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Application of Response Surface Method for Studying the Role of Dissolved Oxygen and Agitation Speed on Gamma-Linolenic Acid Production

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Abstract To study the effect of agitation speed (rpm) and dissolved oxygen concentration (DO) on the production of gamma linolenic acid by *Mucor* sp. RRL001, a central composite design experiment was performed in a 5-L stirred tank bioreactor. The design consisted of a total of 10 runs consisting of runs at five levels for each factor and was divided in two blocks. The ANOVA analysis and Pareto chart of effects suggested agitation speed (p=0.0142) linear effect and DO concentration (p=0.0342) quadratic effects were significant factors with significant contribution to the response. The validation run based on the optimum production zone in response surface plot resulted in the maximum 350.3 mg l^{-1} GLA yield as compared with model predicted value of 340.7 mg l^{-1}. The study suggests that agitation rate is having more pronounced effect on GLA yield than dissolved oxygen concentration by ensuring enhanced mass transfer and by preventing wall growth at elevated agitation speed. Also, it shows that higher GLA yields can be obtained in a simple medium at moderate oxygen saturation and that the *Mucor* sp. RRL001 is resistant to high agitation linked shear stress and suitable for GLA production at higher scale.

Keywords GLA · Fermentation · Response surface methodology · Dissolved oxygen · Agitation speed

Introduction

Omega-3 and omega-6 are the two major series of polyunsaturated fatty acids (PUFAs). The gamma-linolenic acid (GLA) (6, 9, 12, *cis*, *cis*, *cis*-octadecatrienoic acid) comes under omega-6 series. GLA is a precursor of arachidonic acid (AA) and upon sequential oxidation it forms arachidonic acid, which is further used in prostaglandins biosynthesis. The

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prostaglandins play an important physiological role in humans. GLA is formed from linoleic acid (LA) and is the first precursor of AA; delta 6-desaturase enzyme is responsible for the conversion of LA to GLA. In diseased conditions related with old age, delta 6-desaturase is less active and hence affects GLA synthesis, which in turn affects synthesis of prostaglandins [1]. In such a state, exogenous supplementation of GLA has been suggested to fulfill the deficit. The GLA supplementation studies have revealed that dietary GLA retards the development of atherosclerosis [2]. It also exerts clinical efficacy in a variety of diseases, including suppression of chronic inflammation, vasodilation and lowering of blood pressure, and the inhibition of smooth muscle cell proliferation associated with atherosclerotic plaque development [3, 4]. Infants' food formulae containing GLA as precursor of AA could be provided for those not being breast-fed [5]. Hence, interest in this compound arose from its potential in therapeutics, food, and nutritional applications.

Dependence on plants as the sole source of GLA is not sufficient to meet the ever-increasing global demand for GLA, which accounts for the constant search for higher and better producers of this unsaturated fatty acid from alternative sources like microorganisms. Because of its high value, GLA has gained potential interest for its commercial production by fermentation.

The submerged fermentation of microorganisms requires proper distribution of oxygen and nutrients for aerobic cultivation conditions. During fermentation with fungi high shear stress associated with high agitation speed leads to damage of cells resulting in poor production. Also, level of dissolved oxygen and its proper distribution in the fermentation medium is an important aspect for aerobic fermentation. Given the high dissolved oxygen requirement with a need to avoid damage to cells, a proper balance of dissolved oxygen and agitation-linked shear stress is to be found. Hence, a response surface central composite design was employed to find the optimal process settings of agitation speed and dissolved oxygen concentration to achieve the best performance. The central composite designs (CCD) are constructed by adding additional star points and center point runs [6, 7] to two-level full or fractional factorial designs. It allows the determination of both linear and quadratic models and interaction effects of factors. The CCD is a better alternative to the full factorial design as it demands a smaller number of experiments while providing comparable details. Response surface method has been successfully used for fermentation process parameters optimization [8–10]. Our effort in the present study was to investigate the effect of agitation speed (rpm) and dissolved oxygen on total GLA yield using response surface method and to develop a robust model for GLA production using the *Mucor* sp. RRL001.

Materials and Methods

Microorganism and Preparation of Inoculum

The *Mucor* sp. RRL001 used in the present study was isolated from *Western Ghats* of Kerala, India [11] and maintained on the potato dextrose agar (PDA) slants by incubating at 30 °C for 4 days. The fully sporulated fungal mats obtained after 4 days of incubation were used for further experiments. Inoculum was prepared by adding 2 ml of sterile distilled water to each sporulated slant and the spores were dislodged into it by gentle scrapping. The spore suspension consisting of 2×10^7 spores/ml was used to inoculate 50 ml of medium consisting of (g l^{-1}): glucose 100, yeast extract 10, and peptone 1, in a 250-ml sterile Erlenmeyer flask. The pH was adjusted to 5.4±0.1 and incubated at 30 °C for 24 h at 200 rpm. The mycelia obtained after 24 h was used to inoculate the medium in fermenter at 10% (*v*/*v*) [11].

Bioreactor and Experimental Setup for GLA Production

The production medium consisting of glucose at 100 g l^{-1} and yeast extract at 10 g l^{-1} was adjusted to pH=6.5±0.1 using 0.5 M HCl. The experiments were carried out in a bench scale 5-L stirred tank bioreactor-STB (Biostat B-5; B. Braun Biotech-Sartorius) with 3 L of final working volume. The agitation in the fermenter was provided with fixed two-stage Ruston turbine impeller of 64 mm diameter (Di), having six flat blades with an angle between them of 60°. The height: diameter ratio of the bioreactor was 2:1. A stainless steel ring sparger located at the base of the impeller was used to sparge the sterile air/oxygen during the fermentation. The inoculum as above was inoculated into the production medium for GLA production. No pH control was exerted during the fermentation run. The fermentation runs were carried out at different agitation speeds of 100, 200, 400, 600, and 800 rpm. The agitation speed initially adjusted to the desired rpm was kept constant during the entire run. The specific flow rate of the air or oxygen was fixed at 0.5 vvm. The fermentation was run at a temperature of 30 °C. The temperature of the fermentation broth was monitored by temperature probe and controlled by circulating chilled water. The foaming in the fermentation broth was monitored by a ceramic-coated antifoam probe and controlled by adding 20% silicon oil.

The dissolved oxygen (DO) was maintained by supplying the purified oxygen automatically by operating the DO controller in cascade. Hence, under the low dissolved oxygen conditions, purified oxygen was automatically supplied to maintain the desired DO level. Five different minimum dissolved oxygen levels of 5%, 20%, 40%, 60%, and 75% were studied and DO was continuously monitored by a sterilizable polarographic electrode (Mettler-Toledo InPro6000 Series). The DO electrode was calibrated by a two-point calibration method between 0% and 100% oxygen saturation. The fermentation run was performed for 168 h and the total volume of the fermentation broth was measured after the termination of the batch. The samples were withdrawn periodically at an interval of 24 h and microscopically studied for the mycelia breakage, biomass estimation, and for possible contamination.

Cell Dry Mass Determination and Lipid Extraction

The fermentation batches were terminated at the end of 168 h and mycelia were harvested from the medium by the suction filtration through Whatman No. 1 filter paper. The harvested mycelial mass was thoroughly washed with the distilled water, and then freeze-dried at −50 °C in the freeze drier (Operon freeze dryer, Korea). The dried mycelia was collected in an Erlenmeyer flask and suspended in 0.5 N HCl, heated for 30 °C in a boiling water bath. The lipid was extracted from the lysed mycelial suspension by vigorously shaking the suspension in a mixture of chloroform/methanol (2:1) [12]. The lipid extracted in chloroform layer was collected and residual moisture of the chloroform layer was removed by adding anhydrous sodium sulfate and filtering with Whatman filter paper. The lipid was finally collected by distilling the chloroform.

Methyl Ester Preparation and Analysis of Fatty Acid Composition

A portion of the lipid extracted from the biomass was taken and fatty acid methyl ester (FAME) was prepared by refluxing the lipid with boron trifluoride and dried methanol [13]. The methyl esters were extracted twice by adding two volumes of hexane and washed with one volume of distilled water. After mixing (shaking) briefly in a separating funnel, the

hexane layer was separated. The residual moisture was removed from the FAME by adding anhydrous sodium sulfate and then filtered using Whatman No. 1 filter paper [11]. The FAME was concentrated by evaporating the hexane on a boiling water bath and the methyl esters were analyzed by the GC-MS.

Gas Chromatographic Conditions

The fatty acid methyl esters were studied by Agilent 6890 series gas chromatograph equipped with capillary column DB-23 (30 m, 0.25 mm i.d., 0.5-mm film thickness; J & W Scientific, USA) and FID detector. The injector and the detector temperatures were maintained at 230 °C and 250 °C, respectively. The oven was programmed for 2 min at 160 °C, and then increased to 180 °C at 6 °C min^{-1}, maintained for 2 min at 180 °C, increased further to 230 °C at 4 °C min^{-1} and finally maintained for 10 min at 230 °C. Nitrogen was used as a carrier gas at 1.5 ml min^{-1}. The injection volume of the sample was 1 ml, with a split ratio of 50:1. The fatty acid identification was done by using GC-MS performed with Agilent 6890N Gas Chromatograph connected to Agilent 5973 Mass Spectrometer at 70 eV (*m*/*z* 50–550; source at 230 °C and quadrupole at 150 °C) in the EI mode with an HP-5ms capillary column (30 m, 0.25 mm i.d., 0.25-mm film thickness; J & W Scientific, USA). The carrier gas used was helium and maintained at 1.0 ml min^{-1}. The inlet temperature was maintained at 300 °C and the oven was programmed for 2 min at 150 °C, then increased to 300 °C at 4 °C min^{-1}, and maintained for 20 min at 300 °C. The injection volume was 1 ml, with a split ratio of 50:1.

The structural assignments were based on interpretation of the mass spectrometric fragmentation and confirmed by comparison of the retention times as well as the fragmentation pattern of the Methyl gamma-linolenate (Sigma) and the spectral data obtained from the Wiley and NIST libraries [11].

Experimental Design

A central composite experimental design was used to determine the effects of major operating parameters on the GLA production. The central composite design is a response surface design used to evaluate the relationship of a set of controlled experimental factors and observed results. The design included three combinations, the axial (A), factorial (F), and center (C) points. The independent variables were specified at five different levels. The factorial points coded as −1 and +1, axial points as $-\alpha$ and $+\alpha$ along with center point runs were included in the design. The experimental design was developed using Statistica version 7 (StatSoft) and all the analyses were performed using the software. The design consisted of a total of 10 runs including two center point runs and was divided in two blocks. The center point runs coded as 0 levels were used as a test for the entire process. Two operating parameters named as the agitation speed (rpm) and DO concentrations were chosen as the independent variables. The agitation speed was varied between 100 and 800 rpm, whereas the DO concentration was varied from 5% to 75% of oxygen saturation (Table 1). The analysis of variance (ANOVA) was performed for identification of significant factors at $p=0.05$.

Results and Discussion

The experiments were analyzed by considering the GLA yield as the response or a dependent variable. The agitation speed and dissolved oxygen concentrations were

Table 1 Experimental design showing actual and predicted GLA yields.

Run number	Block	Coded values[a]	Coded values[b]	Real values[a]	Real values[b]	Average actual yield (mgl^{-1})[c]	Predicted yield (mgl^{-1})
1	1	0.0	0.0	400	40	286±1.16	311.16
2	2	0.0	1.414	400	75	68±1.82	52.53
3	1	1.0	−1.0	600	20	333±1.27	292.16
4	2	−1.414	0.0	100	40	53±1.12	31.25
5	2	0.0	−1.414	400	5	66±0.62	89.69
6	2	1.414	0.0	800	40	184±1.09	202.53
7	1	−1.0	1.0	200	60	104±0.82	132.27
8	1	−1.0	−1.0	200	20	174±0.41	168.00
9	2	0.0	0.0	400	40	232±1.23	227.014
10	1	1.0	1.0	600	60	292±0.63	285.43

[a] Agitation speed (rpm); [b] DO (dissolved oxygen), [c] Values are mean±SD of two estimations

considered as independent variable. The block was used as blocking variable and responses were analyzed accordingly using Statistica release version 7 (StatSoft, USA) to develop a model based on design, which included main effects of linear and quadratic order and interaction effects. The model proposed based on the analysis related GLA yield with variables of the experiment: agitation speed (RPM) and DO. As the design included two variables, hence the model equation predicted by the software was as given below:

$$Z = -186.122 + 1.09 \times A - 0.001 \times A^2 + 8.93 \times B - 0.127 \times B^2 + 0.002 \times A \times B - 42.07,$$

where Z is the predicted response, −186.122 is intercept, A is agitation speed, and B is DO concentration.

The ANOVA (Table 2) and Pareto chart of effects revealed that agitation speed linear effect (p=0.0142) and DO quadratic effect (p=0.0342) were significant with agitation speed linear effect being the maximum contributor to response. The block effects (p=0.0541), agitation speed quadratic effect (p=0.0565), DO linear effect (p=0.4612), and interaction effect of DO and agitation speed were not significant (p=0.7415). The analyses of effect estimates (Table 2) also suggested that agitation speed linear and DO quadratic effects were

Table 2 ANOVA showing the effect of factors, their interactions and effect estimates.

Factor	Effect	SE	SS	*df*	MS	*F*	*p*
Blocks	−84.143	27.30433	15260.23	1	15260.23	9.4967	0.0541
Agitation speed (L)	138.660	26.94176	42564.00	1	42564.00	26.4882	0.0142
Agitation speed (Q)	−81.574	26.96624	14704.72	1	14704.72	9.1510	0.0565
DO (L)	−21.235	25.19577	1141.36	1	1141.36	0.7103	0.4612
DO (Q)	−101.816	27.48129	22057.25	1	22057.25	13.7265	0.0342
1L × 2L	14.500	40.08622	210.25	1	210.25	0.1308	0.7415
Error			4820.71	3	1606.90		
Total SS			97383.60	9			

L and Q refer to linear and quadratic order of effect; Effect refers to nature of each factor effect on total response with + and – signs referring it to be positive or negative contributor, SE refers to standard error, SS refers to sum of squares, *df* refers to degrees of freedom, MS refers to mean square, *F* and *p* refer to *F* and *p* values, respectively, 1L × 2L refers to interaction of linear effects.

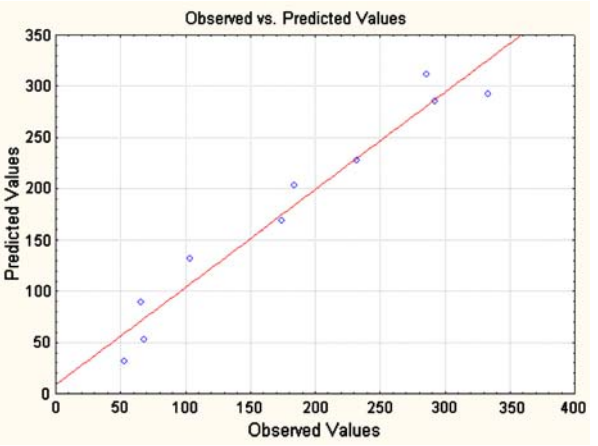

Fig. 1 Graph of observed and predicted values: observed values are experimentally determined yields, whereas predicted values are model predicted yields

significant contributor to response. The regression analysis of the data showed coefficient of determination (R^2) value of 0.951 and adjusted R^2 value was 0.852, which are in close agreement ensuring a satisfactory adjustment of the proposed model with more than 95% variability in response being explained by proposed model. The analyses of model predicted vs. experimentally observed yield values (Table 1, Fig 1) suggested both positive and negative dispersion of observed values. The three-dimensional response surface (Fig. 2) plot based on the proposed model was developed to study the effect of factors on the response and to find out the optimum level of both parameters for maximum GLA production from *Mucor* sp. The response surface plot showed the zone for maximum GLA yield lying within 30% to 50% DO and 450 to 650 rpm with optimum model predicted point being the 39% DO and agitation speed at 569 rpm. The production studies revealed a

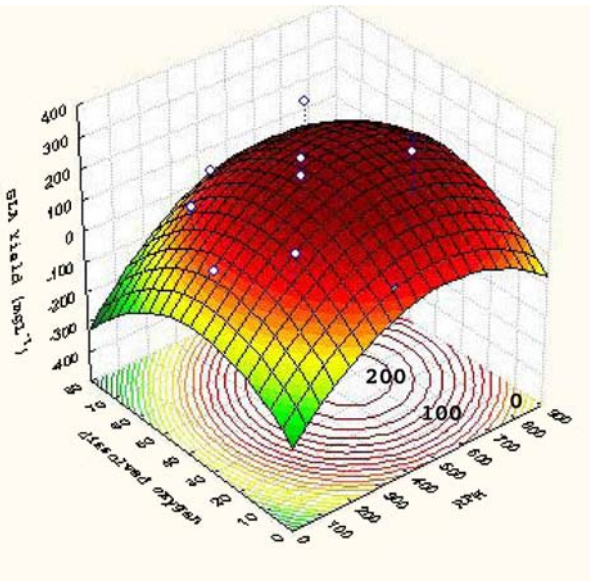

Fig. 2 Response surface plot describing effect of agitation speed (rpm) and DO (dissolved oxygen) on GLA yield. The *Z*-axis refers to predicted GLA yield; values in contour plot (200, 100, and 0) represent GLA yield gradients for corresponding rpm and DO levels. *Hollow squares* in surface plot represent values of rpm and DO for corresponding experiments

maximum GLA production of 333±1.27 mg l^{-1} at 600 rpm and 20% DO, whereas a minimum GLA yield of 53±1.12 mg l^{-1} was observed at 100 rpm and 40% DO (Table 1). It was also observed that with similar agitation speed (400 rpm), the run with very high (75%) and very low (5%) dissolved oxygen level resulted in low GLA yield compared with batch with moderate levels of dissolved oxygen (40%). The validation studies with an agitation speed of 600 rpm and 45% DO resulted in a GLA yield of 350.3 mg l^{-1}, which was very close to the model predicted yield of 340.7 mg l^{-1} from optimum response region.

The GLA yield from the validation study suggested that the contour projecting the optimum production zone was the region where the highest yield of GLA can be obtained, thus validating the robustness of solution provided by the Statistica. Both the agitation speed (linear) and DO (quadratic) are significant effects having major contribution to response (Table 2). This indicated the ability of mold to withstand the high agitation regime as well as the sensitivity of GLA production to high DO. The ability of mold to withstand the high shear regime was also validated by microscopic analysis of mycelia, which showed no breakage over the range of agitation speeds employed. The study of biomass formation revealed rapid biomass formation till 72 h after which there was marginal biomass increase till 168 h. The total dry biomass accumulation showed an increasing trend with increase in agitation speeds with maximum biomass yield of 50 g l^{-1} coming at 800 rpm. This ability to withstand high agitation coupled high shear stress and capacities to reach high biomass level are important properties of this mold. The fermenter studies for 1, 3-PD production also revealed that agitation speed (linear effect) was a significant contributor to response along with aeration quadratic effect being significant [8]. In a similar manner, the studies for inulinase production using *Kluyveromyces marxianus* ATCC 16045 revealed that the enzyme production was strongly influenced by mixing conditions, whereas aeration rate was shown to be less significant. In addition, the increase in the agitation speed was limited by the death rate, which increased drastically at high speeds, lowering the enzyme production [14], suggesting for shear sensitive systems a balance between mass transfer and cell survival needs to be found. The earlier studies with mutated *Mortierella ramanniana* revealed an increase in GLA content of total lipid at a higher agitation speed of 800 rpm [15].

The finding from present study suggests that linear increase in agitation within the design space studied provides sufficient agitation resulting in better distribution of nutrients; also, it retards the wall growth and improves the availability of oxygen to cells by increased mass transfer, making it a significant contributor to response. The interesting observation of sensitivity to high dissolved oxygen suggests that very high oxygen concentration also interferes with the GLA accumulation in cells resulting in lower yield. However, the requirement of moderate levels of dissolved oxygen is a positive attribute of the isolate as any process development with present isolate may lead to less energy consumption for providing compressed air. A similar study for GLA production using *Mortierella ramanniana* resulted in no significant change in the lipid production and the GLA content of the lipid when the DO concentration was varied from 12% to 75%. This again confirms that high DO concentration might not lead to an increase in the GLA content of the lipid [15]. Hence, for optimum production of GLA, oxygen concentration needs to be maintained in the region around the center point of the design. The GLA production yields from present *Mucor* sp. are promising, although there are no comparable reports of GLA production at this scale using *Mucor* sp. as most of the reported GLA yield data are from shake flask studies [16, 17]. The microbial characteristics for fermentative production of omega 3/6 fatty acids and single-cell proteins require that the organism is able to withstand shear stress due to impeller mixing and aeration. The organism should be

capable of high growth rates and exhibit high rates of product formation. The organism should be capable of using low-cost fermentation medium, and bulk products should exhibit high conversion yields of product from substrate. The organism and culture conditions should be such as to facilitate cost effective recovery of the product to desired specifications [18]. The present *Mucor* sp. shows the ability to produce GLA under high shear stress, requires a moderate level of dissolved oxygen, and uses a simple medium. The ability of mold to utilize baker's yeast [11] may make the GLA production more economic. All these findings suggest that the present *Mucor* isolate is a potential candidate for further strain improvement for enhanced GLA content. The suitability of model predicted based on the present study indicates that response surface methodology is useful in evaluating the effects of two important fermentation variables: dissolved oxygen concentration and agitation speed for the production of GLA using the *Mucor* sp.

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