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OPTIMIZATION OF ENVIRONMENTAL GROWTH PARAMETERS FOR BIODIESEL PRODUCING BACTERIA *RHODOCOCCUS OPACUS* USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT

Back ground: Rising level of carbon dioxide, increasing demands and value of fuel, generation of waste are the key issues of the modern society. Biodiesel is an alternate to standard fossil fuel. Currently, it's primarily made from vegetable oils associated consequently it's an adverse impact on food security. Lipids from oleaginous microorganisms (e.g. microalgae, bacteria, fungi and yeasts) may well be an alternate feedstock for biodiesel production and therefore the growth and lipid accumulation of *Rhodococcus opacus* was studied under different environmental conditions.
Results: The current aim of the study is to utilized Box Behnken Design (BBD) of the response surface methodology, for identifying optimum levels of particular variables. BBD design was performed on oleaginous bacterium, *Rhodococcus opacus* considering pH, temperature and incubation period as independent variables. Lipid and biomass contents were analyzed as response variables. A second order polynomial model produced a satisfactory results of the experimental data with regard to biomass yield and lipid content % (R^2 = 98, 04, 96.96 ($P \leq 0.01$). Optimum results of the experiments were 3.82 gL^{-1} biomass and lipid content 33.55% at optimized conditions pH-7, temperature-30^0C and incubation time - 72 hrs. Results of predicted and actual response were differing with 2 to 3 % and desirability of model 98%.

Keywords: *Rhodococcus sp*. 16sRNA, Response surface methodology, Box Behnken design, biomass, lipid

INTRODUCTION

Elevated concentration carbon dioxide (CO_2) emission due to standard fossil fuel burning degrading eological environment; resulted shift in global climate, which is the main concern of the world (**Kumar *et al.*, 2018**). Fossil fuel is depleting very fast and cost is increasing, therefore, there is an urgent need to develop methods for low carbon fuel i.e. biofuel or biodiesel (**Kumar & Thakur, 2018**). Biodiesel is a renewable fuel that can potentially be produced in microbes cost effectively and environment friendly (**Papanikolaou *et al.*, 2008; Easterling *et al.*, 2009; Cho & Park, 2018**). Microbial community such as microalgae cyanobacteria,oleaginous microorganisms, oil from seeds of green plants and waste cooking oils are used for biodiesel production (**Kumar & Thakur, 2018**). Microorganisms contain lipid in their cytoplasmic membrane therefore considered as rich sources of oils and fats for biodiesel production. (**Molina *et al.*, 2017**). Biodiesel comprises of fatty acid methyl esters, originating from vegetable oils and animal fats mainly by trans-esterification of triacylglycerols (TAGs) or from free fatty acids has drawn attention as an ecofriendly, renewable substitute, biodegradable and nontoxic fuel (**Easterling *et al.*, 2009; Papanikolaou *et al.*, 2008**). Furthermore within the field of biodiesel not only algal biofuel of conventional energy resources, microorganism oils even have been gaining a lot of attention as a supplier of novel oils. Bacterial lipids applicable for renewable fuels production and chemicals derived from biological oils or fats (**Castro *et al.*, 2016**). Additionally , they have numerous benefits, such as shorter life span, lesser laborious, season and climate, easy cultivation (**Hidalgo *et al.*, 2013; Shruthi *et al.*, 2014**). According to Papanikolaou and Aggelis, microorganism characterized as oleaginous and their oil as single cell oil, unicellular oil or microbial oil because they have accumulation capabilty of oil more than 20-25% as dry cellular biomass (**Papanikolaou & Aggelis, 2011**)
Further more, certain genera of bacteria belonging to actinomycetes such as Mycobacterium, *Rhodococcus, Gordonia, Streptomyces, Nocardia, Dietzia* have potential of accumulating lipid in their cells and TAGs under nitrogen-stress conditions (**Zhang *et al.*, 2011;Wang & Pan, 2019**). Additionally, oleaginous bacteria having capacity to yield storage lipid, under some growth-restricted conditions also produce special kind of lipids, such as polyhydroxy-alkanoates (PHA) and poly 3-hydroxybutyrate (PHB) and other as cyoplasmic intracellular oil (**Mamatha, 2009; Papanikolaou & Aggelis, 2011; Bajwa & Bishnoi, 2016**). It has various impending commercial applications in pharmaceuticals, nutraceuticals industry, rich source of feed for aquaculture and biofuel production (**Lewis *et al.*, 2000; Peng, & Chen, 2008; Ongmali *et al.*, 2014**). Many environmental factors affect physico-chemical properties of membrane and consequently their functioning which include pressure, pH, temperature, water activity, ions, nutrients, enzymatic activity, microbial growth phase and xenobiotics compounds (**Mrozik *et al.*, 2004**). Many changes in bacterial fatty acid composition and membrane fluidity occur in response to temperature fluctuations. As growth temperature rises, it is common to observe an increase of the proportion of long-chain and saturated fatty acids within the membrane (**Mrozik *et al.*, 2004**)
Response surface methodology is a novel arithmetical design employed to evaluate problems where in the response is dependent on several independent variables with an objective to maximize the process variables for achieving optimum response (**Box & Behnken, 1960**). RSM uses quantitative data from experimental conditions to analysis and solve multivariate equations (**Gorret *et al.*, 2004; Tokcaer *et al.*, 2006**). RSM is very helpful tool in reduction of experiments as compared to manual practises eventually saving chemicals, time and labor. Furthermore, it offers a rapid and unfailing prediction of response, making it a beneficial option for experimental design (**Singh *et al.*, 2013**). The Box Behnken design was taken as it fulfilled most of the requirement for interaction study for various factors (**Gorret *et al.*, 2004; Tokcaer *et al.*, 2006; Sigh *et al.*, 2013**). Thus the aim of present study is to evaluate the various environmental growth parameters *viz.* pH, temperature and incubation on biomass yield and lipid content using Response surface methodology for oleaginous *Rhodococcus sp.*

MATERIAL AND METHODS

Isolation of genomic DNA from bacterial strain and 16s rRNA sequence determination and phylogenetic analysis

Extraction of genomic DNA was from bacterial strain was performed by using Cetyl trimethyl ammonium bromide (CTAB) method (**Ausubel *et al.*,1987**).

After DNA extraction, 800 mg of agarose in 100 ml 1X TAE followed by heating in microwave and added 2 drops of ethidium bromide poured into Gel casting Tray. The amplification was conducted with Universal primers designed to anneal the conserved regions of bacterial 16S rRNA genes (**Khalil, 2011**).The PCR product of 16S rDNA was sequenced by Geneombio Technology Pvt. Ltd. Pune (Maharashtra). Nucleotide sequence was analyzed and compared with Gen Bank nucleotide sequence database using the Basic Local Alignment Tool (BLASTn).

Biomass estimation

Bacterial strain was cultured in MSM broth. Composition of MSM medium given in Table.1.Growth of experimental bacteria in MSM media was measured every 3 hours until 96 hours of the cultivation time and determined dry cell weight and optical density at 600 nm. There was a linear relationship between dry weight and OD 600 nm as linear regression equation. Standard linear regression curve prepared by dilution ranging between 0.2 to 1 (**Tapia *et al.*, 2012**) , y = 0.2425x + 0.2615, R^2 = 0.9923

Table 1 Composition of minimum salt medium

KH_2PO_4	2
K_2HPO_4	7
$ZnCl_2$	0.01
$MgCl_2$	0.20
$FeCl_3$	0.01
$MnCl_2.4H_2O$	0.01
Na_2SO_4	0.20
NH_4NO_3	1.0
Yeast extract	0.006
$CaCl_2$	0.01

Determination of growth and lipid content gravimetrically

Lipid extraction was performed with modified Bligh and Dyer Protocol (Chloroform Methanol: Water) in ratio 1:2:08) respectively for bacterial cells cultivated in Minimal salt medium. Bacterial cells were collected by centrifugation at 5,000 rpm for 15 min. The cell pellet was washed with 40 mL of distilled water. The washed-cell pellet was freeze-dried, held in desiccator until constant mass was attained (usually 24 h) and weighed to estimate its dry cell weight, followed by extraction with a mixture of chloroform, methanol and water (1:2:0.8, volume ratio). Further with addition of chloroform, methanol and water to reach a ratio of 1:1:0.9 (**Papanikolaou *et al.*, 2002**). The solvent mixture containing extracted lipid was centrifuged and lipid layer was pooled by micropipette and the solvent removed in a desiccator. The dry lipid was weighed. Lipid content relative to dry cell weight was determined.

Optimization of pH, temperature and incubation period process variables for the bacterial growth using box behnken design

RSM is a novel arithmetical design employed to evaluate problems wherein the response is dependent on several independent variables with an objective to maximize the process variables for achieving optimum response (**Box, & Behnken, 1960**). The experimental design consisted of factors: pH, incubation temperature (^oC), incubation time (Hours) **(Table 2).** Seventeen experiments were designed by Design Expert 7.0.0 box-Behnken model and conducted at various culture conditions as per experimental set up. Second order polynomial equation was used in order to find relationship between variables and responses. The regression equation coefficients were calculated and the data were fitted to a second order polynomial equation The adequacy of model was evaluated by coefficient of determination (R^2) and model P value. The analysis of variance (ANOVA) of various responses for lipid productivity (dcw %), biomass (gL^{-1}) by using RSM (**Singh *et al.*, 2013**).

Table 2 Three independent process variables used in RSM in terms of coded factors in Box-Behnken design for *Rhodococcus opacus*

Factors	Process variable	Low (−1)	Medium (0)	High (+1)
A	Incubation period (hrs)	24	72	120
B	Temperature (^oC)	20	30	40
C	pH	5	7	9

RESULT AND DISCUSSION

16s rRNA sequence determination and phylogenetic analysis

Comparision of 16S rRNA gene sequence obtained from the experimental species was done with other bacterial sequences by using NCBI mega BLAST. Phylogenetic tree based on 16S RNA was constructed which showed that this isolate has 99% sequences similarity with *Rhodococcus opacus* (**Figure 1**).

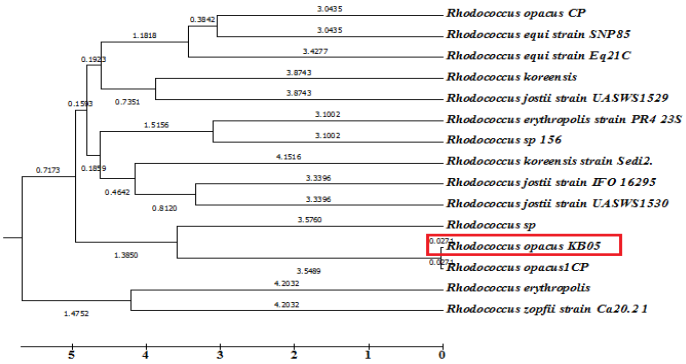


Figure 1 Construction of phylogenetic tree based on 16S rRNA gene sequences by neighbour joining method showing the relationship of *Rhodococcus sp.* with other universal identified species

Table 3 Box Behnken model for combined effect of pH, temperature and incubation period in *Rhodococcus opacus*

Run	Temperature (°C)	pH	Incubation period(Hrs)	Biomass gL^{-1}	Lipid %
1	20	5	24	2.351	27.14
2	30	7	72	3.828	33.55
3	30	9	72	2.86	30.54
4	30	5	72	2.94	29.73
5	20	9	120	3.48	27.69
6	40	7	72	3.5	32.12
7	40	9	120	3.089	31.45
8	20	7	72	3.62	30.65
9	30	7	72	3.83	33.549
10	30	7	72	3.829	33.551
11	30	7	72	3.83	33.55
12	20	9	24	2.661	26.58
13	30	7	120	3.7	31.4
14	40	5	120	3	31.019
15	40	5	24	2.56	26.251
16	30	7	72	3.832	33.55
17	20	5	120	3.299	23.72
18	30	7	24	3.5	28.6
19	40	9	24	2.7	22.51
20	30	7	72	3.83	33.55

Effect of mutual optimized process variables on various growth factors of *Rhodococcus opacus*

The growth of *Rhodococcus opacus* was studied by optimum culture parameter i.e. pH, temperature and incubation period. All experiments were designed by using box behnken model, around 20 sets of experiments designed to fit a second order polynomial equation for optimizing the growth of *Rhodococcus opacus* in respect to concentration of lipid and biomass. The minimum, central and maximum process variables such as pH 5 to 9, temperature 20 to 40°C, incubation period 24 to 120 hrs were used in the BBD for various responses and their impacts on biomass yield and lipid content *Rhodococcus opacus* (Table 3) (**Kirrolia *et al.*,2014**)

ANOVA for the response surface quadratic model of various growth parameters in *Rhodococcus opacus* were depicted in Table 4. The test statistic for lack-of-fit is the ratio between the lack-of-fit mean square and the pure error mean square (**Singh *et al.*,2013; Kirrolia *et al.*,2014**). Lack of fit, non significant value has shown the validity of the quadratic model for various responses of the experimental culture. F-test statistic used in order to determine lack of fit error whether it is significant or not . The value R^2-98.04% , Adj R^2 99.03% for biomass and R^2-99.96%, Adj R^2-99.98% for lipid content showed that they were found to be reasonable agreement with better reliabilty of model. All the R^2 values of the experimental data were found to be close to 1.0 indicated that suitabilty of the model. Signal to noise ratio was measured by adequate precision. Signal to noise ratio greater than 4 is suitable for model validation. A ratio of 7.105, 23.7 of biomass and lipid model indicated an adequate signal which implied that these models can be used to design three dimensional graphs. Low standard deviation value with coefficient variation of biomass, lipid including 0.27, 0.66 and 7.96%, 6.22% which implied that model is suitable for present study. For biomass only linear AB, AC, BC, quadractic (A^2, B^2, C^2) are

significant model terms based on the p-value. For lipid accumulation linear (A, C) mutual (AB, AC, BC) quadractic (A^2, B^2, C^2) are significant model terms. An flat inverted umbrella shaped standard error graph is desirable for BBD design with no sign of data interpretation **(Fig. 2)**.The final responses in term of coded factors for lipid content and biomass yield are depicted in the equations below: (**Kirrolia *et al.*,2013**)

Model equations in terms of coded factors:

Biomass = +3.17+0.42* A+0.14* B+0.27 * C-0.12*A* B+0.1*A* C+0.06* B*C-0.46*A^2-0.4* B^2-0.29* C^2 ……..1

Lipid (%) = +33.53+0.76*A-9.000E-003*B+1.42*C-0.84*A*B+2.00*A*C+1.09*B* C-2.12* A^2-0.87* B^2-3.50*C^2 …….2

Statistical exploration of positive linear coefficient showed that culturing time was the most significant factor affecting the cell growth variables responses in experimental culture **(Eq. 1 to 2)**. Hence the relationship of biomass and lipid with process variables such as pH, temperature and incubation period in *Rhodococcus opacus* can be interpretated from model equations presented in coded factors. Higher values of pH,temperature in linear coefficient term illustrated the significantly positive output of the variables on all the responses (**Singh *et al.*,2014**).Positive linear coefficient value for pH,temperature and incubation period indicated that all three variables showed their maximum effect at various optimum concentrations. Positive values of 0.14, 0.27, 0.06 for linear coefficient of temperature, pH and incubation period illustrated that significantly positivite effect of these factors on biomass production.The negative interactive coefficient data of independent variable (linear C, quadratic, B^2 C^2) on biomass yield was observed as a function of thesse variables by keeping all the variables at a fixed level. Similarly linear coefficients values of pH and temperature (1.42, 2.0,1.09) implied that these factors have significants effects on lipid accumulation. Three dimensional (3D) graphs were used to explore the sensitivity of the responses of two interacting variables by holding the other variables constant at central values (**Kirrolia *et al.*, 2013**).The lipid accumulation and biomass yield assets of *Rhodococcus opacus* under different initial pH, temperature and incubation period were shown in three dimensional graphs. **Fig. 2 (A, B)** which indicated higher temperature 30°C with optimum pH 7 and incubation period of 72 hrs has much significant ($P \leq 0.05$) effects on biomass and total lipid production in *Rhodococcus opacus*.The higher pH and temperature has cellular growth retarding effects on biomass and lipid production (**Leesing, & Baojungharn, 2011; Enshaeieh *et al.*, 2013; Dias *et al.*, 2016; Poontawee *et al.*,2017**). pH has been described as an important factor that strongly interferes in the lipid accumulation in oleaginous microorganism (**Papanikolaou & Aggelis, 2010**)

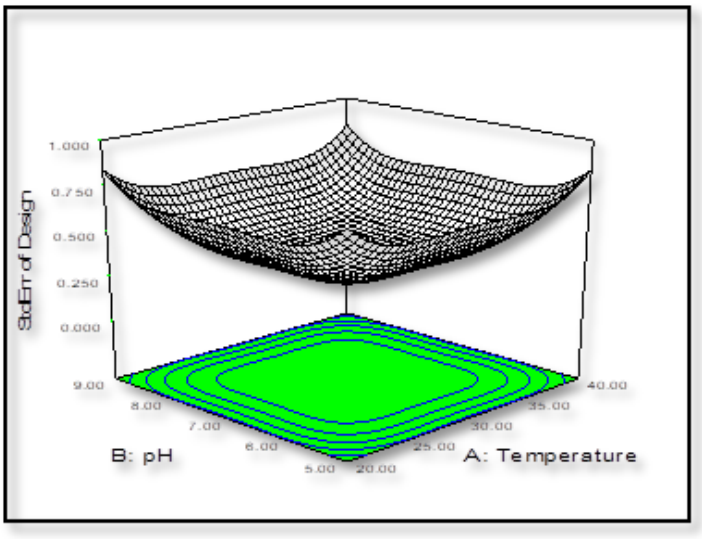
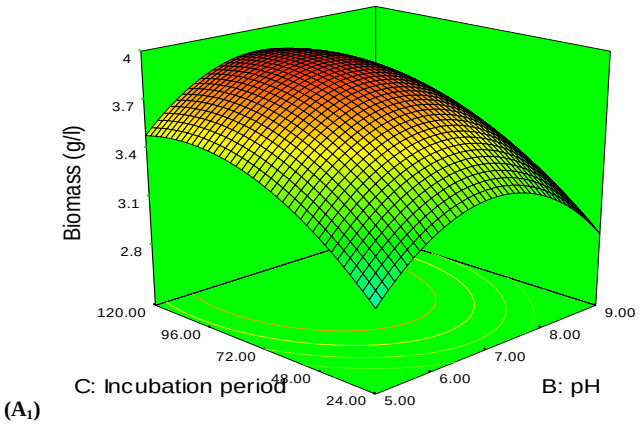
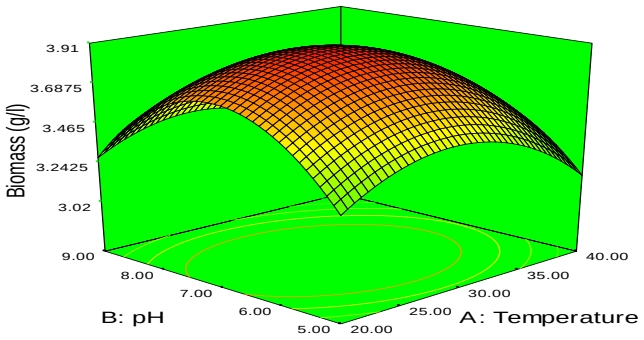


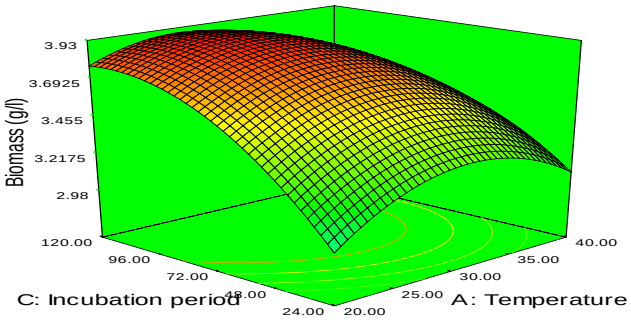
Figure 2 Three dimensional plot of standard error design of the model with pH 7 and temperature 30°C, keeping incubation period constant



(A_1)



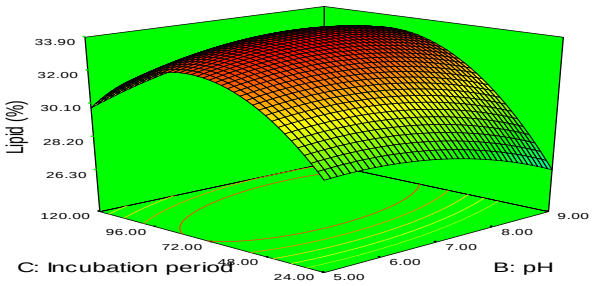
(A_2)

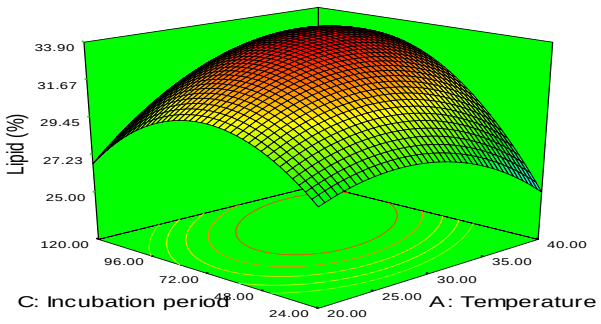


(A_3)

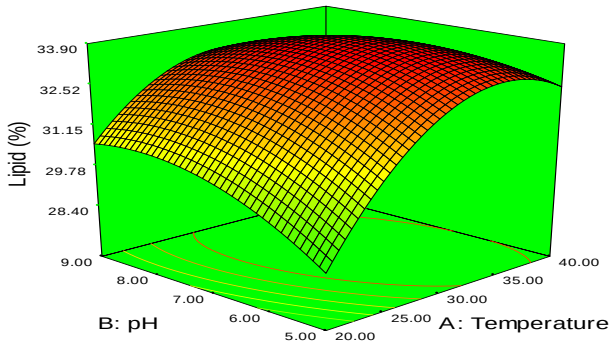
Figure 2 A (A_1,A_2,A_3) Three dimensional response surface plots showing mutual interaction of pH, temperature and incubation period on lipid content %

(B_1)





(B_2)



(B_3)

Figure 2 B (B_1,B_2,B_3) Three dimensional response surface plots showing mutual interaction of pH, temperature and incubation period on lipid content %

As it is cleared from the Figure 2 A (A_1,A_2,A_3) that with increasing pH 5 to 9 there was corresponding increase in biomass yield and optimum result found at pH 7. According to observation of another researchers that temperature has significant effect on free fatty acid of microbial oil and lipid content, the optimum temperature responsible for the synthesis of oleaginous microbial fat, temperature range varies for both fat synthesis and cell growth (**Papanikolaou *et al.*, 2002; Zhao *et al.*, 2010**)

Table 4 ANOVA for the response surface quadratic model of biomass yield and lipid accumulation in *Rhodococcus opacus*

	Response 1				Response 2			
Source	**Sum of Squares**	**Df**	**Mean Square**	**p-value Prob > F**	**Sum of Squares**	**Mean Square**	**F-Value**	**p-value Prob > F**
Model	4.220124	1	0.468903	<0.0001	234.6348	26.07054	5990.365	<0.0001
A-Temperature	0.031584	1	0.031584	<0.0001	5.73049	5.73049	1316.725	<0.0001
B-pH	0.01296	1	0.01296	<0.0001	0.00081	0.00081	0.186118	<0.0001
C-Incubation period	0.781762	1	0.781762	<0.0080	20.15832	20.15832	4631.884	<0.0001
AB	0.00858	1	0.00858	<0.0001	5.6448	5.6448	1297.036	<0.0001
AC	0.109981	1	0.109981	<0.0001	32.07204	32.07204	7369.362	<0.0001
BC	0.00405	1	0.00405	<0.0001	9.465601	9.465601	2174.961	<0.0001
A^2	0.204477	1	0.204477	<0.0001	12.3543	12.3543	2838.713	<0.0001
B^2	0.514837	1	0.514837	<0.0001	2.079301	2.079301	477.7719	<0.0001
C^2	0.148887	10	0.148887	<0.0001	33.77506	33.77506	7760.673	<0.0001
Residual	0.716513	5	0.071651		0.043521	0.004352		
Lack of Fit	0.716504	5	0.143301	<0.0001	0.043519	0.008704	21759.39	<0.0001
Pure Error	8.83E-06	19	1.77E-06		2.00E-06	4.00E-07		
Core Total	4.936637				234.6784			

Response 1- Biomass (R^2- 98.04%, Adj.R^2: 99.03%); Response 2- Lipid (R^2-99.96%, Adj.R^2-99.98%)

Kraisintu *et al.*, (2010), obtained the highest lipid yield (9.26 gL^{-1}) and cellular lipid percentage (71.30% of dry biomass) at medium pH 5.5 in oleaginous yeast. **Komazawa *et al.*, (2007)** reported that for a *Thraustochytrium* strain, pH between 5.0 and 8.0 was optimum. Similar study was reported by Wu et al.,(2005) when pH was varied between pH 5 to 8, a maximum biomass and DHA yield was obtained from *Schizochytrium sp.* at pH 7.0. As presented in **Fig. 2 (B)** with increase in temperature subsequently decrease in lipid content in *Rhodococcus opacus*. According to **Saxena *et al.*, (2009)**, composition of lipid also varied at different temperatures as we found in our present work. **Enshaeieh *et al.*, (2013)** found that 96 hrs is optimum incubation period for lipid accumulation and biomass yield in *Rodotorula sp.* In contrary to our results, Vipra and his co-workers have been observed that highest lipid yield at culturing period (24 hrs) in oleaginous yeasts *viz*. *Lipomyces lipofera* cultures and *Yarrowia lipolytica* (**Vipra *et al.*, 2012**). Hence shorter culture growth time is considered as ideal for potential industrial processes for lipid production (**Holdsworth,1998**). **Papanikolaou *et al.*, (2002)** observed that reduction in lipid accumulation about 1gL^{-1} of *Yarrowia lipolytica* M7 when incubation time incceased from 72 hrs to 96 hrs after increasing of incubation time from. *Yarrowia lipolytica* M7 produced significantly ($P \leq 0.05$) higher biomass and lipid yield upto 72 hours of period of incubation period as we observed in our results **(Papanikolaou *et al.*, (2002)**. Ongmali *et al.*, (2014) found that oleaginous *Aeromonas* sp. showed enhanced cellular dry weight and lipid yield over a period of 72 hours.

MODEL VALIDATION

Box-Behnken design was implemented to screen the key process parameters and identify optimal values that contribute maximum biomass and lipid production (Ghosh *et al.*, 2015). Experiments were run on optimum conditions suggested by model to evaluate the validity of the model **(Sekhar *et al.*, 2014)**. Optimum results of the experiments were 3.82 gL^{-1} biomass and lipid content 33.55%. Results of predicted and actual response were differing with 2 to 3 % and desirability of model 98%. Less than 10 % difference in predicted and experimental response was found to suitable for model validation (**Kumar & Banerjee, 2013)**

CONCLUSION

In conventional one-factor time experiments, a single factor varies; keeping other factors constant and the effect of interaction among the variables is ignored. The RSM is a systematic statistical design approach, aimed at developing the relationships between process variables and responses in order to assemble for a better overall understanding with a minimum number of experiments (**Kirrolia *et al.*,2013**). Most importantly BBD generates appropriate statistical assets so that, for the suitability of quadratic model for data evaluation, only fraction of the trials requisite for a 3-level factorial (**Singh *et al.*,2014**). RSM was successfully implemented for optimization of various culture growth variables such as pH, temperature and incubation time. *Rhodococcus opacus* showed optimum results of the experiments were 3.82 gL^{-1} biomass and lipid content 33.55% at pH 7, temperature 30^0C and incubation period 72 hrs. Results of predicted and actual response were differing with 2 to 3 % and desirability of model 98%. Results revealed that there were no significant variances between the predicted data for studied responses and experimental data obtained with optimum experimental conditions.

Conflict of interest : The authors disclosed that there was no conflicts of interest.

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