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Optimization of Fermentation Conditions for Production of 1, 3-Dihydroxyacetone from Glycerol Obtained as a Byproduct during Biodiesel Production

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Authors' contributions

This work was carried out in collaboration between both authors. Author BLS managed the analyses of the study, performed the statistical analysis and wrote the first draft of the manuscript. Author KRS designed the study, managed the literature searches and wrote the final manuscript. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: Present study was carried out using the glycerol obtained during biodiesel production for the conversion of 1,3-Dihydroxyacetone (DHA) by fermentation using bacterial strains *G. oxydans*, *B. licheniformis* and *Acetobacter sp*.
Study Design: Experiments were carried out to optimize fermentation conditions like media optimization by the addition of metal ions, physical parameters such as pH (3.5 to 7), temperature (20ºC to 45ºC), glycerol concentration (2% to 20%) and fermentation time (24 h to 144 h) were considered for optimization studies.
Methodology: Fermentation was carried in 2000 ml Erlenmeyer flask with agitation speed 120 rpm in shaker incubator (Scigenics Biotech Pvt. Ltd.), starter cultures was adjusted to approximately $5x10^5$ CFU/ml with sterile saline solution. Experiments were carried out in triplicate and standard

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deviation was calculated. Amount of DHA produced in the final fermentation broth was estimated by 3, 5-Dinitrosalicylic acid (DNS) and HPLC method. Coagulation, filtration, lyophilization and vacuum evaporation were carried out to extract DHA from the fermentation broth in a pure form.

Results: The optimum fermentation conditions for conversion of crude glycerol to DHA by *G. oxydans* were found to be fermentation period of 72 h with 10% glycerol concentration using modified MRS media at pH of 5.5 and 30ºC of temperature. The yield of DHA was 92.61 g/l.

Conclusion: The present study can be potentially useful for the efficient DHA production on industrial scale. In 1000 ml of fermentation media that contain 100 g of crude glycerol, and metal ions, were obtained 92.61 g of DHA (92% conversion rate of glycerol to DHA) after 72 h of fermentation with *G. oxydans*.

Keywords: Glycerol; fermentation; 1,3-Dihydroxyacetone (DHA); biodiesel; metal ions; optimization.

1. INTRODUCTION

Due to limited resources of petroleum and environmental concern, plant seed oils and animals fat has increased the demand of biodiesel production. Currently, biodiesel is much more popular as eco-friendly fuel. On the other hand co-products are utilized for the enhancement of economic and sustainable development of biofuel industries. There is a need to give preference for the value addition on the by-products which obtained during the biofuel production process [1].

The biodiesel industry is growing rapidly and continued rapid growth is expected over the next decade. Natural oils are converted to biodiesel by a relatively simple transformation process called transesterification [2]. The process involves combining vegetable oils animal fats, and/or microalgae oils with alcohol (ethanol or methanol) in the presence of a catalyst (sodium or potassium hydroxide) to form fatty esters (ethyl or methyl ester) and glycerol (12%) [3,4]. Utilization of this by-product for the production of high value product is expected to revolutionize biodiesel production and its economics [5,6].

While technologies for the production of biodiesel are available but methods/technologies for by-product utilization are not well developed. If new applications/methods for utilization of by-products like glycerol are developed then the biodiesel production cost would be reduced. The development of processes for the utilization of glycerol produced in biodiesel production will add value to biodiesel production. Moreover, DHA is a oxidation product of glycerol and is an important precursor for the synthesis of various fine chemicals and precursors of pharmaceuticals [7,8], cosmetic industry for making artificial suntans [9-11].

Currently, DHA is industrially produced either by chemical process [12] and or by microbial enzymatic and fermentation process. In turn, the microbial enzymatic methods consist of partial oxidation of glycerol to DHA by some strains of acetic acid bacteria which produces a high activity of glycerol dehydrogenase [13,14]. Microbial fermentation process using *G. oxydans,* is unsurpassed by other organisms. This has ability to completely oxidize a great variety of carbohydrates, alcohols and related compounds [15]. Although the microbial fermentation process can provide high selectivity to DHA when compared to chemical oxidation process but it has some drawbacks such as low productivity and high production cost [16]. In this context the present study investigates the development of microbial process for conversion of glycerol obtained from biodiesel production to DHA and optimization of fermentation conditions.

2. MATERIALS AND METHODS

2.1 Strains and Their Maintenance

The bacterial strains *Gluconobacter oxydans* (MTCC 0904), *Bacillus licheniformis* (MTCC 3054) and *Acetobacter sp.* (MTCC 3245) were obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India. Crude glycerol was obtained from Lab Land Biotech Pvt. Limited, Mysore, India. Solvents used for the sample preparations and chromatography analyses were of HPLC grade and obtained from Fisher Scientific and all the Chemicals and reagents used were of analytical grade. Starter culture was prepared by adding a loop full of bacterial culture (approximately $5x10^5$ CFU/ml) in to autoclaved MRS broth media (100 ml) and incubated at 27ºC for 48 h.

2.2 Optimization of Production Media

Four different selective production Media were used for optimization studies; they are Sorbitol broth media (M1), Glucose, Yeast extract,

Calcium carbonate broth media (GYC) (M2), DeMan, Rogosa and Sharpe (MRS) broth media with reduced metal ions (M3) [17] and MRS broth media (M4).10 ml of starter culture ($5x10^5$ CFU/ml) transferred to 1000 ml of all four production media (M1, M2, M3 & M4) and incubated at 37ºC with the agitation of 120 rpm for 96 h. The amount of DHA yield was estimated in 48 h, 72 h and 96 h by 3, 5-dinitrosalicylic acid (DNS) method. Among the four media tested, Media M4 has kept basis for formulating modified MRS media (M5, M6 & M7) by addition of more metal ions i.e., 5 mM & 10 mM of all metal ions added constantly for M5 & M6 respectively, for M7 varied concentration was added (Table 1). The metal ions were added by dissolving respective concentration of $FeSO_4.4H_2O$, $MgSO_4.7H_2O$, $MnSO_4.4H_2O$, KH_2PO_4, $CaCO_3$, NaCl, K_2HPO_4, K_2SO_4, Na_2SO_4, in 1000 ml pre-prepared MRS broth and MRS media without metal ions served as a control. For all the flasks 10% v/v of glycerol was added and flasks were sterilized at 121ºC for 15 min, cooled to room temperature. Flasks were inoculated with 10 ml of starter culture of *G. oxydance* and incubated at 30°C for 96 h with agitation of 120 rpm in shaker incubator. During fermentation process, DHA yield was estimated in 48 h, 72 h and 96 h by DNS method. Experiments were carried out in triplicate with same conditions. Further the medium M7 was considered for optimization of physical parameters such as glycerol concentration, pH, temperature and time.

2.3 Optimization of Glycerol Concentration in Media

To investigate the effect of glycerol concentration on DHA production, 1000 ml of production media (M7) was mixed with different concentration of glycerol (2, 4, 6, 8, 10, 12, 14, 16, 18 & 20%) and autoclaved at 121^0C for 15 min. 10 ml of starter culture adjusted to approximately $5x10^5$ CFU/ml was inoculated into production media under sterile conditions and incubated at 37ºC for 96 h at 120 rpm in shaker incubator. During fermentation process, DHA yield was estimated in 48 h, 72 h and 96 h by DNS method.

2.4 Optimization Study of Media pH

One thousand milliliter of production media (M7) was prepared with glycerol (10%) and pH of the media was adjusted in between 3.5 to 7 (3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 & 7.0) using 0.1 N HCl/NaOH, autoclaved at 121ºC for 15 min. 10 ml of starter culture ($5x10^5$ CFU/ml) was inoculated into production media under sterile conditions and incubated at 37ºC for 96 h at 120 rpm in shaker incubator (sygenics pvt ltd). During fermentation process, DHA yield was estimated in 48 h, 72 h, and 96 h by DNS method.

Table 1. Production media compositions for optimization

Sl No	Media components In 1000 ml	M1	M2	M3	M4	M5	M6	M7
01	Peptone	10 g	03 g	10 g	10 g	10 g	10 g	10 g
02	Beef extract	1.0 g	---	10 g	10 g	10 g	10 g	10 g
03	Sorbitol 80	5.0 g	---	1.0 g	1.0 g	1.0 g	1.0 g	1.0 g
04	Yeast extract	20 g	10 g	5.0 g	5.0 g	5.0 g	5.0 g	5.0 g
05	Glucose	---	20 g	20 g	20 g	20 g	20 g	20 g
06	Tween 80	---	---	1.0 ml	1.0 ml	1.0 ml	1.0 ml	1.0 ml
07	Sodium acetate	---	---	1.0 g	5.0 g	5.0 g	5.0 g	5.0 g
08	Ammonium Citrate	---	---	0.5 g	2.0 g	2.0 g	2.0 g	2.0 g
09	K_2HPO_4	---	---	0.5 g	2.0 g	5.0 mM	10 mM	5.0 mM
10	$MgSO_4.7H_2O$	0.5 g	---	0.1 g	0.1 g	5.0 mM	10 mM	10 mM
11	$MnSO_4.4H_2O$	---	---	---	0.05 g	5.0 mM	10 mM	5.0 mM
12	NaCl	5.0 g	---	---	---	5.0 mM	10 mM	5.0 mM
13	$FeSO_4.4H_2O$	----	----	---	---	5.0 mM	10 mM	10 mM
14	Na_2SO_4	---	---	---	---	5.0 mM	10 mM	10 mM
15	$CaCO_3$	---	20 g	0.2 g	---	5.0 mM	10 mM	5.0 mM
16	KH_2PO_4	1.5 g	---	0.1 g	---	5.0 mM	10 mM	5.0 mM
17	Glycerol	← 10% (V/V) →						

--- Nil, pH was adjusted to 6.0 for all media

2.5 Optimization of Fermentation Time

1000 ml of production media (M7) was inoculated with 10 ml of starter culture ($5x10^5$ CFU/ml), under sterile conditions and incubated at 37ºC with agitation of 120 rpm in shaker incubator. Samples were taken out at different intervals of time (24 h, 48 h, 72 h, 96 h, 120 h & 140 h) and immediately the DHA yield was estimated using DNS method.

2.6 Optimization of Temperature

To investigate the effect of temperature on DHA production, 1000 ml of production media was prepared with the glycerol (10%) and autoclaved at 121ºC for 15 min. 10 ml of starter culture ($5x10^5$ CFU/ml) was inoculated under sterile conditions. The flasks were incubated at different temperature (20, 25, 30, 35, 40 and 45ºC) with agitation of 120 rpm for 96 h. During fermentation process, DHA yield was estimated in 48 h, 72 h. and 96 h by DNS method.

2.7 Extraction and Recovery of DHA

Fermentation broth was pretreated with low molecular weight chitosan (300 mg/l) for 30 min with agitation speed of 200 rpm. Chitosan solution was prepared by dissolving the 300 mg chitosan flakes in 10 ml of glacial acetic acid. The flakes were pre-soaked in water for overnight, 3% (v/v) activated carbon is added to decolorize and remove other impurities followed by filtered and centrifuged at 5000 rpm for 15 min. The pretreated broth was lyophilized and ethanol was added in the ratio 1:3 to remove salt ions and proteins by precipitation. The liquid phase obtained after precipitation was evaporated under vacuum and DHA residue was recovered using 90% alcohol after evaporation.

2.8 Quantification of DHA

For DHA estimation, DHA residue after extraction was taken and centrifuged at 10,000 rpm for 10 min. Supernatant was reused for further DHA extraction and pallets was used for quantification by colorimetric method [18]. DHA reduces DNS and the resultant compound gives orange red colored complex, the intensity was red at 550 nm [19]. For the confirmation DHA was also analyzed using high performance liquid chromatography (HPLC) method [20,21]. 150 µl of sample was mixed with 50 µl of saturated NaCl solution then 150 µl of Penta-fluorobenzyl hydroxylamine (PFBHA) solution (10 mg/ml) was added to the sample mixture and shaken for 10 min. The above sample was mixed with 150 µl of saturated NaCl solution and 1 ml of acetonitrile for extraction of the DHA. The sample was centrifuged at 10000 rpm for 15 min. Two clear phases were obtained around 20 µl of sample from acetonitrile phases was injected to C^{18} column. HPLC analysis system was setup in binary isocratic gradient using acetonitrile as mobile phase and flow rate was adjusted to 1 ml/min. and carried out with UV detector, where it had the maximum absorption range of 270–285 nm (Fig. 1).

2.9 Statistical Analysis

Data of all experiments were performed in triplicates, statistically analyzed and expressed as the mean ± standard deviation of three replicate experiments. 1000 ml of fermentation media contain 100 g of glycerol, results were obtained in g/l and the conversion rate of glycerol to DHA was expressed in % yield [17].

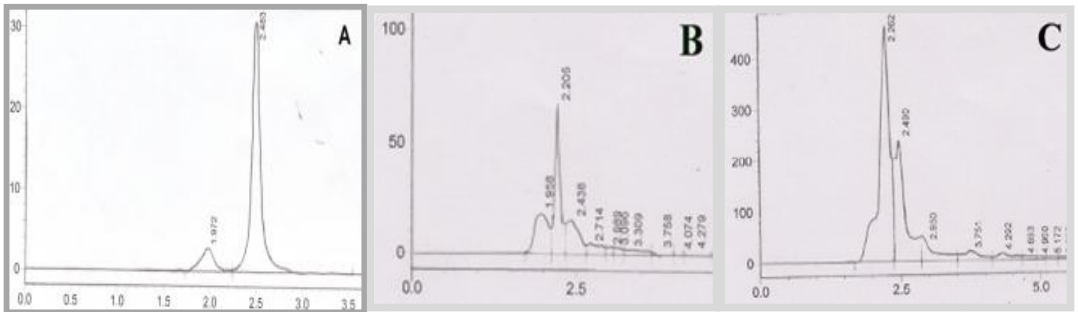

Fig. 1. HPLC Chromatograph of DHA standard g/l (A), culture broth sample solution (B) & (C)
Conditions: mobile phase acetonitrile–water (90:10,v/v), flow rate 1.0 mg/ml, injection volume 20 µl, at 270 nm at room temperature

3. RESULTS

3.1 Selection of Strains

Three strains *B. licheniformis* (MTCC 3054), *Acetobacter sp.* (MTCC 3245) and *G. oxydans* (MTCC 0904) were screened for the maximum yield of DHA from glycerol. Since the *G. oxydance* (MTCC 0904) showed the maximum yield of 69.0±4.6% DHA at 96 h (Table 2), it was selected for further optimization studies.

3.2 Production Media Optimization

Preliminarily four production media (M1, M2, M3 & M4) were evaluated for DHA production, among the four tested media, the MRS broth Media with reduced metal ions (M3) gave slight high yield of 42.1±1.9% of DHA at 96 h, subsequently media M4 gave highest yield of 62.9±2.6% of DHA at 96 h (Table 3). On this basis the media M4 was formulated to media M5, M6, and M7 by the addition of more metal ions in different concentration and evaluated for DHA production. From this the results showed in media M5 with 5 mM metal ions gave high yield than media M6 with 10 mM metal ions (31.5±2.0%) at 96 h, comparatively drastic decrease in the productivity with high concentrated metal ions in M6. Further in media M7 with varied concentration of metal ions showed significantly increased highest production of DHA (92.6±2.1%) when compared to all other media at 72-96 h of incubation (Table 3).

3.3 Optimization of Glycerol Concentration in Production Media

The effect of glycerol concentration on the yield of DHA was carried out using different concentration of glycerol with the medium M7, at three different fermentation times (48 h, 72 h & 96 h). The DHA concentration in production media was gradual increased with the increase in the concentration of glycerol from 2 to 10% v/v, however there was no further increase in the yield of DHA above 10% concentration of glycerol, further decreased in the productivity of DHA after 12% of glycerol concentration (Table 4). High glycerol concentration may leads to inhibitory effect on DHA production by *G. oxydans* [13,22].

3.4 Optimization of Temperature

The results of effect of temperature on production of DHA was carried out at different temperature (20, 25, 30, 35, 40 & 45ºC) showed the optimum temperature for the production of DHA was 30ºC (89.9±3.7%) at 72 h. However there was no significant increase in DHA concentration (90.3± 2.6%) at 96 h of fermentation at 30ºC (Table 5). The yield of DHA decreases as the temperature increase above 35ºC and at 45ºC there was a drastic decrease in DHA yield.

Table 2. Yield of DHA using three strains of bacteria

Bacterial strains	Conversion of glycerol to DHA (%)			
	24 h	48 h	72 h	96 h
Gluconobacter oxydance	33.1±2.8	45.6±3.2	68.1±1.1	69.0±4.6
Acetobacter sp.	18.6±1.6	23.2±1.9	45.3±3.6	48.3±3.8
Bacillus licheniformis	16.8±2.1	21.7±2.4	29.6±2.4	39.2±2.9

± *Standard deviation*

Table 3. Study on yield of DHA using different media

Media	Conversion of glycerol to DHA (%)		
	48 h	72 h	96 h
M1	11.6±2.6	16.2±2.2	21.0±1.9
M2	14.0±3.5	18.4±3.7	22.7±2.7
M3	11.9±3.4	30.0±2.9	42.7±1.9
M4	24.0±4.6	51.4±4.1	62.9±2.6
M5	31.0±3.8	59.2±2.5	63.1±1.3
M6	18.6±2.4	23.8±4.2	31.5±2.0
M7	39.6±3.1	91.8±4.3	92.6±2.1

Table 4. Glycerol concentration and corresponding DHA production

Concentration of glycerol (% v/v)	Conversion of glycerol to DHA (%)		
	48 h	72 h	96 h
2	44.9±4.2	93.8±2.8	94.1±2.1
4	43.5±3.8	93.1±2.5	93.9±2.6
6	43.1±.4.8	93.0±3.1	93.2±2.2
8	42.4±4.3	92.8±3.1	93.0±2.0
10	41.6±3.5	92.8±3.3	93.0±1.8
12	41.2±4.0	92.1±2.9	92.0±1.8
14	41.1±4.3	91.1±3.4	91.1±1.5
16	38.6±3.6	81.4±3.0	82.3±2.0
18	29.4±4.1	66.3±2.8	69.2±2.4
20	21.4±4.0	51.2±2.4	54.6±1.8

Table 5. Study on effect of temperature on DHA production

Temperature (ºC)	Conversion of glycerol to DHA (%)		
	48 h	72 h	96 h
20	10.4±4.1	24.3±3.4	42.9±2.9
25	21.6±3.7	32.1±3.1	73.2±2.4
30	35.9±4.1	89.9±3.7	90.3±2.6
35	33.3±3.9	87.2±3.4	76.2±2.1
40	30.2±4.0	65.6±2.9	54.2±3.1
45	24.0±3.6	32.0±3.0	28.4±2.8

3.5 Optimization of Fermentation Time

The effect of fermentation time on production of DHA, at different time intervals (24 h to 144 h), showed that the conversion of glycerol to DHA begins from 24th h and gradually increased till 96th h, however the yield of DHA steadily decreased after 96th h of fermentation, the optimum fermentation time was 72 h with the yield of 78.9±4.1% of DHA (Table 6).

Table 6. Study on effect of fermentation period on DHA production

Time (h)	Conversion of glycerol to DHA (%)
24	14.7±4.5
48	37.4±4.8
72	78.9±4.1
96	79.4±4.6
120	86.3±4.0
144	86.3±4.2

3.6 Media pH Optimization

The acidic pH was essential for the production of DHA through fermentation by *G. oxydance* [8,23,24]. DHA production at pH 6.0 by immobilized cells was also reported [25]. Usually during fermentation process the acidity increases, since the media contained $CaCO_3$ which neutralizes acid to keep the pH constant, and also the phosphates added in the media have buffer action [17,26]. The results showed that the maximum production of DHA (91.3±3.1%) at 96 h was at pH 5.5 (Table 7). However there is decrease in the DHA yield as the pH increased above 6.0.

4. DISCUSSION

Bioconversion of glycerol to DHA through bacterial fermentation was due to the activity of membrane bound glycerol dehydrogenase. Based on the preliminary evaluation of three strains (*G. oxydance, Acetobacter sp.* and *B. licheniformis*) for the DHA production by fermentation in shake flask method, *G. oxydance* showed highest yield of DHA (69.0±4.6% in 96 h) and we have considered it for further studies. In recent years microbial technologies like expression of glycerol dehydrogenase gene by mutant strains and recombinant strains [27,28], media components optimization [17,26,29] and fermentation reactor designing [29,30] were blooming for the improvement of DHA production. Based on these studies we were introduced metal ions as described by Hu et al. [17] along with carbon sources in media to enhance the rate of production of DHA.

Table 7. Study on effect of pH in fermentation media on DHA production

pH range	Conversion of glycerol to DHA (%)		
	48 h	72 h	96 h
3.5	11.0±3.8	06.4±3.5	0.0
4.0	11.8±4.0	07.0±3.1	04.8±1.8
4.5	16.4±3.5	24.8±3.4	46.7±4.2
5.0	30.6±3.9	88.1±3.0	89.2±3.7
5.5	35.9±4.1	90.9±4.0	91.3±3.1
6.0	33.9±3.4	89.2±3.2	86.2±3.5
6.5	30.2±4.0	74.6±3.5	70.2±2.4
7.0	23.0±4.1	33.1±3.3	28.4±3.0

Media M1 consists of peptone, beef extract, sorbitol, yeast extract and glycerol as carbon sources (g/l) for cell growth low concentration of three metal ions (Mg SO_4, NaCl, & KH_2PO_4) were introduced with it. The yield was constantly increasing with respect to time (11.6±2.6% at 48 h, 16.2±2.2% at 72 h and 21.0±1.9% at 96 h) but productivity was low, this was because more concentration of carbon sources may affected the cell growth and ability of bioconversion of glycerol to DHA [17,31]. Reduced carbon sources in Media M2 was added with $CaCO_3$ to maintain the pH and D-glucose was added in high concentration to boost the microbial enzyme production, which showed slight increased productivity when compared to media M1 (14.0±3.5% at 48 h, 18.4±3.7% at 72 h & 22.7±19 at 96 h) but not much significant rate of production. Further the MRS media (M3) with reduced metal ion concentrations with constant carbon source and original MRS media (M4) were evaluated, here the productivity was increased with increased metal ion concentrations i.e., in M3 it showed 42.7±1.9% at 96 h and in M4 it showed significant result of 62.9±2.6% at 96 h. This leads us to addition of more metal ions in mili Molar range i.e., in media M5 and M6 were added same concentration of carbon sources and 5mM and 10mM of metal ions to M5 and M6 media respectively (Table 1). Use of lesser concentration i.e., 5 mM of metal ions in media M5 showed significant increase in the yield of DHA (63.1±1.3% at 96 h). However the increased metal ions to 10 mM in medium M6 showed drastic decrease in the yield of DHA 31.5±2.0% at 96 h. This may due to the hypertonic pressure of metal ions on the bacterial cells and also the higher concentration of metal ions which will not completely dissolved in the medium [20]. To overcome hypertonic effect on bacterial cells in media M7 with varied concentration of metal ions were used. $MnSO_4.4H_2O$, $CaCo_3$, K_2HPO_4, KH_2PO_4 and NaCl concentration were reduced to 5 mM and rest of the metal ions ($MgSO_4.7H_2O$, $FeSO_4.4H_2O$, and Na2SO4) were maintained 10 mM (Table 1). Results showed media M7 gave highest yield of DHA (91.8±4.3% at 72 h and 92.6±2.1% at 96 h) (Table 3).

Since the media M7 among seven media gave highest yield was considered for the further physical parameters optimization. Due to the addition of more substances as media constituents to formulated media M7, it is necessary to optimize other parameters/conditions like time, temperature, pH and glycerol concentration. Increas in the glycerol concentration exerts the inhibitory effect on DHA production by *G. oxydans* [13,32]. Owing to the inhibitory effect, the glycerol concentration optimization study was determined for DHA production. From the study optimized conditions for the media M7 were obtained; pH 5.5, temperature 30ºC, at 72-96 h with 10% glycerol concentration.

5. CONCLUSION

In conclusion biodiesel derived crude glycerol could be used as an economical and feasible carbon source for the DHA production by *Gluconobacter oxydans*. Metal ions induced G.oxydans produced 92.61 g/l of DHA in 1000ml of fermentation media which consist 100g of crude Glycerol (92.6±2.1% conversion rate of glycerol to DHA) was achieved after 96 h of biotransformation.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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