

Sains Malaysiana 47(3)(2018): 481–488
<http://dx.doi.org/10.17576/jsm-2018-4703-07>

Bioconversion of Biodiesel-derived Crude Glycerol to 1,3-Dihydroxyacetone by a Potential Acetic Acid Bacteria

(Biopenukaran Gliserol Mentah Janaan Biodiesel kepada 1,3-Dihidroksiaseton oleh Potensi Bakteria Asid Asetik)

VARAVUT TANAMOOL, PIYOROT HONGSACHART & WICHAI SOEMPHOL*

ABSTRACT

Acetic acid bacteria (AAB) isolated from natural resources and fermented plant beverages were screened to produce 1,3-dihydroxyacetone (DHA) from non-detoxified crude glycerol. Among them, the isolate NKC115 was identified as Gluconobacter frateurii and produced the highest amounts of DHA. Subsequently, the effects of growth-medium conditions (initial pH, crude glycerol concentration and nitrogen sources) on growth and DHA-production capability were examined. The results showed that the crude glycerol concentration increase to above 100 g/L suppressed growth and DHA production. The highest amount of DHA obtained was 27.50 g/L, from an initial crude glycerol concentration of 100 g/L. Meanwhile, an initial pH of 5.5-7.5 in the YPGc medium did not significantly affect the bacterial growth and DHA production. The optimal nitrogen source was peptone, with DHA production at 34.70 g/L. Furthermore, overexpression of the nhaK2 gene encoding for the Na⁺(K⁺)/H⁺ antiporter from Acetobacter tropicalis SKU1100 in G. frateurii NKC115 improved growth and increased the accumulation of DHA (37.25 g/L) from an initial crude glycerol concentration of 20%. These results indicated that the expression of this antiporter might maintain an optimal intracellular pH and concentration of Na⁺ or K⁺, leading to the cells' ability to tolerate high concentrations of crude glycerol.

Keywords: Acetic acid bacteria; biodiesel; crude glycerol; dihydroxyacetone

ABSTRAK

Bakteria asid asetik (AAB) yang dipencilkan daripada sumber alam dan minuman fermentasi berasaskan tumbuhan telah ditapis untuk menghasilkan 1,3-dihidroksiaseton (DHA) daripada gliserol mentah yang belum disingkirkan toksiknya. Antara mereka, pencilan NKC115 telah dikenal pasti sebagai Gluconobacter frateurii dan menghasilkan jumlah DHA yang tertinggi. Seterusnya, kesan keadaan medium pertumbuhan (pH pemula, kepekatan gliserol mentah dan sumber nitrogen) terhadap pertumbuhan dan kemampuan penghasilan DHA telah dikaji. Keputusan menunjukkan kepekatan gliserol mentah bertambah melebihi 100 g/L pertumbuhan kawalan dan penghasilan DHA. Jumlah DHA tertinggi diperoleh adalah 27.50 g/L, daripada kepekatan gliserol mentah 100 g/L. Sementara itu, pH pemula 5.5-7.5 di dalam medium YPGc tidak mempengaruhi pertumbuhan bakteria dan penghasilan DHA dengan nyata. Sumber optimum nitrogen ialah pepton dengan penghasilan DHA pada 34.70 g/L. Tambahan pula, ekspresi melampau daripada gen pengekodan nhaK2 untuk antipengangkat Na⁺(K⁺)/H⁺ daripada Acetobacter tropicalis SKU1100 dalam G. frateurii NKC115 memperbaiki pertumbuhan dan meningkatkan pengumpulan DHA (37.25 g/L) daripada kepekatan gliserol mentah pemula sebanyak 20%. Keputusan ini menunjukkan ekspresi antipengangkat berkemungkinan mengekalkan pH intrasel yang optimum dan kepekatan Na⁺ atau K⁺, menyebabkan kemampuan sel untuk menerima kepekatan gliserol mentah yang tinggi.

Kata kunci: Bakteria asid asetik; biodiesel; dihidroksiaseton; gliserol kasar

INTRODUCTION

Biodiesel is an essential alternative biofuel derived from trans-esterification of animal and vegetable fats. The worldwide production and consumption of biodiesel has increased dramatically in recent years. As a result, crude glycerol, a major waste by-product of biodiesel production, is generated at approximately 10% (w/w) in the biodiesel production process (Johnson & Taconi 2007). Due to high impurity and low price of the excess crude glycerol, an expensive refining process is required (Almeida et al. 2012). Using crude glycerol directly obtained from biodiesel production as raw material without any

additional treatment and purification to produce a value-added compound would be advantageous. Numerous studies have demonstrated the microbial bioconversion of glycerol into useful products, such as 1,3-propanediol (Xiu et al. 2004), butanol (Taconi et al. 2009), succinic acid (Lee et al. 2001), polyhydroxyalkanoates (Koller et al. 2005) and glyceric acid (Habe et al. 2009b). Similarly, microbial biotransformation of crude glycerol to 1,3-dihydroxyacetone (DHA) has been extensively studied and seems to be more effective than the chemical route (Liu et al. 2013). Dihydroxyacetone (DHA) is commonly used in cosmetic products (Brown 2001) and has been

proposed for use in the pharmaceutical industry (Fesq et al. 2001). Among the known microbial DHA producers, *Gluconobacter* has been widely used in commercial production due to its high productivity and low formation of by-products (Adachi et al. 2003). The effectiveness of oxidative fermentation is well recognized; it is carried out by various dehydrogenase enzymes on the periplasmic membrane, which transfer electrons to the aerobic respiratory chain for bioenergy generation (Matsushita et al. 1994). Such reactions lead to a rapid oxidation of the alternative sugar acids and sugar alcohols and consequently to a high accumulation of the corresponding products in the culture medium. Among these membrane-bound dehydrogenase enzymes, PQQ-dependent glycerol dehydrogenase (PQQ-GLDH) shows versatility in substrate specificity and is responsible for the oxidation of glycerol to dihydroxyacetone (Matsushita et al. 2003). However, the DHA-production capability of *G. oxydans* was seen to be influenced by several parameters such as substrate inhibition, product inhibition and oxygen limitation (Ma et al. 2010). Recently, it was shown that a mutant *G. oxydans* M5 defective in the genes encoding alcohol dehydrogenase and aldehyde dehydrogenase exhibited higher DHA production than the wild type and yielded more DHA than could be obtained by the over-expression of glycerol dehydrogenase (Li et al. 2010). In addition, using crude glycerol, which still contain impurities such as methanol, salts (Na^+ or K^+) and fatty acids, was seen to inhibit growth and production yield (Chatzifragkou & Papanikolaou 2012). One of the most challenging aspects of converting crude glycerol into value-added products, such DHA, is the need to find a highly effective microbial strain that can tolerate the impurities in crude glycerol. Several microorganisms have been investigated by random mutagenesis, for improvements in growth and production (Ma et al. 2010; Sato et al. 2014). This study attempts to screen and characterize a potential isolate of AAB for production of DHA from non-detoxified crude glycerol. In addition, the expression of a gene essential for pH homeostasis in *G. frateurii* NKC 115 will also be discussed.

MATERIALS AND METHODS

BACTERIA AND GROWTH CONDITIONS

YPGD medium consisting of 0.5% (w/v) glucose, glycerol, yeast extract and peptone (Soemphol et al. 2011) was used to prepare the seed culture. The cultivation was carried out on a rotary incubator shaker (Vision Scientific Co., Ltd. Korea) at 200 rpm at 30°C for 24 h. All newly isolated acetic acid bacteria (AAB) were previously isolated from natural resources. Then, the reference AAB strains were maintained on YPGD medium supplemented with 1.5% agar and 0.5% CaCO_3 . Cultures were kept active by periodic transfer to freshly prepared agar medium at 4°C.

CRUDE GLYCEROL

Crude glycerol was provided by the Faculty of Engineering, Khon Kaen University, at a glycerol purity of approximately 50% (w/w) (Teeka et al. 2012).

SCREENING EFFECTIVE ACETIC ACID BACTERIA FOR THE PRODUCTION OF 1,3-DIHYDROXYACETONE (DHA)

To examine the ability to produce DHA from crude glycerol, all isolates were cultured in YPGD medium for 18 h under the same conditions as described above. Five percent (v/v) of each cell culture was then transferred into 5 mL of YPGc, which was modified as described by Soemphol et al. (2011) and consisted of 10% crude glycerol (by weight), 0.5% yeast extract, 0.5% peptone and 0.1% MgSO_4 . Finally, the pH of the medium was adjusted to 6.5 using conc. H_2SO_4 (99%). The cells were then cultivated in culture medium at 30°C shaking at 200 rpm and samples were collected at days 1, 3 and 5 of cultivation. The culture supernatant was obtained by centrifugation at $5,000 \times g$ for 10 min and used for DHA measurement. The isolates that showed high production of DHA were selected for further investigation in YPGc with a final crude glycerol concentration of 10% (w/v).

EFFECT OF CULTURE MEDIUM ON DHA PRODUCTION

The effects of crude glycerol concentration, initial pH and nitrogen source of the YPGc medium were investigated to understand the effect of the medium on growth and DHA production. The initial crude glycerol concentration was adjusted to be 5, 10, 15 and 20% (w/v) in the YPGc medium, as shown above. The effect of the initial pH was investigated by preparing YPGc at a 10% (w/v) crude glycerol concentration and adjusting the pH to 5.5, 6.5 and 7.5 using conc. H_2SO_4 .

In addition, the effects of different nitrogen sources (yeast extract, peptone, $(\text{NH}_4)_2\text{SO}_4$ and urea) were investigated by replacing the nitrogen source in a common YPGc with either one or a mixture of the above nitrogen sources to a final of 1% (w/v). Five percent of the pre-culture was cultivated on YPGD at 30°C for 18 h before being transferred to an Erlenmeyer flask containing 25 mL of the YPGc medium whose composition had been modified as described above. The growth and DHA production were monitored at days 1, 3, and 5 of cultivation.

ANALYTICAL METHODS

DHA concentration was measured using the 3,5-dinitrosalicylic acid (DNS) method as described by Black and Nair (2013). Briefly, the DNS solution was added to the sample at a 1:1 (v/v) ratio and incubated at 100°C for 10 min. The sample was then quickly placed into a water bath for 5 min prior to measurement of the absorbance at 550 nm using a spectrophotometer (Shimadzu UV1800, Japan). The DHA concentration was determined using a calibration curve of standard DHA solutions.

Bacterial growth was measured by reading the optical density at 600 nm (OD_{600nm}) using a spectrophotometer (Shimadzu UV1800, Japan).

IDENTIFICATION OF ACETIC ACID BACTERIA

To identify the selected AAB isolates, the cells were cultured on YPGD medium at 30°C with rotary shaking at 200 rpm until they reached the mid-log phase (24 h). The cells were then harvested by centrifugation and the chromosomal DNA was subsequently isolated as described by a previous report (Soemphol et al. 2008). Approximately 1.5 kb PCR products of 16S rRNA were obtained by amplification using a pair of universal primers, namely, 27F and 1525R and the PCR Go Taq Green Master Mix (Promega, Madison, WI). Multiple sequence alignments were performed using the CLUSTALW program (Thompson et al. 1994). The neighbour-joining phylogenetic tree was constructed using Mega version 6.0 with 1000 bootstrap replicates (Tamura et al. 2013).

EXPRESSION OF *NHA*K2 INTO *GLUCONOBACTER FRATEURII* NKC115

Plasmid pCMnhaK2, carrying *nhaK2*, the gene encoding for the $Na^+(K^+)/H^+$ antiporter of *A. tropicalis* SKU1100, was constructed (Soemphol et al. 2011) and transformed into competent cells of *G. frateurii* NKC115 by electroporation, according to a previous report (Soemphol et al. 2008). The transformant was selected for on YPGD agar medium containing 12.5 mg/mL of tetracycline.

RESULTS AND DISCUSSION

SCREENING NEWLY ISOLATED AAB FOR EFFECTIVE DHA PRODUCTION FROM CRUDE GLYCEROL

The AAB previously isolated from several sources were compared for their ability to produce DHA from crude glycerol. Two groups of 48 AAB isolates were used for preliminary examination. The first group (NKC) was identified within the genus *Gluconobacter* and it did not show over-oxidation but did show lower acetic acid resistance (data not shown). The latter group (FPB) had a strong ability to oxidize ethanol, which is consistent with acetate over-oxidation, and seemed to belong to the genus *Acetobacter*. For comparison, reference AAB strains, *G. frateurii* THD32 and *A. tropicalis* SKU1100, were used in this screen. As shown in Figure 1, the DHA accumulation after cultivation of these AAB on YPGc with 10% crude glycerol was observed in all AAB and clearly divided into 2 groups. The group similar to *G. frateurii* THD32 produced a high amount of DHA at approximately 20–25 g/L. In contrast, the other group produced much less DHA and was similar to *A. tropicalis* SKU1100, with a DHA concentration below 5 g/L. Recently, the whole-genome analysis of *A. pasteurianus* has been reported and has showed that the genes encoding a membrane-bound glycerol dehydrogenase (GLDH), which is well characterized in *Gluconobacter*, do not exist in *A. pasteurianus* (Azuma et al. 2009). This could explain the lower accumulation of DHA in the isolates of the FPB strains, as it might be employed by another cytosolic enzyme, such

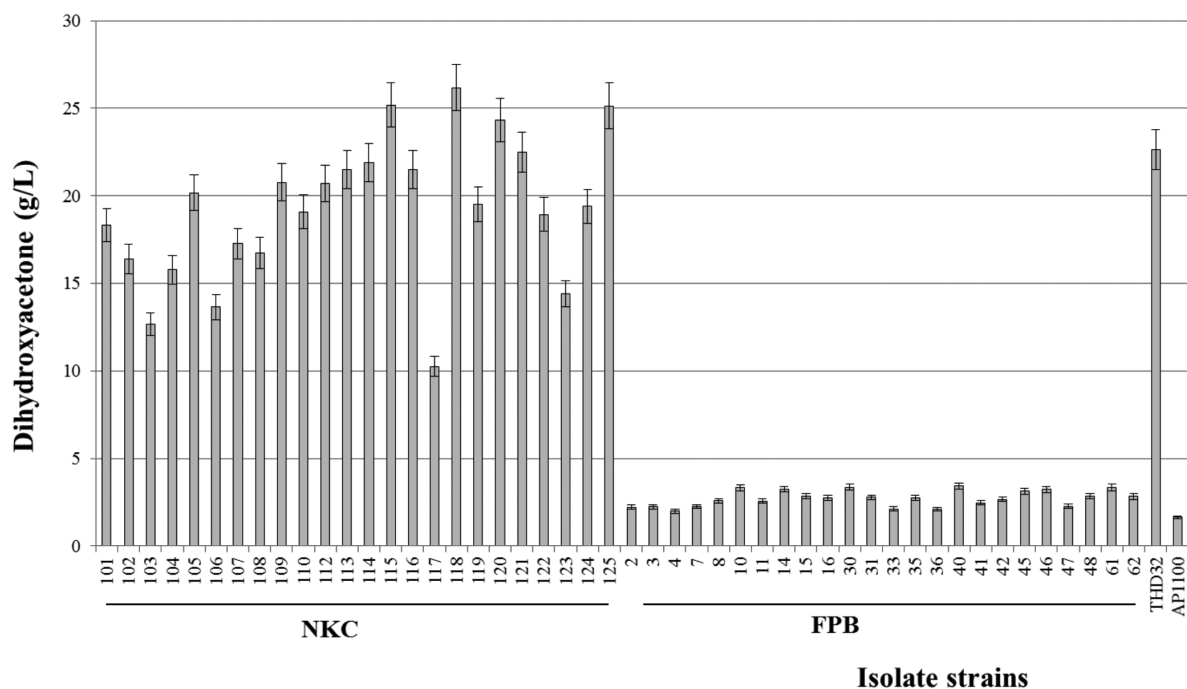


FIGURE 1. Dihydroxyacetone production from crude glycerol by newly isolated acetic acid bacteria. The culture supernatants of each isolate (NKC and FPB) after cultivation for 5 days in 5 mL of YPGc, were collected and measured for DHA concentration. SKU1100; *A. tropicalis* SKU1100, THD32; *G. frateurii* THD32

as NAD⁺-dependent glycerol dehydrogenase (Soemphol et al. 2008).

IDENTIFICATION OF NEWLY ISOLATED AAB

Five isolates were selected for identification based on 16S rRNA. A BLAST search showed that these 5 strains show high similarity to *Gluconobacter* with a similarity score greater than 99%. A phylogenetic tree analysis using neighbour-joining demonstrated that isolates NKC115 and 120 are closely related to *G. frateurii*, while isolates

NKC118, NKC121 and NKC125 belong to the species *G. japonicus* (Figure 2).

The 5 isolates were then selected for further comparison of their growth and DHA production at 10% (w/v) crude glycerol under shake flask conditions (Figure 3). These isolates had the highest DHA production, of approximately 25 g/L, after cultivation for 5 days and there was no significant difference between their yields. However, based on its growth ability, the isolate NKC115 was then selected as the AAB having the most potential for the purposes of this study and was carried forward for further study.

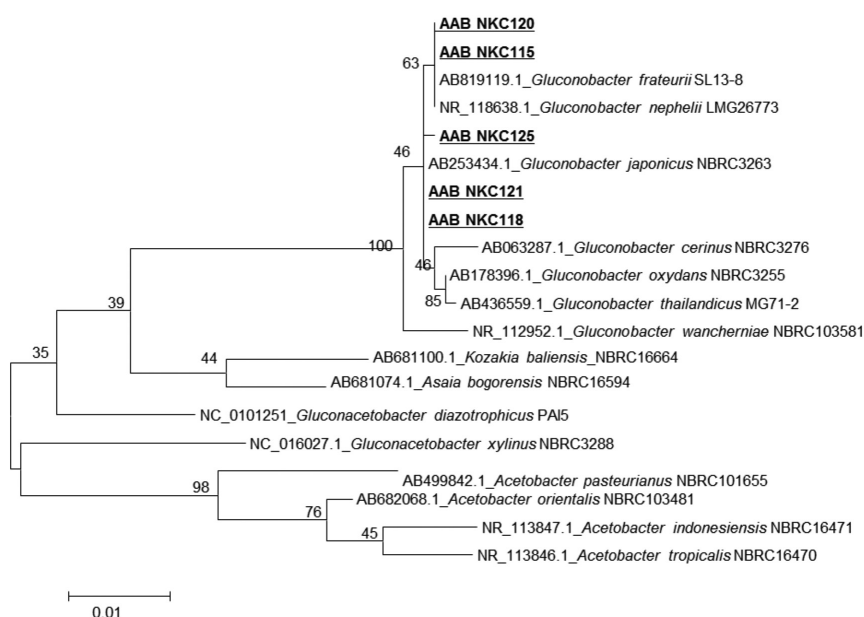


FIGURE 2. Phylogenetic analysis of the effective AAB, producing DHA from crude glycerol, compared with the other related AAB. The neighbour-joining phylogenetic tree was constructed with 1000 bootstrap replicates

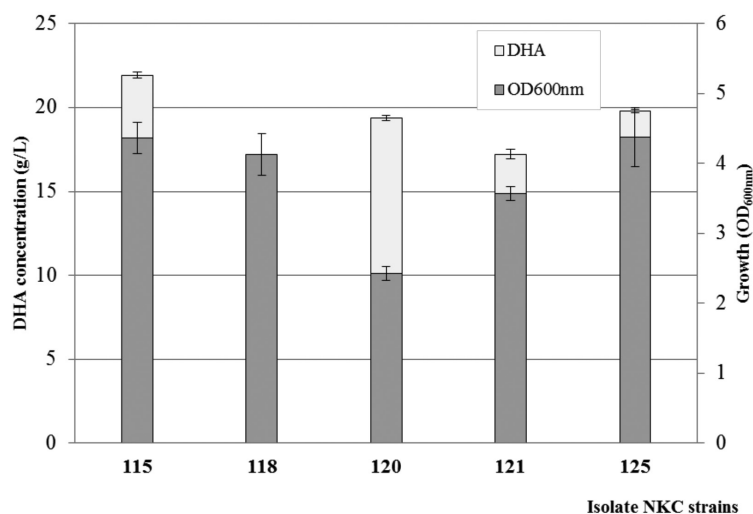


FIGURE 3. Comparison of DHA production (white bars) and growth ability (grey bars) of selected AAB. The 5 selected isolates were cultured in shake flasks at 200 rpm. The culture supernatants were collected after 5 days of cultivation for measurement of DHA and growth (optical density, OD_{600 nm}) as described in the Materials and Methods section

EFFECT OF CRUDE GLYCEROL ON DHA PRODUCTION

In the YPGc medium containing crude glycerol concentrations of 5, 10, 15 or 20% (w/v), the production of DHA increased slightly when going from a glycerol concentration of 5% to 10%, but seemed to be steady at concentrations above 10%, with the highest DHA concentration achieved being 27.5 g/L (Figure 4(a)). Similarly, the growth of this strain improved with an increase in crude glycerol concentration from 5% to 10%. Growth was inhibited at concentrations above 15% (Figure 4(b)). It has been consistently reported that only 10.9 g/L DHA could be produced from 200 g/L of glycerol under shake flask conditions (Liu et al. 2013). The results suggested that higher concentrations of glycerol might suppress the ability of the cells to grow due to hyperosmotic stress (Ma et al. 2010) and may impair the cell division mechanism (Claret et al. 1992). There would also be feedback inhibition caused by the consequent accumulation of high amounts of DHA (Ma et al. 2010). In addition, the increased levels of several impurities that act as inhibitors could possibly affect microbial growth and production (Chatzifragkou & Papanikolaou 2012). Immobilized cells of *Gluconacetobacter xylinus* have been employed to minimize the inhibition of substrate and product (Black & Nair 2013).

EFFECT OF THE INITIAL pH ON DHA PRODUCTION

Growth and DHA production were examined in YPGc medium containing 10% (w/v) crude glycerol with initial pH values adjusted to 5.5, 6.5 or 7.5 (Figure 5). The results showed that the initial pH values tested did not affect the growth or DHA production, with the highest DHA concentration (27.82 g/L) being achieved by a culture with an initial pH of 6.5. However, the optimum pH for DHA production tended to be below neutral pH.

Upon cultivation, the pH values of the culture media from these three conditions were seen to gradually decrease and the pH value was seen to stabilize at approximately 5.0 (data not shown). This may explain the formation of other sugar acids, for instance, DL-glyceric acid, which is another by-product of glycerol oxidation by acetic acid bacteria (Habe et al. 2009a). It has been reported that glycerol dehydrogenase (GLDH) activity has an optimal pH of approximately 5.0 and requires divalent metal ions such as Ca^{2+} or Mg^{2+} (Adachi et al. 2001; Ameyama et al. 1985). Therefore, the optimal pH for cell growth and DHA production is an acidic pH.

EFFECT OF NITROGEN SOURCE ON DHA PRODUCTION

The effect of various organic and inorganic nitrogen sources were compared in shake flasks, including yeast extract mixed with peptone (each 5 g/L), yeast extract (10 g/L), peptone (10 g/L), $(\text{NH}_4)_2\text{SO}_4$ (10 g/L) and urea (10 g/L). As shown in Figure 6, poor cell growth and low DHA production were observed with all the inorganic nitrogen sources tested, while *G. frateurii* NKC115 achieved the highest DHA concentration (34.70 g/L) by using peptone (P). It has been reported that yeast extract and peptone are the optimal nitrogen sources for DHA production by *G. oxydans* ZJB09112 and that there was no remarkable difference in DHA production between the two (Hu et al. 2010). However, among the nitrogen sources tested in this study, peptone seemed to be the most favourable nitrogen source. Since yeast extract and peptone are expensive, it is desirable to find a cheaper complex nitrogen source to produce DHA. Liu et al. (2013) reported that the use of corn steep liquor (CSL) instead of two expensive nitrogen sources resulted in comparable amounts of DHA production by *G. frateurii* CGMCC5397.

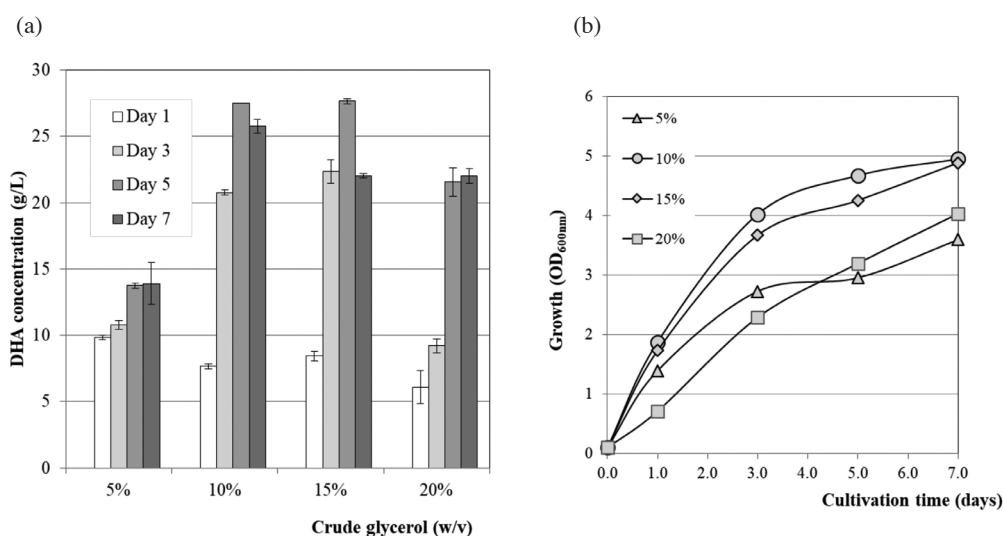


FIGURE 4. Effect of crude glycerol concentration on DHA production (a) and growth of *G. frateurii* NKC115 (b). The preculture of *G. frateurii* NKC115 was transferred into a 250 mL Erlenmeyer flask containing 25 mL of YPGc with different concentrations of crude glycerol, as indicated. DHA concentration and growth were monitored at days 1, 3, 5, and 7 of cultivation

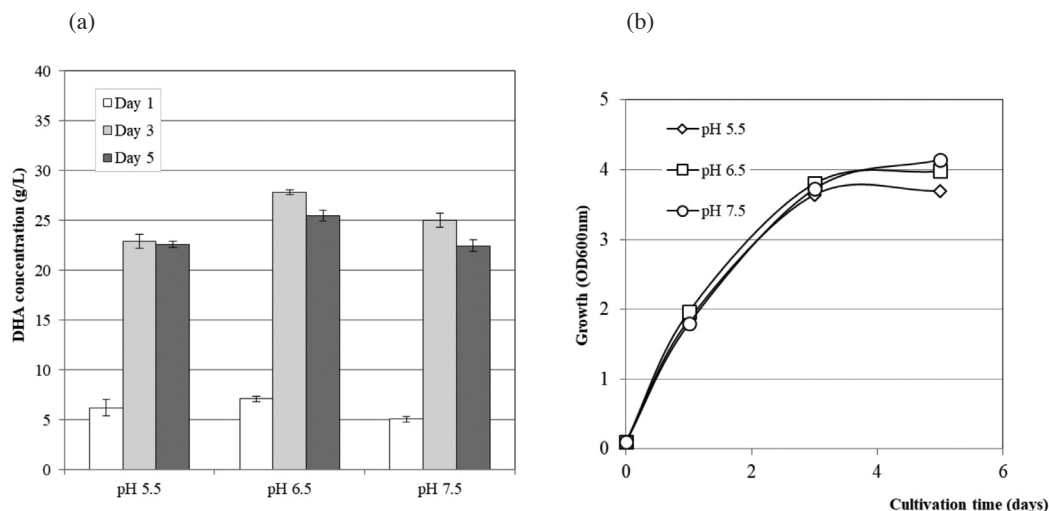


FIGURE 5. Effect of initial pH on DHA production (a) and growth of *G. frateurii* NKC115 (b). The preculture of *G. frateurii* NKC115 was transferred into a 250 mL Erlenmeyer flask containing 25 mL of YPGc at different pH values, as indicated. DHA concentration and growth were monitored at days 1, 3 and 5 of cultivation

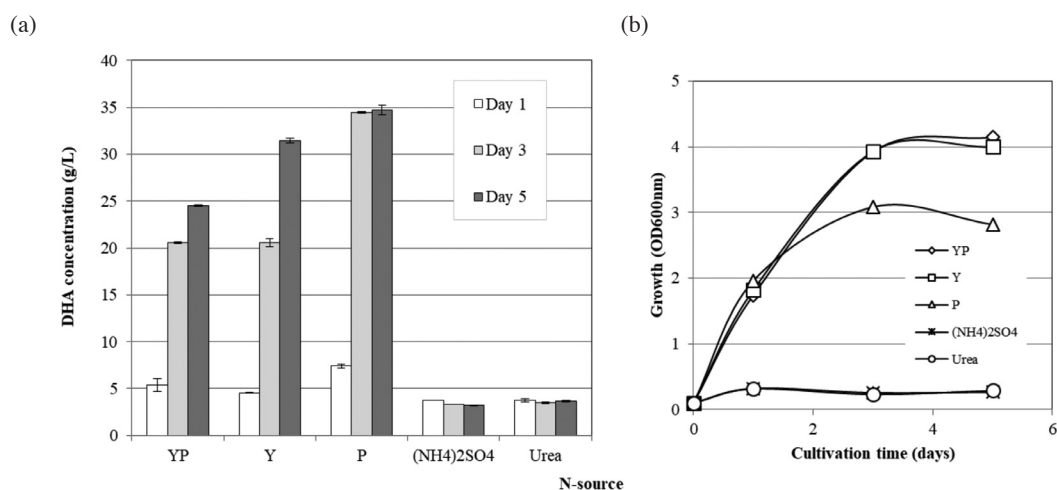


FIGURE 6. Effect of different nitrogen sources on DHA production (a) and growth of *G. frateurii* NKC115 (b). The preculture of *G. frateurii* NKC115 was transferred into a 250 mL Erlenmeyer flask containing 25 mL of YPGc at different pH values, as indicated. DHA concentration and growth were monitored at days 1, 3, and 5 of cultivation

IMPROVEMENT OF GROWTH AND DHA PRODUCTION OF *G. FRATEURII* NKC115

Since an increase in crude glycerol suppresses the growth of *G. frateurii* NKC115, it is possible that it is not only glycerol or DHA that have this effect; several impurities in crude glycerol have been seen to highly inhibit either cellular growth or production (Chatzifragkou & Papanikolaou 2012). Potassium or sodium compounds are used as the catalyst in the transesterification of crude glycerol at a strongly alkaline pH of approximately 12.0 and might have an effect on the bacterial growth. According to our previous report, we found that the gene *nhaK2*, encoding the Na⁺(K⁺)/H⁺ antiporter of *A. tropicalis* SKU1100, plays an important role in the maintenance of intracellular pH and K⁺ homeostasis (Soemphol et al. 2015). Moreover, expression of this gene

was seen to successfully improve the growth of other AAB in media supplemented with potassium, in addition to growth at high temperature. Therefore, the shuttle vector pCMnhaK2 containing this gene was then transformed into *G. frateurii* NKC115, resulting in the strain *G. frateurii* NKC115 (pCMnhaK2), while the empty vector pCM62 was also transformed and showed no effect on growth compared to the wild-type strain (data not shown). As seen in Figure 7, expression of this gene clearly improved the growth of *G. frateurii* NKC115 even though the growth gradually decreased with increasing concentrations of crude glycerol. Furthermore, DHA production improved when crude glycerol was used at 20% and was higher than that at 10% with a maximum concentration of 37.25 g/L. This result suggested that the expression of a Na⁺/H⁺ antiporter might maintain an optimal intracellular pH and

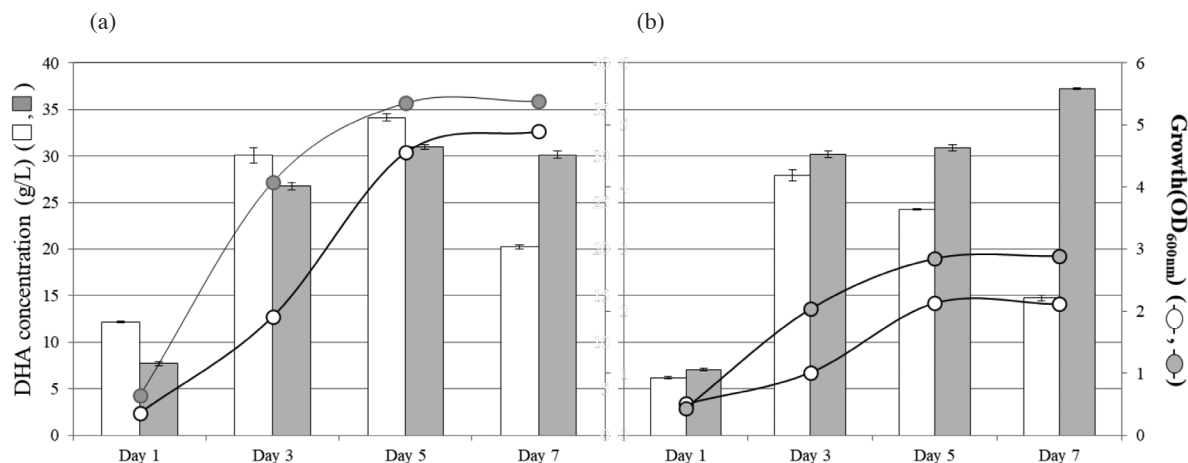


FIGURE 7. Expression of the $\text{Na}^+(\text{K}^+)/\text{H}^+$ antiporter of *A. tropicalis* SKU1100 in *G. frateurii* NKC115. Growth (line graph) and DHA production (bar graph) were examined by comparing expression of wild-type *G. frateurii* NKC115 (open circle and white bar) with the transformant *G. frateurii* NKC115 (pCMnhaK2) (close circle and grey bar). Both strains were cultured in YPGc containing 10% (a) and 20% (b) of crude glycerol

concentration of Na^+ or K^+ resulting in the cells' ability to tolerate higher concentrations of crude glycerol. The chemical mutagenesis of *G. oxydans* by NTG treatment resulted in the cells' ability to tolerate methanol and led to an improvement of growth and DHA production in the presence of methanol (Sato et al. 2014). Low-power laser (He-Ne) irradiation is currently used to construct the mutant *G. oxydans* GM51, which is employed for lower substrate inhibition and the improvement of DHA productivity (Ma et al. 2010).

CONCLUSION

In conclusion, waste glycerol represents a carbon source that is widely available at a relatively low cost and is potentially suitable for many applications. This study demonstrated that NKC115, a new isolate identified as *G. frateurii*, is able to utilize non-detoxified crude glycerol to accumulate a considerable amount of DHA. In this study, the highest amount DHA production was 37.25 g/L from 20% (w/v) crude glycerol by the over-expressed strain, which could also tolerate a higher concentration of crude glycerol than the wild type. However, an increase in the concentration of crude glycerol may result in growth inhibition and decreased DHA production. Obtained from different biodiesel plants, the glycerol by-products might have variable compositions of impurities, making it difficult to control the production of DHA. The use of the most robust microbial strain might fulfill the industrial demands of DHA production from crude glycerol and decrease the production costs. There is potential for efficient DHA production; however, the fermentation process and molecular methods should be optimized in order to improve the efficacy of *G. frateurii* NKC115 for DHA production from crude glycerol.

ACKNOWLEDGEMENTS

This work was supported by funding from Nong Khai Campus, Khon Kaen University. The authors wish to thank Prof. Dr. Kazunobu Matsushita for kindly providing the plasmid pCMnhaK2. We thank Mr. Thanin Kaenthani and Mr. Apisit Nongpho for technical support.

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Varavut Tanamool
Chemistry Program, Faculty of Science and Technology
Nakhon Ratchasima Rajabhat University
Nakhon Ratchasima, 30000
Thailand

Piyorot Hongsachart & Wichai Soemphol*
Faculty of Applied Sciences and Engineering
Khon Kaen University
Nong Khai Campus, Nong Khai, 43000
Thailand

*Corresponding author; email: wichso@kku.ac.th

Received: 7 October 2015

Accepted: 9 October 2017