# Optimization of lipid enriched biomass production from oleaginous fungus using response surface methodology

S P Jeevan Kumar & Rintu Banerjee\*

Microbial and Downstream Processing Laboratory Department of Agricultural and Food Engineering Indian Institute of Technology Kharagpur 721 302, India

Received 9 December 2012; revised 22 February 2013

Oleaginous microorganisms have emerged as potential sources of oils for biodiesel production. To replenish as an alternative to the vegetable oils, higher lipid accumulating strain coupled with process optimization is indispensable. In the present study, response surface methodology (RSM) based central composite design (CCD) was used for optimization of lipid content from oleaginous fungus *Aspergillus* sp. Maximum lipid yield of 73.07% (w/w) was achieved at 3% (v/v) inoculum volume, *p*H 5, glucose 1% (w/v), urea 0.5% (w/v) and incubation time of 5 (days). Biomass (2.08 g/L) having a lipid content of 73.07% (w/w) with major constituents of hexadecanoic acid methyl ester and 9-Octadecenoic acid methyl ester were obtained. The lipid composition signifies that from the oleaginous microbe are highly encouraging and desirable to be considered as diesel substitute.

Keywords: GC-MS, Hexadecanoic acid methyl ester, Lipid, 9-Octadecenoic acid methyl ester, Oleaginous fungus, RSM

In recent years, attention has been paid to the exploration of microbial oils, which might become one of the potential oil sources for biodiesel production in the future. Oleaginous microorganisms are able to accumulate lipids above 20% of their biomass, on dry weight basis<sup>1,2</sup>. Such microbial oils can be used for biodiesel production<sup>1,2</sup>. In comparison to other vegetable oils and animal fats, the production of microbial oil has many advantages: have short life cycle, easily grown in bioreactors, rapid growth rates, easier to scale up and unaffected by space, season or climatic conditions<sup>1,3</sup>. Moreover, oleaginous organisms accumulate lipids, mostly consisting of triacylglycerols (TAG) that form the storage fraction of the cell<sup>4</sup>. On the other hand, it has been reported that cultivation conditions such as C/N ratio, nitrogen temperature, pH, incubation period, sources. inoculums volume, concentration of trace elements and inorganic salt would have varied influence on oil accumulation<sup>5,6</sup>. Hence, optimization of media coupled with statistical design approach is inevitable to understand the effects of various factors and their interactions for oil accumulation. Optimizations of

Telephone +91-3222-283104(O)/+91-3222-283105(R)

Fax: +91-3222-282244

E-mail: rb@iitkgp.ac.in

media are normally carried out by varying one parameter at a time (one factor-at-a-time method) whilst keeping the others constant. The major disadvantage of this technique is that it does not include interactive effects among the variable and eventually, it does not depict the complete effects of the parameter on the process and also it is laborious and time consuming especially for large number of variables<sup>7</sup>. Response surface methodology (RSM) is a technique for studying the effect of several factors acting together and affecting the responses by varying them in a number of experiments<sup>8</sup>. There are several reports on RSM based optimization of medium composition for lipid less biomass production as well as biodiesel production<sup>9,10</sup>. In the present study, RSM has been used to optimize the lipid content from oleaginous fungal isolate, Aspergillus sp. The relationship between the selected factors, their interactions and influences on the measured responses have been established.

## **Materials and Methods**

*Microorganism and culture condition—Aspergillus* sp.<sup>11</sup> was isolated from the local soil samples of Indian Institute of Technology, Kharagpur, India. The cultures were maintained at 4 °C and were sub cultured at regular intervals.

<sup>\*</sup>Correspondent author

*Microbial lipid production*—For systematic studies of microbial lipid accumulation the inoculums of *Aspergillus* sp. was prepared in a nitrogen limiting medium by transferring 1 mL of spore suspension  $(5 \times 10^5 \text{ spores/mL})$  in 100 mL of medium and incubated at 30 °C for 48 h. The media composition was kept similar to that of Kimura *et al*<sup>12</sup>. Seed culture (10 %, v/v) was used as inoculums for batch fermentation in a 250 mL flask containing 100 mL of medium.

*Experimental design*—The basic media constituents were varied based on the different concentration of the macro and micro constituents. Cultivation was then carried out at 30 °C at 120-168 h. Cultures were harvested after completion of fermentation based on the experimental design. Thereafter, the biomass concentration and lipid content were determined.

Optimization of lipid production was carried out by RSM based on three-level-five factor central composite design (CCD) leading to 32 experiments to fit a second order polynomial model<sup>7</sup>. The boundary parameters

selected for lipid production, were incubation time 5-7 (days), *p*H (3-5), inoculum volume 3-5 % (v/v), glucose concentration 0.5-1.5 % (w/v) and urea concentration 0.5-1.5 % (w/v), respectively. Three level central composite design matrix and the experimental responses of the dependent variable (lipid yield) are listed in Table 1. The data were analyzed by the Response Surface Regression (RSREG) method to fit the following second order polynomial equation:

$$Y = \beta_{k0} + \sum_{i=1}^{5} \beta_{ki} x_i + \sum_{i=1}^{5} \beta_{kii} x_i^2 + \sum_{i=1}^{4} \sum_{j=i+1}^{5} \beta k_{ij} x_i x_j \dots (1)$$

where, Y is response of lipid yield % (w/w);  $\beta_{k0}$ ,  $\beta_{ki}$ ,  $\beta_{kii}$  and  $\beta_{kij}$  are constant coefficients and  $x_i$ ,  $x_j$  are the coded independent variables, which influence the response variables Y.

#### Analytical methods

*Determination of cell dry weight*—After completion of fermentation to harvest the cell biomass the fermented broth was passed through a filter paper

	Table 1—Central composite design matrix with actual and predicted responses of lipid yield									
Run order		A2	A3	A4	A5	Lipid yield				
	A1					Experimental	Predicted			
1	5	3	3	1.0	0.5	71.014	70.319			
2	7	3	3	0.5	0.5	57.880	57.214			
3	5	5	3	1.0	0.5	73.070	73.228			
4	7	5	3	0.5	1.5	31.810	31.378			
5	5	3	5	0.5	0.5	54.480	54.415			
6	7	3	5	0.5	1.5	27.890	27.236			
7	5	5	5	1.5	1.5	27.620	27.790			
8	7	5	5	0.5	0.5	33.800	33.999			
9	5	3	3	1.5	1.5	64.800	64.856			
10	7	3	3	1.5	1.5	25.590	25.057			
11	5	5	3	1.5	1.0	42.170	42.461			
12	7	5	3	1.5	0.5	70.070	70.390			
13	5	3	5	1.5	1.0	66.030	66.099			
14	7	3	5	1.5	0.5	53.480	53.578			
15	3	5	5	1.5	1.5	11.000	11.922			
16	7	4	5	1.5	1.5	30.230	30.563			
17	5	4	4	1.0	1.0	56.110	55.204			
18	7	4	4	1.0	1.0	43.660	44.995			
19	6	3	3	1.0	0.5	72.570	74.960			
20	6	5	4	1.0	1.0	64.790	62.829			
21	6	4	3	1.0	1.0	52.100	53.601			
22	6	4	5	1.0	1.0	38.510	37.438			
23	6	4	4	0.5	1.0	34.270	36.255			
24	6	4	4	0.5	1.0	36.480	34.924			
25	6	4	4	1.0	0.5	55.620	54.598			
26	6	4	4	1.0	1.5	40.810	42.261			
27	6	4	4	1.0	1.0	49.230	50.563			
28	6	4	4	1.0	1.0	52.070	50.563			
29	6	4	4	1.0	1.0	50.280	50.563			
30	6	4	4	1	1	51.810	50.563			
31	6	4	4	1	1	52.490	50.563			
32	6	4	4	1	1	49.210	50.563			

(Whatman No.1) and the harvested residual biomass was washed several times to remove unwanted media constituents present on the cell surface. The biomass was kept for drying in a hot air oven at 60 °C till a constant weight was achieved. The cell dry weight was determined gravimetrically according to Devi *et al*<sup>13</sup>.

*Extraction of lipid*—Lipid was extracted from the dried biomass by using chloroform and methanol in a ratio of 2:1 (v/v)<sup>14</sup>. The lipid extract was washed with 150 mL of NaCl (1%) followed by 150 mL of distilled water. The chloroform layer was filtered through Whatman No. 1 filter paper into a previously weighed clean vial (W<sub>1</sub>). Evaporation was carried out using rotary evaporator (BUCHI Rota vapor R-124) and the residue was further dried at 104 °C for 30 min. The weight of the vial was again recorded (W<sub>2</sub>). Lipid content was calculated by subtracting W<sub>1</sub> from W<sub>2</sub>, and was expressed as % dry cell weight<sup>15</sup>.

#### **Identification of lipids**

Sample preparation—The acid-catalyzed transesterification of fungal lipid was carried out using 90:1:3.8 molar ratio of methanol, oil and sulfuric acid as a catalyst<sup>16</sup>. Transesterified lipids were analyzed by gas chromatogram-mass spectrometry (GC/MS) where the identification was carried out based on the mass spectrometry (MS) studies. The interpretation of the obtained results were carried out by using the NIST library based on the relative retention times of the compounds in the profile.

*GC-MS conditions*—The GC-MS analysis for fatty acid methyl esters (FAMEs) was performed using Agilent 6890 N gas chromatography instrument coupled with an Agilent MS-5975 inert XL mass selective detector with an Agilent auto sampler 7683-B injector (Agilent Technologies, Little Fall, NY, USA). A capillary column HP-5MS (5% phenyl methylsiloxane) with dimension of 30 m × 0.25 mm × 0.25 mm film thickness (Agilent Technologies, Palo Alto, CA, USA) was used for the separation of fatty acid methyl esters. The initial temperature of 150 °C was maintained for 2 min raised to 230 °C at the rate of 4 °C/min, and kept at 230 °C for 5 min. The split ratio was 1:50, and helium was used as a carrier gas with the flow rate of 0.8 mL/min. The injector and detector temperatures were 240 and 260 °C, respectively. The mass spectrometer was operated in the electron impact (EI) mode at 70 eV in the scan range of 50–550 m/z.

#### **Results and Discussion**

Using the designed experimental data (Table 1), the second-order polynomial model for the lipid enrichment in terms of coded factors is shown as follows:

 $\begin{array}{l} Y = 454.226 - 34.3A_1 - 163.85A_2 + 19.25A_3 + 41.12A_4 + \\ 36.61 \ A_5 - 0.46A_1{}^2 + 18.33A_2{}^2 - 5.04A_3{}^2 - 59.89A_4{}^2 - \\ 8.53A_5{}^2 + 6.47A_1A_2 + 3.25A_1A_3 + 8.77A_1A_4 - 12.90A_1A_5 - \\ 6.07A_2A_3 - 1.43A_2A_4 - 2A_2A_5 + 6.01A_3A_4 + 11.78A_3A_5 \\ + 6.39A_4A_5 & \dots(2) \end{array}$ 

where Y is the percent of lipid enrichment and  $A_1$ ,  $A_2$ ,  $A_3$ ,  $A_4$ , and  $A_5$  represent incubation time, *p*H, inoculum volume, glucose and urea concentration, respectively.

Based on the experimental response for lipid enrichment, runs 15 and 3 had the minimum and maximum lipid, respectively. The ANOVA results of second-order response surface models for lipid enriched biomass have been given in Table 2. From ANOVA analysis of regression model, at 20 degree of freedom, F-value was 104.09 and P value was <0.001. The F and P values indicated that the quadratic regression model for lipid enrichment was significant. The goodness of fit of the model was checked by the determination coefficient  $(R^2)^{17}$ . The R-squared value provided a measure of the variability in the actual response values that could be explained by the experimental factors and their interactions. A value of one represents the ideal case at which 100% of the variation in the observed value can be explained by

Table 2—ANOVA analysis of the response surface quadratic model									
Source	$\mathrm{DF}^{\mathrm{a}}$	Seq SS <sup>b</sup>	Adj SS <sup>b</sup>	Adj MSc	F	Р			
Regression	20	7453.99	7453.99	372.699	104.09	< 0.001			
Linear	5	2999.67	1011.72	202.343	56.51	< 0.001			
Square	5	1288.57	1288.57	257.714	71.97	< 0.001			
Interaction	10	3165.75	3165.75	316.575	88.41	< 0.001			
Residual Error	11	39.39	39.39	3.581	-	-			
Lack-of-Fit	6	28.65	28.65	4.775	2.22	0.199			
Pure Error	5	10.74	10.74	2.148	-	-			
Total	31	7493.38	-	-	-	-			
$R^2$	99.47%,	98.52%	-	-	-	-			

the model<sup>7,17</sup>. In this case, the mathematical model was found to be reliable with  $R^2$  value of 99.47%, whereas, the adjusted  $R^2$  value of 98.52% indicating that only 1.48% of the total variations were not explained by the model.

The 3D response surface plots are generally the graphical representation of the regression equation. Fig. 1a and b, represent the 3D response surface plot for the optimum conditions of lipid accumulation. Each figure represents effect of two variables on lipid accumulation. From the analysis of the response surface plots, the optimum conditions for lipid accumulation were glucose concentration 1 % (w/v), urea concentration 0.5% (w/v), incubation time 5 (days), pH 5 and inoculums volume 3% (v/v), respectively. Under the optimum conditions, the maximum lipid accumulation was 73.07%, this was close to the predicted response 73.22% respectively. Among the variables of interaction factors, pH and inoculums volume and pH and glucose concentration contributed significantly for lipid content than other factors (Fig. 1a and b).

Papanikolaou *et al.*<sup>18</sup> reported gamma linolenic acid (GLA) production of 7.7 mg/g of dry biomass,



Fig 1—RSM plots depicting (a) effect of pH and inoculum volume, (b) effect of pH and glucose concentration.

with maximum gamma linolenic acid concentration of 801 mg/L of culture medium after 5 days of incubation. This quantity of GLA was remarkable and higher than that obtained from *R. arrizus*  $(0.400 \text{ g/L})^{19}$ . Similarly, *Aspergillus* sp. under investigation achieved 73.07% (w/w) after 5 days of incubation which is higher than the reported lipid content of 57% (w/w) of *Aspergillus*<sup>2</sup>. The possible reason might be that, after inoculation, up to 40–50 h the fungus consumed all of the available nitrogen and then, reserve lipid was synthesized in distinct oil droplets<sup>20</sup>.

Various reports extensively revealed that cultivation conditions such as C/N ratio, nitrogen sources, pH, and inoculums volume would have varied influence on oil accumulation. For instance, Li et al.21 reported 67.5% of lipid accumulation in Rhodosporidium toruloides using glucose by fed batch cultivation at C/N ratio of 10. Similarly, Angerbauer et al.<sup>22</sup> reported that 75% of lipid production from Lipomyces starkeyi using sewage sludge and glucose. Different nitrogen sources also had varied influence on oil production. Huang et al.<sup>23</sup> reported that inorganic nitrogen sources were good for cell growth but not suitable for oil production, while organic nitrogen sources such as peptone was good for oil production but not suitable for cell growth. Therefore, it was well established that the nutritional requirements of microbes varies from one another. Moreover, inoculums size/density has been attributed as one of the major factors for biomass growth and lipid production. Inoculums size/density influences mycelial morphology causes diffusion limitation of nutrients within mycelial flocs<sup>20</sup>. Growth of mycelium in smaller and compact pellet form encourages biomass growth and lipid production via enhanced mass transfer of oxygen and other nutrients<sup>20,24</sup>.

The optimized lipid was transmethylated and the corresponding fatty acid methyl ester samples were analyzed by GC-MS. Palmitate (C16:0) and oleate (C18:1) were the two predominant components in the sample, which were similar to lipid samples from most oleaginous yeasts<sup>5</sup>. Cultivation of oleaginous microorganism has been gained a special attraction because of their ability to accumulate high amounts of intracellular lipid, their relatively high growth rates and the resemblance of their triacylglycerols fraction to plant oils. In particular, for biodiesel production, palmitic acid (C18:1) are potential targets of interest due to the

properties of higher value oils, improve oxidative stability, and have more potential adaptability in the industrial production of biodiesel<sup>2,20</sup>. Thus, response surface methodology coupled with statistical approach has resulted 2.08 g/L of biomass, 73.07 % (w/w) of lipid content was significant in comparison with the previous report of lipid content 57% (w/w) of *Aspergillus* sp.<sup>2</sup>. Furthermore, the composition of lipids of an oleaginous microorganism was similar with the desirable lipids of biodiesel production.

### Conclusion

Optimization of lipid enriched biomass production was carried out by using *Aspergillus* sp. The conditions for lipid enriched biomass were optimized by using RSM-based CCD. Using RSM based CCD maximum lipid content of 73.07% was achieved after 5 days of incubation time at glucose concentration of 1% (w/v), urea concentration of 0.5% (w/v), pH 5 and inoculums volume of 3% (v/v). GC-MS study revealed the fatty acids palmitate and oleate were predominant in the lipid sample. Therefore, it can be concluded that production and optimization of lipid enriched biomass from oleaginous microorganism (*Aspergillus* sp.), could be useful for biodiesel production.

#### References

- Li Q, Du W & Liu D, Perspectives of microbial oils for biodiesel production, *Appl Microbiol Biot Applied*, 80 (2008) 749.
- 2 Meng X, Yang J, Xu X, Zhang L, Nie Q & Xian M, Biodiesel production from oleaginous microorganisms, *Renew Energ*, 34 (2009) 1.
- 3 Kendrick A & Ratledge C, Desaturation of polyunsaturated fatty acids in *Mucor circinelloides* and the involvement of the novel membrane-bound malic enzyme, *Eur J Biochem*, 209 (1992) 667.
- 4 Rossi M, Amaretti A, Raimondi S & Leonardi A, Getting lipids for biodiesel production from oleaginous fungi, *Biodiesel – Feedstocks and Processing Technologies*, (InTech, Italy) 2011, 71.
- 5 Johnson V W, Singh M, Saini V S, Adhikari D K, Sista V & Yadav N K, Utilization of molasses for the production of fat by an oleaginous yeast, *Rhodotorula glutinis* IIP-30, *J Ind Microbiol*, 14(1) (1995) 1.
- 6 Johnson V, Singh M, Saini V S, Sista V R & Yadav N K, Effect of pH on lipid accumulation by an oleaginous yeast: *Rhodotorula glutinis* IIP-30, *World J Microbiol Biotechnol*, 8 (1992) 382.
- 7 Khuri A I & Cornell J A, Response surfaces: Design and analysis, in *Statistics: Textbooks and monographs*, edited by Owen D B, Cornell R G, Kshirsagar A M, Kennedy W J and Shilling, E G (Marcel Dekker, New York) 1987, 3.

- 8 Maddon I S & Richard S H, Use of response surface methodology for rapid optimization of microbiological media, *J Appl Bacteriol*, 43 (1977) 197.
- 9 Aminah S S, Aidil A H & Mohtar W W Y, Medium optimization for the production of lipidless biomass by *Cunninghamella* sp. 2A1 using response surface methodology, *Mal J Microbiol*, 2(1) (2006) 40.
- 10 Boulifi N El, Bouaid A, Martinez M & Aracil J. Process optimization for biodiesel Production from corn oil and its oxidative stability, *Int J Chem Engineer*, 2010 (2010) 1.
- 11 Elbashiti T, Fayyad A & Elkichaoui A, Isolation and Identification of *Aspergillus oryzae* and the production of soy sauce with new aroma. *Pakistan J Nutrition*, 9 (12), (2010) 1171.
- 12 Kimura K, Yamaoka M & Kamisaka Y, Rapid estimation of lipids in oleaginous fungi and yeasts using Nile red fluorescence, *J Microbiol Methods*, 56 (2004) 331.
- 13 Devi P, D'souza L, Kamat T, Rodrigues C & Naik C G, Batch culture fermentation of *Penicillium chrysogenum* and a report on the isolation, purification, identification and antibiotic activity of citrinin, *Indian J Mar Sci*, 38(1), (2009), 38.
- 14 Folch J, Lees M & Stanley G H S, A simple method for the isolation and purification of total lipids from animal tissues, *J Biol Chem*, 226 (1957) 497.
- 15 Christophe G, Kumar V, Nouaille R, Gaudet G, Fontanille P, Pandey A, Ricardo C S & Larroche C, Recent developments in microbial oils production: A possible alternative to vegetable oils for biodiesel without competition with human food?, *Braz. Arch. Biol. Technol*, 55, (2012) 1.
- 16 Zheng S, Kates M, Dube M A & Mc Lean D D, Acidcatalyzed production of biodiesel from waste frying oil, *Biomass Bioenergy*, 30 (2006) 267.
- 17 Haalland P D, *Experimental design in biotechnology*, (Marcel Dekker, Inc. New York) 1989, 250.
- 18 Papanikolaou S, Komaitis M & Aggelis G, Single cell oil (SCO) production by *Mortierella isabellina* grown on highsugar content media, *Bioresource Technol*, 95 (2004) 287.
- 19 Kristofikova L, Rosenberg M, Vlnova A, Sajbidor J & Certik M, Selection of *Rhizopus* strains for L(+)-lactic acid and gamma linolenic acid production, *Folia Microbiol*, 36, (1991) 451.
- 20 Wang L, Ridgway D, Gu T & Moo-Young M, Bioprocessing strategies to improve heterologous protein production in filamentous fungal fermentations, *Biotechnol Advances*, 23 (2005) 115.
- 21 Li Y, Zhao Z K & Bai F, High density cultivation of oleaginous yeast *Rhodosporidium toruloides* Y4 in fedbatch culture, *Enzyme Microbial Technol*, 41 (2007) 312.
- 22 Angerbauer C, Siebenhofer M, Mittelbach M & Guebitz G M, Conversion of sewage sludge into lipids by *Lipomyces* starkeyi for biodiesel production, *Bioresour Technol*, 99 (2004) 3051.
- 23 Huang J Z, Shi Q Q, Zhou X L, Lin Y X, Xie B F & Wu S G, Studies on the breeding of *Mortierella isabellina* mutant high producing lipid and its fermentation conditions, *Microbiol*, 25(4) (1998) 187.
- 24 Hung-Chang, Chen , & Tse-Ming Liu, Inoculum effects on the production of γ-linolenic acid by the shake culture of *Cunninghamella echinulata* CCRC 31840, *Enzyme Microb Tech*, 21 (1997) 137.