

Biotransformation of biodiesel-derived crude glycerol using newly isolated bacteria from environmental consortia



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ABSTRACT

Oversupply of biodiesel-derived crude glycerol, a core by-product of biodiesel production process has become a great environmental and economical concern for growth of biodiesel industries. Biotransformation of this large volume of crude glycerol to value-added bio-products may directly benefit the environment and economic feasibility, encouraging the development of biodiesel plants. In our study, numerous bacterial strains isolated from environmental consortia were screened for their capability of converting crude glycerol to 2,3-BD which is a high worth green product used as a liquid fuel or fuel additive. An aerobic batch flask cultivation was carried out to access the kinetics of bio-product formation and glycerol utilization. The greatest producers of 2,3-butanediol (2,3-BD) identified by 16S rRNA gene sequences were *Klebsiella pneumoniae*, *K. variicola* and *Serratia liquefaciens*. The co-culture constructed by *K. pneumoniae* SRP2 and *K. variicola* SRP3 was capable of simultaneously converting crude glycerol to concurrently produce up to 27.87 g/L of 2,3-BD, yielding 0.73 g/g using 37.0 g/L glycerol under aerobic conditions in batch culture, showing great potential for biotransformation bioprocess.

1. Introduction

Biodiesel has become one of the vibrant renewable fuels produced from animal fats and vegetable oils by reacting with a primary alcohol in the presence of catalyst, generating a significant amount of crude glycerol (10% v/v) as a core by-product. Thus, the crude glycerol produced from biodiesel production process is a global oversupply problem due to lack of refining capacity, and this high volume of crude glycerol has become an environmental problem since it can't be safely disposed in the environment. However, due to its low price, crude glycerol is now considered as a waste instead of a useful product. A high volume of crude glycerol is the crucial problem for the development of biodiesel industries, which is greatly affected on economic viability [1]. In addition to the economic value, conversion of this large amount of biodiesel waste (crude glycerol) would directly benefit the environment by obtaining renewable value-added products, encouraging the development of biodiesel industry and reducing non-renewable fossil fuel use. Glycerol, a simple sugar alcohol, can be used as a carbon and energy source for microbial growth to produce biofuels and other valuable products [2–4]. It has been proved that bioconversion of pure glycerol to fuels or reduced products may result in yields higher than those

obtained with the use of common sugars due to the highly-reduced state of carbon present in glycerol [5]. The ability to conduct fermentative metabolism of pure glycerol in the *Enterobacteriaceae* family is shared by only a few members such as *Enterobacter aerogenes*, *K. oxytoca* and *Klebsiella pneumoniae* [6–8]. Till today, there is no effective microorganism which can convert biodiesel waste (crude glycerol) efficiently to produce high value products 2,3-butanediol (2,3-BD). Moreover, due to lack of biocatalysts, the biodiesel production process remains economically unfeasible thus hindering the process of glycerol conversion. Furthermore, crude glycerol contains methanol, salts, soaps etc. known as matter organic non-glycerol (MONG), and catalysts as the main impurities which can negatively influence the bioconversion process. Almost all the works have been carried out on bioconversion of pure glycerol to biofuels and value-added bio-products through microbial fermentation process [9,10], but very few works have been done on bioconversion of biodiesel waste crude glycerol under anaerobic process [11,12]. Our primary goal is to isolate and develop new strains of bacteria capable of efficiently converting crude glycerol to value-added biotechnological products under completely aerobic process. In oxidative pathway of glycerol metabolisms, *K. pneumoniae* metabolizes glycerol for synthesis of major products including dihydroxyacetone

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(DHA), 2,3-BD, acetoin, acetate and 1,3-propanediol (1,3-PDO) [2,13]. In this oxidative pathway, NAD⁺-dependent glycerol dehydrogenase (GDH) enzyme dehydrogenates glycerol to DHA by generating reducing equivalent NADH [1,14]. Subsequently, DHA is then phosphorylated by ATP or PER-dependent DHA kinase to generate dihydroxyacetone phosphate (DHAP), which is then further metabolized to various products including 2,3-butanediol (2,3-BD), acetoin, lactate, acetate, succinate and ethanol through pyruvate [14–16]. Moreover, microorganisms can continually regenerate NAD⁺ by transferring electrons from NADH to form a reduced end product like 2,3-DB or lactate or ethanol [14].

2,3-BD and acetoin are two significant platform chemicals which can be attained from oxidative pathways of glycerol metabolisms of many bacteria [7,17]. 2,3-BD is a reduced form of acetoin which is widely used as an antifreeze agent, lubricant, liquid fuel or fuel additive, and a precursor of many synthetic materials including polymers and resins [18]. 2,3-BD is used for manufacturing printing ink, perfumes and fumigants, polymer, pharmaceutical carrier, moistening and softening agents, and reagent in different asymmetric chemical synthesis [19,20]. However, acetoin, an important metabolic product is widely used in food, flavor, cosmetics, and chemical synthesis [19,20]. In the recent years, biotechnological production of 2,3-BD from glycerol has been demonstrated for only few bacterial strains including *K. pneumoniae*, *K. variicola*, *K. oxytoca* and *Bacillus amyloliquefaciens* [2,12,13,21]. Now-a-days, the anaerobic fermentation is the most promising option for bioconversion of crude glycerol by *Klebsiella* [22,23]. Several bacterial strains including *Lactobacillus lycopersici* and *Bacillus subtilis* are capable of fermenting sugars producing glycerol, but are unable to further convert glycerol to other product [24]. However, several bacteria including *K. pneumoniae*, *Enterobacter*, *Citrobacter*, and few species of *Clostridium* have already been isolated which are able to ferment glycerol, residual glycerol or mixture of glycerol and sugars, and the main product was 1,3-propanediol (1,3-PDO), while 2,3-BD was not reported along with other products [25–27].

Consequently, more work is needed without any delay if the enormous amounts of surplus glycerol are to be cost effectively converted into value-added commercial products. In this context, the aim of our present work is to make a co-culture from bacterial strains isolated from environmental consortia to increase metabolic product yield of 2,3-BD to make a process relevant for industrial application. The biotransformation kinetics of batch culture processes was studied in detail, and the best co-culture of bacterial strains providing the gain of increased 2,3-BD product yield were evaluated.

2. Materials and methods

2.1. Crude glycerol

Biodiesel-derived crude glycerol was kindly supplied by Dr. Chunbao Xu of Western University (Canada) which was obtained from a small biodiesel plant Centre for Agricultural Renewable Energy and Sustainability (CARES), Guelph, Canada. This crude glycerol (raw glycerol) composition was (in weight based) 50.0 ± 4.6% glycerol, 4.3 ± 0.3% ash, 6.7 ± 1.05% moisture and 36.2 ± 3.3% MONG. The pH of the crude glycerol was 10.6 ± 0.4.

2.2. Isolation of bacterial strains from a microbial consortium

The paper mill sludge, an environmental bacterial consortium was collected from Resolute Forest Products, Thunder Bay, Ontario, Canada. This sample was immediately refrigerated to inhibit the growth of microbes, transported to the research laboratory for glycerol utilization study. For isolation of glycerol degrading strains, 5.0 g of paper mill waste sample was inoculated into in a 250 mL Erlenmeyer flasks containing 100 mL minimal salt (MS) broth medium supplemented with 100 g/L analytical grade glycerol (Sigma Aldrich), incubated at 30 °C

with shaking (200 rpm) for 48 h. MS medium contained glycerol (which is a sole carbon source) and the following components: K₂HPO₄ (0.1 g/L), MgSO₄·7H₂O (0.05 g/L), KCl (0.1 g/L), NaNO₃ (0.1 g/L) and analytical grade glycerol (100 g/L). Subsequent incubation, culture from flasks displaying growth was streaked onto MS agar plates containing 100.0 g/L glycerol, transferred pure culture to LB agar plate, and stored at 4 °C. These isolated strains were further screened for their glycerol assimilation, GDH activity and 2,3-BD production.

2.3. Identification and construction of phylogenetic tree using 16S rRNA partial sequencing

The molecular identification of glycerol degrading bacterial isolates was conducted using 16S rRNA gene sequencing. Genomic DNA of the selected bacterial isolates was extracted using bacterial DNA Genomic Mini Kit (FroggaBio, Canada). The extracted DNA was further amplified using universal primers designed within conserved regions of the 16S rDNA for Eubacteria, which were HDA-1 (5'-GACTCCTACG GGAGGCAGCAGT) and E1115R (5'-AGGGTTGCGCTCGTTGCGGG). The 50 µL PCR mixture contained 0.5 µg template DNA, 0.5 µM forward and reverse primers, 25 µL double strength Tag Mix (0.4 mM dNTPs, 3.2 mM MgCl₂, 2X PCR buffer, 0.25 U/µL Tag DNA polymerase and 0.02% bromophenol blue) and nuclease-free water. The PCR reactions were performed in an automated thermal cycler (Bio-Rad, My Cycler™ thermal cycle) using following thermal cycling conditions: initial denaturation at 94 °C for 3 min followed by 30 cycles at 94 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR products were purified using Geneaid Clean-up kit (FroggaBio, Canada) following the manufacturer's protocol. Confirmation of amplified 16S rRNA gene fragments was validated by a band on a 1% agarose gel. The purified samples were sent to Euroffins Genomics (USA) for sequencing. Sequencing result was inputted in the nucleotide blast tool through the NCBI database (<http://blast.ncbi.nlm.nih.gov/>) for possible identification of bacterial genera. The phylogenetic relationship was analyzed using sequence alignment program ClustalX Omega software. For confirming the species identification, morphological and physiological characteristics were determined [28].

2.4. Biotransformation kinetics

The stock culture of bacterial isolates was maintained at 4 °C by sub-cultured every month, and one set stored at –80 °C with 20% (w/w) glycerol added. For batch culture, the seed culture was prepared from stock culture (1 loopful culture from LB agar plate) by inoculating into a Luria-Bertani (LB) broth (yeast extract 5.0 g/L; peptone 10.0 g/L; NaCl 5 g/L and pH 7.0) medium. The seeds were grown in 125 mL shake flask containing 50 mL medium at 30 °C and 200 rpm under aerobic conditions for 18–20 h. Following incubation, the seed culture was inoculated into appropriate medium for aerobic biotransformation of glycerol. Batch culture was carried out in 250 mL Erlenmeyer flasks with a working volume of 100 mL including 100 µL of 18 h culture. Incubation temperature was maintained at 30 °C when *Serratia* (S1–S3) strains were used and 37 °C when only *Klebsiella* (P1–P5) strains were used. However, agitation was maintained at 200 rpm using a rotary shaker incubator (New Brunswick Scientific, C25 incubator shaker, NJ, USA). The batch culture medium contained glycerol as a sole carbon source. The MS-2 medium supplemented with different concentrations of pure and crude glycerol was used for batch biotransformation comprised (in per L): NaNO₃ (0.1 g), K₂HPO₄ (0.1 g), KCl (0.1 g), and MgSO₄·7H₂O (0.05 g), yeast extract (2.5 g) and peptone (5.0 g). The initial pH (7.0) of the medium was adjusted by using 1 M HCl. All experiments were conducted in triplicates.

2.5. Enzyme activity assay

The membrane-bounded GDH enzyme activity was determined at

room temperature by measuring the initial reduction rate of NAD at 340 nm absorbance according to the method described by Ahrens et al. [29] with some modification. Momentarily, 1.0 mL of culture (OD₆₀₀ of ~0.8) broth was centrifuged at 12,000–15,000 × g for 5 min, and cells were collected. Cells were washed twice using 100 mM potassium phosphate buffer (pH 8.0), re-suspended in 100 mM potassium phosphate buffer containing 50 mM KCl. Using a sonicator (Model CL-18) cells were disrupted at 2–4 °C for 2 min (6 s at a time, and until 2 min). The supernatant was then collected by centrifugation at 12,000–15,000 × g for 4–5 min, kept at low temperature (2–4 °C) or on ice for GDH enzyme activity. Enzyme activity was measured by microplate spectrophotometer (EPOCH, BioTek Gen5™, USA). The GDH activity was determined by the change of substrate-dependent absorbance per minute at 340 nm. Reaction mixture of 0.3 mL for each microplate well contained 0.2 M glycerol, 30 mM ammonium sulfate, 1.2 mM NAD, 50.0 mM potassium phosphate buffer (pH 8.0), and 50 µL cell extract. The absorbance increase (NADH) was followed with a spectrophotometer for 5 min. One unit of GDH activity was defined as the amount of enzyme required to reduce 1 µmole of NAD⁺ to NADH per minute. The specific activity of GDH is defined as enzyme units/mg of cell protein. Protein concentration in the cell free extract was determined according to Bradford method [30] using a microtiter plate reader and bovine serum albumin as the standard.

2.6. Analytical methods

Cell concentration of the culture medium was measured using microplate spectrophotometer (EPOCH, BioTek). The biomass production was expressed as optical density (OD₆₀₀) at 600 nm absorbance.

GC–MS (Varian 1200 Quadrupole GC/MS using helium as the carrier gas) was used to identify the end products. To determine concentrations of glycerol and major metabolic products including 2,3-BD, acetoin and 1,3-PDO, gas chromatograph GC 14A (Shimadzu Corp., Kyoto, Japan) equipped with a flame ionization detector (FID) was used. Briefly, the supernatant of culture broth obtained after centrifugation (accu Spin Micro 17, Fisher Scientific) at 12,000–15,000 × g for 5 min was filtered through a syringe filter (pore size 0.22 µm; Progene, UltiDent Scientific, Canada). DB-WAXetr column was used under the following conditions: sample volume 1 µL; column temperature range from 45 °C (2 min) to 240 °C at the increasing rate of 10 °C/min; the injector and detector temperature 250 °C; carrier gas was nitrogen.

3. Results and discussion

3.1. Glycerol utilizing bacterial strains from environmental consortium

Total eight strains (P1, P2, P3, P4, P5, S1, S2 and S3) were isolated from the sample, a microbial consortium based on their ability to tolerance (100 g/L) and utilize glycerol as the sole carbon source to exhibit GDH enzyme activity under aerobic condition. These highly active eight strains were identified using 16S rRNA gene sequencing, confirmed by their morphological, physiological and biochemical properties. Genomic DNA for 16S rRNA Gene amplification was successfully isolated from all eight GDH producing isolates using DNA isolation kit. Sequencing and sequence analysis results of all eight different 16S rRNA genes were successfully obtained. The partial sequences of 16S rRNA of the isolates/strains P1, P2, P3, P4, P5, S2, and S3 were submitted to the GenBank for their accession numbers. However, the strain S1 reported in this paper is not a new strain which has 100% similarity to the strain *Serratia* sp. 243 (accession No. KT461863). The potential seven isolates reported in our research paper has been nominated as the new strains of *Klebsiella* and *Serratia* sp, and their GenBank accession numbers are released in the NCBI website (Table 1).

However, for the analysis of evolutionary relationship among the newly isolated strains, phylogenetic tree was constructed using 16S

Table 1

List of strains which were identified by 16S rRNA sequencing as well as morphological and biochemical characters.

Isolate No.	Strain identified as	GenBank Accession No.
P1	<i>Klebsiella pneumoniae</i> SRP1	KU550763
P2	<i>K. pneumoniae</i> SRP2	KR092085
P3	<i>K. variicola</i> SRP3	KR092086
P4	<i>K. pneumoniae</i> SRP4	KU550764
P5	<i>K. pneumoniae</i> SRP5	KU550765
S1	<i>Serratia</i> sp. 243.	KT461863
S2	<i>S. liquefaciens</i> SRWQ2	KX602659
S3	<i>S. proteamaculans</i> SRWQ1	KX602658

rDNA sequences of strains *Klebsiella pneumoniae* SRP1, *K. pneumoniae* SRP2, *K. pneumoniae* SRP4, *K. pneumoniae* SRP5, *K. variicola* SRP3, *Serratia liquefaciens* SRWQ2, *Serratia proteamaculans* SRWQ1, *Serratia* sp., and other *Klebsiella* as well as *Serratia* strains retrieved from GeneBank. The phylogenetic tree constructed using the ClustalX Omega software is presented in Fig. 1. The evolutionary history was inferred using the same software (ClustalX Omega). The result of phylogenetic relationship confirmed the identity of our new strains through the distance between all the newly isolated and other strains.

3.2. Glycerol degrading capability of the consortium

All the identified bacterial strains were used to perform the ability of metabolizing crude glycerol from biodiesel production process by cultivating environmental consortia in a batch flask culture at 30 °C. In this aerobic batch culture process, glycerol was only the substrate for GDH and 2,3-BD production. Comparison the capabilities of bacterial consortium to grow in either pure or crude glycerol is shown in Fig. 2. As shown in Fig. 2a, 25.0 g/L of pure glycerol was consumed in 60 h with a production of 14.07 g/L 2,3-BD, yielding 0.56 g/g and productivity of 0.24 g/L/h. The highest GDH activity was 177.21 units/mg protein when the bacterial consortium utilized 11.5 g/L glycerol after 24 h incubation. However, for crude glycerol, the highest GDH activity was 157.31 units/mg protein in 24 h, and utilization of 97.4% (24.3 g/L) glycerol was completed within 60 h of incubation with nearly the same amount (13.44 g/L) of 2,3-BD being produced (Fig. 2b). However, when the culture temperature was maintained at 37 °C the 2,3-BD product yield and glycerol utilization rate was increased using the consortium containing P1, P2, P3, P4 and P5 strains. Isolated strains of *Serratia* (S1, S2 and S3) were not capable of growing as high as 37 °C in batch culture. In case of incubation temperature at 37 °C in a batch culture as shown in Fig. 3, almost 100% (25.0 g/L) glycerol was consumed within 60 h with a product concentration of 15.03 g/L 2,3-BD and a productivity of 0.25 g/L/h, and a product concentration of 14.67 g/L 2,3-BD and a productivity of 0.245 g/L/h in case of pure and crude glycerol respectively. The GDH enzyme activities were also increased at 37 °C of culture temperature compared to that of 30 °C with the consortium contains P1, P2, P3, P4 and P5. The highest GDH activities 195.97 and 187.75 units/mg protein were recorded after 24 h using pure and crude glycerol respectively (Fig. 3a and b). Only one research work has been conducted on the production of 1,3-PDO by environmental consortia using crude glycerol as a sole carbon source until now [22]. Till today, there is no any report on the production of 2,3-BD using environmental consortia or bacterial co-culture. Our findings displayed that some of these strains were present in the bacterial consortia, and were capable of competently converting glycerol or biodiesel derived crude glycerol into 2,3-BD.

3.3. Utilization of glycerol by single cultures

The GDH activity, biomass production and glycerol consumption were observed for 8 bacterial isolates (Table 1) based on previously

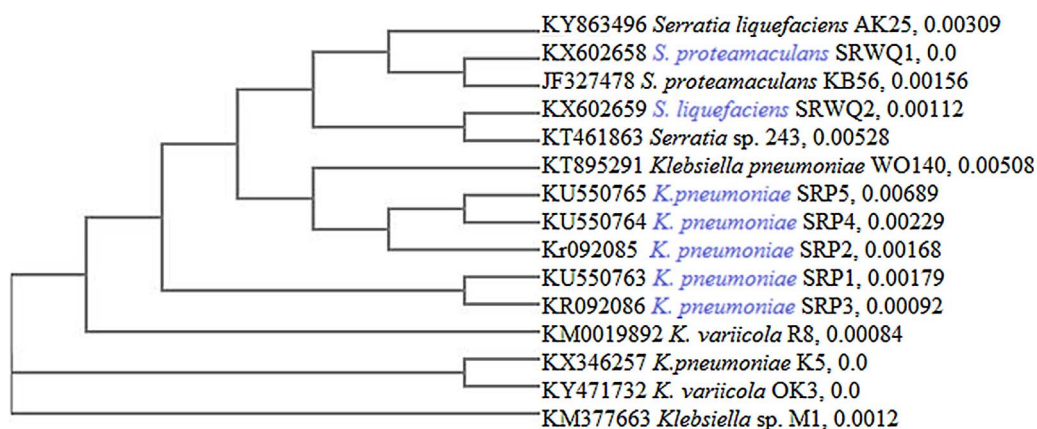


Fig. 1. Evolutionary relationships of the strains: Phylogenetic tree drawn from sequence alignment program using ClustalX Omega software. 16S rRNA gene sequences were retrieved by nucleotide BLAST searches in NCBI. The numbers that follow the names of the strains are accession numbers and bootstraps of published sequences.

characterized glycerol utilizing capabilities. As shown in Fig. 4, none of the bacterial isolates produced significantly demonstrable GDH enzyme in 24 h at 30 °C, nor did they consume significant amount of glycerol after 24 h of incubation under aerobic condition in a batch culture. Moreover, there was an extensive disparity in glycerol utilization, GDH activity and biomass production between individual bacterial strains with both pure and crude glycerol as the sole carbon source. Under aerobic conditions, strains P2, P3 and P4 were the best producers of GDH. As shown in Fig. 4, the best three isolates P2, P3 and P4 displayed the maximum GDH activities of 117.6, 131.7 and 102.1 units/mg protein respectively using pure glycerol as the sole carbon source (Fig. 4a). However, as the sole carbon source crude glycerol, the maximum GDH activities were 102.74, 110.43 and 98.07 units/mg protein with the same isolates P2, P3 and P4 respectively after 24 h at 30 °C (Fig. 4b). The highest GDH activity (131.7 units/mg protein) under aerobic condition was achieved using isolate P3 (*K. variicola*). Furthermore, the isolate P3 exhibited the better glycerol consumption capability of 9.27 g/L in 24 h as well as biomass production (OD_{600} 1.53) with pure glycerol, and almost same results were obtained using crude glycerol. Finally, S1, S2 and S3 isolates displayed a significantly less enzyme activity and glycerol consumption capability, and also these three isolates do not grow at 37 °C. The remaining isolates P1 and P5 did not display significant activity of GDH when compared with the other isolates. Recently, Homann et al. [31] isolated bacterial strains from an environmental consortium, characterized as *K. pneumoniae*, *K. oxytoca* and *Citrobacter freundii*, and these bacterial strains produced between 9.3 and 13.1 g/L of 1,3-PDO in shake flask cultures using 20.0 g/L of pure glycerol. It is notable to point that these researches worked with pure glycerol at low concentrations to produce 1,3-PDO, but there is no report on GDH as well as 2,3-BD production.

3.4. Utilization of glycerol by co-cultures

Likewise, glycerol consumption, GDH activity and production of 2,3-BD were evaluated for bacterial co-cultures constructed by pairing each of the five nominated isolates P1, P2, P3, P4 and P5 with each other once. Like this, a total of ten co-cultures were prepared (Fig. 5). For determination of the best co-culture, the experiment was carried out in shake flask batch culture under aerobic condition at a concentration of 50.0 g/L biodiesel-derived crude glycerol which was contained 25.0 g/L glycerol, incubated at 37 °C for 48 h. In this experiment, the incubation temperature was set at 37 °C based on optimal incubation temperature of P1-P5. Moreover, due to low concentration of 2,3-BD in 24 h incubation in a batch culture stated in Fig. 4, the incubation time was increased to 48 h. Consequently, all co-cultures produced significant amount of 2,3-BD, ranging from 6.89 to 15.73 g/L after consumed 15.27–25.0 g/L glycerol from biodiesel-derived crude glycerol in 48 h at 37 °C in a batch culture (Fig. 5). As shown in Fig. 5, the best productions of 2,3-BD were 15.73, 14.12 and 13.01 g/L obtained from three co-cultures of P2 + P3, P2 + P4 and P3 + P4 respectively. Meanwhile, from these results four co-cultures (P1 + P5, P4 + P5, P1 + P4 and P1 + P2) exhibited less than 10.0 g/L 2,3-BD. In the meantime, the remaining co-cultures, P1 + P3, P2 + P5 and P3 + P5 were showed 10.27–11.3 g/L 2,3-BD using 19.94–22.73 g/L glycerol. Furthermore, co-culture P2 + P3 with the greatest potential towards 2,3-BD production was selected from this ten co-culture groups for the construction of the best bacterial consortia (co-culture), based on its ability to utilized 100.0% (25.0 g/L) of glycerol in 48 h, yielding 0.63 g/g of 2,3-BD as well as 0.33 g/L/h. In regard to the group of ten co-cultures, when looking at their efficacy of glycerol utilization and 2,3-BD production, the isolates P2 and P3 containing co-cultures were demonstrated as the best candidate, and it is readily apparent that the

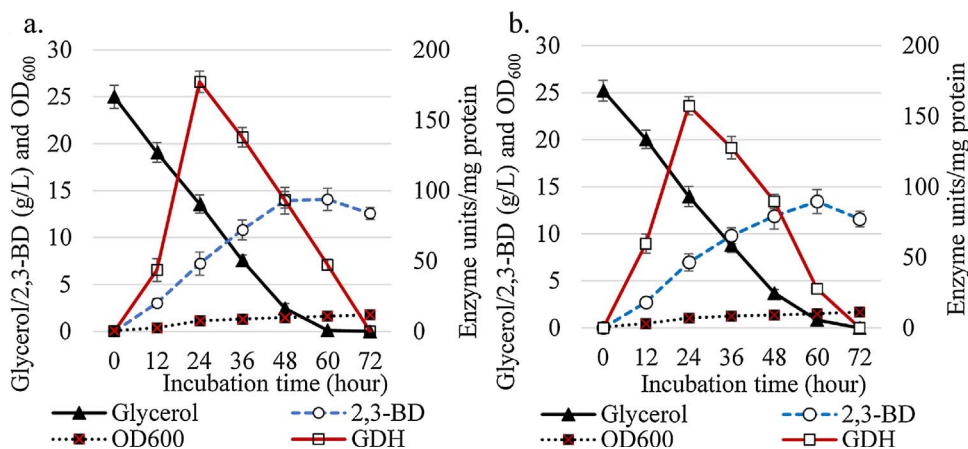


Fig. 2. Profile of glycerol utilization, GDH enzyme activity, biomass and 2,3-BD production of bacterial consortium containing P1 + P2 + P3 + P4 + P5 + S1 + S2 + S3 strains; (a) pure glycerol 25.0 g/L, (b) 50% glycerol containing biodiesel derived raw glycerol 50.0 g/L (25.0 g/L glycerol). MS medium supplemented with pure/crude glycerol, yeast extract 2.5 g/L and peptone 5.0 g/L. Incubation at 30 °C.

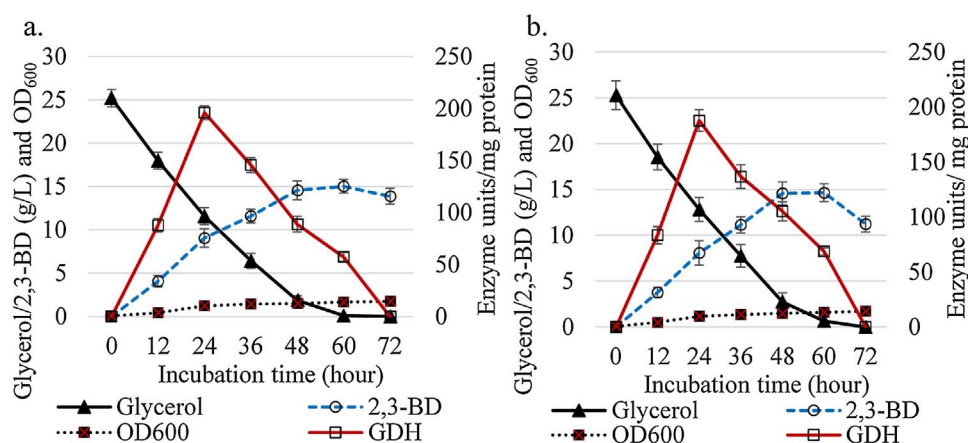


Fig. 3. kinetics of glycerol utilization, GDH enzyme activity, 2,3-BD and biomass production of bacterial consortium containing P1 + P2 + P3 + P4 + P5 isolates (S1 + S2 + S3 strains do not grow at 37 °C); (a) pure glycerol, (b) biodiesel derived crude glycerol. MS medium supplemented with pure glycerol 25 g/L or biodiesel derived crude glycerol 50 g/L, yeast extract 2.5 g/L and peptone 5.0 g/L, and incubation at 37 °C.

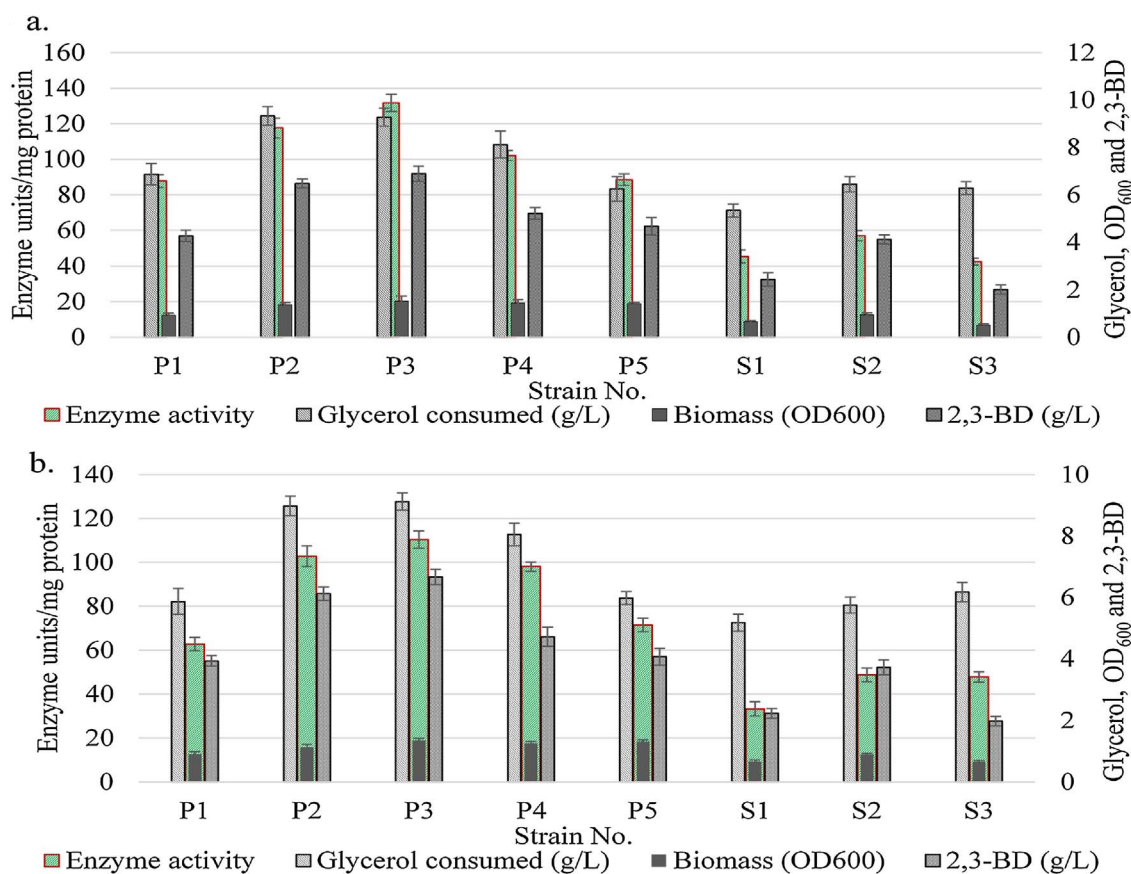


Fig. 4. Profile of glycerol utilization, GDH enzyme activity, 2,3-BD and biomass production of bacterial strains isolated from bacterial consortium; (a) pure glycerol 25.0 g/L, (b) biodiesel derived crude glycerol 50.0 g/L. MS medium supplemented with pure/crude glycerol, yeast extract 2.5 g/L and peptone 5.0 g/L. Incubation at 30 °C for 24 h.

co-culture P2 + P3 was capable of producing 15.73 g/L of 2,3-BD which is the highest amount among ten co-cultures reported in Fig. 5. Specifically, it is clearly seeming that isolates P2 and P3 made the best combination for construction of effectual co-culture (P2 + P3) for a high production of metabolic product 2,3-BD (Fig. 5).

3.5. Utilization of glycerol by a newly constructed bacterial consortium

Based on the outcomes from glycerol utilization in ten co-cultures using biodiesel-derived crude glycerol as only the sole carbon source, the co-culture P2 + P3 produced the greatest yield of 2,3-BD, which was selected as the best bacterial consortium for this study. Aerobic batch biotransformation process was used for a high production of 2,3-BD using 75.0 g/L crude glycerol as a sole carbon source. The kinetics of

batch cultivation of co-culture P2 (*K. pneumoniae* SRP2) + P3 (*K. varicola* SRP3) under aerobic conditions are presented in Fig. 6. Specifically, the biotransformation under aerobic condition (Fig. 6b) displayed a very lower production of acetate (0.14–0.37 g/L) and 1,3-PDO (0.34–0.77 g/L) in 120 h when the glycerol from biodiesel-derived crude glycerol was completely consumed. The maximum production of 2,3-BD was 27.87 g/L, yielding 0.73 g/g (0.29 g/L/h) in 96 h by utilized 97.37% or 37.0 g/L glycerol using the consortium P2 + P3 (Fig. 6a). Additionally, co-culture P2 + P3 exhibited the highest concentration 3.7 g/L of acetoin obtained in 144 h after completely consumed glycerol (carbon source) from the culture medium. The biomass production was increased dramatically until 48 h of incubation, and OD₆₀₀ value reached up to 1.12 (Fig. 6b). After 48 h of incubation, biomass production was increased very slowly, and OD₆₀₀ value reached from

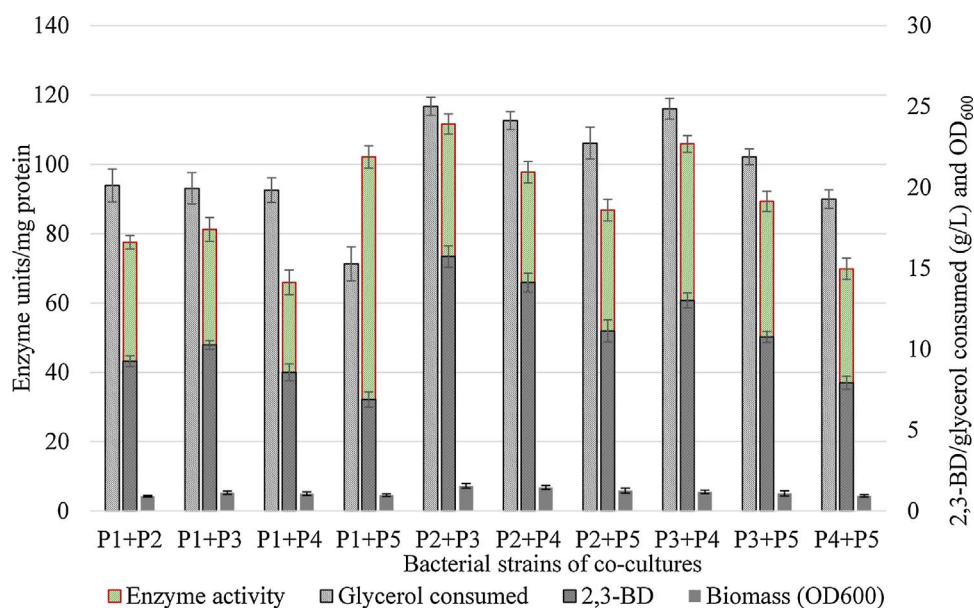


Fig. 5. Profile of glycerol utilization, GDH enzyme activity, 2,3-BD and biomass production of bacterial co-cultures after 48 h at 37 °C on biodiesel waste glycerol. MS-2 medium supplemented with biodiesel derived crude glycerol 50.0 g/L.

1.12 to 1.38 after 120 h incubation (Fig. 6b). However, during the stationary phase (OD_{600} 1.12–1.38) of bacterial co-culture (P2 + P3) under aerobic biotransformation process, the 2,3-BD product yield was increased until 96 h of incubation (Fig. 6a). After 96 h when glycerol was completely consumed by two isolates P2 and P3, the concentration of acetoin was increased and 2,3-BD production was decreased. Once more, when glycerol is absence in the medium, acetoin is produced instead of 2,3-BD, and also 2,3-BD is converting to acetoin. Our results revealed that the co-culture constructed using *K. pneumoniae* SRP2 and *K. variicola* SRP3 was capable of converting biodiesel-derived crude glycerol to 2,3-BD in alike way to results stated in other researchers where the culture was environmental consortium for the production of 1,3-propanediol and monoculture for 1,3-PDO or 2,3-BD [22,32,33], but there is no report on co-culture for production of 2,3-BD using glycerol as a carbon source.

An enteric bacteria *K. pneumoniae* has been known to produce 1,3-PDO through anaerobic fermentation of glycerol [11,34,35]. Moreover, in the recent years, a very few works have been reported on aerobic biotransformation of glycerol to 2,3-BD production [2]. Therefore, Table 2 compares the product yields of 2,3-BD reported earlier and this study in batch fermentation using biodiesel-derived crude glycerol as the only carbon source. Moreover, the use of mixed culture, co-culture or bacterial consortium permits better utilization of the substrate has

gained attention in bioproducts production for their ability to perform more complicated tasks and more readily adapt to changes in the environment than mono-culture [36]. The synergistic effect of enzymes produced by different bacterial strains present in mixed culture could help overcome the lack of effective bio-conversion by a pure culture, because mixed culture's strains may be produced high levels of some but not all enzymes required for effectual biotransformation [36,37]. However, we have confirmed that our co-culture (consortium) constructed from newly isolated strain *K. pneumoniae* SRP2 and *K. variicola* SRP3 could be fermentatively metabolized glycerol to produce notable amount of important liquid fuel or fuel additive product 2,3-BD in a GDH-dependent oxidative (aerobic) pathway.

4. Conclusions

Biotransformation of a core by-product crude glycerol generated from biodiesel production process offers a substantial advantage to produce 2,3-BD in relation to usage of glycerol. Almost all the works have been conducted on pure glycerol as a substrate. Nonetheless, only a few research works have been reported on the possible use of this core by-product crude glycerol generated from biodiesel synthesis process to produce 2,3-BD. Our results consistently revealed that it is possible to isolate novel bacterial strains capable of producing a high yield of 2,3-

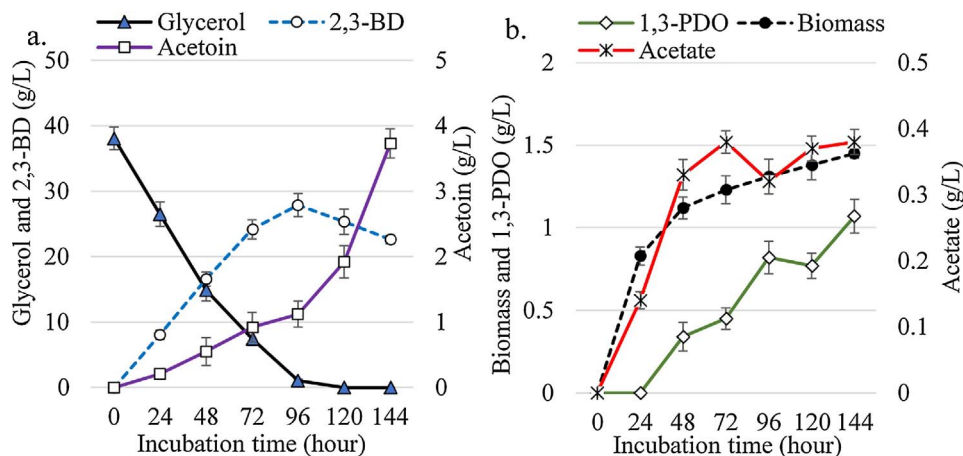


Fig. 6. Kinetics of biotransformation of biodiesel derived crude glycerol to metabolic bioproducts in aerobic batch culture at 37 °C by the co-culture P2 + P3 (*K. pneumoniae* SRP2 + *K. variicola* SRP3); a) glycerol utilization, 2,3-BD and acetoin production, (b) 1,3-PDO, acetate and biomass production. MS-2 medium supplemented with biodiesel-derived crude glycerol 75.0 g/L (glycerol 37.5 g/L).

Table 2

Comparison of 2,3-BD production using crude glycerol as the sole carbon source in batch culture process.

Name of bacterial strains	Carbon source	Product (g/L)	Yield (g/g)	References
<i>Klebsiella oxytoca</i> M1	Crude glycerol	8.9	0.12	[38]
<i>K. oxytoca</i>	Crude glycerol	4.3	0.24	[39]
<i>Enterobacter aerogenes</i>	Crude glycerol	22.0	0.4	[39]
<i>Bacillus amyloliquefaciens</i>	Crude glycerol	18.2	0.35	[41]
<i>K. oxytoca</i> FMCC-197	Crude glycerol	4.8	0.18	[40]
<i>Raoultella terrigena</i> CECT 4519	Crude glycerol	33.6	0.38	[42]
<i>K. pneumoniae</i> SRP2 and <i>K. variicola</i> SW3 (co-culture)	Crude glycerol	27.87	0.73	This study

BD from environmental consortia of microorganisms. We have demonstrated that the co-culture developed by using two strains *K. pneumoniae* SRP2 and *K. variicola* SRP3 isolated newly from natural bacterial consortium could be a potential system for efficiently utilizing glycerol to produce a high product yield of 2,3-BD using low-value or negative – value biodiesel-derived raw glycerol as a feed-stock in industrial bioconversion process. Consequently, further studies with these newly isolated novel strains are granted to increase 2,3-BD production as well as the utilization rate of crude glycerol. Moreover, finding alternatives to chemical methods of crude glycerol conversion remains an important goal biotransformation involving microbes as biocatalysts is an expressively promising and advanced green method.

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