View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Elsevier - Publisher Connector

Available online at www.sciencedirect.com

ScienceDirect

Energy Procedia 61 (2014) 1213 – 1218

Energy Procedia

The 6th International Conference on Applied Energy – ICAE2014

Bioethanol Production from Glycerol by Mixed Culture System

NunthaphanVikromvarasiri[a], ThanapornLaothanachareon[b], VerawatChampreda[b], NiponPisutpaisal[a,c,d,*]

[a]*The Joint Graduate School for Energy and Environment (JGSEE), King Mongkut's University of Technology Thonburi, Bangkok 10140, Thailand*
[b]*Enzyme Technology Laboratory, National Center for Genetic Engineering and Biotechnology, 113 Thailand Science Park, Paholyothin Road, KlongLuang, Pathumthani12120, Thailand*
[c]*Department of Agro-Industrial, Food and Environmental Technology, Faculty of Applied Science, King Mongkut's University of Technology North Bangkok, Bangkok 10800, Thailand*
[d]*Centre of Biosensor and Bioelectronics Technology, King Mongkut's University of Technology North Bangkok, Bangkok 10800, Thailand*

Abstract

Glycerol, a by-product from biodiesel industry, is a promising feedstock for subsequent bioconversion to higher-value products. Potential application of a mixed microbial consortium on the fermentative conversion of glycerol to ethanol was demonstrated in this study. Maximum ethanol concentration of 11.1 g l^{-1} was produced after 72 h fermentation from the initial pure glycerol concentration of 45 g l^{-1}, at 30 °C, and pH 7 under anaerobic conditions, corresponding to the ethanol production rate and yield of 0.34 g l^{-1} h^{-1}, and 0.81 mol ethanolmol^{-1} glycerol, respectively.The microbial consortium yielded lower ethanol concentration (6.5 g l^{-1}) but similar ethanol yield (0.85 mol ethanol mol^{-1} glycerol) when crude glycerol of 45 g l^{-1} was fermented at the same condition. At the optimum fermentative condition of the pure glycerol, phylogenetic analysis of microbial consortium based on 16S rRNA gene sequences indicated that *Gammaproteobacteria* represented 95% of the microbial diversity in the consortium while the rest belonged to *Betaproteobacteria*. The consortium was dominated by bacteria closely related to genera *Enterobacter* and *Klebsiella*, which could play the role on conversion of glycerol to ethanol in this system.

© 2014 Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/3.0/).
Peer-review under responsibility of the Organizing Committee of ICAE2014

Keywords:ethanol;fermentation; glycerol; mixed culture; 16S rRNA gene

* Corresponding author. Tel.: +662-587-8257; fax: +662-587-8257.
E-mail address: npp@kmutnb.ac.th.

1876-6102 © 2014 Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/3.0/).
Peer-review under responsibility of the Organizing Committee of ICAE2014
doi:10.1016/j.egypro.2014.11.1059

1. Introduction

Biodiesel is a new alternative for renewable energy. The depletion of the fossil fuel resources (e.g., petroleum, coal, and natural gas) and the continuous increase of the fossil fuel price cause the rapid increase of biodiesel demand. This situation leads to a swift growth of biodiesel production, which consequently results the generation of a lot of glycerol wastes from the transesterification in the biodiesel production. Since the glycerol has been dramatically increased over demand of consumption, the prices of glycerol are continuously dropped, and will soon become waste by-product, requiring disposal or treatment with costs [1].Several methods based on chemical and biological transformations can convert glycerol into more valuable products such as 1,3-propandiol [2], dihydroxyacetone [3], succinic acid [4], citric acid [5], and ethanol [6-8]. Ethanol was considered the target product in this study. Since the ethanol is a compound, which is easy for utilizations in many applications such as fuel (e.g., gasohol, E20, E80 and pure ethanol), and can be used in the production of biodiesel, beverage, medicine, antiseptic, and so on. Moreover, the ethanol production cost is mainly governed by feedstock and operational costs. Use of crude glycerol, containing alkali salts, alcohol and other impurities, as feedstock instead of traditional feedstock (e.g. sugar, molasses, and lignocellulosic biomass), for the ethanol production could reduce the production cost approximately 40% [1]. Feasibility of using a mixed consortium as starter seed for fermentative conversion of glycerol to ethanol was examined. Kinetics data of the fermentation process and microbial species at the optimal ethanol production were determined.

2. Materials and methods

2.1 Microbial Inoculum

The microbial seed collected from a full-scale upflow anaerobic sludge blanket (UASB) system (MaleeSampran Public Co. Ltd, NakornPrathom, Thailand) was used for entire experiments. Prior to use microbial seed was washed with distilled water twice to remove wastewater and debris coming with the seed, was suspended in the distilled water. The granule suspension was collected for determining the microbial dry weight.

2.2 Culture medium

A cultural medium was prepared in the distilled water with the following constituents (per liter); 7.0 g of K_2HPO_4, 5.5 g of KH_2PO_4, 1.0 g of $(NH_4)_2SO_4$, 0.25 g of $MgSO_4·7H_2O$, 0.021 g of $CaCl_2·2H_2O$, 0.12 g of $Na_2MoO_4·2H_2O$, 2.0 mg of nicotinic acid, 0.172 mg of Na_2SeO_3, 0.02 mg of $NiCl_2$ and 10 ml of trace element solution containing 0.5 g of $MnCl_2·4H_2O$, 0.1 g of H_3BO_4, 0.01 g of $AlK(SO_4)_2·H_2O$, 0.001 g of $CuCl_2·2H_2O$ and 0.5 g of Na_2EDTA [6]. The laboratory grade glycerol (QRec, Auckland, New Zealand) was used as carbon source and energy sources. The crude glycerol waste derived fromtrangpalmoil Co., Ltd, Trang Province, Thailand.The medium was autoclaved at 121°C, 15 psi for 15 min before use.

2.3 Experimental setup for ethanol production by microbial consortium

All experiments were setup in 100ml serum bottles with 50ml working volume. Mixed culture was dispensed into the serum bottle containing sterile cultural medium. The final concentration of biomass in the serum bottle was 0.13 g dry cell weight l^{-1}. Experiments were setup under various conditions, initial glycerol concentrations (0-128 g l^{-1}), and pHs (4-7). The optimum condition was used for investigation of ethanol production fromcrude glycerol. The experiment was conducted in duplicates and was incubatedat

30° C with rotary shaking at 150 rpm. Liquid samples were periodically collected for analysis of glycerol, ethanol and fermentative products.Microbial cells were collected by centrifugation for molecular phylogenetic analysis.

2.4 Analysis of fermentative products

Glycerol concentration in the fermentation broth was determined on a high performance liquid chromatograph (Agilent LC1200 Series, USA) equipped with a UV/RI detector, and an Aminex HPX-87H column of 300 x 7.8 mm. (Bio-Rad, USA).Ethanol and volatile fatty acids (VFAs) were determined with Gas Chromatography (Shimadzu GC-7A, Japan) equipped with a flame ionization detector (FID) and fitted with a Stabilwax DA capillary column (30 m x 0.32 mm, i.d., 0.25 µm film thickness).

2.5 16S rRNA gene sequence analysis of microbial biodiversity

Total genomic DNA was extracted from the microbial cell pellet using the benzyl chloride method [9]. The purified DNA was used as the template for amplification of the full-length 16S rRNA gene using bacterial specific primers BSF8/20 and REVB with DyNAzyme EXT DNA polymerase (Finnzyme, Espoo, Finland), according to Kanokratana*et al.* [10]. Sequences of 16S rRNA gene inserts were grouped according to restriction fragment length polymorphisms (RFLP), determined by *RsaI*and *HhaI* digestion. Representative samples of each pattern were selected for sequence analysis. Chimeric sequences were identified by the CHECK_CHIMERA program provided at the Ribosomal Database Project II website [11]. Taxonomic classification was performed with the BLAST tool of the NCBI-nr database and RDP sequence match tool to determine their approximate phylogeny.

3. Results and discussion

3.1 Influence of pH and initial glycerol concentration on ethanol production

The microbial consortium showed low capable for ethanol production and glycerol utilization in acidic conditions (pH 4-6) (Fig.1A, 1B). Glycerol was negligibly utilized at pH 4 for thewhole range of glycerol concentrations (Fig. 1B), but was completelyutilized at pH 6 ([glycerol]i = 10 g l^{-1}) and pH 6.8 and 7.0 ([glycerol]i = 10, 25 g l^{-1}). These results pointed that the neutral pH favored theglycerol utilization, whereas the glycerol concentration exhibited a reverse trend of the glycerol utilization. In most of cases, glycerol utilization and ethanol production were negligibly changed after 72 h offermentation. The highest ethanol production at pH 5 and 6 were 0.041 and 0.062 mol l^{-1}, respectively, when glycerol concentration was 10 g l^{-1}, whereas that at pH 6.8 and 7 were 0.200 and 0.242 mol l^{-1}, respectively, when glycerol concentration was 45 g/L (Fig. 1. A). Themaximum ethanol concentration was observed in pH 7, when the initial glycerol concentration was 45 g l^{-1}.Moreover, according to the molar fraction of fermentative products at pH 7 and glycerol concentration of 45 g l^{-1}, ethanol was the predominant fermentative product (data not shown). The optimum pH in this study was similar with previous studies regarding the pH-dependent process of glycerol fermentation [8, 12].

3.2 Ethanol production from glycerol waste

The mixed microbial consortium was examined ability of ethanol production from crude glycerol waste. The cultivation condition used same the optimum condition of pure glycerol with 45 g l^{-1}initial glycerol waste concentration, which equated to optimum pure glycerol concentration for the highest

ethanol production. The ethanol production from crude glycerol waste (6.52 g l^{-1}) was quite lower than using pure glycerol (11.1 g l^{-1}) (Fig. 2). This result corresponds with the glycerol utilization, which crude glycerol was used 31.3 %(13.5 g l^{-1}), while pure glycerol was used about 62.1 % (27.7 g l^{-1}). However, both yield of using crude and pure glycerol were quite similar (0.85 and0.81 mol mol^{-1} glycerol, respectively). These results indicated that impurities in crude glycerol waste affected to ethanol production. Previous study found that glycerol waste can inhibit microbial growth [7].

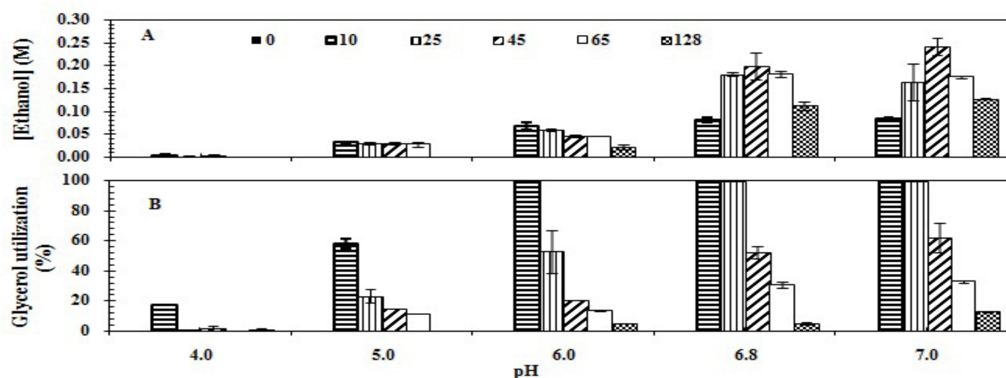


Fig. 1. Ethanol concentration(A) and glycerol utilization (B) after 72 h fermentation obtained at varying pHs and initial glycerol concentrations. Histograms represent mean values of duplicate measurements, and I-bars represent one standard deviation.

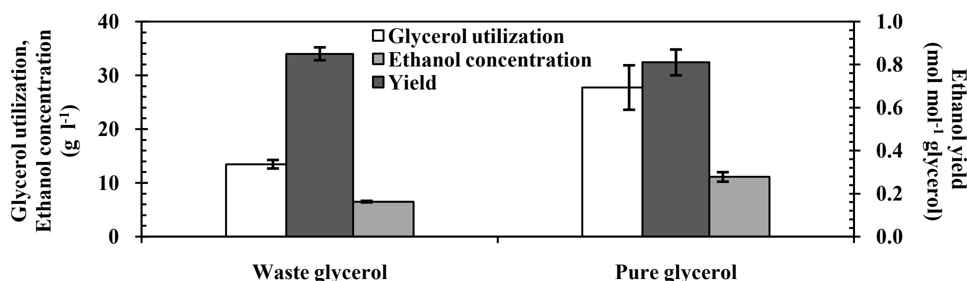


Fig. 2. Ethanol concentration, glycerol utilization, and ethanol yield after 72 h fermentation at the initial glycerol concentration of 45 g l^{-1}. Histograms represent mean values of duplicate measurements, and I-bars represent one standard deviation.

3.3 Microbial community at the optimal ethanol production conditions

Total genomic DNA from microbial sample collected at the optimumethanol production conditions, glycerol concentration of 45 g l^{-1}, pH 7 after 72 h fermentation, was successfully extracted. Approximately 1,500 bp 16S rDNA wasamplified by PCR. A number of 162 positive clones were selected and analyzed withthe restriction fragment length polymorphism (RFLP) resulting in 19 banding patterns with RsaI and HhaI, respectively. Based on RFLP patterns, 19 representative clones were chosen for sequencing andsubmitting to GenBank. Then, Phylogenetic tree of 16S rRNA gene sequences of microbialcommunity was constructed from sequenced data. *Gammaproteobacteria*was the dominant microbes in the community (95%) containing two groups, *Enterobacter*genus (47.5%) and *Klebsiella*genus (47.5%). Minor microbe in the community was *Betaproteobacteria*(5%), *Thauera*genus (*Rhodocyclaceae*family bacterium).Genus *Enterobacter*and *Klebsiella*were reported to capable to covert glycerol to ethanol [7, 13].

4. Conclusion

The current work displayed that *Gammaproteobacteria* in the starting seed obtained from UASB is dominant at the condition where the optimum ethanol production was achieved. The consortium can use glycerol as a feedstock for ethanol production at the optimum pH and glycerol concentration of 7.0 and 45 g l^{-1}. Simultaneous production of ethanol from glycerol and waste reduction of the glycerol waste can be applied to management of the glycerol waste released from the biodiesel industry.

Acknowledgments

This work was financial supported by the Joint Graduate School of Energy and Environment (Grant No. JGSEE 180), King Mongkut's University of Technology Thonburi;S&T Postgraduate Education and Research Development Office (Perdro) (Grant No. JGSEE/PROJECT/007-2011); and King Mongkut's University of Technology North Bangkok.

References

[1]Yazdani SS, Gonzalez R. Anaerobic fermentation of glycerol: a path to economic viability for the biofuels industry. *CurrOpinBiotechnol* 2007;18:213–219.

[2] Cheng KK, Liu DH, Sun Y, Liu WB. 1,3-propanediol production by Klebsiellapneumoniae under different aeration strategies. *BiotechnolLett* 2004; 26:911–915.

[3]Hekmat D, Bauer R, Fricke J. Optimization of the microbial synthesis of dihydroxyacetone from glycerol with Gluconobacteroxydans. *BioprocBiosystEng* 2003; 26:109–116.

[4] Lee PC, Lee WG, Lee SY, Chang HN. Succinic acid production with reduced by-product formation in the fermentation of Anaerobiospirillumsucciniciproducens usingglycerol as a carbon source. *BiotechnoBioeng* 2001;72:41–48.

[5]Imandi SB, Bandaru VVR, Somalanka SR, Garapati HR. Optimization of mediumconstituents for the production of citric acid from byproduct glycerol using Doehlert experimental design. *Enzyme MicrobTechnol*2007; 40:1367–1372.

[6]Vikromvarasiri N, Pisutpaisal N. Fermentative Conversion of Glycerol to Ethanol using Mixed Consortium. *Res. J. Biotech* 2011;6:5-12.

[7] Ito T, Nakashimada Y, Senba K, Matsui T, Nishio N. Hydrogen and ethanol production from glycerol-containing wastes discharged after biodiesel manufacturing process. *J BiosciBioeng* 2005;100:260–265.

[8]Dharmadi Y, Murarka A, Gonzalez R. Anaerobic fermentation of glycerol by Escherichia coli: a new platform for metabolic engineering. *BiotechnolBioeng* 2006; 94: 821–829.

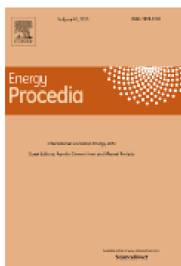
[9] Zhu H, Qu F, Zhu LH. Isolation of genomic DNAs from plants, fungi and bacteria using benzyl chloride. *Nucleic Acids Res*1993;21: 5278–5280.

[10]Kanokratana P, Chanapan S, Pootanakit K, Eurwilaichitr L. Diversity and abundance of bacteria and archaea in the BorKhlueng Hot Spring in Thailand. *J Basic Microbiol*2004;44:430-444.

[11] Cole JR, Chai B, Marsh TL. The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. *Nucleic Acids Res* 2003; 31:442–443.

[12]Murarka A, Dharmadi Y, Yazdani SS, Gonzalez R. Fermentative utilization of glycerol in Escherichia coli and its implications for the production of fuels and chemicals. *App Environ Microbiol* 2008;74:1124–1135.

[13] Jarvis GN, Moore ERB, Thiele JH.Formate and ethanol are the major products of glycerol fermentation produced by a Klebsiellaplanticola strain isolated from red deer. *J ApplMicrobiol*1997;83:166–174.



Biography

Asst. Prof. Nipon Pisutpaisal is a lecturer of Department of Agro-Industrial, Food and Environmental Technology, Faculty of Applied Science, King Mongkut's University of Technology North Bangkok. He is also an affiliate staff at the Joint Graduate School of Environment and Energy (JGSEE), King Mongkut's University of Technology Thonburi. He obtained his Ph.D. degree (Environmental Engineering) from The Pennsylvania State University, USA in 2003. Asst. Prof. Nipon Pisutpaisal is specialized in Biological Wastewater Treatment, Anaerobic Digestion, Bio-hydrogen Energy, and Fermentative Bioenergy Technology.