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Experimental Design as a Tool for the Optimization of Lipid Production by *Meyerozyma guilliermondii* in a Crude Glycerol-based Medium

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Abstract

Biodiesel production, which has been increasing worldwide, transformed crude glycerol, the main byproduct of the reaction, into a commodity of low commercial value, especially due to the high costs involved in its purification process and to the fact that the market cannot account for its generation. Therefore, this study aims at contributing to the search for technological alternatives to the use of surplus crude glycerol so as to add value to this byproduct by using it as the carbon source for the yeast *Meyerozyma guilliermondii*, in order to yield lipids. The Central Composite Rotational Design was proposed to establish empirical models, codified for lipid content, production and productivity as the result of concentrations of Magnesium Sulfate Heptahydrate ($MgSO_4.7H_2O$) and yeast extract. In the optimized conditions, the lipid content in dry basis, lipid production and lipid productivity were 8.25%, 1.10 $g.L^{-1}$ and 0.0092 $g.L^{-1}.h^{-1}$, respectively obtained.

Keywords: Oleaginous yeasts, Byproduct, Biodiesel, Microbial lipids

1. Introduction

Even though biodiesel production from vegetable oil and animal fat has increased

significantly all over the world, its synthesis generates crude glycerol as an unwanted byproduct in considerable proportion (10%), which the market cannot totally account for (Silva et al., 2009). On the other hand, discharge of crude glycerol in the environment may cause serious environmental damage and a purification process that enables its use in several industrial sectors, such as cosmetics, food and pharmaceuticals, is not economically viable because of its low commercial value (Leoneti et al., 2012). Therefore, alternatives must be found to convert crude glycerol into value-added products which may contribute to the sustainability of the biodiesel supply chain. Crude glycerol may be used as the carbon source by different microorganisms in order to yield several products, such as 1,3–propanediol (Chatzifragkou et al., 2011), citric acid (Papanikolaou et al., 2008), succinic acid (Zhang et al., 2010), carotenoids (Silva et al., 2012), single cell protein (Santos et al., 2013) and single cell oil (Spier et al., 2015).

Production of microbial lipids, also known as single cell oil, is attractive, mainly because of the need for alternative biomass as lipid sources which may be applied to biodiesel production (Makri et al., 2010), since increase in plantation areas of oleaginous plants to account for the biodiesel supply chain has led to competition for arable land between biofuel and food (Menezes et al., 2013). Besides, microbial lipids may constitute a source of essential fatty acids in human and animal nutrition (Kot et al., 2016; Francisco et al., 2017).

On the other hand, optimization based on Experimental Design and Surface Response Analysis is a very useful strategy to better understand a process, since it enables the effect of every variable (either synergic or antagonistic) to be analyzed individually in order to maximize production and productivity (Rodrigues & Iemma, 2012). Despite these advantages, in the literature, there are very few studies of the use of this technique in microbial biomass production as the lipid source.

In this study, the Central Composite Rotational Design (CCRD) was proposed to establish optimal cultivation conditions in which *Meyerozyma guilliermondii* yields lipids in shake flasks, with the use of crude glycerol as the carbon source.

2. Materials and Methods

2.1 Microorganism

The yeast *Meyerozyma guilliermondii*, previously isolated and identified by Spier (2014), was kept refrigerated (4ºC) in culture tubes with Yeast Malt (YM) agar, with the following composition ($g.L^{-1}$): 10.0 glucose; 5.0 peptone; 3.0 malt extract; 3.0 yeast extract; and 20.0 agar. In reactivation, successive replicates of stock cultures were carried out in tubes with YM agar and incubated at controlled temperature (30ºC) for 48 h.

2.2 Crude Glycerol

Crude glycerol derived from biodiesel production based on degummed soybean oil by using methanol was applied to the medium preparation, with no purification steps. It was supplied by the BS Bios Indústria e Comércio de Biodiesel Sul Brasil S/A, located in Passo Fundo, RS, Brazil. The crude glycerol contained (%): 5.94 ash; 11.38 moisture; 0.59 nonglyceridic organic matter; 82.09 glycerol; and pH 5.14.

2.3 Inoculum Preparation

The inoculum was prepared with two tubes of reactivated microbial culture. Every tube was

scraped with 10 mL of 0.1% peptone solution. After cell suspension, it was transferred to a 500 mL Erlenmeyer flask with 180 mL YM broth. The resulting suspension was incubated at 30ºC and 180 rpm in a rotary shaker (Tecnal TE-424, Brazil) for 48 h. Cultivation was monitored by Neubauer chamber cell counting (Santos et al., 2013).

2.4 Cultivation in Shake Flasks

Cultivation was carried out in 500 mL Erlenmeyer flasks whose initial volume was 200 mL, as a result of the addition of the culture medium, yeast suspension (inoculum) and sterile distilled water. Flasks were inoculated with the needed volume of the inoculum whose calculation was based on the counting carried out by the Neubauer chamber, so as to reach initial concentration of 10^7cells.mL^{-1}, kept in a rotary shaker (Tecnal TE-424) at 30ºC, at 180 rpm. Aliquots were removed at pre-defined intervals, centrifuged at 3,000 rpm for 15 min in order to have their biomass and lipids determined. The latter were obtained just at the end of the cultivation (120 h).

2.5 Central Composite Rotational Design (CCRD)

The Central Composite Rotational Design (CCRD) was proposed to evaluate three independent variables (concentrations of $MgSO_4.7H_2O$, yeast extract and $ZnSO_4.H_2O$), totaling 17 assays whose levels are shown in Table 1. Concentrations of KH_2PO_4 (5 g.L^{-1}), Na_2HPO_4 (1 g.L^{-1}) and crude glycerol (30 g.L^{-1}), besides temperature (30°C) and pH (5.5), were kept constant.

Variables under evaluation were lipid content in dry basis (%), lipid production (g.L^{-1}) and lipid productivity (g.L^{-1}.h^{-1}) in 120-h cultivation. Data were analysed by the Statistica 5.0 software (Stat Soft Inc, USA).

2.6 Analytical Methods

2.6.1 Biomass

Biomass determination was carried out by the methodology described by Choi & Park (2003), whose absorbance reading was 600 nm by a spectrophotometer (Biospectro SP 22, China) and conversion to the mass concentration by a standard curve found for the microorganism.

2.6.2 Lipids

The dried biomass was treated with 2M HCl for disruption of the cell wall and the lipids were quantified according to Bligh and Dyer (1959). Lipid production was calculated by multiplying biomass concentration by lipid content (dry basis). Lipid productivity was calculated by dividing total lipid production by cultivation time.

3. Results and Discussion

3.1 Microbial Growth

The biomass of *Meyerozyma guilliermondii* was followed throughout cultivation proposed by the CCRD. Results are shown in Figure 1. Assays 5, 6, 7, 8, 9 and 11 were found to have values above 13 g.L^{-1}, demonstrating the strong capability of *Meyerozyma guilliermondii* to assimilate crude glycerol resulting from biodiesel synthesis. It should be highlighted that impurities found in crude glycerol, may exert either positive or negative effect on cell growth and lipid accumulation, depending on the microorganism (Gao et al., 2016).

In the cultivation of *Cryptococcus curvatus*, sweet sorghum bagasse hydrolysate, crude

glycerol, corn cob hydrolysate and activated sludge residue were used as carbon sources and biomass values of 10.83 $g.L^{-1}$ (Liang et al., 2012), 7.11 $g.L^{-1}$ (Cui et al., 2012), 12.60 $g.L^{-1}$ (Chang et al., 2015) and 9.84 $g.L^{-1}$ (Seo et al., 2013), respectively, were reached. Yu et al. (2011) studied the use of wheat straw hydrolysate in the biomass production of *Rhodotorula glutinis*, *Cryptococcus curvatus*, *Lipomyces starkeyi* and *Yarrowia lipolytica* and found biomass values of 13.8 $g.L^{-1}$, 17.2 $g.L^{-1}$, 14.7 $g.L^{-1}$ and 7.8 $g.L^{-1}$, respectively.

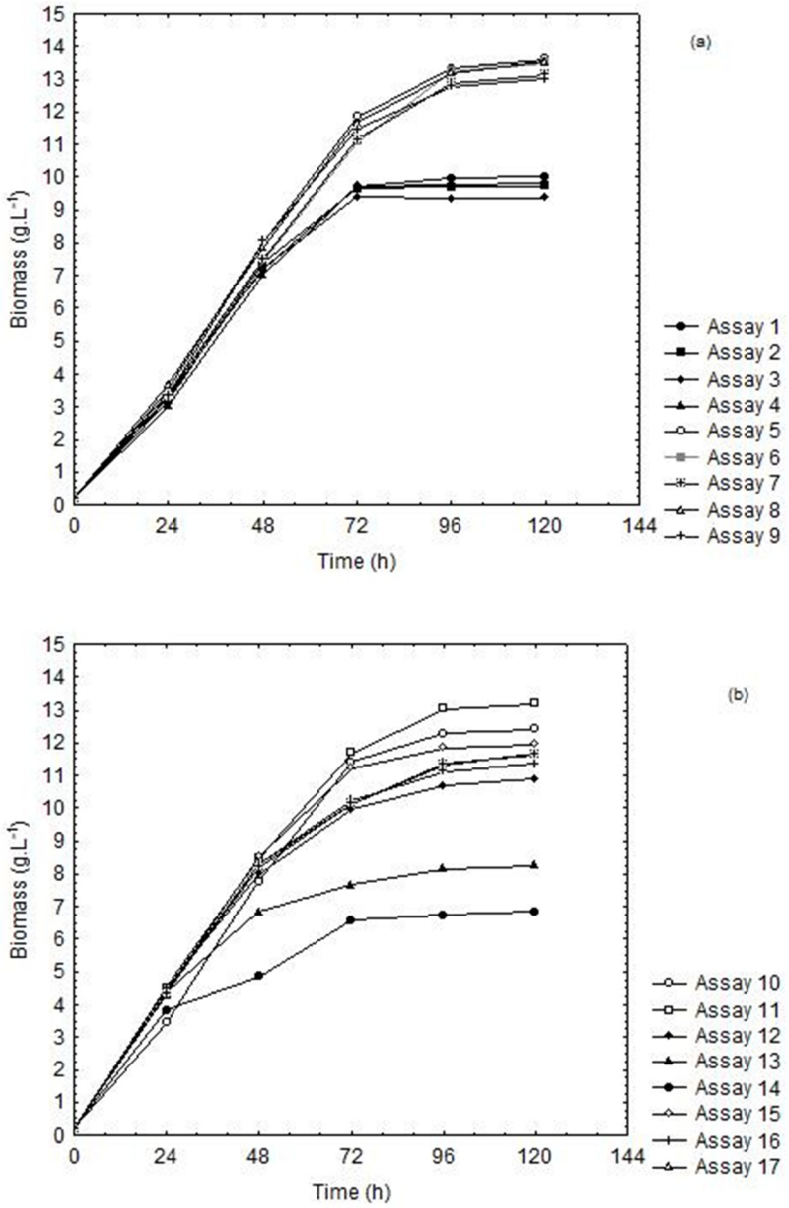

Figure 1. *Meyerozyma guilliermondii* biomass throughout cultivation. (a) Assays 1 to 9; (b) Assays 10 to 17

3.2 Lipid Production

Table 1 shows the CCRD and responses to all 17 assays which aimed at optimizing the culture medium to yield lipids. Lipid contents varied from 3.57% (Assay 8) to 9.68% (Assay

13) whereas lipid production ranged from 0.48 $g.L^{-1}$ (Assay 1 and Assay 8) to 1.33 $g.L^{-1}$ (Assay 11) and lipid productivity was between 0.0040 $g.L^{-1}.h^{-1}$ (Assay 1) and 0.0111 $g.L^{-1}.h^{-1}$ (Assay 11). Canonico et al. (2016), using crude glycerol as carbon source, reached lipid production of 0.45 $g.L^{-1}$ and 0.65 $g.L^{-1}$ for *Metschnikowia pulcherrima* and *Yarrowia lipolytica*, respectively. The optimization of culture medium of *Cryptococcus larentii*, containing cheese whey and molasses, resulted in 2.96 $g.L^{-1}$ lipid production and 0.0082 $g.L^{-1}.h^{-1}$ lipid productivity (Castanha et al., 2014).

Table 1. Matrix of thecentral composite rotational design and responses

Assay	X_1	X_2	X_3	Y_1	Y_2	Y_3
1	-1 (3.4)	-1 (0.0056)	-1 (1.96)	4.79	0.48	0.0040
2	+1 (4.6)	-1 (0.0056)	-1 (1.96)	5.88	0.57	0.0048
3	-1 (3.4)	+1 (0.016)	-1 (1.96)	7.51	0.70	0.0059
4	+1 (4.6)	+1 (0.016)	-1 (1.96)	6.12	0.60	0.0050
5	-1 (3.4)	-1 (0.0056)	+1 (3.46)	5.58	0.76	0.0063
6	+1 (4.6)	-1 (0.0056)	+1 (3.46)	5.24	0.71	0.0059
7	-1 (3.4)	+1 (0.016)	+1 (3.46)	5.00	0.66	0.0055
8	+1 (4.6)	+1 (0.016)	+1 (3.46)	3.57	0.48	0.0040
9	-1.68 (3.0)	0 (0.011)	0 (3.1)	4.35	0.57	0.0047
10	+1.68 (5.0)	0 (0.011)	0 (3.1)	4.20	0.52	0.0044
11	0 (4.0)	-1.68 (0.002)	0 (3.1)	8.21	1.33	0.0111
12	0 (4.0)	+1.68 (0.02)	0 (3.1)	6.83	0.95	0.0079
13	0 (4.0)	0 (0.011)	-1.68 (1.2)	9.68	1.09	0.0091
14	0 (4.0)	0 (0.011)	+1.68 (5.0)	6.08	0.60	0.0050
15	0 (4.0)	0 (0.011)	0 (3.1)	7.95	1.14	0.0095
16	0 (4.0)	0 (0.011)	0 (3.1)	8.00	1.17	0.0098
17	0 (4.0)	0 (0.011)	0 (3.1)	7.25	1.06	0.0089

Variables: X_1: $MgSO_4.7H_2O$; X_2: $ZnSO_4.7H_2O$; X_3: yeast extract. Responses: Y_1: lipid content in dry basis (%); Y_2: lipid production ($g.L^{-1}$); Y_3: lipid productivity ($g.L^{-1}.h^{-1}$). Fixed conditions: 5 $g.L^{-1}$ KH_2PO_4; 1 $g.L^{-1}$ Na_2HPO_4; 30 $g.L^{-1}$ crude glycerol; pH 5.5; temperature 30°C.

In the attempt to check the possibility of constructing empirical models codified with the data found in Table 2, the effect analysis showed that changes in concentrations of $MgSO_4.7H_2O$ and yeast extract had significant effect ($p \leq 0.05$) on all responses under study and that $ZnSO_4.7H_2O$ did not affect responses significantly ($p \geq 0.05$).

In order to check whether the model was predictive, the F-test was carried out. Table 3 shows the ANOVA with the F-test and the correlation coefficient R of the following responses: lipid content, lipid production and lipid productivity. F_{cal}/F_{tab} was above 3 in all responses, a fact that shows that the models are predictive. Correlation coefficients were above 0.8 in all responses.

Table 2. Significant regression coefficients for the construction of models for lipid content (%), lipid production ($g.L^{-1}$) and lipid productivity ($g.L^{-1}.h^{-1}$)

Response	Variable	Regression coefficient	Standard error	*t* (14)	*p*
Y_1	$X_1(Q)$	-1.34	0.2629	-5.11	0.0001
	$X_3(L)$	-0.80	0.2565	-3.13	0.0073
Y_2	$X_1(Q)$	-0.24	0.0499	-4.78	0.0002
	$X_3(Q)$	-0.13	0.0499	-2.66	0.0187
Y_3	$X_1(Q)$	-0.0020	0.0004	-4.79	0.0003
	$X_3(Q)$	-0.0011	0.0004	-2.63	0.0196

Variables: X_1: $MgSO_4.7H_2O$; X_2: $ZnSO_4.7H_2O$; X_3: yeast extract. Responses: Y_1: lipid content in dry basis (%); Y_2: lipid production ($g.L^{-1}$); Y_3: lipid productivity ($g.L^{-1}.h^{-1}$).

Table 3. ANOVA of lipid content, lipid production and lipid productivity

		Lipid content		
Source of Variation	Sum of square	Degrees of Freedom	Mean square	F_{cal}
Regression	32.24	2	16.12	17.91
Residual	12.56	14	0.90	
Total	44.80	16		
		Lipid production		
Regression	0.78	2	0.39	13.00
Residual	0.43	14	0.03	
Total	1.21	16		
		Lipid productivity		
Regression	5.50×10^{-5}	2	2.75×10^{-5}	12.73
Residual	3.02×10^{-5}	14	2.16×10^{-6}	
Total	8.52×10^{-5}	16		

Lipid content ($F_{tab} = 3.74$; $R = 0.8482$); lipid production and lipid productivity ($F_{tab} = 3.74$; $R = 0.8033$).

Equations 1, 2 and 3 represent empirical models codified for lipid content (%), lipid production ($g.L^{-1}$) and lipid productivity ($g.L^{-1}.h^{-1}$), respectively.

Lipid content (%) = + 7.34 - 1.34.X_1^2 – 0.80.X_3 (1)

Lipid production ($g.L^{-1}$) = + 1.09 – 0.24.X_1^2 – 0.13.X_3^2 (2)

Lipid productivity ($g.L^{-1}.h^{-1}$) = + 0.0091 – 0.0020.X_1^2 – 0.0011.X_3^2 (3)

Figure 2 shows the contour plots obtained from the empirical models established for the analysis of the best conditions of $MgSO_4.7H_2O$ and yeast extract concentrations for lipid production.

Figure 2a shows that the highest values of lipid content in dry basis (around 8%) were found at $MgSO_4.7H_2O$ concentration of 4.0 $g.L^{-1}$ (level 0) and at yeast extract ones between1.2 $g.L^{-1}$ (level -1,68) and 1.97 $g.L^{-1}$(level -1). Figure 2b shows that $MgSO_4.7H_2O$ and yeast extract concentrations at 4.0 $g.L^{-1}$ (level 0) and 3.1 $g.L^{-1}$ (level 0), respectively, optimized lipid production and reached around 1 $g.L^{-1}$. Figure 2c shows that the same conditions also led to optimization of lipid productivity (around 0.009 $g.L^{-1}.h^{-1}$).

Based on results found in the optimization by CCRD, a new assay (in triplicate) was proposed with the composition of the production medium established at ($g.L^{-1}$): 5 KH_2PO_4; 1 Na_2HPO_4; 30 crude glycerol; 4 $MgSO_4.7H_2O$; 0.002 $ZnSO_4.7H_2O$; 3.1 yeast extract; temperature 30°C; and initial pH 5.5. In these conditions, lipid content, total lipid production and lipid productivity of 8.25 ± 0.15%, 1.10 ± 0.03 $g.L^{-1}$ and 0.0092 ± 0.0003 $g.L^{-1}.h^{-1}$, respectively, could be found in 120-h cultivation.

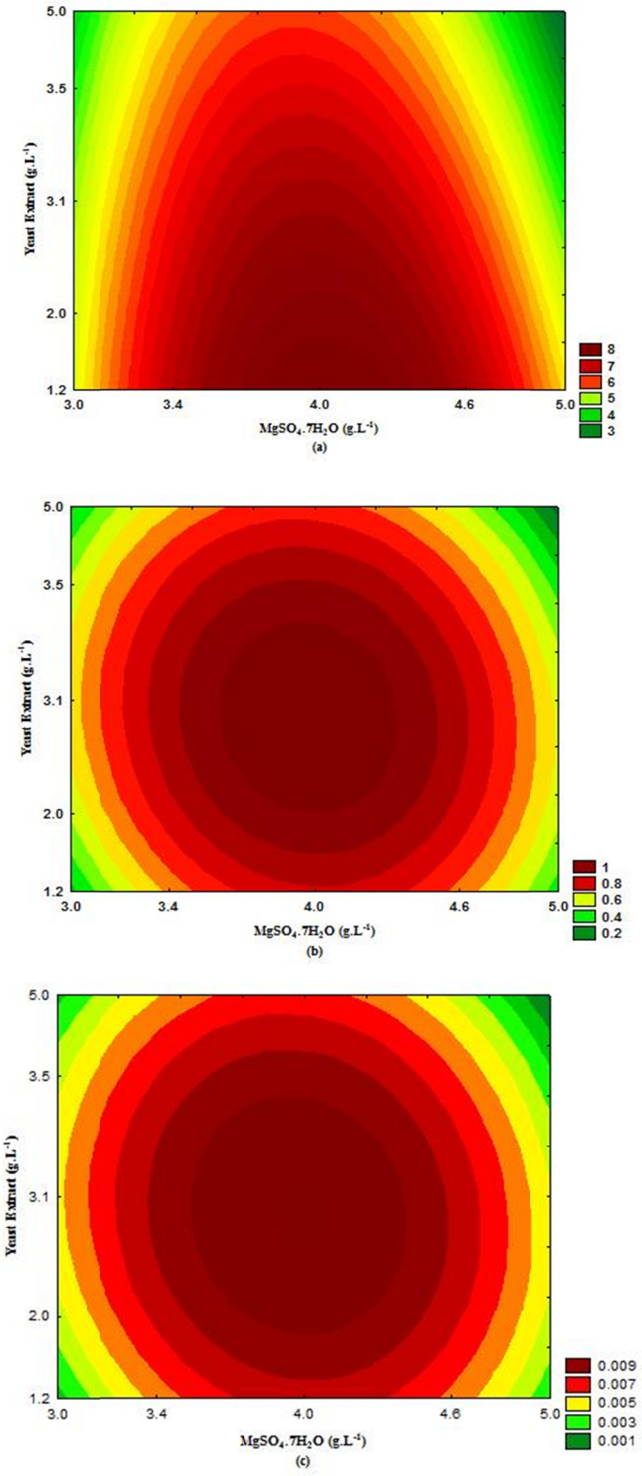

Figure 2. Contour plots for (a) lipid content; (b) lipid production; and (c) lipid productivity. $ZnSO_4.7H_2O$ concentration is 0.011 $g.L^{-1}$ (Level 0)

The relative deviations obtained during the validation of the experimental results and those predicted by the models (Table 4) were 11.03% (lipid content), 0.91% (lipid production) and 1.09% (lipid productivity), all of which were considered to be good for bioprocesses

(Rodrigues & Iemma, 2012). Therefore, Equations 1, 2 and 3 predict the behavior of lipid production by *Meyerozyma guilliermondii* in a crude glycerol-based medium.

Table 4. Validation of the empirical models

Validation responses	Lipid content (%)	Lipid production ($g.L^{-1}$)	Lipid productivity ($g.L^{-1}.h^{-1}$)
Experimental values*	8.25 ± 0.15	1.10 ± 0.03	0.0092 ± 0.0003
Responses predicted by the models	7.34	1.09	0.0091
Deviations of the model (%)	11.03	0.91	1.09

* Results are means of triplicate assays (n=3).

5. Conclusion

This study used crude glycerol as carbon source in *Meyerozyma guilliermondii* cultivation to yield lipids. The CCRD, whose variables were concentrations of $MgSO_4.7H_2O$, yeast extract and $ZnSO_4.7H_2O$, was proposed. *Meyerozyma guilliermondii* showed good growth capacity on a crude glycerol-based medium. Codified empirical models were established for lipid content, lipid production and lipid productivity as the result of yeast extract and $MgSO_4.7H_2O$ concentrations. It enabled lipid production to be maximized and showed that this statistical tool is useful for the optimization of biotechnological processes, such as microbial lipid production.

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