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Challenges in Biochemical Engineering and Biotechnology for Sustainable Environment

Selection of Microbial Strains for Oil Transesterification and Optimization for Higher conversion of alkyl esters

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Abstract: Uninterrupted diminution of fossil fuels due to incessant fossil based energy practices has landed the planet in alert of immediate fuel demands. Alternate fuel was the fervently focused subject for the past few decades since the use of fossil energy resulted in alarming rise of gas particulates. Sustainable energy supply with reduced release of toxic gases was the providential characteristic of biofuels to face the existing fuel demands. Biodiesel is an ester product of transesterification of a fatty acid and a low carbon chain alcohol. Transesterification can be carried out using a lipase producing organism immobilized onto a suitable matrix as whole cell biocatalyst. Selection of a suitable lipase producing organism is an uphill task as it necessitates the fulfillment of several variables such as being a high product yielding strain, thermostable, no side reactions, easy handling and cultivation. The present work focuses on selecting a suitable microorganism among *Rhizopus oryzae*, *Aspergillus oryzae*, *Candida antartica*, *Candida rugosa*, *Psuedomonas cepacia*, *Psuedomonas fluorescens*, *Rhizopus chinensis*, and *Bacillus subtilisis* as a biocatalyst for high yield of biodiesel through methanolysis of waste cooking oil. Maximum conversion of 84% methyl esters were obtained by*Rhizopus oryzae* catalyzed transesterification of oil with low oil to alcohol ratio (1:3) since the organism is known to produce *1,3*-regiospecific lipase that effectively breaks oil to esters than any other lipase. RSM was adopted to optimize the higher conversion of biodiesel from waste cooking oil using *R.oryzae*as whole cell biocatalyst.
Keywords*:* Whole cell biocatalyst, *Rhizopus oryzae*, Biodiesel, Waste cooking oil, Transesterification.

1. Introduction:

In the past decade, the demand for development of alternate fuels has steadily increased due to the depletion in fossil fuel reserves. Also the increased pollution levels and sulfur emission from automobile engines has resulted in the development of new cleaner and greener fuels. One such fuel which is highly studied and researched upon is biodiesel which is chemically a fatty acid alkyl ester. Biodiesel is produced from an oil source which contains either triglycerides or fatty acids by the process of transesterification. Transesterification is defined as the chemical process which happens in the presence of a catalyst in which a triglyceride reacts with an alcohol to form an ester(Biodiesel) and glycerol. The process of transesterification is described by the following equation (Fig.1.). Transesterification process is carried out in the presence of a catalyst (which can either be an acid,alkali or biocatalyst) or in the absence of a catalyst (using supercritical alcohol or ultrasoniation). Catalyst free reaction using supercritical alcohol is energy intensive and requires high reaction

temperature and ultrasound assisted transesterification is still not completely developed and most of it is still not known,making it unreliable. Acid catalyzed transesterification suffers from the drawbackthat it requires a large amount of alcohol, along with being energy intensive and posing difficulty during the separation of the products. Similar to acidcatalysis, alkali catalysis is also energy intensive and will undergo saponification reactioninstead of transesterification and yield soap if thefreefatty acid valueis high in the oil. On the other hand, biocatalyst does not require high temperature or alcohol concentration and catalyze both triglycerides and freefatty acids to yield biodiesel.

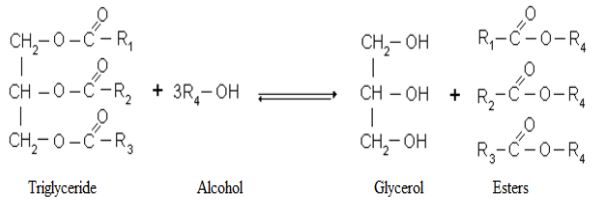

Fig.1. Transesterification reaction

Biocatalysts are of two types- purified lipase and whole cell biocatalyst. Biocatalysts are generally used after immobilizing them onto a suitable matrix which enables easy recovery and separation. Lipases which are also known as triacylglycerol hydrolases are esterases which can hydrolyze the triglycerides[1,2]. Lipases are costly and suffer a disadvantage that they get easily denatured in the biodiesel production process. Whole cell biocatalysts are lipase producing microorganismimmobilized onto to a suitable matrix and used as catalyst for the transesterificationprocess. This type of catalyst is cheap to cultivate and produce good yields with being stable throughout the reaction time.

Several thousands of lipase producing microorganisms has been isolated and all these microorganisms have the potential to be used as whole cell biocatalyst in biodiesel production. Although there is versatility in the microorganisms available,several crucial factors decide the selection of a suitable microorganism[3,4]. Screening of microorganisms is done in two levels based on pathogenicity, doubling time, conversion and lipase activity.

2. Materials and methods:

2.1. Microorganism collection and subculture:

Table. 1. Composition of lipase inducing media

Ingredients	Grams per litre
Peptone	70
Sodium nitrate	1.0
Potassium dihydrogen phosphate	1.0
Magnesium sulphate	0.5
Glucose/olive oil	10/3

Table. 2.Properties of selected microorganisms

Species	Pathogenicity	Doubling Time (hr)	Conversion as WCB at 30°C (Optimum)
Rhizopus chinensis	Pathogenic	3.00	81.3
Aspergillus oryzae	Non - pathogenic	3.85	74.65
Candida antartica	Pathogenic	2.00	76.5
Candida rugosa	Pathogenic	1.50	79.2
Psuedomonas cepacia	Pathogenic	2.45	69.23
Psuedomonas fluorescens	Non-pathogenic	2.00	73.2
Rhizopus oryzae	Moderate	4.53	84.12
Bacillus subtilisis	Non - pathogenic	2.00	63.56

*Conditions: 24hr, 30°C and 1:3 oil to alcohol ratio.

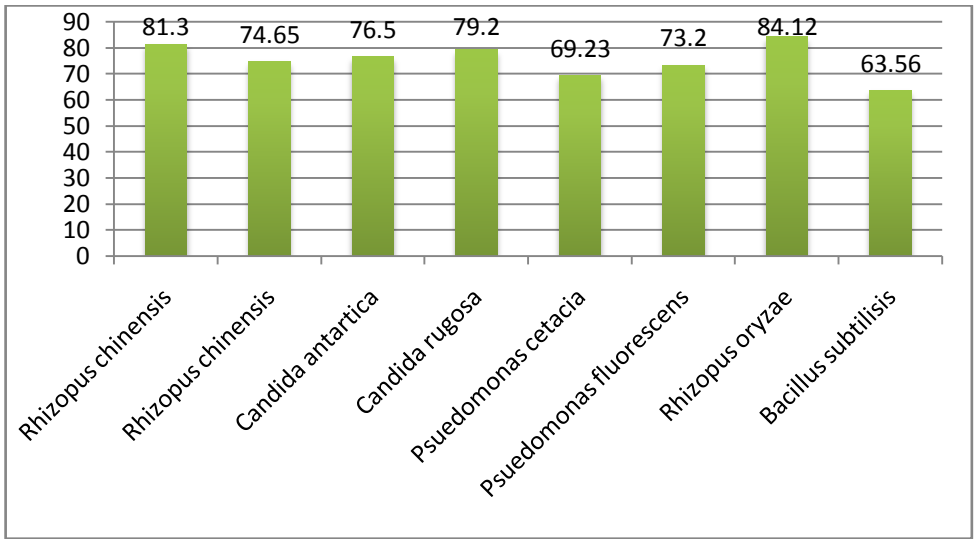

Fig. 2. Percentage Conversion by selected species

All the cultures were obtained from MTCC (Microbial type culture collection center), Chandigarh. *Aspergillus oryzae 634*, *Candida antartica 1644*, *Candida rugosa*, *Psuedomonas cepacia*, *Psuedomonas fluorescens*, *Rhizopus chinensis*, *Bacillus subtilisis* and*Rhizopus oryzae* 262 were obtained. Fungal selective Potato Dextrose Agar (PDA) slants were used for fungi. Medium ingredients include Potato infusion 250g/L, Dextrose 5g/L and Agar 20g/L. The incubation period was 7 days at a temperature of 30^{o}C. The subculture was done regularly at an interval of 30 days. The pH was maintained at 5.6. The media was sterilized in autoclave at 121^{o}C for 21 minutes. The mixing rate was 100 rpm[2]. For bacterial culture revival, nutrient broth was used. pH was maintained at 9.0 and rpm was set at 150 for optimal growth at 30^{o}C.

2.2. Oil Collection:

Waste cooking oil was collected from college canteen at various time intervals. Fine blend of oil mixture was taken for the experiment. The collected oil was filtered using filter paper to get rid of the food waste present as sediments and suspensions. Colored oil can be charcoal treated to remove coloring substances. No acid pretreatment was done to neutralize the acidic pH effect of free fatty acid present in the oil sample. This was done to evaluate the conversion potential of organisms under extreme conditions of reactants. Hence this part remains as a crucial parameter for screening of organisms as biocatalysts.

2.3. Screening parameters:

Basic screening parameters involved testing of organism for its doubling time and pathogenicity. All the organisms selected were found to have good lipase producing ability. Good doubling time increases the rate of catalyst generation and increases the conversion indirectly. Pathogenicity behavior was considered to ensure the safeness of process and ecological hazards after disposal to the environment. Second level of screening was based on conversion efficiency of the organism. The conversion was linked with temperature stability for critical validation of organism as potential biocatalyst. All the organisms can produce lipase but the conversion efficiency of lipase varies with the region specific property and the amount produced. Conversion efficiency of the organism in extreme condition was the mainly significant parameter considered for screening. Considering the conversion parameter, indirectly measures the lipase activity of each organism.

2.4. Immobilization and Transesterification:

Immobilization is a technique for arresting the enzyme/biocatalyst in a polymer material. Calcium alginate is the immobilizing material obtained by the stepwise addition of sodium alginate in drops into calcium chloride solution[3]. The stability and pore size of the beads have greater effects on the activity of the catalyst. Hence the sodium alginate solution was prepared at various concentrations such as 1%, 1.5%, 2%, 2.5%, 3% and 0.5M solution of calcium chloride was prepared. It was observed that the beads formed from 2% sodium alginate showed optimum stability and flexibility.

For entrapment, the whole cell biocatalyst and pure enzyme was mixed well with sodium alginate solution and then the procedure was continued. The beads were suspended in calcium chloride solution. This

was used for the transesterification process. Methanol was used as acyl acceptor throughout the process. Oil to alcohol ratio was maintained as 1:3. 24h was taken as optimal time[4]. Glycerol is separated from biodiesel by phase separation using separation funnel.

2.5. Selection and lipase induction:

The organism with good conversion ability screened out of all selection parameter is subcultured in lipase inducing media. Use of Substrate related compounds in culture medium increases the activity of organism towards the particular substrate[5]. Rhizopus oryzae, selected as efficient whole cell bio catalyst is cultured in media with following ingredients for good lipase induction[6, 7]. The strain was incubated at 30°c at a pH of 5.6 for 7 days.

2.6. Optimization for good conversion:

RSM was used to optimize the parameters for increasing the biodiesel production.The statistical methods have been proved to be effective for investigating the role of key factor[8]. Response Surface Methodology (RSM) is the most commonly used method for the optimization of process over the past few years. RSM was first described by Box and Wilson[9]. It combines various statistical & mathematical techniques which are useful in optimizing processes which are influenced by several variables[9]. The main objective of RSM is to optimize the response generated by the process. RSM reduces the number of experimental trials hence it can be less laborious and time consuming[10]. Also, the enzyme production can be increased through RSM by the optimization of parameters[11]. The parameters taken for the study are those that played a vital role during the preliminary study. Then CCD (Central Composite Design) was performed to determine the interaction of parameters and their combined effect on the process. Statistical analyses were performed using Minitab 15andDesign expert 8.0.7.1. Waste cooking oil was the substrate and methanol was the acyl acceptor in this solvent free system. The coefficients of polynomial model were calculated using the following equation:

$$Y=b_0+\sum b_i X_i+\sum b_{ij} X_i^2+\sum\sum b_{ij} X_i X_j$$

Where Y was predicted response, and i,j were linear, quadratic coefficients, respectively. B and k were regression coefficient and the number of factors studied in the experiment. Three-dimensional plots and their respective contour plots were obtained to study the interaction of one parameter with another. The optimum conditions were identified in the three-dimensional plots

3. Results and discussion:

3.1. Screening based on conversion:

Two layers upper Biodiesel phase and lower glycerol phase were obtained after transesterification reaction. Separation can be done using separation funnel. Large scale separation can be done simply by using separation chambers.Standard procedures were followed to determine their saponification value and acid value[12].The % conversion is calculated using the formula,

$$acid\ value\ \left(\frac{mgKOH}{g}\right)=\frac{titre\ value * normality * 56.1}{weight\ of\ sample}$$

$$\%coversion=\left(1-\frac{AV_{biodiesel}}{AV_{WCO}}\right)*100$$

3.2. Optimization for Biodiesel conversion using RSM:

3.2.1 Experimental Design and Statistical Analysis for *Rhizopus oryzae*

pH (Y_1), olive oil concentration (Y_2) and rpm (Y_3) were chosen as the independent variables as shown in Table 3. Percentage conversion of biodiesel was used as the dependent output variable. 20 runs were performed according to table to optimize the parameters. Among them the center points were 6.

Table. 3. Variables used in the optimization using *Rhizopus oryzae*

Variables	-1.6817	-1	0	+1	+1.6817
pH	5.16	5.30	**5.50**	5.70	3.584
Olive Oil Concentration (G/L)	16.59	20	**25**	30	33.41
Rpm	99.55	120	**150**	180	200.45

3.2.2. Optimization through response surface methodology for *Rhizopus oryzae* catalyzed reaction Statistical analysis and graphical interpretation

For the production of biodiesel using wild *Rhizopus oryzae* the parameters such as pH (Y_1), olive oil concentration (Y_2) and rpm (Y_3) were optimized. The responses were calculated using 20 experimental runs as shown in Table 4.

Table. 4. Responses for the experimental runs

Run	Y_1	Y_2	Y_3	Response
1	5.7	30	120	81.07
2	5.5	25	150	83.76
3	5.16	25	150	75.16
4	5.7	30	180	75.11
5	5.5	25	150	83.16
6	5.7	20	120	69.99
7	5.3	20	180	76.56
8	5.5	25	150	83.76
9	5.5	25	150	83.76
10	5.7	20	180	74.21
11	5.5	25	150	83.76
12	5.84	25	150	78.55
13	5.5	33.41	150	76.09
14	5.3	30	120	74.76
15	5.5	16.59	150	68.33
16	5.3	30	180	74.55
17	5.5	25	99.55	69.78
18	5.5	25	150	83.76
19	5.5	25	200.45	72.3
20	5.3	20	120	67.43

The Model F-value of 92.93 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise.The equation for the quadratic polynomial model can be represented as

$$\mathbf{Y=83.71+0.94Y_1+2.22Y_2+0.84Y_3+0.83Y_1Y_2-1.33Y_1Y_3-2.44Y_2Y_3-2.13Y_1^2-3.77Y_2^2-4.19Y_3^2}$$

From the summary of results, the R^2 value was found to be 0.9882. This signifies that the model is accurate. Also the p-value ($p<0.001$) shows that the results are significant. Thus the optimum conditions for achieving a high conversion of 83.76% was found to be at Y_1=5.5, Y_2=25g/L, Y_3= 150rpm.

Table. 5. Analysis of variance (ANOVA) for the model regression

Source	Sum of squares	df	Mean square	F value	P- value prob<F
Model	602.22	9	66.91	92.93	<0.0001[a]
A	11.96	1	11.96	16.61	0.0022
B	67.45	1	67.45	93.67	<0.0001
C	9.55	1	9.55	13.26	0.0045
AB	5.54	1	5.54	7.70	0.0196
AC	14.20	1	14.20	19.73	0.0013
BC	47.63	1	47.63	66.14	<0.0001
A^2	65.42	1	65.42	90.86	<0.0001
B^2	205.14	1	205.14	284.89	<0.0001
C^2	252.59	1	252.59	350.79	<0.0001

These analysis were made through Central Composite Design and the resulting contour and 3D surface plots were further examined to study the influence of parameters over another (Figs.3-5). The yield increases with increase in olive oil concentration but pH and rpm has only minimal effect on yield.

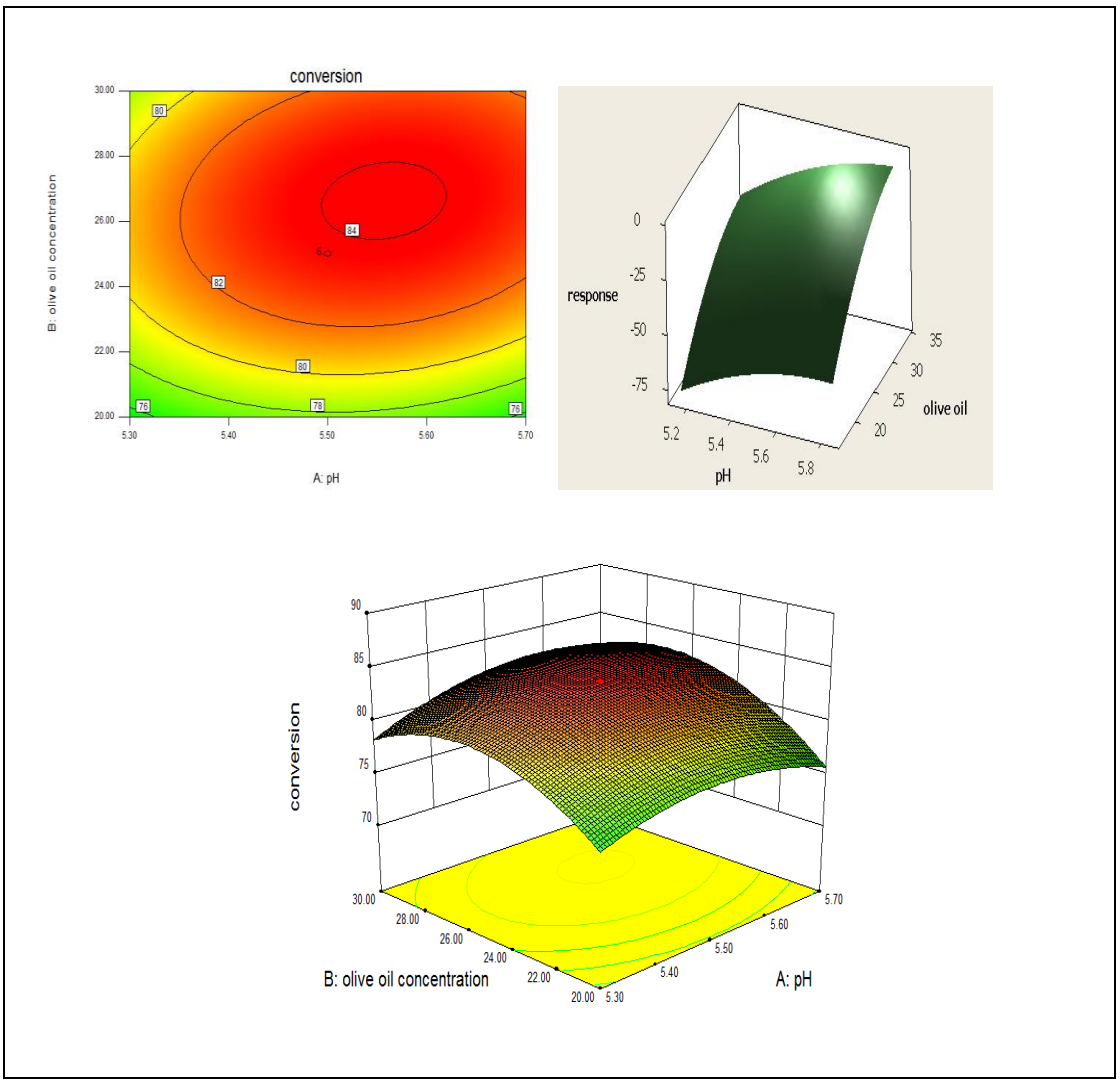

Fig.3. Surface plot, contour plot and 3D graph showing the interaction of pH and olive oil concentration for biodiesel production using *Rhizopus oryzae*.

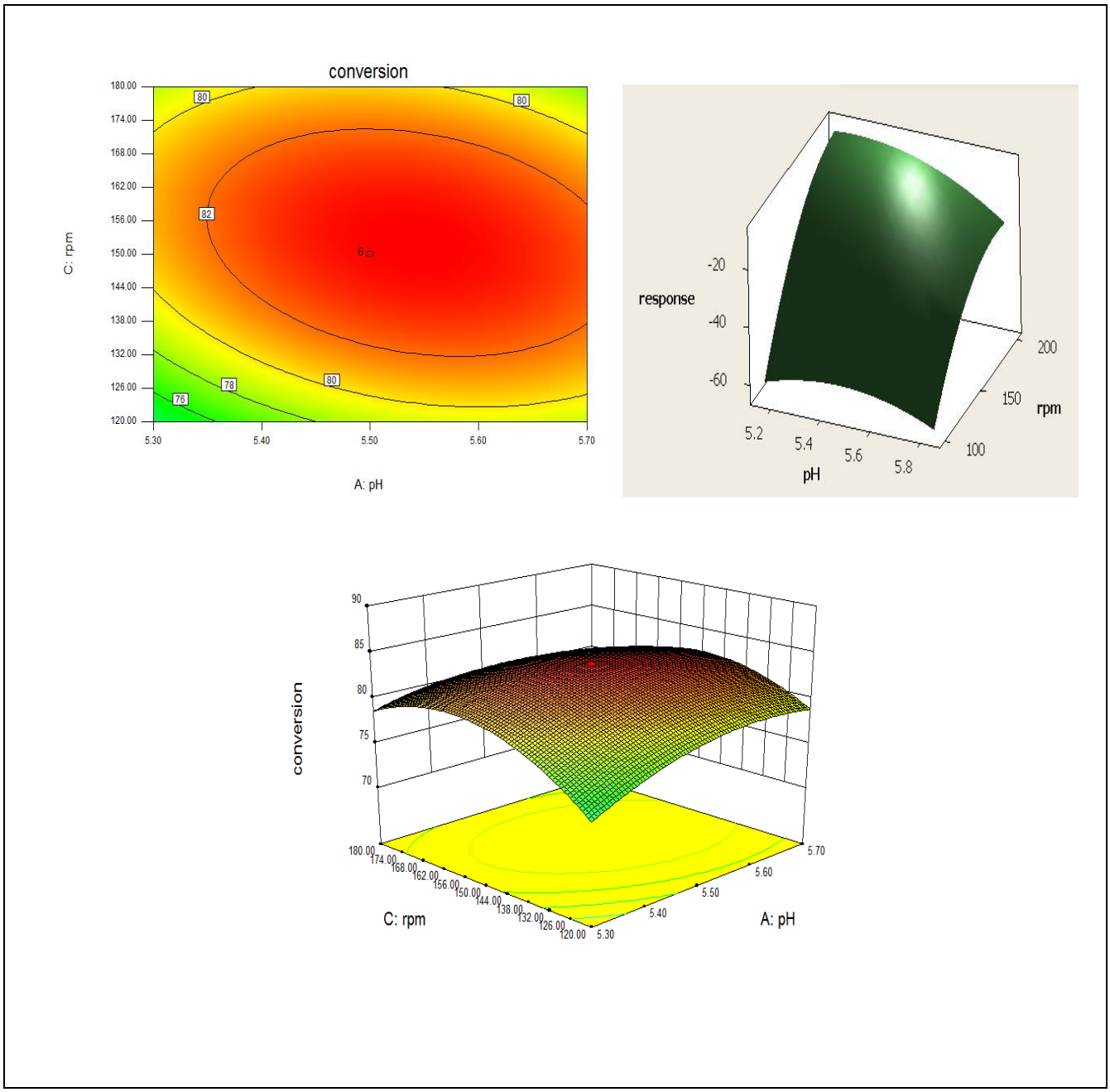

Fig.4. Surface plot, contour plot and 3D graph showing the interaction of pH and rpm for biodiesel production using *Rhizopus oryzae*.

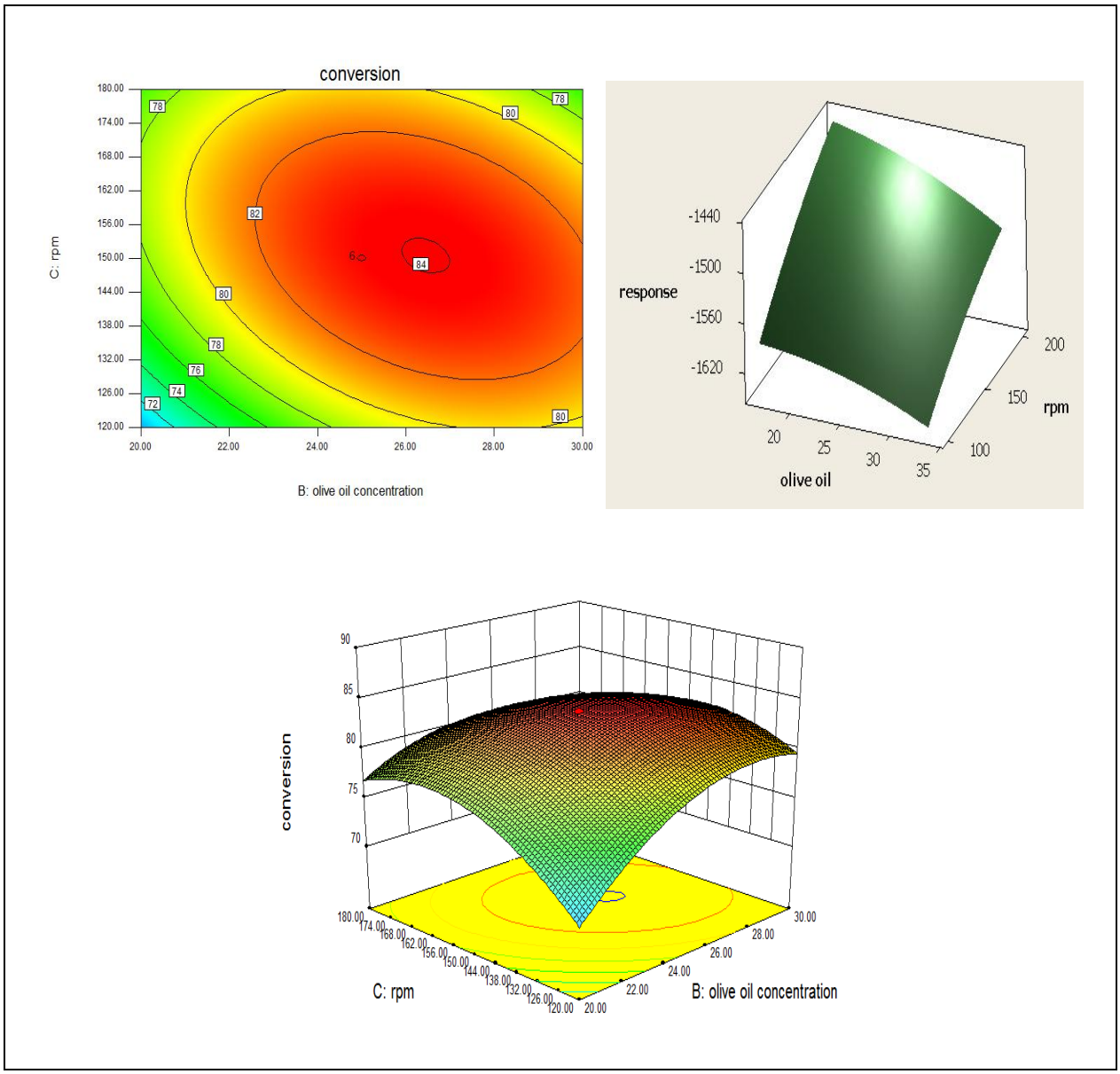

Fig.5. Surface plot, contour plot and 3D graph showing the interaction of olive oil concentration and rpm for biodiesel production using *Rhizopus oryzae*.

4. Conclusion:

Rhizopus oryzae was found to be the efficient organism with good doubling time and moderate pathogenicity. The maximum conversion of Rhizopus oryzae was 84% as whole cell biocatalyst in immobilized calcium alginate beads.The optimization was done through Response Surface Methodology (RSM) procedures. Our research focused on the increased biodiesel yield through the optimized parameters. The preliminary studies needed for choosing the influential parameters were done through the study of one factor at a time. And then the influence of one parameter with another was explored using RSM. Waste cooking oil collected from our college canteen was used as the substrate feedstock and methanol was used as the acyl acceptor. For the process using lipase, the parameters like temperature, time, oil to solvent ratio and enzyme concentration were analyzed through RSM whereas for RO catalyzed reaction, the interaction of pH, olive oil concentration and rpm were explored. The concluded results will provide technical advantages for bioprocess industries relating commercial biodiesel production by biological methods.

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