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Fermentation of an oleaginous yeast *Rhodosporidium Paludigenum* for biofuels production

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Abstract

A third-generation biofuel production was investigated in a 500-L bioreator using an oleaginous yeast *Rhodosporidium paludigenum*. This strain contains a high triglyceride content, and the main fatty acids are C14, C16 and C18 which are very similar to fatty acids found in vegetable oils. The yeast was cultured, harvested, and extracted the oil by using solvent extraction. After fermentation, a combined cross-flow microfiltration (MF) process of concentration and diafiltration mode was investigated for the recovery of yeast cell. The membrane fouling mechanism was investigated using cleaning method in order to evaluate the major factors causing in the flux decline. The flux of pure water was obtained at 80.05 L/m^2.h corresponding to the membrane resistance of 3.1×10^{12} m^{-1}. For MF of the whole broth, the flux sharply decreased and reached its plateau at around 10 L/m^2.h until the end of experiment. Pure water was then added during diafiltration mode for the removal of all impurities. After cell harvesting, a chemical cleaning was applied in order to obtain the cake and adsorption resistances. After cleaning with pure water, the regained flux was obtained at 69.50 L/m^2.h, and the regained flux after 1 wt% NaOH washing was 80.01 L/m^2.h. As a result, the percentage of absorption resistance was 13.24%, and the percentage of cake was 86.76%, respectively. The yeast was dried by a spray dryer prior to extract the oil using diethyl ether as an organic solvent. After solvent removal, the lipid can be used as an appropriate raw material for biofuels production.

Keywords: oleaginous yeast, fermentation, microfiltration, oil extraction, biodiesel production

1. Introduction

Oleaginous yeast species are an alternative for the production of lipids or triacylglycerides (TAGs), which can be an alternative to fossil fuels. Oleaginous yeast, can accumulate oil more than 20% of dry cell weight. It can be used as the so-called second generation biofuels [1]. Moreover, lipids isolated from oleaginous micro-organisms can be used as components in several application such as, coatings, paints, personal care products, and biodiesel [2]. Fatty Acid Methyl Esters originating from the lipids of oleaginous yeast show identical fueling properties compared to conventional diesel [3].

In order to design the production process, microfiltration is an important operation in the processing of bio-products. The solid particle or microbial cells can be separated from a mixture by forcing the fluid though a filter medium. The solid particle can not pass through the membrane, and the particle will be deposited on a filter that cause filter cake increases as well as the resistant to permeate flow. In this experiment, a cross flow microfiltration system was investigated of its efficiency to removal of the oleaginous yeast from fermentation broth.

The objective of this study was to study the microfiltration technique for harvesting of *Rhodosporidium paludigenum*, an oleaginous yeast, from a 500-L bioreactor by using cassava starch as

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the main substrate. The harvested cell was subjected to spray dry, and a solvent extraction was employed for oil extraction.

2. Materials and Methods

2.1. Fermentation study

The oleaginous yeast *R. paludigenum* was isolated from Assoc. Prof. Dr. Mariena Ketudat-Cairns's laboratory. The seed culture was prepared in a 500 mL medium containing (g/L) of; 70 g glucose, 0.75 g yeast extract, 0.55 g $(NH_4)_2SO_4$, 0.4 g KH_2PO_4, and 2.0 g $MgSO_4.7H_2O$, respectively.

Cell cultivation began in a 5-L bioreactor at 30 ℃ for 48 h. After that, the inoculum was transferred into a 50-L bioreactor at 30 ℃ for 48 h. The batch experiment was performed in a 500-L bioreactor with 350-L of working volume (BE Marubishi, Thailand). The fermentation broth for *R. paludigenum* was prepared using cassava starch as the main substrate. The hydrolyzed starch solution was liquefied by an addition of 0.05 wt% of thermostable α-amylase (Termamyl®, Novozymes, Denmark) at the working temperature of 90 ℃ for 2 h [4]. The temperature of the liquefied solution was then decreased to 60 ℃ before being saccharified by an addition of gluco-amylase (Spirizyme® Fuel, Novozymes, Denmark) at 0.05 wt% for 12 h [5]. After sterilization, the inoculum was transferred aseptically into the bioreactor. During fermentation, temperature was automatically maintained at 30 ℃ under aerobic condition. The aeration rate was 0.75 VVM with the agitation rate of 150 rpm. Fig. 1 illustrates the optical microscope picture of the yeast cell (left) showing the budding process, and scanning electron microscope (SEM) of the cell with the size of approximately 5 μm.

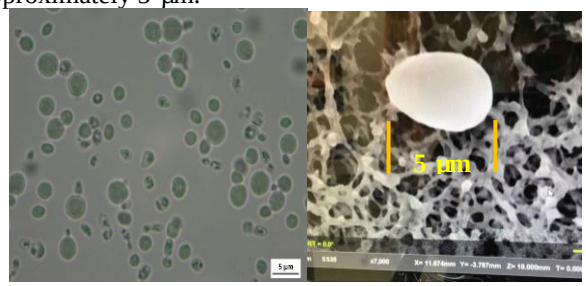


Fig. 1. The optical microscope (left) and SEM picture (right) of *R .paludigenum* used in this work.

2.2. Microfiltration set up

Oleaginous yeast cells from fermentation were separated by using not only a cross-flow microfiltration, but also dead-end filtration for study involving the volume concentration ratio (VCR) in the experiment. The cross-flow MF experiment was investigated using a spiral-wound MF element with the nominal pore size of 0.1 μm (Synder's Filtration, USA) was installed in a stainless steel housing. The diameter, and the length of the membrane element were measured at 10.2 cm, and 96.5 cm, respectively. The feed spacer was 3 mm, and the total filtration area was 4.27 m^2. A 0.75 kW pump was employed to re-circulate the fermentation broth at the volumetric flow rate of 70 L/min corresponding to the calculated linear flow velocity of 0.09 m/s. The feed temperature was controlled at 30 ^oC by using a cooling coil submerged in the MF feed tank. Transmembrane pressure was calculated from the feed, retentate, and permeate pressures using conventional pressure gauges. After each run, the membrane was firstly rinsed with RO water. It was then circulated with 1 wt% NaOH solution for 20 minutes, and rinsed to neutrality with RO water. Finally, it was cleaned with 1.5 wt% phosphoric acid solution before rinsed to pH 7.0 with RO water.

2.3. Resistance analysis

Darcy's Law is used to describe flow through microporous membranes during the microfiltration step. Permeate flux declines owing to the accumulation of oleaginous yeast cell and others particles on the

membrane surface and causing pore clogging [7]. Darcy's law describes liquid passage though the membrane as a function of the applied pressure [8].

Darcy's law

$$Q = \frac{k\Delta p}{\mu l} \tag{1}$$

When Q = Volumatic filtration flowrate (m^3 /s)

k = Darcy's law permeability (m^2)

Δp = Pressure drop across the filter medium (Pa)

μ = Viscosity of the broth (kg/m.s)

l = Thickness of the filter medium (m)

The permeability and thickness of filter can be combine into a medium resistance term .

$$R_m = \frac{l}{k}$$

When R_m = medium resistance (m^{-1})

Equation (1) can be written as;

$$Q = \frac{A\Delta p}{\mu R_m} \text{ or } Q = \frac{dV}{dt} = \frac{A\Delta p}{\mu R_m}$$

At any instant during filtration basing on the Darcy's law, rate of filtration is given by the equation;

$$J = \frac{1}{A}\frac{dV}{dt} = \frac{\Delta p}{\mu R} \tag{2}$$

When $\boldsymbol{J}$ = Flux, and $R = R_m + R_c$

In this case R is a combination of resistance of filter medium (R_m) and resistance of cake solids (R_c). And the resistance of cake solids (R_c) can be written as;

$$R_c = \alpha p_c \frac{V}{A} \tag{3}$$

When ρ_c is the mass of dry cake solids per volume of filtrate and α is specific cake resistance. Combination of equation (2) , and (3) results in the following equation;

$$\frac{1}{A}\frac{dV}{dt} = \frac{\Delta p}{\mu\left[\alpha p_c \frac{V}{A} + R_m\right]} \tag{4}$$

And integration of the eqution (4) results in;

$$\frac{At}{V} = \frac{\mu\alpha p_c}{2\Delta p}\left(\frac{V}{A}\right) + \frac{\mu R_m}{\Delta p} \tag{5}$$

The concentrated cell suspension was further processed by using a spray dryer (Buchi mini spray dryer B-290, Switzerland)

2.4. Analytical methods

The fermentation broth was analyzed by using a HPLC system equipped with a refractive index detector (RID). The column was an AminexR HPX-87H Ion Exclusion-Organic Acid column (300×7.80 mm) with a guard column. The fermentation products were eluted with 5 mM sulfuric acid at a flow rate about 0.4 ml/min, pressure about 57 bar, temperature at 45 ℃ for a total run time around 45 min. The volume 10 µL was injected. The culture samples were prepared by centrifugation at 8000 rpm for 15 min to pellet cells, followed by filtration using cellulose acetate membrane filters (pore size 0.2 mm). The biomass were analyzed to optical density (OD) at 600 nm and cell dry weight. Fatty acid analysis was carried out according to a previous work [1]. The purified fatty acid methyl ester (FAME) was then analyzed via standard EN14103 using Gas Chromatography (GC). An Agilent Technology GC

7890A/MSD 5975C with an Agilent 7693A automated liquid sampler was configured at the following conditions. The column was HP-INNOWAX, 50 m × 0.2 mm id × 0.4 µm, column flow rate of 0.6 mL.min^{-1} and helium was used as the carrier gas. The initial oven temperature was 40 ^{o}C (hold for 2 min) with a programming rate of 14 ^{o}C /min to 140 ^{o}C (hold for 2 min) then 6 ^{o}C /min to 290 ^{o}C (hold for 9 min). The maximum temperature was 310 ^{o}C and run time of 45.143 min.

3. **Resuls and Discussions**

3.1. Fermentation and membrane fouling mechanism

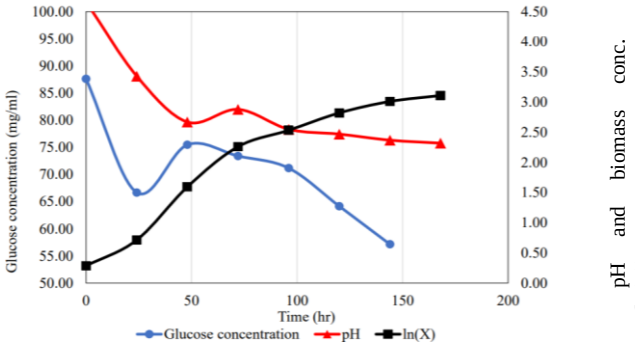


Fig. 2. Time course of *R. paludigenum* fermentation in a 500-L bioreactor.

Batch fermentation *R. paludigenum* was performed in a 500-L bioreactor. Experimental data including pH, dry cell weight, and glucose concentration were shown in Fig. 2. Dry cell weight of cell in natural log was plotted with time at the exponential phase in order to determine the specific growth rate (data not shown). At log phase, the specific growth rate (µ) was achieved at the value of 0.604 day^{-1}. In addition, the doubling time was obtained at approximately 1.15 day. Fermentation experiment finished after 7 days.

The fermentation broth was filtered through a microfiltration unit to remove bacterial cells from fermentation broth containing several dissolved impurities such as proteins, polysaccharide, colouring molecules, etc. These compounds correspond to another resistance called "adsorption resistance, R_a" because they can adsorp on the membrane wall making the permeation flux decrease. The resistance-in-series were measured by using the cleaning method. During the process, the permeate flux (J) decreased at a rate lower than that obtained from the pure water.

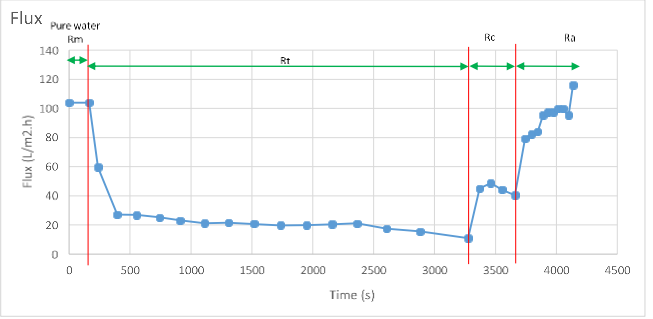


Fig. 3. The change in flux by using the cleaning method

In this experiment, the cell removal rate was of interest. The changing of flux was shown in Fig. 3. *R. paludigenum* culture was transferred to cross- flow filtration system to be ready for the separation. The membrane with 18 m^2 surface area, and 0.1 µm pore diameter was operated at the trans-membrane pressure of 1 bar. Every 25 and 20 L of the permeate was collected and the time used was recorded. The pure water flux was obtained at a constant value of 105 L/m^2.h. However, a flux decline characteristic was observed with the changing of pure water to fermentation broth. The flux decreased rapidly during

the first 500 s followed by a slightly decreased until the end of the experiment. Cells were collected for further downstream processing for oil extraction. In addition, a cleaning method was employed to the machine by RO water followed by 1%NaOH. The recovered flux of water was recorded of each cleaning step until it reached to the original flux value.

3.2. Resistance analysis

Resistance-in-series model that considers membrane resistance, adsorption resistance, and cake resistance has been applied to describe a fouling mechanism of membrane filtration process. This model is particularly applicable for the analysis of flux decline in microfiltration of the present broth because it contains many macromolecules such as proteins, polysaccharides, and peptides [7].

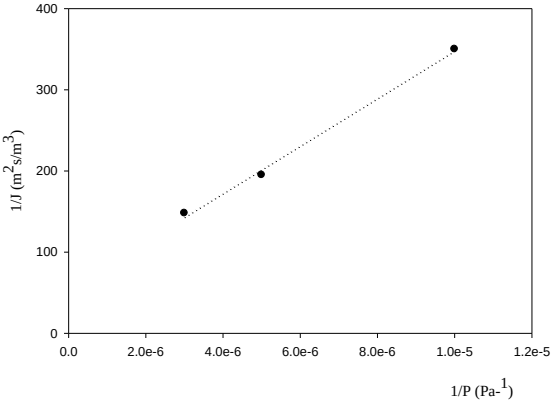


Fig. 4 .Relationship between 1/J Vs 1/P of water.

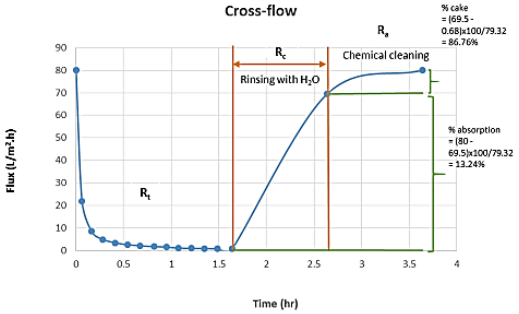
Fig. 4 shows the linear relationship between 1/J and 1/P, indicating the validity of Eq. (2). The membrane resistance, R_m, can be obtained from the flux measurement of pure water. The viscosity of water is 10^{-3} Pa.s. From Fig. 4 show that $1/J(m^2s/m^3)$ increased with increasing 1/P (Pa^{-1}). The value of R_m can be calculated from the slope and R_m is 4×10^{10} (m^{-1}), and correlative coefficient was obtained is 0.989.

3.3. Fouling and cleaning of microfiltration system

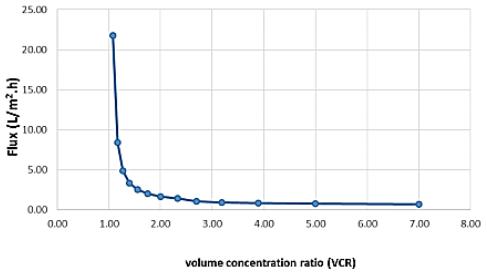
The experimental results were shown in Table 1. Flux value of fermentation broth were used to plot a graph of the relationship between flux and different time (Fig. 5A). In addition, it can be plotted as a graph of the relationship between flux and volume concentration ratio (Fig. 5B).

Table 1. Flux and volume concentration ratio of cross-flow filtration.

Volume per time	permeate volume (L)	Time (hr)	delta time	membrane area (m^2)	Flux	volume concentration ratio (VCR)
0	0	0	0	18	80.00	0
25	25	0.0639	0.0639		21.74	1.08
25	50	0.1647	0.1008		8.43	1.17
25	75	0.2833	0.1186		4.90	1.27
25	100	0.4139	0.1306		3.36	1.40
25	125	0.5422	0.1283		2.56	1.56
25	150	0.6750	0.1328		2.06	1.75
25	175	0.8219	0.1469		1.69	2.00
25	200	0.9497	0.1278		1.46	2.33
20	220	1.0744	0.1247		1.03	2.69
20	240	1.2125	0.1380		0.92	3.18
20	260	1.3475	0.1350		0.82	3.89
20	280	1.4858	0.1383		0.75	5.00
20	300	1.6380	0.1522		0.68	7.00
Washing with H_2O		2.6380	1		69.50	
washing with NaOH		3.6380	1		80.00	



(A)



(B)

Fig. 5. Time course of flux in cross-flow filtration and and time course of volume concentration ratio in crossflow filtration (B).

Theoretically, the efficiency from filtrated with dead-end should be lower at the time because of the thicker of cake layer were formed above of membrane. Flux of samples decreased over time and took around 1.2 hours to get 35 mL of filtrated sample. While the efficiency of cross-flow filtration should be high in the initial time, and continuously decreasing until constant. In the other hand, cake forming was increasing, and stable because of flowing phenomenal was reducing cake thickness. However, 350 L of cross-flow filtration absolutely discord with theory. Apparently, the reason that made cross-flow filtration became a low efficient came from membrane washing. Both of percentage cake formation and absorption literally confirm that membrane must be washing and test the efficiency before applied in industrial. Moreover, there were so many factors to concern when scaling up to industrial scale. Normally, challenging is not occurred in downstream processing compare to upstream that have so much factors to solve. For the water cleaning, the permeate flow rate 1,250 L/h when calculated to the regained flux after water washing as 69.50 L/m^2.h and the regained flux after 1%NaOH washing as 80.01 L/m^2h. Thus, percentage of absorption as 13.24% and percentage of cake as 86.76%. The retained cells accumulated on the membrane surface in a growing cake layer. The thickness of the cake layer can be increased, and cause the increasing in resistance to the permeate flow.

3.4. Spray drying of the yeast cell

Fig. 6. The spray-dried oleaginous yeast *R .Paludigenum.*

The *R. paludigenum* was spray dried at air inlet and outlet temperatures of 130 °C and 72 °C, respectively. The optimum operating parameters in this work were 450 $L.h^{-1}$ of air flow rate, 3.5 $mL.min^{-1}$ feeding rate, and cell concentration of 50 $g.L^{-1}$, respectively. These parameters were chosen based on those found at the scale to yield a powder with a moisture content within specifications of <10%. Cell viability was not the concern of this work since the cells were subjected to oil extraction soon after the drying process. Fig. 6 shows the spray-dried oleaginous yeast *R. Paludigenum.* The typical characteristic of this strain was the red colour obtained from its prefixed name "*Rhodo-*".

3.5. Oil extraction

After cell harvesting, yeast was dried by using a spray dryer. The lipid was extracted from the cell by non-polar solvent such as diethyl ether. The cell breaking process was carried out by sonication, and extraction around 3-4 hours. The cell mixture was centrifuged, and collected only the organic solvent phase (upper layer). The organic solvent, diethyl ether, was removed by using a rotary evaporator at 35 °C under a vacuum pressure of 150 mBar. The extracted lipid from *R. paludigenum* is shown in Fig. 7. The extracted lipid was measured by using gas chromatography (GC), and showed the normal fatty acid profile from C14-C20. The oil can be used as the acyl receptor in trans-esterification with either methanol or ethanol in biodiesel production process.

Fig. 7. The extracted oil from *R. Paludigenum.*

4. Conclusions

In this work, an alternative method for yeast recovery using membrane technique was studied. Yeast was separated from the fermented broth by microfiltration at the end of a batch fermentation. In this experiment, *Rhodosporidium paludigenum* yeast was culture in a 500-L by using cassava starch is substrate. This yeast can produce high lipid for bio-oil production.

Factors affecting the performance of crossflow filtration were investigated. For cleaning process, cells cultivated and suspended in saline, a steady state was attained. When the circulation flow rate was lower than a critical value, a part of the channel of the crossflow filtration module was plugged with cell cake, and thus the steady-state flux was low. In crossflow filtration of suspensions of yeast cell, the flux gradually decreased, and the flux after 8 h of filtration was lower than the value calculated by filtration theory.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

The yeast was screened and stored at Dr. Mariena's lab. Miss Chotika prepared the inoculum. Fermentation, membrane separation, spray drying, and oil extraction were carried out by Mr. Pongsatorn who investigated the experiment, analyzed the data, and also drafted the manuscript. Dr. Apichat approved and revised the manuscript.

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