Asia-Pacific Journal of Science and Technology: Volume: 27. Issue: 02. Article ID.: APST-27-02-05. Research Article

APST

Asia-Pacific Journal of Science and Technology

https://www.tci-thaijo.org/index.php/APST/index

Published by the Research and Graduate Studies,
Khon Kaen University, Thailand

Isolation of bacteria utilizing glycerol as a sole carbon source for potential production of 1,3-propanediol

Nittiyaphorn Sotapong[1,4], Mallika B. Kongkeitkajorn[2,3] and Atcha Oraintara[1,*]

[1]Department of Microbiology, Faculty of Science, Khon Kaen University, Khon Kaen, Thailand
[2]Department of Biotechnology, Faculty of Technology, Khon Kaen University, Khon Kaen, Thailand
[3]Fermentation Research Center for Value Added Agricultural Products, Khon Kaen University, Khon Kaen, Thailand
[4]Graduate School, Khon Kaen University, Khon Kaen , Thailand
[*]Corresponding author: atcha@kku.ac.th

Received 8 June 2021
Revised 26 July 2021
Accepted 8 August 2021

Abstract

Glycerol is a by-product waste from biodiesel production, which can be converted by bacteria to other value-added products. One of these products is 1,3-propanediol (1,3-PDO), an important substrate for synthesis of biopolymer and many others. This research aims to isolate bacteria that can utilize glycerol as a sole carbon source and examine the 1,3-PDO production ability of these isolates using glycerol as a substrate. Bacterial isolates were obtained from soil near biodiesel plant by culturing in mineral salt medium containing glycerol. Growth of selected isolates in a medium containing glycerol as a sole carbon source were monitored and compared with reference bacterial strains. It was observed that 2 isolates grew better than reference strains according to optical density value at 600 nm. Both isolates were identified as *Klebsiella pneumoniae* by 16S rRNA gene sequencing. The isolate 5-3, renamed as *K. pneumoniae* KKU5 (Kp KKU5) was used for further study. The *dhaT* gene of Kp KKU5 shared 99% identity with those of *K. pneumoniae* (CP025630.1). To examine the ability of Kp KKU5 as a 1,3-PDO producer, its fermentation broth containing glycerol was analyzed by high-performance liquid chromatography. Under batch fermentation conditions with 20 g/L initial glycerol, Kp KKU5 produced 0.375 g/L of 1,3-PDO after 7 days. Despite its low production of 1,3-propanediol, we have demonstrated that Kp KKU5 was able to utilize glycerol as a sole carbon source. This study might provide some useful information to contribute in the research area of 1,3-PDO production, that adds value to glycerol waste from biodiesel production.

Keywords: 1,3-Propanediol, Glycerol utilizing bacteria, *Klebsiella pneumoniae*, 1,3-propanediol oxidoreductase

1. Introduction

In a state of energy crisis, the petroleum demand and price keep increasing. Alternative energy options have been sought in order to support demand of world's growing population. One of the current important energy alternative is biodiesel. Its production rate around the world is expanding rapidly. A by-product from biodiesel production is raw glycerol, which may contain up to 10% of the total product [1]. Generally, pure glycerol is an important reagent in industries such as food, pharmaceutical and cosmetics manufacturing. Raw glycerol, however, cannot be used directly in these industries and the cost of glycerol purification process is significantly high. Therefore, researchers find an alternative way to convert raw glycerol into value-added products such as 1,3-propanediol (1,3-PDO), lactic acid (2-hydroxypropionic acid), succinic acid, citric acid, acetic acid, hydrogen, trehalose, glyceric acid (GA), docosahexaenoic acid (DHA), polyhydroxyalkanoates (PHA) from biological conversions [2,3].

Production of 1,3-PDO has drawn more attention from researchers around the world. It can be used as solvent in cosmetics industries and used as monomer for synthesis of biopolymers. A global demand for 1,3-PDO production has been growing continuously, growing at a Compound Annual Growth Rate (CAGR) of 5.8% from 2015 to 2022 [4]. 1,3-PDO can be produced via chemical and biological processes. It can be produced biologically

from fermentation process of microorganisms that can utilize glycerol. The advantages of biological process in 1,3-PDO production is that the product is very specific and it is environmental friendly. Therefore, 1,3-PDO production from glycerol as a source for carbon and energy by microbes has been extensively studied. Generally, bacterial genus *Klebsiella, Clostridium, Citrobacter,* and *Lactobacillus* are natural 1,3-PDO producers. Enzymes involved in glycerol metabolism from these microorganism have been widely reported [5].

There have been two glycerol metabolic pathways reported from bacterial cells so far: an oxidative pathway and a reductive pathway. In oxidative pathway, glycerol is converted to DHA and then DHA is dephosphorylated to pyruvate and further converted to acetate, butyrate, lactate, ethanol, butanol, hydrogen and CO_2, depending on bacteria species and growth conditions. In reductive pathway, conversion of glycerol to 1,3-PDO is regulated by two enzymes, coenzyme B_{12}-dependent glycerol dehydratase (GDHt) and 1,3-propanediol-oxidoreductase (PDOR). GDHt converts glycerol to 3-hydroxypropionaldehyde (3-HPA) followed by a reduction of 3-HPA to 1,3-PDO by PDOR [5,6]. Some microorganism such as *Klebsiella pneumoniae, Clostridium butyricum, Citrobacter freundii, Lactobacillus brevis* and *Clostridium pasteurianum* synthesize enzymes involved in reductive pathway, which are encoded by *dha* regulon [7]. The GDHt and PDOR are encoded by *dhaB* and *dhaT* gene, respectively. Production of 1,3-PDO from glycerol into bacterial cells can occur under both anaerobic or micro-aerobic conditions. To date, 1,3-PDO production from *K. pneumoniae* and *C. butyricum* have been mostly reported. This might be due to their ability to grow well under limited aerobic conditions, because of which high yield and productivity was observed. Researcher currently focus on manipulating genes involved in those aforementioned pathways to improve yield of 1,3-PDO production by used glycerol as renewable substrate.

In this study, we successfully isolated bacteria that can utilize glycerol as a sole carbon source. The isolate was further characterized and 1,3-PDO production of the isolate was observed by high-performance liquid chromatography (HPLC).

2. Materials and methods

2.1 Bacterial strains

To initially compare glycerol-utilizing capability of the isolates and sequential 1,3-PDO production in the future, following bacterial strains capable of producing 1,3-PDO from glycerol were used as positive control. *Klebsiella terrigena* TISTR 1561 and *Lactobacillus buchneri* TISTR 048 were purchased from Thailand Institute of Scientific and Technological Research (TISTR, Thailand). *Klebsiella variicola* TBRC 1174 was purchased from The National Center for Genetic Engineering and Biotechnology (BIOTEC, Thailand). Bacterial strains were stored at -20℃ and afterward were recultivated. For *Klebsiella* sp. were cultivated in Luria-bertani (LB) medium at 37℃ with rotation, *Lactobacillus* sp. was cultivated in De Man, Rogosa and Sharpe (MRS) medium at 30℃ without shacking. MRS medium contains following ingredients: peptone 10 g/L, beef extract 10 g/L, yeast extract 5 g/L, tween-80 1 g/L, K_2HPO_4 2 g/L, ammonium citrate 2 g/L, sodium acetate 5 g/L, $MgSO_4$ 0.2 g/L, and $MnSO_4$ 0.05 g/L, pH 6.2 ± 0.2 [8]. LB medium contains tryptone 10 g/L, yeast Extract 5 g/L, NaCl 10 g/L, pH 7.5 ± 0.2 [9].

2.2 Chemicals and carbon source

1-3-PDO (98% purity) was purchased from Sigma Aldrich (USA). Pure glycerol (> 98% purity) was purchased from KemAus (Australia). Crude glycerol was purchased from the Biodiesel Pilot Plant from Used Vegetable Oil, Khon Kaen University, Khon Kaen, Thailand. The other chemicals used in this study were of analytical grade purity.

2.3 Enrichment and isolation of glycerol-utilizing bacteria from soil

Soil samples contaminated with raw glycerol were collected from the area of Biodiesel Pilot Plant from Used Vegetable Oil, Khon Kaen University, Khon Kaen, Thailand. Ten grams of soil were inoculated into 50 mL of mineral salt medium (MSM), which contains following ingredients: $NaNO_3$ 4 g/L, NaCl 1 g/L, KCl 1 g/L, $CaCl_2.2H_2O$ 0.1 g/L, KH_2PO_4 3 g/L, $Na_2HPO_4.H_2O$ 3 g/L, $MgSO_4$ 0.2 g/L, $FeSO_4.7H_2O$ 1 mg/L, and trace element 2 mL. The composition of trace elements was: $FeCl_3.6H_2O$, $ZnSO_4.7H_2O$, $CoCl_2.6H_2O$, $CuSO_4.5H_2O$, $MnSO_4.H_2O$, H_3BO_3 and $Na_2MoO_4.2H_2O$. Glycerol (> 98% purity, KemAus, Austalia) was added as a sole carbon source to the final concentration of 20 g/L and pH of the medium was adjusted to 6.8 [10]. The soil mixture was incubated at 35℃, 120 rpm for 48 h. After that, the mixture was transferred to fresh MSM with glycerol and further incubated in the same condition. Then it was plated on LB agar and incubated at 30℃, for 24 h. Grown isolates were purified by cross streak and further investigated. Single colony of each isolate were observed under microscope by gram staining technique [11].

2.4 Cultivation of bacterial strains in medium containing glycerol as sole carbon source

All tested bacterial strains and isolates were grown in MSM or LB broth with glycerol. Cell cultivations were carry out in shake flasks at 37℃, 120 rpm overnight, then adjusted to an optical density of 0.2 at 600 nm in 100 mL fresh MSM containing either 20 g/L pure glycerol or 20 g/L crude glycerol. The culture was then incubated at 37℃, 120 rpm and OD at 600 nm that was monitored every 24 h. The condition of bacteria cultivation was modified from Waghmare (2015) [10]. The cultivation with pure glycerol was done in duplicate, each with 3 biological replicates. The rest of all experiments were done in triplicates, each with 3 biological replicates.

2.5 Amplification and identification of 16S rRNA gene and dhaT gene

Genomic DNA was extracted using the rapid method described by Rodriguez [12], and 16S rRNA sequence was PCR-amplified using the universal primers: 27F (5' AGA GTT TGA TCC TGG CTC AG 3') and 1492R (5' TAC GGC TAC CTT GTT ACG ACT T 3') [7]. The reaction was performed with *Taq* DNA polymerase (Vivantis, Malaysia) under following conditions: denaturation at 94℃ for 2 min, 35 cycles of 30 s at 94℃, 30 s at 50℃, 30 s at 72℃, and a final extension 72℃ for 7 min. PCR product was purified by GeneJET PCR Purification Kit (Thermo Fisher Scientific, USA) and sequenced (Macrogen, Korea). Nucleotide sequence of 16S rRNA gene was analyzed by using BLAST tool of NCBI (https://www.ncbi.nlm.nih.gov/). Based on the homology of 16S rRNA sequence, strains with 16S rRNA sequence from relative species were selected, and multiple sequence alignment comparison was performed by clustalW program. A phylogenetic tree was constructed using the PhyML method based on MEGA 7.0 software. A phylogenetic tree was constructed by MEGA program (version X) [13] using the maximum-likelihood method with 1,000 bootstrap replicates.

To identify nucleotide sequence of *dhaT* gene, genomic DNA of the isolates was used as DNA template to amplify the *dhaT* gene with following primers: FW_dhaT_KV/HindIII (5' CCCCC AAG CTT ATG AGC TAT CGT ATG TTT GAT TAT CTG G 3') and RV_dhaT_KV/XhoI (5' CCCGG CTC GAG TCA GAA TGC CTG GCG G 3'). The reaction was performed with KOD One™ PCR master mix (Toyobo, Japan) under the following conditions: denaturation at 98℃ for 20 s, 30 cycles of 10 s at 98℃, 5 s at 57℃, 15 s at 68℃, and a final extension 68℃ for 10 min. The PCR products were purified by Nucleospin® Gel and PCR Clean-Up Kit (Macherey-Nagel, Germany) and sequenced (Macrogen, Korea). The sequence of *dhaT* gene was analyzed based on similarities with closely related sequences within the GenBank database and the sequence was deposited in Genbank database.

2.6 Determination of 1,3-propanediol production

The bacterial isolate was grown in LB broth, incubated at 37℃, 120 rpm overnight, then culture broth was transferred to 50 mL of fresh MSM medium containing 20 g/L pure glycerol as substrate. Optical density was adjusted to 0.2 at 600 nm, and the culture was incubated at 37℃, 120 rpm for 7 days. Culture samples were taken every 24 h. The samples drawn were centrifuged at 16,000 g for 5 min and supernatants were kept for analysis. Determination of 1,3-PDO and glycerol were analyzed using HPLC (LC-20A, Shimadzu, Japan) equipped with Aminex HPX-87H column (Bio-rad, USA) and refractive index detector (RID-6A. Shimadzu, Japan). The working conditions were as follow: 5 mM H_2SO_4 as mobile phase at 40℃ column temperature with flow rate of 0.75 mL/min. Glycerol (> 98% purity, KemAus, Australia) and 1,3-PDO (98% purity, Sigma Aldrich, USA) were used as standard. Glycerol was detected at retention times (RT) of 10.42 min and 1,3-PDO was detected at RT of 13.04 min. The concentration of residual substrate (glycerol) and product (1,3-PDO) were calculated with relationships between peak-area of standard.

2.7 statistical analysis

The cultivation with pure glycerol was done in duplicate, each with 3 biological replicates. The rest of all experiments were done in triplicates, each with 3 biological replicates. The reported data are averaged value. Error bars represent standard deviations from each obtained value.

3. Results

3.1 Isolation of glycerol-utilizing bacteria

Soil samples in biodiesel production plant area were used for isolation of glycerol-utilizing bacteria. A total of 73 colonies were obtained from initial culture on glycerol-containing agar plates. Gram-positive and Gram-negative bacteria with both rod and spherical shaped were obtained from the initial screening as shown in Table 1. After further selection, ten different bacterial isolates were narrowed down from those obtained from initial

screening. The isolates 3-1 and 5-3 could grow well in the medium containing pure glycerol as sole carbon source as shown in Figure 1.

Table 1 colony morphology of isolates.

Source	Isolates	Gram / Shaped	Morphology
Soil	1-1	Positive, shot-rod	Circular, entire, creamy-white, mucoid, convex
	2-1	Positive, rod	Circular, undulate, white, oily
	3-1	Negative, rod	Small circular, undulate, milky white
	3-2	Negative, rod	Circular, undulate, milky white
	4-1	Positive, coccus	Circular, entire, milky white, mucoid, convex
	4-2	Negative, rod	Circular, undulate, milky white
	4-3	Negative, rod	Small circular, undulate, rough, white
	5-1	Negative, rod	Circular, milky white, mucoid, convex
	5-2	Positive, coccus	Circular, undulate, dry white, oily
	5-3	Negative, rod	Circular, entire, milky white, mucoid, convex

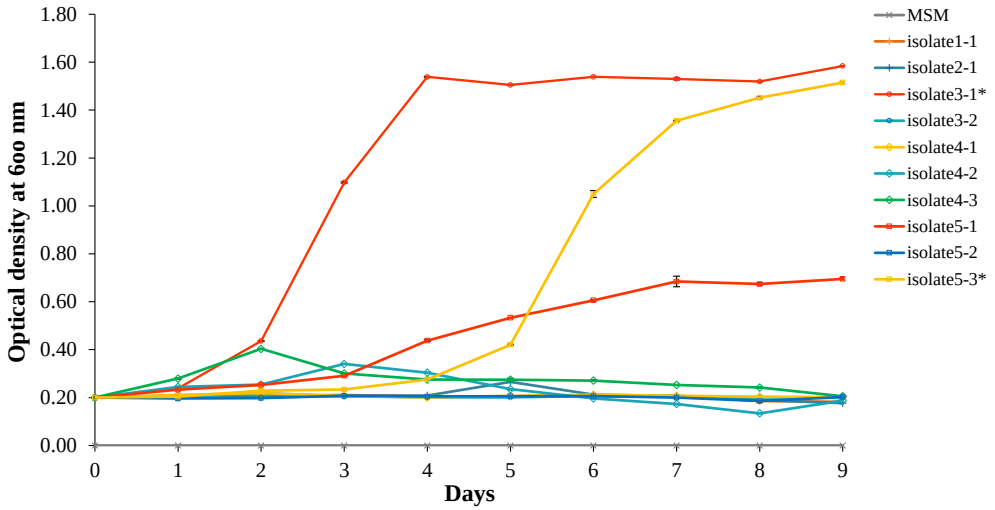

Figure 1 Growth curve of isolated strains when cultivate on MSM containing 20 g/L pure glycerol. The inocula were prepared in LB broth (Gray line labeled MSM = medium incubated without cells).

Since isolate 3-1 and 5-3 showed superior growth than the other isolates, their growth was compared with reference bacteria known for their ability of utilizing glycerol, namely *K. terrigena* TISTR 1561, *K. variicola* TBRC 1174, *L. buchneri* TISTR 048. The result indicated that liquid bacterial culture of the isolate 3-1 reached its highest OD_{600} after overnight incubation in media containing pure glycerol as a sole carbon source, followed by isolate 5-3, *K. variicola* TBRC 1174, *K. terrigena* TISTR 1561 and *L. buchneri* TISTR 048. Optical density of all tested bacterial culture declined after the first day of cultivation (Figure 2A), where OD_{600} reached the highest value.

However, growth of all tested bacteria was differently observed in MSM media containing crude glycerol as a sole carbon source. Their growth curves increased slowly from day 1 to day 7 and rapidly decreased afterwards (Figure 2B). It was found that the isolate 5-3 and *K. variicola* TBRC 1174 were able to grow well under this condition.

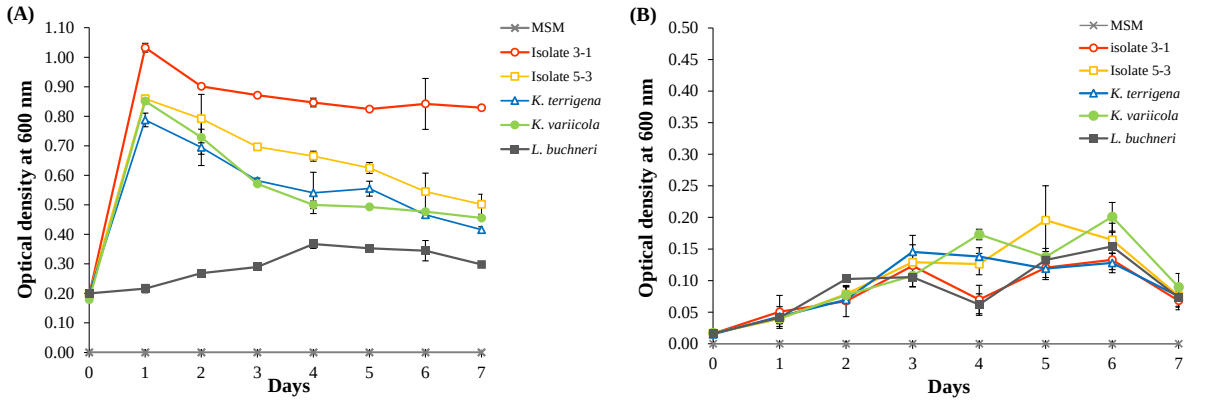

Figure 2 Growth curves of isolate 3-1 and 5-3 comparing with reference bacteria when cultivated in MSM medium containing 20 g/L pure glycerol (A) and 20 g/L crude glycerol (B) as a sole carbon source. The inocula were prepared in MSM broth (Gray line labeled MSM = medium incubated without cells).

3.2 16S rRNA gene and dhaT gene analysis

Nucleotide sequence analysis of 16S rRNA genes of the isolate 3-1 and 5-3 showed 99% identity with *K. pneumoniae* strain YYR54-1 (MF767579.1). The result of phylogenetic tree analysis of both isolates is shown in Figure 3. Both of them were clustered in *K. pneumoniae* subsp. The isolate 5-3 was selected for further study and named *K. pneumoniae* strain KKU5 (Kp KKU5).

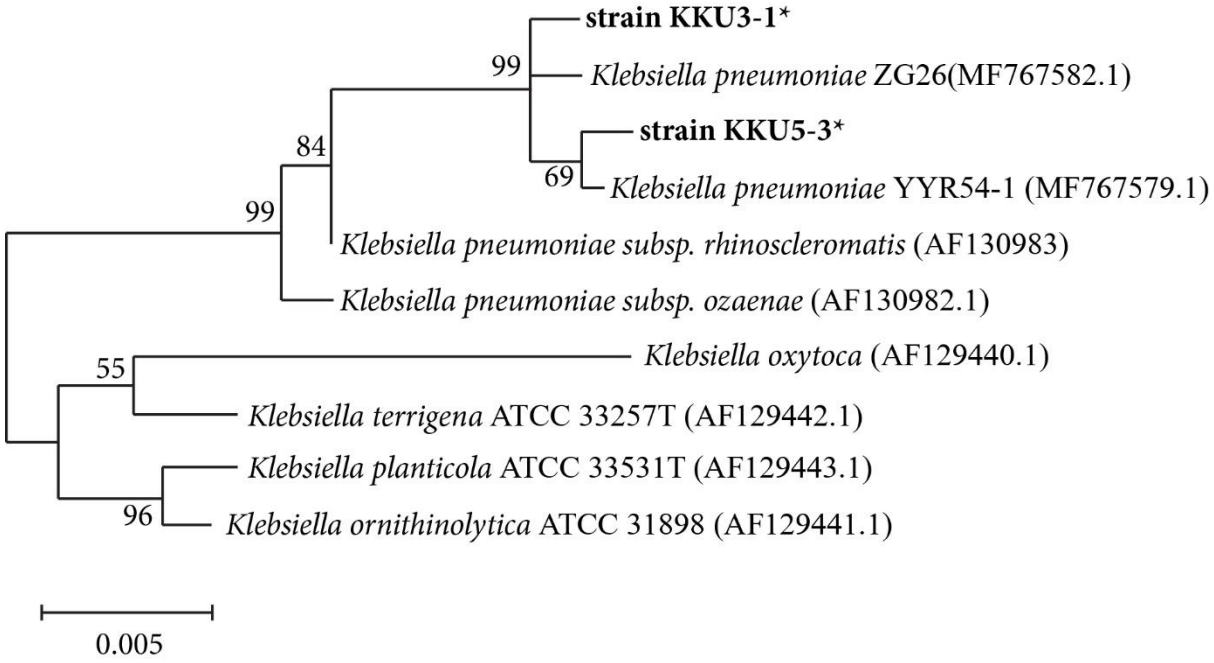

Figure 3 A phylogenetic tree constructed from maximum likelihood method base on 16S rRNA gene of *Klebsiella* sp. comparison showing position of isolate strain 3-1 and 5-3 similarities with *K. pneumoniae*. Bar scale indicate 0.005% of sequence dissimilarity.

Genes in the dha regulon, namely *dhaB* and *dhaT*, which encode enzymes involved in reductive pathway of 1,3-PDO production presented in Kp KKU5 were also analyzed. The complete nucleotide sequence of the *dhaT* gene contains 1,164 nucleotides which encodes a sequence of 387 amino acids. Nucleotide sequence of the *dhaT* gene of Kp KKU5 was close to 99% identity with *K. pneumoniae* (CP025630.1). Amino acid sequence of the encoded gene product shared 99.7% homology with PDOR of *K. pneumoniae* strain MGH78578 (WP_149795854.1). The *dhaT* nucleotide sequence of the isolate KKU5 was deposited in Genbank with accession number MT773418. Analysis of the *dhaB* gene is currently under process.

3.3 Testing for 1,3-propanediol production from glycerol by K. pneumoniae strain KKU5

The ability of *K. pneumoniae* strain KKU5 (Kp KKU5) as a 1,3-PDO producer was verified by batch fermentation in MSM medium containing 20 g/L glycerol under slightly aerated conditions (120 rpm) at 37℃. Supernatant from fermentation broth was analyzed for 1,3-PDO production by HPLC. The bacterial culture reached stationary phase after 2 days of fermentation. The fermentation broth was collected on day 7, by then cells density (OD_{600}) was 10 ± 0.3. As shown in Figure 4A, mixed standard compound of glycerol and 1,3-PDO (1g/L each) could be clearly separated from each other. The result of HPLC analysis of fermentation broth of Kp KKU5 is presented in Figure 4B. It was found that residual glycerol in the fermentation broth was less than 0.9 g/L and 1,3-PDO was produced at a concentration of 0.375 g/L. An unknown substance was detected with the highest amount among these three substances found in the fermentation broth.

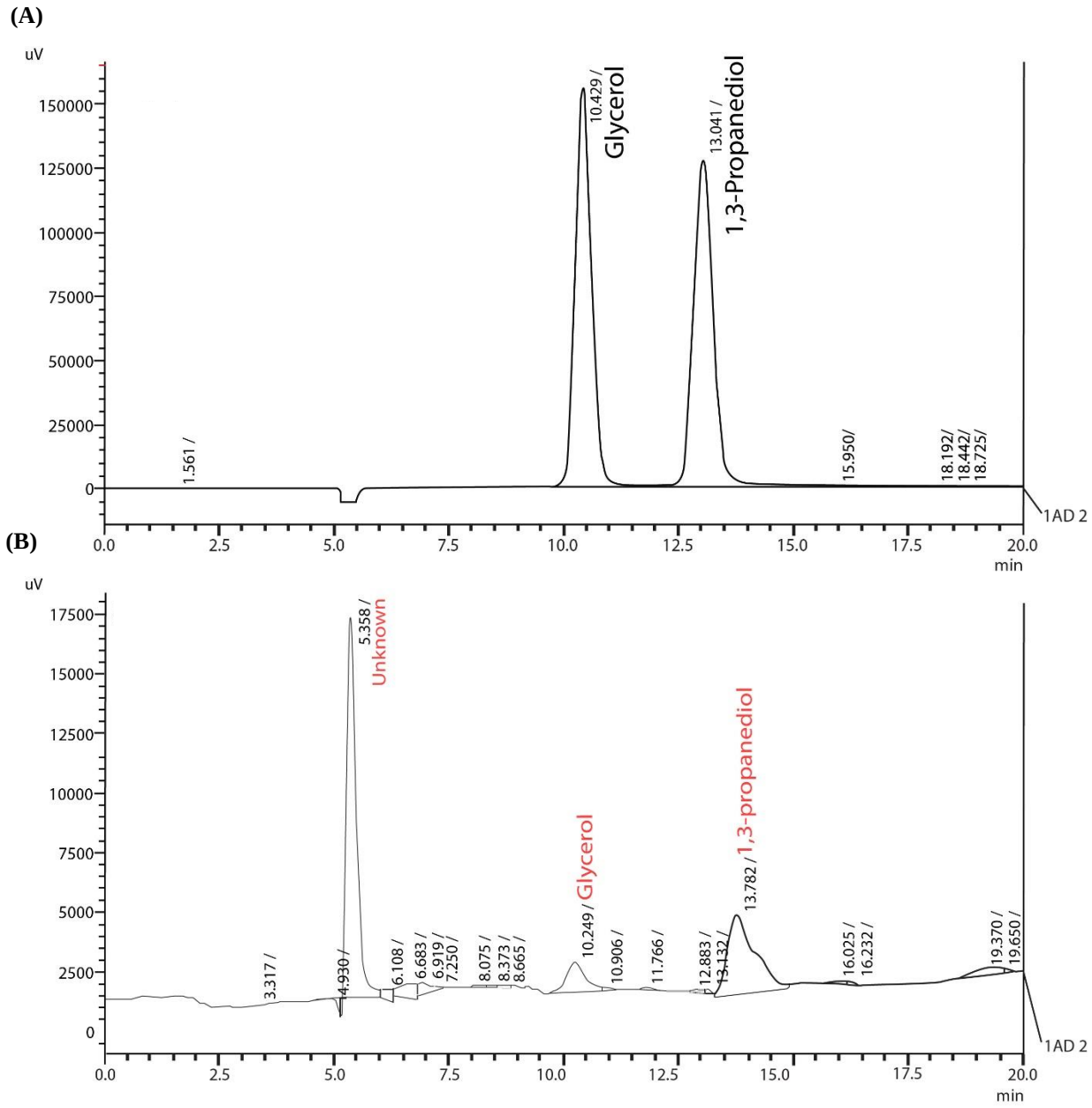

Figure 4 HPLC chromatogram of standard solution containing glycerol and 1,3-PDO (A), fermentation broth of Kp KKU5 on day 7 (B). Bacterial cells were cultured in MSM medium containing 20 g/L glycerol under slightly aerated condition (120 rpm) at 37℃.

4. Discussion

A number of microorganisms able to grow aerobically and anaerobically on glycerol as the sole carbon source have been reported, including *Citrobacter, Klebsiella, Enterobacter, Clostridium* spp. The production of 1,3-PDO from glycerol using bacterial strains has been demonstrated such as *L. brevis, L. buchnerii, Bacillus welchii, C. freundii, K. pneumoniae, C. pasteurianum, C. butyricum* and *Enterobacter agglomerans* [1]. In this study, the isolated strain was identified to *K. pneumoniae*, which is a facultative anaerobic bacterium. Moreover, we analyzed the *dhaT* gene sequence, which encoded 387 amino acids and involves the synthesis of 1,3-PDO oxidoreductase/dehydrogenase. Generally, in the genus of *Klebsiella*, 1,3-PDO is synthesized from glycerol via a reductive pathway. Glycerol is preferably converted to 3-HPA by GDHt, encoded by *dhaB* gene cluster rather than to 1,3-PDO by PDOR, encoded by *dhaT* gene [14]. GDHt activity in a reductive pathway required coenzyme-B_{12} as a precursor. *K. pneumoniae* can naturally synthesize coenzyme B_{12} under anaerobic and micro-aerobic condition, which supports 1,3-PDO production [6,15].

All ten isolates could grow in medium containing glycerol as a sole carbon source. However, when growth in MSM medium with pure glycerol and crude glycerol was compared, all tested bacteria clearly grew much slower in crude glycerol. They never reached their highest OD_{600} on day 6, whereas it took only overnight to reach their highest OD_{600} in pure glycerol. The reason might be that nutrients in crude glycerol are limited and there is the presence of waste and various substances which may be cytotoxic to bacterial cells. Components in crude glycerol generated from biodiesel production contain glycerol, alcohol, inorganic salts, free fatty acid, triacylglycerol, methyl esters and water [16]. These components could affect bacterial growth by interfering with both cells structure and cells metabolism. Effects of crude glycerol on cells metabolism rather than pure glycerol was previously report as well [17]. In addition, fatty acid is the main component of cells membrane and the presence of fatty acid in crude glycerol can also interfere with the synthesis of metabolites within bacterial cells [18].

In this study, *K. pneumoniae* strain KKU5 was able to effectively utilize glycerol as a sole carbon source, since the concentration of glycerol in day 7 dropped drastically from 20 g/L to 0.9 g/L. However, when compared with other previous reports, 1,3-PDO production in this study still needs to be optimized [15,19-21]. Since most studies carried out the production of 1,3-PDO by batch fermentation under anaerobic or micro-aerobic condition, fermentation condition should be another key factor affecting 1,3-PDO production. In our report, *K. pneumoniae* strain KKU5 was cultured with glycerol as a sole carbon source under slightly aerated condition (120 rpm) with limited nutrients (mineral salt medium; MSM), which resulted in low production of 1,3-PDO. One reason for this low product yield might be due to aeration condition. When oxygen is present, facultative anaerobic bacteria makes ATP by aerobic respiration, as a result glycerol might be used in an oxidative pathway to form energy and carbon constituents for cells growth. Therefore, under aerobic condition, glycerol was not brought into reductive pathway, by which 1,3-PDO is produced. Furthermore, it has been reported that the presence of O_2 in fermentation broth would inactivate GDHt activity [22]. GDHt activity is also limited by high concentration of glycerol and 3-HPA, both of these can inhibit GDHt and irreversible suicidal inactivation of GDHt [1]. Besides this, there was a report that stated cells growing under nutrients limitation could result in a reduced 1,3-PDO production [7].

The unknown metabolite in fermentation broth with the highest peak-area might be 3-HPA, an intermediate compound in 1,3-PDO pathway. 3-HPA is generated from a conversion of GDHt. 3-HPA is needed to be further reduced to 1,3-PDO by PDOR). 3-HPA has been reported to be released from HPLC column earlier than glycerol and 1,3-PDO, with RT 6.87, 16.37 and 18.50 min, respectively. The HPLC column in Chen's report [23] was the same as used in this study but with a slightly different condition. A number of small peak-area was observed in the HPLC chromatogram of this study. They could be other metabolites or organic acids that were released during fermentation. All aforementioned factors could affect 1,3-PDO production. Therefore, our further study will focus on optimization of condition for 1,3-PDO production by *K. pneumoniae* strain KKU5.

5. Conclusion

In this study, glycerol-utilizing bacteria were isolated from soil samples in a biodiesel plant. A newly isolated *K. pneumoniae* strain KKU5 was identified. It can grow well in medium containing 20 g/L glycerol and show a comparable growth rate when compared with reference bacteria that are known as natural 1,3-PDO producers. The complete *dhaT* gene sequence of strain KKU5 was deposited in Genbank (Accession number MT773418). The gene is 1,164 nucleotides long encoding 387 amino acids. From initial assessment under limited aeration and mineral medium, *K. pneumoniae* strain KKU5 could effectively utilize glycerol as a sole carbon source and could grow in crude glycerol. The isolate also shows production of 1,3-PDO from glycerol. Therefore, *K. pneumoniae* strain KKU5 could be a potential 1,3-PDO producer and studies to enhance the production by this strain could be further investigated.

6. Acknowledgements

This research was supported by Protein and Proteomics Research Center for Commercial and Industrial Purposes (ProCCI), Khon Kaen University and published in Proceedings of Thai Society for Biotechnology International Conference Online "Green Energy & Zero Waste Society" (TSB Online 2021). The authors thank Ms. Sreyden Hor for her technical assistance in HPLC analysis. We would like to acknowledge Dr. Gurdeep Singh, for editing the manuscript via Publication Clinic KKU, Thailand.

7. References

[1] Jiang W, Wang S, Wang Y, Fang B. Key enzymes catalyzing glycerol to 1,3-propanediol. Biotechnol Biofuels. 2016;9(1):1-19.
[2] Kaur J, Sarma A, Jha M, Gera P. Valorisation of crude glycerol to value-added products: perspectives of process technology, economics and environmental issues. Biotechnol Rep. 2020;27:e00487.
[3] Fokum E, Zabed H, Yun J, Zhang G, Qi X. Recent technological and strategical developments in the biomanufacturing of 1,3-propanediol from glycerol. Int J Environ Sci Technol. 2021;18(8):2467-2490.
[4] 1,3 Propanediol Market Report. https://www.grandviewresearch.com/press-release/global-1,3-propanediol-market; [Accessed 30 June 2021].
[5] Wischral D, Zhang J, Cheng C, Lin M, De Souza L, Pessoa F, et al. Production of 1,3-propanediol by *Clostridium beijerinckii* DSM 791 from crude glycerol and corn steep liquor: process optimization and metabolic engineering. Bioresour Technol. 2016;212:100-110.
[6] Zhou S, Lama S, Sankaranarayanan M, Park S. Metabolic engineering of *Pseudomonas denitrificans* for the 1,3-propanediol production from glycerol. Bioresour Technol. 2019;292:121933.
[7] Guo Y, Dai L, Xin B, Tao F, Tang H, Shen Y, et al. 1,3-Propanediol production by a newly isolated strain, *Clostridium perfringens* GYL. Bioresour Technol. 2017;233:406-412.
[8] Ju J, Wang D, Heo S, Kim M, Seo J, Kim Y, et al. Enhancement of 1,3-propanediol production from industrial by-product by *Lactobacillus reuteri* CH53. Microb Cell Factories. 2020;19(1):1-10.
[9] LB (Luria-Bertani) liquid medium. Cold Spring Harbor Protocols. http://cshprotocols.cshlp.org/content/ 2006/ 1/pdb.rec8141.full?text_only; [Accessed 8 June 2021].
[10] Isolation, screening and characterization of crude glycerol utilizing microorganism for the production of 1, 3-propanediol. https://www.academia.edu/20403847/Isolation_screening_and_characterization_of_crude _glycerol_utilizing_microorganism_for_the_production_of_1_3_propanediol; [accessed 2 June 2021].
[11] Gram Stain Protocols. https://asm.org/getattachment/5c95a063-326b-4b2f-98ce-001de9a5ece3/gram-stain-protocol-2886.pdf; [Accessed 28 July 2021].
[12] How can I extract Lactobacillus DNA without extraction kits?. https://www.researchgate.net/post/How_ can_I_extract_ Lactobacillus_DNA_without_extraction_kits; [Accessed 15 January 2018].
[13] Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol. 2018;35(6):1547-1549.
[14] Li Y, Wang X, Ge X, Tian P. High production of 3-hdroxypropionic acid in *Klebsiella pneumoniae* by systematic optimization of glycerol metabolism. Sci Rep. 2016;6(1):1-10.
[15] Durgapal M, Kumar V, Yang T, Lee H, Seung D, Park S. Production of 1,3-propanediol from glycerol using the newly isolated *Klebsiella pneumoniae* J2B. Bioresour Technol. 2014;159:223-231.
[16] Chatzifragkou A, Papanikolaou S. Effect of impurities in biodiesel-derived waste glycerol on the performance and feasibility of biotechnological processes. Appl Microbiol Biotechnol. 2012;95(1):13-27.
[17] Szymanowska-Powałowska D. The effect of high concentrations of glycerol on the growth, metabolism and adaptation capacity of *Clostridium butyricum* DSP1. Electron J Biotechnol. 2015;18(2):128-133.
[18] Samul D, Leja K, Grajek W. Impurities of crude glycerol and their effect on metabolite production. Ann Microbiol. 2014;64(3):891-898.
[19] Chen W, Chuang C, Chang J, Wang L, Soo P, Wu H, et al. Exploring dual-substrate cultivation strategy of 1,3-propanediol production using *Klebsiella pneumoniae*. Appl Biochem Biotechnol. 2019;191(1):346-359.
[20] da Silva G, de Lima C, Contiero J. Production and productivity of 1,3-propanediol from glycerol by *Klebsiella pneumoniae* GLC29. Catal Today. 2015;257:259-266.
[21] Laura M, Monica T, Dan-Cristian V. The effect of crude glycerol impurities on 1,3-propanediol biosynthesis by *Klebsiella pneumoniae* DSMZ 2026. Renew Energy. 2020;153:1418-1427.
[22] Kumar V, Park S. Potential and limitations of *Klebsiella pneumoniae* as a microbial cell factory utilizing glycerol as the carbon source. Biotechnol Adv. 2018;36(1):150-167.
[23] Chen H, Fang B, Hu Z. Simultaneous HPLC determination of four key metabolites in the metabolic pathway for production of 1,3-propanediol from glycerol. Chromatographia. 2007;65(9-10):629-632.