



***Kozakia baliensis*, THE NEW POTENTIAL ACETIC ACID BACTERIUM USES FOR DIHYDROXYACETONE PRODUCTION**

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Abstract

Many species of acetic acid bacteria are able to oxidize various substrates such as carbohydrates, alcohol, sugar or sugar alcohol and accumulate their oxidation products in the culture medium. The key oxidative products by acetic acid bacteria are applied in industry such as food industry and medicine. Dihydroxyacetone (DHA) is one of attracting oxidation products because it can be blended in chemical, pharmaceutical and cosmetic products. DHA is used as the main active ingredient in self-tanning cream preparations and a precursor for chemical synthesis of various fine chemical compounds. Moreover, DHA is used for the treatment of vitiligo. The aims of this work were to produce and optimize the culture conditions for DHA production by growing cells and resting cells of *Kozakia baliensis*. When using the culture conditions for DHA production by growing cell of *K. baliensis* as follow; 5% inoculum, 5% glycerol, 0.5% yeast extract, 0.5% peptone and incubation at 30°C with shaking at 200 rpm, then production of DHA in shaking flask between Erlenmeyer-flask and baffled-flask was compared. It was clearly shown that production of DHA under the conditions in baffled-flask better than in Erlenmeyer-flask. The yield of DHA was about 36.46 and 29.54 mg/ml at 48 h, respectively. Production of DHA at 30°C and 37°C by *K. baliensis* was investigated. It was interestingly found that *K. baliensis* grew at 37°C and produced DHA with the yield of 29.12 mg/ml at 72 h. However, it was lower than that of the production at 30°C with the yield of 36.46 mg/ml at 48 h. Furthermore, production of DHA by resting cells of *K. baliensis* demonstrated that glycerol concentration for biotransformation should be ranging between 0.5-6.0% (w/v). Increasing in the initial glycerol concentration at 7-10 % (w/v) led to substrate inhibition by which DHA production yield obtained was less than 50%. For resting cell conditions, DHA production at 1 g of wet cell weight in 1% glycerol solution shown to produce the highest DHA of 9.97 mg/ml at 6 h. Therefore, the production of DHA by the resting cell conditions was faster than that of growing cell conditions.

Key words: DHA, oxidative product, glycerol, acetic acid bacteria, *Kozakia baliensis*



Introduction

The main characteristics of acetic acid bacteria are the capacity for oxidize various substrates such as carbohydrates, alcohol, sugar or sugar alcohol and to accumulate oxidation products. Oxidation products are secreted outside cells, which can be isolated directly from a culture medium. This ability is basically due to the dehydrogenase activity in the cell membrane connected to the respiratory chain. (Adachi et al. 2007; Deppenmeier et al. 2002). Oxidation products obtained from acetic acid such as cellulose, gluconic acid, L-sorbose, glyceric acid and DHA etc (Stasiak and Błażejczak 2009). Those compounds are applied in many branches of industry such as food and medicine industry. Among these, dihydroxyacetone (DHA) is of interest for researchers because DHA is a very important for chemical and pharmaceutical product. DHA is used in the cosmetic industry as the main ingredient in self-tanning cream preparations. DHA is a precursor for chemical synthesis of various fine chemical compounds e.g. methotrexate, which is used in the chemotherapeutical treatment of cancer patients (Claret et al. 1994). Moreover, DHA is used for the treatment of vitiligo (Fesq et al. 2001). Dihydroxyacetone is a result of glycerol oxidation catalyzed by glycerol dehydrogenase an enzyme linked with membrane-bound of acetic bacteria (Gätgens et al. 2007). The process for dihydroxyacetone synthesis have two methods; chemical and microbiological synthesis (Gehrer et al. 1995; Charney et al. 1978). The problems involved with chemical synthesis, it may be toxic for environment and the cost of chemical process is expensive (Hekmat et al. 2003). Moreover, isolation and purification of dihydroxyacetone is difficult in the case of chemical route. Therefore, microbiological synthesis is an alternative used for commercial production with the use of acetic acid bacteria because reducing production costs and is not toxic to the environment. According to past research, some strains of acetic acid bacteria, including *Gluonobacter melanogenus*, *Gluconobacter oxydans*, *Acetobacter xylinum* and *Gluconacetobacter xylinus* (Wei et al. 2007a, b; Nabe et al. 1979; Flickinger and Perlman 1977; Asai 1968; Stasiak et al. 2011) are able oxidized glycerol to DHA. However, DHA production by *Kozakia baliensis*, an acetic acid bacterium had never been reported so far. Therefore, in this study, the optimization conditions for DHA production by growing cells and resting cells of *K. baliensis* was performed.

Methodology

Bacterial and Culture conditions

The bacterium used in this study was acetic acid bacteria belonging to the genus *Kozakia baliensis* which kindly supported from Prof. Dr. Kazunobu Matsushita Department of Biological chemistry, Faculty of Agriculture Yamaguchi University, Japan. Growth of *K. baliensis* was cultivated in potato medium at 30°C with shaking at 200 rpm. The stock culture was maintained in potato medium agar slant at 4 °C and the culture was newly transferred every 30 days. Production media for DHA production was the following: 5%, 10%, 15% and 20% glycerol, 0.5% peptone, 0.5% yeast extract and 1% glycerol solution for resting cells.

Quantification of dihydroxyacetone by the colorimetric method

DHA concentration was determined by DNS method according to Burner (1964). The samples were mixed with 3,5-dinitrosalicylic acid solution at the ratio of 1:1 (v/v) and incubated at 100°C for 10 min. Afterwards, the samples were cooled in ice and 2 ml of water was added. Absorbance was measured at a wavelength of 550 nm. DHA concentration was calculated from a standard curve.



Optimization of the conditions for DHA production by growing cells

Effects of seed culture concentrations:

To investigate the effects of initial seed culture concentration of *K. baliensis*, the seed culture of 5%, 10%, 15% and 20% (v/v) were transferred into 500-ml Erlenmeyer-flask containing 100 ml of glycerol medium consisting of 5% glycerol, 0.5% peptone and 0.5% yeast extract. All cultures were incubated at 30°C on a rotary shaker at 200 rpm for 7 days. The supernatant was harvested every 24 h. After the removal of cells by centrifugation, the respective supernatants were analyzed by the colorimetric method.

Effects of glycerol concentrations:

To investigate the effects of initial glycerol concentration on DHA production, seed cultures of *K. baliensis* were transferred into 500-ml Erlenmeyer-flask containing 100 ml of glycerol medium consisting of 5%, 10%, 15% and 20% glycerol, 0.5% peptone and 0.5% yeast extract. All cultures were incubated at 30°C on a rotary shaker at 200 rpm for 7 days. The supernatant was harvested every 24 h. After the removal of cells by centrifugation, the respective supernatants were analyzed by the colorimetric method.

Effects of aeration on DHA production:

To investigate the effects of aeration on DHA production, the culture was incubated in Erlenmeyer-flask and baffled-flasks. The seed cultures of *K. baliensis* were transferred into 500-ml Erlenmeyer and baffled-flasks containing 100 ml of the glycerol medium consisting of 5%, 10%, 15% and 20% glycerol, 0.5% peptone and 0.5% yeast extract. All cultures were incubated at 30°C on a rotary shaker at 200 rpm for 7 days. The supernatant was harvested every 24 h. The supernatants after cells removal were analyzed by the colorimetric method.

Effects of temperature on DHA production:

To investigate the effects of temperature on DHA production, the experiment was performed at 30°C and 37°C. Seed cultures of *K. baliensis* were transferred into 500-ml baffled-flasks containing 100 ml of the glycerol medium consisting of 5% glycerol, 0.5% peptone and 0.5% yeast extract. All cultures were incubated on a rotary shaker at 200 rpm for 7 days. The every day harvested supernatants were analyzed by the colorimetric method.

Detection DHA by Thin Layer Chromatography

After removal of the cells by centrifugation of the culture broth, the supernatant was spotted on a TLC plate (silica gel 60, Merck, Darmstadt, Germany). It was developed with a solvent system of ethyl acetate: acetic acid: methanol: distilled water 6:1.5:1.5:1 ratio. Detection of DHA was done at room temperature by spraying a solution of 2,3,5-triphenyltetrazolium chloride (TTC) over the TLC plate.

Production of DHA by resting cells

Cultivation of *K. baliensis* were performed in 5-L Erlenmeyer-flask containing 1000 ml of potato medium at 30°C and aeration at 200 rpm on a rotary shaker for 24-48 h. Then, the cells were harvested by centrifugation at 8,000 rpm, 4 °C for 20 min. The collected cells were washed and resuspended in 5 mM KPB buffer (pH 6). The wet cell weight of *K. baliensis* of 0.5,1, 2, 3, 4 and 5 g were transferred to 500-ml baffled-flasks containing 100 ml of 1% glycerol solution (w/v). All samples were incubated at 30°C on a rotary shaker at 200 rpm for 7 days. The supernatant was harvest every 3 h. After removal of cells by centrifugation, the supernatants were analyzed by the colorimetric method. For the effects of initial concentration of glycerol solution, wet cells weight were transferred to 500-ml baffled-flasks containing 100 ml of glycerol solution consisting of 1, 2, 3, 4, 5, 6, 8 and 10% glycerol. All samples were incubated at 30°C on a rotary shaker at 200 rpm for 10 days. The supernatant was harvested every 3 h. After the removal of cells by centrifugation, the respective supernatants were analyzed by the colorimetric method.

Results and Discussion

Effects of seed culture concentrations

Seed culture of *K. baliensis* at 5, 10, 15 and 20% was applied for DHA production. The results showed that 5% of seed culture produced the highest DHA at 48 h with the yield of 28.86 mg/ml (Figure 1). Increase of seed culture to 10, 15 and 20% had slightly effected on DHA production. The highest DHA production obtained was 57.72% when incubation at 48 h. Thereafter, the productivity of DHA was decreased.

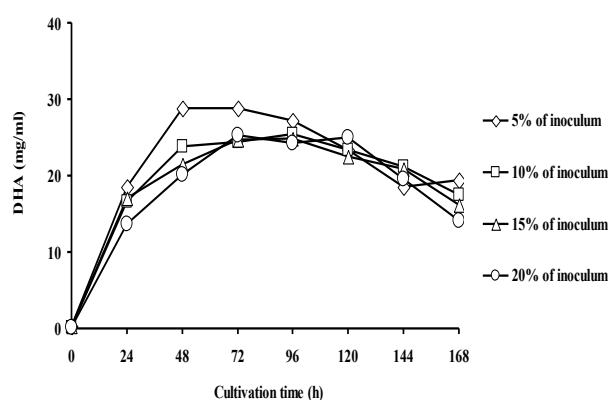


Figure 1 Effects of seed culture concentrations on DHA production from *K. baliensis*

Effects of glycerol concentration

The growth of *K. baliensis* was determined when the cells was grown in the medium containing glycerol at the concentration of 5, 10, 15 and 20% (w/v). *K. baliensis* could utilized glycerol as the carbon source under the culture conditions and produced high amount of DHA at 48 h when 5% glycerol was used. The highest DHA concentration reached a maximum of 29.54 mg/ml (Figure 2A). When high concentration of glycerol was employed for DHA production, it was resulting in decreasing of cell growth and as well as DHA production (Figure 2B). It was assumed that at higher concentration of substrate may inhibit

DHA production. The results was coincided with Ma et al. (2010) indicating that higher concentration of the substrate may be lose growth of acetic acid bacteria which about hyperosmotic stress and lead to decreased biotransformation yield. Therefore, in this experiment the optimum concentration for DHA production was using 5% (w/v) glycerol. Concentration of glycerol which the excessive in the medium may disturb biotransformation of DHA or even make it impossible and inhibit the growth of some strains of acetic acid bacteria. The high content of glycerol may impair mechanisms of cells division (Claret et al. 1992; De Muyck et al. 2007).

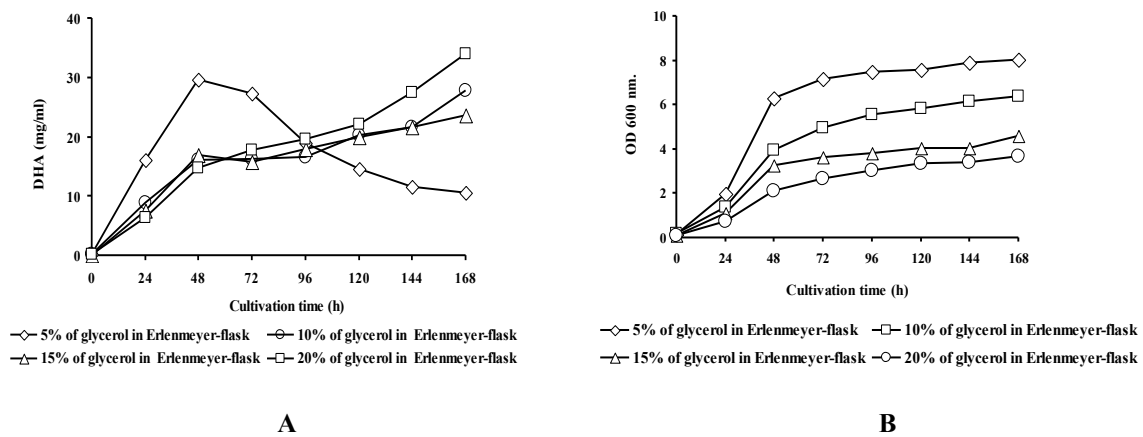


Figure 2 Effects of glycerol concentration on DHA production by *K. baliensis* in Erlenmeyer-flask (A) and Time course of growth (B).

Effects of aeration on DHA production

The experiment was performed in Erlenmeyer and baffled-flasks in order to investigate the effect of aeration on DHA production. In baffled-flask, *K. baliensis* grew well in the culture medium containing 5 and 10% glycerol (Figure 3A). The highest DHA production was reached 36.46 mg/ml at 48 h with baffled-flask containing 5% glycerol medium. After that the amount of DHA gradually decreased. While increasing of the initial glycerol concentration at 10, 15 and 20% the amount of DHA was continuously increased until 120-144 h (Figure 3B). Production of DHA in 10% glycerol revealed that high amount of DHA was detected at 120 h with the yield of 77.87 mg/ml and slightly degraded after 120 h.

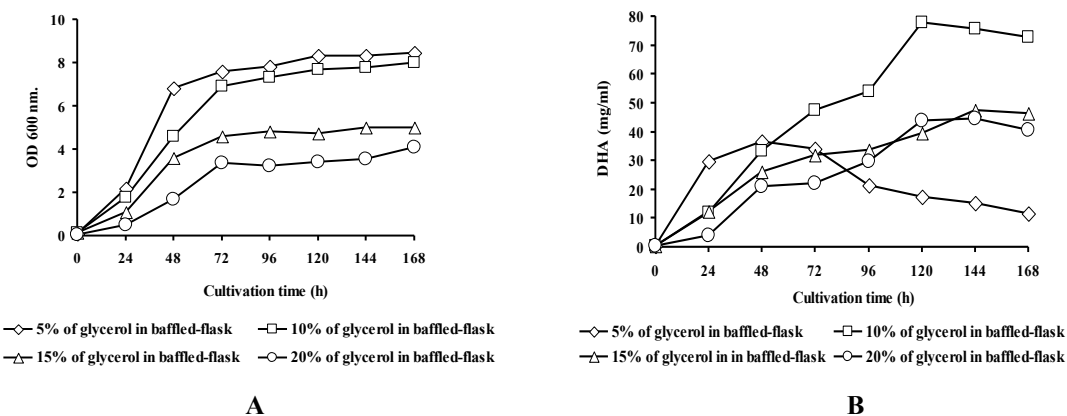


Figure 3 Time course of growth (A) and effects of glycerol concentrations for DHA production (B) by *K. baliensis* in baffled-flask.

As shown in Figure 4A, DHA production in 5% glycerol medium with baffled-flask was better than that of Erlenmeyer-flasks. The yield of DHA at 48 h was about 36.46 mg/ml and 29.54 mg/ml, respectively. Moreover, when increasing the initial glycerol concentration at 10% in baffled-flask, DHA was also produced better than cultured in Erlenmeyer-flasks (Figure 4B). Therefore, DHA could be produced in baffled-flask with increasing amount of substrate. To reduce the production cost, the baffled-flask was suitable for DHA production due to increasing in aeration rate for cell culture that lead to oxidation process increased.

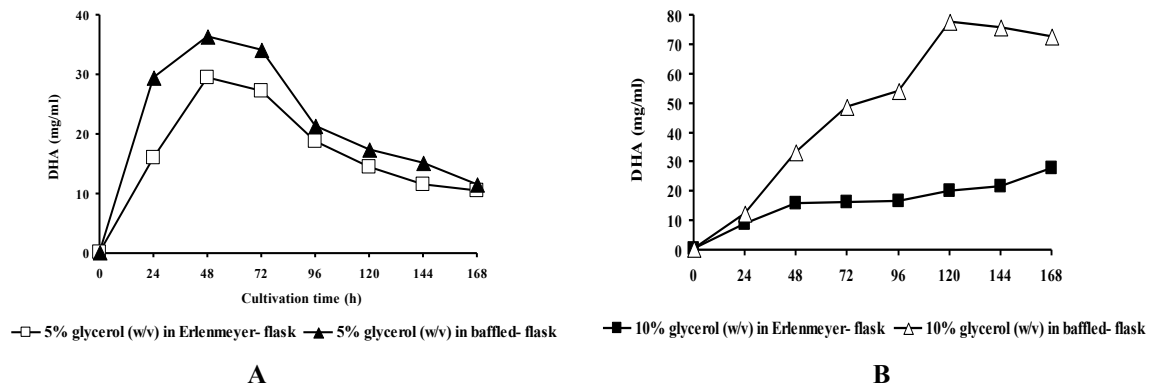


Figure 4 Comparison of DHA production by *K. baliensis* between incubation in Erlenmeyer and baffled-flasks. (A) 5% glycerol medium, (B) 10% glycerol medium

DHA production yield was compared between cultivation in Erlenmeyer and baffled-flasks (Figure 5). Using 5% glycerol medium gave relative yield of DHA at 72.92 % in baffled-flask and 59.08% in Erlenmeyer-flask at 48 h incubation. In 10% glycerol medium, the relative yield of DHA obtained was at 77.87% in baffled-flask and 27.77 % in Erlenmeyer-flask for 120 and 168 h, respectively. At higher concentrations such as 15 and 20% glycerol revealed that DHA production was decreased due to substrate inhibition both in Erlenmeyer and baffled-flasks.

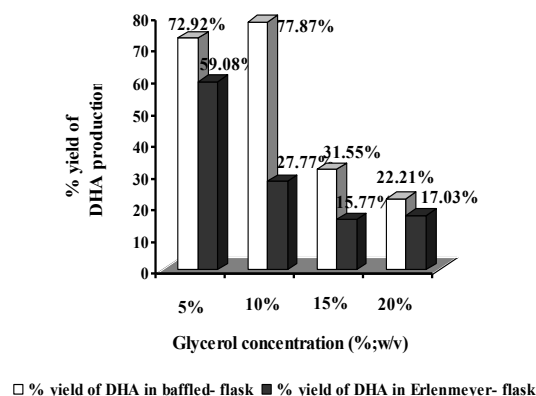


Figure 5 Comparison of DHA production yield by *K. baliensis* between incubation in Erlenmeyer and baffled-flasks.

Effects of temperature on DHA production

DHA production by *K. baliensis* was performed at 30°C and 37°C. As shown in Figure 6, *K. baliensis* grew both at 30°C and 37°C and produce DHA at the yield of 29.12 mg/ml and 36.46 mg/ml for 72 h and 48 h, respectively. As the results, DHA was highly produced at 30°C in which the optimum for growth of *K. baliensis* was reported at 30 °C (Lisdiyanti et al. 2002).

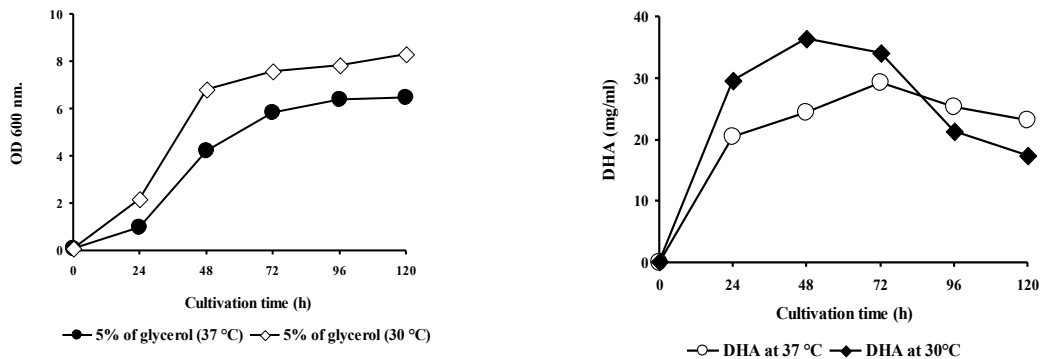


Figure 6 Effect of temperature on growth and DHA production by *K. baliensis*

Detection of DHA by thin layer chromatography

Thin layer chromatography was used as the tool for the monitoring DHA production. As shown in Figure 7, the 0-5 days culture supernatants were spotted onto TLC plate compared to that of authentic DHA (the last lane). The only DHA spot had been detected with the same R_f of authentic DHA.

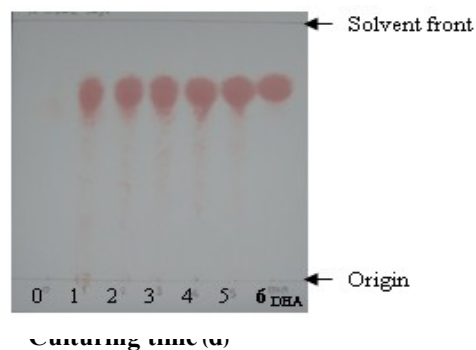


Figure 7 Thin layer chromatogram of DHA produced in culture supernatant by *K. baliensis*, Lane 0: 0 day culture medium; Lane 1: 1 day culture medium; Lane 2: 2 days culture medium; Lane 3: 3 days culture medium; Lane 4: 4 days culture medium; Lane 5: 5 days culture medium; Lane 6: DHA standard.

Biotransformation of glycerol to DHA with resting cells of *K. baliensis*

In order to investigate the effects of wet cell weight of resting cells of *K. baliensis* on DHA production, cell weight 0.5, 1, 2, 3, 4, and 5 g was put into 1% glycerol solution (Figure 8). DHA production at 0.5 g of wet cell weight gave the highest DHA yield at 9.01 mg/ml when incubation at 18 h. When wet cell weight of 1 g was used, the highest DHA yield of 9.30 mg/ml was obtained at 6-21 h. Whereas, DHA yield when using 2 g of wet cell weight reached at 8.31 mg/ml within 3 h.

Using 3, 4 and 5 g of wet cell weight of *K. baliensis*, it was found that within 3 h the highest DHA production yield was at 9.06, 8.03 and 6.30 mg/ml, respectively suggesting that increasing of wet cell weight led to faster oxidation. As the results, DHA was gradually decreased after the highest conversion due to DHA could uptake into cytoplasm of cell and catabolized in the pentose-phosphate pathway. (Bories et al. 1991; Claret et al. 1994)

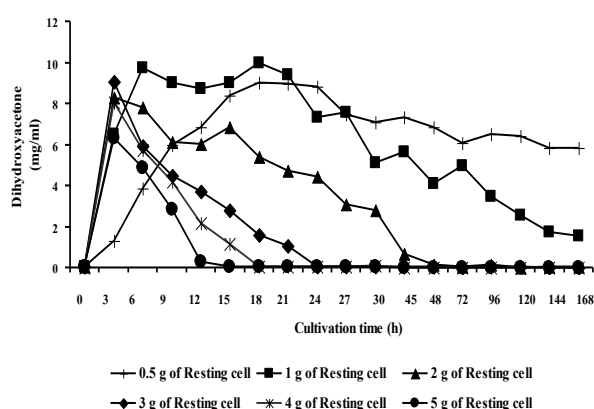


Figure 8 DHA production by resting cells at various wet cell weights.

As shown in Figure 9, to investigate the effects of substrate concentration on DHA production, 0.5% glycerol was used as the substrate. At 9 h incubation gave the highest DHA yield of 3.99 mg/ml or 79.78% conversion. When 1% glycerol was used the productivity of DHA reached 9.97 mg/ml or 99.73% conversion at 6 h. DHA production with 2% glycerol was reached at 19.16 mg/ml or 95.83% conversion at 18 h. The same results could be observed in 3-5% glycerol suggesting that glycerol oxidized to DHA of more than 95% (Figure 9A). However, increasing in the initial substrate concentration led to prolong the conversion rate and time consuming. These results suggest that *K. baliensis* can be oxidized glycerol as the carbon source to DHA under resting cells condition. An increasing in the initial glycerol concentration gave the decreasing of DHA production (Figure 9B). The experiments demonstrated that glycerol concentration for biotransformation should be ranging between 0.5-6.0% (w/v). A higher concentration of the substrate may be cell damage as a result of hyperosmotic stress and to decreased biotransformation yield (Ma et al. 2010).

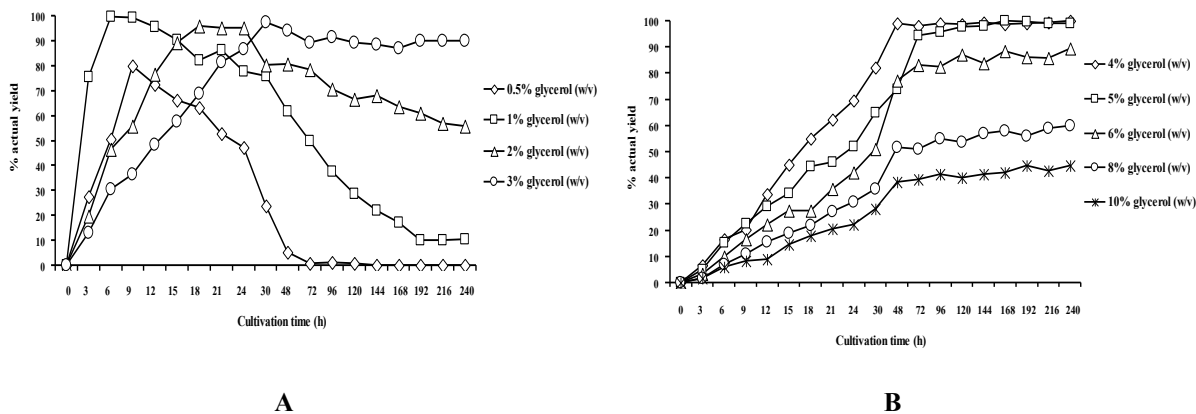


Figure 9 Relative yield of DHA produced by resting cells of *K. baliensis* with various glycerol concentrations. (A) 0.5-3% glycerol, (B) 4, 5, 6, 8 and 10% glycerol

Conclusion

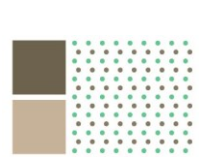
K. baliensis, the new potential acetic acid bacterium can be applied for DHA production. The optimum conditions for DHA production by growing cell was 5% glycerol, 0.5% peptone, 0.5% yeast extract in baffled-flask and incubation with high aeration at room temperature for 48 h. DHA production yield obtained was at 36.46 mg/ml or 72.92% conversion. For resting cells, DHA production using 1 g of cell wet weight can be produced the highest DHA at 9.97 mg/ml or 99.73% conversion within 6 h.

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