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Screening and Preliminary Optimizations for Dihydroxyacetone Production from Glycerol by the *Gluconobacter* and *Asaia* Isolates Found in Thailand

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ABSTRACT

This study aims to investigate the feasibility of using a minimal glycerol medium with inorganic salt supplementation for a low-cost dihydroxyacetone (DHA) production by bacteria. Acetic acid bacterial isolates of *Gluconobacter* and *Asaia*, which are found in the Northern, North-Eastern, Middle, Western, and Southern regions of Thailand, were evaluated for their ability to produce DHA using glycerol as a carbon source. During the qualitative screening, 66 isolates, including *Gluconobacter* (61 isolates) and *Asaia* (5 isolates), from a total of 486 isolates showed highly positive results by the Fehling test. The 5 isolates of *Asaia* gave low DHA production in the quantitative screening, whereas *Gluconobacter* isolates showed DHA production at low (0-5.70 g/L), medium (5.71-11.40 g/L), and high (11.41-16.89 g/L) levels. Preliminary culture medium optimizations for *G. frateurii* BCC 36199, a most promising microorganism for DHA production, were also carried out using a low-cost minimal glycerol medium supplemented with an inorganic salt. *G. frateurii* BCC 36199 produced 18.67 g/L of DHA with y_{sp} of 95.44% (DHA moles/glycerol moles) at 30°C, 20 g/L of glycerol, and pH 4.5. The cultivation of *G. frateurii* BCC 36199 in the developed minimal glycerol medium is practical and can be further optimized in order to apply for industry.

Keywords: Dihydroxyacetone; Glycerol; *Gluconobacter*; *Asaia*; Acetic acid bacteria

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1. Introduction

Glycerol is currently generated in excess amounts as a major by-product of biodiesel production processes [1]. Consequently, the price of glycerol has declined [2]. Glycerol has been studied as a carbon source for microbial fermentation to generate many useful chemicals [1]. Among the chemicals obtained from glycerol fermentation, dihydroxyacetone (DHA) is a promising product because of its high price and versatility [3]. DHA is used in cosmetic products, pharmaceuticals, and various kinds of chemicals syntheses [4].

Acetic acid bacteria are commonly used for DHA production using glycerol as a carbon source. Microbial production of DHA from glycerol was first observed in the case of the bacteria sorbose *bacillus* [5]. Other acetic acid bacteria such as *Acetobacter xylinum* [6,7], *Acetobacter suboxydans* [8], and *Gluconobacter melanogenus* [9] have also been reported for DHA production from glycerol. In recent years, *G. oxydans* has become the most commonly used microorganism for converting glycerol to DHA [4, 10-12].

Asaia, another genus of the acetic acid bacteria, also shows the ability to produce DHA from glycerol [13,14]. Acetic acid bacteria are normally found in fruits and flowers [15]. These bacteria are also present in alcoholic beverages [16]. A diversity of acetic acid bacteria in the genera of *Gluconobacter and Asaia* has been found in Thailand [17]. These two bacterial genera, which have been reported to consume glycerol, could be promising microorganisms that have the ability to produce DHA from glycerol. Therefore, this study aimed to evaluate the feasibility of DHA production from glycerol using the acetic acid bacteria genera of *Gluconobacter* and *Asaia* to determine whether these bacteria are promising DHA- producing microorganisms.

2. Materials and Methods

2.1 Microorganisms

The *Gluconobacter* (256 isolates) and *Asaia* (230 isolates) genera used in this study were obtained from the BIOTEC Culture Collection (BCC), BIOTEC, National Science and Technology Department Agency (NSTDA), Thailand. Before using, the microorganisms, which were kept at -20°C, were activated by culturing them on a Glucose- Ethanol- Calcium carbonate Agar (GECA) medium (20 g/L of glucose, 5 g/L of ethanol, 1.2 g/L of peptone, 3 g/L of yeast extract, and 7 g/L of calcium carbonate) at 30°C for 24 h.

2.2 Qualitative Screening for DHA Production from Glycerol

The 486 isolates of *Gluconobacter* and *Asaia* were cultured in test tubes containing 10 mL of an organic screening medium (glycerol 60 g/L, peptone 10 g/L, and yeast extract 5 g/L) at 30°C for 24 h. After 24 hours, the fermented screening media were tested for DHA generation by reacting with the Fehling's reagent [18]. Yellow to red precipitate was inspected in the cultured media with the generated DHA. The non-fermented organic screening medium added with the DHA standard was used as a positive control, and the negative control was the unfermented organic screening medium.

2.3 Quantitative Screening for High DHA Production Microorganisms

The acetic acid bacteria which showed highly positive results from the qualitative screening step were selected to be studied in this section. Microbial inoculums were prepared by culturing the bacteria in a 150-mL Erlenmeyer flask containing 30 mL of the inoculum medium (30 g/L of glycerol, 10 g/L of peptone, and 5 g/L of yeast extract) at 30°C for 24 h with a shaking speed of 150 rpm. The inoculum (1 mL), with absorbance at 560 nm (OD_{560}) around 1.3, was then transferred into a 150-mL Erlenmeyer flask containing a minimal medium (50 mL) composed of only glycerol (60 g/L) and

diammonium phosphate [$(NH_4)_2HPO_4$, 2.8 g/L]. $(NH_4)_2HPO_4$ was used as an inorganic nitrogen source for the microorganisms in place of the usual organic nitrogen sources (yeast extract and peptone) in order to observe the capability of the screened bacteria for DHA production in a low cost culture medium. The inoculated inorganic screening medium was cultured at 30°C for 24 h with a shaking speed of 150 rpm, and the cultured medium was then monitored for DHA and glycerol concentrations by high performance liquid chromatography (HPLC). The DHA production of the screened bacteria was presented as DHA concentration (p, g/L) and DHA yield (y_{sp}, % of DHA moles/glycerol moles). The y_{sp} was calculated by Eq. (2.1) :

$$y_{sp} = (DHA\ moles/initial\ glycerol\ moles) \times 100 \quad \text{(Eq. 2.1)}$$

2.4 Preliminary Optimizations for DHA Production from Glycerol

Gluconobacter frateurii BCC 36199, which showed the highest DHA production from the screening, was cultivated under 8 different culture conditions based on the full factorial design of experiment in order to primarily optimize for DHA production by *G. frateurii* BCC 36199. The culture parameters that were studied in the preliminary optimization were glycerol concentration, pH, and temperature (Table 1). The media were supplemented with $(NH_4)_2HPO_4$ as an inorganic nitrogen source. The cultivation was done at 30°C for 72 h with a shaking speed of 250 rpm in 150-mL Erlenmeyer flask containing 50 mL of the optimizing media. Fermentation samples were collected daily and analyzed for DHA production.

Regression models to describe the relationship between the significant cultivation parameters and the DHA production are derived based on a full factorial design [19]. Statistical analysis of the responses was performed by least squares fitting using DOE PRO XL 2007 (Sigma zone) software. Two levels of three numerical factors (Table 1) were contributed in the statistical calculations.

2.5 Determination of Glycerol and DHA in Glycerol Fermentation

Fermentation samples were centrifuged (5,000 rpm, 10 min) and then analyzed for the amounts of DHA and glycerol by HPLC. The HPLC system was equipped with a CarboSep CHO 682 (7.8 mm × 300 mm, Transgenomic) column as the stationary phase operated at 80°C. De-ionized (DI) water was used as the mobile phase with a flow rate of 0.5 mL/min. Sucrose was used as an internal standard. Culture samples were filtered (VertiClean™ PTFE syringe Filters, 13mm, 0.2µm) before the analysis. A Refractive Index Detector (RID) was used as a detector for the HPLC system.

Table 1. Culture parameters for preliminary optimization.

parameter	code	level	
		-1	1
Glycerol (g/L)	*A*	20	60
pH	*B*	4.5	7.0
Temperature (°C)	*C*	25	30

3. Results and Discussion

3.1 Qualitative Screening for Glycerol Utilizing Microorganisms with the Ability to Generate DHA

Qualitative screening was used as a first step to select DHA- producing microorganisms using the Fehling's reagent test. The unfermented glycerol medium without DHA products, which showed a purple color with the Fehling's test reagent, was used as the negative control. The unfermented glycerol medium with the added DHA (30 g/L), which showed a yellow color with the Fehling's test reagent, was used as the positive control. Cultured samples that showed negative, low positive, and highly

positive results for DHA production gave purple, yellow- green, and yellow colors, respectively, with the Fehling's test reagent. Table 2 shows the bacterial isolates which gave positive results for the qualitative screening. From a total of 486 isolates of acetic acid bacteria, including 230 isolates of *Asaia* and 256 isolates of *Gluconobacter*, only 61 isolates of *Gluconobacter* and 5 isolates of *Asaia*, gave highly positive results, evidenced by the orange color obtained using the Fehling test reagent, as shown in Table 2. These 66 isolates were selected for further quantitative screenings.

According to the qualitative screening results, a larger number of *Gluconobacter* isolates, which expressed positive results for DHA production, were obtained compared to that of *Asaia* isolates. This finding is similar to most of the studies on DHA production using glycerol as a source of carbon; most of these studies commonly use *Gluconobacter* strains, such as *G. melanogenus* [9] , *G. oxydans* [10-12, 20-23], and *G. frateurii* [24, 25] . *Asaia* strains, including *As. bogorensis*, *As. siamensis*, *As. krungthepensis*, and *As. lannaensis*, have been previously reported for their ability to produce DHA from glycerol [13, 14] . However, in these studies, these bacterial strains were not used further for the production of DHA because the ability to produce DHA from glycerol by these bacteria was low, based on the Fehling test [13, 14].

3.2 Quantitative Screening for High DHA-Producing Microorganisms

The 66 isolates of acetic acid bacteria, including the 5 isolates of *Asaia* and the 61 isolates of *Gluconobacter* which gave highly positive results in the qualitative screening, were further studied for their DHA production using glycerol in the quantitative screening step. Based on the DHA concentration (*p*, g/L) produced, the 66 bacterial isolates were categorized into three groups (Table 3) which are 1) low DHA-producing isolates (0 – 5.05 g/L), 2) medium DHA-producing isolates (6.25 – 10.06 g/L), and 3) high DHA-producing isolates (11.38 – 16.89 g/L). *Asaia* sp., *As. bogorensis*, *As. siamensis*, *As. krungthepensis*, and *G. cerinus* produced DHA at low levels. Isolates of *Gluconobacter* sp. and *G. frateurii* produced DHA at low, medium, and high levels. Variations in DHA production levels by the same species, but of different strains, were observed. These results emphasize the need to evaluate the available bacterial isolates for the strain that can result in maximum DHA production. This study found only 7 isolates from a total of 486 isolates screened for their capability of producing a high concentration of DHA from glycerol.

Based on HPLC (Table 3), there were 9 isolates of *G. frateurii* which did not show any DHA production. These 9 isolates, which gave highly positive results on the Fehling test, might have produced very small amounts of DHA that could not be detected by HPLC. The Fehling test is probably more sensitive than the HPLC method because the coloring substrate $CuSO_4$ was added in a substantial amount for the redox chemical reaction to generate the color.

Table 2. Bacterial isolates tested in qualitative screening

Species	Location [17]	Number of isolates	
		Total	Positive in Fehling
G. albidus	*W*	11	-
G. cerinus	*S*	4	2
G. frateurii	*N, NE, M, W*	111	46
G. kanchanaburiensis	*W*	1	-
G. oxydans	N/A	1	-
G. thailandicus	*M*	1	-
G. uchimurae	*M*	1	-
G. wancherniae	*NE*	2	-
Gluconobacter sp.	*N, NE, M, W, S*	128	13
As. astilbis	*S*	2	-
As. bogorensis	*N, S*	143	2
As. krungthepensis	*M, W*	19	1
As. lannensis	*N, S*	3	-
As. siamensis	*N, M, W, S*	21	1

As. spathodeae	*S*	4	-
Asaia sp.	*N, W, S*	38	1

Note: *N* = Northern, *NE* = North-*Eastern*, *M* = Middle, *W* = Western, *S* = Southern, N/A = Not Available (for Thailand)

The remaining 52 isolates of *Gluconobacter*, including 37 isolates of *G. frateurii*, 13 isolates of *Gluconobacter* sp., and 2 isolates of *G. cerinus*, showed variations in DHA productions from 0.31 - 16.89 g/L (Table 3). DHA production yields (y_{sp}, % of DHA moles/glycerol moles) in Table 3 are based on the initial concentration of glycerol (60 g/L) supplied in the screening medium. The yields represent the efficiency of the tested microorganisms for producing DHA from glycerol. The 5 isolates of *Asaia*, including *As. bogorensis* BCC 15913, *Asaia* sp. BCC 15940, *As. siamensis* BCC 26080, *As. bogorensis* BCC 15917, and *As. krungthepensis* BCC 15659, gave low DHA production with concentrations of 0.68 g/L, 0.71 g/L, 3.03 g/L, 3.54 g/L, and 4.17 g/L respectively. *G. cerinus* BCC 7033 and *G. cerinus* BCC 26094 also produced low DHA concentrations of 0.76 g/L and 2.11 g/L respectively. In this study, *G. frateurii* BCC 36199 produced the highest DHA concentration (p) of 16.89 g/L with a DHA production yield (y_{sp}) of 28.78%. *G. frateurii* BCC 36199 was, therefore, selected for further primary optimization.

Table 3. DHA concentration and production yield from quantitative screening.

No.	Genus	Species	BCC	Group of DHA production	p [a] (g/L)	$y_{p/s}$ [a] (%)
1	*Gluconobacter*	*frateurii*	BCC 15685		ND	ND
2	*Gluconobacter*	*frateurii*	BCC 15752		ND	ND
3	*Gluconobacter*	*frateurii*	BCC 15781		ND	ND
4	*Gluconobacter*	*frateurii*	BCC 15799		ND	ND
5	*Gluconobacter*	*frateurii*	BCC 15854		ND	ND
6	*Gluconobacter*	*frateurii*	BCC 15858		ND	ND
7	*Gluconobacter*	*frateurii*	BCC 15859		ND	ND
8	*Gluconobacter*	*frateurii*	BCC 15870		ND	ND
9	*Gluconobacter*	*frateurii*	BCC 15898		ND	ND
10	*Asaia*	*bogorensis*	BCC 15913		0.68 ± 0.00	1.16 ± 0.00
11	*Asaia*	sp.	BCC 15940		0.71 ± 0.01	1.22 ± 0.02
12	*Asaia*	*siamensis*	BCC 26080	Low	3.03 ± 0.04	5.16 ± 0.07
13	*Asaia*	*bogorensis*	BCC 15917		3.54 ± 0.63	6.02 ± 1.07
14	*Asaia*	*krungthepensis*	BCC 15659		4.17 ± 0.34	7.11 ± 0.59
15	*Gluconobacter*	sp.	BCC 26078		0.31 ± 0.37	0.52 ± 0.62
16	*Gluconobacter*	*frateurii*	BCC 15748		0.45 ± 0.22	0.77 ± 0.37
17	*Gluconobacter*	*cerinus*	BCC 7033		0.76 ± 0.08	1.30 ± 0.14
18	*Gluconobacter*	*frateurii*	BCC 15894		0.77 ± 0.40	1.32 ± 0.68
19	*Gluconobacter*	*frateurii*	BCC 15896		0.93 ± 0.05	1.59 ± 0.08
20	*Gluconobacter*	sp.	BCC 49193		1.30 ± 0.09	2.21 ± 0.16
21	*Gluconobacter*	sp.	BCC 49194		1.36 ± 0.20	2.31 ± 0.34
22	*Gluconobacter*	*frateurii*	BCC 15757		1.49 ± 0.31	2.54 ± 0.53
23	*Gluconobacter*	*frateurii*	BCC 15777		1.52 ± 0.31	2.59 ± 0.54

[a] data presented as average ± standard deviation of three independent experiments; ND: not detectable

Table 3. DHA concentration and production yield from quantitative screening. (Continued)

No.	Genus	Species	BCC	Group of DHA production	p [a] (g/L)	$y_{p/s}$ [a] (%)
24	*Gluconobacter*	*frateurii*	BCC 15776		1.62 ± 0.12	2.75 ± 0.20
25	*Gluconobacter*	*frateurii*	BCC 15892		1.75 ± 0.39	2.99 ± 0.67
26	*Gluconobacter*	*frateurii*	BCC 15771		1.99 ± 0.19	3.40 ± 0.33
27	*Gluconobacter*	*frateurii*	BCC 15786		2.00 ± 0.79	3.41 ± 1.34
28	*Gluconobacter*	*cerinus*	BCC 26094		2.11 ± 0.04	3.59 ± 0.07
29	*Gluconobacter*	*frateurii*	BCC 15785		2.13 ± 0.03	3.64 ± 0.06
30	*Gluconobacter*	*frateurii*	BCC 15831		2.17 ± 1.09	3.70 ± 1.85
31	*Gluconobacter*	*frateurii*	BCC 15861		2.29 ± 0.17	3.91 ± 0.29
32	*Gluconobacter*	*frateurii*	BCC 15860		2.38 ± 0.21	4.05 ± 0.35
33	*Gluconobacter*	*frateurii*	BCC 15768		2.69 ± 0.52	4.58 ± 0.88
34	*Gluconobacter*	*frateurii*	BCC 15679		2.81 ± 0.32	4.79 ± 0.54
35	*Gluconobacter*	*frateurii*	BCC 15705	Low	3.02 ± 0.57	5.14 ± 0.97
36	*Gluconobacter*	*frateurii*	BCC 15794		3.10 ± 1.49	5.28 ± 2.54
37	*Gluconobacter*	*frateurii*	BCC 15680		3.36 ± 0.67	5.72 ± 1.14
38	*Gluconobacter*	sp.	BCC 26074		3.49 ± 0.20	5.95 ± 0.34
39	*Gluconobacter*	*frateurii*	BCC 15886		3.49 ± 0.63	5.95 ± 1.07
40	*Gluconobacter*	sp.	BCC 49412		3.61 ± 0.37	6.16 ± 0.63
41	*Gluconobacter*	sp.	BCC 26099		4.01 ± 1.30	6.83 ± 2.22
42	*Gluconobacter*	sp.	BCC 27784		4.08 ± 0.20	6.96 ± 0.34
43	*Gluconobacter*	sp.	BCC 49413		4.31 ± 0.06	7.35 ± 0.10
44	*Gluconobacter*	*frateurii*	BCC 15801		4.73 ± 0.01	8.06 ± 0.03
45	*Gluconobacter*	*frateurii*	BCC 15853		4.97 ± 0.47	8.47 ± 0.80
46	*Gluconobacter*	*frateurii*	BCC 6611		5.05 ± 0.18	8.61 ± 0.30
47	*Gluconobacter*	*frateurii*	BCC 15828		6.25 ± 0.88	10.66 ± 1.50
48	*Gluconobacter*	*frateurii*	BCC 15797		6.80 ± 1.54	11.59 ± 2.62
49	*Gluconobacter*	*frateurii*	BCC 6304		6.91 ± 0.09	11.78 ± 0.14
50	*Gluconobacter*	*frateurii*	BCC 15716		7.35 ± 0.27	12.53 ± 0.47
51	*Gluconobacter*	sp.	BCC 31375		7.60 ± 2.88	12.95 ± 4.91
52	*Gluconobacter*	sp.	BCC 26120		8.32 ± 0.99	14.18 ± 1.68
53	*Gluconobacter*	sp.	BCC 26162	Medium	8.71 ± 0.16	14.85 ± 0.27
54	*Gluconobacter*	*frateurii*	BCC 15855		9.31 ± 0.24	15.86 ± 0.41
55	*Gluconobacter*	*frateurii*	BCC 15789		9.56 ± 0.39	16.28 ± 0.67
56	*Gluconobacter*	*frateurii*	BCC 15766		9.74 ± 1.12	16.59 ± 1.91
57	*Gluconobacter*	*frateurii*	BCC 15755		9.76 ± 0.31	16.64 ± 0.53
58	*Gluconobacter*	*frateurii*	BCC 15738		9.79 ± 0.42	16.68 ± 0.72
59	*Gluconobacter*	sp.	BCC 26083		10.06 ± 0.55	17.14 ± 0.93

[a] data presented as average ± standard deviation of three independent experiments; ND: not detectable

Table 3. DHA concentration and production yield from quantitative screening. (Continued)

No.	Genus	Species	BCC	Group of DHA production	p [a] (g/L)	$y_{p/s}$ [a] (%)
60	*Gluconobacter*	*frateurii*	BCC 15856		11.38 ± 0.22	19.39 ± 0.37
61	*Gluconobacter*	*frateurii*	BCC 15793		11.80 ± 0.36	20.10 ± 0.61
62	*Gluconobacter*	*frateurii*	BCC 15714		12.44 ± 1.24	21.20 ± 2.11
63	*Gluconobacter*	*frateurii*	BCC 15682	High	13.20 ± 0.89	22.49 ± 1.51
64	*Gluconobacter*	sp.	BCC 36733		13.33 ± 1.00	22.71 ± 1.71
65	*Gluconobacter*	*frateurii*	BCC 15796		13.77 ± 1.17	23.46 ± 2.00
66	***Gluconobacter***	***frateurii***	**BCC 36199**		**16.89 ± 2.85**	**28.78 ± 4.85**

[a] data presented as average ± standard deviation of three independent experiments; ND: not detectable

3.2 Preliminary optimizations for DHA production by *G. frateurii* BCC 36199

DHA productions by *G. frateurii* BCC 36199 in 8 different culture conditions are shown in Fig. 1. As shown in Fig. 1, *G. frateurii* BCC 36199 cultured in pH 4.5 produced significantly higher DHA concentrations than those in culture conditions with pH 7.0. In the culture conditions with pH 4.5, *G. frateurii* BCC 36199 cultivated in 20 g/L of glycerol generated significantly higher DHA amounts than those in conditions with 60 g/L of glycerol. The highest p and y_{sp} of 18.67 g/L and 95.44%, respectively, were obtained at 30°C, pH 4.5, and 20 g/L of glycerol.

3.3 Regression Model of DHA Production by *G. frateurii* BCC 36199

The significances of the flask culture conditions, including glycerol concentration (A, g/L), pH (B) and temperature (C, °C), on the responses of interest, which are p (g/L), and y_{sp} (%), were determined using the statistical program DOE PRO XL 2007 (Sigma Zone). The regression models of p and y_{sp}, as shown in Equations 2 and 3, respectively, were fitted with the experimental data shown in Fig. 1. Factor

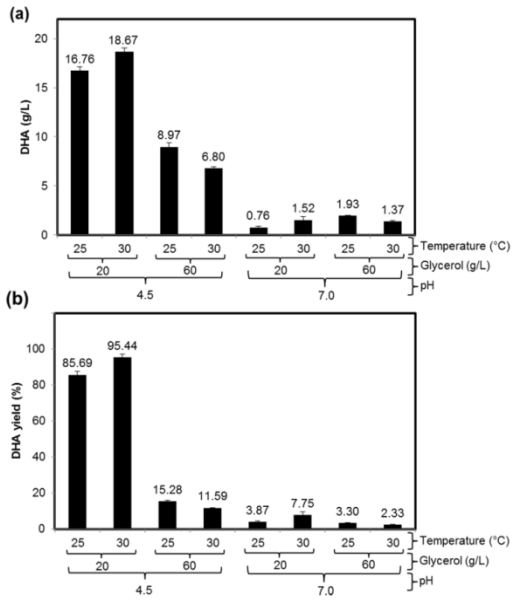

Fig. 1. DHA productions by *G. frateurii* BCC 36199 in 8 culture conditions; (a) DHA concentration (g/L) and (b) DHA production yield (%) from three independent experiments.

coefficients, which represent the effects of the factors on the responses of interest, are shown in the regression models. Positive coefficients present direct positive effects of the factors on the responses. Negative factor coefficients indicate adverse effects of the factors on the responses of interest. The determination coefficients (R^2) of the p and y_{sp} models, which are 0.9991 and 0.9994,

indicate that more than 99% of the total variation in the responses can be explained by the regression models. The statistical analysis of variance (ANOVA) of the models is shown in Table 4. According to Table 4, the calculated *F* values of *p* and y_{sp} (from the models) are 1304.78 and 2009.75, respectively. The calculated *F* values of the developed regression models were greater than 6.0 (theoretical *F* value); therefore, the regression models developed in this study are reliable at the 95% confidence level [26].

$$p = 7.10 - 2.33A - 5.70B + 2.59AB - 0.68AC + 0.34ABC$$

$R^2 = 0.991$ (Eq. 3.1)

$$y_{sp} = 28.16 - 20.03A - 23.84B + 1.12C + 18.53AB - 2.29AC$$

$R^2 = 0.9994$ (Eq. 3.2)

where p: DHA concentration (g/L); y_{sp}: DHA yield (% of DHA moles/glycerol moles); A: glycerol concentration (-1, 1); B: pH (-1, 1); and C: temperature (-1, 1)

According to the models, there are five (*A*, *B*, *AB*, *AC*, and *ABC*) and six (*A*, *B*, *C*, *AB*, *AC*, and *ABC*) significant linear terms for the *p* and y_{sp} models, respectively. Coefficients of *A* for *p* (-2.33) and y_{sp} (-20.03) may indicate an inhibition effect on DHA production due to a high glycerol concentration. The inhibition effect of high glycerol concentration on DHA production by *G. oxydans* is commonly found [27]. *B* also expressed a negative effect on *p* (-5.70) and y_{sp} (-23.84). Since *Gluconobacter* is acetic acid bacteria which normally produce acetic acid during their growth, the bacteria prefer culture conditions wherein the pH is low [17]. *G. oxydans*, which is the most common bacteria for DHA production, was also cultivated under low pH conditions [3, 28]. Temperature (*C*) has no significant effect on *p* but the factor shows a small positive effect on y_{sp} (1.12). *G. frateruii* BCC 36199 was isolated from a tropical country, Thailand [17]; thus, the bacteria prefer higher temperature conditions. Commonly, DHA productions using acetic acid bacteria are carried out at 28°C - 30°C [10, 12, 23, 25]. Significantly positive interaction effects of *A* and *B* were observed on both *p* (2.59) and y_{sp} (18.53). The significant interaction effects of *A* and *B* on *p* and y_{sp} indicate that the utilization of higher glycerol concentration is enhanced under lower pH culture conditions. However, the interaction effects between *A* and *C* are very small at -0.68 for *p* and -2.29 for y_{sp}.

The three dimensional response surface plots of the two interaction factors (Fig. 2) show that *A* and *B* have synergistic effects on the responses of interest. *p* (Fig. 2 (a)) and y_{sp} (Fig. 2 (c)) increased significantly at the lower ends of glycerol and pH axes. This indicates the interaction effects of the factors on the responses. In addition, the regression models show that there are small interactions among the three factors positively affecting *p* (0.34) and y_{sp} (1.07) (Eq. (3.1), Eq. (3.2)).

The optimum flask culture conditions for DHA production by *G. frateurii* BCC 36199 were predicted by the developed regression models. The models predicted that *p* and y_{sp} of 18.66 g/L and 95.38%, respectively, can be obtained at 20 g/L of glycerol, pH 4.5, and 30°C (Table 5). The highest *p* of 18.67 g/L and y_{sp} of 95.44% were obtained with an actual experiment, under the same culture conditions as predicted by the regression models. Therefore, the developed optimization models were able to accurately predict the optimal conditions and the results of DHA production.

3.4 Kinetics of DHA Production in Flask Cultivation

The time profiles of glycerol consumption and DHA production of *G. frateurii* BCC 36199, cultured at 30°C, 20 g/L of glycerol, and pH 4.5 are shown as Fig. 3.

Table 4. Y-hat regression table of DHA production in the flask culture calculated using DOE PRO XL (Sigma Zone).

Factor	DHA (g/L)				Yield (%)			
	Coefficient	***P* (2 Tail)**	**Tolerance**	**Sig.**	**Coefficient**	***P* (2 Tail)**	**Tolerance**	**Sig.**
Constant	7.10	0.0000			28.16	0.0000		
A (glycerol, g/L)	-2.33	0.0000	1	X	-20.03	0.0000	1	X
B (pH)	-5.70	0.0000	1	X	-23.84	0.0000	1	X
C (temperature, °C)	-0.01	0.9039	1		1.12	0.0064	1	X
AB	2.59	0.0000	1	X	18.53	0.0000	1	X
AC	-0.68	0.0000	1	X	-2.29	0.0001	1	X
BC	0.06	0.4441	1		-0.39	0.2359	1	
ABC	0.34	0.0012	1	X	1.07	0.0080	1	X
R^2	0.9991				0.9994			
Adj R^2	0.9984				0.9989			
Std Error	0.28				1.23			
F	1304.78				2009.75			

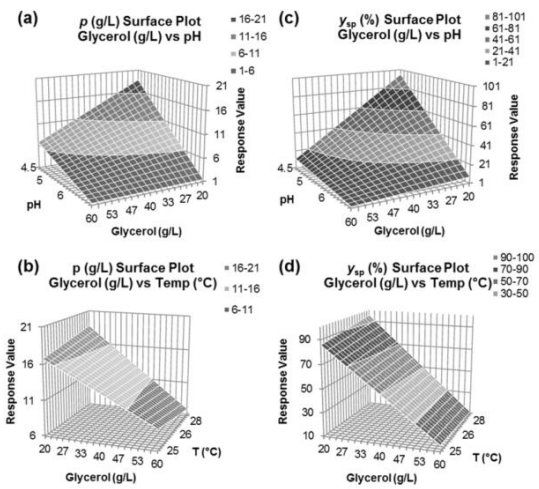

Fig. 2. Graphical surface plots of DHA production by *G. frateurii* BCC 36199 in flask culture: (a) glycerol and pH for DHA (g/L), (b) glycerol and temperature for DHA (g/L), (c) glycerol and pH for DHA yield (%), and (d) glycerol and temperature for DHA yield (%)

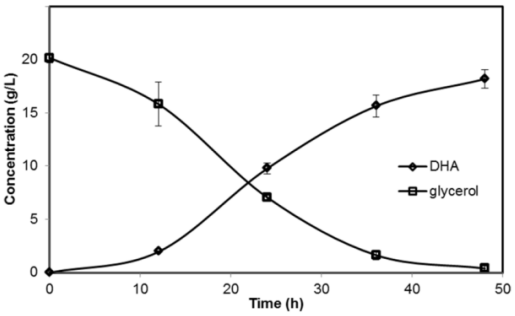

Fig. 3. Time profiles of DHA production in flask cultivation by *G. frateurii* BCC 36199

Table 5. Comparisons between the predicted outcomes by the developed optimization models and the experimental results.

Response	Optimization		Error (%)
	Predicted Values	**Experimental Results**	
p_{max} **(g/L)**	18.66	18.67	0.054
y_{sp} **(%)**	95.38	95.44	0.063

Table 6. Kinetic parameters of DHA production from glycerol by *G. frateurii* BCC 36199 in flask cultivation.

p_{max} (g/L)	y_{sp} (%)	$r_{s\,max}$ (g/Lh)	$r_{p\,max}$ (g/Lh)
18.17 ± 0.85	92.86 ± 4.36	0.59 ± 0.11	0.57 ± 0.04

Data: average ± standard deviation of three independent experiments.

The exponential growth phase of DHA production began at the 12th hour of the cultivation. Glycerol was completely consumed by the 48th hour. The maximum DHA concentration (p_{max}) of 18.17 g/L with y_{sp} of 92.86% was obtained (Table 6). A glycerol consumption rate ($r_{s\,max}$) and a DHA production rate ($r_{p\,max}$) of 0.59 g/Lh and 0.57 g/Lh, respectively, were obtained. The high y_{sp} observed in this study suggests that the optimum culture conditions, established in this study, are suitable for DHA production by *G. frateurii* BCC 36199.

In comparison, *G. frateurii* BCC 36199 (as per the experiments carried out in this study) produced lower DHA than did *G. oxydans* [4, 10-12, 29] and *G. frateurii* [24, 25] in studies reported previously. *G. oxydans* was cultivated under suitable culture conditions which were optimized for DHA production in a bioreactor [12,29]. Mutants of *G. oxydans* were also created in order to improve the DHA production ability of the bacteria [4,10,11]. *G. frateurii* was also cultured under optimum culture conditions, which served as the optimized fed-batch fermentation for DHA production by *G. frateurii* [25]. For this study, *G. frateurii* BCC 36199 was cultivated in a preliminary optimized minimal medium containing only glycerol and $(NH_4)_2HPO_4$ as carbon and nitrogen sources, respectively. The culture medium may not be the most preferable set of conditions for the bacteria. Therefore, the culture conditions can be further optimized to maximize DHA production by *G. frateurii* BCC 36199

This study evaluated DHA production using new isolates of acetic acid bacteria in the genera of *Gluconobacter* and *Asaia* found in tropical countries. *G. frateurii* BCC 36199 was the most promising DHA producing microorganism observed in this study. Although *G. frateurii* BCC 36199 produced lower DHA than in experiments reported previously, DHA production by *G. frateurii* BCC 36199 could be maximized by further study on the optimization of culture conditions in a flask and a bioreactor.

4. Conclusions

Certain isolates (66) of screened acetic acid bacteria from a total of 486 isolates showed highly positive results in the small scale qualitative screenings. Quantitative screening in flask cultivation showed that *Asaia* produced low concentrations of DHA; *Gluconobacter* produced DHA in the range of low to high levels. *G. frateurii* BCC 36199 was selected for preliminary optimization and the bacterial culture produced 18.67 g/L of DHA with y_{sp} of 95.44% at 30 °C, 20 g/L of glycerol, and pH 4.5. *G. frateurii* BCC 36199 is a promising microorganism for DHA production in a low-cost culture medium which contains only glycerol as a carbon source and $(NH_4)_2HPO_4$ as an inorganic nitrogen source. Culture conditions could be further optimized in order to obtain the highest DHA production by *G. frateurii* BCC 36199.

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References

[1] da Silva GP, Mack M, Contiero J. Glycerol: A Promising and Abundant Carbon Source for Industrial Microbiology. Biotechnol Adv 2009;27(1):30-9.

[2] alibaba. com [Internet] . Hangzhou: Chemicals; c1999- 2017 [updated 2017; cited 2017 Feb 8] . Available from: https://www.alibaba.com/trade/search?fsb=y&IndexArea=product_en&CatId=&SearchText=glycerine.

[3] Hekmat D, Bauer R, Fricke J. Optimization of the Microbial Synthesis of Dihydroxyacetone from Glycerol with *Gluconobacter oxydans.* Bioproc and Biosys Eng 2003;26(2):109-16.

[4] Li MH, Wu J, Liu X, Lin JP, Wei DZ, Chen H. Enhanced Production of Dihydroxyacetone from Glycerol by Overexpression of Glycerol Dehydrogenase in an Alcohol Dehydrogenase Deficient Mutant of *Gluconobacter oxydans.* Biores Technol 2010;101(21) : 8294-99.

[5] Virtanen AI, Nordlund M. An Improved Method for the Preparation of Dihydroxyacetone. Biochem Jol 1933;27(2):442-4.

[6] Nabe K, Izuo N, Yamada S, Chibata I. Conversion of Glycerol to Dihydroxyacetone by Immobilized Whole Cells of *Acetobacter xylinum.* Appl and Environ Microbiol 1979;38(6):1056-60.

[7]. Yamada Y, Yukphan P, Lan Vu HT, Muramatsu Y, Ochaikul D, Tanasupawat S, et ai. Description of *Komagataeibacter* gen. nov. , with Proposals of New Combinations (*Acetobacteraceae*). Jol of Gen and Appl Microbiol 2012;58(5):397-404.

[8] Hauge JG, King TE, Cheldelin VH. Alternate Conversions of Glycerol to Dihydroxyacetone in *Acetobacter suboxydans.* Jol of Bio Chem 1955;214(1):1-9.

[9] Flickinger MC, Perlman D. Application of Oxygen- Enriched Aeration in the Conversion of Glycerol to Dihydroxyacetone by *Gluconobacter melanogenus* IFO 3293. Appl and Environ Microbiol 1977;33(3):706-12.

[10] Hu ZC, Liu ZQ, Xu JM, Zheng YG, Shen YC. Improvement of 1,3- Dihydroxyacetone Production from *Gluconobacter oxydans* by Ion Beam Implantation. Prepar Biochem and Biotechnol 2012;42(1) : 15-28.

[11] Ma L, Lu W, Xia Z, Wen J. Enhancement of Dihydroxyacetone Production by a Mutant of *Gluconobacter oxydans.* Biochem Eng Jol 2010;49(1):61-67.

[12] Zhou X, Zhou X, Xu Y, Yu S. Improving the Production Yield and Productivity of 1,3- Dihydroxyacetone from Glycerol Fermentation Using *Gluconobacter oxydans* NL71 in a Compressed Oxygen Supply- Sealed and Stirred Tank Reactor (COS- SSTR) . Bioproc and Biosys Eng 2016;39(8):1315-8.

[13] Malimas T, Yukphan P, Takahashi M, Kaneyasu M, Potacharoen W, Tanasupawat S, et al. *Asaia lannaensis* sp. nov., a New Acetic Acid Bacterium in the *Alphaproteobacteria,* Biosci Biotechnol and Biochem 2008;72(3):666-71.

[14] Yukphan P, Potacharoen W, Tanasupawat S, Tanticharoen M, Yamada Y. *Asaia krungthepensis* sp. nov., an Acetic Acid Bacterium in the *α-Proteobacteria*. Int Jol of System and Evolution Microbiol 2004; 54(2):313-6.

[15] Gupta A, Singh VK, Qazi GN, Kumar A. *Gluconobacter oxydans*: Its Biotechnological Applications. J Mol Microbiol Biotechnol 2001;3(3):445-56.

[16] Battey AS, Schaffner DW. Modelling Bacterial Spoilage in Cold-Filled Ready to Drink Beverages by *Acinetobacter calcoaceticus* and *Gluconobacter oxydans.* Jol of Appl Microbiol 2001;91(2):237-47.

[17] Lisdiyanti P, Katsura K, Potacharoen W, Navarro RR, Yamada Y, Uchimura T, et al. Diversity of Acetic Acid Bacteria in Indonesia, Thailand, and the Philippines. Microbiol and Cult Coll 2003;19(2):91-9.

[18] Hu ZC, Zheng YG. A High Throughput Screening Method for 1,3- Dihydroxyacetone- Producing Bacterium by Cultivation in A 96-Well Microtiter Plate. Jol of Rap Meths & Automat in Microbiol 2009;17(2):233-41.

[19] Montgomery DC, Runger GC. Applied Statistics and Probability for Engineers. 6th ed. United State of America: Wiley; 2014.

[20] Bories A, Claret C, Soucaille P. Kinetic Study and Optimisation of the Production

of Dihydroxyacetone from Glycerol Using *Gluconobacter oxydans.* Proc Biochem 1991;26(4):243-8.

[21] Claret C, Salmon JM, Romieu C, Bories A. Physiology of *Gluconobacter oxydans* During Dihydroxyacetone Production from Glycerol. Appl Microbiol and Biotechnol 1994;41(3):359-65.

[22] Gätgens C, Degner U, Bringer-Meyer S, Herrmann U. Biotransformation of Glycerol to Dihydroxyacetone by Recombinant *Gluconobacter oxydans* DSM 2343. Appl Microbiol and Biotechnol 2007;76(3):553-9.

[23] Hekmat D, Bauer R, Neff V. Optimization of the Microbial Synthesis of Dihydroxyacetone in a Semi-Continuous Repeated-Fed-Batch Process by In Situ Immobilization of *Gluconobacter oxydans.* Proc Biochem 2007;42(1):71-6.

[24] Liu YP, Sun Y, Tan C, Li H, Zheng XJ, Jin KQ, et al. Efficient Production of Dihydroxyacetone from Biodiesel-Derived Crude Glycerol by Newly Isolated *Gluconobacter frateurii*. Biores Technol 2013;142:384-9.

[25] Zheng XJ, Jin KQ, Zhang L, Wang G, Liu YP. Effects of Oxygen Transfer Coefficient on Dihydroxyacetone Production from Crude Glycerol. Brazil Jol of Microbiol 2016;47:129-35.

[26] James G, Witten D, Hastie T, Tibshirani R. An Introduction to Statistical Learning. 1st ed. New York: Springer; 2013.

[27] Claret C, Bories A, Soucaille P. Glycerol Inhibition of Growth and Dihydroxy-acetone Production by *Gluconobacter oxydans.* Curr Microbiol 1992;25(3):149-55.

[28] Leonhard Ohrem H, Voß H. Process Model of the Oxidation of Glycerol with *Gluconobacter oxydans.* Proc Biochem 1996;31(3):295-301.

[29] Hu ZC, Zheng YG, Shen YC. Dissolved-Oxygen-Stat Fed-Batch Fermentation of 1,3-Dihydroxyacetone from Glycerol by *Gluconobacter oxydans* ZJB09112. Biotechnol and Bioproc Eng 2010;15(4):651-6.