Renewable Energy 114 (2017) 1272–1280

Contents lists available at ScienceDirect

Renewable Energy

journal homepage: www.elsevier.com/locate/renene

Utilization of by-product glycerol from bio-diesel plants as feedstock for 2,3-butanediol accumulation and biosynthesis genes response of *Klebsiella variicola* SW3

Md. Shafiqur Rahman [a, b], Chunbao (Charles) Xu [c], Kesen Ma [d], Haipeng Guo [e], Wensheng Qin [a, *]

[a] Department of Biology, Lakehead University, 955 Oliver Road, Thunder Bay, Ontario, P7B 5E1, Canada
[b] Department of Microbiology, University of Chittagong, Bangladesh
[c] Institute for Chemicals and Fuels from Alternative Resources (ICFAR), Western University, London, Ontario, N6A 5B9, Canada
[d] Department of Biology, University of Waterloo, 200 University Avenue West, Waterloo, Ontario, N2L 3G1, Canada
[e] College of Life Sciences, Zhejiang University, China

ARTICLE INFO

Article history:
Received 27 January 2017
Received in revised form
16 June 2017
Accepted 17 July 2017
Available online 18 July 2017

Keywords:
Crude glycerol
2,3-Butanediol
Biodiesel
Liquide fuel
Fuel additive
Klebsiella variicola

ABSTRACT

The booming of biodiesel industry all over the world has led to generate a large amount (10% v/v) of crude glycerol, created an oversupply problem. Herein, we compared the product concentrations of major metabolic products attained from pure and crude glycerol biotransformation process using an adapted mutant strain *Klebsiella variicola* SW3. Real-time qPCR and glycerol dehydrogenase (GDH) enzyme activity assay revealed that the overexpression of GDH gene resulted in an increased GDH enzyme activity, led to a markedly boosted 2,3-butanediol (2,3-BD) production. Based on these results, the SW3 strain obtained from wild type strain *Klebsiella variicola* SRP3 displayed a 1.39-fold increased 2,3-BD production of 82.5 g/L from 59.3 g/L, yielding 0.62 g/g using pure glycerol. However, in a batch culture, a final 33.5 g/L of 2,3-BD was accumulated within 96 h from 50 g/L glycerol. Moreover, the strain SW3 withstanding high concentration (200 g/L) of crude glycerol displayed 64.9 and 29.25 g/L 2,3-BD in fed-batch and batch cultures respectively. Therefore, this bioconversion of crude glycerol to 2,3-BD —a value-added green product with potential industrial applications as a liquid fuel or fuel additive would represent a remarkable alternative to add value to the biodiesel production helping biodiesel industries development.

1. Introduction

In recent years, the demand of biofuels has increased dramatically. Biodiesel, a renewable vibrant biofuel, is produced from vegetable oils and animal fats by transesterification. A large amount (10% v/v) of crude glycerol is generated as a core by-product during the synthesis of biodiesel. Therefore, crude glycerol produced from biorefineries is a worldwide overflow problem due to a nonexistence of purifying facility. The increasing market for biodiesel has conspicuously altered the cost and obtainability of glycerol generated from biodiesel manufacturing plants. Consequently, a high volume of this biodiesel derived crude glycerol is a crucial problem for development and economic feasibility of biodiesel industry [1]. Moreover, with rising biodiesel plants, a considerable number of glycerol production industries will be shut down within few years due to price drop by overflow of glycerol [2,3] that require new profitable products from glycerol conversion. Besides this, the purification or disposal of non-biodegradable biodiesel by-product is intricate, costly, and it is becoming a noteworthy cost factor for the biodiesel industries [4]. Consequently, due to its low price, biodiesel derived crude glycerol is considered as waste and it is, therefore, important that new and sustainable applications should be explored for glycerol utilization. Therefore, biotechnological approach, a biological process of glycerol conversion could help evade the disadvantages of chemical conversion process, whereas offering the opportunity to produce numerous value-added products [5,6]. Glycerol, a simple three carbon sugar alcohol would be a potential carbon

* Corresponding author.
E-mail address: wqin@lakeheadu.ca (W. Qin).

http://dx.doi.org/10.1016/j.renene.2017.07.080
0960-1481/

and energy source for microbial growth to synthesize a number of valuable biotechnological products [6]. A large number of microorganisms is capable of utilizing glycerol as a sole carbon source in the synthesis of a lot of value-added products including 1,3-PDO (1,3-propanediol), ethanol, hydrogen, 2,3-BD, and organic acids [6–8]. There is no metabolically efficient microorganism which can convert glycerol effectually to produce high value products. The major microbial conversion route of glycerol leads to produce of 1,3-PDO through an anaerobic fermentation process [4,5,9]. Biodiesel derived crude glycerol contains methanol, salts, catalyst and organic acids as the main impurities which are negatively influenced on bioconversion process [10]. If the crude glycerol is used as a renewable waste substrate to produce value-added products through bioconversion process would offer a substantial option for the inventive and profitable waste management of biodiesel plants. Bioconversion of glycerol to 2,3-BD is a promising and alternative to chemical synthesis. Our main objective is high production of 2,3-BD from glycerol as well as crude glycerol by developing an adapted mutant strain(s) with increased tolerance toward crude glycerol under completely aerobic process.

2,3-BD, a glycol, is an expensive and important bulk chemical which is used in pharmaceutical, plastic, antifreeze solution and solvent preparation. Furthermore, as a platform chemical, 2,3-BD is widely used as reagent for the synthesis of a number of chemicals, and it could be converted to liquid fuel or fuel additive [13,14]. 2,3-BD is also a reduced from of acetoin. A metabolic product acetoin is an important platform chemical with many applications in cosmetics, food, and synthesis of many chemicals [15]. 2,3-BD and acetoin can be transferred to each other in cells [15].

The metabolic reactions proposed for glycerol assimilation in *K. pneumoniae* basically follow oxidative and reductive pathways (Fig. 1), which results in the synthesis of major products including dihydroxyacetone (DHA), 2,3-BD, 1,3-PDO, acetoin, ethanol, acetate and other organic acids. In the oxidative branch, an important cell-bounded (intracellular) and NAD^+-dependent enzyme GDH converts glycerol to DHA, producing $NADH_2$ as a reducing equivalent [11,12]. Subsequently, dihydroxyacetone phosphate is generated from DHA by adenosine triphosphate-depended dihydroxyacetone kinase, which is then further undergoes glycolysis to form various products through pyruvate [12]. Meanwhile, $NADH_2$ produced after receiving electrons from oxidation of substrate organic compounds could stimulates 1,3-PDO production in aerobic process [12]. Moreover, glycerol is firstly converted to 3-hydroxypropionaldehyde by the coenzyme B_{12}-dependent glycerol dehydratase through the parallel reductive pathway, and it is then converted by $NADH_2$-linked 1,3-PDO dehydrogenase to the major product 1,3-PDO [12].

Several species of *Klebsiella* are known to produce 1,3-PDO as a core metabolic product through glycerol bioconversion process [4,5,9,10]. Our intention of this research is to study the development of a new strain of bacteria capable of competently accumulation and convert by-product glycerol from biodiesel plant into value-added products under completely aerobic condition. Moreover, the aim of this study is to pinpoint transcriptional profiles of key enzyme genes related to the biosynthesis pathways of 2,3-BD. Instead, through investigation of metabolic activity, we developed an adapted mutant strain (*K. variicola* SW3) that is withstanding a high concentration of biodiesel waste (residual glycerol). In the current study, we have, therefore, chosen a newly developed strain of *K. variicola* SRP3 with increased tolerance towards crude glycerol by evolutionary adaptation (mutagenesis). Here, we report a high product concentration of 2,3-BD from glycerol as a sole carbon source by an adapted mutant strain SW3 obtained from our newly isolated strain *K. variicola* SRP3.

2. Materials and methods

2.1. Biodiesel by-product glycerol/crude glycerol

Crude (raw) glycerol obtained from biodiesel synthesis was supplied by CARES (Centre for Agricultural Renewable Energy and Sustainability) biodiesel plant (Guelph, Canada), which uses vegetable oil as the raw material. In this small plant, about 600,000 L/year biodiesel production is obtained by the transesterification of vegetable oil using a catalyst methanol. This crude glycerol was

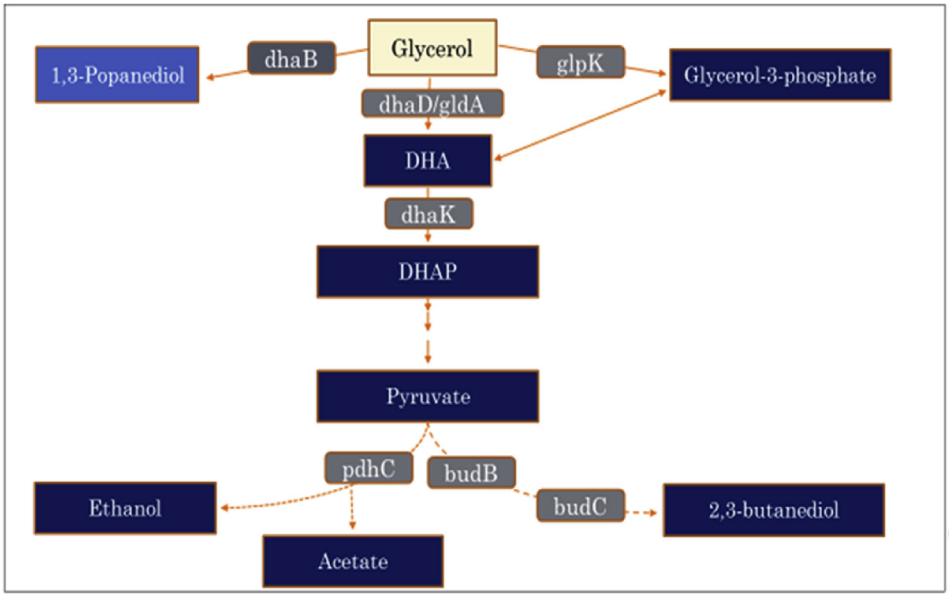

Fig. 1. Schematic representation of initial steps in glycerol metabolism pathway of *K. pneumoniae*. The key enzymes/representative genes related to glycerol metabolism are glycerol dehydratase (dhaB), glycerol dehydrogenase (dhaD and gldA), dihydroxyacetone kinase (dhaK), glycerol kinase (glpK), acetolactate synthatase (budB), acetoin reductase (budC) and pyruvate dehydrogenase (pdhC).

directly used as a feedstock for microbial growth. The composition of the crude glycerol was determined as mass fractions (glycerol 50.0%, ash 4.2%, MoNG 36.2%, water 6.7%, and pH 10.6).

2.2. Bacterial strains and culture conditions

K. variicola SW3 (GenBank Accession No. KY437692), an adapted mutant strain was used throughout the study. *K. variicola* SRP3 (GenBank Accession No. KR092086), a wild type strain isolated from paper mill (Resolute Forest Products, Canada) waste, reported in our earlier paper [13] was used to develop the adapted mutant SW3. Seed culture for batch and fed-batch process was prepared from stock slant culture by inoculating into a Luria-Bertani (LB) broth medium contained (g/L): peptone 10.0, yeast extract 5.0, NaCl 5.0. The seed LB broth culture was incubated at 37 °C and 200 rpm under aerobic conditions for 20 h. For batch and fed-batch cultures, the seed culture was inoculated into MS-2 medium contained K_2HPO_4 (0.1 g/L), $NaNO_3$ (0.1 g/L), $MgSO_4{\cdot}7H_2O$ (0.05 g/L), KCl (0.1 g/L), yeast extract (2.5 g/L) and peptone (5.0 g/L). Different concentrations of pure or crude glycerol were used in the MS-2 medium as a carbon source. All the growth parameters were performed in triplicates. The batch and fed-batch cultures were carried out in 125 ml Erlenmeyer flasks (50 ml medium, 100 μl of 20 h culture) at 37 °C and 200 rpm. In case of fed-batch culture, the glycerol concentrations were varied. In our fed-batch culture, an initial concentration of glycerol was 50 g/L, and then a concentrated solution containing 800 g/L of pure glycerol was fed into the culture as required. However, in fed-batch culture, the initial crude glycerol was 100 g/L, and this crude glycerol was directly fed into the culture as required.

2.3. Strain development and identification

The wild type strain *K. variicola* SRP3 was mutagenized by evolutionary adaptation technique using pure glycerol which was described in our earlier work [13]. One of the utmost 2,3-BD producing strains achieved from this evolutionary adaptation process was further gradually developed (adapted) with increased tolerance toward crude glycerol using the same protocol [13]. This evolutionary developed adapted mutant strain nominated as *K. variicola* SW3 could grow at a concentration of 200 g/L crude glycerol. The partial sequence of 16S ribosomal RNA gene of this adapted mutant SW3 was input into NCBI BLAST tool, compared with the sequences in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/). The phylogenetic relationship was analyzed using sequence alignment program of the Neighbor-Joining method with MEGA6. The partial sequence of 16S rDNA of the strain SW3 was submitted to the NCBI GenBank for accession number.

2.4. RNA isolation and quantitative real-time PCR

For isolation of RNA, seed cultures of SRP3 and its adapted mutant SW3 were prepared from stock slant culture by inoculating into a LB broth medium, incubated at 37 °C and 200 rpm for 20 h. Following incubation, 1% (v/v) of the LB broth seed culture was inoculated into 125 ml flash containing 50 ml MS-2 medium supplemented with different concentrations (0 g/L, 25 g/L, 50 g/L and 75 g/L) of glycerol, incubated at 37 °C and 200 rpm into a shaker incubator for 72 h. Subsequent incubation, the cells collected after centrifugation at 5000*g* and 4 °C for 5 min was used for RNA isolation. RNA was isolated according the protocol of PureLink^{Tm} RNA extraction kit (Ambion, Thermo Fisher Scientific, USA). The concentration of each RNA sample was determined using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). The first-stand cDNA was synthesized using cDNA synthesis kit (Tetro cDNA synthesis Kit, Bioline, UK) and RNA as a template. Quantitative gene expression was carried out using SensiFAST™ SYBR No-ROX Kit (Bioline, UK) on C1000™ thermal cycler quantitative real-time PCR (qRT-PCR) detector system (BioRad, USA). The 16S rRNA, obtained based on the primers 5′-GCGGTTGTTACAGTCAGATG-3′ and 5′-GCCTCAGCGTCAGTATCG-3′ was used as an internal standard. The primer sequences of glycerol metabolism genes used in this study are presented in Table 1. The $2^{-\Delta\Delta CT}$ method was used to analyze the fold change gene expression over control [16].

2.5. Enzyme assay and toxicity test

GDH enzyme activity was determined at room temperature using microplate spectrophotometer (EPOCH, BioTek) by measuring the initial reduction rate of NAD at 340 nm as described in our earlier report [13]. Protein was quantified by the standard protocol of Bradford [17], using bovine serum albumin as a standard protein.

For toxicity test, the minimum inhibition concentration (MIC) of glycerol were performed in batch culture. A series of MS-2 culture medium tubes were prepared with different concentrations of glycerol ranging from 0 to 350 g/L, inoculated with 50 μL of a 20 h culture, and incubated at 37 °C for 120 h. Following incubation, growth was evaluated spectrophotometrically based on the absorbance at 600 nm, and the dry cell mass was measured by using a standard curve (a linear correlation between the dry cell mass and absorbance of cell suspension). All tests were conducted in at least triplicates.

2.6. Quantification of biomass, glycerol and metabolic products

Biomass or cell dry weight (g/L) was calculated from absorbance at 600 nm using the calibration curve. Optical density at 600 nm (OD_{600}) obtained from microplate spectrophotometer (EPOCH, BioTek) was converted to cell dry weight using a calibration equation ($y = 1.9175x + 0.0183$, where $y = OD_{600}$, x = biomass g/L). This calibration equation was obtained from the standard curve of a linear correlation between the dry cell mass and absorbance (OD_{600}) of cell suspension. Metabolic products were identified by GC-MS (Varian 1200 Quadrupole). The concentrations of major metabolic products 2,3-BD, 1,3-PDO, acetoin and acetate, as well as glycerol were quantified using a GC-FID (Shimatzu GC 14A) under the following conditions: sample volume 1 μl; column temperature range from 45 °C (2 min) to 240 °C at the rate of 10 °C/min; the injector and detector temperature 250 °C; carrier gas nitrogen;

Table 1
List of primers designed for gene expression studies by qRT-PCR.

Genes	Enzymes	Primer sequences
dhaD	Glycerol dehydrogenase	F: AGCGGGCGAGTTTGAAGAGTATCTG
		R: GCCAGCAGCGTATCATAGCACAGG
gldA	Glycerol dehydrogenase	F: CGAACACTCACCGCCAAACGG
		R: TCGCATTATTCAATCACCAGGCAAAT
dhaB	Glycerol dehytratase	F: ACCAGTGCCACGTCACCAATCTC
		R: CGGACTGCGAACCGACCAACA
dhaK	Dihydrohyacetone kinase	F: GCTGAGATGGCGATTCTGACCC
		R: GGTGGATGGCACGACGTTGATAG
glpK	Glycerol kinase	F: CGGGTCCAGACCGCTCAAAT
		R: GGGCTGGGCGAACAGGTAAA
budB	Acetolacteate synthatase	F: ACCAGTGCCACGTCACCAATCTC
		R: TGGTGGACCTGTTTGGCTTTATCG
budC	Acetoin reductase	F: TGTTTCTGACCGCGACCAGGTATT
		R: CCCGCCGTGACCCTCTTTCTTA
pdhC	Puruvate dehydrogenase	F: TTCTCCTGTTGCTGGGACGAT
		R: GTGCTACTCCGCCTTGCTTTG

F: Forward; R: Reverse.

column DB-WAXetr. The injecting sample was purified by centrifugation (Fisher Scientific, Germany, accu Spin Micro 17,) and membrane filter (0.22 μm pores size) respectively.

3. Results

3.1. Strain development, phylogenetic relationship and toxicity

An **a**dapted mutant strain *K. variicola* SW3 with increased tolerance toward pure and crude glycerol developed from wild type strain *K. variicola* SRP3 able to grow at 20% (200 g/L) of crude glycerol or pure glycerol. The partial sequence of 16S rDNA of the strain SW3 inputted into NCBI Basic Local Alignment Search Tool (BLAST) showed 99% sequence similarity with its wild type strain SRP3. After submitted the partial sequence (16S rDNA) of SW3 in GeneBank, the accession number is released as KY437692.

Nevertheless, the 16S rDNA sequence of SRP3 and SW3, and other *Klebsiella* strains retrieved from GeneBank are presented as a phylogenetic tree (Fig. 2) for their phylogenetic relationship analysis. The phylogenetic tree was constructed using the Neighbor-Joining method. The evolutionary history was inferred using the Neighbor-Joining method through NCBI website. The result of phylogenetic relationship confirmed mutagenesis through the distance between the *K. variicola* SRP3 and its adapted mutant *K. variicola* SW3.

To assess pure and crude glycerol utilization competence, toxicity test was performed. *K. variicola* SW3, an evolutionary adapted mutant was used for a toxicity test towards different concentrations of pure and crude glycerol (Table 2). Growth of SW3 was detectable after 120 h incubation at the initial concentrations of 300 g/L glycerol. Based on this result, the strain SW3 appeared most tolerant to pure and crude glycerol. MIC of glycerol was determined as 325 g/L (Table 2).

Table 2
Minimum inhibitory concentration (MIC) of glycerol after 120 h incubation in MS-2 medium supplemented with glycerol.

Glycerol (g/L)	Biomass[a] (g/L)	Growth in broth medium	MIC (g/L)
0	0.485 ± 0.021	+	325
50	0.619 ± 0.043	+	
100	0.513 ± 0.032	+	
150	0.277 ± 0.028	+	
200	0.129 ± 0.017	+	
250	0.044 ± 0.006	+	
300	0.022 ± 0.004	+	
325	0	−	
350	0	−	

[a] Dry weight of bacterial cell biomass.

3.2. Quantitative real-time PCR analysis and enzyme activity

To demonstrate the expression of the genes responsible for 2,3-DB accumulation in SRP3 and SW3 strains, the experiment was carried out with qRT-PCR. With the aim of target, the genes related to 2,3-BD accumulation and learn about the transcriptional changes in response to different concentrations of glycerol in a batch culture, a preliminary effort has been made to search differentially expressed genes. Therefore, the expression levels of genes accountable for important enzymes in 2,3-BD, acetoin, 1,3PDO and acetate metabolic pathway were studied using real-time qPCR. For gene expression study, all the bacterial cells were cultured for a constant period to minimize its effect on the analysis of RT-PCR. The quantitative relationship among the expression levels of these genes with the strains SRP3 and SW3 at different concentrations of glycerol are presented in Fig. 3. In this study, we presented a comparison of expression levels of a key gene (dhaD) and its enzyme (GDH) activity between the two strains SRP3 and SW3 at different concentrations of glycerol (Fig. 4a). However, for the productions of 2,3-BD and biomass, the comparison between SRP3 and SW3 strains is shown in Fig. 4b. As shown in Fig. 3, the results indicated that 50 g/L (5.0%) glycerol could stimulate relative expression levels of the genes dhaD, gldA, glpK, budB and budC in the 2,3-BD metabolic pathway, and the mutant strain SW3 displayed higher expression levels compared to that of its wild type strain SRP3.

3.3. Biotransformation of glycerol in batch and fed-batch culture

To evaluate glycerol assimilation and 2,3-BD production using *K. variicola* SRP3 and *K. variicola* SW3, aerobic bioconversion was performed with pure and crude glycerol as the carbon sources. Biotransformation of glycerol to 2,3-BD and other metabolic products was carried out in shake flasks at initial pH 7.0. Batch and fed-batch process were carried out under optimized conditions for 2,3-BD production. Experiments were set at 37 °C in a shaker incubator. MS-2 medium supplemented with specified concentrations of pure or crude glycerol were used for both batch and fed-batch cultures. This biotransformation process revealed two major metabolic products 2,3-BD and acetoin with other minor products viz., acetate and 1,3-PDO. The concentrations of glycerol (substrate) as well as end (metabolic) products as a function of incubation time in a batch culture process of pure glycerol biotransformation by the SRP3 and its adapted mutant SW3 are presented in the Fig. 5a and b. The wild type *K. variicola* SRP3 reported this article, used 100% of glycerol within 120 h, producing 22.72 g/L 2,3-BD, 2.11 g/L 1,3-PDO, 6.44 g/L acetoin and 0.82 g/L acetate in batch culture under aerobic condition. The maximum

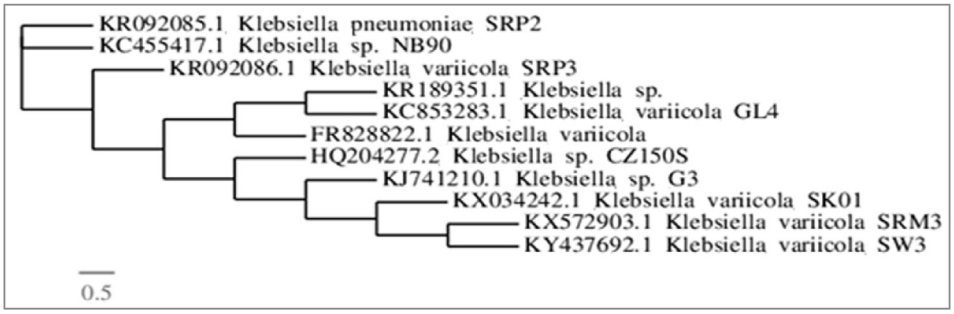

Fig. 2. Evolutionary relationships of the strains: Phylogenetic tree drawn from sequence alignment program using Neighbor-Joining method with MEGA6, presented in TreeView. 16S rRNA gene sequences were retrieved by nucleotide BLAST searches in NCBI. The numbers that follow the names of the strains are accession numbers of published sequences. The scale bar represents 0.5 substitutions per nucleotide position.

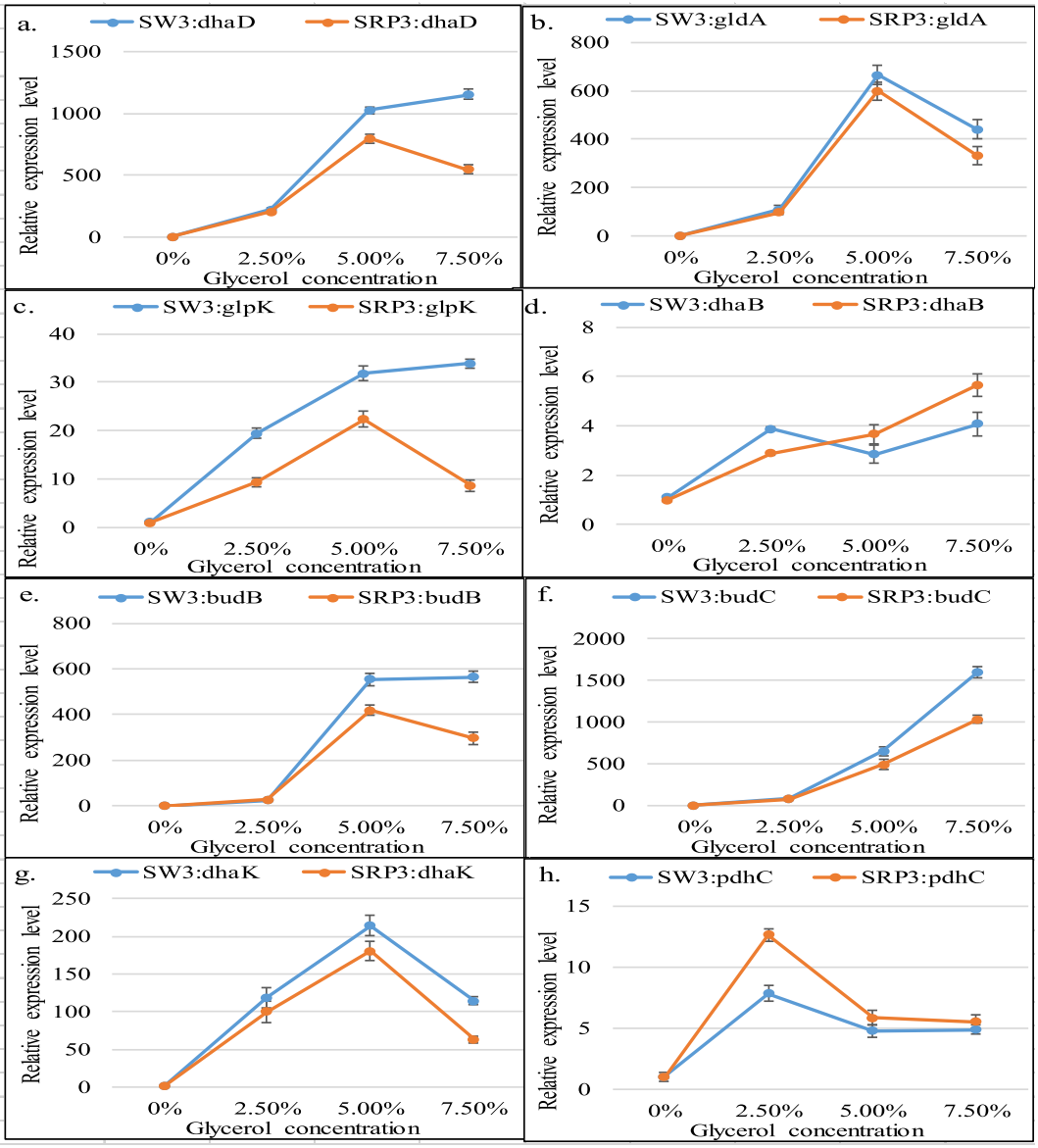

Fig. 3. Expression level of glycerol dehydratase (dhaB), glycerol dehydrogenase (dhaD and gldA), dihydroxyacetone kinase (dhaK), glycerol kinase (glpK), acetolactate synthatase (budB), acetoin reductase (budC) and pyruvate dehydrogenase (pdhC) genes in glycerol metabolism.

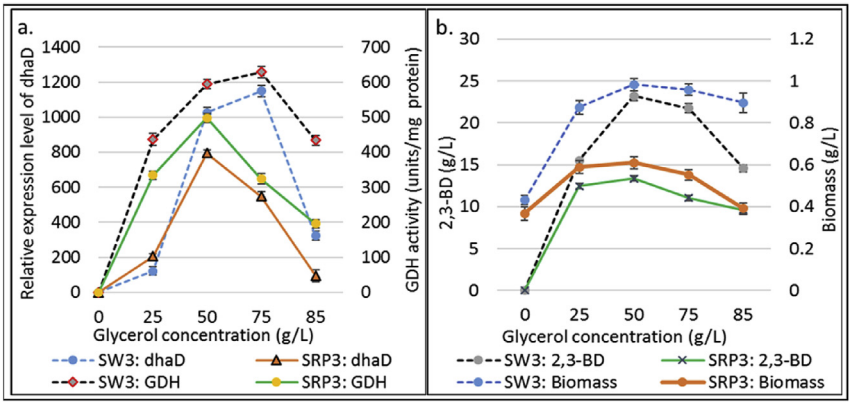

Fig. 4. Comparison between SRP3 and SW3 strains: (a) GDH activity and relative expression level of dhaD; (b) 2,3-BD and biomass productions.

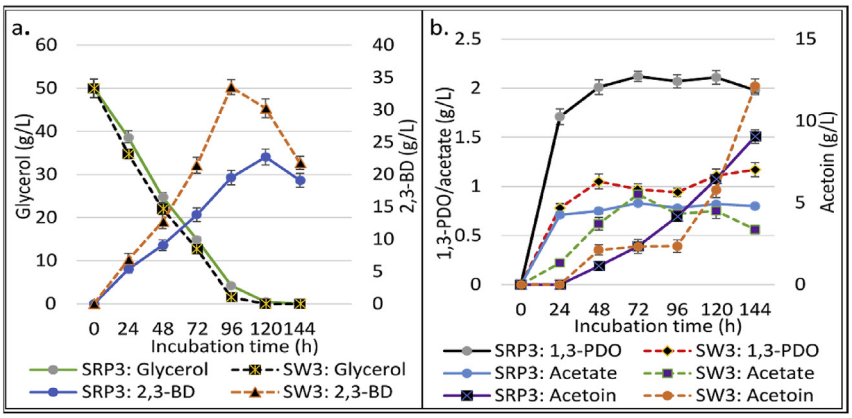

Fig. 5. Comparison of time course data and metabolic products of *K. variicola* SRP3 and *K. variicola* SW3 in batch culture using pure glycerol: (a) glycerol, and 2,3-B concentrations; (b) concentrations of 1,3-PDO, acetate and acetoin. MS-2 medium with glycerol 50.0 g/L; incubation temperature 37 °C, and initial pH 7.0.

concentration and yield of 2,3-BD obtained after 120 h incubation were 22.72 g/L and 0.45 g/g respectively (Fig. 5a). However, a maximum 33.52 g/L of 2,3-BD was achieved from 50.0 g/L glycerol after 96 h incubation by SW3, and the product yield was 0.65 g/g (Fig. 5a). Conspicuously, 100% of glycerol consumed after 96 h incubation by the strain SW3, and the concentrations of foremost end products other than 2,3-BD obtained from our batch culture were acetoin, acetate and 1,3-PDO, with concentrations, 2.35 g/L, 0.72 g/L and 0.94 g/L respectively (Fig. 5b). Notably, as shown in Fig. 5a, the adapted mutant strain *K. variicola* SW3 exhibited less concentrations of 1,3-PDO and acetate compared to that of *K. variicola* SRP3 in batch culture. The SW3 strain attained from SRP3 using adapted mutagenesis was capable to grow in a high concentration of pure glycerol or crude glycerol, up to 200 g/L. Consequently, this adapted strain *K. variicola* SW3 was further investigated as a possible effectual strain for high production of 2,3-BD using crude glycerol as a carbon source. Moreover, this SW3 strain was better adapted for utilizing crude glycerol, noticeably convert it to 2,3-BD compared to its wild type strain SRP3.

To explore the feasibility of crude glycerol utilization and a high concentration of 2,3-BD production in a batch culture by *K. variicola* SW3, the experiment was performed in shake flask (200 rpm) using MS-2 medium containing 100 g/L biodiesel-derived crude (raw) glycerol as a carbon source. To investigate whether the impurities present in biodiesel derived crude glycerol would inhibit 2,3-BD production as well as cell growth of *K. variicola* SW3, batch culture process using crude glycerol was performed, and the results are presented in Fig. 6. When biotransformation was performed for 144 h using crude glycerol, *K. variicola* SW3 effectively produced 2,3-BD at the concentration up to 29.25 g/L with the yield of 0.59 g/g (Fig. 6a). In case of *K. variicola* SRP3, the product concentration and product yield of 2,3-BD were obtained up to 26.51 g/L and 0.53 g/g respectively (Fig. 6a). Furthermore, the maximum biomass (cell dry weight) of *K. variicola* SW3 was also higher than that of *K. variicola* SRP3 (1.51 g L^{-1} vs. 1.13 g L^{-1}). The foremost metabolic products other than 2,3-BD were acetoin and 1,3-PDO with little acetic acid (Fig. 6b).

In fed-batch process, the biotransformation kinetics displayed that SW3 produced high concentration of the principal product 2,3-BD in aerobic condition. Table 3 compares the production of principal product 2,3-BD as well as other co-products in fed-batch process by *K. variicola* SRP3 and *K. variicola* SW3 using pure and crude glycerol. As shown in Table 3, a high concentration (82.5 g/L) of 2,3-BD was attained from fed-batch culture using pure glycerol, utilized 134.0 g/L glycerol by the adapted mutant strain *K. variicola* SW3. However, for the crude glycerol used in fed-batch culture, the product concentration of 2,3-BD was achieved up to 64.93 g/L by the same strain SW3, consumed 102.33 g/L glycerol. The product yields of the principal metabolic product 2,3-BD were obtained up to 0.62 g/g and 0.63 g/g using pure and crude glycerol respectively (Table 3 and 4).

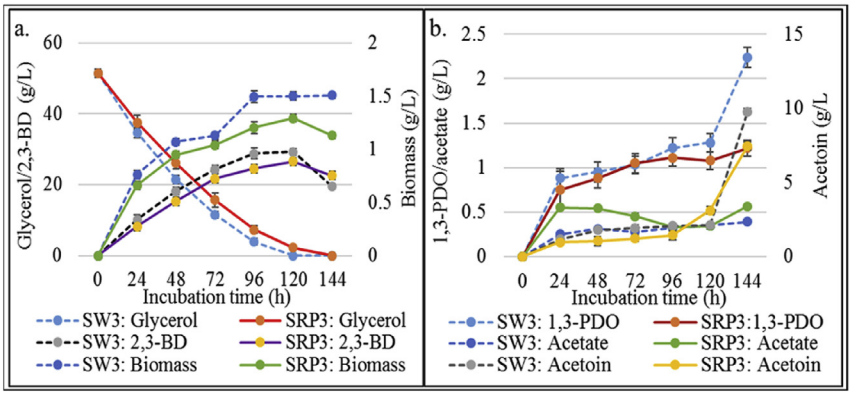

Fig. 6. Comparison of time course data and metabolic products of *K. variicola* SRP3 and *K. variicola* SW3 in batch culture using crude glycerol: (a) glycerol, 2,3-B and biomass concentrations; (b) concentrations of 1,3-PDO, acetate and acetoin. MS-2 medium with raw (crude) glycerol 100.0 g/L; incubation temperature 37 °C, and initial pH 7.0.

Table 3
Comparison of fed-batch bio-transformations by *K. variicola* SRP3 and *K. variicola* SW3 using pure glycerol and biodiesel derived raw (crude) glycerol.

Culture parameters/products	*K. Variicola* SRP3		*K. Variicola* SW3	
	Pure glycerol	Crude glycerol	Pure glycerol	Crude glycerol
Final pH	5.25	6.48	5.42	6.23
Dry weight of cell (g/L)	2.08	1.87	2.53	2.37
Glycerol utilized (g/L)	101.2	76.65	134.0	102.33
2,3-BD (g/L)	59.3	43.79	82.5	64.93
2,3-BD yield (g/g)	0.59	0.57	0.62	0.63
Acetoin (g/L)	8.1	5.71	5.9	6.32
Acetoin yield (g/g)	0.08	0.07	0.07	0.06
1,3-PDO (g/L)	3.12	4.83	2.4	2.85
1,3-PDO yield (g/g)	0.03	0.06	0.03	0.03
Incubation time (h)	192	168	216	168

All experimental values presented are mean values from triplicate experiments.

Table 4
Comparison of 2,3-BDO production using crude and pure glycerol as the sole carbon source.

Name of bacterial strains	Culture	Carbon source	Product (g/L)	Yield (g/g)	References
Klebsiella pneumoniae G31	Fed-batch	Pure glycerol	70.00	0.39	[30]
Bacillus amyloliquefaciens	Fed-batch	Crude glycerol	43.1	0.45	[24]
K. variicola SRP3	Batch	Pure glycerol	29.9	0.60	[13]
K. oxytoca	Batch	Crude glycerol	4.8	0.14	[18]
Klebsiella sp. Ana-WS5	Fed-batch	Pure glycerol	30.1	–	[33]
K. variicola SW3	Fed-batch	Pure glycerol	82.5	0.62	This study
K. variicola SW3	Batch	Pure glycerol	33.5	0.67	This study
K. variicola SW3	Fed-batch	Crude glycerol	64.9	0.63	This study
K. variicola SW3	Batch	Crude glycerol	29.3	0.57	This study

4. Discussion

With the flourishing of biodiesel manufacturing plants, global oversupply of biodiesel derived crude glycerol is increasing dramatically [1,3]. A large amount (10% v/v) of crude glycerol generated from biodiesel production process is the main economical as well as environmental concern for development of biodiesel industries [3]. Therefore, given the difficulties and economic cost of recovering glycerol biomass produced from biodiesel production process, an appreciated and alternative approach to utilizing the glycerol as a renewable resource would be the bioconversion process using microorganisms. Consequently, biotransformation of glycerol to renewable energy like 2,3-BD, an expensive product used as a liquid fuel or fuel additive would lead to both environmental and economic dividends of biodiesel plant. However, in the recent years, bioconversion of glycerol to value-added metabolic products is an interesting topic for many researchers [18–21]. Unfortunately, until now, there is no available effective strain nominated for utilizing of glycerol to produce 2,3-BD successfully. In the last few years, numerous attempts have been made on bioconversion of glycerol to biofuels and other various products including ethanol, 1,3-PDO, 1,2-PDO, and H_2 under microaerophilic or anaerobic culture process [22,23]. However, very little studies have been done on aerobic bioconversion of glycerol to biofuels and other value-added metabolic products until now [24–26].

Considering aerobic biotransformation of glycerol to value-added metabolic products, *K. variicola* SW3, an efficient adapted mutant strain developed from a newly isolated strain *K. variicola* SRP3 was used for high production of 2,3-BD using pure and crude glycerol. Only two species of *Klebsiella* (*K. pneumoniae* and *K oxytoca*) has been reported to utilize glycerol with producing 2,3-BD and 1,3-PDO in aerobic and anaerobic processes respectively [18,27,28]. There is no report except our earlier report [13] on the bioconversion of glycerol to value-added product by *K. variicola*. In our study, however, we have confirmed that the adapted mutant strain *K. variicola* SW3 developed from our newly isolated strain *K. variicola* SRP3 could aerobically convert glycerol to substantial amount of biotechnologically important product 2,3-BD along with other minor co-products through a GDH-dependent oxidative pathway. Recently Yang et al. [23] and Cho et al. [29] reported high concentrations of 2,3-BD 83.3 g/L by *Bacillus amyloliquefaciens* B10-127 and 131.5 g/L by *K. oxytoca* M1 respectively using molasses or casamino acid as a co-substrate (carbon source) in addition to glycerol. Nevertheless, when glycerol was used as the sole carbon source, *B. amyloliquefaciens* B10-127 produced only 43.1 g/L of 2,3-BD [23]. Furthermore, Cho et al. [29] stated the highest yield 0.44 g/g of 2,3-BD by metabolically engineered *K. oxytoca* M3 strain using a co-substrate casamino acid in addition to crude glycerol where fed-batch fermentation parameters were controlled by bioreactor. However, we reported 82.2 g/L 2,3-BD using a different organism *K. variicola* SW3 in a flask culture without control of fermentation parameters including pH where glycerol was only the substrate (carbon source), and the product yield was as high as 0.63 g/g. Furthermore, in batch culture, considering that the 2,3-BD product yield obtained from crude glycerol fermentation by *K. variicola* SW3 (29.25 g/L and 0.58 g/g glycerol) was much higher than that reported (8.9 g/L and 0.12 g/g glycerol) by Cho et al. [29] using crude glycerol. Rebecchi et al. [30] demonstrated 2,3-BD production using *Bacillus licheniformis* by supplying glucose and sucrose respectively as the carbon source instead of glycerol, and the highest product yield was as low as 8.7 g/L. Moreover, considering our earlier report Rahman et al. [13] that the 2,3-BD product yield 29.87 g/L was obtained from pure glycerol biotransformation in batch culture by *K. variicola* SRM3, and crude glycerol was not reported. Keeping in mind under the above discussion, our report on 2,3-BD product yield from biodiesel-derived crude glycerol is much higher than those obtained using glycerol or crude glycerol as the sole (only) carbon source. Lately, Petrov and Petrova [31] reported the highest concentration (70.0 g/L) of 2,3-BD with a yield of 0.39 g/g in a fed-batch culture using pure glycerol as a carbon source by *K. pneumoniae* G31. Table 4 compares the product concentrations

of 2,3-BD reported earlier and this study in batch and fed-batch cultures using glycerol as the only carbon source.

Interestingly, our adapted mutant strain *K. variicola* SW3 able to grow on and tolerated a high glycerol concentration of pure or crude glycerol up until 200.0 g/L. Moreover, in the meantime, keeping the conditions defined in this study, concentration (82.5 g/L) and yield (0.62 g/g) of 2,3-BD were higher than that of previously published reports by other researchers using glycerol as the only substrate. Likewise, 64.93 g/L with a yield of 0.63 g/g, a high production of 2,3-BD was also a significant amount obtained using biodiesel derived raw glycerol as the sole carbon source in our fed-batch process. Furthermore, *K. variicola* SW3, an adapted strain able to tolerate a high concentration of the glycerol up to 20% (v/v). Overall, our results confirmed that a high yield (0.63 g/g) of 2,3-BD obtained from crude glycerol biotransformation could be the new prospect for economic feasibility of biodiesel industries. Further improvement on 2,3-BD production would make it more feasible to produce 2,3-BD from biodiesel-derived crude glycerol for industrial use. Consequently, a general viewpoint on the fundamental mechanism of 2,3-BD biosynthesis response to glycerol as a feedstock, quite a few genes that could play an important role in 2,3-BD biosynthesis were designated as promising candidates for metabolic engineering (Fig. 3). It was therefore, undoubtedly observed that the relative expression level of key enzyme (GDH) gene dhaD responsible for conversion of glycerol in SW3 strain was higher compared to that of SRP3. The overexpression of GDH displayed a significant enhancement on budB and budC gene expression levels (Fig. 3). However, at a concentration of 50.0 g/L (5.0%) glycerol, both strains SW3 and SRP3 exhibited higher expressions of dhaD/gldA. Moreover, the wild type strain SRP3 displayed higher expression levels for the genes dhaB and pdhC than that of its adapted mutant SW3 at the same concentration (50.0 g/L) of glycerol. Information obtained from our enzyme genes expression study would indicate further study on metabolic engineering.

Evolutionary adaptation is a method for increasing tolerance towards an inhibitor of a microbial strain. Consequently, this increased tolerance may lead down-regulation of gene(s) responsible for the predictable product [32]. Our attainment with increased tolerance towards glycerol and increased 2,3-BD production by adapted mutagenesis confirmed the competence of evolutionary adaptation for development of *K. variicola* SW2 mutant. As shown in Fig. 6, the utilization capability of crude glycerol in batch process by *K. variicola* SW3 was almost similar as compared to that for pure glycerol. As comparison with the efficacy of SRP3 and SW3 strains, the bio-conversion rate of pure and crude glycerol to 2,3-BD with the strain SW3 was much higher than that of SRP3. However, a high product yield (0.59 g/g) of 2,3-BD obtained from Batch culture process was the highest amount using crude glycerol as a feed stock until now. We have proved, however, that an adapted mutant *K. variicola* SW3 could utilize glycerol to produce 2,3-BD through oxidative pathway in a GDH-dependent manner. Evidently, the GDH enzyme plays a vital role in oxidative pathway of glycerol metabolism and 2,3-BD formation.

According to the above exposed results, our report has for the first time proved that this *K. variicola* species can efficiently convert biodiesel derived crude glycerol, and also 1st time report on biosynthesis gene expression study of glycerol metabolisms for metabolic engineering. This gene expression study is the key point of genetic engineering for developing the microbial strains to produce high concentration of metabolic products including 2,3-BD, 1,3-PDO, acetate and ethanol. The gene expression study in this report demonstrated the gene's function in biotransformation of glycerol metabolic pathway of the bacteria. It is our achievement that the mutant strain *K. variicola* SW3 has the capability of 1.29 (1025.75 vs. 795.31), 1.32 (553.0 vs. 417.83) and 1.55 (696.8 vs. 449.55) fold increasing gene expressions of dhaD, budB and budC genes respectively at 50.0 g/L of glycerol compared to that of its wild type strain *K. variicola* SRP3 (Fig. 3). Therefore, these three genes (dhaD, budB and budC) directly involve for 2,3-BD biosynthesis. Furthermore, our mutant strain SW3 withstanding as high as 200.0 g/L of biodiesel-derived crude glycerol which is the highest concentration to date. However, to the best of our knowledge, the product yield of 0.63 g/g of 2,3-BD attained in our report is the highest point on bioconversion of biodiesel-derived crude glycerol until now.

Nowadays, biodiesel-derived crude glycerol is treated as waste instead of product, and it is also the major concern to develop biodiesel industry. Obviously, it could be the cost-effective process if we use this crude glycerol as a substrate for production of biofuel and other value-added products. Our results also representing that without using any expensive co-substrate in biotransformation process, biodiesel-derived crude glycerol could be used directly as a sole substrate to produce 2,3- BD cost-effectively in industrial bioconversion process. In this research work, crude glycerol was used as the sole carbon source in batch and fed-batch flask cultures to produce 2,3-BD efficiently in a cost-effective way where low-value or negative-value biodiesel waste was only the substrate.

5. Conclusion

Even though the bioconversion of biodiesel derived crude glycerol to 2,3-BD offers a noteworthy advantage in relation to the use of pure glycerol, most of the research works have been directed using pure glycerol. Therefore, in our present report, the metabolic product 2,3-BD achieved from biotransformation of crude glycerol compared to that of pure glycerol showing that this biodiesel by-product could be used with a great potential to produce 2,3-BD. Our result also showed that it is possible to discover novel microbial strains to produce 2,3-BD 1,3-PD using crude glycerol. Moreover, the gene expression study of glycerol metabolism was a primary step to investigate the culture parameters influencing gene regulation in the syntheses of metabolic products. It is demonstrated that the adapted mutant *K. variicola* SW3 able to tolerate a high concentration of biodiesel derived raw (crude) glycerol, have a high glycerol utilization rate, and high product yield of 2,3-BD. Therefore, further studies with our newly developed adapted strain SW3 are granted to boost the utilization rate of glycerol, and the production of 2,3-BD. Until now, the high production and product yield (0.63 g/g) of 2,3-BD reported in this study using glycerol as the sole carbon source is the highest amount obtained from biotransformation process.

Acknowledgement

This research work was supported by the grant of BioFuelNet Canada (Project No. 67) and Lakehead University, Canada. We would like to thank Professor Dr. David Law of Biology Department at Lakehead University (Canada) allowing us to use his equipment.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.renene.2017.07.080.

References

[1] F. Yang, M.A. Hanna, R. Sun, Value-added uses for crude glycerol, a byproduct of biodiesel production, Biotechnol. Biofuels 5 (2012) 13–22.
[2] M. McCoy, Glycerol surplus: plants are closing and new uses for the chemical are being found, Chem. Eng. News 84 (2006) 87–97.
[3] M.R. Nanda, Z. Yuan, W. Qin, H.S. Ghaziaskar, M. Poirier, C. Xu,

Thermodynamic and kinetic studies of a catalytic process to convert glycerol into solketal as an oxygenated fuel additive, Fuel 117 (2014) 470–477.
[4] T. Willke, K. Vorlop, Biotransformation of glycerol into 1,3-propanediol, Eur. J. Lipid Sci. Technol. 110 (2008) 831–840.
[5] A. Heimath, M. Kannabiran, V. Rangaswamy, 1,3-propanediol production from crude glycerol from Jatropha biodiesel process, New Biotechnol. 28 (2010) 19–23.
[6] S.S. Yazdani, R. Gonzalez, Anaerobic fermentation of glycerol: a path to economic viability for the biofuels industry, Curr. Opin. Biotechnol. 18 (2007) 213–219.
[7] P.F. Amaral, T.F. Ferreira, G.C. Fontes, M.A.Z. Coelho, Glycerol valorization: new biotechnological routes, Food Bioprod. Proc. 87 (2009) 179–186.
[8] S. Cho, K.D. Kim, J.H. Ahn, J. Lee, K.W. Kim, Y. Um, Selective production of 2, 3-butanediol and acetoin by a newly isolated bacterium *Klebsiella oxytoca* M1, Appl. BiochemS Biotechnol. 170 (2013) 1922–1933.
[9] C. Moon, J.H. Ahn, S.W. Kim, B.I. Sang, Y. Um, Effect of biodiesel-derived raw glycerol on 1, 3-propanediol production by different microorganisms, Appl. Biochem. Biotechnol. 161 (2010) 502–510.
[10] A. Chatzifragkou, D. Dietz, M. Komaitis, A.P. Zeng, S. Papanikolaou, Effect of biodiesel derived waste glycerol impurities on biomass and 1,3-propanediol production of *Clostridium butyricum* VPI 1718, Biotechnol. Bioeng. 107 (2010) 76–84.
[11] Q. Zhang, Z. Xiu, Metabolic pathway analysis of glycerol metabolism in *Klebsiella pneumoniae* incorporating oxygen regulatory system, Biotehnol. Prog. 25 (2009) 103–115.
[12] A.P. Zeng, H. Biebl, H. Schlieker, W.D. Deckwer, Pathway analysis of glycerol fermentation by *Klebsiella pneumoniae*: regulation of reducing equivalent balance and product formation, Enzyme Microb. Technol. 15 (1993) 770–779.
[13] M.S. Rahman, Z. Yuan, K. Ma, C.C. Xu, W. Qin, Aerobic conversion of glycerol to 2,3-butanediol by a novel *Klebsiella variicola* SRP3 strain, J. Microb. Biochem. Technol. 7 (2015) 299–304.
[14] L. Li, L. Zhang, K. Li, Y. Wang, C. Gao, B. Han, A newly isolated *Bacillus licheniformis* strain thermophilically produces 2,3-butanediol, a platform and fuel bio-chemical, Biotechnol. Biofuels 6 (2013) 123–135.
[15] X. Zhang, T. Yang, Q. Lin, M. Xu, H. Xia, Z. Xu, Isolation and identification of an acetoin high production bacterium that can reverse transform 2,3-butanediol to acetoin at the decline phase of fermentation, World J. Microbiol. Biotechnol. 27 (2011) 2785–2790.
[16] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real time quantitative PCR and $2^{-\Delta\Delta CT}$ method, Methods 25 (2001) 402–408.
[17] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[18] M. Mesoviti, K. Paraskevaidi, A. Koutinas, A.P. Zeng, S. Papanikolaou, Production of 1, 3-propanediol, 2, 3-butanediol and ethanol by a newly isolated *Klebsiella oxytoca* strain growing on biodiesel-derived glycerol based media, Process Biochem. 47 (2012) 1872–1882.
[19] D.M. Rossi, E.A. de Souza, M.A. Zachia, Conversion of residual glycerol from biodiesel synthesis into 1,3-propanediol by a new strain of *Klebsiella pneumoniae*, Renew. Energy 55 (2013) 404–409.
[20] H. Huang, C.S. Gong, G.T. Tsao, Production of 1,3-propanediol by *Klebsiella pneumoniae*, Appl. Biochem. Biotechnol. 98 (2002) 687–698.
[21] Y. Wang, F. Tao, P. Xu, Glycerol dehydrogenase plays a dual role in glycerol metabolism and 2,3-butanediol formation in *Klebsiella pneumoniae*, J. Biol. Chem. 289 (2014) 6080–6090.
[22] Y. Mu, H. Teng, D.J. Zhang, W. Wang, Z.I. Xiu, Microbial production of 1,3-propanediol by *Klebsiella pneumoniae* using crude glycerol from biodiesel preparations, Biotechnol. Lett. 78 (2006) 1755–1759.
[23] F. Liu, B. Fang, Optimization of bio-hydrogen production from biodiesel wastes by *Klebsiella pneumoniae*, Biotechnol 2 (2007) 374–380.
[24] T.W. Yang, Z.M. Rao, X. Zhang, M.J. Xu, Z.H. Xu, S.T. Yang, Fermentation of biodiesel-derived glycerol by *Bacillus amyloliquefaciens*: effects of co-substrates on 2,3-butanediol production, Appl. Microbiol. Biotechnol. 97 (2013) 7651–7658.
[25] G. Zhang, B. Ma, X. Xu, C. Li, L. Wang, Fast conversion of glycerol to 1,3-propanediol by a new strain of *Klebsiella pneumoniae*, Biochem. Eng. 37 (2007) 256–260.
[26] Y. Nakashimada, B. Marwoto, T. Kashiwamura, T. Kakizono, N. Nishio, Enhanced 2,3-butanediol production by addition of acetic acid in *Paenibacillus polymyxa*, J. Biosci. Bioeng. 90 (2000) 661–664.
[27] W. Mateusz, R. Alberto, R. Vanessa, E.S. Victoria, L.G. José, G.O. Félix, 1,3-Propanediol production by *Klebsiella oxytoca* NRRL-B199 from glycerol. Medium composition and operational conditions, Biotechnol. Rep. 6 (2015) 100–107.
[28] X. Chen, Z. Xiu, J. Wang, D. Zhang, P. Xu, Stoichiometric analysis and experimental investigation of glycerol bioconversion to 1,3-propanediol by *Klebsiella pneumoniae* under microaerobic conditions, Enzyme Microb. Tech. 33 (2003) 386–394.
[29] S. Cho, T. Kim, H.M. Woo, Y. Kim, J. Lee, Y. Um, High production of 2,3-butanediol from biodiesel-derived crude glycerol by metabolically engineered *Klebsiella oxytoca* M1, Biotechnol. Biofuels 8 (2015) 146–157.
[30] S. Rebecchi, G. Zanaroli, F. Fava, 2,3-Butanediol production from bio-wastes with *Bacillus licheniformis*: a preliminary study, Chem. Eng. Trans. 49 (2016) 379–384.
[31] K. Petrov, P. Petrova, Enhanced production of 2, 3-butanediol from glycerol by forced pH fluctuations, Appl. Microbiol. Biotechnol. 87 (2010) 943–949.
[32] J.R. Borden, E.T. Papoutsakis, Dynamics of genomic-library enrichment and identification of solvent tolerance genes for *Clostridium acetobutylicum*, Appl. Environ. Microbiol. 73 (2007) 3061–3068.
[33] H.W. Yen, F.T. Li, J.S. Chang, The effects of dissolved oxygen level on the distribution of 1,3-propanediol and 2,3-butanediol produced from glycerol by an isolated indigenous *Klebsiella* sp. Ana-WS5, Bioresour. Technol. 153 (2014) 374–378.