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Batch and Fed-Batch Cultivations of Oleaginous Bacterium *Rhodococcus erythropolis* for Lipid Production Using Glucose Medium with Auxiliary Nitrogen Source

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Abstract. Biodiesel is produced from vegetable oils and animal fats, it can use and replace the petroleum diesel. Recently, alternative biodiesel feedstock as microbial oils has received much more attention for biodiesel production. The oleaginous bacterium *Rhodococcus erythropolis* were studied by using shake flask batch and fed-batch cultivations with adding yeast extract as auxiliary nitrogen source. Glucose as a sole carbon source reached dry biomass, and lipid yield of 3.5 g/L and 1.6 g/L, and 4.7 g/L and 2.0 g/L, respectively. The lipid production of *R. erythropolis* increased to 25 % for flask fed-batch cultivation. The major fatty acids produced by two cultivations were palmitic acid (C16:0) and oleic acid (C18:1). Based on the results, accumulated lipids from *R. erythropolis* are a potential alternative oil resource for biodiesel production.

Keywords: *rhodococcus erythropolis*, batch cultivation, fed-batch cultivation, lipid production, biodiesel feedstock

1. Introduction

With the limitation of fossil fuel resources, the price of crude oil sharply increasing and the environmental problems, alternative, renewable, biodegradable and nontoxic fuel as biodiesel have been received much more attention in the last decade. Biodiesel is produced from vegetable oils, animal fats and wasting oils mainly by transesterification of triacylglycerols (TAGs) [1], [2]. In South East Asia, Europe, United States and China, palm oil, rapeseed oil, transgenic soybeans and wasting oil were used to produce biodiesel, respectively. Vegetable oils are use for human consumption thus, the price of food-grade oils can increase, causing biodiesel to become expensive, likewise, animal fat oils need to feed these animals [3]. Although the usage of wasting oils can reduce the costs, the process requires additional cheap raw materials. Therefore, to satisfy the global demand, environmental sustainability and to avoid from conflict between fuels and food [4], the oleaginous microorganisms involving bacteria, yeasts, mold and algae maybe suitable alternatives due to they can accumulate lipids more than 20% of their total cell dry weight and their composition of fatty acids are similar to vegetable oils and animal fats [5]. They have the capacity to convert a number of raw materials into accumulated oils in their cells, even inexpensive material, such as residues from agriculture or industry [6]. In addition to, they have many advantages, such as short life cycle, less labor required, less affection by venue, season and climate, easier to scale up. Leftover biomass after lipid extraction can even serve as fertilizers for crop cultivation [7].

Bacteria belonging to actinomycetes group –*Streptomyces*, *Norcardia*, *Rhodococcus*, *Mycobacterium* are able to accumulate lipids under nitrogen-limiting condition [8]. Especially, well-known oily bacterium *Rhodococcus opacus* PD630 is capable of accumulating lipids up to 93 % of total dry biomass when grown on sugar cane molasses medium and lipid bodies were mainly triacylglycerols (TAGs) [9], while, *Rhodococcus erythropolis* has been reported is capable of synthesizing lipid in their cells 45.8 % of total dry biomass under limiting nitrogen medium using glycerol as carbon source and the extracted lipids of both strains contain long-chain fatty acids that are comparable to conventional plant oils [10].

A number of the cultivation modes have been examined to increase the cellular lipid content of oleaginous microbes; in particular, the fed-batch cultivation mode has been proven effective in increasing both of cell density and cellular lipid content yeasts [11]. The general objective of this study was to

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investigate the ability of *R. erythropolis* to grow in glucose medium where the auxiliary nitrogen source, firstly, flask batch cultivations were carried out to point out the actual the concentration of carbon source and nitrogen source requirements of the biomass and lipid yield. Secondly, flask fed-batch cultivations were performed in order to observe the amount of lipids and fatty acid composition of *R. erythropolis* and to generate ready supply of diesel fuel substitute using bacteria.

2. Materials and Methods

2.1. Bacterial strain and culture medium

Rhodococcus erythropolis IGTS8 was purchased from the American type Culture Collection (ATCC) and maintained at 4 ˚C on LB agar plates. The composition of minimal salt medium (MSM) used in this experiment was as follow (g/L): KH_2PO_4 2; K_2HPO_4 7; $ZnCl_2$ 0.01; $MgCl_2.6H_2O$ 0.2; $FeCl_3.6\ H_2O$ 0.01; $MnCl_2.4\ H_2O$ 0.01; $CaCl_2.2\ H_2O$ 0.01; Na_2SO_4 0.2; NH_4NO_3 1 and yeast extract 0.005 [10] and this was used for all growth experiments. Glucose was used as sole carbon source.

2.2. Culture conditions

A loopful of cells from single colony on LB agar plate was aseptically transferred to 50 mL of culture medium containing 10 g/L glucose as carbon source in a 125-mL Erlenmeyer flask as a seed culture. The culture was incubated at 30˚C with shaking at 150 rpm for 48 h. Then, 5% (v/v) of a 48-h preculture (2.0 x 10^7 – 3.0 x 10^7 cells/mL) was transferred into 250-mL Erlenmeyer flask containing 45 mL culture medium with a total glucose concentration 30 g/L and 50 g/L, respectively, and incubated at 30˚C in an orbital shaker at 150 rpm for 96 h. For flask fed-batch cultivation were carried out 500 mL flask containing 100 mL of the MSM medium and incubated at incubated at 30˚C in an orbital shaker at 150 rpm for 168 h, glucose was maintained at 30 g/L by feeding into the culture medium after 48 h.

2.3. Analytical methods

The cell dry matter was determined according to Gouda et al. [12]. 10 mL of the culture were centrifuged at 6,000xg for 10 min. The pellet was washed three times with deionized water and dried at 80℃ until constant weight (typically 24 h). The dry cell weight was determined gravimetrically.

Total lipids were extracted from whole bacterial cells by the method of Folch et al. [13] with some modifications and according to Sriwongchai et al. [10]. A known wet weight 100- to 1000-mg of the pellet was extracted with 3.75 mL of chloroform/methanol solution (2:1, v/v), the mixture was vortexed for 15 min at room temperature. To this was added 1.25 mL of chloroform. The mixture was vortexed for 1 min followed by addition of 1.25 mL of 1 M NaCl to the mixture and vortexed again for 1 min. The mixture was centrifuged at 3000xg for 15 min. The combined extract was evaporated by drying at 60˚C as described by Xue et.al. [14] and lipid weight was determined gravimetrically. The total lipids were saponified and methyl esterified to yield fatty acid methyl esters which were analyzed using an Agilent 6890N/5973 GC/MSD fitted with a capillary column (30.0 m x 0.32 m x 0.25 μm) under the following conditions: oven temperature initiated at 45 ℃, followed by a 1 min hold, then 2 ℃/min to 260 ℃; injector temperature of 250 ℃; detector temperature of 280 ℃ and carrier gas (N_2) flow rate of 2 mL/min [10].

To determine the amount of residual glucose, the glucose content in the supernatant was analyzed by the dinitrosalicylic acid method as described by Miller [15], with glucose as a standard solution.

2.4. Statistical analysis

Design of experiment (DOE) was done by factorial design. One-way analysis of variance (ANOVA) was applied. The significant difference F-test was analyzed under the study. Tukey's HSD (Honestly Significant Difference) test was chosen to determine which specific pair/pairs are differentially expressed. The statistical analysis was used at 95% confidence interval ($p < 0.05$) using SPSS version 11.5 statistical software to test for the significant differences.

3. Results and Discussion

3.1. Flask batch and flask fed-batch cultivations of *R. erythropolis*

To determine the growth and lipid production of *R. erythropolis*, batch flask experiments were performed using MSM medium containing 30 g/L and 50 g/L of glucose as the sole carbon source with varying concentrations of yeast extract. This experiment was tested the possibility of auxiliary nitrogen source as yeast extract might stimulate biomass and lipid production by the bacterium. Based on MSM medium, the concentrations of yeast extract were supplemented as shown in Table 1. The results indicated

that the bacterial cell biomass at 96 h ranged from 2.6 g/L to 4.2 g/L. The bacterium *R. erythropolis* consumed about 28.8 g/L and 44.9 g/L of glucose out of the initial concentration of 30 g/L, and 50 g/L respectively. The maximum lipid yield was obtained 1.6 g/L and the highest lipid content was found in the presence of 30 g/L of glucose and 0.05 g/L of yeast extract (M3) as 45.7 % of total dry biomass. There was apparent trend towards greater biomass and lipid yield with increasing yeast extract concentrations. Zhang et al. [11] reported that *Cryptococcus curvatus* O3 accumulated more biomass and lipids when an organic nitrogen source as yeast extract was included in the medium. Likewise, the two oleaginous yeast strains *Yarrowia lipolytica* JDC 335 and *Yarrowia lipolytica* DSM 70561 were grown on medium supplementing yeast extract and lend more biomass and lipid yield [16].

Table 1. The growth and lipid production of *R. erythropolis* in different media.

Medium	Substrate (g/L)			Biomass (g/L)	Lipid yield (g/L)	Glucose consumption (g/L)
	Glucose	Ammonium nitrate	Yeast extract			
M1*	30	1	0.005	3.2 ± 0.08^{d}	1.5 ± 0.03^{a}	28.7 ± 0.13^{cd}
M2	30	1	-	2.6 ± 0.02^{e}	1.0 ± 0.01^{b}	26.2 ± 0.22^{d}
M3	30	1	0.05	3.5 ± 0.08^{c}	1.6 ± 0.04^{a}	29.6 ± 0.31^{c}
M4	30	1	0.5	3.6 ± 0.09^{c}	1.6 ± 0.02^{a}	29.8 ± 0.15^{c}
M5	30	1	1	3.8 ± 0.07^{b}	1.6 ± 0.07^{a}	29.5 ± 0.18^{c}
M6*	50	1	0.005	4.1 ± 0.02^{a}	1.6 ± 0.08^{a}	44.2 ± 0.10^{a}
M7	50	1	-	3.1 ± 0.02^{d}	1.2 ± 0.05^{b}	40.7 ± 0.17^{b}
M8	50	1	0.05	4.2 ± 0.04^{a}	1.5 ± 0.07^{a}	46.8 ± 0.34^{a}
M9	50	1	0.5	4.2 ± 0.02^{a}	1.5 ± 0.03^{a}	45.9 ± 0.23^{a}
M10	50	1	1	4.2 ± 0.02^{a}	1.5 ± 0.02^{a}	46.7 ± 0.11^{a}

* based on MSM medium with varying glucose concentrations at 30 g/L and 50 g/L, respectively.
** Data presented as mean value ± standard error (n = 3)
*** Values followed by the same letter are statistically the same; different letters indicated significant differences ($P \leq 0.05$, Turkey's HSD test)

Next step, the different pulse-feeding as fed-batch cultivation was investigated with the aim of avoiding the osmotic stress. Another reason, fed-batch cultivation could significantly improve lipid production [17]. *R. erythropolis* could grow in MSM medium containing glucose up to 50 g/L. Kurosava et al. [18] demonstrates that *R. opacus* PD630 grown in batch-culture with a high concentration of 240 g/L glucose and lend biomass 77.6 g/L composed of approximately 38% TAGs.

Fig. 1 shows the typical time-course of glucose concentration, biomass and lipid yield in flask fed-batch culture containing an initial 30 g/L glucose with supplementing 0.05 g/L yeast extract and an addition of glucose over 2 d. The concentration of glucose was sharply consumed and decreased from 30 g/L to 13.5 g/L within 48 h, whereas, the biomass rapidly increased after 48 h. The amounts of glucose 16.5 g/L and 18.3 g/L were added to the culture at 48 h and 96 h, respectively, which corresponds to the total glucose concentration of 30 g/L. Biomass rapidly increased and reached 4.7 g/L and for 168 h. The residual of glucose concentration was not consume by *R. erythropolis* was 2.8 g/L. The lipid yield gradually increased and accurately accumulated to 2.0 g/L at 168 h, as well as, lipid content was estimated to be 42.6 % of total dry biomass. Zhang et al. [11] explained to their studied at the end of oleaginous yeast *C. curvatus* O3 fed-batch cultivation, glucose consumption was couple with lipid content reduction due to the yeast's utilization of lipid as a reserved store of carbon and energy. The present study is concerned with the investigation of the quantity of lipid accumulation *R. erythropolis* which cultivated in flask fed-batch process. The results indicate that *R. erythropolis* had the ability to grow in sugar medium containing yeast extract as auxiliary

nitrogen source with the fed-batch cultivation. The amount of lipids is higher than flask batch cultivation. Likely, the fed-batch culture of cyanobacterium *Spirulina platensis* led to better growth and provided biomass and lipid content more than batch culture [19]. Whilst, the oleaginous yeast *Cryotococcus* sp. accumulated intercellular lipids up to 60.9% of total dry biomass when incubated in corncob hydrolysate-based medium with the fed-batch culture [1], another oleaginous yeast *Rhodosporidium toruloides* Y4 in flask fed-batch culture lend biomass and lipid content of 151.5 g/L and 48% of total dry biomass, respectively [17].

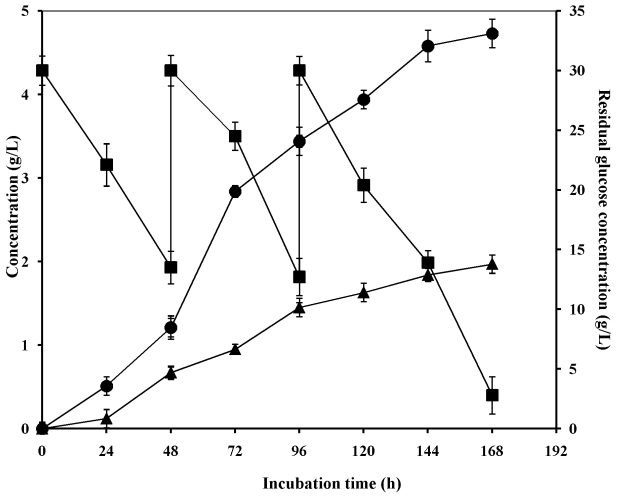

Fig. 1: Time course of biomass, lipid yield and residual glucose during the flask fed-batch cultivation in glucose medium with the auxiliary nitrogen source. ●: Biomass; ▲: Lipid yield; ■: Residual glucose.

3.2. Fatty acid composition

Table 2 showing the profile of fatty acid composition of lipids obtained by *R. erythropolis* using glucose as carbon source and yeast extract as auxiliary nitrogen source in flask batch and fed-batch cultivation. The even-numbered fatty acids were predominant with the major fatty acids for palmitic acid (C16:0) and oleic acid (C18:1). With batch cultivation, high percentages of palmitic and oleic acid were 24.7 % and 30.0 %, and 21.5 % and 29.5 % of fed-batch cultivation. Other fatty acids, presented in the lower amounts such as stearic acid (C18:0) (13.0 % and 15.7 %) and palmitoleic acid (C16:1) (11.4 % and 13.4%), respectively. Identically, the fatty acid composition had been found in the cellular lipid of *R. erythropolis*, content high proportion of C16 and C18 fatty acids, consisted 20.2 % of palmitic acid and 18.9 % of oleic acid [10]. The significance of earned bacterial oil, the fatty acid composition of the cellular lipid of *R. erythropolis* closed to canola oil [20], palm oil or sunflower oil [21]. So these results suggest that the oleaginous bacterium *R. erythropolis* may have a potential for biodiesel production.

Table 2. Relative percentage of fatty acid composition of *R. erythropolis* in batch and fed-batch cultivation.

Cultivation	C14:0	C14:1	C15:0	C15:1	C16:0	C16:1	C18:0	C18:1
Batch	8.3±0.70	5.1±0.15	3.4±0.95	4.1±0.21	24.7±1.75	11.4±1.01	13.0±1.60	30.0±1.44
Fed-batch	6.7±0.43	4.2±0.57	5.2±0.31	3.8±0.45	21.5±1.95	13.4±1.12	15.7±1.38	29.5±1.44

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