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Microbial Oil Production by Isolated Oleaginous Yeast *Torulaspora globosa* YU5/2

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***Abstract*—**Microbial oil was produced by soil isolated oleaginous yeast YU5/2 in flask-batch fermentation. The yeast was identified by molecular genetics technique based on sequence analysis of the variable D1/D2 domain of the large subunit (26S) ribosomal DNA and it was identified as *Torulaspora globosa*. *T. globosa* YU5/2 supported maximum values of 0.520 g/L/d, 0.472 g lipid/g cells, 4.16 g/L, and 0.156 g/L/d for volumetric lipid production rate, and specific yield of lipid, lipid concentration, and specific rate of lipid production respectively, when culture was performed in nitrogen-limiting medium supplemented with 80g/L glucose. Among the carbon sources tested, maximum cell yield coefficient ($Y_{X/S}$, g/L), maximum specific yield of lipid ($Y_{P/X}$, g lipid/g cells) and volumetric lipid production rate (Q_P, g/L/d) were found of 0.728, 0.237, and 0.619, respectively, using sweet potato tubers hydrolysates as carbon source.

***Keywords*—** Microbial oil, oleaginous yeast, *Torulaspora globosa* YU5/2, sweet potato tubers, kinetic parameters.

I. Introduction

BIODIESEL, mixture of mono-alkyl esters of long chain fatty acids derived from triacylglycerol (TAG), can be produced from renewable resources such as vegetable oil or animal fat, the first generation feedstock [1]. Recently, there has been an increasing interest in looking for new oil feedstock for biodiesel production especially non-food feedstock to avoid the food-fuel conflict. Among them, microbial oils, lipid produced from oleaginous microorganisms are now considered as promising feedstock because of their similar fatty acid composition to that of vegetable oils, the culture of these microbe species is affected neither by seasons nor by climates and can accumulate lipids within a short period of time as well as grow well on a variety of substrates [2, 3]. Some oleaginous yeast strains, such as *Rhodosporidium* sp., *Rhodotorula* sp., can accumulate intracellular lipids to level exceeding 70% of their biomass under nutrient limitation condition [4, 5]. It is known that lipid production requires medium with an excess of sugars and limited other nutrients, usually nitrogen [6]. It was also reported that lipid production by oleaginous yeasts has many advantages due to their high growth rate, high oil content and the resemblance of their TAG fraction to plant oil [7]. The cost of lipid production currently is relatively high and therefore, it is prudent to search for inexpensive carbon sources, which are nutritionally rich enough to support the growth of the microorganism as well as the production of lipid such as agricultural product or agro-industry by product.

The Monod model is the most widely used and considered the basic equation of an unstructured model [8]. This model introduced the concept of growth-limiting substrate (S), relating the specific growth rate (μ) to the concentration of a single growth-limiting substrate via two parameters, the maximum specific growth rate (μ_{max}), and the Monod's constant or saturation constant (K_s). The growth rate has been shown by Monod to be related to the concentration of substrate medium by the equation:

$$\mu = \frac{\mu_{\max} S}{K_s + S} \tag{1}$$

With the linearization method, the specific growth rate is determined by calculating the difference in the natural log of the biomass concentrations over time, corresponding to the exponential growth phase was plotted:

$$\mu = \frac{\ln(X_t - X_0)}{t} \tag{2}$$

where X_0 is the biomass concentration (g/L) at the beginning of the exponential growth phase, Xt is the biomass concentration at time t.

The Hanes plot is based in based in the linearization of the Monod equation.

$$\left(\frac{S}{\mu}\right) = \left(\frac{Ks}{\mu \max}\right) + \left(\frac{1}{\mu \max}\right) S \tag{3}$$

The slope is $\left(\frac{1}{\mu_{\max}}\right)$ and the intercept is $\left(\frac{K_s}{\mu_{\max}}\right)$ of the plot $\left(\frac{S}{\mu}\right)$ versus S.

The method is the most recommended for many different situations, because it minimizes the distortions in

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experimental error.

In fermentation, variables which are of great relevance to the economic evaluation of biotechnological processes are the cell yield on a substrate ($Y_{X/S}$), specific growth rate (μ), volumetric substrate consumption rate (Q_S), specific substrate consumption rate (q_s), product yield based on substrate ($Y_{P/S}$), specific product yield ($Y_{P/X}$) and volumetric product formation rate (Q_P). All these kinetic parameters have major technological importance in up scaling the fermentation process [8].

The objective of this study is to investigate the effects of different concentration of glucose and different carbon source on growth kinetics of the isolated oleaginous yeast in batch fermentation and to develop a kinetic model incorporating substrate inhibition which would be used to assist the design and scale-up of the cultivation process.

Abbreviations

K_s : Monod's constant (g/L)
P : Lipid concentration (g/L)
Q_P : Volumetric lipid production rate (g/L/d)
Q_S : Volumetric substrate consumption rate (g substrate/L/d)
Q_X : Volumetric cell mass production rate (g cells/L/d)
q_p : Specific rate of lipid production (g lipid /g cells/d)
q_s : Specific rate of substrate consumption (g substrate/g cells/ d)
S : Substrate concentration (g/L)
X : Cell mass concentration (g/L)
$Y_{P/S}$: Process product yield (g lipid/g substrate)
$Y_{P/X}$: Specific yield of lipid (g lipid/g cells)
$Y_{X/S}$: Cell yield coefficient (g cells/g substrate)
μ : Specific growth rate coefficient (1/d)
μ_{max} : Maximum specific growth rate (1/d)

II. Materials and Methods

A. Yeast Strain

Yeast strains YU5/2 was isolated from soil samples collected from a sugarcane field crop, UdornThani Province, Thailand, by enrichment culture technique, briefly, 5.0 g of soil was added into a 250-mL flask containing 50 mL of glucose-enriched medium (g/L): glucose 70, NH_4Cl 2.5, KH_2PO_4 7.0, $MgSO_4.7H_2O$ 1.0, $CaCl_2$ 0.2, $FeCl_3.6H_2O$ 0.0005, $CuSO_4.5H_2O$ 0.0005, $ZnSO_4.7H_2O$ 0.7, yeast extract 2.0, and supplemented with streptomycin 100 mg, and incubated in an incubator shaker at 30°C for 2 days with shaking speed at 150 rpm. Ten-fold serial dilutions were made from the enriched-culture broths, 0.1mL from each dilution ranging from 10^{-2} to 10^{-4} were spread onto YPG agar plates containing (g/L): glucose 20, peptone 10, yeast extract 10, agar 15. The plates were then incubated at 30°C for 2 to 3 days. Yeasts were then isolated by cross streak each isolate on the YPG agar plates, and purified yeast strain was maintained on YPG agar for further study.

B. Identification and Genetic Characterization

In order to identify the isolated yeast, sequence analysis of the variable D1/D2 domain of the large subunit (26S) ribosomal DNA was performed. The divergent D1/D2 domain of 26S rDNA was amplified with primers NL-1 (5'- GCA TAT CAA TAA GCG GAG GAA AAG-3') and NL4 (5'-GGT CCG TGT TTC AAG ACG G-3') [9]. The polymerase chain reaction (PCR) reactions were performed in a final volume of 100 µL reaction mixtures conditioning 100 ng of genomic DNA, 2.5 U of Taq polymerase, 20 mM of each dNTP, 40 mM of each primer, 10 mM Tris-HCl and 1.5 mM $MgCl_2$. The reaction was pre-denatured at 94°C for 5 min, then repeated for 30 PCR cycles with denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2.5 min and then followed by the final extension at 72°C for 10 min. The amplified product was then purified and sequenced. The 26S sequence of the isolated yeast was used for a BLAST search in the EMBL/GenBank database.

C. Lipid Production by Yeast Strains

The seed culture of isolated yeasts were initially cultivated onto YPG broth for 1 day at 30°C, then 5mL of these culture were transferred to 250-mL flasks with 50mL of nitrogen-limiting medium containing (g/L): glucose 70, $(NH_4)_2SO_4$ 0.2, KH_2PO_4 0.4, $MgSO_4.7H_2O$ 1.5, $ZnSO_4$ 4.4 mg, $CaCl_2$ 25 mg, $MnCl_2$ 0.5 mg, $CuSO_4$ 0.3 mg, and yeast extract 0.75 and grown at 30°C in an incubator shaker at a shaking speed of 150 rpm for 1 day.

To study of different glucose concentrations, the seed culture (10%, v/v) was inoculated into nitrogen-limiting medium, supplemented with glucose to formulate a medium with an initial concentration of 40, 50, 60, 70, 80 and 90 g/L.

To study the effect of different carbon sources, the seed culture (10%, v/v) was inoculated into nitrogen-limiting medium, supplemented with agro-industry product, sugarcane molasses, sugarcane juice, potato tubers hydrolysate, and sweet potato tubers hydrolysate. Carbon source concentration effect was studied using 30 g/L of total sugar available.

The hydrolysates were prepared by dilute acid hydrolysis and stream explosion, briefly, potato tubers and sweet potato tubers were smashed to 2 cm and mixed with 1.5% HCl to give a mixture with a solid loading of 10% (w/v). The mixture was treated in an autoclave at 121°C for 30 min and the liquid fraction was separated by centrifugation and neutralized by adding 5N NaOH, stored at 4°C prior to use.

D. Analytical Methods

Duplicate samples were analyzed for cell dry weight, and residual glucose. The culture broth (5 mL) was centrifuged at 5,000 rpm for 5 min. The supernatant was analyzed for glucose concentration according to DNS method. Harvested biomass was washed twice with 5 mL of distilled water and then dried at 90°C to constant weight. The biomass was determined gravimetrically.

The total lipids were determined by the modified method of Know and Rhee (1986) with modifications [10]. Lipid content was expressed as gram lipid per gram dry biomass.

E. Determination of Growth Kinetic

Volumetric lipid production rate (Q_P) was determined from a plot between lipids (g/L) and fermentation time, process product yield ($Y_{P/S}$) was determined from dP/dS, and specific product yield ($Y_{P/X}$) was determined using relationship dP/dX, while volumetric rate of substrate consumption (Q_S) was determined from a plot between substrate (g/L) present in the fermentation medium and fermentation time. Volumetric cell mass production rate (Q_X) was determined from a plot of dry cells (g/L) versus time of fermentation (d).

The specific growth rate is the slope determined by plotting the natural log of biomass versus time for each substrate concentration during the initial phase of exponential growth (equation 2) before the substrate concentration decreases significantly while specific rate of lipid production (q_P) was a multiple of μ and $Y_{P/X}$. Then, the determined values of specific growth rate and substrate concentration determined are used to estimate the kinetics parameters, maximum specific growth rate (μ_{max}) and Monod's constant (K_s), with Hanes linear methods (equation 3).

III. Results and Discussion

A. Identification and Genetic Characterization

BLAST analysis of the 26S rRNA gene sequence of the yeast isolate YU5/2 was revealed it to be a perfect match with that of *Torulaspora globosa* CBS 764 type strain. The alignment and comparison of the 26S sequence of the isolate to the published 26S rRNA sequences belonging to five reference strains of phenotypically close species of Torulaspora confirmed the 99% correspondence to the *T. globosa* CBS 764, *T. globosa* SSK9, *T. globosa* SSK8, *T. globosa* LY10, and *Torulaspora* sp. WB17 type strains.

B. Lipid Production by Yeast Strains

The time course of cell growth of two yeasts in nitrogen-limiting medium supplemented with 50g/L glucose were shown in Fig. 1. Apparently glucose was used mainly for cell growth at the beginning of cultivation. Biomass, lipid content and utilized glucose gradually increased and lipid yield reached the maximum of 3.12 g/L or 36.2%CDW were obtained respectively. A slight decrease was found in biomass and lipid yield after day 8 while utilized glucose increased. The possible reason may be that nitrogen source was exhausted and a great deal of glucose consumption led to a decrease of pH, thus inhibiting cell growth. The similar changes were also observed in lipid content of *Trichosporon fermentans*, after exhaustion of the carbon source in the growth environment [11].

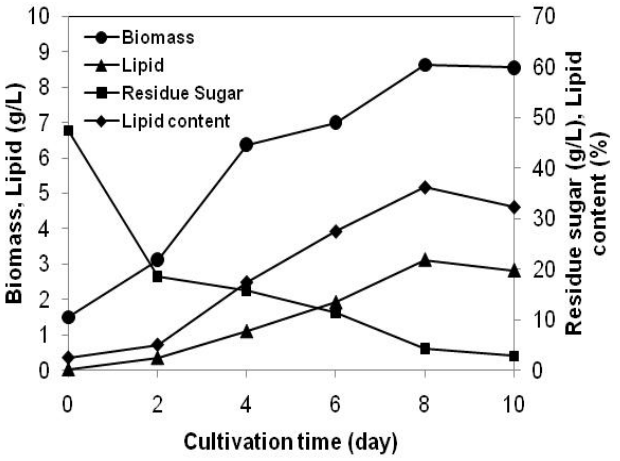

Fig. 1 Kinetic growth of *Torulaspora globosa* YU5/2 on nitrogen-limiting medium supplemented with 50 g/L glucose and 0.2 g/L $(NH_4)_2SO_4$ at 30°C for 10 days.

C. Effect of Glucose Concentration

Batch cultures were investigated to determine the suitable glucose concentration of the initial medium. Therefore, to study of glucose concentrations on cell growth and lipid accumulation, the concentration of glucose at 40, 50, 60, 70, 80 and 90 g/L with 0.2 g/L $(NH_4)_2SO_4$ were investigated. As shown in Fig. 2 and Table 1. No significant difference in cell growth biomass using different glucose concentration. Whereas, the cellular lipid accumulation was quite low at low level of glucose concentration, then showed an increase when glucose concentration increased. Lipid production of *T. globosa* YU5/2 reached the maximum of 4.31 g/L with 45.7%CDW at 80 g/L glucose were obtained. Indeed, glucose concentration has been found to be the major impact factor for oil accumulation by the oleaginous microorganisms [4, 6].

The increase in glucose concentration resulted in a decrease in cell yield coefficient values ($Y_{X/S}$), and an increase in lipid concentration (P). Maximum cell yield coefficient ($Y_{X/S}$, g/L) was found of 0.283 using 40 g/L glucose, whereas maximum specific yield of lipid ($Y_{P/X}$, g lipid/g cells) and volumetric lipid production rate (Q_P , g/L/d) of 0.472 and 0.520 were obtained using 80g/L glucose. Further increase in glucose beyond 80g/L resulted in a slight drop in lipid concentration and biomass, suggesting that a considerable glucose inhibitory effect had occurred. The determined values of specific growth rate and glucose concentration determined are used to estimate the kinetics parameters, maximum specific growth rate (μ_{max}) and Monod's constant or halt saturation constant (K_s), with Hanes linear methods. With fitted by linear regression of Hanes plot (y =2.6483x –13.799,

R^2=0.96), μ_{max} of 0.368 (1/d), and K_s of 5.21 g/L were obtained.

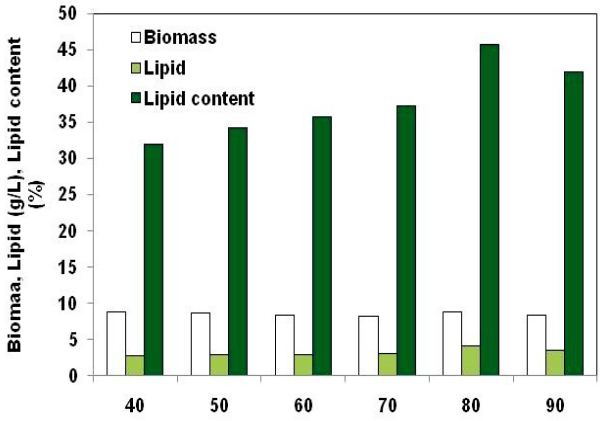

Fig. 2 Effects of glucose concentration on biomass, lipid, and lipid content of *T. globosa* YU5/2 on nitrogen-limiting medium supplemented with different concentration of glucose, at 30°C for 8 days.

TABLE I
COMPARATIVE FERMENTATIVE KINETIC PARAMETERS OF TOTULOSPORA GLOBOSA YU5/2 ON INTROGEN-LIMITING MEDIUM SUPPLEMETED WITH 0.2 G/L $(NH_4)_2SO_4$ (PH 5.0) IN THE PRESENCE OF DIFFERENT GLUCOSE CONCENTRATION IN 250ML FLASK AT 30°C

Kinetic parameters	Glucose concentration (g/L)					
	40	50	60	70	80	90
X	8.79	8.69	8.45	8.28	8.81	8.44
P	2.80	2.97	3.02	3.08	4.16	3.53
μ	0.312	0.318	0.333	0.341	0.331	0.330
Q_S	3.875	4.821	5.714	6.607	8.571	9.464
Q_X	0.035	0.028	0.023	0.019	0.016	0.014
Q_P	0.350	0.372	0.377	0.385	0.520	0.442
$Y_{X/S}$	0.283	0.225	0.185	0.157	0.129	0.111
$Y_{P/X}$	0.319	0.342	0.357	0.372	0.472	0.419
$Y_{P/S}$	0.090	0.077	0.066	0.058	0.061	0.047
q_S	0.441	0.555	0.676	0.798	0.973	1.122
q_P	0.099	0.109	0.119	0.127	0.156	0.138

However, the comparison of process product yield ($Y_{P/S}$) in batch fermentation at high substrate concentration, it was obvious that increase of glucose concentration resulting in decrease of this kinetic parameter, suggesting to difficult for up scaling of lipid production by the oleaginous organisms due to high substrate consumption rate and high concentration of glucose with lower level of nitrogen source could be effect the cell growth, because nitrogen source supported the cell growth, thus, depleted of nitrogen may result to low biomass. To solve these phenomena, further fed-batch fermentation should investigated with initial nitrogen-rich medium to obtain high biomass or high cell density at the early stage of cell growth, then high concentration of carbon source will feed onto culture medium for stimulate the cellular lipid accumulation. Fed-batch fermentation modes have been widely applied for microbial lipid production [12].

D. Effects of Various Carbon Sources

Glucose is the most commonly used carbon source for microbial oil production. Therefore, it is important to use a low cost carbon source or alternative carbon source instead of glucose in order to reduce the cost of microbial oil production. Thus, different carbon substrates were used for lipid production by *T. globosa* YU5/2. After cultivation on nitrogen-limiting medium supplemented with sugarcane molasses, sugarcane juice, potato tuber hydrolysates, and sweet potato tuber hydrolysates as a carbon substrate with pH 5.0 at 30°C for 6 days, *T. globosa* YU5/2 grew well on several types of carbon source (Fig. 3) and Table 2.

Among the carbon sources tested, potato hydrolysate supported the maximum biomass of 10.75 g/L with lipid content of 19.47 %CDW, followed by sweet potato tuber hydrolysates of 10.43 g/L with lipid content of 23.73%CDW. Maximum cell yield coefficient ($Y_{X/S}$, g/L), maximum specific yield of lipid ($Y_{P/X}$, g lipid/g cells) and volumetric lipid production rate (Q_P, g/L/d) were found of 0.728, 0.237, and 0.619, respectively, using sweet potato hydrolysates as carbon source. However, lower process product yield ($Y_{P/S}$, g lipid/g substrate) of 0.091 was obtained using sugarcane juice as carbon source. The obtained result suggested that oil production from *T. globosa* YU5/2 can be performed with lower cost production process because sweet potato is a cheap and available agriculture product, contains a large amount of starch, which is a suitable feedstock for industrial fermentation. Zhao et al. (2010) found that *Rhodotorula mucilaginosa* TJY15a could accumulate 48.8% (w/w) oil from hydrolysate of inulin and its cell dry weight reached 14.8 g/l during the batch cultivation while it could accumulate 48.6% (w/w) oil and 52.2% (w/w) oil from hydrolysate of extract of Jerusalem artichoke tubers [13].

It has been known that, the costs of microbial oil production are currently higher than those of vegetable oil but there are many methods to improve the low cost of microbial oil production processes. For example, the more economic carbon source should be employed to replace pure glucose such as sweet potato, agro-industrial waste residues. In addition, potential and realistic progress in transforming of lignocelluloses to fermentable carbon sources might provide an optimal way to reduce the cost of microbial oils production. Process engineering that leads to a higher lipid production rate and cellular lipid content may also contribute in this regard. Thus, to realize the large-scale production of biodiesel from microbial oils, it was necessary to obtain a large amount of biomass and lipid content as well as the low cost of cultivation process.

For further study, fatty acids profile of extracted microbial oil of *T. globosa* YU5/2 will be investigate and fed-batch

fermentation using the hydrolysates of sweet potato tubers will be study.

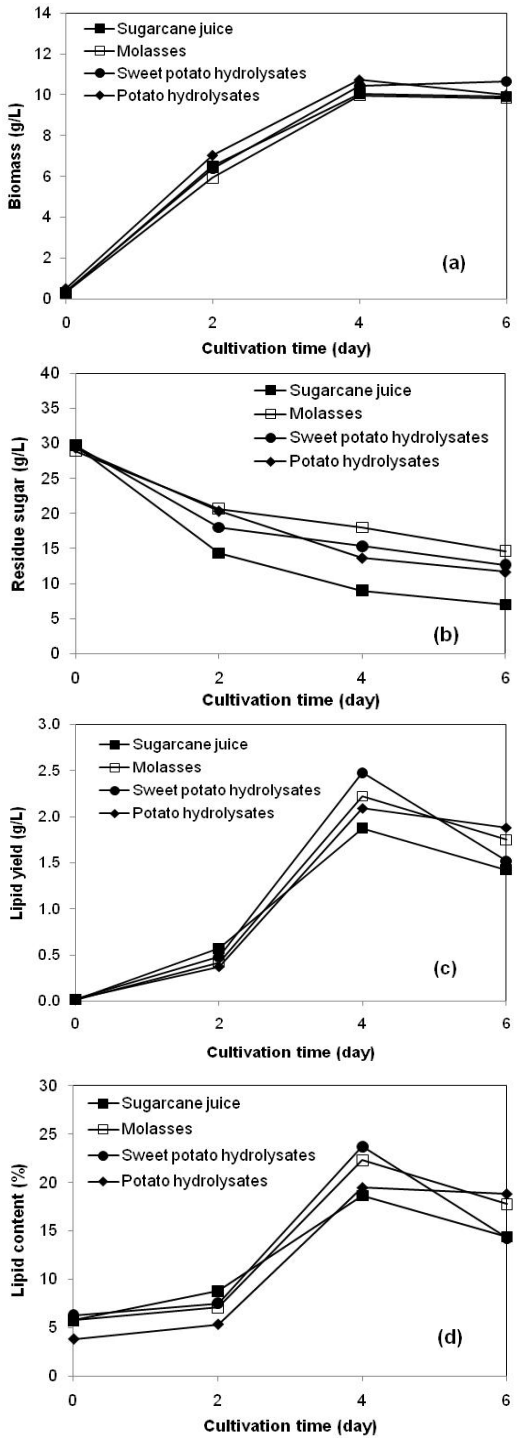

Fig. 3 Effect of different carbon sources on biomass concentration (a), residual sugar (glucose) (b), lipid yield (c), and lipid content (d) during fermentation of *T. globosa* YU5/2.

TABLE II
COMPARATIVE FERMENTATIVE KINETIC PARAMETERS OF TOTULOSPORA GLOBOSA YU5/2 ON INTROGEN-LIMITING MEDIUM SUPPLEMETED WITH 0.2 G/L $(NH_4)_2SO_4$ (pH 5.0) IN THE PRESENCE OF DIFFERENT CARBON SOURCES AT 30°C

Kinetic parameters	Glucose concentration (g/L)			
	Sugarcane juice	Molasses	Sweet potato hydrolysates	Potato hydrolysate
X	10.06	9.96	10.43	10.75
P	1.88	2.22	2.48	2.09
μ	0.218	0.259	0.244	0.211
Q_S	5.175	4.275	3.583	3.917
Q_X	2.514	2.489	2.608	2.686
Q_P	0.469	0.555	0.619	0.523
$Y_{X/S}$	0.486	0.582	0.728	0.686
$Y_{P/X}$	0.186	0.223	0.237	0.195
$Y_{P/S}$	0.091	0.130	0.173	0.134
q_S	0.515	0.429	0.344	0.365
q_P	0.041	0.058	0.058	0.041

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