Optimization of Nitrogen Sources and Carbon Source for Recombinant Omega-3 Fatty Acid-Containing Biomass Production

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Abstract: The compositions of the medium for recombinant *Pichia pastoris* producing omega-3 fatty acid were determined for maximum biomass production using crude glycerol as a carbon source and ammonium salts as nitrogen sources. The optimization of the medium was done using Box-Behnken a design software with 3 factors, 3 levels, 1 response for an optimal level of crude glycerol, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$. The optimum medium condition was 40 g/L crude glycerol, 3 g/L $(NH_4)_2SO_4$, and 7 g/L $(NH_4)_2HPO_4$. The highest biomass in flasks cultivation after 7 days production was 6.064 g/L DCW and 0.1516 g biomass/ g crude glycerol.

Key words: *Pichia pastoris*, recombinant omega-3 fatty acid, crude glycerol, Box-Behnken.

1. Introduction

Pichia pastoris, a yeast that is an established protein expression host mainly applied for the production of biopharmaceuticals and industrial enzymes. It has easily been applied to produce recombinant proteins such as an omega-3 fatty acid biosynthesis pathway protein [1]. In the past few decades, many clinical studies have been conducted on the health benefits of omega-3 LC-PUFAs. DHA is a structural component of the human brain, cerebral cortex, skin, and retina [2]. EPA can reduce heart attacks, strokes, and cardiovascular deaths [3]. Omega-3 LC-PUFAs are important for human health, and have been highly recommended for human diet. Normally, omega-3 LC-PUFAs can be acquired from a variety of sources such as fish oil, plant oil, and krill oil. However, the primary sources of omega-3 in the human diet is often derived from fish oil. The amount of omega-3 in fish is usually limited, and is difficult to collect. Therefore, they have been considered as a promising alternative recombinant omega-3 fatty acid *P. pastoris* for fish oil as source of these fatty acids. Firstly, the optimization of the medium is really important to support for collecting high biomass product. For carbon sources, *P. pastoris* can be consumed some sources such as glucose from cassava, glycerol, etc. Nevertheless, crude glycerol, a kind of carbon sources that have a lot of benefits, is a by-product from biodiesel reaction. In biodiesel reaction, depending on sources of oil and the catalyst of reaction, the quality of crude glycerol would be different. However, *P. pastoris* is a kind of recombinant yeast that is easy to grow, can easily use crude glycerol [4].

Reusing crude glycerol can protect the environment and also reduce the cost of a final product. In previous research, some nitrogen sources such as yeast extract, peptone, ammonium salts have been used for the cultivation of oil yeasts [5], [6]. However, the amount of the nitrogen sources strongly affected to biomass production, other studies have agreed that lipid accumulation increases with nitrogen limitation [7]. Therefore, the ammonium salts were supplanted by nitrogen sources in this study.

The experiments for the optimization of the medium components for the biomass production of recombinant omega-3 fatty acid *P. pastoris* was designed by Box-Behnken with 3 factor including crude glycerol, $(NH_4)_2SO_4$ and $(NH_4)_2HPO_4$, respectively. Finally, the optimization of the medium was done with 7 g/L $(NH_4)_2HPO_4$, 3 g/L $(NH_4)_2SO_4$, 40 g/L crude glycerol.

2. Materials and Methods

2.1. Strains

The recombinant omega -3 fatty acid *P. pastoris* was used in this study [1]. The EPA and ARA accumulation were detected in recombinant *P. pastoris* containing pGAPZ:Δ6&Δ5:E6. Stock culture was maintained in YPD include yeast extract 1%, peptone 2%, glucose 2%.

2.2. Media and Growth Conditions

P. pastoris was cultured at 30^0C and 200 rpm in flask 250 mL containing 100 mL with different level of crude glycerol (20 - 40 g/L), $(NH_4)_2SO_4$ (3 - 9 g/L), $(NH_4)_2HPO_4$ (2 - 7 g/L). Crude glycerol, a by-product from biodiesel production form palm oil, was kindly provided by the BioSynergy Company, Thailand. Besides that, 4.6 mL/L PTM4 trace salts were added in broth. The compositions of PTM4 trace salts were (in g/L): 2 $CuSO_4.5H_2O$; 0.08 KI; 3 $MnSO_4.H_2O$; 0.2 $NaMoO_4.2H_2O$; 0.02 H_3BO_3; 0.5 $CoCl_2.6H_2O$; 6.7 $ZnCl_2$; 21.7 $FeSO_4.7H_2O$; and 0.2 biotin, respectively [4]. All mediums with different levels of crude glycerol and ammonium salts were adjusted to pH 5 by citric acid that have supported to increasing produce oil inside *P. pastoris* [8] and was sterilized by autoclave before inoculation.

2.3. Experimental Design and Statistical Analysis

Composition of the medium was optimized for biomass production of recombinant omega-3 fatty acid *P. pastoris* by using crude glycerol as carbon source, $(NH_4)_2SO_4$, and $(NH_4)_2HPO_4$ as nitrogen sources. The concentration of crude glycerol, $(NH_4)_2SO_4$, and $(NH_4)_2HPO_4$ were optimized by response surface methodology using three factors, three-levels using a Box-Behnken design. The three variables selected for the statistical analysis were designated as X_1, X_2, X_3 and the predicted response in the form of biomass production was designated as Y (Table 1). The experimental design protocol for response surface methodology was developed using design-expert Software. A total of fifteen experiments with different composition of three factors were carried out (Table 1). As a dependent variable, biomass production was measured at the end of cultivation, 7 days for cultivation, when glycerol completely consumed.

2.4. Analytical Method

For dry cell weight estimation, samples after 7 days for cultivation were centrifuged at 4000 rpm, 10 min. The cell pellet was washed with DI water and then dried by freeze dryer to a constant weight. Dry cell weight of 1 OD cells corresponded to 0.475 g/L DCW. Crude glycerol was determined for the elements Na, Mg, K, Ca, Fe, P by ICP-OES (PerkinElmer Optima 8000 ICP-OES, USA). Crude glycerol (0.207 – 0.2114g) was digested using 10 mL of HNO_3 in a microwave digester (Ethos One, Italy). The digester temperature was ramped to 200°C in 15 min, maintained for 15 min, and cooled down to 25°C. The digested crude glycerol solution was transferred into a 100mL volumetric flask and filled to mark using DI water. The solution was mixed and

analyzed using ICP-OES. Amount of Glycerol from crude glycerol was performed by HPLC using Aminex HPX-87H column, RID-10A [9].

Table 1. Box-Behnken Design Matrix for the Three Variables and Experimental and Predicted Results under Different Composition and Same Condition

Runs	$(NH_4)_2SO_4$ (X_1) (g/L)	$(NH_4)_2HPO_4$ (X_2) (g/L)	Crude Glycerol (X_3) (g/L)	Biomass (Y) (g/L)	
				Experimental	Predicted
1	9	4.5	20	4.142	4.146
2	3	7	30	5.416	5.406
3	6	4.5	30	4.614	4.609
4	6	7	20	4.137	4.159
5	9	7	30	4.678	4.660
6	3	2	30	4.042	4.028
7	9	4.5	40	4.289	4.299
8	9	2	30	4.209	4.223
9	3	4.5	20	4.122	4.129
10	6	2	40	4.408	4.569
11	6	4.5	30	4.502	4.569
12	6	7	40	4.797	4.567
13	3	4.5	40	5.05	5.042
14	6	4.5	30	5.285	5.303
15	6	2	20	3.912	3.889

3. Results and Discussion

3.1. Analyses of Crude Glycerol

Amount of glycerol in crude glycerol was measured around 72.6% (v/v) by using HPLC, Aminex HPX-87H column, RID-10A, H_2SO_4 5mM mobile phase (Fig. 1). The results indicated that amount of glycerol in crude glycerol was relatively similar with other crude glycerol [9], [10], [4], appeared at 12.870 min reaction time.

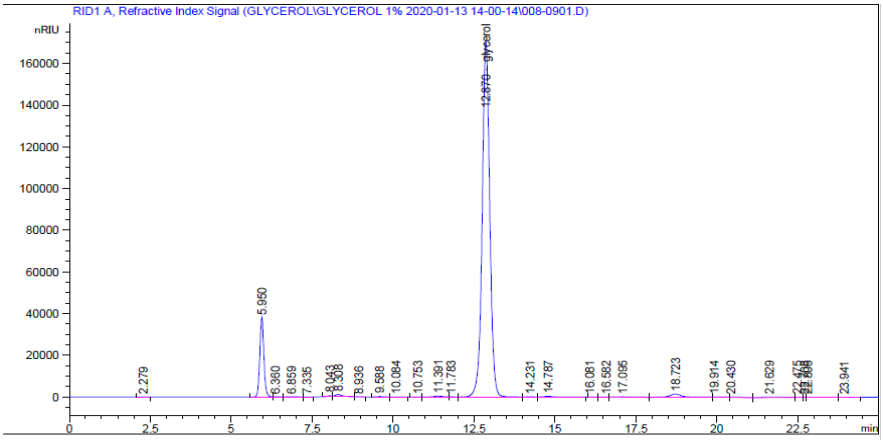

Fig. 1. HPLC profiles of glycerol from crude glycerol.

For elements analyst of crude glycerol, the findings were shown in Table 2. Nevertheless, biodiesel reaction of BioSynergy Company was used acid catalysis, elemental of crude glycerol were quite lower more than base catalysis [11].

Table 2. Elements Analyses of Crude Glycerol

Elements	Crude glycerol
Na (ppm)	11.996 ± 1.572
K (ppm)	19.888 ± 1.289
Ca (ppm)	7.073 ± 1.299
Mg (ppm)	8.631 ± 6.557
Fe (ppm)	4.801 ± 5.286
P (ppm)	0.675 ± 0.103

3.2. Response Surface Methodology for Biomass Production

The amount of dry cells weight was measured in fifty-five experiments with different composition and corresponding results were shown in Table 1. The results were exhibited in quadratic model using a multiple regression analysis as shown in the following equation;

$$\text{Biomass} = 4.36428 + 0.372789X_2 - 0.0558X_3 - 0.053358X_1 - 0.0552X_2X_3 - 0.003467X_2X_1 + 0.01711X_3X_1 - 0.011255X_2^2 - 0.004727X_3^2 + 0.000140\,X_1^2 \quad (1)$$

The ANOVA for quadratic model was demonstrated in Table 3. The coefficient of multiple determinations, R^2 was found to be 0.9716, which was shown that the model could be explained 97.16% of the variability in the system. For a good statistical model, the R^2 value should be close to 1.0. The relatively high value of R^2 indicated that second order polynomial equation is capable of representing the system under the given experimental domain. The significance of the model terms was evaluated by applying the analysis of variance (ANOVA) test using a design expert software. The P-value of less than 0.05 indicated that the model term was significant. The contour plot was created as shown in Fig. 2 to evaluated the combined effect of two ammonium salts on the biomass production.

Table 3. ANOVA for Quadratic Model

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	2.90	9	0.3223	19.03	0.0023	significant
A-(NH4)2SO4	0.9467	1	0.9467	55.91	0.0007	
B-(NH4)2HPO4	0.3507	1	0.3507	20.71	0.0061	
C-CRUDE GLYCEROL	0.1006	1	0.1006	5.94	0.0589	
AB	0.6856	1	0.6856	40.49	0.0014	
AC	0.0433	1	0.0433	2.56	0.1708	
BC	0.7319	1	0.7319	43.23	0.0012	
A^2	0.0379	1	0.0379	2.24	0.1949	
B^2	0.0032	1	0.0032	0.1903	0.6808	
C^2	0.0007	1	0.0007	0.0425	0.8448	
Residual	0.0847	5	0.0169			
Lack of Fit	0.0024	3	0.0008	0.0194	0.9952	not significant
Pure Error	0.0823	2	0.0411			
Cor Total	2.99	14				

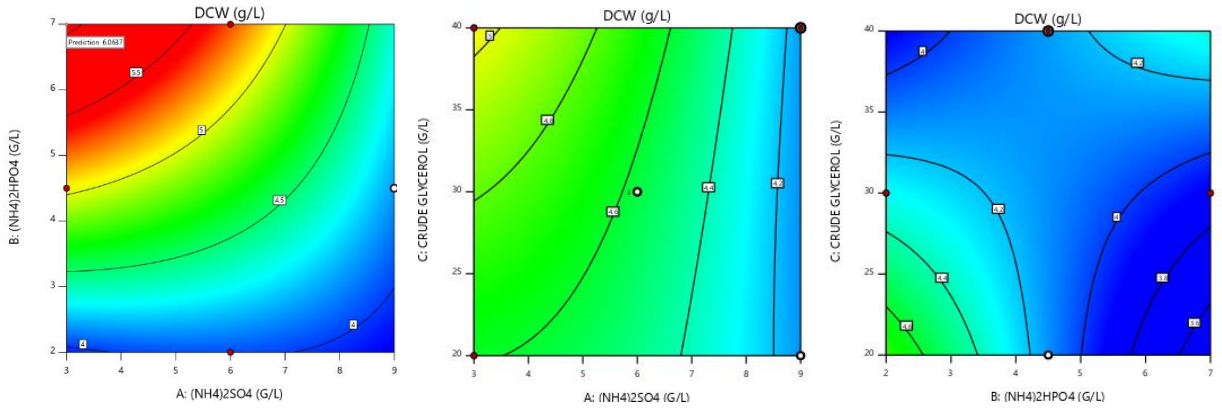

Fig. 2. Contour plots of the combined effects of $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$, and crude glycerol, respectively.

4. Conclusion

A waste-to-profit concept was successfully illustrated for the production of omega-3 fatty acid containing biomass production from crude glycerol as a by-product of biodiesel production process. The composition of optimized medium was obtained at 40 g/L crude glycerol, 3 g/L $(NH_4)_2SO_4$, and 7 g/L $(NH_4)_2HPO_4$, respectively. This medium will be applied for 500L fermentation and then the purification of omega-3 fatty acid will be investigated in the future work.

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Reference

[1] Chotika, G. (2013). *The Construction of Pichia Pastoris to Produce Polyunsaturated Fatty Acids (PUFAS)*. Institute of Agricultural Technology Biotechnology Suranaree University of Technology, Thailand.

[2] Connor, W. E., & Neuringer, M. (1988). The effects of n-3 fatty acid deficiency and repletion upon the fatty acid composition and function of the brain and retina. *Progress in Clinical and Biological Research, 282*, 275-294.

[3] Xie, D., Jackson, E. N., & Zhu, Q. (2015). Sustainable source of omega-3 eicosapentaenoic acid from metabolically engineered *Yarrowia lipolytica*: From fundamental research to commercial production. *Applied Microbiology and Biotechnology, 99(4)*, 1599-1610.

[4] Robert, J. M., Lattari, F. S., Machado, A. C., Castro, A. M., Almeida, R. V., Torres, F. A. G., *et al.* (2017). Production of recombinant lipase B from *Candida antarctica* in *Pichia pastoris* under control of the promoter PGK using crude glycerol from biodiesel production as carbon source. *Biochemical Engineering Journal, 118*, 123-131.

[5] Çelik, S. E., Ozbay, N., Oktar, N., & Çalik, P. (2008). Use of biodiesel byproduct crude glycerol as the carbon source for fermentation processes by recombinant *Pichia pastoris*. *Industrial and Engineering Chemistry Research, 47(9)*, 2985-2990.

[6] Lima, P. B. A., Mulder, K. C. L., Melo, N. T. M., Carvalho, L. S., Menino, G. S., Mulinari, E., *et al.* (2016). Novel homologous lactate transporter improves L-lactic acid production from glycerol in recombinant strains of *Pichia pastoris*. *Microbial Cell Factories, 15(1)*, article 158.

[7] Hassan, M., Blanc, P. J., Granger, L. M., Pareilleux, A., & Goma, G. (1996). Influence of nitrogen and iron limitations on lipid production by Cryptococcus curvatus grown in batch and fed-batch culture. *Process Biochemistry, 31(4)*, 355-361.

[8] Adrio, J. L. (2017). Oleaginous yeasts: Promising platforms for the production of oleochemicals and biofuels. *Biotechnology and Bioengineering, 114(9)*, 1915-1920.

[9] Anastácio, G. S., Santos, K. O., Suarez, P. A. Z., Torres, F. A. G., De Marco, J. L., & Parachin, N. S. (2014). Utilization of glycerin byproduct derived from soybean oil biodiesel as a carbon source for heterologous protein production in *Pichia pastoris*. *Bioresource Technology, 152*, 505-510.

[10] Tang, S., Boehme, L., Lam, H., & Zhang, Z. (2009). *Pichia pastoris* fermentation for phytase production using crude glycerol from biodiesel production as the sole carbon source. *Biochemical Engineering Journal, 43(2)*, 157-162.

[11] Hu, S., Luo, X., Wan, C., & Li, Y. (2012). Characterization of crude glycerol from biodiesel plants. *Journal of Agricultural and Food Chemistry, 60(23)*, 5915-5921.

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