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PRODUCTION D'HYDROGÈNE PAR BIOCONVERSION DU GLYCÉROL BRUT ET UTILISATION DURABLE DES REJETS LIQUIDES GÉNÉRÉS AU COURS DU PROCESSUS

Par

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DÉDICACE

This thesis is dedicated in memory of my father (Late) Karmi Dev Sarma

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(Saurabh Jyoti Sarma)

RÉSUMÉ

Le biohydrogène est une source d'énergie renouvelable, qui a l'avantage d'être le carburant le plus propre pour l'avenir. La combustion de l'hydrogène ne produit pas de CO_2. La seule émission du processus est de l'eau et, par conséquent, ce combustible a un potentiel de réduction des émissions de gaz à effet de serre (GES) très élevé. De même, l'hydrogène a une densité d'énergie massique supérieure à celle des combustibles conventionnels tels que l'essence ou le diesel. Sur une note similaire, le biodiesel est l'un des carburants renouvelables principalement étudiés et disponibles au niveau commercial. Au cours de la production du biodiesel par transestérification des lipides (par exemple, les huiles végétales, les graisses animales et les lipides des algues), du glycérol brut (GB) est généré comme sous-produit et représente 10% du poids du produit. En raison de la présence de nombre d'impuretés ainsi que l'augmentation continue de la production mondiale, la valeur de marché actuelle du GB est aussi bas que $ 0.11/kg. De plus, le coût d'élimination efficace et durable du GB contenant du méthanol, du sel et d'autres impuretés est un défi pour les fabricants de biodiesel. En variante, le GB peut être utilisé comme matière première (substrat) pour la production de biohydrogène. De façon conventionnelle, l'hydrogène peut également être produit par des techniques chimiques tels que le reformage à la vapeur du gaz naturel, l'oxydation partielle du pétrole et du charbon par la gazéification. Contrairement à la production biologique d'hydrogène, toutes ces techniques utilisent des combustibles fossiles comme matière première et ne sont donc pas considérés comme des processus durables.

Dans le cadre de ce projet de doctorat, la faisabilité technico-économique de la production d'hydrogène par la bioconversion du GB a été évaluée. Le coût le plus élevé de production se situe au niveau du procédé de fermentation. Néanmoins, certaines options alternatives pour la réduction du coût du procédé ont été identifiées. Une recherche a été effectuée afin de déterminer le potentiel maximum de production de H_2 à partir du GB en l'absence de tout nutriment coûteux supplémentaire. Une production maximale de 2023 mL-H_2/L-milieu a été atteint et 10 g/L de GB a été jugée la concentration initiale optimale. En outre, l'ajout au milieu d'une quantité de biomasse

endogène (50 mg/L) provenant d'une fermentation antérieure et dont les ressources sont épuisées a permis d'améliorer la production de 32,5%. De même, l'application de suppléments moins chers, tels que les rejets liquides d'abattoir (LA), les déchets de brasserie (BDB) et l'urée ont amélioré la production de 19±4, 27±3 et 39±4%, respectivement. Le citrate ferrique et ses particules séchées par un atomiseur ont également amélioré la production d'hydrogène de 17,2% à 31,7%. La production d'hydrogène utilisant des concentrations extrêmement faibles de GB initiale de 100 mg/L a permis de produire 22,7 mol-H_2/kg GB soit une valeur de 2,75 fois plus élevées que la valeur de 8,25 mol-H_2/kg GB connu pour les systèmes de fermentation sombre.

L'effet inhibiteur des impuretés contenues dans le GB d'origine industrielle, telles que le methanol, NaCl et le savon a été étudié. Sur la base de la vérification effectuée en utilisant la méthodologie de réponse de surface, il a été observé que le savon est le facteur le plus significatif (valeur p = 0,0104 < 0,05) pour inhiber la production d'hydrogène. Afin de réduire l'effet inhibiteur du savon, le GB a été traité avec du $MgSO_4$ pour convertir le savon en une forme inactive (écume). L'approche a permis d'augmenter la production cumulative de H_2 (34,7%) ainsi que l'utilisation du glycérol (près de 2,5 fois). Du même coup, le traitement permet d'augmenter la teneur en Mg (un nutriment) dans le milieu passant d'une concentration de 0,57 mg/L à 201,9 mg/L.

Pour éviter l'inhibition du processus métabolique ou décalage possible en raison de l'accumulation d'acide organique dans le milieu, lors de la fermentation, un ajustement rigoureux du pH est nécessaire. Comme alternative, pour la première fois, un système de bioréacteur anaérobie à deux phases a été mis au point pour l'extraction *in situ* des différents acides organiques générés lors de la fermentation. Sur la base de biocompatibilité, l'alcool oléique a été sélectionné en tant que phase organique du système et une augmentation de la production d'hydrogène de 26,6 mmol/L à 29,6 mmol/L-milieu de culture a été obtenu.

En ce qui concerne les expérimentations finales, un procédé semi-continu a été utilisé pour éviter l'inhibition du substrat, ainsi que réduire l'accumulation de sous-produits pendant la production d'hydrogène. Le processus a été optimisé pour produire un rendement volumique aussi haut que 5.18 L-H_2/L-milieu de culture, ce qui est nettement

supérieur à celui de 2,02 à 2,68 L-H_2/L-milieu précédemment rapporté pour la bioconversion du GB.

Quant au volet visant la valorisation des rejets de fermentation l'aspect solubilisation du phosphate a été abordé. Certains bio-fertilisants commerciaux solubilisant le phosphate (PSB) utilisent des acides organiques pour chélater les cations de phosphates insolubles (comme $Ca_3(PO_4)_2$) présents dans le sol pour solubiliser le phosphate et le rendre disponible pour les plantes. Il a été observé que le liquide organique des rejets de fermentation du GB est riche en acide organique et pourrait servir à la solubilisation du phosphate. Des essais en laboratoire ont donc été réalisés. Lors de son ajout au sol, une augmentation de l'assimilation du phosphore de 2,18 à 2,74 fois a été mesuré par les plantes de soya. Ces résultats suggèrent que les déchets liquides de fermentation du GB ont le potentiel de remplacer les PSB disponibles dans le commerce. De même, lors de la production d'hydrogène par fermentation, en plus de H_2, divers produits chimiques industriels, y compris l'éthanol, le 1,3 propanediol et de l'acide butyrique sont produits. Si ces produits ne sont pas récupérés, le liquide résiduel peut être utilisé comme matière première pour la production de polyhydroxyalcanoates, des lipides, CH_4, H_2 et de l'électricité. Ainsi, dans le cadre de la présente recherche, un processus de production de biohydrogène a été évalué comme une bioraffinerie ayant le potentiel de produire du biocarburant, des produits de la chimie fine et des biomatériaux. La stratégie pourrait être utile pour réduire le coût global du processus en générant des revenus de sources multiples.

ABSTRACT

Biohydrogen is the renewable energy source, which has been postulated to be the cleanest fuel for future. Hydrogen combustion does not produce CO_2; sole emission of the process is water and hence, it has very high greenhouse gas (GHG) emission reduction potential. Likewise, H_2 has a gravimetric energy density higher than conventional fuels such as gasoline, diesel. On a similar note, biodiesel is one of the mostly studied and commercially available renewable fuels. During biodiesel production by transesterification of lipids (e.g. vegetable oils, animal fats and algal lipids) crude glycerol (CG) is generated as a byproduct; and by weight, it is around 10 % of the product. Due to presence of number of impurities as well as continuous increase in its global production, the present market value of CG is as low as $ 0.05/pound. Additionally, cost effective and sustainable disposal of CG containing methanol, salt and other impurities is a challenge for biodiesel manufacturers. Alternatively, CG can be used as a feedstock for microbial hydrogen production. Hydrogen can also be produced by chemical techniques such as steam reforming of natural gas; partial oxidation of heavy oil and coal gasification. Unlike biological hydrogen production, all these techniques use fossil fuels as the feedstock and thus are not considered as sustainable processes.

As a part of the present investigation, techno-economic feasibility of hydrogen production by CG bioconversion has been evaluated and high process cost was found to be the major bottleneck. Nevertheless, certain achievable alternative options for reduction of process cost have been identified. An investigation has been carried out to determine maximum H_2 production potential of CG in the absence of any additional expensive supplement. A maximum production of 2022.5 mL H_2/L medium has been achieved and 10 g/L was found to be the optimum initial CG concentration. Further, addition of spent biomass (50 mg/L) of the process into a subsequent process was found to improve the production by 32.50%. Similarly, application of less expensive supplements, such as slaughterhouse liquid waste (SL), brewery waste biomass (BWB) and urea was found to improve the production by 18.81 ± 3.56, 27.30 ± 3.54 and 38.57 ± 3.66 %, respectively. Ferric citrate and its nano-spray dried particles have also been

found to enhance hydrogen production by 17.18% to 31.71%. Hydrogen production using extremely low initial CG concentration of 100 mg/L has been found to produce 22.7 mol-H_2/kg CG; which is 2.75 folds higher than the value of 8.25 mol-H_2/kg CG known for dark fermentation.

The effect of CG impurities, such as methanol, NaCl and soap on hydrogen production and substrate (glycerol) utilization has been studied. Based on the investigation carried out using response surface methodology, it has been observed that soap is the most significant factor (p value = 0.0104 < 0.05) to inhibit hydrogen production. In order to reduce the inhibitory effect of soap, CG was treated with $MgSO_4$ to convert the soap to its inactive form (scum). The approach was found to increase cumulative H_2 production (34.70%) as well as glycerol utilization (nearly 2.5 folds). Additionally, the treatment can increase the Mg (a nutrient) content of the medium from 0.57 ppm to 201.92 ppm.

To avoid process termination or possible metabolic shift due to organic acid accumulation in the medium, during fermentative hydrogen production a rigorous pH adjustment is necessary. Alternatively, for the first time, a two phase anaerobic bioreactor system has been developed for *in situ* extraction of different organic acid byproducts. Based on biocompatibility, oleyl alcohol has been chosen as the organic phase of the system and an increase in hydrogen production from 26.58 mmol/L to 29.61 mmol/L-medium has been achieved. Further, a semi-continuous process has been used to avoid substrate inhibition as well as to minimize byproducts accumulation during hydrogen production. The process was found to produce as high as 5.18 L-H_2/L-medium, which is significantly higher than 2.02 to 2.68 L-H_2/L-medium previously reported for CG bioconversion.

Organic acids produced by commercial phosphate solubilizing bio-fertilizer (PSB) can chelate the cations of insoluble phosphates (such as Ca_3 $(PO_4)_2$) present in soil to solubilize the phosphate and make it available for plants. It has been observed that organic acid-rich liquid waste of fermentative hydrogen production process also has excellent phosphate solubilizing ability. Upon its addition to soil, 2.18 to 2.74 folds increase in phosphorus uptake by soybean plants has been observed. The finding indicates that the liquid waste has the potential to be an alternative for PSB. Likewise,

during fermentative hydrogen production, in addition to H_2, various industrial chemicals including ethanol, 1,3 propanediol and butyric acid are produced. If these products are not recovered, the liquid waste can be used as the feedstock for production of polyhydroxyalkanoates, lipid, CH_4, H_2 and electricity. Thus, as a part of present investigation, biohydrogen production process has been evaluated as a potential biorefinery producing biofuels, fine chemicals and biomaterials. The strategy could be useful to reduce overall cost of the process by generating revenue from multiple sources.

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AS	apple pomace sludge
BLW	brewery liquid waste
CAGR	compound annual growth rate
CFR	code of federal regulations
CG	crude glycerol
COD	chemical oxidation demand
CSTR	continuous stirred tank reactor
DHA	docosahexaenoic acid
EPA	Environmental protection agency, US
EU	European Union
Fd	ferredoxin
GDHt	glycerol dehydratase
GHG	greenhouse gas
GWh	gigawatt hour
HAP	hydroxyapatite
ICP-AES	inductively coupled plasma atomic emission spectroscopy
kWh	kilowatt hour
MFC	microbial fuel cell
mM	millimolar
MMt	million metric tonnes
MONG	matter organic non-glycerol
MSDS	material safety data sheet

NA	North America
NAD^+	Nicotinamide adenine dinucleotide
NAPL	non-aqueous phase liquid
PD	1,3-propanediol
PDOR	1, 3-propanediol oxidoreductase
POTW	publicly owned treatment works
PSB	phosphate solubilizing bio-fertilizer
RFS2	revised renewable fuel standard 2
RSM	response surface methodology
SIW	starch industry wastewater
SL	slaughterhouse liquid wastes
SS	secondary sludge
TOC	total organic carbon

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CHAPITRE 1

SYNTHÈSE

PARTIE I. PRODUCTION D'HYDROGENE PAR GLYCÉROL BRUT BIOCONVERSION-REVUE DE LA LITTÉRATURE

1.1. Introduction

Les combustibles fossiles sont les principales sources d'énergie naturelles, qui couvrent près de 80% de la demande mondiale d'énergie (Ni *et al.*, 2006). Cependant, le stock mondial de carburant fossile s'épuise rapidement et la combustion de combustibles accroît les impacts négatifs mondialement sur le climat (Aksoy, 2011, Gnana Kumar *et al.*, 2010). Une telle situation a déclenché une recherche rapide des sources d'énergie de remplacement dans le monde entier. "L'énergie durable pour tous" est une initiative des Nations Unies et "doubler la part des énergies renouvelables dans le mix énergétique mondial" est l'un de ses trois objectifs principaux à remplir d'ici 2030 (http://www.unfoundation.org/what -we-do/issues/energy-and-climate/clean-energy-development.html. consulté le 8 mai 2013). Cette initiative est l'un des exemples de l'intérêt de la communauté mondiale vers le développement de sources d'énergie renouvelables pour la sécurité énergétique de l'avenir. Les biocarburants étant une source d'énergie renouvelable ils peuvent être un substitut approprié aux combustibles fossiles.

Le biodiesel est un des biocarburants les plus couramment étudiés et il peut être produit par trans-estérification d'acides gras naturels (Muniyappa et al., 1996). En raison de la disponibilité des matières premières, la mise en œuvre simple des technologies pour sa production et son rôle dans le bilan des gaz à effet de serre (GES); la production mondiale de biodiesel est en augmentation rapide. À l'heure actuelle (2013), la production mondiale de biodiesel est d'environ 30 milliards de litres, et il devrait atteindre 42 milliards de litres d'ici 2021 (http://www.fao.org/fileadmin/templates/est/COMM_MARKETS_MONITORING/Oilcrops/Documents/OECD_Reports/biofuels_chapter.pdf. Consulté le 9 mai 2013.). En outre, la production de biodiesel a été promue par les différents gouvernements par diverses subventions et politiques. Le programme de norme sur les carburants renouvelables révisé (RFS2) a été mis au point, selon lequel, à partir de 2012, chaque année un minimum de 1 milliard de gallons de biodiesel sera mélangé dans le carburant diesel et

il faudra environ 36 milliards de litres de carburant renouvelable d'ici 2022 (http: / / www.biodieselmagazine.com/articles/4080/report-12-billion-gallons-of-biodiesel-by-2020. consulté le 27 décembre 2011). De même, le Canada est un important producteur de biodiesel en Amérique du Nord et le gouvernement du Canada a fixé un objectif de réduction des GES de 17 pour cent d'ici 2020 et de 2% de contenu renouvelable sera obligatoire pour le diesel (http://www.ec.gc.ca/default.asp?lang=En&n=714D9AAE-1&news=BDE26528-CBCB-4008-949D-CDFDFBD054FB. consulté le 27 décembre 2011) (Règlement modifiant le Règlement sur les carburants renouvelables, de l'environnement canadien loi sur la protection, 1999, ministère de l'Environnement, Canada. https://tsapps.nist.gov/notifyus/docs/wto_country/CAN/corrigenda/pdf/CAN311_add_3 (anglais). pdf. Consulté le 27 décembre 2011).

Au cours du processus de production du biodiesel, un grand volume de glycérol brut (GB) est produit comme sous-produit. En poids, la proportion de glycérol est de 10% de biodiesel produit (Selembo *et al*., 2009a, Yazdani *et al*., 2007). Le glycérol est une matière première importante pour l'alimentation, l'industrie pharmaceutique et cosmétique, et autres processus de fabrication (Guerrero - Pérez *et al*., 2009). Toutefois, en raison de la présence d'une grande quantité d'impuretés (méthanol, savon, sels, huiles et matières organiques solides) (Chi *et al.*, 2007, Ngo *et al.*, 2011, Selembo *et al*., 2009b); le glycérol brut (GB) ne peut pas être directement utilisé dans les industries alimentaires ou pharmaceutiques et sa purification est très coûteuse (Escapa *et al*., 2009). En outre, le GB, considéré comme un déchet, s'il est directement rejeté dans l'environnement sans traitement approprié, pourrait avoir des conséquences néfastes (da Silva *et al*., 2009, Selembo *et al*., 2009a). Comme alternative, le GB contenant des déchets d'effluent d'usine de production de biodiesel peut être valorisé comme un substrat moins coûteux pour la production de différents produits à valeur ajoutée (PHVA), tels que 1, 3 -propanediol, l'éthanol, et les aliments pour animaux, (da Silva *et al*., 2009, Wang *et al.*, 2001). Toutefois, en raison de la présence d'impuretés, la récupération et purification des PHVA produits sont également complexes et coûteuses (Pachauri *et al.*, 2006). Par contre, le GB peut être utilisé comme substrat pour la production d'hydrogène, dans ce cas, le produit recherché (H_2) est

automatiquement séparé du milieu et il peut être récupéré en utilisant des techniques simples.

La production de H_2 en utilisant le GB généré par l'industrie du biodiesel a déjà été étudiée par plusieurs chercheurs (Ito *et al.*, 2005, Ngo *et al.*, 2011, Selembo *et al.*, 2009a). Il est établi que l'H_2 a un contenu très élevé en énergie et il peut être utilisé comme biocarburant (Ngo *et al.*, 2011). La chaleur de combustion de l'hydrogène est -285,8 kJ/mol et il a une teneur en énergie de 142,9 kJ/g (disponible à partir de: http://www.creative-chemistry.org.uk/gcse/documents/Module21/N-m21- 10.pdf).

En outre, si l'H_2 est utilisé comme combustible, le seul sous-produit généré est l'eau (Selembo *et al.*, 2009a). Par conséquent, compte tenu de la préoccupation croissante sur le réchauffement climatique, l'H_2 peut être considéré comme le carburant le plus propre pour l'avenir. Par conséquent, la bioconversion du GB en hydrogène est une approche avec un avenir prometteur. L'H_2 peut aussi être réalisé par différentes techniques chimiques, tels que le reformage du gaz naturel (Chen *et al*., 2008, Roh *et al.*, 2010.); l'oxydation partielle de l'huile lourde (Liu, 1999) et la gazéification du charbon (Stiegel *et al*., 2006). Contrairement à la production de l'H_2 biologique, toutes ces techniques utilisent des combustibles fossiles comme matière première pour la production d'hydrogène et ils ne sont pas considérés comme des processus durables. Ainsi, dans le cadre de l'intérêt mondial pour le développement des sources d'énergie renouvelables, la production de biohydrogène par la bioconversion du GB peut être considéré comme une technologie prometteuse.

La fermentation sombre, la photofermentation, ou les deux étapes en série et l'application de l'électrolyse microbienne sont les différentes méthodes couramment utilisées pour la production biologique d'hydrogène (Call *et al*., 2008, Meher Kotay *et al*., 2008). Jusqu'à ce jour, diverses stratégies ont été appliquées pour la production de H_2 par bioconversion du GB dont la fermentation sombre; celle-ci est le processus le mieux étudié. Ngo *et al* (2011) ont rapporté une production de 2,73 ± 0,14 mol-H_2/mol glycérol par *Thermotoga neapolitana* (Ngo *et al.,* 2011). De même, Fernandes *et al* (2010) ont rapporté une production de 200 mL -H_2/g COD (Fernandes *et al.*, 2010). Dans une autre étude similaire, Marques *et al* (2009) ont utilisé *Enrerobacter aerogenes* et les auteurs

ont rapporté une production de 2,5 dm^3- H_2/dm^3 milieu de culture (Marques *et al.*, 2009). Cependant, dans tous ces cas, malgré un rendement très élevé en hydrogène, l'application technologique a encore beaucoup de problèmes à surmonter pour une application industrielle potentielle.

Dans cette recherche, une section de revue de la littérature présente une étude systématique et comparative des rapports actuellement disponibles sur la production de bio-hydrogène à partir de glycérol brut. Cette section cible également les orientations futures de recherche.

Tout d'abord, les caractéristiques du GB généré à partir du procédé de fabrication du biodiesel sont examinées suivies du potentiel de production du biohydrogène. Ensuite, divers procédés de pré-traitement, les types de bioréacteurs utilisés, ainsi que le potentiel de bioconversion du glycérol par différents micro-organismes ont été abordés. Finalement, diverses stratégies pour améliorer la production d'hydrogène ont été proposées. Pour une présentation plus détaillée, l'article scientifique présenté dans le chapitre 2 de cette thèse (Sarma *et al*., 2012) peut être consulté.

1.2. Glycérol: sous-produit de la production du biodiesel

1.2.1. Transestérification d'huiles végétales et des graisses animales: Le glycérol est un sous-produit du processus de production du biodiesel. Pour la production du biodiesel, de façon classique, les triglycérides, telles que les huiles végétales et les graisses animales sont mélangées avec du méthanol dans un réacteur. De l'hydroxyde de sodium est ajouté comme catalyseur de la réaction et le mélange est agité et chauffé à la température d'ébullition du methanol (Kim *et al*., 2004, Muniyappa *et al*., 1996, Schuchardt *et al*., 1998). Lors de l'arrêt de l'agitation, le mélange réactionnel se sépare en deux phases. La couche supérieure est l'ester méthylique d'acides gras soit le biodiesel et la couche inférieure est le GB contenant du méthanol n'ayant pas réagi, le sel et les substances solides présentes dans les matières premières. La Figure 2.1.1 représente une vue schématique de transestérification et le processus de production du biodiesel. La Figure 2.1.2 montre l'équation chimique pour la production de glycérol par transestérification de triglycérides (Muniyappa *et al.*, 1996). La phase de GB contient généralement près de 75 % de glycérol (Muniyappa *et al*., 1996). Toutefois, le contenu

de glycérol dans les déchets de production du biodiesel peut varier d'une usine à l'autre (Tableau 2.1.1).

1.2.2. Impuretés présentes dans le glycérol brut: En raison de sa haute teneur en énergie, différents chercheurs du monde entier ont étudié la bioconversion du GB en produit à valeur ajoutée (Ito *et al.*, 2005, Mu *et al*., 2006, Ngo *et al*., 2011, Sakai *et al*., 2007, Selembo *et al*., 2009a, Selembo *et al*., 2009b). Ils ont caractérisé le GB provenant des usines de fabrication du biodiesel pour obtenir une vue d'ensemble des impuretés présentes. Chi *et al* (2007) ont rapporté que les impuretés principalement présentes dans GB sont le savon, les acides gras libres, le méthanol, les triglycérides qui n'ont pas réagi, les diglycérides et monoglycérides (Chi *et al*., 2007) (Tableau 2.1.1). Le GB du processus de fabrication du biodiesel peut également contenir une petite quantité de l'huile utilisée pour la transestérification (Chi *et al*., 2007) et d'autres impuretés présentes dans cette huile.

1.2.3. Problèmes environnementaux reliés aux rejets du glycérol provenant de la production de biodiesel: Comme mentionné ci-dessus, le GB généré à partir des usines de fabrication du biodiesel peut contenir jusqu'à 25% (p/p) de méthanol (Ito *et al.*, 2005). À partir de différentes études, il a été conclu que les déchets du biodiesel contenant du méthanol sont inhibiteurs de la croissance microbienne (Athalye *et al*., 2009, Chi *et al*., 2007) et peuvent être toxiques pour l'environnement, s'ils sont libérés sans traitements appropriés. L'excès de méthanol présent dans le GB peut contaminer l'eau souterraine. De même, en raison de l'addition de NaOH dans le processus de fabrication du biodiesel, le pH des déchets produits est généralement trop élevé et doit être neutralisé avant l'élimination des déchets. De plus, les eaux usées à salinité et alcalinité élevées peuvent prendre plus de temps pour être dégradées par les microorganismes indigènes du milieu récepteur.

1.3. Glycérol: un substrat pour la production de bio-hydrogène

1.3.1. Glycérol comme matière première pour la production d'hydrogène: Le glycérol est un substrat qui peut être utilisé par de nombreux microorganismes pour leur croissance et qui a été largement étudié pour son potentiel de production d'hydrogène. Le contenu énergétique du glycérol pur est de 19,0 MJ/kg; cependant, il est de 25,30

MJ/kg pour le GB en raison de la présence de méthanol et des traces de biodiesel (Disponible à partir de: http://www.esru.strath.ac.uk/EandE/Web_sites/06-07/Biodiesel/experiment.htm). Cette haute teneur en énergie du glycérol brut indique un fort potentiel comme substrat pour la production d'hydrogène. En outre, contrairement à la plupart des matériaux des déchets cellulosiques, il ne nécessite pas de prétraitement supplémentaire pour le rendre disponible pour les micro-organismes produisant de l'hydrogène. Également, des substrats tels que le lactosérum et la mélasse sont en forte demande en raison de leur large éventail d'applications dans les fermentations industrielles. En contrepartie, des substrats tels que les déchets alimentaires contiennent généralement des matières solides d'origines différentes et ont besoin de broyage et de mélange approprié avant de les soumettre à la fermentation. Ainsi, pour la production d'hydrogène à grande échelle, le GB semble être le support idéal sans contraintes de pré-traitements majeurs. Différents rapports sur la production d'hydrogène par bioconversion du glycérol pur et brut ont été résumées dans le Tableau 2.1.3.

1.3.2. Métabolisme du glycérol et de la production d'hydrogène: Les métabolismes oxydatifs et réducteurs du glycérol sont connus pour certaines espèces telles que *Klebsiella, Citrobacter, Clostridium, et Enterobacter* (Drożdżyńska *et al.*, 2011). Comme le montre la Figure 2.1.4, dans la voie oxydative, le glycérol est d'abord converti en dihydroxyacétone sous l'activité catalytique de la glycérol déshydrogénase. Dans la prochaine étape, la dihydroxyacétone est phosphorylé par l'enzyme glycolytique, la dihydroxyacétone kinase. Enfin, le produit phosphorylé est métabolisé par la glycolyse (Daniel *et al*., 1995, Drożdżyńska *et al*., 2011, Luers *et al*., 1997). Dans la voie de la réduction, le glycerol est finalement converti en PD (1,3 -propanediol) par l'intermédiaire de la production du produit 3-hydroxypropionaldéhyde.

D'après la Figure 2.1.4, on peut voir que pendant le métabolisme oxydatif du glycérol, le pyruvate est formé comme produit intermédiaire; qui, à son tour, peut être métabolisé en divers produits finaux tels que l'éthanol, le butanol, l'acétone, l'acétate, le butyrate et le lactate (da Silva *et al*., 2009, Drożdżyńska *et al*., 2011). Dans la plupart des voies de bioconversion du glycérol, ainsi que les différents métabolites, de l'H_2 est également

produit au cours du métabolisme oxydatif. À partir des équations stœchiométriques présentées dans la Figure 2.1.5, on peut conclure que la production du produit final très réduit s'accompagne d'un rendement de de production faible de H_2. Cette réaction peut être vue à partir de la Figure 2.1.6 où le pyruvate est décomposé en acétyl -CoA par l'intermédiaire d'une réduction de la ferrédoxine (Fd) catalysée par la pyruvate ferrédoxine oxydoréductase. La ferrédoxine réduite (Fd) est ensuite oxydée par une hydrogénase qui reproduit la Fd oxydée et de l'hydrogène gazeux (Mathews *et al.* 2009, Nath *et al.*, 2004). Une quantité supplémentaire d'hydrogène peut être produite par la glycolyse lorsque le NADH est oxydé par la réduction de la Fd et NADH-ferrédoxine réductase (Mathews *et al.*, 2009, Vardar-Schara *et al.,* 2008). Théoriquement, un maximum de 4 moles de H_2 peuvent être produites par mole de glucose lorsque l'acétate est le produit final de fermentation. Cependant, seulement deux moles de H_2 par mole-glucose peuvent être générées lors de la production du butyrate et autres produits finaux réduits tels que les alcools et l'acide lactique (Levin *et al* ., 2004, Mathews *et al*., 2009).

1.4. Prétraitement du glycérol brut

1.4.1. Importance du prétraitement du glycérol brut: Tel que mentionné précédemment, le processus de transestérification pour la production de biodiesel implique un mélange de graisses animales et/ou végétales avec du méthanol et de l'hydroxyde de sodium comme catalyseur (Kim *et al*., 2004, Muniyappa *et al*., 1996, Schuchardt *et al*., 1998). Par conséquent, en plus du glycérol, un rejet typique de production de biodiesel peut contenir des concentrations élevées en sels, en méthanol, en savon et en précipités solides (Athalye *et al*., 2009, Yazdani *et al*., 2007), ce qui peut affecter la croissance microbienne lors de la bioconversion (Chi *et al*., 2007, Ngo *et al*., 2011). Afin d'éviter de tels incidents, le GB doit être prétraité avant d'être utilisé comme matière première pour la production d'hydrogène.

1.4.2. Différentes méthodes de prétraitements du GB: Pour utiliser le GB comme matière première pour la bioconversion, le méthanol peut être retiré à l'autoclave (Athalye *et al*., 2009, Ethier *et al*., 2011). Selon Pyle *et al* (2008), le méthanol s'évapore à 65 °C, d'où le processus de passage à l'autoclave durant 15 min à 121°C afin

d'éliminer une quantité importante de méthanol à partir du GB (Pyle *et al.*, 2008). Dans une récente recherche sur la production d'hydrogène à partir de glycérol brut, Ngo *et al* (2010) ont rapporté l'élimination du méthanol et de l'éthanol à l'évaporateur rotatif à 45 °C, où les solides ont été récoltés par centrifugation à 15 000 rpm pendant 15 min (Ngo *et al*., 2011). Le savon est l'autre impureté majeure présente dans le GB, qui peut être éliminé par précipitation à partir du milieu liquide à l'aide d'un ajustement du pH (Athalye *et al*., 2009, Ethier *et al*., 2011). Ainsi, diverses méthodes d'élimination des impuretés ont été évaluées et la possibilité d'utiliser une seule méthode d'élimination collective pour toutes les principales impuretés présentes dans le GB devraient être explorées.

1.5. Génie biologique pour la production d'hydrogène

1.5.1. Évaluation de différents types de réacteurs pour la production de bio-hydrogène: Pour évaluer la production d'hydrogène en utilisant différents déchets organiques, y compris le GB généré dans les usines de production de biodiesel, différents types de réacteurs ont été utilisés. Le bioréacteur utilisé à cette fin peut être une petite fiole à sérum de 10 mL de volume de travail ou un fermenteur de 2,5-L. Un résumé des types de bioréacteurs utilisés pour la production d'hydrogène a été présenté dans le tableau 2.1.4. Dans un rapport sur la production d'hydrogène à partir de déchets de la transformation du biodiesel, Ito *et al* (2004) ont indiqué que les déchets de production de biodiesel contenant du glycerol doivent être dilués avec un milieu synthétique pour augmenter le taux d'utilisation du glycérol. Ils ont rapporté que la production de H_2 diminue avec une augmentation de la concentration des déchets du biodiesel (Ito *et al.,* 2005). Par conséquent, pour la bioconversion d'un grand volume de GB dilué, un système continu peut être plus approprié qu'un système de traitement en mode discontinu. D'ailleurs, au niveau de la recherche d'informations il y a moins d'études qui traite des systèmes en lots.

1.5.2. Paramètres importants du procédé et leur niveau optimal:

Selon le tableau 2.1.4, il est clair que la production d'hydrogène microbienne a été étudiée en utilisant différents types de réacteurs et les paramètres optimaux des procédés ont été choisis pour des cas spécifiques. La gamme de température utilisée

par différents chercheurs pour la production d'hydrogène varie de 25º C à 75º C (Escapa *et al.*, 2009, Ngo *et al*., 2011). Pour le pH des valeurs de 4,5 à 7,5 ont été signalées (Fang *et al.*, 2006, Ngo *et al.*, 2011). Les paramètres du procédé peuvent varier en fonction du mode de fonctionnement ou les types de microorganismes utilisés dans le procédé. Par conséquent, une température ou un pH particulier ne peuvent pas être considérées comme optimales pour le procédé de production d'hydrogène dans son ensemble.

1.6. Microorganismes utilisés pour la bioconversion du glycerol

1.6.1. Microorganismes utilisés actuellement pour la bioconversion du glycérol. Comme le glycérol a été utilisé pour la production de différents produits; divers microorganismes ont été évalués pour leur capacité de bioconversion du glycérol. Différents microorganismes utilisés actuellement pour la bioconversion du GB ont été répertoriés dans le Tableau 2.1.5. À haute température, la production d'hydrogène est plus exergonique et les microorganismes hyper thermophiles présentent une résistance à des pressions partielles d'hydrogène élevées (van Niel *et al.*, 2003). L'un des avantages de la fermentation à des températures extrêmes, c'est que le procédé est moins sensible aux contaminations, mais d'autre part, il est plus complexe de réaliser une relation économique positive entre l'énergie utilisée pour chauffer et maintenir le réacteur à des températures élevées et la production de H_2. En outre, les bactéries anaérobies thermophiles extrêmes se développent habituellement en faibles densités résultant d'un taux de production faibles. Les chercheurs ont étudié la bioconversion du glycérol en utilisant la monoculture ou un consortium microbien mixte. Phowan *et al* (2010) ont démontré que la co-culture de *C. butyricum* TISTR 1032 et *E. aerogenes* TISTR 1468 était capable de réduire la phase de latence de la production d'hydrogène (Phowan *et al.,* 2010). Comme il est montré dans le Tableau 2.1.5, *Enterobacter aerogenes* est l'organisme le plus étudié pour la production d'hydrogène à l'aide du GB. Les cultures microbiennes mixtes à partir de sources environnementales sont les autres inoculums les plus utilisés pour la bioconversion du GB. Cependant, certaines contraintes au niveau de la stabilité du processus existent et doivent être considérées

surtout si des eaux usées industrielles avec des variations de composition sont utilisées.

1.6.2. Génie génétique et métabolique pour l'amélioration de la production d'hydrogène: Le génie génétique et métabolique peut être considéré comme un outil potentiel d'amélioration de la production d'hydrogène par bioconversion du GB. Maeda *et al* (2007) ont rapporté qu'une souche de *E. coli* recombinant est capable d'exprimer une enzyme bidirectionnel la [NiFe]-hydrogénase de *Synechocystis* sp. PCC 680 (Maeda *et al.,* 2007). Une augmentation de la production d'hydrogène de facteur de 41 fois par rapport au type sauvage a été signalé pour la souche recombinante. Chittibabu et al (2006) ont également mis au point une souche génétiquement modifiée de *E. coli* contenant le gène [FeFe]-hydrogénase de *Enterobacter cloacae* IIT BT- 08. Avec cette souche génétiquement modifiée, un rendement de 3.12 mol H_2 par mole de glucose a été obtenu, ce qui était beaucoup plus élevé que le type sauvage (Chittibabu *et al.,* 2006). Ainsi, un certain nombre de succès ont été obtenus à partir des tentatives de génie génétique pour améliorer la production de biohydrogène. Toutefois, le potentiel de production d'hydrogène d'une souche recombinante, en utilisant différents déchets organiques en tant que substrat n'est pas encore documenté. Dans une étude récente, Mathews et Wang (2009) ont suggéré que, pour accroître la viabilité économique de la production d'hydrogène, différentes voies d'utilisation du substrat doivent être conçues dans des organismes produisant de l'hydrogène (Mathews *et al.,* 2009). Par conséquent, une souche microbienne issue du génie génétique et qui présente une bioconversion efficace du GB peut améliorer la viabilité économique de la production de biohydrogène. Récemment, en utilisant l'évolution adaptative et la mutagenèse chimique, suivie par l'expérience de croissance sur le glycérol, Hu et Wood (2009) ont mis au point une amélioration de la souche d'*Escherichia coli* (HW2), qui produit 20 fois plus d'hydrogène dans du glycérol contenant un supplément (0,68 ± 0,16 mmol-H_2 L^{-1} h^{-1}) (Hongbo Hu *et al*., 2010). Shams Yazdani et Gonzalez (2008) ont également décrit une souche recombinante d '*Escherichia coli* capable de bioconvertir du glycérol en éthanol et en hydrogène (Yazdani *et al.,* 2007).

PARTIE II. PROBLÈMES, HYPOTHÈSE, OBJECTIFS ET ORIGINALITÉ

2.1. Problèmes

À partir de la revue de la littérature (Sarma *et al.*, 2012) et l'analyse de la faisabilité technico-économique de la production de biohydrogène (Sarma *et al.*, 2013) présentée au chapitre 2, les problématiques suivantes ont été soulevées

2.1.1. Coût des composantes et média supplémentaires

Le coût de fonctionnement des processus récemment évalués pour la production d'hydrogène par bioconversion du GB est très élevé et, par conséquent, ils ne sont pas économiquement viables pour des applications commerciales. Dans l'analyse de faisabilité, il a été mis en évidence que le coût des composantes des milieux est d'environ 82% du coût total du procédé. Ceci est dû au fait qu'une grande quantité de milieu nutritif (50-400 L/kg GB) doit être ajoutée pour diluer le GB à une très faible concentration de départ pour sa bioconversion. D'autre part, le coût de développement de l'inoculum est de près de 27% du coût total de production.

2.1.2. Effet inhibiteur des impuretés dans le GB (comme le savon, le méthanol, NaCl) sur la production de H_2

Le GB n'est pas un substrat pur pour la production d'hydrogène et il peut contenir une variété d'impuretés. Du méthanol (23,4 à 37,5% p/p) (Tang *et al.,* 2009, Thompson *et al*., 2006), le sel (3,12% p/p) (http://rendermagazine.com/articles/2008-issues/2008 - august/2008-08-crude-glycerin / (consulté le 03_09_2012) et du savon (de 20,5 à 31,4% p/p) (Shengjun Hu *et al*., 2012) sont trois des principales impuretés présentes dans le GB et ils pourraient avoir certains effets sur la production d'hydrogène (Venkataramanan *et al*., 2012, Yang *et al*., 2012). Les impuretés, telles que le méthanol et le savon, peuvent également être utilisées comme sources de carbone, leur présence ou leur absence dans les milieux pourraient affecter les performances du processus. Par conséquent, l'effet de ces trois impuretés du GB sur la production d'hydrogène doit être évalué et des stratégies appropriées devraient être élaborées pour atténuer leurs effets inhibiteurs.

2.1.3. Co-production d'un grand volume de déchets liquides pendant la production de biohydrogène

Sur la base de notre estimation (Sarma *et al*., 2013), près de 50-400 L de déchets sont générés lors de la production de H_2 à partir d'un kg de GB. Compte tenu du volume important de milieu, il est à noter que la gestion durable de ces déchets liquides sera nécessaire.

2.1.4. Effet inhibiteur du pH sur la production du H_2 en fermentation sombre

Lors de la production de biohydrogène par fermentation sombre, une gamme d'acides organiques sont produits et ils sont généralement considérés comme des déchets (Shida *et al*., 2009). En outre, la production d'acide organique peut consommer une partie importante du substrat disponible pour la production de H_2 et diminuer le rendement et aussi abaisser le pH du milieu ce qui peut inhiber le processus de fermentation. Par conséquent, il est considéré comme le principal frein technico-économique de la fermentation sombre pour produire du H_2 (http://www.slideshare.net/kumarhukkeri/finalppt-em-1 (consulté le 02/03/2013)).

2.1.5. Auto inhibition de la production par accumulation de H_2

Il est bien connu que le mode de traitement peut fortement influencer le rendement en hydrogène. Dans un procédé discontinu, l'H_2 s'accumule dans l'espace de tête du réacteur pour augmenter la pression partielle de H_2, qui, à son tour, peut inhiber la production de H_2 (Oh *et al*., 2009, Van Andel *et al*., 1985). Avec l'hydrogène, le CO_2 est produit et même l'accumulation de CO_2 a un effet négatif sur la production d'hydrogène (Oh *et al*., 2009, Park *et al*., 2005). Avec l'augmentation de la concentration en hydrogène, la voie métabolique est décalée vers la production de solvants et augmente la production de produits réduits, tels que le butanol, le lactate ou de l'acétone, résultant en une diminution de la production d'hydrogène (Junghare *et al*., 2012, Levin *et al*., 2004). Dans une étude utilisant *Clostridium butyricum* TM-9A, Junghare *et al* (2012) ont montré qu'il est possible d'augmenter la production d'hydrogène de 2,6 fois par la diminution de la pression partielle de 33,9 à 10,13 kPa (Junghare *et al*., 2012).

2.2. Hypothèse

Le projet de recherche comprend différentes hypothèses:

2.2.1. Le GB est un sous-produit de la production de biodiesel par transestérification. Sur la base de la nature de la charge, le catalyseur et les conditions de réaction utilisées pour ce procédé, la composition du GB pourrait varier. Le processus de production de biodiesel génère le GB qui nécessite une bonne caractérisation avant son utilisation pour la production d'hydrogène.

2.2.2. Le GB est une bonne source de carbone et il a le potentiel pour soutenir la croissance microbienne. Par conséquent, *E. aerogenes*, la bactérie choisie pour la présente étude doit se développer sur le GB en l'utilisant comme seul substrat et produire le H_2. Si elle peut être cultivée avec succès, aucun nutriments supplémentaires ne sera nécessaire et le coût des processus de production de H_2 pourrait être réduit. En outre, si la biomasse produite pendant la fermentation pourrait être ré-utilisée dans le processus ultérieur, soit comme inoculum ou comme source de nutriment supplémentaire, une nouvelle réduction du coût de traitement pourrait être possible.

2.2.3. Comme mentionné dans la section de revue de la littérature, le GB est généralement complété par des suppléments de milieu synthétique pour une meilleure production de H_2. Dans ce contexte, les déchets riches en éléments nutritifs, tels que les boues secondaires provenant des usines de traitement des eaux usées, les eaux usées d'abattoir et les rejets de brasserie entre autres pourraient être évalués en tant que remplacement des composantes de milieux synthétiques.

2.2.4. Outre le glycérol, le GB contient une série d'autres composés, tels que le savon, le méthanol, NaCl etc. Selon la revue de la littérature, il est évident que certains de ces composés pourraient avoir un effet négatif sur la croissance microbienne alors que certains d'entre eux pourraient avoir un apport bénéfique pour le processus. Ainsi, une étude doit être réalisée en utilisant le glycérol pur en tant que substrat et les différents composantes du GB pourraient être ajoutées de façon externe afin d'évaluer leur effet sur la production de H_2. Pour réduire le nombre d'expériences nécessaires pour cette

recherche et avoir une idée claire des effets interactifs des différentes composantes, l'approche de la méthodologie de réponse de surface pourrait être utilisée.

2.2.5. Le savon est l'une des principales impuretés présentes dans le GB et il est reconnu pour avoir des effets négatifs sur la croissance microbienne. En augmentant la salinité du GB, le savon peut être précipité et être éliminé par centrifugation. Ainsi, le NaCl peut être utilisé pour l'enlèvement du savon et son effet sur la production d'hydrogène pourrait être étudié. De même, le Mg^{2+} peut réagir avec des molécules de savon et les inactiver pour former de l'écume. Ainsi, l'addition de $MgSO_4$ pour un milieu à base de GB pourrait détruire les propriétés fonctionnelles du savon et réduire son effet inhibiteur et améliore la production d'hydrogène.

2.2.6. Le fer est un cofacteur de l'hydrogénase, l'enzyme clé responsable de la production d'hydrogène. Par conséquent, la production d'hydrogène peut être améliorée par l'addition d'un composé contenant du fer dans le milieu utilisé à cet effet. Les composés, tels que le $FeCl_2$ et $FeSO_4$ ont été rapportés comme ayant un potentiel d'améliorer la production d'hydrogène. Dans ce contexte, le citrate ferrique peut être évalué en tant que supplément pour améliorer la production d'hydrogène à partir du GB. En plus d'être une source de fer, il est capable d'agir comme une source de carbone supplémentaire et facilement accessible pour le métabolisme et par conséquent, on peut prévoir qu'il y aura de meilleurs rendements que le $FeCl_2$ et $FeSO_4$, traditionnellement utilisé dans ce but.

2.2.7. Pendant la production d'hydrogène en fin de fermentation, essentiellement des acides organiques accumulent dans le milieu. Ces acides peuvent diminuer le pH du milieu, initier des changements métaboliques et réduire la perfomance du processus. Par conséquent, un système de bioréacteur de séparation à deux phases peut être développé pour éliminer in situ les acides organiques générés lors de la fermentation. Dans ce cas, les acides organiques produits au cours de la fermentation seront solubilisés dans la phase organique, de sorte que les bactéries présentes dans la phase aqueuse ne subiront pas l'effet néfaste de ces acides.

2.2.8. Le mode de fermentation adopté pour la production d'hydrogène a une forte influence sur les rendements de production. Dans le cas du procédé classique

discontinu fermé, le gaz produit est accumulé dans l'espace gazeux du réacteur avec une augmentation graduelle de la pression partielle du H_2. Cependant, l'augmentation de la pression partielle du H_2 est inhibitrice pour le procédé. La production de H_2 pourrait être améliorée par une libération continue du gaz produit. Également, une fermentation continue presente certains avantages par rapport à une fermentation par lots. En particulier, lorsque l'accumulation de sous-produits est inhibitrice pour le procédé, ceux-ci sont évacués en continu. Par conséquent, un bioréacteur de 7,5 L a été utilisé pour la production continue d'hydrogène en libérant de façon continue le gaz produit. Cependant, dans ce cas, la surveillance de la concentration du gaz produit sera difficile. Par conséquent, un capteur d'hydrogène peut être utilisé pour la surveillance en temps réel de la production de H_2.

2.2.9. Conformément à l'estimation, lors de la production d'hydrogène par fermentation pour 1 kg de GB, un volume aussi élevé que 50 à 400 litres d'effluents liquides contenant une gamme d'acides organiques seront générés. Fait intéressant, ils existent sur le marché des bio-engrais commerciaux (PSB) contenant des acides organiques qui peuvent chélater les cations de phosphates insolubles (comme $Ca_3(PO_4)_2$) présents dans le sol pour solubiliser le phosphate et le rendre disponible pour les plantes. Ainsi, la capacité de solubilisation du phosphate du rejet liquide de fermentation du GB pourrait être évaluée. En cas de succès, une plante modèle pourrait être utilisée pour vérifier son efficacité comme une alternative potentielle de PSB.

2.3. Objectifs

L'objectif principal de cette étude est de renforcer la production d'hydrogène par bioconversion du glycérol brut en réduisant les coûts de fermentation et utiliser de façon durable les déchets générés au cours du processus afin d'offrir une solution " zéro déchet " pour la gestion du glycérol brut.

Sur la base de ce qui précède, une revue de la littérature et des recherches préliminaires ont été réalisées et ont permis d'élaborer les objectifs spécifiques suivants :

2.3.1. Caractérisation du glycérol brut

Le GB est caractérisé pour la teneur en glycérol, le savon total, la quantité totale de carbone, l'azote total et les différents éléments, notamment le Fe, Mg, S, P, Ni, Mn, Cu, Zn, etc.

2.3.2. Production de biohydrogène à partir de la bioconversion du GB provenant d'une usine de production de biodiesel : évaluation technico-économique

Sur la base des données disponibles dans la littérature sur la bioconversion du GB et la production biologique d'hydrogène, une étude de faisabilité technico-économique du processus sera effectuée.

2.3.3. Évaluation de GB en tant que seule substrat pour la production d'hydrogène et valorisation des déchets dans le processus ultérieur

Afin de déterminer le potentiel réel du GB comme matière première pour la production de biohydrogène, et de déterminer l'effet de la concentration initiale de GB sur la production de $H_{2,}$ différentes concentrations de GB seront testées comme substrat. De plus, les déchets (comme la biomasse) générés au cours du processus seront réutilisés (soit comme inoculum ou comme source d' éléments nutritifs) dans le processus ultérieur.

2.3.4. Screening de différents déchets en tant que source de nutriments supplémentaires

Similaire à l'objectif 2, si les déchets tels que les boues des stations d'épuration des eaux usées, les eaux usées d'abattoir ou les déchets de brasserie pouvaient être utilisés comme remplacement des suppléments synthétiques coûteux, une réduction des coûts du procédé de fermentation serait possible. Par conséquent, une gamme de ces matériaux sera testée pour leur application possible dans le processus de bioconversion du GB.

2.3.5. Étude de l'effet des différentes impuretés du glycérol brut sur la production d'hydrogène et de l'utilisation du glycérol par *E. aerogenes* utilisant du glycérol pur comme substrat

En utilisant la méthodologie de surface deréponse , l'effet individuel et interactif de trois principales impuretés de GB (savon, méthanol, NaCl) sera évalué. À cet effet, des expériences de production de H_2, en utilisant le glycérol pur en mélange avec des quantités définies (selon la conception expérimentale) des impuretés seront utilisées pour formuler des milieux.

2.3.6. Utilisation du NaCl et $MgSO_4$ comme stratégie de base pour l'atténuation de l'effet inhibiteur du savon présent dans le GB

Selon la première stratégie, le NaCl est utilisé pour éliminer le savon par relargage. Dans le second cas, le $MgSO_4$ sera utilisé pour détruire la propriété tensioactve du savon en le convertissant en écume. Toutefois, le savon ne sera pas réellement retiré du GB, de sorte que son rapport C/N restera constant. Les deux approches seront optimisées pour une meilleure production de H_2.

2.3.7. Étude des effets de citrate de fer et de ses nanoparticules vaporisés (spray dryer) sur la production d'hydrogène par bioconversion du GB

Le citrate de fer solubilisé en utilisant une solution d'eau chaude/NaOH ou formulé comme des nanoparticules à l'aide d'un "nanospray dryer" sera évalué comme un supplément de fer et de source de carbone.

2.3.8. Développement d'un système à deux phases pour l'amélioration de la production d'hydrogène par élimination simultanée des acides organiques du milieu

Un nouveau système de bioréacteur à deux phases sera développé pour l'extraction simultanée d'acide organique lors de la production de H_2 par bioconversion du GB. À cet effet, un liquide de phase non aqueuse biocompatible (PNA) sera sélectionné et testé en bioréacteur à petite échelle (125 mL flacons à sérum). Le système sera optimisé pour améliorer la production d'hydrogène et l'utilisation maximale du glycérol.

2.3.9. Production d'hydrogène en utilisant un bioréacteur de 7,5 L avec une libération continue du gaz produit et la surveillance en ligne de la production d'hydrogène par un capteur spécifique au H_2

Un procédé semi-continu sera développé où la fermentation sera lancée comme un processus de traitement par lots à une concentration initiale optimale de GB. Après la consommation d'une partie du substrat, une solution d'alimentation contenant des concentrations élevées de GB (par exemple 60-120 g/L) est ajoutée lentement dans le réacteur de 7,5 L. Pour éviter la perte de biomasse active, un long temps de rétention hydraulique sera maintenu. Le gaz produit sera périodiquement libéré de l'espace de tête du réacteur afin de minimiser l'inhibition des réactions.

2.3.10. Évaluation de la capacité de solubilisation du phosphate des rejets liquides du bioréacteur de production de biohydrogène et son application comme une alternative au bioengrais utilisé pour solubiliser le phosphate

Les déchets liquides seront recueillis à la fin de la fermentation et ils seront testés comme agent potentiel de solubilisation du phosphate. Le $Ca_3(PO_4)_2$, un phosphate insoluble présent dans le sol est couramment utilisé comme modèle pour déterminer la capacité de solubilisation du phosphate par les biofertilisants solubilisateurs de phosphate (PSB). Le déchet est mélangé avec le composé dans des proportions différentes et la concentration en phosphate soluble est mesurée afin de déterminer l'efficacité de ces déchets. En cas de succès, un modèle végétal (de plante de soja) pourrait être utilisé pour vérifier son efficacité comme une alternative potentielle de PSB.

2.4. Originalité

Basé sur la revue de la littérature ci-dessus l'originalité des travaux prévus pourrait être décrite comme suit:

a. Aucune recherche n'est disponible sur l'analyse biochimique et élémentaire détaillée du glycérol brut généré lors de la production du biodiesel à partir des déchets de viande et des graisses de restaurants.

b. Afin de réduire le coût du procédé, le GB doit être évalué en tant que seule composante pour la production d'hydrogène par fermentation et la possibilité de recyclage de la biomasse, soit comme inoculum ou comme source de nutriment, doit être évaluée. De même, afin d'assurer une utilisation maximale de la charge, une relation possible entre la concentration initiale de GB, la production de H_2 et le taux d'utilisation du glycérol doit être étudiée.

c. Le GB est essentiellement une source de carbone et en le complétant avec les différentes composantes de milieux synthétiques, la production de H_2 pourrait être améliorée. Cependant, aucune tentative n'a été faite pour évaluer l'efficacité des différents déchets organiques riches en éléments nutritifs (par exemple boues d'épuration, les eaux usées d'abattoir, rejets des brasseries).

d. Jusqu'à présent, aucune tentative n'a été faite pour évaluer l'effet interactif des différentes composantes du GB sur la production de biohydrogène. Dans ce contexte, la méthodologie de surface de réponse pourrait être adoptée pour évaluer toutes les interactions possible entre le savon, le méthanol et le NaCl, la production de H_2 et l'utilisation du glycérol.

e. Le savon peut être retiré du GB par relargage à l'aide de NaCl. De même, si le $MgSO_4$ est ajouté à une solution de GB, l'ion Mg^{2+} réagit avec les molécules de savon présentent et détruit les propriétés tensio-actif en les convertissant en écume. Ainsi, pour se débarrasser de l'effet inhibiteur du savon sur le processus de bioconversion du GB, ces deux nouvelles stratégies pourraient être appliquées.

f. Le $FeCl_2$ et $FeSO_4$ ont été rapportés comme ayant une meilleure capacité de production d'hydrogène, car ils peuvent servir comme source de fer. Nous tenons à proposer le citrate de fer comme une alternative de ces deux composés, car, en plus d'être une source de fer, il est capable d'agir comme une source de carbone supplémentaire pour le procédé. De plus, afin d'améliorer sa biodisponibilité (il est insoluble dans l'eau) le citrate ferrique peut être fourni sous forme de nanoparticules (nanospraydried).

g. Mise au point d'un nouveau système de bioréacteur à deux phases qui pourrait être développé pour l'élimination *in situ* des acides organiques et améliorer la production de H_2.

h. Nous avons proposé un procédé de fabrication semi-continu de H_2 en réacteur de 7,5 L. En outre, le gaz produit sera périodiquement libéré de l'espace de tête du réacteur pour éviter toute inhibition due à l'accumulation du H_2. Un capteur d'hydrogène sera testé pour la surveillance en temps réel de la production de H_2.

i. Jusqu'à ce jour, la capacité de solubilisation du phosphate à partir des rejets liquides de la production d'hydrogène par fermentation n'a pas été étudiée. Ainsi, dans la présente étude les rejets seront utilisés comme solubilisateur de phosphate et en cas de succès, un modèle d'usine sera utilisé pour déterminer son potentiel comme une alternative aux biofertilisant servant à solubiliser le phosphate.

Dans l'ensemble, l'originalité de la recherche proposée est, *"**le développement de procédés de production de biohydrogène nouveaux et efficaces pour une meilleure bioconversion du glycérol brut issu de la production de biodiesel avec l'utilisation durable des déchets secondaires générés au cours du processus**".*

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

Un aperçu simplifié de l'ensemble du projet, sous forme de procédé, est illustré à la Figure 1.1. En outre, des études spécifiques menées dans le cadre de cette thèse ont été divisées en chapitres suivants :

3.1. Production de biohydrogène: Principes, possibilité et défis (trois articles publiés, un soumis)

3.2. Production d'hydrogène par la bioconversion du glycérol brut: Une synthèse pratique (deux articles publiés)

3.3. Effet des impuretés du glycérol brut: spéculations et faits (deux articles publiés)

3.4. Application de la nanotechnologie pour améliorer la bioconversion du glycérol brut (un article soumis)

3.5. Application potentielle des déchets liquides de fermentation pour la solubilisation du phosphate (un article publié, un soumis)

3.6. Conception et optimisation de nouveaux bioprocédés pour améliorer la production d'hydrogène en utilisant le glycérol brut (deux articles soumis)

3.1. Production de biohydrogène: Principes, possibilité et défis (trois articles publiés, un soumis)

3.1.1. Production microbienne d'hydrogène par bioconversion du glycérol brut: revue de l'information (chapitre 2, partie I)

Les déchets de procédé de fabrication du biodiesel contenant le glycérol sont un produit de base ayant un potentiel de production d'hydrogène par fermentation microbienne. Différents chercheurs ont évalué sa performance en tant que substrat peu coûteux pour la production d'hydrogène. ces recherches ont montre que potentiel de production est comparable à tout autre déchet organique actuellement utilisé pour la production

d'hydrogène. L'avantage le plus important de l'utilisation du GB sur d'autres substrats est l'augmentation du bénéfice global des usines de fabrication de biodiesel. Une telle situation peut encourager la production et l'utilisation des biocarburants, ce qui est bénéfique pour l'environnement. Cependant, le GB contient de nombreuses impuretés qui sont des inhibiteurs potentiels de la croissance microbienne. Les informations scientifiques sur le prétraitement de GB sont limitées. Ainsi, des efforts additionnels sont encore nécessaires pour optimiser le prétraitement du glycérol brut pour la production de biohydrogène. Une méthode d'élimination collective pour les différents types d'impuretés ainsi qu'une étude de faisabilité de son application à l'échelle industrielle s'avère essentielles pour l'avenir de la production d'hydrogène à grande échelle à partir du glycérol brut. Quelques études réalisées en mode continu ont donné un meilleur rendement de production d'hydrogène que les expériences de traitement par lots. Ainsi, une recherche plus poussée sur la production d'hydrogène par fermentation en utilisant le mode continu est recommandée.

Actuellement, le microorganisme le plus étudié pour la production d'hydrogène à partir du GB brut est *Enterobacter aerogenes*. D'autres inoculums fréquemment utilisés sont la microflore indigène mixte contenue dans les déchets organiques.

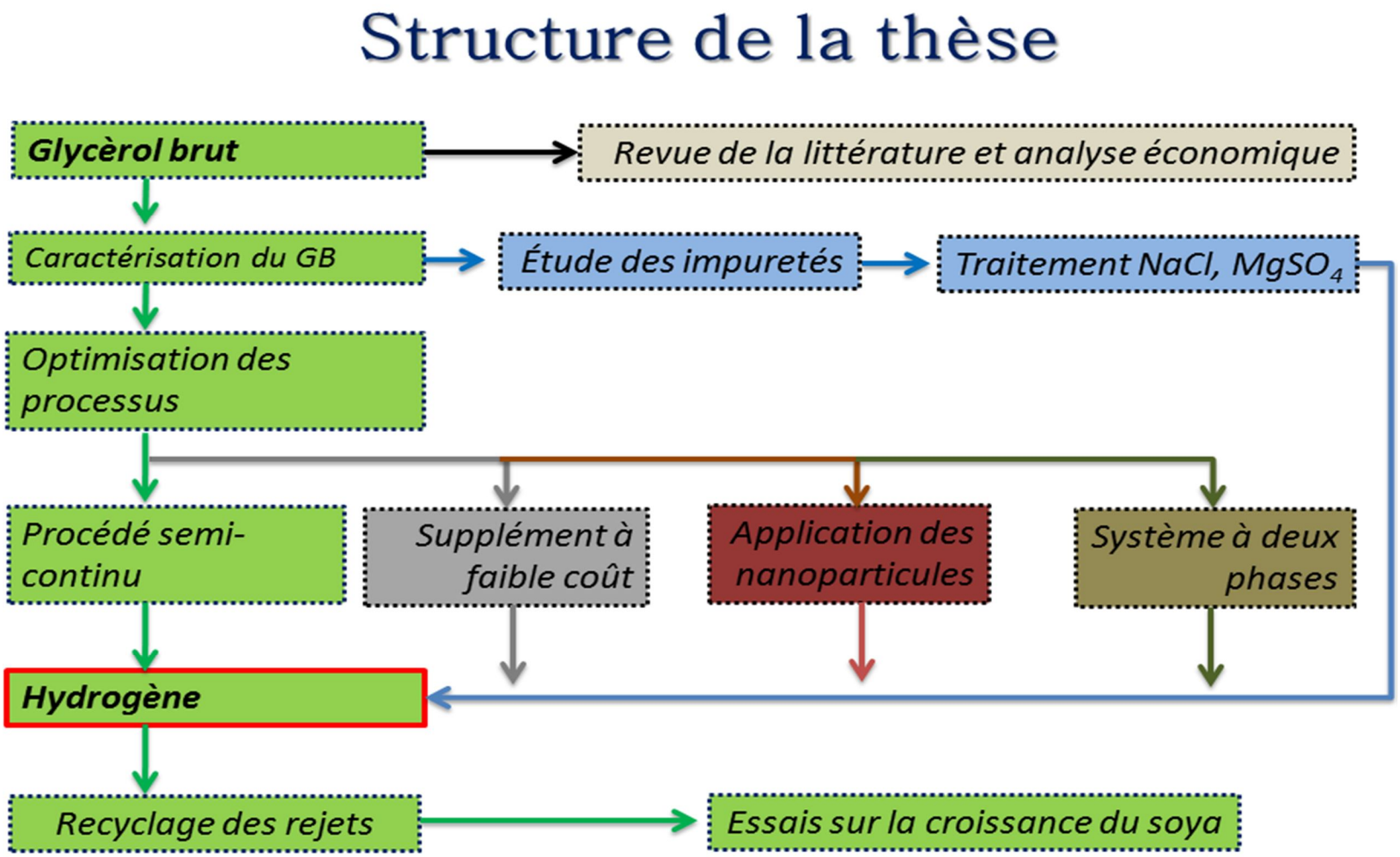

Figure 1. 1: Présentation de la thèse.

3.1.2. Enzymes clés: amélioration de la bioconversion du glycérol brut en biohydrogène (chapitre 2, partie II)

Le métabolisme oxydatif et réducteur est possible pour la bioconversion du glycérol. La plupart des microorganismes testés pour la production d'hydrogène à partir du GB sont connus pour avoir des enzymes pour les deux voies. Si le glycérol est principalement métabolisé par la voie réductrice, la plupart des atomes d'hydrogène présents dans le glycérol seront utilisés pour générer le 1,3 -propanediol résultant en un rendement de production d'hydrogène pauvre par mole de glycérol utilisée. Par conséquent, choisir la bonne souche et optimiser les paramètres du procédé afin de réduire la production de 1,3 -propanediol peut être une stratégie intéressante pour améliorer le rendement de l'hydrogène à partir du GB. Une fois le glycérol décomposé en pyruvate, son métabolisme peut procéder par la voie du Pyruvate formate lyase (PFL) ou Pyruvate ferredoxine oxido reductase (PFOR). Par contre la voie PFL n'a pas le système enzymatique nécessaire pour avoir accès au NADH générateur d'hydrogène. Par conséquent, le rendement en hydrogène maximale possible pour les microorganismes ayant une seule voie PFL est seulement de 2 moles de H_2 par mole de glucose. De même, lors de la production d'hydrogène par l'intermédiaire de la voie PFOR ou PFL, les produits finaux de la fermentation tels que les acides organiques et les solvants sont produits et la disponibilité des protons pour la production d'hydrogène est limitée. Cependant, la voie des pentose-phosphates (PPP) est une autre voie efficace qui permet la conversion de sucres en H_2O et CO_2 sans la formation de tout autre produit final de fermentation. Par conséquent, si le métabolisme du glycerol peut être dévié du métabolisme glycolytique classique pour voie du pentose phosphate (PPP), un rendement maximal d'hydrogène peut être possible. En outre, des éléments tels que le Ni, Fe, Mo et S sont essentiels pour la synthèse des différents enzymes produisant/consommant l'hydrogène (hydrogénases et nitrogénases). Par conséquent, un bon équilibre de ces éléments dans le milieu de culture est nécessaire pour améliorer le rendement en hydrogène.

3.1.3. Production de biohydrogène et concept de bioraffinerie: Utilisation potentielle des déchets liquides de la production d'hydrogène (DLPH) par fermentation (chapitre 2, partie III)

Au cours de la production d'hydrogène par fermentation sombre, 60-70% du substrat est transformé en différents sous-produits. L'éthanol, le 1,3 propanediol et l'acide butyrique sont des sous-produits qui ont une très grande importance commerciale. Si on considère la production d'hydrogène couplée avec la récupération de l'éthanol en simultanée; en plus de 1,58 moles de H_2, 0,90 mole de l'éthanol pourrait être récupérée pour chaque mole de glucose transformée. Compte tenu du fait que l'hydrogène est le principal produit récupéré et que l'éthanol extrait du DLPH n'a pas de coût direct de production; le rendement est important.

En s'inspirant de cette approche, selon une estimation de bioconversion de 1 kg de glycérol brut, il est possible de produire 48,14 L de H_2 et 307,06 g de 1-3 Propanediol (PD). Considérant la valeur de marché actuelle de H_2 (7,75 à 11,50$/m^3) et le PD (2.43$/Kg- $ 288/L), la quantité de PD qui pourrait être récupérée à partir DLPH a une importance industrielle. Par bioconversion de 1 kg de glucose, en plus de la production d'hydrogène de 326,44 L, 299,37 g de butyrate en vrac avec un prix plus élevé que 50$/Kg ou 74,6$/L, peuvent être récupérés comme sous-produit. En variante, le DLPH est un agent ayant un potentiel de solubilisation du phosphate. En effet, des essais en laboratoire ont permis d'accroître la concentration en phosphate soluble d'un milieu aqueux contenant du phosphore inorganique insoluble par un facteur de 36 fois. Par conséquent, il doit être évalué comme un substitut des biofertilsants solubilisateurs de phosphore (PSB) commercialement disponible. Comme (i) le PSB est un engrais biologique avec un succès commercial, (ii) le DLPH n'a pas besoin de traitement en aval pour cette application, (iii) il n'a pas de coût direct de production et (iv) par rapport au PSB, il est prévu avoir une durée de vie supérieure. Cette stratégie a un intérêt considérable en tant que source de revenus supplémentaires pour la production d'hydrogène par fermentation. Le méthane peut également être produit à partir du DLPH. Toutefois, cette approche va consommer tous les sous-produits du processus de production de l'hydrogène qui, autrement, auraient un meilleur marché que le méthane. Dans l'ensemble, l'approche de la bioraffinerie d'hydrogène porte un potentiel industriel considérable et, par conséquent, au lieu de se concentrer sur un sous-produit particulier, l'ensemble du processus doit être évalué comme une stratégie intégrée et une analyse coûts-avantages appropriée de l'approche est nécessaire.

3.1.4. Production de bio-hydrogène par la bioconversion du glycérol brut dérivé de la production du biodiesel: Une évaluation technico-économique (chapitre 2, partie IV)

La fermentation en deux étapes, comprenant la fermentation sombre et la photo-fermentation est une des options les plus prometteuses disponibles pour la production de biohydrogène. Dans la présente étude, la faisabilité technico-économique d'un tel processus en deux étapes a été évaluée. L'analyse a été faite sur la base des avancées récentes dans la production d'hydrogène par fermentation à l'aide de GB comme matière première. L'étude a été réalisée avec une référence particulière sur le marché du biodiesel en Amérique du Nord et, plus précisément, les données disponibles pour les provinces canadiennes et le Québec ont été utilisées. Considérant le matériel, la puissance, les installations et les coûts d'opération, le coût total associé avec le procédé a été calculé. De cette analyse, il est ressorti que les composantes de milieux de culture requis pour le procédé couvrent près de 82 % du coût total de production. Sur la base de cette analyse, des propositions ont été développées pour la réduction du coût du procédé.

Également, le procédé a été jugé très bénéfique au point de vue de l'environnement. Il a été estimé que la bioconversion de 1 kg de GB a le potentiel de réduire de presque 7,66 kg de GES. Aussi, le processus a le potentiel pour atténuer les risques environnementaux possibles dues à une mauvaise élimination du GB.

3.2. Production d'hydrogène par la bioconversion du glycérol brut: Une synthèse pratique (deux articles publiés)

3.2.1. Production d'hydrogène à partir du glycérol brut généré lors de la production de biodiesel à partir de graisses animales et des déchets de restaurant par fermentation anaérobie et ré-utilisation d'une culture carencée (chapitre 3, partie I)

Le but de la présente étude était de caractériser le GB afin de déterminer son potentiel maximal de production de H_2, en l'absence de tout nutriment coûteux supplémentaire.

En outre, l'utilisation durable des déchets des procédés de production de H_2 est l'un des objectifs. Des différences significatives de la teneur en glycérol, du pH et de la composition élémentaire ont été observées entre l'huile de soja et le GB généré à partir d'une usine de biodiesel. Une production maximale de 2022,5 H_2 mL/L milieu a été réalisée par la bioconversion du GB (sans éléments nutritifs supplémentaires) et 10 g/L de GB a été jugée optimale. De plus, l'addition d'une culture âgée et carencée (50 mg/L) provenant d'une fermentation antérieure dans le procédé permet d'améliorer la production d'hydrogène de 32,50 %, avec un taux maximum de 1,040 mL/L/jour. De même, près de 75 % du H_2 total a été produit à un pH aussi bas que 3,8, indiquant une tolérance élevée en acide de la souche utilisée (*Enterobacter aerogenes* NRRL B407).

3.2.2. Évaluation des différents nutriments supplémentaires pour une meilleure production de biohydrogène par Enterobacter aerogenes NRRL B 407 à partir du glycérol brut provenant de déchets de production du biodiesel (chapitre 3, partie II)

La bioconversion du GB, un sous-produit de la production de biodiesel, est un processus relativement nouveau et prometteur pour la production d'hydrogène. Cependant, la transformation est fortement limitée par le coût prohibitif attribué aux composantes de milieux de culture. Dans la présente étude, les déchets, tels que des BDB (biomasse de déchets de brasserie), RLA (rejets liquides d'abbatoirs), BS (boues secondaires), MP (marc de pomme), EUIA (Eaux usées de l'industrie de l'amidon) et de l'urée, une source d'azote moins coûteuse, ont été évalués comme substitut aux composantes du milieu de culture. L'Espèce *E. aerogenes* a été utilisée pour l'étude de cas. Le GB à 10 g/L était la seule source de carbone utilisé et les observations en laboratoire indiquent que l'addition de RLA, BDB et l'urée peut améliorer la production de 18,81 ± 3,56, 27,30 ± 3,54 et 38,57 ± 3,66 %, respectivement. De même, la production cumulée de 116,41 ± 3,72 mmol H_2/L milieu de culture et un taux de 965 ± 35 mL/L de production de H_2 maximale/jour ont été obtenus en utilisant l'urée à une concentration aussi basse que 10 mg/L comme source d'azote supplémentaire. Les résultats obtenus se sont avérés comparables à ceux provenant d'autres études disponibles sur la bioconversion du GB, démontrant la pertinence d'utiliser des rejets

tels que les BDB, RLA et l'urée comme produit de remplacement pour les composantes coûteuses du milieu de culture.

3.3. Effet des contaminants du glycérol brut: spéculations et faits (deux articles publiés)

3.3.1. Étude de l'effet des différentes composantes du glycérol brut sur la production d'hydrogène par Enterobacter aerogenes NRRL B- 407 (chapitre 4, partie I)

En utilisant la méthodologie de surface de réponse, le rôle des trois principaux contaminants du GB (savon, méthanol et NaCl) sur la production d'hydrogène et la bioconversion du glycérol a été évalué. Parmi ces contaminants, le savon (1 à 3 g/L) s'est avéré avoir un effet inhibiteur significatif sur la production d'hydrogène (valeur $p = 0,0104<0,05$). De même, le méthanol (1.3 à 3.3 g/L) a également montré une réponse similaire dans la plage des concentrations considérées. Fait intéressant, pour tous les contaminants étudiés, la réponse entre la consommation de glycérol et la production d'hydrogène n'était pas similaire (comme prévu). La co-utilisation des contaminants avec le glycérol ainsi qu'un changement métabolique due à la présence de ces contaminants ont été identifiés comme étant la raison la plus probable pour une telle observation. Finalement, le NaCl a généré un effet bénéfique à la fois pour la production de H_2 et pour l'utilisation du glycérol. Sur la base de ces observations, une nouvelle stratégie impliquant du NaCl a été proposé pour l'enlèvement du savon combiné à l'amélioration de la capacité tampon du milieu à base de GB.

3.3.2. Atténuation de l'effet inhibiteur du savon par traitement au sel de magnésium du glycérol brut : Une nouvelle approche pour une meilleure production de biohydrogène à partir des déchets de l'industrie du biodiesel (chapitre 4, partie II)

Tel que déjà mentionné, le GB contient une série de contaminants et le savon est l'un d'eux. La teneur en savon de différents échantillons de GB peut varier de 20,5 ± 0,1 à 31,4 ± 0,1 % (p/p). Malheureusement, le savon est un inhibiteur potentiel de la croissance microbienne. Par conséquent, l'élimination du savon du GB peut avoir un effet bénéfique sur la croissance microbienne et une production accrue d'hydrogène

peut être attendue. Par une stratégie de relargage (salting out), jusqu'à 42 % du savon a été retiré du GB et l'approche a eu un effet bénéfique sur la production de H_2. Toutefois, l'élimination de plus de 7% de savon a été jugée inhibitrice. En fait, le savon est utilisé comme co-substrat et, en raison de son enlèvement, le rapport carbone-azote du milieu peut avoir diminué affectant ainsi la production de biohydrogène. Sans modifier le rapport carbone-azote du GB, le traitement au $MgSO_4$ peut convertir le savon en sa forme inactive (écume). Cette approche a permis d'augmenter le taux de production du H_2 (33,82%), la production cumulative de H_2 (34,70%) ainsi que l'utilisation du glycérol (près de 2,5 fois). En outre, le traitement peut augmenter le Mg (un nutriment) contenu dans le milieu de culture de 0,57 mg/L à 201,92 mg/L.

3.4. Application de la nanotechnologie pour améliorer la bioconversion du glycérol brut (un article soumis)

3.4.1. Amélioration de la production d'hydrogène par bioconversion des rejets de l'industrie du biodiesel supplémentés avec du citrate ferrique et de ses nanoparticules générées par un atomiseur à sec (chapitre 5)

Des nanoparticules de citrate ferrique, de $FeSO_4$, $NiCl_2$ et de Ni-acétate ont été produites par un atomiseur à sec et évaluées pour leur potentiel d'application en tant que supplément pour la production d'hydrogène par bioconversion du GB. Parmi ces suppléments, les nanoparticules de citrate ferrique ont permis d'obtenir un effet bénéfique sur la production d'hydrogène. Deux types de particules de citrate ferrique différentes ont été préparés : soit par dissolution dans de l'eau chaude ou dans une solution de NaOH. Entre ces deux particules, les particules à base de NaOH ont été inhibitrices pour la production d'hydrogène. Au contraire, les particules à base d'eau ont amélioré la production d'hydrogène par 31,71 %. En dehors des effets de ces suppléments, la concentration initiale en substrat a été identifiée comme un facteur essentiel pour déterminer le rendement en hydrogène. À très faibles concentrations initiale en GB de 100 mg/L, et en supplémentant la culture avec des nanoparticules de citrate ferrique, un rendement en hydrogène aussi élevé que 29,9 mol-H_2/kg GB a été atteint. Ce rendement est nettement plus élevé que 8,25 mol-H_2/kg GB déjà répertorié pour la production de biohydrogène par fermentation sombre.

3.5. Application potentielle des rejets liquides, riches en acide organique, provenant du procédé de production du biohydrogène pour la solubilisation du phosphate insoluble (un article publié, un autre soumis)

3.5.1. Rejets liquides de la production de biohydrogène-une alternative commercialement intéressante en remplacement des biofertilisants solubilisant le phosphate (chapitre 6, partie I).

Les rejets liquides issus de la production de biohydrogène sont un excellent agent de solubilisation du phosphate. De plus, ce rejet a un fort potentiel pour être une alternative commercialement intéressante aux biofertilisants solubilisant les phosphates. Pour des essais réalisés en système liquide, une augmentation de la concentration en phosphate soluble de 23 fois en moins d'une minute et 36 fois après 10 j suivant son application a été obtenue. Des valeurs supérieures pourraient être obtenues en fonction du procédé de production d'hydrogène adopté. Contrairement aux biofertilisants traditionnels solubilisant le phosphate, la solution proposée n'est pas une formulation de microorganismes vivants et, par conséquent, il devrait avoir une durée de conservation supérieure. Enfin, le processus de production de biofertilisant proposé pourrait être intégré au procédé actuel de production du biodiesel afin de mettre à niveau l'industrie du biodiesel à une branche "profits élevés et zéro déchets".

3.5.2. Solubilisation du phosphate en utilisant les rejets liquides provenant de la production de biohydrogène (RLPH) et amélioration de l'absorption du phosphore par les plants de soya (chapitre 6, partie II)

La production cumulative d'hydrogène par le procédé de bioconversion du GB utilisant *Enterobacter aerogenes* NRRL B-407 a été établie à près de 0,5 mmol/L milieu de culture. Une forte diminution de pH du milieu a été observée; ce qui indique que le début du processus de production d'hydrogène correspond également à une production d'acides organiques. Le RLPH généré au cours du processus a été testé sur un sol comme agent de solubilisation du phosphate. Les plants de soya cultivés dans le sol enrichi avec le RLPH ont montré de 2,18 à 2,74 fois plus d'absorption de phosphore. L'observation suggère une éventuelle commercialisation du RLPH comme une alternative aux biofertilisants commerciaux solubilisant le phosphore. Cependant, la

période d'application appropriée ainsi que la dose de RLPH doivent être déterminées par une étude au champ plus complète où les rendements des produits agricoles pourront être mesurés de façon plus précise.

3.6. Conception et optimisation de nouveaux bioprocédés pour améliorer la production d'hydrogène en utilisant le GB (deux articles soumis)

3.6.1. Nouveau système anaérobie à deux phases pour la production de biohydrogène et l'extraction *in-situ* des acides organiques produits (chapitre 7, partie I).

Lors de la production d'hydrogène par fermentation sombre anaérobie, l'accumulation de sous-produits tels les acides organiques favorise l'acidification du milieu de culture diminuant ainsi les performances du procédé. Pour éliminer ce problème, un nouveau réacteur en deux phases a été expérimenté afin de produire du biohydrogène et extraire en simultanée les acides organiques générés lors de la fermentation. En raison de sa biocompatibilité, l'alcool oléique a été choisi pour l'extraction *in situ* des acides organiques. Un rapport de la phase organique:aqueuse de 1: 50 a été jugée optimale pour le procédé qui permet d'augmenter la production d'hydrogène de 26.6 mmol/L à 29.6 mmol/L-milieu de culture. Pour une fermentation de concentration initiale en GB de 25 g/L, un rendement aussi élevé que 11,7 mmol-H_2/L-milieu de culture a été obtenu pour le système à deux phases. En comparaison, un système de fermentation conventionel à une phase génère dans ces conditions 1,48 mmol-H_2/L-milieu de culture. Avec une concentration de substrat initiale élevée de 25 g/L de GB, la concentration d'acide organique dans le milieu est importante et, par conséquent, l'approche du réacteur en 2 phases est plus appropriée.

3.6.2. Mise au point d'un bioprocédé en semi-continu à faible coût et suivi en continu de la production d'hydrogène à partir du glycérol brut (chapitre 7, partie II)

L'acétone, le butanol et l'éthanol ont été détectés dans le GB utilisé comme matière première pour cette recherche. Les résultats indiquent qu'en raison de la période de stockage du GB, une fermentation naturelle à partir des microorganismes indigènes s'amorce et une production d'acétone, butanol et éthanol se produit. Cette étude a montré que par dilution avec de l'eau distillée, le GB peut être utilisé comme le seul

composant du milieu de culture permettant ainsi de concevoir un procédé peu coûteux pour la production d'hydrogène. Des rendements aussi haut que 5.18 L-H_2/L- milieu de culture ont été observés, ce qui équivaut à 210 mmol-H_2/L-milieu de culture. Cette quantité est nettement supérieure à celle de 2,02 à 2,68 L-H_2/L-milieu de culture connue pour la production d'hydrogène à partir du GB. L'éthanol est le principal sous-produit détecté lors de ces expérimentations. En réduisant la concentration en GB en passant de 120 g/L à 60 g/L, l'utilisation du glycerol peut être améliorée de 65 % à 91 %.

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CHAPTER 2

BIOHYDROGEN PRODUCTION: PRINCIPLES, POSSIBILITY AND CHALLENGES

PART I

MICROBIAL HYDROGEN PRODUCTION BY BIOCONVERSION OF CRUDE GLYCEROL: A REVIEW

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Résumé

L'hydrogène est une source d'énergie propre, sans production de sous-produits nocifs lors de sa combustion. La bioconversion de différents déchets organiques en hydrogène est une technologie durable pour la production de bioénergies et a été étudiée par plusieurs chercheurs. Le glycérol brut généré pendant le procédé de production de biodiesel peut servir comme matière première pour la production d'hydrogène en utilisant des procédés par fermentation microbienne. Cet article présente en revue les recherches réalisées sur le sujet. Une étude actualisée sur la production mondiale de biodiesel et de glycérine brute et de leur potentiel de marché a également été réalisée. De même, différentes contraintes techniques de bioconversion de glycérol brut ont été minutieusement examinées et des stratégies pour améliorer le rendement de production en hydrogène ont également été proposées. Il a été soulevé que l'utilisation de glycérol brut provenant de la production du biodiesel afin de produire de l'hydrogène présente de nombreux avantages par rapport à l'utilisation d'autres déchets organiques. De façon notable, la bioconversion du glycérol brut en hydrogène a des avantages économiques directs pour les industries de fabrication de biodiesel. Cependant, les différentes impuretés présentes dans le glycerol brut sont connues pour inhiber la croissance microbienne. Par conséquent, le pré-traitement approprié du glycérol brut est recommandé pour un rendement maximal de production d'hydrogène. De même, en utilisant le système de bioréacteur approprié et en adoptant un mode de fonctionnement continu, une recherche plus approfondie sur la production d'hydrogène, en utilisant du glycérol brut comme substrat, doit être entreprise. En outre, l'isolement de souches plus productives ainsi que le développement de micro-organismes plus productif est recommandé. Des stratégies pour l'utilisation de co-culture de micro-organismes appropriés comme inoculum pour la bioconversion du glycérol brut et l'amélioration de la production d'hydrogène ont également été proposées.

Mots clés: biodiesel; bioconversion; glycérol brut; hydrogène; prétraitement

Abstract

Hydrogen is a clean source of energy with no harmful byproducts produced during its combustion. Bioconversion of different organic waste materials to hydrogen is a sustainable technology for hydrogen production and it has been investigated by several researchers. Crude glycerol generated during biodiesel manufacturing process can also be used as a feedstock for hydrogen production using microbial processes. The possibility of using crude glycerol as a feedstock for biohydrogen production has been reviewed in this article. A review of recent global biodiesel and crude glycerol production and their future market potential has also been carried out. Similarly, different technical constraints of crude glycerol bioconversion have been elaborately discussed and some strategies for improved hydrogen yield have also been proposed. It has been underlined that use of crude glycerol from biodiesel processing plants for hydrogen production has many advantages over the use of other organic wastes as substrate. Most importantly, it will give direct economic benefit to biodiesel manufacturing industries, which in turn will help in increasing biofuel production and it will partially replace harmful fossil fuels with biofuels. However, different impurities present in crude glycerol are known to inhibit microbial growth. Hence, suitable pre-treatment of crude glycerol is recommended for maximum hydrogen yield. Similarly, by using suitable bioreactor system and adopting continuous mode of operation, further investigation of hydrogen production using crude glycerol as a substrate should be undertaken. Furthermore, isolation of more productive strains as well as development of engineered microorganism with enhanced hydrogen production potential is recommended. Strategies for application of co-culture of suitable microorganisms as inoculum for crude glycerol bioconversion and improved hydrogen production have also been proposed.

Key words: Biodiesel; Bioconversion; Crude glycerol; Hydrogen; Pretreatment

Introduction

Among the natural energy sources, fossil fuels are the major energy sources, which cover almost 80 % of global energy demand (Ni *et al.*, 2006). However, the storage of fossil fuels is being rapidly exhausted and additionally combustion of fossil fuels has encumbered negative impacts on global climate (Aksoy, 2011, Gnana kumar *et al.*, 2010). Such a situation has triggered a rapid search of alternative energy sources all over the globe. Bio-fuels being a renewable energy source can be a suitable substitute of fossil fuels. Biodiesel is one of the mostly investigated bio-fuel, which can be produced by transesterification of natural fatty acids (Muniyappa *et al.*, 1996). Presently, in developed nations, numerous plants have been established for commercial biodiesel production from cheap organic materials. According to Selembo et al. (2009) till 2008-09, in US alone, there were nearly 176 biodiesel plants producing 9.8 billion liters of biodiesel per year (Selembo *et al.*, 2009a). During biodiesel production process, a large volume of glycerol is produced as a byproduct. One volume of glycerol is generated for every 10 volumes of biodiesel produced (Selembo *et al.*, 2009a, Yazdani *et al.*, 2007). The concentration of glycerol in biodiesel manufacturing waste may vary from plant to plant, which may be 1% to 85% (v/v) or higher (Marques *et al.*, 2009, Mu *et al.*, 2006, Ngo *et al.*, 2011). The crude glycerol produced during biodiesel production generally contains impurities, such as methanol, soap, salts, oils and solid organic materials (Chi *et al.*, 2007, Ngo *et al.*, 2011, Selembo *et al.*, 2009b). Glycerol is an important raw material for food, pharmaceutical and cosmetic manufacturing process (Guerrero-Pérez *et al.*, 2009). However, due to presence of large amount of impurities; crude glycerol cannot be directly used to food or pharmaceutical industries and its purification is also expensive (Escapa *et al.*, 2009). Hence, crude glycerol generated during biodiesel manufacturing process is of less commercial value (Escapa *et al.*, 2009). Moreover, if directly released to the environment without proper treatment, it may be hazardous to the environment (da Silva *et al.*, 2009, Selembo *et al.*, 2009a). Hence, it cannot be disposed to the environment as a waste byproduct of biodiesel manufacturing process (da Silva *et al.*, 2009). Alternatively, the glycerol containing waste effluent of biodiesel manufacturing plant can be used as a cheap substrate for production of different valuable products, such as 1, 3-propanediol, ethanol, and animal feed, among others

(da Silva *et al.*, 2009, Zhengxiang Wang *et al.*, 2001). Production of hydrogen may be another alternative means for utilization of crude glycerol. Bio-hydrogen production using crude glycerol form biodiesel manufacturing industry has already been investigated by various researchers (Ito *et al.*, 2005, Ngo *et al.*, 2011, Selembo *et al.*, 2009a). It is established that bio-hydrogen has very high energy content and it can be used as a bio-fuel (Ngo *et al.*, 2011). Heat of combustion for hydrogen is -285.8 KJ/ mol and it has an energy content of 142.9 KJ/g (http://www.creative-chemistry.org.uk/gcse/documents/Module21/N-m21-10.pdf (accessed on 01/06/2014). Moreover, if hydrogen is used as a fuel, only water is generated as a byproduct (Selembo *et al.*, 2009a). Hence, considering the increasing concern over global warming, hydrogen may be considered as the cleanest fuel for future. Therefore, bioconversion of crude glycerol to hydrogen is an interesting upcoming green field.

In this pursuit, this review presents a systematic and comparative study of presently available reports on bio-hydrogen production from crude glycerol and tries to find out the future research that needs to be carried out. Firstly, characteristics of crude glycerol generated from biodiesel manufacturing processes are reviewed followed by hydrogen production potential of crude glycerol, various pretreatment methods, bioreactor systems used for microbial hydrogen production as well as the glycerol bioconversion potential of different microorganisms. Short comings of crude glycerol bioconversion are discussed elaborately and various strategies for improved hydrogen production have been suggested.

Glycerol: by-product of biodiesel production

Transesterification of vegetable oils and animal fats*:* Glycerol is a byproduct of biodiesel production process. For biodiesel production, conventionally, triglycerides, such as vegetable oils and animal fats are mixed with methanol in a reactor. Sodium hydroxide crystals are added as catalyst of the reaction and the mixture is agitated and heated to the boiling temperature of methanol (Hak-Joo Kim *et al.*, 2004, Muniyappa *et al.*, 1996, Schuchardt *et al.*, 1998). When agitation stops, the reaction mixture gets separated into two phases. The upper layer is methyl ester of fatty acids, which is biodiesel and the lower layer is crude glycerol containing un-reacted methanol, salt and

solid substances present in the raw materials. Figure 2.1.1 represents a schematic overview of transesterification and biodiesel production process. For every 10 volume of biodiesel production, one volume of glycerol generated as a byproduct (Selembo *et al.*, 2009a, Yazdani *et al.*, 2007). Figure 2.1.2 shows the chemical equation for glycerol production by transesterification of triglycerides (Muniyappa *et al.*, 1996). The crude glycerol phase generally contains almost 75% glycerol (Muniyappa *et al.*, 1996). However, glycerol content in biodiesel manufacturing waste may differ for different manufacturing plants. In an investigation, Ngo et al (2010) have used biodiesel waste containing 1% (w/v) glycerol for hydrogen production (Ngo *et al.*, 2011). In another investigation, Selembo et al (2009) have reported the presence of 69.5% (w/v) glycerol in the waste generated by a biodiesel manufacturing plant (Selembo *et al.*, 2009a). Similarly, Ito et al (2005) have reported the presence of 41 % (w/v) glycerol in biodiesel waste collected from biodiesel manufacturing factory, Hiroshima prefecture, Japan (Ito *et al.*, 2005).

Global biodiesel market and crude glycerol production: Germany, US, France, Argentina, and Brazil are the top five biodiesel producing nations in the world. Together, nearly 68.4% of the world's total biodiesel is produced by these countries (http://biodiesel-news.com/index.php/2010/03/22/global-biodiesel-market-analysis-and-forecasts-to-2020/). According to Global Biodiesel Market (2009 – 2014), a recent market research report, published by "Markets and Markets"; the total global biodiesel market is expected to be worth US$12.6 billion by 2014, out of which the European and American market will share nearly 55.6 % and 28.6 % of the total revenues, respectively. It is expected that the biodiesel market will grow from $8.6 billion in 2009 to $12.6 billion in 2014 (http://www.marketsandmarkets.com/PressReleases/global-biodiesel-market.asp). From Figure 2.1.3, it can be seen that Europe is the leading biodiesel market in the world with a production share of 49.8% which is followed by the Americas with a production share of 32.8% and Asia Pacific with a share of 4.4% in 2009 (http://biodiesel-news.com/index.php/2010/03/22/global-biodiesel-market-analysis-and-forecasts-to-2020/). According to European Biodiesel Board, presently there are approximately 120 plants in the EU producing up to 6,100,000 tons of biodiesel annually. These plants are mainly located in Germany, Italy, Austria, France and

Sweden (http://www.ebb-eu.org/biodiesel.php). North America is the other front runner in global biodiesel production. According to a recent survey, in 2010 total biodiesel consumption in North America was 327 million barrels and during the period of 2006-2010, biodiesel consumption was increased by a compound annual growth rate (CAGR) of 23.9% (http://www.businesswire.com/news/home/20110713006409/en/Research-Markets-Guide-Biofuel-Consumption-North-America). Similarly, in the same report it has been estimated that by the end of 2015, total biodiesel consumption revenue of North America will reach to $ 116.8 billion with a highly significant CAGR (http://www.businesswire.com/news/home/20110713006409/en/Research-Markets-Guide-Biofuel-Consumption-North-America). The United States of America is the second largest producer of biodiesel in the world which produced 17.7% of global biodiesel production in 2009 (http://biodiesel-news.com/index.php/2010/03/22/global-biodiesel-market-analysis-and-forecasts-to-2020/). There were nearly 170 biodiesel plants in the US by 2008 and soy was mainly used as raw material for biodiesel production (http://news.cnet.com/8301-11128_3-9930000-54.html). The US market for biodiesel is expected to reach 6,453 million liters in 2020 (http://biodiesel-news.com/index.php/2010/03/22/global-biodiesel-market-analysis-and-forecasts-to-2020/). For every ten volume of biodiesel produced nearly one volume of crude glycerol is generated, hence, a very rapid increase of global crude glycerol production in recent future can be estimated.

The impurities present in crude glycerol: Due to its high energy content, different researchers all over the globe have investigated bioconversion of crude glycerol to valuable products (Ito *et al.*, 2005, Mu *et al.*, 2006, Ngo *et al.*, 2011, Sakai *et al.*, 2007, Selembo *et al.*, 2009a, Selembo *et al.*, 2009b). They have characterized crude glycerol from biodiesel manufacturing plants to obtain a broader idea of the impurities present in it. Chi et al. (2007) reported presence of 16% of impurities in a sample of crude glycerol collected from a biodiesel refinery. According to them, the impurities mainly present in the sample were soap, free fatty acids, methanol, unreacted triglycerides, diglycerides, and monoglycerides (Chi *et al.*, 2007). For a crude glycerol sample collected from a biodiesel manufacturing plant in Japan; Ito et al. (2005) have reported the presence of 540 g/L total organic carbon (TOC) with ash (8%, w/v), methanol (25%, w/w), 0.04%

(w/w) diacylglycerol and 0.01% (w/w) monoacylglycerol (Ito *et al.*, 2005). In another report by Tang et al. (2009), presence of sodium sulfate 25.8 ± 2.7 % (w/w), methanol 1.5 ± 0.2 % (w/w), water and other compound 5.2 ± 1.0 % (w/w), were confirmed for a crude glycerol sample (Tang *et al.*, 2009). A crude glycerol sample from biodiesel manufacturing process may also contain small amount of the oil used for transesterification (Chi *et al.*, 2007) and any other impurities present in such oil.

Environmental problems of glycerol containing biodiesel waste: As mentioned above, crude glycerol from biodiesel manufacturing plant may contain up to 25% (w/w) methanol (Ito *et al.*, 2005) and from different studies it has been concluded that biodiesel waste containing methanol is inhibitory to microbial growth (Chi *et al.*, 2007, Tang *et al.*, 2009) and may be toxic to the environment, if released without proper treatment. Excess methanol present in crude glycerol may contaminate the ground water. Similarly, due to addition of NaOH in biodiesel manufacturing process, pH of the generated waste is generally higher and it should be neutralized before waste disposal. The waste water having high pH, if released without treatment may be harmful to the biotic community of surrounding environment. Moreover, the waste water with high salinity and alkalinity may take longer time to be recycled by indigenous microorganisms of recipient environment which may result in continuous release of offensive odor and air pollution for longer time.

Different applications of crude glycerol: As mentioned earlier, purification of crude glycerol is costly (Escapa *et al.*, 2009) and hence its applications in food, pharmaceutical and personal care industries are not economically significant. Alternatively, it can be used as a substrate for bioconversion to valuable products. Different researchers have investigated bioconversion of crude glycerol to numbers of valuable products and it is summarized in Table 2.1.1. Barbirato et al. (1998) have reported bioconversion of crude glycerol obtained from colza oil transesterification process, for production of 1, 3-propanediol. A maximum 0.69 mol 1, 3-propanediol per mol glycerol was produced. The maximal diol production rate was 1.85 g l^{-1} h^{-1}, and butyrate (7.5 g l^{-1}) and acetate (8.1 g l^{-1}) were the only by-products (Barbirato *et al.*, 1998). Mu et al. (2006) have also reported bioconversion of crude glycerol (85% w/v),

collected after lipase-catalyzed transesterification of soybean oil (Mu *et al.*, 2006). Tang et al. (2009) have reported production of phytase by *Pichia pastoris*, where crude glycerol containing methanol from biodiesel processing plant was the sole carbon source (Tang *et al.*, 2009). Chi et al. (2007) have investigated the production of docosahexaenoic acid (DHA) by microalgae *Schizochytrium limacinum* (Chi *et al.*, 2007). Liu et al. (2011) have described glycolipid production by *Ustilago maydis* using crude glycerol as a feedstock (Yanbin Liu *et al.*, 2011). Similarly, Nitayavardhana and Khanal (2011) have showed that edible fungus *Rhizopus microsporus* could be grown using crude glycerol as a carbon source. The resulting biomass contains very high amount of threonine and can be used as an animal feed supplement (Nitayavardhana *et al.*, 2011). The above discussion clearly shows that crude glycerol has been successfully used for different bioconversion processes. However, there is a need to find out yet another economically attractive and environmentally sound bioconversion technology for crude glycerol. Production of hydrogen by bioconversion of crude glycerol may be a suitable option, since, bio-hydrogen has high energy content and it is a pollution free source of energy, having the potential to be an alternative of increasingly depleting fossil fuels.

Glycerol: a substrate for bio-hydrogen production

Different substrates presently used for bio-hydrogen: Burning fossil fuel as an energy source results in global warming (Shaffer, 2009), which is one of the most serious environmental problems for the world. The burning also causes the acid rain problem by its combustion gas (Tanisho *et al.*, 1995). In such a situation an alternative clean energy source is urgently needed. One great advantage of the fermentative hydrogen production is the broad spectrum of applicable substrates, allowing the possibility of coupling the energetic utilization of biomass to hydrogen with the simultaneous treatment of waste materials. Hydrogen produced via bioconversion of these organic waste materials may be a suitable alternative as combustion of hydrogen generates only water as byproduct (Selembo *et al.*, 2009a), drastically reducing CO_2, NO_x, particulate and other emissions that accompany the use of fossil fuels. Different plant derived organic materials have been evaluated as feedstock by various

researchers for their biohydrogen production potential. Perego et al. (1998) have evaluated bioconversion of corn starch hydrolysate by *Enterobacter aerogenes* for its hydrogen production potential (Perego *et al.*, 1998). Mizuno et al. (2000) have investigated bean curd manufacturing waste as a substrate for hydrogen production by anaerobic micro flora (Mizuno *et al.*, 2000). Shin et al. (2003) have reported production of hydrogen by the mesophilic and thermophilic acidogenic culture using food waste as a substrate (Shin *et al.*, 2004). Yu et al. (2002) have studied production of hydrogen using rice winery wastewater as a feedstock. They have reported a maximum yield of 1.9 mol hydrogen per mol substrate used, in an upflow anaerobic reactor by using mixed anaerobic cultures (Hanqing Yu *et al.*, 2002). Similarly, Tanisho and Ishiwata (1995) have evaluated molasses for its hydrogen production potential (Tanisho *et al.*, 1995). Also, numbers of other feedstock were evaluated by different workers for their hydrogen production potential and they are summarized in Table 2.1.2. From the above discussion it is clear that a range of cheap and easily available substrates have been evaluated by various researchers, albeit the yields have been pretty low. At this behest, there is a need to find an alternative which can give overall yield and lesser mess.

Glycerol as a feedstock for hydrogen production: Glycerol is another feedstock which can be utilized by many microbes for their growth and has been extensively studied for its hydrogen production potential. Energy content of pure glycerol is 19.0 MJ/ kg; however, it is 25.30 MJ/kg for crude glycerol which may be due to presence of methanol and traces of biodiesel (http://www.esru.strath.ac.uk/EandE/Web_sites/06-07/Biodiesel/experiment.htm). Such high energy content of crude glycerol indicates its high potential to be an effective substrate for hydrogen production. Additionally, unlike most cellulosic waste materials it does not require additional pretreatment to make it available for the hydrogen producing microorganisms. Moreover, substrates such as whey and molasses have high demand due to their wide range of application in industrial fermentations. Further, substrates such as food waste generally contains solid materials of different origin and need proper grinding and mixing before subjecting it to fermentation. Hence, for large scale hydrogen production, crude glycerol seems to be the ideal substrate without having afore mentioned constraints.

Kivistö et al. (2010) have demonstrated hydrogen production by *Halanaerobium saccharolyticum* utilizing pure glycerol as a substrate (Kivistö *et al.*, 2010). Escapa et al. (2009) have reported hydrogen production from glycerol by using a microbial fuel cell (MFC). According to them, if heat-treated anaerobic sludge was used as inoculum, a maximum volumetric rate of hydrogen production of 0.6 l l^{-1} d^{-1} could be achieved (Escapa *et al.*, 2009). Similarly, Ito et al. (2005) have investigated hydrogen production from pure glycerol. By using *Enterobacter aerogenes* HU-101 as inoculum, they have reported a maximum hydrogen yield rate of 80 mmol l^{-1} h^{-1} (Ito *et al.*, 2005). Wu et al. (2011) also have studied the potential of glycerol as a feedstock for hydrogen production by *Klebsiella* sp. HE1 (Wu *et al.*, 2011). Similarly, Seifert et al. (2009) have evaluated pure glycerol as a substrate for hydrogen production. For their study, a 60 mL glass reactor with working capacity of 30 ml was operated in batch mode and anaerobic digested sludge was used as inoculum. A maximum 0.41 mol H_2 per mol glycerol was obtained for a medium containing 10 g l^{-1} glycerol (K Seifert *et al.*, 2009). Thus, there have been numbers of reports on high level of hydrogen production using glycerol suggesting its suitability as a feedstock for hydrogen production and they are summarized in Table 2.1.3. However, pure glycerol is expensive and its use as a substrate for commercial production of hydrogen will not be economically significant.

Alternatively, crude glycerol produced during biodiesel production may be a good feedstock for microbial hydrogen production. Crude glycerol from biodiesel manufacturing plant is a waste byproduct of biodiesel refinery and it needs proper treatment prior to its disposal (da Silva *et al.*, 2009, Selembo *et al.*, 2009a). Meanwhile, it is a good carbon source and it has the potential to support microbial growth as sole carbon source. Therefore, it can be used as an alternative feedstock for microbial hydrogen production. Various investigators have studied the hydrogen production potential of crude glycerol and reported very high hydrogen yield. Ngo et al. (2010) have investigated hydrogen production by *Thermotoga neapolitana* DSM 4359 by using crude glycerol as a substrate. In a small scale batch experiment using 120-mL serum bottles, they observed a maximum 0.14 mol-H_2 yield per mol-glycerol used (Ngo *et al.*, 2011). Sakai and Yagishita (2007) have used *Enterobacter aerogenes* NBRC 12010 to produce hydrogen from crude glycerol by using bioelectrochemical cells (Sakai *et al.*,

2007). Fernandes et al. (2010) have reported production of hydrogen by anaerobic sludge using crude glycerol as a substrate (Fernandes *et al.*, 2010). Sabourin-Provost et al., (2009) have reported hydrogen production using photo-fermentation of pure and crude glycerol by *Rhodopseudomonas palustris.* According to them, a maximum of 6 moles of H_2 was obtained per mole of glycerol consumed in the process (Sabourin-Provost *et al.*, 2009). This amount is 75% of theoretical maximum 8 moles H_2 production per mole glycerol (Sabourin-Provost *et al.*, 2009). It indicates that in this case, intermediate products such as acetic acid, ethanol and butyric acid were further metabolized to hydrogen, which is otherwise accumulated during dark fermentation. Chin et al (2003) have also pointed out that intermediate products, such as organic acid can be further metabolized to CO_2 and H_2 by photosynthetic organisms (Chin *et al.*, 2003). Therefore, a two stage fermentation of crude glycerol where dark fermentation will be followed by photo fermentation by suitable photosynthetic organism should be considered for further investigation. Further, during bioconversion of crude glycerol, both H_2 and CO_2 are produced and CO_2 should be removed from the gas mixture to obtain H_2 which can be used as a fuel. In laboratory, NaOH solution is used to remove CO_2 from the biogas mixture and in industrial scale, pressure swing adsorption technology can be used (Junyapoon *et al.*, 2011, Manish *et al.*, 2008).

From the above discussion based on Table 2.1.2 it is clear that bioconversion of organic materials for hydrogen production has been extensively studied and feasible. A range of cheap and waste carbonaceous materials have been investigated as a substrate for biohydrogen production and good hydrogen yield has been reported for them. Many workers have used pure glycerol as a feedstock for biohydrogen production reporting high hydrogen yield potential of glycerol. However, due to higher cost of pure glycerol, crude glycerol from biodiesel manufacturing process would be a preferred feedstock for hydrogen production.

Glycerol metabolism and hydrogen production: Both oxidative and reductive metabolisms of glycerol are known for some species of *Klebsiella, Citrobacter, Clostridium*, and *Enterobacter* (Drożdżyńska et al., 2011). As shown in Figure 2.1.4, in the oxidative pathway, glycerol is first converted to dihydroxyacetone under catalytic

activity of glycerol dehydrogenase. In the next step dihydroxyacetone is phosphorylated by the glycolytic enzyme dihydroxyacetone kinase. Finally the phosphorylated product is metabolized through glycolysis (Daniel *et al.*, 1995, Drożdżyńska *et al.*, 2011, Luers *et al.*, 1997, Macis *et al.*, 1998). In the reducing pathway, glycerol is finally converted to PD via production of the intermediate product 3-hydroxypropionaldehyde. Conversion of glycerol to 3-hydroxypropionaldehyde is catalyzed by B_{12}-dependent glycerol dehydratase and related diol dehydratases, which is then reduced to PD by the $NADH^+H^+$- dependent enzyme 1,3-propanediol dehydrogenase (Ahrens *et al.*, 1998, Drożdżyńska *et al.*, 2011, Nemeth *et al.*, 2003).

From Figure 2.1. 4 it can be seen that during oxidative metabolism of glycerol, pyruvate is formed as an intermediate. According to Drożdżyńska et al (2011) pyruvate formed during glycerol bioconversion may be metabolized to different end-products by different organism (Drożdżyńska *et al.*, 2011). According to da Silva et al (2009) ethanol, butanol, acetone, acetate, butyrate and lactate are some of the possible metabolites of oxidative metabolism of glycerol (da Silva *et al.*, 2009). In most of the glycerol bioconversion pathways, along with different metabolites H_2 is also produced during oxidative metabolism. From the stoichiometric equations presented in Figure 2.1.5 it can be concluded that production of highly reduced end product is accompanied by leaser H_2 yield. Microbial hydrogen production is based on a simple redox reaction: $2H^+ + 2e^- \leftrightarrow H_2$ (Mathews *et al.*, 2009). This reaction is catalyzed by some hydrogen-producing enzymes found in hydrogen producing organisms. Three main such hydrogen producing enzymes which catalyze most biohydrogen generating reactions are nitrogenases, [NiFe]- hydrogenases, and [FeFe]-hydrogenases (Mathews *et al.*, 2009, Vignais *et al.*, 2001). Mathews et al (2009) have illustrated the different metabolic pathways present in *Clostridium acetobutylicum* which produce H_2 during anaerobic fermentation of glucose (Mathews *et al.*, 2009). It can be seen from the Figure 2.1.7 that similar to oxidative metabolism of glycerol, first pyruvate is produced which is then converted to different metabolites and H_2 via different pathways. Pyruvate is broken down to acetyl-CoA via reduction of a ferredoxin (Fd) catalyzed by pyruvate ferredoxin oxidoreductase. Reduced ferredoxin (Fd) is then oxidized by a hydrogenase that reproduces oxidized Fd and hydrogen gas (Mathews *et al.*, 2009, Nath *et al.*, 2004). Some additional amount of

hydrogen can be produced during glycolysis when NADH is oxidized by Fd reduction and NADH-ferredoxin reductase (Mathews *et al.*, 2009, Vardar-Schara *et al.*, 2008). Theoretically a maximum of 4 mole H_2 can be produced per mole glucose when acetate is the fermentation end product. However, only 2 mol H_2 per mol glucose can be generated during butyrate production and for reduced end-products such as alcohols and lactic acid yet lower H_2 generation is known (Levin *et al.*, 2004, Mathews *et al.*, 2009).

Justification of crude glycerol bioconversion to hydrogen: From the above discussion, it is clear that 1, 3-propanediol (PD) and H_2 are the two major products which can be obtained by bioconversion of crude glycerol. However, due to following reasons, H_2 production is found to be more suitable than PD production by crude glycerol bioconversion: (a) Glycerol has high hydrogen content (8 numbers) and energy content of hydrogen is as high as 142.9 KJ/g (http://www.creative-chemistry.org.uk/gcse/documents/Module21/N-m21-10.pdf), which is suitable to use it as a fuel, (b) It will encourage the use of H_2 as an alternative fossil fuel which in turn will reduce greenhouse gas emission, (c) Low yield and low productivity is the major constraint of bioconversion of glycerol to PD (Zeng *et al.*, 2002). In an investigation, Abbad-Andaloussi et al. (1998) found that when glycerol alone was used as substrate, 43% of glycerol was oxidized to organic acids to obtain energy for growth and only 57% was utilized to produce PD (Abbad-Andaloussi *et al.*, 1998, Saxena *et al.*, 2009). However, very high bioconversion efficiency has been reported for hydrogen production using crude glycerol (Sabourin-Provost *et al.*, 2009). (d) Glycerol fermentation to PD is a two-step reaction catalyzed by Vit-B_{12}-dependent glycerol dehydratase (GDHt) and 1, 3-propanediol oxidoreductase (PDOR) enzymes. Therefore, addition of high cost molecule, Vit-B_{12} to the medium is required for the production of PD (Laffend *et al.*, 1997, Saxena *et al.*, 2009). Moreover, when glycerol is used as a substrate, GDHt undergoes irreversible inactivation by glycerol (Daniel *et al.*, 1995, Corinna Seifert *et al.*, 2001). e) At the end of bioconversion of crude glycerol to PD, the fermentation broth generally contains water, residual glycerol, by-products such as acetate, lactate, ethanol; macromolecules such as proteins, polysaccharides; and unused medium components. Moreover, PD is known to be hydrophilic in nature and has a high boiling

point (Saxena *et al.*, 2009). Because of aforesaid reasons purification of PD from a fermentation broth is very complex. According to Xiu et al (2008), commonly used methods for purification of PD are reactive extraction, liquid–liquid extraction, evaporation, distillation, membrane filtration, pervaporation and ion exchange chromatography, however, all these methods are known to have numbers of limitations (Saxena *et al.*, 2009, Xiu *et al.*, 2008). Meanwhile, such constraints are hardly reported for H_2 production as relatively insoluble H_2 spontaneously partitions to the gas phase of a reactor (Sabourin-Provost *et al.*, 2009). Thus, bioconversion of crude glycerol to H_2 can be considered as the most suitable option for utilization of biodiesel industry derived crude glycerol.

Challenges to be addressed: It is clear that akin to other cheap organic substrates, crude glycerol from biodiesel manufacturing process may also be a potential feedstock for industrial production of hydrogen using microbial process. However, to convert it to be a reliable and economically significant option for microbial hydrogen production, the following issues should be addressed successfully:

(a) From Figure2.1. 4 it can be concluded that in many microorganism there exist two biochemical pathways for glycerol metabolism. H_2 gas is generated during bioconversion of glycerol through oxidative pathway; however, PD is produced during reductive metabolism. When both the pathways exist in the same organism, some amount of H_2 is consumed for PD production. According to Selembo et al. (2009), during anaerobic fermentation of glycerol, for production of 1 mol of PD, 1 mol of H_2 is consumed (Selembo *et al.*, 2009a). Therefore, final yield of H_2 is reduced by directly consuming the produced H_2 for simultaneous production of PD and also by making most of the substrate unavailable for H_2 production by reducing it to PD. Continuous removal of produced hydrogen from fermentation broth by pursing N_2 may result in improved hydrogen production by making it unavailable for subsequent PD production. Similarly, genetic engineering strategies for blocking PD production pathway in H_2 producing organism may also be a viable option for improved H_2 production. Another alternative possibility is to further break down of PD produced during glycerol metabolism to H_2 by developing novel strategies. These strategies may involve chemical treatment of PD,

produced during anaerobic fermentation of crude glycerol, to ensure its availability as a substrate for hydrogen production. Otherwise, suitable metabolic engineering strategies may also be devised for the same purpose.

(b) Usually, heat pretreatment is used for hydrogen producing inocula to inhibit hydrogen consuming organisms, such as methanogens without reducing the activity of hydrogen producing organism (Ginkel *et al.*, 2001, Selembo *et al.*, 2009a). Other methods for pretreatment of hydrogen producing inocula are ultra-sonication, acidification, sterilization, and freezing/thawing (Cai *et al.*, 2004, Jan *et al.*, 2007, Selembo *et al.*, 2009a, CC Wang *et al.*, 2003). However, according to Selembo et al. (2009) efficiency and effect of these different pretreatment methods on hydrogen production and glycerol fermentation is not well documented (Selembo *et al.*, 2009a). Therefore, all such methods need to be verified for glycerol fermentation and hydrogen production efficiency for better hydrogen yield.

(c) Accumulation of fermentation end product such as acetic acid, butyric acid resulted in lower yield of hydrogen during dark fermentation (Mathews *et al.*, 2009, Van Ginkel *et al.*, 2005). Undissociated acid concentration of 19 mM in the fermentation broth is enough to initiate solventogenesis and cease of hydrogen production (Mathews *et al.*, 2009, Van Ginkel *et al.*, 2005). Therefore, further conversion of end products of glycerol fermentation to hydrogen is necessary for maximum hydrogen yield. Two stage fermentation of glycerol where dark fermentation is followed by photo fermentation or microbial fuel cell technology to produce hydrogen may be a suitable option. Other alternative is to use membrane bioreactors in continuous processes, allowing biomass to be retained and fluid (containing volatile fatty acids) to be washed out (Oh *et al.*, 2004). Alternatively, co-culture of hydrogen producing organism and microbes that can produce hydrogen from glycerol fermentation end products may be another option.

(d) It can be seen from the Figure 2.1. 5 that high hydrogen yield is possible when acetic acid is produced as fermentation end product instead of butyric acid and other solvents. Morimoto et al (2005) have reported that in *C. paraputrificum* M-21 improved hydrogen yield has been achieved by over-expression of hydA gene which encodes [FeFe]- hydrogenase (Mathews *et al.*, 2009, Morimoto *et al.*, 2005). Almost 1.7-fold increase in hydrogen production over wild strain was accompanied by increased acetic

acid production due to over-oxidation of NADH (Mathews *et al.*, 2009, Morimoto *et al.*, 2005). Other similar strategies should be developed for increased acetic acid production during glycerol fermentation for improved hydrogen yield.

(e) From different reports, it can be found that methanol and soap, the major impurities present in crude glycerol are inhibitory towards microbial growth (Chi *et al.*, 2007, Ngo *et al.*, 2011). Therefore for improved hydrogen production by bioconversion of crude glycerol suitable pretreatment technology for biodiesel industry derived glycerol should be developed. Such technology should be suitable for scaling up to industrial scale and economically sound.

(f) Round the year availability of crude glycerol should be ensured for large scale production and use of hydrogen as a fuel. Government agencies should take the initiative for popularization of use of hydrogen energy as well as to develop proper market for biohydrogen to ensure economic viability of large scale bioconversion of crude glycerol for hydrogen production.

Pretreatment of crude glycerol

Importance of crude glycerol pretreatment: As it is mentioned earlier, the transesterification process for biodiesel production involves mixing of vegetable oil and animal fats with methanol and addition of sodium hydroxide as catalyst (Hak-Joo Kim *et al.*, 2004, Muniyappa *et al.*, 1996, Schuchardt *et al.*, 1998). Therefore, apart from glycerol, a typical biodiesel waste may contain high concentrations of salts (e.g., sodium chloride), methanol, and precipitated solids (Vicente *et al.*, 2004, Yazdani *et al.*, 2007), which may affect the microbial growth, when added as a substrate for bioconversion (Chi *et al.*, 2007, Ngo *et al.*, 2011). Ngo et al., (2010) have reported that when crude glycerol was directly mixed with culture medium for bioconversion purpose, soaps precipitated from the liquid media, which was found detrimental to cell growth (Chi *et al.*, 2007, Ngo *et al.*, 2011). Athalye et al. (2009) have reported the negative effect of crude glycerol containing methanol and soap on production of eicosapentaenoic acid by *Pythium irregular* (Athalye *et al.*, 2009). Similarly, Pyle et al. (2008) have also reported the inhibitory effect of crude glycerol containing methanol and soap on production of docosahexaenoic acid by *Schizochytrium limacinum* (Pyle *et al.*, 2008). In order to avoid

such incidences, crude glycerol should be pre-treated before using as a feedstock for hydrogen production. To investigate the effect of pretreatment of crude glycerol on hydrogen production, Ngo et al. (2010) had conducted a study where pre-treated glycerol and untreated glycerol waste were used as the substrate for *T. neapolitana*. According to the authors, the level of H_2 produced from pre-treated glycerol waste, from where alcoholic compounds are removed by rotary evaporation and solid substances were removed by centrifugation, was much higher than that from glycerol waste without treatment (Ngo *et al.*, 2011).

Different methods of crude glycerol pretreatment: As discussed earlier, soap and methanol are the major impurities present in crude glycerol and they are inhibitory to microbial growth (Athalye *et al.*, 2009, Chi *et al.*, 2007). To use the crude glycerol as a feedstock for bioconversion, methanol can be removed by autoclaving (Athalye *et al.*, 2009, Ethier *et al.*, 2011). According to Denver et al (2008) methanol evaporates at 65 °C, hence the autoclave process lasting for 15 min at 121 °C is capable of removing significant amount of methanol from crude glycerol (Pyle *et al.*, 2008). Alternatively, since boiling point of methanol is only 65°C which is far lower than that of water and glycerol; methanol removal by controlled distillation of crude glycerol can also be considered. A very simple distillation set up will be required for such a process and it will be easy to scale up for industrial application. In a recent investigation on hydrogen production from crude glycerol, Ngo et al. (2010) have reported the removal of methanol and ethanol by rotary evaporation at 45^{o}C, where solids were precipitated by centrifugation at 15,000 rpm for 15 min (Ngo *et al.*, 2011).

Soap is the other major impurity present in crude glycerol, which can be removed by precipitation from the liquid medium through pH adjustment (Athalye *et al.*, 2009, Ethier *et al.*, 2011). Chi et al. (2007) have used the following procedure to remove soap from crude glycerol: (i) crude glycerol was first mixed with distilled water at a ratio of 1:4 (v/v) to reduce the viscosity; (ii) pH of the solution was adjusted to 6.5 with hydrochloric acid to convert the soluble soap into insoluble free fatty acids; (iv) finally precipitated fatty acid was removed from the crude glycerol solution by centrifugation at 5000 rpm (Chi *et al.*, 2007). The main advantage of this method is that the final pH of resulting solution

(6.5) is suitable for H_2 production by wide range of microorganisms. Similarly, Ethier et al. (2011) have adopted the following procedure: (i) crude glycerol was mixed with distilled water at a ratio of 1:4 (v/v) to reduce the viscosity of the solution, (ii) soap was precipitated by converting it to insoluble free fatty acid by adjusting the pH of the solution to 3 by using sulfuric acid, (iii) then the fatty acid-precipitated solution was kept static for 30 min to allow free fatty acid and glycerol to separate into two phases, (iv) finally, the upper free fatty acid phase was removed from the crude glycerol phase through a separation funnel (Ethier *et al.*, 2011). Sodium chloride is another major impurity present in crude glycerol from biodiesel production process (Vicente *et al.*, 2004, Yazdani *et al.*, 2007). Chiu et al. (2006) have evaluated a method for removal of sodium from glycerol solutions by crystallization/precipitation of hydroxyapatite (HAP) through the co-addition of lime [Ca (OH) $_2$] and phosphoric acid (Chiu *et al.*, 2006). However, presence of sodium chloride in crude glycerol also offers a unique opportunity to use salt resistant H_2 producing microorganisms for crude glycerol bioconversion without having much competition for substrate utilization. Therefore, before recommending any method for sodium chloride removal, the possible advantage offered by its presence should also be considered.

Thus, various methods have been evaluated for removal of major impurities from crude glycerol. However, no attempt has been made to scale up any of above mentioned process for impurity removal from crude glycerol. Therefore, information on suitability of such method for application in large scale bioconversion of crude glycerol is lacking. Hence, detailed study on optimization of such pretreatment methods, investigation of effect of such pretreatments on bioconversion of crude glycerol and their economic feasibility for industrial application should be evaluated. Moreover, the possibility of a collective removal method for all the major impurities present in crude glycerol should also be explored.

Bioreactor systems for hydrogen production

Different types of reactors evaluated for bio-hydrogen production: To evaluate hydrogen production using different organic waste materials including crude glycerol from biodiesel manufacturing plant, a range of reactor types have been used. A

bioreactor used for this purpose may be a small serum vial of 10 mL working volume or a 2.5-L fermentor. Kivistö et al. (2010) have reported the use of airtight glass tube of 10 mL working volume for hydrogen production by using *Halanaerobium saccharolyticum*. It has been reported that by using pure glycerol as a substrate, nearly 1.61 mol-H_2 could be generated from each mol of glycerol, where the study was carried out in batch mode (Kivistö *et al.*, 2010). Fernandes et al. (2010) have studied hydrogen production from crude glycerol by using a 2-L glass flasks consisting of 1-L of liquid volume and 1-L of headspace. The flask was inoculated with the inoculum derived from a packed-bed reactor used to produce hydrogen from a sucrose-based synthetic substrate and anaerobic environment was maintained using N_2 (Fernandes *et al.*, 2010). Ito et al. (2004) have described a packed-bed reactor of 60 mL working volume which was operated in continuous mode. Crude glycerol containing fresh medium was supplied from the bottom, and the resulting effluent was discharged from the top of the reactor, with the help of a peristaltic pump (Ito *et al.*, 2005). In another study, Yu et al. (2002) reported hydrogen production from rice winery wastewater using a 3.0-L up-flow reactor. The Plexiglas made reactor was of 90 mm diameter (internal) and a height of 480 mm. Using mixed anaerobic culture, under continuous operation of the reactor, a maximum 2.14 mol-H_2 per mol hexose was reported by the authors (Hanqing Yu *et al.*, 2002). Use of bio-electrochemical cell is another widely investigated method for hydrogen production. Different workers have evaluated the use of pure or crude glycerol as a substrate for hydrogen production using bio-electrochemical cells (Escapa *et al.*, 2009, Sakai *et al.*, 2007, Selembo *et al.*, 2009b). Sakai and Yagishita (2007) have described a bio-electrochemical two-compartment reactor with a cation-exchange membrane separating the compartments and crude glycerol was used as a feedstock for hydrogen production by *Enterobacter aerogenes* NBRC 12010 (Sakai *et al.*, 2007). Figure 2.1.6 represents a microbial electrolysis cell used for H_2 production by bioconversion of glycerol. The basic principle of the system is that during bioconversion of glycerol, electron is donated to the anode, and with the help of an external power supply, free protons are reduced on the cathode to produce H_2 (Escapa *et al.*, 2009, Hongqiang Hu, 2009, Hong Liu *et al.*, 2005, Logan *et al.*, 2008).

From the above discussion (Table 2.1.4), it is clear that a range of bioreactor setup has been evaluated for hydrogen production from cheap organic materials including crude glycerol from biodiesel industry. They may be simple serum bottles of different size (Akutsu *et al.*, 2009, Kivistö *et al.*, 2010, Marques *et al.*, 2009, Sabourin-Provost *et al.*, 2009, Selembo *et al.*, 2009a), laboratory scale fermentor with agitation and pH control (Wu *et al.*, 2011, Yokoyama *et al.*, 2007a), pack-bed or up flow reactor constructed from Plexiglas (Ito *et al.*, 2005, Hanqing Yu *et al.*, 2002), or may be bio-electrochemical cells (Escapa *et al.*, 2009, Sakai *et al.*, 2007, Selembo *et al.*, 2009b). However, based on the hydrogen yield potential reported, a particular setup could not be considered superior to the rest. It is also observed that most of the experiments were carried out in batch mode using serum vials (Akutsu *et al.*, 2009, Kivistö *et al.*, 2010, Ngo *et al.*, 2011), as the studies are still in their infancy stage. In a report on hydrogen production from biodiesel processing waste, Ito et al. (2004) have indicated that the biodiesel wastes containing glycerol should be diluted with a synthetic medium to increase the rate of glycerol utilization. They have reported that the production of H_2 decreased with an increase in the concentrations of biodiesel wastes (Ito *et al.*, 2005). Therefore, for bioconversion of large volume of diluted crude glycerol, a continuous system may be more suitable than a batch system, once it assures maximum mixing and homogeneity and controlled concentration of volatile fatty acids (based on organic loading rate and hydraulic retention time), on which relatively less reports are available. Further, unlike mostly reported free cell systems for crude glycerol bioconversion, suitable reactor with immobilized cell system, such as packed bed and membrane reactors may offer dual benefits of higher initial cell density and protection of microorganisms from impurities present in crude glycerol.

Important process parameters and their optimum levels: From Table 2.1.4, it is clear that microbial hydrogen production has been investigated using different reactor types and chosen optimum process parameters have been different for separate cases. Ngo et al. (2010) have investigated hydrogen production potential of *Thermotoga neapolitana* DSM 4359 using biodiesel manufacturing waste as feedstock. They have reported high hydrogen yield by the hyperthermophilic eubacterium, where the experiment was carried out at 75ºC and pH 7.5 (Ngo *et al.*, 2011). Selembo et al. (2009)

have investigated the hydrogen production by anaerobic fermentation of crude glycerol with heat-treated mixed cultures. They have carried out the experiment at 30º C and pH 6.2 (Selembo *et al.*, 2009a). Perego et al. (1998) have studied hydrogen production by *E. aerogense,* using corn starch hydrolysate as a substrate. The reactor was operated at 40±0.5 ºC and pH 5.5 and a high yield of 1.8 mmol H_2 per mmol glucose was reported by the authors (Perego *et al.*, 1998). From the above discussion, it is clear that the temperature range used by various researchers for hydrogen production varied from 25 º C to 75 º C (Escapa *et al.*, 2009, Ngo *et al.*, 2011), and similarly pH 4.5 to 7.5 was reported (Fang *et al.*, 2006, Ngo *et al.*, 2011). The process parameters may vary according to the mode of operation or the biological agent (microorganism) used in the process. Therefore, a particular temperature or pH cannot be considered optimum for hydrogen production process as a whole. However, when the process is carried out using a particular strain, there will be an optimum level for such parameters. Environmental conditions and media composition when working with industrial complex wastewaters plays an important role in process development because it influences drastically hydrogen production. Many studies reported the influence of Zn, Cu, nitrogen, phosphorous, magnesium and iron in dark fermentation processes (Chong *et al.*, 2009, Oztekin *et al.*, 2008, Winter, 2005, Han Qing Yu *et al.*, 2001). Hawkes et al. (2007) reviewed the media composition for hydrogen production and found that apart from N and P source, only K, Mg and Fe are common in all recipes analyzed so far (Hawkes *et al.*, 2007). More details of microelements consumption and dark fermentation evolution might be evaluated but it is clear that by knowing media composition supplementation of micronutrients and/or dilution with water can be predicted (saving time and money).

Microorganisms used for glycerol bioconversion

Microorganisms presently used for glycerol bioconversion: As glycerol has been used for production of different products; various microorganisms have been evaluated for their glycerol bioconversion ability. Ngo et al. (2010) reported glycerol bioconversion and hydrogen production using *Thermotoga neapolitana* DSM 4359, a hyperthermophilic eubacterium. They have reported nearly 2.73 mol-H_2 production for

each mol glycerol used (Ngo *et al.*, 2011). Selembo et al. (2009) have investigated bioconversion of glycerol by heat treated mixed microbial culture. They have reported a maximum production of 0.31 mol H_2 per mol-glycerol consumed as well as CO_2 and PD as byproduct (Selembo *et al.*, 2009a). Barbirato et al. (1998) have used *Klebsiella pneumoniae ATCC 25955* to produce PD by bioconversion of glycerol (Barbirato *et al.*, 1998). Similarly, Tang et al., (2009) used *Pichia pastoris* to produce Phytase by using crude glycerol as a substrate (Tang *et al.*, 2009). Nitayavardhanaa et al (2011) have recently reported the possibility of production of animal feed supplement from crude glycerol by using edible fungus, *Rhizopus microspores* (Nitayavardhana *et al.*, 2011).

At high temperature, hydrogen production is more exergonic, extreme- and hyper-thermophiles show resistance to high hydrogen partial pressures (van Niel *et al.*, 2003), which otherwise would cause a metabolic shift to production of more reduced products. One advantage of fermentation at extreme temperatures is that the process is less sensitive to contaminations but on the other hand it is complicated to achieve a positive economic relation between the energy used to heat and maintain the reactor at high temperatures and the H_2 production. Moreover, extreme thermophilic anaerobic bacteria usually grow low densities resulting in low production rates. Researchers have investigated the bioconversion of glycerol using monoculture or mixed microbial consortia. As it is shown in Table 2.1.5, *Enterobacter aerogenes* is the most studied organism for hydrogen production by using crude glycerol. Mixed microbial culture from environmental sources is the other mostly used inoculums for glycerol bioconversion; however, some constraints of process stability do exist and might be considered especially when industrial wastewaters with continuous composition variation are used. Phowan et al. (2010) have demonstrated that co-culture of *C. butyricum* TISTR 1032 and *E. aerogenes* TISTR 1468 was capable of reducing the lag phase of hydrogen production from cassava pulp hydrolysate with the maximum cumulative hydrogen production of 458 ml. They have reported that production of H_2 was approximately 14.50% higher than that of using only *C. butyricum* TISTR 1032 alone (Phowan *et al.*, 2010). Similarly, Wang et al. (2008) have demonstrated a co-culture of *Clostridium acetobutylicum* X9 and *Ethanoligenens harbinense* B49 capable of producing 1810 ml H_2/L-medium using microcrystalline cellulose as substrate (Aijie Wang *et al.*, 2008).

However, the use of co culture of two suitable strains for bioconversion of crude glycerol to hydrogen has not been explored completely.

Use of co-culture for glycerol bioconversion to hydrogen may play a significant role in improving hydrogen yield. It is observed that during glycerol metabolism along with hydrogen numbers of fermentation end products such as acetic acid, butyric acid, ethanol and butanol may be produced (Figure 2.1. 7). However, accumulation of such products in the fermentation broth is known to be inhibitory towards hydrogen production (Mathews *et al.*, 2009). Li et al (2007) have shown that intermediate products of glycerol fermentation such as butyric acid and ethanol can be further converted to hydrogen by acetogenic bacteria (Li *et al.*, 2007). Therefore, co-culture of acetogenic bacteria along with hydrogen producing organism for glycerol bioconversion may reduce the accumulation of metabolites and improve hydrogen yield. However, acetogenic strain to be used for glycerol bioconversion as co-culture should be verified for preferential metabolism of fermentation end products in presence of glycerol as major substrate.

Methanol and soap are the two major impurities present in crude glycerol and they are known to have inhibitory effect on microbial growth (Chi *et al.*, 2007, Ngo *et al.*, 2011). Bacteria, such as *Sporomusa acidovorans* is known to have methanol degradation potential (Cord-Ruwisch *et al.*, 1986). Similarly, some species of Klebsiella, Escherichia, Enterobacter are known to have soap degradation potential (Amund *et al.*, 1997). Therefore, a co-culture of hydrogen producing bacteria and methanol or soap degrading bacteria may be helpful for improve bioconversion of crude glycerol to hydrogen. However, soap or methanol degradation efficiency of proposed co-culture should be verified in presence of crude glycerol as alternative substrate, before using it for glycerol bioconversion.

Similarly, PD is another product which may be produced during glycerol bioconversion to hydrogen. Therefore, a co-culture of suitable microorganism which can simultaneously utilize PD or intermediate product of PD pathway and can produce hydrogen via glycerol fermentation may be a suitable option of crude glycerol bioconversion.

Genetic and metabolic engineering for improved hydrogen production: Genetic and metabolic engineering may be seen as potential tools for improvement of hydrogen production by bioconversion of crude glycerol. In recent years, various researchers have investigated microbial hydrogen production by using engineered microorganisms and have reported increased hydrogen yield (Chittibabu *et al.*, 2006, Eui-Jin Kim *et al.*, 2008, Maeda *et al.*, 2007, Morimoto *et al.*, 2005). Kim et al. (2008) have reported hydrogen production by recombinant *Rhodobacter sphaeroides* containing the genes for pyruvate metabolism of *Rhodospirillum rubrum*. They have observed that under photoheterotrophic conditions, H_2 production by the recombinant *Rhodobacter sphaeroides* increased up to two fold and produced 4 mol H_2 per mol of glucose compared to 2 mol H_2 per mol of glucose by the strain without the gene from *R. rubrum* (Eui-Jin Kim *et al.*, 2008). Similarly, Maeda et al (2007) have reported a recombinant *E. coli* strain expressing the bidirectional [NiFe]-hydrogenase from Synechocystis sp. PCC 6803. A 41 fold increase in hydrogen production over the wild type has been reported for the recombinant strain. According to the authors, by using an optimized medium, the recombinant *E. coli* cells are also capable of producing 2 folds higher hydrogen than the well-studied *Enterobacter aerogenes* HU-101 (Maeda *et al.*, 2007). Chittibabu et al. (2006) also have developed a genetically modified strain of *E. coli* containing [FeFe]-hydrogenase gene from *Enterobacter cloacae* IIT BT-08. 3.12 mol-H_2 yield per mol glucose has been achieved for the genetically modified strain, which was much higher than its wild type (Chittibabu *et al.*, 2006).

A number of successful genetic engineering attempts have been made for improvement of microbial hydrogen production. However, hydrogen production potential of a recombinant strain by using different organic wastes materials as feedstock is not yet documented. In a recent review, Mathews et al (2009) suggested that in order to increase the economic viability of hydrogen production, various substrate utilization pathways should be engineered into hydrogen-producing organisms (Mathews *et al.*, 2009). Therefore, a genetically engineered microbial strain capable of efficient bioconversion of crude glycerol may improve the economic viability of microbial

hydrogen production. Recently, by using adaptive evolution and chemical mutagenesis followed by growth experiment on glycerol, Hu et al (2009) have developed an improved *Escherichia coli* strain (HW2) that produces 20-fold more hydrogen in glycerol containing medium (0.68 ± 0.16 mmol-H_2 l^{-1} h^{-1}) (Hongbo Hu *et al.*, 2010). Yazdani et al (2007) also have described a recombinant *Escherichia coli* strain capable of bioconversion of glycerol to ethanol and hydrogen (Yazdani *et al.*, 2007).

Conclusion and future perspectives

Hydrogen can be considered as the cleanest alternative of fossil fuels as no harmful byproduct generated during its combustion and it can be produced from renewable energy sources. Glycerol containing waste from biodiesel manufacturing process is a potential feedstock for microbial hydrogen production. Different researchers have evaluated its performance as a cheap substrate for hydrogen production and indicated that its hydrogen production potential is comparable to any other organic waste presently used for hydrogen production. The most important advantage of using crude glycerol over other substrates for hydrogen production is that it will increase the overall profit of biodiesel manufacturing plants. Such a situation may encourage the production and utilization of biofuels, which is environmentally beneficial. Biohydrogen can be burned for heat generation and/or converted to electricity through fuel cells and be used in the biodiesel process, replacing and avoiding dependence of other non green energy sources. However, crude glycerol contains many impurities which are inhibitory to microbial growth and hydrogen production. Scarce literature reports are available on pretreatment of crude glycerol used for hydrogen production. Hence, further investigation is still required to optimize crude glycerol pretreatment for biohydrogen production. A collective removal method for different types of impurities and feasibility study of its industrial scale application may be helpful for crude glycerol bioconversion and large scale hydrogen production in future. Accumulation of fermentation end products, such as acetic acid and butyric acid is known to have negative effect on overall hydrogen yield. Hence, alternative strategy, such as further conversion of fermentation end product into CO_2 and H_2 by photo fermentation should be investigated in detail. It should be noted that maximum production of 6 mole H_2 per mole glycerol

has been achieved by photo fermentation which is 75% of theoretical maximum 8 mole H_2 per mole glycerol.

Similarly, most investigations on crude glycerol bioconversion have been carried out in serum bottle scale batch reactors. Only, a few studies carried out in continuous mode have given better yield of hydrogen than batch experiments. Hence, further investigation of microbial hydrogen production using continuous mode is recommended. The most investigated microorganism for hydrogen production from crude glycerol is *Enterobacter aerogenes.* Other frequently used inoculum is indigenous mixed micro-flora of organic waste materials. It is a common practice that when a microbial consortium is used as inoculum, pretreatment of such inoculum is made to eliminate hydrogen consuming organisms, such as methanogens. Ultrasonic, thermal and acid treatments are some common method for inoculum pretreatment. However, their role on overall hydrogen production is not well documented. Hence, detailed study and optimization of such methods may play a vital role large scale hydrogen production in future. Alternatively, co-culture of two different strains can also be evaluated for crude glycerol bioconversion. Application of a co-culture, which is capable of reducing the accumulation of fermentation end products by simultaneously metabolizing it to H_2, is an interesting subject for future investigation. Further, genetically engineered microbial strains may enhance the hydrogen production potential of crude glycerol.

Abbreviations

PD= 1, 3-propanediol, EU= European Union, GWh= gigawatt hour, TOC= total organic carbon, DHA= docosahexaenoic acid, MFC= microbial fuel cell, COD= chemical oxygen demand, mM= millimolar, HAP= hydroxyapatite, GDHt= glycerol dehydratase, PDOR= 1, 3-propanediol oxidoreductase

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Table 2.1. 1: Different valuable products obtained from crude glycerol generated during bio-diesel production

Source of bio-diesel production waste containing glycerol	**Glycerol content (w/w)**	**Impurities present in crude glycerol**	**Maximum product yield using crude glycerol as feedstock**	**Ref.**
M Energy Co., Pyeongteak city, South Korea.	1%	Methanol and/ethanol; solid substances	2.73±0.14 mol-H_2 mol^{-1} glycerol consumed	(Ngo *et al.*, 2011)
Biodiesel manufacturing factory, Hiroshima prefecture, Japan	41%	Total organic carbon (TOC) 540 g/l, ash (8%, w/v), methanol (25%, w/w), 0.04% (w/w) diacylglycerol and 0.01% (w/w) monoacylglycerol.	63 mmol-H_2.l^{-1}.h^{-1} and 0.85 mol-ethanol.mol^{-1} glycerol consumed.	(Ito *et al.*, 2005)
BDF plant, Ishikawa Prefecture, Japan		__	0.77 mol-H_2 .mol^{-1} glycerol consumed	(Sakai *et al.*, 2007)
Nittany Biodiesel, State College, PA	69.5%	Chemical oxygen demand (COD) of 1,300 ± 100 mg/l.	0.31 mol-H_2 .mol^{-1} glycerol and 0.59 mol-1, 3-propanediol (PD). mol^{-1} glycerol consumed	(Selembo *et al.*, 2009a)
Nittany Biodiesel, State College, PA	69.5%	Methanol, sodium hydroxide, sodium methylate and a chemical oxygen demand (COD) of 1160 ± 100 mg/l	0.41 ± 0.1 m^3 H_2. m^{-3} d^{-1}	(Selembo *et al.*, 2009b)

Source of bio-diesel production waste containing glycerol	**Glycerol content (w/w)**	**Impurities present in crude glycerol**	**Maximum product yield using crude glycerol as feedstock**	**Ref.**
ROBBE, Compie`gne, France	80%	__	0.69 mol-PD. mol^{-1} glycerol consumed and butyrate 7.5 $g\text{-}l^{-1}$ and acetate 8.1 $g\text{-}l^{-1}$ were the by-products	(Barbirato *et al.*, 1998)
Prepared in laboratory by using transesterification of soybean oil	85%	__	1, 3-propanediol 53 $g\text{-}l^{-1}$ crude glycerol.	(Mu *et al.*, 2006)
Integrity Biofuels, Indiana, USA	67.5 ± 3.2 %	Sodium sulfate 25.8 ± 2.7 %, methanol 1.5 ± 0.2 %, water and other 5.2 ± 1.0 %.	Phytase (1125 $U\text{-}ml^{-1}$ supernatant)	(Tang *et al.*, 2009)
Virginia Biodiesel Refinery (West Point, VA, USA) and Seattle Biodiesel (Seattle,WA, USA)	84%	16% of impurities containing soap, free fatty acids, excess methanol, unreacted triglycerides, diglycerides, or monoglycerides	Docosahexaenoic acid (4.91 $g\ l^{-1}$)	(Chi *et al.*, 2007)
Se Quential Pacific Biodiesel, Inc. (Salem, OR, USA)		__	Animal feed (0.83± 0.02 g biomass increase-g^{-1} initial biomass)	(Nitayavardhana *et al.*, 2011)
Biofuel Research Pte. Ltd. (Singapore)		__	Glycolipid (3.91 $g\text{-}l^{-1}\ d^{-1}$)	(Yanbin Liu *et al.*, 2011)

Source of bio-diesel production waste containing glycerol	**Glycerol content (w/w)**	**Impurities present in crude glycerol**	**Maximum product yield using crude glycerol as feedstock**	**Ref.**
Rothsay, Guelph, ON	70–75%	__	6 mol-H_2 mole^{-1} glycerol consumed	(Sabourin-Provost *et al.*, 2009)
Biocapital, Charqueada, SP, Brazil	__	__	0.53 kg-H_2 .100 m^{-3} h^{-1}	(Fernandes *et al.*, 2010)
Biodiesel factory, Portugal	86%	MONG 6.2% (w / w), ash (4.6% w / v) and methanol (0.03% w /w)	710.0 cm^3-H_2dm^{-3} medium	(Marques *et al.*, 2009)

Table 2.1. 2: Maximum hydrogen production by different inocula using different non-glycerol feedstock (Hak-Joo Kim *et al.*, 2004)

Substrate	**Inocula**	**Operation Mode**	**Maximum hydrogen yield**	**Ref.**
Bean curd manufacturing waste	Micro-flora of fermented soybean-meal	Batch	130 ml-H_2 h^{-1} l^{-1} culture	(Mizuno *et al.*, 2000)
Food waste	Anaerobic sludge	Batch	1.8 mol-H_2 mol^{-1} hexose	(Shin *et al.*, 2004)
Food waste	Anaerobic sludge	Batch	101 ml-H_2 g^{-1} COD	(Chen *et al.*, 2006)
Beer lees	Micro-flora of cow dung compost	Batch	68.6 ml- H_2 g^{-1} total volatile solid	(Fan *et al.*, 2006a)
Rice winery wastewater	Municipal sewage sludge	Continuous	1.9 mol- H_2 mol^{-1} hexose	(Hanqing Yu *et al.*, 2002)
Rice slurry	Anaerobic sludge	Batch	346 ml- H_2 g^{-1} carbohydrate	(Fang *et al.*, 2006)
Cornstalk wastes	Micro-flora of cow dung compost	Batch	149.69 ml-H_2 g^{-1} total volatile solid	(Zhang *et al.*, 2007)
Corn starch hydrolisate	*Enterobacter aerogenes*	Batch	1.8 mmol-H_2 $mmol^{-1}$glucose	(Perego *et al.*, 1998)
Corn starch hydrolisate	*E. coli*	Batch	0.36 mmol-H_2 $mmol^{-1}$glucose	(Perego *et al.*, 1998)
Molasses	*Enterobacter aerogenes* E 82005	Continuous	3.5 mol- H_2 mol^{-1} sugar	(Tanisho *et al.*, 1995)
Palm oil mill effluent	Anaerobic sludge	Batch	6.33 l-H_2 l^{-1} substrate	(O-Thong *et al.*, 2008)

Substrate	**Inocula**	**Operation Mode**	**Maximum hydrogen yield**	**Ref.**
Cow dung	Micro-flora of cow dung	Batch	743 ml-H_2 kg^{-1} cow dung	(Yokoyama *et al.*, 2007b)
Cow waste slurry	Micro-flora of cow waste slurry	Batch	392 ml-H_2 l^{-1}slurry	(Yokoyama *et al.*, 2007a)
Grass silage	Micro-flora from a full-scale landfill	Batch	16 ml-H_2 g^{-1} volatile solid	(Karlsson *et al.*, 2008)
Wheat straw	cow dung compost	Batch	68 ml-H_2 g^{-1} volatile solid	(Fan *et al.*, 2006b)
Sugarcane bagasse	*Caldicellulosiruptor saccharolyticus*	Batch	19.6 ml-H_2 g^{-1} volatile solid	(Ivanova *et al.*, 2009)
Pig slurry	Sludge from a mesophilic methanogenic reactor	Continuous	3.65ml-H_2 g^{-1} volatile solid	(Kotsopoulos *et al.*, 2009)
Cheese whey	Indigenous micro-flora	Continuous	2.9 ml-H_2 ml^{-1} cheese whey	(Venetsaneas *et al.*, 2009)

Table 2.1. 3: Maximum hydrogen production by different inocula using pure and crude glycerol as feedstock

Substrate	**Inocula**	**Mode of experiment**	**Maximum Hydrogen yield**	**Ref.**
Pure glycerol	*Halanaerobium saccharolyticum* (subspecies saccharolyticum)	Batch	0.62 ± 0.02 mol-H_2 mol^{-1} glycerol	(Kivistö *et al.*, 2010)
Pure glycerol	*Halanaerobium saccharolyticum* (subspecies senegalensis)	Batch	1.61 ± 0.28 mol-H_2 mol^{-1} glycerol	(Kivistö *et al.*, 2010)
Pure glycerol	Heat-treated anaerobic sludge,	Continuous	0.6 l-H_2 l^{-1} d^{-1}	(Escapa *et al.*, 2009)
Pure glycerol	Domestic wastewater	Batch	3.9 mol-H_2 mol^{-1} glycerol	(Selembo *et al.*, 2009b)
Pure glycerol	*Klebsiella* sp. HE1	Batch	0.345 mol-H_2 mol^{-1} glycerol	(Wu *et al.*, 2011)
Pure glycerol	Anaerobic digested sludge	Batch	0.41 mol-H_2 mol^{-1} glycerol	(K Seifert *et al.*, 2009)
Pure glycerol	Different heat-pretreated natural micro-flora.	Batch	11.5–38.1 ml-H_2 g^{-1} COD	(Akutsu *et al.*, 2009)
Pure glycerol	*Enterobacter aerogenes* HU-101	Continuous	80 mmol-H_2 l^{-1} h^{-1}	(Ito *et al.*, 2005)
Crude glycerol	*Thermotoga neapolitana* DSM 4359	Batch	2.73±0.14 mol-H_2 mol^{-1} glycerol	(Ngo *et al.*, 2011)
Crude glycerol	Mixed culture	Batch	0.31 mol-H_2 mol^{-1} glycerol	(Selembo *et al.*, 2009a)

Substrate	**Inocula**	**Mode of experiment**	**Maximum Hydrogen yield**	**Ref.**
Crude glycerol	*Enterobacter aerogenes* HU-101	Continuous	63 mmol-H_2 l^{-1} h^{-1}	(Ito *et al.*, 2005)
Crude glycerol	Anaerobic sludge	Batch	200 ml-H_2 g^{-1} COD	(Fernandes *et al.*, 2010)
Crude glycerol	*Enterobacter aerogenes*	Batch	710.0 cm^3-H_2dm^{-3} medium	(Marques *et al.*, 2009)
Crude glycerol	*Enterobacter aerogenes* NBRC 12010	Batch	0.77 mol-H_2 mol^{-1} glycerol	(Sakai *et al.*, 2007)
Crude glycerol	domestic wastewater	Batch	0.41±0.1 m^3 -H_2 m^{-3} d^{-1}	(Selembo *et al.*, 2009b)
Crude glycerol	*Rhodopseudomonas palustris*	Batch	6 mol-H_2 mol^{-1} glycerol	(Sabourin-Provost *et al.*, 2009)

Table 2.1. 4: Different bioreactor systems used for H_2 production using glycerol and other feedstock

Substrate	Bioreactor type	Bioreactor operation conditions			Maximum Hydrogen yield	Ref.
		Temp. (°C)	Optimum pH	Mode of operation		
Pure glycerol	Small sealed glass tube	37	7.4	Batch	0.62 ± 0.02 mol-H_2 mol^{-1} glycerol	(Kivistö *et al.*, 2010)
Pure glycerol	Small sealed glass tube	37	7	Batch	1.61 ± 0.28 mol-H_2 mol^{-1} glycerol	(Kivistö *et al.*, 2010)
Pure glycerol	Microbial electrolysis cell(MEC) with a 250 ml anodic chamber and a gas-phase cathode	25	7	Continuous	0.6 l-H_2 l^{-1} d^{-1}	(Escapa *et al.*, 2009)
Pure glycerol	Single-chamber membrane less MEC reactors consisted of a 4-cm long by 3-cm diameter cylindrical chambers	30	7	Batch	3.9 mol-H_2 mol^{-1} glycerol	(Selembo *et al.*, 2009b)
Pure glycerol	2.5 l fermenter containing 1.5 l media	35	6	Batch	0.345 mol-H_2 mol^{-1} glycerol	(Wu *et al.*, 2011)
Pure glycerol	Glass reactors (60 cc) with working capacity of 30 cc	37	6	Batch	0.41 mol-H_2 mol^{-1} glycerol	(K Seifert *et al.*, 2009)

Substrate	**Bioreactor type**	**Bioreactor operation conditions**			**Maximum Hydrogen yield**	**Ref.**
		Temp. (°C)	**Optimum pH**	**Mode of operation**		
Pure glycerol	120 ml vials containing 50 ml media	35	6.5	Batch	11.5–38.1 ml-H_2 g^{-1}COD	(Akutsu *et al.*, 2009)
Pure glycerol	Packed-bed reactor of 60 ml working volume	37	pH not controlled	Continuous	80 mmol-H_2 $l^{-1}h^{-1}$	(Ito *et al.*, 2005)
Pure glycerol	500 ml serum glass bottles filled with 250 ml media	30	6.2	Batch	0.28 mol-H_2 mol^{-1} glycerol	(Selembo *et al.*, 2009a)
Pure glycerol	670 cm^3 flasks containing 290 cm^3 of medium	37	6.5	Batch	608.7 cm^3-H_2dm^{-3} medium	(Marques *et al.*, 2009)
Pure glycerol	125 ml serum bottles	30	__	Batch	6 mol-H_2 mol^{-1} glycerol	(Sabourin-Provost *et al.*, 2009)
Crude glycerol	120-ml serum bottles containing 40 ml media	75	7.5	Batch	2.73 ± 0.14 mol-H_2 mol^{-1} glycerol	(Ngo *et al.*, 2011)
Crude glycerol	500 ml serum glass bottles filled with 250ml of media	30	6.2	Batch	0.31 mol-H_2 mol^{-1} glycerol	(Selembo *et al.*, 2009a)
Crude glycerol	Packed-bed reactor of 60 ml working volume	37	pH not controlled	Continuous	63 mmol-H_2 $l^{-1}h^{-1}$	(Ito *et al.*, 2005)

Substrate	**Bioreactor type**	**Bioreactor operation conditions**			**Maximum Hydrogen yield**	**Ref.**
		Temp. (°C)	**Optimum pH**	**Mode of operation**		
Crude glycerol	2-l glass flasks, consisting of 1 l of liquid volume	25.0±0.5	5.5	Batch	200 ml-H_2 g^{-1} COD	(Fernandes *et al.*, 2010)
Crude glycerol	670 cm^3 flasks containing 290 cm^3 of medium	37	6.5	Batch	710.0 cm^3-H_2dm^{-3} medium	(Marques *et al.*, 2009)
Crude glycerol	Bio-electrochemical two-compartment reactor	30	6	Batch	0.77 mol-H_2 mol^{-1} glycerol	(Sakai *et al.*, 2007)
Crude glycerol	Single-chamber membrane less MEC reactors consisted of a 4-cm long by 3-cm diameter cylindrical chambers	30	7	Batch	0.41 ± 0.1 m^3-H_2 m^{-3} d^{-1}	(Selembo *et al.*, 2009b)
Crude glycerol	125 ml serum bottles	30	__	Batch	4 mol-H_2 mol^{-1} glycerol	(Sabourin-Provost *et al.*, 2009)
Other substrates						
Bean curd manufacturing waste	1.2-litre serum vial with a 1-litre culture volume	35±1	6	Batch	130 ml-H_2 $h^{-1}l^{-1}$ culture	(Mizuno *et al.*, 2000)
Food waste	715-ml serum bottles	55 ±1	5.5	Batch	1.8 mol-H_2 mol^{-1} glycerol	(Shin *et al.*, 2004)

Substrate	**Bioreactor type**	**Bioreactor operation conditions**			**Maximum Hydrogen yield**	**Ref.**
		Temp. (°C)	**Optimum pH**	**Mode of operation**		
Food waste	250-mL serum bottles	36±1	5.5 ± 0.1	Batch	101 ml-H_2 g^{-1} COD	(Chen *et al.*, 2006)
Beer lees	250-ml serum vials	36±1	6-7	Batch	68.6 ml-H_2 g^{-1} total volatile solid	(Fan *et al.*, 2006a)
Rice winery wastewater	3.0-l up-flow reactor	55	5.5	Continuous	2.14 mol-H_2 mol^{-1} hexose	(Hanqing Yu *et al.*, 2002)
Rice slurry	280 ml serum bottles	37	4.5	Batch	346 ml-H_2 g^{-1} carbohydrate	(Fang *et al.*, 2006)
Cornstalk wastes	250 ml serum vials	36±1	7	Batch	149.69 ml-H_2 g^{-1} total volatile solid	(Zhang *et al.*, 2007)
Corn starch hydrolysate	2.0 l bioreactor with a working volume of 1.5 l	40±0.5	5.5	Batch	1.8 mmol-H_2 $mmol^{-1}$ glucose	(Perego *et al.*, 1998)
Corn starch hydrolisate	2.0 l bioreactor with a working volume of 1.5 l	37 ± 0.5	5.5	Batch	0.36 mmol-H_2 $mmol^{-1}$ glucose	(Perego *et al.*, 1998)
Molasses	300 ml hand-made fermenter having 250 ml culture fluid	___	6	Continuous	3.5 mol-H_2 mol^{-1} sugar	(Tanisho *et al.*, 1995)

Substrate	**Bioreactor type**	**Bioreactor operation conditions**			**Maximum Hydrogen yield**	**Ref.**
		Temp. (°C)	**Optimum pH**	**Mode of operation**		
Palm oil mill Effluent	150 ml serum bottles	60	5.5	Batch	6.33 l-H_2 l^{-1} substrate	(O-Thong *et al.*, 2008)
Cow dung	625 ml glass bottle tightly sealed with butyl rubber cap	60	6.6	Batch	743 ml-H_2 kg^{-1}cow dung	(Yokoyama *et al.*, 2007b)
Cow waste slurry	2-l fermentor	60	7	Batch	392 ml-H_2 l^{-1} slurry	(Yokoyama *et al.*, 2007a)
Grass silage	Continuous stirred tank reactors constructed from 2 l-glass bottles	55	7	Batch	16 ml-H_2 g^{-1}volatile solid	(Karlsson *et al.*, 2008)
Wheat straw	250 ml serum vials	36 ± 1	7	Batch	68.1 ml-H_2 g^{-1} volatile solid	(Fan *et al.*, 2006b)
Sugarcane bagasse	Anaerobic 50 ml hypo-vials	70	__	Batch	19.6 ml-H_2 g^{-1} volatile solid	(Ivanova *et al.*, 2009)
Pig slurry	Continuous stirred tank reactor having 750 cm^3 working volume	70	__	Continuous	3.65 cm^3-H_2 g^{-1} of volatile solid	(Kotsopoulos *et al.*, 2009)
Cheese whey	500 ml active volume mesophilic CSTR-type digester	35	5.2	Continuous	2.9 ml-H_2 ml^{-1} cheese whey	(Venetsaneas *et al.*, 2009)

Table 2.1. 5: Microorganisms used for bioconversion of pure and crude glycerol to H_2 and other products

Strain (s)	**Maximum hydrogen yield**	**Other by-product (s)**	**Ref.**
Thermotoga neapolitana DSM 4359	2.73 ± 0.14 mol-H_2 mol^{-1} glycerol		(Ngo *et al.*, 2011)
Halanaerobium saccharolyticum *(subspecies Saccharolyticum)*	0.62 ± 0.02 mol-H_2 mol^{-1} glycerol	1,3-propanediol (PD), butyrate, ethanol and 0.58 ± 0.03 mol-CO_2 mol^{-1} glycerol	(Kivistö *et al.*, 2010)
Halanaerobium saccharolyticum (subspecies senegalensis)	1.61 ± 0.28 mol-H_2 mol^{-1} glycerol	Acetate and 1.11 ± 0.21 mol-CO_2 mol^{-1} glycerol	(Kivistö *et al.*, 2010)
Mixed culture	0.31 mol-H_2 mol^{-1} glycerol	0.59 mol-PD mol^{-1} glycerol and 39% CO_2 in gas phase	(Selembo *et al.*, 2009a)
Heat treated anerobic sludge	0.6 l-H_2 l^{-1} d^{-1}		(Escapa *et al.*, 2009)
Enterobacter aerogenes HU-101	63 mmol-H_2 $l^{-1}h^{-1}$	0.85 mol-ethanol mol^{-1} glycerol	(Ito *et al.*, 2005)
Domestic wastewater	0.41 ± 0.1 m^3-H_2 m^{-3} d^{-1}		(Selembo *et al.*, 2009b)
Klebsiella pneumoniae ATCC 25955	__	1,3-propanediol	(Barbirato *et al.*, 1998)
Citrobacter freundii ATCC 8090	__	1,3-propanediol	(Barbirato *et al.*,

Strain (s)	**Maximum hydrogen yield**	**Other by-product (s)**	**Ref.**
			1998)
Enterobacter agglomerans CNCM 1210	__	1,3-propanediol	(Barbirato *et al.*, 1998)
Clostridium butyricum CNCM 1211	__	1,3-propanediol	(Barbirato *et al.*, 1998)
Rhodopseudomonas palustris	6 mol-H_2 mol^{-1} glycerol	__	(Sabourin-Provost *et al.*, 2009)
Mixed micro flora obtained from fixed-bed anaerobic reactors	200 ml-H_2 g^{-1} COD	__	(Fernandes *et al.*, 2010)
Enterobacter aerogenes	710.0 cm^3-H_2dm^{-3} medium	345.0 cm^3-CO_2dm^{-3} medium	(Marques *et al.*, 2009)
Enterobacter aerogenes NBRC 12010	0.77 mol-H_2 mol^{-1} glycerol	0.80 mol-CO_2 mol^{-1} glycerol	(Sakai *et al.*, 2007)
Mixed micro flora of organic waste or soil	11.5–38.1 ml-H_2 g^{-1}COD	1,3-propanediol	(Akutsu *et al.*, 2009)
Klebsiella sp. HE1	0.345 mol-H_2 mol^{-1} glycerol		(Wu *et al.*, 2011)
Anaerobic digested sludge	0.41 mol-H_2 mol^{-1} glycerol	0.784 ± 0.063 l-CO_2 l^{-1} media	(K Seifert *et al.*, 2009)

Strain (s)	**Maximum hydrogen yield**	**Other by-product (s)**	**Ref.**
Klebsiella pneumoniae	__	53 g-PD l^{-1} crude glycerol	(Mu *et al.*, 2006)
Pichia pastoris	__	Phytase	(Tang *et al.*, 2009)
Microalga *Schizochytrium limacinum*	__	Docosahexaenoic acid	(Chi *et al.*, 2007)
Edible fungus*, Rhizopus microsporus*	__	Animal feed	(Nitayavardhana *et al.*, 2011)
Ustilago maydis	__	Glycolipids	(Yanbin Liu *et al.*, 2011)

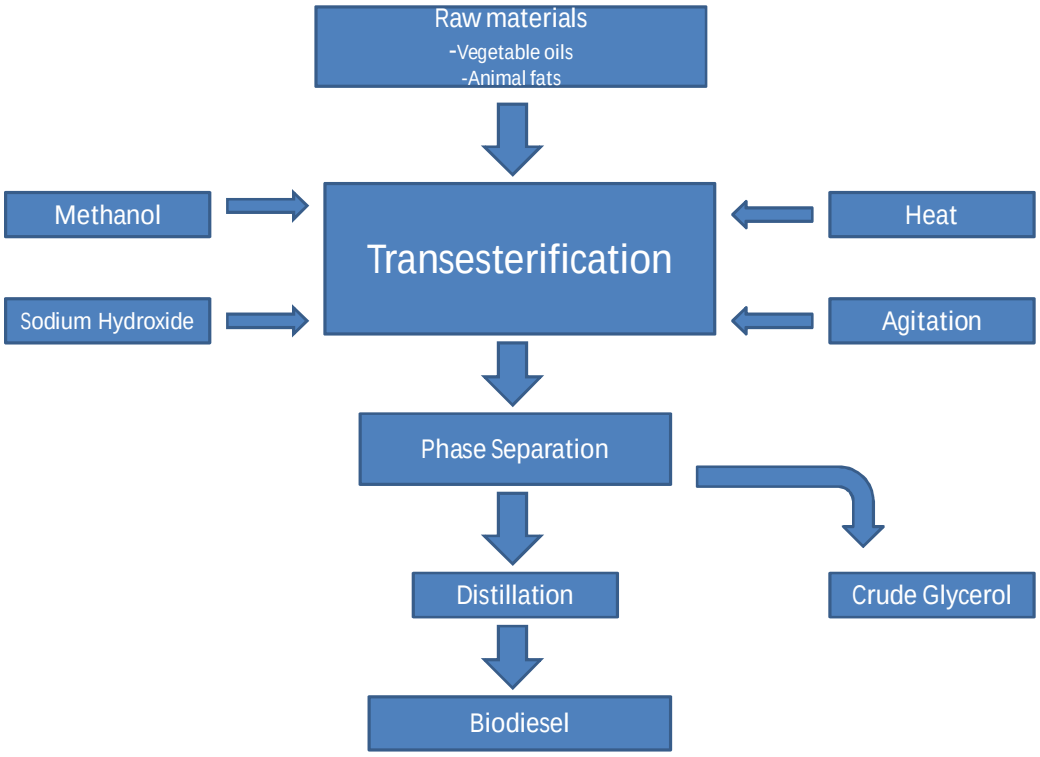

Figure 2.1.1: Schematic representation of a general biodiesel production process using transesterification of vegetable oil and fats (adapted from (Hak-Joo Kim *et al.*, 2004, Muniyappa *et al.*, 1996, Schuchardt *et al.*, 1998))

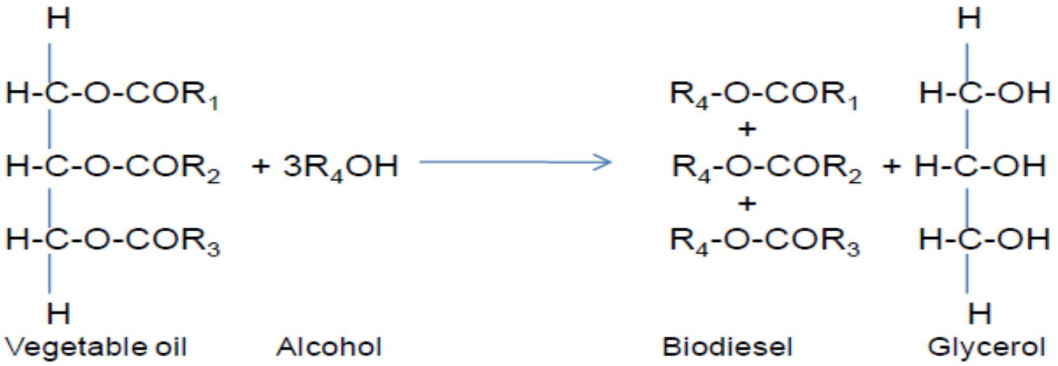

Figure 2.1.2: **Equation showing transesterification of large branched triglyceride molecule to biodiesel and glycerol (Muniyappa *et al.*, 1996)**

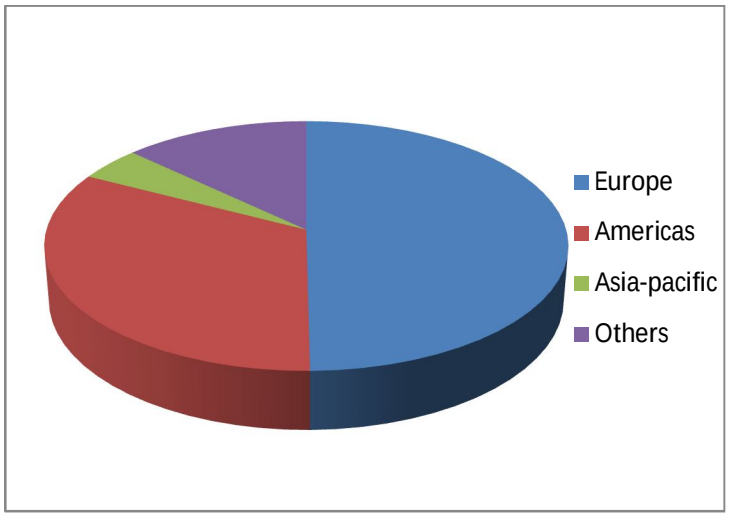

Figure 2.1.3: **Global biodiesel production share of different geographic regions of the world till 2009**

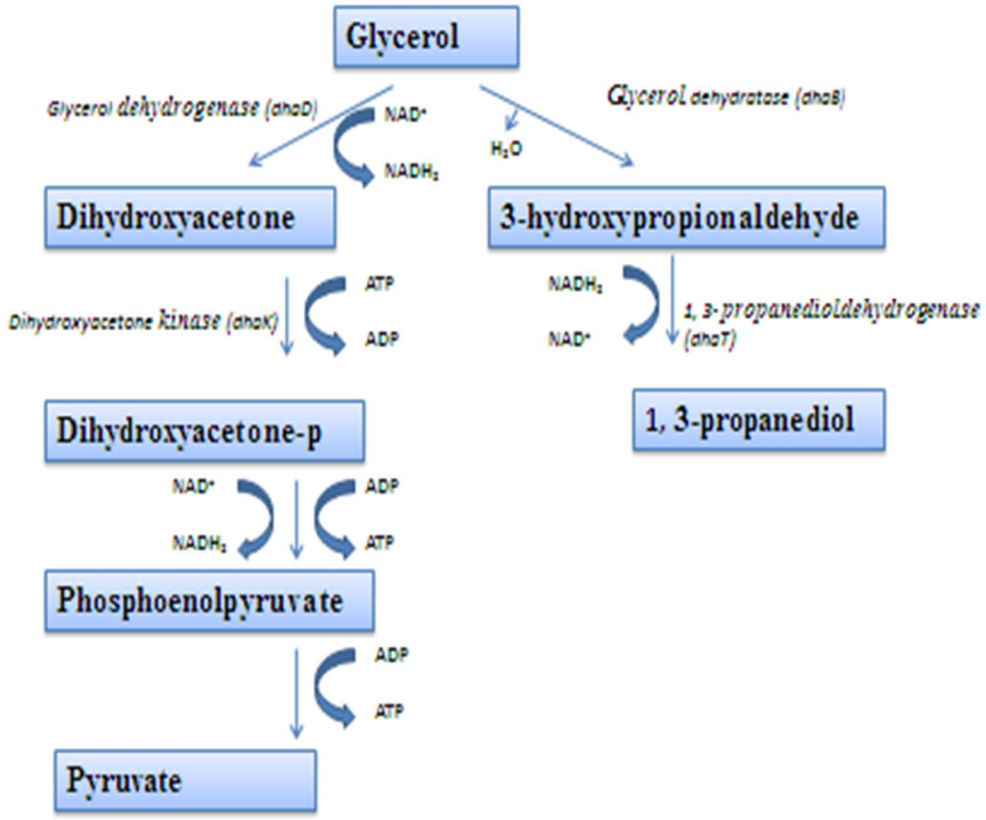

Figure 2.1.4: **Schematic representation of glycerol metabolism during anaerobic fermentation (Adapted from (Biebl *et al.*, 1999, Drożdżyńska *et al.*, 2011))**

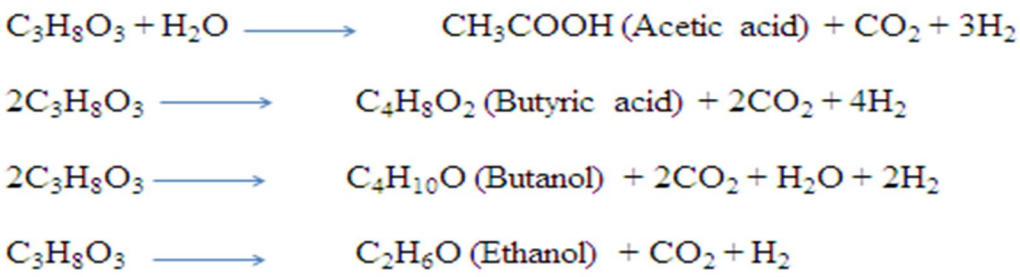

Figure 2.1.5: Stoichiometric equations showing hydrogen yield during glycerol bioconversion

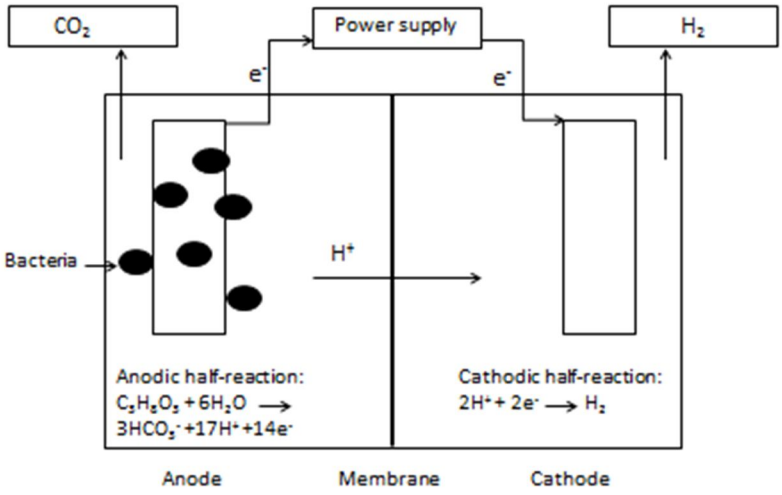

Figure 2.1.6: **A two-chambered microbial electrolysis cell used for H_2 production by bioconversion of glycerol (Escapa *et al.*, 2009, Hongqiang Hu, 2009, Hong Liu *et al.*, 2005, Logan *et al.*, 2008)**

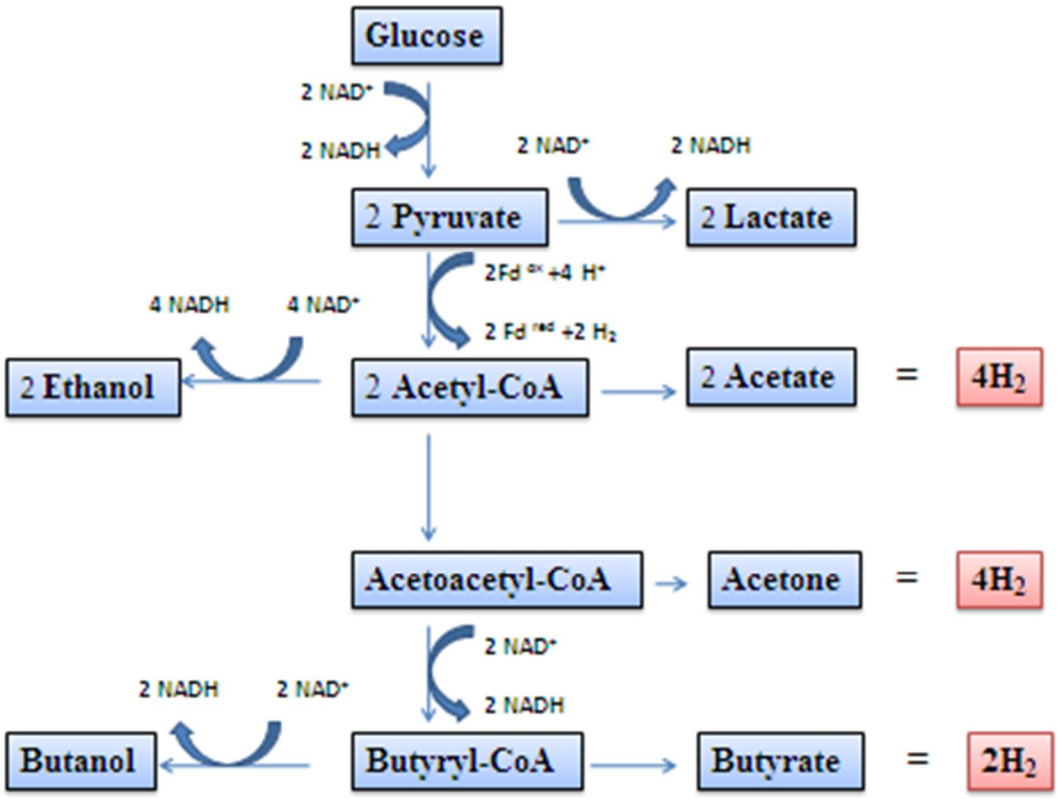

Figure 2.1.7: **Anaerobic metabolism of glucose and pyruvate and hydrogen production (Adapted from (Chin *et al.*, 2003, Mathews *et al.*, 2009)**

PART-II

KEY ENZYMES IN VALUE-ADDITION OF CRUDE GLYCEROL TO BIOHYDROGEN

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Enzymes in value-addition of waste (2014)

PART-III

HYDROGEN BIOREFINERY: POTENTIAL UTILIZATION OF THE LIQUID WASTE FROM FERMENTATIVE HYDROGEN PRODUCTION

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Résumé

En termes de potentiel de réduction des émissions de gaz à effet de serre, l'hydrogène est supérieur aux biocarburants commerciaux et aux combustibles fossiles, car il a une densité d'énergie élevée et il génère principalement que de l'eau. La production de biohydrogène a un avantage environnemental supplémentaire puisque les différents déchets organiques peuvent être valorisés au cours du processus. Cependant, en raison du coût élevé du procédé de bioconversion, sa production commerciale n'est pas encore réaliste.

Lors de la fermentation sombre, en plus de l'hydrogène, environ 60% de la charge d'alimentation peut être convertie en divers produits chimiques industriels, y compris l'éthanol, le 1-3 propanediol et l'acide butyrique. Si ces substances ne sont pas récupérées, les déchets liquides générés pendant le processus peuvent être utilisés comme matière première pour la production de polyhydroxyalcanoates, des lipides, du méthane, de l'hydrogène et de l'électricité. Le rejet liquide est aussi un substitut potentiel des engrais biologiques de solubilisation du phosphate. Ainsi, dans cette étude, le processus de production de biohydrogène est évalué comme une bioraffinerie potentiel pour la production de biocarburants, la chimie fine et des biomatériaux. La stratégie pourrait être utile pour réduire le coût global du procédés en générant des revenus de sources multiples.

Mots clés: biohydrogène; bioraffinerie; acide butyrique; éthanol; 1, 3 propanediol; déchets liquides

Abstract

In terms of greenhouse gas emission reduction potential, hydrogen is superior to commercial biofuels and fossil fuels because, it has high energy density and it generates only water as major emission. Biohydrogen production has additional environmental benefit as different organic wastes can be valorized during the process; however, because of high process cost, its commercial production is yet to start. During dark fermentation, in addition to hydrogen, around 60 % of the feedstock may convert to various industrial chemicals including ethanol, 1, 3 propanediol and butyric acid. If these products are not recovered, the liquid waste generated during the process can be used as the feedstock for production of polyhydroxyalkanoates, lipid, methane, hydrogen and electricity. The liquid waste is also a potential substitute of phosphate solubilizing biofertilizers. Thus, in this review, biohydrogen production process is evaluated as a potential biorefinery producing biofuels, fine chemicals and biomaterials. The strategy could be useful to reduce overall cost of the process by generating revenue from multiple sources.

Key words: Biohydrogen; biorefinery; butyric acid; ethanol; 1, 3 propanediol; liquid waste

Introduction

Depleting fossil fuel reserves and the concern of global climate change have accelerated the development of renewable energy sources. As a result of greenhouse gas emission reduction awareness and significant success in the field of renewable energy research, presently, some of the renewable energy carriers are being used in commercial level. Bioethanol, biodiesel and biogas are the common non-fossil renewable fuels derived from biomass. Apart from biofuels, biomass based feedstock can also be used for production of chemicals or biomaterials. In future, mostly due to unavailability of raw materials, petroleum based refinery, presently involved in the production of fuels, chemicals and materials; will have to reduce their production level. In this context, biorefinery has emerged as a potential alternative of petroleum based refinery. Biorefinery is a concept where biomass is used as an alternative of petroleum based raw materials and a range of products such as biofuel, industrial chemicals and biomaterials including commercially important biopolymers are produced.

Biohydrogen is a biofuel which has different advantages over presently commercialized biofuels such as, bioethanol, biodiesel and biogas. It is a cleaner energy carrier releasing water and negligible amount of nitrogen oxides during its combustion. In terms of greenhouse gas emission reduction, therefore, it has tremendous advantage over fossil fuels as well as other biofuels. Compared to petrol, a vehicle using CNG containing 30 % of hydrogen can reduce the particulate matter emission by 55 %, nitrogen oxides and hydrocarbon (non-methane) emission by 60 %, and CO_2 emission by 35 % http://ec.europa.eu/dgs/jrc/index.cfm?id=1410&obj_id=17140&dt_code=NWS&lang=en &ori=HLN (accessed on 31/01/2014). Although, relatively low volumetric energy density is one of major technological concerns of hydrogen fuel; the energy yield of hydrogen is 122 KJ/g, which is nearly 2.75 times higher than that of conventional hydrocarbon based fuels (Chang *et al.*, 2004). Biohydrogen can be produced from any biomass, ranging from those used as food/feed, forest biomass, algae/fungi to agro-industrial wastes. Dark fermentation is commonly used, rapid and relatively simple method of biohydrogen production. However, during hydrogen production by this method, only 30-

40 % of the substrate is utilized in hydrogen production and remaining 60-70 % are converted to various other metabolites (Venkateswar Reddy *et al.*, 2013). Ethanol, butyric acid, and 1, 3 propanediol are some commercially attractive metabolites produced during hydrogen production. Acetic acid, succinic acid, lactic acid and propionic acid are a few other metabolites accumulated during hydrogen fermentation. If these products are not recovered, the fermentative hydrogen production liquid waste (HPLW) can be converted to other value-added products such as, polyhydroxyalkanoates (bioplastic) (Venkata Mohan *et al.*, 2010), lipid (Venkata Mohan *et al.*, 2012), biobutanol (WH Chen *et al.*, 2011), methane and even hydrogen (Su *et al.*, 2009b). Additionally, organic acid containing HPLW is a potential substitute of phosphate solubilizing bio-fertilizer (Sarma *et al.*, 2013b). Therefore, the concept of hydrogen production from biomass based substrate can be extended to hydrogen biorefinery; where, in addition to hydrogen, different other products of commercial interest could be simultaneously produced (Figure 2.3.1). Incomplete substrate utilization is one of the major drawbacks of dark fermentative hydrogen production. The proposed strategy will help to get rid of this disadvantage by ensuring maximum biomass utilization. On the same note, it will generate additional revenue to support biological hydrogen production, industrial production of which is otherwise not possible due to high process cost.

Characteristics of the liquid waste from biohydrogen production process

HPLW from crude glycerol (CG) based hydrogen production process has been characterized for organic carbon content, total nitrogen, dry cell weight, elemental composition, and pH (Sarma *et al.*, 2013c). It has been observed that biohydrogen production using 1 Kg of CG can generate as high as 400 L of HPLW (Sarma *et al.*, 2013a). According to this estimation, production of 1 Kg of hydrogen may generate as high as 8771.92 L of HPLW (Sarma *et al.*, 2013a). Depending on the substrate used, COD of HPLW may be as high as 55±1.5 % of original COD (75,200–96,300 mg/L) of the substrate (O-Thong *et al.*, 2008). As a result of acidic byproduct accumulation, HPLW generally has a low pH which may be as low as 3.1 (Sung *et al.*, 2002). This pH

value may vary according to initial pH of the medium, process mode and duration, and microorganism selected for the process. In this context, how the acidic pH affects value-addition or disposal of HPLW will be interesting to know. It must be explored whether such a low pH will be helpful for cost competitive recovery of certain byproducts. Based on the type of substrate, microorganism used and process mode applied; the composition of HPLW may also vary. Major variation may be observed in terms of the types of the fermentation end products, their concentrations as well as the residual substrate concentration. As mentioned in the introduction section, acetic acid, butyric acid, propionic acid, succinic acid, lactic acid, ethanol and PD are some of the metabolites which may be present in the HPLW (Rajhi *et al.*, 2013, Selembo *et al.*, 2009). Reported concentrations of different metabolites in HPLW and their market price are presented in Figure 2.3.2 (Han *et al.*, 2010, Lalaurette *et al.*, 2009, Selembo *et al.*, 2009). It has been observed that all the metabolites have very high commercial value and the amount of PD, ethanol, acetic acid, butyric acid, and succinic acid present in HPLW is worthy to be recovered. In this context, it should be noted that the market prices of different byproducts of hydrogen production process mentioned in Figure 2.3.2 are the prices of their highly pure form. It implies that these are the maximum commercial values of such products one can expect; however, very high purification cost may be the major bottleneck in recovering such pure products. Therefore, proper cost benefit analysis of the recovery process of each of these products is a prerequisite of proposed hydrogen biorefinery approach. In Figure 2.3.3, a comparison of Gibbs free energies of formation (ΔG_f^o) for different byproducts of hydrogen production process is presented https://www.e-education.psu.edu/drupal6/files/be497b/pdf/Bioenergetics_AppA.pdf (01/02/2014)http://highered.mcgraw-hill.com/sites/dl/free/0072849606/315014/physical_properties_table.pdf (accessed on 01/02/2014). Figure 2.3.3 showed that among different byproducts, succinic acid has highest tendency to be produced. For microbial processes, however, the enzymatic pathways exist in the microorganism and the levels of expression of different enzymes are the crucial factors to determine the type and amount of different products. Hence, succinic acid may not be the dominant byproduct of a process chosen/optimized for

maximum hydrogen production. Moreover, hydrogen may be utilized during succinic acid production (Krishnakumar, 2013, Murarka *et al.*, 2008); which, in turn may reduce the hydrogen yield. Therefore, in order to develop a hydrogen based biorefinery, the process should be designed in such a way that the hydrogen production is not compromised due to the production of particular byproduct. In next sections, potential utilization of HPLW for the recovery of certain important products or its potential utilization as a feedstock for the production of different valuable products has been elaborately discussed.

Ethanol recovery from HPLW

Ethanol produced from renewable materials is a commercially successful biofuel. Since 1980s, bioethanol produced from corn has been used by blending with gasoline (Sun *et al.*, 2002). For conventional internal combustion engines, gasoline with 5-26% anhydrous ethanol can be used as fuels (Gnansounou *et al.*, 2005); however, suitable modification of the engine is required to use only hydrated ethanol (approximately 95% ethanol and 5% H_2O) as the fuel. The average octane number or the anti-knock index (AKI) of ethanol is 99; which is significantly higher than that of (88) gasoline (Gnansounou *et al.*, 2005). In order to increase the octane number of gasoline, conventionally, methyl tertiary butyl ether (MTBE) is added; however, it is known to be toxic and a potential contaminant of groundwater (Sun *et al.*, 2002). Alternatively, ethanol could be used as an eco-friendly replacement of MTBE.

For bioethanol production, the common feedstock, such as sugarcane, corn and wheat and barley are used by the commercial biofuel producers in Brazil, US or Europe (Balat *et al.*, 2009). Similarly, cassava, sweet sorghum, sweet potatoes, and lignocellulosic biomass are the other common substrates (Balat *et al.*, 2009). Alternatively, as mentioned in the introduction section, ethanol can also be recovered from the liquid waste of dark fermentative hydrogen production. Table 2.3.1 summarizes a number of studies on simultaneous hydrogen and ethanol production. From Table 2.3.1, it can be concluded that the amount of ethanol and hydrogen produced during dark fermentative hydrogen production varies from 0.29 mol ethanol and 1.04 mol H_2/mol glucose to 0.90 mol ethanol (equivalent to 0.23g ethanol/g glucose) and 1.58 mol H_2/mol glucose (Ken-

Jer Wu *et al.*, 2007, Zhao, 2009). Even hydrogen production as high as 2.49 mol H_2/mol glucose is also known where fermentation end-product was mostly ethanol (You-Kwan Oh *et al.*, 2003). Moreover, hydrogen and ethanol yield may vary according to the type of substrate as well as microbial strain and process mode used for the purpose. For example, production of 0.96 mol ethanol and 1.12 mol H_2/mol-glycerol has been reported for *Enterobacter aerogenes* HU-101(Ito *et al.*, 2005). From Table 2.3.1, it is also clear that the microbial agents used for the purpose are either facultative/strict anaerobic bacteria or anaerobic mixed culture, which could be thermophilic or mesophilic. Glucose, α-cellulose, acid treated cellulose hydrolysates, cellobiose, xylose, sucrose, fructose, pure glycerol, biodiesel manufacturing process liquid waste containing glycerol, molasses or wastewater containing molasses are the different substrates evaluated for simultaneous hydrogen and ethanol production. For comparison purpose, different dedicated bioethanol production processes with no hydrogen production have been summarized in Table 2.3.2. From the table it is clear that unlike, simultaneous hydrogen and ethanol production mostly yeasts are used for dedicated bioethanol production processes. Similarly, the ethanol yield of different processes listed in Table 2.3.2 ranges from 0.41 g/g glucose to 0.48 g/g substrate. These values are nearly twice the yield of 0.90 mol ethanol/mol glucose (equivalent to 0.23g ethanol/g glucose) known for simultaneous hydrogen and ethanol production. Thus, the ethanol yield of a simultaneous hydrogen and ethanol production process is obviously lesser than a dedicated process. In the former case, apart from ethanol, hydrogen is also produced and the fermented broth containing ethanol and other byproducts is mainly considered as waste. Therefore, although the ethanol yield of a hydrogen production process is not comparable to that of a dedicated ethanol production process, still recovery of such ethanol may be helpful in reducing the overall process cost of hydrogen production. However, this possibility needs to be verified by a thorough cost benefit analysis of simultaneous hydrogen and ethanol production and purification. At present number of reports are available only on concomitant hydrogen and ethanol production; whereas, ethanol purification from HPLW is largely unexplored.

1,3-propanediol recovery from HPLW

1, 3-properndiol (PD) is an important platform chemical with global production of more than 200,000 tons per annum (Szymanowska-Powałowska *et al.*, 2013). Different commercially important applications of PD are as follows: (i) polytrimethylene terephthalate, a biodegradable polymer widely used in textile fiber production can be produced from PD (Dabrowski *et al.*, 2012, Drożdżyńska *et al.*, 2011, Saxena *et al.*, 2009), (ii) it is also used as monomer for the production of different other polymers, such as polyurethane, and polyether (Barbirato *et al.*, 1995, Drożdżyńska *et al.*, 2011, Ferreira *et al.*, 2012), (iii) it is an important material for the production of different cosmetics, detergents, and adhesives (Drożdżyńska *et al.*, 2011, Ferreira *et al.*, 2012) and (iv) as solvent and for production of dioxanes (Biebl *et al.*, 1999).

PD can be produced by chemical synthesis and biotechnological methods. These methods comprise: (i) catalytic synthesis using ethylene oxide, carbon monoxide, and hydrogen (Dabrowski *et al.*, 2012, Saxena *et al.*, 2009), (ii) chemical synthesis using acrolein (Drożdżyńska *et al.*, 2011, Ken-Jer Wu *et al.*, 2007), (iii) bioconversion of crude glycerol (Drożdżyńska *et al.*, 2011, Ferreira *et al.*, 2012, Selembo *et al.*, 2009), and (iv) bioconversion of glucose (Biebl *et al.*, 1999). Out of these methods, utilization of renewable materials, no toxic byproduct formation, no need of any catalyst, simultaneous treatment of industrial waste, and mild process conditions are some of the benefits of microbial PD production. Additionally, as shown in Table 2.3.3, during PD production by bioconversion of organic materials, such as pure/crude glycerol, hydrogen can be recovered as a byproduct or vice-versa. Therefore, liquid waste generated during fermentative hydrogen production using pure/crude glycerol could be considered as a potential renewable source of PD. Crude glycerol is a byproduct of biodiesel manufacturing by trans-esterification process and nearly 10 Kg of crude glycerol is generated for every 100 Kg of biodiesel produced (Santibáñez *et al.*, 2011). Although it is an expensive process (Santibáñez *et al.*, 2011); glycerol can be recovered from crude glycerol (Yong *et al.*, 2001). Owing to the increase in global biodiesel demand, crude glycerol production is increasing day by day. In turn, due to availability of large amount of crude glycerol, compared to demand, production of glycerol is increasing. Therefore,

a sharp decrease in glycerol price has been seen (Chunfei Wu *et al.*, 2013). Thus, considering all these facts, hydrogen production by crude glycerol bioconversion and subsequent recovery of PD form the liquid waste stream could be an interesting option. As shown in Table 2.3.3, using crude glycerol, simultaneous production of 0.31 mol-H_2/mol-glycerol and 0.59 mol-PD/mol-glycerol is possible. Therefore, from 1 Kg crude glycerol (derived from trans-esterification of soybean oil) containing 63 % (w/v) of glycerol (Hu *et al.*, 2012), nearly 48.14 L (2.12 mol) of H_2 and 307.06 g (4.03 mol) of PD could be produced. Additionally, aforementioned crude glycerol also contains nearly 37 % (w/v) of non-glycerol materials; therefore, actual hydrogen and PD production from 1 Kg of crude glycerol could be more that the projected value. Present market price of analytical grade PD is $288/L https://www.fishersci.ca/coupon.do?cid=4509876&lang=en&itemId= (accessed on 06/02/2014). Likewise, based on cylinder size, the current (2013) market value of industrial grade hydrogen is around $7.75-$11.50/cubic meter http://infopage.gem.wa.gov.au/docs/Price_Schedule_-_Industrial_Gases_49009.xls? (accessed on 17/02/2014). Therefore, commercial potential of simultaneous recovery of both the products by an eco-friendly process such as bioconversion of organic waste needs to be explored.

Butyric acid recovery from HPLW

Butyric acid has different commercially important applications which include: (i) production of cellulose acetate butyrate fiber suitable for textile industry (Zhang *et al.*, 2009), (ii) potential application as a raw material for biotechnological production of butanol (Dwidar *et al.*, 2012), (iii) preparation of polymeric fiber with enhanced heat, sunlight and cold resistance (Dwidar *et al.*, 2012, Zhang *et al.*, 2009), (iv) for bio-plastic (β-hydroxybutyrate) production (Dwidar *et al.*, 2012, Zhang *et al.*, 2009), (v) for improved biobutanol production efficiency (Tashiro *et al.*, 2004, Xuepeng Yang *et al.*, 2013, Zhang *et al.*, 2009), (vi) for the production of fuels such as ethyl butyrate or butyl butyrate (Dwidar *et al.*, 2012, Salihu *et al.*, 2013), (vii) as calcium butyrate for leather tanning purpose (Zhang *et al.*, 2009), (viii) as tributyrin for potential treatment of malignancy (Zi-Xing Chen *et al.*, 1994), (ix) butyric acid esters as aroma compounds

and flavoring agent (Dwidar *et al.*, 2012, Larios *et al.*, 2004, Zhang *et al.*, 2009), and (x) as indole-3-butyric acid- a plant hormone. Butyric acid is commonly produced by butyraldehyde oxidation (Zigova *et al.*, 2000). The starting material for this process is propylene, which in turn is synthesized from crude oil (Dwidar *et al.*, 2012). However, this method has certain disadvantages: (i) as it is a chemical process, the butyric acid produced by this method is not preferred for the processes where the product of natural origin is required, (ii) propylene is one of the mostly used crude oil derived raw materials having wide range of other applications; therefore, by using renewable sources for butyric acid production considerable amount of propylene could be saved and rout to other more valuable products, and (iii) crude oil is not a renewable resource; therefore, there is a need to look into other more sustainable opportunities. In this context, organic acid rich liquid waste of dark fermentative hydrogen production could be considered as a renewable and less expensive source of butyric acid. As shown in Table 2.3.4, during hydrogen production by bioconversion of organic materials/organic waste materials, the amount of butyric acid accumulated in the fermentation medium could be as high as 61.0 % of total soluble microbial products (Abreu *et al.*, 2009). Apart from producing 3.2 mol-H_2/mol-hexose, by weight the amount of butyrate produced as the byproduct of the process could be as high as 3793 ± 671 mg-butyrate/L (Hafez *et al.*, 2009). Similarly, hydrogen and butyrate yield as high as 2.59 mol-H_2/mol-hexose and 0.62 mol-butyrate/mol-hexose have been recorded during bioconversion of the wastewater from sugar factory (Ueno *et al.*, 1996). From these observations it could be concluded that by bioconversion of 1 Kg of glucose (5.55 mol); at the same time and at same production cost, nearly 28.75 g (326.44 L at STP) of hydrogen gas and 299.37 g of butyrate can be produced. As already mentioned, present market value of hydrogen is around $7.75-$11.50/cubic meter. Similarly, present market value of bulk quantity of natural butyric acid is around $2-$50/Kg http://www.alibaba.com/product-gs/703515617/natural_Butyric_acid_107_92_6.html (accessed on 30/10/2013). Whereas, the price of analytical grade butyric acid could be as high as $74.6/L https://www.fishersci.ca/coupon.do?cid=AC108110010&lang=en&itemId=,AC108110010 (accessed on 06/02/2014). Therefore, instead of concentrating on production of only hydrogen or only butyric acid, in order to ensure maximum utilization of the feedstock as

well as to simultaneously reduce the production cost of both the products; more investigations should be carried out for developing an efficient integrated hydrogen and butyric acid production process. From Table 2.3.4 and above discussion it is clear that a significant amount of hydrogen and butyric acid could be produced by an integrated bioconversion process by using either pure feedstock such as glucose or even highly complex materials such as industrial wastewaters. Therefore, instead of merely trying to improve the yield of one product; more effort should be given to develop an efficient process for simultaneous industrial production of both the products. Similarly, instead of concentrating on reducing only production cost or only purification cost of hydrogen and butyric acid; more effort should be given to reduce total production and purification cost. For example, as shown in Table 2.3.4, to reduce the process cost instead of expensive feedstock, such as glucose or sucrose industrial waste materials, such as corn syrup waste and wastewater from sugar factory have been used. However, application of a relatively complex feedstock, such as wastewater may increase the purification cost of butyric acid by complicating the purification process. Similarly, in order to bypass the sterilization of the feedstock required for a process involving pure microbial culture, processes with mixed microbial culture are often considered. However, in the case of mixed culture based processes, number of different gaseous and liquid products will be produced. Thus, there is a chance that product purification cost will increase.

Methane production from HPLW

Compared to methane, hydrogen is more valuable fuel. Therefore, although methane production from organic waste material is a traditional practice, yet hydrogen production is another commercially significant option. Instead of combusting methane alone, application of a mixture of H_2 and methane can reduce nitrogen oxide emission (Zhu *et al.*, 2008). As already discussed, unlike methane production, during fermentative hydrogen production different solvents and organic acids such as ethanol, butanol, PD, acetic acid, and butyric acid are produced as fermentation end products. These end products can be further converted to methane by conventional anaerobic digestion. Alternatively, both hydrogen and methane production can be simultaneously carried out in a single process; however, optimum hydrogen and methane production conditions

are not exactly same (Zhu *et al.*, 2008). Likewise, in a single-stage hydrogen and methane production process, methane producers (methanogens) can consume hydrogen. Therefore, methane recovery from hydrogen production liquid waste by a two-stage hydrogen and methane production process has obvious technical advantage over only methane production or single-stage methane and hydrogen production using organic waste. In Table 2.3.5 some recent studies on two-stage hydrogen and methane production have been summarized. From the table, it is evident that continuous hydrogen and methane production process is quite popular among the researchers. One major reason for such preference is that a continuous process can prevent the accumulation of organic acids produced as byproducts of hydrogen production; thereby, early termination of the process due to sharp pH drop can be avoided. Additionally, decrease in medium pH may initiate metabolic shift to reduce the cumulative hydrogen production (Khanal *et al.*, 2004). Likewise, mixed microbial culture from waste material is the preferred inoculum over pure strains. Additionally, experiments have been commonly carried out at larger scale using 10L to 500L reactors. It indicates that considerable success that has been made in the field of biomethane production using HPLW. However, major drawback of this strategy is that valuable acids and solvents present in HPLW are converted to methane, which otherwise, would have been recovered.

Bioplastic (PHA) production from HPLW

Polyhydroxyalkanoates (PHAs) are the polyesters which can be produced by microorganisms by utilizing organic substrates including waste materials (Balaji *et al.*, 2013, Du *et al.*, 2012, Zinn *et al.*, 2001). Considering the depletion of fossil based feedstock, PHAs produced from biomass or agro-industrial waste based substrates have significance in terms of sustainability. Additionally, owing to their biodegradability, in comparison to petroleum based polyesters they are more eco-friendly. In addition to biodegradability, biocompatibility is the other important feature of PHAs which make them suitable for biomedical applications such as tissue engineering (Guo-Qiang Chen *et al.*, 2005, Samy *et al.*, 2013). PHAs are presently commercialized as low volume high value products and they have the potential to be used for packaging and textile industry

(Keshavarz *et al.*, 2010). Presently, pure substrates, such as glucose, soybean oil (He *et al.*, 1998); biomass based feedstock, such as hemicellulose (Ramsay *et al.*, 1995), forest biomass (Keenan *et al.*, 2006); industrial waste materials such as paper industry wastewater (Bengtsson *et al.*, 2008), molasses (Albuquerque *et al.*, 2007) are known to be used for microbial polyester, such as PHAs production.

In this context, the liquid waste generated during biohydrogen production process could be a potential substrate for PHAs production (Patel *et al.*, 2011, Venkata Mohan *et al.*, 2010). As already an elaborate description has been made, during fermentative hydrogen production, volatile fatty acids (VFAs), such as acetic acid and butyric acid are produced as byproducts. Different microorganisms can convert these VFAs to biopolymers, such as PHAs (Patel *et al.*, 2012, Venkateswar Reddy *et al.*, 2013). Application of VFAs containing liquid of biohydrogen production process has certain advantages. Firstly, the waste contains large amount of acetic and butyric acid and their conversion to structurally close PHAs will be relatively convenient, as simpler metabolic pathways with fewer number of steps will be involved (Venkata Mohan *et al.*, 2010). Secondly, COD of the liquid waste will be significantly removed and its acidic pH will be neutralized by conversion of the organic acids, which in turn will omit treatment requirement for hydrogen production liquid waste. Thirdly, biomass based feedstock needs proper pre-treatment for PHAs production (Bowers *et al.*, 2013); however, if the liquid waste is used, other than the pH adjustment, no other pre-treatment will be needed. Considering the fact that high process cost is the major bottleneck of fermentative hydrogen production; the proposed approach has the potential to provide an additional source of revenue for hydrogen manufacturers to improve the economic feasibility of the process. Additionally, the technology can offer ecofriendly/sustainable and renewable polyester in the form of PHAs.

As per the authors' knowledge, presently only four reports are available on this topic (Patel *et al.*, 2011, Porwal *et al.*, 2008, Venkata Mohan *et al.*, 2010, Venkateswar Reddy *et al.*, 2013). Therefore, further investigations are encouraged which should determine maximum theoretical PHAs production potential of the hydrogen fermenter liquid waste (HPLW), technological barriers in achieving that limit as well as the process cost involved. According to Reddy et al. (2013), during fermentative hydrogen production by

using organic feedstock, 60-70 % of the organic materials are left as fermentation end-products (Venkateswar Reddy *et al.*, 2013), most of which are organic acids. Thus, it will be interesting to know how much of this organic acid could be converted to PHAs and what is the rate of conversion.

Organic acids present in HPLW may be inhibitory for the microorganism used for PAHs production (Venkateswar Reddy *et al.*, 2013). In this context, application of acid tolerant PHAs producing recombinant strain could be considered as a potential strategy for industrial production of PHAs using HPLW. Due to similar reason, HPLW will not be suitable for PHAs production by using batch process. Therefore, more attention should be given to continuous or fed-batch fermentation of HPLW. Chemical analysis of PHAs produced from HPLW was found to have around 89 % of hydroxy butyrate and 9 % hydroxy valerate (Venkateswar Reddy *et al.*, 2013). The composition of HPLW having relatively higher amounts of acetic and butyric acid and relatively lower amount of propionic acid have been mentioned as a probable reason for high hydroxy butyrate content (Venkateswar Reddy *et al.*, 2013). The copolymer made up of hydroxy butyrate and hydroxy valerate has better physical and thermal properties than the homo polymer, poly-hydroxy butyrate (PHB) (Venkateswar Reddy *et al.*, 2013). Therefore, being a mixture of different organic acids, HPLW has additional advantage as feedstock for commercially important PHAs production. Porwal et al. (2008) have reported that microorganisms, such as *Bacillus thuringiensis* EGU45 can produce as high as 0.63 mol H_2/mol glucose and 435 mg PHB/L of medium (Porwal *et al.*, 2008). Patel et al. (2011) have reported that under batch cultivation, *Bacillus thuringiensis* EGU45 can produce 1.67 mol H_2/mol glucose and PHB as high as 11.3 % of dry biomass could be produced by utilizing the waste generated during the process (Patel *et al.*, 2011). Thus, it will be interesting to investigate continuous H_2 production followed by continuous PHAs production using HPLW.

Potential application of HPLW as phosphate solubilizer

Plants can take up the soluble phosphorus present in soil; however, the amount of soluble phosphorus present in soil is relatively low (Park *et al.*, 2009). In the form of different chemical fertilizers, soluble phosphorus can be applied into the agricultural

field. However, a significant portion of applied soluble phosphorus reacts with different cations present in soil and is converted to insoluble forms, such as, $Ca_3 (PO_4)_2$, $FePO_4$, and $AlPO_4$ to become unavailable for plants (Park *et al.*, 2009). Moreover, the soil itself contains insoluble inorganic phosphorus as the salt of calcium, iron and aluminum (Yadav *et al.*, 2012). Application of phosphate solubilizing bio-fertilizers (PSBs) is one method commonly used to increase phosphorus availability of soil. Commercially used PSBs are the microorganisms which can solubilize the insoluble phosphates present in soil to make them available for the utilization of plants. These microbes can release different organic acids which, in turn, chelate the cations of insoluble inorganic phosphorus present in soil to release bioavailable phosphorus (YP Chen *et al.*, 2006).

As described in previous sections, during hydrogen production by dark fermentation a range of organic acids are produced; hence, HPLW containing different organic acids could be used as an alternative of PSB. Laboratory study using insoluble $Ca_3 (PO_4)_2$ have shown that application of HPLW can increase the soluble phosphorus concentration by nearly 36 times (Sarma *et al.*, 2013b). It indicates that the approach has tremendous potential as a substitute of commercial PSBs. Conventional PSBs have relatively shorter shelf-life of nearly 6 months, because they are nothing but some microbial cells; viability of which might be lost during storage. On the contrary, HPLW could have superior shelf-life as it has no relation with microbial cell viability (Sarma *et al.*, 2013b). Similarly, HPLW is a waste material which has no direct production cost; however, PSB production process needs expensive chemicals. In general, PSB formulations are comprised of microbial cell, nutrient and carrier material; whereas, HPLW could be applied in agricultural field without further formulation. Thus, HPLW has certain advantages over conventional PSBs; however, its practical applicability should be verified by field trials. Residual COD and BOD of HPLW might be a concern over its potential application to the agricultural field. Therefore, prior to its land application, removal of microbial biomass might be needed.

Other products from HPLW

A report is available on lipid production by microalgae using HPLW as the substrate. According to Mohan et al. (2012), from each mL of volatile fatty acids (VFAs) rich

HPLW, as high as 1.42 mg of wet algal biomass could be produced of which 26.4% are lipids of commercial interest (Venkata Mohan *et al.*, 2012). This study demonstrated possible application of HPLW for lipid production. However, algae are capable of producing as high as 3.2 g biomass /L medium, which is higher than the amount reported for HPLW (Li *et al.*, 2008). Therefore, further investigation should be undertaken to achieve maximum biomass/lipid production using HPLW. As already mentioned, HPLW contains only 60-70 % of the organic matter used for hydrogen production. Hence, better results can be expected by using HPLW as a co-substrate with another substrate commonly used for algal lipid production. HPLW can also be used as a feedstock for hydrogen production by photo-fermentation (Chun-Yen Chen *et al.*, 2008, Su *et al.*, 2009a, Honghui Yang *et al.*, 2010). Chen et al. (2008) have reported that by dark fermentation, hydrogen yield as high as 3.80 mol H_2/mol sucrose could be obtained. If the HPLW generated during the process is used as a substrate for photo-fermentation, cumulative hydrogen yield could be increased up to 10.02 mol H_2/mol sucrose (Chun-Yen Chen *et al.*, 2008). Thus, in terms of substrate utilization and hydrogen yield, the approach has very high potential; however, it has various concerns too. Firstly, for this process a light source is required and the culture vessel should be designed in such a way that sufficient light is available for the microorganisms. This will increase the process cost and the process will be difficult to scale up; especially, when the final product is gaseous hydrogen and requires a close reactor with large headspace. Secondly, the approach is suitable only for pure substrate, such as sucrose where the fermentation medium is relatively transparent and light can easily penetrate through it. However, in the case of waste based feedstock, such as wastewater/wastewater sludge, the fermentation medium is relatively opaque and hence, due to limited light access, the approach may not be able to show similar efficiency. Owing to high availability and less expensive nature, agro-industrial waste and biomass based substrates are recognized as most suitable for potential industrial production of hydrogen. Thus, the approach will have limited industrial significance. Thirdly, before starting the photo-fermentation, sterilization of HPLW may be needed; which will increase the process cost and energy consumption. Additionally, compared to dark fermentation, photo-fermentative hydrogen production process is relatively slow

and may take as long as 7-8 days to complete a batch process (Sabourin-Provost *et al.*, 2009). Hence, this strategy needs a thorough techno-economic analysis to determine its commercial feasibility. Akin to photo-fermentation, HPLW can also be used as a substrate to produce hydrogen by using microbial electrolysis cell (MEC) (Lalaurette *et al.*, 2009). However, requirement of expensive catalyst, such as platinum is one disadvantage of this approach. Performance of a microbial electrolysis cell may be reduced by the pH gradient established between anode and cathode chamber (Rozendal *et al.*, 2008). On a similar note, efficiency of the hydrogen producing microorganism present in the anode may be altered by the presence of hydrogen consumer such as, methanogen (Hong Liu *et al.*, 2010, Rozendal *et al.*, 2008). Relatively low proton concentration of a MEC may increase the internal resistance (Hong Liu *et al.*, 2010). In order to reduce the resistance, the distance between anode and cathode should be reduced (Hong Liu *et al.*, 2010). Therefore, pilot scale operation of the process is challenging. Alternatively, HPLW can be directly converted to electricity by using a microbial fuel cell (SangEun Oh *et al.*, 2005). However, the approach also has concerns similar to MEC. Electrode should have very large surface area to support the biofilm of anodophilic microorganisms (Logan *et al.*, 2006). Cathodic reaction with poor reducing ability is also considered as a limiting factor of electricity generation by microbial fuel cell technology (Byung Hong Kim *et al.*, 2007). Thus, application of HPLW for lipid, hydrogen and electricity production are at natal stage and these technologies will take some time to attain commercial interest.

Concluding statements

In terms of gravimetric energy density and greenhouse gas reduction potential, hydrogen produced from biomass based feedstock has clear advantage over contemporary biofuels, such as biodiesel, bioethanol and biomethane. Dark fermentation is mostly investigated, straight forward and a rapid method of biohydrogen production. During hydrogen production by dark fermentation, 60-70 % of the substrate is channeled to different byproducts. Ethanol, 1,3 propanediol and butyric acid are such byproducts which have immense commercial importance. If simultaneous hydrogen production and ethanol recovery are considered; in addition to 1.58 mol H_2, 0.90 mol

ethanol could be recovered for each mol of glucose. This ethanol yield is equivalent to 0.23g ethanol/g glucose which is almost half of 0.41 g ethanol/g glucose produced by conventional bioethanol production process. Considering the fact that hydrogen is the main product and ethanol recovered from HPLW has no direct production cost; the yield is significant. According to an estimation bioconversion of 1 Kg of crude glycerol, a waste generated during biodiesel production, can produce 48.14 L of H_2 and 307.06 g of PD. Considering present market value of H_2 (\$7.75-\$11.50/m^3) and PD (\$2.43/Kg-\$288/L), the amount of PD which could be recovered from HPLW has industrial importance. By bioconversion of 1 Kg of glucose, apart from producing 326.44 L of hydrogen, 299.37 g of butyrate with a bulk price as high as \$50/Kg - \$74.6/L, can be recovered as a by-product. During hydrogen production, at the same time more than one by-product can be recovered from the HPLW. In this case, the yield of one product may have negative effect on the yield of others. Therefore, metabolic engineering strategies should be considered to direct the material flow towards certain important products. Alternatively, HPLW is a potential phosphate solubilizer, which can increase the soluble phosphate concentration of an aqueous medium containing insoluble inorganic phosphorus by 36 times. Therefore, it should be evaluated as a substitute of PSB. As (i) PSB is a commercially successful biofertilizer, (ii) HPLW needs no downstream processing for this application, (iii) it has no direct production cost involved, and (iv) compared to PSB it is projected to have superior shelf life; the strategy has considerable interest as additional revenue source for fermentative hydrogen production. PHA and lipid production are other different indirect but attractive options for sustainable utilization of HPLW. Methane can also be produced from HPLW; however, this approach will consume all the byproducts of hydrogen production process which, otherwise, would have better a market than methane. Moreover, methane production needs further fermentation of HPLW; which is not the case for other byproducts. Similarly, photo-fermentation and microbial electrolysis cell can be used for further conversion of HPLW to hydrogen. However, high process cost and concern over its scalability are the issues yet to be resolved. Overall, hydrogen biorefinery approach carries tremendous industrial potential and hence, instead of concentrating on one

particular by-product, the entire process should be evaluated as an integrated strategy and a proper cost benefit analysis of the approach is required.

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Table 2.3. 1: Summary of different reports on simultaneous hydrogen and ethanol production

SL. No.	Microorganism	Substrate	Process operation	Hydrogen production	Ethanol production	Reference
1	Mixed anaerobic culture from hot spring	Glucose (9 g/L) and additional nutrients	1L CSTR (Anaerobic)	0.80 mol-H_2/mol-glucose	0.50 mol-ethanol/mol-glucose	(Karadag *et al.*, 2010)
2	Microorganisms from cow dung sludge	α-cellulose and xylose	Batch study using 125 mL serum bottles	2.8 mmol-H_2/g-cellulose and 0.3 mol-H_2/mol-xylose	5.8 mmol-ethanol/g-cellulose and 1 mol-ethanol/mol-xylose	(Lin *et al.*, 2008)
3	Enrichment microbial cultures from hot spring	Glucose/cellulose	0.45L continuous flow reactor	0.32 mol-H_2/mol-glucose	0.69 mol-ethanol/mol-glucose	(Koskinen *et al.*, 2007)
4	*Citrobacter* sp. Y19	Glucose	Batch study using 165 mL serum bottles	2.49 mol H_2/mol glucose	30.6±2.4% carbons of the substrate were directed toward ethanol production	(You-Kwan Oh *et al.*, 2003)
5	Anaerobic sludge immobilized on polyethylene-octane elastomer	Sucrose	Fluidized-bed reactor and packed-bed reactor	0.64 mol-H_2/mol-hexose	0.24 mol-ethanol/ mol-hexose	(Ken-Jer Wu *et al.*, 2007)
		Glucose		1.04 mol-H_2/mol-hexose	0.29 mol-ethanol/mol-hexose	
		Fructose		0.56 mol-H_2/mol-	0.65 mol-ethanol/ mol-	

SL. No.	Microorganism	Substrate	Process operation	Hydrogen production	Ethanol production	Reference
				hexose	hexose	
6	*Enterobacter aerogenes* NBRC 12010	Biodiesel industry effluent containing glycerol	Two-compartment bio electrochemical reactor (liquid volume 300 mL x 2)	0.77 mol-H_2/mol-glycerol	0.88 mol-ethanol/mol-glycerol	(Sakai *et al.*, 2007)
7	Extreme thermophilic microbial mixed culture	Glucose (2g/L) and additional nutrients	Batch process using 250 mL serum bottles	1.58 mol-H_2/mol-glucose	0.90 mol-ethanol/mol-glucose (equivalent to 0.23g-ethanol/g-glucose)	(Zhao, 2009)
8	*Enterobacter aerogenes* HU-101	Biodiesel industry effluent containing glycerol	Batch process using 125 mL serum bottles	1.12 mol-H_2/mol-glycerol	0.96 mol-ethanol/mol-glycerol	(Ito *et al.*, 2005)
9	Newly isolated strain (strain AK54) close to *Thermo anaerobacterium aciditolerans*	Acid treated cellulose hydrolysates	Batch process using 117.5 mL serum bottles	56.6 ± 2.1 mmol /L	29.6 ± 0.5 mmol /L	(Sigurbjorns dottir *et al.*, 2012)
10	*Escherichia coli* K12	Glycerol (20 g/L)	Study using 2.25 L bioreactor	1.40 mmol /L	0.32 mmol /L	(Chaudhary *et al.*, 2011)

SL. No.	Microorganism	Substrate	Process operation	Hydrogen production	Ethanol production	Reference
11	Immobilized sludge	Molasses	Study using 12.5L CSTR	10.74 mmol H_2/h/L	11.72 mmol ethanol/h/L	(Han *et al.*, 2011)
12	*Clostridium* sp. strain URNW	Cellobiose (2 g/L)	Batch study using 27 mL Balch tubes	14.2 mmol-H_2/L-medium	0.4 mmol-H_2/L-medium	(Ramachandran *et al.*, 2011)
13	Mixed culture (maintained on wastewater sludge)	Glucose	Semi-continuously operated reactor	100–200 mL-H_2/g-glucose (25–40% H_2)	Not specified	(Hwang *et al.*, 2004)
14	Mixed culture	Wastewater having molasses	Expanded granular sludge bed	3.47 mol-H_2/mol-sucrose	62–128 mg-ethanol/L (after 10 days)	(Guo *et al.*, 2008)
15	Mixed culture	Wastewater with molasses	CSTR	0.45 L-H_2/g-COD removed (35 days)	Not specified	(Nanqi Ren *et al.*, 2007b)

Table 2.3. 2: Ethanol production by microbial conversion of different organic substrates

SL. No.	Microorganism	Substrate	Process	Ethanol production	Reference
1	Baker's yeast (*Saccharomyces cerevisiae*)	Hydrothermal and enzymatically hydrolyzed wheat straw	Batch process using fermentation flask (200 mL)	0.41 g/g (glucose)	(Kaparaju *et al.*, 2009)
2	*Saccharomyces cerevisiae*	Marine macro algal (*Sacchorina latissima*) biomass	Batch process using Erlenmeyer flask (100 mL)	0.45 % (v/v)	(Adams *et al.*, 2009)
3	*Saccharomyces cerevisiae; Zymomonas mobilis; Kluyveromyces fragilis*	Processed tuber of *Jerusalem artichoke*	Batch process using Erlenmeyer flask (500 mL)	0.48 g/g	(Szambelan *et al.*, 2004)
4	*Pichia stipitis*	Rice straw hydrolysate	Batch process using Erlenmeyer flask (250 mL)	0.45 g/g	(Huang *et al.*, 2009)
5	*Candida tropicalis*	Rice straw hydrolysate	Batch process using polycarbonate flask (150 mL)	0.48 g/g	(Oberoi *et al.*, 2010)

Table 2.3. 3: Summary of different reports on simultaneous hydrogen and 1, 3-propanediol production

Sl. No.	Microorganism	Substrate	Process	Hydrogen production	1,3-propanediol production	Reference
1	Mixed culture (heat-treated)	Pure glycerol	Batch process using serum bottles (500 mL)	0.28 mol-H_2/mol-glycerol	0.69 mol-PD/mol-glycerol	(Selembo *et al.*, 2009)
2		Crude glycerol		0.31 mol-H_2/mol-glycerol	0.59 mol-PD/mol-glycerol	
3		Glucose		1.06 mol-H_2/mol-glucose	No PD production	
4	Microbial culture from organic soil	Biodiesel waste containing glycerol	Batch process using serum bottles (160 mL)	0.75 mol-H_2/mol-glycerol (at shorter hydrogen retention time)	0.65 mol-PD/mol-glycerol (at longer hydrogen retention time)	(Bingchuan Liu *et al.*, 2013)
5	*Halanaerobium saccharolyticum (saccharolyticum)*		Batch process using serum bottles (25 mL)	2.06 mol-H_2/mol-glycerol (without vitamin B_{12})	0.39 mol-PD/mol-glycerol (with vitamin B_{12})	(Kivistö *et al.*, 2011)
6	*Clostridium* spp. from Colombian soil	Pure glycerol	Batch process using bioreactor (4 L)	0.1962 mol-H_2/mol-glycerol	Not specified	(Bernal *et al.*, 2013)
7	*Enterobacter agglomerans*	Pure glycerol	Batch process using 120 mL	Negligible H_2 production	0.51mol-PD/mol-glycerol	(Barbirato *et al.*, 1995)

Sl. No.	Microorganism	Substrate	Process	Hydrogen production	1,3-propanediol production	Reference
			penicillin flasks			
8	Recombinant *Escherichia coli*	Pure glycerol	Batch process using 100 mL of medium	-	0.3 g-PD/g-glycerol	(Dabrowski *et al.*, 2012)
9	*Citrobacter freundii* ATCC 8090	Crude glycerol (20 g/L)	Batch process using 500 mL flasks	-	4.85 g-PD/L-medium	(Ferreira *et al.*, 2012)
10	*Clostridium butyricum* CNCM 1211	Crude glycerol	-	-	63.4 g-PD/L	(Drożdżyńska *et al.*, 2011)
11	*Klebsiella pneumoniae* DA-1HB	Pure glycerol	-	-	70.5 g-PD/L	

Table 2.3. 4: Summary of different reports on simultaneous hydrogen and butyric acid production

SL. No.	Microorganism	Substrate	Process	Hydrogen production	Butyric acid production	Reference
1	*Clostridium tyrobutyricum* 3T	Microalgae (*Scenedesmus obliquus*) hydrolysate	-	1.36 mol-H_2/mol-sugar	0.49 mol-butyric acid/mol-sugar	(Ortigueira *et al.,* 2012)
2	*Clostridium butyricum* W5	Molasses	Batch process using a bioreactor with a working volume of 1.5 L	1.85 mol-H_2/mol-hexose	0.27 g-butyric acid/g-hexose	(Xiaoyi Wang *et al.*, 2009)
3	*Clostridium tyrobutyricum* PAK-Em	Glucose	5 L stirred-tank bioreactor operated in fed-batch mode	0.024 g-H_2/g-glucose	0.42 g-butyrate /g-glucose	(Xiaoguang Liu *et al.*, 2006)
4	Anaerobic sludge of reactor used for the treatment of swine wastewaters	Glucose 2 g/L	Anaerobic fluidized bed reactor with a HRT of 2 hours	2.49 mol-H_2/mol-glucose	40.99 % of total soluble microbial products	(de Amorim *et al.*, 2012)
5	Mixed culture from activated sludge	Molasses	3.1L continuous acidogenic reactor	~ 40 %	720 mg/L	(NQ Ren *et al.*, 2007a)
6	*Clostridium beijerinckii*	Food waste	Batch H_2 production using	231 mL H_2/200 mL of	3,546 mg/L	(Jung Kon

SL. No.	Microorganism	Substrate	Process	Hydrogen production	Butyric acid production	Reference
	KCTC 1785		500 mL serum bottle	medium		Kim *et al.*, 2008)
7	Sludge from anaerobic digester	Cassava wastewater	Batch H_2 production using 75 mL serum bottle	340.19 mL-H_2/ g-COD	36.24% of VFS produced	(Reungsang *et al.*, 2007)
8	Sludge compost	Wastewater from sugar factory	Continuous fermentation using a 5L vessel	2.59 mol-H_2/mol-hexose	0.62 mol-butyrate/mol-hexose	(Ueno *et al.*, 1996)
9	*Clostridium acetobutylicum* ATCC 824 (mutant strain M5)	Glucose (10 g COD/L)	Continuous fermentation using a 2L vessel	2.44 ± 0.16 mol-H_2/mol-glucose	2870 ± 60 mg/L	(Sang-Eun Oh *et al.*, 2009)
10	Heat treated sludge	Glucose (25 g COD/L)	Batch H_2 production using 150 mL serum bottle	~ 375 mL-H_2/g-cell	554.3 mg-COD/L	(Cheong *et al.*, 2006)
11	*Clostridium* sp. AK_{14}, isolated from hot spring	Glucose (20 mM)	Batch H_2 production using 23 mL serum bottle	30.5 mM-H_2	10.7 mM-butyrate	(Almarsdottir *et al.*, 2010)
12	Anaerobic digester sludge enriched with supplementary fat	L-Arabinose (30 g COD/L)	Batch H_2 production using 125 mL serum bottles	2.5 mol-H_2/mol-arabinose utilized	61.0 % of soluble microbial products	(Abreu *et al.*, 2009)

SL. No.	Microorganism	Substrate	Process	Hydrogen production	Butyric acid production	Reference
13	Anaerobically digested sludge from wastewater treatment plant	Corn syrup waste	A novel continuous fermentation process using a 5L bioreactor	3.2 mol-H_2/mol-hexose	3793 ± 671 mg-butyrate/L	(Hafez *et al.*, 2009)
14	*Enterobacter aerogenes* st. E. 82005	Glucose (10 g/L)	Batch process using a 2.5L bioreactor	~15 mmol/g-cell/h	0.36 mol -butyrate/mol-glucose	(Kurokawa *et al.*, 2005)
15	*Clostridium sp HN001*	Starch	Continuous fermentation using a 1L bioreactor operated at HRT of 9h	2.0 mol-H_2/mol-hexose	9.3 mmol- butyrate/L-culture/ h	(Yasuda *et al.*, 2010)
16	Seed sludge from laboratory scale anaerobic digester	Sucrose (10 g/L)	Batch process using glass bottles	131.9 mL-H_2/g-sucrose	131.7 mg-butyrate/g-sucrose	(Young Joon Lee *et al.*, 2001)

Table 2.3. 5: Bio-methane production using dark fermentative hydrogen production liquid waste

Sl. No.	Original substrate	Process	Microorganism/inoculum		Hydrogen production	Methane production	Reference
			H_2 production	**CH_4 production**			
1	Potato processing waste	Continuous two stage process using one 1L and one 5L bioreactor	Sludge	Digested sludge	119 mL-biogas/h with 45% (v/v) H_2	141 mL-biogas/h with 76% (v/v) CH_4	(Zhu *et al.*, 2008)
2	Food waste	Continuous two stage process using one 10L (H_2 production) and one 40L (CH_4 production) bioreactor	Sludge from anaerobic digester of wastewater treatment plant		205 mL-H_2/g-volatile solid	464 mL-CH_4/g-volatile solid	(Chu *et al.*, 2008)
3	Sucrose	Continuous two stage process using one 11L CSTR (H_2 production) and one 20L upflow filter (CH_4 production)	Sludge from mesophilic digester	Digested sludge supplemented with sucrose	1.62 ± 0.2 mol-H_2/mol-hexose	323 mL-CH_4/g-COD	(Kyazze *et al.*, 2007)
4	Food waste	Continuous two stage process using one 10L (H_2 production) and one 40L (CH_4 production) bioreactor	Heat treated sludge from anaerobic digester	Sludge from anaerobic digester (without treatment)	11.1 L-H_2/L-fed/d	47.4 L-CH_4/L-fed/d	(Dong-Yeol Lee *et al.*, 2010)

Sl. No.	Original substrate	Process	Microorganism/inoculum		Hydrogen production	Methane production	Reference
			H_2 production	**CH_4 production**			
5	Partially processed garbage and paper waste	Continuous two stage process using one 200L CSTR (H_2 production) and one 500L internal recirculation packed-bed reactor (CH_4 production)	Thermophilic microbial consortium from activated sludge compost	Anaerobic sludge from a thermophilic methanogenic process	5.4 L-H_2/L/d	6.1 L-CH_4/L/d	(Ueno *et al.*, 2007)
6	Cassava stillage	Continuous two stage H_2 and CH_4 production using 300 mL reactors	Anaerobic granular sludge		73.7 ± 3.2 mL-H_2/g-volatile solid	379.6 ± 13.0 mL-CH_4/g-volatile solid	(Wen Wang *et al.*, 2011)

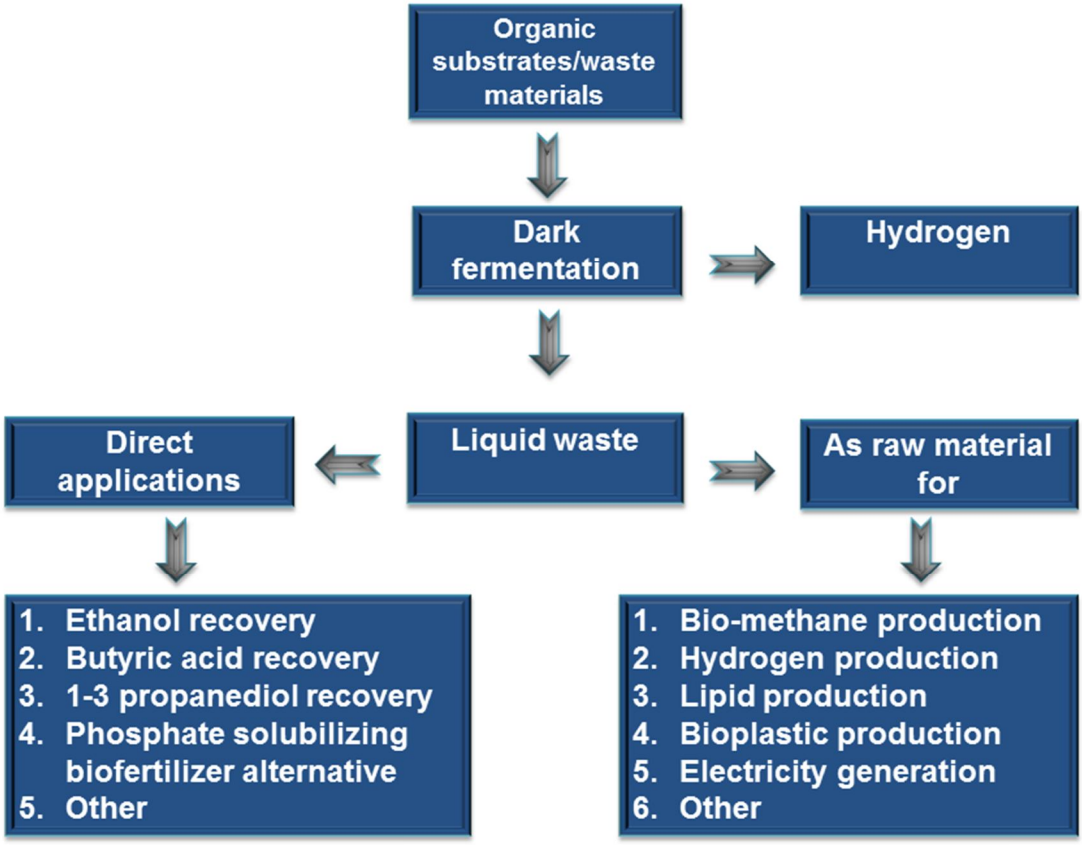

Figure 2.3. 1: Possible applications of the liquid waste from dark fermentative hydrogen production.

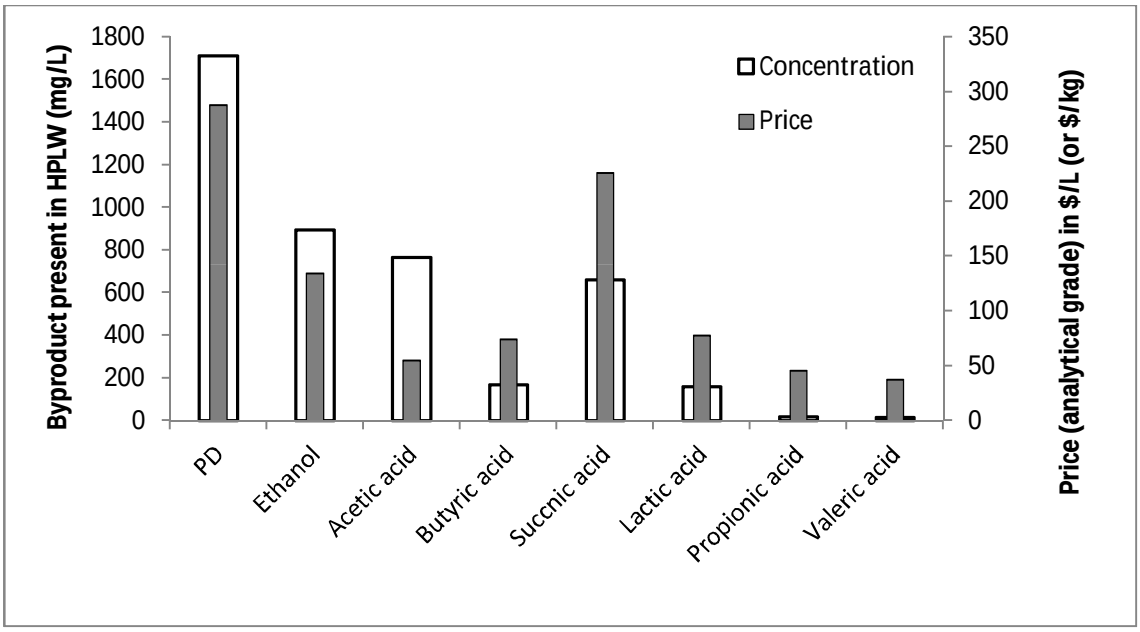

Figure 2.3. 2: Reported concentration levels of different metabolites present in HPLW and their market price in US dollars (Han *et al.*, 2010, Lalaurette *et al.*, 2009, Selembo *et al.*, 2009).

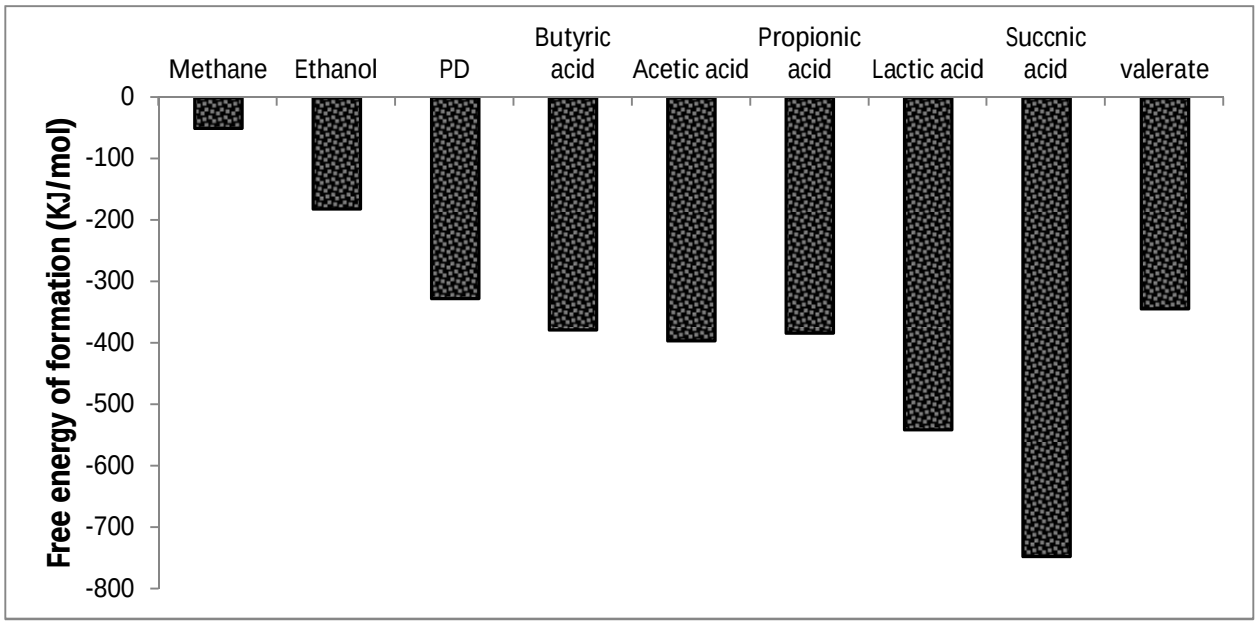

Figure 2.3. 3: Free energy of formation (25º C) of different metabolites produced during biohydrogen production.

PART-IV

BIO-HYDROGEN PRODUCTION BY BIODIESEL DERIVED CRUDE GLYCEROL BIOCONVERSION: A TECHNO-ECONOMIC EVALUATION

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Résumé

La production mondiale de biodiesel est en constante augmentation et il est proportionnellement accompagné d'une énorme quantité de glycérol brut (GB) comme sous-produit. En raison de sa nature brute, le GB a un intérêt commercial très bas, bien que son homologue pur a des applications industrielles. En variante, le GB est une très bonne source de carbone et peut être utilisé comme matière première pour la production d'hydrogène par fermentation. En outre, son utilisation présente un double avantage, à savoir qu'elle offre une méthode durable pour l'élimination des déchets de fabrication du biodiesel, et qu'elle produit des biocarburants à faible émission de gaz à effet de serre (GES). La fermentation en deux étapes, comprenant la phase sombre et la photo-fermentation est une des options les plus prometteuses pour la production de biohydrogène. Dans la présente étude, la faisabilité technico-économique d'un tel processus en deux étapes a été évaluée. L'analyse a été faite sur la base des avancées récentes dans la production d'hydrogène par fermentation à l'aide du GB comme matière première. L'étude a été réalisée avec une référence particulière sur le marché du biodiesel en Amérique du Nord et, plus précisément, les données disponibles pour les provinces canadiennes et le Québec ont été utilisées. Sur la base de notre analyse technico-économique, l'augmentation du coût de production a été démontrée comme étant le principal goulot d'étranglement dans la production commerciale de l'hydrogène par fermentation. Cependant, certaines options alternatives réalisables pour la réduction des coûts de procédé ont été identifiées. En plus des réductions des émissions de GES, le processus de bioconversion du GB dans son ensemble offre des avantages environnementaux supplémentaires. Les estimations calculées démontrent que la bioconversion d'un kg de glycérol brut (70% p/v) permet de réduire les émissions de GES de 7,66 kg d'équivalent CO_2.

Mots clés: biodiesel; biohydrogène; glycérol brut; gaz à effet de serre; l'évaluation technico-économique

Abstract

Global biodiesel production is continuously increasing and it is proportionally accompanied by a huge amount of crude glycerol (CG) as by-product. Due to its crude nature, CG has very less commercial interest; although it's pure counterpart has different industrial applications. Alternatively, CG is a very good carbon source and can be used as a feedstock for fermentative hydrogen production. Further, a move of this kind has dual benefits, namely it offers a sustainable method for disposal of biodiesel manufacturing waste as well as produces biofuels and contributes in greenhouse gas (GHG) reduction. Two-stage fermentation, comprising dark and photo-fermentation is one of the most promising options available for bio-hydrogen production. In the present study, techno-economic feasibility of such a two-stage process has been evaluated. The analysis has been made based on the recent advances in fermentative hydrogen production using CG as a feedstock. The study has been carried out with special reference to North American biodiesel market; and more specifically, data available for Canadian province, Québec City have been used. Based on our techno-economic analysis, higher production cost was found to be the major bottleneck in commercial production of fermentative hydrogen. However, certain achievable alternative options for reduction of process cost have been identified. Further, the process was found to be capable in reducing GHG emissions. Bioconversion of one Kg of crude glycerol (70 % w/v) was found to reduce 7.66 Kg CO_2 eq (equivalent) GHG emission, and the process also offers additional environmental benefits.

Key words: Biodiesel; bio-hydrogen; crude glycerol; greenhouse gas; techno-economic evaluation

Introduction

Biodiesel is one of the renewable alternatives of fossil fuel which is mainly produced from vegetable oils and animal fat. Due to availability of feedstock and requirement of very simple technology for its production and its role in greenhouse gas (GHG) reduction; global production of biodiesel is rapidly increasing. European Union is the largest producer of biodiesel in the world followed by North America (NA). NA's biodiesel market is mainly dominated by the U.S., which is the second largest biodiesel producing nation with production of nearly 17.7 percent of the world's biodiesel in 2009. Further, biodiesel production in US has been promoted by US government through various subsidies and policies. The revised renewable fuel standard (RFS_2) program has been devised, according to which by 2012 annually a minimum of 1 billion gallons of biodiesel is to be blended into U.S. diesel fuel and it will require approximately 36 billion gallons of renewable fuel by 2022 (http://www.biodieselmagazine.com/articles/4080/report-12-billion-gallons-of-biodiesel-by-2020. Aaccessed 27 Dec 2011). Similarly, Canada is a major biodiesel producer in NA and Canadian government has set a target of 17 percent reduction of GHG by 2020 and 2 % renewable content will be mandatory for diesel oil (http://www.ec.gc.ca/default.asp?lang=En&n=714D9AAE-1&news=BDE26528-CBCB-4008-949D-CDFDFBD054FB. Aaccessed 27 Dec 2011; Regulations amending the renewable fuels regulations, Canadian environmental protection act, 1999, department of the environment, Canada. https://tsapps.nist.gov/notifyus/docs/wto_country/CAN/corrigenda/pdf/CAN311_add_3(english).pdf. Aaccessed 27 Dec 2011). Hence, biodiesel production in NA will be expected to increase significantly in the coming decade. It is well established that biodiesel production is accompanied by generation of glycerol as a by-product, by weight, which is almost 10 % of the total biodiesel produced (Thompson *et al.*, 2006). Therefore, there will be a sharp increase in crude glycerol (CG) production in recent future and management of such a huge amount of waste will be a problem for biodiesel manufacturers. Interestingly, CG can be used as a feedstock for production of number of valuable products; however, due to presence of various impurities in biodiesel derived glycerol, product recovery is expensive (Pachauri *et al.*, 2006). Alternatively, CG

can be used as a substrate for hydrogen production, in which case, produced H_2 is automatically separated from the media and it can be recovered using simple technology such as, pressure swing adsorption. Moreover, produced H_2 can be used as a fuel with no harmful emissions during its combustion (Auer *et al.*, 2000); and hence, it has the potential of reducing GHG emission by replacing conventional fossil fuels. Further, the microbial biomass generated during H_2 production process can be used for biogas production by anaerobic digestion. There are number of different strategies for H_2 production that have been investigated (Das *et al.*, 2001, Hawkes *et al.*, 2007, Nath *et al.*, 2004); however, based on comparison of different processes, very high H_2 production potential has been reported with a two-stage fermentation process. In an investigation by Yokoi et al (2002), dark fermentation was shown to produce 2.7 mol H_2 mol^{-1} glucose and subsequent photo-fermentation of spent media by *Rhodobacter* sp. M-19 was reported to produce additional 4.5 mol H_2 mol^{-1} glucose (Manish *et al.*, 2008, Yokoi *et al.*, 2002). Further, by production of 1 Kg of hydrogen, such two-stage process has the potential to reduce GHG emissions by 7.31–9.37 Kg CO_2 eq (Manish *et al.*, 2008). Therefore, the purpose of this article is to make a techno-economic evaluation of a two-stage fermentation process for H_2 production by CG bioconversion based on different assumptions corresponding to the recent technological progresses and available literature data.

Biodiesel and CG production in NA

Considering global biodiesel market, US and Canada are the two major representatives of North America (NA). Therefore, for convenience of estimation, data available for US and Canada together will be considered as the data for whole NA. Soybean oil, canola oil and animal fat (tallow) are common feedstock used for biodiesel production in NA. Total annual production of soybean oil in NA is estimated to be 8.84 million metric tonne (http://www.soystats.com/2011/page_21.htm. Aaccessed 27 Dec 2011; http://www.indexmundi.com/agriculture/?country=ca&commodity=soybean-oil&graph=production. Aaccessed 27 Dec 2011). Tallow is the second major biodiesel feedstock in NA and its annual production is 2.73 million metric tonne (http://www.petfoodindustry.com/Columns/Ingredient_Issues/2390.html Aaccessed 27

Dec 2011; C.B. Prakash, A critical review of biodiesel as a transportation fuel in Canada. A technical report. http://www.dieselduck.ca/library/05%20environmental/1998%20Biodiesel%20in%20Canada.pdf. Aaccessed 27 Dec 2011). Similarly, Canada is the topmost canola oil producer in the world and annual production of canola oil in NA is 2.06 million metric tonne (http://www.canolacouncil.org/ind_overview.aspx. Aaccessed 27 Dec 2011; The CRB Commodity Yearbook 2007. By Commodity Research Bureau. http://books.google.ca/books?id=YqKVT3w67KoC&pg=PA317&lpg=PA317&dq=The+CRB+Commodity+Yearbook+2007&source=bl&ots=sbgW7MZjEj&sig=rZlGYVgWr8zce3M8YDZ45CmuDAo&hl=en&sa=X&ei=JJH7ToSwM8ru0gG9xsC5Ag&ved=0CDkQ6AEwAg#v=onepage&q=The%20CRB%20Commodity%20Yearbook%202007&f=false.
Aaccessed 27 Dec 2011). Currently, in US alone, there are nearly 170 biodiesel manufacturing plants (Glycerin becomes “The Newest Biofuel” with the MK Glycerin Burner from AlterHeat. http://www.glycerinburners.com. Aaccessed 27 Dec 2011) and annually they are producing around 2839.05 million liters of biodiesel (http://www.earth-policy.org/datacenter/xls/book_wote_ch9_biofuels_9.xls. Aaccessed 27 Dec 2011). Annual production of biodiesel and biodiesel feedstock in leading North American nations are summarized in Table 2.4.1 and by weight, the total annual biodiesel production in NA is found to be only 19.51 % of the available feedstock. Therefore, NA has the potential to further increase its annual biodiesel production. As mentioned earlier, during biodiesel production, a huge amount of CG is produced as waste by-product and annually around 0.2 million metric tonne of CG have been produced in NA (http://www.biodieselmagazine.com/articles/1123/combating-the-glycerin-glut.
Aaccessed 27 Dec 2011). Considering annual production of excess feedstock and favourable government policy for increased biodiesel production and well established market, it can be expected that in the coming years, North American biodiesel production will eventually increase. Therefore, in near future, CG production will also be further increased and a sustainable method of CG management will be necessary.

CG bioconversion process overview

Till date, various strategies have been applied for H_2 production by CG bioconversion among which dark anaerobic fermentation is the most well studied process. Ngo et al (2011) have reported the production of 2.73 ± 0.14 mol-H_2 mol^{-1} glycerol by *Thermotoga neapolitana* (Ngo *et al.*, 2011). Similarly, Fernandes et al. (2010) have reported the production of 200 ml-H_2 g^{-1} COD (Fernandes *et al.*, 2010). In another similar investigation, Marques et al (2009) have used *Enrerobacter aerogenes* and the authors have reported the production of 2.5 dm^3-H_2 dm^{-3} medium (Marques *et al.*, 2009). However, in all such cases despite very high hydrogen yield, bioconversion efficiency is still limited by accumulation of fermentation end products. Alternatively, hydrogen production efficiency of a two-stage process is reported to be highest among other microbiological processes (Manish *et al.*, 2008). In this case, end-products accumulated during first phase of fermentation are further utilized by subsequent fermentation. Therefore, in this study we will investigate the techno-economic feasibility of a two-stage process, where dark fermentation will be followed by photo-fermentation for maximum bioconversion of CG. Figure 2.4.1 represents the proposed process and bioconversion of one Kg of CG will be evaluated considering the cost of materials and power requirement in various steps.

In general, CG from biodiesel manufacturing plant contains approximately 70 % (w/w) glycerol (Sabourin-Provost *et al.*, 2009, Priscilla A. Selembo *et al.*, 2009b, Tang *et al.*, 2009). For most bioconversion study, around 0.25 % to 1 % (w/v) glycerol has been successfully utilized for bio-hydrogen production (Ito *et al.*, 2005, Kivisto *et al.*, 2010, Ngo *et al.*, 2011, P. A. Selembo *et al.*, 2009a). However, high H_2 yield has been reported at relatively low initial glycerol concentration (Ito *et al.*, 2005). Therefore, in this study 0.25 % (w/v) glycerol will be considered as initial glycerol concentration to be used for H_2 production. Hence, for bioconversion of one kilogram of CG, it should be diluted to 400 L (0.25 % w/v) solution. Therefore, a 1000 L continuous stirred tank reactor (CSTR) will be considered for entire bioconversion process and different calculations will be based on this assumption.

Inoculum preparation

5% (v/v) inoculum will be considered for proposed bioconversion study and hence, 20 L inoculum will be required for 400 L (20 L + 380 L) media. It will be developed in a 50 L CSTR containing 20 L MD media. The MD medium contains per liter of deionized water: glucose monohydrate (5 g), casein peptone (5 g), yeast extract (0.5 g), KH_2PO_4 (2 g), $MgSO_4.7H_2O$ (0.5 g) and L-cysteine hydrochloride (0.5 g) (Thonart *et al.*, 2010). Based on the catalogue (2011) of a professional chemical supplier (VWR, Canada); total cost for 20 L media component will be $44.88. Similarly, electricity charges will be applicable for sterilization of media and incubation of inoculum at 37±1ºC. It will take approximately 2 h to sterilize the total media and approximately 15 h of incubation for inoculum development. For a production scale bioreactor, power input requirement is around 1-3 kW/m^3 (Scale-up of bioprocess, Chapter 11 in NV&L. http://www.chemeng.lth.se/kte071/Arkiv/scale-up.pdf. Aaccessed 27 Dec 2011). Hence, considering a 50 L CSTR having *in situ* sterilization facility and with 0.05 kW requirements, 0.85 kWh of power will be needed for entire inoculum development process. According to the present electricity tariff of Hydro-Quebec, which is 4.46 cent/ kWh; electricity cost for inoculum development will be $ 0.037 (http://www.hydroquebec.com/residential/tarif-affaires.html. Aaccessed 27 Dec 2011).

CG pre-treatment

It is well known that CG from biodiesel manufacturing plant contains different impurities. Methanol and soap are the two major impurities present in CG and they may be inhibitory to microbial growth (Athalye *et al.*, 2009, Chi *et al.*, 2007, Ngo *et al.*, 2011). However, methanol evaporates at 65 ±1 °C; hence, the sterilization of CG is capable of removing significant amount of methanol (Ethier *et al.*, 2011, Pyle *et al.*, 2008). Similarly, the other major impurity present in CG is soap and it can be removed by precipitation through pH adjustment (Athalye *et al.*, 2009, Ethier *et al.*, 2011). As suggested by Chi et al. (2007), to reduce the viscosity, CG will be first diluted with distilled water at 1:4 ratio. Later, HCl will be added to reduce the pH to 6.5 to convert the soluble soap into insoluble free fatty acids and precipitated solid will be discarded (Chi *et al.*, 2007). In general, initial pH of CG is around 11-12 and after dilution with

distilled water, total volume of the CG solution will be around 5L. Hence, at least 200 ml of HCl will be required for its pH adjustment and it will cost approximately $ 2.38.

Media preparation

It can be found from the literature that when CG is diluted with only deionized water, no significant microbial growth and hydrogen production was observed (Ito *et al.*, 2005). However, after dilution with suitable buffer solution containing supplementary nutrient, significant improvement in cell growth and hydrogen production has been observed (Ito *et al.*, 2005). Similarly, Selembo et al (2009) and some other workers have also used a suitable nutrient solution for CG bioconversion experiment (Fernandes *et al.*, 2010, Sabourin-Provost *et al.*, 2009, P. A. Selembo *et al.*, 2009a). Therefore, for bioconversion, CG will be diluted with phosphate buffer containing 4.58 g/L Na_2HPO_4 and 2.45 g/L $NaH_2\ PO_4$. H_2O (pH 7.0) (P. A. Selembo *et al.*, 2009a). Further, addition of yeast extract was found to increase the CG consumption rate (Ito *et al.*, 2005); hence 1g/L yeast extract will be added to the media containing CG. For 380 L media, about 1740.4 g Na_2HPO_4, 931 g $NaH_2\ PO_4.H_2O$ and 380 g yeast extract will be required. The cost for these items will be $ 96.97, $ 38.82 and $ 25.25, respectively.

Anaerobic dark fermentation

Prepared media will be sparged with N_2 for 5 to 10 min followed by sterilization at 121±1^oC for 20 min. A nitrogen generator will be used for obtaining pure N_2 flow and the reactor vessel along with the media will be sterilized *in situ*. Ito et al (2005) have reported complete consumption of glycerol within one day in a batch experiment for initial concentration as high as 2.5 % (Ito *et al.*, 2005). Further, Selembo et al (2009) have observed nearly 18 h of lag phase in H_2 production when CG bioconversion was investigated using mixed microbial culture (P. A. Selembo *et al.*, 2009a). However, they have shown that by using a freshly collected inoculum from previous CG fermentation, the lag phase of H_2 production could be reduced to 5 h. Similarly, Ngo et al (2011) have shown that when *Thermotoga neapolitana* was used as inoculum, H_2 production rapidly increased after 6 h of incubation. However, after 24 h of cultivation, the process pH dropped and the rate of H_2 production was also declined (Ngo *et al.*, 2011). From these observations, it can be assumed that a batch of H_2 production process using CG as

feedstock will be completed within 48 h. Further, Mahanty et al (2010) have shown that charge of electricity for a 3 L bioreactor operated at constant agitation and aeration was $ 0.38 per hour, however, nearly half of the cost was due to aeration (Mahanty *et al.*, 2010). In the case of anaerobic bioconversion, aeration will not be required and the charges will be even lower. As it is mentioned earlier, maximum electricity requirement of a 1000 L CSTR is 1 kW, hence, electricity charge for entire dark fermentation can be calculated to be around $ 2.14.

Photo fermentation

For 400 L spent media of dark fermentation; 20 L inoculum (5% v/v) of phototrophic bacteria will be used. Hence, cost of inoculum development can be assumed to be the same as compared to that of dark fermentation. For photo- fermentation, pH adjustment of the spent media and addition of supplementary media component may be necessary (Tao *et al.*, 2007). Therefore, we will assume that 0.5 g/L yeast extract and 200 g NaOH will be added to the spent media before sterilization. It is known from the literature that during photo-fermentation, after 6^{th} day, the rate of H_2 production decreased (Sabourin-Provost *et al.*, 2009, Tao *et al.*, 2007). In this investigation, we will assume that fermentation will be stopped at 144 h. Usually, 4000 to 5000 lux light intensity is used for photo-fermentation (Tao *et al.*, 2007, Yokoi *et al.*, 2002). Sabourin and Hallenbeck (2009) have used a 50 W halogen light for a 125 ml reactor used for CG bioconversion and reported very high hydrogen yield (Sabourin-Provost *et al.*, 2009). In the present market, 50 L photo-bioreactor (Applicon ®, Netherlands) are available with 120 watt lighting panel (http://www.applikon-bio.com. Aaccessed 27 Dec 2011). Hence, for convenience of calculation, a 1000 L photo-bioreactor with 2400 W lighting panel will be considered for bioconversion of 400 L spent media and electricity charges for the entire operation will be $21.83.

Treatment of waste and biogas production

Based on the estimation made so far, nearly 400 L of waste will be generated during H_2 production from one Kg of CG. Considering the volume, possible microbial biomass and nutrient content of CG bioconversion waste, it can be easily concluded that a proper treatment will be necessary before it can be released to the environment. In a recent

report, Manish and Banerjee (2008) have concluded that biological hydrogen production is very efficient only when recovery and utilization of by-products is considered (Manish *et al.*, 2008). Therefore, the waste will be further digested in an anaerobic digester for biogas generation. Considering the media composition of bioconversion experiments, the spent media should contain approximately 5.52 Kg dry biomass. Siswo (2010) have reported that by anaerobic digestion of effluent, 726.43 ml biogas /gram total solid can be produced (Siswo, 2010). Hence, nearly 4010.9 L biogas can be produced using the spent media. Further, 1 m^3 (1000 L) biogas is equivalent to 0.6 L of diesel oil (Michael AM, Khepar SD, Sondhi SK. Water wells and pumps. http://www.mcgrawhill.ca/professional/products/9780071591201/water+wells+and+pumps. Aaccessed 27 Dec 2011). Therefore, produced biogas can replace 2.40 L fossil diesel of around $ 3.27 (136.2 cents/ L).

Building construction, equipment & labour

Following equipment will be required for bioconversion of 1 Kg of CG: N_2 generator, 50 L CSTR, 1000 L CSTR, 1000 L photo-bioreactor, pressure swing adsorption technology (for H_2 separation) and anaerobic digester. In general, instruments have 3-4 years warranty and remain in good condition for 10 years with minimum maintenance and all buildings have at least 30 years of lifespan (Lardon *et al.*, 2009). Based on such facts, for present estimation it will be assumed that cost of construction, equipment and labour add 10 % additional expenses in H_2 production process.

Hydrogen yield

Still only a few reports are available on H_2 production by CG bioconversion and maximum H_2 yield reported in all such available reports are summarized in Table 2.4.3. Dark fermentation with pure and mixed culture, photo-fermentation as well as microbial electrolysis cell has been used so far for H_2 production from CG. Culture conditions and experiment setups in all such methods are different from each other and hence, maximum H_2 yield in all such cases are different. From Table 2.4.3 it can be seen that Sabourin-Provost (2009) have reported that a maximum yield of 6 moles of H_2 per mole glycerol has been achieved, which is 75 % of maximum possible theoretical yield (Sabourin-Provost *et al.*, 2009). Hence, based on molecular weight, from 92.09 g

glycerol, maximum 6 g H_2 could be generated. Therefore, from one Kg of CG (70 % w/v), using presently available technology, approximately 45.6 g of H_2 could be generated.

A cost benefit analysis of CG bioconversion process

Table 2.4.2 and Figure 2.4.2 represent the estimated cost distribution of CG (1 Kg) bioconversion process. From this analysis, it can be summarized that the operation cost of the processes considered in this study is very high. Similarly, different CG bioconversion and bio-hydrogen production processes till now followed by different researchers are more or less identical to the process assumed here and hence, they are also not economically feasible for commercial application due to very high process cost involved. However, these processes offer number of different options for reduction of the process cost. Firstly, from Figure 2.4.2, it is evident that cost of media constituents is about 82 % of the total process cost. It is only because of the large volume of nutrient media that has to be added to dilute the CG to a very low initial concentration for its bioconversion. This problem can be easily solved if the experiment can be carried out at higher initial CG concentration, so that, total volume of supplementary media needed for the process can be reduced. In the present estimation, initial concentration of CG was considered to be 2.5 g/L; however, numbers of reports are available, where H_2 production was demonstrated at 10 g/L or higher initial concentrations (Kivisto *et al.*, 2010, Ngo *et al.*, 2011, P. A. Selembo *et al.*, 2009a). Even report on bioconversion of CG at 50 g/L initial concentration is also available (Liu *et al.*, 2011). Therefore, 40 times or more reduction in process cost will be possible if bioconversion process is carried out at higher initial CG concentration. However, at high initial substrate concentration, H_2 production rate will be decreased and it will take longer incubation time to complete a batch of bioconversion reaction. Interestingly, from Figure 2.4.2, it is clear that cost of electricity is only 8% of the total process cost (Quebec City, Canada scenario). Therefore, a longer incubation time will not have much effect on the total process cost. Secondly, the cost of inoculum development is nearly 27 % of the total production cost. However, if the residual biomass of previous batch is used as inoculum, the process cost can be further reduced by 27 %. Similarly, without

any supplementary material, CG alone can also support microbial growth and it can be fermented for H_2 production. In that case, more than 92 % reduction in process cost is possible; although, there is a possibility of reduction in H_2 yield. However, before ruling out the possibility of fermentation of CG without any supplementary media, maximum H_2 yield of such process should be experimentally determined and its economic feasibility should be evaluated. Further, cheap nitrogen sources such as, slaughter house wastewater; brewery wastewater as well as activated sludge from wastewater treatment plant should also be evaluated as supplementary nutrient for CG bioconversion process. This option has the potential of maintaining high H_2 yield as well as reduction of process cost. From Figure 2.4.3 it can be seen that the electricity requirement of photo-fermentation is more than 90 % of total electricity requirement of the process. It is mainly because of the high intensity light source which needs to be maintained throughout the fermentation period. Interestingly, from Table 2.4.2 and Figure 2.4.3, it can be concluded that the electricity cost for dark fermentation is nearly 10 times lesser than that of photo-fermentation. Therefore, instead of two-stage dark and photo-fermentation, repeated batch dark fermentation may be a suitable option, where one cycle of dark fermentation will be followed by another cycle of dark fermentation at different environmental conditions. For example, spent media (containing residual glycerol) of first dark fermentation can be diluted with fresh media before second cycle of dark fermentation; pH, temperature and microorganism used for first cycle may be different in second cycle. Such an approach may be helpful in maximum utilization of glycerol and increasing H_2 yield per mole glycerol utilized; and also will cut the additional electricity cost of photo-fermentation. However, it is only an assumption and it needs to be established by further investigation.

One Kg of mass of hydrogen has an energy value of 120-142 MJ (http://hypertextbook.com/facts/2005/MichelleFung.shtml. Aaccessed 27 Dec 2011). Alternatively, energy density for diesel fuel ranges from 32 to 40 MJ/L (http://hypertextbook.com/facts/2006/TatyanaNektalova.shtml. Aaccessed 27 Dec 2011). Therefore, 1 Kg of H_2 can replace 3.55 L of conventional diesel. Similarly, it has been already mentioned that using presently available technology, by bioconversion of 1 Kg of CG, 45.6g H_2 and 4010.9 L biogas can be obtained. Considering the energy

content of the two fuels, hydrogen and biogas, biofuels produced from 1 Kg of CG are capable of replacing 2.56 L of fossil diesel. Hence, 0.2 million metric tonne CG annually produced in NA can replace 512 million liters of fossil diesel worth of 697.34 million dollar (Table 2.4.5).

Estimation of environmental benefit of CG bioconversion process

GHG emission reduction

Methane yield coefficient of crude glycerol is 0.306 m^3 CH_4/Kg glycerol (Siles Lopez *et al.*, 2009). Therefore, if the total CG produced in NA is disposed to ultimate landfill, annually at least 61.2 million m^3 CH_4 will be generated. Alternatively, if CG is converted to animal feed, it will generate 659 Kg CO_2eq/tonne crude glycerol (Carbon Life Cycle Calculation for Biodiesel, Home Grown Cereals Authority, United Kingdom. http://www.co2star.eu/final-documents/Annex3-4-WP2-D3_Life%20cycle%20biodiesel.pdf. Aaccessed 27 Dec 2011). In such a situation, where very few literature data on CG GHG potential are available, for present estimation, these figures will be assumed as CG GHG emission potential. Further, during production (refining) of 1 Kg fossil diesel approximately 360.41 g CO_2 is released to the atmosphere; therefore, production of 2.56 L diesel will generate 0.77 Kg CO_2 eq GHG (Sheehan *et al.*, 1998). From Table 2.4.2, total electricity requirement for bioconversion of 1 Kg of CG can be estimated to be 539.3 kWh. According to a recent report by Environment Canada, CO_2 emission coefficient for electrical power (Quebec, Canada, 2008) is 2 g CO_2 equivalent per kWh (http://www.ec.gc.ca/ges-ghg/default.asp?lang=En&n=EAF0E96A-1. Aaccessed 27 Dec 2011). Therefore, during bioconversion process of 1 Kg of CG, nearly 1.07 Kg of CO_2 will be generated. This estimation will be valid only when it is considered that CG produced in a biodiesel manufacturing plant is used in the same plant for H_2 production and GHG emission due to CG transportation is negligible. As a fuel, liquid H_2 produces very low or zero emissions and the only waste product while burning hydrogen is water vapour, with a small amount of NO_x (Nojoumi *et al.*, 2009). Similarly, during combustion of 1 Kg biogas, 748.02 g GHG (CO_2 and CH_4) is generated (Yu *et al.*, 2008). Therefore, few grams of biogas produced during bioconversion of 1 Kg CG will have a very negligible

contribution on GHG emission, and hence, it is not considered for present estimation. Similar to economic cost benefit analysis, GHG emission due to CG bioconversion plant construction is assumed to be 10 % of the same of CG bioconversion process. From previous discussion it can be found that H_2 and biogas produced during bioconversion of 1Kg CG is capable of replacing 2.56 L of diesel. Further, combustion of one liter diesel produced around 2.9 Kg CO_2 (http://www.abc.net.au/tv/carboncops/factsheets/cc_cars.pdf. Aaccessed 27 Dec 2011). Therefore, as shown in Table 2.4.4, biofuels produced during bioconversion of 1 Kg of CG has the potential to reduce nearly 7.42 Kg CO_2eq of GHG by replacing the fossil fuel. However, supplementary media components needed to be used in both dark and photo-fermentation will also make a small contribution towards GHG emission. Therefore, net GHG reduction potential of the present process will be slightly lower than the estimated value. However, if any industrial waste could be used as supplementary material, as previously discussed, GHG emission reduction potential of the proposed process will further increase. Thus, overall environmental and economic benefit of the proposed process is summarized in Table 2.4.5.

An outlook on other environmental benefits

Crude glycerol is an environmental hazard and it has objectionable effect on human health. According to material safety data sheet (MSDS) of CG, it can enter the body by inhalation, ingestion or through skin and may cause irritation in respiratory track, skin and eye. Ingestion may cause diarrhea, nausea and headache and chronic exposure may cause kidney injury. Persons suffering from skin and eye problems and with liver and kidney disorder are more prone to the adverse effect CG (Material safety data sheet (crude glycerol): http://www.biodieselgear.com/documentation/MSDS_Glycerol.pdf. Aaccessed 27 Dec 2011). According to US-EPA, land filling, composting, land application, subsurface disposal, direct discharge to surface water (dumping), treatment at a publicly owned treatment works (POTW) and anaerobic digestion are the different options of CG management (Environmental laws applicable to construction and operation of biodiesel production facilities, U.S. Environmental Protection Agency. http://www.epa.gov/region07/priorities/agriculture/pdf/ethanol_plants_manual.pdf.

Aaccessed 27 Dec 2011). However, all such methods have certain limitations. Firstly, due to the presence of methanol, CG may have a flash point well below 140º F, which will make it an ignitable hazardous waste. If the particular crude glycerin waste is considered as a hazardous waste, for landfill disposal it must meet land disposal restriction universal treatment standards of 40 CFR (Code of Federal Regulations) 268.48 and it will further increase biodiesel waste disposal cost. Additionally, some landfills refuge CG due to the concern of spontaneous combustion. Secondly, CG is purely a carbon source and cannot be composted alone and it requires a source of nutrient. Also, because of very high BOD, high energy content, and viscosity of CG, vigorous management of the compost would be required to maintain its aerobic nature (Environmental laws applicable to construction and operation of biodiesel production facilities, U.S. Environmental Protection Agency. http://www.epa.gov/region07/priorities/agriculture/pdf/ethanol_plants_manual.pdf. Aaccessed 27 Dec 2011). Additionally, pH of CG is very high and it has little buffering capacity [54]; hence, during composting, pH should be externally maintained in composting range. Similarly, either due to high salt content, high methanol content or limited transpiration due to physical barrier posed by viscous CG, direct land application can potentially burn the plant cover of the particular land. Further, due to high BOD, CG may be harmful to aquatic organisms and its direct discharge to surface water is prohibited.

Alternatively, presently proposed system of bioconversion of CG to produce hydrogen and anaerobic digestion of the biomass obtained from the process may be a substitute of these different CG treatment methods. Therefore, from environmental point of view, the present method has dual benefit of reducing environmental pollution by proper disposal of CG as well as potential contribution towards GHG reduction by production renewable and eco-friendly fuels i.e., hydrogen and biogas.

Conclusions

Crude glycerol is the major by-product of biodiesel production process equivalent to 10% by weight. Due to global warming concerns and strict policies of leading nations, global biodiesel production is rapidly increasing. However, such increase demands a

sustainable method for management of glycerol rich biodiesel manufacturing waste. Alternatively, crude glycerol from biodiesel production plant can be used as a feedstock for biological hydrogen production. In the present investigation, techno-economic feasibility of a two-stage fermentation process for CG bioconversion and hydrogen production has been evaluated. Considering the materials, power, facility and labour requirement, total cost associated with the process was calculated. From this analysis, it was observed that the media components required for the process covered nearly 82 % of total production cost. Based on the analysis, few propositions have been developed for reduction of the process cost. Further, the process was found to be very beneficial from environmental point of view. It was observed that bioconversion of 1 Kg of CG has the potential to reduce nearly 7.66 Kg of GHG. Moreover, the process has the potential to mitigate possible environmental hazards due to improper disposal of CG.

Abbreviations

CFR (Code of federal regulations), CG (crude glycerol), CSTR (continuous stirred tank reactor), EPA (Environmental protection agency, US), GHG (greenhouse gas), kWh (kilowatt hour), MSDS (material safety data sheet), RFS (Revised renewable fuel standard)

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Table 2.4. 1: Feedstock availability and annual biodiesel and CG production in North America

Item	**Annual production (MMt / million liter)**		
	US	**Canada**	**Total**
Soya bean oil	8.6 MMt	0.24 MMt	8.84 MMt
Canola oil	0.46 MMt	1.6 MMt	2.06 MMt
Animal fat (Tallow)	2.53 MMt	0.2 MMt	2.73 MMt
Biodiesel	2839.05 million liter	190 million liter	3029.05 million liter
CG	0.181 MMt	0.019 MMt	0.2 MMt

Table 2.4. 2: **Cost distribution of crude glycerol (1 Kg) bioconversion and hydrogen production using a two-stage process**

SL No.	Process	Item required	Amount	Cost $
1.	Inoculum development (20 L)	Glucose monohydrate (5 g/L)	100 g	4.6
		Casein peptone (5 g/L)	100 g	22.51
		Yeast extract (0.5 g/L),	10g	1.2
		KH_2PO_4 (2 g/L),	40g	8.15
		$MgSO_4.7H_2O$ (0.5 g/L)	10g	0.45
		L-cysteine hydrochloride (0.5 g/L)	10g	7.97
		Sterilization & incubation (17 h)	Electricity 0.85 kWh	0.037
2.	Pretreatment of CG	HCl	200 ml	2.38
3.	Media preparation (380 L)	Na_2HPO_4 (4.58 g/L)	1740.4 g	96.97
		$NaH_2\ PO_4.H_2O$ (2.45 g /L)	931 g	38.82
		Yeast extract (1g/L)	380 g	25.25
4.	Anaerobic dark fermentation	Sterilization & incubation (48h)	Electricity 48 kWh	2.14
5.	Photo-fermentation	Inoculum development (20 L)	Same to dark fermentation	44.92

SL No.	Process	Item required	Amount	Cost $
		NaOH	200 g	1.46
		Yeast extract (0.5 g/L)	190 g	24.42
		Illumination and incubation (144 hours)	Electricity 489.6 kWh	21.83
6.	Treatment of waste	Biogas will be produced	4010.9 L	(-) 3.27
7.	Equipment and labour	10 % additional cost	-	29.98

Table 2.4. 3: **Hydrogen production by different fermentation techniques using crude glycerol as a cheap substrate**

Fermentation	**Inoculum**	**Experiment conditions**			**Maximum hydrogen yield**	**References**
		Initial CG concentration (g/L)	**Incubation time (h)**	**Incubation temperature (°C)**		
Dark fermentation (pure culture)	*Thermotoga neapolitana* DSM 4359	05	200	75	1.98 ± 0.10 mol-H_2 mol^{-1} glycerol	*(Ngo et al., 2011)*
	Enterobacter aerogenes HU-101	10	12	37	63 mmol-H_2 L^{-1} h^{-1}	*(Ito et al., 2005)*
	Enterobacter aerogenes	20	-	37	2448.27 ml-H_2 L^{-1} medium	*(Marques et al., 2009)*
Dark fermentation (mixed culture)	Anaerobic sludge	COD 3.67	25	25.0±0.5	200 ml-H_2 g^{-1} COD	*(Fernandes et al., 2010)*
	Heat treated mixed culture	03	72	30	0.31 mol-H_2 mol^{-1} glycerol	*(P. A. Selembo et al., 2009a)*
Photo fermentation (pure culture)	*Rhodopseudomonas palustris*	~ 0.9	240	30	6 mol-H_2 mol^{-1} glycerol	*(Sabourin-Provost et al., 2009)*
Microbial electrolysis cell (pure culture)	*Enterobacter aerogenes* NBRC 12010	09.9	36	30	0.77 mol-H_2 mol^{-1} glycerol	*(Sakai et al., 2007)*

Fermentation	**Inoculum**	**Experiment conditions**			**Maximum hydrogen yield**	**References**
		Initial CG concentration (g/L)	**Incubation time (h)**	**Incubation temperature (°C)**		
Microbial electrolysis cell (mixed culture)	Domestic wastewater	01	36	30	2.3 mol-H_2/mol-glycerol	*(Priscilla A. Selembo et al., 2009b)*

Table 2.4. 4: **Estimation of environmental benefit from bioconversion of 1 Kg crude glycerol using a two-stage process**

GHG emission sources	**GHG emission**	**References**
GHG emission from electricity consumed during bioconversion of 1 Kg CG	1.07 Kg of CO_2eq	Present estimation and (http://www.ec.gc.ca/ges-ghg/default.asp?lang=En&n=EAF0E96A-1. Aaccessed 27 Dec 2011)
GHG emission due to permanent constructions (10 % of CG bioconversion process)	0.107 Kg CO_2eq	
GHG emission potential of 1 Kg CG (if disposed or used for other purpose)	0.65 Kg CO_2eq	Present estimation and (Carbon Life Cycle Calculation for Biodiesel, Home Grown Cereals Authority, United Kingdom. http://www.co2star.eu/final-documents/Annex3-4-WP2-D3_Life%20cycle%20biodiesel.pdf. Aaccessed 27 Dec 2011)
GHG emission during combustion of hydrogen and biogas obtained from 1 Kg CG	Negligible	Present estimation
GHG emission during production of 2.56 L (equivalent to 1 Kg CG) fossil diesel	0.77 Kg CO_2 eq	Present estimation and (Sheehan *et al.*, 1998)

GHG reduction by replacing fossil fuel combustion with H_2 and biogas from 1 Kg CG	7.42 Kg CO_2eq	Present estimation and (http://hypertextbook.com/facts/2005/MichelleFung.shtml. Aaccessed 27 Dec 2011; http://hypertextbook.com/facts/2006/TatyanaNektalova.shtml. Aaccessed 27 Dec 2011; http://www.abc.net.au/tv/carboncops/factsheets/cc_cars.pdf. Aaccessed 27 Dec 2011)
Total GHG reduction by 1 Kg CG bioconversion process	7.66 Kg CO_2eq	Present estimation

Table 2.4. 5: **Table summarizing possible benefits of the crude glycerol bioconversion process considered in the present study**

Crude glycerol	**Estimated H_2 production**	**Estimated biogas production**	**Estimated fossil fuel replacement**	**Estimated GHG reduction** (Kg CO_2eq)	**Estimated value of the process**
1 Kg CG	45.6 g	4010.9 L	2.56 L	7.66 Kg	$ 3.49
CG produced in NA/year	09.12 million Kg	802180 million liter	512 million liter of diesel	1532 million Kg	$ 697.34 M/year

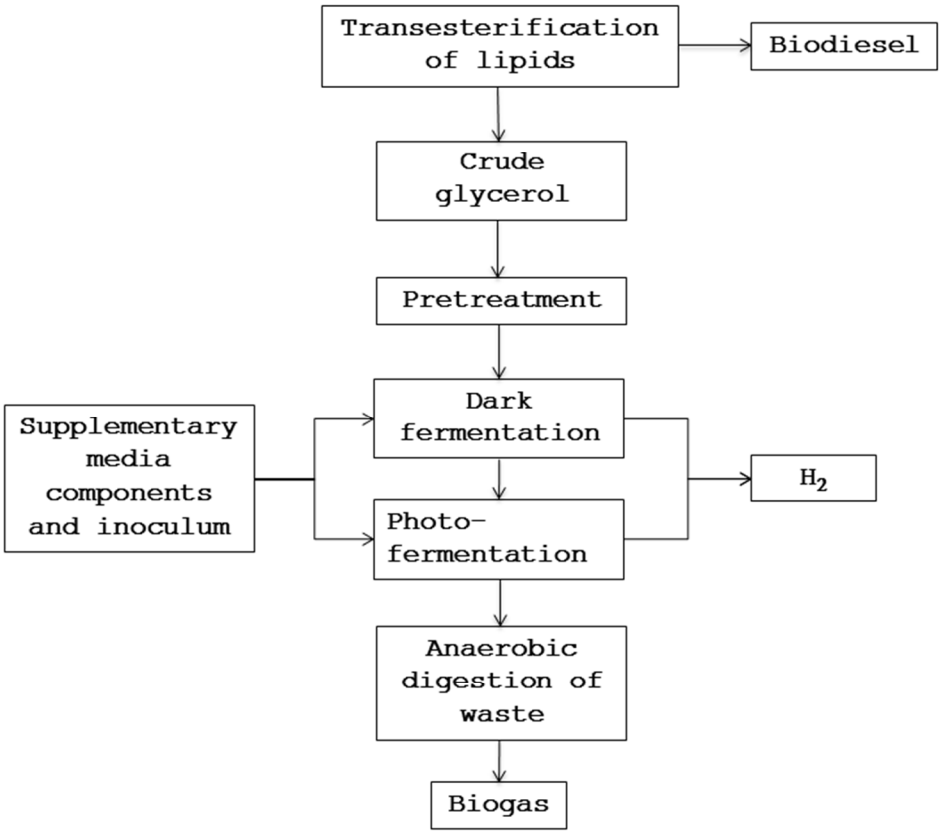

Figure 2.4. 1: Schematic representation of the process considered for the present estimation

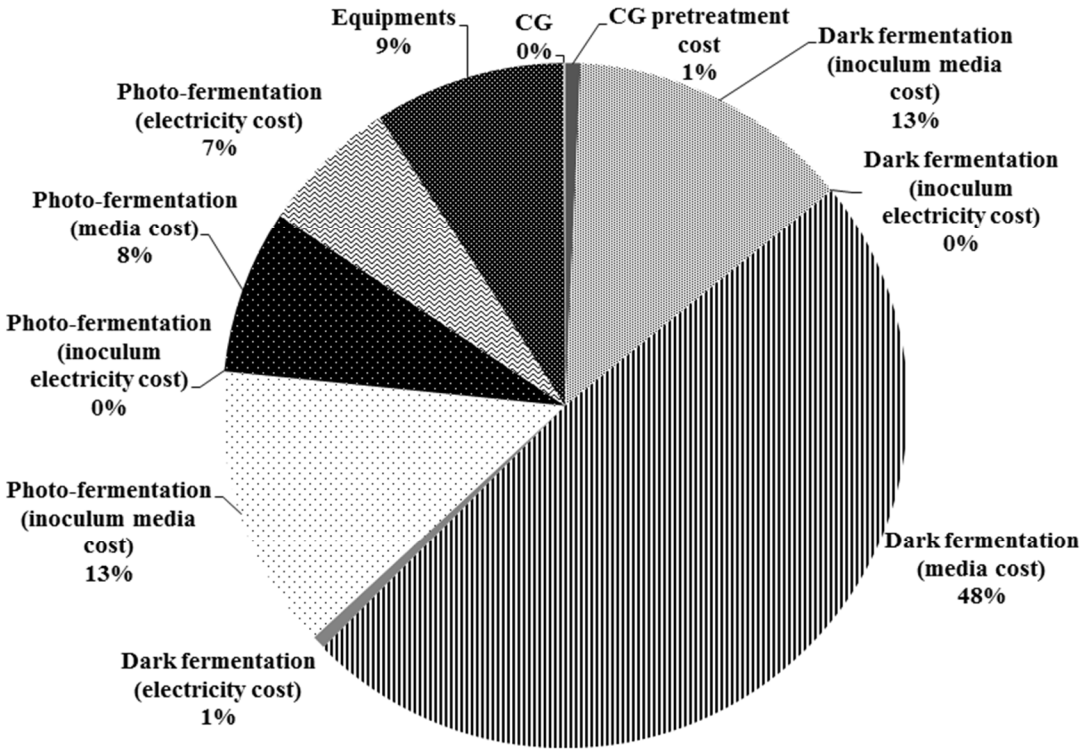

Figure 2.4. 2: Cost distribution of the crude glycerol bioconversion process

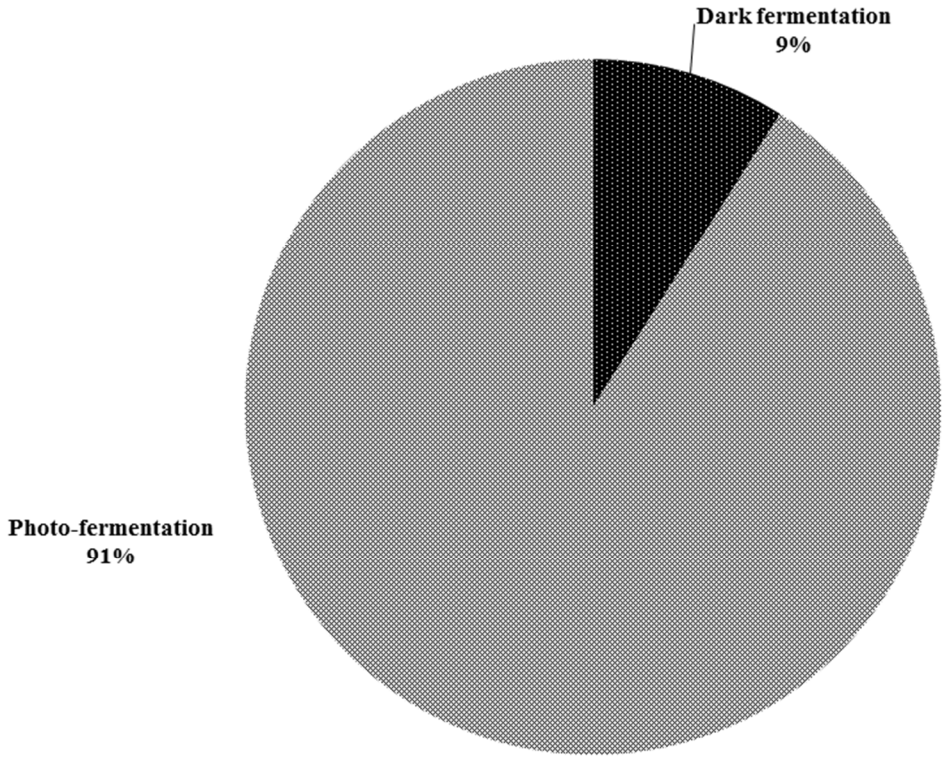

Figure 2.4. 3: Electricity requirement of a two-stage fermentation process for CG bioconversion and H_2 production

CHAPTER 3

HYDROGEN PRODUCTION BY CRUDE GLYCEROL BIOCONVERSION: A PRACTICAL SUMMARY

PART I

HYDROGEN PRODUCTION FROM MEAT PROCESSING AND RESTAURANT WASTE DERIVED CRUDE GLYCEROL BY ANAEROBIC FERMENTATION AND UTILIZATION OF THE SPENT BROTH

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Résumé

CONTEXTE: Le glycérol brut (GB), le principal sous-produit de processus de production de biodiesel pourrait être utilisé pour la production de biohydrogène. Cependant, la production d'hydrogène par fermentation est fortement limitée par le coût des solutions tampons et des nutriments supplémentaires nécessaires à la fermentation. Ainsi, le but de la présente étude était de déterminer le potentiel maximal de production de H_2 à partir du GB et ce, en l'absence de tout supplément coûteux supplémentaire. De plus, la valorisation des rejets de fermentation (post production de H_2) est un autre objectif qui a été abordé.

RÉSULTATS: Un maximum de production de H_2 de 2022,5 mL/L milieu de culture a été réalisé par bioconversion du GB seul (sans éléments nutritifs supplémentaires). Une concentration de 10 g/L de GB a été jugée optimale. En outre, l'addition d'une petite quantité de 50 mg/L de biomasse microbienne en phase endogène (carencé) provenant d'une fermentation antérieure a permis d'améliorer la production de 32,50%, avec un taux maximum de 1,040 mL/L/jour. De même, près de 75% du H_2 totale a été produit à un pH aussi bas que 3,8, indiquant une tolérance élevée au milieu acide par la souche *Enterobacter aerogenes* NRRL B407 utilisée.

CONCLUSION: Le glycérol brut provenant des procédés de transformation des graisses animales et des déchets de restaurant a été caractérisé et évalué pour son potentiel de production de H_2. L'utilisation d'une biomasse âgée en carence de substrat provenant d'une fermentation antérieure (comme complément) a permis d'améliorer les performances du procédé.

Mots clés: biodiesel, glycérol brut, production d'hydrogène, pH, culture carencée

Abstract

BACKGROUND: Crude glycerol (CG), the major by-product of biodiesel production process could be used for biohydrogen production. However, fermentative hydrogen production is highly limited by the cost of buffer and additional nutrients required for the process. Thus, the purpose of the present study was to determine maximum H_2 production potential of CG in the absence of any additional expensive supplement. Further, sustainable utilization of the waste of H_2 production process was the other objective.

RESULTS: A maximum production of 2022.5 mL H_2/L media has been achieved by CG bioconversion (without any additional nutrient) and 10 g/L CG was found to be optimum. Further, addition of spent biomass (50 mg/L) of the process into a subsequent process was found to improve the production by 32.50% with a maximum rate of 1040 mL/L/d. Similarly, nearly 75% of total H_2 were produced at a pH as low as 3.8, indicating high acid tolerance of the strain (*Enterobacter aerogenes* NRRL B407) used.

CONCLUSION: Meat processing and restaurant waste based CG has been characterized and evaluated for maximum H_2 production potential. Likewise, utilization of spent biomass of CG bioconversion process (as supplement) was found to improve the process performance.

Key words: Biodiesel, crude glycerol, hydrogen production, pH, spent broth

Introduction

Owing to the concern of global warming and limited storage of fossil fuel, global production of biodiesel is rapidly increasing (Hasheminejad *et al.*, 2011, Pachauri *et al.*, 2006). European Union and USA are the two leading biodiesel producers and presently they have 120 and 176 active biodiesel manufacturing facilities, respectively (Sarma *et al.*, 2012). Further, biodiesel production has been promoted by different governments through various subsidies and policies. US government has devised the revised renewable fuel standard (RFS_2) program, according to which by 2022, approximately 36 billion gallons of renewable fuel has to be blended into U.S. diesel fuel (Sarma *et al.*, 2013). Similarly, Canadian government has set a target of blending the diesel with at least 2 % renewable fuel and 17 % reduction of greenhouse gas emission by 2020 (Sarma *et al.*, 2013). Therefore, further increase in global biodiesel production is expected in the coming years. Biodiesel is mainly produced by the transesterification of vegetable oil and animal fats (Endalew *et al.*, 2011). However, increased application of these materials for biodiesel production may increase the price of these materials in food market (Mata *et al.*). Therefore, low cost materials such as, the waste of meat processing industry containing animal fat and waste cooking oil from restaurants are becoming popular as the substrate for biodiesel production (Mata *et al.*, Meng *et al.*, 2008). During biodiesel production, for every 100 Kg of biodiesel, 10 Kg of crude glycerol (CG) is generated as by-product (Selembo *et al.*, 2009, Yazdani *et al.*, 2007). However, due to the presence of large amount of impurities, unlike its pure counterpart, CG has very low commercial value (Pachauri *et al.*, 2006). Moreover, CG derived from complex waste materials such as, meat processing and restaurant waste may have even more impurities than the CG derived from pure substrates and its commercial application will be further limited. Further, direct release of CG to the environment, without proper treatment, is known to be unacceptable for the environment (da Silva *et al.*, 2009, Selembo *et al.*, 2009). Alternatively, CG is a good carbon source and its application for fermentative hydrogen production may be a sustainable means for its value added utilization (Ngo *et al.*, 2011, Selembo *et al.*, 2009). Likewise, water is the

only major byproduct of H_2 combustion process (Selembo *et al.*, 2009) and hence, the proposed process will contribute in reducing the greenhouse gas emission.

As already mentioned, due to high glycerol content, CG is mainly a carbon source and hence, addition of other nutrient source is required for its enhanced bioconversion. Further, during H_2 production a range of organic acids are produced to change the media pH (Jame *et al.*, 2011) and the process quickly approaches towards termination. Therefore, the media is supplemented with buffering agents to avoid any drastic pH change. However, we have made a techno-economic evaluation of the process (Sarma *et al.*, 2013) and found that the cost of the buffer and synthetic media components used for the process may be as high as 82% of total process cost. Similarly, during H_2 production by bioconversion of 1Kg of CG, 400 L of spent media will be generated (Sarma *et al.*, 2013) which will be composed of a range of organic acids, residual CG, residual nutrient and biomass. Thus, the present investigation has three objectives- (i) evaluation of H_2 production by bioconversion of meat processing and restaurant waste derived CG without applying any buffering agent and synthetic media components, (ii) screening of less expensive nutrient source for the process and; (iii) utilization of spent broth of the process. Further, *Enterobactor aerogenes* is a well-known microorganism for its CG bioconversion and H_2 production ability (Ito *et al.*, 2005, Marques *et al.*, 2009, Sakai *et al.*, 2007) and it was considered for the present investigation. Thus, it is an integrated study to improve the CG bioconversion process in terms of H_2 yield, as well as to develop a sustainable process for utilization of the spent media generated during H_2 production to develop a minimum waste sustainable process which can be integrated into the glycerol refinery in a typical biodiesel production plant.

Materials and methods

Microorganism and inoculum development

The microorganism considered for this study is *Enterobacter aerogenes* NRRL B-407, collected from ARS, USDA, USA. It is a gram-negative, rod-shaped facultative anaerobic bacteria belonging to the family Enterobacteriaceae. A complex media containing glucose (5 g/L), casein peptone (5 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 to grow and maintain the microorganism (Beckers *et al.*, 2010). For this purpose, 125 mL serum bottles (working volume 50 mL) were used and anaerobic environment was created by sparging the media with pure N_2 gas for 2 min. The culture was incubated in orbital incubator shaker maintained at 150 rpm and 30°C (Marques *et al.*, 2009) and the log phase culture broth was used as inoculum throughout the study.

Chemicals and reagents

Crude glycerol (CG) used in the present study was collected from Rothsay®, Canada. Rothsay uses the fat containing waste from meat processing plants and used grease from restaurants to produce biodiesel (http://www.rothsay.ca/products/biodiesel/, accessed on 19/07/2013). Chemicals, such as glucose, $MgSO_4.7H_20$, NaOH, sodium periodate, acetylacetone, ethanol and acetic acid were purchased from Fisher scientific, Canada. Casein peptone and KH_2PO_4 was purchased from VWR, Canada. Yeast extract used in the present investigation was a gift from Lallemand, Canada.

Characterization of CG

Glycerol content and physical properties

Glycerol content of the CG was determined by UV-Vis spectrophotometer and the detailed method has been separately described later. The density of crude glycerol was determined from the volume and weight of crude glycerol measured at room temperature (24 ± 0.5 °C) (Hu *et al.*, 2012). Similarly, to determine the pH, 1 g of crude glycerol was dissolved in 50 mL of deionized water and at room temperature (24 ± 0.5 °C), the pH of the solution was measured by a digital pH meter (EcoMet®, Istek Inc.) (Hu *et al.*, 2012).

Elemental analysis

The CG was analyzed for different elements, such as Na, K, Fe, Ca, Mg, Mn, Al, P, Co, Cu, and Zn by using ICP-AES (Varian VISTA-AX, CCD simultaneous ICP-AES). Prior to the analysis, the sample was digested by a method slightly modified from the original method used for acid digestion of soil and sediment

(http://www.epa.gov/osw/hazard/testmethods/sw846/pdfs/3050b.pdf). Briefly, 500 mg of sample was taken in a glass tube and placed in a block digester (Technicon BD-40). About 5 mL of HNO_3 (50%) was added to the sample, the temperature of the block was increased up to 95 ± 1°C and left for 10 min. After removing the tube, it was allowed to cool down for 15 min and 2.5 mL of concentrated HNO_3 was added. Later, the tube was again incubated for 1 hour at 95 ± 1°C, which was subsequently allowed to cool down for 30 min. Further, 3 mL H_2O_2 was added and the tube was incubated for 10 min at the same condition as already mentioned and allowed to cool down for 5 min. This step was repeated two more times following which the tube was left for two hours at 95 ± 1°C. After this period, 2.5 mL of concentrated HNO_3 was added and the tube was again left for 15 min in the same digestion block operated at 95 ± 1°C. Finally, the sample was cooled down and the volume was adjusted to 50 mL using distilled water before analysis.

Carbon and nitrogen content of the CG sample was analyzed by total organic carbon/total nitrogen measuring unit (Shimadzu TOC-VCPH/TNM-1) using standard protocol (http://www2.shimadzu.com/apps/appnotes/tocan102.pdf). The important conditions of the analysis were as follows: injection volume (500µL), sparging time (7 min), carrier gas flow rate (150 mL/min) and furnace temperature (720 °C).

Investigation of the effect of CG concentration on hydrogen production

To determine the effect of initial CG concentration on hydrogen production, five different concentrations of CG were tested, viz., 2.5 g/L, 5g/L, 10 g/L, 15 g/L and 20 g/L. Similar to inoculum development, 125 mL serum bottles (working volume 50 mL) were used for hydrogen production experiments and CG was diluted with deionized water to achieve different initial concentrations. At pH 6.0, high hydrogen production by the microorganism *E. aerogenes* has been reported (Sakai *et al.*, 2007) and hence, in all cases, the pH of the media was adjusted to 6.0. Media was sparged with pure N_2 gas as already described and the bottles were sealed with aluminum crimp seal containing silicone septum (Fisher scientific, Canada). After autoclaving, the media was allowed to cool to room temperature and inoculated with 5 % (v/v) inoculum equivalent to 0.25 mg

dry cell mass. Bottles were incubated in the same way as described in previous section and 1 mL of aqueous sample was collected at 12 h interval. Composition of the produced gas was analyzed by gas chromatography; the volume of produced H_2 was measured at 12 h interval as described later.

Characterization of spent media fraction

To determine the cell mass present, 10 mL of the spent media was centrifuged at 5000 x g for 15 min, cell pellet was collected, dried at 105° C, weighed and the value was expressed as mg dry cell/L media (Chang *et al.*, 2005). pH of the broth was determined by a digital pH meter (EcoMet®, Istek Inc. Korea). The spent broth was also subjected to an elemental analysis including carbon and nitrogen content using ICP-AES and TOC/TN analyzer, respectively. The method of sample preparation and analysis used for this investigation was similar to that used for CG and already discussed in details.

Utilization of spent media fraction in subsequent process for H_2 production

To evaluate the possibility of utilizing the spent media of H_2 production process for CG bioconversion, a separate set of experiment was carried out. Media containing 10 g/L CG (pH: 6.0) was prepared, inoculated with *E. aerogenes* and incubated for 7 d as stated previously. The spent broth generated from this batch process was mixed with 10 g/L sterile CG taken in different serum bottles, at different proportions (2, 5, 10 and 20 % v/v) however, the final working volumes of all the reactors were kept constant at 50 mL. All the bottles were inoculated with 5% (v/v) inoculum, however, a control experiment was also carried where, 10 g/L CG was mixed with only spent broth (5 % (v/v) and no fresh inoculum was used. As during first cycle of fermentation, CG was almost completely utilized, the final glycerol concentration in all the culture bottles was nearly constant. Further, the incubation conditions and sampling interval were same as already described.

Utilization of active biomass from spent media

To evaluate the possibility of recycling the living biomass of H_2 production process, a small scale batch H_2 production experiment was carried out. Five different serum bottles

containing 10 g/L CG were prepared in the same way as already described. Further, the spent broth of CG (10 g/L) fermentation was centrifuged at 4000 x g for 5 min at 4°C (Sorvall® RC 5C plus, Sorvall instrument, Du pont) and the cell pellet was collected. Collected biomass ranging from 5 to 30 mg/L (equivalent dry cell mass) was mixed with 5 % (v/v) fresh inoculum and distributed into all the different serum bottles containing sterilized media. However, similar to the previous study, one control experiment was performed using active biomass equivalent to 5 mg dry cell mass/L, where no fresh inoculum was used. All the bottles were incubated and the gas and liquid samples were collected and analyzed in the same way as described in previous sections.

Utilization of spent biomass as a supplementary nutrient

To evaluate the possibility of recycling the residual biomass of H_2 production process, *E. aerogenes* cells were grown in a media containing 10 g/L CG, using the same experimental setup and conditions used for H_2 production experiments. After 7 days of fermentation, the cells were harvested by centrifugation (5000 x g for 5 min) and mixed with 10 g/L CG at different concentrations ranging from 5 to 150 mg/L. The different serum bottles containing the media and the cell pellet was then sterilized, inoculated and incubated in the same way as described in previous sections.

Analysis

Glycerol analysis

During hydrogen production experiments, residual glycerol concentration was measured by the spectrophotometric method described by (Bondioli *et al.*, 2005). The method is based on periodate oxidation of glycerol where, 1 mL of sample was mixed with 1 mL of ethanol (47.5% v/v) followed by the reaction with 1.2 mL each of acetylacetone solution (0.2 M) and sodium periodate solution (10 mM) at 70 ± 1°C for 1 min. Following the reaction, the samples were immediately cooled to 20-25 °C using tap water and maintained in the same temperature until analyzed using a Uv-Vis spectrophotometer (Carry 100 Bio®, Varian USA) at 410 nm.

Hydrogen analysis

The composition of produced gas was analyzed by a gas chromatograph (Varian 3800, USA) fitted with PoraPLOT Q® column (Agilent technology, USA) and thermal conductivity detector (TCD). The column temperature was 100 °C and Nitrogen was used as the carrier gas. The gas flow rate used in this analysis was 3.5 mL min^{-1} and the retention time was 4.5 min. The volume of produced H_2 was measured by the inverted cylinder –NaOH (10%) method as described by previous researchers (Junyapoon *et al.*, 2011, Maeda *et al.*, 2008). Briefly, the produced gas was passed through 100 mL NaOH (10% w/v) and collected in a graduated glass cylinder filled with water and placed in inverted position in a beaker containing water. Volume of H_2 produced was calculated from the displacement of water level in the cylinder (Junyapoon *et al.*, 2011, Maeda *et al.*, 2008) and, where applicable, considering the temperature and atmospheric pressure, volume of produced gas was converted to mmol (http://www.easycalculation.com/chemistry/ideal-gas.php).

Results and discussion

Crude glycerol characterization

The meat processing waste and waste grease derived CG fraction used in the present study was found to be composed of at least 23.63 ± 2.5 % (w/w) glycerol and the detailed characterization of the CG sample is presented in Table 3.1.2. The glycerol content of the CG sample investigated in this study was found to be lower than used food oil based CG (45 % glycerol, w/w) (Sakai *et al.*, 2007) and soybean oil based CG (63.3 % glycerol, w/w) (Hu *et al.*, 2012). However, total organic carbon content (35.9 % (w/w) ± 0.4) is notably higher than the total glycerol content of the CG investigated, indicating the presence of considerable amount of carbonaceous material other than glycerol. Similarly, compared to soybean oil based CG (0.3 ± 0.1 %, w/w), relatively higher nitrogen content (3.25 ± 0.1 %, w/w) has been observed; however, the finding is still comparable to soybean oil- waste vegetable oil based CG (1.2 ± 0.1%, w/w) (Hu *et al.*, 2012). The difference in nitrogen content indicates the possible presence of proteinaceous materials in the meat processing and restaurant waste grease derived

CG. Further, from the elemental analysis, presence of relatively higher amount of S (8827 ± 33 ppm) has been observed for the first time (as per the knowledge of the authors) in a CG sample. Moreover, the Fe content of the CG sample was also higher than soybean oil derived CG (Table 3.1.2). Coincidently, these two elements are the core structural components of Fe-S clusters of major H_2 producing enzymes (hydrogenases) (Kim *et al.*, 2011) and play a vital role in electron transfer during H_2 production (Marseille, 1998). Their presence, therefore, might have beneficial effect for a CG bioconversion process dedicated for H_2 production. Similarly, pH of the CG sample was found to be 3.4 (at 24 ± 0.5 °C), which is significantly lower than different reported values for soybean oil based CG (pH 6.9), waste vegetable oil based CG (pH 10.1) as well as pure glycerol (pH 6.4) (Hu *et al.*, 2012, Sakai *et al.*, 2007). Interestingly, such a low pH was not a problem for hydrogen production as CG was always diluted with distilled water before bioconversion process and the resulting solution had a pH of ~5.5. Further, the density of CG was determined to be 1106 g/L which is nearly similar to different reported values (Table 3.1.2) and lower than pure glycerol (Hu *et al.*, 2012).

Hydrogen production by CG bioconversion

CG at different concentrations (2.5 to 20 g/L) was tested as a feedstock for H_2 production and different profiles are presented in Figure 3.1.1. From Figure 3.1.1, it can be seen that for all concentrations, the rate of H_2 production was identical in the first 24 h. Further, in the case of 2.5 g/L CG, no H_2 production was observed after 24 h of incubation. Similarly, in the case of 5 g/L CG, the rate of H_2 production decreased after 48 h and no production was observed after 60 h. For 10, 15 and 20 g/L CG, H_2 production rates were almost similar and constant up to 60 h which were then decreased and no significant production was observed after 168 h of incubation. From these observations, it can be concluded that by increasing the CG concentration from 2.5 g/L to 10 g/L, the cumulative H_2 production can be increased from 580 mL/L to 2022.5 mL/L media. However, when the concentration was increased from 10 to 20 g/L, no significant improvement in H_2 production was observed. Such observation may be due to substrate inhibition or the impurities of CG, some of which are known to inhibit microbial growth. Similarly, Figure 3.1.1 shows the profiles of glycerol utilization during

H_2 production by using different concentrations of CG. From the Figure 3.1.1, it can be observed that in the case of 2.5 g/L CG, within 36 h, the glycerol was almost completely utilized by the microorganism. Similarly, in the case of 5 g/L CG, it was almost completely utilized within 120 h and in case of 10 g/L, 94.08 % glycerol was consumed within 7d. However, in the case of 15 and 20 g/L CG, only 71.96 and 59.05 % of the glycerol was utilized during the investigation period (7d). Therefore, considering both H_2 production and glycerol utilization, 10 g/L CG is chosen to be the optimal concentration to be used for further investigations.

Further, as the feedstock used in the present investigation is a waste material and contains different impurities; hence, pretreatment of the substrate may be needed prior to its application as a fermentation media. Methanol and solid particles present in the CG can be removed by rotary evaporation and centrifugation, respectively (Ngo *et al.*, 2011). Similarly, soap present in CG may be inhibitory for microbial growth and can be removed by precipitation using acid followed by centrifugation (Athalye *et al.*, 2009, Chi *et al.*, 2007). Similarly, as shown in Table 3.1.1, fermentation media can be supplemented with different synthetic media components. However, these additional steps of pretreatment and addition of supplementary nutrients can further increase the process cost. Hence, another objective of the present investigation was to evaluate H_2 production without any substrate pretreatment and synthetic media component. Moreover, the maximum cumulative H_2 production obtained in this investigation has been compared with different literature reports and presented in Table 3.1.1. Productivity of the present system was found comparable to different recent investigations and more importantly, addition of a large amount of supplementary media components and additional feedstock preparation have been successfully avoided without compromising the product yield (Table 3.1.1).

Figure 3.1.2 represents the pH profile of the media during H_2 production using different concentrations of CG. From the profiles, it is observed that in all cases, within first 24 h, the media pH dropped to below 4. Further, there is a correlation between the media pH at the end of the process and the initial CG concentrations. In the case of 20 g/L CG, the final pH of the broth was as low as 3.65; however, in the case of 2.5 g/L CG, the pH

was 4.06. Such observation may be due to the fact that in the case of 20 g/L CG, the substrate was available to the microorganism till the end of the fermentation and the amount of different organic acids produced was more than that of 2.5 g/L CG. In the latter case, the substrate was completely utilized within 24 h and the amount of organic acid produced was not sufficient to drop the pH below a certain level. Further, in this case, towards the end of the incubation period, the media pH was increased from 3.74 to 4.06. A possible reason for such observation may be that in the absence of available carbon source, the organic acids produced during first 24 h were utilized by the microorganism to increase the media pH. However, it is only an assumption and further investigation will be necessary to prove it. Further, as shown in Table 3.1.1, different buffering agents are commonly added to the CG based culture media used for H_2 production (Ngo *et al.*, 2011, Selembo *et al.*, 2009). The main reason of such practice is to prevent any drastic change in media pH due to simultaneous production of different organic acids during H_2 production, in turn, which may inhibit the process. Figure 3.1.3 represents the H_2 production and pH profiles for 10 g/L CG. It can be seen that nearly 75 % of total H_2 was produced when the media pH was around 3.8. Interestingly, cumulative H_2 production is still comparable with different recent investigations carried out in the presence of buffering agents (Table 3.1.1). Therefore, it can be concluded that *E. aerogenes* can continue H_2 production in a comparable rate at a pH as low as 3.8 and addition of buffering agents to the process may be avoided. This observation may be important for future commercialization of the process, where the requirement of a large amount of buffering agents could be avoided to boost the economic feasibility of such processes.

Further, to have an idea about the importance of the waste materials generated during the process, a mass balance analysis has also been made. From the analysis, it was observed that during hydrogen production process, 10.65 g of media components (CG and the constituents of inoculum) present in 1L of media is converted to 50 mg dry cell mass and about 169.4 mg H_2 gas. Rest of the material is either unutilized nutrients or different organic acids, solvents and waste gases (mostly CO_2) generated during the

process. Thus, this observation indicates that recycling of the waste liquid and gas is necessary for maximum utilization of the CG.

Utilization of spent broth of H_2 production process

Unlike most industrial processes where the fermented broth is subjected to downstream processing for recovery of the final product, during H_2 production, the gas is readily separated from the media and the fermented broth has no further use. Moreover, as already mentioned, 400 L of such spent media (SM) may be generated for every Kg of CG used (Sarma *et al.*, 2013). Further, the SM of the process may contain the living and dead biomass, residual nutrients including glycerol and biomolecules such as, proteins released to the media during fermentation and a detail characterization of the spent media is presented in Table 3.1.2. Further, it may be possible that the living biomass of SM still retains its H_2 production potential and the residual nutrients and biomolecules present in the broth can be used as a supplementary nutrient source for a subsequent H_2 production process. Unfortunately, because of the acidic pH and negligible glycerol content (Table 3.1.2), such spent broth will not be suitable as a sole medium component for a subsequent process. However, if the SM is mixed in small quantity with a fresh media containing CG, improved H_2 production may be observed for this new process. Based on this assumption, as already mentioned, an experiment was carried out where 10 g/L CG was mixed with different volumes of spent broth and the result of this investigation is shown in Figure 3.1.4. Figure 3.1.4 shows H_2 production profiles for the different runs of the experiment. From Figure 3.1.4, it can be seen that SM fraction (5% v/v), when used without any fresh inoculum can also produce H_2 by using a media containing only CG (10 g/L). However, the level of H_2 production was very low. Interestingly, when fresh inoculum was used along with the SM, the production was increased by several folds. Similar results were observed for different SM fractions ranging from 2 to 10 % v/v, however, when the SM fraction was increased to 20 % (v/v), production was inhibited. Similar results were observed when the SM fraction was increased beyond 20 % (data not shown).

Further, as compared to H_2 production by using only CG (10 g/L) (Figure 3.1.1), addition of SM (2-10 % v/v) was found to enhance the production by 13.37 %. As already discussed, there may be two reasons for such observation; firstly, in addition to the living cells present in inoculum, the SM also had living *E. aerogenes* cells which might help in enhanced H_2 production. Similarly, CG is mainly a carbon source, hence, proteins and other nutrients present in SM might help in increased cell proliferation leading to enhanced H_2 yield. Further, as elaborated in Table 3.1.1 and already discussed, cumulative H_2 production obtained in the present study is comparable with different investigations carried out in the presence of expensive buffering agents and synthetic supplementary nutrients.

From the previous study, we found that if appended with fresh media, the active biomass (AB) present in the spent media of H_2 production process is still has the potential to produce H_2. However, if the spent media containing the living cells are directly mixed with fresh media in small quantity, the number of viable cells present in this volume may not be sufficient for significant improvement in H_2 production. Moreover, when larger volume of such spent media was used (20 % v/v or beyond), it was found to be inhibitory for H_2 production. Therefore, it was decided that the cells will be separated from the spent by centrifugation and such cell pellet will be added to the fresh media, instead of whole spent broth. For this purpose, AB ranging from 5-30 mg/L was tested and the results are presented in Figure 3.1.5. From Figure 3.1.5, it can be seen that the cell pellet (5 mg/L) collected form spent broth, when mixed with fresh media was also able to produce a small amount of H_2. However, upon mixing the cell pellets with fresh inoculum (5% v/v), H_2 production was greatly improved. Further, there is a correlation between the amount of cell pellet used and the cumulative H_2 production. When the amount of cell pellet used was increased from 5 mg/L to 30 mg/L, production of H_2 was found to be improved by 57.98% (Figure 3.1.5).

Furthermore, as a fresh media containing 10 g/L CG was used for all these investigations, the H_2 production profile obtained for 10 g/L CG (Figure 3.1.1) was considered as the control profile and the results were compared. From this comparison, it was observed that, in the case of 5 mg/L and 10 mg/L AB, the amount of H_2 produced

was lower than the control. The possible reason for this observation may be that prior to the inoculation, the inoculum used in this investigation was mixed with the cell pellet from previous fermentation and this step might alter the efficiency of the inocula. However, when the addition of cell pellet was increased to 30 mg/L, due to higher cell density in the inoculum, improved H_2 production was observed over the control profile.

Microbial cells are good source of protein (Dhanasekaran *et al.*, 2011) and it may be possible to use them as a nitrogen source during fermentation. As already mentioned, CG is mainly a carbon source and addition of a nitrogen source may result in improved H_2 production. Therefore, one more investigation was carried out to evaluate the possibility of utilizing the SB of H_2 production process as a nitrogen/nutrient source for subsequent fermentation. Cell pellets of previous fermentation (5- 150 mg/L) was mixed with the media containing 10 g/L CG and H_2 production experiments were carried out as described in materials and methods section. Figure 3.1.6 shows the H_2 production profiles obtained from this investigation. The results were compared with the profile obtained for 10 g/L CG (without any supplementary nutrient, (Figure 3.1.1), and in the case of 50 mg/L SB, cumulative H_2 production was found to increase by 32.50%. However, SB in the range of 5-10 mg/L was not sufficient to produce any significant improvement in cumulative H_2 production. Further, in the case of 150 mg/L SB, H_2 production was improved by 25.59 %; however, the improvement was lower than that of 50 mg/L SB. The most probable reason for such observation may be that the addition of 150 mg/L SB might decrease the C-N ratio (increase the nitrogen content) of the media to inhibit the process (Androga *et al.*, 2011, Zhu *et al.*, 1999). The results are compared with different reports on H_2 production by CG bioconversion and presented in Table 3.1.1. From Table 3.1.1, it can be concluded that SB (50 mg/L) of previous fermentation can be reused as a supplementary nutrient for improved H_2 production and a large amount of synthetic media components can be replaced.

Conclusions

Significant difference in glycerol content, pH and elemental composition was observed between soybean oil based and meat processing and restaurant waste based CG. 10

g/L of the latter was found to be optimum for H_2 production (as feedstock). In this case, nearly 75 % of the total H_2 was produced when media pH was around 3.8, indicating the possibility of H_2 production in the absence of any expensive buffering agents. Further, application of spent biomass (50 mg/L) as nutrient source for a subsequent process can improve H_2 production by 32.50% with a maximum rate of 1040 mL/L/d.

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Table 3.1. 1: Summary of different recent investigations on CG bioconversion and H_2 production

Sl. no	Microorganisms	CG concentration (g/L)	Additional media components/buffering agent	H_2 production	Reference
1	*Thermotoga neapolitana*	5.0	*2.0 g/L soybean flour as the N-source, 0.05 M HEPES buffer*	518.25 ± 25.9 mL/L	(Ngo *et al.*, 2011)
2	*Enterobacter aerogenes* ATCC 13048	20	7.0g of K_2HPO_4, 5.5g of KH_2PO_4, 5g of tryptone, 5g of yeast extract , 1.0g of $(NH_4)_2SO_4$, 0.25g of $MgSO_4.7H_2O$, 0.021g of $CaCl_2.2H_2O$, 0.12g of $Na_2MoO_4.2H_20$, 0.002g of nicotinic acid, 0.000172g of Na_2SeO_3 and 0.00002g of $NiCl_2$ per liter medium	2500 mL/L	(Marques *et al.*, 2009)
3	Mixed culture from anaerobic reactors used for hydrogen production	COD 3.6	Sodium bicarbonate (500mg), chloridric acid (10 mol), CH_4N_2O (6 mg), $NiSO_4{\cdot}6H_2O$(0.15 mg), $FeSO_4{\cdot}7H_2O$ (0.75 mg), $FeCl_3{\cdot}6H_2O$ (0.075 mg), $CoCl_2{\cdot}2H_2O$ (0.012 mg), $CaCl_2{\cdot}6H_2O$ (0.618 mg), SeO_2 (0.0108 mg), KH_2PO_4 (1.608 mg), $KHPO_4$ (0.39 mg), $Na_2HPO_4{\cdot}2H_2O$ (0.828 mg) per liter	200 mL/g COD	(Fernandes *et al.*, 2010)
4	Mixed culture from wheat soil	3.0 (COD 1.3 g/L)	70mM MES buffer, NH_4Cl (0.31 g/L), KCl (0.13 g/L), trace vitamins and minerals	43 mL/g COD	(Selembo *et al.*, 2009)

Sl. no	**Microorganisms**	**CG concentration (g/L)**	**Additional media components/buffering agent**	**H_2 production**	**Reference**
5	*E. aerogenes* NRRL B407	10	No additional media component	2022.5 mL/L (equivalent to 84.08 mmol/L)	Present study
6			Spent biomass (50 mg/L)	2680 mL/L (equivalent to 111.42 mmol/L)	

Table 3.1. 2: Characterization of crude glycerol and spent broth of H_2 production process

Composition/property	**Soybean oil based CG (from literature)**	**Meat processing and restaurant (grease) waste based CG (present study)**	**Spent broth of CG (10 g/L) based H_2 production (present study)**	**References**
Glycerol content	69.5 % / 63.0 ± 0.3 % (w/w)	23.63 ± 2.5 % (w/w)	0.014 % (w/w)	(Hu *et al.*, 2012, Selembo *et al.*, 2009)
pH	6.9	3.4 (24 ± 0.5 °C)	3.8 (24 ± 0.5 °C)	(Hu *et al.*, 2012)
Density (g/L)	1010-1200	1106	-	(Hu *et al.*, 2012)
Total carbon content % (w/w)	24.3 ± 0.2	35.9 ± 0.3	2.88 ± 0.2	(Hu *et al.*, 2012)
Total nitrogen content % (w/w)	0.3 ± 0.1	3.25 ± 0.1	0.031 ± 0.1	(Hu *et al.*, 2012)
S (ppm)	-	8827 ± 33	152.5 ± 28	-
Na (ppm)	11769 ± 1561	7508 ± 36	686.9 ± 35	(Hu *et al.*, 2012)
P (ppm)	38.7 ± 4.8	1179 ± 20	837.9 ± 20	(Hu *et al.*, 2012)
Ca (ppm)	Below detection limit	215.1 ± 19	191.6 ± 22	(Hu *et al.*, 2012)

Composition/property	**Soybean oil based CG (from literature)**	**Meat processing and restaurant (grease) waste based CG (present study)**	**Spent broth of CG (10 g/L) based H_2 production (present study)**	**References**
K (ppm)	118.8 ±26.8	125 ± 19	39.41 ± 18	(Hu *et al.*, 2012)
Mg (ppm)	Below detection limit	57.41 ± 18	51.73 ± 18	(Hu *et al.*, 2012)
Fe (ppm)	31.6 ±19.0	44.41 ± 16	5.13 ± 16	(Hu *et al.*, 2012)
Zn (ppm)	-	~1.74	~1.05	-
Ni (ppm)	-	~0.94	~0.49	-

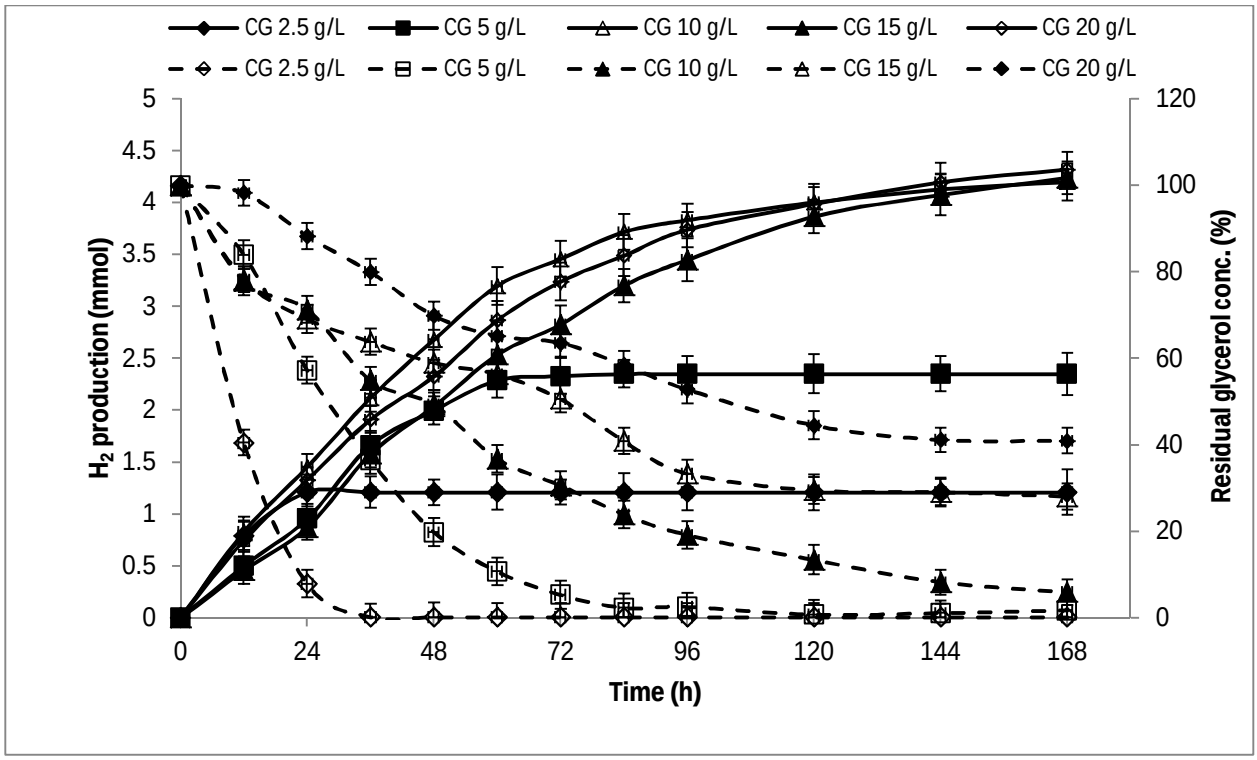

Figure 3.1. 1: Batch H_2 production (solid lines) and glycerol utilization (dotted lines) profiles obtained for different initial CG concentrations

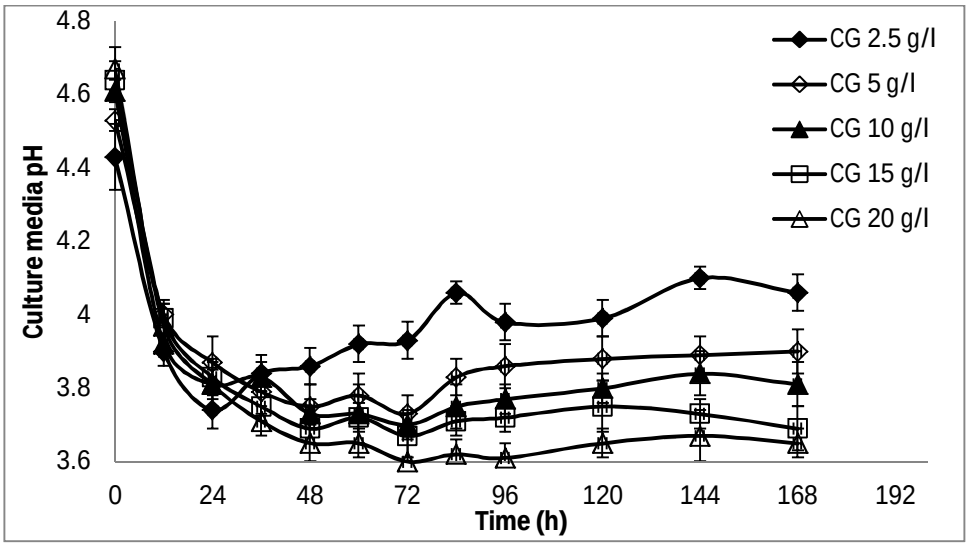

Figure 3.1. 2: pH profiles obtained from the batch experiments conducted for different (initial) CG concentrations

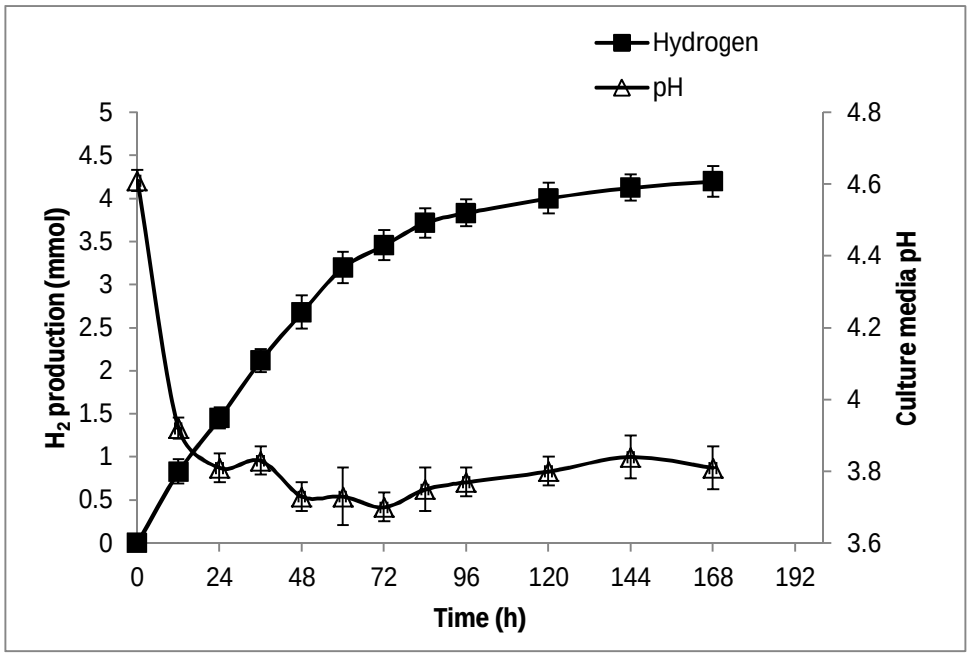

Figure 3.1. 3: H_2 production and pH profile obtained for 10 g/L CG showing the production of nearly 75% of H_2 at pH 3.8

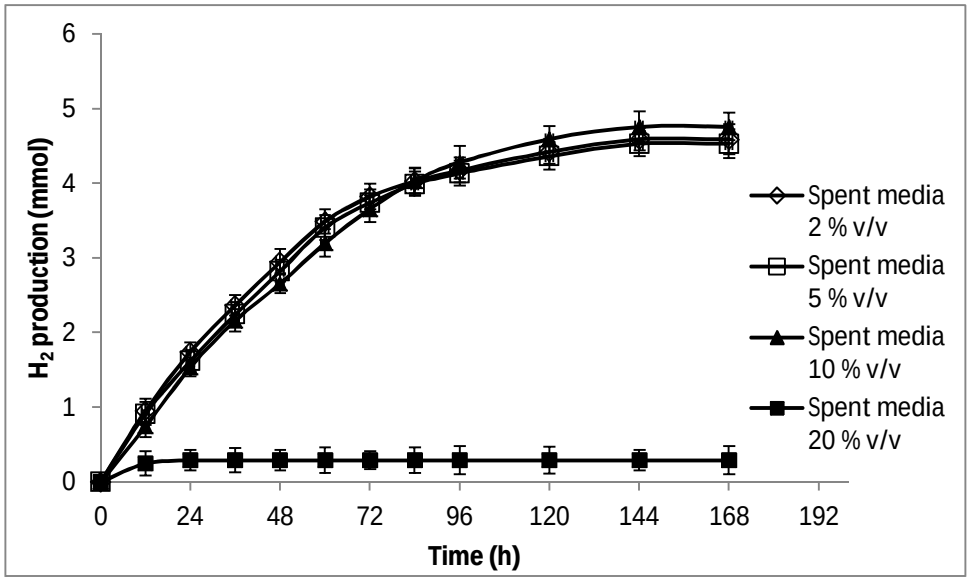

Figure 3.1. 4: Batch H_2 production profiles obtained by addition of different spent media fractions

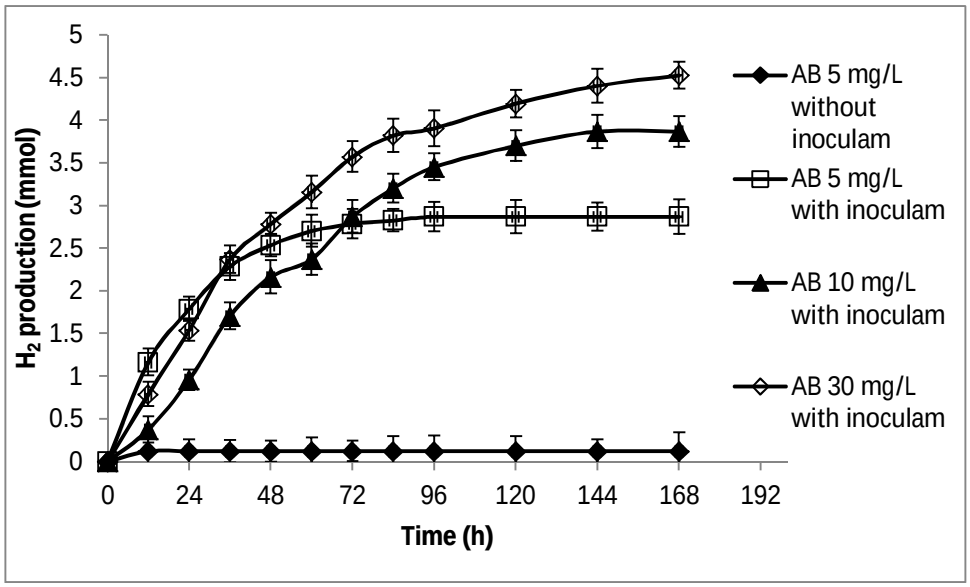

Figure 3.1. 5: Batch H_2 production profiles obtained by addition of different amount of active biomass from previous fermentation

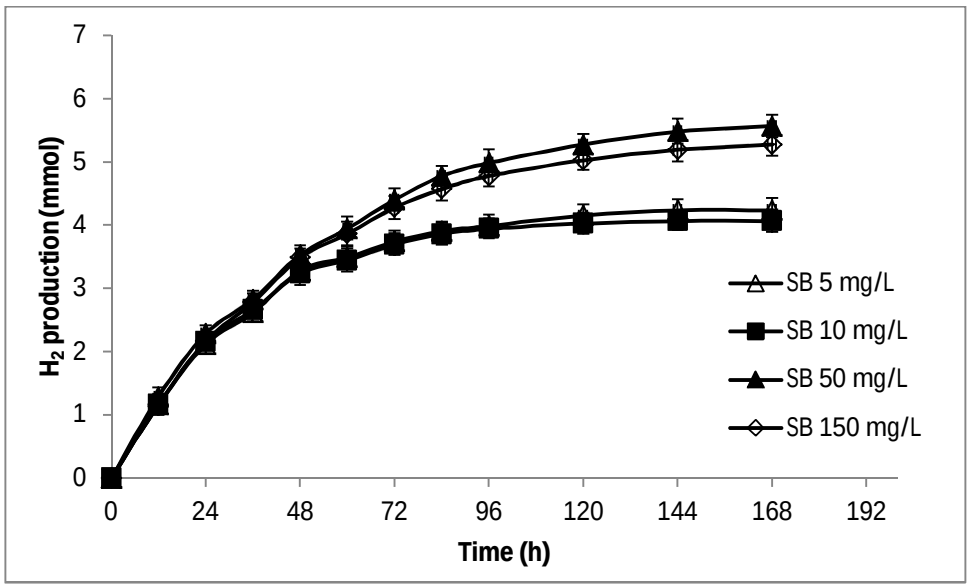

Figure 3.1. 6: Batch H_2 production profiles obtained by addition of different concentrations of spent biomass (autoclaved) from previous fermentation

PART II

EVALUATION OF DIFFERENT SUPPLEMENTARY NUTRIENTS FOR ENHANCED BIOHYDROGEN PRODUCTION BY *ENTEROBACTER AEROGENES* NRRL B 407 USING WASTE DERIVED CRUDE GLYCEROL

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Résumé

Le glycérol brut (GB) provenant de l'industrie du biodiesel (graisse animal et de restaurant) a plusieurs avantages comme substrat de culture pour la production de biohydrogène par *Enterobacter aerogenes* NRRL B 407. Également, les coûts élevés du processus de production en raison des additifs chimiques (nutriments) nécessaires à la fermentation est une préoccupation. Par conséquent, des nutriments moins coûteux, principalement composés de divers résidus d'origine agroalimentaire, ont été évalués comme additifs supplémentaires pour augmenter la production de H_2. Parmi les résidus choisis, les rejets liquides d'abattoir (AL), la biomasse des déchets de brasserie (BDB) et l'urée ont permis d'améliorer la production de 18,8 ± 3,6, 27,3 ± 3,5 et 38,6 ± 3,7%, respectivement. Dans le cas de l'urée (10 mg/L), une production cumulative aussi élevée que 116,4 ± 3,7 mmol H_2/L milieu a été obtenue, ce qui se compare à d'autres recherches portant sur la bioconversion du GB. Ainsi, cette étude démontre que le remplacement de nutriments chimiques conventionnels et coûteux, utilisés en grande concentration (~ 5 à 6 g/L) par des rejets agroalimentaires ajoutés en quantité négligeable (~ 10 mg/L), n'affecte pas les rendements cumulatifs en H_2. Également, la souche utilisée dans la présente étude a été apte à se développer à un pH acide aussi bas que 3,3, indiquant de bonne perspective d'application pour la production de H_2 par fermentation sombre.

Mots clés: déchets de brasserie, glycérol brut, production d'hydrogène, éléments nutritifs supplémentaires, urée

Abstract

Crude glycerol (CG) has several advantages over a range of conventional substrates used for biohydrogen production by *Enterobacter aerogenes* NRRL B 407. Meanwhile, high process cost due to requirement of expensive supplementary media component is a concern. Therefore, different less expensive (or wastes) materials have been evaluated as supplementary nutrient for H_2 production by CG (meat processing and restaurant waste based biodiesel derived) bioconversion. Among the materials selected, slaughterhouse liquid waste (SL), brewery waste biomass (BWB) and urea was found to improve the production by 18.81 ± 3.56, 27.30 ± 3.54 and 38.57 ± 3.66 %, respectively. Further, in the case of urea (10 mg/L), cumulative production as high as 116.41 ± 3.72 mmol H_2/L media has been achieved; which is comparable to other reports available on CG bioconversion. Thus, present study demonstrates successful replacement of large amount (~5-6 g/L) of expensive nutrients/buffering agents by negligible amount (~10 mg/L) of different waste materials, without compromising the cumulative H_2 yield. Further, the strain used in the present study was found to grow at an acidic pH as low as 3.3, indicating its prospective application for dark fermentative H_2 production.

Key words: Brewery waste, crude glycerol, hydrogen production, supplementary nutrient, urea.

Introduction

Hydrogen combustion does not produce CO_2 (greenhouse gas) and water is the only byproduct of the process. Therefore, it is projected as the cleanest future alternative of the fossil fuels (Özgür *et al.*, 2010). Steam reforming of natural gas and ethanol, coal gasification and partial oxidation of heavy oil are a few methods available for hydrogen production (Dicks, 1996, Vasudeva *et al.*, 1996, Yurum, 1995). However, such processes have different concerns, such as dependence on fossil fuels as well as elevated thermal demand. Alternatively, bioconversion of organic feedstock is a sustainable method for H_2 production. A number of agricultural waste or plant based materials, such as sugarcane bagasse, corn stover biomass and lignocellulosic energy crops have already been successfully used as the major feedstock for the purpose (Datar *et al.*, 2007, de Vrije *et al.*, 2009, Pattra *et al.*, 2008). However, most of the waste materials and lignocellulosic materials, in particular require proper pre-treatment prior to fermentation (Abreu *et al.*, 2012).

Crude glycerol (CG) generated during biodiesel manufacturing process is a readily usable substrate for biohydrogen production. Actually, during biodiesel production (trensesterification), for every 100 Kg biodiesel, 10 Kg of CG is generated as byproduct (Chatzifragkou *et al.*, 2012, Santibáñez *et al.*, 2011). Owing to its crude nature, CG does not have any significant commercial application and further stricter environmental regulations are applied for its disposal (http://www.epa.gov/region7/priorities/agriculture/pdf/biodiesel_manual.pdf (accessed on 28_08_2012)). Therefore, considering the increase in global biodiesel production, cost effective management of large amounts of byproduct (CG) could be an emerging challenge for biodiesel manufacturers. Hence, CG bioconversion for hydrogen production could be an alternative option for its sustainable management. Meanwhile, CG is mainly a carbon source for industrial fermentation and hence, additional nutrient sources are generally applied for its improved bioconversion (Liu *et al.*, 2011, Ngo *et al.*, 2011, Wilkens *et al.*, 2012). However, according to an analysis, the cost of the supplementary nutrients used for H_2 production by CG bioconversion may be as high as

82 % of total process cost (Sarma *et al.*, 2013a). Therefore, the major objective of the present investigation is to evaluate different less expensive nutrient sources for potential application as a replacement of costly synthetic media components required for the process. H_2 production could be realized either by dark fermentation or by photo-fermentation. However, owing to its higher hydrogen production rate, dark fermentation is considered more advantageous over photo-fermentation (Ciranna *et al.*, 2011) and hence, dark fermentation has been considered for the present investigation. Similarly, *Enterobacter aerogenes,* well-known facultative anaerobic bacterium for CG bioconversion (Ito *et al.*, 2005, Marques *et al.*, 2009, Sakai *et al.*, 2007), has been used in the present study. Moreover, increased hydrogen partial pressure in the headspace of the reactor is known to be thermodynamically unfavorable for H_2 synthesis and metabolic shift towards the production of more reduced end products could be a consequence (Ciranna *et al.*, 2011, Levin *et al.*, 2004). Therefore, the present investigation was carried out at relatively low H_2 partial pressure by periodically removing the produced H_2 from the reactor headspace.

Materials and Methods

Microorganism and inoculum development

Enterobacter aerogenes NRRL B 407 (ARS, USDA, USA), a gram-negative, facultative anaerobic, rod-shaped bacterium belonging to the family Enterobacteriaceae was considered for the present investigation. A nutrient medium composed of glucose (5 g/L), casein peptone (5 g/L), KH_2PO_4 (2 g/L), yeast extract (0.5 g/L) and $MgSO_4.7H_2O$ (0.5 g/L) (Beckers *et al.*, 2010) was used to maintain the microorganism (21 ± 1°C) and the same medium was also used for inoculum development throughout the investigation. For both purposes, 125 mL serum bottles (working volume 50 mL) were used and the media was sparged with ultrapure N_2 gas for 2 min to create anaerobic environment for supporting the growth of the microorganisms. Following N_2 sparging, immediately the bottles were sealed with aluminum crimp seal containing silicone septum (Fisher scientific, Canada) by using a hand operated crimper (E-Z Crimper™, VWR, Canada). Prior to inoculation, the bottles were autoclaved and cooled to room

temperature. Inoculation was made by using a sterile syringe which was followed by incubation of the bottles in orbital incubator shaker maintained at 150 rpm and 30±1°C (Marques *et al.*, 2009). Freshly prepared culture media containing the microorganism in its exponential growth phase was used as inoculum (5%, v/v) for all the experiments conducted in this investigation.

Chemicals and other materials

Chemicals, such as glucose, urea, $MgSO_4$ $7H_2O$, NaOH, sodium periodate, acetone, acetylacetone, bromophenol blue, HCl, ethanol and acetic acid were purchased from fisher scientific, Canada. Casein peptone and KH_2PO_4 were purchased from VWR, Canada and the yeast extract was a kind gift from Lallemand, Canada. Crude glycerol (CG), a by-product of biodiesel production by transesterification was collected from Rothsay®, Canada and used as the major feedstock for present study. Rothsay mainly produces biodiesel and CG by using the waste materials from meat processing plants and used grease from restaurants (http://www.rothsay.ca/products/biodiesel/; (accessed on 06-08-2012)). Secondary sludge (SS) used in the present investigation was collected from Quebec urban community (CUQ) wastewater treatment plant, Quebec, Canada (DP Mohapatra *et al.*, 2012). Brewery liquid waste (BLW) used in the present investigation was kindly provided by La Barberie (Quebec), Canada. The slaughter house liquid (SL) waste material was collected from Gibiers Canabec, Quebec, Canada. It was mainly composed of blood and other liquid waste of meat processing operation, which was used without any pretreatment. Similarly, starch industry wastewater (SIW) from ADM Ogive; Candiac, Quebec, Canada and the apple pomace sludge (AS) was obtained from Lassonde Inc., Rougemont, Montreal, Canada (see reference for detailed characterization) (Dhillon *et al.*, 2011).

Characterization of crude glycerol

As specifically described in later section, glycerol content of the CG sample (Sarma *et al.*, 2013b) was determined by spectrophotometric method. To determine the total suspended solids, the CG sample (10 g) was diluted with 1 L of deionized water and passed through a glass fiber filter paper (G6, Fisher, Canada). Finally, the filter paper

was dried in oven (105 ±1 °C to constant weight) to remove the water and weighed after cooling. From the difference of filter paper weight before and after filtration, total suspended solids were calculated and the result was expressed as mg/Kg.

Soap present in CG was determined by titration and expressed as sodium oleate. Briefly, a mixture of 60 mL acetone and 0.15 mL of 0.5 % (w/v) bromophenol blue (in 95 % ethanol) was prepared and neutralized with 0.1 M NaOH (www.oiv.int/oiv/files/CODEX_2012_EN.pdf (accessed on 11_09_2012)). The solution was mixed with 10 g of CG sample and heated in a water bath (70 ±1°C) for 1 min. Later, the mixture was titrated using hydrochloric acid solution (0.1M). On complete neutralization of the soaps present in the CG sample by the acid used, blue color of the solution (pH indicator) disappeared. Further, the amount of the acid needed for the experiment was recorded and used to calculate the soap content of the CG sample.

Hydrogen production experiments

As already outlined for inoculum development, 125 mL serum bottles (working volume 50 mL) were used as anaerobic bioreactor for hydrogen production experiments. Other conditions of media preparation and further incubation were kept the same. In a previous investigation, different initial concentrations of CG have been evaluated and 10 g/L CG was found to be optimum for bio-hydrogen production (unpublished result). Therefore, for present investigation, 10 g/L CG (CG diluted with deionized water) was used as the basic media for all hydrogen production experiments, which was supplemented with different nutrient sources according to the demand of the investigation (as described below). Further, pH 6.0 is reported to be the optimum for hydrogen production by *E. aerogenes,* the microorganism used in the present investigation (Sakai *et al.*, 2007). Hence, all the experiments were carried out at pH 6.0. Similarly, all the experiments were carried out using 5 % (v/v) aforementioned inoculum, which was equivalent to 0.25 mg dry cell mass. At 12 h interval, 1 mL of the aqueous media was collected by sterile syringe and pH and residual glycerol concentration was measured. Accordingly, the composition of produced gas mixture was analyzed by gas chromatography and the volume of produced H_2 was measured at 12 h interval and

cumulative H_2 production was expressed as millimoles per liter (mmol/L) (see below). Reported H_2 concentrations are the average of the readings from duplicate experiments.

Evaluation of organic waste materials as supplementary nutrient source for CG bioconversion

In the present investigation, a range of waste materials, such as secondary sludge (SS) from waste treatment plant, brewery liquid waste (BLW), starch industry wastewater (SIW), apple pomace sludge (AS) and slaughterhouse liquid (SL) wastes have been evaluated as a supplementary nutrient source for improved H_2 production by CG bioconversion. No pretreatment was made (unless specifically mentioned) for such waste materials prior to their use in media preparation. The pH, total solids, total organic carbon and ammoniacal nitrogen content of the sludge sample used in the present investigation were 6.19±0.2, 17.46±2.3 (g/L), 282±10 (g/L) and 0.17±11 (g/L), respectively. Detailed characterization of the wastewater sludge could be found in Mohapatra *et al*, 2011 (D.P. Mohapatra *et al.*, 2011). Similarly, physical properties and elemental composition of SIW and AS considered for present investigation could be found in Dhillon *et al,* 2011 (Dhillon *et al.*, 2011). To investigate the effect of these materials on H_2 production using separate serum bottles, SS (10, 20, 40 and 80 mg /L), SIW (0.2, 1, 3 and 5 %, v/v), AP (10, 20, 100, 300 and 500 mg/L) and SL (10, 20, 100, 300 and 500 mg/L) were mixed with 10 g/L CG and the resulting media types were autoclaved. All the other conditions of media preparation, inoculation, incubation and sample collection were the same as discussed in previous section.

In the case of brewery waste, it mainly contains yeast biomass and liquid material left after product recovery. Two separate investigations were carried out to evaluate the wastes for H_2 production. For the first set of experiments, 10 g/L CG was separately mixed with 20, 60, 120 and 240 mg/L of the liquid waste (BLW) and autoclaved. Alternatively, for second set of experiments, the waste was mixed with distilled water to have a final concentration of 100 g/L. Resulting mixture was centrifuged at 5000 *x g* for 5 min, the supernatant was discarded and the solid fraction containing biomass was

collected. The purpose of this step was to remove the alcoholic component that may be present in the brewery waste and could be inhibitory for microbial growth. However, it was an assumption and no experiment was conducted to verify the necessity of this step. Thus, the biomass collected (BWB) after centrifugation was supplemented with 10 g/L CG at different concentrations ranging from 10 to 160 mg/L, autoclaved and hydrogen production experiment was carried out as outlined in previous sections.

Evaluation of urea as supplementary nitrogen source for CG bioconversion

This investigation was conducted to determine the potential of urea as a less expensive nitrogen source for H_2 production by CG bioconversion and six different concentrations of urea ranging from 10 to 180 mg/L have been evaluated. As already mentioned for different waste materials, 10 g/L CG was enriched with different concentrations of urea, pH was adjusted, sparged with N_2 (see above) and separately autoclaved. All the conditions of incubation, sample collection and analysis were similar as already described.

Analytical methods

Glycerol

Glycerol concentration of the aqueous samples collected during hydrogen production experiments were measured by the spectrophotometric method (Bondioli *et al.*, 2005). The method is based on periodate oxidation of glycerol followed by optical density measurement at 410 nm. In practice, 1 mL of the aqueous sample was mixed with 1 mL of ethanol (47.5% v/v) followed by addition of 1.2 mL each of acetylacetone solution (0.2 M) and sodium periodate solution (10 mM). Subsequently, using a water bath shaker, the reaction mixture was incubated at 70 ± 1 °C for 1 min and immediately cooled to 20-25 °C by using cold water. The samples were maintained at the same temperature until analyzed by UV-Vis spectrophotometer (Carry 100 Bio®, Varian USA) (Sarma *et al.*, 2013a).

Hydrogen analysis

H_2 and CO_2 are principal gases produced during CG bioconversion (Marques *et al.*, 2009) and H_2 in the mixture was analyzed by a gas chromatograph (Varian 3800, USA) fitted with PoraPLOT Q® column (Agilent technology, USA) and thermal conductivity detector (TCD). The column temperature was fixed at 100 °C and nitrogen was used as the carrier gas. The gas flow rate was maintained at 3.5 mL min^{-1} with a retention time (H_2) of 4.5 min. As described by previous investigators, the volume of produced H_2 was measured by the inverted cylinder –NaOH (10%) method (Junyapoon *et al.*, 2011, Maeda *et al.*, 2008). Briefly, to remove CO_2 fraction from the gas mixture, the produced gas was passed through 100 mL NaOH (10% w/v). Subsequently, it was collected in a water filled inverted graduated glass cylinder placed in a partially filled (with water) beaker. Based on displacement of water level in the cylinder, volume of produced H_2 was calculated (Junyapoon *et al.*, 2011, Maeda *et al.*, 2008) and, where applicable, considering the temperature and atmospheric pressure, volume of produced gas was expressed as millimoles/L media (http://www.easycalculation.com/chemistry/ideal-gas.php (accessed on 16_07_2012).

Results and discussion

Crude glycerol characterization

The CG fraction considered for the present investigation contains 23.63 % (w/w) glycerol and it has a pH of 3.4 (at 24 ± 0.5 °C) (Sarma *et al.*, 2013b). In addition, it was found to contain 300 mg suspended solids per Kg of CG. Similarly, the soap content of the CG sample was found to be 0.364 ± 0.05 % (w/w). This value is significantly lower than 20-31 % (w/w) of soap reported for different CG samples (Hu *et al.*, 2012). Considering relatively low pH (3.4) of present CG sample, such observation may be due to possible acidification of soap. Meanwhile, soap has potential inhibitory effect on bacterial growth (http://www.enterprise-europe-network.ec.europa.eu/src/matching/templates/completerec.cfm?bbs_id=172911 (accessed on 11_09_2012)). Therefore, CG with negligible soap content could be considered ideal for bioconversion purposes, such as hydrogen production.

Evaluation of different waste materials as nutrient supplements

CG is mainly a carbonaceous material and hence, addition of supplementary nutrient source could have improved its potential as a feedstock. In fact, improve bioconversion efficiency has been observed by addition of supplementary nutrient sources, such as peptone, yeast extract and a range of minerals (micro and macro nutrients) (Fernandes *et al.*, 2010, Ito *et al.*, 2005, Ngo *et al.*, 2011, Sakai *et al.*, 2007, Selembo *et al.*, 2009). However, most of the synthetic or semisynthetic ingredients have commercial importance and hence are expensive. Further, production of such compounds, similar to any other manufacturing processes might have potential negative effect on the environment. Therefore, extensive application of such compounds for environmentally sustainable management of CG may not be a feasible concept. On the contrary, a range of agro-industrial organic waste materials could be a suitable alternative of such synthetic compounds. Further, this strategy not only has the potential to replace the expensive synthetic materials from the process, but also could be a mean for sustainable management of different agro-industrial wastes.

For this purpose, different nutrient rich waste materials, ranging from secondary sludge of municipal wastewater treatment plant to wastewater from slaughter house have been evaluated. Commonly, these materials are rich in proteinaceous compounds and minerals and should be a good source of nutrients for the proposed process. However, considering their crude nature, inhibitory effect of such compounds could not be ruled out and hence, each of them was separately evaluated. Batch H_2 production experiments were conducted where 10 g/L CG was supplemented with different concentrations of these materials and cumulative H_2 production was considered as the measure of their potential application. Moreover, an experiment was carried out where cumulative H_2 production using 10 g/L CG (without any supplementary component) was reported (Sarma *et al.*, 2013b). This result was considered as the negative control and all results from subsequent investigations were compared with this value to quantify the improvement in H_2 production (see below).

Figure 3.2.1(a) shows the H_2 production profiles obtained for different concentrations of slaughter house liquid waste used as a supplementary nutrient. Cumulative H_2 production, after the period of the experiment was found to be almost similar for 10-100 mg/L SL; where, 10 mg/L gave the highest cumulative production. Further increment in SL concentration (300-500 mg/L) was found to be highly inhibitory for the process. Similar response trend was found for apple pomace sludge (AS) too (Figure 3.2.1 (b). Higher production was obtained for the concentration range of 10-20 mg/L, whereas, at relatively higher concentrations (300-500 mg/L) cumulative H_2 production was diminished.

Further, Figure 3.2.1 (c) shows the H_2 production profiles for different concentrations of SS supplemented to the process. Similar to the previous cases, SS in the range of 10-80 mg/L was showing nearly similar response in terms of H_2 production and at relatively higher concentration (500 mg/L and beyond), H_2 production was considerably inhibited (data not shown). Figure 3.2.1 (d, e and f) show the profiles obtained for BLW, BWB and SIW, respectively. Overall, for all the waste materials tested in the present study, relatively lower concentration gave better response and was found to be inhibitory at higher concentrations. Further, as shown in Figure 3.2.2, best response from each independent investigation was compared with response from a controlled experiment (10 g/L without any supplementary component) (Sarma *et al.*, 2013b). From Figure 3.2.2, it could be concluded that SS, SL, BLW and BWB (at relatively low concentration) were found to improve the cumulative H_2 production over the control condition. Among these materials, BWB (10 mg/L) was the most successful supplement. As obvious from Figure 3.2.2, BWB was able to improve the cumulative H_2 production by 27.30 ± 3.54 %. Similarly, SL was the other material to improve the production by 18.81 ± 3.56 %. Actually, brewery and slaughterhouse wastes are nitrogen rich (proteinaceous) materials. Hence, improved H_2 production could be attributed to improved cell proliferation in the presence of nitrogenous compounds. However, these wastes were also found to inhibit H_2 production when used at relatively higher concentration (usually more than 100 mg/L) (Figure 3.2.1 (a). The possible reason for such observation may be that the addition of these wastes at higher concentration could decrease the C-N

ratio of the broth leading towards diminished H_2 production (Lin *et al.*, 2004). Similarly, a metabolic pathway shifting in the presence of relatively higher concentration of these wastes (BWL, BWB, and SL) resulting in increased production of reduced end-products and decreased H_2 yield could not be ignored. However, it is only a possibility and for further explanation, detailed investigation could be conducted to get the information on the types as well as amounts of different fermentation end-products. On the contrary, AP and SIW were unable to impart any improvement in H_2 production at any of the tested concentrations (Figure 3.2.2). Considering the crude nature of all the materials evaluated in the present study, presence of growth inhibitors (such as, heavy metals) could be the reason for their negative effect. Similarly, shifting of metabolic pathway may be the other most possible reason. Further, in the special cases of AP and SIW, their non-proteinaceous origin (unlike BWB, SL and SS) may be a reason for their failure to improve the CG bioconversion process even at lower concentrations.

Most of the reports presently available on CG bioconversion and H_2 production are summarized in Table 3.2.1. As it is obvious from the Table 3.2.1, a range of synthetic media components were used in all such studies. Further, to maintain the process pH during all the H_2 production studies, either commercial buffering agents were used or automatic pH controlling system applied. However, the application of any such synthetic media component can increase the process cost by up to 82 % (Sarma *et al.*, 2013a). On the contrary, in the present investigation the synthetic media components were successfully replaced by waste materials, such as BWB and SL. Similarly, no buffering agent was used for maintaining a constant pH during the process. Still, as obvious from Table 3.2.1, the results are highly comparable with any available report on H_2 production by CG bioconversion. Unlike, conventional pure substrate based CG, special chemical composition of the CG (meat processing and restaurant waste based) used in present investigation (Sarma *et al.*, 2013b), its inherent buffering property as well as various supplementary nutrients could be attributed to observed better productivity. Thus, the present finding has depicted that CG supplemented with small amount of (10-20 mg/L) nitrogen rich waste (especially BWB) could be as effective as a process augmented with synthetic media (~5-6 g/L) components (Table 3.2.1).

As summarized in Figure 3.2.2, glycerol utilization and H_2 production (maximum) profiles obtained for different investigations was found to have good correlation with media pH recorded at the end of different processes. Compared to the control experiment (without any supplement), the investigations carried out with BWB and SL were found to have lower final broth pH. Such observation could be due to increased acetic acid production corresponding to enhanced H_2 production in those cases (Van Ginkel *et al.*, 2005). Further, corresponding to relatively low glycerol utilization for SIW and AP (61 ± 3.3 and 72.1 ± 3.4 % respectively; Figure 3.2.2), final media pH in those cases were higher than the cases of any other supplements. Most importantly, the strain (*E. aerogenes* NRRL-B407) used in the present study was found to produce H_2 at a pH well below 4 (around 3.3). Such finding could be beneficial, as in the case of dark fermentative H_2 production the process (batch) is usually terminated by pH change due to organic acid production. Hence, improved H_2 production could be expected by application of this acid tolerant strain.

Effect of urea on H_2 production using CG

Similar to different waste materials, urea was also evaluated as a possible low cost nitrogen source for CG bioconversion. Figure 3.2.3 shows the H_2 production profiles obtained for different concentrations of urea supplemented to the process. From Figure 3.2.3, it could be summarized that among different concentrations of urea, maximum cumulative H_2 production was obtained for 10 mg/L urea. Further, H_2 production was found to be diminished with the increase in urea concentration as well as, a prominent lag phase (in H_2 production) was observed at relatively higher concentration (180 mg/L urea). Similar to the case of different nitrogen rich waste materials, a possible reason for such observation may be that with the increase in urea concentration, the C-N ratio of the culture media decreased and H_2 production has been repressed (Kapdan *et al.*, 2006, Rojas *et al.*, 2010). Meanwhile, at relatively low urea concentration (10 mg/L), H_2 production was found to be improved by 38.57 ± 3.66 % (Figure 3.2.2). As already mentioned, different reports on H_2 production by CG bioconversion have been summarized in Table 3.2.1. It could be observed from the table (Table 3.2.1) that the performance of the present process is highly comparable with different previous

investigations. Further, the significance of the present observation is that a small amount of urea (10 mg/L) was successfully used to replace around 5-6 g/L of costly synthetic media components (nutrients and buffering agents) without compromising the H_2 production efficiency of the process (Table 3.2.1). Hence, reduced process cost could be an advantage of the present process over different presently known equally successful processes (Table 3.2.1).

Thus, less expensive nutrient sources such as SL, BWB and urea could be considered as potential additive for sustainable bio-refinery of CG. Most importantly, application of slaughterhouse liquid waste for improved bioconversion of meat processing waste based biodiesel derived CG has particular significance as both the industries are interlinked. Hence, waste generated in one process could be sustainably applied for waste valorization of the other. Based on this concept and considering proteinaceous nature of SL, its possible application in inoculum development (preparation of protein rich medium) for CG bioconversion process could be considered as a promising option for future investigation.

Conclusions

Bioconversion of biodiesel byproduct (CG) is a relatively new and promising process to be introduced for hydrogen production. However, the process is highly limited by prohibitory process cost attributed by expensive supplementary media components. In the present investigation, waste materials, such as BWB, SL, SS, AP, SIW and urea, less expensive nitrogen sources, have been evaluated as the replacement of expensive media components. *E. aerogenes* has been used for the study where, 10 g/L CG was the sole carbon source and it was observed that addition of SL, BWB and urea can enhance the production by 18.81 ± 3.56, 27.30 ± 3.54 and 38.57 ± 3.66 %, respectively. Similarly, cumulative production of 116.41 ± 3.72 mmol H_2/L media and a maximum H_2 production rate of 965 ± 35 mL/L/d have been achieved by using urea as low as 10 mg/L as the supplementary nitrogen source. Present findings were found to be comparable to any other reports available on CG bioconversion, demonstrating the

suitability of BWB, SL and urea as the replacement of expensive media component used for the process.

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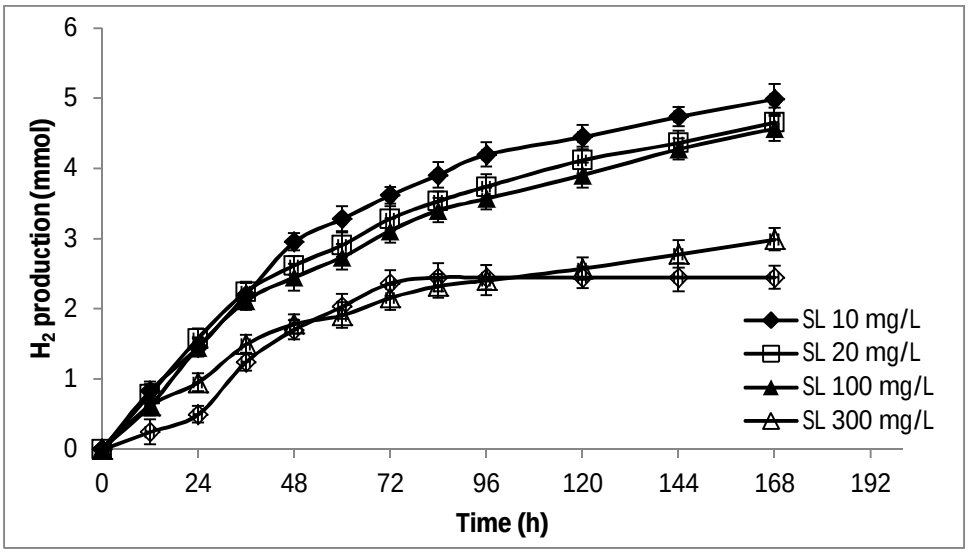
Table 3.2. 1: **The summary of different reports available on H_2 production by CG bioconversion**

Sl. no	Microorganism	Inoculum size	CG concentration (g/L)	CG origin	Additional nutrient	pH controlled/ buffering agent added	H_2 production	Ref.
1	*Enterobacter aerogenes NBRC 12010*	4% (v/v)	CG containing 9.9 g/L glycerol	*used food oils*	*Synthetic media*	yes	138.6 mmol/L*	(Sakai *et al.*, 2007)
2	*Enterobacter aerogenes* ATCC 13048	0.1 g/L (biomass)	20	-	Synthetic media	yes	2500 mL/L	(Marques *et al.*, 2009)
3	*Enterobacter aerogenes HU-101*	4 %	CG containing 1.7 g/L glycerol	-	Synthetic media	yes	20.67 mmol/L*	(Ito *et al.*, 2005)
4	Mixed culture	10 % (v/v)	COD 3.6	Vegetable oil	Synthetic media	yes	200 mL/g COD	(Fernandes *et al.*, 2010)
5	*Thermotoga neapolitana*	10 % (v/v)	05	-	*Soybean flour*	yes	518.25 ± 25.9 mL/L	(Ngo *et al.*, 2011)
6	Mixed culture	1g/L (soil)	03	Soybean oil	Synthetic media	yes	7.018 mmol/L*	(Selembo *et al.*, 2009)
7	*E. aerogenes* NRRL B407	5% (v/v)	10 (2.3 g/L glycerol)	Meat processing and restaurant	Slaughter house liquid waste (10 mg/L)	No	2400 mL/L (99.78 mmol/L)	Present study

8				waste	Brewery waste biomass (10 mg/L)		2570 mL/L (106.84 mmol/L)	
9					Urea (10 mg/L)		2800 mL/L (116.41 mmol/L)	

(a)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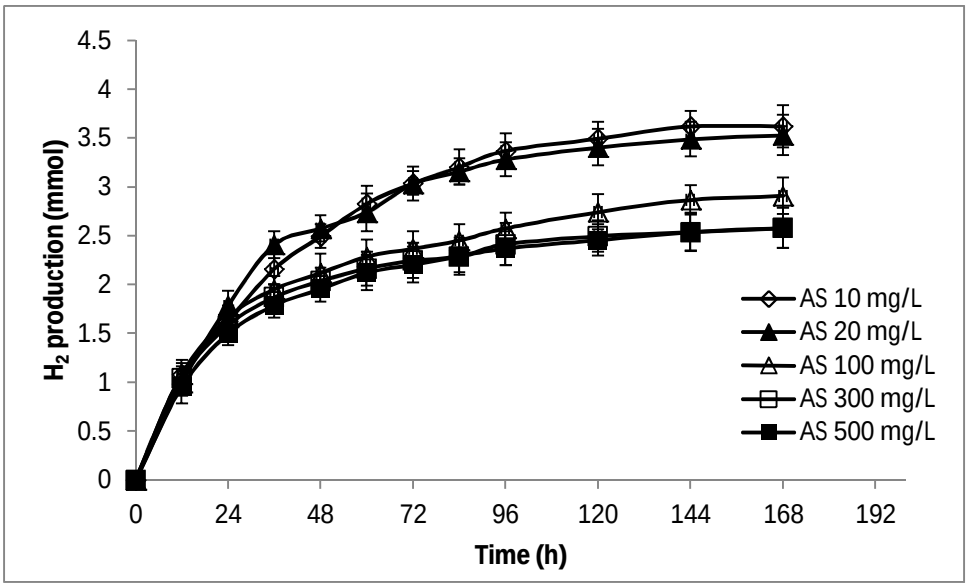
5
4
3
2
1
0
H_2 production (mmol)
0
24
48
72
96
120
144
168
192
Time (h)
SL 10 mg/L
SL 20 mg/L
SL 100 mg/L
SL 300 mg/L

(b)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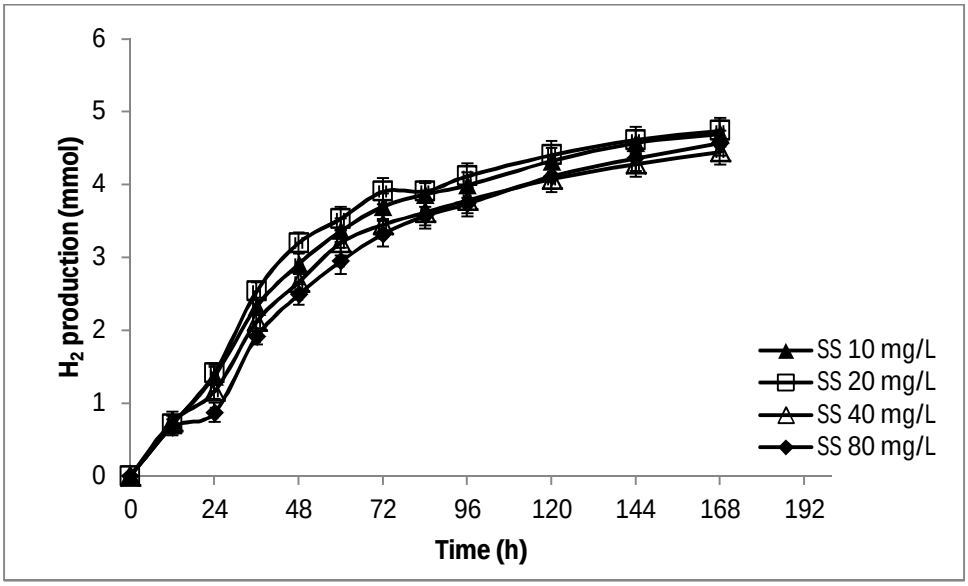
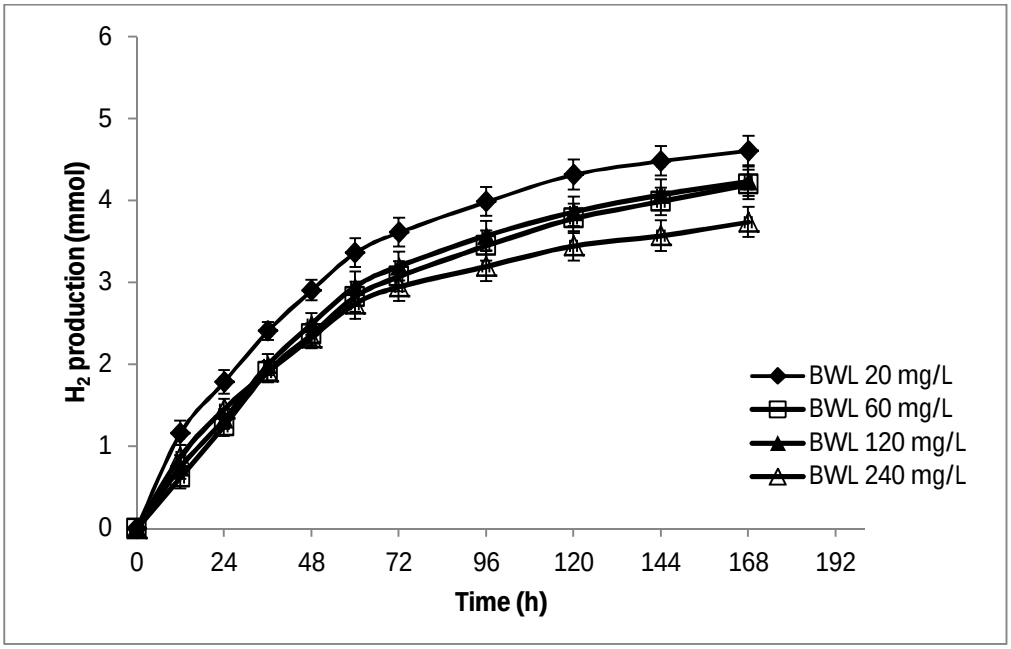
3.5
3
2.5
2
1.5
1
0.5
0
H_2 production (mmol)
0
24
48
72
96
120
144
168
192
Time (h)
AS 10 mg/L
AS 20 mg/L
AS 100 mg/L
AS 300 mg/L
AS 500 mg/L

(c)


6
5
4
3
2
1
0
H_2 production (mmol)
0
24
48
72
96
120
144
168
192
Time (h)
SS 10 mg/L
SS 20 mg/L
SS 40 mg/L
SS 80 mg/L

(d)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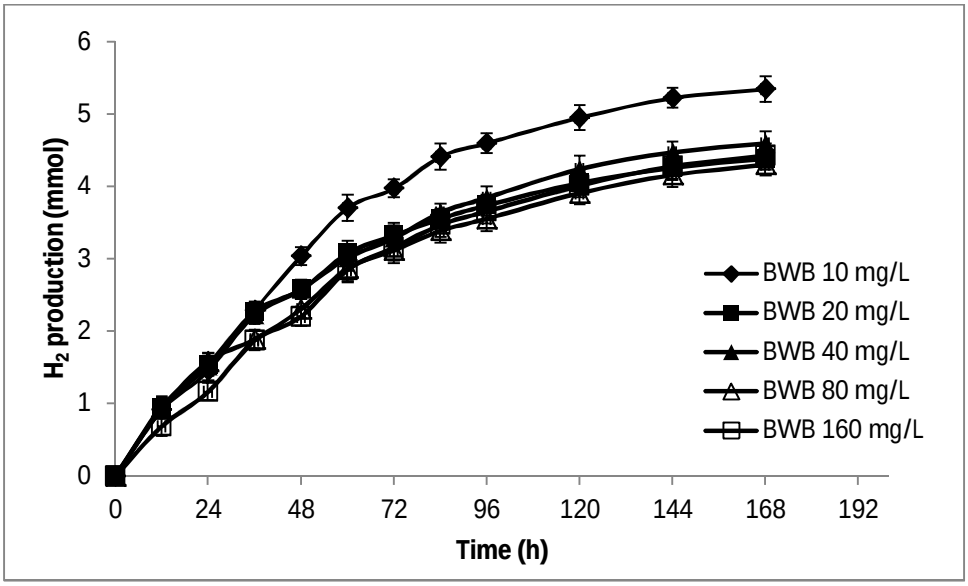
5
4
3
2
1
0
H_2 production (mmol)
0
24
48
72
96
120
144
168
192
Time (h)
BWL 20 mg/L
BWL 60 mg/L
BWL 120 mg/L
BWL 240 mg/L

(e)


6
5
4
3
2
1
0
H_2 production (mmol)
0
24
48
72
96
120
144
168
192
Time (h)
BWB 10 mg/L
BWB 20 mg/L
BWB 40 mg/L
BWB 80 mg/L
BWB 160 mg/L

(f)

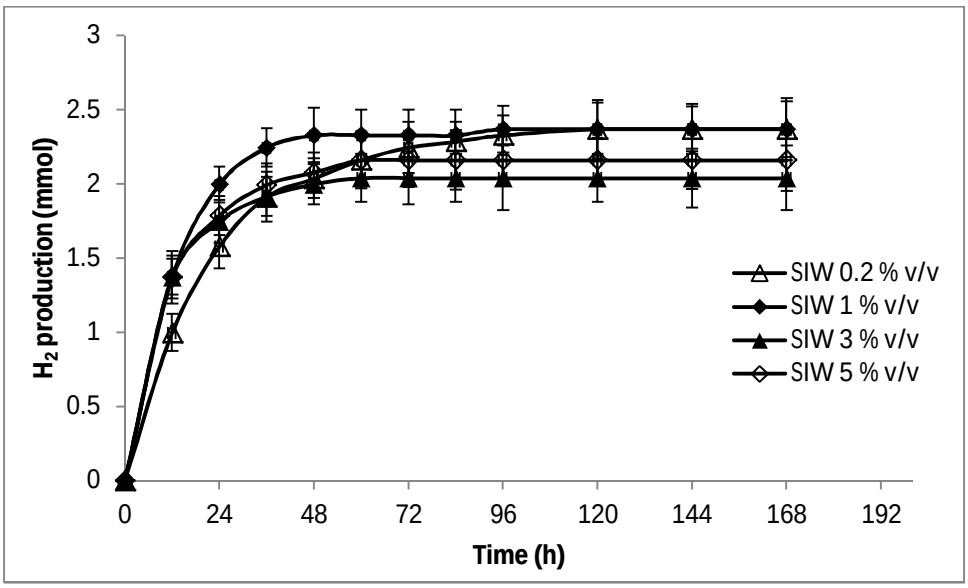


Figure 3.2. 1: H_2 production profiles obtained for different concentrations SL (a), AS (b), SS (c), BWL (d), BWB (e) and SIW (f) supplemented to 10 g/L CG

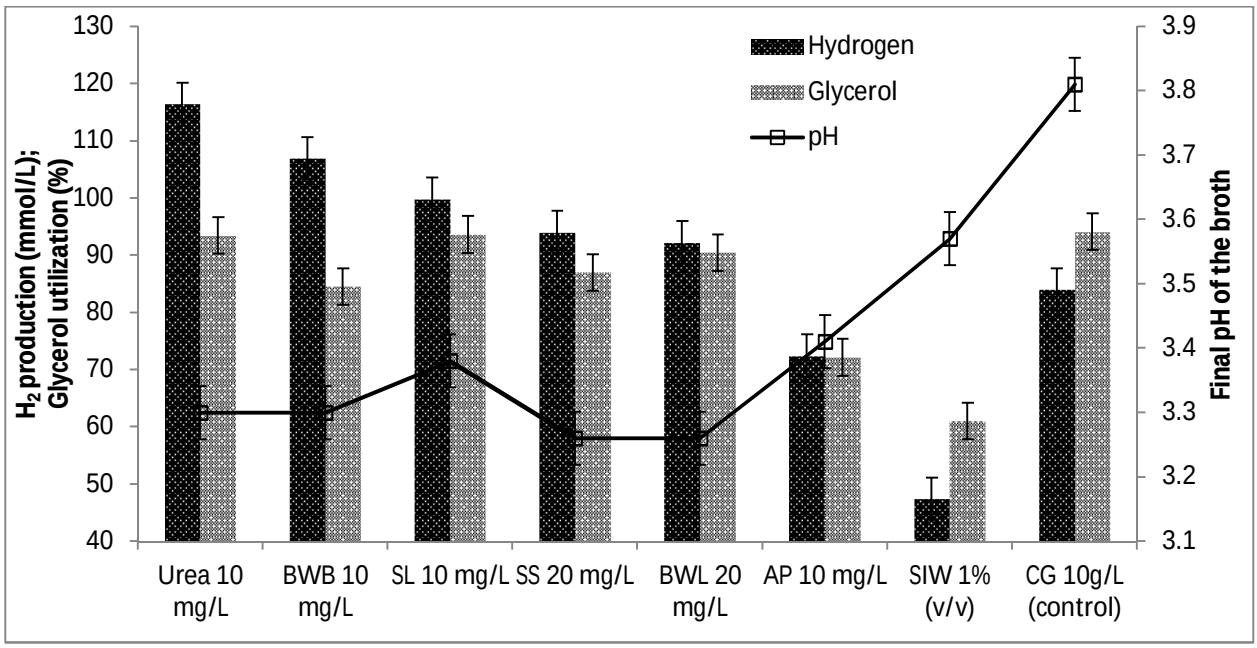


Figure 3.2. 2: Maximum cumulative H_2 production (mmol/L), glycerol utilization (%) and broth pH recorded at the end of fermentation for different material supplemented to 10 g/L CG. The control indicates the results obtained for 10 g/L CG without any additive.

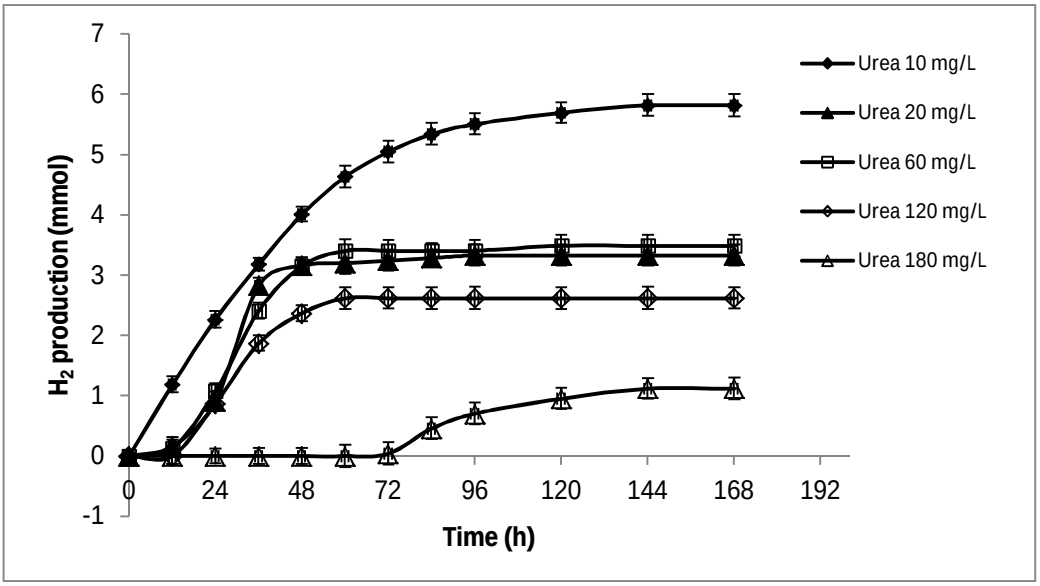


Figure 3.2. 3: H_2 production profiles obtained for different concentrations urea supplemented to 10 g/L CG

CHAPTER 4

EFFECT OF CRUDE GLYCEROL IMPURITIES: SPECULATIONS AND FACTS

PART I

INVESTIGATION OF THE EFFECT OF DIFFERENT CRUDE GLYCEROL COMPONENTS ON HYDROGEN PRODUCTION BY *ENTEROBACTER AEROGENES* NRRL B-407

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Résumé

Le glycérol brut (GB) est le principal sous-produit (déchets) de la production de biodiesel (par transestérification de lipides) et en poids, il pourrait être aussi élevé que 10% (poids/poids) du produit final. Considérant l'intérêt mondial pour la production de biodiesel, il existe un besoin pour une gestion durable de ces déchets. Dans ce contexte, la production de biohydrogène par l'utilisation du GB pourrait être une option intéressante. De plus, l'hydrogène est considéré comme étant la plus propre des énergies renouvelables pour le remplacement des combustibles fossiles. Cependant, le GB contient quelques impuretés, tels que le méthanol, le NaCl les savons et leurs effets individuel et interactif sur la production d'hydrogène doivent être examinés. Ainsi, cette étude traite de l'effet des contaminants du GB tels que le méthanol, le NaCl et le savon sur la production de biohydrogène. Sur la base de la vérification effectuée en utilisant la méthodologie de surface de réponse, on a observé que le savon est le facteur le plus significatif (valeur $p = 0,0104 < 0,05$) pour inhiber la production d'hydrogène. De façon similaire, le méthanol a un effet négatif sur la production d'hydrogène dans la gamme de concentration de 1.3 à 3.3 g/L. En contrepartie, le NaCl à des concentrations de 0.15 à 0.55 g/L a montré un effet positif sur le processus de bioconversion. Dans l'ensemble, la présence des différents contaminants contenus dans le GB a eu un effet inhibiteur réduisant la production cumulative de H_2 de 21,6 à 95,3%. En outre, sur la base des observations présentes une nouvelle stratégie intégrée pour l'enlèvement du savon et la gestion du pH lors de la fermentation a été émise afin d'améliorer la production de H_2 à partir du GB.

Mots-clés: biodiesel; biohydrogène; glycérol brut; impureté; savon

Abstract

Crude glycerol (CG) is the main by-product (waste) of biodiesel production (by transesterification of lipids) and by weight it could be as high as 10 % (w/w) of the final product. Considering global interest for biodiesel production, there is a need for sustainable management of the waste. In this context, bio-hydrogen production by utilization of CG could be an interesting option. Moreover, hydrogen is considered as the cleanest renewable alternative of fossil fuels. However, CG contains a few impurities, such as methanol, NaCl and soap and their individual and interactive effect on hydrogen production should be investigated. Thus, present study deals with the investigation of the effect of methanol, NaCl and soap on hydrogen production and substrate (glycerol) utilization. Based on the investigation carried out using response surface methodology, it was observed that soap is the most significant factor (p value = 0.0104 < 0.05) to inhibit hydrogen production. Similarly, methanol was also found to have negative effect on hydrogen production in the concentration range (1.3 to 3.3 g/L) considered. Meanwhile, NaCl (0.15- 0.55 g/L) showed positive effect on the process. Overall, presence of different impurities was found to reduce the cumulative H_2 production by 21.56 to 95.31 %. Furthermore, based on present observations a novel integrated strategy for soap removal and process pH management has been hypothesized for improved H_2 production by CG bioconversion.

Keywords: biodiesel; bio-hydrogen; crude glycerol; impurity; soap

Introduction

Shrinking fossil fuel reserves, the concern of greenhouse gas emission and global climate change, strict regulations for mandatory renewable content in transportation fuels as well as subsidies offered by different governments are some of the reasons for wide exploration of biodiesel production technologies. Transesterification of vegetable oils, animal fats and other lipids (microbial origin) is the core process involved in biodiesel production. During trans-esterification process, crude glycerol (CG) is generated as a waste by-product, which is around 10 % by weight of the biodiesel produced by the process (Chatzifragkou *et al.*, 2012). Considering possible increase in global biodiesel production and the amount of CG produced by the process, there is a need for a sustainable strategy of CG management. Utilization of CG as a feedstock for microbial production of commercially important products could be an interesting option (Dobson *et al.*, 2012). In this context, production of bio-hydrogen by bioconversion of CG has been evaluated by various researchers (Sabourin-Provost *et al.*, 2009, Sakai *et al.*, 2007, Selembo *et al.*, 2009). Actually, H_2 is considered as an ideal renewable energy source for future because it does not produce any greenhouse gas during combustion. In fact, it is the only carbon free fuel to produce only water as combustion by-product as well as it has highest energy content among gaseous fuels of equivalent weight (Meher Kotay *et al.*, 2008). Similarly, it can be easily fed to fuel cells or internal combustion engines for energy conversion (Fang *et al.*, 2006). However, CG is not a pure substrate for hydrogen production and it may contain a range of impurities. Methanol, salt and soap are three of the major impurities present in CG and they might have certain effect on hydrogen production by CG bioconversion (Venkataramanan *et al.*, 2012, Yang *et al.*, 2012). In fact, type of substrate and carbon-nitrogen ratio of the media has significant influence on hydrogen production (Zhao *et al.*, 2008). As the impurities such as methanol and soap could also be utilized as carbon sources, their presence or absence in the media might affect the performance of the process. Moreover, all three impurities might show certain interactions among themselves and these possibilities are yet to be explored for a bio-hydrogen production process. Thus,

the main objective of the present study was to evaluate the effect of these three CG impurities on hydrogen production by glycerol bioconversion.

The investigation has been carried out using response surface methodological approach and the experimental plan was prepared by using central composite design. Batch bio-hydrogen production experiments were carried out using small scale anaerobic bioreactors by employing *Enterobacter aerogenes* (Nakashimada *et al.*, 2002, Rachman *et al.*, 1997).

Materials and methods

Chemicals

Crude glycerol (CG) used in the present investigation is a by-product of biodiesel production process which was collected from Rothsay®, Canada. Other chemicals, such as glucose, ethanol, NaOH, HCl, $MgSO_4.\ 7H_2O$, sodium periodate, acetone, acetylacetone, bromophenol blue and acetic acid were purchased from Fisher scientific (Missisauga, Ontario, Canada). Casein peptone and KH_2PO_4 were supplied by VWR International (Ontario, Canada) and the yeast extract used in the present study was a gift from Lallemand, Canada.

Microorganism and inoculum preparation

Enterobacter aerogenes NRRL B 407, the microorganism considered for the present investigation was provided by ARS, USDA, USA. It is a gram-negative, facultative anaerobic, rod-shaped bacterium known to produce hydrogen by using a range of organic substrates. The microorganism was grown and maintained in a culture medium composed of glucose (5 g/L), casein peptone (5 g/L), KH_2PO_4 (2 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L) and yeast extract (0.5 g/L) (Beckers *et al.*, 2010). For the entire investigation, the same growth medium was used to prepare the inocula. For inoculum development, the microorganism was anaerobically grown at 30 ±1° C (150 rpm) using a rotary shaker incubator and 5% (v/v) freshly prepared (exponential growth phase) cell suspension was used as inoculum for all the experiments conducted in the present investigation.

Saponification of crude glycerol and separation of soap

The soap used in the present investigation was separated from a CG sample collected from biodiesel production plant. The CG sample was first saponified by a slightly modified method based on the original method reported by (Hu *et al.*, 2012). Briefly, 10 g CG was dissolved 100 mL water and the solution was mixed with 100 mL of 50 g/L NaOH-ethanol solution. The mixture was taken in a screw cap bottle and kept in a rotary shaking water bath (90 ± 1°C/50 rpm) for 1h. After cooling the mixture to room temperature equal volume of deionized water was added and the soap present in the solution was precipitated by salting out http://people.cedarville.edu/employee/gollmers/gsci1020/labs/08g1020lab5.pdf (accessed on 29/11/2012) (using 10% w/v NaCl). Precipitated soap was separated from the solution by centrifugation (5000 g for 20 min) and lyophilized for 24h prior to its application in the investigation. Entire process of soap separation has been shown in Figure 4.1.1.

Experimental design and hydrogen production experiments

As already mentioned, methanol, soap and NaCl are the three impurities of crude glycerol which were evaluated in the present investigation for their role in CG bioconversion and hydrogen production. A central composite design was employed to study their effect using response surface methodology (RSM). In Table 4.1.1, the amount of the impurities which could be present in a CG sample has been summarized based on literature data. Similarly, 10 g/L CG has been found to be optimum for biohydrogen production (Sarma *et al.*, 2013). Therefore, as presented in Table 4.1.1 and 2, the minimum concentrations of the impurities that could be present in 10 g/L CG have been considered as the central points of the independent variables (impurities) for present experimental design. Further, the experimental design has been constructed by using Design-Expert® -7 software (Stat-Ease Inc. Minneapolis, MN) and the design has been extended up to + α and – α level (Table 4.1.2). Based on the design, 20 different runs of experiments were performed using different combinations of the independent variables (impurities) as presented in Table 4.1.3. Cumulative hydrogen production

(mmol/L after 48h) and percent utilization of glycerol have been considered as the two responses (dependent variables) of the investigations (Table 4.1.3).

All the experiments were carried out by using 125 mL serum bottles with 50 mL working volume. The experiments were performed using (separate serum bottles) a culture media composed of pure glycerol (5 g/L), casein peptone (1 g/L) and yeast extract (0.05 g/L) with which different concentrations of methanol, soap and NaCl were mixed according to the experimental plan (Table 4.1.3). One control experiment was carried out using the same media composition (but devoid of any impurity) and this experiment was not a part of the present experimental design. Further, pH 6 was found to be optimum for H_2 production by *E. aerogenes* (Sakai *et al.*, 2007). Hence, the pH of the culture media taken in different serum bottles was adjusted to 6 by using 0.1 N NaOH/HCl solutions. Subsequently, the media were sparged with ultrapure N_2 gas for 2 min to create anaerobic environment for supporting the growth of the facultative anaerobic bacteria (*E. aerogenes*). The bottles sparged with N_2 were immediately sealed with aluminum crimp seal containing silicone septum (Fisher scientific, Canada) with the help of a hand operated crimper (E-Z Crimper™, VWR International, Ontario, Canada). Sealed bottles were sterilized by autoclaving and cooled to room temperature following which they were inoculated using the inoculum mentioned in previous section (section 2.2). Inoculation was made through the silicone septum of the crimp seal of the serum bottle by using a sterile syringe fitted with a fine needle. Finally, the bottles were incubated at 30±1°C by using an orbital incubator shaker maintained at 150 rpm (Sarma *et al.*, 2013). By using a 5 mL gastight syringe gas samples (1 mL each) were collected from the headspace of the culture bottle and kept in gas sampling vial (Exetainer®, Labco, UK) for gas chromatographic analysis. Similarly, at the end of the fermentation (H_2 production process), 1 mL of liquid sample was also collected from each bottle for residual glycerol content analysis.

Analytical techniques

Hydrogen analysis

After 48h of incubation, H_2 gas produced in the case of each experiment was collected (as already mentioned in previous section) in gas sampling vial (Exetainer®, Labco, UK) and analyzed by a gas chromatograph (Varian 3800, USA) fitted with PoraPLOT Q® column (Agilent technology, USA) and thermal conductivity detector (TCD). Likewise, the column temperature was fixed at 100 °C and nitrogen was used as the carrier gas with a gas flow rate was 3.5 mL/min and the retention time was 4.5 min. Obtained value was expressed as mmol H_2/L media.

Glycerol analysis

During hydrogen production process aqueous samples were collected at regular interval (24h). Glycerol concentration of such samples were measured by using a spectrophotometric method of glycerol analysis proposed by (Bondioli *et al.*, 2005). Briefly (please see the original method for further information), 1 mL of the aqueous sample (after proper dilution with distilled water) was mixed with 1 mL ethanol (47.5% v/v). Further, 1.2 mL each of 0.2 M acetylacetone solution and 10 mM sodium periodate solution (both were prepared by using a 1:1 mixture of 1.6 M acetic acid and 4.0 M ammonium acetate stock solution) were added to the mixture. Subsequently, the reaction mixture was incubated at 70 ± 1°C for 1 min by using a rotary shaker water bath and immediately cooled to 20 ± 1°C by using cold water. The samples were maintained at the same temperature (by changing the water, if needed) until the optical density (410 nm) was measured by using an UV-Vis spectrophotometer (Carry 100 Bio®, Varian USA) (Sarma *et al.*, 2013).

Results and discussion

The results and discussion portion of the present investigation could be divided into two parts based on the two different responses considered in the study. In the first part, the effect of the impurities on hydrogen production has been discussed and the second part deals with glycerol utilization.

Effect of the impurities on cumulative H_2 production

The responses (hydrogen production and glycerol utilization) obtained for different runs of experiments carried out using different concentrations of the impurities have been presented in Table 4.1.3. Obtained responses of the experiments were analyzed by using the same software (Design-expert®) used for designing of the experiments. Response surface linear model was the suggested model for the response observed in terms of hydrogen production. The ANOVA report for the response surface linear model has been presented in Table 4.1.4. The model was significant for the observed response of hydrogen production with insignificant *lack of fit* and factor C (soap) was the most significant factor. Similarly, the graphs obtained by analysis of the response for hydrogen production have been presented in Figure 4.1.2, 4.1.3 & 4.1.4. Figure 4.1.2 represents the response obtained as a function of methanol (factor A) concentration. Methanol had a strong negative effect on H_2 production and the increase in methanol concentration from 1.3 g/L to 3.3 g/L can result in the decrease in cumulative H_2 production. Meanwhile, in an investigation of docosahexaenoic acid production by bioconversion of crude glycerol, Pyle et al (2008) have mentioned that due to the increase in methanol concentration from 0 to 20 g/L in the culture, media biomass productivity could be decreased from 1.92 ± 0.08 g/L-d to 0.94 ± 0.08 g/L-d (Pyle *et al.*, 2008). Therefore, known inhibitory effect of methanol on microbial growth could be the most possible reason of present observation.

Likewise, Figure 4.1.3 represents the response of hydrogen production obtained as a function of NaCl (factor B) concentration. By increasing the NaCl concentration from 0.15 g/L to 0.55 g/L, cumulative H_2 production could be improved from around 1.3 mmol/L to 1.6 mmol/L. There may be two reasons for such observation. Firstly, NaCl might be helping in maintaining the osmotic balance of the system leading to improved H_2 production. Secondly, NaCl may form the salt of organic acids (e.g. sodium acetate with the acetic acid) produced during the process by which it may improve the buffering capacity of the system. As pH change due to organic acid production can significantly inhibit the process, improvement of buffering capacity (due to NaCl) of the media could enhance the hydrogen production. The present observation is strongly supported by a

previous study by (Ngo *et al.*, 2011) where the authors have reported the beneficial effect of NaCl on H_2 production using pre-treated crude glycerol. Thus, it can be concluded that although NaCl is an impurity in CG, its presence in the concentration level tested in the present study has no negative effect on the process.

Unlike NaCl, soap (factor C) was found to be inhibitory for hydrogen production. Moreover, as shown in Table 4.1.4 it is found to be the most significant factor (p value = 0.0104 < 0.05) to influence the process. Figure 4.1.4 is the graphical representation of the influence of the factor on hydrogen production. As it is clear from the figure, an increase in soap concentration from 1g/L to 3 g/L can decrease the cumulative hydrogen production by nearly 33% and reduce the production from around 1.8 mmol/L to 1.2 mmol/L. Meanwhile, in an investigation of eicosapentaenoic acid production by bioconversion of biodiesel derived CG Athalye et al (2009) have shown that even a soap concentration as low as 1 g/L can significantly reduce the biomass as well as the product yield and it supports our findings (Athalye *et al.*, 2009). Further, possible role of soap molecules in the disruption of microbial cell membranes could be a reason of our observation. Thus, in the concentration ranges tested in the present study, methanol and soap was found to have negative effect on hydrogen production. Therefore, proper pre-treatment of CG for soap and methanol removal could be the best option for improved H_2 production.

Effect of the impurities on glycerol utilization

Similar to the responses for hydrogen production, glycerol utilization responses obtained for 20 different runs of experiments have been presented in Table 4.1.3 and analyzed by Design Expert-7 software. Unlike the data for hydrogen production, response surface linear model could not be fitted to the data obtained for glycerol utilization indicating that the factors might have interaction among themselves. All the graphs generated during the analysis of the data obtained for glycerol utilization have been shown in Figure 4.1.5. Figure 4.1.5 (a) shows the 3 dimensional response surface indicating the effect of methanol and NaCl (factor A & B) on glycerol utilization. It could be determined that the glycerol utilization has been significantly reduced by initial

methanol concentration higher than 2.3 g/L. Similar to other alcohols, methanol could be toxic to bacterial cell either by destroying their cell wall or by altering the functions of intracellular proteins Whas's toxic and what's not. http://2the4.net/toxic.htm (accessed on 10/12/2012). Hence, reduced glycerol utilization at higher methanol concentration could be attributed to detrimental effect of the alcohol to the process organism. Likewise, maximum glycerol utilization could be observed for initial methanol concentration of around 2 g/L. Alcohol concentration dependent improvement in cell permeability (Volpe *et al.*, 2008) and probable improvement in glycerol uptake rate could be a reason for such response of glycerol utilization. Moreover, at methanol concentration less than 1.5 g/L slight decrease in glycerol utilization has been observed. A possible reason for finding of this kind could be that at relatively low methanol concentration improvement in cell permeability was not sufficient to significantly improve the substrate utilization; on the contrary, at such concentration methanol may compete with the substrate for preferential utilization. However, it is a general assumption and it has to be established by proper investigation. Similarly, from Figure 4.1.5 (a) corresponding to the increase in NaCl concentration improvement in glycerol utilization could be observed. Considering NaCl, it is an identical response obtained for both glycerol utilization and hydrogen production which has been discussed in previous section. Likewise, a similar response (increase in glycerol utilization) has been observed (Figure 4.1.5-b) when both soap and NaCl concentration have been simultaneously increased to maximum. Further, from Figure 4.1.5 (c) it could be summarized that at constant NaCl (0.3 g/L) and relatively low methanol (around 1.8 g/L) concentration, percent utilization of glycerol could be slightly increased by increasing the soap concentration. Meanwhile, surfactant is known to improve the bioavailability of organic substrate (Sarma *et al.*, 2011); hence, soap mediated improved glycerol utilization could be the most probable reason of present observation. However, unlike the present observation (response 2), as already mentioned, a strong negative effect of soap could be observed for response 1 (hydrogen production) Figure 4.1.4. Thus, it has been noted that by increasing the soap concentration from 1g/L to 3 g/L glycerol utilization could be slightly improved however corresponding increase in hydrogen production has not been

observed. Metabolic shift, a phenomenon commonly observed in fermentative hydrogen production (Srikanth *et al.*, 2010) may be a reason for reduced hydrogen production in presence of relatively higher amount of soap.

Thus, soap and methanol in the initial concentration ranges investigated in the present study were found to have very strong negative effect on hydrogen production by CG bioconversion process. Further, Figure 4.1.6 shows the hydrogen production profile obtained for a control experiment carried out without the addition of any impurity. Cumulative hydrogen production obtained for the control experiment was higher than all 20 runs of experiment carried out using different concentration of the impurities. Actually, compared to the control experiment (Figure 4.1.6) 21.56 to 95.31 % reduction in cumulative H_2 production could be observed in the case of 20 runs of experiments carried out using different concentration of the impurities (Table 4.1.3). This observation further confirmed the inhibitory effect of the impurities. Meanwhile, the present investigation was planned considering that 10 g/L CG is optimum for hydrogen production. Therefore, from the responses obtained from the experimental design (Table 4.1.3), it could be summarized that hydrogen production could be improved by diluting the CG below 10 g/L. However, it should also be considered that by diluting the CG, the glycerol present in CG will also be diluted and it will have a negative effect on cumulative H_2 production of the process. Thus, selective removal of methanol and soap could be the most obvious option for enhanced CG bioconversion. Methanol could be evaporated from a CG sample by increasing the temperature beyond 65 °C (Jasuja *et al.*, 2010, Pyle *et al.*, 2008). Likewise, as it is already described salting out (by adding NaCl) of soap could be an interesting option. Moreover, NaCl has showed beneficial response for hydrogen production as well as it might have a role in improving the buffering capacity (as already discussed good buffering capacity of the media is beneficial for fermentative H_2 production) of the process. Hence, by applying NaCl, a combined soap removal and pH management strategy could be developed for enhanced H_2 production by CG bioconversion. Presently we are working on this strategy and it will be communicated as a separate report.

Conclusions

Using response surface methodology, role of three different CG impurities (soap, methanol and NaCl) on hydrogen production and glycerol bioconversion has been evaluated. Among these impurities, soap (1 to 3 g/L) was found to have significant (p value = 0.0104 < 0.05) inhibitory effect on hydrogen production. Likewise, methanol (1.3 to 3.3 g/L) has also showed similar response in the concentration range considered. Interestingly, for both the impurities, the response for glycerol utilization was not similar to the response obtained hydrogen production (as expected). Co-utilization of the impurities along with glycerol as well as a metabolic shift due to the presence of the impurities was identified as most probable reason for such observation. Meanwhile, NaCl was found to have beneficial effect for both H_2 production and glycerol utilization. Based on the observations a novel strategy involving NaCl has been proposed for combined soap removal and buffer capacity improvement of CG based media.

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Table 4.1. 1: Distribution of different impurities of biodiesel derived crude glycerol

CG impurity	Presence in CG	Minimum conc. of the impurity present in CG	Minimum conc. present in 10 g/L CG	Reference
Methanol	23.4-37.5 % (w/w)	23%w/w	2.3 g/L	(Tang *et al.*, 2009, Thompson *et al.*, 2006)
Sodium chloride	3.12 % (w/w)	3.5% w/w	0.35 g/L	http://rendermagazine.com/articles/2008-issues/2008-august/2008-08-crude-glycerin/ (accessed on 03_09_2012)
Soap	20.5-31.4 % (w/w)	20 % w/w	2.0 g/L	(Hu *et al.*, 2012)

Table 4.1. 2: Central composite design ranges of the three factors considered for present investigation.

Sl. no.	Variables	Symbol	Variable concentration (coded level) g/L				
			-α	Low	Mid	High	+α
1	Methanol	A	0.62	1.30	2.3	3.3	3.98
2	Sodium chloride	B	0.01	0.15	0.35	0.55	0.69
3	Soap	C	0.32	1.00	2.0	3.00	3.68

Table 4.1. 3: Experimental design and the responses obtained for 20 different experiments carried out during the present study

Run	Independent variables			Responses	
	Methanol (g/L)	**NaCl (g/L)**	**Soap (g/L)**	**H_2 (mmol/L)**	**Glycerol utilization (%)**
1	2.30	0.35	2.00	1.68	78.57
2	3.30	0.55	3.00	1.75	81.42
3	3.30	0.15	3.00	1.11	68.57
4	2.30	0.35	0.32	2.51	80
5	2.30	0.35	2.00	1.34	65.71
6	2.30	0.35	2.00	1.75	68.57
7	0.62	0.35	2.00	1.27	58.57
8	1.30	0.55	1.00	1.96	55.71
9	3.30	0.55	1.00	2.01	65.71
10	2.30	0.35	2.00	1.84	64.28
11	2.30	0.69	2.00	1.68	80

Run	Independent variables			Responses	
	Methanol (g/L)	**NaCl (g/L)**	**Soap (g/L)**	**H_2 (mmol/L)**	**Glycerol utilization (%)**
12	1.30	0.55	3.00	1.28	72.85
13	1.30	0.15	3.00	1.28	77.14
14	3.30	0.15	1.00	1.65	78.57
15	2.30	0.35	3.68	1.09	78.57
16	1.30	0.15	1.00	1.81	74.28
17	2.30	0.35	2.00	1.09	71.42
18	2.30	0.01	2.00	0.9	51.42
19	2.30	0.35	2.00	1.4	60
20	3.98	0.35	2.00	0.15	18.57

Table 4.1. 4: ANOVA for response surface linear model used for response 1 (hydrogen production)

Source	Sum of squares	Degree of freedom	Mean square	F value	P value
Model	2.07	3	0.69	4.10	0.0245*
A-Methanol g/L	0.21	1	0.21	1.25	0.2803
B-NaCl g/L	0.44	1	0.44	2.64	0.1239
C-Soap g/L	1.42	1	1.42	8.42	0.0104*
Residual	2.69	16	0.17	-	-
Lack of Fit	2.28	11	0.21	2.51	0.1598**
Cor Total	4.76	19	-	-	-

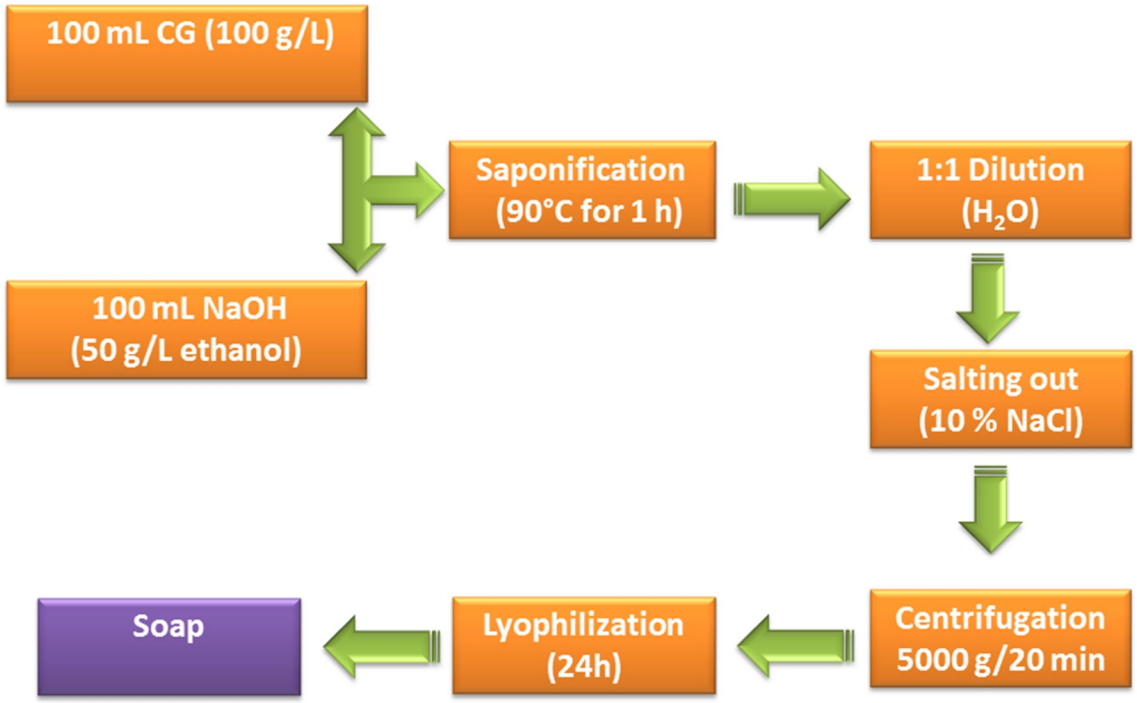


Figure 4.1. 1: Schematic representation of soap separation from crude glycerol sample

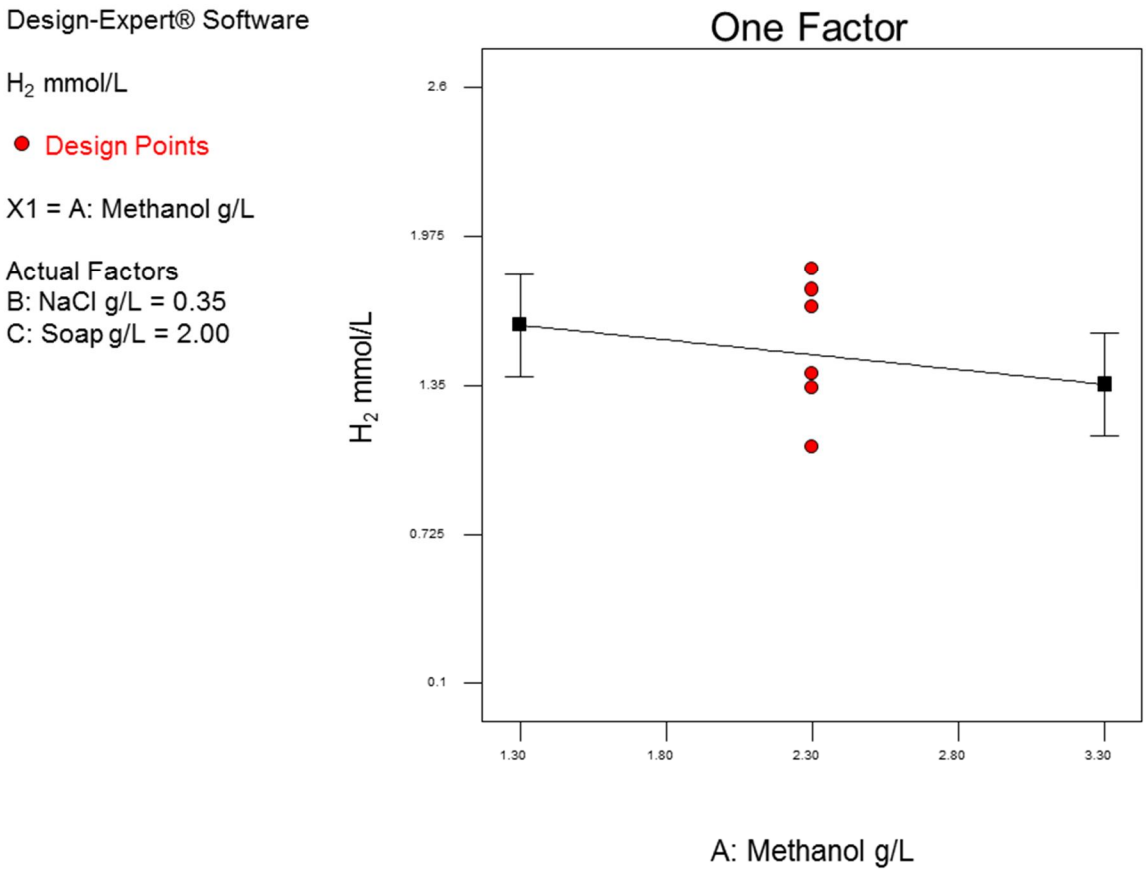


Figure 4.1. 2: Response surface graph showing the effect of methanol on hydrogen production

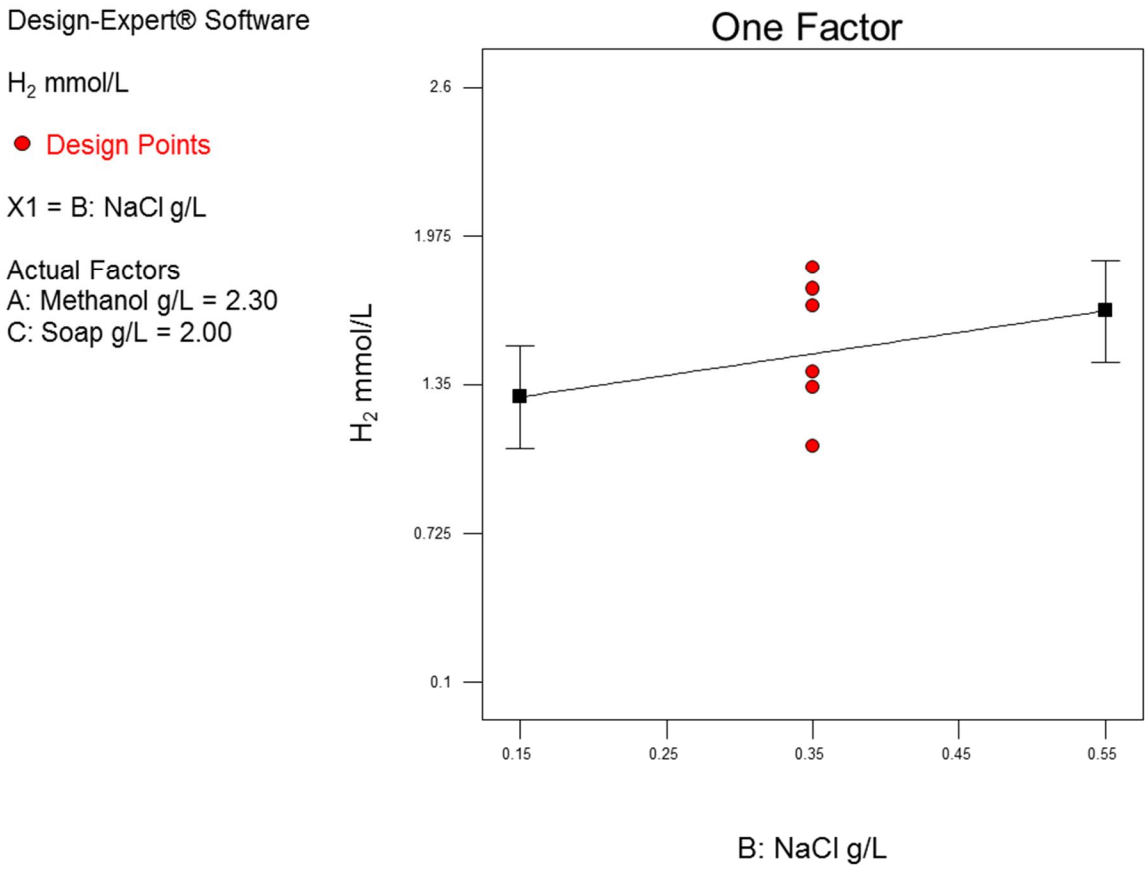


Figure 4.1. 3: Response surface graph showing the effect of NaCl on hydrogen production

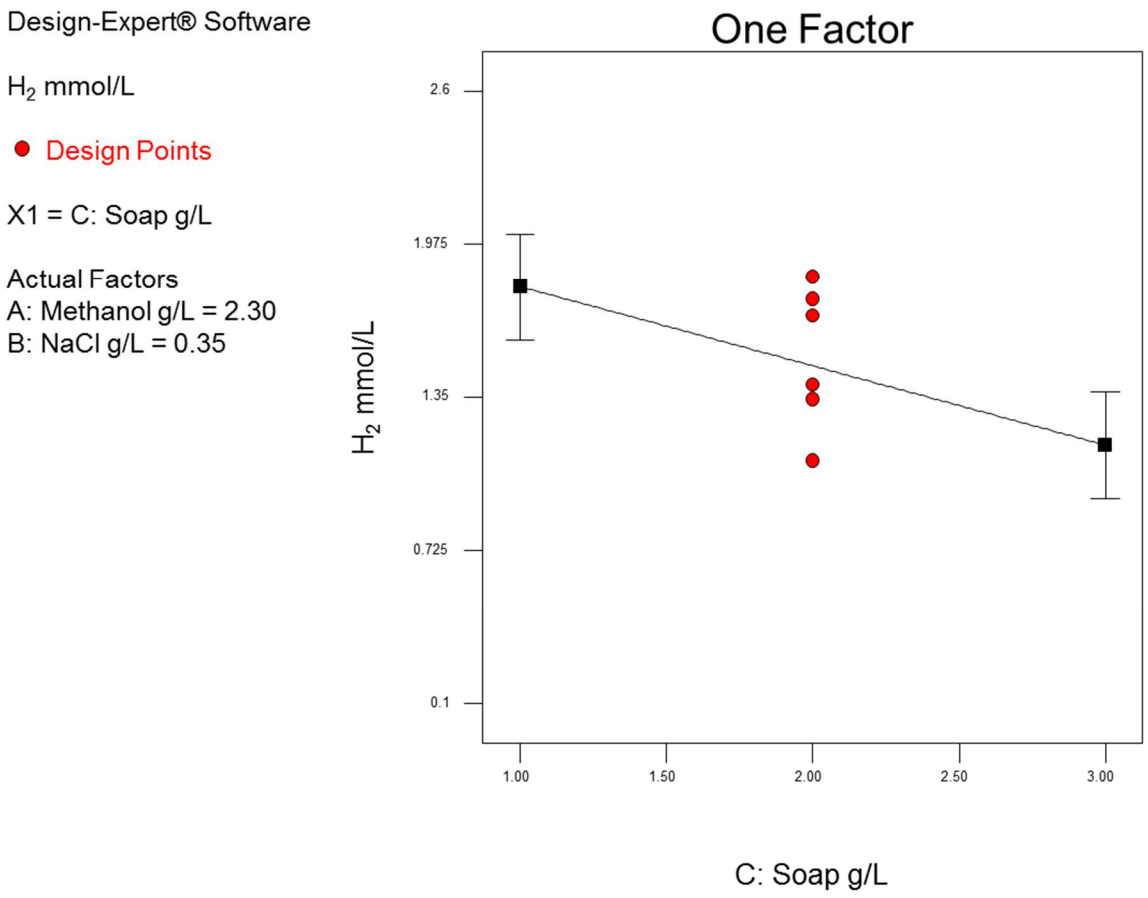
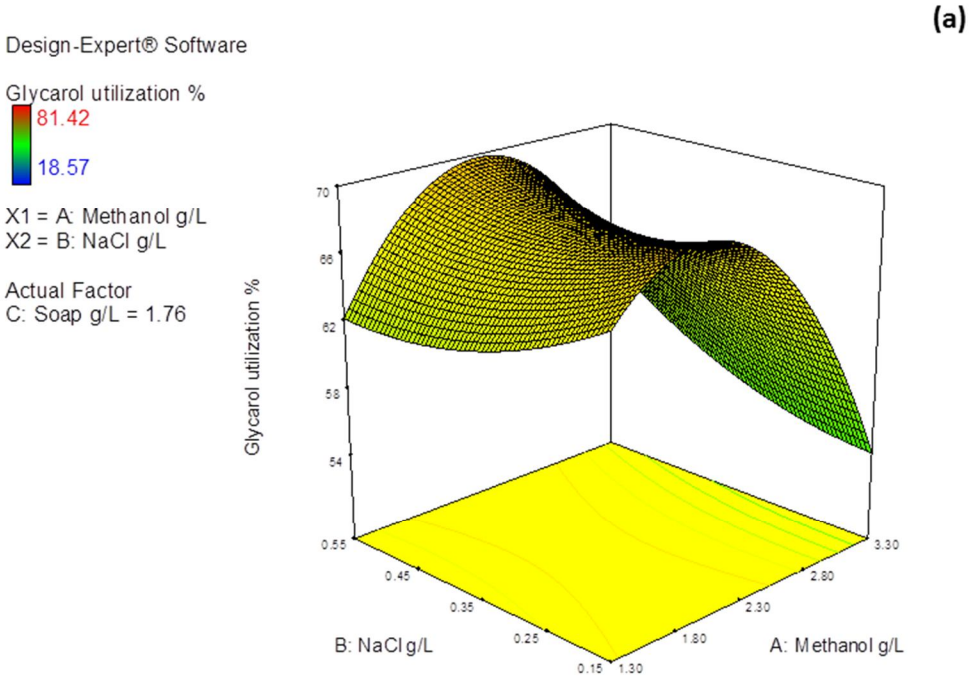
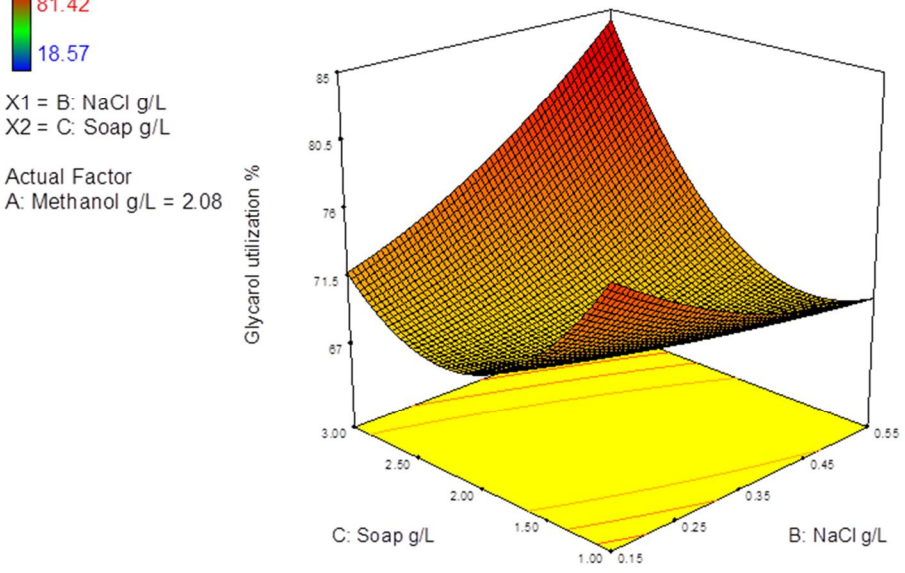


Figure 4.1. 4: Response surface graph showing the effect of soap on hydrogen production


(a)
Design-Expert® Software
Glycarol utilization %
81.42
18.57
X1 = A: Methanol g/L
X2 = B: NaCl g/L
Actual Factor
C: Soap g/L = 1.76
Glycarol utilization %
70
66
62
58
54
0.55
0.45
0.35
0.25
0.15
B: NaCl g/L
1.30
1.80
2.30
2.80
3.30
A: Methanol g/L

(b)


Design-Expert® Software
Glycarol utilization %
81.42
18.57
X1 = B: NaCl g/L
X2 = C: Soap g/L
Actual Factor
A: Methanol g/L = 2.08
Glycarol utilization %
85
80.5
76
71.5
67
3.00
2.50
2.00
1.50
1.00
C: Soap g/L
0.55
0.45
0.35
0.25
0.15
B: NaCl g/L

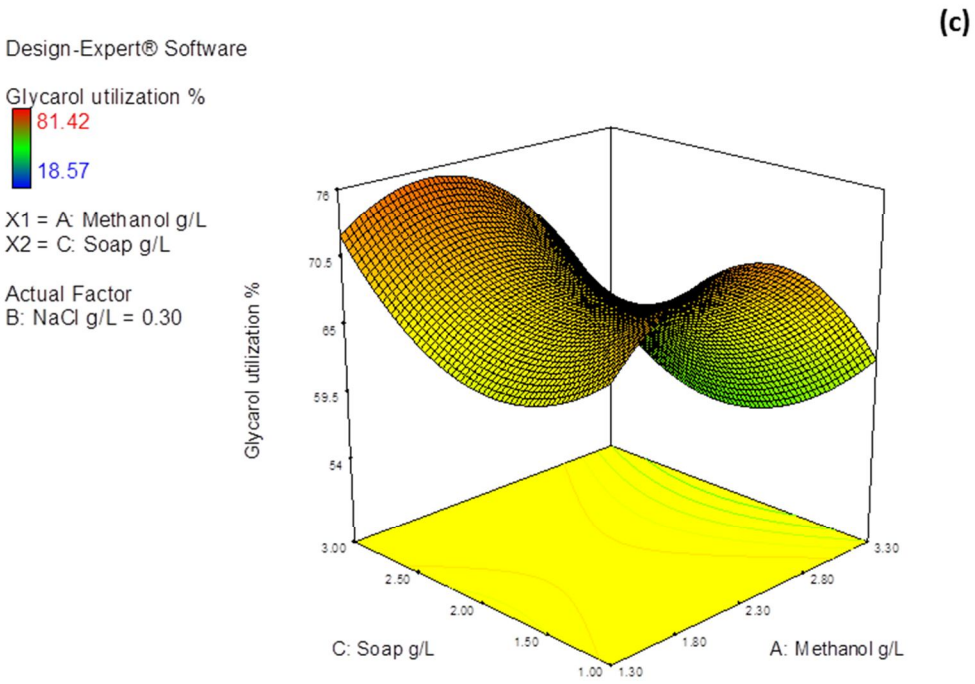


Figure 4.1. 5: Response surface graphs showing the effect of (a) methanol and NaCl; (b) NaCl and soap and (c) soap and methanol on glycerol utilization

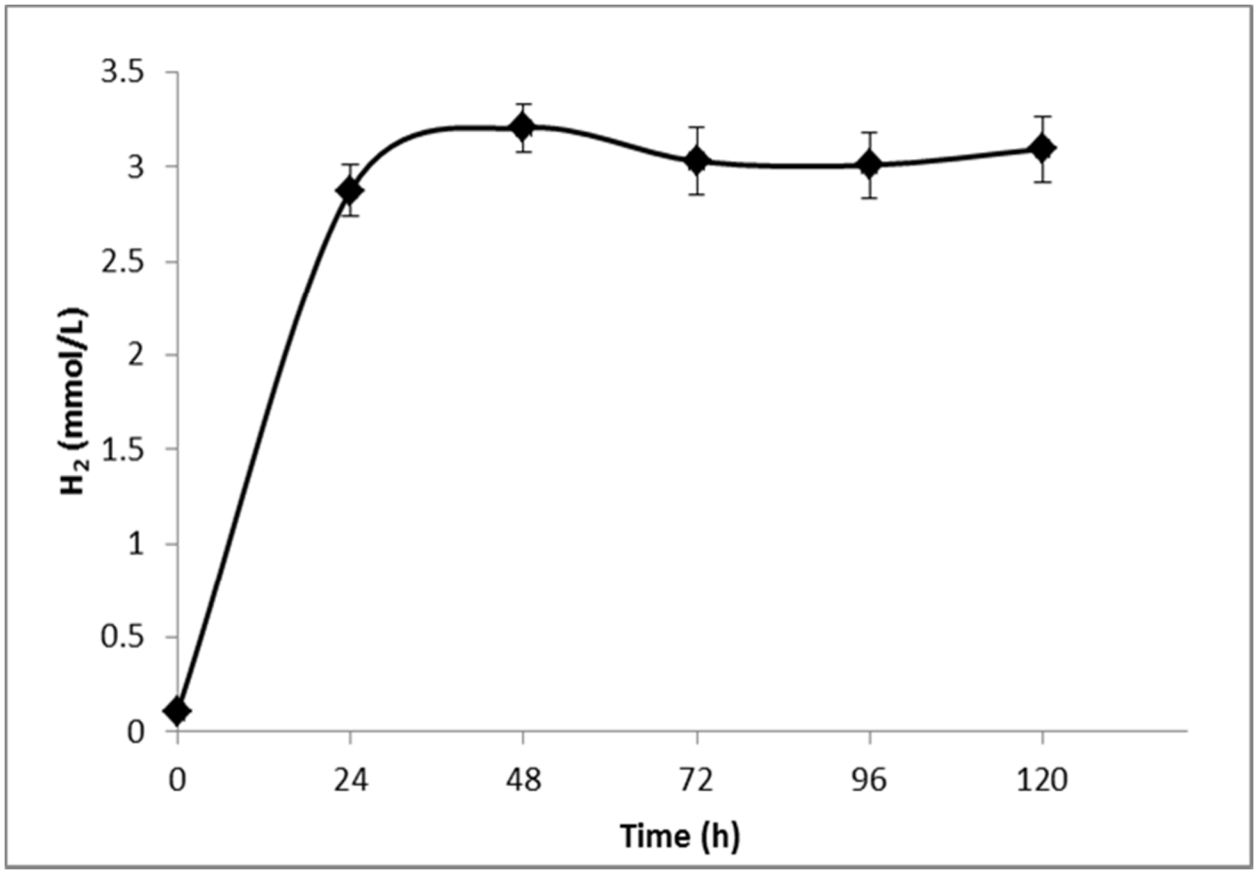


Figure 4.1. 6: Hydrogen production profile obtained for the control experiment carried out in the absence of any impurity

PART II

MITIGATION OF THE INHIBITORY EFFECT OF SOAP BY MAGNESIUM SALT TREATMENT OF CRUDE GLYCEROL- A NOVEL APPROACH FOR ENHANCED BIOHYDROGEN PRODUCTION FROM THE BIODIESEL INDUSTRY WASTE

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Résumé

En raison de son effet inhibiteur sur la croissance microbienne, le savon présent dans le glycérol brut (GB) est un sujet de préoccupation pour la valorisation biologique des déchets de fabrication du biodiesel. Par une stratégie de relargage (Salting out), jusqu'à 42% du savon a été retiré du milieu et l'approche a un effet bénéfique sur la production de H_2. Toutefois, les expérimentations montrent que l'élimination de plus de 7% du savon a été défavorable à la production de H_2. En fait, le savon, sous certaines concentrations, est utilisé comme co-substrat et son élimination affecterait le rapport carbone-azote du milieu diminuant ainsi la production de H_2. Sans modifier le rapport carbone-azote du GB, le traitement au $MgSO_4$ permet de convertir le savon en sa forme inactive (écume). L'approche a permis d'augmenter le taux de production de H_2 de 33,8%, la production cumulative de H_2 de 34,7% et la consommation du glycérol (près de 2,5 fois). Également, le traitement permet d'augmenter le Mg (un nutriment) contenu dans le milieu de culture support de 0,57 mg/L à 202mg/L.

Mots clés: biodiesel; biohydrogène; glycérol brut; impureté; savon; magnésium

Abstract

Owing to its inhibitory effect on microbial growth, soap present in crude glycerol (CG) is a concern in biological valorization of the biodiesel manufacturing waste. By salting out strategy, up to 42% of the soap has been removed and the approach has beneficial effect on H_2 production; however, removal of more than 7% of the soap was found to be inhibitory. Actually, soap is utilized as a co-substrate and due to removal; the carbon-nitrogen ratio of the medium might have decreased to reduce the production. Alternatively, without changing the carbon-nitrogen ratio of CG, $MgSO_4$ treatment can convert the soap to its inactive form (scum). The approach was found to increase the H_2 production rate (33.82%), cumulative H_2 production (34.70%) as well as glycerol utilization (nearly 2.5 folds). Additionally, the treatment can increase the Mg (a nutrient) content of the medium from 0,57 mg/L to 202 mg/L.

Key words: Biodiesel; biohydrogen; crude glycerol; impurity; soap

Introduction

At present, global biodiesel production is around 30 billion liter and it is projected to reach 42 billion liter mark by 2021 (www.fao.org, 2013). During biodiesel production by transesterification of lipids (e.g. vegetable oils, animal fats and algal lipids, among others), crude glycerol (CG) is generated as a byproduct; and by weight, it is around 10% of the biodiesel produced (Johnson *et al.*, 2007). Due to presence of numbers of impurities as well as continuous increase in its global production (corresponding to the increase in biodiesel production), the present market value of CG is very low. According to a report by Yang *et al.* (2012), the price of CG is only around $ 0.05/pound. Likewise, due to high pH, high COD/BOD, presence of methanol as well as its viscous nature, environmental disposal of CG is a challenge for biodiesel manufacturers (Dobson *et al.,* 2012). Alternatively, CG can be used as a feedstock for microbial hydrogen production and in the context of global interest for the development of renewable energy sources, it can be considered as a promising technology.

As already mentioned, CG contains a range of impurities and soap is one of them (Hu *et al*., 2012; Pyle *et al*., 2008). According to Hu *et al*. (2012), soap content of different CG sample may vary from 20.5 ± 0.1 to 31.4 ± 0.1 % (w/w). Unfortunately, soap is a potential inhibitor of microbial growth (Athalye *et al*., 2009). Therefore, removal of the soap from CG may have beneficial effect on microbial growth and enhanced hydrogen production can be expected by CG bioconversion. Thus, in the present study NaCl and $MgSO_4$ based treatment strategies to mitigate the inhibitory effect of soap present in CG has been demonstrated. Enhancement in hydrogen production and glycerol utilization has been determined to evaluate the approaches. Additionally, potential beneficial effect of soap present in CG on hydrogen production has also been explored. *Enterobacter aerogenes,* a well-known hydrogen producer (Fabiano and Perego, 2002) has been used in the present study.

Materials and methods

Chemicals and reagents

Biodiesel manufacturing waste (crude glycerol) was supplied by Rothsay, Canada and after proper dilution (as specified below) it was used as the substrate for hydrogen production. Glucose, NaCl, $MgSO_4$, NaOH, HCl, acetone, ethanol, bromophenol blue, sodium periodate, acetylacetone, ammonium acetate and acetic acid were purchased from Fisher scientific (Mississauga, Ontario, Canada). Yeast extract was provided by Lallemand (Montreal, Canada) and casein peptone and KH_2PO_4 was purchased from VWR (Mississauga, Ontario, Canada).

Microorganism and culture conditions

Enterobacter aerogenes NRRL B 407, a facultative anaerobic bacterium provided by ARS, USDA, USA, was used for all the hydrogen production experiments conducted in this study. The microorganism was maintained by regular sub-culturing using a nutrient medium containing glucose (5 g/L), casein peptone (5 g/L), KH_2PO_4 (2 g/L) and yeast extract and $MgSO_4$ (0.5 g/L each). For this purpose, 125 mL serum bottles were used and in each bottle, about 50 mL of the medium was taken. The serum bottles containing the media were converted to anaerobic bioreactor by passing ultrapure N_2 gas (for 2 min) through the media and they were immediately sealed with aluminum crimp seal containing silicone septum. A hand operated crimper (E-Z Crimper™, VWR, Canada) was used for sealing the bottles. After autoclaving, the bottles were cooled to room temperature and they were inoculated (using sterile syringe fitted with fine needle) with 5 % (v/v) microbial suspension from preceding culture. Finally, the bottles were incubated in an incubator shaker operated at 150 rpm and 30°C. A bacterial cell suspension obtained by the above procedure with the microorganisms in their exponential phase of growth was used as inoculum (5% v/v) for all hydrogen production experiments conducted during the present investigation (Sarma *et al*., 2013).

CG pretreatment using NaCl

A crude glycerol sample with initial pH of 11.37 has been considered for this investigation. Due to high viscosity, the original sample was diluted with distilled water to prepare a CG solution of 200 g/L. To determine the effect of NaCl concentration on soap removal from crude glycerol, 50 mL of 200 g/L CG was separately mixed with different concentrations of NaCl (1, 5, 10, 20, 50, 100, 200 and 300 g/L) by using a magnetic stirrer. After complete solubilization of NaCl, precipitated soaps were removed by centrifuging the mixtures at 5000 g for 20 min. Soap concentration of untreated and NaCl treated CG samples were determined (as described later) and soap removal efficiency of different NaCl treatment was expressed as percent soap removal. NaCl containing supernatants were properly diluted (as specified in next sections) with distilled water to get required concentration of CG and used as the feedstock for hydrogen production.

Bio-hydrogen production using NaCl treated CG

Optimum CG concentration for biohydrogen production by *E. aerogenes* NRRL B 407 was found to be 10 g/L (Sarma *et al*., 2013). Therefore, to determine the effect of NaCl treatment, CG treated with different concentrations of NaCl (viz. 1, 5, 10, 20, 50, 100 and 200 g/L of NaCl) were diluted with distilled water to obtain a final CG concentration of 10 g/L. For each experimental run, 25 mL of NaCl treated diluted CG (i.e. 10 g/L) was taken in 125 mL serum bottle (VWR, Canada) and the medium pH was adjusted to 6.0 (which is optimum for hydrogen production by *E. aerogenes* (Marques *et al*., 2009)). An experiment was also conducted using untreated CG of similar concentration and it was considered as negative control. As already mentioned, to achieve anaerobic condition, oxygen was driven out of the medium by passing ultrapure N_2 gas using a fine needle fitted silicon tube and the bottle was immediately sealed. Sealed bottles were sterilized (by autoclaving) and after cooling to room temperature, they were inoculated with 5 % (v/v) fresh microbial suspension. Inoculum preparation, inoculation technique and other culture conditions were same as mentioned above. During incubation period (120h), at 12/24h interval, 1 mL of the produced gas was collected from the headspace of each

serum bottle by using a 5 mL gastight syringe (SGE Analytical Science Pvt. Ltd, Australia). Likewise, 1 mL of aqueous sample was collected at 24 h interval by using a sterile syringe. Collected gas and liquid samples were analyzed by standard analytical techniques which are separately described.

Bio-hydrogen production using $MgSO_4$ treated CG

For this experiment, 20 g/L of CG was used as the substrate for hydrogen production and four different concentrations of $MgSO_4$ (viz. 0.5, 1, 2.5 and 5 g/L) have been tested. Specified amount of $MgSO_4$ was separately mixed with 25 mL of CG taken in different serum bottles (125 mL) and to evaluate the effect of $MgSO_4$ treatment on hydrogen production, the bottles were further prepared, inoculated and incubated in the same manner as described for NaCl treated CG.

Analytical methods

Determination of soap concentration in CG samples

To determine the amount of soap present in CG, a titrimetric method was used and the concentration was expressed as sodium oleate equivalent (Sarma *et al*., 2013). Briefly, 150 µL of 0.5% (w/v) bromophenol blue prepared in 95% ethanol was mixed with 60 mL of acetone. Further, the solution was neutralized with 0.1 M NaOH and was mixed with 10 g of CG sample (NaCl treated and untreated). Subsequently, for 1 min the solution was placed in a water bath maintained at 70°C and after cooling to room temperature, it was titrated using 0.1 M HCl. Disappearance of blue color of the solution was an indication of completion of the titration and the amount of the acid consumed was used for calculating the soap content of different CG samples (Sarma *et al*., 2013).

Hydrogen analysis

To determine the concentration of hydrogen accumulated in the headspace of the serum bottles (used for hydrogen production), the samples were analyzed by gas chromatography. As already mentioned, 1 mL of gaseous sample was collected from each bottle, at 12/24h interval for 120h by using gastight syringe and they were kept in 5.9 mL gas sampling vial (Exetainer®, Labco, UK). The gas chromatographic analysis

was performed by using a Varian 3800 gas chromatograph (Varian, USA) and the column and detector used for the analysis were PoraPLOT Q® (Agilent technology, USA) and thermal conductivity detector (TCD), respectively. The carrier gas used for this analysis was nitrogen, the flow rate was 3.5 mL/min and the column temperature was 100 °C (Sarma *et al*., 2013).

Glycerol analysis

The liquid samples collected during hydrogen production were analyzed for residual glycerol concentrations by using the same method as already described in (Sarma *et al*., 2013; Bondioli and Della Bella, 2005). Briefly, the original sample was diluted with distilled water and 1 mL of the same was mixed with 1 mL of ethanol (47.5 % v/v). The resulting solution was mixed with 1.2 mL of acetylacetone solution (0.2 M) and 1.2 mL of sodium periodate solution (10 mM). Further, the reaction mixture was placed in a water bath maintained at 70°C (for 1 min) and subsequently transferred to another water reservoir containing cold water (20°C). Finally, the samples were analyzed (410 nm) by an UV-Vis spectrophotometer (Varian, USA) (Sarma *et al*., 2013).

Results and discussion

NaCl pretreatment and bio-hydrogen production using treated CG

Addition of salt (NaCl) can increase the solubility of glycerol (glycerol is more soluble in saline water) in a crude glycerol sample and therefore, the soap present in the solution can be precipitated and removed by centrifugation (PCE, 2013). Due to high viscosity, the original sample of CG was not suitable for centrifugal removal of precipitated soap. Therefore, it was first diluted with distilled water to prepare a CG solution of 200 g/L and then treated with different concentrations of NaCl. Eight different concentrations of NaCl (1, 5, 10, 20, 50, 100, 200 and 300 g/L) were evaluated for soap removal. The concentration, 300 g/L is almost close to the solubility limit of NaCl; hence, more than 300 g/L was not considered. 1 g/L NaCl was unable to remove any soap; whereas, 42 % soap removal was achieved for 300 g/L of NaCl. Further, crude glycerol treated with different concentrations of NaCl was evaluated as the feedstock for hydrogen production. Figure 4.2.1 shows cumulative hydrogen production obtained (after 120h of

incubation) by bioconversion of 10 g/L of crude glycerol treated with different concentrations of NaCl. From Figure 4.2.1, it can be concluded that treatment of CG by 5, 10 or 20 g/L of NaCl has beneficial effect on cumulative hydrogen production; where, a maximum 19.28 % improvement in hydrogen production has been observed for CG treated with 10 g/L NaCl (corresponding to 5% soap removal). However, further increase in NaCl concentration can drastically inhibit the production; where, maximum 93.03 % decrease in hydrogen production (Figure 4.2.1) has been observed for CG treated with 200 g/L NaCl (corresponding to 31% soap removal). One probable reason for such observation can be that 9-31 % removal of soap (corresponding to 50-200 g/L NaCl treatment) might decrease the C-N (carbon-nitrogen) ratio of CG based media to an inhibitory level. It is well known that slight change in C-N ratio can significantly affect the hydrogen production process (Argun *et al*., 2008; Lin and Lay, 2004). This observation also indicates that although, soap is largely considered as an impurity of CG; still it has a role in hydrogen production and most possibly, along with glycerol it is used as a co-substrate by the bacterium.

It should be noted that during NaCl treatment of CG, NaCl was completely dissolved in the CG solution. Therefore, apart from CG, the hydrogen production media (prepared by diluting the CG treated with different concentrations of NaCl) also contained different concentrations of NaCl (Figure 4.2.1). Hence, presence of relatively high concentration of NaCl in the media might be a reason for reduced hydrogen production in the case of 50-200 g/L NaCl treated CG. To verify this possibility, an experiment was conducted where 200 g/L NaCl treated CG was diluted with distilled water to an extent so that it contained only 0.5 g/L NaCl in the final media (as maximum hydrogen production was obtained for 10 g/L NaCl treated CG where NaCl concentration in the final medium was 0.5 g/L -Figure 4.2.1). As shown in Figure 4.2.2, the results clearly indicate that irrespective of NaCl concentration in the medium (within the concentration range tested in the present investigation) separation of the soap from CG has negative effect on hydrogen production. Thus, as already mentioned non-availability of soap as a co-substrate and change in C-N ratio of the media due to separation of the soap from CG, can be the main reasons of observed reduced hydrogen production.

Final pH of the media left after hydrogen production by bioconversion of CG treated with different concentrations of NaCl can vary from 3.96 ± 0.03 to 3.75 ± 0.03. It has been observed that corresponding to an increase in soap removal due to NaCl treatment, there was a decrease in final pH of the media. The result indicates that soap might have played a role of buffering/acid neutralizing agent in CG based media and it has the capacity to minimize the pH drop due to organic acid production during bio-hydrogen production.

Biohydrogen production using $MgSO_4$ treated CG

From aforementioned findings, it is clear that although soap is considered as an impurity of CG and it has known inhibitory effect on microorganism; yet, presence of soap in CG is beneficial for fermentative hydrogen production. Therefore, if without separating it from CG, the functional property of the soap molecule could be destroyed (so that its inhibitory effect could be mitigated); enhanced H_2 production by CG bioconversion may be possible. It is well known that hard water contains Mg^{2+} or Ca^{2+} ions which react with soap (www.mrwa.com, 2013). Mg^{2+} ion binds with soap molecules to form scum, which is an ineffective form of soap. Therefore, $MgSO_4$ was added to the CG based medium so that soap molecules present in the solution could be converted to scum and at the same time they remain as a part of the solution (unlike salting out). Figure 4.2.3a & 4.2.3b show the hydrogen production and glycerol utilization profiles obtained by bioconversion of CG treated with four different concentrations of $MgSO_4$ (0.5 g/L, 1 g/L, 2.5 g/L and 5 g/L). It is clear that 0.5 g/L $MgSO_4$ was unable to show any significant improvement in hydrogen production. In the case of 1 g/L $MgSO_4$ treated CG, during active production phase, the hydrogen production rate was 33.82% higher than that of untreated CG. Similarly, cumulative hydrogen production was found to be enhanced by 34.70%. Thus, the approach has the potential to significantly reduce the duration of the fermentation with simultaneous increase in cumulative hydrogen production. Apart from its role as a “killer of soap”, $MgSO_4$ might have played a role of additional nutrient for the bacterium. This assumption is further supported by the fact that Mg is a part of the culture media used for Enterobacter (Zhen-qian and Chun-yun, 2009) and crude glycerol contains only 57.41 ± 18 ppm of the same (Sarma *et al*., 2013). For hydrogen

production, CG has to be diluted to a fermentable concentration (e.g. 10 g/L) and during this process Mg concentration is further decreased to around 0.57 ppm. Moreover, this small concentration of Mg naturally present in CG can react with soap to form scum, and become unavailable for microbial utilization. Thus, application of $MgSO_4$ has dual benefits for the process. However, further increase in $MgSO_4$ concentration was not found to have any beneficial effect, in fact CG treated with 5 g/L $MgSO_4$ was found to inhibit the hydrogen production by 75.94%. The glycerol utilization profiles presented in Figure 4.2.3b shows that compared to 8.65% glycerol utilization in the case of untreated CG (control) nearly 2.5 folds increase (around 20.94%) in glycerol utilization has been observed in the case of 1 g/L $MgSO_4$ treated CG. Similarly, in the case of 0.5 g/L, 1 g/L and 2.5 g/L $MgSO_4$ treated CG, the rates of glycerol utilization were found to be higher than that of untreated CG. A possible explanation of improved glycerol utilization is that due to $MgSO_4$ treatment, soap scum was formed and a portion of the soap molecules became temporarily unavailable (as co-substrate) for the bacterium; hence, more glycerol was utilized. This observation further ascertains the assumption that although soap is commonly considered as an impurity of CG (Pyle *et al*., 2008; Athalye *et al*., 2009; Ethier *et al*., 2011); during hydrogen production along with glycerol it is also utilized as a co-substrate.

The purpose of the present investigation was to determine the effect of NaCl and $MgSO_4$ treatment on hydrogen production by CG bioconversion. Both the treatments have shown beneficial effect on the process. Especially, owing to negligible Mg content of CG, application of magnesium salt seems to be indispensable for improved CG bioconversion. Similarly, for the first time, the evidence of beneficial role of soap (present in CG) on CG bioconversion has been obtained. Thus, the present investigation has covered an unexplored side of CG bioconversion process. However, these are only preliminary findings and to be suitable for industrial application, the process needs further improvement in a numbers of aspects. Firstly, the concentration of $MgSO_4$ and CG should be optimized in such a way, so that the requirement of $MgSO_4$ can be reduced and complete utilization of CG can be achieved. Secondly, the closed batch mode of fermentation used for the present investigation is suitable only for the

study of the effect of different treatments. However, hydrogen accumulation in the headspace of the reactor is inhibitory for the process (van Niel *et al*., 2003). Hence, continuous separation of produced gas should be considered for large scale hydrogen production. Similarly, in the present investigation, sterilized CG was used as the feedstock. However, considering the cost and energy requirement, feedstock sterilization may not be a feasible option for large scale hydrogen production. Compared to conventional feedstock such as, wastewater/wastewater sludge, CG is expected to have a lesser load of indigenous microorganism. Therefore, in the case of hydrogen production by CG bioconversion, presence of potential hydrogen consumers such as, methanogens is not expected. Thus, it will be interesting to study the hydrogen production potential of unsterile CG.

Conclusions

Salting out can remove around 42% of the soap present in a CG; however, along with glycerol it is utilized as a co-substrate and its removal may inhibit the H_2 production by decreasing the C-N ratio of the medium. Alternatively, without removing it from the medium, $MgSO_4$ treatment of CG can convert the soap to its inactive form (scum). The novel approach can increase the cumulative H_2 production by 34.70% and glycerol utilization by nearly 2.5 folds. Additionally, as CG contains only 57.41 ± 18 ppm of Mg, $MgSO_4$ addition can also improve the nutritional quality of the feedstock.

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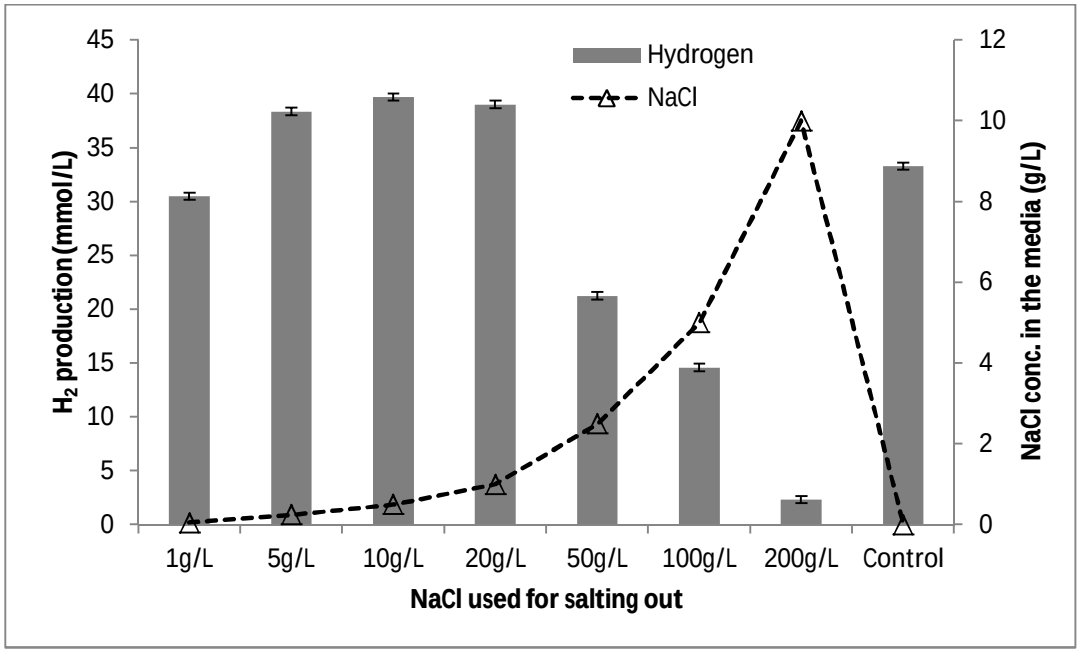
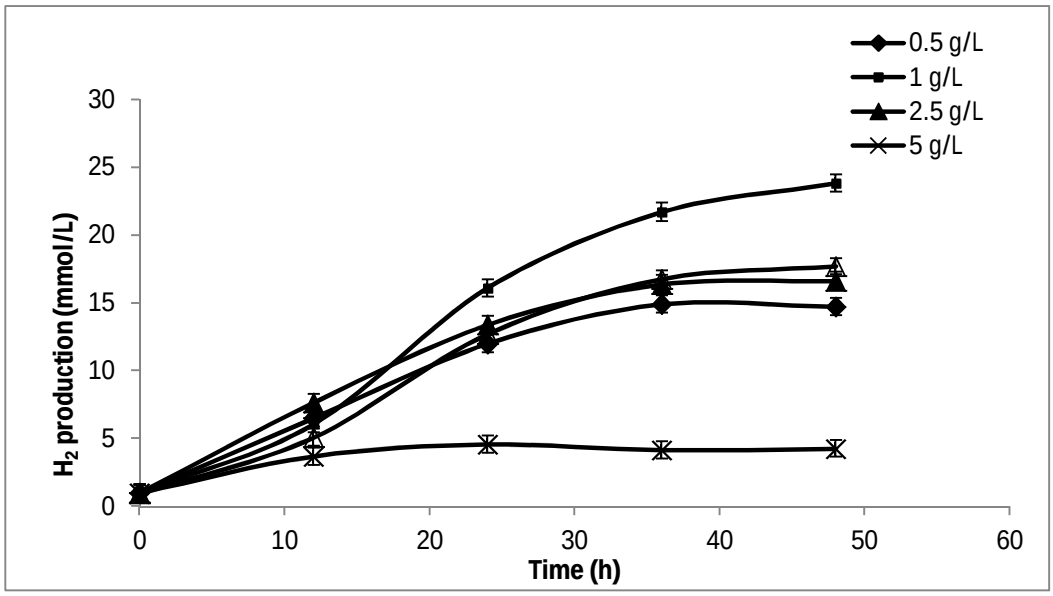
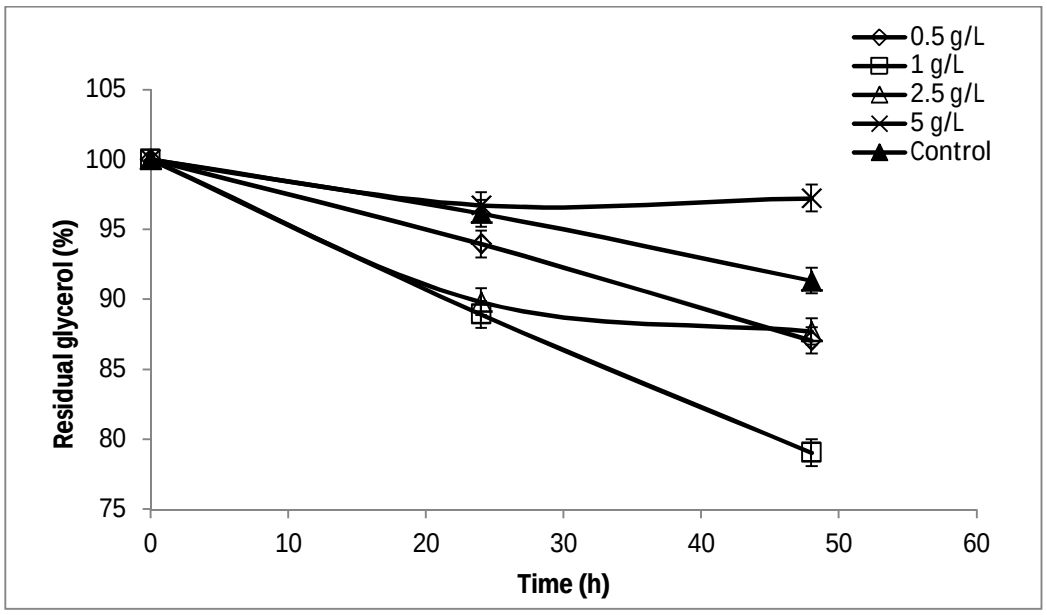


Figure 4.2. 1: Cumulative H_2 production (after 120h) obtained using 10 g/L CG (treated with different concentration of NaCl). Initial NaCl concentration in the medium of each run of experiment was also shown

Figure 4.2. 2: Cumulative H_2 production obtained using 200 g/L NaCl treated CG diluted to an initial NaCl concentration of 0.5 g/L (as from Figure 1, 0.5 g/L NaCl was found to be optimum). Control represents result obtained for equivalent amount of untreated CG


0.5 g/L
1 g/L
2.5 g/L
5 g/L
30
25
20
15
10
5
0
H_2 production (mmol/L)
0
10
20
30
40
50
60
Time (h)

3a



3b

Figure 4.2. 3: H_2 production (a) and glycerol utilization (b) profiles obtained for 20 g/L CG treated with different concentrations of $MgSO_4$. Control represents result obtained for untreated CG

CHAPTER 5

APPLICATION OF NANOTECHNOLOGY FOR IMPROVED CRUDE GLYCEROL BIOCONVERSION

ENRICHED HYDROGEN PRODUCTION BY BIOCONVERSION OF BIODIESEL WASTE SUPPLEMENTED WITH FERRIC CITRATE AND ITS NANO-SPRAY DRIED PARTICLES

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Résumé

L'augmentation de la consommation des combustibles fossiles non renouvelables ainsi que l'inquiétude suscitée par la pollution et les changements climatiques ont accéléré le développement de l'industrie des biocarburants durables. Le biodiesel, le bioéthanol et le biométhane sont disponibles au niveau commercial comme des alternatives des combustibles fossiles. Actuellement, des recherches visent à trouver un biocarburant plus respectueux de l'environnement, de préférence produit à partir de matières premières non alimentaires et capable de satisfaire aux exigences énergétiques des transports. Dans ce contexte, le biohydrogène produit à partir des déchets organiques produit de l'eau comme seule émission importante lors de la combustion. De plus, l'hydrogène est un candidat idéal en raison de son contenu énergétique massique plus élevé par rapport aux carburants fossiles et biocarburants actuels. Dans la présente recherche, le glycérol brut (GB) comme sous-produit de production de biodiesel a été utilisé comme matière première pour la production de biohydrogène et différents suppléments ont été testés pour augmenter le rendement de la fermentation. Le citrate ferrique et ses nanoparticules produites par atomisation à sec ont permis d'améliorer la production d'hydrogène par 50,5 %. La production d'hydrogène utilisant des concentrations extrêmement faibles en GB de 100 mg/L a permis de produire 22,7 mol-H_2/kg GB ce qui est supérieur à 2,75 fois la valeur de 8,3 mol-H_2/kg GB répertoriée pour la fermentation sombre.

Mots clés: biohydrogène; biodiesel; glycérol brut; Le citrate ferrique; nanoparticule

Abstract

Increasing consumption of non-renewable fossil fuels as well as the concern over pollution and global climate change has accelerated the development of sustainable biofuel industry. Biodiesel, bioethanol and biomethane are already commercially available as alternatives of fossil fuels and the search for a more environmental friendly biofuel, preferentially produced from non-food raw materials and capable of fulfilling the transportation energy requirement of the globe for longer duration, is going on. In this context, biohydrogen produced from waste biomass; which produces water as the only major emission during combustion and has higher energy content compared to fossil and biofuels of equivalent mass, is an ideal candidate. In the present investigation, crude glycerol (CG) generated as a by-product of biodiesel production process has been used as a feedstock for biohydrogen production and different supplements have been tested for increasing the product yield. Ferric citrate and its nano-spray dried particles have been found to enhance the hydrogen production by 50.45 %. Hydrogen production using extremely low CG concentration of 100 mg/L has been found to produce 22.7 mol-H_2/kg CG; which is 2.75 folds higher than 8.25 mol-H_2/kg CG known for dark fermentation.

Key words: Biohydrogen; Biodiesel; Crude glycerol; Ferric citrate; Nanospray dried particles

Introduction

Considering global energy security as well as greenhouse gas emission reduction potential, increased production and application of biofuels is inevitable. At this juncture, biofuel production is not equally profitable in all countries and hence, different governments have applied encouraging policies, such as tax exemption (Banse *et al.*, 2013). Moreover, in order to ensure increased biofuel consumption and replacement of fossil fuel, mandatory blending of fossil fuels with 2-10 % of biofuel has been targeted in many countries (Banse *et al.*, 2013). There are different types of biofuels presently in use; however, greenhouse gas emission reduction potential, availability of raw materials and energy content of these biofuels are not similar. Therefore, for long term production and use, all of them may not be equally suitable (Hay *et al.*, 2013). In this context, biohydrogen could be considered as an emerging biofuel with very high sustainability. It can be produced from a diverse group of renewable raw materials and hence, does not necessarily compete with food crop based biomass for feedstock. Additionally, during combustion, hydrogen is converted to water and does not produce any carbon based emission (Hay *et al.*, 2013, Mohd Yasin *et al.*, 2013, Sydney *et al.*). Energy content of hydrogen is 142 kJ /g, which is higher than any fossil fuel of equivalent mass (Hay *et al.*, 2013, Sydney *et al.*). Thus, hydrogen production by bioconversion of low cost agro-industrial organic waste materials has commercial importance. In the present study, enhanced hydrogen production by bioconversion of crude glycerol has been investigated. In the course of biodiesel production by trans-esterification of vegetable oil, animal fat or any other lipid based feedstock; crude glycerol (CG) is produced as a by-product. By weight, the amount of glycerol generated by the process could be as high as 10 % of the biodiesel produced (Yang *et al.*, 2012). Corresponding to increased global biodiesel production, crude glycerol production is also increasing and consequently, CG price has decreased to around $ 0.05/pounds (Yang *et al.*, 2012). Thus, application of CG as a feedstock for hydrogen production by anaerobic fermentation could be considered as a lucrative option for its value-addition.

[NiFe]-hydrogenase and [FeFe]-hydrogenase are the key enzymes responsible for fermentative hydrogen production (Schmidt *et al.*, 2011). Ni and/or Fe cofactors are indispensable part of the active sites of these enzymes (Volbeda *et al.*, 1996). Therefore, it has been postulated that sufficient amount of Ni and Fe should be present in the fermentation media for improved hydrogen production. Based on this assumption, in the present study, fermentation media prepared using CG have been supplemented with ferric citrate, $FeSO_4$, nickel acetate, and $NiCl_2$ and their effects on hydrogen production have been investigated. Owing to their very large specific surface area, nanoparticles are generally considered to have higher reactivity (Van Leeuwen *et al.*, 2013). Therefore, aforementioned supplements were used in nanospray dried form. For the present investigation, *Enterobacter aerogenes,* a well-known hydrogen producing microorganism has been used (Tanisho, 1998, Zhang *et al.*, 2011). CG used in this investigation was supplied by Rothsay®(Canada) and its detailed characterization could be found in Sarma *et al.* (2013).

Materials and Methods

Microorganism and inoculum preparation

Enterobacter aerogenes NRRL B-407, a rod-shaped, gram-negative, facultative anaerobic bacterium from Enterobacteriaceae family has been used for hydrogen production experiments. It was collected from ARS, USDA, USA in freeze-dried form. To subculture the organism and to prepare inoculum for the study, a synthetic medium composed of glucose and casein peptone (5 g/L each); KH_2PO_4 (2 g/L); and yeast extract and $MgSO_4.7H_2O$ (0.5 g/L each) was used. Serum bottles of 125 mL with 50 mL of working volume were used to culture the microorganism. Media taken in these bottles were first sparged with N_2 gas for 2 min; the bottles were immediately closed with aluminum crimp seals having silicon septa and sterilized by autoclaving. After inoculation, the culture bottles were incubated in an incubator shaker operated at 30°C and 150 rpm and 5 % (v/v) of microbial culture at its exponential phase of growth was used as an inoculum for hydrogen production.

Preparation and characterization of different nano-spray dried particles

Ferric citrate, $FeSO_4$, nickel acetate, and $NiCl_2$ particles used in the present investigation have been prepared by using a nano-spray dryer (Buchi B-90, Switzerland). For the preparation of $FeSO_4$, nickel acetate, and $NiCl_2$ particles, a solution of 50 g/L of each compound was separately prepared in distilled water and subjected to nano-spray drying process. For all particles, the respective solution was fed to the nano-spray dryer at a flow rate of nearly 20 mL/h (flow level 3). Similarly, in each case, the maximum temperature of the spray dryer was kept constant at 120 °C and the air flow rate was maintained at 120L/min. The spray cap used for entire spray drying process was of 4.0 µm mesh size. During the process, particles were accumulated on the internal surface of the collecting electrode which was subsequently recovered by particle collecting accessories supplied with the instrument. Collected nano-spray dried particles were used in hydrogen production experiments. For preserving the particles, airtight glass vials were used. For the preparation of ferric citrate particles, almost similar process was applied. However, as ferric citrate solution could not be prepared using water in room temperature; either hot water or 1N NaOH was used.

Zeta potential distribution of nano-spray dried ferric citrate particles prepared using hot water was determined by a zetasizer nano ZS system (Malvern instruments Ltd., UK). For this purpose, small amount of the particles was dispersed in Milli-Q water and 1 mL of this solution was analyzed.

A scanning electron microscope (Zeiss EVO® 50 Smart SEM) has been used for morphological characterization of the nano-spray dried particles prepared for this investigation. The ferric citrate nano-spray dried particles were first dispersed in Milli-Q water; a small amount of dispersed nano-spray dried particles was taken on a piece of aluminum foil, air dried and finally transferred to SEM stub. Prior to SEM analysis, samples were coated with gold by using a sputter coater.

Screening of different nano-spray dried particles for biohydrogen production

Purpose of this investigation was to investigate the effect of nano-spray dried particles prepared using ferric citrate, $FeSO_4$, nickel acetate, and $NiCl_2$, on hydrogen production by CG bioconversion. All the particle types were prepared using aqueous solution as already mentioned in previous section (2.2). CG stock solution was distributed in different 125 mL serum bottles in such a way that after inoculating with 5 % (v/v) inoculum, total volume of the medium and concentration of CG in each bottle would become 25 mL and 12 g/L, respectively. About 50 mg/L of each nano-spray dried particle type was separately added to different bottles and the medium pH of each bottle was adjusted to 6. Nitrogen was passed through the media; bottles were sealed, sterilized, inoculated, and incubated in the same manner as described in section 2.1. One mL of biogas containing hydrogen was collected from the headspace of each bottle using a 5 mL gastight glass syringe (SEG, Australia) and stored in 5.9 mL airtight glass vials (Exetainer®, Labco, UK). Prior to storing the gas samples, glass vials were flashed with ultrapure nitrogen gas and evacuated using a needle fitted vacuum pump.

Investigation of the effect of Fe-citrate particle preparation method on hydrogen production

As mentioned in section 2.2, ferric citrate was dissolved either in hot water or in NaOH (1N); which gave two different types of solutions. From these two solutions, two different types of ferric citrate particles were prepared by nano-spray drying process. About 10, 50, 100, 300 and 500 mg/L of both particles were separately added to different serum bottles containing 12 g/L CG and their effect on hydrogen production was investigated by similar method mentioned in section 2.3.

Investigation of the effect of CG concentration on H_2 production using media supplemented with ferric citrate particles

The purpose of this study was to investigate the effect of ferric citrate nano-spray dried particles on hydrogen production by using different concentrations of CG. From previous investigation (section 2.4), 100 mg/L of ferric citrate particles was selected for this investigation. Different concentrations of CG, viz., 100 mg/L, 500 mg/L, 1000 mg/L,

2000 mg/L, 5000 mg/L and, 10000 mg/L were separately distributed to different serum bottles (125 mL). From a stock solution prepared using water, 100 mg/L of ferric citrate particles was added to each bottle in such a way that after inoculating the bottle with 5% (v/v) inoculum, total volume of the medium would become 25 mL. Similar to different hydrogen production experiments already discussed, serum bottles were processed, inoculated, incubated, and gas samples were collected, stored and analyzed (section 2.1 & 2.3).

Hydrogen analysis

Gas samples stored in 5.9 mL airtight glass vials were analyzed by gas chromatography. The GC system used for this purpose was of Varian 3800 model (USA), equipped with automatic sample injector. The detector was a thermal conductivity detector (TCD) and the column used was PoraPLOT Q® (Agilent technology, USA). For this analysis, N_2 was used as the carrier gas and column temperature was kept constant at 100 °C. The carrier gas flow rate of 3.5 mL/min was used and the retention time for hydrogen was determined to be 4.5 min.

Results and Discussion

Characterization of nano-spray dried particles

SEM images of the ferric citrate particles used in the present investigation have been presented in Figure 5.1.1. A very broad particle size distribution has been observed with the diameters ranging from around 1.73 µm to 468 nm or smaller. Particles were completely spherical and an aggregation tendency was observed among them. Zeta potential distribution of these particles has been presented in Figure 5.1.2. From Figure 5.1.2, the zeta potential was determined to be more than ± 5 mV, which ruled out the possibility of instant coagulation of the particles. However, it was lesser than ± 40 mV, which implied that aggregation among the particles was possible. Therefore, the observed aggregation behavior of ferric citrate particles can be considered as normal.

Screening of different nano-spray dried particles for biohydrogen production

NiFe-hydrogenase and FeFe-hydrogenase are two crucial enzymes responsible for microbial hydrogen production in anaerobic environment (Kim *et al.*, 2010, Marseille, 1998, Xu *et al.*, 2013). The active site of NiFe-hydrogenase has one Ni and one Fe atom; whereas, two Fe atoms are present in the active site of FeFe-hydrogenase (Sarma et al 2014. Key Enzymes in Value-Addition of Glycerol to Biohydrogen. In: Enzymes in value-addition of waste. https://www.novapublishers.com/catalog/product_info.php?products_id=47561&osCsid=cdc47dcd8e6201708571788e5dabc903 (accessed on 22/02/2014). Therefore, hydrogen production media should contain sufficient amount of Ni and Fe, so that synthesis of these enzymes are not affected. In CG, only a negligible amount of Ni may be present; whereas, the amount of Fe presents in CG could be as low as 31.6 ± 19.0 ppm to 44.41 ± 16 ppm (Sarma *et al.*, 2013). For hydrogen production by crude glycerol bioconversion, the feedstock needs to be diluted to an optimum concentration; which is usually around 10 g/L (Sarma *et al.*, 2013). Hence, final concentration of Fe in a biohydrogen production medium prepared using only CG (10 g/L) will be as low as 0.31 to 0.44 ppm. In this context, CG supplemented with Ni and Fe might have beneficial effect on biohydrogen production by CG bioconversion. Therefore, the purpose of the present study was to evaluate the effect of ferric citrate, $FeSO_4$, nickel acetate, and $NiCl_2$ supplements supplied in nano-spray dried form, on hydrogen production by CG bioconversion. In Figure 5.1.3 cumulative hydrogen production by bioconversion CG supplemented with different nano-spray dried particles has been presented. As shown in Figure 5.1.3, presence of relatively small amount of different particles in the media was found to significantly alter the hydrogen production ability of the microorganism. As there are different probable mechanisms for nickel toxicity in microorganisms; nickel may be toxic to the bacterium used in this investigation (Macomber *et al.*, 2011). It could be a reason for reduced hydrogen production in the case of nickel acetate, and $NiCl_2$. Compared to nickel containing particles, more hydrogen production has been observed in the case of iron containing particles. Between iron containing particles, particles made up of ferric citrate were found to produce more amount (nearly 2.5 folds more) of

hydrogen (Figure 5.1.3) than $FeSO_4$ particles. Citrate moiety of ferric citrate containing 6 carbon and 5 hydrogen atoms might have served as an additional/easily accessible substrate for the process and it could be a reason for improved hydrogen production. During fermentation some bacteria can use Fe (III) as electron acceptor (Pham *et al.*, 2003); thus ferric citrate might have additional beneficial role for the process. Based on this observation, ferric citrate particles have been selected for further investigation.

Effect of Fe-citrate particle preparation method on hydrogen production

The purpose of the present investigation was to compare the effect of hot water based and NaOH based nano-spray dried ferric citrate particles on hydrogen production. Different amounts of these particles, ranging from 10 mg/L to 500 mg/L were tested as supplement for hydrogen production by CG bioconversion and the results have been presented in Figure 5.1.4. From Figure 5.1.4, it is clear that compared to ferric citrate particles prepared using NaOH, hot water based particles are more favorable as a supplement for hydrogen production. As shown in Figure 5.1.4, corresponding to increased concentration of NaOH based ferric citrate from 10 mg/L to 500 mg/L, decreased hydrogen production has been observed. In addition to ferric citrate, NaOH based particles may also contain Na^+ ions in large number. Therefore, unfavorable ionic strength of the medium supplemented with such particle could be one reason of observed reduced hydrogen production. On the contrary, by increasing the concentration of hot water based ferric citrate particles from 10 mg/L to 500 mg/L, hydrogen production was increased by nearly 50.45 % (Figure 5.1.4). Based on this observation, 100 mg/L of hot water based ferric citrate particles have been selected for further investigation. If relatively higher concentration of the supplement (ferric citrate particles) is used, medium pH will decrease and presence of higher concentration of the particles might have negative effect on the microorganisms used for hydrogen production. Thus, choosing a relatively lower concentration of the particles will be an appropriate option.

Effect of CG concentration on H_2 production using media supplemented with nano-spray dried ferric citrate particles

The purpose of this experiment was to determine the effect of initial CG concentration on hot water based ferric citrate particle supplemented hydrogen production process. Results of this investigation have been presented in Figure 5.1.5 and from the figure it can be inferred that at constant ferric citrate particle concentration (100 mg/L), by increasing the initial CG concentration from 100 mg/L to 10 g/L, cumulative hydrogen production can be increased by nearly 12 folds. It is quite obvious as optimum CG concentration for a batch hydrogen production process has been found to be around 10 g/L (Sarma *et al.*, 2013).

As shown in Figure 5.1.3, however, if total hydrogen production by bioconversion of 1 kg of CG is considered; the amount projected to be produced at initial CG concentration of 100 mg/L was found to be nearly 8.20 folds more than that of 10 g/L. In the case of 100 mg/L CG, the amount of ferric citrate particles (100 mg/L) added to the process was equal to that of substrate (CG). Therefore, for hydrogen production media prepared using one kg of CG, nearly 1 kg of ferric citrate particles would be needed. However, if an initial CG concentration of 10 g/L is used and 100 mg/L of the particles is added as supplement; for 1 kg of CG, only 10 g of the particles would be required. Therefore, 8.20 folds increase in hydrogen production projected for 100 mg/L CG supplemented with 100 mg/L of ferric citrate particles could be attributed to the additional amount of the particles required for the approach.

In Figure 5.1.6, cumulative hydrogen productions by only CG (100 mg/L); CG (100 mg/L) supplemented with ferric citrate (100 mg/L) as well as CG (100 mg/L) supplemented with nano-spray dried ferric citrate particles (100 mg/L) have been presented. Figure 5.1.6 demonstrates that by supplementing the CG bioconversion process with ferric citrate or its nano-spray dried particles, 17.18% to 31.71 % percent improvement in hydrogen production can be achieved. However, supplementation process will involve additional process cost; hence, a proper cost benefit analysis of the approach is a prerequisite for its industrial trial. Moreover, it should also be determined,

whether the application of ferric citrate or its nano-spray dried particle will be appropriate for large scale hydrogen production using CG. Likewise, process mode and hydrogen partial pressure in the headspace of the reactor are known to have influence on cumulative hydrogen production. Thus, in order to determine the potential of the approach, unlike the closed batch process used in the present investigation, hydrogen production using CG supplemented with ferric citrate or its nano-spray dried particle should also be evaluated using process with proper pH control and at relatively low hydrogen partial pressure.

A summary of different reports on hydrogen production by microbial conversion of crude glycerol have been presented in Table 5.1.1. From the table, it is evident that compared to reported maximum hydrogen yield by dark fermentation of CG (8.25 mol-H_2/kg CG); by using an initial CG concentration of 100 mg/L with no supplementary nutrient, 2.75 folds more hydrogen (22.7 mol-H_2/kg CG) could be produced. This observation indicated that hydrogen production by using an initial substrate concentration as low as 100 mg/L can significantly improve cumulative hydrogen production by maximum feedstock utilization. This approach may enhance hydrogen yield not only in the case of CG bioconversion but also for any other substrate. Three possible reasons for this observed improved hydrogen yield are discussed below. (i) Volatile fatty acids (VFAs), such as acetate, butyrate etc., are produced as by-products of fermentative hydrogen production (Oh *et al.*, 2003). Production of these byproducts can quickly decrease the pH of the medium to trigger a metabolic shift, which may lead to decreased cumulative hydrogen production (Khanal *et al.*, 2004). At low initial substrate concentration (100 mg/L), lesser amount of these VFAs will be produced; hence, the concentration of these compounds in the medium will be lower than a process with high initial substrate concentration. Thus, inhibitory effect of VFAs could be avoided if a relatively low initial substrate concentration is used. This, approach has industrial significance as it may reduce the requirement of large amount of alkali and acid for process pH control. (ii) Hydrogen partial pressure buildup in the headspace of the reactor can reduce cumulative hydrogen production and by periodically flushing out the accumulated gas, hydrogen production can be increased (Ngo *et al.*, 2011). If an initial substrate

concentration as low as 100 mg/L is used, only a small amount of hydrogen will be produced per liter of medium; therefore, hydrogen accumulation in the headspace will be far lower than a process with high initial substrate concentration such as, 10 g/L. Thus, low partial pressure of hydrogen in the headspace could be one of the reasons of improved hydrogen yield per kg of substrate observed in the present investigation. (iii) In the case of present investigation, initial CG concentrations ranging from 100 mg/L to 10 g/L were used; however, inoculum size was kept constant at 5 % (v/v). Therefore, in the case of the process with 100 mg/L of CG, the amount of active microbial biomass introduced per kg of substrate (CG) was 100 times more than that of the process with 10 g/L CG. Thus, the role of excess amount of biomass is undeniable.

Conclusions

Among ferric citrate, $FeSO_4$, $NiCl_2$ and Ni-acetate nano-spray dried particles, only ferric citrate particles were found to have beneficial effect on hydrogen production. Two different types of ferric citrate particles were prepared, either by dissolving it in hot water or in NaOH solution. Between these two particles, hot water based ones were found to enhance the hydrogen production by 50.45 %. Apart from supplementation, initial substrate concentration has been identified as a crucial factor to determine the hydrogen yield. At extremely low initial CG concentration of 100 mg/L, by supplementing the process with ferric citrate particles, a hydrogen yield as high as 29.9 mol-H_2/kg CG has been achieved. This yield is significantly higher than the value of 8.25 mol-H_2/ kg CG; previously known for dark fermentative hydrogen production.

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Table 5.1. 1: Summary of different reports on hydrogen production by crude glycerol bioconversion

SL. No.	Fermentation type	Substrate	Initial substrate concentration	Microorganism	Supplement	H_2 production (reported)	H_2 production (mol/kg CG)	Reference
1	Photo-fermentation	CG containing 70-75 % glycerol	CG containing 0.92 g/L (10 mM) glycerol	*Rhodopseudomonas palustris*	RCV medium, biotin, para-aminobenzoic acid	6 mol/mol glycerol	48.86 mol-H_2/kg CG*****	(Sabourin-Provost *et al.*, 2009)
2	Dark fermentation	CG containing 41 % (w/w) glycerol	CG containing 10 g/L glycerol	*Enterobacter aerogenes* HU-101	Complex medium, phosphate buffer	0.71 mol-H_2/mol-glycerol	3.16 mol-H_2/kg CG*****	(Ito *et al.*, 2005)
3		CG with 86% (w/w) glycerol	CG containing 10 g/L glycerol	*Enterobacter aerogenes* ATCC 13048	Complex medium with buffering agents	624.8 mL-H_2/256 mL-medium	8.25 mol-H_2/kg CG*****	(Marques *et al.*, 2009)
4		CG containing nearly 1.0% (w/v) glycerol	5 g/L pretreated CG	*Thermotoga neapolitana* DSM 4359	2 g/L yeast extract, 0.05M HEPES buffer	2.73 ± 0.14 mol-H_2 / mol-glycerol consumed	0.296 mol-H_2/kg CG*****	(Ngo *et al.*, 2011)
5		CG containing 45 % (w/w)	CG containing 14.8g/L	*Enterobacter aerogenes* NBRC	NBRC 702 medium, thionine	0.66 mol-H_2/mol-	3.22 mol-H_2/kg CG*****	(Sakai *et al.*, 2007)

SL. No.	**Fermentation type**	**Substrate**	**Initial substrate concentration**	**Microorganism**	**Supplement**	**H_2 production (reported)**	**H_2 production (mol/kg CG)**	**Reference**
		glycerol	glycerol	12010		glycerol consumed		
6		CG containing 69.5 % glycerol	3 g/L CG	Mixed culture	70 mM MES buffer, nutrient solution	0.31 mol-H_2/mol-glycerol	2.33 mol-H_2/kg CG*****	(Selembo *et al.*, 2009)
7		*CG containing 23.63 ± 2.5% (w/w) glycerol*	***100 mg/L CG***	*Enterobacter aerogenes NRRL B-407*	***No supplement***	*2.27 mmol-H_2/L*	***22.7 mol-H_2/kg CG***	***Present study***
8		*CG containing 23.63 ± 2.5% (w/w) glycerol*	***100 mg/L CG***	*Enterobacter aerogenes NRRL B-407*	***100 mg/L ferric citrate NP***	*2.99 mmol-H_2/L*	***29.9 mol-H_2/kg CG***	***Present study***

*Calculated from literature data by assuming complete consumption of glycerol present in a CG based medium.

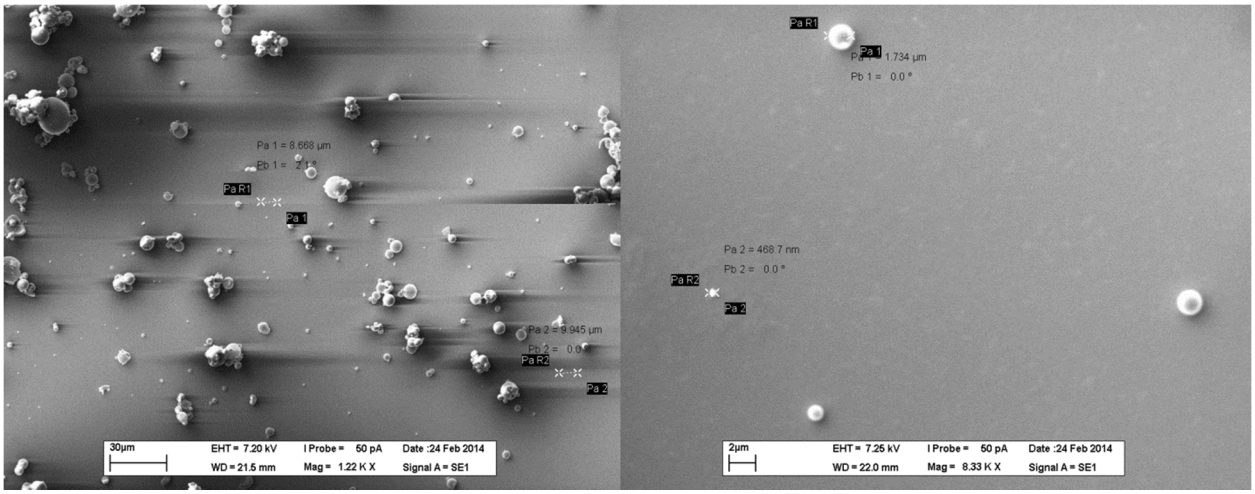

Figure 5.1. 1: SEM images (at different magnification) of ferric citrate particles prepared using hot water

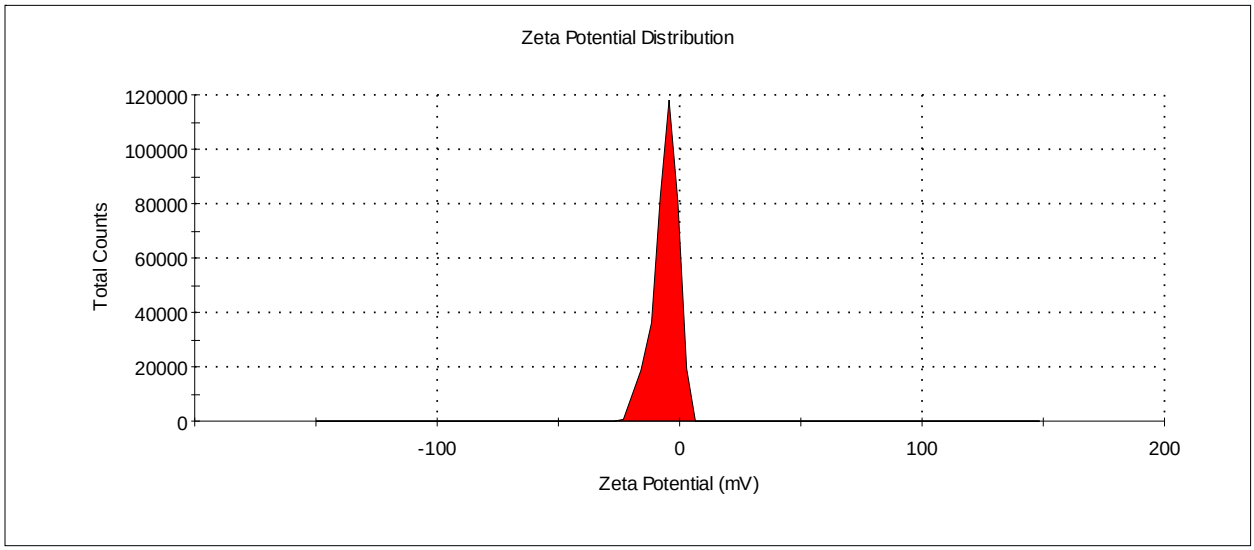

Figure 5.1. 2: Zeta potential distribution of ferric citrate particles prepared using hot water

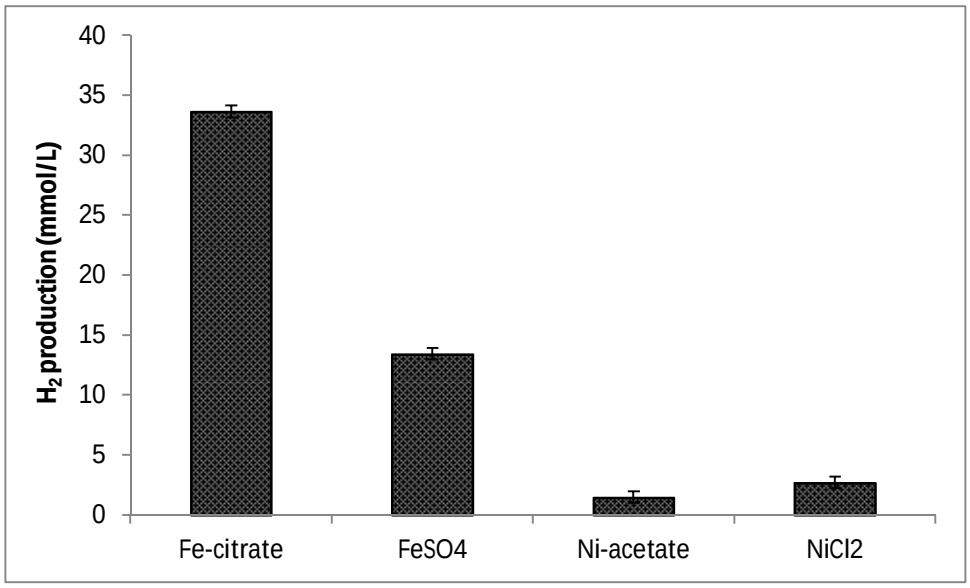

Figure 5.1. 3: Cumulative hydrogen production obtained after 48 h of incubation. 50 mg/L of respective nanospray dried particles were added to 12 g/L of CG

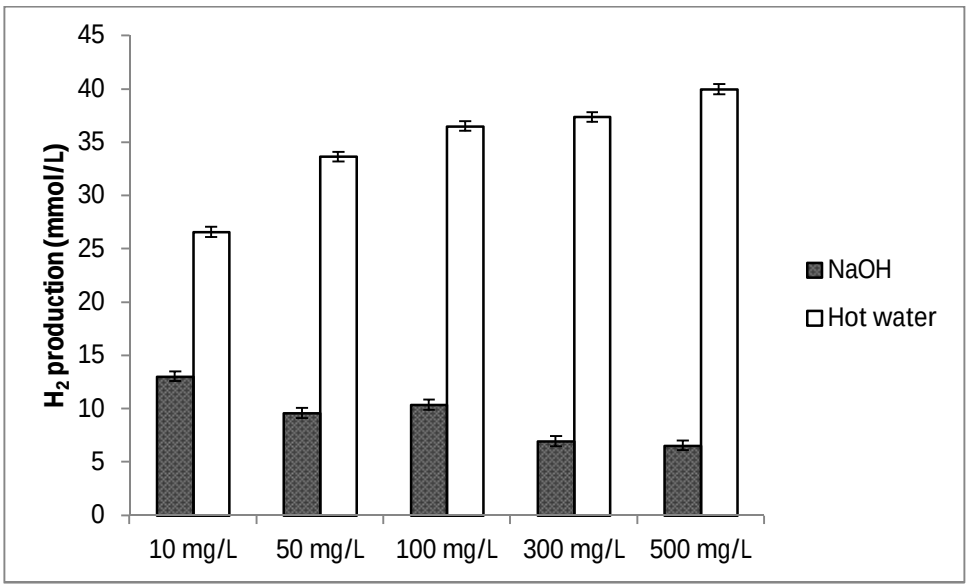

Figure 5.1. 4: Cumulative hydrogen production obtained after 48 h of incubation. Different concentrations of two different types of ferric citrate particles were added to 12 g/L of CG

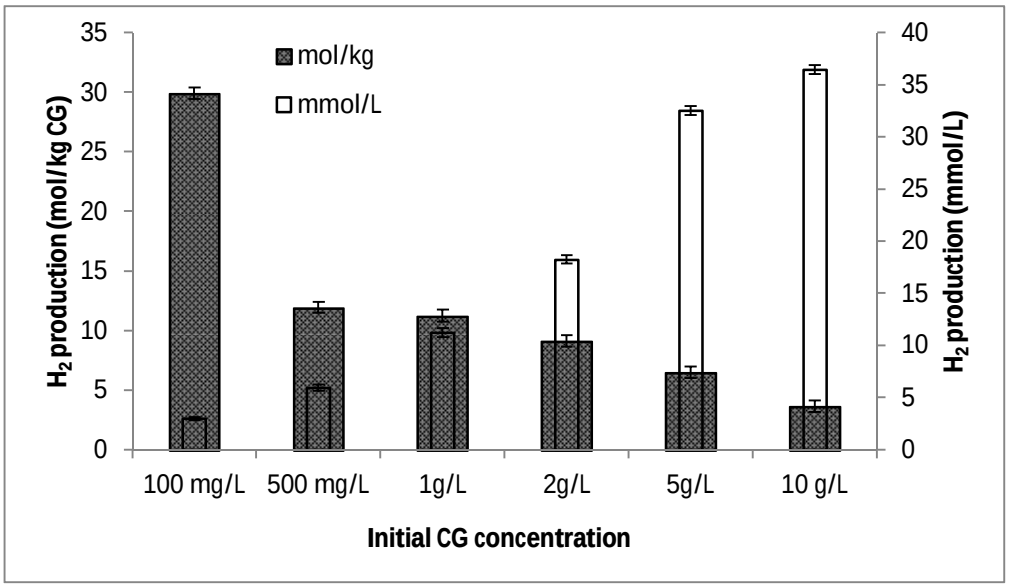

Figure 5.1. 5: Cumulative hydrogen production obtained after 48 h of incubation. 100 mg/L of ferric citrated particles (prepared with hot water) were added to different concentrations of CG. Projected hydrogen yields by bioconversion of 1 kg of CG have also been shown for each initial CG concentration

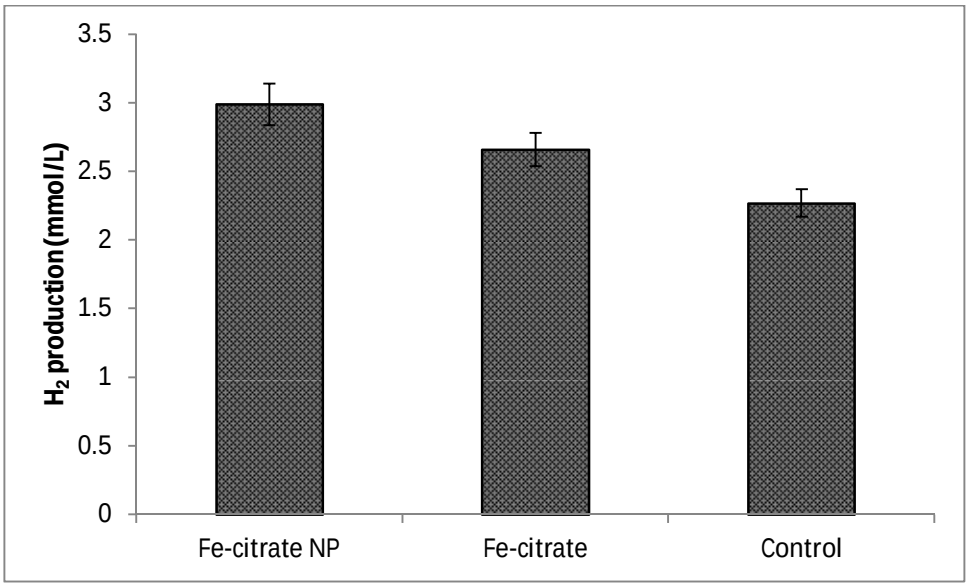

Figure 5.1. 6: Cumulative hydrogen production obtained after 48 h of incubation. Control is only CG (100 mg/L)

CHAPTER 6

POTENTIAL APPLICATION OF ORGANIC ACID RICH LIQUID WASTE FROM HYDROGEN PRODUCTION PROCESS FOR PHOSPHATE SOLUBILIZATION

PART I

LIQUID WASTE FROM BIO-HYDROGEN PRODUCTION - A COMMERCIALLY ATTRACTIVE ALTERNATIVE FOR PHOSPHATE SOLUBILIZING BIO-FERTILIZER

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Résumé

Au cours de la production de H_2 par fermentation sombre des acides organiques différents sont produits comme des sous-produits indésirables. Ces rejets liquides, riches en acides organiques, ont une excellente capacité de solubilisation du phosphate (augmentation de 36,1 fois) et pourraient être considérés comme une alternative potentielle pour les bio-engrais commerciaux solubilisateurs de phosphate (PSB). Les acides organiques produits par les bioengrais commerciaux solubilisateurs de phosphate (PSB) sont reconnus pour chélater les cations de phosphates insolubles (comme $Ca_3(PO_4)_2$) présents dans le sol, favorisant la solubilisation du phosphate et le rendre disponible pour les plantes.

Il est connu que le GB généré lors de la fabrication du biodiesel pourrait être utilisé comme substrat pour la production d'hydrogène. Le déchet secondaire riche en acides organiques pourrait être utilisé comme agent de solubilisation du phosphate. Ainsi, cette nouvelle approche pourrait: (i) offrir un engrais vert; (ii) améliorer la viabilité économique de la production de biohydrogène et; (iii) devenir un apport financier pour les fabricants de biodiesel en leur offrant une stratégie de gestion des déchets rentable.

Mots-clés: biodiesel; biohydrogène déchets liquides; glycérol brut; acide organique; phosphate solubilisation bioengrais; gestion des déchets

Abstract

Organic acids produced by commercial phosphate solubilizing bio-fertilizer (PSB) can chelate the cations of insoluble phosphates (such as Ca_3 $(PO_4)_2$) present in soil to solubilize the phosphate and make it available for plants. Meanwhile, during H_2 production (dark fermentation) different organic acids are produced as unwanted by-products. Interestingly, the acid-rich liquid waste of the process has excellent phosphate solubilizing ability (36.12 folds increase) and it could be considered as a potential alternative for PSB. Studies have demonstrated that biodiesel manufacturing waste (crude glycerol) could be used as a substrate for hydrogen production and the secondary waste could be used as a phosphate solubilizing agent. Thus, this novel approach could: (i) offer a green fertilizer; (ii) improve economic sustainability of bio-hydrogen production and; (iii) become a financial booster for biodiesel manufacturers by offering them a profitable waste management strategy.

Keywords: biodiesel; bio-hydrogen liquid waste; crude glycerol; organic acid; phosphate solubilizing bio-fertilizer; waste management

Introduction

Hydrogen is a renewable energy carrier which is postulated to be one of the clean fuels of future (http://www.alternative-energy-news.info/technology/hydrogen-fuel/ (accessed on 26/04/2013)). Water is the only by-product of its combustion and hence, it has the potential to significantly reduce the greenhouse gas emission. As a fuel it is suitable for fuel cells or internal combustion engines and energy content of H_2 is higher than any other gaseous fuels of equivalent weight (Meher Kotay *et al.*, 2008). During bio-hydrogen production by dark fermentation, organic acids such as acetic, butyric and lactic acid are produced (Shida *et al.*, 2009), which are generally considered as waste. Actually, production of organic acid can drop the pH of the medium to terminate the process. Similarly, conversion of a significant portion of the substrate to different organic acids results in reduction of H_2 yield. Therefore, organic acid production is considered as the major bottleneck of dark fermentative H_2 production (http://www.slideshare.net/kumarhukkeri/finalppt-em-1 (accessed on 02/03/2013)). Interestingly, some of the organic acids can chelate the cations of insoluble mineral phosphates (present in soil) and release soluble phosphate to make it available for plants (Kucey, 1983). In fact, phosphate solubilization by production of organic acid is the main plant growth promoting mechanism (Vyas *et al.*, 2009) of commercially used PSB. Thus, organic acid rich bio-hydrogen production wastes could be a potentially less expensive alternative for PSB. Moreover, PSBs have relatively shorter shelf-life as they are actually a preparation of living microorganisms and the organisms must be alive when it is applied to the field. Alternatively, the performance of the proposed fertilizer does not depend on the number viable cell it contains and hence, an extended shelf-life could be expected. Thus, production, packaging, storage and application of proposed fertilizer should be easier than conventional PSBs.

In general, the liquid waste of dark fermentative H_2 production using any substrate/any microorganism should be suitable as the proposed fertilizer. If the proposed strategy could be integrated into an industrial process, such as biodiesel production, apart from hydrogen production, it could become a profitable strategy for waste management. For

example, as presented in Figure 6.1.1, crude glycerol (CG), the major by-product (10 % by weight) of biodiesel production process (Chatzifragkou *et al.*, 2012) could be used (as a substrate) for bio-hydrogen production and the final liquid waste could be used as an alternative of phosphate solubilizing bio-fertilizers. In fact, crude glycerol is a hazardous waste and its proper disposal is an additional expense for biodiesel manufacturers. Therefore, if successful, the proposed approach may be a huge breakthrough for financial/environmental sustainability of biodiesel industry.

Materials and methods

Hydrogen production

Dark fermentative hydrogen production experiments were carried out using 10 g/L crude glycerol (Rothsay, Canada) as the only media component. Actually, 10 g/L crude glycerol was found to be optimum for hydrogen production (Sarma *et al.*, 2012, Sarma *et al.*, 2013). Likewise, *Enterobactor aerogenes* NRRL B 407 (ARS, USDA, USA) was used in this study and all other conditions of this investigation (H_2 production) was similar to our previous report (Sarma *et al.*, 2012). Briefly, for each experiment, 50 mL CG solution (10 g/L) was taken in 125 mL serum bottle and anaerobic condition was created by passing ultrapure N_2 gas through the medium. The bottles were properly sealed (using aluminum crimp seal having silicon septum) and autoclaved. Finally, they were inoculated using 5% (v/v) fresh *Enterobactor aerogenes* culture and incubated (150 rpm/30°C). Unlike the previous study, in the present case, H_2 gas produced during the process was not periodically released form the serum bottles used for this purpose (Sarma *et al.*, 2012).

Phosphate solubilization study

To determine phosphate solubilization potential of the spent media of bio-hydrogen production process, experiments were conducted using 6 different conical (glass) flasks (125 mL). In each flask, 50 mg of $Ca_3 (PO_4)_2$ (tricalcium phosphate) was taken and 50 mL of 0 %, 10 %, 20%, 40%, 60% and 100 % (v/v) of the spent media of crude glycerol based bio-hydrogen production process were added (1 g $Ca_3 (PO_4)_2$ in 1L liquid). Finally, the flasks were incubated (150 rpm and 28° C) in an orbital incubator shaker

(Infors HT, Switzerland) and liquid samples were collected at 3 h interval for the first 12h (and final samples were collected after 10 d).

Analysis

Gaseous samples collected during the investigation were analyzed by a gas chromatograph (Sarma *et al.*, 2012). Likewise, to determine the soluble phosphate concentration, 1 mL of aqueous sample collected during the phosphorus solubilization study was centrifuged (5000 g for 10 min.) and the suspended tricalcium phosphate present in the solution was removed. About 0.1 mL of the supernatant was collected, diluted 10 times with distilled water and soluble phosphate present in the solution was analyzed by using a method described by (Murphy *et al.*, 1958). As described by the authors, all glass-wares used in the analysis were pretreated with concentrated H_2SO_4 (Murphy *et al.*, 1958).

Results and discussion

Cumulative H_2 production using 10 g/L crude glycerol as the only substrate was found to be 20.61 mmol/L (H_2 production profile is not shown). This value is lower than our previous finding (84.08 mmol/L) using same concentration of the crude glycerol. It is well known that process mode can strongly influence the hydrogen yield and the observed difference is due to two variable process modes adopted. In the present case, a closed batch system was used where H_2 produced during the process was accumulated in the headspace of the vessel, whereas, in previous case, produced gas was periodically released from the headspace. Increase in H_2 partial pressure in the headspace is known to negatively affect the production and hence the final concentrartion in the two cases were different.

Liquid waste generated during crude glycerol bioconversion and H_2 production process has been evaluated for phosphate solubilization potential. Figure 6.1.2 summarizes the results of phosphate solubilization study involving the bio-hydrogen production waste. In fact, soluble phosphate concentration of the spent medium itself was found to be 14.96 mg/L. As a control experiment, when only distilled water (0% (v/v) of the waste) was used, soluble phosphate concentration was found to be only 1.38 mg/L. In contrast,

soluble phosphate concentration as high as 32.06 mg/L had been achieved by using bio-hydrogen production waste (100 %, v/v), which is 23.17 folds higher than the control experiment. This finding (23.17 folds increase within 1 minute) is significant in a situation where conventional PSBs were known to liberate similar amount of phosphate in a period of 2-3 days (Trivedi *et al.*, 2008). As shown in Table 6.1.1, (Zhu *et al.*, 2011) have reported that a newly isolated strain of phosphate solubilizing bacteria can solubilize 283.16 mg/L of phosphorus within an incubation period of 11 days. However, it should be noted that the authors performed the phosphate solubilization study using chemically defined liquid media in optimum laboratory conditions. Hence, the performance of the strain could be different in real field application. Moreover, during storage, the cell count of a biofertilizer formulation can sharply decrease (Menaka *et al.*, 2007) and further reduce its phosphate solubilization ability. Thus, considering the fact that in the case of biohydrogen production, liquid waste phosphate solubilization is instant as well as it has nothing to do with microbial cell viability, performance of the present approach is comparable to that of a conventional PSB. A detailed comparison of the two fertilizers has been presented in Table 6.1.1. Likewise, the phosphate solubilization processes was monitored at 3 h interval for 12h, and phosphate solubilization achieved within 1 minute was only slightly different to that observed after 12h (data not shown). However, after 10 d of incubation enhancement in cumulative soluble phosphate concentration was recorded to be as high as 18.59, 25.86 and 36.12 folds for 40, 60 and 100 % (v/v) of the waste, respectively. Interestingly, any significant improvement in soluble phosphate concentration was not observed for 10 and 20 % (v/v) of the waste. One possible explanation for such observation could be that a small number of hydrogen producing bacteria (facultative anaerobe) were still active in the spent media and they played certain role (e.g. production of organic acid or enzyme such as phosphatase) in phosphate solubilization. In the case of 10 and 20 % (v/v) of the waste, such microbial activities were not sufficient (assuming that only a small fraction of the bacteria was active) for a significant response. Moreover, for these two cases, a slight decrease in soluble phosphate concentration was observed. It might be due to incorporation of bioavailable phosphate into the biomass (synthesis of DNA,

RNA, phospholipids etc.). However, these are simple assumptions and further investigation is required to verify them. Thus, it could be concluded that bio-hydrogen production waste has excellent potential as a phosphate solubilizing agent.

In general, a commercial PSB is capable of increasing the phosphate availability by 25-30 kg per hectare and it is sufficient to increase the product yield up to 20% (Bionics Technology, New Delhi, India. http://www.indiamart.com/bionics-technology/fertilizers.html (accessed on 21/02/2013)). Meanwhile, according to the estimated hydrogen production, liquid waste generated by bioconversion of 1 kg of crude glycerol would be sufficient to liberate 20 g of phosphate. Hence, for each hectare, waste generated from 1250 kg of CG will be required to completely replace a commercial PSB (Table 6.1.1). Therefore, for convenience of application, pre-concentration of the waste may be required. Although, phosphate solubilizing ability of bio-hydrogen production waste has been demonstrated its actual plant growth promoting potential needs to be verified by field scale experiment using food crop.

Conclusions

Bio-hydrogen production liquid waste is an excellent phosphate solubilizing agent and it has high potential to be a commercially important alternative of phosphate solubilizing bio-fertilizer. It can increase the soluble phosphate concentration by 23.17 folds within 1 minute and 36.12 folds after 10 d (of incubation) of its application to a liquid system and this value may be even better depending on the hydrogen production process adopted. Unlike traditional phosphate solubilizing bio-fertilizers, the proposed alternative is not a formulation of living microorganisms and hence, it is postulated to have superior shelf-life. Finally and most importantly, the proposed fertilizer production process could be integrated to biodiesel manufacturing process (aka biorefinery) to upgrade the biodiesel industry to a “high profit-zero waste” industry.

Acknowledgments

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Table 6.1. 1: Possible benefit of proposed technology compared to conventional PSB

Fertilizer	**Phosphate solubilization ability**	**Phosphate solubilization time**	**Amount required to solubilize 25 kg phosphate** (for one hectare of land)	**Chemicals required**	**Shelf-life**	**Production Cost**	**Additional benefit** (other than being a sustainable fertilizer)	**Pollution/GHG emission**
Conventional PSB	283.16 mg/L media (after 11days of incubation)*	Long incubation period (10 to 30 days)	Equivalent to 88339.22 L of fermented broth	Yes, expensive chemicals as media components	Only 6 months as it is a formulation of living microorganism**	Includes fermentation cost (around $0.25/L)*** and formulation cost	No	Manufacturing waste may need separate treatment
Bio-hydrogen production waste	49.98 mg/L waste	Instant solubilization	Bio-hydrogen production waste generated from 1250 kg of CG	No, only H_2O and CG required	Expected to be superior as it is not a formulation of living microorganism	No production cost (waste by-product)	Production of carbon free biofuel	Zero waste; high GHG emission reduction potential due to additional biofuel production

* (Zhu *et al.*, 2011), **as for average bio-fertilizer, *** (Sethi *et al.*, 2012)

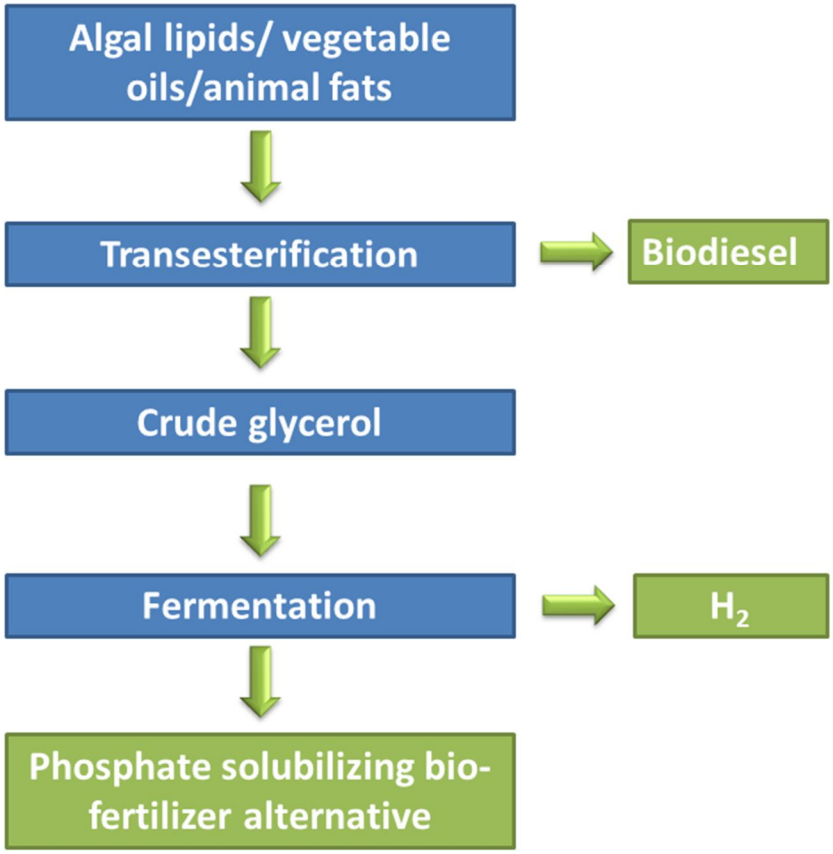

Figure 6.1. 1: A schematic representation of the proposed concept

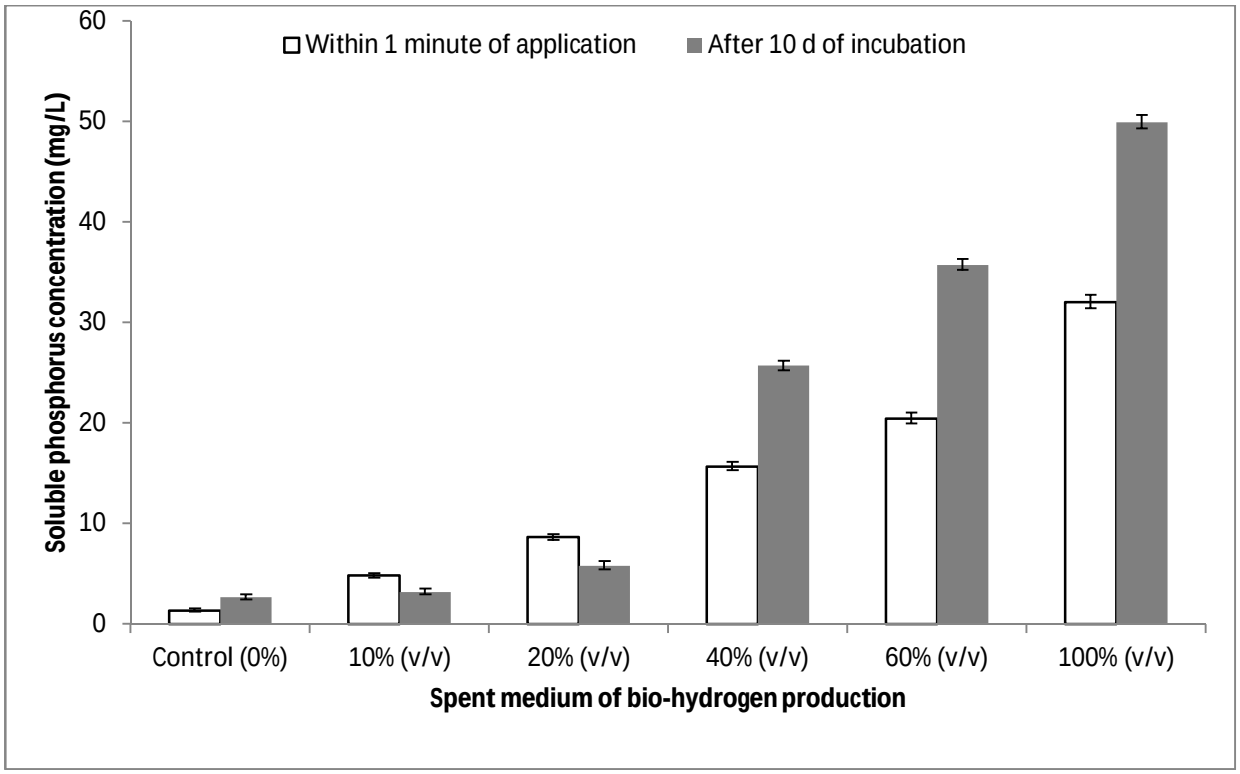

Figure 6.1. 2: Phosphate solubilization achieved using different concentrations of bio-hydrogen production liquid waste. The control represents soluble phosphate concentration obtained using only water as the solubilizing agent

PART II

NOVEL ENVIRONMENTAL AND AGRICULTURAL USES OF BIOHYDROGEN PRODUCTION WASTEWATER-A STUDY USING SOYBEAN PLANTS

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Résumé

Avec l'absence d'émission de CO_2 et une densité d'énergie massique plus élevée que les carburants conventionnels, tels que l'essence, le diesel, le biodiesel et le bioéthanol, le biohydrogène est une option prometteuse en terme d'énergie renouvelable. Cependant, la production commerciale de biohydrogène est principalement limitée par le coût élevé des processus impliqués. En outre, au cours de la production d'hydrogène par fermentation sombre, 60-70% de la charge d'alimentation est convertie en différents sous-produits, dominés par des acides organiques. Afin de faire face à l'utilisation inefficace des substrats ainsi que le coût élevé des étapes du procédé, une approche simple de valorisation des déchets liquides de la production d'hydrogène (HPLW) a été démontrée. Les microorganismes présents dans le sol peuvent produire des acides organiques pour solubiliser les différentes formes de phosphates inorganiques, tels que $Ca_3(PO_4)_2$, en chélatant les cations et libérant ainsi le phosphore soluble. Ce phosphore soluble peut être ensuite utilisé par les végétaux. De façon expérimentale, le rejet HPLW riche en acide organique a été ajouté à un sol contenant des semences de soya. Une augmentation de l'assimilation du phosphore par les plantes de soja de 2,2 à 2,7 fois a été observée.

Mots-clés: Biohydrogène; solubilisation du phosphore; acides organiques; rejets de fermentation, soya

Abstract

With CO_2 free emission and a gravimetric energy density higher than conventional fuels, such as gasoline, diesel, biodiesel and bioethanol; biohydrogen is a promising option as a green renewable energy carrier. However, commercial production of biohydrogen is mostly limited by high process cost involved. Moreover, during hydrogen production by dark fermentation, 60-70 % of the feedstock is converted to different byproducts, dominated by organic acids. In order to cope with inefficient substrate utilization as well as high process cost; a simple approach of value-addition of hydrogen production liquid waste (HPLW) has been demonstrated. Phosphate solubilizing microorganisms present in soil can produce different organic acids to solubilize inorganic phosphates, such as $Ca_3 (PO_4)_2$, by chelating the cations, and release soluble phosphorus to be utilized by plants. Thus, organic acid rich HPLW has been added to soil and 2.18 to 2.74 folds increase in phosphorus uptake by soybean plants has been observed.

Keywords: Biohydrogen; phosphorus solublization; organic acids; spent medium; soybean plants

Introduction

Biohydrogen can be used as a CO_2 free fuel, since its combustion releases only water as major emission to the atmosphere. This property makes it more environmentally friendly than commercial biofuels, such as biodiesel, bioethanol and biomethane. Moreover, the gravimetric energy density of hydrogen is 142 MJ/kg; which is nearly 3 times of major fossil fuels, such as gasoline (47 MJ/kg), and diesel (43 MJ/kg) (Şensöz *et al.*, 2000, Sydney *et al.*, 2014). Biohydrogen can be produced from a diverse group of renewable raw materials including non-food crop based biomass and agro-industrial organic wastes. Dark fermentation of these materials is relatively rapid, simple and most commonly used technique for biohydrogen production. However, during this process, only 30-40 % of the feedstock is actually used; rest is converted to different byproducts (Venkateswar Reddy *et al.*, 2014). These byproducts include mainly different volatile fatty acides (VFAs), in the form of acetate (15.3-34.1%), butyrate (31.2-45.6%), propionate (0.9-15.9%), lactate (2-4.6%) and alcohols such as ethanol (4.6-10.1%), among others (Fang *et al.*, 2002). Value addition of the hydrogen production liquid waste (HPLW) containing these byproducts may give an additional revenue source to commericial biohydrogen production; which is otherwise impared by high process cost. In this context, as a part of present investigation, acid rich HPLW has been evaluated as phosphate solubilizer for soybean cultivation.

By weight, phosphorus content of a typical soil is around 0.05%, of which, only around 0.1% is directly available to plants (Mardad *et al.*, 2013). In soil, a significant amount of phosphorus is present in its insoluble forms, such as $Ca_3(PO_4)_2$, $CaHPO_4$, $FePO_4$ and $AlPO_4$. Moreover, soluble phosphorus supplied to agricultural fields in the form of chemical fertilizer, may react with cations, such as Ca^{2+}, Fe^{3+}, Al^{3+} to precipitate immediately after its use and become unavailable (Mardad *et al.*, 2013, Zhu *et al.*, 2012). Application of phosphate solubilizing biofertilizer (PSB) is a solution to this problem. PSBs are microorganisms which can secret a range of organic acids in order to chelate the cations of insoluble inorganic phosphate present in soil such as, $Ca_3(PO_4)_2$ and solubilize them to be available for plants. Thus, soil application of organic

acid rich HPLW has been postulated to have similar beneficial effect on phosphorus uptake by plants.

For the present investigation, crude glycerol (CG) has been used as the feedstock for hydrogen production by *Enterobacter aerogenes*. CG is a waste generated during biodiesel manufacturing by transesterification of lipid (Pott *et al.*, 2014, Zhou *et al.*, 2014). As high as 10 kg of CG may be generated for every 100 kg of biodiesel produced (Santibáñez *et al.*, 2011). Commercially CG is mainly used as a source of glycerol. However, due to increase in global CG production, market price of glycerol has been dropped by nearly 20 folds (Pott *et al.*, 2013). Therefore, researchers are looking for an alternative option for CG valorization. In this context, as shown in Figure 6.2.1, CG has been used as a feedstock for hydrogen production. Liquid waste generated during this process has been applied to soil as phosphate solubilizer and its effect on phosphorus uptake by soybean plants has been determined.

Materials and methods

Microorganism and inoculum preparation

Enterobacter aerogenes NRRL B-407 collected from ARS (USDA, USA) was used for hydrogen production by CG bioconversion. It is a facultative anaerobic, gram-negative rod-shaped bacterium, which belongs to Enterobacteriaceae family. For maintenance of the strain, it was periodically grown in a complex medium composed of glucose (5 g/L), casein peptone (5 g/L), KH_2PO_4 (2 g/L), yeast extract (0.5 g/L) and $MgSO_4.7H_2O$ (0.5 g/L). About 50 mL of the medium was taken in serum bottle (125 mL), sparged with N_2 gas for 2 minutes and closed with crimp seal having silicon septum. After sterilization, these serum bottles were used to grow the microorganism. The incubation condition routinely followed for growing the organism was 30°C and 150 rpm and an incubator shaker was used for incubating the bottles.

For inocula preparation for the present investigation, serum bottles containing a medium made up of 12 g/L of CG was used. Other conditions of media preparation and incubation were similar to those mentioned above. After 24 h of incubation, 5 % (v/v) of

microbial culture were used as the inocula for hydrogen production experiments carried out in a 7.5 L bioreactor.

Biohydrogen production from crude glycerol by using a 7.5 L bioreactor

Hydrogen production experiments were carried out in a 7.5 L bioreactor (Labfors 5, Infors-HT, Canada). About 3 liters of medium containing 12 g/L of CG as the only medium component was used for the present study. The bioreactor vessel along with the medium was autoclaved at 121 ºC for 20 minutes. After cooling, ultrapure nitrogen gas was passed through the medium to bring the dissolved oxygen concentration to zero. pH of the medium was adjusted to 6.6 and inoculated with 5 % (v/v) inoculum, which has been prepared as described in previous section (section 2.1). The temperature of the bioreactor was maintained at 30 ºC and the agitation used for the process was 200 rpm. The bioreactor was connected to a computer interfaced with process control software (Iris, Infors-HT, Canada); the signals of different sensors were recorded online. In order to record real time pH change of the process, after inoculation, the automatic pH control was inactivated.

Investigation of phosphorus uptake by soybean plant in presence of HPLW

Soybean (*Glycine max*) was selected as the model plant for phosphorus uptake behavior study and the seeds were collected from local market. Plastic pots containing 120 g of soil (Floral premium potting mix; Canadian tire) were used for the present investigation. In Table 6.2.1, the amount of HPLW and tricalcium phosphate ($Ca_3(PO_4)_2$) mixed with the soil of different pots has been presented. A pot containing only soil (pot 3) was used as control for the experiment. 15 soybean seeds were placed in each pot and subsequently covered with nearly 1 cm thick layer of soil. Distilled water was daily provided to each pot to keep the soil sufficiently wet. All pots containing soybean seeds were incubated in a plant growth chamber (Conviron®, Canada) maintained at 25 ºC. Inside the chamber, the pots were exposed to constant illumination by using two 160 watt Philips tubes. After one month, plants were harvested and analyzed for phosphorus content as explained in section 2.4.3.

Analytical techniques

Hydrogen analysis

In order to determine the hydrogen concentration in the biogas accumulated in the headspace of the reactor, gas samples were collected by using a gastight syringe (SGE Analytical Science, Australia), stored in 5.9 mL gas sampling vial (Exetainer®, Labco, UK) and subsequently analyzed by gas chromatography. A Varian 3800 gas chromatograph (Varian, USA), fitted with PoraPLOT Q® column (Agilent technology, USA) and thermal conductivity detector (TCD) was used for this purpose. Ultra-pure nitrogen at a flow rate of 3.5 mL/min was used as the carrier gas and the column temperature was 100 °C (Sarma *et al.*, 2014).

Characterization of HPLW

In order to identify the major byproducts of hydrogen production, at the end the process liquid sample was collected from the fermenter and analyzed by gas chromatography. Prior to the analysis, the sample was centrifuged for 5 minutes at 10000 rpm and the supernatant was filtered by 0.45 µ syringe filter. The GC (GC7890B, Agilent Technologies, USA) used for this analysis was fitted with HP-INNOWax column (Agilent Technologies, USA) and a flame ionization detector (FID). The column temperature gradient used for this analysis was 50-250ºC and the injection volume was 0.8 µl. Isobutanol was used as the internal standard.

Phosphorus analysis

For this analysis, a standard method for persulfate digestion for total phosphorus analysis (https://uwlab.soils.wisc.edu/files/procedures/DNR_TotalP.pdf (accessed on 02/04/2014)) was followed by a colorimetric method for phosphate determination (Murphy *et al.*, 1958, Sarma *et al.*, 2013a). Briefly, a digestion solution was prepared by dissolving 12.8 g ammonium persulfate in 32 mL of 5.6 M H_2SO_4 and total volume of the solution was adjusted to 100 mL by adding Mili-Q water. Stem of the soybean plants grown in different pots were cut into small pieces and 50 mg form the plant of each pot were used to analyze the phosphorus content. The stem pieces were taken in a 15 mL

screw cap thick walled teflon acid digestion tube and 8 mL of water was added. 0.5 mL of the digestion solution was added to each tube and tightly closed. The digestion tubes containing the samples were autoclaved at 121ºC for 30 minutes. After cooling, phosphorus concentration in the solution was determined as reported earlier (Sarma *et al.*, 2013a).

Measurement of biomass production

In order to investigate the effect of HPLW addition to soil on soybean plant biomass production, plants were picked from all different pots. The roots were carefully washed with soap solution as well as distilled water to remove the soil and spread on tissue paper for two hours to dry. Finally, the plants were weighed by using a digital balance.

Determination of soil pH

After harvesting the plants, one g of soil was collected from each pot and dissolved in 20 mL of distilled water. The samples were thoroughly mixed by using a vortex mixture and pH of the solutions was recorded by a pH meter.

Results

Biohydrogen production from crude glycerol

A 7.5 L bioreactor was used for hydrogen production by CG bioconversion. During fermentation, changes in the values of different process parameters, such as pH, dissolved oxygen concentration (DO), temperature, and agitation were recorded online. These profiles have been presented in Figure 6.2.2a. From Figure 6.2.2a, it is evident that other than pH, all parameters were kept constant throughout the fermentation period. There was a sharp decrease in medium pH until nearly 48-50 h; and even after that it continued to decrease at a slower rate until termination of the process. As already mentioned, during hydrogen production by dark fermentation, different volatile fatty acids (VFAs) are produced as byproducts (Sydney *et al.*, 2014). Therefore, quick decrease in medium pH indicates the accumulation of these products. A gas chromatogram of HPLW has been presented in Figure 6.2.2b. The chromatographic analysis showed that the HPLW contains acetic acid (734.59 mg/L), butyric acid (90.23

mg/L) as well as residual glycerol (retention time 11.403 min.). VFAs containing liquid waste can solubilize inorganic phosphate such as tricalcium phosphate ($Ca_3(PO_4)_2$) to release soluble phosphate (Sarma *et al.*, 2013a). Hence, the HPLW of present investigation was tested as a phosphate solubilizer to facilitate phosphorus uptake by soybean plants and the results were discussed in section 4.2.

Investigation of phosphorus uptake by soybean plant in presence of HPLW

Soybean plants grown in different pots containing soil or soil amended with HPLW or tricalcium phosphate (TCP) are analyzed for phosphorus uptake during one month growth period. Based on this analysis, phosphorus content per g of biomass was calculated for different plant samples; which has been presented in Figure 6.2.3. As shown in Figure 6.2.3, compared to control (only soil), the plants grown in soil amended with 50 and 100 mL of HPLW, respectively, have shown 2.18 and 2.74 folds increase in phosphorus uptake. From this observation, it is evident that HPLW is capable of improving the phosphorus uptake behavior of soybean plants. It has been demonstrated that HPLW can solubilize insoluble inorganic phosphate to release the orthophosphate group; which is available for plants (Sarma *et al.*, 2013a). Thus, improved phosphorus uptake can be considered as a consequence of its increased bioavailability due to HPLW's presence in the soil.

Discussion

Biohydrogen production from crude glycerol

As mentioned in section 2.4.1, gaseous samples were collected from the headspace of the reactor and analyzed by GC. At the end of the fermentation (120h), hydrogen concentration in the headspace was found to be nearly 0.5 mmol/L-medium. Based on this observation, certain approaches can be considered for further improvement in hydrogen production. Firstly, glycerol is the major component of CG and hence, it is a good carbon/energy source for fermentative hydrogen production. However, it has relatively lower amount of other nutrients such as, nitrogen rich compounds, Mg, Fe, among others (Sarma *et al.*, 2013b). Therefore, introduction of additional nutrient to the process; either by direct addition of supplements to the medium or by growing the

inoculum in a nutrient rich medium, further improvement in hydrogen production might be possible. Similarly, if no buffering agents are used, hydrogen production medium containing only CG has very low buffering capacity. Therefore, in the present case, a sharp decrease in medium pH was observed. Metabolic shift is a well-known phenomenon in fermentative hydrogen production which can reduce the product yield. Accumulation of VFAs and change in media pH is one major factor which can initiate a metabolic shift. Hence, change in media pH and subsequent metabolic shift could be a reason of reduced hydrogen production observed in the present study. For improved hydrogen production, therefore, further optimization of the process parameters is needed.

Phosphorus uptake by soybean plant in presence of HPLW

As shown in Figure 6.2.3, for pot 5 the plants were found to uptake nearly 2.84 folds more phosphorus in presence of 1 g of TCP; even in the absence of HPLW. These observations indicate that although TCP is sparingly soluble in water (6.4 $x10^{-25}$ µg/L); if applied on soil at relatively high TCP: soil ratio of 1:120, a considerable amount may become available to the plants. Additionally, slightly acidic pH of the soil might have helped in solubilizing a portion of the inorganic phosphate; since literature data suggests that application of insoluble rock phosphate may be helpful in improving the soluble phosphorus level of acidic soil, although, it is unlikely for neutral and alkaline soil (Kumar *et al.*, 2001). Further, as shown in Figure 6.2.3, phosphorus uptake observed for pot 1 and 5 was almost similar. This observation has ascertained that application of phosphorus rich material to the soil can be avoided by the addition of HPLW and the latter has the potential to increase the bioavailability of phosphorus already present in soil.

Further, compared to the plants grown in soil containing TCP (pot 5); plants grown in soil amended with both HPLW (50 mL) and TCP (pot 4) have shown 8.86 % improvement in phosphorus uptake. However, it should be noted that in this case of pot 4 as high as 1 g of insoluble TCP was added to the soil and 50 mL of HPLW was used as phosphate solubilizer; hence, expected phosphorus uptake was more than observed

uptake. This is more so because, in the case of pot 2, addition of only HPLW (50 mL) was found to improve the phosphorus uptake by 2.18 folds (Figure 6.2.3). At the same time, as shown in Figure 6.2.3, among all the plant samples analyzed, the plants of pot 4 have taken up maximum amount of phosphorus (43.34 µg/g biomass). This observation indicates that in the case of pot 4, probably a considerable amount of soluble phosphorus remained unutilized and unlike the present case, a growth period of more than one month would be more appropriate to observe a significant difference in phosphorus uptake between pot 4 and pot 5.

For each pot, biomass production was estimated after one month of growth period and the results have been presented in Figure 6.2.4. Among all the pots containing soil with different additives, best biomass production was observed in the case of un-amended soil. In this context it should be considered that the soil used in the present investigation was actually a commercial potting mix designed for gardening. Therefore, in spite of observed better phosphorus uptake by the plants of other pots, the one containing only soil was capable of supporting relatively better biomass production. If pot 1, 2 and 3 are considered; from Figure 6.2.4 it is evident that addition of 100 mL of HPLW to the soil resulted in reduced biomass production. Corresponding to a decrease of the amount of HPLW from 100 to 50 mL; an increase in biomass production was observed. The observation indicated that the HPLW has inhibitory effect on biomass production and the inhibition can be reduced by decreasing the amount added. Therefore, the dose of HPLW beneficial for the plants should be determined by further optimization. In this context, it should be considered that HPLW was mixed with the soil at the beginning of the experiment; hence, delicate seedlings were exposed to large amount of HPLW at an early stage of growth. Therefore, there is a possibility that the plants will behave differently if the HPLW is applied to the field once the plants are mature enough to grow in its presence.

Interestingly, as shown in Figure 6.2.4, addition of 50 mL of HPLW was found to have beneficial effect on seed germination (33.33% improvement). Similarly, 1 fold increase in seed germination was observed for soil containing both TCP and 50 mL of HPLW. However, negative effect on seed germination has been observed for 100 mL of HPLW.

It has been observed that presence of ethanol in the soil can improved the germination rate in some cases (Salehi *et al.*, 2008). Ethanol may present in HPLW as a byproduct of light independent fermentative hydrogen production process (Rosa *et al.*, 2014). Additionally, HPLW also contains acetic acid (734.59 mg/L), butyric acid (90.23 mg/L) and residual glycerol. Combine effect of these chemicals, therefore, could be a reason of improved germination rate observed in the present study. Most probably, soybean seeds were protected by these chemicals from harmful microbial attack, thereby, increasing their chance to germinate. However, this hypothesis needs experimental evidence.

Equal amount of HPLW (50 mL) was added to pot 2 and pot 4; however, relatively higher biomass production has been observed in the latter case (Figure 6.2.4). As already discussed, in addition to HPLW, 1 g of TCP was also mixed with the soil of pot 4. Thus, present observation indicates that in presence of sufficient amount of insoluble inorganic phosphate, inhibitory effect of HPLW on biomass production is reduced. Most probable reason for such observation could be the reaction between TCP and the organic acids of HPLW, which might have neutralized the acids immediately after their introduction to the soil to release soluble orthophosphate. Therefore, in order to avoid the inhibitory effect of unused acids and for improved plant biomass production, the dose of HPLW needs further optimization.

Over their growth period, phosphate solubilizing microorganisms present in rhizosphere can produce small quantities of different organic acids, and in typical soil solution the concentration of these acids may vary from 1 to 50 mM (Mardad *et al.*, 2013). It indicates that if organic acids are applied to the agricultural field at relatively low concentration, in terms of phosphate solubilization, they may be beneficial for the plants and will have no negative consequences. HPLW may contain as high as 44.06 mM of acetate, 31.03 mM of butyrate, 8.21 mM propionate and other VFAs in small quantities (Foglia *et al.*, 2011). Therefore, instead of applying large amount of HPLW at the beginning of cultivation, its multiple small doses over the growth period of plant could be a viable option for reducing its potential inhibitory effect and for maintaining its phosphate solubilization efficiency. Alternatively, similar to controlled drug delivery

(Brannon-Peppas, 1995), a carrier can be designed for sustained delivery of these organic acids to agricultural soil. However, such approach needs proper cost benefit analysis before considering.

After harvesting the plants, the pH of the soil of different pots was measured. It has been observed that soil pH was almost constant even after addition of the organic acid rich HPLW (data not shown). Therefore, the possibility of decreasing the soil pH post HPLW application was negligible. Thus, HPLW could be a good alternative of phosphate solubilizing biofertilizer. In fact, it has been postulated to be lesser expensive as well as have better shelf life than commercial phosphate solubilizing biofertilizers (Sarma *et al.*, 2013a). The present investigation revealed that HPLW can improve phosphorus uptake by plants; however, its positive effect on the yields of agricultural products needs to be demonstrated by detailed investigation.

In conclusion, nearly 0.5 mmol-H_2/L-medium has been produced by CG bioconversion in the absence of any supplementary media components. Further optimizations of different process parameters are needed to improve the hydrogen yield. Acetic acid (734.59 mg/L) and butyric acid (90.23 mg/L) were the major byproducts of this process. The liquid waste containing these acids has been evaluated as soil phosphate solubilizer. Soybean plants grown in this liquid waste enriched soil have shown 2.18 to 2.74 folds more phosphorus uptake. The observation suggested possible commercialization of hydrogen production liquid waste as an alternative of phosphate solubilizing biofertilizer. However, further field scale investigations are required to optimize the approach.

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Table 6.2. 1: Soil composition of different pots used to grow soybean plants (HPLW, hydrogen production liquid waste; TCP, Tricalcium phosphate).

Pot number	Soil (g)	HPLW (mL)	TCP (g)
Pot 1	120	100	0
Pot 2		50	0
Pot 3 (control)		0	0
Pot 4		50	1
Pot 5		0	1

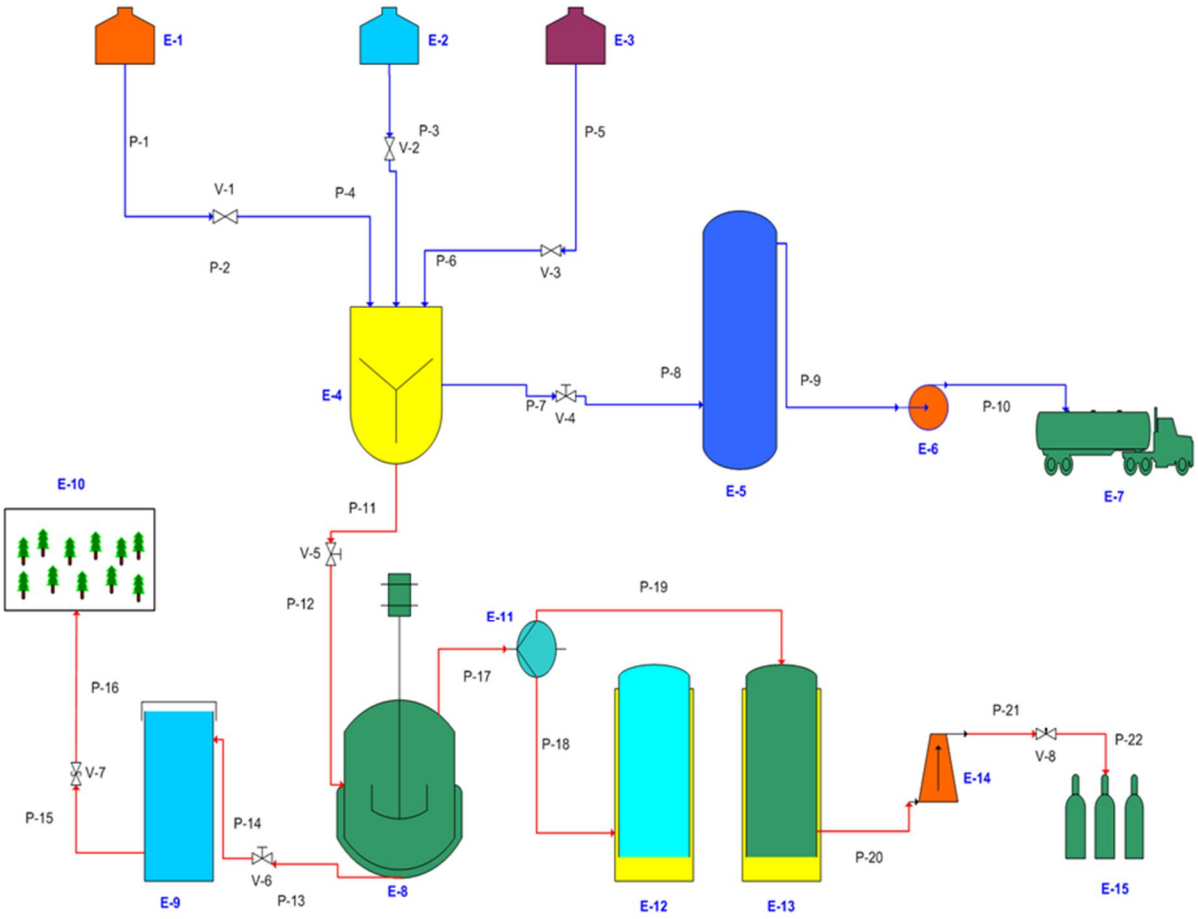

Figure 6.2. 1: A schematic representation of the approach considered for the present investigation. E-1 (Lipid), E-2 (Methanol), E-3 (NaOH/catalyst), E-4 (Transesterification), E-5 (Biodiesel processing), E-6 (Pump), E-7 (Biodiesel distribution), E-8 (Crude glycerol fermentation), E-9 (Organic acid rich liquid waste), E-10 (Agricultural field), E-11 (Gas separator), E-12 (Gas holder-CO_2), E-13 (Gas holder-H_2), E-14 (Compressor), E-15 (H_2-cylinders); P (Pipeline); V (Valve).

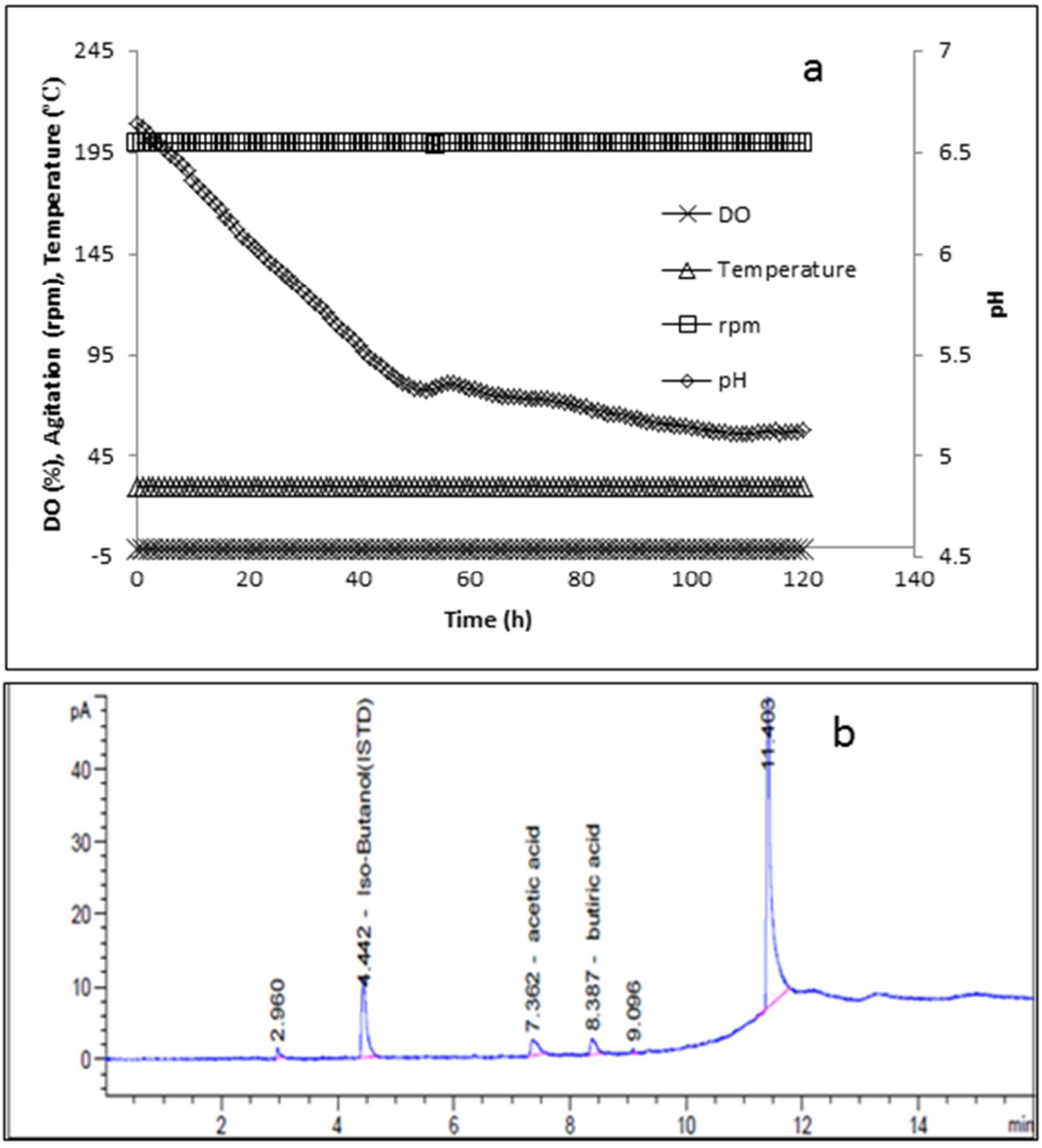

Figure 6.2. 2: (a) Values of different process parameters recorded online during hydrogen production using a 7.5L fermenter. (b) Gas chromatogram of hydrogen production liquid waste (HPLW) showing the presence of acetic acid and butyric acid as major byproducts.

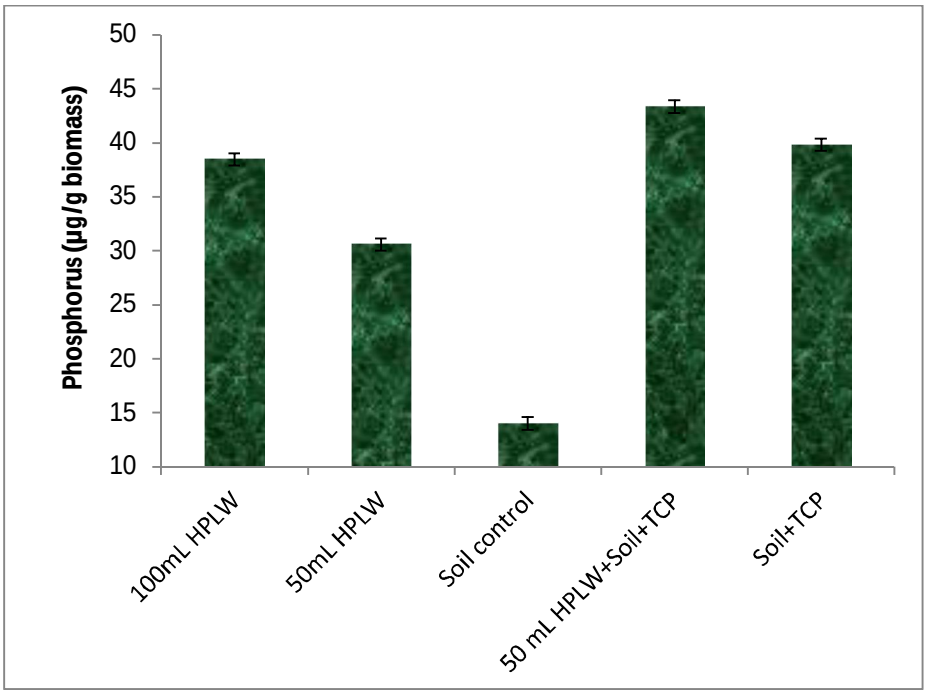

Figure 6.2. 3: Phosphorus uptake by soybean plants cultivated in the presence of different amounts of hydrogen production liquid waste (HPLW) and tricalcium phosphate (TCP).

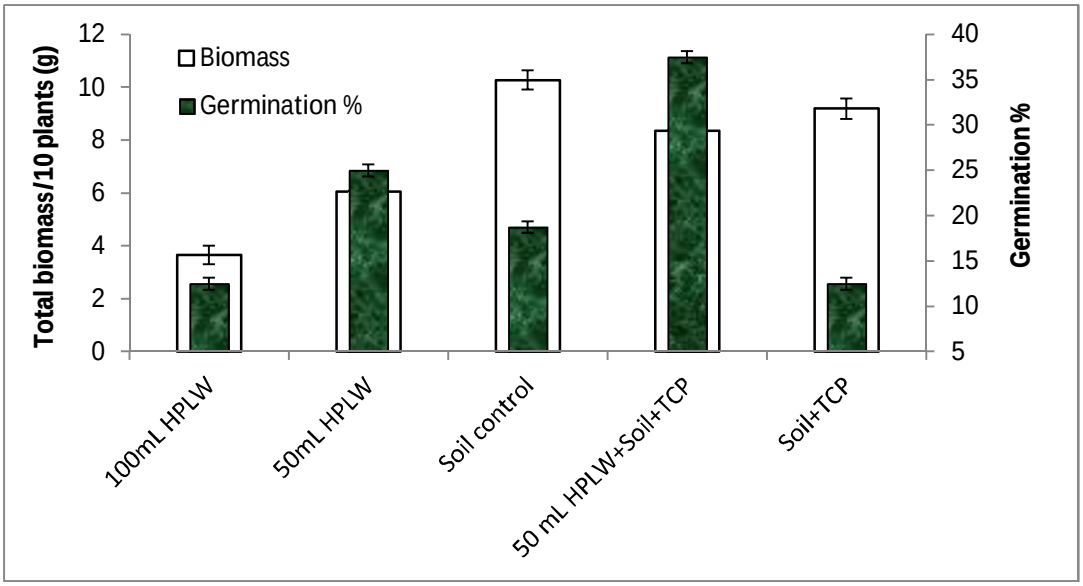

Figure 6.2. 4: Effect of hydrogen production liquid waste (HPLW) and tricalcium phosphate (TCP) treatment of soil on soybean plant biomass production and seed germination.

CHAPTER 7

DESIGNING AND OPTIMIZATION OF NOVEL BIOPROCESSES FOR IMPROVE HYDROGEN PRODUCTION USING CG

PART I

A NOVEL ANAEROBIC TWO PHASE SYSTEM FOR BIOHYDROGEN PRODUCTION AND *IN SITU* EXTRACTION ORGANIC ACID BYPRODUCTS

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Bioprocess and Biosystems Engineering (submitted)

Résumé

Avec l'absence d'émission de CO_2 et une densité d'énergie massique plus élevée que les carburants conventionnels, tels que l'essence, le diesel, le biodiesel et le bioéthanol, le biohydrogène est une option prometteuse en terme d'énergie renouvelable. Par conséquent, la production biologique d'hydrogène, a une importance commerciale lorsqu'il est produit à partir de matières premières provenant de déchets industriels à faible coût. La fermentation sombre anaérobie est une méthode simple, efficace et largement étudiée pour la production de biohydrogène. Au cours de la production d'hydrogène par ce procédé, divers acides organiques sont accumulés diminuant ainsi le pH du procédé. Par conséquent, pour éviter l'inhibition métabolique ou diminution de rendement lors de la période de fermentation, un ajustement de pH rigoureux est nécessaire. Pour la première fois, un système de bioréacteur anaérobie à deux phases a été testé pour la production de biohydrogène et l'extraction *in situ* d'acides organiques. Parmi les différents solvants testés, l'alcool oléique a été choisie en tant que phase organique dans le procédé en raison de sa biocompatibilité. Un rapport de la phase organique : aqueuse de 1: 50 a été déterminé comme étant optimal pour augmenter la production d'hydrogène de 26.6 mmol/L à 29.6 mmol/L- milieu de culture.

Mots-clés: Biohydrogène; glycérol brut; alcool oléique; acide organique; bioréacteur en deux phase

Abstract

Owing to CO_2 free emission, hydrogen is considered as a potential green alternative of fossil fuels. Water is the major emission of hydrogen combustion process and gravimetric energy density hydrogen is nearly three times more than that of gasoline and diesel fuel. Biological hydrogen production, therefore, has commercial significance; especially, when it is produced from low cost industrial waste based feedstock. Light independent anaerobic fermentation is simple and mostly studied method of biohydrogen production. During hydrogen production by this method, a range of organic acids are accumulated resulting in sharp decrease in process pH. Therefore, to avoid process termination or possible metabolic shift, throughout the fermentation period a rigorous pH adjustment is necessary. Alternatively, for the first time, a two phase anaerobic bioreactor system has been reported for biohydrogen production and in situ extraction of different organic acids. Among different solvents, based on biocompatibility oleyl alcohol has been chosen as the organic phase of the process. An organic: aqueous phase ratio of 1: 50 has been found to increase the hydrogen production from 26.58 mmol/L to 29.61 mmol/L-medium.

Keywords: Biohydrogen; crude glycerol; oleyl alcohol; organic acid; two phase bioreactor

Introduction

Fossil fuels are extensively used to fulfill global energy demand. Unfortunately, it is a limited resource and most importantly, over exploration of fossil fuel has adverse effect on the environment (Ntaikou *et al.*, 2010). In this context, a search for renewable and sustainable energy source is going on throughout the world. As a result of this attempt, biofuels such as biodiesel and bioethanol have become commercially available and lately, biohydrogen is receiving much attention as a potential alternative of fossil fuels. There are different reasons behind researchers' the attention towards biohydrogen as a renewable fuel. Firstly, almost all biomass, including different agro industrial wastes, can be converted to hydrogen by microbial processes; although, hydrogen yield may vary from feedstock to feedstock. Secondly, it is CO_2 emission free fuel; major emission of which is only water. Finally, gravimetric energy density of hydrogen is 142 MJ/kg; which is approximatly three times of gasoline (47 MJ/kg) and diesel fuel (43 MJ/kg) (Şensöz *et al.*, 2000, Sydney *et al.*, 2014).

Crude glycerol (CG) is a byproduct generated during trans-esterification of lipid used for biodiesel production. For every 100 kg of biodiesel, nearly 10 kg of CG is produced which may contain different compounds such as methanol, soap, monoglyceride, diglyceride etc (Johnson *et al.*, 2007). Mainly because of continuous global production and crude nature, market value of CG could be as low as 0.05$/pound (Yang *et al.*, 2012). Therefore, researchers are looking for an economically sound method for CG valorization. In this context, crude glycerol has been evaluated as a feedstock for biohydrogen production by light independent anaerobic fermentation. During this process along with hydrogen, a range of organic acids are produced as byproduct. Accumulation of these acids results in sharp decrease in fermentation medium pH; which may lead to termination of the process. Moreover, accumulation of organic acids may initiate metabolic shift in an anaerobic process to produce more reduced end products such as solvents (butanol) (Hawkes *et al.*, 2002, Yusof *et al.*, 2012), as a result of which hydrogen production may decrease. Therefore, a novel two phase process has been evaluated for potential application in hydrogen production. As shown in Figure

7.1.1, the organic phase of the process could be a biocompatible organic solvent capable of extracting the organic acids produced during hydrogen production. The aqueous phase will be CG based medium containing the microorganism. Thus, the purpose of present investigation was to screen different organic solvent to identify the most biocompatible one and to optimize process parameter such as volume of the organic phase, for enhanced hydrogen production.

Materials and methods

Microorganism and culture conditions

Enterobacter aerogenes NRRL B 407, a rod-shaped, gram-negative, facultative anaerobic bacterium has been used for the present investigation. The bacterium, which is known to produce hydrogen from a range of feedstock including waste biomass, was provided by ARS (USDA, USA). The organism was routinely grown in a culture medium composed of the following components (g/L): glucose (5), casein peptone (5), KH_2PO_4 (2), $MgSO_4 \cdot 7H_2O$ (0.5) and yeast extract (0.5). For inoculum development, 50 mL of aforementioned medium taken in serum bottle (125 mL) was bubbled with pure nitrogen gas for 2 minutes, so that anaerobic condition can be created. After sterilization, the bottles were inoculated and incubated using an incubator operated at 30 ±1° C and 150 rpm. For each experiment, 5% (v/v) of the medium containing freshly grown microbial cell was used as inoculum (Sarma *et al.*, 2013c).

Screening of different organic solvents for two phase system

Isopropyle ether ("Extraction of Acetic Acid from Water Using Isopropyl Ether" from the Wolfram Demonstrations Project http://demonstrations.wolfram.com/ExtractionOfAceticAcidFromWaterUsingIsopropylEther/ (accessed on 23/04/2014)), Tri-*n*-octyle amine (Rasrendra *et al.*, 2011), Ethyle ether (Kalaichelvi *et al.*, 2007), Oleyl alcohol (Malinowski, 2001), 2-ethyle-1-hexanol (Rasrendra *et al.*, 2011) and Aliquat 336 (Katikaneni *et al.*, 2002); a few solvents commonly used for organic acid extraction, have been selected for present investigation. This experiment was designed to evaluate the possibility of growing *Enterobacter aerogenes* NRRL B 407 in presence of these solvents. Initial CG

concentration used for this investigation was 12 g/L. Sterile CG solutions were distributed into different serum bottles in such a way that after inoculating with 5 % (v/v), there would be 25 mL medium in each bottle. 2 mL of each solvent were filter sterilized by 0.22 µ (25 mm) syringe filter (Fisher scientific, Canada) and separately added to different serum bottles containing CG solutions. The two phase systems so obtained were bubbled with nitrogen gas for 2 minutes to drive out the oxygen dissolved in the liquid and the bottles were immediately closed with aluminum crimp seals and silicone septa. By using a syringe fitted with hypodermic needle, 1.25 mL of inoculum prepared using the procedure mentioned in section 2.1 was added to each bottle. Followed by inoculation, the bottles were incubated for 96 hours in an incubator maintained at 30 ºC and 150 rpm. In a similar manner, a serum bottle was prepared without any organic solvent and used as control for comparison purpose. At the end of the experiment, samples were collected from the aqueous phases of the systems and cumulative biomass production and final pH was measured.

Hydrogen production with selected organic solvents

Based on the result of previous experiment, tri-n-octyle amine, oleyl alcohol and aliquat 336 were selected for further investigation. As mentioned in section 2.2; 23.75 mL of CG solution was taken in serum bottle in such a way that after addition of 1.25 mL of inoculum, total volume of the aqueous phase medium would become 25 mL and CG concentration would be 12g/L. Different organic solvents were distributed in separate serum bottles containing the CG solutions in such a way that for each organic solvent three different organic: aqueous phase ratios, namely, 1: 25, 1: 12.5, and 1: 6.25, could be obtained. One bottle was used as control where none of the solvents was added. Liquids of each bottle was bubbled with nitrogen gas for 2 minutes and immediately closed with crimp seal and septum as mentioned in section 2.2. All bottles were sterilized in closed condition; cooled, inoculated with 1.25 mL of inocula and incubated as described above. Using a gas tight syringe (SGE Analytical Science, Australia), from the headspace of each bottle, 1 mL of gas sample was collected after 48 hours of incubation and stored in gas tight vials until analyzed.

Investigation of the effect of oleyl alcohol and CG concentration on hydrogen production

Based on the result of previous experiment (section 2.3), oleyl alcohol was selected to be used in biohydrogen production process. It was observed that in the case of oleyl alcohol, a relatively low organic: aqueous phase ratio was more favorable for hydrogen production by *Enterobacter aerogenes* NRRL B 407. Therefore, hydrogen production at different relatively low oleyl alcohol (organic phase): aqueous phase ratios, namely, 1: 100, 1: 50 and 1: 25 were compared with that of control medium with no organic phase. Media preparation, sterilization, inoculation, incubation and sample collection conditions were similar to the ones mentioned in section 2.3. Moreover, in an attempt to determine the effect of CG concentration on hydrogen production by oleyl alcohol based novel two phase system, another experiment was conducted using 25 g/L of CG. A set of serum bottles containing only CG (25 g/L) were used as control whereas, in another set oleyl alcohol was added at an organic: aqueous phase ratio of 1:25. Other conditions of the experiments were similar to those mentioned above.

Analysis

Hydrogen analysis

In order to determine the hydrogen content, biogas samples which had been collected during the experiments were analyzed by gas chromatography. The gas chromatography system used for this analysis was of Varian 3800 model (USA), capable of headspace analysis. The column and detector used for this analysis were PoraPLOT Q® (Agilent technology, USA) and thermal conductivity detector (TCD), respectively. N_2 at a flow rate of 3.5 mL/minute was used as the carrier gas whereas; the column temperature was 100 °C.

Analysis of fermentation end products

In order to analyze the fermentation end products, at the end of each experiment liquid samples were collected from the aqueous phase of the system. They were centrifuged for 5 minutes at 10000 rpm; supernatants were filtered by 0.45 µ syringe filter and

analyzed by GC. The GC (GC7890B, Agilent Technologies, USA) used for this analysis was fitted with flame ionization detector (FID). The column temperature gradient used for this analysis was 50-250 ºC. It was achieved by increasing the temperature at a rate of 20 ºC per minute. Injection volume was 0.8 µl and isobutanol was used as internal standard for all samples.

Results and discussion

Screening of different organic solvents for two phase system

Biocompatibility of the organic phase is highly desirable for a two phase reactor system used for bioconversion purpose. Therefore, six different solvents considered for the present investigation were tested for possible inhibitory effect on biomass production of *Enterobacter aerogenes* NRRL B 407, the hydrogen producing bacterium used in this study. The results of this investigation have been presented in Figure 7.1.2. From Figure 7.1.2 it is evident that, in the case of oleyl alcohol biomass production was found to be nearly 60 mg/L; which is 50 % more than the control experiment conducted in the absence of any solvent. However, presence of other solvents was found to have adverse effect on biomass production. Among rest of the solvents biomass production was slightly higher in the case of tri-n-octyle amine and aliquat 336; hence, along with oleyl alcohol, they were also considered for further investigation. At the end of the process, aqueous phase pH of the two phase systems corresponding to different solvents was measured. As shown in Figure 7.1.2, compared to pH 4.65 found for control, for oleyl alcohol the same was found to be 5.15. This observation indicates that oleyl alcohol was capable of extracting the organic acids accumulated in the aqueous phase to resist sharp decrease in media pH; which is, otherwise, commonly observed in the case of batch hydrogen production processes. From Figure 7.1.2 it is also observed that final media pHs of two phase systems involving tri-n-octyle amine, ethyle ether, 2-ethyle-1-hexanol, and isopropyle ether were also higher than single phase control. However, in those cases microbial growth was negligible; which indicates that only a small amount of organic acids were there in the aqueous phase of those systems.

Hydrogen production with selected organic solvents

The purpose of this investigation was to determine the effect of organic phase volume on hydrogen production. As mentioned in previous section, oleyl alcohol, tri-n-octyle amine and aliquat 336 were chosen for this investigation and organic phase: aqueous phase ratios considered were 1: 25, 1: 12.5, and 1: 6.25. The results of this investigation have been presented in Figure 7.1.3. From Figure 7.1.3 it is clear that at all organic phase: aqueous phase ratios, tri-n-octyle amine and aliquat 336 were inhibitory for hydrogen production and hence they were not considered for further investigation. On the contrary, in the case of oleyl alcohol, corresponding to decrease in organic: aqueous phase ratio from 1: 6.25 to 1.25; gradual improvement in hydrogen production has been observed (Figure 7.1.3). Probably, a thick organic layer on top of the aqueous phase medium posed as a barrier for hydrogen to release into the headspace of the reactor. Additionally, a large volume of the organic phase may interfere in N_2 bubbling process, preventing sufficient removal of oxygen from the media. Based on this observation it has been postulated that by further decreasing the organic phase volume, improved hydrogen production could be possible. Thus, as explained in next section (3.3) another experiment was carried out using oleyl alcohol with yet lower organic phase volume.

Investigation of the effect of oleyl alcohol volume and CG concentration on hydrogen production using proposed two phase system

As a part of this investigation, the effect of oleyl alcohol phase volume on hydrogen production by *E. aerogenes* has been investigated and the results have been presented in Figure 7.1.4. Figure 7.1.4 shows that application of an organic phase comprising of oleyl alcohol has beneficial effect on hydrogen production; which can be improved by reducing the organic: aqueous phase ratio from 1:25 to 1: 50. By using a phase ratio of 1: 50, hydrogen production can be increased from 26.58mmol/L to 29.61mmol/L-medium. However, further increase in the ratio to 1: 100 did not show any significant enhancement in hydrogen production. Probably, at this ratio, organic acid extraction by the organic phase was not sufficient to make any significant impact on hydrogen

production process. As already mentioned in section 2.5.2, aqueous samples were analyzed by GC to determine the fermentation end products. In Figure 7.1.5, fermentation end product profiles of control and two phase system involving oleyl alcohol at a phase ratio of 1: 50 have been presented. From Figure 7.1.5 it is evident that, the butyric acid peak seen at 8.45 min of the control sample became unrecognizable due to presence of oleyl alcohol phase. Likewise, the peak at 5.48 min, most probably for a low molecular weight organic acid such as formic acid, also disappeared in the aqueous sample collected from the proposed two phase reactor. This observation indicates that the organic phase was capable of extracting the organic acids from the aqueous phase of the reactor and it could be related to observed increase in hydrogen production.

CG concentration is also a crucial factor in hydrogen production by CG bioconversion. It has been observed that for a batch process, 10 g/L CG is optimum for hydrogen production (Sarma *et al.*, 2013b). Increase in CG concentration beyond this concentration resulted in almost no increase in hydrogen production. Moreover, corresponding to an increase in CG concentration from 2.5 g/L to 20 g/L, gradual decrease in final media pH was observed (Sarma *et al.*, 2013b). It suggests that at relatively high initial CG concentration, more amounts of organic acids accumulate in the medium. Thus, simultaneous extraction of these organic acids might have beneficial effect on cumulative hydrogen production. Therefore, proposed two phase system was tested for hydrogen production; where, initial CG concentration was as high as 25 g/L. Results of this investigation has been presented in Figure 7.1.6. From Figure 7.1.6 it is evident that in contrast to 1.48 mmol hydrogen produced per liter medium of a single phase process; it was as high as 11.65 mmol per liter medium of proposed two phase system. High glycerol concentration is known to have inhibitory effect on hydrogen production (Ito *et al.*, 2005); therefore, for further improvement in product yield substrate inhibition should be avoided by using a continuous process. Overall, the observation suggests that simultaneous extraction of organic acid accumulated in the medium has positive effect on hydrogen production. Therefore, the concept deserves further exploration. For instance, instead of carrying out fermentation and extraction in the

same vessel; a dedicated extraction vessel can be introduced for continuous extraction of organic acids and extracted medium can be recirculated to the fermentation vessel for further fermentation. Additionally, if the organic acid byproducts are extracted, without further sterilization remaining water can be reused. It may save both energy and water required for hydrogen production. For bioconversion of 1 kg of CG as high as 400 L of water is required (Sarma *et al.*, 2013a); therefore, reutilization of water is inevitable for large scale hydrogen production. However, direct application of large volume (more than 10 % v/v) of organic acid rich wastewater from preceding batch was found to be inhibitory for hydrogen production (Sarma *et al.*, 2013b). Thus, further investigation on simultaneous extraction of organic acid will be beneficial for fermentative hydrogen production.

Conclusions

During hydrogen production by light independent anaerobic fermentation, organic acid byproducts are accumulated in the medium; which is detrimental for the process. To get rid of the problem, a novel two phase process for hydrogen production and simultaneous extraction of organic acid has been proposed. Owing to its biocompatibility, oleyl alcohol has been chosen for *in situ* extraction of organic acids. An organic: aqueous phase ratio of 1: 50 was found to be optimum for the process. For a process with initial CG concentration of 25 g/L, compared to 1.48 mmol-H_2/L-medium of a single phase process; as high as 11.65 mmol-H_2/L-medium has been obtained by proposed two phase system. At high initial substrate (CG) concentration such as 25 g/L, organic acid concentration in the medium is more; therefore, the approach should be more appropriate for such process. Moreover, the strategy has the potential to reduce the volume of water required for large scale hydrogen production.

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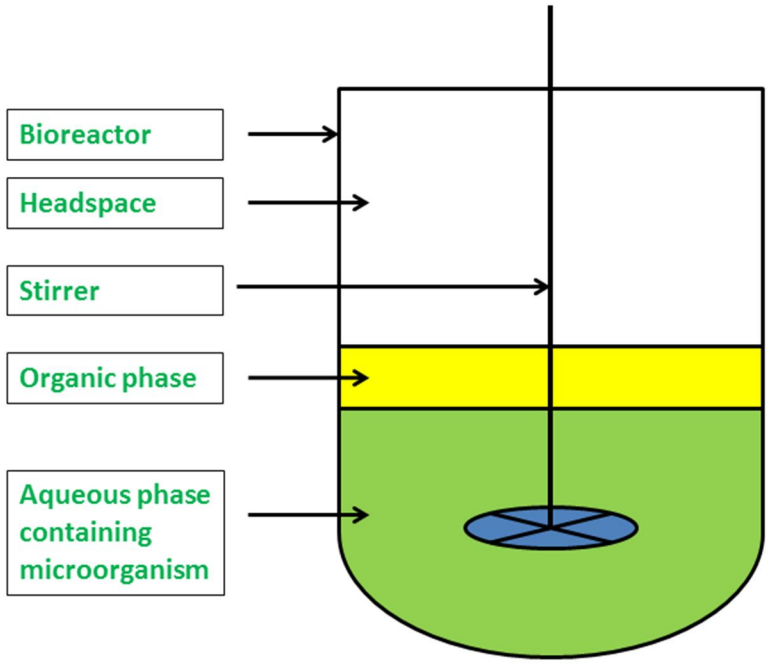

Figure 7.1. 1: Schematic representation of the proposed method

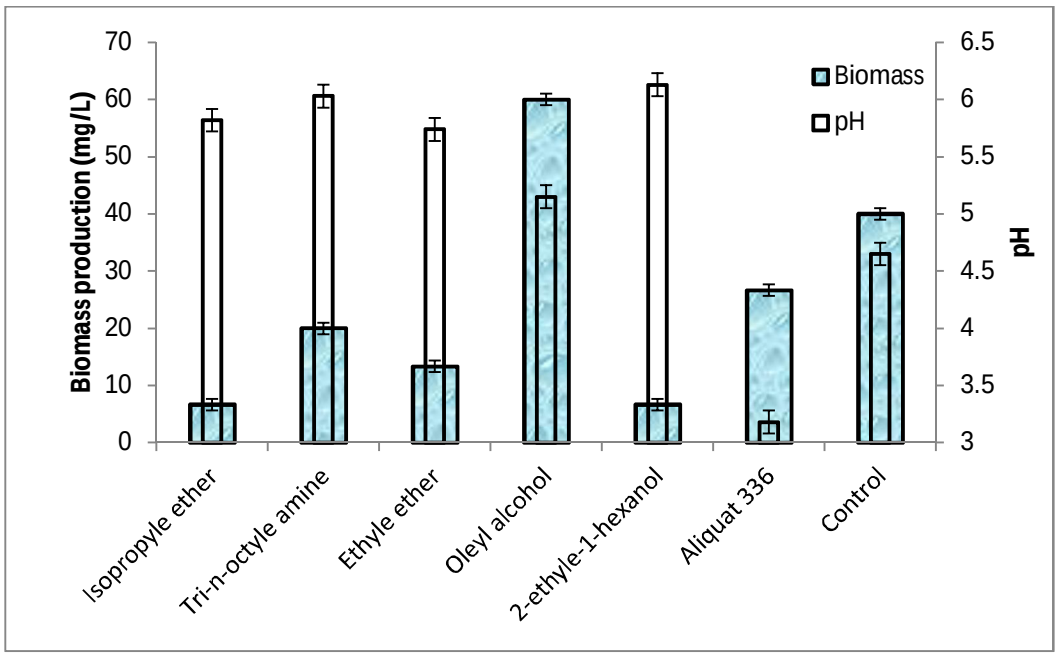

Figure 7.1. 2: Screening of organic phase based on biocompatibility & final media pH

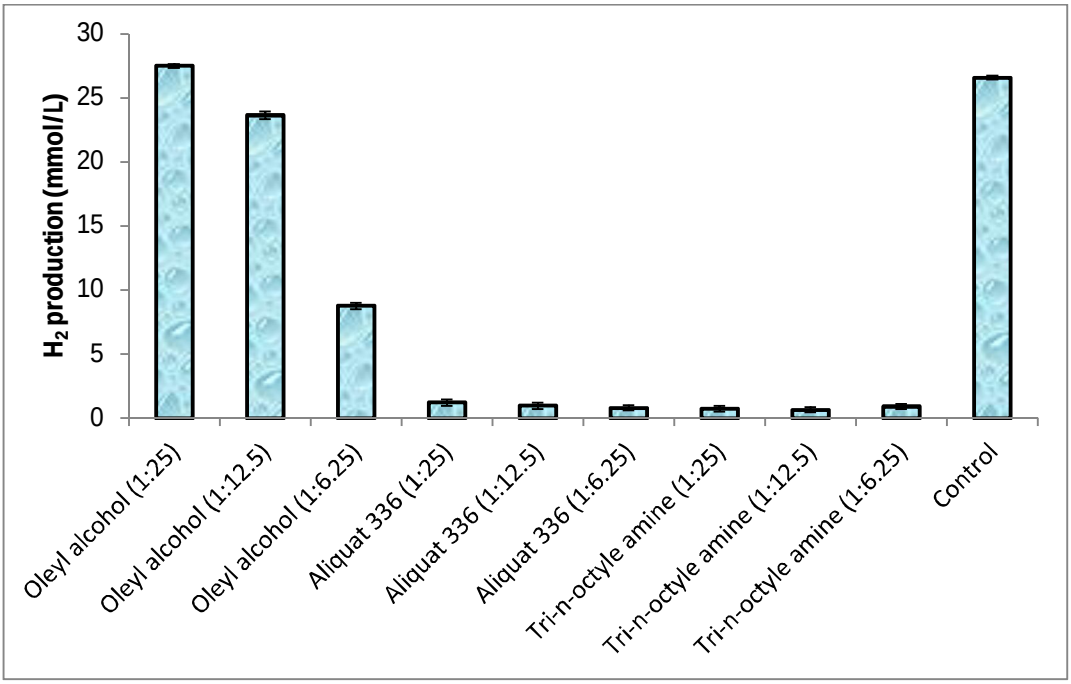

Figure 7.1. 3: Screening of organic phases based on hydrogen production

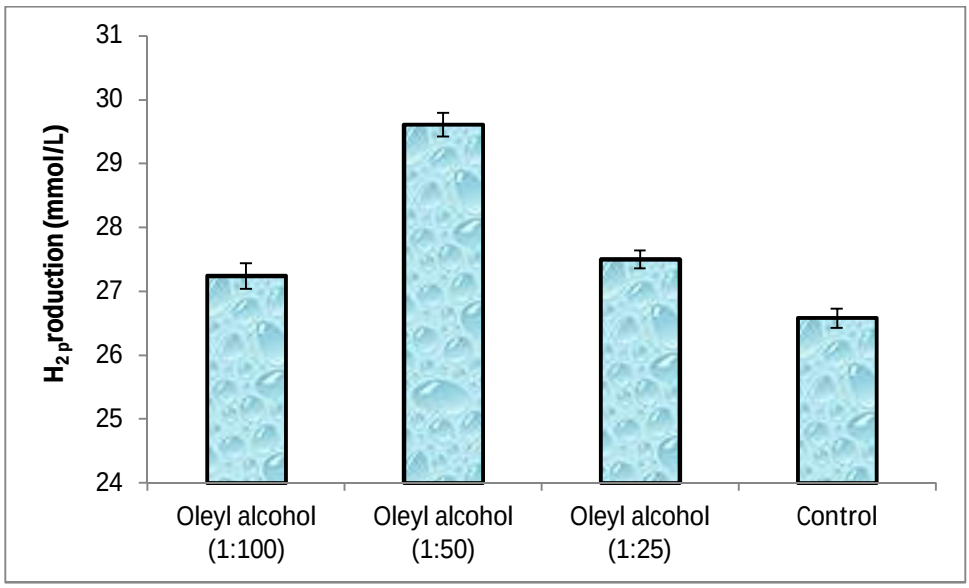

Figure 7.1. 4: Investigation of the effect of oleyl alcohol: aqueous phase ratio on hydrogen production

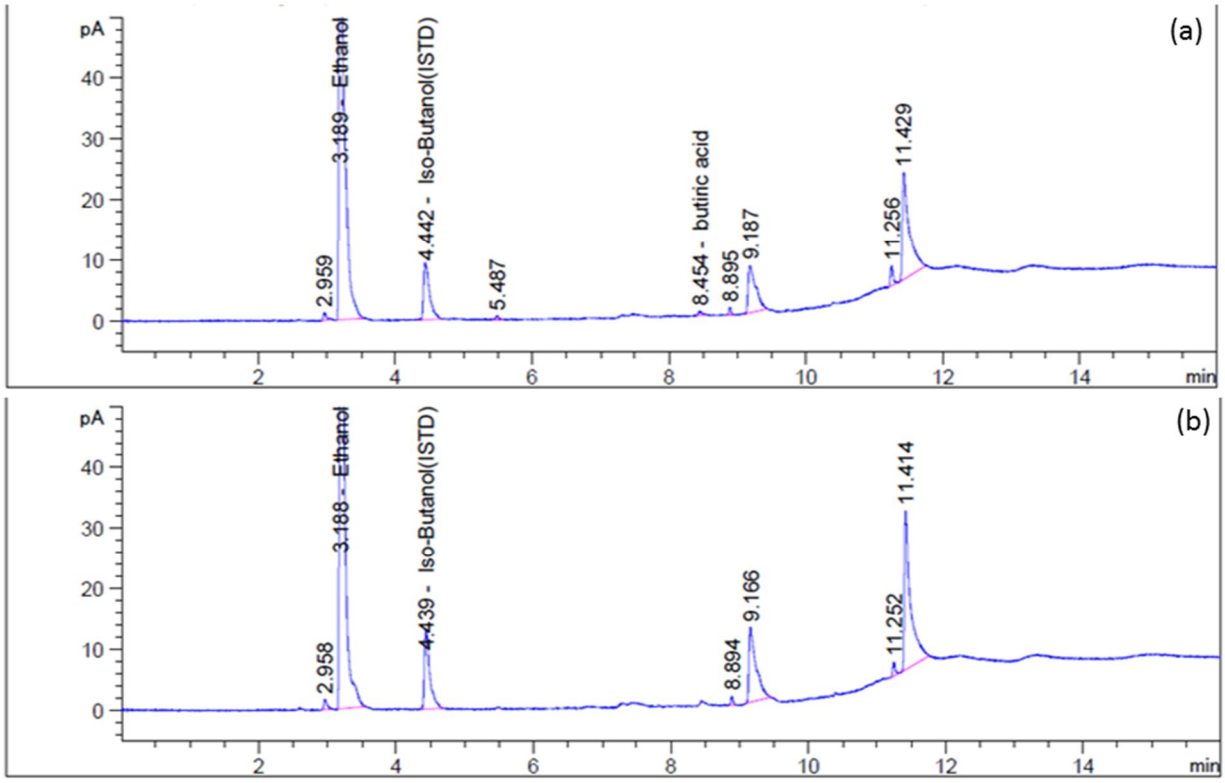

Figure 7.1. 5: Fermentation end products detected in the aqueous phase of the reactor. (a) Single phase control, (b) two phase process containing oleyl alcohol at an organic: aqueous phase ratio of 1: 50.

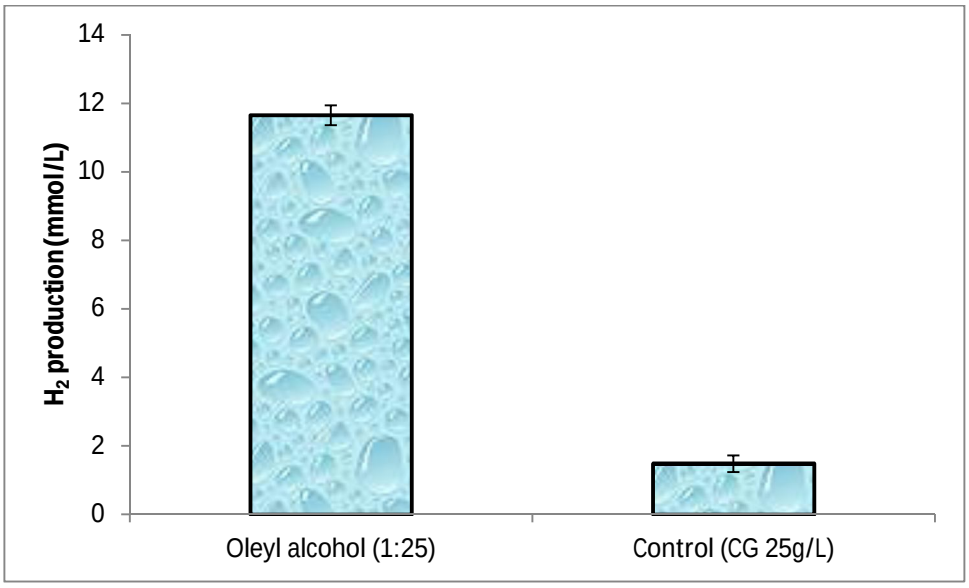

Figure 7.1. 6: Investigation of the effect of high CG concentration on hydrogen production using novel two phase system

PART II

LOW COST SEMI-CONTINUOUS BIOPROCESS AND ONLINE MONITORING OF HYDROGEN PRODUCTION FROM CRUDE GLYCEROL

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Environmental Science and Technology (submitted, possibility of US patent)

CHAPITRE 8

CONCLUSIONS ET RECOMMANDATIONS

Conclusions

Les principales conclusions de la thèse de doctorat sont présentées ci-dessous:

1. Les rejets de l'industrie de biodiesel, particulièrement riche en glycérol, représentent un milieu favorable pour la production d'hydrogène par voie microbienne. Dans les travaux réalisés dans cette thèse, 10 g / L de glycérol brut (GB) s'est avérée la concentration optimale pour la production de H_2.
2. Le Ni, le Fe, le Mo et le S sont les éléments essentiels pour la synthèse des enzymes (hydrogénases et nitrogénases) intervenant dans la production de l'hydrogène. Un milieu équilibré en ces éléments favorise et améliore la production d'hydrogène.
3. L'éthanol, le 1,3 propanediol, l'acide butyrique et le méthane sont les principaux sous-produits à grande importance commerciale générés durant la production d'hydrogène par voie fermentaire.
4. Le coût des différents suppléments ajoutés au GB pour la production d'hydrogène tel que l'addition de rejets solides de l'industrie de bière (50 mg / L) comme source d'éléments nutritifs permet d'améliorer la production de H_2 de 32,50% avec un taux maximum de production journalier de 1040 mL/L. Il a été également observé que l'addition des déchets liquides d'abattoir (SL), de la biomasse des déchets de brasserie (BWB) et de l'urée au GB peut améliorer la production d'hydrogène de respectivement 18,81 ± 3,56; 27,30 ± 3,54 et 38,57 ± 3,66%.
5. En utilisant la méthodologie de surface de réponse, l'effet du savon, du méthanol et du NaCl sur la production d'hydrogène et la bioconversion du glycérol a été évalué. le savon (1 à 3 g/L) avait un effet inhibiteur significatif (p = 0,0104 < 0,05) sur la production d'hydrogène. De même, le méthanol (1.3 à 3.3 g / L) avait un effet similaire dans la plage de concentration étudiée.
6. La désalaison peut éliminer jusqu'à environ 42% de savon présent dans le GB. Cependant, son élimination affecte négativement la production de H_2 (le savon est utilisé comme co-substrat pour la production de H_2). le traitement du GB par du $MgSO_4$ permet de convertir le savon en sa forme inactive (écume). Cette nouvelle

approche permet d'augmenter la production de H_2 de 34,70% et la bioconversion du glycérol de 2,5 fois. L'utilisation de $MgSO_4$ permet également d'améliorer la qualité nutritionnelle du GB vu qu'il contient seulement 57 ± 18 mg/L.

7. Des nanoparticules de citrate de fer, de $FeSO_4$, $NiCl_2$ et de l'acétate de nickel ont été préparées en utilisant la technologie de séchage par nano-pulvérisation. L'effet de ces nanoparticules sur la production de H_2 a été étudié. Seules les particules de citrate ferrique avaient un effet bénéfique sur la production de H_2. Deux types de nanoparticules de citrate de fer ont été préparés, par dissolution dans de l'eau chaude, et dissolution dans une solution de NaOH. Les nanoparticules préparées par dissolution dans de l'eau chaude se sont avérées capables d'améliorer la production d'hydrogène de 50, 5%.
8. La concentration initiale en GB est le facteur principal qui affecte le rendement en hydrogène. À des concentrations initiales extrêmement faibles (de l'ordre de 100 mg/L) et en utilisant les particules de citrate de fer, le maximum du rendement en hydrogène atteint était de 29,9 mol H_2/kg GB. Ce rendement est nettement supérieur à 8,25 mol H_2/kg GB, valeur obtenue dans le cas de fermentation obscure.
9. Les rejets liquides issus de la production du biohydrogène (HPLW) ont d'excellentes propriétés de solubilisation du phosphate. Ces rejets ont un fort potentiel commercial puisqu'ils peuvent être utilisés comme une alternative aux bioengrais. Avec ces rejets, on peut augmenter la concentration en phosphate soluble 23,17 fois en 1 minute d'application. Contrairement aux bioengrais traditionnelles, ces rejets ne renferment pas de microorganismes vivants et par conséquent, la durée de conservation est plus longue pour ces produits (rejets) proposés.
10. Des plantes de soya cultivés dans du sol enrichi avec du HPLW ont présenté une capacité d'absorption du phosphore 2,18 à 2,74 fois supérieure par rapport au contrôle (plantes cultivées dans du sol sans HPLW). Grâce à ces résultats, les HPLW peuvent être présentés comme étant un produit à fort pouvoir commercial et une alternative intéressante aux bioengrais phosphatés actuellement sur le marché.
11. Durant la production du biohydrogène, l'accumulation des acides organiques (sous-produits de cette fermentation obscure et anaérobie) entraîne une diminution

considérable du pH du milieu ce qui est critique pour le procédé. Pour remédier à cette problématique, un nouveau procédé biphasique a été développé, permettant la production d'hydrogène et, en même temps, l'extraction de l'acide organique généré. En raison de sa biocompatibilité, l'alcool oléique a été sélectionné pour l'extraction *in situ* de ces acides organiques. Un rapport phase organique/phase aqueuse de 1: 50 était l'optimal pour ce procédé, ce qui permet d'augmenter la production d'hydrogène de 26,58 mmol / L à 29,61 mmol / L.

12. L'acétone, l'éthanol et le butanol ont été détectés dans le GB (substrat) utilisé dans la présente étude. L'acétone-butanol-éthanol (ABE) sont convertis en GB par action de la microflore indigène durant le stockage.
13. En le diluant avec de l'eau, le GB peut être utilisé seul, sans aucun ajout de suppléments pour la conception d'un procédé de production de H_2 en mode semi-continu et à faible coût, avec une production améliorée d'hydrogène. Avec ce procédé, il est possible d'atteindre 5,18 L-H_2 /L, ce qui est équivaut à 210 mmol-H_2 /L. Cette productivité est nettement plus élevée que 2,02 à 2,68 L H_2 / L, valeur moyenne de productivité d'hydrogène à partir du GB.

Recommandations pour l'avenir

Sur la base des résultats issus de cette thèse de doctorat, les recommandations suivantes peuvent être proposées pour la continuité de la recherche.

1. Pour parvenir à une production optimale en biohydrogène à partir du glycérol brut (GB) et, à partir de n'importe quel autre substrat d'une façon générale, les procédés de fermentation devraient assurer le maximum de bioconversion de cette matière première. Par exemple, les procédés fonctionnant en mode "fed-batch " et en mode "en continu " devront être considérés.
2. Une grande quantité d'eau est utilisée durant la production de biohydrogène, cette quantité pourrait atteindre jusqu'à 400 L/kg de substrat. Pour une production durable d'hydrogène, cette eau devrait être réutilisée.
3. D'autres études supplémentaires devront être réalisées afin d'exploiter au maximum les avantages du procédé biphasique proposé dans cette thèse. Au lieu d'utiliser un seul compartiment au sein du bioréacteur pour les deux phases aqueuse et organique, l'étape d'extraction peut être effectuée dans un deuxième compartiment dédié pour ce fait. En utilisant cette approche, Il est possible de contourner les problèmes dûs à la présence de la phase organique durant l'extraction d'biohydrogène.
4. Pour atteindre un taux de production d'hydrogène élevé ainsi qu'une productivité améliorée, le processus devrait être étudié à haute densité initiale de cellules.
5. Afin de réduire la quantité d'énergie nécessaire pour la production biologique d'hydrogène, la stérilisation classique à la chaleur peut être remplacée par de nouvelles technologies de stérilisation. : la destruction sélective des microorganismes indésirables par traitement chimique peut être une technologie intéressante.
6. Il sera intéressant si toute l'énergie nécessaire à la production d'hydrogène biologique pourrait être obtenue à partir d'une source d'énergie renouvelable telle que l'énergie solaire.

ANNEXES

ANNEXE I

Data: Hydrogen biorefinery: Potential utilization of the liquid waste from fermentative hydrogen production

Figure 2.3.2: Reported concentration levels of different metabolites present in HPLW and their market price in US dollars (Han *et al.*, 2010, Lalaurette *et al.*, 2009, Selembo *et al.*, 2009).

	1,3-propandiol	Ethanol	Acetic acid	Butyric acid	Succnic acid	Lactic acid	Propionic acid	Valeric acid
yield (mg/L)	1710.28	895.69	764.64	168.86	660	160	18.6	14.72
Price ($/L)	288	134.6	55.25	74.6	226	78	45.5	37.4

Figure 2.3.3: Free energy of formation (25º C) of different metabolites produced during biohydrogen production.

Methane	-50.79
Ethanol	-181.75
1,3-propandiol	-326.93
Butyric acid	-377.8
Acetic acid	-396
Propionic acid	-383.5
Lactic acid	-540
Succnic acid	-746.38
valerate	-344.34

ANNEXE II

Data: Bio-hydrogen production by biodiesel derived crude glycerol bioconversion: a techno-economic evaluation

Figure 2.4.2: Cost distribution of the crude glycerol (1 kg) bioconversion process

Item/process	**Cost ($)**
CG pretreatment	2.38
Dark fermentation (inoculum media cost)	44.88
Dark fermentation (inoculum electricity cost)	0.037
Dark fermentation (media cost)	161.04
Dark fermentation (electricity cost)	2.14
Photo-fermentation (inoculum media cost)	44.88
Photo-fermentation (inoculum electricity cost)	0.037
Photo-fermentation (media cost)	25.88
Photo-fermentation (electricity cost)	21.83
Equipment	29.98
CG	0.33
Total Cost	333.414

Figure 2.4.3: Electricity requirement of a two-stage fermentation process for CG (1 kg) bioconversion and H_2 production

Process	**Electricity required (kWh)**
Dark fermentation	48.85
Photo-fermentation	490.45

ANNEXE III

Data: Hydrogen production from meat processing and restaurant waste derived crude glycerol by anaerobic fermentation and utilization of the spent broth

Figure 3.1.**1**: Batch H_2 production and glycerol utilization profiles obtained for different initial CG concentrations

Time (h)	Hydrogen production (mmol)					Residual glycerol concentration (%)				
	2.5 g/L CG	5 g/L CG	10 g/L CG	15 g/L CG	20 g/L CG	2.5 g/L GC	5 g/L CG	10 g/L CG	15 g/L CG	20g/L CG
0	0	0	0	0	0	100	100	100	100	100
12	0.79	0.49	0.83	0.45	0.74	40.50	83.93	77.87	78.25	98.24
24	1.21	0.95	1.45	0.87	1.32	7.84	57.2	71.25	69.10	88.26
36	1.21	1.66	2.11	1.57	1.91	0.17	36.4	54.88	63.82	79.93
48	1.21	1.99	2.67	2.03	2.32	0.17	19.8	49.28	58.94	69.82
60	1.21	2.28	3.19	2.53	2.86	0.17	10.73	36.76	56.30	65.2
72	1.21	2.32	3.45	2.82	3.24	0.17	5.32	30.60	50.60	63.59

84	1.21	2.35	3.71	3.20	3.48	0.17	2.34	23.92	40.85	58.43
96	1.21	2.35	3.83	3.45	3.73	0.17	2.55	19.17	33.33	52.80
120	1.21	2.35	4.00	3.86	3.98	0.17	0.77	13.38	29.47	44.51
144	1.21	2.35	4.12	4.07	4.19	0.17	1.11	8.24	29.06	41.15
168	1.21	2.35	4.2	4.24	4.32	0.17	1.74	5.92	28.04	40.95

Figure 3.1.2: pH profiles obtained from the batch experiments conducted for different (initial) CG concentrations

Time (h)	2.5 g/L CG	5 g/L CG	10 g/L CG	15 g/L CG	20 g/L CG
0	4.43	4.53	4.61	4.64	4.67
12	3.9	4	3.92	4	3.97
24	3.74	3.87	3.81	3.83	3.81
36	3.84	3.79	3.83	3.75	3.71
48	3.86	3.75	3.73	3.69	3.65
60	3.92	3.78	3.73	3.72	3.65
72	3.93	3.73	3.7	3.67	3.6
84	4.06	3.83	3.75	3.71	3.62
96	3.98	3.86	3.77	3.72	3.61
120	4	3.88	3.8	3.75	3.65
144	4.1	3.89	3.84	3.73	3.67
168	4.06	3.9	3.81	3.69	3.65

Figure 3.1.6: Batch H_2 production profiles obtained by addition of different concentrations of spent biomass (SB) (autoclaved) from previous fermentation

Time (h)	SB 5 mg/L	SB 10 mg/L	SB 50 mg/L	SB 150 mg/L
0	0	0	0	0
12	1.16	1.16	1.28	1.16
24	2.12	2.15	2.28	2.16
36	2.61	2.65	2.82	2.78
48	3.28	3.23	3.53	3.49
60	3.49	3.44	3.94	3.86
72	3.74	3.69	4.40	4.28
84	3.90	3.86	4.78	4.57
96	3.99	3.94	4.98	4.78
120	4.15	4.02	5.27	5.03
144	4.24	4.07	5.48	5.19
168	4.24	4.07	5.57	5.28

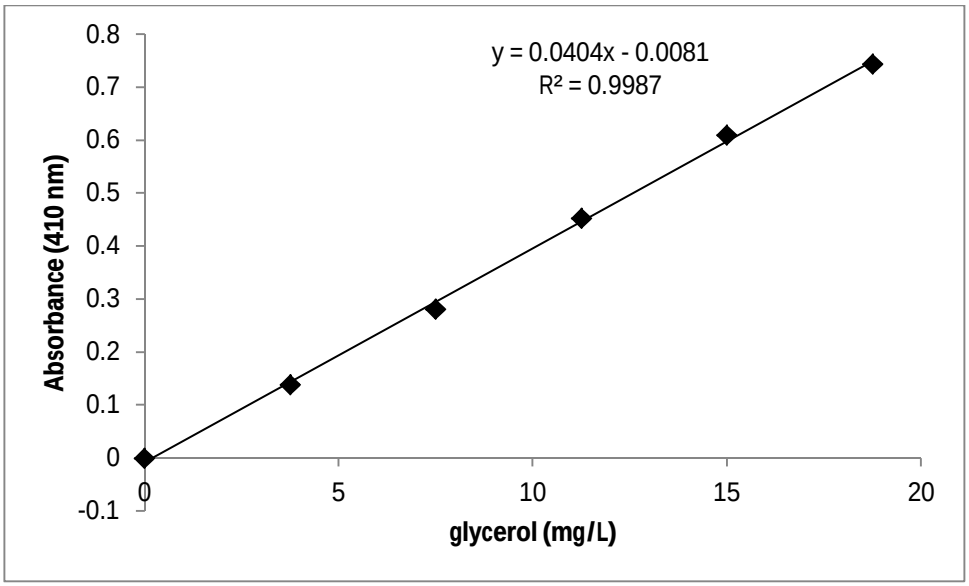

Figure: Standard curve for determination of glycerol concentration

ANNEXE IV

Data: Evaluation of different supplementary nutrients for enhanced biohydrogen production by *Enterobacter aerogenes* NRRL B 407 using waste derived crude glycerol

Figure 3.2.2: Maximum cumulative H_2 production (mmol/L), glycerol utilization (%) and broth pH recorded at the end of fermentation for different material supplemented to 10 g/L CG. The control indicates the results obtained for 10 g/L CG without any additive.

Response	**Urea 10 mg/L**	**BWB 10 mg/L**	**SL 10 mg/L**	**SS 20 mg/L**	**BWL 20 mg/L**	**AP 10 mg/L**	**SIW 1% (v/v)**	**CG 10g/L (control)**
H_2 production (mmol/L)	116.4	106.92	99.8	94.00	92.2	72.4	47.4	84
Final pH	3.3	3.3	3.38	3.26	3.26	3.41	3.57	3.81
Glycerol utilization (%)	93.40	84.50	93.60	87	90.5	72.135	61.00	94.08

Figure 3.2.3: H_2 production profiles obtained for different concentrations urea supplemented to 10 g/L CG

Time (h)	**10 mg/L**	**20 mg/L**	**60 mg/L**	**120 mg/L**	**180 mg/L**
0	0	0	0	0	0
12	1.18	0.16	0.12	0	0
24	2.26	0.91	1.08	0.9	0
36	3.18	2.83	2.40	1.9	0
48	4.01	3.16	3.15	2.37	0
60	4.63	3.20	3.40	2.62	0
72	5.05	3.24	3.40	2.62	0.04
84	5.34	3.28	3.40	2.62	0.45
96	5.50	3.33	3.40	2.62	0.70
120	5.7	3.33	3.49	2.62	0.95
144	5.82	3.33	3.49	2.62	1.12
168	5.82	3.33	3.49	2.62	1.12

ANNEXE V

Data: Mitigation of the inhibitory effect of soap by magnesium salt treatment of crude glycerol- a novel approach for enhanced biohydrogen production from the biodiesel industry waste

Figure 4.2.1: Cumulative H_2 production (after 120h) obtained using 10 g/L CG (treated with different concentration of NaCl). Initial NaCl concentration in the medium of each run of experiment was also shown

Response	1g/L	5g/L	10g/L	20g/L	50g/L	100g/L	200g/L	Control
Cumulative H_2 production: mmol/L	30.49	38.38	39.72	39.00	21.23	14.58	2.32	33.3
NaCl (g/L) in final medium	0.05	0.25	0.5	1.00	2.5	5.00	10	0

Figure 4.2.3: H_2 production (a) and glycerol utilization (b) profiles obtained for 20 g/L CG treated with different concentrations of $MgSO_4$. Control represents result obtained for untreated CG

(a) Cumulative H_2 production (mmol/L)					
Time (h)	**$MgSO_4$ (0.5g/L)**	**$MgSO_4$ (1g/L)**	**$MgSO_4$ (2.5g/L)**	**$MgSO_4$ (5g/L)**	**Control (CG 20 g/L)**
0	0.99	0.99	0.99	0.99	0.92
12	6.50	6.06	7.63	3.69	5.07
24	11.98	16.09	13.37	4.55	12.70
36	14.9	21.7	16.37	4.13	16.74
48	14.71	23.83	16.62	4.25	17.7

(b) Residual glycerol (%)					
Time (h)	**$MgSO_4$ (0.5 g/L)**	**$MgSO_4$ (1 g/L)**	**$MgSO_4$ (2.5 g/L)**	**$MgSO_4$ (5 g/L)**	**Control (CG20 g/L)**
0	100	100	100	100	100
24	93.96	88.93	89.83	96.74	96.15
48	87.06	79.05	87.71	97.24	91.34

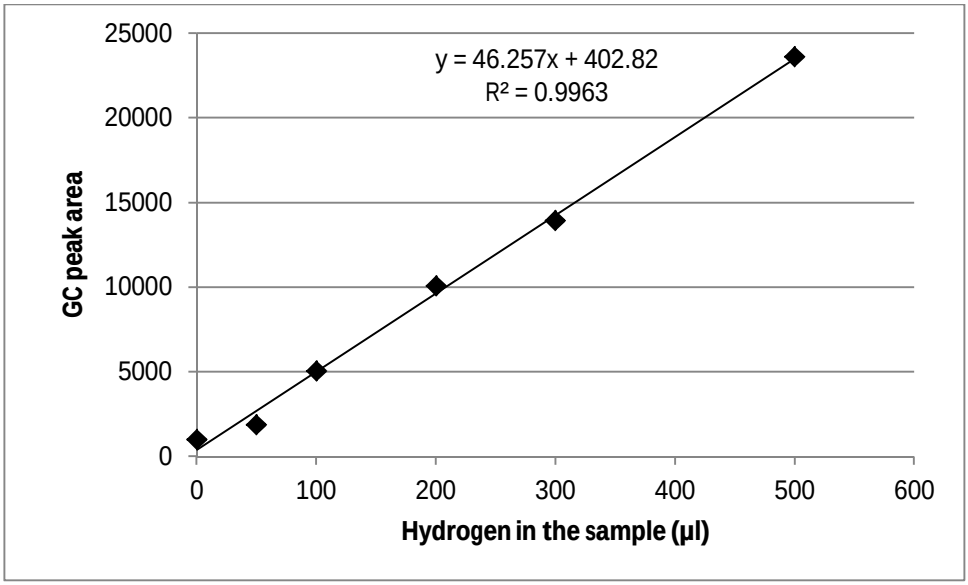

Figure: Standard curve for determination of hydrogen concentration

ANNEXE VI

Data: Enriched hydrogen production by bioconversion of biodiesel waste supplemented with ferric citrate and its nano-spray dried particles

Figure 5.1.4: Cumulative hydrogen production obtained after 48 h of incubation. Different concentrations of two different types of ferric citrate particles were added to 12 g/L of CG

Particle type	**10 mg/L**	**50 mg/L**	**100 mg/L**	**300 mg/L**	**500 mg/L**
NaOH based particles	13.03	9.6	10.34	6.95	6.54
Water based particles	26.58	33.64	36.49	37.37	40.00

Figure 5.1.5: Cumulative hydrogen production obtained after 48 h of incubation. 100 mg/L of ferric citrated particles (prepared with hot water) were added to different concentrations of CG. Projected hydrogen yields by bioconversion of 1 kg of CG have also been shown for each initial CG concentration

Response	**100 mg/L**	**500 mg/L**	**1 g/L**	**2 g/L**	**5 g/L**	**10 g/L**
Cumulative H_2 production (mmol/L)	3.00	5.97	11.23	18.24	32.53	36.45
Cumulative H_2 production (mol/kg)	29.9	11.94	11.23	9.12	6.50	3.64

ANNEXE VII

Data: Liquid waste from bio-hydrogen production - a commercially attractive alternative for phosphate solubilizing bio-fertilizer

Figure 6.1.2: Phosphate solubilization achieved using different concentrations of bio-hydrogen production liquid waste. The control represents soluble phosphate concentration obtained using only water as the solubilizing agent

Response	**Bio-hydrogen production liquid waste**					
	Control 0% (v/v)	**10% (v/v)**	**20% (v/v)**	**40% (v/v)**	**60% (v/v)**	**100% (v/v)**
Soluble phosphate (mg/L)	1.38	4.83	8.65	15.71	20.50	32.06
	2.7	3.23	5.84	25.73	35.78	49.98

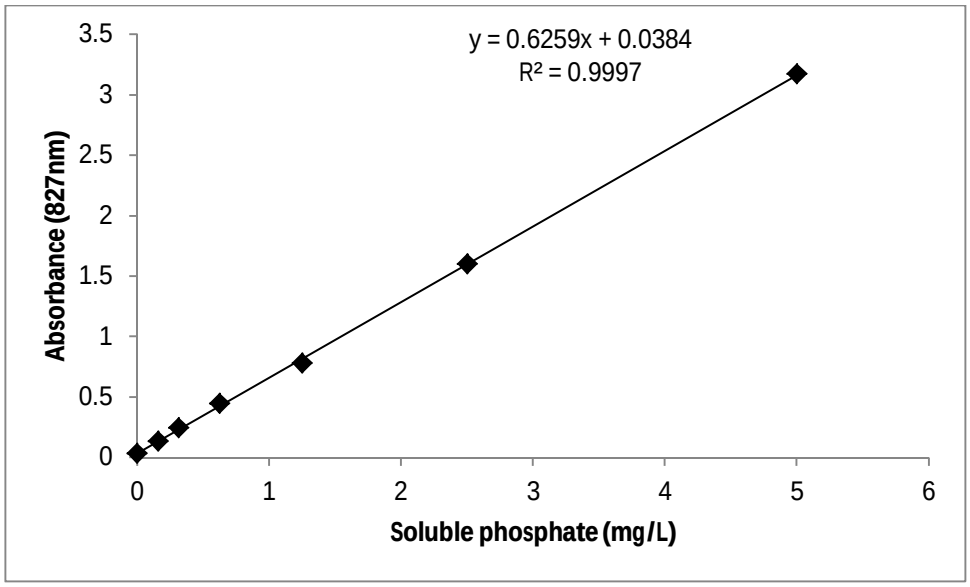

Figure: Standard curve used to determine the soluble phosphate concentration

ANNEXE VIII

Data: Phosphate solubilization using biohydrogen production liquid waste and improved phosphorus uptake by soybean plant

Figure 6.2.3: Phosphorus uptake by soybean plants cultivated in the presence of different amounts of hydrogen production liquid waste (HPLW) and tricalcium phosphate (TCP).

Response	**100mL HPLW**	**50mL HPLW**	**Soil control**	**50 mL HPLW+Soil+TCP**	**Soil+TCP**
Phosphate (µg/g biomass)	38.46	30.58	14.01	43.35	39.81

Figure 6.2.4: Effect of hydrogen production liquid waste (HPLW) and tricalcium phosphate (TCP) treatment of soil on soybean plant biomass production and seed germination.

Response	**100mL HPLW**	**50mL HPLW**	**Soil control**	**50 mL HPLW+Soil+TCP**	**Soil+TCP**
Total biomass (g) of 10 plants	3.66	6.04	10.27	8.35	9.19
Germination (%)	12.5	25	18.75	37.5	12.5

ANNEXE IX

Data: A novel anaerobic two phase system for biohydrogen production and in situ extraction organic acid byproducts

Figure 7.1.2: Screening of organic phase based on biocompatibility & final media pH

Organic phase (2 mL)	Isopropyle ether	Tri-n-octyle amine	Ethyle ether	Oleyl alcohol	2-ethyle-1-hexanol	Aliquat 336	Control
Biomass production (mg/L)	6.66	20.00	13.33	60.00	6.66	26.66	40
pH	5.82	6.03	5.74	5.15	6.13	3.18	4.65

Figure 7.2.3: Screening of organic phases based on hydrogen production

Organic phase: medium	Oleyl alcohol (1:25)	Oleyl alcohol (1:12.5)	Oleyl alcohol (1:6.25)	Aliquat 336 (1:25)	Aliquat 336 (1:12.5)	Aliquat 336 (1:6.25)	Tri-n-octyle amine (1:25)	Tri-n-octyle amine (1:12.5)	Tri-n-octyle amine (1:6.25)	Control
H_2 production (mmol/L)	27.50	23.64	8.78	1.22	0.97	0.80	0.73	0.64	0.91	26.58