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OPTIMIZATION OF FATTY ACID GENERATION USING PARAMETRIC ANALYSIS

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Abstract

The objective of this study is to optimize the different parameters to carry out analysis of fatty acids. A kinetic was observed for first order enzymatic hydrolysis of flax seed methyl ester was carried out by using Rhizomucor michei. In this study the analysis of hydrolysis was carried out by varying the temperature (30-40^{o}C) and enzyme load (2-5%). The optimal condition were found to temperature 50^{o}C, 6h reaction time, buffer to flax seed methyl ester ratio 1.5:1(v/w) and 4% enzyme load to achieve a maximum hydrolysis conversion of 97.56%. The effect of temperature on the reaction rate constant and equilibrium constant has been determined using Arrhenius equation. The heat of reaction was found 14.516 KJ/mol. Taguchi's design of experiment L_{16} and L_9 orthogonal array was performed to optimize hydrolysis reaction conditions. Rate of reaction, effect of temperature, enzyme modifier, pH and oil to buffer ratio were considered as a primary influencing parameters which effects the percentage of hydrolysis and fatty acid formed. From the analysis of variance, the influencing parameters on production of fatty acid were reaction time and enzyme modifier. The predicted conversion was found in good rectification with experimental values having R^2=0.9945 and R^2=0.983. Maximum fatty acid formed was 98.76% from methyl ester and 98.92% from oil.

Keywords: Hydrolysis; Kinetics; Flax Seed Oil; Flax Seed Methyl Ester; Optimization; Taguchi Method.

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1. Introduction

The polyunsaturated (PUFAs) are the essential nutrients for human health. Long chain polyunsaturated fatty acids such as docosahexaenoic acid (DHA) and eicosatetraenoic acid (EPA) are the most important structural components of cell membranes in kidney, retina, liver, vital

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tissues of brain and play an important role in maintenance of structural integrity of the cell membranes.[1][2] Recently there has been a growing survey in the biological properties of flax and its beneficial effect on coronary heart disease and hormonal disorders[3].

Oils and fats are triglycerides and their hydrolysis involves reactions with water to produce valuable free fatty acids and glycerol. Fatty acids play very important role in naturally produced renewable raw materials which includes oils from vegetable origin. Manufacturing of high-value product such as lubricating oils, adhesives, coating and cosmetics require huge amount of fatty acids. There are mainly three methods of hydrolysis of oil in the production of fatty acids involves: high pressure steam splitting, enzymatic hydrolysis and alkaline hydrolysis. [4]

Enzymatic hydrolysis of triglycerides may be carried out at ambient conditions and making it energy efficient process as compare with other process [4]. In order to maximize the conversion of yield of esters, attention was given to the optimization of the system parameters (such as enzyme concentration and temperature [5]. The formation of water present in the reaction problem in hydrolysis reaction as it favors the reverse[6]. Use of enzymatic hydrolysis has main technical advantage such as economic, ecological, toxicological and environment free [7].

The previous investigations reveal a merge study on the hydrolysis of flax seed oil or flax seed methyl ester for preparation of fatty acids such as alpha linolenic fatty acid; the literature shows the information on the hydrolysis of flax seed oil using different enzymes. Banin Rupani et al. [1] mentioned that Candida rugosa lipase reacted with triglycerides by hydrolysis process resulted in fatty acids that was enriched in alpha linolenic to about 72% and further reached to 80%. It was observed that concentration of linolenic acid in the product mixture increased from 50.45 to 72.33%. Candida rugosa lipase showed maximum hydrolysis percentage while Rhizomucor michei showed the minimum hydrolysis of flax seed oil. Patricia de O. et al (2009) [8] studied the hydrolysis of acylglycerol using three kinds of native microbial lipases (Aspergillus niger, Rhizopus javanicus and Penicillium solitum). The optimum conditions were enzyme concentration of 500U/g oil, reaction temperature of 45oC and water/oil mass rate 2:1(m/m) after 24 h reaction which gives the degree of hydrolysis 60% led to an increase the docosahexaenoic acid (DHA)[11].

Akhila Rajan et al. (2013) [9] used the novel alkaline lipase from Aspergillus fumigates MTCC 9657 for removal of unwanted fatty acids and enrichment of ω-3 fatty acids. The free fatty acid content was slightly increased after 4hours and it continued up to the end of the process. It was clearly mentioned that enzyme was active for 12h and free fatty was increased from 0.02 to 15%. Sulaiman Al-Zuhair et al. (2013) [13] investigated the kinetics of the enzymatic hydrolysis of palm oil using in a batch reactor. Shau-Wei Tsai et al. (1991) [14] examined the optimal conditions using the lipase-catalyzed hydrolysis of high concentration olive oil in biphasic isooctane-aqueous system. The maximum equilibrium conversion was attained higher than 98% for 0.1 g/mL olive oil.

Generally alpha linolenic acid is present in flax seed oil (50-52%) which can be obtained through hydrolysis. Because of the off flavor and rancidity flax seed oil is not used as a cooking oil in India. As per the literature the study was carried out on the hydrolysis of flax seed oil. In the present study a novel method was developed for the enzymatic hydrolysis of flax seed oil methyl ester as a substrate. The kinetics for enzymatic hydrolysis [12] of flax seed oil methyl ester is examined

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the influence of experimental parameters like reaction temperature, time and enzyme concentration using immobilized lipase Rhizomucor michei as a catalyst. Taguchi method was used to design the best model.

2. Materials and Methods

2.1. Materials

Flax seed methyl ester used in the experiments was 100% pure with a saponification value of 193.82 mg KOH/gm. Lipozyme RMIM (Rhizomucor michei) was obtained from Novozyme A/S, Krogshoejvej 36-2880 Bagsvaerd, Denmark. All the chemicals as methanol, ethyl acetate, potassium hydroxide and phenolphthalein indicator were of analytical grade procured from M/s. Sd Fine Chem. Pvt. Ltd., Mumbai. A phosphate buffer of 7.5 pH was prepared in the laboratory.

2.2. Methods

The fatty acids composition of flax seed methyl ester was analyzed using a Gas Chromatograph Agilent 6890 series equipped with a flame ionization detector in accordance with the AOCS official method Ce le-91. The stationary phase used was a capillary column, DB-225 MS (i.d. 0.25mm, length 30m, 0.5 µm). The oven temperature from 150 to 300oC at 5oC per minute with nitrogen at a flow rate of 35mL.min-1. The injector and detector temperature was programmed from 160 oC for 2 min and 230oC at 5oC per minute and held at this temperature for 20 min. The carrier gas used was nitrogen with a flow rate of 1 mL min-1. The injector and detector temperature were maintained at 230 and 270oC respectively. The area percentage was recorded using an HP Chem Station Data System. The methyl ester was found to be 100% pure. Acid value and Saponification value were determined as per AOCS official method Cd 3-25 (AOCS 2004a and AOCS 2004b).

Method for Preparation of Flax Seed Methyl Ester
Transesterification [21] of flax seed oil was carried out in presence of 1% H2SO4 and methanol under condensation at temperature of 60oC for a reaction time 3h. The samples were drawn at regular interval of time and monitoring the progress of the reaction by determining the thin layer chromatography (TLC). The fatty acids compositions were analyzed by Gas Chromatography. The acid value of the product sample was found to be 0.1034.

2.3. Experimental Procedure

Hydrolysis of flax seed methyl ester was carried out using Rhizomucor michei in presence of phosphate buffer (1.5:1) (v/w). The reaction mixture was incubated at 50oC for 6 h. Samples were drawn at regular time intervals to check the progress of the reaction. After that sample was filtered by filter paper, neutralized with water, dried and analyzed. The amount of fatty acid in the product was estimated by the calculating the acid value.0.5g of sample obtained by hydrolysis reaction were subjected to titration against a 0.1N KOH solution and acid value was calculated. This acid value was used to calculate the percentage hydrolysis.

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The percentage hydrolysis was tabulated using the following formula.

$$\% \text{ Hydrolysis} = \frac{\text{AV of product} - \text{AV of methyl ester}}{\text{SV of methyl ester} - \text{AV of methyl ester}} \times 100 \quad \text{Eq. 1}$$

Where AV is the acid value and SV is the saponification value. Different sets of experiments were carried out with the help of several operating conditions. The first set of experiment were carried out by varying time from 30min to 6h, while the other parameters remain constant i.e. 4% enzyme modifier, buffer to flax seed methyl ester 1.5:1(v/w) and temperature at 50oC. A second set of experiment was carried out by varying temperature range 30 to 60oC, while maintaining other parameters constant i.e. 4% enzyme load and buffer to flax seed methyl ester ratio 1.5:1(v/w), time period of 6h. The third set of experiment was conducted at varying enzyme concentration from 2 to 5% by keeping a same buffer to flax seed methyl ester ratio 1.5:1(v/w) at 60oC.

2.4. Statistical Analysis

The experiment was carried out in duplicate for experimental error estimation and the data was analyzed by a paired student's t-test to evaluate the level of statistical significance. A p-value < 0.05 was considered significance. A p-value of 0.0006 was estimated which was considered as significance.

2.5. Kinetic Model

For this reaction system, first order reversible model was considered (Knezevic et al. 1998). The reaction mechanism for the kinetic model involving reversible reaction is as follows:

$$A + B \Leftrightarrow C + D \quad \text{Eq.2}$$

The rate of reaction is expressed as:

$$r_A = -\frac{dC_A}{dt} = k_1 C_A - k_2 C_c C_D \quad \text{Eq.3}$$

where CA, CC and CD denote the concentration of methyl ester, concentration of mixture of fatty acid and concentration of methanol formed during the reaction, respectively. CB is the concentration of water which was not considered for the developing the kinetic model. k1 and k2 are kinetic rate constants for the forward and backward reactions, respectively.

$$\frac{dX_A}{dt} = k1(1 - X_A) - k_2 C_{AO} X_A^2 \quad \text{Eq.4}$$

At equilibrium, $\frac{dX_A}{dt} = 0$ and XA=XE, and from Eq.4, we get:

$$k_2 = \frac{k_2(1 - X_A)}{C_{A0} - X_E^2} \quad \text{Eq.5}$$

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By substituting the value of k2 in Eq. 4 and rearranging the terms, we get:

$$\frac{dX_A}{dt} = \frac{k_1}{X_E^2}[(X_E - 1)X_A^2 - X_E^2 X_A + X_E^2] \quad \text{Eq. 6}$$

Integrating of Eq. 6 yields and we get:

$$\ln[X_A - X_E] - \ln[X_A(X_E - 1) - X_E] = \frac{(X_E - 2)}{X_E} k_1 t \quad \text{Eq. 7}$$

The conversion as a function of time can be deduced from Eq. 7 as follows:

$$X_A = \frac{X_E(1 - e^{\beta t})}{[1 - X_A e^{\beta t} + e^{\beta t}]} \quad \text{Eq.8}$$

Where $\beta = [k1*(XE-2)/XE]$ Eq.9

Rearranging the terms gives rate constant.

$$k_1 = \frac{\beta X_E}{(X_E - 2)} \quad \text{Eq. 10}$$

2.6. Design of Experiment

Taguchi method [15] is a robust design of experiment [17] method which works on the basis of orthogonal array. It provides the fewest experiments which will give maximum information about influencing parameters present on the system. The experimental results are represented in the terms of signal-to-noise (S/N) ratio. There are three categories of quality characteristic in the analysis of S/N ratio, i.e. the-lower-the-better, the-higher-the-best, and the nominal-the-better. Therefore the optimum condition of the process parameter is the level having the greatest S/N ratio. A statistical analysis of variance (ANOVA)[16] is carried out to identify which process parameters are statistically significant.

2.6.1. Optimization of Percentage Hydrolysis of Flax Seed Methyl Ester Varying the Parameters

Selection of the injecting parameters and their levels (flax seed methyl ester)

In the present study, the experiments were incubated in the 50 ml round bottom flask. The maximum percent hydrolysis was defined by varying the process parameters, i.e. temperature in the range 30-60oC,enzyme load in the range 2-4(wt %), reaction time in the range 2-8 hour, flax seed oil to aqueous ratio in the range 1:0.25- 1:2 (v/w) and pH in the range 5-8.

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Table1: Experimental results for percentage hydrolysis and S/N ratio

Run	A	B	C	D	E	% Hydrolysis	S/N RATIO	Mean
	Temperature	Enzyme load	Reaction time	Methyl ester-aqueous ratio	pH			
1	1	1	1	1	1	87.09	38.7994	87.09
2	1	2	2	2	2	91.54	39.2322	91.54
3	1	3	3	3	3	95.98	39.6436	95.98
4	1	4	4	4	4	98.79	39.8943	98.79
5	2	1	2	3	4	95.72	39.6201	95.72
6	2	2	1	4	3	94.20	39.4810	94.20
7	2	3	4	1	2	92.96	39.3659	92.96
8	2	4	3	2	1	91.44	39.2227	91.44
9	3	1	3	4	2	90.08	39.0926	90.08
10	3	2	4	3	1	88.70	38.9585	88.70
11	3	3	1	2	4	98.11	39.8343	98.11
12	3	4	2	1	3	96.72	39.7103	96.71
13	4	1	4	2	3	92.74	39.3453	92.74
14	4	2	3	1	4	96.75	39.7130	96.75
15	4	3	2	4	1	89.70	39.0558	89.70
16	4	4	1	3	2	93.71	39.0558	93.71

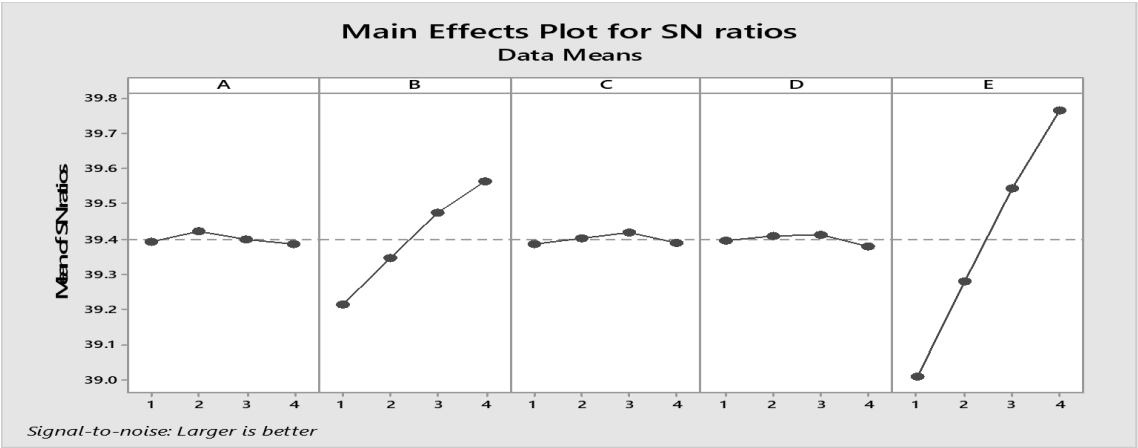

Figure1: S/N ratio graph

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Development of Regression Model

The complete design matrix of the experiments coupled with the experimental yield, S/N ratio and mean was shown in Table 1. The Minitab 17 was generated the best fitted model as shown in Table 2. According to the sequential model sum of square, ...The regression model equation representing the effect of temperature, enzyme load, reaction time, buffer to methyl ester ratio and pH in terms of their levels, is given as,

% Conversion = 83.643 - 0.0553 A + 1.2662 B + 0.0202 C - 0.0492 D + 2.7167 E

The value of regression of determination R2 for the above equation is 0.9945. The regression model explained 99.45% of the total variability in percent hydrolysis conversion.
Analysis of variance (ANOVA) was further carried out to determine the significance and the fitness of the linear model.

Table 2: Sequential model sum of squares

Source	**DF**	**Adj. Sum of Square**	**Adj. Sum of square**	**F-Value**	**P-Value**
Regression	5	179.800	35.960	358.55	0.000
Temperature	1	0.061	0.061	0.61	0.453
Enzyme Load	1	32.068	32.068	319.74	0.000
Reaction Time	1	0.008	0.008	0.08	0.781
Methyl ester and buffer ratio	1	0.049	0.048	0.48	0.503
pH	1	147.615	147.615	1471.82	0.000
Error	10	1.003	0.100		

Confirmation Test

The confirmation test is used to verify estimated result compare with the experimental results. If the optimal combination of parameter and their levels coincidently match with one the experiment in the orthogonal then the confirmatory test is not required.

Confirmation test was required in the present study because combination A2B4C3D3E4 did not correspond to any experiment of the orthogonal array. One set of experiment at an optimal combination of parameter and their levels i.e. A2B4C3D3E4 was produced on the same method and from the same material. Percent error was calculated by using following formula.

$$\% \text{ Error} = \frac{\text{Theoretical Value} - \text{Experimental Value}}{\text{Theoretical Value}} \times 100$$

Table 3: Result from confirmation experiments

	Optimum Condition		
	Estimated	**Experiment**	**Difference**
Level	A1B4C4D4E4	A2B4C3D3E4	-
% Hydrolysis	98.79	98.92	0.1315

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3. Results and Discussion

3.1. Effect of Reaction

The rate of hydrolysis of the reaction depends on the reaction time. Reaction time was optimized by keeping experiments at an enzyme concentration of 4%, 50oC, buffer to flax seed methyl ester 1.5:1(v/w). Figure shows a linear plot as a function of conversion and reaction rate. From figure it can be concluded that reaction proceeds faster during the initial 30min and conversion increases as the reaction time increased. The most suitable period for the hydrolysis of flax seed methyl ester was found to be 6h.

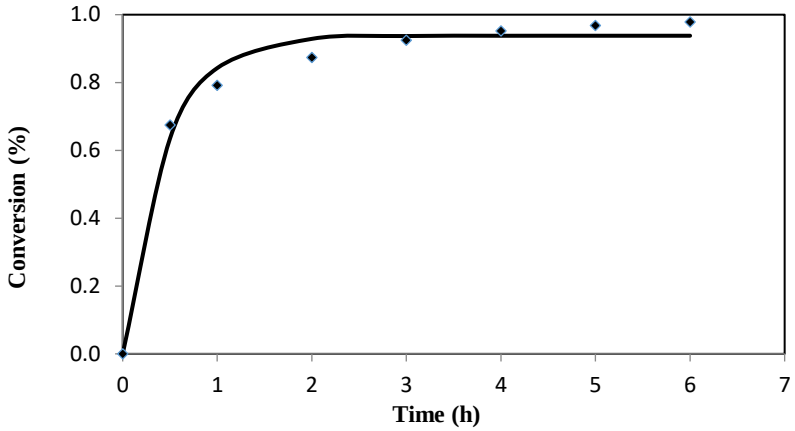

Figure 2: Effect of rate of reaction on hydrolysis of flax seed methyl ester.(Reaction temperature 60oC, enzyme concentration 4%, buffer to flax seed methyl ester ratio of 1.5:1(v/w))
♦ experimental values −−−model values

3.2. Effect of Enzyme Load

Generally, lipase catalyzed reaction take place at the interface, and the amount of enzyme available at the interface is very important. To determine the effect of enzyme on the system, the enzyme concentration was varied from 2 to 5% with buffer to methyl ester ratio 1.5:1(v/w) at 50oC for 6 h. The effect of enzyme on the given process is shown in figure. When the enzyme concentration was 2% the conversion was 89.45%, at 4% enzyme load, the conversion was 97.74%. A further increase in enzyme concentration to 5% did not yield any further increase in conversion. Hence this value was considered as optimum condition.

3.3. Effect of Reaction Temperature

The temperature effect on the hydrolysis reaction at 4% enzyme concentration, buffer to methyl ester 1.5:1(v/w) and 6 h reaction time is shown in Figure. It was concluded that the reactant temperature increases the conversion rate also increases. At 30oC, the conversion was 80.78% and as the temperature increases the conversion also increases at 40oC, the conversion 89.42%, a maximum conversion reached at 50oC, 97.41% was measured. A further increase in the temperature 60oC, there was sudden fall on conversion to 85.43%.

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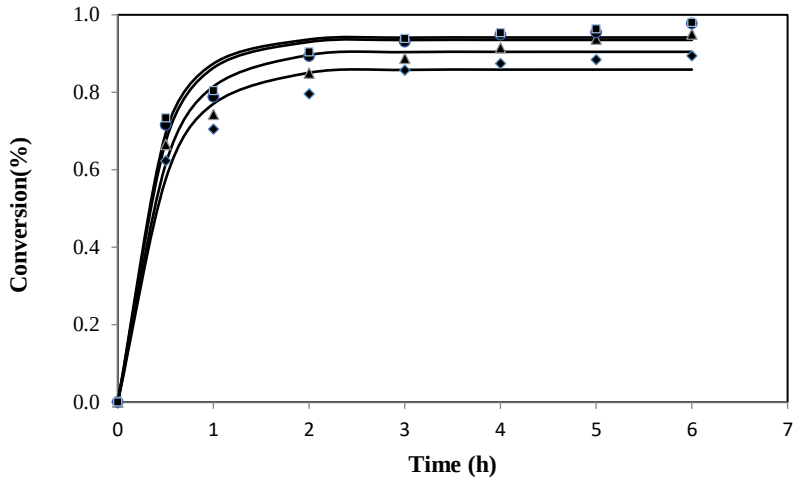

Figure 3: Effect of enzyme concentration on hydrolysis of flax seed methyl ester.
(Reaction time 6 h, buffer to methyl ester ratio of 1.5:1(v/w), temperature 60oC)
♦2% ▲3% ■4% ●5% ––– model values

3.4. Applying the Kinetic Model

The derived kinetic model equation for first order reversible reaction was fitted to the experimental data and the two parameters, Xe and β of Eq.9 were determined by non linear regression with a Levenberg-Marquardt[19] algorithm using statistical software. A regression co-efficient value is 0.969, it seems model is statistically significant and inter predict the relationship between the experimental and theoretical parameters. The values of rate constant k1 and K2 for the forward reaction and backward reaction was calculated from Eq.10 and Eq.5 respectively. The results obtained for the rate constants and the equilibrium conversion have been tabulated in Table 3.

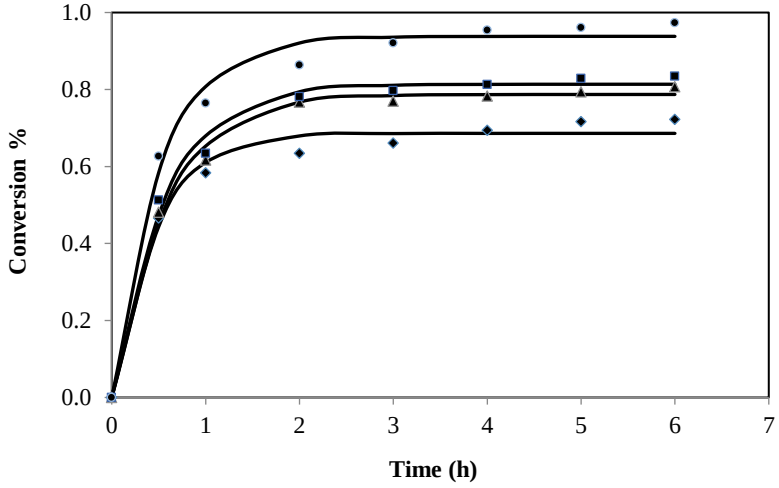

Figure 4: Effect of rate of reaction on hydrolysis of flax seed methyl ester.
(Reaction temperature 60oC, enzyme concentration 4%, buffer to flax seed methyl ester ratio of 1.5:1(v/w))
♦ experimental values –––model values

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Table 4: Equilibrium conversion, kinetic rate constants and equilibrium constant for the hydrolysis of methyl ester at different reaction temperatures

Temperature(oC)	Xe	k1	k2	K
30	0.78715	1.253479	0.671094	1.867816
40	0.84637	1.590571	0.511203	3.111427
50	0.93814	1.78824	1.195177	1.496214
60	0.81886	1.457673	0.72494	2.01075

The effect of temperature on the forward reaction rate constant was obtained by keeping k1 to the Arrhenius equation (Eq.11 and Eq. 12).

$$k = Ae^{\left[\frac{-\Delta E}{RT}\right]} \qquad \text{Eq.11}$$

And

$$\ln k_1 = \frac{-\Delta E}{RT} + \ln A \qquad \text{Eq.12}$$

For forward reaction, rate constant k1 is calculated using Eq.10. From the plot of lnk1 as a function of the reciprocal temperature, as shown in Figure 5, for 4% enzyme concentration, the frequency factor, A, and the energy of activation, ΔE, were formed to be 0.404×103 and 14.516 KJ/mol, respectively.

The fitting of the experimental data to the proposed model is also assessed by comparing the experimental conversion values with the theoretically predicted conversions using Eq.7, and is presented in Figure.5. A good agreement between the experimental conversion and values calculated from Eq. 7 was absorbed. Since the p-value for the model was lower than 0.05 there was statistical relation between the response and the selected variables at 95% confidence level. It can be inferred that the proposed model represented the present reaction system satisfactorily.

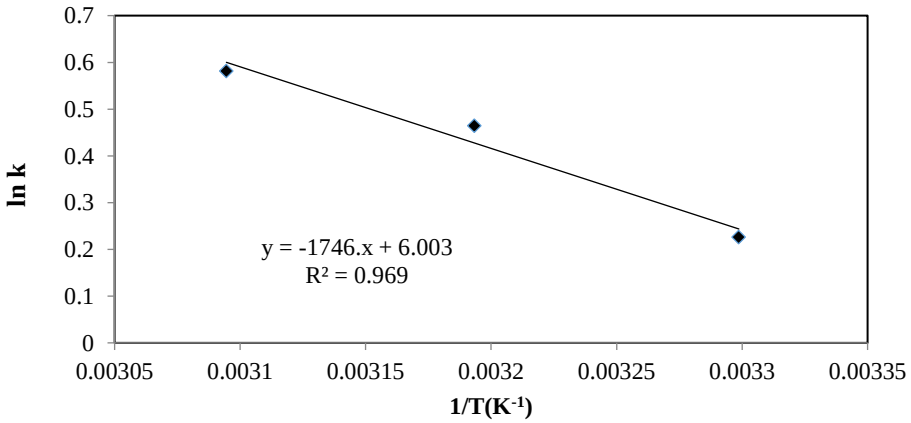

Figure 5: Effect of reaction temperature on reaction rate constant

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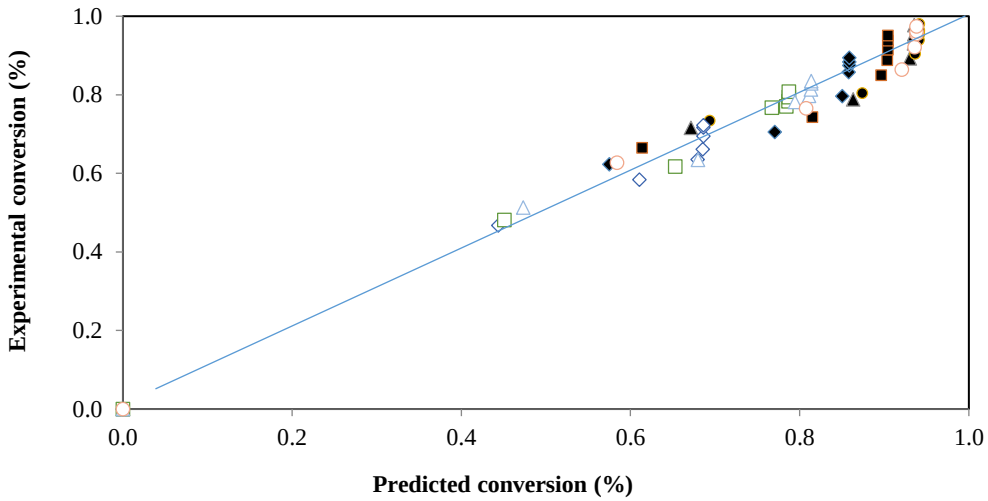

Figure 6: Comparison of experimental and predicted conversion

4. Conclusion

The experimental results and the analysis of the same has clearly shown that, the predicted values match closely with the observed values of fatty acid generation using the proposed optimization method. The results depicted in Figure 6 show the correlation between the predicted and observed conversion percentages. It is evident that the major contributing parameters for the conversion percentage are reaction time and enzyme modifier. Controlling the same as per requirement will always yield the necessary volume of fatty acids with a considerable accuracy. Thus it can be concluded that the proposed method of optimization fares well in controlled environment for production of fatty acids.

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