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Selection of *Pichia kudriavzevii* Strain for the Production of Single-Cell Protein from Cassava Processing Waste

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***Abstract*—**A total of 115 yeast strains isolated from local cassava processing wastes were measured for crude protein content. Among these strains, the strain MSY-2 possessed the highest protein concentration (>3.5 mg protein/mL). By using molecular identification tools, it was identified to be a strain of *Pichia kudriavzevii* based on similarity of D1/D2 domain of 26S rDNA region. In this study, to optimize the protein production by MSY-2 strain, Response Surface Methodology (RSM) was applied. The tested parameters were the carbon content, nitrogen content, and incubation time. Here, the value of regression coefficient (R^2) = 0.7194 could be explained by the model which is high to support the significance of the model. Under the optimal condition, the protein content was produced up to 3.77 g per L of the culture and MSY-2 strain contains 66.8 g protein per 100 g of cell dry weight. These results revealed the plausibility of applying the novel strain of yeast in single-cell protein production.

***Keywords*—**Single cell protein, response surface methodology, yeast, cassava processing waste.

I. Introduction

THE increasing of population growth worldwide leads to the tremendous demand for food production to supply the needs in both human food and animal feed sectors. This situation motivates research and process development to formulate and investigate the alternative food sources. Single cell protein (SCP) production is one of the solutions to be used for supplementation of a staple diet. SCP is defined as the protein extracted from cultivated microbial biomass, including algae, fungi, yeast, and bacteria.

However, high fermentation costs prevent their further possibility in industrialization. Therefore, various low-cost substrates were investigated and utilized as the materials for SCP production. Cassava processing is one of the large and growing industry because cassava products and by-products have been used widely for production of foods and biofuels [1], [2]. Cassava processing generally generates large volume of organic wastes which can impact the environments. Regarding to the abundance of cassava processing waste, it is interesting to search for the microorganisms that are well capable of utilizing cassava waste to produce SCP.

Currently, many species of yeasts, such as *Saccharomyces cerevisiae*, *Candida utilis*, and the species of genera *Pichia* and *Torulopsis* can be used for single cell protein production. Yeast SCP have many advantages, for example, the probiotics effect on enhancement of animal weight and growth [3], high protein content [4], capability to utilize various types of substrates [5], [6]. Therefore, conversion of raw biomass or waste into yeast biomass and SCP is useful because of the high nutritional quality and it provide the potential uses in animal feed formulations.

This study was aimed at the screening and identification of yeast strains isolated from cassava processing wastes that have potential for SCP production. Then to optimize the culturing condition to achieve highest SCP production, the Response Surface Methodology (RSM) is applied to generate mathematic model that explains the correlations of culturing parameters and SCP production. The statistical analysis of this study could be used to find the optimal condition that is readily to be applied in up-scale process.

II. Materials and Methods

A. Screening of Yeast Strains with High Protein Content

Cassava processing wastes were obtained from local cassava processing plants in Maha Sarakham province, Thailand. Each sample was washed and serial diluted with 50 mM phosphate buffer saline (PBS). Then the diluted samples were plated on Yeast Malt (YM) agar, and incubated at 30°C for 48 h. Total 115 separated yeast colonies on the YM plates were collected, re-streaked in slants separately, and kept as the stock cultures. To screen for yeast strains that produce high protein content, each isolates were inoculated in 10 ml of YM broth and incubated at 30°C for 48 h. Yeast cells were collected by centrifugation at 8,000 rpm for 10 min. Cell pellets were washed with distilled water three times, and resuspended in 100 µl lysis buffer [7], [8] (containing 5.0 ml/L of Triton X-100, 0.372 g/L of thylenediaminetetraacetic acid (EDTA), and 0.0345 g/L of Phenylmethylsulfonyl fluoride (PMSF)). Then the cell suspension was incubated for 20 min

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under shaking condition at room temperature to lyse the cells. After incubation, the sample was sonicated for 5 min with ultrasonic homogenizer (Cole-Parmer Instruments, Vernon Hills, Illinois, US) to increase the cell lysis and the release of the protein. The protein content then was measured by using Bio-Rad Protein Assay Kit based on Bradford dye-binding method [9] (Bio-Rad Laboratories, Inc., CA, US). Triplicate protein measurements for each sample were performed in this study.

B. DNA Extraction, PCR, and Yeast Identification

After screening experiment, only one yeast isolate, MSY-2, that produced the highest protein content among 115 isolates was selected for further identification. First, single colony of MSY-2 was inoculated in 5 ml of YM broth medium in a test tube, and incubated at 30°C in a rotary shaker for 24 h. Cells were harvested by centrifugation at 6,000 rpm for 10 min. Then, genomic DNA was extracted by using PureLink™ Genomic DNA Mini Kit (Invitrogen, Life Technologies, CA, US). The cell pellet was resuspended in 500 µl Zymolase buffer (containing 15 U of Zymolase enzyme, 1 M sorbitol, 10 mM sodium EDTA, 14 mM β-mercaptoethanol) and incubated at 37°C for 1 h. The sample was centrifuged again at 3,000 rpm for 10 min at room temperature to pellet, and then resuspended again in 180 µl PureLink Genomic Digestion Buffer. 20 µl of Proteinase K was added and sample was vortexed briefly. After incubation at 55°C for 45 h, 20 µl RNase A was added to the sample, and incubated at room temperature for 2 min. Then 200 µl of PureLink Genomic Lysis/Binding buffer was mixed with sample to obtain a homogeneous solution. 200 µl of 96% ethanol was added to the lysate and sample was mixed briefly by vortexing. To purify genomic DNA, yeast lysate was transferred into PureLink Spin column, the column was centrifuged at 10,000 rpm for 1 min at room temperature, and the flow-through was discarded. 500 µl of Wash buffer 1 was added into the column and the column was centrifuged at 10,000 rpm for 1 min at room temperature, and the flow-through was discarded. Then 500 µl of Wash buffer 2 was added into the column and the column was centrifuged at 10,000 rpm for 3 min at room temperature. The DNA sample was eluted by adding 100 µl of sterile distilled water, and column was centrifuged to collect DNA sample. The quantity of DNA sample was determined using a UV spectrometry at 260 nm.

Next, the D1/D2 domain of 26S rDNA region was amplified using the specific primers NL1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and NL4 (5′-GGT CCG TGT TTC AAG ACG G-3′) [10]. 50 ng of genomic DNA was used as a template. The components of 50 µl of polymerase chain reaction (PCR) (RBC Bioscience, containing 1X PCR reaction buffer, 0.1 µM dNTP mix, 0.2 µM primers, 1.25 units RBC Taq DNA polymerase) were mixed together. Then the sample was placed in thermal cycler machine (MS Major Science). Amplification was carried out using a thermal cycling protocol consisting of predenaturation for 10 min at 94°C followed by 30 cycles of 60 s at 94°C, 60 s at 52°C, and 90 s at 72°C. The PCR product was separated in 1% agarose gel electrophoresis and detected under UV lamp after staining with ethidium bromide.

To purify and concentrated PCR products, gel/PCR fragments extraction kit (RBC Bioscience) was used. 250 µl of DF buffer was added into 50 µl of PCR product, and mixed with vortexing. The mixed sample then was transferred into DF column, and centrifuged at 13,000 rpm for 1 min. 600 µl of wash buffer was added into DF column, and centrifuged at 13,000 rpm for 1 min. To completely discard flow-through, DF column was additionally centrifuged for 2 more min. 20 µl of sterile distilled water was added into DF column to elute purified PCR product. Then the purified PCR product was submitted for sequencing, and nucleotide sequences were analyzed to identify yeast strain using BLAST algorithm provided by National Center for Biotechnology Information (NCBI).

C. Experimental Design and Fermentation

Optimization of culturing condition for yeast protein production (Y) was carried out using RSM with Box-Behnken design [11]. Three of independent variables were studied here including incubation time (X_1), carbon content (X_2), and nitrogen content (X_3). For each variables, three levels (max = +1, mid = 0, min = -1) was selected for the optimization, with a total of 17 runs. The range and levels of independent variables and code values were represented in Table I. Experimental data were analyzed using the Design-Expert software (version 7.0.0, STAT-EASE Inc., USA), to fit the second-order polynomial regression model as shown in (1):

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \quad (1)$$

where Y is the response variable (protein production), β_0 is the constant, β_i is the linear coefficient, β_i is the quadratic coefficient, and β_{ij} is the two factor interaction coefficient. The accuracy and general ability of the above polynomial model could be evaluated by the coefficient of determination (R^2).

TABLE I
CODED FACTORS AND ACTUAL VALUE OF INDEPENDENT VARIABLES

Factor	Name	Range of variables		
		Low (-1)	Mid (0)	High (+1)
X_1	Incubation time (h)	16	32	48
X_2	Carbon content (g/L)	20	50	80
X_3	Nitrogen content (g/L)	5	15	25

To culture the MSY-2 strain as designed based on RSM, one colony of MSY-2 was inoculated in 10 ml of YM broth media and incubated at 30°C for 48 h. The inoculum was added into 90 ml of minimal media (containing 0.05% $MgSO_4$, 0.01% NaCl, 0.01% $CaCl_2$, 0.01% yeast extract), which was supplemented with glucose, and ammonium sulfate as carbon and nitrogen source (with the amounts as indicated in Tables I and II) respectively. Then all the MSY-2 cultures

were incubated at 30°C in shaking incubation (with 200 rpm shaking) until incubation times were reached as designed. The cell pellets were collected by centrifugation at 8,000 rpm for 10 min, and proteins were extracted and quantified as described above again.

III. Results and Discussion

A. Screening and Identification of the Yeast Strains with High Protein Content

A total 115 yeast isolates (MSY1 - MSY115) were obtained from cassava processing wastes, and protein contents were extracted and measured (Fig. 1). Most numbers of yeasts (77 isolates) here produce proteins less than 2 mg/ml, and only one isolates (MSY-2) produce proteins more than 3.5 mg/ml. Based on the protein production, we therefore selected MSY-2 strain that produced the highest amount of protein (3.53 mg/ml) among all yeast isolates in our collection as the candidate for further experiment.

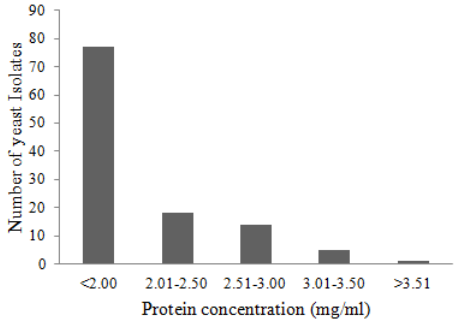

Fig. 1 Total cellulase activity of fraction 2 extracted from microbial consortium isolated from cattle manure using rice straw as substrate

To identify yeast MSY-2 strain, partial sequences of D1/D2 domain of 26S rDNA region was amplified from genomic DNA, analyzed, and aligned by using BLAST analysis. The result showed that the sequences of PCR product have 100.00% similarity to the sequences of 26S rDNA fragment of *Pichia kudriavzevii* (Accession: U76347). Therefore, it could be suggested that the MSY-2 strain is the new isolate of *Pichia kudriavzevii*. Note that two strains of *Pichia kudriavzevii* have been recently whole-genome sequenced [12], [13]. Interestingly, a strain of *Pichia kudriavzevii* were reported to have potential in ethanol and phytase production [12], [14]. So, it is interesting to further investigate the other applications of MSY-2 in biomass conversion to other useful products, i.e. biofuels, as well.

B. Optimization of Protein Production using Response Surface Methodology

Although among 115 yeast strains, the MSY-2 strain produce the highest amounts of proteins. We aimed to perform optimization experiment to enhance the protein production using RSM with Box-Behnken design. Here, incubation time of 16-48 h (X_1), carbon content of 20-80 g/L (X_2), and nitrogen content of 5-25 g/L (X_3) were tested to optimize the protein production. The experiment trials were designed for 17 runs with three different coded levels (high (+1), medium (0), low (-1)) based on Box-Behnken design as shown in Table II. A response factor, protein content, was measured for each of the culturing conditions.

TABLE II
Experimental Design to Test the Effect of Independent Variables on Protein Production (Y)

Run	X_1	X_2	X_3	Y (g)
1	0	-1	+1	0.222
2	+1	+1	0	0.361
3	-1	-1	0	0.185
4	-1	0	-1	0.319
5	0	-1	-1	0.198
6	0	0	0	0.215
7	-1	+1	0	0.197
8	0	0	0	0.248
9	+1	0	+1	0.311
10	0	+1	-1	0.245
11	+1	0	-1	0.196
12	0	0	0	0.272
13	-1	0	+1	0.218
14	+1	-1	0	0.242
15	0	+1	+1	0.377
16	0	0	0	0.214
17	0	0	0	0.228

The statistic software package, Design-Expert software version 7.0.0 (STAT-EASE Inc., USA), was used for regression analysis of experimental data and to plot response surface. One-way analysis of variance (ANOVA) was used to estimate the statistical parameters. The responses of Box-Behnken design were well fitted with the second-order polynomial equation as shown in (2):

$$Y = 0.26264 - (3.55454 \times 10^{-3})X_1 + (1.38692 \times 10^{-3})X_2 - (8.68929 \times 10^{-3})X_3 + (3.37591 \times 10^{-4})X_1X_3 \quad (2)$$

In this equation, Y is the protein content, and X_1, X_2 and X_3 are the coded value of the test variables as incubation time, carbon content, and nitrogen content, respectively. The statistical significance of the model was evaluated by the F-test for ANOVA (Table III). ANOVA of the model (2FI vs. Linear) suggested that the model is significant with the value of "Prob > F" was < 0.05, which indicated that the computed model was statistically significant with a confidence interval of 99.95%. The model F-value (4.78) implied that the model was significant and there was only a 1.54% chance that a "Model F-value" could occur because of noise. The "Lack of Fit" test also confirmed the statistical significance of the model because the "Prob>F" value was > 0.05.

The cut-off criteria of statistically significance with "Prob > F" with < 0.1 was applied to each "model term", and, in this case, X_2, and X_1X_3 are significant model parameters to the protein production in MSY-2. The coefficient of determination (R^2) of the model was 0.7194, which indicated that the model was suitable for representing the relationships among the selected variables and advocated a high significance of the model.

TABLE III
ANOVA ANALYSIS OF THE DESIGN

Source	Mean square	F-value	Prob>F
Model	8.440×10^{-3}	4.78	0.0154
X_1	4.665×10^{-3}	2.64	0.1300
X_2	0.014	7.84	0.0160
X_3	3.574×10^{-3}	2.02	0.1803
X_1X_3	0.012	6.61	0.0245

Response surface plots of the RSM as a function of two variables at a time are helpful in understanding both the main and the interaction effects of these variables (Fig. 2). The maximal protein yield (0.3768 g with 66.8% g-protein per 100 g of cell dry weight) was obtained when 47.47 h of shaking speed, 72.97 g/L of nitrogen content, and 24.77 g/L of carbon content were used.

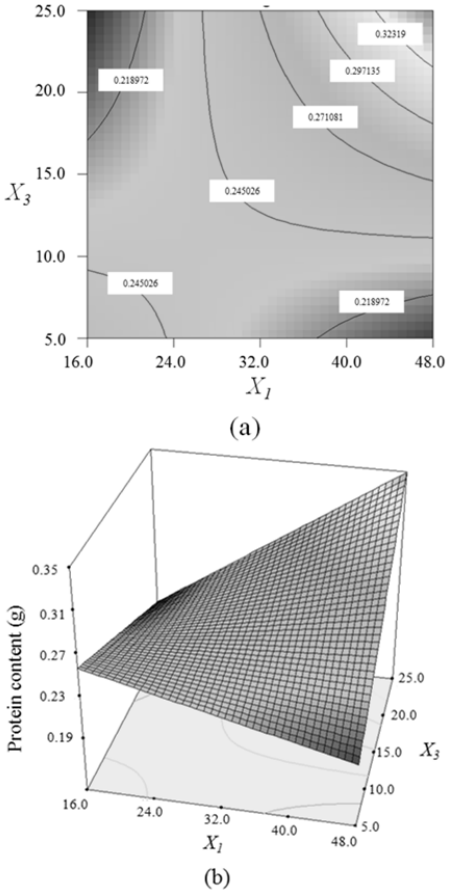

Fig. 2 Response surface plots showed the effect of interaction between the two independent variables (incubation time (X_1) and nitrogen content (X_3)) to the protein production. (a) Contour plot and (b) 3D surface plot

Based on the ANOVA analysis of model term, only X_2 and X_1X_3 are statistically significant. It is interesting that X_1, X_2, X_3 have positive effects on protein production, however the effects of interactions between two variables, X_1X_3, on protein production are complicated (Fig. 2). At lower nitrogen content, protein production decreased when incubation time increased, while, at higher nitrogen content, it became further increased when incubation time increased. These interactions suggest that finding the optimal balance point of these parameters for culturing MSY-2 is necessary to maximize protein production and to avoid the negative interaction effect from the interacting factors.

IV. CONCLUSION

Bioconversion of agricultural waste to value-added products is a growing area of research and process development. In this study, a new strain of yeast, *Pichia kudriavzevii* MSY-2, isolated from cassava processing waste was identified. This yeast was proven to be a promising microorganism for the high production of SCP. This research suggested the potential use of the MSY-2 to produce SCP from cassava processing waste. Additionally, the results from optimization experiment preliminarily indicated the significance of culturing parameters on SCP production. Availability of the results from the optimization experiment and the modeling will be useful information for the industries to include SCP production for conversion of agricultural waste to valued-added products in near future.

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