aerogenes* produced ethanol (1200 ± 17 mg/L), succinate (360 ± 27 mg/L) and also 1,3-PD (300 ± 49 mg/L). *C. butyricum* was able to produce ethanol (1010 ± 14 mg/L), succinate (430 ± 22 mg/L). However, co-culture followed the pathway behaviour similar to *E. aerogenes* producing ethanol (540 ± 67 mg/L), succinate (310 ± 38 mg/L) along with 1,3-PD (300 ± 54 mg/L) as seen in Table 3.2.2. The formation of ethanol, succinate and 1,3-PD consume NADH and decrease the production of hydrogen (Pachapur *et al.*, 2015a; Wang *et al.*, 2007).

In the presence of salts of succinate, the hydrogen production shifted from ethanol production towards succinate and 1,3-PD during co-culture system. The observation was in accordance with (Wilkens *et al.*, 2012) who reported production and accumulation of metabolites caused a shift in glycerol fermentation pathways.

The choice of buffering agents plays a very important role for co-culture system to maintain optimum condition for specific product, such as hydrogen from complex substrate crude glycerol. The main objective of carrying out co-culture study was to study the effect of additional media components along with crude glycerol on hydrogen production. To identify the media components necessary for hydrogen production, crude glycerol at 1% along with 0.2% KH_2PO_4 and 0.05% $MgSO_4.7H_2O$ at the same concentration as in the basal media was carried out. Other ingredients from the media, such as yeast and peptone were excluded. The concentration of 0.2% KH_2PO_4 and 0.05% $MgSO_4.7H_2O$ are optimum and studied across co-culture system of hydrogen production using glucose and sweet potato starch residue (Alshiyab *et al.*, 2008; Yokoi *et al.*, 2001).

The hydrogen production using these media components for both mono and co-culture experiments are presented in Figure 3.2.3. The maximum hydrogen production in presence of media components for *E. aerogenes, C. butyricum* and co-culture was around 2.9 ± 0.22, 3.2 ± 0.22 and 3.8 ± 0.39 mmol/L respectively as seen in Figure 3.2.3. The hydrogen production in presence of media components (KH_2PO_4 and $MgSO_4.7H_2O$) was the lowest. The results are in accordance to (Yokoi et *al.*, 2001), where in presence of media components (KH_2PO_4 and $MgSO_4.7H_2O$) using starch residue there was no hydrogen production. However, the pH of media after the fermentation was within the optimum growth (pH 6.0–6.3), which favored the bacterial biomass growth. The optimum growth condition in presence of media components drive the carbon flux towards biomass production, leading to poor metabolite formation and decreased hydrogen production (Nwachukwu *et al.*, 2013). The carbon was integrated into cellular mass, as 80% of glycerol was utilized and absorbance reading at 600 nm (data not shown) was visibly turbid at the end of fermentation. In case of metabolite analysis in the presence of media components (KH_2PO_4 and $MgSO_4.7H_2O$) for mono and co-culture system is presented in the Table 3.2.2. With carbon flux shifting towards more of cellular mass formation there was very poor formation of metabolite (below detection limits as seen in Table 3.2.2) across mono and co-culture system. The

residual glycerol concentration ranged from 2100 ± 424 to 3200 ± 251 mg/L, suggesting utilization of glycerol for biomass synthesis instead of metabolites.

The presence of media components acts as activator to increase enzyme activity and also as cofactors for different enzymes during hydrogen production (Alshiyab *et al.*, 2008). However, along with media components additional nitrogen source is also essential for hydrogen production (Yokoi et *al.*, 2001). The requirements of carbon, nitrogen and phosphorous are necessary in prevailing stoichiometry for hydrogen yield (Argun *et al.*, 2008). To determine the hydrogen production in presence media components, the co-culture system was cultured in modified basal medium.

Hydrogen production in batch cultures using modified basal media

To investigate the importance of media components, modified basal media with replacement of glucose with the exact percentage of crude glycerol was tested. The purpose was to determine the effect of media components, such as peptone and yeast along with KH_2PO_4 and $MgSO_4.7H_2O$ in presence of *E. aerogenes* as reducing agent. The hydrogen production in the presence and absence of reducing agent for mono- and co-culture using modified basal medium is presented in Figure 3.2.4.

The co-culture system resulted in increased hydrogen production reaching a maximum of around 19.46 ± 0.95 mmol H_2/L of medium in comparison to *E. aerogenes* (15.64 ± 0.47 mmol/L) and *C. butyricum* (17.44 ± 0.38 mmol/L) in the presence of reducing agent. *C. butyricum* was able to produce 15.60 ± 0.31 mmol/L after a lag time of 48 h in the absence of reducing agent. However, in the absence of reducing agent *E. aerogenes* produced around 17.19 ± 0.40 mmol/L as presented in Figure 3.2.4. In presence of *E. aerogenes* as reducing agent during co-culture system, *C. butyricum* produced hydrogen without any lag time. The increase in percentage of co-culture in comparison to mono-culture was around 27.02% in the case of *E. aerogenes* and 11.90% in the case of *C. butyricum,* respectively. After diluting crude glycerol with media components, rate of glycerol utilization increased reaching 85% and with addition of media components H_2 production reached maximum. The percentage increase in this study was comparable to 9% increase across sweet potato starch residue in the presence of media components carried out by Yokoi *et al* using similar cultures (Yokoi

et *al.*, 2001). *C. butyricum* was able to produce increased hydrogen in the presence of modified basal medium consisting of crude glycerol and not in its absence. With the use of media components, the optimum condition for the growth of *C. butyricum* was maintained and the media pH was stable within the optimum pH. The final pH was within pH 5.4–5.6 for both mono- and co-culture system, which is near to the optimum pH (5.2) for co-culture as observed by (Yokoi et *al.*, 1998) for increased hydrogen production.

During crude glycerol fermentation, the end metabolite concentrations are presented in the Table 3.2.2. Use of media components suggested an optimum system for hydrogen production accompanied by the production of metabolites (acetate, ethanol and 1,3-PD) (Phowan *et al.*, 2010). The metabolite profile for mono and co-culture suggest both oxidative and reductive pathway working simultaneously as presented in the Table 3.2.2. The production of acetate was higher (1120 ± 97 mg/L) along with 1,3-PD (2800 ± 106 mg/L) formation for *E. aerogenes*. The similar trend even for *C. butyricum* with acetate (830 ± 74 mg/L) and 1,3-PD (3100 ± 222 mg/L) formation was observed. In case of co-culture followed both oxidative pathway with acetate (980 ± 70 mg/L) and reductive pathway with 1,3-PD (2700 ± 78 mg/L) formation. The decreased production of 1,3-PD (2700 ± 78 mg/L) for co-culture system in comparison to monoculture (2800 ± 106 and 3100 ± 222 mg/L) resulted in increased H_2 production as seen in Table 3.2.2. Production of 1,3-PD utilizes the $NADH_2$ necessary for hydrogen production and liberates water as by-product (Pachapur *et al.*, 2015a). The optimized conditions led to efficient substrate utilization (residual glycerol <1500 mg) below detection level for both mono- and co-culture system. The maximum hydrogen production is generally associated with the production of organic acids indicating enriched microbial community producing H_2 proficiently (Mangayil *et al.*, 2012). The results of increased hydrogen with metabolite production was in accordance with (Phowan *et al.*, 2010) who carried out using cassava pulp hydrolysate in a co-culture system. Hydrogen production along with formation of ethanol (by *E. aerogenes*) and 1,3-PD (by *C. butyricum*) determined the syntrophic consortium in a co-culture system to carry out glycerol fermentation by both oxidative (acetate, ethanol, butyrate etc) and reductive pathway (1,3-PD production)

(Pachapur *et al.*, 2015a). This implied the fact that the co-culture system co-existed and thus induced the hydrogen production at the expense of media components for optimal conditions. The complete shift of glycerol fermentation from reductive to oxidative pathway will further increase the hydrogen production.

Hydrogen production at varying inoculum ratio of co-culture

E. aerogenes played the role of reducing agent by consuming oxygen to attain anaerobic conditions optimum for *C. butyricum* growth for hydrogen production. The hydrogen production depends on the syntrophic performance of one culture with the other in the co-culture system (Pachapur *et al.*, 2015). To evaluate the optimum inoculum ratio, different inoculum ratio of *E. aerogenes* and *C. butyricum* were considered with constant inoculum size of 5% (*v/v*). Considering the inoculum ratio used in the literature (Phowan *et al.*, 2010; Yokoi *et al.*, 2002; Yokoi *et al.*, 1998), with few more dominant ratios for both the cultures were planned. The hydrogen production using different inoculum ratio for the co-culture system is presented in Figure 3.2.5.

The extreme end for *E. aerogenes* with 1.88 mL at inoculum ratio of (3:1) resulted in lower hydrogen production of around 14.01 ± 0.24 mmol/L. More number of fast growing *E. aerogenes* outplayed and created difficult conditions for growth of *C. butyricum* resulting in reduced hydrogen production. The other extreme end for *C. butyricum* with 2.29 mL at inoculum ratio of (1:11) resulted in higher hydrogen production of around 19.29 ± 0.70 mmol/L as presented in Figure 3.2.5. The amount of *E. aerogenes* used in (1:11) is optimum to create an anaerobic condition resulting in favorable condition for the growth of *C. butyricum* (Yokoi *et al.*, 2002). The other ratios (0.5:1, 1:7, 1:1, 1:3, and 1:0.5) resulted in constant hydrogen production of around 17.74 to 18.41 mmol/L. Higher inoculum ratio for *C. butyricum* resulted in increased percentage (37.68%) of hydrogen production in comparison to higher inoculum ratio of *E. aerogenes*. The increase in the hydrogen production depends on the dominance of *C. butyricum* over *E. aerogenes.* Even in the case of higher ratio of *E. aerogenes*, the hydrogen production will be lower if *C. butyricum* is not predominant (Yokoi et *al.*, 2001). At higher ration of *E. aerogenes*, substrate utilization will be around 20-30% of glycerol in the first 24 h with release of metabolites causing deviation in optimum pH resulting in decreased

hydrogen production (Sarma *et al.*, 2013b; Yokoi *et al.*, 1997b). Comparing the metabolite production in the Table 3.2.2, the concentration of acetate (1100 ± 53 mg/L) was higher for the inoculum ratio (1:11) in comparison to ratio (1:1) with 980 ± 70 mg/L respectively. Thus, the inoculum ratio (1:11) was found to be optimum for the co-culture system for increased hydrogen production.

Hydrogen production across different substrates

The results of co-culture system using different substrates, considering the experimental results are presented in Table 3.2.3. The H_2 yield (mol H_2/mol substrate) represents the molar ratio of H_2 evolved for the substrate utilized during the fermentation (Yokoi et *al.*, 1998).

Yokoi *et al*. reported hydrogen yield of around 0.93 using starch, considering the highest hydrogen evolved in a batch experiment at around 525 mL (Yokoi et *al.*, 1998). In the case of Phowan *et al*., the yield was around 0.46 with highest H_2 evolved of around 428 mL for 5.64 g/L of substrate utilization (Phowan *et al.*, 2010). In this study, the highest hydrogen evolved was around 512 mL from 8.50 g/L of glycerol utilization resulting in 0.95 hydrogen yield. The hydrogen yield obtained in this study are among the best reported so far with co-culture system in comparison to mixed-culture yield of 0.96 (Varrone *et al.*, 2012). The modified basal media in presence of pure glycerol was also investigated using co-culture system for hydrogen production. In presence of pure glycerol with 99% purity, hydrogen production resulted with 14.43 ± 0.43 mmol/L in comparison to 19.46 ± 0.95 mmol/L using crude glycerol. The presence of MONG in crude glycerol contributes to increased production in comparison to pure glycerol (Sarma *et al.*, 2012). However in case of apple pomace hydrolysate and pure glucose the hydrogen production was higher around 19.33 ± 0.78 to 29.13 ± 0.56 mmol/L. The apple pomace hydrolysate was obtained from acid hydrolysis of apple pomace at 121 °C for 20 min in autoclave with a mass ratio of solid (g dry weight) to liquid (mL) at 2:15 using 10 N H_2SO_4 at 0.5% (w/v) (Pachapur *et al.*, 2015b). The co-culture system possesses advantages over pretreatment methods for complex substrates and enriching activity sludge inoculum for mixed culture (Pachapur *et al.*, 2015). However, using different substrates across single co-culture system platform, the hydrogen

production for crude glycerol was higher. Given the highly reductive nature of glycerol, hydrogen production at higher yields from crude glycerol is possible in comparison to common sugars, such as glucose and xylose (Murarka *et al.*, 2008).

The analysis of the spent media for the end metabolite using GC and LC-MS analysis provided vast amount of information over glycerol fermentation. The summary of the metabolite production under different experimental conditions resulting in various metabolite productions is represented in Figure 3.2.6. In the case of crude glycerol as stand-alone substrate during glycerol fermentation resulted in production of acetate and ethanol. The route of glycerol fermentation followed the oxidative pathway with production of acetate. However, at these condition there was no reductive pathway of glycerol fermentation resulting in no production of 1,3-PD. In the presence of salts of succinic acid, the glycerol fermentation followed both oxidative and reductive pathway with production of ethanol and 1,3-PD as presented in Figure 3.2.6. Presence of media components (KH_2PO_4 and $MgSO_4.7H_2O$) resulted in the cellular mass formation along with butyric acid production. The presence of media components (peptone, yeast, KH_2PO_4 and $MgSO_4.7H_2O$) along with crude glycerol resulted in the production of more value added products, such as 1,3-PD, ethanol, acetic acid and lactate. Presence of media components suggested that the selected functional consortium mainly followed hydrogen and metabolite type fermentation. The production of acetic acid and ethanol generated higher ATP for the cells, which meant a higher thermodynamic efficiency and competitive advantage of product formations derived glycerol fermentation (Varrone *et al.*, 2012). The production of these value-added products can be achieved using crude glycerol instead of pure glycerol, resulting in 50% reduction in the price of these products (Amaral *et al.*, 2009). The spent media after hydrogen production using crude glycerol is evaluated as potential substitute for fine chemicals, biomaterials and phosphate solubilizing bio-fertilizers (Sarma *et al.*, 2015). Therefore, crude glycerol would be a good alternative to low cost substrates to produce hydrogen and potential in-house energy source for biodiesel industry in future.

E. aerogenes growth requirement is very simple and can grow in presence of only crude glycerol as carbon source during H_2 production (Sarma *et al.*, 2013b).

However, on other hand *C. butyricum* growth requirement is complex and needs the presence of minimal media components to utilize crude glycerol as carbon source (peptone, yeast extract, KH_2PO_4 and $MgSO_4.7H_2O$). The presence of media components (peptone and yeast extract) provide optimum growth for the co-culture system and also nullify the substrate inhibitor concentration during hydrogen production. On other hand presence of media components (KH_2PO_4 and $MgSO_4.7H_2O$) regulate the fermentation pH during release of acids/solvents and provide the nutrient value for co-culture system during hydrogen production. In case of culturing well known H_2-producing bacteria (*E. aerogenes* and *C. butyricum*) in a co-culture system using crude glycerol as sole substrate, presence of minimal media components is necessary for increased hydrogen production. However, using *E. aerogenes* along with *C. butyricum* performs the role of reducing agents in a co-culture system and avoids the use of expensive reducing agent during hydrogen production. Using *E. aerogenes* and *C. butyricum* in combination not only decreased the media cost, but also utilized biodiesel waste crude glycerol without any additional pretreatment steps to produce renewable energy source hydrogen.

Conclusions

Crude glycerol, is abundant in renewable biomass and promising raw material and not been investigated using the co-culture for hydrogen production. The functional characteristics and abilities of pure strains in a microbial consortium need to possess conditions for higher conversion efficiency of crude glycerol. During crude glycerol fermentation, in comparison to mono-culture of 15.64 ± 0.47 (EA) and 17.44 ± 0.38 mmol-H_2/L (CB) as high as 19.46 ± 0.95 mmol-H_2/L has been obtained by proposed co-culture system. The hydrogen yield was around 0.95 mmol-H_2/mol of crude glycerol was comparable to the other co-culture studies using the same combination of pure cultures over starch and cassava pulp hydrolysate. Each of the media components played important role to produce higher hydrogen yield for co-culture system in comparison to mono-culture. The complete shift of glycerol fermentation from reductive to oxidative pathway with decreased 1,3-PD production will further increase the hydrogen yield. Along with hydrogen, the production of high value-added products has several

advantages with increasing industrial applications. The co-culture system helped in reducing the overall process cost with hydrogen production in the absence of pretreatment, expensive reducing agent and thus providing process stability with reduction of lag-time for hydrogen production. Therefore, the co-culture of *E. aerogenes* and *C. butyricum* will be an effective combination for hydrogen production from crude glycerol. Valorization of crude glycerol as low cost substrate with efficient co-culture system of hydrogen production can aid in-house energy source for biodiesel industry in future.

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Table 3.2.1: Literature studies carried out for hydrogen production using co-culture and mixed culture system

Microorganisms	Co-culture approach	Source	Operating conditions	Process	H_2 yield/production	Ref.
E. aerogenes HO-39, *C. butyricum* IFO13949	Anaerobic condition and avoid use of reducing agents	Starch	200 mL working volume with starch 1% in synthetic media, initial pH 6.5, 4+8 mL inoculum at 36 °C	Co-immobilized cells	2.6 mol/mol of glucose	(Yokoi *et al.*, 1998)
E. aerogenes HO-39, *C. butyricum* IFO13949[a]	Increased utilization of starch and avoid use of reducing agents	Sweet potato starch residue	200 mL working volume with 0.5-2% starch residue and media components, 1+11 mL inoculum with controlled pH 5.25 at 37 °C	Mono-culture [a]	2.2 mol/mol of glucose	(Yokoi *et al.*, 2001)
				Co-culture	2.4 mol/mol of glucose	
E. aerogenes HO-39, *C. butyricum* IFO13949	Use of organic waste rich in nitrogen source for effective and economical H_2 production	Sweet potato starch residue	200 mL working volume with 1% starch residue and media components and nitrogen source, 1+11 mL inoculum with controlled pH 5.25 at 37 °C	Co-culture	2.7 mol/mol of glucose	(Yokoi *et al.*, 2002)
E. aerogenes TISTR 1468, *C. butyricum* TISTR 1032	Anaerobic condition and avoid use of reducing agents	Cassava pulp hydrolysate	100 mL working volume with glucose 25 g COD/L and media component, with 2+4 mL inoculum at initial pH 5.5 at 36 °C	Co-culture	345.8 mL H_2/g COD	(Phowan *et al.*, 2010)
E. aerogenes NRRL B-407, *C. butyricum* NRRL B-41122	Avoid nitrogen sparging step	Crude glycerol	50 mL working volume with 1% crude glycerol and media component, initial pH 6.5, 1:1 inoculum ratio at 36 °C with gas release set-up	Co-culture	1.5 mol H_2/mol of glycerol	(Pachapur *et al.*, 2015a)

Microorganisms	**Co-culture approach**	**Source**	**Operating conditions**	**Process**	**H_2 yield/production**	**Ref.**
E. aerogenes NRRL B-407, *C. butyricum* NRRL B-41122	Addition of co-substrate for hydrogen production	Crude glycerol and apple pomace hydrolysate	50 mL working volume with 15 g/L crude glycerol and media component, initial pH 6.5, 15% Inoculum size, 1:1 inoculum ratio at 36 °C with gas release set-up	Co-culture	1.7 mol H_2/mol of glycerol	(Pachapur *et al.*, 2015b)
Mixed culture	Conversion of glycerol into high value products	Crude glycerol	250 mL working volume with 0.3% glycerol and media components, 0.1% inoculum size with initial pH 6.2 at 30 °C	Mixed-culture	0.31 mol/mol of glycerol	(Selembo *et al.*, 2009)
Mixed culture	H_2 generation with digester sludge	Pure[b] and crude glycerol[c]	30 mL working volume with 1% glycerol and media components, 1.16 g VSS/L inoculum amount with initial pH 6.0 at 37 °C	Mixed-culture	0.41[b] and 0.71[c] mol/mol of glycerol	(Seifert *et al.*, 2009)
Mixed culture	H_2 generation with upflow anaerobic sludge	Crude glycerol	30 mL working volume with 3% glycerol and media components and trace elements, 10% inoculum size with initial pH 7.0 at 36 °C	Mixed-culture	1.2 mol/mol of glycerol	(Rossi *et al.*, 2011)
Mixed culture	Efficiently convert crude glycerol into H_2	Crude glycerol	45 mL working volume with 1.5% glycerol only, 10% inoculum size, initial pH 7.9, 5 mL inoculum at 37 °C	Mixed-culture	0.96 mol/mol of glycerol	(Varrone *et al.*, 2012)
Mixed culture	Conversion of glycerol into	Crude glycerol	250 mL working volume with 1% glycerol and	Mixed-culture	1.1 mol/mol of glycerol	(Mangayil *et al.*, 2012)

Microorganisms	**Co-culture approach**	**Source**	**Operating conditions**	**Process**	**H_2 yield/production**	**Ref.**
	high value products		media components, 10% inoculum size with initial pH 6.5 at 37 °C			
E. aerogenes NRRL B-407, *C. butyricum* NRRL B-41122	Avoid pre-treatment and use of expensive reducing agents	Crude glycerol	50 mL working volume with crude glycerol 1% and media component, initial pH 6.5, 1:11 inoculum ratio at 36 °C	Mono-culture	15.64 ± 0.47 mmol H_2/L	This study
				Mono-culture	17.44 ± 0.38 mmol H_2/L	
				Co-culture	19.46 ± 0.95 mmol H_2/L	

Table 3.2.2: End metabolite production along with glycerol concentration in mg/L across 1% (w/v) concentration of crude glycerol used for the mono culture and co- culture experiments.

Experimental conditions	**Microorganisms used**	**End metabolites (mg/L)**								
		Glycerol	**Acetic acid**	**Butyric acid**	**1,3-propandiol**	**Methanol**	**Ethanol**	**Lactate**	**Succinate**	**Final pH**
Using only Crude glycerol	*E. aerogenes*	3400 ± 353	360 ± 84	<150	<150	<50	150 ± 70	88 ± 22	16 ± 16	3.6 ± 0.06
	C. butyricum	2100 ± 494	620 ± 42	<150	<150	87 ± 26	1050 ± 43	170 ± 17	<4	3.8 ± 0.04
	Co-culture	5500 ± 424	360 ± 88	<150	<150	<50	1400 ± 35	210 ± 14	98 ± 19	4.2 ± 0.03
Using salts of succinic acid	*E. aerogenes*	3900 ± 459	300 ± 62	<150	300 ± 49	<50	1200 ± 17	140 ± 19	360 ± 27	4.2 ± 0.06
	C. butyricum	5100 ± 141	330 ± 60	<150	<150	<50	1010 ± 14	270 ± 20	430 ± 22	4.4 ± 0.05
	Co-culture	4300 ± 282	<100	230 ± 28	300 ± 54	<50	540 ± 67	< 20	310 ± 38	4.7 ± 0.05
Using salts of buffering agent	*E. aerogenes*	3200 ± 251	<100	160 ± 17	<150	<50	<50	< 20	< 4	6.3 ± 0.05
	C. butyricum	2100 ± 424	<100	190 ± 28	<150	<50	100 ± 70	< 20	< 4	6.0 ± 0.06
	Co-culture	2300 ± 415	<100	270 ± 50	<150	<50	95 ± 70	< 20	< 4	6.2 ± 0.08
Using media component	*E. aerogenes*	<1500	1120 ± 97	<150	2800 ± 106	<50	700 ± 106	570 ± 20	190 ± 24	5.6 ± 0.06
	C. butyricum	<1500	830 ± 74	<150	3100 ± 222	<50	485 ± 81	530 ± 24	390 ± 16	5.6 ± 0.06

	Co-culture (1:1)	<1500	980 ± 70	<150	2700 ± 78	<50	830 ± 70	470 ± 13	370 ± 12	5.4 ± 0.06
	Co-culture (1:11)	<1500	1100 ± 53	<150	3000 ± 77	<50	960 ± 63	730 ± 17	500 ± 62	5.4 ± 0.06

Table 3.2.3: Hydrogen production across the studies using co-culture system with details on the pretreatment steps, source content, microorganism used and hydrogen yield.

Pretreatment step	**Source content**	**Microorganisms details**	**Process**	**H_2 production mmol/L of medium**	**H_2 yield (mmol/mol of substrate)**	**Ref.**
No treatment step	Commercial starch	*E. aerogenes* HO-39 *,* *C. butyricum* IFO13949	Co-culture	20.7	0.93	(Yokoi et *al.*, 1998)
Acid hydrolysis step	Cassava Pulp Hydrolysate: 80.2% moisture, 66.4% starch, 2.1% protein, 28.8% fiber, 0.2% fat and 2.5% ash	*E. aerogenes* TISTR 1468, *C. butyricum* TISTR 1032	Co-culture	18.06	0.46	(Phowan *et al.*, 2010)
No treatment step	Crude Glycerol: 23.63% glycerol, 3.06% ash, 5.75%, moisture and 67.56% MONG	*E. aerogenes* NRRL B-407[a], *C. butyricum* NRRL B-41122[b]	Mono-culture [a]	15.64 ± 0.47	0.82	This study
			Mono-culture [b]	17.44 ± 0.38	0.83	
			Co-culture	20.19 ± 0.48	0.95	

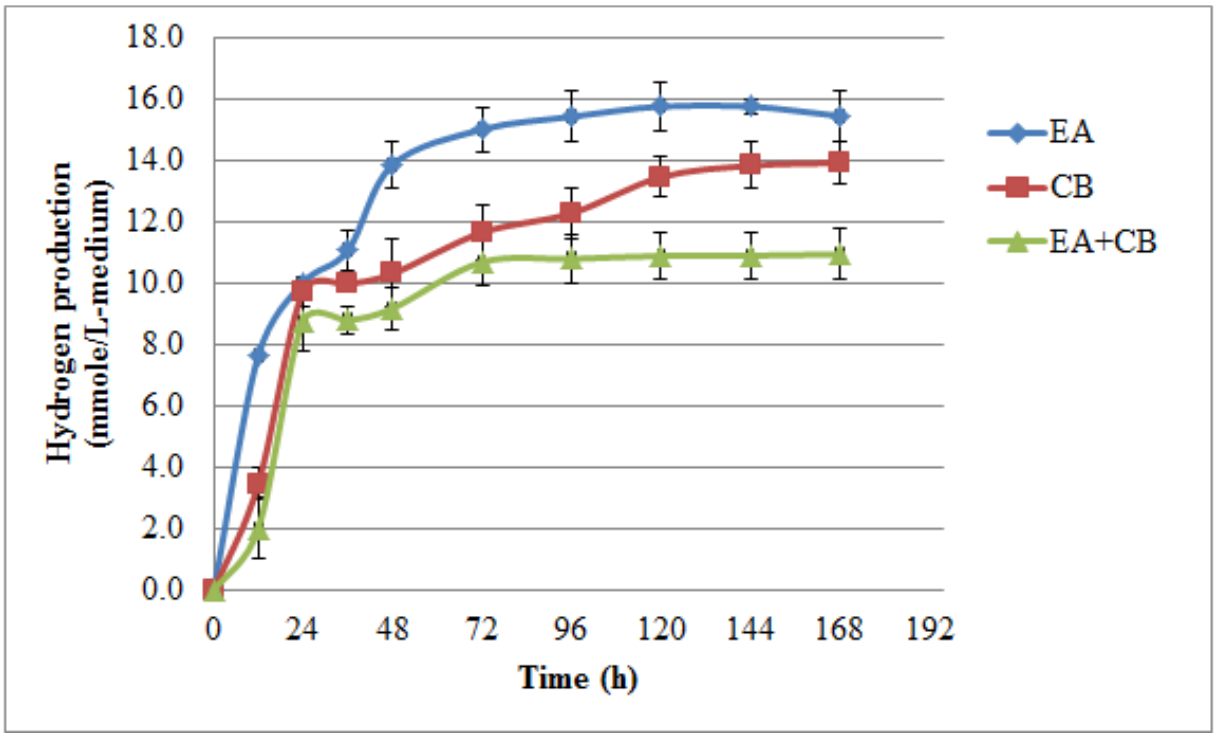

Figure 3.2.1: Hydrogen production in mmol/L of medium across 1% (w/v) concentration of crude glycerol as stand-alone substrate for the mono culture (EA) and (CB) and co-culture (EA+CB) experiments. EA: *Enterobacter aerogenes,* CB: *Clostridium butyricum.*

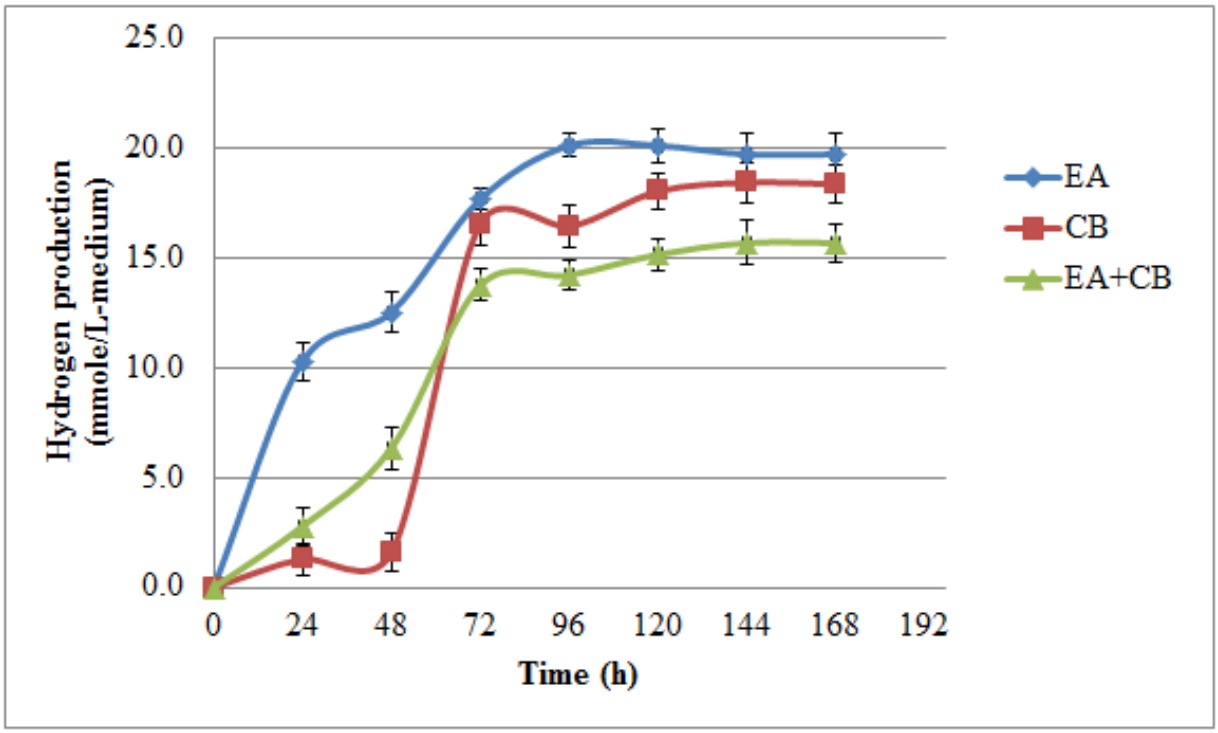

Figure 3.2.2: Hydrogen production in (mmol/L of medium) for 1% (w/v) of crude glycerol substrate for the mono culture (EA) and (CB) and co- culture (EA+CB) experiments using salt of succinic acid. EA: *Enterobacter aerogenes,* CB: *Clostridium butyricum.*

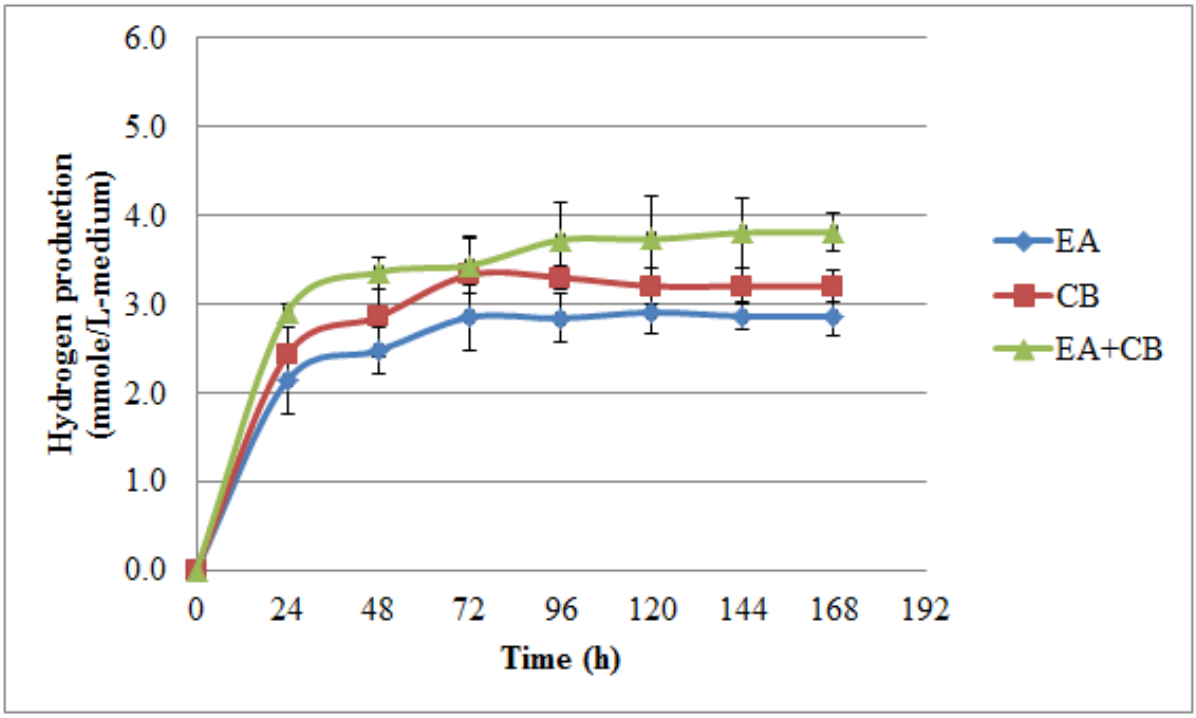

Figure 3.2.3: Hydrogen production in (mmol/L of medium) across 1% (w/v) concentration of crude glycerol substrate for the mono culture (EA) and (CB) and co- culture (EA+CB) experiments using buffering agent (KH_2PO_4 and $MgSO_4.7H_2O$). EA: *Enterobacter aerogenes,* CB: *Clostridium butyricum.*

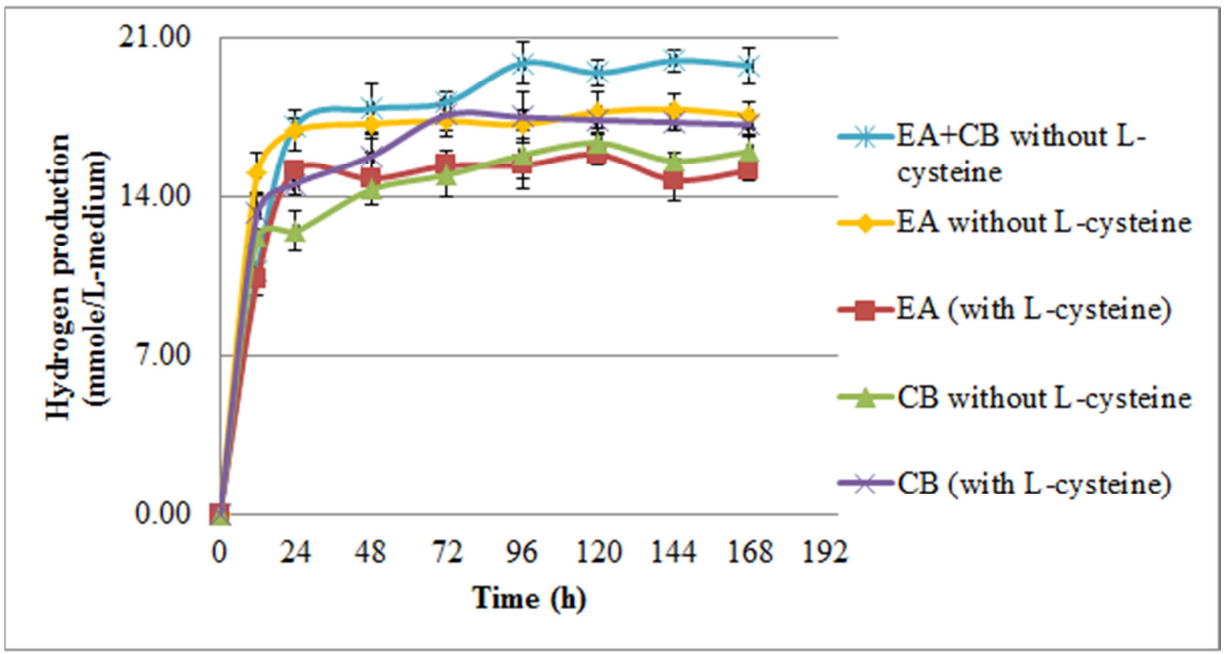

Figure 3.2.4: Hydrogen production in (mmol/L of medium) for 1% (w/v) of crude glycerol substrate with modified basal media for the mono culture (EA) and (CB) and co- culture (EA+CB) experiments in the presence and absence of reducing agent (L-cysteine). EA: *Enterobacter aerogenes*, CB: *Clostridium butyricum*.

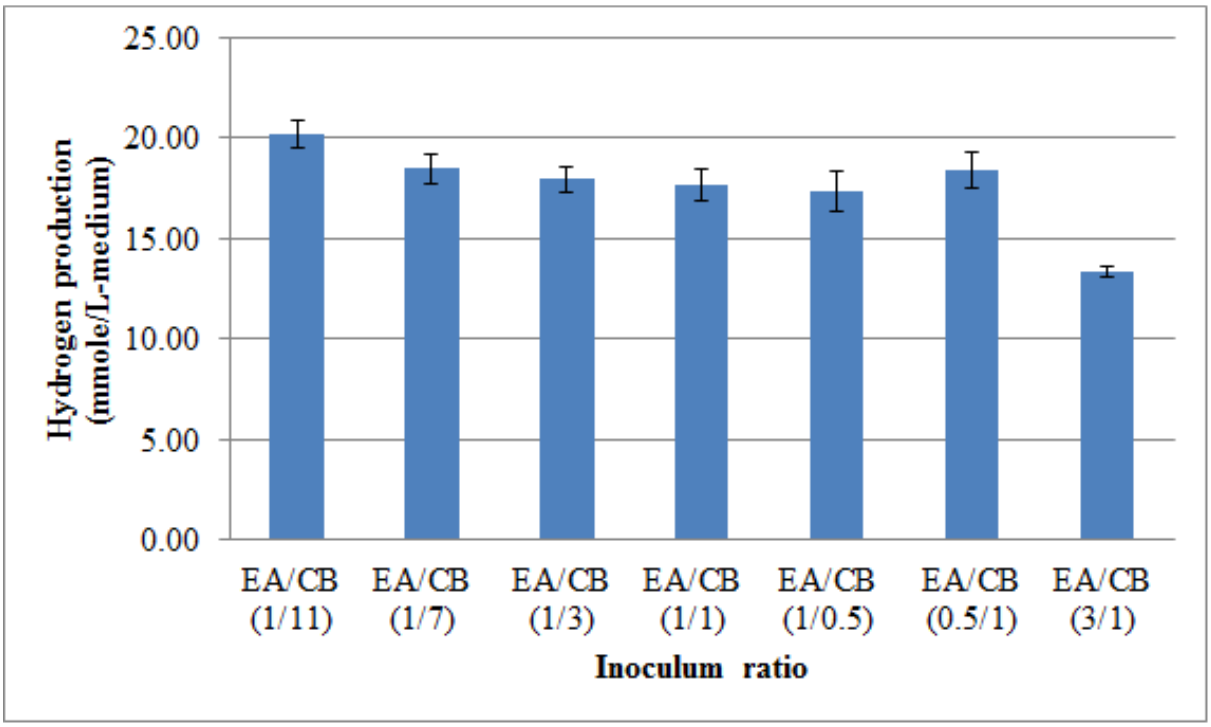

Figure 3.2.5: Hydrogen production averaged for 96-120 h in (mmol/L of medium) for 1% (w/v) crude glycerol substrate with media components for the co-culture experiment at different inoculum ratios of (EA) and (CB). EA: *Enterobacter aerogenes,* CB: *Clostridium butyricum.*

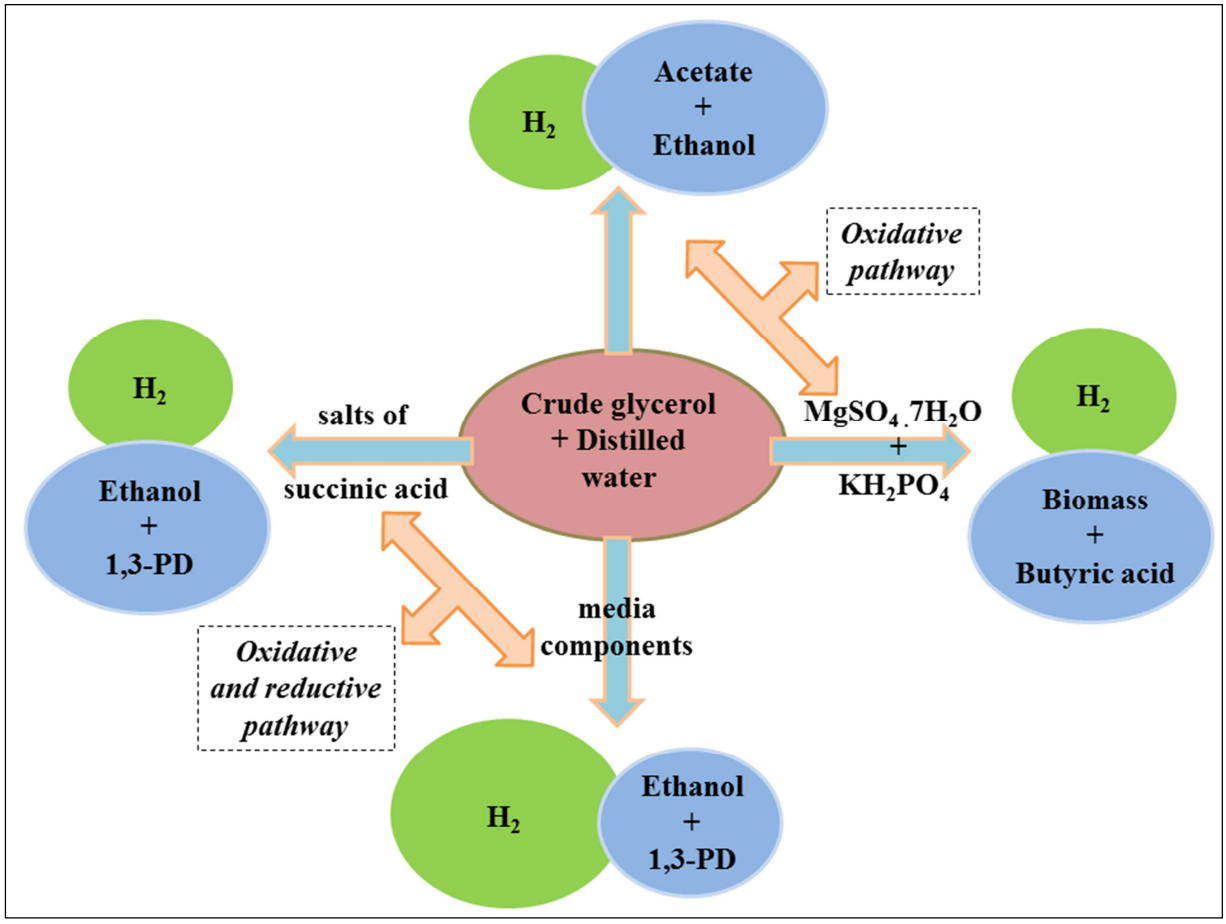

Figure 3.2.6: Effect of media components (synthetic) on crude glycerol fermentation into hydrogen production along with production of valuable end metabolites.

CHAPTER 4

HYDROGEN PRODUCTION BY CO-CULTURE SYSTEM WITH MULTIPLE SUBSTRATE

PART 1

BIOHYDROGEN PRODUCTION BY CO-FERMENTATION OF CRUDE GLYCEROL AND APPLE POMACE HYDROLYSATE USING CO-CULTURE OF *ENTEROBACTER AEROGENES* AND *CLOSTRIDIUM BUTYRICUM*

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Résumé

L'utilisation de co-substrat provenant de divers déchets présentant des caractéristiques complémentaires peut fournir un support complet pour une plus grande production d'hydrogène. Cette étude a permis d'évaluer le potentiel de l'hydrolysat de marc de pomme (APH) co-fermenté avec du glycérol brut (GB) pour augmenter la production de H_2 et une diminution des sous-produits formés. L'utilisation d'un plan experimental avec design central composante (CCD) et une méthodologie surface de réponse de (RSM) a été utilisée comme outil d'optimisation et 15 g/L de GB, 5 g/L d'APH et 15% (v/v) d'inoculum ont été definies comme étant les conditions optimales. Dans ces conditions la production en H_2 a été aussi haute que 26,07 ± 1,57 mmol H_2/L de milieu. La valeur *p* de 0,0017 a indiqué que l'APH à faible concentration a eu un effet significatif sur la production de H_2. Le GB utilisé comme unique source de carbone, favorise le métabolisme de la voie réductrice du glycérol et permet une production de 19,46 mmol H_2/L. Cependant, avec l'APH, la voie oxydative a été favorisée par une plus grande production de H_2 (26,01 mmol/L) et la diminution de la formation des sous-produits réduits tels que (1,3-propanediol et de l'éthanol). L'APH augmente la production de H_2, et diminue l'inhibition du substrat.

Mots clés: Hydrolysat de marc de pomme, Biohydrogen, Co-culture, Co-substrat, Glycérol brut

Abstract

Co-substrate utilization of various wastes with complementary characteristics can provide a complete medium for higher hydrogen production. This study evaluated potential of apple pomace hydrolysate (APH) co-fermented with crude glycerol (CG) for increased H_2 production and decreased by-products formation. The central composite design (CCD) along with response surface methodology (RSM) was used as tool for optimization and 15 g/L of CG, 5 g/L of APH and 15% (v/v) inoculum were found to be optimum to produce as high as 26.07 ± 1.57 mmol H_2/L of medium. The p-value of 0.0017 indicated that APH at lower concentration had a significant effect on H_2 production. By using CG as sole carbon source, reductive pathway of glycerol metabolism was favored with 19.46 mmol H_2/L. However, with APH, oxidative pathway was favored with higher H_2 production (26.07 ± 1.57 mmol/L) and decrease in reduced by-products (1,3-propanediol and ethanol) formation. APH inclusion enhanced H_2 production, and decreased substrate inhibition.

Keywords: Apple pomace hydrolysate, Biohydrogen, Co-culture, Co-substrate, Crude glycerol

Introduction

With high energy content, hydrogen is already a fuel of choice and guarantees environmentally clean transport in the future. Biological hydrogen production through dark fermentation is less expensive, economically friendly, coupling energy production with waste treatment and is one of the sustainable methods of hydrogen production (Gilroyed *et al.*, 2010; Pachapur *et al.*, 2015b).

Pure glucose and sucrose use as substrates of dark fermentation are not economically feasible and currently, carbon rich wastes have been explored as suitable substrates. Co-substrate utilization of different wastes with complementary characteristics can provide a complete medium for hydrogen production (Lateef *et al.*, 2012). Different co-substrate utilization studies for hydrogen production have been presented in Table 4.1.1. Based on substrate selection, co-substrate fermentation may help in maintaining recommended pH levels to avoid the need for external alkali (Tenca *et al.*, 2011), as a nitrogen source (Yokoi *et al.*, 2002), as inoculum seed to produce hydrogen (Perera *et al.*, 2011), as a co-substrate for effective management of the process (Marone *et al.*, 2015) altogether to improve net H_2 yield. However, to date, there is no report on H_2 production from crude glycerol with apple pomace hydrolysate (apple industry waste) as co-substrate.

In 2012, Canada had produced 190 million liter of biodiesel to substitute 5% of the diesel, which generated more than $1.1 billion farm income with around 19 million liter of crude glycerol as waste (Sarma *et al.*, 2013a) (www.eia.gov, 2015; www.greenfuels.org, 2015). In EU, crude glycerol is classified as hazardous waste, and its refining is no longer a cost-effective process (Pott *et al.*, 2014). Exploitation of crude glycerol as a cost-effective feedstock in dark fermentation for hydrogen production is the best alternative thereby decreasing the cost of waste disposal (Sarma *et al.*, 2012).

In 2013, out of world total apple production (80×10^6 tons), Canada had contributed around 0.3×10^6 tons (www.faostat3.fao.org, 2015). The processing of apples leads to production of tons of apple pomace (AP) (25-30%) and apple pomace sludge (AS) as waste every year. Apple processing industries are subjected to additional losses for the

treatment of waste and disposal into landfills, a practice that is environmentally questionable (Dhillon *et al.*, 2011a). The composition of AP with total carbon of around 128 g/L (Dhillon *et al.*, 2011b) can be lucrative fermentable substrate for hydrogen production, which will be economically sound and environmentally beneficial alternative for apple processing industries.

Theoretically, glycerol is an interesting substrate for H_2 production (Heyndrickx *et al.*, 1991) but for 50 years, glycerol is fermented by anaerobic bacteria to 1,3-propanediol (1,3-PD), ethanol, acetic acid and lactic acid (Biebl *et al.*, 1992). The higher reduction state of glycerol needs excess of reducing equivalents, which can be accomplished by diverting NADH consuming pathway towards reduced or neutral end-products (ethanol or 1,3-PD) or via H_2 production (Heyndrickx *et al.*, 1991). Research carried out using the *Enterobacter* and *Clostridium* with crude glycerol as substrate either produced ethanol (Ito *et al.*, 2005; Jitrwung *et al.*, 2011; RES Nwachukwu *et al.*, 2012; R. E. Nwachukwu *et al.*, 2013) or 1,3-propanediol (Chatzifragkou *et al.*, 2011; Gonzalez-Pajuelo *et al.*, 2004; Gonzalez-Pajuelo *et al.*, 2005; Szymanowska-Powałowska, 2014; Zeng, 1996), which needs to be produced at higher concentration at the expense of media cost (Jitrwung *et al.*, 2011). Additionally a co-culture of facultative (*E. aerogenes*) and strict anaerobe (*C. butyricum*) can ensure high H_2 yield by using glucose as carbon source in the absence of expensive reducing agent (Phowan *et al.*, 2010; Yokoi *et al.*, 1998).

Till date, only few studies have reported H_2 production by co-culture using starch/glucose as substrate and process parameters, such as pH and media supplements have been optimized (Phowan *et al.*, 2010, Yokoi *et al.*, 2002). As seen from Table 4.1.1, studies dealing with co-substrate using crude glycerol are very scarce; they are focused mainly on the use of pure synthetic substrate, with no statistical optimization of the fermentation of crude glycerol using co-culture. The optimization of substrate and co-substrate concentration along with inoculum size are of primary importance for bioprocess development during hydrogen production (Pan *et al.*, 2008).

Heyndrickx *et al*. (1991), indicated that glycerol was an excellent substrate for 1,3-PD production using *Clostridium* species. The purpose of this study was to evaluate the

hydrogen production potential of crude glycerol when co-fermented with apple pomace hydrolysate at different inoculum size and to increase substrate utilization rate with reduced 1,3-PD and ethanol formation. Since CG can cause 66% of substrate inhibition (Szymanowska-Powałowska, 2014; Viana *et al.*, 2012), the concept of co-substrate addition deals with such inhibition and will allow the process to run at higher substrate concentration. An integrated co-substrate technique opens up opportunity in coming years to use mixtures of various kinds of organic wastes to increase the H_2 production.

Materials and Methods

Microorganisms, pre-culture media and inoculum development

Microorganisms (*Enterobacter aerogenes* NRRL B-407 and *Clostridium butyricum* NRRL B-41122) used in this study were procured from ARS, USDA, USA. *E. aerogenes* was pre-cultured anaerobically in basal synthetic medium consisting of glucose (10 g/L), casein polypeptone (20 g/L), KH_2PO_4 (2 g/L), yeast extract (0.5 g/L) and $MgSO_4.7H_2O$ (0.5 g/L) at 30 ºC. *C. butyricum* was pre-cultured anaerobically in modified basal medium supplemented with L-cysteine-$HCl.H_2O$ (1 g/L) as a reducing agent at 36 ºC (Yokoi et *al.*, 1998). In distilled water, different media components were mixed; initial pH was adjusted to 6.5 by using NaOH and final media volume of 47.5 mL was transferred into 125 mL serum bottles. To create anaerobic conditions, the media was sparged with pure N_2 gas for 5 min and later sealed using aluminum crimp seals with pre-inserted septa (Pachapur *et al.*, 2015a). The sterilization was carried out using autoclave (Tuttnauer 3870-Heidolph) at 121 °C for 20 min. Logarithmic phase culture broth was used as inoculum at 5% (v/v) and it was transferred using sterile syringe and the cultures were incubated at 150 rpm in an orbital incubator shaker (INFORS HT – multitron standard) (Pachapur *et al.*, 2015a).

Source of substrate, chemicals and reagents

Crude glycerol was supplied by Rothsay®, Canada. CG contained (w/w): glycerol (23.6%), carbon (35.9%), nitrogen (3.2%), ash (3.06%) moisture content (5.75%) and amount of matter organic non-glycerol (MONG) at 67.56% (Pachapur *et al.*, 2015a).

The chemicals and reagents used in this study are the same as mentioned in (Sarma *et al.*, 2014).

Pretreatment of apple pomace

Apple pomace (AP) used in this study was obtained from Lassonde Inc., Rougemont, Montreal, Canada. Detailed characterization can be found in (Dhillon *et al.*, 2011b). The apple pomace sludge (AS) used by Sarma *et al*. (2013a), reported no improvement in H_2 production which may be due to lack of fermentable sugar (Sarma *et al.*, 2013b). In this study, therefore, it has been decided to use apple pomace hydrolysate as a co-substrate during crude glycerol fermentation for H_2 production. Acid hydrolysis of apple pomace was conducted at 121 °C for 20 min in autoclave with a mass ratio of solid (g dry weight) to liquid (mL) at 2:15 using 10 N H_2SO_4 at 0.5% (w/v). After hydrolysis step, the solid material of apple pomace was separated from hydrolysate by filtration through a thin layer cloth. The pH of the hydrolysate was adjusted to 10 using $Ca(OH)_2$, the precipitate was removed by centrifugation at 381 x *g* for 15 min. The supernatant collected was re-acidified to pH 7.0 with further centrifugation to discard any sediment (Phowan *et al.*, 2010). The final hydrolysate was stored at -20 °C and used as a co-substrate along with CG for hydrogen production.

Experimental design and hydrogen production

Central Composite Design (CCD) was used to investigate the hydrogen production as a function of crude glycerol (substrate), apple pomace hydrolysate (co-substrate) and inoculum size using response surface methodology (RSM). Crude glycerol at 10 g/L has been found to be optimum for hydrogen production (Pachapur *et al.*, 2015a; Sarma *et al.*, 2013b). Thus, 10 g/L has been considered as the central point with 15 g/L as higher value and 5 g/L as lower value for the present experimental design. Design-Expert®-7 software (Stat-Ease Inc. Minneapolis, MN) was used in constructing the experimental design resulting in 20 runs of experiments having different concentrations of crude glycerol (CG), apple pomace hydrolysate (APH) and various inoculum sizes (InS). The central composite design matrix comprising varied crude glycerol and apple pomace hydrolysate concentration along with different inoculum size (InS) is presented in Table

4.1.2. Each individual parameter varied from low value: 5 and high value 15, resulted in only two alpha values (-α= 1.59 and +α= 18.41). Using alpha value, the design generated 6 axial points (runs: 1, 2, 4, 6, 10 and 13), 6 central points (runs: 7, 9, 15, 17, 19 and 20) and rest factorial points.

Exact concentration of crude glycerol and apple pomace hydrolysate along with media supplement (casein polypeptone, KH_2PO_4, yeast extract and $MgSO_4.7H_2O$) was mixed in distilled water and initial pH was adjusted to 6.5 using NaOH. The final volume with inoculum addition was transferred to serum bottle and subsequently flushed with nitrogen to ensure anaerobic condition. The serum bottles for each run in triplicates were sealed, autoclaved and experiments were conducted at 37 °C and 150 rpm. Gas and liquid samples were collected every 24 h and analyzed, as described below.

The H_2 production (mmol/L), ethanol (g/L) and 1,3-propanediol (g/L) measured for each run was chosen as the response variable. The relationship between the parameters and the response variable was determined by design matrix evaluation, by taking into consideration the response surface quadratic model for interactions. The significance of the regression was tested, models with a p-value higher than 0.05 were not considered. The best fit of the polynomial models was evaluated from R^2 values (adjusted coefficient of determination) and final equation in terms of factors and lack of fit (LOF) test was obtained from the analysis of variance (ANOVA).

Analytical techniques

Total reducing sugar estimation by DNS method

The formation of the reducing sugars in the apple pomace hydrolysate were measured by dinitrosalicylic acid (DNS) method (Miller, 1959). About 1 mL of apple pomace hydrolysate and 2 mL of DNS reagent were mixed in labelled tubes in duplicates, which were subsequently shaken and placed in a boiling water bath for 5 min. The tubes were eventually cooled on ice thoroughly for 5 min and about 7 mL of distilled water was added to each tubes and the absorbance was read at 550 nm against a blank reagent with known glucose sample as standard.

Hydrogen analysis by GC

Hydrogen analysis was carried out using gas chromatography (Varian 3800, USA) equipped with a PoraPLOT Q® column (Agilent technology, USA) fitted with thermal conductivity detector (TCD). The column and detector temperature was fixed at 100 ºC and nitrogen was used as carrier gas at a flow rate of 3.5 mL/min resulting in H_2 retention time of 4.5 min (Sarma *et al*., 2014).

Analysis of by-products by GC-FID

The concentrations of substrate (glycerol) and by-products (ethanol and 1,3-propanediol) were verified by gas chromatography (GC) (7890B GC-Agilent, Santa Clara, CA) with ZB-WAX plus column fitted with flame ionization detector (FID) detector. The GC conditions comprised: helium carrier gas at a flow rate of 1 mL/min in a temperature range of 80–240 ºC for 8.4 min method run time (Pachapur *et al.*, 2015a).

Results and Discussion

Hydrogen production using co-substrate

The experimental runs using different substrate composition of crude glycerol and apple pomace hydrolysate at varied inoculum size that were tested, along with the results obtained for H_2, ethanol and 1,3-PD production for each run have been presented in Table 4.1.2. H_2 response ranged from about 6.95 mmol/L (obtained with CG: 10, APH: 18.41, InS: 10) to a maximum of 26.07 ± 1.57 mmol/L (obtained with CG: 15, APH: 5, InS: 15). APH was thus found to be a suitable co-substrate for the conversion of crude glycerol into H_2.

The model has a p-value of 0.0078, which is lesser than 0.05 indicating a statistically significant model. The "Lack of Fit F-value" of 14.73 implies the Lack of Fit is significant. There is only a 0.52% chance that a "Lack of Fit F-value" this large could occur due to noise.The final model equation (Eq. (1)) that best fitted the data has been shown below:

$$\text{Hydrogen} = 20.45 + 0.44 \times \text{CG} - 3.26 \times \text{APH} + 0.79 \times \text{InS} - 0.04 \times \text{CG} \times \text{APH} + 0.08 \times \text{CG} \times \text{InS} - 0.02 \times \text{APH} \times \text{InS} - 0.05 \times \text{CG} \times \text{CG} - 0.12 \times \text{APH} \times \text{APH} - 0.06 \times \text{InS} \times \text{InS} \quad (1)$$

The coefficient of APH was much higher than the other ones, indicating that the

quadratic effect of APH in the studied range had a dominant effect on H_2 production. ANOVA for the fitting model for H_2 equation with p-value has been presented in Table 4.1.3. The p-value of 0.0017 and 0.0016 indicated that the linear dependence of APH had a significant effect on H_2 production. The p-values > 0.05 for crude glycerol, inoculum size and from their interaction did not have any significant effect on H_2 production.

In Figure 4.1.1, the experimental values of H_2 production have been plotted with the fitting function of crude glycerol, apple pomace hydrolysate and inoculum size. At minimum concentration of APH (5 g/L) with increasing concentration of crude glycerol, the production of hydrogen increased as seen in Figure 4.1.1(a). This is seen in the run 18 at CG concentration of 15 g/L with lower concentration of APH (5 g/L) and the H_2 production reached a maximum of 26.07 ± 1.57 mmol/L of medium. At higher concentration of APH (18.41 g/L), the H_2 production reached a minimum of 6.95 ± 0.06 mmol/L. At increasing concentration of CG along with increasing inoculum size, the production of H_2 increased as seen in Figure 4.1.1(b). At a higher concentration of CG (15 g/L) with maximum inoculum size (15%), H_2 production reached 26.07 ± 1.57 mmol/L. On the contrary, with maximum concentration of CG (15 g/L) at lower inoculum size (5% v/v), H_2 production was around 11.00-16.17 mmol/L and vice-versa for run (8 and 16) where it was around 10.98-14.53 mmol/L. H_2 production increased with increasing inoculum size from 5 to 15% v/v along with increasing concentration of crude glycerol. Crude glycerol and inoculum size showed a parabolic relationship for hydrogen production. On the contrary, inoculum size in relation with APH as presented in Figure 4.1.1(c) showed a different trend as compared to crude glycerol for H_2 production. At 15 g/L of APH with maximum inoculum size of 15%, produced 10.98 ± 0.04 mmol H_2/L. The best result at minimum concentration of APH (5 g/L) with inoculum size of 15% v/v resulted in H_2 production of 26.07 ± 1.57 mmol/L. Thus, 15 g/L of crude glycerol at inoculum size of 15%, within the concentration range of 1.49-5 g/L for APH suggested that the optimum point was indeed within this range for maximum H_2 production with lower 1,3-PD and ethanol production. These results suggested the fact that during glycerol fermentation, APH was used as indirect H-acceptor, as was the case with

acetate addition during glycerol fermentation which diverted the route from1,3-PD towards H_2 formation (Heyndrickx *et al.*, 1991).

1,3-PD production using co-substrate

1,3-PD production ranged from about 0.40 g/L (obtained with CG: 10, APH: 18.41, InS: 10) to a maximum of 4.14 ± 0.04 g/L (obtained with CG: 10, APH: 1.59, InS: 10). APH was found to be a suitable co-substrate for minimizing the end-product formation during conversion of crude glycerol into H_2. The model showing p-value of 0.0009 was statistically significant. The final model equation (Eq. (2)) that best fitted the data has been shown below:

$$1{,}3-\mathrm{PD} = 1.19 + 0.30 \times \mathrm{CG} - 0.77 \times \mathrm{APH} - 0.16 \times \mathrm{InS} - 0.03 \times \mathrm{CG} \times \mathrm{APH} - 0.09 \times \mathrm{CG} \times \mathrm{InS} - 0.06 \times \mathrm{APH} \times \mathrm{IS} - 0.05 \times \mathrm{CG} \times \mathrm{CG} + 0.37 \times \mathrm{APH} \times \mathrm{APH} + 0.08 \times \mathrm{InS} \times \mathrm{InS} \quad (2)$$

The coefficient of APH was much higher than the other ones with p-value of <0.0001 and 0.0041 which indicated that the linear dependence from APH had a significant effect on 1,3-PD production. In Figure 4.1.2, the experimental values of 1,3-PD production have been plotted with the fitting function of crude glycerol, APH and inoculum size. At a maximum concentration of CG (18.41 g/L), the production of 1,3-PD was about 1.52 ± 0.18 g/L, at maximum concentration of APH (18.41 g/L) the production was minimum of 0.40 ± 0.13 g/L and at maximum InS (18.41%) production was around 1.28 ± 0.34 g/L. At maximum range and around the central point of all the three factors, the production of 1,3-PD was minimum as seen in Figure 4.1.2, suggesting co-substrate utilization of APH with crude glycerol for hydrogen production resulting in minimum production of 1,3-PD. This is beneficial for hydrogen production, because glycerol conversion to 1,3-PD releases H_2O (Barbirato *et al.*, 1995) and consumes NADH needed for H_2 production (Da Silva *et al.*, 2009; Heyndrickx *et al.*, 1991; Sarma *et al.*, 2012; Viana *et al.*, 2012).

Ethanol production using co-substrate

Ethanol production ranged from about 0.63 ± 0.03 g/L (obtained with CG: 10, APH: 18.41, InS: 10) to a maximum of 2.51 ± 0.52 g/L (obtained with CG: 10, APH: 1.59, InS: 10). The model showing p-value of 0.0021 was statistically significant with R^2 of 0.8709. The final model equation (Eq. (3)) that best fitted the data has been shown below:

$$\text{Ethanol} = 1.04 + 0.09 \times \text{CG} - 0.48 \times \text{APH} + 0.07 \times \text{InS} - 0.16 \times \text{CG} \times \text{APH} - 0.01 \times \text{CG} \times \text{InS} - 0.08 \times \text{APH} \times \text{InS} + 0.01 \times \text{CG} \times \text{CG} + 0.21 \times \text{APH} \times \text{APH} + 0.01 \times \text{InS} \times \text{InS} \quad (3)$$

The coefficient of APH was much higher than the other factors with p-value of <0.0001 and 0.0097 indicated that the linear dependence from APH had a significant effect on ethanol production. In Figure 4.1.3, the experimental values of ethanol production have been plotted with the fitting function of crude glycerol, APH and inoculum size. At maximum concentration of CG (18.41 g/L), the production of ethanol was only 0.90 ± 0.47 g/L, at maximum concentration of APH (18.41 g/L), the production was 0.63 ± 0.03 g/L and at maximum InS (18.41 %) production was around 1.00 ± 0.19 g/L. At the maximum range and around the central point of all the three factors, the production of ethanol remained minimum as seen in Figure 4.1.3, suggesting the co-substrate utilization of APH with crude glycerol for hydrogen production resulting in minimum production of ethanol. This was again beneficial for H_2 production, because glycerol conversion to ethanol produced formic acid (Barbirato *et al.*, 1995) and consumed NADH, which would otherwise be used by NADH dependent hydrogenase for H_2 production (Da Silva *et al.*, 2009; Heyndrickx *et al.*, 1991; Sarma *et al.*, 2012; Viana *et al.*, 2012).

Hydrogen production at optimum conditions

Hydrogen production at optimum conditions using co-substrate (set: 1 using CG: 15 g/L, APH: 5 g/L, InS: 15%) along with control condition using single substrate as APH (set: 2 with CG: 0 g/L, APH: 5 g/L, InS: 15%) and for CG as single substrate (set: 3 with CG: 15 g/L, APH: 0 g/L, InS: 15%) for co-culture and mono-culture system was carried out. Hydrogen responses for tested parameters for all the sets are presented in Figure 4.1.4.

The optimum condition from the central design (set: 1) resulted in highest hydrogen production (26.49 ± 1.49 mmol/L of medium) in comparison to other set: 2 (19.39 ± 1.59 mmol) and set: 3 (20.07 ± 1.09 mmol), respectively. The concentration of the 1-3PD for the set: 1 was minimum at around 1.51 ± 0.30 g/L resulting in oxidative pathway with production of butyrate at around 3.5 ± 0.89 g/L favoring increased hydrogen production in comparison to other sets. The hydrogen and metabolite yield for the utilization of the glycerol in presence (set: 1) and absence of apple pomace hydrolysate (set: 2) as co-substrate was carried out. The hydrogen yield was around 1.7 ± 0.1 mol/mol of glycerol in presence of co-substrate and in its absence, the yield decreased to 0.6 ± 0.2 mol/mol of glycerol. Thus, fermentation of crude glycerol along with apple pomace hydrolysate as co-substrate increased hydrogen yield in co-culture system.

APH was found to be the most influential factor for H_2, 1,3-PD and ethanol production. As shown in Figure 4.1.1, the results indicated the possibility to use APH within 1.59–5.00 g/L range, with H_2 production reaching an optimal range (light yellow). The reason of using ethanol and 1,3-PD data was to monitor the utilization of crude glycerol in presence of APH for increased H_2 production with minimum end-product formation. Ethanol and 1,3-PD are the most common metabolites of glycerol metabolism by *E. aerogenes* (Ito *et al.*, 2005; Jitrwung *et al.*, 2011; Nwachukwu *et al.*, 2012; Nwachukwu *et al.*, 2013) and *C. butyricum* (Chatzifragkou *et al.*, 2011; Gonzalez-Pajuelo *et al.*, 2004; Gonzalez-Pajuelo *et al.*, 2005; Szymanowska-Powałowska, 2014; Zeng, 1996). Ito *et al*. (2005), indicated that a higher initial concentration of crude glycerol decreased the yield of H_2 and ethanol. However, by using 1.7 g/L of crude glycerol in the presence of additional 5 g/L of yeast and 5 g/L tryptone resulted in 0.71 mol H_2/mol glycerol with 3.2 g/L of ethanol production (Ito *et al.*, 2005). Jitrwung *et al*. (2011), used 15 g/L glycerol with additional ammonia and salt concentration of 8 g/L Na_2HPO_4, 0.00625 g/L $FeSO_4$ and 1.5 g/L NH_4NO_3 at 18% inoculum size to produce 0.67 mol H_2/mol glycerol with 0.78 ethanol/mol glycerol production (Jitrwung *et al.*, 2011). Nwachukwu *et al*. (2012), recovered glycerol (69.8% pure) from 85% H_3PO_4 pretreatment at 17.8 g/L in presence of tryptic soy broth using mutant *E. aerogenes* strain to produce only ethanol at 12.8 g/L. Gonzalez-Pajuelo *et al*. (2004), at 30 g/L with 65% glycerol used complex

synthetic medium in presence of vitamin supplement, to produce 1,3-PD at 0.60 mol/mol glycerol consumption, suggesting the economic viability of the process dependent on crude glycerol price and availability (Gonzalez-Pajuelo *et al.*, 2004). Chatzifragkou *et al*. (2011), used 20 g/L of crude glycerol along with 1% yeast extract with continous N_2 sparging during fed-batch fermentation for the production of 1,3-PD (Chatzifragkou *et al.*, 2011). The production of ethanol and 1,3-PD from crude glycerol required additional media supplements and need to be produced at higher concentration to reduce purification cost (Gonzalez-Pajuelo *et al.*, 2005; Ito *et al.*, 2005). Use of synthetic media components sacrificed the benefit of hydrogen production by increasing the media cost (Jitrwung *et al.*, 2011). Therefore, instead of simultaneously producing H_2 and these by-products, if overall metabolic activity could be shifted towards minimum reduced by-product formation, H_2 production will definitely increase. On the other hand, apple pomace represented the most suitable, low-cost and readily available source for co-fermentation with crude glycerol to maximize H_2 production.

The common glycerol fermentation pathway and its conversion into 1,3-PD, ethanol, butyrate along with release of H_2 and other components is presented in Figure 4.1.4. The substrate consumption along with butyrate and acetic acid concentration across each run is presented in Table 4.1.4. The formation of each end-product from glycerol with formation of 1,3-PD released H_2O and in the case of ethanol released formic acid with no H_2 production in both the cases (Barbirato *et al.*, 1995). However, during glycerol conversion to butyrate, H_2 production along with reducing equivalents ($NADH+H^+$) are generated (Biebl *et al.*, 1992). This is also true in the case of acetate production, but in this study, higher butyrate concentration (3.9 ± 0.81 g/L) was obtained in comparison to acetate (0.6 ± 0.10 g/L). The regeneration of reducing equivalents via butyric acid pathway increased H_2 production along with substrate losses in the form of CO_2 (Papanikolaou *et al.*, 2004). The H_2 production pathway is in relation with the ratio of $NADH/NAD^+$ and acetyl CoA/CoA (Heyndrickx *et al.*, 1991). With APH as co-cubstrate, the most active butyrate pathway is reponsible for high $NADH/NAD^+$ turnover rate and favourable acetyl-CoA/CoA ratio for H_2 production. This assumption is supported by the results reported in Heyndrickx *et al*. (1991) for using acetate as co-

substrate during glycerol fermentation. In the presence of *Clostridium* species, acetate was used as indirect H-acceptor, it regulated the redox balance and gradually diverted 1,3-PD production towards H_2 and butyrate production (Heyndrickx *et al.*, 1991). With this model, the use of low-cost APH as co-substrate not only increased the hydrogen production, but also minimized the end-product concentration. In our earlier studies using the co-culture system with 10 g/L of crude glycerol as sole substrate, the concentration of 1,3-PD was on higher side of around 3.0 g/L resulting in lower H_2 production of 19.46 mmol/L and lower amount of butyrate (<0.15 g/L). Further, with higher concentration of crude glycerol, the H_2 production decreased (Pachapur *et al.*, 2015a). Hence, crude glycerol caused substrate inhibition effect on H_2 production and favored reductive pathway of glycerol metabolism resulting in 1,3-PD formation instead of H_2 production. However, in the presence of APH as co-substrate and at higher concentration of crude glycerol (15 g/L), the system followed the oxidative pathway with formation of more butyrate (3.9 ± 0.81 g/L) favoring the production of H_2 (26.07 ± 1.57 mmol/L) with minimum concentration of 1,3-PD (2.2 ± 0.46 g/L) formation. With lower concentration of APH (<5 g/L), the substrate conversion rate increased with minimum production of reduced end-metabolites (as seen from Table 4.1.4), so that optimum concentration was on the border of design boundary for maximum H_2 production.

The optimum condition from the design was carried out in presence (set: 1) and absence of apple pomace hydrolysate (set: 3) along with apple pomace as sole substrate for set: 2. The hydrogen production in case of apple pomace as only substrate resulted in decreased hydrogen production (19.39 ± 1.59 mmol/L) in comparison to other set:1 (26.49 ± 1.98 mmol/L) and set: 3 (20.07 ± 1.09 mmol/L). The hydrogen production even with 15% inoculum size for set: 3 produced similar hydrogen production (19.46 mmol/L) at 10 g/L using 5% inoculum size in our previous studies (Pachapur *et al.*, 2015a). The hydrogen production was maximum for set: 1 resulting in 26.49 ± 1.98 mmol/L with minimum concentration of 1,3-PD as a co-substrate being around 1.51 ± 0.30 g/L in comparison to 5.5 ± 0.40 g/L, obtained in its absence. Similarly, the concentration of ethanol in presence of co-substrate was around 1.9 ± 0.54 g/L in comparison to 2.9 ± 0.71 g/L found in its absence. The decrease in concentration of

these metabolites resulted in increase in butyrate concentration from 1.9 ± 0.03 g/L in absence of co-substrate to 3.5 ± 0.89 g/L in presence of co-substrate. The results from the sets showed that hydrogen production could be promoted by addition of apple pomace hydrolysate as co-substrate. However, presence of apple pomace hydrolysate as only substrate showed inhibitory influence with decreased hydrogen production (as seen from Figure 4.1.5). Addition of apple pomace hydrolysate as co-substrate at optimum concentration acted as an indirect H-acceptor and favored oxidative pathway of butyric acid production instead of reductive pathway with production of 1,3-PD during glycerol fermentation. The oxidative pathway favored hydrogen production with butyrate formation releasing hydrogen during glycerol fermentation (Biebl *et al.*, 1992).

In presence of pure carbon source, *Enterobacter* and *Clostridium* sp. have produced 1 and 2 mol H_2/mol of glucose (Yokoi et *al.*, 1998). Maximum hydrogen was produced by these microorganisms using glucose as carbon source. But during crude glycerol fermentation, both *E. aerogenes* and *C. butyricum* mainly produced ethanol and 1,3-PD (Gonzalez-Pajuelo *et al.*, 2005; Ito *et al.*, 2005; R. E. Nwachukwu *et al.*, 2013; Zeng, 1996). By using these microorganisms at higher concentration of crude glycerol, the yield of 1,3-PD and ethanol was maximum, but by using co-substrate, the contrasting results were obtained . The respective yield in presence and absence of co-substrate for co-culture and mono-culture system is calculated and presented in Table 4.1.5. In case of mono-culture studies, 15 g/L of crude glycerol in the absence of co-substrate acted as substrate inhibitor and produced more 1,3-PD resulting in decreased hydrogen with increased yield of metabolites (as seen from Table 4.1.5). Nevertheless, with availability of co-substrate, the metabolic pathway for both mono-cultures shifted, resulting in increased hydrogen and butyric acid yield. The optimum condition for mono-culture presented a metabolic pattern of acetate production by *E. aerogenes* and butyrate production by *C. butyricum* in presence of co-substrate. In the absence of co-substrate, *E. aerogenes* produced more 1,3-PD and for *C.butyricum*, the concentration of CG resulted in substrate inhibition (with only 30 ± 2% of substrate degradation). *C.butyricum* preferred APH over CG producing more hydrogen and *E. aerogenes* preferred CG over APH during mono-culture studies (as seen from Figure 4.1.5). In

case of glycerol metabolism with co-substrate using co-culture of *E. aerogenes* and *C. butyricum* resulted in increased hydrogen production by degradation of both the substrates. The inhibition towards utilization of crude glycerol as a sole substrate was minimized by co-substrate of glucose source showing improved tendency for *E. aerogenes* and *C. butyricum* towards increased hydrogen production. The hydrogen yield for *C.butyricum* (1.4 ± 0.1 mol/mol) and *E. aerogenes* (0.8 ± 0.2 mol/mol) almost reached theoretical maximum of hydrogen yield. These results justified the selection of best hydrogen producing microorganisms for the co-culture system in agreement with previous studies (Phowan *et al.*, 2010; Yokoi *et al.*, 1998).

Addition of apple pomace hydrolysate as co-substrate resulted in the shift of metabolite pathway from reductive to oxidative causing decreased production of 1,3-PD with increase in production of butyrate favoring hydrogen production. The addition of co-substrate not only increased hydrogen yield (from 0.6 ± 0.2 to 1.7 ± 0.1 mol/mol of glycerol) but also increased the glycerol utilization, which was acting as substrate inhibiter at higher concentration. This approach of co-substrate fermentation leads to a new perspective of increased substrate utilization at substrate inhibition concentration for increased hydrogen yield.

The predominant glycerol fermentation pathway using *E. aerogenes* and *C. butyricum* cultivated under anaerobic condition is used for 1,3-propanediol biosynthesis (Papanikolaou *et al.*, 2004). However, in this study, glycerol fermentation followed the pathway of butyric acid (for *Clostridium*) and acetate biosynthesis (for *Enterobacter*) along with H_2 production. The ability of producing hydrogen in presence of co-substrate indicated the microbial capacity to shift the metabolic pathways during glycerol fermentation. Hence, a symbiotic relationship between *E. aerogenes* and *C.butyricum* in co-culture system was significant with the pattern of the metabolites obtained during glycerol fermentation in the presence of APH as co-substrate for increased H_2 production. Until date, hydrogen production has been mostly carried out using single or dual pure carbon source; however pure carbon source are far too expensive as feedstock. Again, renewable waste materials are attractive potential alternative with increasing industrial and municipal wastes containing mixed organic source. The

sustainable production of hydrogen with introduction of co-substrate by mixing two or more industrial wastes, such as biodiesel derived crude glycerol and apple industry waste will lead to practical application in near future.

Conclusion

In order to increase the hydrogen production and decrease 1,3-propanediol formation and shift the glycerol metabolism towards oxidative pathway, apple pomace hydrolysate was included as a co-substrate. The statistical model helped in obtaining higher H_2 production of around 26.07 ± 1.57 mmol/L at (crude glycerol: 15 g/L, apple pomace hydrolysate: 5 g/L, InS: 15%) favored oxidative pathway instead of reductive pathway with trace amount of 1,3-propanediol production. Apple pomace hydrolysate (p-value: 0.0017) even at lower concentration had a significant effect on H_2 production. Co-substrate utilization, thus decreased substrate inhibition, reduced by-product production and offered additional benefit of waste treatment for apple industry.

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Table 4.1.1: Comparison of optimized conditions and results of recent studies dealing with co-substrate utilization for H_2 production.

Substrate used	Purpose	Inoculum	Operating conditions	Design	Contribution	H_2 production (mmol/L of medium)	Ref.
Starch-manufacturing wastes and corn steep liquor (CSL)	Use of organic waste rich in nitrogen source for effective and economical H_2 production	*E. aerogenes* HO-39, *C. butyricum* IFO13949	200 mL stirred reactor with pH 5.25 at 37 °C	-	Highest H_2 evolution obtained at 0.5% CSL concentration in the medium	6.7	(H Yokoi *et al.*, 2002)
Acetate medium with propionate and butyrate	Investigate photo-H_2 production by mixed substrate	*Rhodopseudomonas faecalis* RLD-53 and mixed bacteria (*Rhodopseudomonas genus*)	100 mL serum bottle with pH 7 at 35 °C, 120 rpm using 4000 lux	-	Influence of butyrate on enhancing H_2 production is more than propionate	116	(Wang *et al.*, 2014)
Cheese whey (CW), crude glycerol and buffalo slurry (BS)	Improve H_2 yield and stability of the process with different substrate	*Buttiauxella* sp. 4, *Rahnella* sp. 10, *Raoultella* sp. 47 and F210: a microbial mixed culture	125 mL serum bottle with pH 6.5 at 37 °C using 120 rpm	Simplex-lattice design	Buffalo slurry shown: buffer capacity, reduced lag phase and increased degradation efficiency	18.1	(Marone *et al.*, 2015)
Crude glycerol and apple pomace	To improve H_2 production by shifting the	*E. aerogenes*, *C. butyricum*	125 mL serum bottle with pH 6.5	Central Composite design	Increased H_2 production with minimum	26.07 ± 1.57	This study

hydrolysate	metabolic pathway		at 37 °C using 150 rpm		production of by-products		

Table 4.1.2: Central composite design matrix defining crude glycerol concentration, apple pomace hydrolysate concentration, inoculum size and results on H_2 production, end metabolites concentration for each run.

Run	Crude glycerol (g/L)	Apple pomace hydrolysate (g/L)	Inoculum size (%)	H_2 in (mmol/L)	1,3-propanediol (g/L)	Ethanol (g/L)
1	10	10	18.41	16.20 ± 1.22	1.28 ± 0.34	1.00 ± 0.19
2	10	10	1.59	12.51 ± 0.55	1.62 ± 0.17	1.02 ± 0.23
3	5	5	5	21.49 ± 0.50	1.80 ± 0.14	1.24 ± 0.41
4	10	1.59	10	13.23 ± 0.02	4.14 ± 0.40	2.51 ± 0.52
5	5	15	5	11.83 ± 0.76	0.90 ± 0.20	0.73 ± 0.49
6	18.41	10	10	15.87 ± 2.38	1.52 ± 0.18	0.90 ± 0.47
7	10	10	10	19.90 ± 1.32	1.57 ± 0.25	0.95 ± 0.42
8	5	5	15	14.53 ± 3.34	1.69 ± 0.06	1.51 ± 0.48
9	10	10	10	20.71 ± 1.46	1.00 ± 0.67	0.86 ± 0.16
10	10	18.41	10	6.95 ± 0.06	0.40 ± 0.13	0.63 ± 0.03
11	15	5	5	16.17 ± 0.05	2.66 ± 0.31	1.81 ± 0.89
12	15	15	5	11.00 ± 0.90	1.68 ± 0.32	0.95 ± 0.62
13	1.59	10	10	15.21 ± 2.69	0.61 ± 0.14	1.12 ± 0.47
14	15	15	15	10.55 ± 0.05	0.99 ± 0.07	0.92 ± 0.29
15	10	10	10	20.71 ± 1.65	1.30 ± 0.01	1.09 ± 0.29
16	5	15	15	10.98 ± 0.04	0.59 ± 0.15	0.95 ± 0.30
17	10	10	10	19.67 ± 0.74	0.93 ± 0.26	0.84 ± 0.14
18	15	5	15	26.07 ± 1.57	2.22 ± 0.46	2.40 ± 0.90
19	10	10	10	22.36 ± 2.30	1.24 ± 0.01	1.33 ± 0.44
20	10	10	10	19.81 ± 0.45	1.09 ± 0.09	1.15 ± 0.41

Table 4.1.3: Summarized ANOVA results of the fitting model for H_2, 1,3-propanediol and ethanol.

Source	**p-value**		
	H_2	**1,3-propanediol**	**Ethanol**
A-Crude glycerol	0.5737	0.0153	0.1983
B-APH	0.0017	< 0.0001	< 0.0001
C-Inoculum	0.4695	0.1630	0.2963
AB	0.3711	0.8492	0.1062
AC	0.0560	0.5221	0.9244
BC	0.6071	0.6756	0.3555
A^2	0.1240	0.5983	0.8477
B^2	0.0016	0.0041	0.0097
C^2	0.0488	0.4426	0.8477

Table 4.1.4: Substrate consumption along with the butyric and acetic acid concentration across each run.

Run	CG (g/L)	APH (g/L)	Inoculum size (%)	Glycerol utilization (%)	Total reducing sugar (TRS) utilization (%)	Acetate (g/L)	Butyrate (g/L)
1	10	10	18.41	64 ± 3	41 ± 3	1.1 ± 0.16	2.9 ± 0.44
2	10	10	1.59	59 ± 1	21 ± 9	1.8 ± 0.04	0.1 ± 0.08
3	5	5	5	65 ± 3	55 ± 2	0.6 ± 0.09	2.5 ± 0.15
4	10	1.59	10	76 ± 7	69 ± 1	0.4 ± 0.01	3.6 ± 0.63
5	5	15	5	52 ± 2	25 ± 3	1.9 ± 0.18	0.1 ± 0.13
6	18.41	10	10	52 ± 5	28 ± 1	1.2 ± 0.26	2.9 ± 0.46
7	10	10	10	63 ± 3	25 ± 5	1.4 ± 0.02	3.4 ± 0.13
8	5	5	15	63 ± 5	45 ± 2	5.0 ± 0.07	3.0 ± 0.51
9	10	10	10	67 ± 3	29 ± 1	1.1 ± 0.15	2.3 ± 0.98
10	10	18.41	10	40 ± 8	12 ± 3	1.8 ± 0.25	0.1 ± 0.13
11	15	5	5	43 ± 7	41 ± 1	2.1 ± 0.61	0.2 ± 0.07
12	15	15	5	12 ± 6	19 ± 1	2.6 ± 0.77	0.2 ± 0.17
13	1.59	10	10	30 ± 3	15 ± 3	1.9 ± 0.37	2.4 ± 0.33
14	15	15	15	38 ± 3	17 ± 2	1.8 ± 0.01	0.5 ± 0.08
15	10	10	10	47 ± 3	7 ± 1	1.4 ± 0.24	2.8 ± 0.27
16	5	15	15	42 ± 8	18 ± 4	1.9 ± 0.22	0.5 ± 0.02
17	10	10	10	58 ± 3	41 ± 3	1.1 ± 0.08	2.0 ± 0.31
18	15	5	15	39 ± 5	50 ± 2	0.6 ± 0.10	3.9 ± 0.81
19	10	10	10	44 ± 6	10 ± 1	1.5 ± 0.16	2.7 ± 0.05
20	10	10	10	42 ± 5	19 ± 4	1.4 ± 0.24	2.5 ± 0.27

Table 4.1.5: Responses for hydrogen and metabolites yield (per mol of glycerol) across the optimum condition in presence (set: 1) and absence (set: 3) of co-substrate for co-culture system using *E. aerogenes* and *C. butyricum* (EA+CB) along with mono-culture system *E. aerogenes* (EA) and *C. butyricum* (CB).

Optimum condition	Microorganisms	H_2 (mol/mol)	1,3-propanediol (mol/mol)	Ethanol (mol/mol)	Acetic acid (mol/mol)	Butyric acid (mol/mol)
SET: 1 (CG[a]: 15 g/L, APH[b]: 5 g/L, InS[c]: 15)	EA+CB	1.7 ± 0.1	0.27 ± 0.04	0.20 ± 0.03	0.07 ± 0.01	0.54 ± 0.04
	EA	0.8 ± 0.2	0.32 ± 0.01	0.19 ± 0.02	0.22 ± 0.02	0. 03 ± 0.01
	CB	1.4 ± 0.1	0.07 ± 0.03	0.06 ± 0.01	0.07 ± 0.01	0. 41 ± 0.02
SET: 3 (CG: 15 g/L, APH: 0 g/L, InS:15)	EA+CB	0.6 ± 0.2	0.54 ± 0.1	0.28 ± 0.01	0.07 ± 0.01	0.16 ± 0.02
	EA	0.7 ± 0.1	0.29 ± 0.1	0.21 ± 0.03	0.08 ± 0.01	0.02 ± 0.01
	CB	0.3 ± 0.1	0.08 ± 0.1	0.07 ± 0.02	0.03 ± 0.02	0.05 ± 0.02

[a] crude glycerol.

[b] apple pomace hydrolysate.

[c] inoculum size.

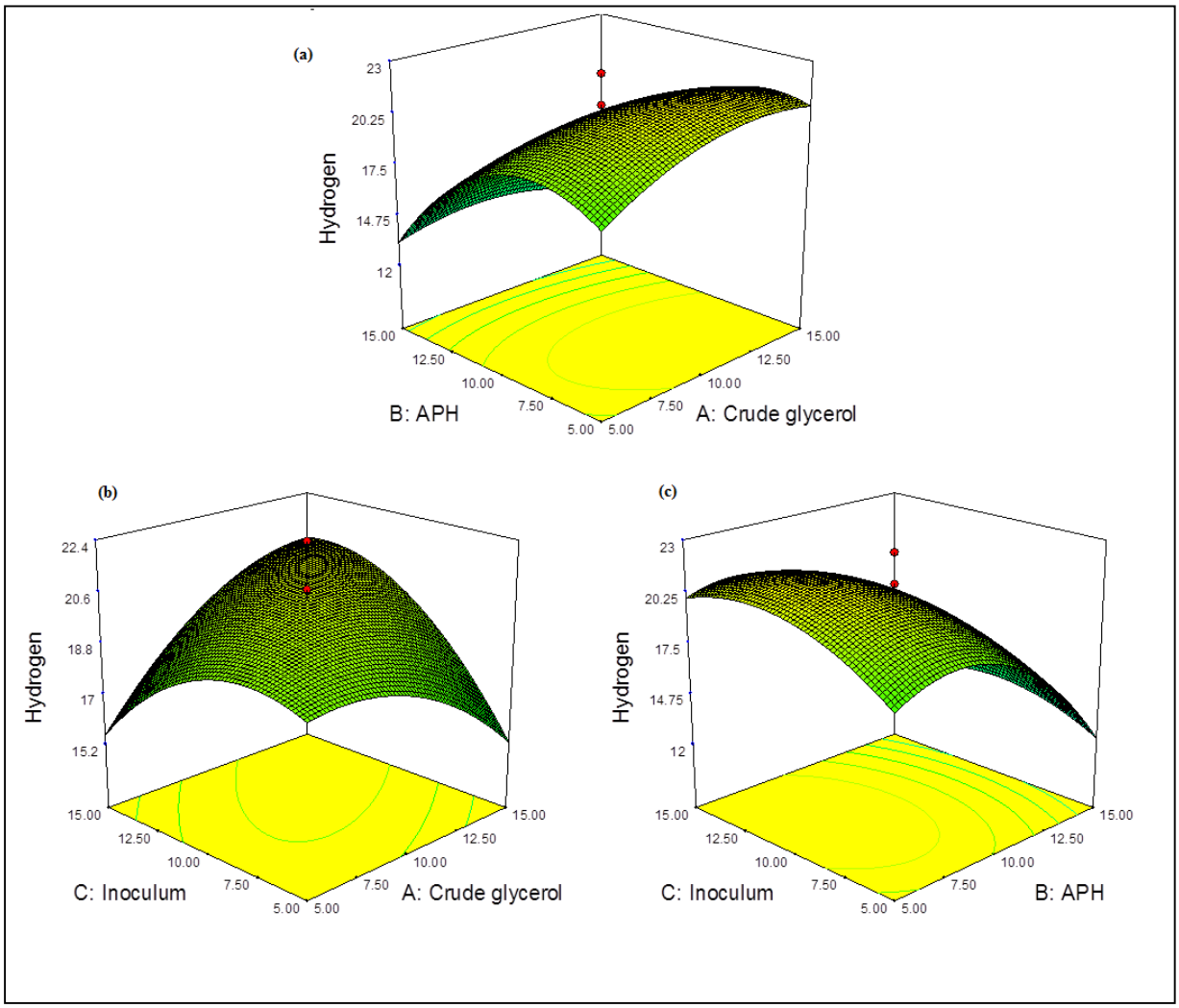


Figure 4.1.1: Response surface plots showing the interactive effect on H_2 production (a) interaction between apple pomace hydrolysate (APH) and crude glycerol (CG), (b) interaction between inoculum size and CG and (c) interaction between inoculum size and APH.

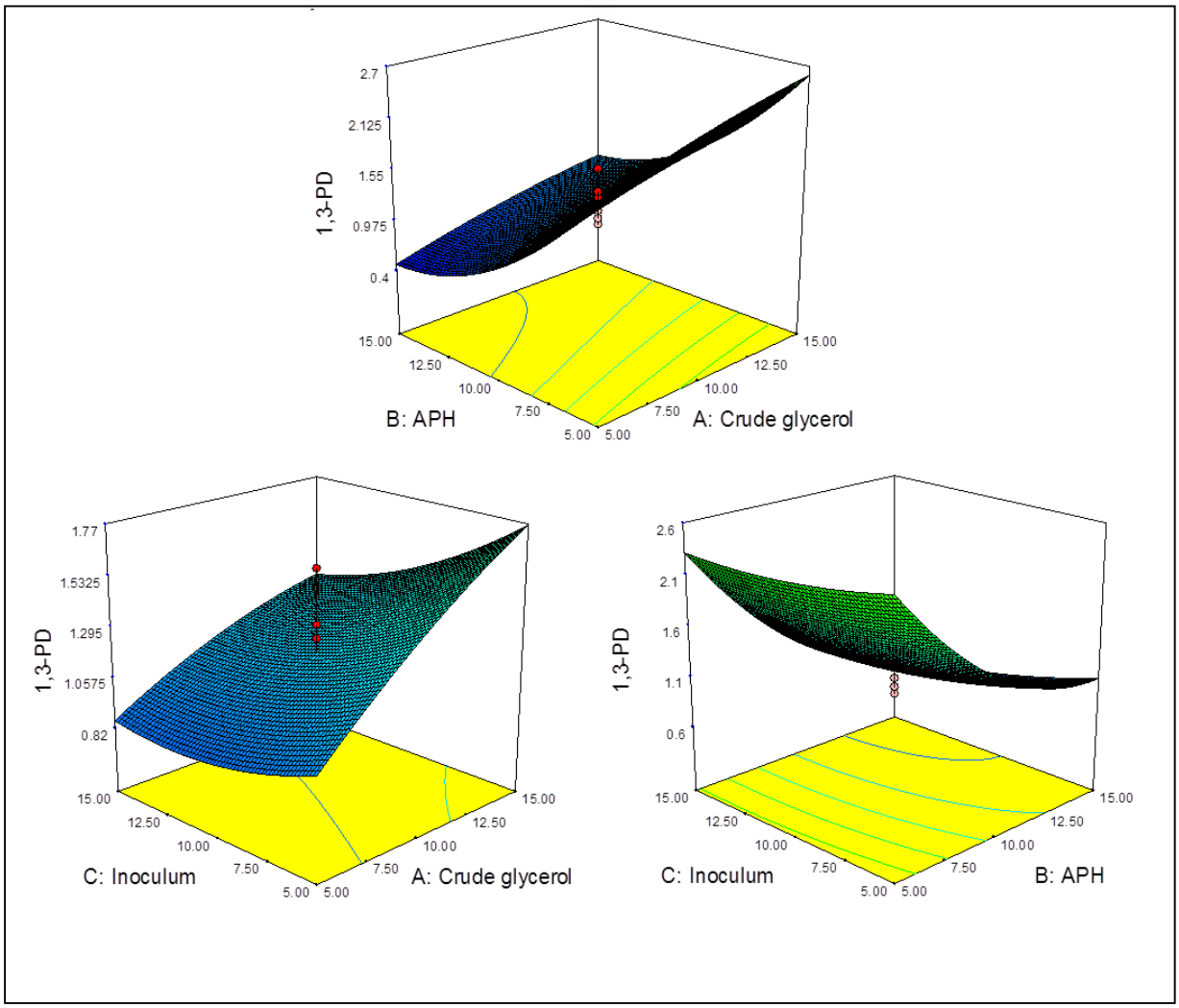


Figure 4.1.2: Response surface plots showing the interactive effect on 1,3-propanediol production.

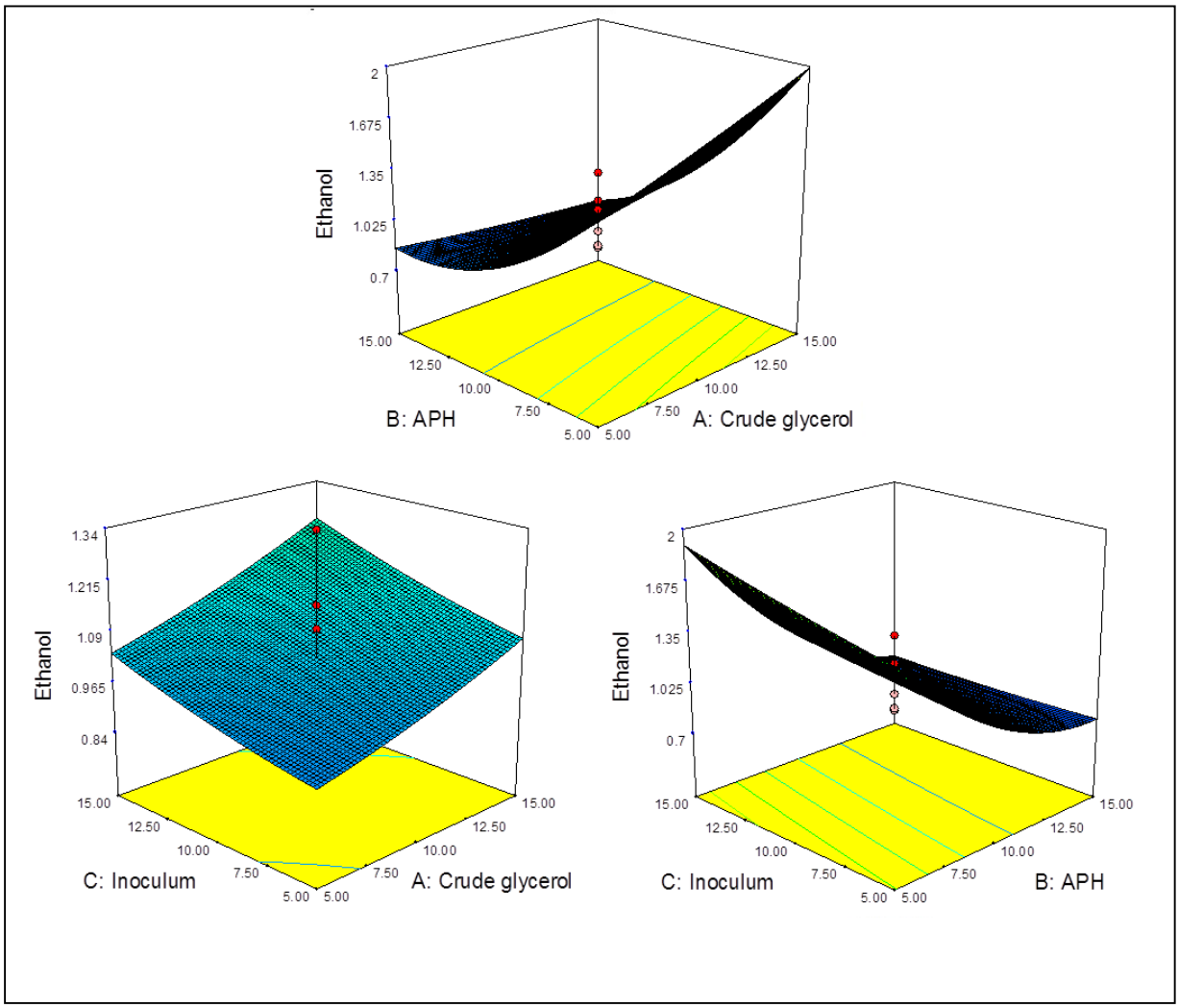


Figure 4.1.3: Response surface plots showing the interactive effect on ethanol production.

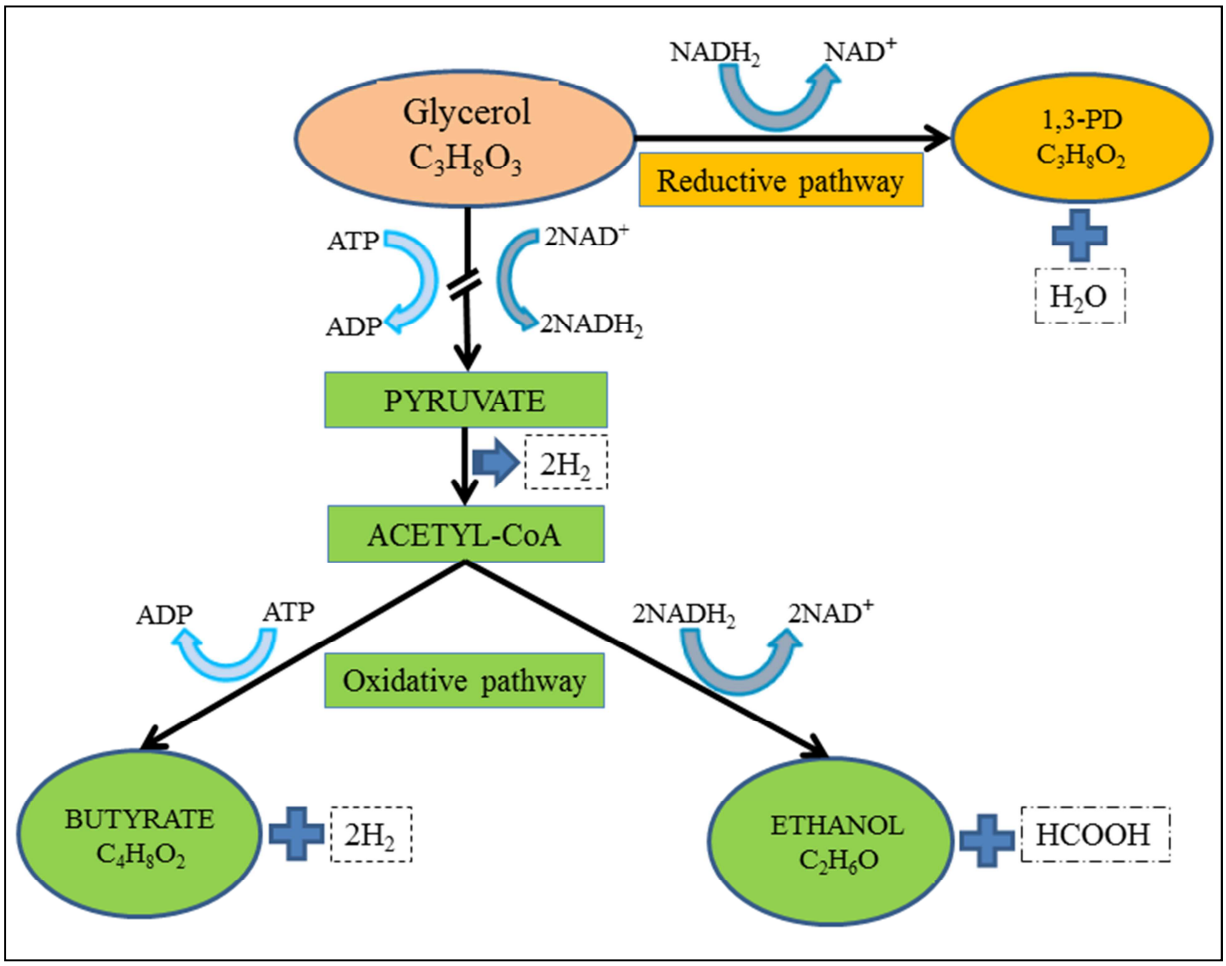
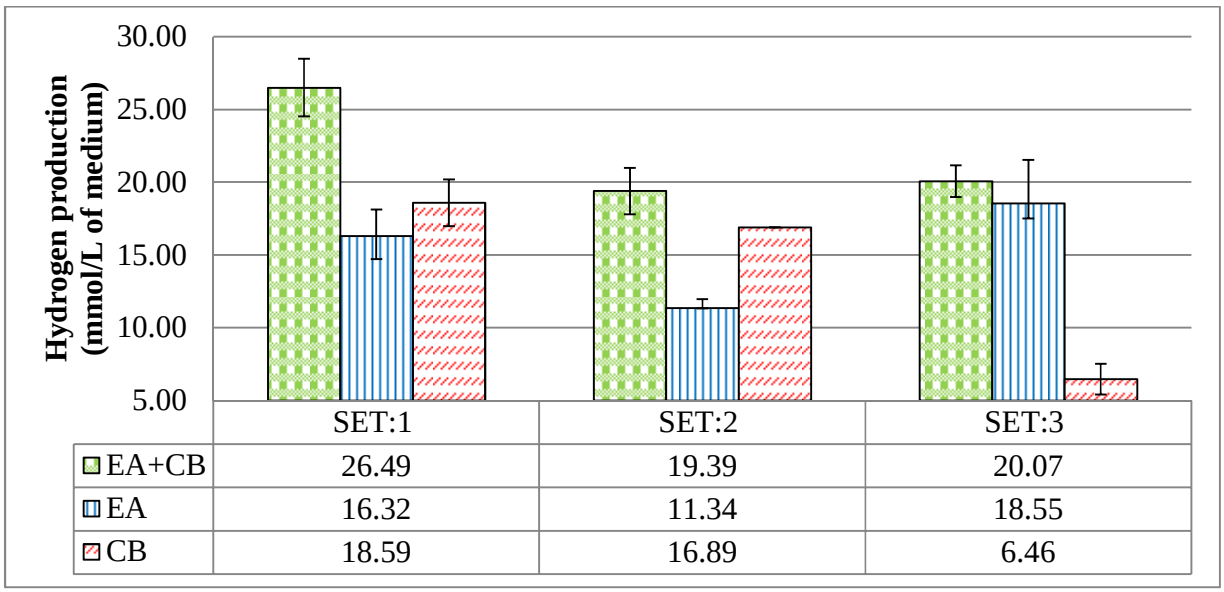


Figure 4.1.4: Glycerol fermentation pathway by co-culture of *E. aerogenes* and *C. butyricum* in the presence of apple pomace hydrolysate as co-substrate following oxidative pathway for H_2 production along with butyrate formation instead of following reductive pathway for 1,3-propanediol production.



	SET:1	SET:2	SET:3
EA+CB	26.49	19.39	20.07
EA	16.32	11.34	18.55
CB	18.59	16.89	6.46

Figure 4.1.5: The hydrogen production at optimum conditions using co-substrate (set: 1 using CG: 15 g/L, APH: 5 g/L, InS: 15%), along with control condition using single substrate as APH (set: 2 with CG: 0 g/L, APH: 5 g/L, InS: 15%) and CG as single substrate (set: 3 with CG: 15 g/L, APH: 0 g/L, InS: 15%) for co-culture system *E. aerogenes* and *C. butyricum* (EA+CB) and mono-culture system *E. aerogenes* (EA) and *C. butyricum* (CB). (CG: crude glycerol, APH: apple pomace hydrolysate, InS: Inoculum size).

CHAPTER 5

HYDROGEN PRODUCTION BY CO-CULTURE SYSTEM WITH PROCESS OPTIMIZATION

PART 1

EVIDENCE OF METABOLIC SHIFT ON HYDROGEN, ETHANOL AND 1,3-PROPANEDIOL PRODUCTION FROM CRUDE GLYCEROL BY NITROGEN SPARGING UNDER MICRO-AEROBIC CONDITIONS USING CO-CULTURE OF *ENTEROBACTER AEROGENES* AND *CLOSTRIDIUM BUTYRICUM*

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Résumé

L'hydrogène (H_2), un futur porteur d'énergie propre, nécessite des voies d'amélioration fondées dans les procédés de production afin de réduire les coûts de production. L'impact du barbotage à l'azote (N_2) sur la production de H_2 à partir du GB en système de co-culture de *E. aerogenes* et *C. butyricum* a été étudié pour réduire le coût global du procédé. La production de H_2 en utilisant 1% GB sous des conditions de barbotage du milieu à l'azote avant l'autoclavage a abouti à une production de 1,2 mol-H_2/mol de glycérol par rapport à 1,5 mol-H_2/mol de glycérol sans barbotage. En présence d'air variant de 5 à 75 mL dans le volume d'espace de tête, la production de H_2 a augmenté jusqu'à un maximum de 26,14 mmol/L et 1,4 g/L pour la production d'éthanol. La concentration en 1,3-propanediol lors du barbotage avec le N_2 était d'environ 3,0 g/L et a diminué à 0,5 g/L en raison de la présence de 75 mL d'air dans l'espace de tête. Cette observation peut être attribuée à un changement du métabolisme réducteur et oxydant pour le glycérol. Une stratégie d'amélioration basée sur les procédé pour optimiser la formation H_2 a entraîné un changement de voie métabolique de réducteur à oxydant avec l'augmentation de la production de H_2. L'influence synergique du système de co-culture en l'absence d'agent réducteur coûteux et sans étape de barbotage à l'azote peut offrir une meilleure stratégie économique. Cette approche permet de minimiser la production de métabolites et accroît l'application sur le terrain à l'échelle industrielle.

Mots clés: Co-culture, glycérol brut, éthanol, hydrogène, barbotage à l'azote, 1,3-propanediol

Abstract

Hydrogen (H_2), a possible future clean energy carrier, requires process-based improvement routes for cutting down the production cost. The impact of nitrogen (N_2) sparging on H_2 production during co-culture system of Enterobacter aerogenes and Clostridium butyricum from crude glycerol (CG) was studied to reduce the overall process cost. H_2 production using 1% CG under nitrogen sparged medium before autoclaving resulted in 1.2 mol-H_2/mol of glycerol in comparison to 1.5 mol-H_2/mol of glycerol without sparging. In the presence of air ranging from 5 mL to 75 mL in the headspace volume, H_2 production increased to a maximum of 26.14 mmol/L with 1.4 g/L of ethanol production. The concentration of 1,3-propanediol with N_2 sparging was around 3.0 g/L and decreased to 0.5 g/L due to presence of 75 mL of air in the headspace. This observation can be attributed to a shift from reductive to oxidative metabolism of glycerol. A process-based improvement strategy to optimize H_2 formation resulted in metabolic pathway shift from reductive to oxidative with increase in H_2 production. Synergistic influence of co-culture system in absence of expensive reducing agent and without nitrogen sparging step can offer a better process-based economic strategy for H_2 production, minimize the metabolite production and increase field-scale application of biodiesel plant.

Keywords: Co-culture, crude glycerol, ethanol, hydrogen, nitrogen sparging, 1,3-propanediol

Introduction

Hydrogen gas is a clean fuel, and with development of storage technologies as metal hydrides, it can be used for multipurpose application as replacement for petroleum-based fuels (Mizuno *et al.*, 2000). Nations importing petroleum-based fuels are supporting research on hydrogen production. Hydrogen production with current physico-chemical methods is energy-intensive and expensive, thus considerable possibilities as a future fuel are short (Kim *et al.*, 2006; Mizuno *et al.*, 2000).

Alternate ways of biological production of hydrogen are potentially more attractive with use of inexpensive and abundant organic waste or biomass as substrate for fermentation. Dark fermentation of hydrogen production has advantages over photo-fermentation in terms of faster production rate, simple technique, waste reduction and no requirement of light energy (Kim *et al.*, 2006).

There are several ways to increase the economic feasibility of hydrogen production by dark fermentation. Initial step is to utilize various organic wastes as substrate to increase the hydrogen producing efficiency. The rise in biodiesel contributes to crude glycerol availability as an abundant, inexpensive and excellent carbon source (Chatzifragkou *et al.*, 2011; Wilkens *et al.*, 2012). Microbial conversion of crude glycerol (CG) to various compounds, such as H_2 (Ito *et al.*, 2005; Jitrwung *et al.*, 2011; Marques *et al.*, 2009; Sarma *et al.*, 2013b), ethanol (Choi *et al.*, 2011; Ito *et al.*, 2005) and 1,3-propanediol (Barbirato *et al.*, 1995; Chatzifragkou *et al.*, 2011; Papanikolaou *et al.*, 2004; Wilkens *et al.*, 2012) have been investigated using microbial species belonging to *Enterobacter, Klebsiella, Citrobacter* and *Clostridium* (Ito *et al.*, 2005; Sarma *et al.*, 2012). H_2 production from CG is an attractive approach where produced H_2 can be easily separated from media and requires no additional purification cost in comparison to ethanol and 1,3-PD (Ito *et al.*, 2005; Sarma *et al.*, 2013a).

The second step is to reduce the cost of the fermentation processes by minimizing the media components, avoiding use of expensive reducing agents and making improvements in the process (Morsy, 2014). In this co-culture system, *E. aerogenes* acts as reducing agent for *C. butyricum* exhibiting minimal media requirement for

inhibited growth with increased and improved H_2 production efficiency by eliminating use of expensive media component (Pachapur *et al.*, 2015; Phowan *et al.*, 2010; Yokoi *et al.*, 1998).

During dark fermentation, creation of anaerobic conditions is carried out with sparging of media with inert gases (Sarma *et al.*, 2013b; Selembo *et al.*, 2009; Sittijunda *et al.*, 2012). Nitrogen usage during the initial step of media sparging is a tedious process and sparging of nitrogen after inoculum step poses high risk for contamination (Morsy, 2014). Complete anaerobic condition during glycerol metabolism shifts *Enterobacter and Clostridium* strains to increased 1,3-PD production and decreased H_2 production (Barbirato *et al.*, 1995; Chatzifragkou *et al.*, 2011; Choi *et al.*, 2011; Jitrwung *et al.*, 2011). The high reduction state of glycerol predominates *Enterobacter* and *Clostridium* strains towards reductive pathway production of 1,3-PD under anaerobic conditions (Barbirato *et al.*, 1995; Papanikolaou *et al.*, 2004). In order to shift from reductive to oxidative pathway, acetate as co-substrate acted like an electron acceptor with increased H_2 production (Heyndrickx *et al.*, 1991).

Therefore, to minimize the production cost, without co-substrate, nitrogen sparging step was eliminated and microaerobic conditions were created which were investigated for hydrogen production using the co-culture system. In this study, improvement in the process by eliminating nitrogen sparging step during H_2 production led to strain-based metabolic-pathway shift with increased H_2/ethanol/lactate and decreased 1,3-PD production. In fact, this is the very first report of using crude glycerol with co-culture system for hydrogen production in the presence and absence of nitrogen sparging and detailing the metabolic pathway shift.

Materials and Methods

Microorganisms, pre-culture media and inoculum development

Microorganisms (*Enterobacter aerogenes* NRRL B-407 and *Clostridium butyricum* NRRL B-41122) used in this study are collected from ARS (USDA, USA). Basal synthetic medium consisting of (w/v): 1.0 % glucose, 2.0 % casein polypeptone, 0.2 % KH_2PO_4, 0.05 % yeast extract and 0.05 % $MgSO_4.7H_2O$ at pH 6.5 was used to pre-culture *E. aerogenes* anaerobically at 30 ± 1 ºC. Modified basal medium supplemented

with 0.1 % L-cysteine-HCl.H_2O as a reducing agent, was used to pre-culture *C. butyricum* anaerobically at 36 ± 1 ºC (Yokoi *et al.*, 1998). The media components were mixed in distilled water; initial pH was adjusted to 6.5 ± 0.3 by NaOH and final volume of 47.5 mL was transferred into 125 mL serum bottles. The total working volume was 50 mL including 5 % (v/v) inoculum and remaining 75 mL of headspace volume was used for gas collection (Pachapur *et al.*, 2015).

The media was sparged with pure N_2 gas for 3 min to create anaerobic conditions (Sarma *et al.*, 2013b), later sealed using seals with pre-inserted septa (Headspace 20mm Crimp Seals with Septa, Thermo Scientific™) and sterilization was carried out using autoclave (Tuttnauer 3870-Heidolph). The log phase culture broth was transferred using sterile syringe (All-Plastic Norm-Ject™ Syringes, Thermo Scientific™) at 5 % (v/v) inoculum and the cultures were incubated at 150 rpm at 36 ± 1 ºC in an orbital incubator shaker (INFORS HT–multitron standard) (Pachapur *et al.*, 2015; Sarma *et al.*, 2013b).

Source of substrate, chemicals and reagents

Crude glycerol was supplied by Rothsay®, Canada, contained (w/w) up to 23.6 % of glycerol, 35.9 % carbon and 3.2 % nitrogen content and the chemicals, reagents used in this study are the same as referred in Sarma *et al*. (Sarma *et al.*, 2013b).

Hydrogen production using modified basal media

Hydrogen production by co-culture was investigated using modified basal media, containing 1 % crude glycerol (instead of glucose used during inoculum step), 2 % casein polypeptone, 0.2 % KH_2PO_4, 0.05 % yeast extract and 0.05 % $MgSO_4.7H_2O$. The media preparation, nitrogen sparging and sterilization step was carried out in a similar manner as that of inoculum development. The experiment was set up using mono- and co-culture at the inoculum size of 5 % (v/v) and with 1:1 ratio of precultured broth of *E. aerogenes* and *C. butyricum* for co-culture system. During sampling of gas, 15 mL of gas sample using gas tight syringe was released simultaneously at different intervals until 168 h to increase the production of hydrogen (Ananyev *et al.*, 2012; Marques *et al.*, 2009) with 1 mL of gas sample collected for hydrogen analysis. Aqueous sample at the end of fermentation was analyzed for glycerol and end metabolite concentration.

Hydrogen production with and without nitrogen sparging

To study the effect of nitrogen sparging during hydrogen production, modified basal media preparation was sparged with pure nitrogen (for anaerobic condition) for 3 min into test samples and a set without sparging (for aerobic condition) was carried out. The serum bottles were immediately sealed with pre-inserted septa. Subsequently, autoclave step, inoculum step and sampling step using co-culture system was carried out as described earlier.

Hydrogen production under micro-aerobic conditions

To study the amount of oxygen in headspace necessary for the H_2 production microaerobic condition was carried out. To create microaerobic condition in nitrogen sparged sample sets, about 5 mL of headspace nitrogen was replaced with 5 mL of air using air tight syringe, which is 6.67 % of 75 mL headspace volume. Likewise, sample set with 10 (13.33 %), 20 (26.66 %), 37.5 (50 %) and 50 mL (66.66 %) air in headspace was created. Subsequently, autoclave step, inoculum step and sampling step using co-culture system was carried out as described earlier.

Hydrogen analysis by GC

Gas chromatography (Varian 3800, USA) equipped with a thermal conductivity detector (TCD) fitted with a 3 m PoraPLOT Q® column (Agilent technology, USA) was used for gas sample analysis. Nitrogen gas with a flow rate of 3.5 mL/min with injector, column temperature and detector temperature set at 100 ºC, respectively was used. The volume of gas produced was converted to mmol, considering the experimental temperature and atmospheric pressure (Sarma *et al.*, 2013b).

Analysis of by-products by GC-FID

The aqueous samples at the end of fermentation were centrifuged (Minispin plus, Eppendorf) at 6708 x g for 10 min and filtered through 0.2 µm syringe filters prior to analysis. The concentrations of substrate (glycerol) and by-products (ethanol, acetic acid, butyric acid, 1,3-propanediol (1,3-PD)) were verified by gas chromatography (GC) (7890B GC-Agilent, Santa Clara, CA) with a flow rate of 1 mL/min over the ZB-WAXplus column (30 mm x 0.25 mm, 0.25 µm film thickness) using flame ionization detector (FID)

detector with a helium carrier gas at a temperature profile of 80–240 ºC with 8.4 min run time (Heyndrickx *et al.*, 1991).

Results and Discussion

Hydrogen production with and without nitrogen

In this experiment, hydrogen production using 1 % CG with modified basal media components with (anaerobic condition) and without (aerobic condition) nitrogen sparging was tested. The hydrogen production for the co-culture system with and without nitrogen sparging is presented in Figure 5.1.1. The effect of nitrogen sparging resulted in lower hydrogen production (21.40 mmol/L) in comparison to without sparging (26.14 mmol/L). The sufficient amount of oxygen present inside the headspace necessary for the increased H_2 production is assessed by carrying out microaerobic condition.

Hydrogen production under micro-aerobic condition

After sparging the media with nitrogen gas, the volume of gas inside the sealed serum bottle was replaced with increasing volume of air from 5 mL to 50 mL. The results obtained for hydrogen, 1,3-PD and ethanol in the presence of different volumes of air are represented in Figure 5.1.2. Increasing amount of air in the headspace of the serum bottle resulted in marginal increase in amount of hydrogen production. However, the amount of cumulative hydrogen reached a maximum of 139.6 mmol/L of media without sparging. This indicated that in the presence of complete air in the headspace, the co-culture system was able to produce the maximum amount of hydrogen. Soon after the end of the fermentation, the liquid samples were analyzed for metabolites (1,3-propandieol and ethanol).

From Figure 5.1.2, it can be seen that 1,3-PD production decreased from 3 g/L (under nitrogen sparged condition) with increasing amount of air in the headspace lowest production of 0.5 g/L in the case of without sparging. The pattern for ethanol and lactate production was completely contrary to 1,3-PD. In the case of media sparged with nitrogen, the amount of ethanol and lactate production was around 0.6 and 0.4 g/L and for media without sparging, it was around 1.4 and 1.0 g/L. The production of hydrogen,

1,3-PD, ethanol and lactate followed a trend depending on the presence of air, which can be properly explained using fermentation of glycerol by reductive and oxidative pathways.

Glycerol metabolism and hydrogen production followed oxidative and reductive pathway for *Enterobacter* and *Clostridium* species. In the oxidative pathway, NAD^+ gets reduced to NADH with glycerol entering glycolysis to produce pyruvate (Sarma *et al.*, 2012). The pyruvate, depending upon the microorganism will be broken down to various products (ethanol, lactate, acetate, butyrate, among others) along with hydrogen production. Ethanol is the common by-product produced by *Enterobacter* (Ito *et al.*, 2005, Jitrwung *et al.*, 2011) and *Clostridium* (Heyndrickx *et al.*, 1991) species during hydrogen production using CG. During the reductive pathway, NADH is reoxidized to NAD^+ with glycerol getting dehydrated into 1,3-propanediol (Sarma *et al.*, 2012). The summarized pathway of both the microorganisms is presented in Figure 5.1.3.

During anaerobic condition, 1,3-PD regenerates NAD^+ required for the oxidative pathway, which results in production of H_2. Since both the hydrogen producing microorganisms synthesize 1,3-PD to regenerate NAD^+, the reductive pathway becomes an alternative source of electron sink (Choi *et al.*, 2011). These two pathways work simultaneously during the anaerobic conditions resulting in the production of 1,3-PD along with H_2 and ethanol as seen in Figure 5.1.1. Production of 1,3-PD releases H_2O with no release of H_2 and consumes NADH during reductive pathway, causing decrease in H_2 production (Heyndrickx *et al.*, 1991; Sarma *et al.*, 2012). The shift from anaerobic to aerobic extends the lag phase of H_2 production (Morsy, 2014), but the co-culture system of *E. aerogenes* and *C. butyricum* possesses the ability to utilize oxygen to create aerobic condition to recover H_2 production (Yokoi *et al.*, 1998). However, during aerobic condition, oxygen served as electron acceptor that consumed NADH to regenerate NAD^+, providing an optimal condition for *E. aerogenes* to produce H_2 and ethanol. The results obtained in Figure 5.1.1 was supported by Choi *et al*. (Choi *et al.*, 2011), and Jitrwung and Yargeau (Jitrwung *et al.*, 2011). In the case of *C. butyricum* due to insufficient anaerobic environment under self-generated anaerobic conditions seems to impose phosphoroclastic reaction with the reductive pathway of pyruvate to

lactate is favored resulting in decreased production of 1,3-PD in the absence of nitrogen sparging. The concentration of lactate under anaerobic conditions was around 0.47 g/L in comparison to 0.96 g/L under aerobic condition (data not shown). The results obtained in Figure 5.1.1 was supported by Chatzifragkou *et al*. (Chatzifragkou *et al.*, 2011). The diversity of glycerol fermentation products depends on NADH/NAD^+ ratio. Higher ratio favors the reductive pathway in both the strains with production of 1,3-PD. During anaerobic to aerobic switch, the ratio significantly decreases and favors oxidative pathway. In the case of *C. butyricum* under acidogenic conditions, NADH gets reoxidized with H_2 production along with ferredoxin oxidoreductase (FD ox) and hydrogenase enzymes are produced (as seen in Figure 5.1.3). The FD ox regulates the ratio of NADH/NAD^+ and acetyl-CoA/CoA with extra reducing powers getting synthesized into H_2. In the case of *E. aerogenes*, the hydrogenase produces H_2 from the formate derived by splitting pyruvate-formate lyase (Nakashimada *et al.*, 2002). The glycerol fermentation with possible route of H_2 release along with metabolites production through carbon balance is shown as below in equations 1-6 (Barbirato *et al.*, 1995; Biebl *et al.*, 1992):

1,3-Propanediol: $C_3H_8O_3 + NADH + H^+ \rightarrow C_3H_8O_2 + H_2O + NAD^+$ (1)

Acetate: $C_3H_8O_3 + H_2O + 2NAD^+ + 2ADP \rightarrow C_2H_4O_2 + HCOOH + H_2 + 2NADH + 2H^+ + 2ATP$ (2)

Butyrate: $2\ C_3H_8O_3 \rightarrow C_4H_8O_2 + 2CO_2 + 2H_2 + 2NADH + 2H^+$ (3)

Ethanol: $C_3H_8O_3 + ADP \rightarrow C_2H_6O + HCOOH + ATP$ (4)

Formic acid: $C_3H_8O_3 + H_2O + 2Co\text{-}A \rightarrow 2(CH_2O_2) \rightarrow 2CO_2 + 2H_2 + 2CH_3\text{-}CoA$ (5)

Lactate: $C_3H_8O_3 + NAD^+ + ADP \rightarrow C_3H_6O_3 + NADH + H^+ + ATP$ (6)

In co-culture system, the production of hydrogen, 1,3-PD and ethanol depends on the presence or absence of oxygen to follow either reductive or oxidative pathway. From Figure 5.1.2, it was confirmed that amount of oxygen present in the headspace was the optimum requirement for the co-culture system for increased production of hydrogen

and ethanol from crude glycerol. Jitrwung *et al*. [6], reported that during glycerol fermentation, *E. aerogenes* required externally-supplied oxygen at a lower concentration to produce hydrogen and ethanol (Jitrwung *et al.*, 2011). Choi *et al*. (Choi *et al.*, 2011), used *Kluyvera cryocrescens* S26 bacteria belonging to *Enterobacteriaceae* family to convert CG into ethanol under microaerobic condition where lower concentration of oxygen served as electron acceptor and favored ethanol production (Choi *et al.*, 2011). Excess of oxygen supply integrates carbon mass into cellular mass for CO_2 production and high oxygen concentration regulates more carbon flux towards biomass production (Choi *et al.*, 2011). In order to eliminate this condition, the experiment for micro-aerobic and aerobic conditions was carried out within the availability of oxygen inside the headspace. The presence of oxygen converted NADH into NAD^+ and maintained the carbon flux towards the oxidative pathway producing more of ethanol and hydrogen by the co-culture system as seen in the Figure 5.1.2. The main function of 1,3-PD was to regenerate NAD^+ so that NAD^+ gets reduced during oxidative pathway. In the absence of oxygen when media was sparged with nitrogen, the production of 1,3-PD was higher

The effect of nitrogen sparging on hydrogen, 1,3-PD and ethanol production for both *Enterobacter* and *Clostridium* are presented in the Figure 5.1.4. The results confirmed that amount of oxygen in headspace was sufficient by *E. aerogenes* for optimum production of hydrogen and ethanol. In the case of nitrogen sparged media, *E. aerogenes* was able to produce higher 1,3-PD (3245 mg/L) with low hydrogen (9.52 mmol/L) and ethanol (370 mg/L) and in its absence, lower 1,3-PD (450 mg/L) with higher hydrogen (15.87 mmol/L) and ethanol (675 mg/L) was produced. Under anaerobic condition, to regenerate NAD^+, *E. aerogenes* synthesized 1,3-PD and during aerobic condition, oxygen regenerated NAD^+ was used by *E. aerogenes* to synthesize hydrogen and ethanol. These results are well supported by Jitrwung and Yargeau *et al*. and Choi *et al*., with *E. aerogenes* and *K. cryocrescens* S26 requiring lower oxygen to produce hydrogen and ethanol. They also suggested that oxygen played a key role in shift between metabolite production and excess oxygen played a dominant role in biomass formation. The effect of nitrogen on hydrogen production was almost similar

with respect to *Clostridium*, suggesting a similar kind of behaviour to that of *E. aerogenes*. *Clostridium* species during glycerol fermentation followed reductive pathway to produce 1,3-PD (González-Pajuelo *et al.*, 2005) under anaerobic condition as seen in Figure 5.1.3. However, for *C. butyricum*, in the presence of oxygen, concentration of 1,3-PD was decreased (from 2580 to 720 mg/L) with increase in the production of hydrogen (from 16.2 to 17.5) and lactate (from 347 to 560 mg/L). The glycerol metabolism by *Clostridia* can be regulated for the production of either reduced product 1,3-PD (Nakashimada *et al.*, 2002) or oxidized product lactic acid (Wilkens *et al.*, 2012). The presence of reducing agent (L-cysteine) in the media helped mono-culture of *C. butyricum* to overcome the aerobic condition and caused a shift in the metabolic pathway towards H_2 production instead of 1,3-PD production under anaerobic condition. The co-culture system of *E. aerogenes* and *C. butyricum* possesses the characteristic to utilize oxygen to create anaerobic condition and to recover H_2 production (Yokoi *et al.*, 1998). Even evolution of oxygen tolerance mechanism was seen in strict anaerobes (Leja *et al.*, 2014) with additional lag phase of 10 h observed before H_2 production (Yokoi *et al.*, 1998). Leja *et al*. (Leja *et al.*, 2014), investigated the aero-tolerance of *Clostridium bifermentans* and its ability for 1,3-PD production in the presence of oxygen. Leja *et al*. (Leja *et al.*, 2014), concluded that some of these strict anaerobic bacteria were able to survive short-time oxygenation period and they continued their growth with metabolite production after resuming the anaerobic condition (Leja *et al.*, 2014). The mono-culture of *E. aerogenes* and *C. butyricum* under aerobic condition was able to produce more hydrogen with reduced 1,3-PD in comparison to anaerobic conditions. The amount of H_2 produced during co-culture was higher in comparison to mono-culture, suggesting the co-existence of both cultures during co-culture system.

The summary of glycerol fermentation in the presence and absence of nitrogen sparging is presented in the Figure 5.1.5. During anaerobic process, H_2 is produced along with 1,3-PD production, however the co-culture system possesses the ability to produce more H_2 under aerobic condition with trace amount of 1,3-PD production. The yield of 1,3-PD during N_2 sparging was around 0.43 (mol/mol of glycerol) and sudden shift towards aerobic process resulted in decreased yield of 0.04. The decrease in the

yield of 1,3-PD resulted in increased ethanol and lactate yield from 0.15 and 0.06 (N_2 sparged) to 0.33 and 0.12 (mol/mol of glycerol) under aerobic conditions (as shown in Figure 5.1.5). Similarly, hydrogen yield during N_2 sparged was around 1.2 mol-H_2/mol of glycerol in comparison to 1.5 mol/mol of glycerol, indicating the increased yield with aerobic condition. The shift of carbon flux from reductive pathway production of 1,3-PD towards oxidative pathway resulted in increased H_2, ethanol and lactate yield. This supported the findings for anaerobic to aerobic process shift, the metabolic pathway shift from reductive towards oxidative pathway for increased production of hydrogen under co-culture system of *E. aerogenes* and *C. butyricum* was achieved. The symbiotic relation existed in co-culture system, with *E. aerogenes* acting as reducing agent to promote the growth conditions of *C. butyricum* and *C. butyricum* stimulating *E. aerogenes* to reach high experimental H_2 yield by eliminating nitrogen sparging step without expensive reducing agent. Nowadays, nitrogen sparging step is eliminated during H_2 production to reduce process cost and simplify the fermentation process (Morsy, 2014) and application during commercial scale production is relatively expensive and uneconomical (Vatsala *et al.*, 2008), separated CO_2 from H_2-containing gas mixture produced during H_2 fermentation can be used for sparging as an alternative replacement for costly high purity N_2 (Kim *et al.*, 2012). Eliminating the nitrogen sparging step during H_2 production will minimize the overall process cost and will increase the field-scale application of biodiesel plant.

Conclusion

The current study investigated the nitrogen sparging effect on hydrogen production by *E. aerogenes* and *C. butyricum* in a co-culture system from crude glycerol to reduce the hydrogen fermentation costs. This study can be used to characterize a set of optimal environmental conditions in order to favor the metabolic pathway that leads to the increased H_2 production. Eliminating the use of nitrogen sparging without affecting the hydrogen production would be cost effective process for industrial application. Using 1 % of crude glycerol without nitrogen sparging resulted in hydrogen yield of 1.5 mol-H_2/mol of glycerol in comparison to 1.2 for nitrogen sparged media. Similarly, ethanol yield during N_2 sparging was around 0.15 in comparison to 0.33, lactate yield was 0.06

to 0.12 indicating the increased yield with aerobic condition. The yield of 1,3-PD during N_2 sparging was around 0.43 and sudden shift towards aerobic process resulted in decreased yield of 0.04. Decision on tedious nitrogen sparging step during co-culture system can offer a new improved strategy for H_2 production at lower cost and reduce the metabolite production, bringing down the product recovery cost and streamline the fermentation process. The process based improvement strategy demonstrated the ability to shift the carbon flux towards H_2 production that will make H_2 production from crude glycerol more efficient and economical process. This co-culture strategy without nitrogen sparging makes a potential breakthrough for industrial conversion of crude glycerol into hydrogen since the improvement is very simple and not subjected to use of genetically engineered microorganism for increasing hydrogen production.

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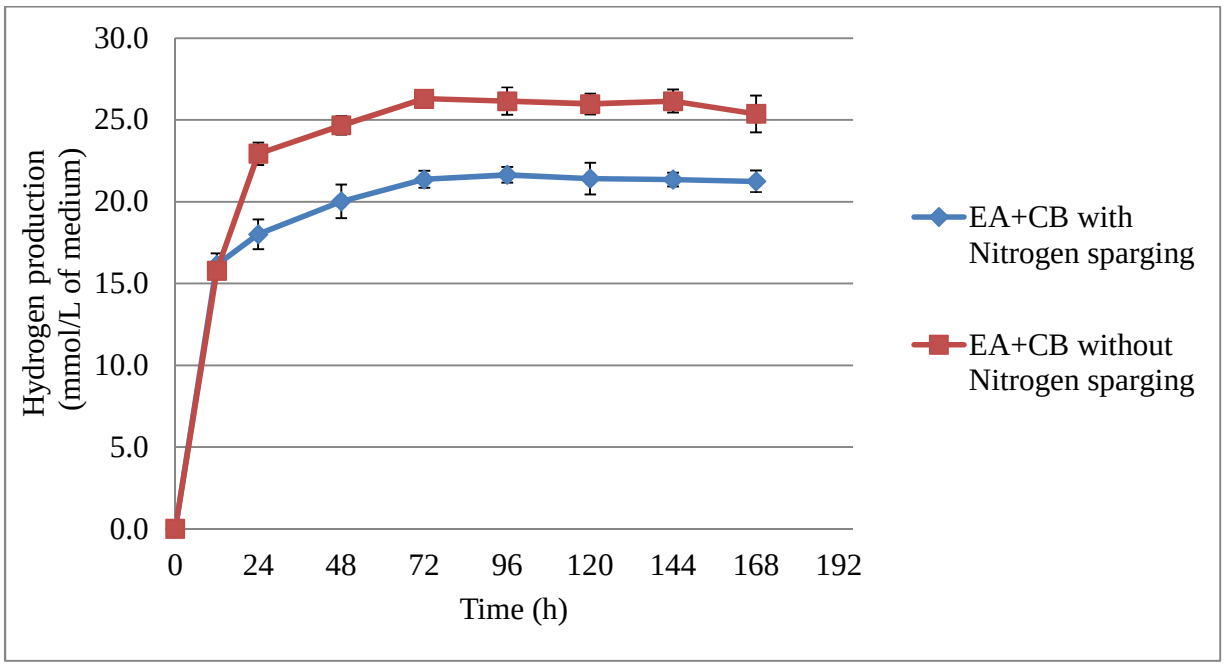

Figure 5.1.1: Effect of nitrogen sparging on hydrogen production using *Enterobacter aerogenes* (EA) and *Clostridium butyricum* (CB) in modified basal media.

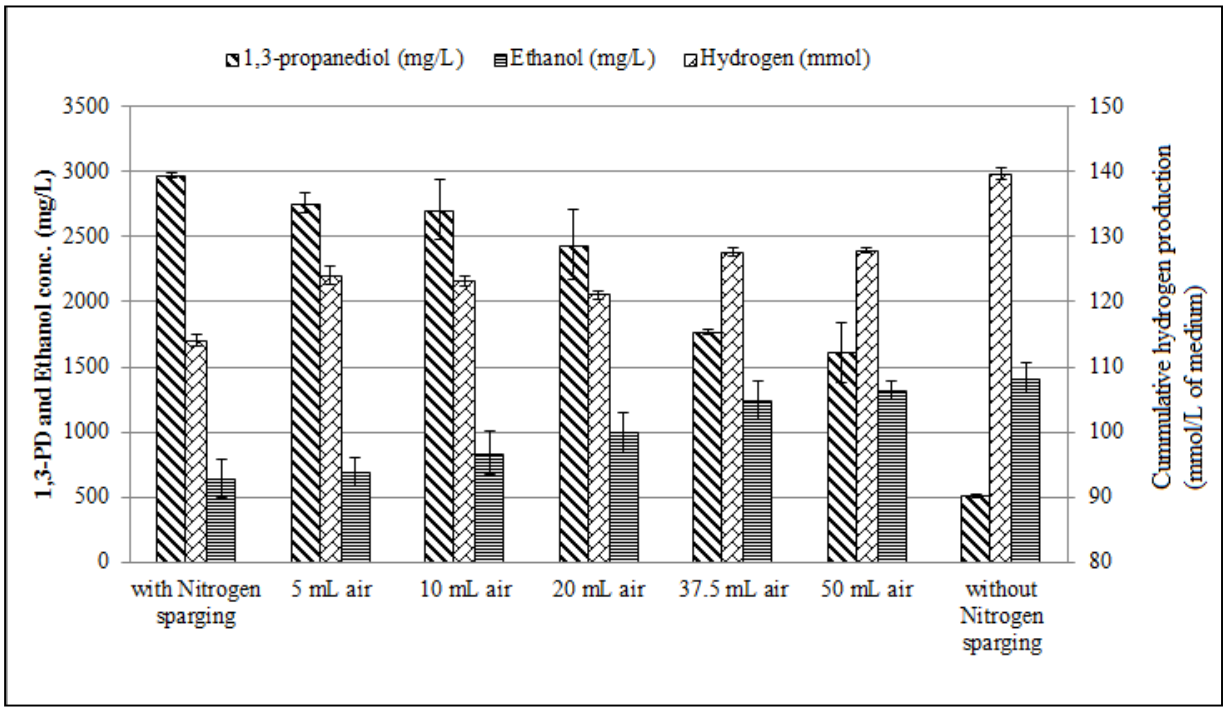

Figure 5.1.2: Effect of different amounts of micro-aerobic conditions on cumulative hydrogen (mmol/L of medium) along with 1,3-PD and ethanol production using *Enterobacter aerogenes* (EA) and *Clostridium butyricum* (CB) in modified basal media.

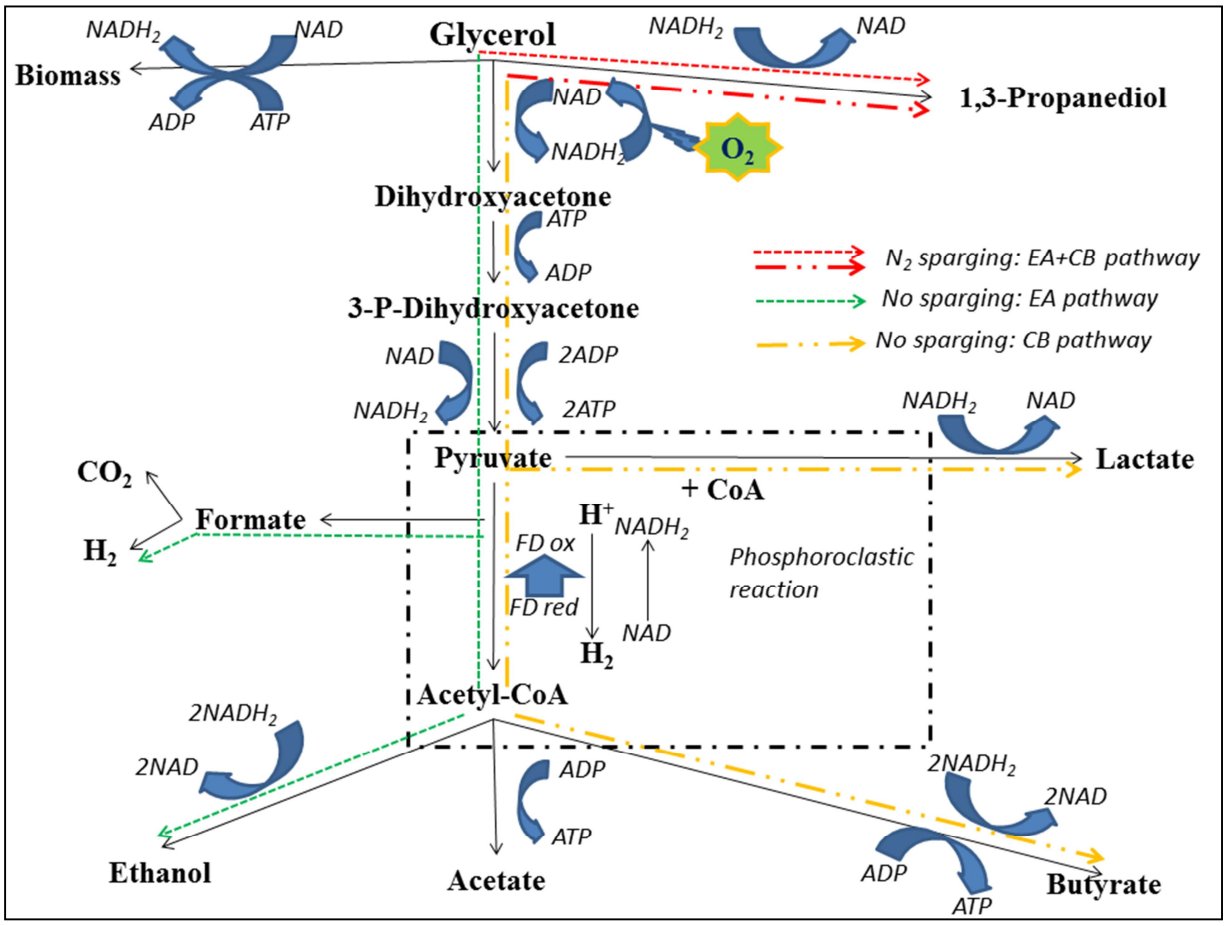

Figure 5.1.3: Common pathways of glycerol metabolism observed in the *Enterobacter* (Barbirato *et al.*, 1995) and *Clostridium* species (Ayure *et al.*, 2010; Zeng, 1996). Effect of nitrogen sparging on metabolic pathways observed for *Enterobacter aerogenes* (EA) and *Clostridium butyricum* (CB).

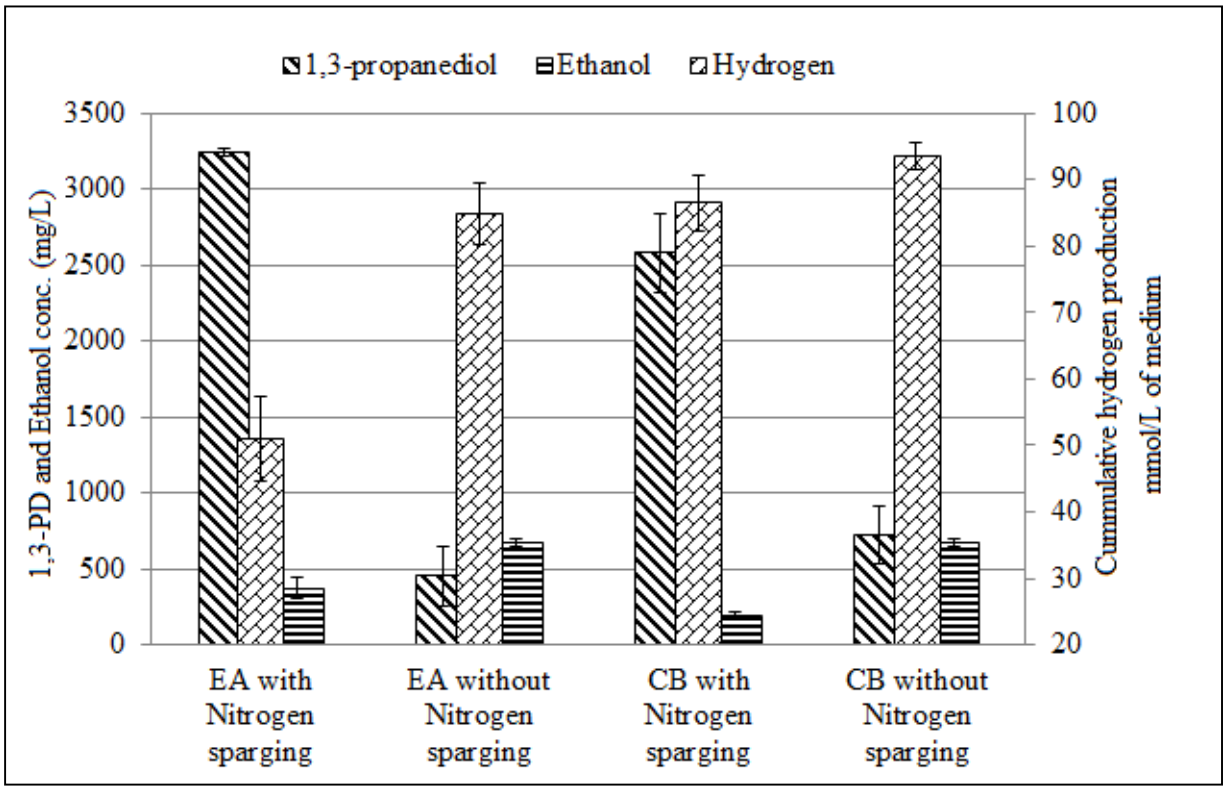

Figure 5.1.4: Hydrogen in mmol/L along with 1,3-PD and ethanol production in mg/L using 1 % crude glycerol substrate for *Enterobacter aerogenes* (EA) and *Clostridium butyricum* (CB) in modified basal media under (+) and (-) sparging condition.

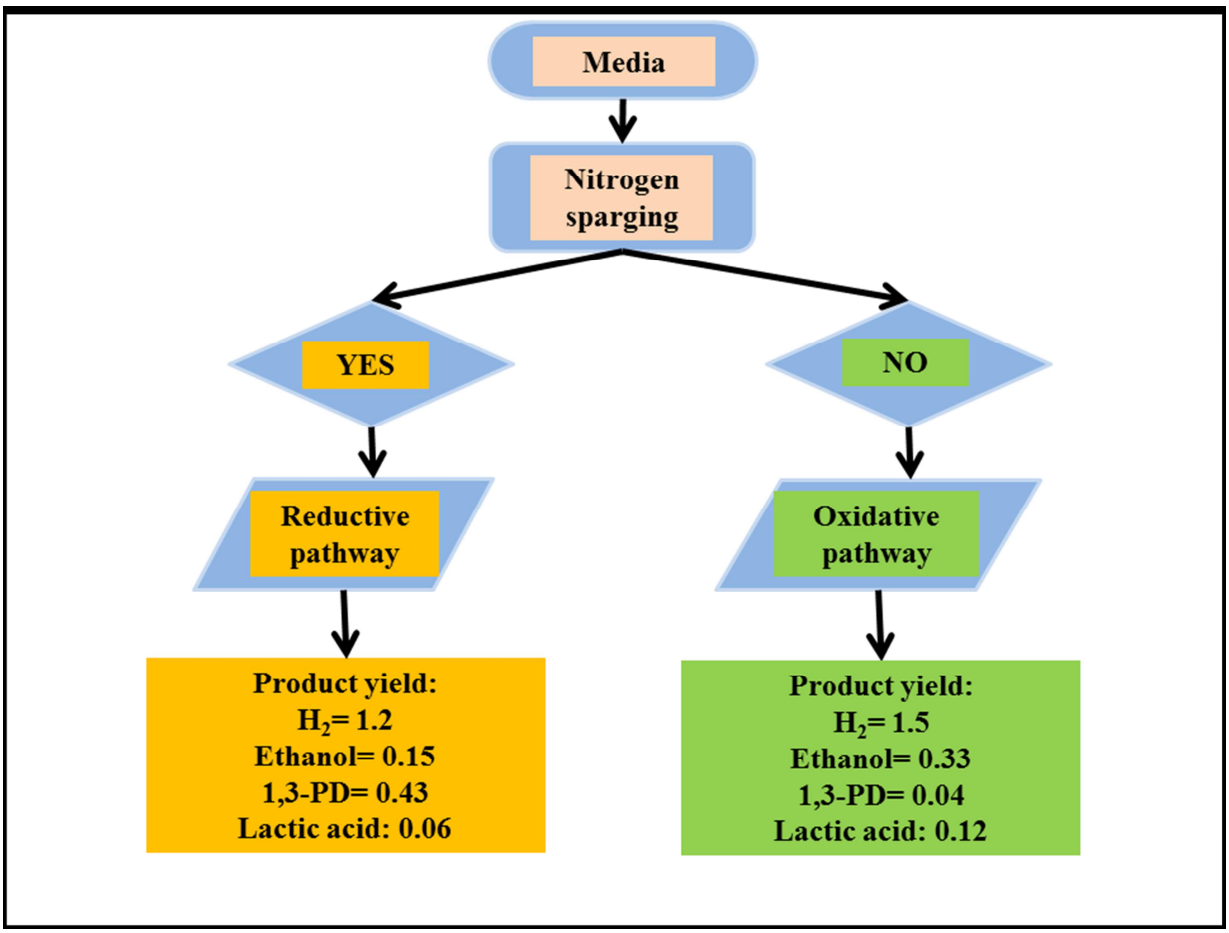

Figure 5.1.5: Summary of glycerol fermentation: in the presence of oxygen, it follows oxidative pathway for production of hydrogen with ethanol and lactate in the absence of oxygen, it follows reductive pathway for production of 1,3-PD.

PART 2

SURFACTANT MEDIATED ENHANCED GLYCEROL UPTAKE AND HYDROGEN PRODUCTION FROM BIODIESEL WASTE USING CO-CULTURE OF *ENTEROBACTER AEROGENES* AND *CLOSTRIDIUM BUTYRICUM*

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Résumé

Dans la présente étude, le Tween 80, un agent tensioactif non ionique, a été utilisé pour améliorer la production d'hydrogène à partir du GB en utilisant la bioconversion par une co-culture de *Enterobacter aerogenes* et *Clostridium butyricum*. Le but de l'introduction de l'agent tensio-actif est de diminuer la viscosité du glycérol brut, de sorte que la solubilité et la biodisponibilité apparente du glycérol puissent être améliorées au détriment des étapes de prétraitement. Des expériences ont été planifiées en utilisant le concept plan central composite (CCD); les concentrations de GB et Tween 80 ont été optimisées alors que la production de H_2, l'utilisation du glycérol et de la viscosité des médias ont été considérés comme des réponses. La surface de réponse pour le modèle quadratique montre que la concentration de Tween 80 a un effet significatif ($p < 0,05$) sur les trois réponses. En utilisant les conditions optimisées à 17,5 g/L de GB et 15 mg/L de Tween 80, la production de H_2 a atteint un maximum de 32,1 ± 0,03 mmol/L de milieu. L'augmentation de la production de H_2 est d'environ 1,25 fois, en présence de Tween 80 par rapport à son absence, avec 25,56 ± 0,91 mmol/L de production. Des conditions optimales sélectionnées ont également été validées par rapport à l'absence de GB (4,69 ± 0,76 mmol/L), l'utilisation de GB prétraitée (20.06 ± 0.51) et à travers le système de mono-culture (15,43 ± 0,79 à 22,14 ± 0,94). L'introduction du Tween 80 dans le milieu de fermentation améliore le taux d'utilisation du glycérol, ce qui entraîne une augmentation de la production de H_2 et permet d'éliminer les étapes de prétraitement.

Mots clés: Co-culture; glycérol brut; hydrogène; tween 80; viscosité

Abstract

In the present study, Tween 80, a non-ionic surfactant, has been used for enhanced hydrogen production by crude glycerol bioconversion using co-culture of *Enterobacter aerogenes* and *Clostridium butyricum*. The purpose of introducing the surfactant was to decrease the crude glycerol viscosity, so that apparent solubility and bioavailability of glycerol could be improved at the expenses of pretreatment steps. Experiments were planned using central composite design (CCD); crude glycerol and Tween 80 concentrations were optimized whereas, hydrogen production, glycerol utilization and viscosity of the media were considered as responses. The response surface for quadratic model showed, Tween 80 concentration had significant effect ($p < 0.05$) on all the three responses. Using the optimized conditions at 17.5 g/L crude glycerol and 15 mg/L Tween 80, hydrogen production reached a maximum of 32.1 ± 0.03 mmol/L of medium. The increase in hydrogen production was around 1.25-fold in presence of Tween 80 in comparison to its absence with 25.56 ± 0.91 mmol/L production. Selected optimum conditions were also validated against absence of crude glycerol (4.69 ± 0.76), with pretreated crude glycerol (20.06 ± 0.51) and across mono-culture system (15.43 ± 0.79 to 22.14 ± 0.94). Introduction of Tween 80 to the fermentation medium improved the glycerol utilization rate, resulting in increased hydrogen production and eliminated pretreatment steps.

Keywords: Co-culture; crude glycerol; hydrogen; tween 80; viscosity

Introduction

Biodiesel is an abundant, secure, 100% renewable and sustainable alternative fuel option for fossil fuels (Ayoub *et al.*, 2012; Mangayil *et al.*, 2015). Potential market of biodiesel with improved governmental policies and incentives is promoting global biodiesel demand (Ayoub *et al.*, 2012; Chatzifragkou *et al.*, 2010; Nwachukwu *et al.*, 2013). With increasing annual growth of 42%, the global biodiesel production would reach 159 billion liters by 2020. The surplus production of biodiesel will generate 16 billion liters of crude glycerol as waste by-product by 2020 (Nwachukwu *et al.*, 2013, Pachapur *et al.*, 2015a). Sustainable production with commercialization of biodiesel requires transformation of crude glycerol into value-added products (Chatzifragkou *et al.*, 2010; Mangayil *et al.*, 2015). Emerging research areas are concentrating on ways to increase crude glycerol as feedstock for appreciable applications to indirectly support and strengthen the biodiesel industry (Ayoub *et al.*, 2012).

Increasing abundance and decreasing market price, makes crude glycerol a potential substrate for biological fermentation process to produce hydrogen and ethanol using microorganisms (Ito *et al.*, 2005; Nwachukwu *et al.*, 2013). Hydrogen production is more attractive for its high energy content and considered as future clean energy source (Dutta *et al.*, 2014; Ito *et al.*, 2005; Selembo *et al.*, 2009). Microbial hydrogen production using co-culture delivers advantages with higher yield in lesser time, perform complex functions, ensure stability and better performance in comparison to mono- or mixed-culture systems (Pachapur *et al.*, 2015b). Previous studies by authors using co-culture system resulted in increased hydrogen production in comparison to monoculture system (Pachapur *et al.*, 2015a; Pachapur *et al.*, 2015c; Pachapur *et al.*, 2015d).

Hydrogen production depends on crude glycerol characteristics and composition of major impurities, such as methanol and soap (Ito *et al.*, 2005; Ngo *et al.*, 2011; Nwachukwu *et al.*, 2013). Methanol inhibition is taken care of during media sterilization (Ito *et al.*, 2005; Sarma *et al.*, 2012). Effect of soap is lessened by initial pretreatment step of decreasing viscosity of crude glycerol by mixing with distilled water (1:4 *v/v*), followed by pH adjustment and centrifugation step to remove precipitated free fatty

acids (Athalye *et al.*, 2009; Chi *et al.*, 2007; Ethier *et al.*, 2011; Ngo *et al.*, 2011). Removal of soap resulted in decreased hydrogen production, suggesting that soap presence played a role of buffering agent and had beneficial effect on glycerol utilization (Sarma *et al.*, 2014). Addition of surfactant at low concentration in the glycerol media reduces the surface tension thereby decreases the viscosity of the solution (Wang *et al.*, 1996). Application of surfactant during ethanol production have potentially reduced the operating costs by reducing the fermentation time and amount of external enzyme used for hydrolysis of complex substrate (Eriksson *et al.*, 2002; Kaar *et al.*, 1998; Sarkar *et al.*, 2012). In case of anaerobic digestion in presence of surfactant resulted in improved biodegradation along with 70% increase in gas production (Patel *et al.*, 1998).

In this study, along with mixing of crude glycerol and distilled water to decrease the viscosity, addition of the surfactant to further improve the glycerol utilization by microorganisms has also been investigated. With the help of statistical modeling at varying concentrations of crude glycerol and surfactant as input parameters, glycerol utilization and viscosity of the fermentation media as response factors was studied for hydrogen production. The present investigation covers the unexplored side of utilization of surfactant at very low concentration for bioconversion of crude glycerol into hydrogen production using co-culture system of *Enterobacter aerogenes* (*E. aerogenes*) and *Clostridium butyricum* (*C. butyricum*).

Materials and Methods

Microorganisms, pre-culture media and inoculum development

E. aerogenes (NRRL B-407) and *C. butyricum* (NRRL B-41122) were procured from ARS, USDA, USA. Basal synthetic medium consisting of glucose (10 g/L), casein polypeptone (20 g/L), KH_2PO_4 (2 g/L), yeast extract (0.5 g/L) and $MgSO_4.7H_2O$ (0.5 g/L) was used for pre-anaerobic culturing *E. aerogenes* at 30 ºC. Modified basal medium supplemented with L-cysteine-$HCl.H_2O$ (1 g/L) as a reducing agent was used for pre-culturing *C. butyricum* anaerobically at 36 ºC (Pachapur *et al.*, 2015d; Yokoi *et al.*, 1998). Different media components were mixed in distilled water to make-up the volume to 47.5 mL. The initial pH was adjusted to 6.5 by using NaOH and 47.5 mL final medium

was transferred into serum bottles (125 mL). Medium was sparged with pure N_2 gas for 5 min and later sealed using aluminum crimp seals with pre-inserted septa to create anaerobic conditions (Pachapur *et al.*, 2015a). The serum bottles containing medium was sterilized using autoclave (Tuttnauer 3870-Heidolph) at 121 °C, for 20 min. About 5% (*v/v*) inoculum at log phase was transferred using sterile syringe and culture incubation was carried out at 150 rpm in an orbital incubator shaker (INFORS HT–multitron standard) (Pachapur *et al.*, 2015a). According to previous studies, the co-culture system resulted in increased hydrogen production in comparison to mono-culture (Pachapur *et al.*, 2015a; Pachapur *et al.*, 2015c; Pachapur *et al.*, 2015d). Hence, all experiments were carried out using co-culture system in triplicates and the presented values are the averages of triplicates with standard deviation (±) values as error bars.

Source of substrate, chemicals and reagents

Crude glycerol was supplied by Rothsay®, Canada which comprises restaurant and meat processing industry containing cooking oil and animal fats for biodiesel production. The crude glycerol composition contained (*w/w*): glycerol (23.6 %), carbon (35.9 %), nitrogen (3.2 %), ash (3.06 %), and moisture content (5.75 %) with 67.56 % of matter organic non-glycerol (MONG). The crude glycerol pH was around 3.4 ± 0.07 and with methanol recovery carried out by Rothsay Inc. led to methanol fraction of around <1-0.5% (Sarma *et al.*, 2013b). The chemicals and reagents used in this study are the same as mentioned in Pachapur *et al*. (Pachapur *et al.*, 2015a).

Effect of surfactant on hydrogen production

Surfactants, such as Tween 80 have been used in fermentation to assist growth, entry of substrate into cells (Reese *et al.*, 1969), increase performance during fermentation (Lee *et al.*, 1996) with highest bacterial growth (Goes *et al.*, 1999) during enzymes (Goes *et al.*, 1999; Reese *et al.*, 1969), degrade high molecular weight hydrocarbons (Boonchan *et al.*, 1998), increase solubility/yield of substrate (Sarkar *et al.*, 2012) and ethanol (Lee *et al.*, 1996) production. To the best of authors' knowledge, the effect of surfactant on hydrogen production using crude glycerol has not been studied so far. Hence, Tween 80 at varying concentrations (10, 20, 50, 100 and 1000 mg/L) in

presence of (1% crude glycerol + media components), along with positive control (1% crude glycerol + media components) without Tween 80 and negative control (1% Tween + media components) without crude glycerol was tested. The range of Tween 80 concentration was referred to the concentrations used across the fermentation studies for enzyme and ethanol production (Goes *et al.*, 1999; Lee *et al.*, 1996; Reese *et al.*, 1969). In the previous study, 1% crude glycerol resulted in increased hydrogen production across co-culture system in comparison to mono-culture system (Pachapur *et al.*, 2015a). Hence, to study the effect of surfactant on hydrogen production, 1% of crude glycerol was fixed with varying concentrations of Tween 80.

Optimization of hydrogen production using Response Surface Methodology

In order to optimize Tween 80 and crude glycerol concentrations for maximum hydrogen production, response surface methodology (RSM) has been used. Central Composite Design (CCD) was used to design the experiments where hydrogen production was investigated as a function of crude glycerol (substrate) and Tween 80 concentration. Crude glycerol at 10 g/L had been found to be optimum for hydrogen production (Pachapur *et al.*, 2015a; Sarma *et al.*, 2013a). Thus, 10 g/L of crude glycerol has been considered as low value with 25 g/L as high value for the present experimental design. Similarly, Tween 80 at 5 mg/L and 25 mg/L were considered as low and high actual value, respectively. The CCD ranges of the two input factors considered for the study are presented in Table 5.2.1.

Experimental design was developed using Design-Expert®-7 software (Stat-Ease Inc. Minneapolis, MN) resulted in 13 experimental runs. Depending upon each run requirement, exact concentration of crude glycerol and Tween 80 was mixed in distilled water to make-up working volume (47.5 mL) along with media components (casein polypeptone, KH_2PO_4, $MgSO_4.7H_2O$ and yeast extract). After mixing, pH of the final volume for each run was adjusted to 6.5, later steps similar to inoculum development at 36 °C and 150 rpm were followed. During the fermentation, gas were collected every 24 h and liquid samples at the end was analyzed, as described in analytical technique section. Each individual parameter varied from low value: 5 and high value 15, resulted

in only four alpha values (-α= 6.89 and 0.86 and +α= 28.11 and 29.14). Using alpha value, the design generated 4 axial points (runs: 1, 4, 7 and 12), 5 central points (runs: 2, 3, 8, 11 and 13) and rest factorial points.

The relationship between the factors considered in this study, i.e., crude glycerol (g/L) and Tween 80 (mg/L) and the response variables: H_2 production (mmol/L), glycerol utilization (%) and viscosity (cP) of the fermentation media was determined by design matrix evaluation. The response surface for significant interactions in terms of quality of model fit, model term coefficient within p value (≤ 0.05), adjusted R^2 values, final equation and statistical significance was verified for the analysis of variance (ANOVA).

Validation experiments using CCD optimized values

Different concentrations of crude glycerol and Tween 80 were used to generate an optimum value for hydrogen production using CCD. Validation of the optimized conditions (crude glycerol: 17.5 g/L and Tween 80: 15 mg/L) was tested for the mono-culture studies in presence of *E. aerogenes* and *C. butyricum* only. The optimized condition in absence of Tween 80 was also tested. The effect of yeast extract and peptone as carbon source for hydrogen production was determined using optimized conditions in the absence of crude glycerol. In order to determine the effect of crude glycerol pretreatment on hydrogen production, the optimized conditions in presence of pretreated crude glycerol was carried out. The pretreatment method was in accordance to pH adjustment and centrifugation steps carried out by Sarma *et al*. (Sarma *et al.*, 2014) and Athalye *et al*. (Athalye *et al.*, 2009).

Analytical Methods

Hydrogen analysis by GC-TCD

The gas sample collected manually using gas tight syringe (5 mL, SGE Analytical Science, Australia) in sample vials. The hydrogen analysis was carried out using gas chromatography (Varian 3800, USA) setup. A PoraPLOT Q® column (Agilent technology, USA) fitted with thermal conductivity detector (TCD) equipped within carrier gas was nitrogen at a flow rate of 3.5 mL/min with column and detector working

temperature fixed at 100 ºC. H_2 retention time was around 4.5 min and volume of gas produced was converted to mmol/L (Pachapur *et al.*, 2015d).

By-products analysis by GC-FID

The liquid sample for determination of glycerol utilization and by-products (ethanol, butyric acid and 1,3-propanediol) were analyzed using ZB-WAX plus column (30 m x 0.25 mm, 0.25 µm film thickness) fitted with flame ionization detector (FID) within gas chromatography (GC) (7890B GC-Agilent, Santa Clara, CA) setup. The carrier gas, helium at a flow rate of 1 mL/min with a 80–240 ºC temperature profile under 8.4 min method run time was developed for the analysis (Pachapur *et al.*, 2015a).

2.5.3. Analysis of media viscosity

Viscosity measurement of the fermentation media, before and after addition of Tween 80 was performed using a rotational viscometer (Fungilab, Premium Series, USA) setup. The L1 spindle was cleaned using dust-free Kim wipes, sample cup containing 25 mL of fermentation media at constant shear rate (305.7 per sec) with 2 min analysis time (adapted and optimized for the entire study) (Brar *et al.*, 2005).

Results and Discussion

Effect of surfactant on hydrogen production

The effect of surfactant on hydrogen production, using different Tween 80 concentrations (10, 20, 50, 100 and 1000 mg/L) at fixed crude glycerol concentration of (10 g/L) along with positive and negative control is presented in Figure 5.2.1. In positive control, the hydrogen production was around 21.4 ± 0.99 mmol/L of medium, highest production among other Tween 80 concentrations. With further increase in concentration of Tween 80, the hydrogen production started decreasing from 25.7 ± 0.34 to 19.8 ± 0.78 mmol/L and in case of negative control; the hydrogen production was the least with 2.1 ± 0.55 mmol/L as seen from the Figure 5.2.1. The least production of hydrogen was in presence of only Tween 80 (negative control) suggested that the co-culture system depended on crude glycerol as substrate source for hydrogen production. The glycerol utilization among different concentrations of Tween 80 was

within close range of (99.85 ± 0.12 to 88.59 ± 0.45) for positive control (90.47 ± 0.33%) and for negative control was below detection level. In this set of experiments, hydrogen production was the main parameter in pre-selecting the concentration of Tween 80 (10-20 mg/L) for further optimization along with media viscosity measurement. Presence of Tween 80 (10 mg/L) resulted in 20.20% increase in hydrogen production in comparison to 21.4 ± 0.99 mmol/L obtained for the positive control. The crude glycerol concentration of 10 g/L for co-culture system (Pachapur *et al.*, 2015a) in presence of minimum concentration of Tween 80 (10 mg/L) resulted in increased hydrogen production. Hence, the effect of Tween 80 at higher concentration of crude glycerol was investigated using CCD. The effect of Tween 80 across 10 and 20 mg/L resulted in similar glycerol utilization values ranging from 99.85 ± 0.12 % to 98.69 ± 0.48 %. Thus, crude glycerol concentration (10 to 25 g/L) and Tween 80 concentration (5 to 25 mg/ L) were varied across the CCD model.

Optimization of hydrogen production using RSM

The experimental design involving different concentrations of crude glycerol and Tween 80, along with the results obtained for hydrogen, glycerol utilization and viscosity for each experimental run has been presented in Table 5.2.2. The hydrogen production ranged from about 23.7 ± 1.58 mmol/L (run: 4, CG: 17.5 and Tween 80: 29.14) to a maximum of 32.1 ± 0.03 mmol/L (run: 11, CG: 17.5 and Tween 80: 15).

The model p-value of 0.0036 implied statistically significant relation. The "Lack of Fit F-value" of 6.24 implies there is a 5.46% chance that a "Lack of Fit F-value" of this magnitude could occur due to noise.The final model equation (Eq. (1)) in terms of coded factors that best fitted the hydrogen production response is shown below:

$$\text{Hydrogen} = 31.14 - 0.24 \times \text{CG} - 1.92 \times \text{Tween} - 0.63 \times \text{CG} \times \text{Tween} - 2.64 \times \text{CG} \times \text{CG} - 1.74 \times \text{Tween} \times \text{Tween} \quad (1)$$

The coefficient of Tween 80 (1.92) as seen in (Eq. (1)) was much higher than the other ones, indicating that the studied range had a dominant effect on hydrogen production. Summarized ANOVA for the response surface quadratic model in case of H_2 production

with p-value has been presented in Table 5.2.3. The p-value of 0.0047 indicated that the linear dependence of Tween 80 had a significant impact on H_2 production.

The experimental responses of hydrogen production are plotted with the fitting function of crude glycerol and Tween 80 concentration in Figure 5.2.2. At minimum concentration of Tween 80 (5 mg/L) with increasing concentration of crude glycerol, the production of hydrogen increased. However, further increase in the concentration of Tween 80 (> 15 mg/L) decreased the production of hydrogen as seen in Figure 5.2.2.

Crude glycerol and Tween 80 have a parabolic relationship for hydrogen production. At maximum concentration of Tween 80 (29.14 mg/L) for the run 4 in presence of 17.5 g/L of crude glycerol resulted in minimum value of 23.7 ± 1.58 mmol/L of hydrogen production. The optimum concentration of crude glycerol for the co-culture study in our earlier studies was around 10 g/L, further increase in crude glycerol concentration resulted in substrate inhibition causing a decrease in hydrogen production (Pachapur *et al.*, 2015a). In case of minimum concentration of Tween (0.86 mg/L) in presence of 17.5 g/L of crude glycerol, the hydrogen production further increased to 29.8 ± 0.05 mmol/L. The results at two extreme ends of Tween 80 concentration suggested that the optimum condition of Tween 80 for increased hydrogen production was within 5 to 15 mg/L. This is seen in run 11 at crude glycerol concentration of 17.5 g/L with Tween 80 concentration of (15 mg/L) resulting in maximum H_2 production reaching 32.1 ± 0.03 mmol/L. In case of run 7 and 13, the hydrogen production values were closer while considering standard deviation. However, in case of run 7, with lower amount of Tween 80 (0.86 mg/L) resulted in decreased glycerol utilization with 79.4 ± 0.91 % and increased production of 1,3-PD around 4.15 ± 0.94 g/L in comparison to optimized conditions of run 7 with 87.7 ±0.50 % utilization and 3.78 ± 0.18 g/L production. The increased concentration of Tween 80 for run 13 resulted in increased rate of hydrocarbon degradation with increased hydrogen production, and in lower concentration of Tween 80 for run 7 resulted in substrate inhibitor with 1,3-PD production. The increased production of 1,3-PD results in decreased production of hydrogen, as reducing equivalents for hydrogen production are utilized during 1,3-PD production (Pachapur *et al.*, 2015c; Pachapur *et al.*, 2015d). On the contrary, with

further increase in the concentration of crude glycerol to 28.11 g/L in presence of 15 mg/L of Tween 80, hydrogen production reached 25.5 ± 0.63 mmol/L. Using crude glycerol at higher concentration in the fermentation media resulted in decreased hydrogen production and increased production of by-products (Ito *et al.*, 2005) such as 1,3-PD (5.15 ± 0.48 g/L). The presence of Tween 80 in the fermentation media helped to increase the bioavailability of the substrate to the microorganisms (Goes *et al.*, 1999, Sarma *et al.*, 2013b), increase hydrocarbon degradation (Boonchan *et al.*, 1998), increased growth, improved fermentation activity (Lee *et al.*, 1996) and increased substrate utilization rate (Sarma *et al.*, 2011). The effect of Tween 80 on hydrogen production is also well supported by the model p-value of 0.0047 as seen from Table 5.2.3.

The central design points of the model with optimum conditions of crude glycerol: 17.5 g/L and Tween 80: 15 mg/L resulted in increased hydrogen production reaching a maximum value of 32.1 ± 0.03 mmol/L.

Effect of Tween 80 on glycerol utilization

The residual glycerol after fermentation was analyzed by GC-FID; later glycerol utilization in terms of percentage was calculated and used for ANOVA analysis. The quadratic model for glycerol utilization with p-value of < 0.0001 was statistically significant with R^2 value of 0.95 as seen from Table 5.2.3. The final equation (Eq. (2)) that best fitted the response and to compare coefficient of parameters for glycerol utilization, is shown below:

$$\text{Glycerol utilization} = 86.92 - 15.53 \times \text{CG} + 2.32 \times \text{Tween} + 3.55 \times \text{CG} \times \text{Tween} - 2.93 \times \text{CG} \times \text{CG} - 2.50 \times \text{Tween} \times \text{Tween} \quad (2)$$

The coefficient of crude glycerol (15.53) was much higher indicating that the linear dependence of crude glycerol had a significant impact on glycerol utilization as seen from Table 5.2.3. The response of glycerol utilization across the concentrations of crude glycerol and Tween 80 using response surface plot is presented in Figure 5.2.3. The

glycerol utilization ranged from about 58 ± 0.71 % (run: 6, CG: 25 g/L, Tween: 5 mg/L) to a maximum of 99.8 ± 0.02 % (run: 9, CG: 10, Tween: 25) as seen from Table 5.2.2. The increased production of hydrogen resulted in optimized conditions of 17.5 g/L crude glycerol and 15 mg/L of Tween with glycerol utilization of around ~90 %. Increase in the concentration from the optimized conditions for crude glycerol resulted in decreased glycerol utilization. In case runs 1 and 2, 5 and 6, 12 and 13, with increasing concentration of crude glycerol from 15.5 to 28.11, 10 to 25, 6.89 to17.5 g/L, there was decrease in glycerol utilization as seen from Figure 5.2.3 and Table 5.2.2. Across the runs with crude glycerol as input parameter played a very important role in case of glycerol utilization and is well supported by model p-value of <0.0001 (A) and 0.0256 (A^2) as seen from Table 5.2.3.

At maximum concentration of Tween 80 (29.14 mg/L) in presence of 17.5 g/L of crude glycerol, around 82.5 ± 0.25 % of glycerol was utilized. However, at minimum concentration of Tween 80 (0.86 mg/L) at same concentration of crude glycerol (17.5 g/L), the glycerol consumption decreased to 79.4 ± 0.91%. At maximum concentration of crude glycerol (28.11 g/L), in presence of 15 mg/L of Tween 80, the glycerol utilization reached lower value of 60.7 ± 0.66 % indicating substrate inhibition. This suggested higher amount of Tween 80 within 5-15 mg/L required for increased utilization of crude glycerol at 17.5 g/L. Thus, at 17.5 g/L of crude glycerol in presence of 15 mg/L of Tween 80 resulted around~ 87% utilization of crude glycerol with increased hydrogen production suggesting the optimum concentration of Tween 80. The main objective of the study was to produce increased hydrogen production at higher concentration of crude glycerol in presence of Tween 80. Thus, the optimized conditions for run 2, 3, 8, 11 and 13 of crude glycerol (17.5 g/L) and Tween 80 (15 mg/L) together improved the glycerol utilization, which resulted in increased hydrogen production.

Presence of Tween 80 in the fermentation media assisted microorganism growth, increased enzyme activity and improved microorganism-substrate interaction resulting in increased substrate utilization (Goes *et al.*, 1999). The effect of surfactant might have played a role of buffering/acid neutralizing agent by eliminating pH drop and its

presence had beneficial effect with 2.5-fold increase in glycerol utilization (Sarma *et al.*, 2014).

Compared to other approaches, such as immobilization, repeated batch fermentation and two-stage system to increase the substrate utilization for hydrogen production (Yokoi *et al.*, 2001; Yokoi *et al.*, 1998), using less-expensive mode of adding Tween 80 at minimum concentration resulted in increased hydrogen production along with increased substrate utilization rate.

Effect of Tween 80 on media viscosity

The media viscosity after addition of Tween 80 was used in the study. The model terms for viscosity with p-value of 0.0249 (< 0.05) was found to be statistically significant. The final equation (Eq. (3)) that best fitted the response data and to compare coefficient of parameters for viscosity, is shown below:

$$\text{Viscosity} = 2.28 + 0.026 \times \text{CG} - 0.018 \times \text{Tween} + 2.500\text{E}-3 \times \text{CG} \times \text{Tween} + 2.875\text{E}-3 \times \text{CG} \times \text{CG} + 2.875\text{E}-3 \times \text{Tween} \times \text{Tween} \quad (3)$$

The coefficient of crude glycerol (0.026) was slightly higher in comparison to the coefficient of Tween 80 (0.018), indicating the fact that viscosity is dependent on the concentration of crude glycerol. The response of viscosity value across the interaction of crude glycerol and Tween 80 using response surface plots are presented in Figure 5.2.4.

At maximum concentration of Tween 80 (29.14 mg/L) in presence of 15 g/L of crude glycerol showed viscosity of 2.25 ± 0.04 cP. At minimum concentration of Tween 80 (0.86 mg/L) in presence of crude glycerol (15 g/L), the viscosity measurement (2.30 ± 0.03 cP) remained close to the viscosity of fermentation media without addition of Tween 80 at around (2.35 ± 0.02 cP). At minimum concentration of crude glycerol (6.89 g/L) in presence of 15 mg/L of Tween 80 resulted in lowest viscosity value around 2.22 ± 0.02 cP. However, at maximum concentration of crude glycerol (28.11 g/L) in presence of 15 mg/L of Tween 80 resulted in slight lowering of viscosity (2.33 ± 0.04 cP) from (2.35 ± 0.02 cP). Thus, the media viscosity was largely dependent upon the

concentration of crude glycerol at first place, higher the concentration of crude glycerol higher the viscosity as seen from the Figure 5.2.4. The observations are well supported with significant (p-values of 0.0040) contribution of crude glycerol on model response for viscosity.

The concentration of crude glycerol above the optimum value (17.5 g/L) for the run 1 (28.11 g/L) and 10 (25 g/L) even at optimum and increased value of Tween 80 (15 and 25 mg/L) showed minimum reduction in viscosity (2.33 ± 0.04 and 2.29 ± 0.06 cP) with decreased glycerol utilization (60.7 ±0.66 and 72.2 ± 0.75 %) resulting in decreased hydrogen production (24.4 ± 1.43 to 25.5 ± 0.63 mmol/L).

The concentration of crude glycerol above the optimum value caused minimum lowering of viscosity and also resulted in substrate inhibition so that there was decrease in substrate utilization resulting in decreased hydrogen production (as seen for run: 1, 6 and 10 in Table 5.2.2). In case of crude glycerol concentration below the optimum value for the run 5 (10 g/L), 9 (10 g/L) and 12 (6.89 g/L) at varying concentrations of Tween 80 (5, 25 and 15 mg/L) showed complete glycerol utilization (99.5-99.8%) reaching maximum hydrogen to 29.6 ± 1.93 mmol/L. The concentration of crude glycerol below the optimum value was almost completely utilized by the microorganism, so that there was no further increase in hydrogen production even at optimum Tween 80 concentration. However, the effect of surfactant cannot be neglected in decreasing the media viscosity. Optimum crude glycerol concentration (17.5 g/L) and Tween 80 concentration (15 mg/L) for run (2, 3, 8, 11 and 13) helped in lowering the viscosity to minimum (2.27-2.28 cP) in comparison to without addition of Tween 80 at around (2.35 ± 0.02 cP). The optimized condition showed near to complete utilization of crude glycerol reaching (84.7-87.8%) resulting in remarkable 1.5 increase from 21.4 to 32.1 ± 0.03 mmol/L of hydrogen production.

During pretreatment of crude glycerol before hydrogen production, the viscosity of crude glycerol is decreased by mixing with distilled water (Sarma *et al.*, 2012). During additional pre-treatment step with decreasing the viscosity of crude glycerol, the pretreated crude glycerol will be promising feedstock for microbial fermentation in

producing value-added products (Athalye *et al.*, 2009; Chi *et al.*, 2007; Ethier *et al.*, 2011). In case of heavy crude oil for pipeline transportation, additional surfactants are used to decrease the viscosity by suspending heavy oil in a water phase achieving viscosity reduction (Alomair *et al.*, 2013; Guo *et al.*, 2014; Hasan *et al.*, 2010; Qi *et al.*, 2013). On a similar note, improvement of hydrogen production in presence of Tween 80 could be explained as follows: presence of surfactant in the medium would reduce its surface tension, which in turn would help to increase the apparent solubility of glycerol and as a consequence its microbial availability would be enhanced. Due to presence of three -OH groups, glycerol is highly hygroscopic. Therefore, at relatively high concentration, it might have difficulty in passing through the bacterial cell wall. The surfactant-glycerol interactions depend on the concentration of glycerol and critical concentration of surfactant will form micelle on glycerol molecules (Wang *et al.*, 1996). The surfactant hydrophilic groups surround and interact with glycerol hydroxyl group by pointing hydrophobic group outward to form micelles (Wang *et al.*, 1996). The formation of micelle decreases the surface/interfacial tension of water, increases solubility of glycerol and enhances the accessibility of glycerol to microbes for increased utilization rate (Eckard *et al.*, 2012; Eckard *et al.*, 2013; Eriksson *et al.*, 2002). Thus, presence of a surfactant might have beneficial effect during its transportation by increasing its bioavailability. This is just a hypothesis which needs further experimental evidence. However, such detailed investigation is beyond the scope of this study.

The by-product analysis at the end of fermentation resulted in the identification of ethanol, butyric acid and 1,3-PD concentration for different runs presented in Table 5.2.2, (the details of p-value along with response surface plots are presented as supplementary data). Glycerol fermentation by oxidative pathway results in hydrogen production with by-product formation, such as ethanol, acetic acid and butyric acid (Pachapur *et al.*, 2015c). In case of reductive pathway glycerol gets reduced into 1,3-PD with no production of hydrogen (Pachapur *et al.*, 2015c; Pachapur *et al.*, 2015d). For butyric acid formation, 2 mol of hydrogen are produced during glycerol fermentation (Sarma *et al.*, 2012). In this study, the co-culture system followed the oxidative pathway and also reductive pathway with hydrogen production along with ethanol, butyric acid

and 1,3-PD formation as seen in Table 5.2.2. The optimized condition for runs (2, 3, 8, 11 and 13) produced increased concentration of butyric acid averaging around ~3.86 g/L in comparison to other runs. Under optimized condition for runs (2, 3, 8, 11 and 13) the concentration of 1,3-PD was also minimum.1,3-PD formation at 5.15 ± 0.48 to 5.85 ± 0.71 g/L resulted in decreased hydrogen production, which is well supported for the run 1, 6 and 10 for crude glycerol concentration from 25 to 28.11 g/L. Higher concentration of crude glycerol for *E. aerogenes* and *C.butyricum* followed reductive pathway to reduce highly reductive crude glycerol into 1,3-PD (Jitrwung *et al.*, 2013, Szymanowska-Powalowska, 2014). The ethanol production across the optimized values for runs (2, 3, 8, 11 and 13) was within the range of 2.58 to 2.91 g/L. In case of maximum concentration of crude glycerol for runs (1, 6 and 10), the formation of ethanol was on higher side ranging from 4.04 to 4.67 g/L. The increased production of ethanol directly co-related to decreased hydrogen production, as ethanol formation consumed NADH necessary for hydrogenase dependent hydrogen production (Heyndrickx *et al.*, 1991; Pachapur *et al.*, 2015d). The optimum crude glycerol (17.5 g/L) and Tween 80 concentration (15 mg/L) not only favored the glycerol utilization but also channelized the oxidative and reductive pathway in co-culture system with minimum by-products formation for increased hydrogen production.

The analysis of by-product at the end of the fermentation delivered clarity on the pathway followed during glycerol fermentation and also provided the information on the rate limiting by-products formed during hydrogen production.

Hydrogen production across validation experiments

In order to prove the model hypothesis, the validation experiments across different sets using the optimized conditions were tested as presented in Table 5.2.4. The optimized run in the absence of Tween 80 resulted in the decreased hydrogen production around 25.56 ± 0.91 mmol/L in comparison to its presence was 31.07 ± 0.92 mmol/L. The glycerol utilization in absence of Tween was around 77.71 ± 0.45% in comparison to 91.91 ± 0.71% in presence of Tween. The results obtained followed the same trend of hydrogen production at 10 g/L crude glycerol in presence and absence of Tween 80 as

shown in Figure 5.2.1. The presence of Tween 80 supported the hypothesis with increased glycerol utilization along with increased hydrogen production. In the absence of Tween, degradation of crude glycerol is reduced and at a concentration higher than 15 g/L, it results in substrate inhibition for hydrogen production resulting in a pathway shift towards 1,3-PD production (Pachapur *et al.*, 2015d). The concentration of 1,3-PD is at higher range in the absence of Tween 80 at around 3.07 ± 0.14 g/L in comparison to 2.17 ± 0.08 g/L in presence of Tween. In order to determine the effect of yeast extract and polypeptone as a source of substrate on hydrogen production, crude glycerol was omitted from the fermentation medium. In absence of crude glycerol, around 4.69 ± 0.76 mmol/L of hydrogen was produced suggesting the dependency of co-culture system on crude glycerol as substrate for hydrogen production. In the absence of yeast extract and polypeptone, in the earlier studies, the co-culture system was unable to produce increased hydrogen production (Pachapur *et al.*, 2015a). The co-culture system requires addition of these media components (yeast extract and polypeptone) as nitrogen source to maintain balanced carbon/nitrogen ratio during hydrogen production (Yokoi *et al.*, 2001). The purpose of using co-culture system was to obtain increased hydrogen production and to minimize the use of costly reducing agents (Pachapur *et al.*, 2015a). Using a lone hydrogen producer in the case of *C. butyricum* required the addition of costly media component (L-cysteine) (Yokoi *et al.*, 1998). *E. aerogenes* (EA) acted as reducing agent possess the property to remove oxygen and generate anaerobic condition for the growth of *C. butyricum* (CB) (Yokoi *et al.*, 2001). The combination of these two microorganisms resulted in increased hydrogen production during co-culture system in comparison to monoculture across previous studies (Pachapur *et al.*, 2015a; Pachapur *et al.*, 2015c; Pachapur *et al.*, 2015d). The results obtained using optimized conditions for mono-culture (EA: 22.14 ± 0.94 and CB: 15.43 ± 0.79 mmol/L) and co-culture system (31.07 ± 0.92 mmol/L), justified the hypotheses of using co-culture system for increased hydrogen production. *E. aerogenes* prefers crude glycerol over glucose source (Sarma *et al.*, 2012) and was able to produce higher hydrogen in comparison to *C. butyricum. C. butyricum* prefers glucose derived substrate (Pachapur *et al.*, 2015d) and at higher concentration of crude glycerol resulted in

substrate inhibition (with only 13.49 ± 1.10% of glycerol utilization) with decreased hydrogen production. In the present study, the co-culture system showed improved growth in comparison to mono-culture system with increased hydrogen production.

Utilization of crude glycerol requires initial pretreatment step for production of value-added products across studies presented in Table 5.2.5. The pretreatment step of mixing with distilled water, adjusting the pH for precipitation of solids, followed by centrifugation step and addition of media components is necessary to reduce the viscosity of crude glycerol before being used for fermentation (Athalye *et al.*, 2009; Chi *et al.*, 2007; Ethier *et al.*, 2011). Additional pretreatment steps are tedious and increase the production cost, however crude glycerol pretreatment step carried out for hydrogen production resulted with decreased production around 20.06 ± 0.51 mmol/L in comparison to without pretreatment (31.07 ± 0.92 mmol/L). The pretreatment step is carried out to remove crude glycerol inhibitor, such as soap, however soap removal resulted in 93.03% decreased hydrogen production (Sarma *et al.*, 2014). The presence of soap maintained the carbon-nitrogen ratio balance of the medium, acted as buffering/acid neutralizing agent, thus pretreatment of soap removal resulted in decreased hydrogen production (Sarma *et al.*, 2014).

In this study, in the absence of pretreatment step with minimum utilization of Tween 80 of around 0.0015% (*v/v*) resulted in increase in hydrogen production along with increased glycerol utilization, covering the unexplored side of bioconversion of crude glycerol. Addition of very small amounts of Tween 80 will be economical solution to pretreatment issues, further addition of surfactant eliminates contamination in bioreactors (Goes *et al.*, 1999), improves medium characteristics, and enhance nutrient availability (Brar *et al.*, 2005). The improved distribution and solubility of substrate with simple addition of Tween (Sarma *et al.*, 2011; Zhu *et al.*, 2013) can induce a significant evolution in hydrogen production. Bioconversion of crude glycerol by energy intense pretreatment steps (such as pH adjustment, precipitation and centrifugation) and costly time-consuming techniques (such as immobilization and repeated fed-batch fermentation) for increased substrate utilization was eliminated by a simple addition of very small amount of surfactant. This resulted in economic solution for increased

bioconversion and utilization rate of crude glycerol along with increased hydrogen production.

Conclusions

To increase glycerol utilization along with hydrogen production and decrease the viscosity of fermentation media, Tween 80 was selected as a surfactant. The CCD model helped to focus on determining the optimal concentration of crude glycerol and Tween 80 concentrations along with responses of hydrogen production, glycerol utilization and viscosity. Concentration of Tween ($p< 0.05$) had a dominant effect on hydrogen production and crude glycerol had dominant effect on glycerol utilization and viscosity. The optimized conditions of crude glycerol: 17.5 g/L and Tween 80: 15 mg/L resulted in increased hydrogen production reaching a maximum of 32.1 ± 0.03 mmol/L with 87.7% of glycerol utilization rate. The validation experiments in absence of Tween (25.56 ± 0.91 mmol/L of H_2), in absence of crude glycerol (4.69 ± 0.76), with pretreated crude glycerol (20.06 ± 0.51) and across mono-culture system (15.43 ± 0.79 to 22.14 ± 0.94) resulted in decreased hydrogen production in comparison to increased hydrogen production (31.07 ± 0.92) for the optimized conditions. The surfactant addition resulted in increased hydrogen production at higher concentration of crude glycerol with elimination of additional energy-intense pretreatment steps and costly time-consuming techniques to increase substrate utilization. Inclusion of the most suitable, low-cost, readily available surfactant source represented an excellent measure for viscosity reduction of crude glycerol for increased H_2 production and increased substrate utilization rate. Additionally, the utilization of Tween 80 at minimum concentration offered low-cost improved strategy to boost biodiesel industry through increased hydrogen production from crude glycerol.

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Table 5.2.1: Central composite design ranges of the two input factors considered for the hydrogen production

Factor	Name	Units	Low Actual	Mean	High Actual	-α	+α
A	Crude glycerol	g/L	10	17.5	25	6.89	28.11
B	Tween	mg/L	5	15	25	0.86	29.14

Table 5.2.2: Experimental design matrix defining concentration of crude glycerol and Tween 80 with the response on H_2 production, residual glycerol, viscosity and by-products concentration for each run.

Run	A:Crude glycerol	B:Tween 80	Hydrogen	Glycerol utilization	Viscosity	1-3PD	Butyric acid	Ethanol
	g/L	mg/L	mmol/L	%	(cP)	g/L	g/L	g/L
1	28.11	15	25.5 ± 0.63	60.7 ± 0.66	2.33 ± 0.04	5.15 ± 0.48	1.79 ± 0.35	4.67 ± 0.74
2	17.5	15	30.7 ± 0.14	84.7 ± 0.25	2.27 ± 0.04	3.64 ± 0.86	3.96 ± 0.48	2.54 ± 0.51
3	17.5	15	30.7 ± 0.09	87.4 ± 0.60	2.28 ± 0.03	3.23 ± 0.34	3.63 ± 0.47	2.87 ± 0.96
4	17.5	29.14	23.7 ± 1.58	82.5 ± 0.25	2.25 ± 0.04	3.82 ± 0.44	1.65 ± 0.91	2.49 ± 0.43
5	10	5	29.6 ± 1.14	99.8 ± 0.34	2.30 ± 0.02	3.98 ± 0.70	3.64 ± 0.50	2.14 ± 0.89
6	25	5	29.1 ± 1.93	58.0 ± 0.71	2.32 ± 0.02	5.65 ± 0.53	2.99 ± 0.78	4.15 ± 0.45
7	17.5	0.86	29.8 ± 0.05	79.4 ± 0.91	2.30 ± 0.03	4.15 ± 0.94	3.01 ± 0.38	2.11 ± 0.81
8	17.5	15	31.7 ± 0.02	87.8 ± 0.54	2.28 ± 0.03	3.71 ± 0.78	3.96 ± 0.86	2.78 ± 0.69
9	10	25	27.5 ± 1.38	99.8 ± 0.02	2.26 ± 0.06	2.58 ± 0.90	2.98 ± 0.40	1.88 ± 0.49
10	25	25	24.5 ± 1.43	72.2 ± 0.75	2.29 ± 0.06	5.85 ± 0.71	1.71 ± 0.21	4.04 ± 0.41
11	17.5	15	32.1 ± 0.03	87.7 ± 0.50	2.28 ± 0.03	3.75 ± 0.18	3.82 ± 0.61	2.91 ± 0.78
12	6.89	15	24.4 ± 0.83	99.5 ± 0.37	2.22 ± 0.02	2.71 ± 0.75	1.85 ± 0.45	1.69 ± 0.12
13	17.5	15	30.5 ± 1.23	87.0 ± 0.28	2.28 ± 0.03	3.78 ± 0.38	3.92 ± 0.57	2.83 ± 0.39

Table 5.2.3: Summarized ANOVA for the response surface quadratic model for H_2, residual glycerol and viscosity.

Source	***p*-value**		
	H_2	**Glycerol utilization**	**Viscosity**
Model	0.0036	< 0.0001	0.0249
A-Crude glycerol	0.6208	< 0.0001	0.0040
B-Tween	0.0047	0.0473	0.0236
AB	0.3780	0.0357	0.7805
A^2	0.0012	0.0256	0.6737
B^2	0.0107	0.0465	0.6737
R^2	0.80	0.95	0.79

Table 5.2.4: Hydrogen production across the validation experiments using the optimized values from the central composite design.

Optimum condition (CG:17.5 g/L, Tween 80: 15 mg/L)	**Hydrogen (mmol/L)**	**Ethanol (g/L)**	**Butyric acid (g/L)**	**1,3-propaediol (g/L)**	**Glycerol utilization (%)**
with Tween 80	32.07 ± 0.92	1.96 ± 0.16	2.73 ± 0.04	2.17 ± 0.08	91.91 ± 0.71
without Tween 80	25.56 ± 0.91	1.88 ± 0.10	2.63 ± 0. 07	3.07 ± 0.14	77.71 ± 0.45
pretreated crude glycerol	20.06 ± 0.51	2.03 ± 0.15	1.43 ± 0.70	2.67 ± 0.59	55 .27 ± 1.18
without crude glycerol	4.69 ± 0.76	0.43 ± 0.03	0.82 ± 0.11	0.51 ± 0.06	Nil
E. aerogenes only	22.14 ± 0.94	1.72 ± 0.10	0.21 ± 0.13	2.48 ± 0.35	4 4.27 ± 0.82
C. butyricum only	15.43 ± 0.79	0.22 ± 0.06	1.39 ± 0.26	2.19 ± 0.19	13 .49 ± 1.10

Table 5.2.5: Utilization of crude glycerol with initial pretreatment step during fermentation for different microorganisms to produce value-added products.

Crude glycerol pretreatment steps	Microorganism used	Final product	Final output	Ref.
(1) Crude glycerol mixed with distilled water at 1:4 (*v/v*), (2) pH adjusted to 6.5 using HCl, (3) centrifugation at 5000 rpm and (4) addition of media nutrients	*Schizochytrium limacinum* SR21(ATCCMYA-1381)	Docosahexaenoic acid (DHA)	DHA yield of 4.91 g/L	(Chi *et al.*, 2007)
(1) Crude glycerol mixed with distilled water, (2) pH adjusted to 3 using HCl, (3) centrifugation at 5000 rpm and (4) Addition of yeast extract	*Pythium irregulare*	Eicosapentaenoic Acid (EPA)	14.9 mg/L-day	(Athalye *et al.*, 2009)
(1) Crude glycerol mixed with distilled water at 1:4 (*v/v*), (2) pH adjusted to 3 using H_2SO_4, (3) Static time of 30 min for two phase and (4) glycerol recovery using separation funnel, (5) addition of media nutrients	*Schizochytrium limacinum*	Docosahexaenoic acid (DHA)	0.52 g/L-day	(Ethier *et al.*, 2011)
(1) Crude glycerol mixed with distilled water at 1:4 (*v/v*), (2) pH adjusted to 11.37 (3) NaCl addition (4) centrifugation at 5000 rpm, (5) soap removal and addition of media nutrients	*Enterobacter aerogenes*	Hydrogen	93.03% decreased hydrogen production	(Sarma *et al.*, 2014)
(1) Crude glycerol mixed with distilled water at 1:2.7 (*v/v*), (2) Addition of Tween 80 0.0015% (v/v) along with media component (3) pH adjusted to 6.5 using HCl	*Enterobacter aerogenes* and *Clostridium butyricum*	Hydrogen	32.1 ± 0.03 mmol/L (1.25-fold) increased production	This study

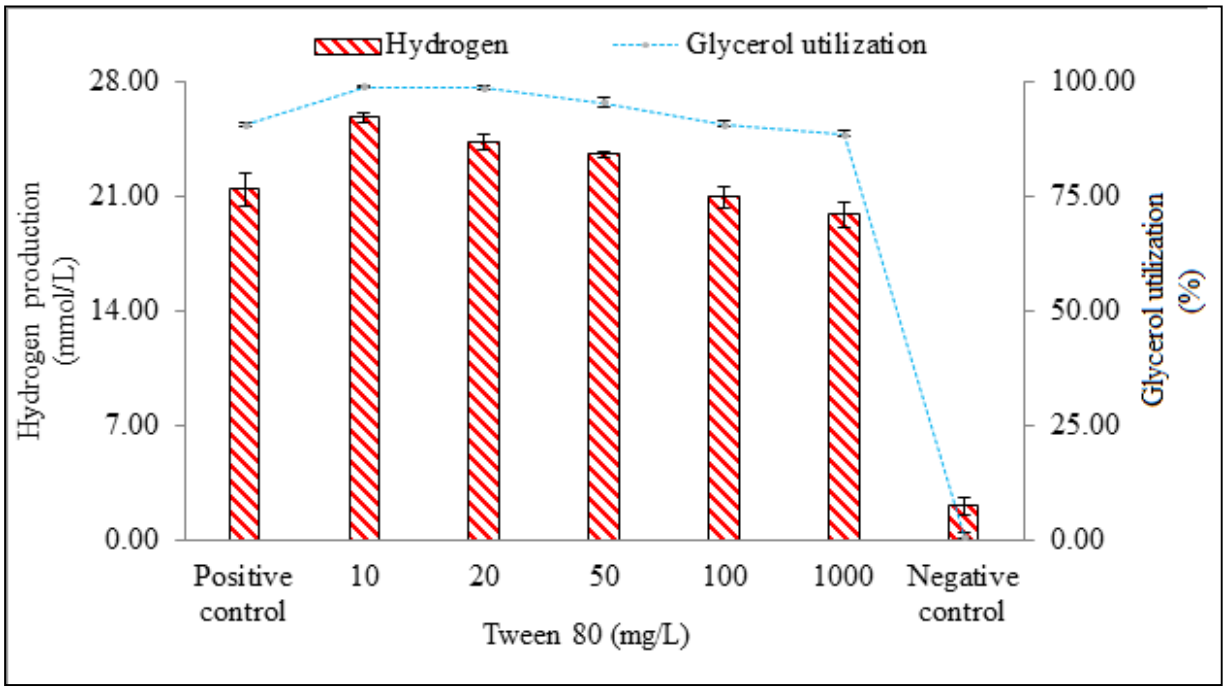

Figure 5.2.1: Hydrogen production along with glycerol utilization (%) in the presence of varying concentrations of Tween 80 (1% crude glycerol + media components), positive control (1% crude glycerol + media components) with no Tween 80 and in presence of negative control (1% Tween + media components) with no crude glycerol was tested.

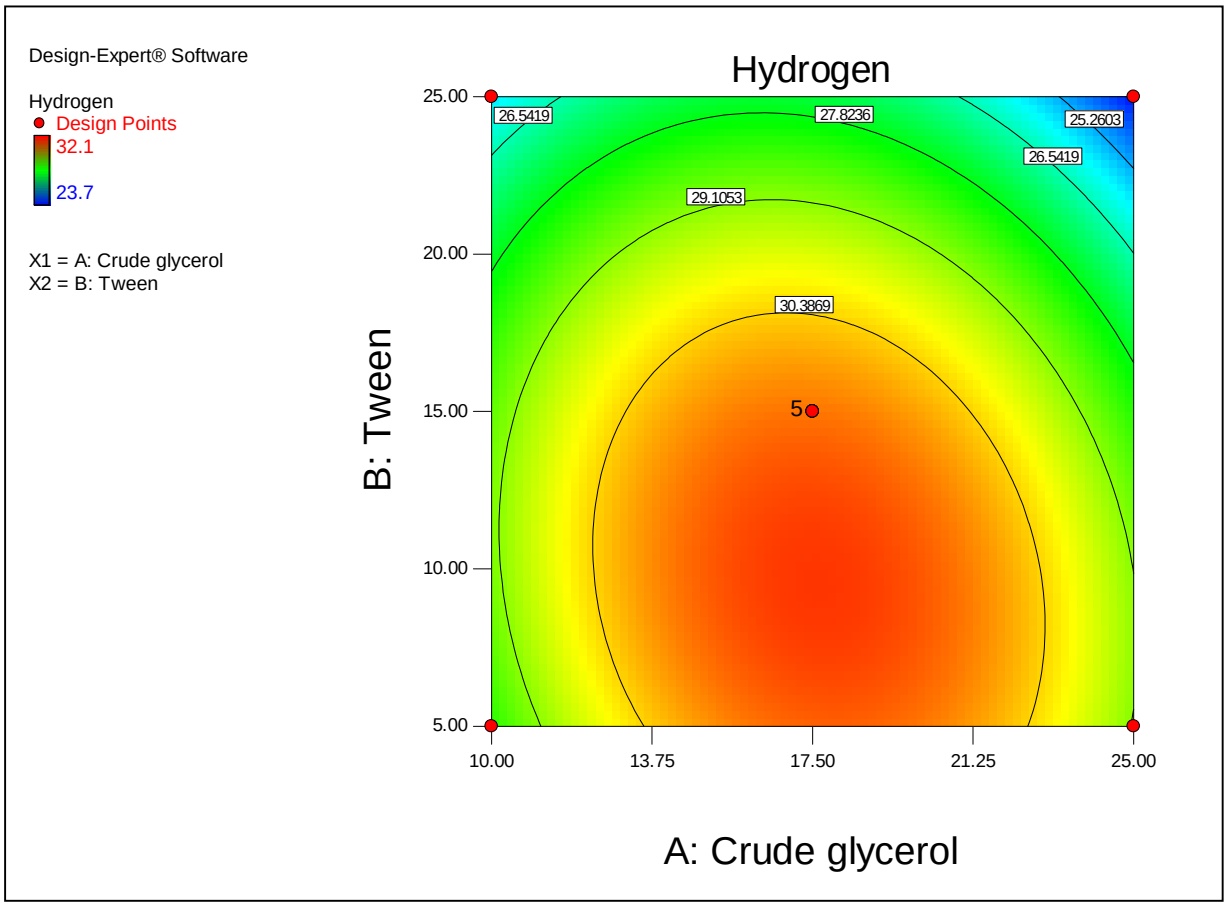

Figure 5.2.2: Experimental responses of hydrogen production (mmol/L) with the fitting function of crude glycerol (g/L) and Tween concentration (mg/L) using response surface plots.

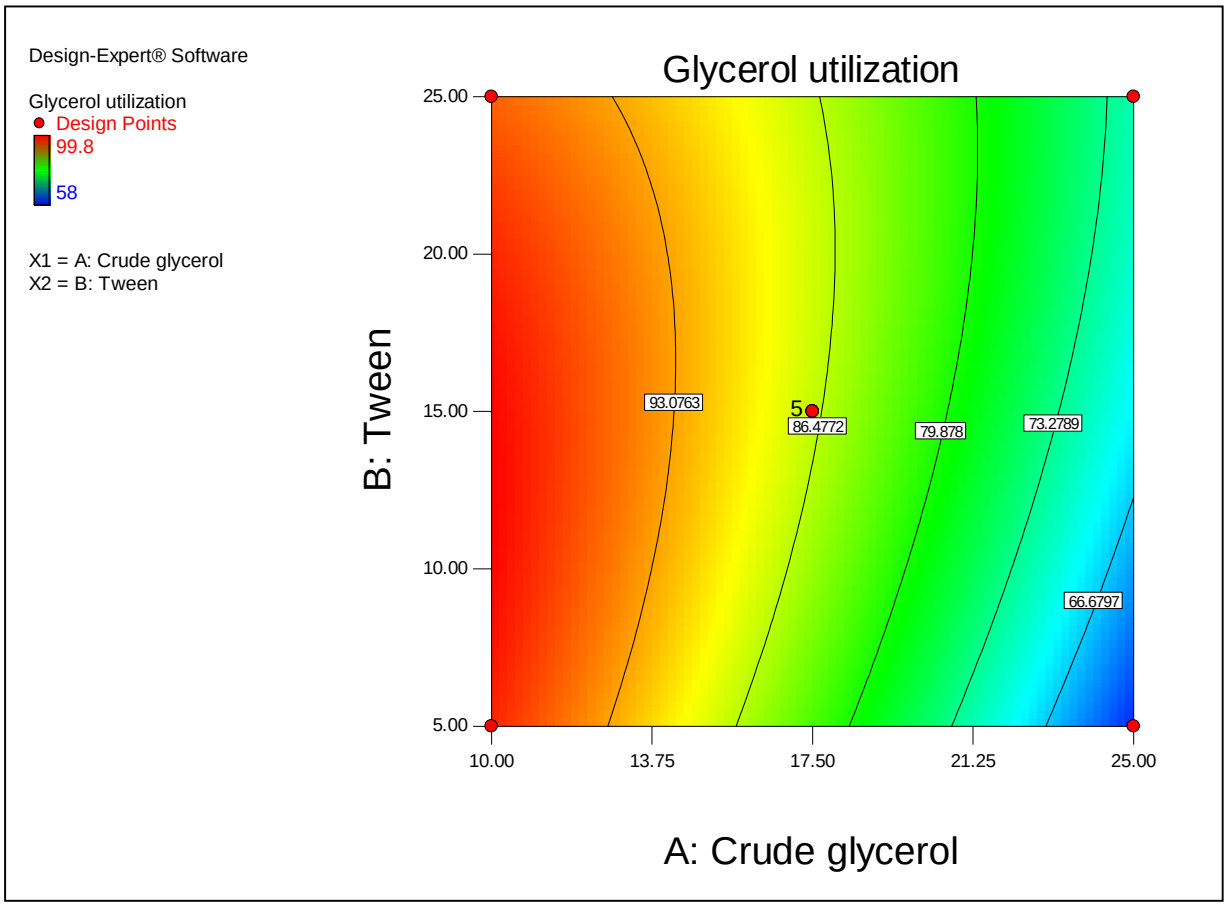

Figure 5.2.3: Response of glycerol utilization (%) across the concentration of crude glycerol (g/L) and Tween 80 (mg/L) using response surface plots.

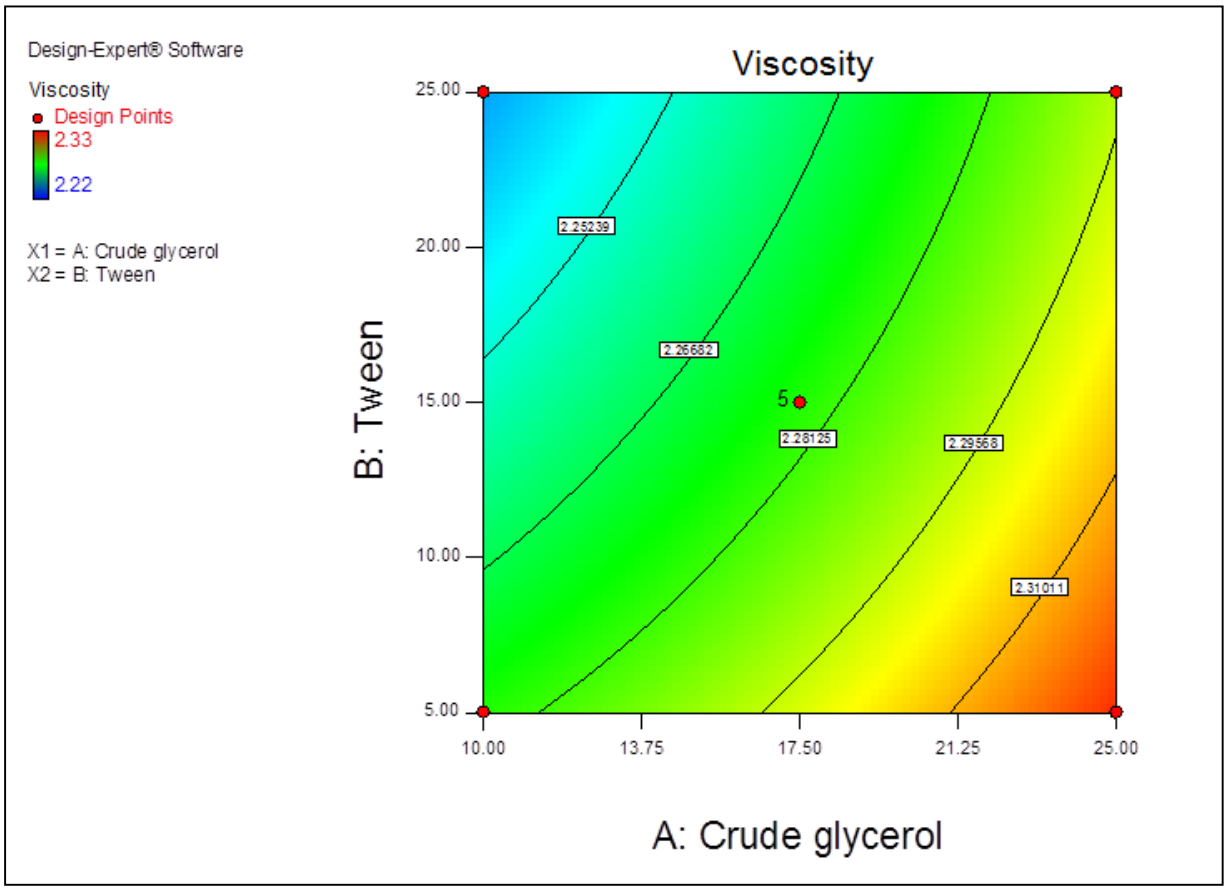

Figure 5.2.4: Response of viscosity (cP) value across the interactions of crude glycerol (g/L) and Tween 80 (mg/L) using response surface plots.

CHAPTER 6

HYDROGEN PRODUCTION BY CO-CULTURE SYSTEM WITH IMMOBILIZED CELLS

PART 1

VALORIZATION OF CRUDE GLYCEROL AND EGGSHELL BIOWASTE AS MEDIA COMPONENTS DURING HYDROGEN PRODUCTION: A SCALE-UP STUDY USING CO-CULTURE SYSTEM

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Résumé

Les propriétés des coquilles d'oeufs comme agent immobilisant neutralisant ont été examinées lors de la production de H_2 en utilisant le GB par co-culture de *Enterobacter aerogenes* et *Clostridium butyricum*. Différentes tailles de coquilles d'œufs à plusieurs concentrations ont été testées lors de la réalisation des lots de fermentation discontinue répétée. La production de H_2 maximum selon la taille des coquilles d'œufs de 33 um < x_5 < 75 um avec 36,53 ± 0,53 mmol/L pendant le lot suivie par 41,16 ± 0,95 mmol/L pendant la seconde fermentation répétée. La production de l'H_2 a augmenté avec la diminution de la taille de la coquille d'œuf qui maintient aussi le pH de fermentation (6,00-6,30). De plus, la propriété d'immobilisation a été vérifiée par des images de microscopie électronique à balayage. La concentration en coquille d'oeuf de 0,25% (v/v) a été jugée optimale et il a complété le rôle de support microbien et de substance de remplacement à des composés chimiques du milieu de culture avec 31,66 ± 0,55 mmol/L par rapport à la présence de composantes du milieu avec 32,07 ± 0,92 mmol/L lors de la production de H_2. L'étude de mise à l'échelle avec des conditions optimisées à l'aide d'un bioréacteur de 7,5 L a entraîné près de 1,5 fois plus de H_2 par rapport à la mono-culture. Une production de 312,12 mmol ou 7,69 L H_2/L de milieu avec 86,65% de glycérol utilisé ont été mesurés. L'utilisation des coquilles d'œufs en remplacement des composants des milieux de culture peut faire baisser le coût des médias de 85-90% pour la production de H_2. La valorisation des coquilles d'œufs comme un agent neutralisant, agent immobilisant et comme source d'éléments nutritifs peut réduire de façon significative le coût de production du H_2. En outre, cette valorisation réduit les effets des biodéchets sur l'environnement faisant ainsi l'industrie du biodiesel et de H_2 plus compétitive.

Mots clés: Glycérol brut; la co-culture; eggshell, hydrogel; immobilisation; métabolites

Abstract:

The properties of eggshells (EGS) as neutralizing and immobilizing agent were investigated for hydrogen (H_2) production using crude glycerol (CG) by co-culture of *Enterobacter aerogenes* and *Clostridium butyricum*. Eggshells of different sizes and concentrations were used during batch and repeated-batch fermentation. For batch and repeated-batch fermentations, the maximum H_2 production (36.53 ± 0.53 and 41.16 ± 0.95 mmol/L, respectively) were obtained with the EGS size of 33 μm<x_5<75 μm. Hydrogen production increased with the decreased size of EGS. Eggshells maintained the fermentation pH (6.00-6.30) and provided immobilization support for both the bacterial strains as confirmed by scanning electron microscopy. As media components, the EGS concentration of 0.25% (w/v) was found to be optimum for maximum H_2 production (31.66 ± 0.55 mmol/L) and the production profile was comparable to H_2 production (32.07 ± 0.92 mmol/L) obtained with all media components. In the scale-up study with semi-continuous bioreactor (7.5 L), almost 1.5-fold increase (in comparison to mono-culture) i.e. 7.69 L H_2/L of medium with 86.65% glycerol utilization were obtained. Valorization of EGS can reduce the environmental biowaste effects, reduce production cost of H_2 by 85-95%, improve H_2 yield making biodiesel and H_2 industry competitive.

Keywords: Crude glycerol; co-culture; eggshell, hydrogen; immobilization; metabolites

Introduction

Bio-fuel economy comprising biodiesel and biohydrogen are the possible types of low-carbon and post-petroleum economy in the near future (Lee, 2015). Biodiesel industry growth in the last decade led to 100% increase in crude glycerol (CG) production globally (Valerio *et al.*, 2015). Biodiesel industry generates large quantities of crude glycerol (by-product) at 1:10 during biodiesel production (Len *et al.*, 2014). For sustainable growth in the near future, biodiesel industry needs to utilize crude glycerol for synthesis of bio-based fuels/chemicals in order to extend renewable resource utilization (Valerio *et al.*, 2015). Glycerol properties with three hydroxyl group, higher redox potential and different reactivity for biological conversion into high value added chemicals interests biodiesel industry (Len *et al.*, 2014).

The complexity of CG with the amount of impurities present and glycerin purification is no longer cost-effective and production of value-added products such as 1,3-propanediol, succinic acid, and ethanol require additional expensive recovery steps (Pachapur *et al.*, 2016a; Valerio *et al.*, 2015). Bioconversion of CG to hydrogen is a widely explored approach in comparison to the production of other value-added products (Pachapur *et al.*, 2016c). Biological hydrogen production can be carried out with renewable and sustainable technologies with combined waste treatment in comparison to chemical methods associated with consumption of fossil fuels (Ao *et al.*, 2016; Sivagurunathan *et al.*, 2016).

Dark fermentation of H_2 production attributes towards broad spectra of organic wastes, requiring simple reactor set-up and in absence of light, favour efficient H_2 production in comparison to photo-fermentation (Pachapur *et al.*, 2016a; Pachapur *et al.*, 2016c; Sivagurunathan *et al.*, 2016). During dark fermentation, production of organic acids causes a sharp decrease in the medium pH and results in lower H_2 production. Thus, to maintain the fermentation pH, addition of external buffering agents is unavoidable (Sarma *et al.*, 2015a; Tenca *et al.*, 2011). Improvements during fermentation requiring additional media components (Tenca *et al.*, 2011), co-substrate utilization and

immobilization techniques are necessary for increased H_2 production (Sivagurunathan *et al.*, 2016) with increased production cost.

Dark fermentation of organic wastes for commercialization at large scale requires new routes for decreasing the cost of H_2 production (Lee, 2015; Morsy, 2015). The recent trends of replacing the media components with organic wastes to increase H_2 production and to decrease production cost are represented in Table 6.1.1. In presence of additional acetate, *Clostridium* strains utilized twice the amount of glycerol with 9% increase in H_2 production in comparison to without acetate (Heyndrickx *et al.*, 1991). In a repeated batch culture with polypeptone resulted in increase in H_2 yield from 2 to 2.4 mol/mol of glucose (Yokoi et *al.*, 2001). The expensive polypeptone was replaced with corn steep liquor, a nitrogen-rich organic waste also resulted with increase in H_2 yield from 2.4 to 2.7 mol/mol of glucose (Yokoi *et al.*, 2002). Likewise, two industrial wastes, crude glycerol and apple pomace co-fermented resulting in 2.83 fold increase in H_2 production (Pachapur *et al.*, 2015b). Fruit and vegetable market waste was mixed with swine manure; an alkali-rich material resulted in process stability and eliminated exogenous adjustments of pH for H_2 production (Tenca *et al.*, 2011). Furthermore, the immobilization technique of using porous glass bead (Yokoi *et al.*, 1998) found an alternative in using dried ligno-cellulosic materials (such as Banana leaves , coconut coir , groundnut shells or pea shells) resulting with 6.4 fold improvement in H_2 production (Patel *et al.*, 2014; Patel *et al.*, 2010). The replacement of costly media components, utilization of industrial wastes and better strategy with low-cost immobilizing material resulted in increased substrate availability for effective and economical way of H_2 production.

In the year 2015, Canada produced around 610 million dozen eggs and with the growth in the demand, the production is anticipated to increase 4% per year (eggfarmers.ca). Food processing and manufacturing plants across worldwide generate eggshells (10% of total mass) as solid waste and is commonly disposed in landfills without any pretreatment (Wei *et al.*, 2009). The composition of eggshell (EGS) with 94-97% of $CaCO_3$ acted as a natural neutralizing agent in maintaining fermentation pH (Wei *et al.*, 2009). In recent years, the application of eggshell as low-cost catalyst for biodiesel

production (Chen *et al.*, 2014; Wei *et al.*, 2009) and as neutralizing agent for fumaric acid production (Das *et al.*, 2015) has been conducted. In the latest application, eggshell have been used as inexpensive calcium-based sorbent for enchanced CO_2 capture (Sacia *et al.*, 2013).

The added cost of media components, external buffering agents and material cost for immobilization can be eliminated by using a cost-effective and environmental approach of recycling eggshell during H_2 production. As the literature suggests, utilization of eggshell in the production of H_2 is yet to be explored. In future, H_2 demand is expected to increase with growing needs of utilization of by-product CG for biodiesel industry to make a competitive biodiesel with petroleum diesel (Kumar *et al.*, 2015; Pachapur *et al.*, 2016a; Thengane *et al.*, 2014; Wei *et al.*, 2009). Therefore, the requirements of sustainable development for solid waste recycle of using CG and eggshell as media replacement will result in cost-effective process of H_2 production. In this study along with CG as substrate, the property of EGS as neutralizing agent and also as low-cost immobilizing support will be explored for the first time during H_2 production.

Materials and Methods

Crude glycerol as substrate

The CG used in this study was supplied by Rothsay®, Canada (Rothsay) that recycles food and animal by-products for biodiesel production. The composition of CG (by w/w) is given as follows: glycerol (23.6%), carbon (35.9%), nitrogen (3.2%), ash (3.06%), moisture (5.75%) and matter organic non-glycerol (67.56%). Chemicals and reagents used in this study were purchased from Fisher scientific, VWR and Lallemand, Canada (Pachapur *et al.*, 2016c).

Microorganisms, pre-culture media and inoculum development

The co-culture system of *Enterobacter aerogenes* and *Clostridium butyricum* considered in this study were purchased from USDA, USA. The basal synthetic medium consisting (w/v) of glucose (1%), casein polypeptone (2.0%), KH_2PO_4 (0.2%), yeast extract (0.05%) and $MgSO_4.7H_2O$ (0.05%) maintained anaerobically at pH 6.5 was used for *E.*

aerogenes pre-cultured at 30 ºC. The modified basal medium supplemented with 0.1% L-cysteine-HCl.H_2O was used for C. *butyricum* precultured at 36 ºC (Yokoi et *al.*, 1998). In distilled water, the exact concentrations of media components was dissolved using magnetic stirrer set-up. The pH of pre-culture media was set to 6.5 using NaOH, degassing by N_2 gas for 3 min and bottles sealed using pre-inserted septa followed by autoclave (Pachapur *et al.*, 2015a).

In case of inoculum development, log phase culture broth at (5% *v/v*) was used as inoculum throughout the study and cultures were incubated at 36 ºC at 150 rpm in an orbital incubator shaker (INFORS HT multitron standard) (Pachapur *et al.*, 2015a).

***Hydrogen production in presence of different sized* EGS**

To study the effect of variation in size of EGS on H_2 production, the eggshells were first oven dried and then broken into small sizes using a mortar and pestle. The small pieces of eggshells were later sieved through a metal sifters and EGS of varying sizes from 1.7 mm < x_1< 3.35 mm, 850 µm < x_2< 1.7 mm, 300 µm < x_3< 850 µm, 75 µm < x_4< 300 µm and 33 µm < x_5< 75 µm, were obtained. The collected eggshells of varying sizes was collected separately and stored at 4 ºC before use. In presence of modified basal media containing (*w/v*): CG (1.75%), casein peptone (2%), yeast extract (0.05%), KH_2PO_4 (0.2%), $MgSO_4.7H_2O$ (0.05%) and addition of eggshell at concentration of 0.25% (w/v).The pH of the fermentation media was maintained at a pH=6.5 and transferred to serum bottles with a 50 mL working volume. The N_2 sparging, sealing, autoclave and incubation steps are similar to the inoculum development step as explained earlier. The concentration of CG at 1.75% found to be optimum in authors earlier studies for H_2 production (Pachapur *et al.*, 2016d) and addition of eggshell at concentration of 0.25% (w/v) equivalent to exact concentration of (0.2% KH_2PO_4 and 0.05% $MgSO_4.7H_2O$) was carried out in triplicates. The experimental runs were performed in triplicates and the presented values are the averages of triplicates with standard deviation (±) values as error bars.

Repeated batch culture of hydrogen production in presence of different sized eggshells

After the batch culture of H_2 production in presence of varying size of eggshells, 47.5 mL of spent media was drawn out using a syringe with long needle and syringe filter (0.45 µm) fitted to a peristaltic pump (Yokoi *et al.*, 2002). The idea behind using syringe filter is to restore the eggshells inside and drawn out only spent media from the serum bottle. Later, 47.5 mL of fresh degassed modified medium was added and the serum bottle was vacuumed to zero down H_2 concentration before repeated batch culture (Yokoi *et al.*, 2002). The spent media of 2.5 mL was used as inoculum for the fresh degassed medium during repeated batch culture fermentation. The steps to draw out spent media, addition of fresh media and vacuum creation was carried out in laminar air flow under sterilized conditions.

EGS as immobilizing support material

The immobilizing property of eggshell provides necessary compatibility for attachment, adsorption and growth of microorganisms (Das *et al.*, 2015). To determine the immobilizing potential of eggshells in the co-culture system during H_2 production, the eggshells were collected from the spent media at the end of the fermentation, processed and were analyzed by scanning electron microscopy.

Hydrogen production at different concentrations of EGS

To determine the exact concentration of egg-shells of size x_5 (33 µm $<x_5<$ 75 µm) for increased H_2 production, the amount of eggshell was varied from 0.5, 1, 2 and 4% (*w/v*). In presence of modified basal media at CG 1.75% with yeast, peptone and at increasing concentration of egg-shells of size x_5 from 0.5 to 4% was carried out in triplicates.

Effect of EGS in presence and absence of media components during H_2 production

To study the effect of eggshells during H_2 production with modified basal media consisting of (*w/v*): CG (1.75%), eggshell of size x_5 (0.25%) and in presence and absence of casein peptone (2%), yeast extract (0.05%), KH_2PO_4 (0.2%), $MgSO_4.7H_2O$

(0.05%) was carried out. To determine the role of eggshell during H_2 production, the experimental settings in the absence and as a replacement of each component in presence of eggshell was carried out. The fermentation media in presence and absence of each component was carried out as explained above in inoculum development step. A control experiment for synthetic $CaCO_3$ at exact concentration of eggshells was carried out for comparative study. In addition validation experiments in presence and absence of CG were also carried out in triplicates.

Semi-continuous hydrogen production using 7.5 L bioreactor

The co-culture system of hydrogen production using eggshells in the absence of media components using 7.5 L bioreactor (Labfors, IINFORS-HT, Switzerland) was carried out. The semi-continuous approach eliminated substrate inhibition and successfully developed a low-cost bio-engineering system for H_2 production (Sarma *et al.*, 2015a). The initial CG concentration in the bioreactor was around 10 g/L, which was diluted using distilled water to make-up for the working volume of 3 L. The external feeding CG concentration was around 120 g/L was feed into the bioreactor after 8 hours to start of fermentation. The real time values of different parameters such as (pH, rpm, dissolved oxygen, temperature and hydrogen) were monitored and recorded using Iris software (Labfors, IINFORS-HT, Switzerland) operated over a system. To account for hydrogen partial pressure, once the hydrogen concentration reached 30-35% (*v/v*) in the headspace of the reactor, pure N_2 was sparged. The operating conditions (pH: 6.5, temperature: 36 ºC and rpm: 100) were kept constant during fermentation as described across (Sarma *et al.*, 2015a).

Analytical techniques

Hydrogen analysis by GC

At the end of each fermentation experiment, the gas samples were collected in vacuumed sample vials using gas tight syringe and later were analyzed by gas chromatography (GC). The technical specifications of the GC instrument were: Model: Varian 3800, USA, fitted with a 3 m PoraPLOT Q® column (Agilent technology, USA) and equipped with a thermal conductivity detector (TCD). The GC set-up with injector,

column temperature and detector temperature were set at 100 ºC and carrier gas N_2 was used at a flow rate of 3.5 mL/min (Pachapur *et al.*, 2015). Considering the temperature and atmospheric pressure during the experimental runs, the volume of H_2 gas produced was calculated and expressed in mmol concentration unit, (Sarma *et al.*, 2013a).

End-metabolites/by-products analysis by GC-FID

The concentrations of ethanol, butyric acid, acetic acid and 1,3-propanediol (1,3-PD) was analyzed on ZB-WAX plus column fitted with flame ionization detector (FID) detector in a gas chromatography (GC) (7890B GC-Agilent, Santa Clara, CA) set-up. The GC condition at a flow rate of 1 mL/min using helium carrier gas at a temperature profile of 80–240 ºC under 8.4 min method run time was developed (Pachapur *et al.*, 2016c).

Bacterial morphology analysis by Scanning Electron Microscopy (SEM)

The ESG at the end of the repeated batch fermentation were recovered and washed twice in potassium phosphate buffer (50 mM, pH 6.5). The bacterial cells were fixed, washed and dehydrated according to the sample preparation steps mentioned earlier and later the fixed cells were analyzed by scanning electron microscopy (SEM, Carl Zeiss EVO® 50) (Das *et al.*, 2015), [32].

Results and Discussion

Hydrogen production in presence of different sizes of EGS

To determine the role of eggshells during hydrogen production using CG (1.75%) in presence of modified basal medium, the co-culture studies was carried out in presence of different sizes of eggshells. At the end of batch fermentation, the hydrogen production and end-metabolite production obtained with the different sizes of eggshell are presented in Table 6.1.2. The different sizes of eggshells obtained using metal sieves were labelled as x_1 to x_5. For different sizes of eggshells experimented, the highest hydrogen production (36.53 ± 0.53 mmol/L) was obtained for the size x_5 around and the lower production (29.33 ± 0.38 mmol/L) resulted with x_1 size . The pH of the

spent media at the end of fermentation using eggshell was within 6.00 to 6.50 in comparison to ~5.5 in absence of eggshells. During the fermentation with the accumulation of different organic acids (acetate and butyrate) along with solvents (ethanol), the fermentation pH decreases to around ~5.5 from 6.5 causing pathway shift with lower hydrogen production. Addition of eggshells helped to maintain near to the optimum pH required for the growth of *E. aerogenes* and *C. butyricum.* The hydrogen concentration (36.53 ± 0.53 mmol/L) obtained with eggshells was higher in comparison to 32.1 ± 0.03 mmol/L without eggshells (Pachapur *et al.*, 2016d). In case of size x_5, acetic acid was produced at higher concentration (2.92 ± 0.01 g/L) in comparison to other applied sizes of eggshells, which resulted in increased hydrogen production for size x_5. The $CaCO_3$ (Hydrogen Bond Acceptor Count=3) present in the eggshell acted as indirect H-acceptor and gradually diverting the fermentation from 1,3-PD towards acetate and H_2 formation. Similar results were observed during addition of sodium acetate (Hydrogen Bond Acceptor Count=2) as co-substrate acted as indirect H-acceptor favored acetyl CoA/CoA ratio towards acetate with increased H_2 production (Heyndrickx *et al.*, 1991). The increased production of acetate can also be due to the decalcification reaction accounted during conversion of calcium carbonate present in eggshell into calcium acetate (Nakano *et al.*, 2001). The hydrogen production increased with increased production of acetic acid, as 3 mole of hydrogen are released from 1 mole of acetate (Hong Liu *et al.*, 2005; Pachapur *et al.*, 2016c). The decreased size of eggshell resulted in the increased production of hydrogen. The larger size in case of x_1 to x_4, tend to settle down to form heaps at the bottom of the serum bottle during the fermentation. However, in case of size x_5, there was no settling down of EGS resulting in constant contact with media for sufficient mixing system resulting in increased hydrogen production. The increase size of EGS accentuated the mixing problem with formation of dead zones (with insufficient mixing) resulting in local accumulation of EGS heaps. The dead zone across the fermentation with limited media exposure tends to decrease the fermentation performance resulting in decreased hydrogen production. Homogenous mixing directly influences the uniform distribution of media components by

keeping microorganisms in suspension with no dead zone formation desirable to increase the performance of bioreactor (Schäpper *et al.*, 2009)..

The eggshell addition maintained the media pH, resulted in enhanced hydrogen production with decreased sizes of eggshells. In order to determine the reuse property of eggshell, repeated batch fermentation was carried out.

Repeated batch culture of hydrogen production in presence of different sizes of eggshells

In addition to maintaining the pH of the fermentation, eggshells also act as immobilizing agent. Hydrogen production at the end of batch fermentation with different sizes was later supplemented with fresh degassed media and repeated batch fermentation was carried out. The repeated batch cycle determined the immobilizing property of eggshells and ability to reuse eggshells during hydrogen production. The hydrogen and end-metabolites production obtained in the repeated batch fermentation in presence of different varying sizes of eggshells are presented in Table 6.1.2. The hydrogen and metabolite production profile in case of repeated batch was similar to batch fermentation. The highest hydrogen production was around 41.16 ± 0.95 mmol/L for size x_5 and the lowest production of 34.93 ± 0.81 mmol/L for size x_1. In case of repeated fermentation, the concentration of end-metabolite was higher in comparison to batch fermentation. In case of repeated fermentation, the spent media as inoculum attributed to increased cell proliferation and higher adaptation to the CG resulting in enhanced hydrogen production. The spent media at 5-10% inoculum size in case of *E. aerogenes* resulted in 13.37% increased hydrogen production (Sarma *et al.*, 2013b). The 2^{nd} cycle of repeated batch fermentation resulted in decreased hydrogen production with increased production 1,3-PD (data not shown). In case of 2^{nd} cycle of repeated fermentation, the incoming CG with different impurities tend to inhibit the hydrogen production pathway resulting in 1,3-PD formation with decreased hydrogen production. The reuse property of eggshell property was determined with increased hydrogen production across repeated fermentation.

The potential of eggshells as immobilizing support materials for the two bacterial strains was confirmed from the SEM micrographs. The eggshell possesses the property of immobilization compatibility for attachment, adsorption and proliferation of microorganisms. SEM micrograph of mono-culture and co-culture system in absence and presence of eggshell are represented in the Figure 6.1.1. The rod-shaped morphology of *E. aerogenes* and *C. butyricum* can be seen in the mono-culture system in the Figure 6.1.1 (A and B). The co-culture system of *E. aerogenes* and *C. butyricum* in the absence of eggshell can be seen in Figure 6.1.1 (C),The figure also represents the growth/presence of both the microorganisms during hydrogen production in the co-culture system. The blunt end of *C. butyricum* can be easily differentiated from round end *E. aerogenes* in the co-culture. The co-culture system was carried out in the presence of eggshell as represented in the Figure 6.1.1 (D). The bacterial immobilization on the surfaces of eggshells can be seen in the Figure 6.1.1(D). The increase in immobilized bacterial biomass suggested the role of eggshells as immobilizing surface during co-culture.

The advantages of co-culture over mono-culture were justified with increased H_2 production (Pachapur *et al.*, 2015a), increased co-substrate utilization (Pachapur *et al.*, 2015b), increased glycerol uptake and decreased by-product production (Pachapur *et al.*, 2016d). However, for the first time the simultaneous growth of *E. aerogenes* and *C. butyricum* in the co-culture system was justified at molecular level using SEM in this study.

Hydrogen production in presence of increasing concentration of eggshells

In comparison to the other four applied size ranges (x_1, x_2, x_3, and x_4) of eggshells, the size range x_5 resulted in the highest hydrogen production in both batch and repeated fermentation conditions. To determine the optimum concentration for maximum hydrogen production, different concentrations (0.5 to 4 %, *w/v*) of eggshell was used. The production profiles of hydrogen and end-metabolite obtained with different concentrations (%) of x_5 (33 µm $<x_5<$ 75 µm) are presented in Table 6.1.3. The highest hydrogen production was around 37.03 ± 0.32 mmol/L in case of 1% w/v of eggshell and minimum was around 32.73 ± 0.22 mmol/L for 4% of eggshell of size x_5. The

hydrogen production in case of increasing concentration of eggshell followed both oxidative with production of acetate, butyrate and ethanol along with reductive pathway with production of 1,3-PD as seen from the Table 6.1.3. The increase in the production of hydrogen 37.58 ± 0.32 mol/L while using 1% (*w/v*) eggshell was marginal increase in comparison to 36.53 ± 0.53 mmol/L using 0.25% (*w/v*) of eggshell concentration as seen in Table 6.1.2. The hydrogen production in presence of 0.125% (*w/v*) of eggshell was lower in comparison to hydrogen production at 0.25% (*w/v*) eggshell. The concentration of 0.25% (*w/v*) eggshell in case of 1.75% (*w/v*) CG resulted in the optimum conditions for increased hydrogen production for the co-culture system. The optimum condition of 0.25% (*w/v*) of eggshell matched the total (*w/v*) of KH_2PO_4 (0.2%) and $MgSO_4.7H_2O$ (0.05%) used in the basal media for the inoculum growth.

Increase in the concentration of eggshell resulted in the marginal increase in the hydrogen production and 0.25% (*w/v*) of eggshell of size x5 was the optimum condition. The optimum condition of 0.25% (*w/v*) of eggshell of size x_5 was fixed and used in the later studies.

Hydrogen production with and without different media components

In order to determine the role of eggshell, each of the media components was replaced during hydrogen production. The optimum condition of modified basal media CG (1.75%), and in presence and absence of casein peptone (2%), yeast extract (0.05%), KH_2PO_4 (0.2%), $MgSO_4.7H_2O$ (0.05%) was studied at eggshell size of x5 with 0.25% w/v concentration. The experimental sets, along with hydrogen and end-metabolite production with and without f media components are presented in Table 6.1.4. The highest hydrogen production was around 30.73 ± 0.32 mmol/L in case of media composition without KH_2PO_4 and $MgSO_4.7H_2O$ and minimum was around 21.86 ± 0.44 mol/L in case of media composition without yeast extract. The decrease in pH towards weakly alkaline favors methanogenesis, homoacetogensis and direct consumption of produced hydrogen, requiring addition of external buffering agents (Tenca *et al.*, 2011). The purpose of this study was to determine the role of eggshell as a replacement for any of the possible media supplements. The eggshell is used as neutralizing agent during the fumaric acid production as replacement of synthetic $CaCO_3$ (Das *et al.*,

2015). In case of hydrogen production, the property of eggshell as neutralizing agent is justified and can be used as possible supplement to replace costly media components (KH_2PO_4 and $MgSO_4.7H_2O$). The role of KH_2PO_4 is to regulate the pH during fermentation (Liu *et al.*, 2007) and $MgSO_4.7H_2O$ addition as trace metal necessary for biomass generation during hydrogen production (Alshiyab *et al.*, 2008). The composition of eggshell with $CaCO_3$ (94%), magnesium carbonate (1%), calcium phosphate (1%) acts as a natural neutralizing agent for the microorganisms (Wei *et al.*, 2009). In support of using eggshell resulted with higher production (30.73 ± 0.32 mmol/L) in comparison to synthetic $CaCO_3$ with only 26.43 ± 0.23 mmol/L hydrogen production. In the absence of nitrogen source, such as yeast extract and peptone, the co-culture system was able to produce around 22.08 ± 0.29 mmol/L of hydrogen. The presence of organic matter (4%) (Wei *et al.*, 2009) in the eggshell along with 3% nitrogen content in CG supplemented the nitrogen source during hydrogen production.

The eggshell with the role of maintaining the fermentation pH, immobilizing agent along with possible replacement as neutralizing agent can be used for hydrogen production from crude glycerol.

Hydrogen production in comparative studies

In the presence and absence of media components using the eggshell, the co-culture system was able to produce the increased hydrogen production. In order to determine the role of eggshell in complete absence of media components, comparative studies were carried out. The conditions used across the studies included CG at 1.75%, eggshell sixe x_5 (0.25%) along with media components (casein peptone (2%), yeast extract (0.05%), KH_2PO_4 (0.2%), $MgSO_4.7H_2O$ (0.05%)). The experimental plan along with hydrogen and end-metabolites production across the comparative studies is presented in the Table 6.1.5. In the absence of the eggshell using 1.75% of CG, the co-culture system was able to produce around 32.07 ± 0.92 mmol/L of hydrogen. In this case, the fermentation followed the reductive pathway with butyric acid production (2.73 ± 0.04 g/L) with trace amount of acetic acid (0.27 ± 0.03 g/L) production. Butyric acid theoretical yield from CG is only 2 mole of hydrogen in comparison to acetic acid production with 3 mole of hydrogen. In case of eggshell, the co-culture system was able

to produce increased hydrogen production around 36.53 ± 0.53 mmol/L following the reductive pathway with production of acetic acid (2.92 ± 0.01 g/L) along with butyric acid (1.44 ± 0.04 g/L). In presence of eggshell, the glycerol fermentation favored the acetic acid production along with increased hydrogen production (as explained in section 3.1). The same condition was studied for the mono-culture system. In case of *E. aerogenes*, the hydrogen production was around (24.21 ± 0.52 mol/L) along with acetic acid (1.54 ± 0.02 g/L) and trace amount of 1,3-PD production (2.70 ± 0.03 g/L). In case of *C. butyricum,* only hydrogen production was around (20.14 ±0.38 mmol/L) with butyric acid (1.81 ± 0.03 g/L) and 1,3-PD production (2.80 ± 0.08 g/L). The concentration of CG at 1.75% *w/v* tend to act as substrate inhibitor during mono-culture studies resulting in increased production of 1,3-PD for both *E. aerogenes* and *C. butyricum*. The co-culture system in the presence of CG with eggshell and in the absence of media components resulted in (31.66 ± 0.55 mmol/L) of hydrogen production along with acetic acid (2.67 ± 0.03 g/L) and butyric acid (1.16 ±0.01 g/L) production.

The H_2 concentration (31.66 ± 0.55 mmol/L) obtained without media components were comparable to without eggshell (32.07 ± 0.92 mmol/L). In the absence of media components, the limiting conditions such as neutralizing property and nutrient/organic source were supplemented by EGS and CG for hydrogen production. The media components might have masked the available nutrients from both CG and eggshell, which resulted in marginal hydrogen production (32.07 ± 0.92 mmol/L). However, with EGS as replacement of media components the co-culture system was able to produce sufficient hydrogen (31.66 ± 0.55 mmol/L). The conditions of absence of CG and in presence of eggshell and media components resulted in 6.35 ± 0.05 mol/L of hydrogen production.

The comparative studies at optimized conditions validated the replacement of media components by EGS during H_2 production in presence of CG by co-culture system.

Semi-continuous hydrogen production using 7.5 L bioreactor

The maximum benefit in terms of substrate inhibition, product inhibition, maintaining pH during generation of metabolites and elimination of media components was achieved

with semi-continuous low-cost approach of hydrogen production (Sarma *et al.*, 2015a). The study was carried out using mono-culture (*E. aerogenes*) in presence of only CG along with distilled water without media components. The approach was modified with addition of 0.25% (*w/v*) EGS and carried out using co-culture system (*E. aerogenes* and *C. butyricum*). The purpose was to scale-up the results obtained during serum bottle of 125 mL volume to bioreactor with 7.5 L volume. During the comparative study, the co-culture system produced more hydrogen in comparison to mono-culture system. As seen from the cost analysis for semi-continuous process considered for each of the comparative studies as seen in Table 6.1.6. The total cost for bioconversion of 1 kg of CG into hydrogen by co-culture system using only EGS resulted around $24.24 in comparison to using media components was around $408.22.

In context to the above results and to develop cost-effective process, co-culture system without media components using 0.25% (*w/v*) eggshell with semi-continuous approach of hydrogen production was carried out using. The online monitored data of the parameters (pH, dissolved oxygen, temperature, agitation and hydrogen) during semi-continuous fermentation (7.5 L) using co-culture system for hydrogen production are represented in the Figure 6.1.2.

Using *E. aerogenes* with semi-continuous approach produced around 210 mmol or 5.18 L H_2/ L of medium with 65% glycerol utilization (Sarma *et al.*, 2015a). In this study with co-culture system using 0.25% (*w/v*) eggshell result obtained was almost 1.5-fold increase with 312.12 mmol or 7.69 L H_2/ L of medium with 86.65% glycerol utilization. The co-culture system in absence of eggshell resulted with only 1.15-fold increase in absence of eggshell (data not shown). The results obtained across the serum bottle study with 125 mL volume matched the results obtained using bioreactor of 7.5 L volume. Different types of bioreactors with varying volumes, with batch, fed-batch and continuous and stirred reactors have been used for H_2 production (Vatsala *et al.*, 2008). The semi-continuous bioreactor works with CG diluted only with distilled water along with eggshell to reduce substrate inhibition, increase glycerol utilization and reduce process cost. Based on the results parallel treatment of crude glycerol along with

eggshell can be achieved with sustainable state of H_2 production using semi-continuous bioreactor.

Every year around ± 5700 tons of crude glycerol is produced across Quebec region (Pachapur *et al.*, 2016b). With the optimized condition from this study around 814 tons of eggshells are required at ratio of 1:7. Food processing industry spends about $12 per hour to dry one ton on eggshell waste before landfilling or composting with increasing problems of disposal cost, distant landfills with excess field capacity (eggshell-waste-into-income, 2015). EU has already banned landfill disposal or composting of eggshell waste with moisture content of over 4% (eggshell-waste-into-income, 2015). However, with valorization approach of using eggshell will save around $ 100,000/year for small and medium sized food processing industry (eggshell-waste-into-income, 2015). In addition, the biodiesel industry will generate revenue from the waste crude glycerol with efficient process of hydrogen production as a viable alternative energy fuel.

The method of using the eggshell during the hydrogen production from CG by co-culture system is a novel approach. The ability of eggshell to neutralize and maintain the pH during the hydrogen production can replace the cost of the neutralizing agent, which in turn will be a huge boost to the hydrogen production research. In other application of the eggshell as immobilizing agent, it will not only act as cost-effective source of immobilizing agent but also replace the complex steps of immobilization technique(Pachapur *et al.*, 2015c) with simple step of addition during fermentation. Eggshells can also act as immobilizing support for attachment and growth of microorganisms (Das *et al.*, 2015). The eggshell as the source of $CaCO_3$ possesses the property to maintain the pH, with supplementation in the media tending to increase the cell growth and substrate conversion efficiency resulting in increased hydrogen production. In the absence of the media components, the co-culture system has the ability to utilize the nutrients across both CG and eggshell for increased hydrogen production. The addition of eggshell as a replacement of media component will uplift the hydrogen production industry. In terms of cost calculation, with elimination of glucose, casein polypeptone, KH_2PO_4, yeast extract and $MgSO_4.7H_2O$ across the media with only utilization of crude glycerol and eggshell, the media cost will reduce to 85-90%

during hydrogen production (as seen from Table 6.1.6). The media chemical cost can be reduced by using the eggshell, which in turn will benefit the hydrogen production industry. Even after the end of fermentation the spent media along with eggshell can be a perfect phosphate solubilizing biofertilizer (Sarma *et al.*, 2015b). The role of eggshell during hydrogen production is limitless ranging from fermentation pH neutralizing agent, immobilizing agent, media supplement and nutrient source with added advantage as biofertilizer. In the recent research, eggshell was used as low-cost solid catalyst for biodiesel production (Wei *et al.*, 2009), and as promising biosorbent for enchanced carbon dioxide capture (Sacia *et al.*, 2013). The use of eggshell will help valorize the solid waste of food processing industry and additional economic benefit of replacing costly media components during hydrogen production. Use of eggshell with biodiesel synthesis to generate crude glycerol followed by in-house hydrogen production using eggshell and using the spent media with eggshell as fertilizer will make biodiesel industry competitive with petroleum industry.

Conclusion

The property of eggshell as immobilization for attachment, adsorption and growth of microorganisms can be exploited during hydrogen production using crude glycerol by co-culture system. Use of different sizes of eggshells at 0.25% (*w/v*) increased hydrogen production with decrease in the size of eggshells. The maximum hydrogen production resulted with eggshell size of x_5 with 36.53 ± 0.53 mmol/L during batch followed by 41.16 ± 0.95 mmol/L during repeated-batch fermentation. The eggshell also played the role of neutralizing agent by maintaining the pH (within 6.00 to 6.30) of the fermentation media, exhibited the role of immobilizing agent and reusability during repeated-batch fermentation. The use of eggshell can be a possible media supplement with hydrogen production of (31.66 ± 0.55 mmol/L) in its absence in comparison to presence of media supplement producing 32.07 ± 0.92 mmol/L. Using eggshell can reduce the media chemicals component cost by 85-90% during hydrogen production.

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Table 6.1.1: The trend of using additional media components, co-substrate utilization and immobilization techniques for increased H_2 production.

Material	**Microorganisms**	**Concentration**	**Purpose**	**H_2 increase**	**Ref.**
Acetate	*Clostridium* spp.	3.2 g/L	Additional media component	9%	(Heyndrickx *et al.*, 1991)
Polypeptone	*C. butyricum* and *E. aerogenes*	10 g/L	Additional media component	3%	(Yokoi et *al.*, 2001)
Corn steep liquor	*C. butyricum* and *E. aerogenes*	10 g/L	Media replacement	0.74%	(Yokoi *et al.*, 2002)
Apple pomace hydrolysate	*C. butyricum* and *E. aerogenes*	5 g/L	Co-substrate	2.83 fold	(Pachapur *et al.*, 2015b)
Porous glass beads	*C. butyricum* and *E. aerogenes*	100 mL (3-5 mm in diameter, 60–300 mm in pore size) packed 2 mm mesh)	Immobilization	1.3 fold	(Yokoi et *al.*, 1998)
Banana leaves (BL), coconut coir (CC), groundnut shells (GS) or pea shells (PS)	*Bacillus* and *Enterobacter* spp.	3 g/L packed in polyvinylchloride tube (3X2 cm)	Immobilization	2.5-6.4 fold	(Patel *et al.*, 2014; Patel *et al.*, 2010)
Eggshells (EGS)	*C. butyricum* and *E. aerogenes*	0.25% (*w/v*)	Neutralizing and immobilizing agent	1.5 fold	This study

Table 6.1.2: Hydrogen (mmol/L) and end-metabolite concentration (g/L) obtained with varying sizes of eggshells at 0.25% (*w/v*) concentration during batch and repeated batch fermentation.

Batch fermentation eggshell size	**Hydrogen (mmol/L)**	**Ethanol (g/L)**	**Butyric acid (g/L)**	**Acetic acid (g/L)**	**1,3-PD (g/L)**
1.7 mm<x_1< 3.35 mm	29.33 ± 0.38	1.11 ± 0.12	1.23 ± 0.01	1.33 ± 0.11	3. 56 ± 0.34
850 µm<x_2< 1.7 mm	30.97 ± 0.84	1.31 ± 0.07	1.28 ± 0.02	1.91 ± 0.04	3. 53 ± 0.17
300 µm<x_3< 850 µm	32.21 ± 0.96	1.16 ± 0.20	1.27 ± 0.02	2.29 ± 0.06	3. 53 ± 0.17
75 µm <x_4< 300 µm	33.57 ± 0.82	1.14 ± 0.24	1.31 ± 0.04	2.49 ± 0.06	3. 51 ± 0.28
33 µm <x_5< 75 µm	36.53 ± 0.53	1.27 ± 0.11	1.44 ± 0.04	2.92 ± 0.01	3.87 ± 0.15
Repeated-batch fermentation	**Hydrogen (mmol/L)**	**Ethanol (g/L)**	**Butyric acid (g/L)**	**Acetic acid (g/L)**	**1,3-PD (g/L)**
1.7 mm<x_1< 3.35 mm	34.93 ± 0.81	1.23 ± 0.37	1.54 ± 0.10	2.78 ± 0.08	3. 78 ± 0.24
850 µm<x_2< 1.7 mm	35.76 ± 0.91	1.45 ± 0.10	1.74 ± 0.09	2.89 ± 0.06	3. 99 ± 0.15
300 µm<x_3< 850 µm	36.50 ± 0.82	1.64 ± 0.03	1.79 ± 0.04	2.95 ± 0.03	3. 83 ± 0.16
75 µm <x_4< 300 µm	37.45 ± 0.70	1.79 ± 0.28	2.32 ± 0.03	3.14 ± 0.22	4. 16 ± 0.28
33 µm <x_5<75 µm	41.16 ± 0.95	1.68 ± 0.36	2.44 ± 0.11	3.46 ± 0.14	4.28 ± 0.31

Table 6.1.3: Hydrogen (mmol/L) and end-metabolite concentration (g/L) in presence of increasing concentration (*w/v*) (%) of eggshells of size x_5 (33 µm <x_5< 75 µm).

Batch fermentation eggshell of size x_5 concentration (%)	Hydrogen (mmol/L)	Ethanol (g/L)	Butyric acid (g/L)	Acetic acid (g/L)	1,3-PD (g/L)
0.25	36.53 ± 0.53	1.27 ± 0.11	1.44 ± 0.04	2.92 ± 0. 01	3.87 ± 0.15
0.5	37.05 ± 0.41	1.11 ± 0.02	1.22 ± 0.01	2.75 ± 0.05	3.62 ± 0.12
1	37.58 ± 0.32	1.42 ± 0.03	2.15 ± 0.02	3.08 ± 0.02	3.75 ± 0.01
2	36.81 ± 0.11	1.46 ± 0.13	1.99 ± 0.04	2.97 ± 0.01	3.81 ± 0.02
4	32.73 ± 0.22	1.69 ± 0.04	1.38 ± 0.01	2.84 ± 0.05	3.94 ± 0.03

Table 6.1.4: Hydrogen (mmol/L) and end-metabolite concentration (g/L) using co-culture system in presence and absence of media components at 17.5 g/L of CG with eggshell x_5 at 0.25% (*w/v*) concentration.

Batch fermentation	Hydrogen (mmol/L)	Ethanol (g/L)	Butyric acid (g/L)	Acetic acid (g/L)	1,3-PD (g/L)
Without yeast extract	22.08 ± 0.29	1.34 ± 0.34	0.21 ± 0.01	0.79 ± 0.23	2. 94 ± 0.40
Without casein peptone	21.86 ± 0.44	2.12 ± 0.01	0.17 ± 0.02	1.62 ± 0.06	4. 46 ± 0.15
Without KH_2PO_4	27.57 ± 0.32	0.90 ± 0.01	0.19 ± 0.02	1.84 ± 0.09	3 .62 ± 0.02
Without $MgSO_4.7H_2O$	30.18 ± 0.24	1.06 ± 0.02	1.24 ± 0.01	2.21 ± 0.10	3. 14 ± 0.08
Without KH_2PO_4 and $MgSO_4.7H_2O$	30.73 ± 0.32	1.29 ± 0.50	1.22 ± 0.03	1.79 ± 0.17	2. 22 ± 0.14
With synthetic $CaCO_3$	26.43 ± 0.23	1.18 ± 0.03	0.21 ± 0.10	2.12 ± 0.02	3. 55 ± 0.08

Table 6.1.5: Hydrogen (mmol/L) and end-metabolite concentration (g/L) for comparative study across mono- and co-culture system at 17.5 g/L of CG with eggshell x_5 at 0.25% (*w/v*) concentration.

Batch fermentation	Hydrogen (mmol/L)	Ethanol (g/L)	Butyric acid (g/L)	Acetic acid (g/L)	1,3-PD (g/L)
CG + media components, without eggshell for co-culture	32.07 ± 0.92	1.96 ± 0.16	2.73 ± 0.04	0.27 ± 0.03	2. 17 ± 0.08
CG + media components + eggshell for co-culture	36.53 ± 0.53	1.27 ± 0.11	1.44 ± 0.04	2.92 ± 0.01	3. 87 ± 0.15
CG + media components + eggshell for *E. aerogenes* only	24.21 ± 0.52	1.25 ± 0.01	0.90 ± 0.01	1.54 ± 0.02	2. 70 ± 0.03
CG + media components + eggshell for *C. butyricum* only	20.14 ± 0.38	1.13 ± 0.02	1.81 ± 0.03	0.84 ± 0.02	2. 80 ± 0.08
CG + eggshell, without media components for co-culture	31.66 ± 0.55	1.94 ± 0.06	1.16 ± 0.01	2.67 ± 0.03	2. 56 ± 0.16
Media components + eggshell, without CG for co-culture	6.35 ± 0.05	0.02 ± 0.03	0.32 ± 0.02	0.13 ± 0.04	0. 42 ± 0.12

Table 6.1.6: Cost analysis for bioconversion of 1 kg of crude glycerol into hydrogen for each of the comparative studies using co-culture system.

Process and materials required	**Amount for bioconversion of 1 kg CG**	**Cost ($)**		
		CG + media components, without EGS	**CG + media components + EGS**	**CG + EGS, without media components**
Inoculum development (2.5 L)				
Glucose monohydrate (1%)	25 g	1.14	1.14	1.14
Casein peptone (2%)	50 g	19.00	19.00	19.00
KH_2PO_4 (0.2%)	5 g	1.01	1.01	1.01
Yeast extract (0.05%)	1.25 g	0.15	0.15	0.15
$MgSO_4$ (0.05%)	1.25 g	0.05	0.05	0.05
L-cysteine (0.1%)	2.5 g	2.13	2.13	2.13
Media preparation (47.5 L)				
Crude glycerol	1 kg	0.10	0.10	0.10
Casein peptone (2%)	950 g	361.00	361.00	NIL
KH_2PO_4 (0.2%)	95 g	19.19	19.19	NIL
Yeast extract (0.05%)	23.75 g	2.85	2.85	NIL
$MgSO_4$ (0.05%)	23.75 g	0.95	0.95	NIL
EGS (0.25 %)	118.75 g	NIL	0.01	0.01
Fermentation (143 h)	13.15 kWh	0.65	0.65	0.65
Total cost ($) for bioconversion of 1 kg CG		408.22	408.23	24.24

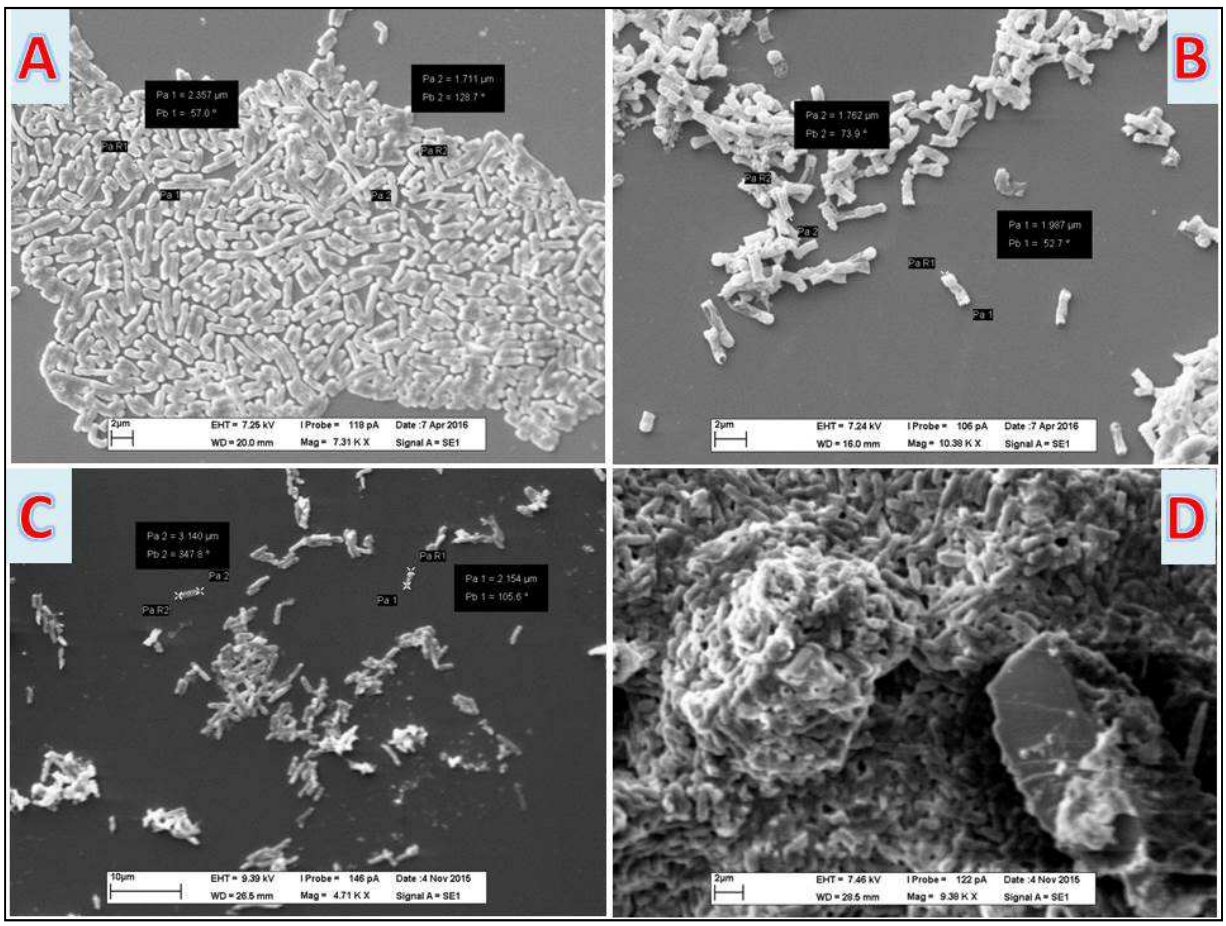

Figure 6.1.1: Scanning electron micrograph of the (A) mono-culture *E. aerogenes* without eggshell; (B) mono-culture *C. butyricum* without eggshell; (C) co-culture of *E. aerogenes* and *C. butyricum* without eggshell; (D) co-culture with eggshell.

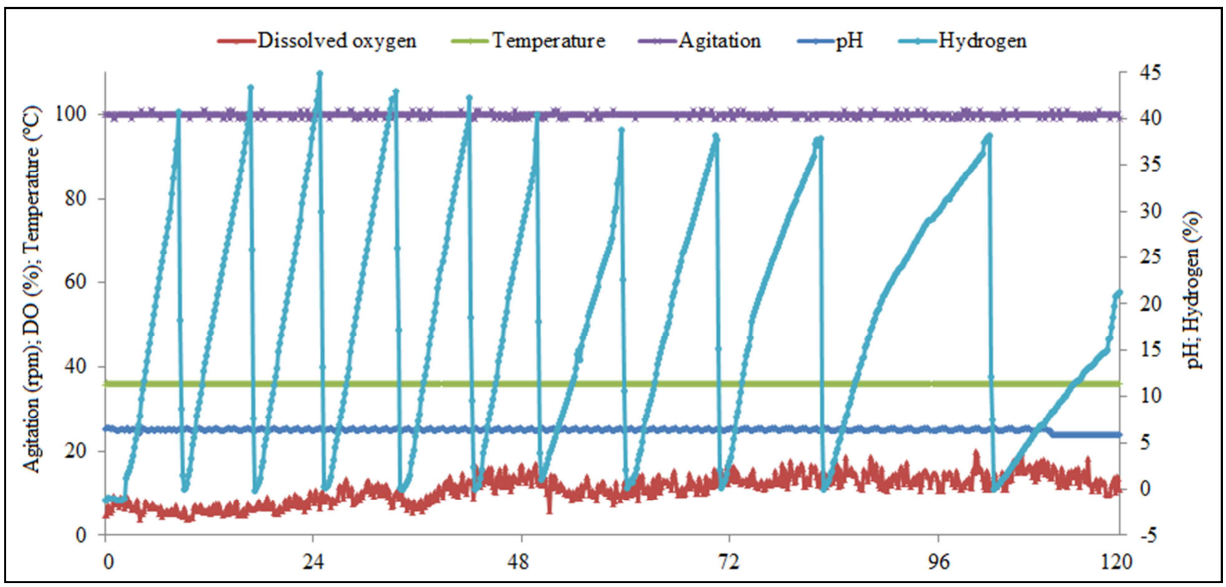

Figure 6.1.2: The online monitored data of the parameters (pH, dissolved oxygen, temperature, agitation and hydrogen) during semi-continuous fermentation (7.5 L) using co-culture system for hydrogen production.

CHAPTER 7

HYDROGEN PRODUCTION BY CO-CULTURE SYSTEM WITH DARK AND PHOTO-FERMENTATION

PART 1

ALTERNATE APPROACH FOR CO-CULTURE SPENT MEDIA UTILIZATION ACROSS MIXED-CULTURE AND PHOTOFERMENTATION

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Résumé

En vue d'élever l'industrie du biodiesel en tant que vecteur énergétique majeur, une nouvelle approche pour minimiser les déchets et utiliser ces déchets générés a été explorée. Les milieux usés générés par le système en co-culture peuvent servir comme supplément renouvelable pour les cultures mixtes afin de produire du H_2. Ce milieu épuisé peut également remplacer les milieux utilisés en photo fermentation pour produire des liplides. La conversion directe des médias usés avec le glycérol brut à 20 g/L à l'aide de boues de station d'épuration traitées par choc thermique a donné lieu à 38,12 ± 0,84 mmol/L de H_2. Dans une autre approche, les médias usés ont été utilisés comme co-supplément avec un milieu frais à un rapport de 3:2 pour la croissance des algues, ce qui entraîne un rendement de 0,098 ± 0,007 g/L de production de lipides. Les médias usés contiendraient de la biomasse morte, des nutriments résiduels, des biomolécules et du glycérol inutilisé. Cette matrice pourrait agir comme source supplémentaire au cours de la production de l'hydrogène et des lipides. L'hydrogène produit peut être utilisé comme source d'énergie interne dans l'industrie et les lipides peuvent être utilisés comme matière de première génération. L'étude explore l'utilisation du glycérol brut, des milieux usés et des boues primaires vers une approche en système fermé efficace pour l'industrie du biodiesel afin de minimiser la production de déchets et augmenter la valeur commerciale du GB.

Mots clés: Biodiesel; glycérol brut; lipide; mixte -culture; photo-fermentation

Abstract:

In a view to uplift the biodiesel industry as a major energy carrier, new approach of minimizing the waste and utilizing generated waste needs to be explored. The spent media generated from the co-culture system is cost effective and can serve as renewable supplement for mixed-culture of hydrogen (H_2) production and media replacement for lipid production. Direct conversion of spent media along with crude glycerol (CG) at 20 g/L using heat-shock pretreated wastewater sludge resulted in 38.12 ± 0.84 mmol/L of H_2 production. In another approach, the spent media was used as co-supplement along with fresh media at 3:2 for algal growth, resulting in 0.098 ± 0.007 g/L of lipid. The spent media contained dead biomass, residual media nutrients, biomolecules and unutilized glycerol together acting as supplementary source during H_2 and lipid production. According to the closed system results, the H_2 produced ($1.47x10^9$ L of H_2) can be converted into energy ($1.87\ x10^4$ GJ) for electricity ($1.77\ x10^4$ GJ) and heat ($4.32\ x10^3$ GJ). The produced H_2 can be used as in-house energy source and the lipids can be used as third generation feedstock. The study explores the utilization of CG, spent media and biodiesel primary sludge (BPS) towards an efficient closed system approach for biodiesel industry to utilize the waste generation and to increase the commercial value of CG.

Keywords: Biodiesel; crude glycerol; lipid; mixed-culture; photo-fermentation

Introduction

Significant success in renewable energy has resulted in the commercial utilization of biohydrogen in comparison to fossil fuels utilization with concerns of global climate change (Sarma *et al.*, 2015b). The advantages of hydrogen over commercial fuels resulted with strong support from government policies and increased incentives worldwide (Sarma *et al.*, 2015b). Across the world, a new initiative of bioconversion of crude glycerol (CG) (by-product of biodiesel industry) to hydrogen (H_2) production is carried out to expand biodiesel industry (Ayoub *et al.*, 2012). The characteristics of crude glycerol, such aslow market value, feedstock availability, with increased reduction state in comparison to other organic wastes make it best suited for microbial conversion to H_2 production over other value-added products (Pachapur *et al.*, 2016a).

The bioconversion of CG to H_2 can be carried out using co-culture, mixed-culture and photo-fermentation systems. Each of the systems has advantages and disadvantages. The co-culture system works in harmony, reduces the fermentation time, performs complex functions and produces higher H_2 in comparison to mono-culture system (Pachapur *et al.*, 2015). The dark fermentation carried out using mixed-culture system have broader variety of potential substrate, including residuals and waste products during hydrogen production (Nath *et al.*, 2005). Photo-fermentation offers typical advantages with high theoretical conversion ability and utilization of organic acids (acetate, butyrate) or solvents (acetone, butanol) produced during dark fermentation (Nath *et al.*, 2005).

The accumulation of organic acids and solvents results in sharp drop in fermentation pH and limit H_2 production during dark fermentation. The spent media containing organic compounds and unutilized substrate with media components is of high interest as promising choice for waste utilization (Sargsyan *et al.*, 2016). Researchers have carried out combined dark and photo-fermentation for complete utilization of chemical energy in spent media by using unconverted metabolites to resolve the problem of waste utilization (Nath *et al.*, 2005; Sargsyan *et al.*, 2016). Sustainable utilization of active biomass and spent media resulted in improved H_2 production (Sarma *et al.*, 2013) along

with one-pot green synthesis of nanoparticles (Morsy, 2014). The ethanol and beer producing industry spent waste is valorized into lactic acid production by utilizing the free nitrogen content in wastes and eliminating the necessary addition of nitrogen supplement (Djukić-Vuković *et al.*, 2016). Production of ethanol can also be carried out by using a simple step of acid pretreatment on waste algal biomass resulting in almost 2-fold increased yield in comparison to control experiment using glucose (Fathima *et al.*, 2016). The spent media generated across different systems with purpose of production of value-added compounds is presented in Table 7.1.1.

The H_2 production is only able to utilize 30-40% of substrate with remaining 60-70% used across metabolite production. Bioconversion of CG to 1 kg of H_2 generates 8700 L of spent media consisting of organic carbon, total nitrogen, media components, biomass, metabolites in distilled water (Sarma *et al.*, 2015b). In order to match the production level and market value of H_2 with other commercial fuels, it is mandatory to valorize the spent media. Thus, spent media utilization will minimize the additional media components usage and the distilled water used for the next batch of H_2 production.

In the present study, the spent media after the co-culture system of H_2 production was proposed as media supplement during mixed-culture of H_2 production. The mixed-culture system was studied for the first time during H_2 production with biodiesel primary sludge (BPS) as inoculum along with spent media and CG as substrate. In another approach, the fresh media was replaced with spent media and used for algal growth for lipid production. In the interest of algae as third generation feedstock for the biodiesel production and to minimize the cost of TAP (Tris-Acetate-Phosphate) growth media, spent media was used for *Chlamydomonas reinhardtii* growth during lipid production. The approach was to utilize the spend media generated during H_2 production in an efficient closed system approach to uplift the commercialization of H_2 and biodiesel fuel.

Materials and Methods

Crude glycerol as substrate

The animal by-products from food processing, superstores and restaurants are recycled for biodiesel production by Rothsay®, Canada (http://www.rothsay.ca/environment/, 2016). The generated crude glycerol and wastewater sludge by Rothsay®, Canada are used in this study. The composition of crude glycerol with (w/w): 23.6% glycerol, 35.9% carbon, 5.7% moisture, 3.2% nitrogen, 3.1% ash, <1.0-0.5% methanol and 67.56% matter organic non-glycerol (MONG). The pH of the crude glycerol was around 3.4 ± 0.1.

Chemicals and reagents used in this study are purchased from Fisher scientific, VWR and Lallemand, Canada.

Seed inoculum for mixed-culture

Rothsay®, carries out rigorous wastewater treatment prior to discharge of recycled water in local waterbodies (http://www.rothsay.ca/environment/, 2016). The purification system prior to discharge generates biodiesel primary sludge (BPS) (), which was used as seed inoculum to carry out mixed-culture system of H_2 production. The BPS was stored at 4 ^{0}C, prior to pretreatment to produce H_2 using CG. Likewise, wastewater secondary sludge (WSS) collected from Quebec Urban Community (QUC) wastewater treatment plant (WWTP) (Quebec, QC, Canada) was analyzed as possible seed inoculum along with BPS from biodiesel industry.

A comparative study of acid, alkali, chloroform and microwave pretreatment on wastewater sludge was carried out using CG as substrate. The increased H_2 production resulted in heat-shock pretreatment in comparison to other methods (Pachapur *et al.*, 2016a). In this study, BPS and WSS was subjected to heat pretreatment by transfer of 50 mL into two separate 150 mL serum bottles, pure nitrogen gas was sparged (3-4 min) to obtain anaerobic environment; the bottle was sealed using pre-inserted septa and transferred to pre-set 100 ^{0}C Isotemp Standard Lab Ovens for 15 min (Pachapur *et*

al., 2016a). The cooled treated BPS and WSS was used as inoculum and transferred using sterile syringe at varying volume for H_2 production.

Algae pre-culture media and inoculum development for photo-fermentation

The green algae, *Chlamydomonas reinhardtii* is being currently used in the H_2 production (Pachapur *et al.*, 2015) and considered as model organisms for accumulation of energy rich compounds, such as lipids (Siaut *et al.*, 2011) (Work *et al.*, 2010). The green algae, *C. reinhardtii* was grown using 100 mL of TAP growth medium (Gibco®, ThermoFisher Scientific, USA) ready-to-use 1X with pH 7.0, under constant agitation of 60 rpm at 20 ± 1^0C incubation temperature with continuous illumination of 60-80 µmol/m^2/s throughout 7 days(Lavoie *et al.*, 2014).

Hydrogen production using spent media by mixed-culture system

The optimum condition of 20 g/L crude glycerol (CG), 20% inoculum size (InS) and pH 7.0 from our previous study using wastewater secondary sludge as seed inoculum was utilized (Pachapur *et al.*, 2016a). Proposed addition of spent media during the fermentation was carried out for increased H_2 production. The spent media characteristics are presented in Table 7.1.2. The spent media obtained after H_2 production was used to make-up the final volume to replace the addition of distilled water. With the presence of unutilized CG in spent media, the CG concentration in the fermentation media was varied across 15, 20 and 25 g/L. The spent media with increasing concentration of CG was mixed with spent media to make-up the working volume to 40 mL. A control experiment using distilled water in absence of spent media was also carried out. The pH was set at 7.0, transferred to serum bottles, sparged with nitrogen, sealed with pre-inserted septa followed by sterilization at 121 ^{0}C for 15 min in autoclave. The pretreated sludge at 20% (*v/v*) inoculum size was transferred to the sterilized media using sterile syringe under laminar hood. The H_2 production was carried out at 150 rpm at 37 ^{0}C for five days and all the experiments were performed in triplicates. The presented values are the average of triplicates and error bars represent the standard deviation (±) values. During fermentation, every 24 h, gas sample using a gas tight syringe (1 mL) was collected from the headspace into vacuumed sample vials

for hydrogen analysis by gas chromatography (GC). Likewise, after five days, the fermented sample was analyzed for glycerol and end metabolite concentration by GC.

Lipid production using spent media by Photo-fermentation

The TAP growth media is optimized for *C. reinhardtii* culture and is ready-to-use, eliminates the procurement of individual media components, trace elements with tedious media preparation steps. The TAP media was replaced from (50 to 0 mL) with addition of spent media (autoclaved) at different volume from (0 to 50 mL) and final mixture was transferred under laminar flow chamber to serum bottles. The experimental set-up was carried out in aerobic (for lipid production) in triplicates. At the end of incubation, lipid estimation was carried out.

Material and Energy balance calculation

An efficient closed system approach is designed to support the biodiesel industry in order to valorize the wastes crude glycerol into in-house self-sufficient energy source. In this study, the energy and mass balance are calculated based on 45 million liter (average: 10-75 million L) of crude glycerol production across biodiesel industry in Canada (Pachapur *et al.*, 2016b). Bioconversion of CG into H_2 using semi-continuous fermentation with capacity conversion (240-356 L of cumulative H_2/kg of CG) (Sarma *et al.*, 2015a) are considered in the calculation. Further, energy value of produced H_2 in-terms of energy, electricity and heat (Pachapur *et al.*, 2016b; Zhang *et al.*, 2016) are calculated in this study.

Analytical techniques

Hydrogen analysis by GC

During the mixed-culture system, the hydrogen gas sample collected was analyzed using gas chromatography (Varian 3800, USA) with a set-up of thermal conductivity detector (TCD). The PoraPLOT Q® column (Agilent technology, USA) of 3 m width under carrier gas nitrogen at flow rate of 3.5 mL/min was used. During the method run, the injector, column temperature and detector temperature are set at 100^0C. The area under the curve was converted to volume of gas produced (mmol) in consideration of

the experimental conditions, such as temperature and atmospheric pressure (Pachapur *et al.*, 2015a).

End-metabolites/by-products analysis by GC-FID

The concentrations of glycerol and end-metabolites were determined using GC (7890B GC-Agilent, CA) with flame ionization detector (FID) system. The column used was ZB-WAX plus with carrier helium gas at 1 mL/min flow rate in a 80–240 ^{0}C temperature profile for 8.4 min run time (Pachapur *et al.*, 2015b).

Estimation of lipid production

The total lipids at the end of fermentation was extracted from *C. reinhardtii* biomass and determined using gravimetric method as described in (Chen *et al.*, 2012) (Sarma *et al.*, 2014). Around 35 mL of fermented media was subjected to centrifugation (4000 *x* g) for 15 min, the cell pellet was separated from the supernatant. Around 800 µL phosphate buffer (0.05 M, pH 7.4) and 400 µm glass beads was added and transferred to cell disruptor for 10 min. To the lysed mixture, 800 µL phosphate buffer, 4 mL of chloroform, 2 mL of methanol was mixed and the lipid was extracted by 15 min of sonication. After sonication, 2 mL each of chloroform and methanol was added and the resulting mixture was made to settle for separation. The bottom organic phase containing the lipids was transferred and equal volume of 5% NaCl solution (1:1 *v/v*) was added. The solvent was subjected to nitrogen evaporation; the left over lipid was calculated and expressed in g/L of medium (Chen *et al.*, 2012) (Sarma *et al.*, 2014).

Results and Discussion

Hydrogen production using spent media by mixed-culture system

Hydrogen production using the spent media in the absence of distilled water with biodiesel primary sludge (BPS), wastewater secondary sludge (WSS) and mix 1:1 (BPS:WSS) as seed inoculum by mixed-culture system was carried out. The mixed inoculum was composed of BPS and WSS at 1:1 ratio. The H_2 production using the optimized condition of (InS: 20% and pH:7) in case of different CG concentrations of 15, 20 and 25 g/L are presented in the Table 7.1.3. The maximum H_2 production was

around 38.12 ± 0.84 mmol/L for WSS at 20 g/L of CG. The minimum H_2 production was around 18.96 ± 0.13 for the mix at 15 g/L of CG as seen from Table 7.1.3.

In case of BPS as seed inoculum, the maximum H_2 production was around 27.09 ± 0.83 mmol/L with minimum of around 22.39 ± 0.23 mmol/L. Across the three seed inoculum types, the H_2 production increased from 15 to 20 g/L. However, with further increase at 25 g/L CG the H_2 production decreased. The CG concentration of 20 g/L was found to be optimum for the seed inoculum for the increased H_2 production. The optimum condition of (CG: 20 g/L, InS: 20% and pH 7.0) in case of heat treated WSS in absence of spent media utilization resulted in 29.43 ± 0.71 mmol/L of H_2 production (Pachapur *et al.*, 2016a). In this study, the spent media containing the unutilized CG along with media components benefited with 29.53% increased H_2 production (38.12 ± 0.84 mmol/L) .The results matched 32.5% obtained during use of spent media along with CG across *Enterobacter aerogenes* of H_2 production (Sarma *et al.*, 2013). The spent media contains dead biomass, residual media nutrients, biomolecules and unutilized glycerol together which act as supplementary source for the mixed-culture system for H_2 production.

The advantage of mixed-culture system to grow on broader choice of organic waste feedstock requires easy and simple pretreatment conditions. The ability to reuse the spent media during mixed-culture system uplifts the H_2 production making it economical. The impurities in CG have increased inhibition effect on co-culture at concentrations of 15-20 g/L and above (Pachapur *et al.*, 2015b). However, the seed inoculum from WSS nullified the inhibition effect at CG concentration of 20 g/L with increased H_2 production. The choice of WSS as seed inoculum along with heat-shock treatment proved to be the best combination for the utilization of spent media along with CG as substrate for increased H_2 production. In case of seed inoculum of BPS, the H_2 production reached a maximum of 27.09 ± 0.83mmol/L at 20 g/L of CG. The objective of using the BPS was to identify the microorganism community able to degrade glycerol at higher concentration, as BPS is in contact with residual glycerol after biodiesel production. However, the BPS possessed the ability to produce higher 1,3-propanediol (1,3-PD) (6.72 ± 0.51g/L) across other seed inocula as seen in Table 7.1.3. While monitoring the

H_2 production in most cases, production of 1,3-PD is also determined as they are important metabolites of the glycerol fermentation pathway. The production of 1,3-PD increased during the glycerol fermentation as reductive pathway was favored over oxidative pathway with decreased production of H_2 (Pachapur *et al.*, 2015a). This was true as CG was 25 g/L, H_2 production decreased to 22.39 ± 0.23 mol/L with increased production of 1,3-PD reaching a high of around 6.72 ± 0.51g/L in case of BPS as seed inoculum. The ability to degrade glycerol at higher concentration and produce a value-added compound 1,3-PD, in one way or the other will help the biodiesel industry. The combination of two sludges (BPS: WSS) at 1:1 ratio as seed inoculum was also investigated for H_2 production. The maximum H_2 production was around 24.06 ± 0.45 mmol/L and for 1,3-PD the maximum was around 3.45 ± 0.14 g/l in case of mix sludge as seen from the Table 7.1.3. In order to exploit the property of H_2 production from WSS and 1,3-PD from BPS, the mixed seed inoculum was investigated. The ratio of 1:1 was not sufficient in exploiting the property of both the seed inocula. A combination of different ratios can be tested for increased H_2 and 1,3-PD production.

The WSS is the final repository of various complex microorganisms possessing the property of working at higher substrate concentration with ability to degrade complex substrate and hence providing capability to reutilize the spent media with ease (Pachapur *et al.*, 2016a; Xiao *et al.*, 2009). The sludge generated from wastewater treatment plant is composed of microbial matter beneficial for anaerobic digestion during H_2 production along with H_2 consuming microorganisms (Phowan *et al.*, 2014; Xu *et al.*, 2009). Heat-shock pretreatment has been currently tested as a simple pretreatment step to screen and accelerate growth rate of H_2-producing species for increased H_2 production (Datar *et al.*, 2007; Phowan *et al.*, 2014).

Lipid production using spent media by algae

C. reinhardtii emerged as model organism for the synthesis of bioenergy carriers for the efficiency conversion of light, water and CO2 into renewable energy applications, such as H_2 and lipids (Work *et al.*, 2010). *C. reinhardtii* are gaining increasing attention to test cultivation strategies in increasing lipid yields for biodiesel production (Siaut *et al.*, 2011). The growth conditions of *C. reinhardtii* were optimized by (Lavoie *et al.*, 2014)

and used for the photofermentation using different volumes of spent media for lipid production and are presented in Table 7.1.4.

Lipid production using *C. reinhardtii* at different volumes of spent and fresh media (TAP growth media) was carried out and is presented in Table 7.1.4. The purpose was to reduce the utilization of fresh media (FM) and utilize the spent media (SM) during lipid production. The maximum lipid production was around 0.098 ± 0.07 g/L for the mixture of (SM: 30, FM: 20) and the minimum was around 0.010 ± 0.02 g/l in case of (SM: 0, FM: 50). In the presence of completely fresh media (50 mL), the lipid production was around 0.045 ± 0.006 g/L in comparison to 0.010 ± 0.002 g/L in presence of complete spent media (50 mL). With the increase in the concentration of the spent media from (0 to 30 mL), the production of lipid increased from 0.045 to 0.098 g/L. However, with further increase from 30 mL of spent media, the lipid production decreased reaching a minimum of 0.010 ± 0.002 g/L. The maximum lipid production of 0.098 ± 0.007 g/L was on higher side in comparison to (0.05 g/L) (Sarma *et al.*, 2014) and matched the results across different studies (Work *et al.*, 2010) (Zhao *et al.*, 2016). The spent media composition with organic/solvents and unutilized glycerol at minimum concentration tend to favor the growth of *C. reinhardtii*. With further increase in the volume of the spent media, the concentration of these compounds increased resulting in an inhibition of the growth of *C. reinhardtii* with decreased lipid production.

The spent media is composed of acetate and butyrate, which are utilized as substrate during photofermentation. In case of *C. reinhardtii* growth media, the external addition of organic acids is carried out along with complex media and micronutrients requirement during the lipid production. *C. reinhardtii* possesses the ability to grow on the acetate and was supplemented with glacial acetic acid with 20 mM (1.2 g/L) of carbon source in the minimal medium (Kim *et al.*, 2006). In order to determine the metabolite utilization across the spent media during lipid production, analysis of acetate, butyrate along with glycerol concentration before and after lipid production was carried out. The results of the metabolite concentration (g/L) along with utilization percentage (%) at the end of photofermentation (7-days) for lipid production are presented in the Table 7.1.5.

The results across the lipid production suggested that the mixture volume M4 (SM: 30, FM: 20) produced maximum lipid in comparison to other mixture volumes. In case of M4, the percentage utilization for glycerol was around 55.66%, butyrate was around 47.77% and acetate was highest with 99.03%. The percentage utilization of these compounds was highest across other mixture volumes, supporting the results with lipid production. The metabolite ethanol and 1,3-PD was also analyzed, however there was not much change in the percentage utilization (data not shown). The percentage of glycerol, acetate and butyrate utilization increased with the spent media volume till 30 mL. However, with further increase in the volume from 40 to 50 mL, there was decrease in the percentage utilization, similar to lipid production as presented in Table 7.1.4. The optimum concentration of acetate for the growth of *C. reinhardtii* was around 20 mM (1.2 g/L) (Kim *et al.*, 2006), in case of volume mixture of M5 and M6, the acetate limits the optimum conditions and inhibits the growth resulting in decreased lipid production. In the case of glycerol, the optimum concentration was around 30-50 mM (Pachapur *et al.*, 2015), which in case of M4 was within the limits. However, for M5 and M6, the concentration of glycerol reached the limiting concentration resulting in decreased lipid production. The optimum mixture volume (3:2) of spent media (30 mL) and fresh media (20 mL) resulted in the increased lipid production along with maximum percentage utilization of the metabolites from the spent media.

The spent media from the dark fermentation utilized around 60-70% of substrate for metabolite generation during H_2 production (Sarma *et al.*, 2015b). The presence of acetate and butyrate in the spent media, tend to act as carbon source and help towards the growth of *C. reinhardtii* for lipid production. The effective approach of utilizing the spent media will bring down the cost of microalgae cultivation and decrease the overall cost, making microalgae biodiesel competitive.

Efficient closed system approach for biodiesel industry

Treatment plants are facing increasing challenges in disposal of excess sludge due to rapidly shrinking landfills, stringent environmental standards, awareness from governing bodies and increasing disposal cost (Xiao *et al.*, 2009; Xu *et al.*, 2009). Researchers are exploring the sludge treatment and disposal methods for maximum energy recovery (Xu

et al., 2009). The proposed closed system is an alternative approach for sludge stabilization with reduction in the volume and weight of excess sludge through sustainable harvest into biofuels.

In a proposed efficient closed system for biodiesel industry as represented in figure 7.1.1, the approach was to minimize the waste generated during hydrogen production and utilize in an efficient approach to uplift biodiesel industry. In this study, the spent media obtained after co-culture system of hydrogen production was utilized for media preparation instead of distilled water for the mixed-culture system of hydrogen. The efficient closed system can use the secondary wastewater sludge as seed inoculum with simple heat-shock treatment. The mixed-culture successfully resulted in utilization of crude glycerol, spent media to produce hydrogen, which can be used as in-house energy fuel for biodiesel industry. In another approach of closed system, the spent media was successfully replaced by the fresh media and also resulted in increased lipid production. The utilization of spent media helped to minimize the use of fresh media, thereby decreasing the media cost for lipid production. In addition, the produced lipid can be used as third generation feedstock for the biodiesel industry.

Bioconversion of CG to 1 kg of H_2 utilizes 30-40% of substrate and generates 8700 L of spent media available as potential for energy recovery. With efficient closed system, the recovered energy can be utilized as fuel or electricity or heat to create an in-house self-sufficient energy source. This is based on an assumption that a biodiesel industry with capacity of 45 million liter production generates CG around $4x10^6$ L per year (http://www.rothsay.ca/environment/, 2016). In case of detailed methodology of bioconversion of 1 kg of CG into H_2 (Sarma *et al.*, 2015a; Vatsala *et al.*, 2008), material balance of microalgae cultivation (Zhang *et al.*, 2013) and energy balance equivalent (Pachapur *et al.*, 2016b) can be consulted. The 75% of CG (i.e $3x10^6$ L) is used for semi-continuous of H_2 production using co-culture system with capacity of (356 L of cumulative H_2/kg of CG) produces around $1.2x10^9$ L of H_2 with $7.4x10^{11}$ L of spent media. The spent media is divided into half and utilized for semi-continuous of H_2 production using mixed-culture system with capacity of (240 L of cumulative H_2/kg of CG) produces around $0.27x10^9$ L of H_2. According to the effective closed system

results, the H_2 produced ($1.47x10^9$ L of H_2) can be converted into energy ($1.87\ x10^4$ GJ) for electricity ($1.77\ x10^4$ GJ) and heat ($4.32\ x10^3$ GJ). The spent media ($3.7x10^{11}$ L), used for microalgae cultivation with capacity of 1200 m^3 to produce algae at 50 $kg/m^3/d$ with lipid content of 35-50% (*w/w*) (Zhang *et al.*, 2013) and can be used as third generation feedstock for biodiesel industry. The sludge treatment cost in case of 100 m3 reactor is around $270,864/year to reduce the organic loading rate in the effluent (Pachapur *et al.*, 2015; Vatsala *et al.*, 2008). Using effective closed system, the organic loading rate with H_2 production by co-/mixed-culture is reduced to 30-40% and further 60% reduction by photofermentation. The effective closed system was encouraging in terms of crude glycerol utilization, H_2 production, spent media reuse, sludge as inoculum, photo-fermentation for lipid production and sludge treatment. The decreasing market value of crude glycerol with the approach of efficient closed system will have a new market value. The small- and medium-scale biodiesel industry approach is to recycle around 99-100% of its input into value-added products. The efficient closed system can help to reach the figures requiring minor production modification and the long term result will uplift small- and medium-scale biodiesel industry.

Conclusions

The spent media generated during dark fermentation containing organic compounds and unutilized substrate with media components presents promising choice for waste utilization. The spent media can be used across different platforms to generate value-added chemicals. In this study, the spent media is used to replace distilled water used across media preparation during hydrogen production using mixed-culture system. The heat-shock pretreatment of wastewater sludge at 20% inoculum volume in presence of crude glycerol at 20 g/L resulted in increased hydrogen production of around 38.12 ± 0.84 mmol/L. In another approach, the spent media was replaced with fresh media across *C. reinhardtii* growth during lipid production. The mixture volume of spent media (30 mL) and fresh media (20 mL) resulted in 0.098 ± 0.007 g/L of lipid production. The spent media was used for both mixed-culture and photo-fermentation for hydrogen and lipid production. The effective closed system approach of utilizing crude glycerol, H_2 production, spent media valorization, sludge as inoculum, photo-fermentation for lipid

production and sludge treatment can make the biodiesel industry competitive in the biofuels market.

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Table 7.1.1: Spent media generated across different systems for production of value-added compounds.

Purpose	Spent media type	Process details	Product	Ref.
To complete utilization of chemical energy stored in spent media	After dark fermentation of H_2 production: unconverted metabolites	Photofermentation using *Rhodobacter sphaeroides* O.U.001	Hydrogen production: higher yield in combined process	(Nath *et al.*, 2005)
Sustainable utilization of waste from hydrogen production	After dark fermentation of H_2 production: spent and active biomass	Dark fermentation using *Enterobacter aerogenes*	Hydrogen production: improved from 13.37 to 57.98%	(Sarma *et al.*, 2013)
Fast mass scale one-pot green synthesis	After dark fermentation of H_2 production: waste culture	Bioreduction of silver ions into silver nanoparticles (AgNPs)	Silver nanoparticle: improved formation of AgNPs	(Morsy, 2014)
Bioconversion of waste algal biomass into ethanol	After the growth of algae: harvested algal biomass	Ethanol production using *Clostridium phytofermentans*	Ethanol production: improved production with acid pretreated algal biomass	(Fathima *et al.*, 2016)
To resolve the problem of waste utilization	After ethanol production: distillers grain	Dark- and Photo-fermentation for hydrogen production	Hydrogen production: distillers grain as effective and valuable source of organic substances	(Sargsyan *et al.*, 2016)
Valorization of waste substrates from bioethanol and beer productions	After ethanol and beer production: wasted bread, wasted potato stillage and brewers spent grain hydrolysate	Lactic acid production by *Lactobacillus rhamnosus* ATCC 7469.	Lactic acid production: appropriate use of free alpha amino nitrogen content in wastes	(Djukić-Vuković *et al.*, 2016)

Table 7.1.2: Characterization of spent media.

Composition of spent media	
Ethanol (g/L)	0.58 ± 0.18
Acetate (g/L)	2.03 ± 0.06
Butyrate (g/L)	2.37 ± 0.80
1,3-Propanediol (g/L)	0.92 ± 0.39
Residual glycerol (g/L)	5.02 ± 0.50
pH	5.56 ± 0.13

Table 7.1.3: Hydrogen (mmol/L) and 1,3-Propanediol (g/L) production across different seed inocula using variable crude glycerol concentrations (g/L).

Seed inoculum type	Crude glycerol g/L	Hydrogen mmol/L	1,3-Propanediol g/L
BPS	15	24.51 ± 0.20	2.83 ± 0.31
	20	27.09 ± 0.83	5.62 ± 0.11
	25	22.39 ± 0.23	6.72 ± 0.51
WSS	15	25.44 ± 0.62	3.17 ± 0.49
	20	38.12 ± 0.84	5.46 ± 0.37
	25	33.04 ± 0.61	6.52 ± 0.26
MIX	15	18.96 ± 0.13	1.87 ± 0.08
	20	24.06 ± 0.45	2.98 ± 0.09
	25	19.68 ± 0.52	3.45 ± 0.14

Table 7.1.4: Photofermentation using different media composition using varied volumes of spent (mL) and fresh media (mL) for lipid production.

Media composition	Spent Media mL	Fresh Media mL	Lipid g/L
M1	0	50	0.045 ± 0.006
M2	10	40	0.067 ± 0.006
M3	20	30	0.072 ± 0.002
M4	30	20	0.098 ± 0.007
M5	40	10	0.036 ± 0.004
M6	50	0	0.010 ± 0.002

Table 7.1.5: Glycerol and metabolite concentration (g/L) along with percentage of utilization across different media composition at the end of photofermentation (7-days) for lipid production.

Media composition	Acetate (g/L)	Butyrate (g/L)	Glycerol (g/L)
Before fermentation-M1	1.083 ± 0.01	0.021 ± 0.02	2.420 ± 0.03
After fermentation-M1	0.325 ± 0.01	0.020 ± 0.01	1.778 ± 0.08
% utilization	70.00 ± 1.37	3.58 ± 1.50	26.52 ± 3.25
Before fermentation -M2	1.157 ± 0.02	0.395 ± 0.01	2.667 ± 0.10
After fermentation -M2	0.053 ± 0.01	0.294 ± 0.01	1.631 ± 0.03
% utilization	95.39 ± 0.47	25.52 ± 1.79	38.84 ± 1.06
Before fermentation -M3	1.314 ± 0.02	0.760 ± 0.05	3.010 ± 0.07
After fermentation -M3	0.034 ± 0.01	0.544 ± 0.02	1.727 ± 0.10
% utilization	97.44 ± 0.56	28.45 ± 1.14	42.62 ± 2.12
Before fermentation -M4	1.387 ± 0.01	1.153 ± 0.02	3.712 ± 0.10
After fermentation -M4	0.013 ± 0.01	0.601 ± 0.01	1.646 ± 0.07
% utilization	99.03 ± 0.38	47.88 ± 0.73	55.66 ± 1.18
Before fermentation -M5	1.489 ± 0.02	1.529 ± 0.03	4.614 ± 0.05
After fermentation -M5	0.018 ± 0.01	0.894 ± 0.05	3.279 ± 0.06
% utilization	98.81 ± 0.41	41.56 ± 1.45	28.92 ± 1.38
Before fermentation -M6	1.527 ± 0.01	1.635 ± 0.02	4.846 ± 0.08
After fermentation -M6	0.049 ± 0.01	1.365 ± 0.05	3.720 ± 0.02
% utilization	96.80 ± 0.42	16.50 ± 1.29	23.23 ± 0.49

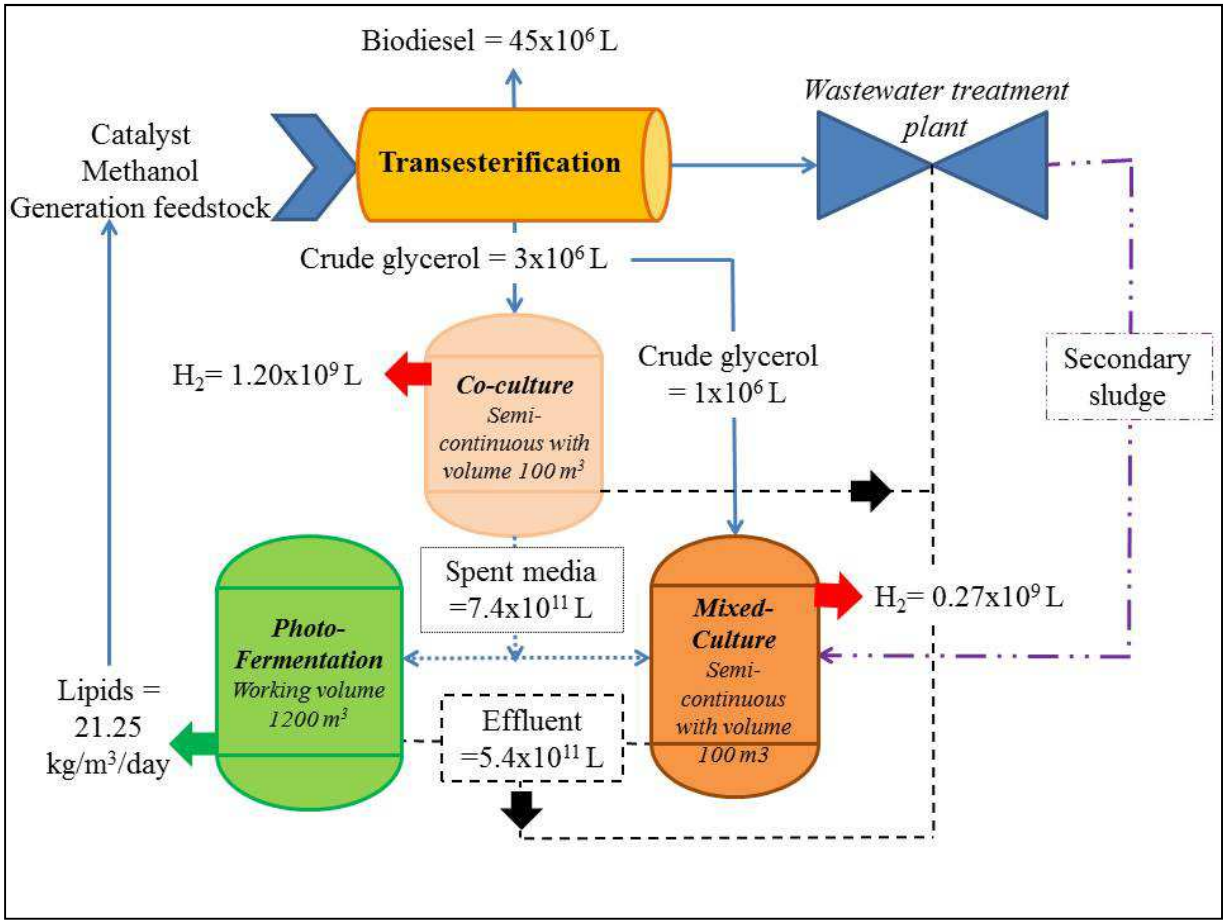


Figure 7.1.1: Efficient closed system approach for biodiesel industry for valorization of by-product crude glycerol into hydrogen production by co-culture system, utilization of the generated spent media across mixed-culture for hydrogen and photo-fermentation for lipid production.

Chapter 8

Conclusions et Recommandations

Conclusions

Les conclusions suivantes peuvent être tirées à partir de ce travail de recherche :

1. **Chapitre 2, Partie I**: Dans les prochaines années, la production de déchets organiques dépassera 250 milliards de tonnes dans par an, le monde entier. Les déchets organiques offrent une source abondante de substrats facilement disponibles et peu coûteux pour la production d'hydrogène par fermentation. Une approche durable pour la production d'hydrogène à partir de diverses méthodes, telles que photo-, fermentation en absence de lumière et séquentielle à deux étapes présente des avantages importants pour compléter le procédé traditionnel de production de biodiesel pendant la fermentation d'hydrogène, des microorganismes consortium bien définis et bien caractérisés sont étudiés. Des efforts de recherche importants ont été menés pour augmenter la production d'hydrogène en utilisant le système de co-culture, qui offre l'avantage d'une augmentation accrue dans le rendement en hydrogène et de taux de production par rapport aux mono-cultures. Le concept du système de co-culture est une simple étape de mélange de différentes souches microbiennes pour améliorer les propriétés individuelles manquantes dans les autres souches. Le système en co-culture est rentable, éliminant potentiellement l'étape de pré-traitement et évite l'utilisation d'un agent réducteur coûteux. En éliminant ces deux étapes, le coût total du procédé peut être réduit sans affecter le rendement en hydrogène. Le système en co-culture hydrolyse directement les substrats organiques complexes en sucres fermentescibles avec un rendement amélioré de 94,1% par rapport aux mono-cultures. La co-culture offre plusieurs avantages, par exemple, la réduction de la phase de latence, la résistance aux fluctuations de production et assure la stabilité de la vitesse de la production sans interruption d'hydrogène, ce qui est huit fois plus rapide en comparaison avec les mono-cultures. Le système de production d'hydrogène en co-culture est aussi une méthode alternative de traitement des effluents avec une réduction de 60% du niveau de la demande chimique en oxygène (DCO) et peut être facilement intégré dans une échelle pilote pour réaliser une production continue d'hydrogène. Les

performances du système de co-culture suggèrent un potentiel énorme de production d'hydrogène en utilisant des déchets organiques complexes applicable dans l'industrie.

2. **Chapitre 2, Partie II**: La production d'hydrogène à partir de déchets organiques biologiques est une approche moins coûteuse, moins exigeante en énergie, et un procédé respectueux de l'environnement. La mono-culture pure fournit un contenu d'hydrogène bas et un faible rendement. Ces limitations sont surmontées par l'utilisation d'un système de co-culture qui surpasse les cultures mixtes avec un rendement d'hydrogène amélioré. Les stratégies utilisées dans les systèmes de co-culture pour augmenter la production d'hydrogène ont été discutées dans cette revue. Ces stratégies incluent l'hydrolyse de divers substrats complexes tels que la cellulose, la mélasse, le glycérol brut et de la biomasse algale en sucres fermentescibles simples pour améliorer le rendement en hydrogène en éliminant l'utilisation d'enzymes exogènes. Les stratégies peuvent apporter des microorganismes isolés à partir de différentes sources géographiquement éloignées pouvant coexister pour une utilisation simultanée du substrat et de métabolites dans la production d'hydrogène de 99,9% de pureté, sans dépenser dans les agents réducteurs. Dans le cas de la production maximale d'hydrogène, l'utilisation des stratégies de co-culture, *Clostridium*, *Enterobacter*, et les bactéries de photo-fermentation dans un système de bioprocédés consolidé se traduira par un rendement d'hydrogène amélioré. Un système de co-culture est plus faisable et permet d'obtenir un rendement théorique d'hydrogène avec une conversion plus efficace des déchets organiques, d'améliorer la viabilité économique de la production d'hydrogène, de fournir une meilleure qualité des effluents de traitement, et en même temps répondre aux limitations de la production d'hydrogène.
3. **Chapitre 3, Partie I**: La production de biodiesel à travers le monde a entraîné une augmentation proportionnelle du glycérol brut comme déchets de production. L'utilisation de glycérol brut améliorera la viabilité économique de l'industrie du biodiesel. Cette étude a comparé le bilan énergétique de l'utilisation du glycérol brut pendant la fermentation en absence de lumière pour la

production d'hydrogène à la purification du glycérol. Le bilan énergétique pour chacun des matériaux utilisés lors de l'inoculum, les étapes de préparation des médias et de l'électricité consommée dans les deux méthodes ont été calculées. La consommation d'énergie par la purification de glycérol (872,4 MJ) était de 2,5 fois plus élevée par rapport à la consommation d'énergie maximum totale du glycérol brut dérivé de matière première végétale (344,3 MJ). La valeur commerciale du glycérol pur diminue par rapport à l'augmentation de la valeur de l'hydrogène comme carburant. Pour une utilisation efficace du glycérol brut, la bioconversion à la production d'hydrogène par fermentation en absence de lumière peut être considérée comme une option de production de combustibles durable et énergétiquement efficace. L'énergie nette (MJ) pour différentes matières premières (source végétale 158,9, divers matières premières 113,2 et déchets animaux 83.1) lors de la production d'hydrogène varie avec la teneur en glycérol. Effectuer une fermentation en absence de lumière dans des conditions ambiantes et l'utilisation de l'électricité produite à partir de la capture de biogaz réduisent la consommation totale en énergie. Ce faisant, l'énergie nette pour différentes matières premières aura une valeur positive.

4. **Chapitre 3, Partie II**: La valorisation du glycérol brut, un déchet du procédé de production de biodiesel, a été étudiée pour augmenter la production d'hydrogène par co-culture en utilisant les *Enterobacter aerogenes* NRRL B-407 et le *Clostridium butyricum* NRRL B-41122. Les capacités des *E. aerogenes* en tant que remplacement d'un agent réducteur coûteux afin de maintenir des conditions anaérobies pour la croissance du *C. butyricum* ont été évaluées. La co-culture a donné lieu à une croissance accrue de la production d'hydrogène, atteignant un maximum de 19,46 ± 0,95 mol-H_2/L-medium par rapport à la monoculture de *E. aerogenes* (15,64 ± 0,47) et *C. butyricum* (17,44 ± 0,38) en présence d'un agent réducteur. L'effet du rapport de l'inoculum a été étudié et le rendement d'hydrogène était de 0,95 mol-H_2/mol de glycérol pour un rapport d'inoculum de 1:11 avec une utilisation du substrat de plus de 85% ce qui est comparable à d'autres études de culture mixtes et de co-culture. En plus de l'hydrogène, des sous-produits à valeur ajoutée tels que le 1,3-propanediol, l'acide acétique,

l'acide butyrique et d'éthanol ont également été produits. La co-culture a permis la valorisation du glycérol brut en tant que consortium fonctionnel stable avec une plus grande efficacité de conversion pour l'augmentation de la production d'hydrogène ainsi que d'autres sous-produits à haute valeur ajoutée. En outre, l'étude a fourni la preuve du changement de voie métabolique contrôlée par composition durant la bioconversion du glycérol brut. La valorisation du glycérol brut à coût faible avec une co-culture efficace de production d'hydrogène peut aider une source énergétique interne de biodiesel.

5. **Chapitre 4:** L'utilisation de co-substrat de divers déchets présentant des caractéristiques complémentaires peut donner un medium complet pour une plus grande production d'hydrogène. Cette étude a évalué le potentiel de l'hydrolysat de déchet de pomme (APH) co-fermenté avec du glycérol brut afin d'augmenter la production de H_2 et diminuer la formation de sous-produits. Le plan central composite (CCD) ainsi que la méthodologie de surface de réponse (RSM) ont été utilisés comme outil d'optimisation et 15 g/L de glycérol brut, 5 g/L d'APH et 15% (v/v) d'inoculum ont été jugés optimales pour la production de jusqu'à 26,07 ± 1,57 mmol-H_2/L de medium. La valeur-p de 0,0017 indique que l'APH en basse concentration a eu un effet significatif sur la production d'hydrogène. En utilisant le glycérol brut comme unique source de carbone, la voie métabolique réductrice du glycérol a été favorisée avec 19,46 mmol-H_2/L. Cependant, avec l'APH, la voie oxydative a été favorisée avec une production de H_2 plus élevé (26,07 ± 1,57 mmol/L) et une diminution de la formation de sous-produits (1,3-propanediol et de l'éthanol). L'inclusion de l'APH a renforcée la production de H_2 et a diminué l'inhibition du substrat.
6. **Chapitre 5, Partie I:** L'hydrogène (H_2), un futur porteur d'énergie propre, nécessite des voies d'amélioration fondées sur le processus et la méthodologie utilisée pour réduire les coûts de production. L'impact du barbotage de l'azote (N_2) sur la production de H_2 dans un système de co-culture d'*Enterobacter aerogenes* et *Clostridium butyricum* à partir du glycérol brut (CG) a été étudié pour réduire le coût global du procédé. La production d'hydrogène en utilisant 1% de glycérol brut dans un medium barboté d'azote, avant autoclavage, a

abouti à 1,2 mol-H_2/mol de glycérol par rapport à 1,5 mol-H_2/mol de glycérol sans barbotage. En présence d'air allant de 5 à 75 mL de volume dans l'espace de tête, la production de H_2 a augmenté jusqu'à un maximum de 26,14 mmol/L avec production de 1,4 g/L d'éthanol. La concentration de 1,3-propanediol avec un barbotage d'azote est d'environ 3,0 g/L et a diminué à 0,5 g/L en raison de la présence de 75 mL d'air dans l'espace de tête. Cette observation peut être attribuée à un changement du métabolisme du glycérol de réductif à oxydatif. Une stratégie d'amélioration basée sur les procédé pour optimiser la formation d'hydrogène a entraîné un changement de voie métabolique de réductive à l'oxydative avec augmentation de la production d'hydrogène. L'influence synergique du système de co-culture en l'absence d'agent réductif coûteux et sans étape de barbotage d'azote peut offrir une meilleure stratégie économique basée sur les procédé pour la production d'hydrogène, minimiser la production de métabolites et améliorer son application dans un site de production de biodiesel à grande échelle.

7. **Chapitre 5, Partie II**: Dans la présente étude, le Tween 80, un agent tensioactif non ionique, a été utilisé pour améliorer la production d'hydrogène par bioconversion de glycérol brut à l'aide de co-culture de *Enterobacter aerogenes* et *Clostridium butyricum*. Le but de l'introduction de l'agent tensioactif est de diminuer la viscosité du glycérol brut, de sorte que la solubilité apparente et la biodisponibilité du glycérol peuvent être améliorées au détriment des étapes de prétraitement. Des expériences ont été planifiées en utilisant un plan central composite (CCD); les concentrations du glycérol brut et du Tween 80 ont été optimisées alors que la production d'hydrogène, l'utilisation du glycérol et la viscosité des médias ont été considérés comme des réponses. La surface de réponse pour le modèle quadratique a montré que la concentration du Tween 80 a eu un effet significatif ($p < 0,05$) sur l'ensemble des trois réponses. En utilisant les conditions optimisées à 17,5 g/L de glycérol brut et de 15 mg/L de Tween 80, la production d'hydrogène a atteint un maximum de 32,1 ± 0,03 mmol/L de milieu. L'augmentation de la production d'hydrogène est d'environ 1,25 fois, en présence de Tween 80 par rapport à son absence, avec une production de 25,56

± 0,91 mmol/L. Les conditions optimales sélectionnées ont également été validées par rapport à l'absence de glycérol brut (4,69 ± 0,76 mmol/L), avec le glycérol prétraité brut (20,06 ± 0,51) et à travers le système en mono-culture (15,43 ± 0,79 à 22,14 ± 0,94). L'introduction de Tween 80 dans le medium de fermentation améliore le taux d'utilisation du glycérol, ce qui entraîne une augmentation de la production d'hydrogène et l'élimination des étapes de prétraitement.

8. **Chapitre 6:** Les propriétés des coquilles (EGS) comme neutralisant et agent immobilisant pour la production d'hydrogène (H_2) en utilisant la production de glycérol brut par co-culture d' *Enterobacter aerogenes* et *Clostridium butyricum* ont été étudiées. Des coquilles d'œufs de différentes tailles et concentrations ont été utilisées pendant la fermentation de batch et de batch-répétés. Lors de la fermentation de batch et de batch-répétés, la production d'hydrogène maximale (36,53 ± 0,53 et 41,16 ± 0,95 mmol/L, respectivement) a été obtenue avec des coquilles de taille 33 µm < x_5 <75 µm. La production d'hydrogène a augmenté avec la réduction de la taille des coquilles d'œufs. Les coquilles d'œufs ont maintenu le pH (6,00 à 6,30) de la fermentation et ont fourni un appui pour l'immobilisation des deux souches bactériennes et ceci a été confirmé par balayage à microscopie électronique. En tant que composante des milieux, la concentration des coquilles d'œufs de 0,25% (w/v) a été jugée optimale pour une production maximale d'hydrogène (31,66 ± 0,55 mmol/L) et le profil de production était comparable à la production d'hydrogène (32,07 ± 0,92 mmol/L) obtenue avec toute les composantes des milieux. Dans l'étude de mise à l'échelle avec bioréacteur semi-continu (7,5 L), une augmentation (en comparaison la mono-culture) de près de 1,5 fois (à savoir 7,69 L H_2/L de medium avec l'utilisation de 86,65% de glycérol) a été obtenue. La valorisation des coquilles d'œufs peut réduire les effets des bio-déchets sur l'environnement, réduire les coûts de production d'hydrogène de 85-95%, améliorer le rendement en hydrogène et rendre l'industrie du biodiesel et de l'hydrogène plus compétitive.

9. **Chapitre 7:** En vue d'élever l'industrie du biodiesel en tant que vecteur énergétique majeur, une nouvelle approche de minimisation et d'utilisation des déchets doit être explorée. Le milieu pré-utilisé par le système de co-culture est rentable et peut servir de supplément renouvelable pour la culture mixte de production d'hydrogène (H_2) et de milieu de remplacement dans la production de lipides. La conversion directe des milieux pré-utilisés ainsi que le glycérol brut à 20 g/L en utilisant de la boue d'eau usée traitée au choc thermique a donné lieu à la production de 38,12 ± 0,84 mmol/L d'hydrogène. Dans une autre approche, le milieu pré-utilisé a été réutilisé comme supplément pour la culture des algues avec un medium frais à 3:2, ce qui a donné 0,098 ± 0,007 g/L de lipide. Le milieu pré-utilisé contenait de la biomasse morte, des nutriments résiduels, des biomolécules et du glycérol inutilisé ensemble agissant comme une source supplémentaire au cours de la production d'hydrogène et de lipides. Selon les résultats du système fermé, l'hydrogène produit ($1.47x10^9$ L de H_2) peut être convertie en énergie ($1,87\ x10^4$ GJ) électrique ($1,77\ x10^4$ GJ) et thermique ($4,32\ x10^3$ GJ). L'hydrogène produit peut être utilisé comme source d'énergie interne et les lipides peuvent être utilisés comme matière première de troisième génération. L'étude explore l'utilisation du glycérol brut, des milieux usés et de la boue primaire du biodiesel (BPS) dans une approche système-fermé efficace pour l'industrie du biodiesel afin d'utiliser les déchets et d'augmenter la valeur commerciale du glycérol brut.

Recommendations futures

À partir des résultats présentés dans cette thèse, les recommandations suivantes peuvent être présentées pour la continuité de la recherche:

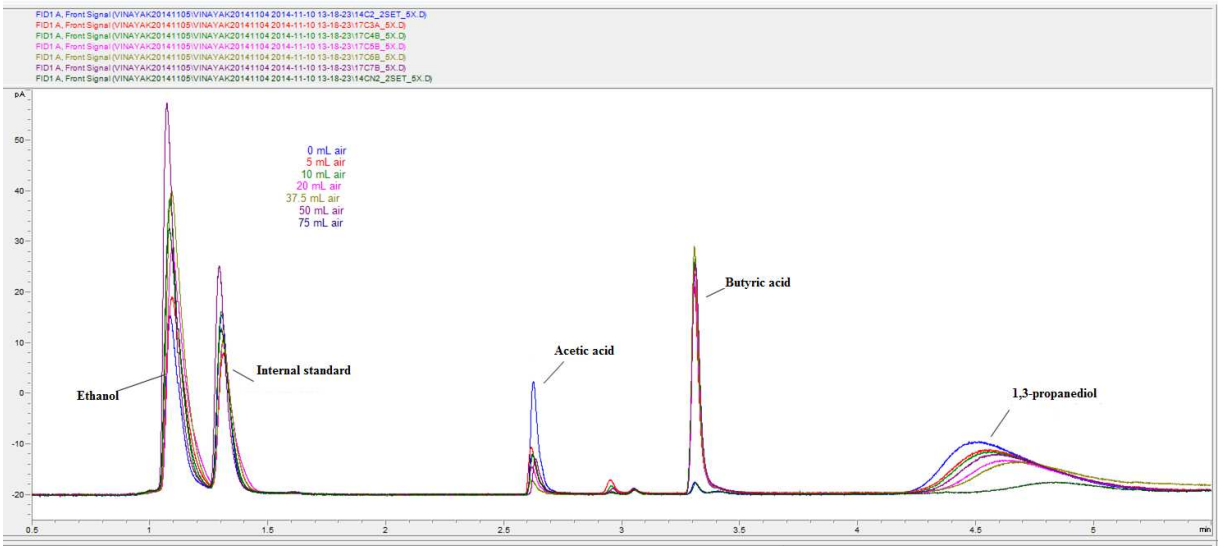
1. Il serait pertinent d'évaluer si la fermentation peut être effectuée à température ambiante, ce qui permettrait une économie significative d'énergie.
2. L'utilisation de systèmes de co-cultures pour la fermentation du glycérol brut en provenance de différentes matières primaires appert être une voie à explorer pour l'obtention d'un meilleur rendement en hydrogène.
3. L'utilisation des co-substrats, par le mélange d'au moins deux déchets industriels, constitue une voie à explorer afin de permettre une production à grande échelle dans un proche futur.
4. Il est aussi proposé d'évaluer une stratégie basée sur la minimisation de la production des métabolites en vue d'augmenter la production d'hydrogène.
5. Il faut également viser l'utilisation potentielle des déchets liquides issus de la production d'hydrogène fermentatif dans une approche de bioraffinerie d'hydrogène.

ANNEXES

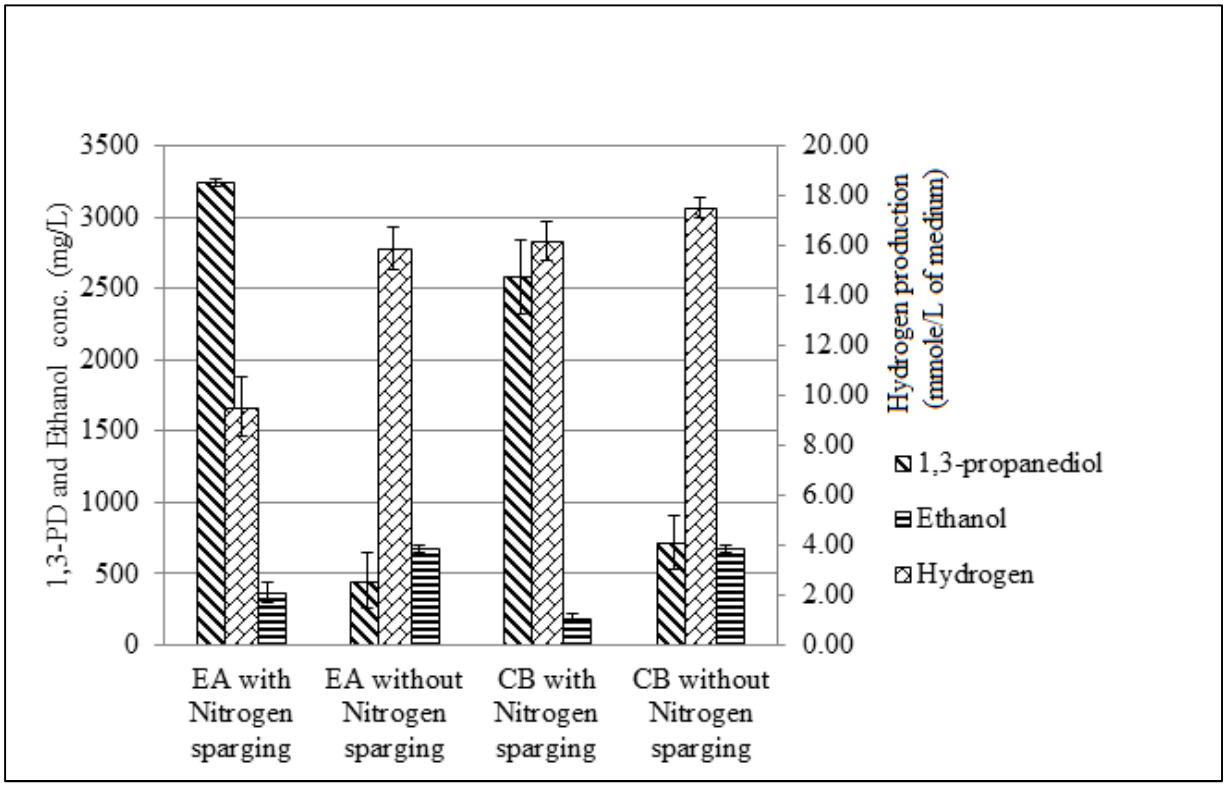
ANNEXES I

CHAPTER 5 PART 1

DATA: The overlay chromatogram results of the sample set. Indicates the 1,3-PD production decreased with increasing amount of air in the headspace of the serum bottle. The pattern for ethanol production was completely opposite to 1,3-PD.



DATA: Biohydrogen in mmol/L along with 1,3-PD and ethanol production in mg/L using 1 % crude glycerol substrate for *Enterobacter aerogenes* (EA) and *Clostridium butyricum* (CB) using modified basal media under (+) and (-) sparging condition.



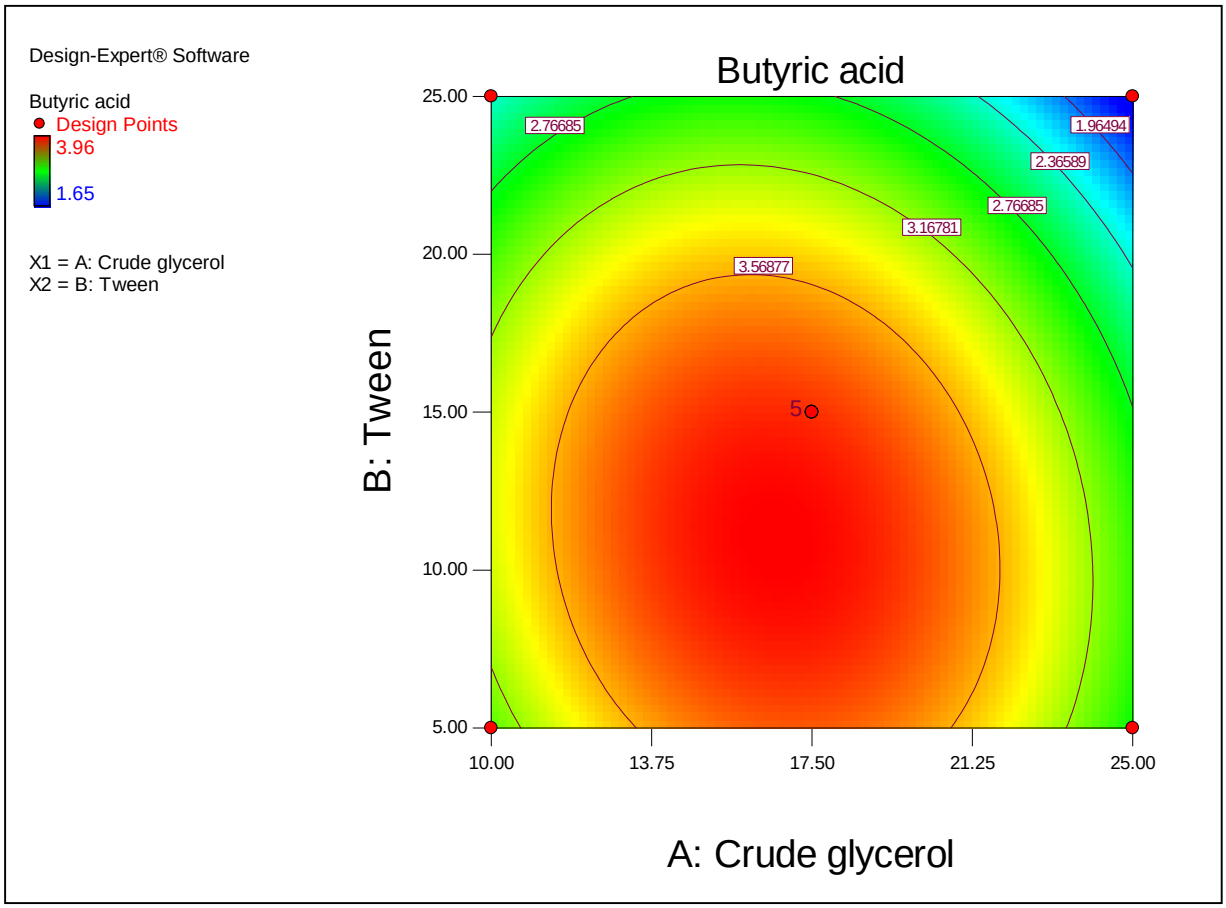
ANNEXES II

CHAPTER 5 PART 2

DATA: Summarized ANOVA for the response surface quadratic model for butyric acid, 1,3-Propanediol and ethanol.

Source	*p*-value		
	Butyric acid	**1,3-Propanediol**	**Ethanol**
Model	0.0098	0.0015	< 0.0001
A-Crude glycerol	0.1864	< 0.0001	< 0.0001
B-Tween	0.0257	0.5832	0.8076
AB	0.5422	0.4879	0.7580
A^2	0.0027	0.1809	0.0173
B^2	0.0165	0.1403	0.1055
R^2	0.73	0.84	0.93

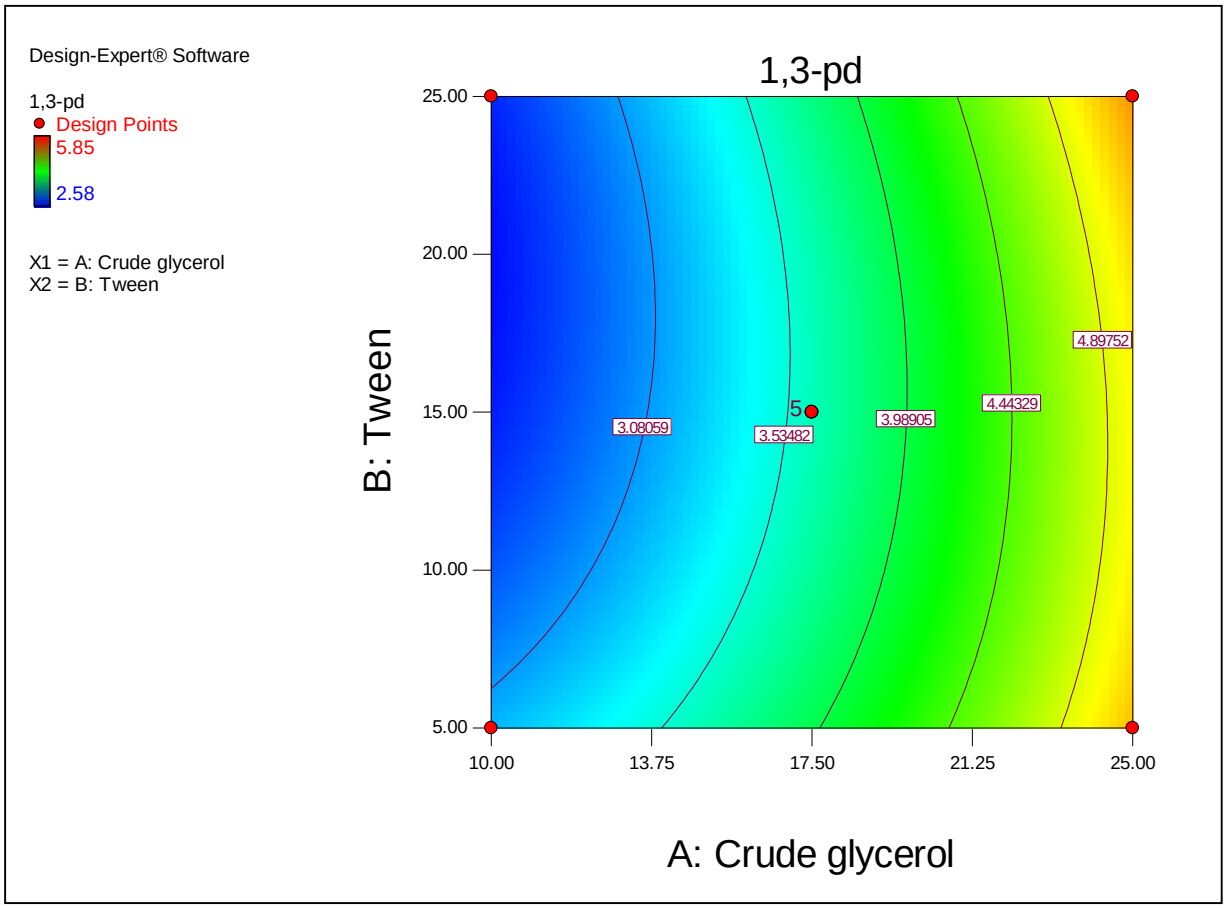
DATA: Experimental responses of butyric acid production (g/L) with the fitting function of crude glycerol (g/L) and Tween concentration (mg/L) using response surface plots. (SF1).



Final model equation (Eq. SF1)

$$\text{Butyric acid} = 3.86 - 0.25 \times \text{CG} - 0.48 \times \text{Tween} - 0.16 \times \text{CG} \times \text{Tween} - 0.83 \times \text{CG} \times \text{CG} - 0.58 \times \text{Tween} \times \text{Tween} \quad (SF1)$$

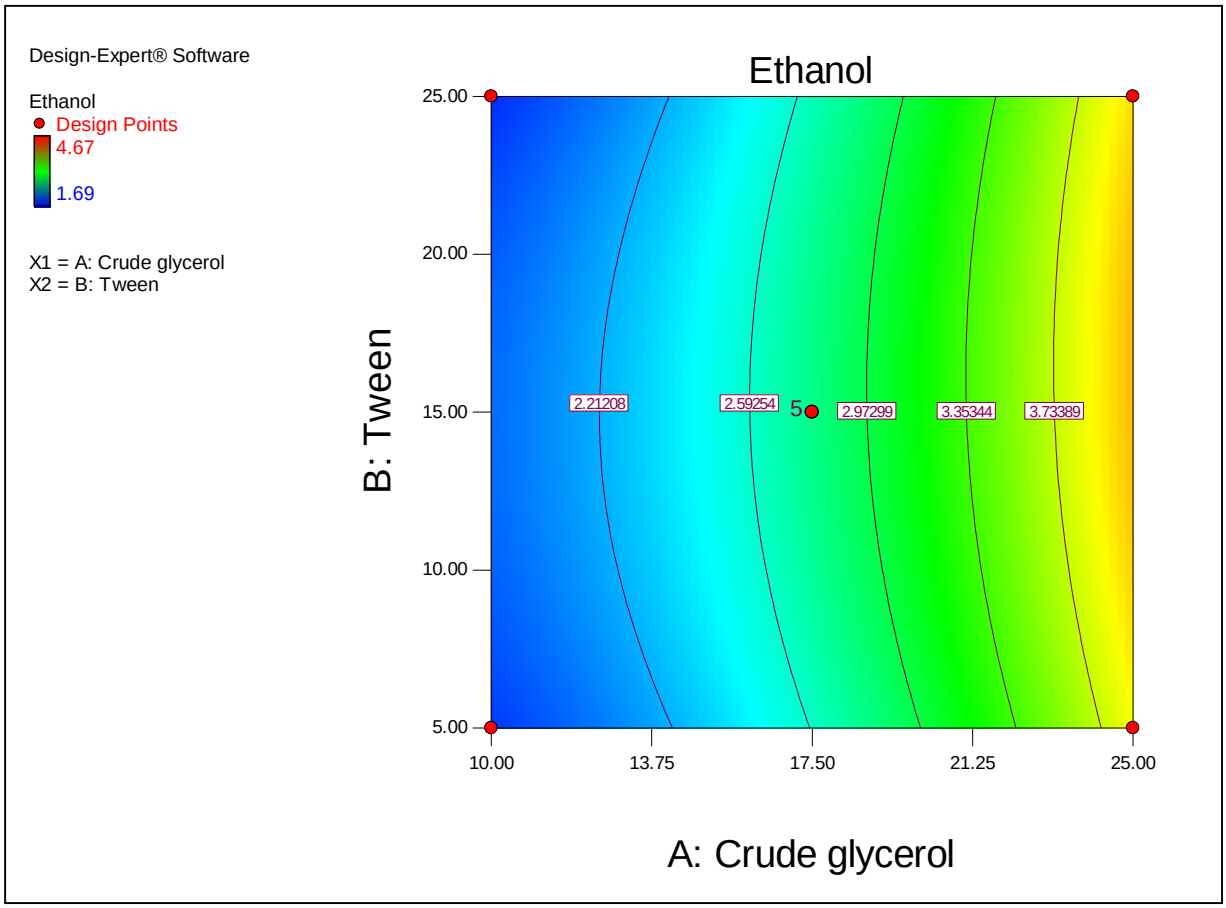
DATA: Experimental responses of 1,3, Propanediol (1,3-pd) production (g/L) with the fitting function of crude glycerol (g/L) and Tween concentration (mg/L) using response surface plots.



Final model equation (Eq. (SF2))

$$1{,}3-\text{Propanediol} = 3.62 + 1.17 \times \text{CG} - 0.083 \times \text{Tween} + 0.15 \times \text{CG} \times \text{Tween} + 0.23 \times \text{CG} \times \text{CG} + 0.26 \times \text{Tween} \times \text{Tween} \quad \text{(SF2)}$$

DATA: Experimental responses of ethanol production (g/L) with the fitting function of crude glycerol (g/L) and Tween concentration (mg/L) using response surface plots.



Final model equation (Eq. (SF3))

$$\text{Ethanol} = 2.79 + 1.05 \times \text{CG} - 0.021 \times \text{Tween} + 0.037 \times \text{CG} \times \text{Tween} + 0.28 \times \text{CG} \times \text{CG} - 0.16 \times \text{Tween} \times \text{Tween} \quad \text{(SF3)}$$