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ORIGINAL PAPER

Oil wastes management: medium optimization for the production of alpha-linolenic acid in *Mucor circinelloides*

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Abstract The neutraceutical and pharmaceutical application of essential fatty acids is much cleared. Alpha-linolenic acid (ALA) is omega-3 fatty acid and generally known to have beneficial effects in CVS, CNS and other diseases. The purpose of the present investigation is to produce essential fatty acid, especially ALA by *Mucor circinelloides* from oil wastes. Five oil wastes collected from food industries were used as carbon sources, and the contents of total lipids, biomass and fatty acids were examined during 168 h. The ability of oil waste degradation was determined by measuring of biochemical oxygen demand (BOD) and chemical oxygen demand (COD). Interestingly, 76 % reduction in BOD and 68 % reduction in COD by this strain were achieved, and *M. circinelloides* could be a good candidate for oil waste treatment. In order to enhance ALA production, fermentation variables were chosen in accordance with the fractional design and further optimized by the response surface method. The statistical model was constructed via central composite design. Following the optimization step, ALA production increased by approximately 44.3 %, when compared to the screening step. The results indicate that carrying out the fermentation under the conditions of oil waste 4.37 %, yeast extract at 0.65 g/l, $(NH_4)_2SO_4$ at 0.38 g/l, an agitation rate of 180 rpm and fermentation time of 3 days will increase the ALA production up to 108.57 mg/l. In this study, a new renewable source of ALA was employed and optimized successfully for the production of valuable fatty acids.

Keywords Alpha-linolenic acid (ALA) · Biochemical oxygen demand (BOD) · Chemical oxygen demand (COD) · Essential fatty acids · *Mucor circinelloides*

Introduction

Essential fatty acids (omega-3/6) have crucial roles in the structure and biological functions required for conserving the homeostasis in all active organisms, which in turn convenes fluidity and modulates the behavior of certain membrane-bound proteins (Gill and Valivety 1997; Vigh et al. 2005). Human health and development has been related to dietary intake of essential fatty acids (Simpoulos 2002). These are precursors for various metabolites such as prostaglandins and leukotrienes and regulating critical biological functions (Gill and Valivety 1997; Dyaneshwar et al. 2006; Yongmanitchai and Ward 1989). The essential fatty acids are important for brain development, immune system function and blood pressure regulation (Avijit et al. 1999; Nakahara et al. 1996; Ratledge 1992; Certik and Shimizu 1999).

Some nuts, seeds and vegetable oils contain alpha-linolenic acid and may be converted to docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) in the body (Chapman et al. 1983; Burdge and Calder 2005). Microorganisms are good candidates for essential fatty acid production. Among these, special attention has been paid to the use of various oleaginous zygomycetes fungi like species of *Mortierella*, *Mucor* and *Cunninghamella* and marine fungi like *Trichoderma pseudokoningii*, *Crvalaria lunata* and *Aspergillus niveus* which are capable of

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producing lipids containing linolenic acid (Certik and Shimizu 1999; Devi et al. 2006). Microorganisms can convert not only a broad range of common substrates such as glucose and cellulose, but also agro-residues and some industrial and organic wastes into lipid (Amaral et al. 2012; Chatzifragkou et al. 2010). The fungus *Mucor circinelloides* was found to be capable of synthesizing EPA when cultivated in thin stillage and centrifuged thin stillage from corn to ethanol production (Liang et al. 2012). The highest concentration of γ-linolenic acid was obtained with *M. circinelloides* in culture containing vegetable oil (Tauk-Tornisielo et al. 2009). The contents of total lipids, biomass and various essential fatty acids were examined during 168 h. The ability of oil waste degradation was determined by measuring biochemical oxygen demand (BOD) and chemical oxygen demand (COD) that is novel in this research. We aimed to design an optimal medium for an efficient alpha-linolenic acid (ALA) production using response surface methodology (RSM). RSM is a collection of statistical techniques suitable for experimental design, model construction, evaluating the effects of factors and screening optimum conditions of factors for fascinating responses. Central composite design (CCD) is the most extensively used response surface designs. Although ratability is a desirable property of a central composite design, a face-centered design can be used when there is a difficulty in remaining the start points beyond the experimental region defined by the upper and lower limits of each factor (Box et al. 1978; Haaland 1990). The objective of the present study was using oil wastes as carbon sources for oleaginous zygomycetes fungi *M. circinelloides* DSM1175 for bioconversion of cheap substrate to valuable products like essential fatty acids and pollution control. Various studies have been managed to optimize the production of fatty acids by fungi (Aminah et al. 2006; Mamatha et al. 2008). In the first step, the factorial design was used to investigate the effects of medium components; then, the concentration of these factors was optimized using RSM. This study was performed in a 15-month period between September 2013 and February 2015, on laboratory scale at University of Isfahan.

Materials and methods

Microorganisms and media

Mucor circinelloides DSM 1175 (Leibniz Institute DSMZ, Germany) was used for essential fatty acid production. This strain was maintained on potato dextrose agar (PDA) (Merck, Darmstadt, Germany). The inoculated production medium contained (per liter of distilled water) 7.0 g KH_2PO_4, 2.5 g Na_2HPO_4, 1.5 g $MgSO_4$, 0.06 g $MnSO_4$, 0.15 g $CaCl_2$, 0.15 g $FeCl_3$, 0.5 g yeast extract, 0.5 g $(NH4)_2SO_4$, pH 6.0 and 2 % (V/V) oil waste as a carbon source (Papanikolaou et al. 2007). One milliliter of spore suspension (around 1×10^7 spores) was inoculated in 250-ml Erlenmeyer flask containing 50 ml of basal fermentation medium. This was incubated in a rotary shaker incubator at 180 rpm and 28 °C for 72 h.

Cell dry mass

The mycelia were harvested from the medium by the filtration through Whatman No. 1 filter paper and thoroughly washed with distilled water. To remove unconsumed oil, an additional washing was carried out using ethanol and then dried at 105 °C to constant weight. The dry cell weight was measured gravimetrically (Papanikolaou et al. 2004).

BOD and COD determinations

BOD was determined using the modified iodometric method, and COD was determined by the standard closed reflux method according to the procedures described by Clesceri et al. (1998). The BOD and COD degradation efficiency was defined as the reducing amount of BOD and COD versus the amount of initial values.

Analytical methods

Extraction and modification of lipids

Lipid extraction was performed according to the modified procedure of Bligh and Dyer. To assay the lipid and fatty acid content, cells were disrupted by acid hydrolysis and lipid extraction was performed using chloroform/methanol mixture (1:1). The lipid-containing fractions were separated and modified to obtain fatty acid methyl esters (FAMEs) (Pan et al. 2009).

The extracted fatty acids were modified to fatty acid methyl esters (FAMEs) according to the method of Christie (1993). The FAMEs were subjected to analysis using gas chromatography (GC), and the chromatograms were studied based on the standard peaks obtained from the standard FAMEs, which included C18:2 (linolenic acid), C18:3 (GLA), C18:3 (alpha-linolenic acid), C20:4 (ARA), C20:5 (EPA), C22:6 (DHA).

FAMEs analysis by gas chromatography (GC)

GC was performed on Agilent 19091J-413 series gas chromatograph equipped with a FID and the capillary column HP5 (30 m, 0.25 mm i.d., 0.25-μm film thickness; USA). Injector and detector temperatures were maintained at 260 and 300 °C, respectively. The oven was

programmed for 2 min at 100 °C, then increased to 160 °C at 3 min, maintained for 2 min at 215 °C, increased further to 217 °C at 2 min, then maintained for 2 min at 218 °C and finally increased to 260 °C at 2 min. The carrier gas, nitrogen, was used at a flow rate of 1.5 ml/min. The injection volume was 1 µl, with a split ratio of 100:1.

Experimental design and statistical analysis

The samples of different oil wastes from various industries including restaurants and fried food factories were collected in Isfahan, Iran. The samples were abbreviated as F1 and F2 for oil wastes from fried food factory of Shilan Kish, F3 for the sample from Naz plant oil factory, and R1 and R2 from two different fast food restaurants. In order to select the significant variables for ALA production, the independent variables such as oil waste, mineral nitrogen sources (ammonium sulfate), organic nitrogen sources (yeast extract), pH and inoculation were considered and screened via 1/2 fractional factorial design. A total of five variables were included for screening, and each independent variable was tested at two levels: high level (+1) and low level (−1). For a 1/2 fractional factorial with the five variables, 48 experimental runs with three replications of the center points are required. After fractional design, the fundamental factors influencing the essential fatty acid and lipid production were selected and subjected to RSM to obtain maximum concentration and level of individual factors.

Three independent variables, carbon sources (oil wastes) (*A*), yeast extract (*B*) and ammonium sulfate concentrations (*C*), and the dependent response variable ALA, total lipid, biomass, GLA and linoleate were studied. The experimental design of CCD is listed in Table 1. By using this design, the experimental data were fitted according to the equation. The response data obtained after central composite design were analyzed by Minitab version 16 and Design Expert version 7 software which generated 3D contour plots and standard analysis of variance (ANOVA) indicating the optimum concentrations and interaction among these factors.

Results and discussion

In the present study, the impact of *M. circinelloides* on the production of essential oil, especially ALA, and alterations in BOD and COD was studied. Five oil wastes were used as carbon sources, and biochemical aspects of media such as the total amount of lipids, biomass and essential fatty acids were examined by optimization of media by fractional design and response surface methods.

Table 1 Design of experiments: central composite design of variable

Run number	Oil waste (%)	Yeast extract (g/l)	$(NH_4)_2SO_4$ (g/l)
1	4.00	0.55	0.55
2	4.00	1.31	0.55
3	9.05	0.55	0.55
4	4.00	0.55	0.55
5	7.00	1.00	1.00
6	4.00	0.55	0.55
7	4.00	0.55	0.55
8	1.00	0.10	0.10
9	4.00	0.55	1.31
10	4.00	0.55	−0.21
11	7.00	0.10	0.10
12	1.00	1.00	0.10
13	1.00	1.00	1.00
14	1.00	0.10	1.00
15	4.00	0.55	0.55
16	4.00	−0.21	0.55
17	7.00	1.00	0.10
18	−1.05	0.55	0.55
19	7.00	0.10	1.00

Lipid and fatty acid determination

To assay the lipid and fatty acid content, cells were disrupted and lipid extraction was performed subsequently. The FAMEs were subjected to analysis using gas chromatography (GC). Table 2 shows a comparison between the produced lipids, biomass, yield of production (rate of lipid to biomass) and essential fatty acids by the strain in medium containing different oil wastes. The results show that R1 is the main substrate for lipid production and its final product is about 60 %. Furthermore, ALA is the important fatty acid that produces by the strain. The onset of lipid turnover occurred rapidly after transition from nitrogen-limitation to carbon-starvation conditions (Fakas et al. 2008; Kendrick and Ratledge 1992). Zygomycete fungi like *Mortierella* spp., Rhizopus spp. and Cunninghamella sp. are good strains for essential fatty acids production, but ALA was found in low concentrations in the produced oils (Certik and Shimizu 1999; Chatzifragkou et al. 2010; Jangbua et al. 2009). However, in *M. circinelloides* DSM 1175, when large amounts of oil were accumulated in the mycelium, ALA was present in large concentrations (Table 2).

BOD and COD assay

BOD and COD removal by the strain in oil wastes was significant when compared with control. Comparison of

Table 2 Comparison of lipid(g/g), biomass(g/l), yields (%lipid/biomass w/w), essential fatty acid production (mg/g) by two *Mucor circinelloides* in media containing different oil wastes as carbon sources (F1, 2, 3 oil wastes from factories and R1, 2 oil wastes from restaurant)

Oil waste in media	Biomass (g/l)	Total lipid (g/g)	Yield (w/w %)	Essential fatty acid concentrations in total lipid (mg/g)			
				C18:2 linoleate (n-6)	C18:3 gamma linoleate (GLA) (n-6)	C 18:3 linoleate (n-3)	C22:6 docosahexaenoate (DHA) (n-3)
F1	10.3	4.2	40.7	84.22	10.85	78.6	6.78
F2	13.78	5.1	37	15.15	7.35	64.5	–
F3	12.49	4.3	34.42	64.8	3.131	34.3	–
R1	10.63	6.4	60.21	75.85	13.4	91.4	3.23
R2	11.73	4.92	35	50.12	12.48	87.5	7.21

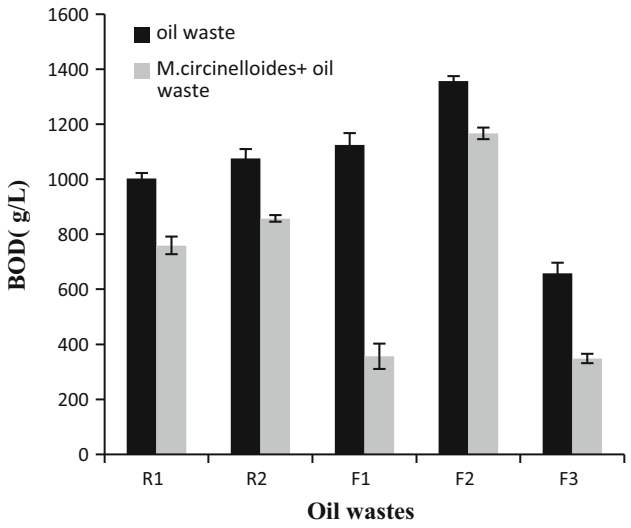

Fig. 1 Biochemical oxygen demand (BOD_5) values (g/l) in different oil wastes and oil wastes treatment by oleaginous fungus *M. circinelloides* DSM 1175 (F1, 2, 3 oil wastes from factories and R1.2 oil wastes from restaurant)

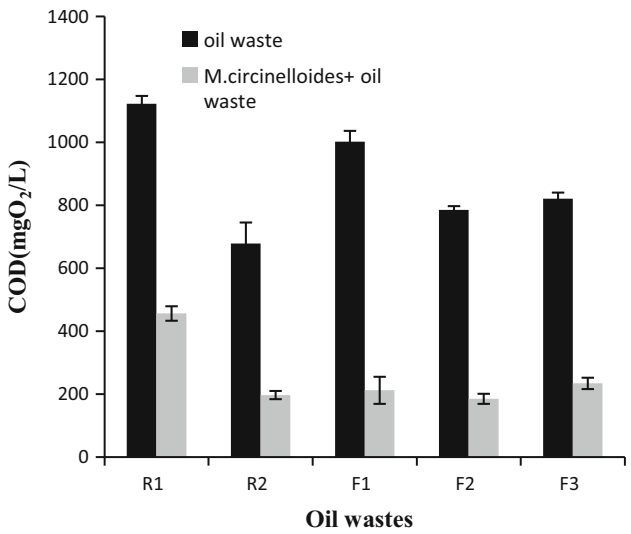

Fig. 2 Chemical oxygen demand (COD) values (mgO_2/l) in different oil wastes and oil wastes treatment by oleaginous fungus *M. circinelloides* DSM 1175 (F1, 2, 3 oil wastes from factories and R1.2 oil wastes from restaurant)

BOD_5 and COD in oil wastes and oil waste treated by *M. circinelloides* is shown in Figs. 1, 2. Maximum reduction percent of BOD and COD was about 68 and 76 % in different oil waste, respectively. Tomato waste hydrolyses was used by these fungi for fatty acid production (Fakas et al. 2008). Food waste, olive oil mill wastewaters, industrial fats and whey and starch hydrolyses were other wastes for microbial lipid production by oleaginous fungi (Amaral et al. 2012; Fakas et al. 2008; Zhu et al. 2003). Strong (2009) used four fungi to reduce COD in distillery wastewater. This study determined that using fungi could reduce oil waste pollutions and production of valuable products.

Optimization by using the factorial design

The results of the 1/2 fractional factorial design indicate that three factors including yeast extract, $(NH_4)_2SO_4$ and carbon sources have a significant effect on ALA production by *M. circinelloides*. Figure 3 indicates that three factors including pH and the inoculation have not significant effects on ALA production by the strain. The analysis of variance of the variables on the response in the screening step confirmed these results (not showed). The *p* values less than 0.05 were considered to show a significant effect on the response.

Optimization of the significant variables using RSM

Results showed that carbon source, yeast extract and ammonium sulfate were the major nutrients for the enhancement of ALA. The data obtained were subjected to regression analysis using the second-order regression equation. The coefficients of the regression equation were calculated using the Design Expert software provided in the following regression equation.

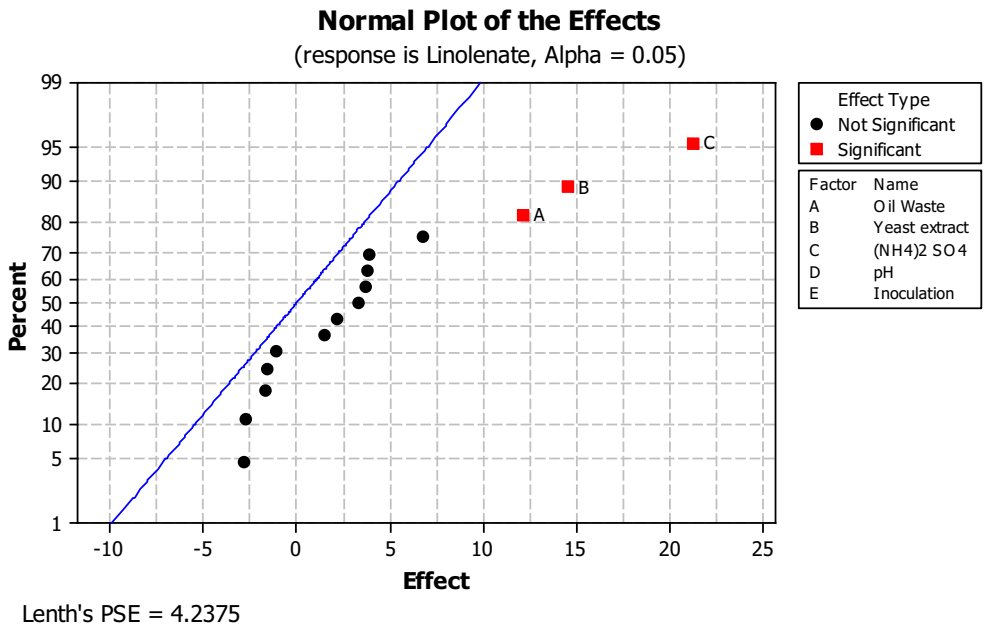

Fig. 3 Normal plot of effective factors in alpha-linolenic acid production by *M. circinelloides* DSM 1175

$$Y = 98.42 + 18.61A + 16.22B - 15.74C + 16.39AB + 14.7AC - 1.28BC - A^2 - 19.66B^2 - 6.99C^2$$

where Y is ALA concentration, A oil waste concentration, B yeast extract concentration and C $(NH_4)_2SO_4$. The results of the second-order regression model fitting in the form of ANOVA are given in Table 3. A good fit of the regression model was checked by the coefficient of determination (R^2), correlation coefficient (R) values and total regression F test. This is an estimate of the fraction of the overall variation in the data accounted by the model, and thus, the model is capable of explaining 89.1 % of the variation in response. It ensures a satisfactory adjustment of the quadratic model to the experimental data. The 'adjusted R^2' was found to be 0.7821, indicating that the model is good. Also, the 'model F value' of 49.6 implies that the model is significant. The ANOVA results (Table 3) indicated that the independent variable oil waste is influencing the ALA production very significantly ($p < 0.05$).

The contour plots and 3D response surface curves were created by plotting the response against each of the two independent variables for determination of optimal levels of each variable for maximum ALA production, while maintaining the other variables at their fixed (zero) levels. The main interaction effects of these two factors were understood by this manner. These plots were easily

Table 3 ANOVA for RSM analysis for response linoleate (ALA) surface quadratic model obtained from experimental designs

Source	Sum of squares	*df*	Mean square	*F* value	*p* value prob. > *F*	Significance
Model	23,840.83	9	2648.98	8.19	0.0022	Significant
A—oil waste	4728.71	1	4728.71	14.62	0.0041	Significant
B—yeast extract	3593.94	1	3593.94	11.11	0.0088	Significant
C—$(NH_4)2SO_4$	3384.75	1	3384.75	10.46	0.0102	Significant
AB	2149.91	1	2149.91	6.65	0.0298	Significant
AC	1727.72	1	1727.72	5.34	0.0462	Significant
BC	13.12	1	13.12	0.041	0.8449	Not significant
A^2	4082.82	1	4082.82	12.62	0.0062	Significant
B^2	5274.05	1	5274.05	16.30	0.0029	Significant
C^2	667.68	1	667.68	2.06	0.1847	Not significant
Residual	2911.79	9	323.53			
Pure error	46.22	4	11.55			
Cor total	26,752.62	18				

obtained by calculating from the model values taken by one factor where the second factor varies with restriction of a given Y value.

The yield values for different concentrations of the variables are also predicted from the respective response surface plots (Figs. 4, 5, 6). The maximum predicted yield was indicated by the surface confined in the response surface diagram.

The response surface plot was obtained as a function of oil waste concentration versus yeast extract concentration, while the third variable ammonium sulfate was maintained at zero level (coded; Fig. 4). An increase in ALA yield with an increase in concentration of oil waste versus yeast extract was observed. Among the two independent variables, oil waste plays a major role in the production of ALA. Its level was very critical as evidenced from the 3D surface graph. The optimum value was near the center point of oil waste level, and the interaction of oil waste and ammonium sulfate did not result in any further beneficial effect on the system. The response surface plot was obtained as a function of concentration of oil waste versus yeast extract when the third variable ammonium sulfate was kept at its mid-level (Fig. 5). An increased ALA with increased concentration of oil waste versus ammonium sulfate was also observed. As indicated in Fig. 1a, the same trend was also observed here. The role of oil waste is very critical and significant when compared with ammonium sulfate. The interaction effect of both independent variables resulted in a decrease in the production of ALA after the mid-level. The effect of concentration of yeast extract versus ammonium sulfate, when the third variable is oil waste, was maintained at zero level and not significant (Fig. 6). An increase in ALA yield with increased concentration of ammonium sulfate was also observed. From the response surface, ANOVA revealed that the increase in concentration of ammonium sulfate or yeast extract was

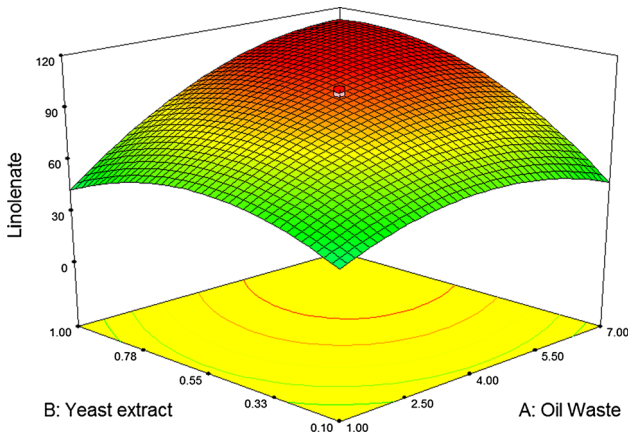

Fig. 4 Contour plot of ALA productivity (g/l) as a function of oil waste (g/l) and yeast extract (g/l) concentrations

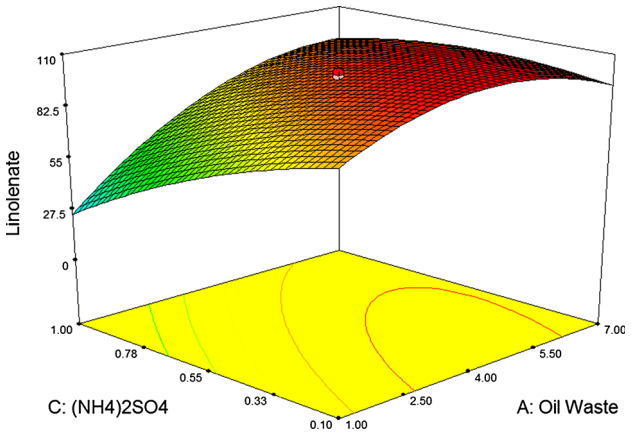

Fig. 5 Contour plot of ALA productivity (g/l) as a function of oil waste (g/l) and ammonium sulfate (g/l) concentrations

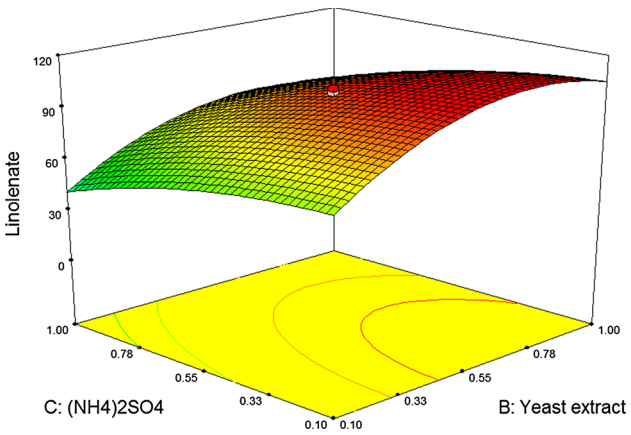

Fig. 6 Contour plot of ALA productivity (g/l) as a function of yeast extract (g/l) and ammonium sulfate (g/l) concentrations

only marginal for ALA increase in the total lipid of mycelium.

The results supported the predicted values and the effectiveness of the model. A 10.4 % increase in production of ALA was achieved when compared to that in the basal medium. The results of the conditions for maximizing the ALA production within the experimental region were predicted and given. The results were validated by doing the experiments with the predicted levels by the model. The results indicated that the predicted and observed values for ALA did not differ significantly ($p > 0.05$). This indicated that the optimized medium components concentration favored the production of ALA in the mycelium. Figure 7 represents the differences between actual and predicted responses that completed results in Table 3. These results cleared that the model is acceptable for biomass, GLA and linoleate like ALA which are explained in this paper. In this study, low production of ALA was obtained by low concentration of nutrients. In contrast, inhibition of secondary metabolite synthesis resulted in

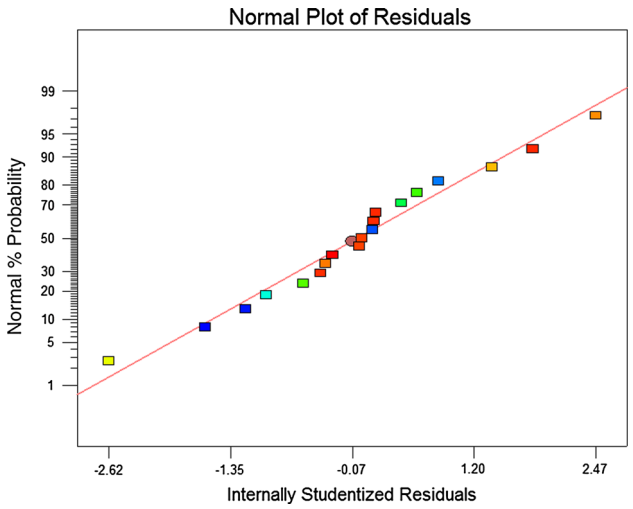

Fig. 7 Representation of predicted versus actual response of experimental runs under RSM design

higher concentration (ALA increase in the fatty acids). This was observed in other findings on carbon and nitrogen repression effects (Certik and Shimizu 1999; Ratledge 1992). To obtain optimum yield of secondary metabolite production in this work, a multifactorial statistical method was used for showing the conditions. CCD exploits the amount of information that can be obtained while seeing the interaction of independent variables and limiting the number of individual experiments required. The response surface methodology that is commonly satisfying the optimization of many microbial processes is a smaller and less time-consuming experimental design (Chang et al. 2006; Linder et al. 2005). Aminah et al. (2006), Mamatha et al. (2008), and Rocky-Salimi et al. (2011) applied RSM for optimization of fatty acid production by fungi. The ideal culture medium found in this work could be applied as a basis for further study with batch or fed-batch cultivation.

Conclusion

For determination of the conditions causing the maximum yield of ALA, the RSM was found to be functional. The use of an experimental design allowed the rapid screening of a large experimental domain in search of the best condition and levels of the ALA production. To the best of our knowledge, there were no reports available on the ALA production by media engineering. This research focused chiefly on an attempt to reveal the application of statistical design and RSM to maximize ALA production. Using cheap substrate like oil wastes and reduction in BOD and COD were the other findings of this work.

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