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Fatty acid methyl ester production from industrial waste by *Rhodococcus erythropolis* IGTS8 and *Rhodococcus gordoniae* R3

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ABSTRACT: Experiments were performed to test the ability of *R. erythropolis* IGTS8 and *R. gordoniae* R3 to accumulate non-polar lipids from industrial waste (molasses and glycerol) to yield fatty acid methyl esters (FAMEs). The bacterium strain IGTS8 grew well in glycerol and yielded 0.11 g/l per day of FAMEs, while the strain R3 used only molasses as a carbon source and yielded 0.03 g/l per day of FAMEs. Fatty acids produced by both strains had 10–22 carbon atoms. Most were C16 fatty acids that are suitable for biodiesel production. Most fatty acids produced are saturated fatty acids with even-numbered carbons, while *R. gordoniae* R3 grown in molasses produced unsaturated-fatty acids as the major fatty acids. Fatty acids containing two or more double bonds were barely observed. Nitrogenous compounds in molasses had an effect on *R. gordoniae* R3 growth, yield of FAMEs, and composition of fatty acid produced. In the case of *R. erythropolis* IGTS8, only the growth of bacteria was noticeably affected. The effect of incubation time on the type of fatty acid was also noted in molasses grown cells. Glycerol and molasses were selected for the large-scale cultivation of *R. erythropolis* IGTS8 and *R. gordoniae* R3, respectively, in a 2-l continuously stirred tank bioreactor. The lipid contents produced by *R. erythropolis* IGTS8 grown in glycerol were increased, whereas the lipid contents produced by *R. gordoniae* R3 grown in molasses was found to decrease with increasing feeding time. In conclusion, the large-scale experiments gave higher lipid productivity than those performed in the laboratory scale, with the FAMEs productivity of 40 and 0.27 g/l per day for *R. erythropolis* IGTS8 and *R. gordoniae* R3, respectively.

KEYWORDS: oleaginous bacteria, biodiesel, triacylglycerol, bioreactor

INTRODUCTION

As a consequence of economic growth, the demand for energy particularly liquid fuels has increased significantly over the past decade[1]. Increased in oil prices and an awareness of the shortage of energy supply have made it necessary to search for new sources of liquid fuel. Biomass is an attractive source of energy because it is clean, renewable, and helps to reduce greenhouse gas and CO_2. Biodiesel is a prominent alternative fuel to petroleum. Fuel production from plants however is time consuming, requiring long cultivation time, large agricultural area, which affect the global food supply. Non-food materials or non-edible oils are therefore more attractive materials as sources of energy production in the future[2,3].

Plants[4], microalgae[5], yeast[6], bacteria[7], and fungi[6] are all high potential materials for biodiesel production. Bacteria can accumulate various types of lipids and wax esters. Lipids produced by bacteria are predominantly found as polyhydroxyalkanoates and poly(3-hydroxybutyrate)[7,8]. Neutral lipids or non-polar lipids such as triacylglycerol suitable for biodiesel production are found mostly in actinomycete bacteria such as *Rhodococcus*, *Gordonia*, *Streptomyces*, and *Mycobacterium*[9–12]. Triacylglycerol (TAG) is an excellent non-toxic storage compound. Bacteria reserve TAG for a carbon source and as a depositary of free fatty acids. TAG produced by bacteria can vary in yield, types, and pattern of fatty acids, depending on bacterial strains and growth substrates[7,13].

Rhodococcus are saprophytic soil microorgan-

isms that are dispersed widely in the environment. Many *Rhodococcus* strains are useful for environmental and industrial applications due to their broad metabolic activities and capability to degrade and transform various substrates, particularly organic compounds[14–17]. Certain bacterial strains such as *R. opacus*, *R. erythropolis*, and *R. facians* are capable to accumulate TAG from various organic compounds and are considered as oleaginous[7,18]. *R. erythropolis* and *R. gordoniae* exhibit both biodegradation and lipid accumulation properties, despite most research has hitherto focused mainly on their biodegradation ability.

In this study, experiments were performed in order to observe the ability of *R. erythropolis* and *R. gordoniae* in synthesizing and accumulating TAG using organic wastes including molasses and glycerol as the carbon sources. The influences of growth factors on bacterial growth, lipid contents, distribution of fatty acids, and lipid productivity were examined. Finally, lipid production from organic wastes was studied using a larger scale two-litres stirred tank bioreactor under suitable substrate conditions.

MATERIALS AND METHODS

Bacterial strains and culture conditions

R. erythropolis IGTS8 (ATCC 53968) was purchased from the American Type Culture Collection, USA; and *R. gordoniae* R3 was isolated using dibenzothiophene as a sole sulphur source from an oil-contaminated area in Bangkok, Thailand[19]. Luria-Bertani medium was used for the enrichment medium. For lipid production, bacteria were cultured in low-nitrogen mineral salt medium (Low-N MSM) pH 7.0 containing (per litre) 2 g $(NH_4)_2SO_4$; 4 g KH_2PO_4; 6 g Na_2HPO_4; 0.2 g $MgSO_4 \cdot 7H_2O$; 0.05 g $CaCl_2 \cdot 2H_2O$; 0.01 g $FeSO_4 \cdot 7H_2O$[20]. Molasses solution or glycerol at various concentrations were added as a carbon source. The medium was sterilized by autoclaving at 121 °C, 15 psi for 15 min. Molasses solution (20% v/v) was prepared by dissolving molasses in distilled water and sterilized separately with syringe filter before adding to the sterilized medium. Bacteria from the enrichment culture were harvested by centrifugation at 4 °C, 10 600*g*, washed twice with phosphate buffered saline (PBS) and resuspended in phosphate buffer (PB) to obtain an OD_{600} of 2. One millilitre of cell suspension was inoculated into a 250-ml Erlenmeyer flask containing fresh low-N MSM. Bacteria were grown in a 250-ml Erlenmeyer flask at 30 °C on a rotary shaker with an agitation rate of 150 rpm.

Scale-up experiments

Scale-up experiments were carried out in a 2-l stirred tank bioreactor (New Brunswick Bioflo 110 Fermenter/Bioreactor) with an active volume of 1.5 l. The reaction started at pH 7.0 with 100% oxygen saturation. Temperature and agitation rate were maintained at 30 °C and 100 rpm, respectively. Oxygen concentration was controlled at 20% of saturation using an aeration rate of 0.1–5.0 l per minute. Bacteria were cultivated on low-N MSM. Glycerol (0.5% v/v) and molasses solution (2% v/v) were used as the carbon sources for *R. erythropolis* IGTS8 and *R. gordoniae* R3, respectively. Cell density was observed by measuring the absorbance at 600 nm. Lipid contents and pattern of fatty acids were determined at the end of each batch. An additional carbon source was fed to the culture three times during the lag phase; Glycerol was fed to the culture of *R. erythropolis* IGTS8 to obtain a concentration of 0.5% (v/v), whereas molasses solution was fed to the culture of *R. gordoniae* R3 to obtain a concentration of 2% (v/v).

Growth measurement and lipid contents

Bacterial cells from low-N culture (1 ml) were collected by centrifugation at 4 °C, 10 600*g* for 10 min. The cell pellet was washed with PBS and resuspended in 1 ml of PB. Lipid contents in the bacterial cells are expressed as yields of fatty acid methyl ester (FAMEs) by direct transesterification determined by gravimetric method and calculated as percentage (w/w) of cell dry matter.

Direct transesterification

Direct transesterification was performed according to Bligh and Dyer method with modification[21]. Briefly, the bacterial cell pellet was washed twice with PBS, and the intact bacterial cells were lyophilized. Transmethylation reaction was carried out by adding 2 ml of 6% H_2SO_4 in methanol directly to the dried bacterial cells, mixing vigorously with a vortex mixer, then heating at 80 °C for 14–16 h. When the reaction was completed and the temperature was cooled down to room temperature, 2 ml of petroleum ether and 1 ml of deionized water was successively added to the reaction mixture. At each step, the suspension was mixed vigorously and thoroughly with a vortex mixer. The petroleum ether layer containing FAME was collected, followed by evaporation using N_2 gas at 38 °C. FAME was redissolved in 1 ml of hexane and stored at 4 °C for further analysis.

Table 1 Growth of *R. erythropolis* IGTS8 and *R. gordoniae* R3.

	Time	Concentration (%)	Optical density at 600 nm				
			glucose	sucrose	ethanol	molasses	glycerol
R. erythropolis IGTS8	72 h	0.1	0.54 ± 0.19^{a}	0.45 ± 0.07^{a}	0.77 ± 0.15^{a}	0.05 ± 0.01^{a}	0.51 ± 0.07^{a}
		0.5	1.53 ± 0.15^{b}	0.72 ± 0.23^{ab}	1.57 ± 0.11^{b}	0.17 ± 0.01^{b}	0.23 ± 0.07^{b}
		1	1.66 ± 0.07^{b}	0.99 ± 0.31^{bc}	1.59 ± 0.09^{b}	0.41 ± 0.06^{c}	0.30 ± 0.17^{b}
		2	1.77 ± 0.06^{b}	1.17 ± 0.10^{c}	1.38 ± 0.10^{b}	0.98 ± 0.02^{d}	0.20 ± 0.03^{b}
	120 h	0.1	0.56 ± 0.02^{a}	0.94 ± 0.08^{a}	0.74 ± 0.06^{a}	0.05 ± 0.01^{a}	0.83 ± 0.11^{a}
		0.5	1.76 ± 0.08^{a}	1.30 ± 0.10^{b}	1.48 ± 0.12^{b}	0.20 ± 0.01^{b}	2.04 ± 0.01^{b}
		1	1.89 ± 0.11^{b}	1.61 ± 0.10^{c}	1.47 ± 0.05^{b}	0.44 ± 0.04^{c}	2.07 ± 0.04^{b}
		2	1.86 ± 0.10^{b}	1.81 ± 0.07^{d}	1.00 ± 0.13^{a}	0.99 ± 0.01^{d}	1.68 ± 0.34^{a}
R. gordoniae R3	72 h	0.1	0.11 ± 0.02^{a}	0.06 ± 0.03^{a}	0.32 ± 0.06^{a}	0.06 ± 0.03^{a}	ND*
		0.5	0.13 ± 0.01^{a}	0.13 ± 0.01^{b}	0.43 ± 0.13^{ab}	0.55 ± 0.02^{b}	ND
		1	0.14 ± 0.02^{a}	0.13 ± 0.02^{b}	0.55 ± 0.13^{b}	1.03 ± 0.02^{c}	ND
		2	0.11 ± 0.02^{a}	0.16 ± 0.02^{b}	0.46 ± 0.05^{ab}	1.44 ± 0.06^{d}	ND
	120 h	0.1	0.38 ± 0.10^{a}	0.09 ± 0.02^{a}	0.30 ± 0.09^{a}	0.07 ± 0.04^{a}	ND
		0.5	0.50 ± 0.06^{ab}	0.14 ± 0.06^{a}	0.31 ± 0.06^{a}	0.56 ± 0.02^{b}	ND
		1	0.54 ± 0.07^{b}	0.16 ± 0.05^{a}	0.51 ± 0.08^{b}	1.05 ± 0.03^{c}	ND
		2	0.36 ± 0.08^{a}	0.27 ± 0.08^{b}	0.29 ± 0.15^{a}	1.48 ± 0.03^{d}	ND

* ND = not determined.
The ANOVA and LSD test were used to compare differences between means (see text). Means within a group followed by the same letter are not significantly different ($p > 0.05$).

Analysis of fatty acids

Fatty acid compositions were analysed by gas chromatography-mass spectroscopy (Agilent Technologies Network GC system model 6890 N) equipped with Agilent Technologies mass spectrometer detector model 5973 (MSD). A carrier helium gas was controlled with constant pressure mode. Sample (1 μl) was injected to the DB-5MS column (0.25 mm × 30 m × 0.25 μm) with split mode (split ratio = 20:1). The initial temperature of the oven was held at 45 °C for 1 min then increased to 300 °C with the first ramp rate at 25 °C/min (runtime = 7 min), the second ramp rate at 5 °C/min and held for 4 min (runtime = 20 min). The final temperature of 300 °C was held for 3 min followed by post runtime (≈ 23 min). For mass selective detector, MS source temperature and scanning mass range were 230 °C and 50–550 a.m.u., respectively.

Statistical analysis

One-way analyses of variance (ANOVA) were applied to evaluate the data with one given factor. Testing for normality was performed with the Kolmogorov-Smirnov test. The difference of means between two independent samples was determined by the least-significant difference (LSD) method. Statistical analyses were performed at 95% confidence interval ($p < 0.05$) using SPSS (version 17.0) statistical software.

RESULTS AND DISCUSSION

Effect of different substrates and substrate concentrations on bacterial growth

Bacteria were cultivated in low-N MSM supplemented with 0.1, 0.5, 1, and 2% (v/v) molasses solution or glycerol as a sole carbon source. Growth of bacteria was measured twice, at 72 h and 120 h, because the substrates might be differentially toxic to bacteria and could affect different phase of the growth. A substantial growth of *R. erythropolis* IGTS8 was achieved by cultivation on 2% molasses solution or 0.5% glycerol, whereas *R. gordoniae* R3 exhibited the highest growth when cultivated on 2% molasses solution only (Table 1).

Growth of *R. erythropolis* IGTS8 and *R. gordoniae* R3 in molasses was increased upon increasing the substrate concentrations. In contrast, growth of *R. erythropolis* IGTS8 was suppressed at high concentrations of glycerol (Table 1). The results are in agreement with an earlier report[22], which showed that the growth of bacteria in glycerol decreased upon addition of excess amounts of substrates due to the toxicity to bacteria. Indeed, the previous study[23] also showed that the growth of *R. erythropolis* IGTS8 in S-free MSM containing 30, 50, 70, and 100 g/l glycerol as a carbon source was decreased.

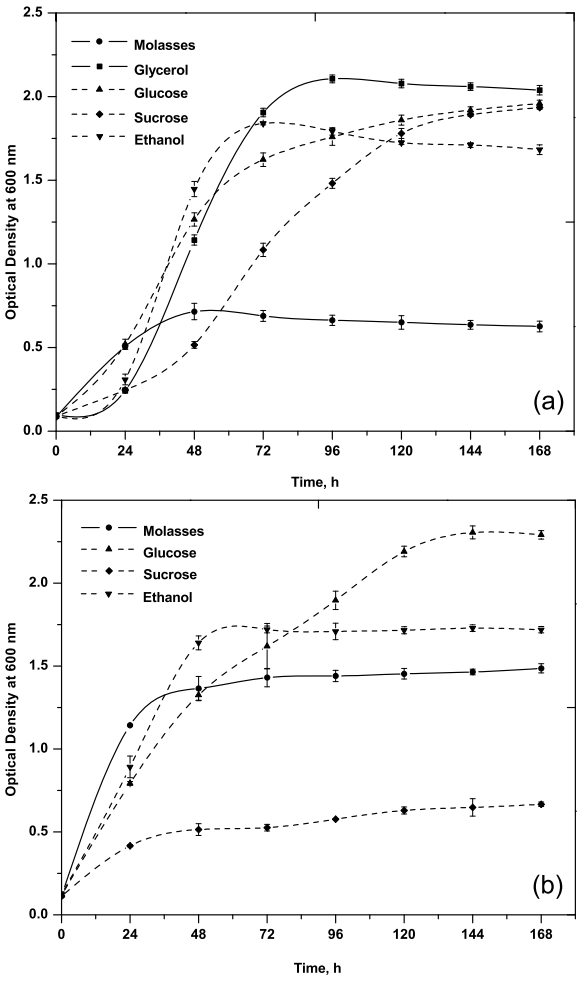

Fig. 1 Growth of (a) *R. erythropolis* IGTS8 on low-nitrogen mineral salt medium containing molasses, glycerol, glucose, sucrose, and ethanol; (b) *R. gordoniae* R3 on low-nitrogen mineral salt medium containing molasses, glucose, sucrose, and ethanol. The data are given as mean ± SD ($n = 3$).

Characterization of bacterial growth

Cell density, growth rate and duration of each growth phase were found to be varied among the bacterial strains and growth substrates (Fig. 1). To obtain the optimal growth of bacteria, 0.5% glycerol and 2% molasses solution were selected for the cultivation of *R. erythropolis* IGTS8 and *R. gordoniae* R3, respectively. The growth of *R. erythropolis* IGTS8 and *R. gordoniae* R3 in glycerol and molasses were also compared with the growth in common sources of carbon such as glucose, sucrose which is a major component of molasses and ethanol which is a good carbon source for *R. erythropolis* strain IGTS8[24,25], N1-36[26], and KA2-5-1[27,28]. The optimal concentrations of glucose, sucrose, and ethanol were also studied. The highest growth of the strain IGTS8 was obtained when the media was supplemented with 1% glucose, 2% sucrose, and 0.5% ethanol, whereas the highest growth of the strain R3 was obtained when the media was supplemented with 1% glucose, 2% sucrose, and 1% ethanol (Table 1).

R. erythropolis IGTS8 grew well in a number of common substrates, including glucose, sucrose, and ethanol. We found however that glycerol gave a better result (Fig. 1a). Glucose and ethanol could promote better growth of *R. gordoniae* R3 comparing to molasses (Fig. 1b). Both bacterial strains had high growth rate in media containing ethanol (Fig. 1) because the added ethanol can be converted to acetaldehyde, and yields NADH which is a high energy molecule that plays a role in several metabolic pathways and enzyme regulations. Likewise, the study of *R. erythropolis* N1-36 growth revealed that the addition of 0.1–1% ethanol to the culture in S-free-MSM supplemented with glucose could promote higher growth rate and shorter lag-phase of bacteria[28–30]. Nevertheless, excess concentration of ethanol could inhibit growth of both *R. erythropolis* IGTS8 and *R. gordoniae* R3 (Table 1). This is in good agreement with earlier report that a high concentration of ethanol was harmful to *R. erythropolis* IGTS8[24,25] and *R. erythropolis* N1-36 cells in the desulphurization experiment[31].

Although sucrose is the major component of molasses, the growth of *R. gordoniae* R3 in molasses was much higher than that in pure sucrose (Fig. 1b). One possible reason for this disparity is the multifarious composition of molasses, which varies depending on raw materials, season, and the refining process[32]. In general, sugar cane molasses is composed of 30–40% sucrose, 20% water, 10% glucose, 10% fructose, 1% raffinose, and 5–10% non-sugar components. Molasses may therefore provide more than one carbon source for bacteria. Furthermore, a variety of non-sugar components in molasses such as CaO, SiO_2, Al_2O_3, Fe_2O_3, KCl, NaCl, CaO, and MgO are frequently added to the mineral salt medium as trace elements[32,33].

Fatty acid analysis

For pilot-scale production of biodiesel, the yield of the final product (FAMEs), the cost effectiveness, time, and solvent consumption are the concerns. Due to the complexity of the regular method described by Bligh and Dyer[21], compounded with its

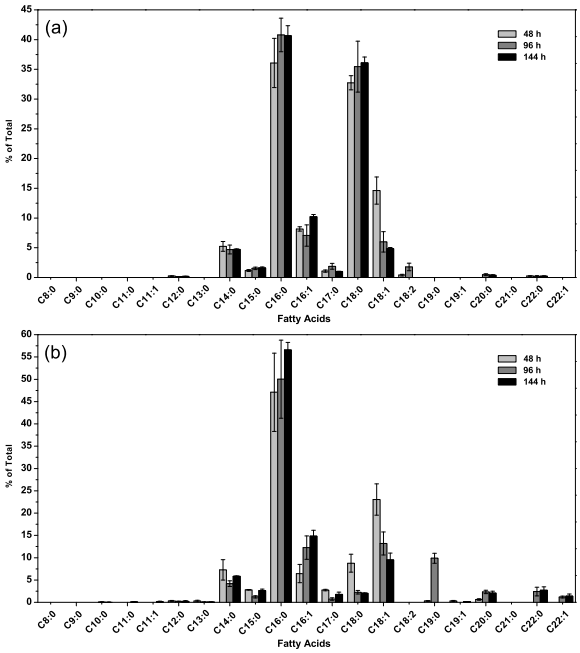

Fig. 2 Distribution of fatty acids found in *R. erythropolis* IGTS8 grown with (a) molasses and (b) glycerol after 48, 96, and 144 h of incubation under nitrogen-limited condition. Bars represent mean ± SD % of total fatty acids ($n = 3$).

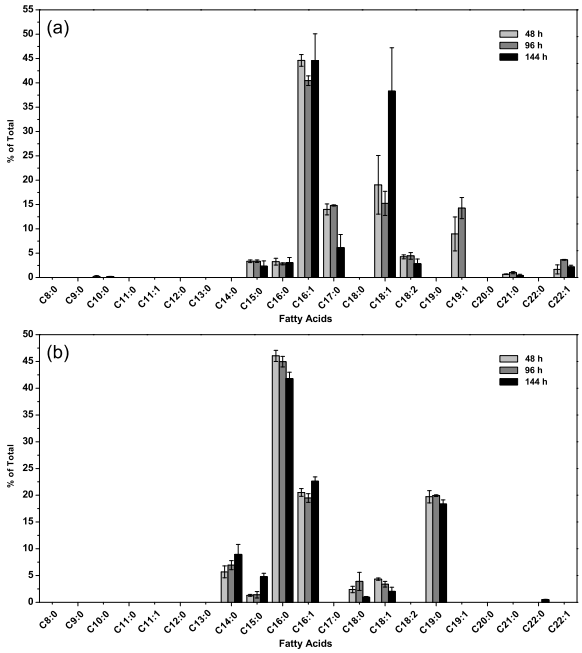

Fig. 3 Distribution of fatty acids found in *R. gordoniae* R3 grown with (a) molasses and (b) sucrose after 48, 96, and 144 h of incubation under nitrogen-limited condition. Bars represent mean ± SD % of total fatty acids ($n = 3$).

large consumption of solvent, direct transesterification method was employed in this study. This method is simpler, requires small amount of solvent, consumes less time, and yields FAMEs as the final product directly.

Fatty acids obtained from *R. erythropolis* IGTS8 and *R. gordoniae* R3 ranged from 10–22 carbon atoms and were odd-numbered, even-numbered, saturated, and unsaturated fatty acids. The even-numbered fatty acids, particularly C16 fatty acids, predominated in both strains. The fatty acids that contained two or more double bonds were barely observed (Figs. 2 and 3). Saturated fatty acids were also abundant in these two strains (Table 2). It is noteworthy that only the most abundant fatty acids from *R. gordoniae* R3 grown in molasses were unsaturated fatty acids (Table 2).

Incubation time was found to have an impact on type (Table 2) and distribution of fatty acids (Figs. 2 and 3) of *R. erythropolis* IGTS8 and *R. gordoniae* R3 grown in sucrose and molasses. The relation of incubation time and type of fatty acids however could not be clearly observed in *R. erythropolis* IGTS8 grown in glycerol (Table 2). The differences in the type of fatty acids (Table 2) and fatty acid distribution (Fig. 3) between cells grew in molasses and those grew in sucrose were also detected. It is possible that at the different time interval, bacteria accumulated lipids from different components in molasses. The study of lipid accumulation by *R. opacus* PD630 at the pilot-plant scale also revealed that during the exponential growth phase, components of molasses excluding sucrose were used as carbon sources. Sucrose was utilized predominantly in the stationary growth phase[32]. It is likely that other components in molasses are more preferable for *R. gordoniae* R3 than sucrose since *R. gordoniae* R3 grew slowly on sucrose (Fig. 1b).

In general, biodiesel is produced from fatty acids containing 14–24 carbon atoms. Because the general properties of biodiesel such as cetane number and viscosity are affected by the number of carbon atoms in order to meet desired specifications, the feedstock should not contain a high percentage of long chain fatty acids[2,6]. It may be concluded that FAMEs obtained by *R. erythropolis* IGTS8 and *R. gordoniae* R3 are the suitable feedstock for biodiesel production.

Table 2 Effect of incubation time on the type of fatty acid produced.

Carbon sources	Time (h)	Fatty acids (% of total)[†]			
		R. erythropolis IGTS8		*R. gordoniae* R3	
		Saturated-fatty acids	Even-numbered	Saturated-fatty acids	Even-numbered
Sucrose	48	70.6 ± 0.5^{a}	84.4 ± 5.5^{a}	75.2 ± 0.5^{a}	80.0 ± 1.1^{a}
	96	80.5 ± 1.8^{b}	84.8 ± 3.6^{a}	77.1 ± 0.6^{b}	78.7 ± 0.6^{a}
	144	75.4 ± 0.9^{c}	96.9 ± 0.6^{b}	75.3 ± 0.4^{a}	76.9 ± 0.7^{b}
Molasses	48	76.8 ± 1.9^{a}	97.7 ± 0.3^{a}	21.5 ± 0.8^{a}	73.0 ± 3.1^{a}
	96	85.2 ± 3.6^{b}	96.6 ± 0.7^{b}	22.0 ± 0.6^{a}	69.6 ± 2.4^{a}
	144	85.0 ± 0.3^{b}	97.4 ± 0.1^{ab}	12.2 ± 3.7^{b}	91.1 ± 1.0^{b}
Glycerol	48	70.2 ± 5.7^{a}	93.9 ± 0.4^{a}	ND	ND
	96	73.4 ± 5.2^{a}	88.1 ± 1.1^{b}	ND	ND
	144	74.0 ± 0.2^{a}	95.2 ± 0.8^{a}	ND	ND

[†] Values are means ± SD ($n = 3$). ND = not determined. Means within a group followed by the same letter are not significantly different ($p > 0.05$).

Table 3 Effect of incubation time on yields of fatty acid methyl esters by *R. erythropolis* IGTS8 and *R. gordoniae* R3.

Bacteria	Carbon source	Yields of FAMEs (% w/w)[†]		
		48 h	96 h	144 h
IGTS8	Molasses	14.1 ± 2.0^{a}	10.3 ± 2.9^{ab}	8.9 ± 0.8^{b}
	Glycerol	11.2 ± 3.2^{a}	10.3 ± 0.3^{a}	7.4 ± 1.8^{b}
	Sucrose	12.8 ± 1.4^{a}	13.5 ± 0.2^{a}	13.5 ± 0.8^{a}
R3	Molasses	8.3 ± 1.0^{a}	9.2 ± 0.7^{a}	11.8 ± 1.7^{b}
	Sucrose	18.8 ± 4.8^{a}	15.2 ± 1.4^{a}	12.8 ± 1.8^{a}

[†] Values are means ± SD, in % w/w of cell dry matter ($n = 3$).
The ANOVA and LSD test were used to compare differences between means of yields of FAMEs by time. Means within a row followed by the same letter are not significantly different ($p > 0.05$).

Effect of substrates and incubation time on the yield of FAMEs

According to the previous experiment, *R. erythropolis* IGTS8 and *R. gordoniae* R3 were cultivated under N-limited condition with 0.5% glycerol and 2% molasses solution, respectively. The maximum FAMEs yield achieved by the strain IGTS8 and R3 were 11.16 and 12%, respectively, during the late-log phase and the stationary phase (Table 3).

Lipid content of *R. erythropolis* IGTS8 grown with glycerol decreased progressively during the stationary phase as shown by the decrease in yield of FAMEs from 10% at 96 h to 7% at 144 h (Table 3). Similar results was observed in *R. opacus* PD63 and *Gordonia* sp. DG during the stationary growth phase. When carbon source provided in the medium was completely consumed, the bacteria then started to use the accumulated lipids as a carbon source in order to maintain cell activities and growth[7,10,13].

As described above, the highest lipid content of strain R3 grown in molasses was achieved during the stationary phase of growth, whereas the lipid content of the strain IGTS8 grown in glycerol decreased gradually during this phase (Table 3). Because most bacteria accumulate lipid under N-limited condition but molasses contains up to 1–2% nitrogenous compounds[33], the highest lipid content in strain R3 was possibly obtained after the bacteria had completely consumed the nitrogenous compounds in the medium including molasses. To confirm this assumption, we decided to cultivate both *R. gordoniae* R3 and *R. erythropolis* IGTS8 in sucrose, which is an N-free major component of molasses, and then compared the outcomes afterwards.

Unfortunately, we found that *R. erythropolis* IGTS8 yielded the highest amount of FAMEs at the late-log phase and the amount of FAMEs declined gradually during stationary phase when the bacteria was grown in either glycerol or molasses (Table 3). The FAMEs yields obtained by *R. erythropolis* IGTS8 grown in molasses and sucrose were not significantly different, whereas in *R. gordoniae* R3, the yield obtained from bacteria grown in molasses was much lower than that of sucrose (Table 3). It seems that lipid synthesis in *R. erythropolis* IGTS8 was not strongly affected by the nitrogenous compounds in molasses because the bacteria can utilize nitrogen from $(NH_4)_2SO_4$ and nitrogenous compounds in molasses which are the N sources in the low-N MSM. Furthermore, *R. erythropolis* IGTS8 was able to accumulate a high quantity of lipid dur-

Table 4 Productivity of fatty acids methyl esters by *R. erythropolis* IGTS8 and *R. gordoniae* R3.

Bacteria	Carbon source	Productivity (mg/l per day)[†]		
		48 h	96 h	144 h
IGTS8	Molasses	39 ± 5^{ac}	26 ± 8^{b}	26 ± 5^{c}
	Glycerol	26 ± 14^{c}	106 ± 13^{c}	72 ± 21^{ab}
	Sucrose	32 ± 5^{ac}	75 ± 31^{a}	89 ± 18^{b}
R3	Molasses	22 ± 3^{b}	24 ± 2^{d}	31 ± 5^{d}
	Sucrose	17 ± 3^{b}	18 ± 3^{b}	15 ± 5^{b}

[†] Values are means ± SD ($n = 3$). Means within a group followed by the same letter are not significantly different ($p > 0.05$).

ing the cultivation in N-rich molasses even though bacteria generally accumulate lipid under N-limited condition (Table 3). This finding is in good agreement with an observation reported earlier[23] which showed that there were no significant differences in growth, lipid content and lipid productivity of *R. erythropolis* IGTS8 grown in NH_4NO_3, NH_4Cl, or urea.

In some cases, significant lipid content was observed, while growth of bacteria seemed to be very low. The effectiveness of bacteria in biodiesel production could therefore neither be judged from the yield nor lipid content alone. Lipid productivity was calculated and demonstrated in Table 4. The highest FAMEs productivity of *R. erythropolis* IGTS8 grown in glycerol (0.11 g/l per day) and *R. gordoniae* R3 grown in molasses (0.03 g/l per day) were achieved at 96 h and 144 h, respectively. Nevertheless, the highest productivity of strain R3 grown on molasses was significantly greater than those of pure sucrose, which is approximately 0.02 g/l per day.

R. erythropolis IGTS8 and *R. gordoniae* R3 were cultivated under N-limited condition in 0.5% glycerol and 2% molasses solution, respectively. A strong relationship between bacterial growth, oxygen concentration and aeration rate may be seen clearly in Figs 4 and 5. Oxygen demand increased when the bacteria grew at a high rate. Because the oxygen content was controlled at 20% of saturation, an increase in oxygen consumption could be investigated as an increase in aeration rate. Batch culture of *R. erythropolis* IGTS8 reached the stationary phase at 103 h with the maximum cell density at OD_{600} of 1.75. For the fed-batch cultivation, feeding of a certain concentration of substrate (0.5%) was performed three times at 103, 152, and 218 h of cultivation. The maximum optical densities achieved by the 1st, 2nd, and 3rd feeds were 2.09, 2.46, and

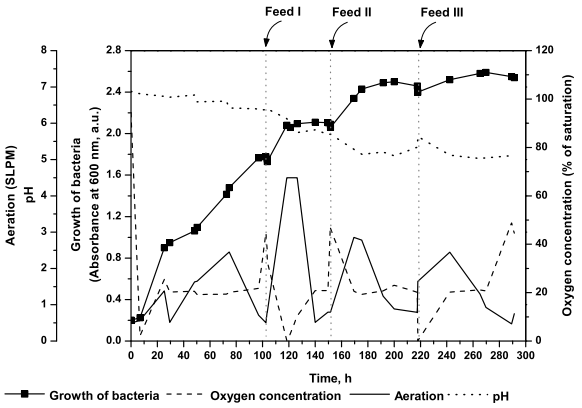

Fig. 4 Fed-batch cultivation of *R. erythropolis* IGTS8 in low-N MSM containing glycerol as a carbon source.

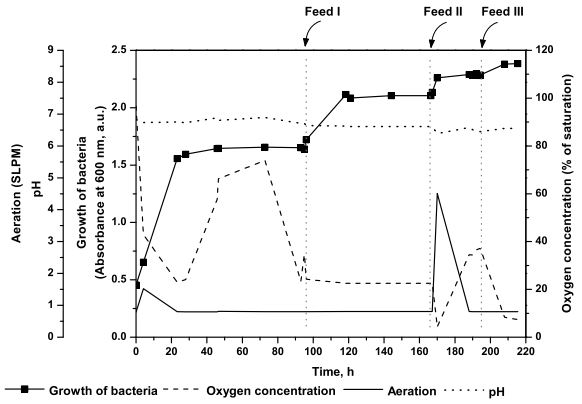

Fig. 5 Fed-batch cultivation of *R. gordoniae* R3 in low-N MSM containing molasses as a carbon source.

2.54, respectively. The duration between feeding times increased as the number of feeds increased (from 49 h to 66 h). This result implies that glycerol consumption decreased gradually after the 1st, 2nd, and 3rd feeds, respectively (Fig. 4).

Bioreactor scale-up experiment

For *R. gordoniae* R3, batch culture reached the stationary phase at 95 h with the maximum cell density at OD_{600} of 1.640. Feeding of a certain concentration of substrate (2%) was performed for three times at 95, 167, and 194 h of cultivation. The maximum optical densities achieved at the 1st, 2nd, and 3rd feeds were 2.11, 2.28, and 2.29, respectively. In contrast to *R. erythropolis* IGTS8, the duration between the feeding time decreased as the number of feeds increased (from 72 h to 27 h). This result implies that carbon source consumption increased gradually after each feed (Fig. 5). More-

over, an increase in cell density of both strains was barely observable after the 3rd feed.

Growth of *R. erythropolis* IGTS8 in batch culture stopped when the bacteria completely consumed the carbon source. Feeding of a carbon source during the cultivation allowed the bacteria to grow continuously. While after the second feed, the growth of bacteria increased slightly. After the third feed, the bacterial cell density did not increase even though the carbon source was supplied. A possible explanation is that the nitrogen source necessary for cell proliferation[7,32] became insufficient. Nevertheless, the growth of *R. gordoniae* R3 also showed the same trend, even though nitrogenous compounds were fed to the culture as molasses. This result suggests that growth of bacteria was also limited by the accumulation of their metabolites such as organic acids. Bacterial growth and cultivation time were related to pH of the culture (Figs. 4 and 5). The higher cell density indicates higher bacterial activity and results in decrease in pH. There is some evidence which showed that the growth of *R. erythropolis* IGTS8 was affected by acetate accumulation during incubation with ethanol. Bacterial growth was inhibited when acetic acid accumulated at levels above 3 g/l[24]. Furthermore, a study on fed-batch cultivation of *R. rhodochrous* revealed that the bacterial growth terminated after 28 h of incubation even though carbon sources were provided, and no accumulation of organic acid was detected[34].

The lipid contents of *R. erythropolis* IGTS8 increased as the number of feeds increased, approaching a maximum after the 2nd feed (Fig. 4). Nevertheless, the lipid contents of *R. gordoniae* R3 on molasses decreased gradually as the number of feeds increased. Batch culture of *R. gordoniae* R3 gave the maximum yield (Fig. 5). The highest FAMEs yield obtained by fed-batch culture of *R. erythropolis* IGTS8 was approximately 19% (w/w) of dry cell matter (Table 5), while the maximum yield of fatty acid methyl ester achieved by lab-scale experiments was only 11%. In comparison to the flask experiments, fed-batch bioreactor experiment gave higher yields because a carbon source was fed to the culture during cultivation without either a nitrogen source or fresh medium feeding, resulting in an imbalance in the C:N ratio. Under nitrogen-limited conditions, if the culture medium contains sufficient carbon sources for bacteria, the main activity of cell will change from cell proliferation to the accumulation of storage compounds[7,32].

R. gordoniae R3 showed different outcomes; the maximum FAMEs yield obtained during bioreactor batch culture of *R. gordoniae* R3 was 9% (w/w) of cell dry matter (Table 5), while flask experiments yielded up to 12%. As the growth of *R. gordoniae* R3 was extremely high in the bioreactor compared to the lab-scale experiment, a large amount of carbon source would be utilized for the cell growth rather than the synthesis of storage compounds[7,32]. In addition, Voss and Steinbüchel[32] also suggested that molasses, which is nitrogen-rich media, should be fed to the culture only on the first feeding occasion since nitrogen is an obstacle for lipid synthesis and accumulation[32,33]. Subsequently, this could be replaced by feeding sucrose. Because of the complex composition of molasses, it is difficult to investigate and follow the nutrient consumption of bacteria.

Table 5 Yield of fatty acid methyl ester obtained during batch and fed-batch cultivation of *R. erythropolis* IGTS8 and *R. gordoniae* R3.

Bacteria	Fermentation	Yield (% w/w)[†]
R. erythropolis IGTS8	Batch	11.21 ± 0.14^{a}
	1st feed	11.05 ± 0.09^{a}
	2nd feed	19.09 ± 0.07^{b}
	3rd feed	15.94 ± 0.11^{c}
R. gordoniae R3	Batch	8.75 ± 0.09^{a}
	1st feed	8.26 ± 0.07^{b}
	2nd feed	7.80 ± 0.13^{c}
	3rd feed	7.44 ± 0.11^{d}

[†] Values are means ± SD ($n = 3$). Means within a group followed by the same letter are not significantly different ($p > 0.05$).

Fed-batch bioreactor cultivation of *R. erythropolis* IGTS8 and *R. gordoniae* R3 gave the maximum FAMEs productivity of 0.40 and 0.27 g/l per day, respectively. Laboratory scale cultivation of *R. erythropolis* IGTS8 and *R. gordoniae* R3, however, yielded 0.11 and 0.03 g/l per day of FAMEs, respectively. It was also noted that the fatty acids obtained by large-scale cultivation are more variable and contain a wider range of carbon atoms. Fatty acids found in fed-batch culture of *R. erythropolis* IGTS8 and *R. gordoniae* R3 ranged from 8–22 and 10–22 carbon atoms, respectively. These fatty acids were predominantly found as saturated and even-numbered with the majority of C16:0 fatty acids. However, the relationship between pattern of fatty acids and number of feed has yet to be precisely determined.

R. erythropolis IGTS8 and *R. gordoniae* R3 were able to accumulate lipids from various organic compounds. In this study, industrial wastes including

molasses from sugar industry and glycerol from biodiesel industry were used as the media for the growth of bacteria with the aim to reduce the cost of FAMEs production. Direct transesterification method was also exploited to minimize the use of solvent and complications during the conventional lipid extraction procedure.

Fatty acids from *R. erythropolis* IGTS8 and *R. gordoniae* R3 comprised 10–22 carbon atoms; the majority of fatty acids have 16 carbons. The fact that the bacteria accumulated significant proportions of even-numbered and saturated fatty acids could render the bacteria a suitable feedstock for biodiesel production. The findings also indicated that lipid contents, FAMEs productivity and pattern of fatty acids may change depending upon the bacterial strain used, growth factors such as incubation time, and type of substrate.

Generally, large-scale production under controlled oxygen concentrations and substrate levels will give a better understanding of the requirements of the two bacterial strains. Cultivation of *R. erythropolis* IGTS8 and *R. gordoniae* R3 in a stirred tank bioreactor was found to give higher lipid productivity, and therefore is a potential means for low-cost production of FAMEs. For commercial scale production, further studies such as finding new substrates and optimizing the culture conditions are required in order to reduce the cost and enhance the productivity of lipid. The production process could also be improved in many steps, i.e., lipid extraction, transesterification, and recovery of FAMEs. Reusable catalysts, alternative reaction media and emerging technologies should be employed. In addition, energy efficiency, waste minimization and the control of environmental impacts should be considered.

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