GREEN SYNTHESIS OF THE FLAVOR ESTER BUTYL BUTYRATECATALYZED BY NOVOZYME 435: A STATISTICAL APPROACH

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Abstract

Butyl butyrate (C8H16O2) is one of esters of short chain fatty acids and alcohols that are important components for flavoring having fruity aromas. Adhering to green chemistry, enzymatic synthesis was performed for the production of butyl butyrate catalyzed by recombinant*Candida Antarctica* expressed in*Aspergillusniger.* The acrylic resin immobilized commercial lipase (Novozyme435) was the biocatalysts of choice to catalyze the esterification reaction to produce butyl butyrate. The enzymeassisted esterification was optimized using the statistical approach of Response Surface Methodology by the Box-Behnken Design (BBD). In this study, the reaction parameters evaluated were substrate molar ratio, time and temperature, while the response of each parameter was measured as percentage conversion yields. UsingtheDesign Expert 7.1.6 optimization function, the conditions with the highest desirabilityand percentage conversionvalues were selected to obtain the optimized conditions for the Novozyme 435 catayzed esterification reaction. Two sets of optimum conditions were selected and afforded the highest conversions of 99.62% and 99.55% using (i) molar ratio butyric acid:butanol 1:3.93, 9.93 h at 56.09°C and (ii) molar ratio butyric acid:butanol1:3.35, 9.79 h at 53.90°C, respectively. The esterification of butanol and butyric acid to produce butyl butyrate was monitored using Thin Layer chromatography (TLC). The production of butyl butyrate was confirmed by various methods which include FTIR Spectroscopy: Attenuated Total Reflectance (ATR) and Gas chromatography (GC).

Keywords: butyl butyrate;Novozyme 435; Box-Behnken Design; response surface methodology

INTRODUCTION

Butyric acid is commonly used as an industrial intermediate in the preparation of various butyrate esters and food additives. The ester form of the compound is not genotoxic and generally recognized as a safe food additive used in accordance with good manufacturing practice (Karimiand Vahabzadeh, 2014).However, the chemical route to produce the esters is often linked with numerous shortcomings which include the use of environmentally unfriendly corrosive acids such as hydrogen fluoride or sulphuric acid as catalyst, and metal halides (Mohamad*et al*., 2015). Ester synthesis *via*the biotechnological route offers high activities in organic solvents and conversion of a broad number of substrates with high stereospecificity (Ferraz*etal*., 2014).

Lipases (triacylglycerol ester hydrolysis EC 3.1.1.3)are medicinally, industrially and commercially important enzymes that have grown increasingly popular as biocatalysts (Saleh*et al*., 2007) over inorganic catalysts due to its high specificity, efficient reaction rate, non-toxicity, biodegradability and reproducibility under normal laboratory conditions (Zhou *et al*., 2012).The immobilization of enzymes to solid support materials confers great benefits such as improvement of the activity and stability of enzymes, possibility recovery of the immobilized enzyme at the end of the reaction and thus, its potential reuse of the enzyme-catalyzed reaction under continuous process (Kharrat*etal*., 2011). Furthermore, catalysis in a solvent-free system offers the added benefits of minimizing environmental impact by avoiding the use of flammable organic, toxic solvents and simpler downstream processing (Wahab*et al*.,2014).

EXPERIMENTAL

Esterification of butyl butyrate

The solventlesssynthesis of butyl butyrate was carried out in a 100 ml round bottom flask that consisted of butanol (1.5 M) and butyric acid (0.5 M). The Novozyme 435 were added to initiate the reaction and refluxed at 150 rpm, temperature (35–65°C). Sample (0.2 ml) waswithdrawn and titrated with NaOH, 0.05 M using phenolphthalein as indicator.In this reaction, the following analyses were performed to confirm the production of butyl butyrate. Three instruments were used to monitor production of butyl butyrate namely as Thin Layer Chromatography (TLC), FTIR Spectroscopy: Attenuated Total Reflectance (ATR) and Gas chromatography (GC).

Experimental design and optimization forNovozyme 435 catalyzed esterification

A three-factor, five-level Box-Behnken design (BBD) that required 17 experiments was used in this study to evaluate the parameters. Experimental data obtained for the lipase-catalyzed synthesis of butyl butyrate are

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shown in Table 1. The variable and level selected for the Novozyme 435 catalyzed esterification production of butyl butyrate were molar ratio acid to alcohol 1:1–1:5, incubation time 2–10 h and temperature 35–65°C.

Table 1: Process variables and their levelsused in Box-BehnkenDesign

Variables		Ranges and levels		
		−1	0	+1
A:	molar ratio (alcohol/acid)	1	3	5
B:	Incubation time (h)	2	6	10
C:	temperature (°C)	35	50	65

A software package by Design Expert Version 7.1.6 (State-Ease Inc., Statistics Made Easy, Minneapolis, MN, USA) was used to fit this model to the independent variables using model equation to predict the optimum conditions and subsequently elucidate the interaction between the variables. The quadratic polynomial equation model for predicting the optimal point are expressed according to Equation 1:

$$y = b_0 + \sum_{i=1}^{4} b_i x_i + \sum_{i=1}^{4} b_{ii} x_i + \sum_{i=j}^{3} \sum_{j=i+1}^{4} b_{ij} x_{ij} + e \qquad \text{(Equation 1)}$$

Where$y$ is the response variable to be modeled; x_i and x_j are independent variables (time, temperature, molar ratio and stirring rate); b_0, b_i, b_{ii} and b_{ij} are the regression coefficients of the model and e is the error of the model.The coefficient of determination (R^2) could be used to evaluate the accuracy and general ability of the second order multiple regression models. The fitted polynomial equation was expressed as response surface and contour plots in order to visualize the relationshipbetween the response and experimental levels of each factor, as well as to deduce the optimum conditions.

RESULTS AND DISCUSSION

Employment of statistical tools such as RSM is particularly beneficial as the method relatively simplifies the reaction parameters needed to establishthe optimum conditions of a particular reaction. In this study, the Box-Behnken design consisted of threefactors and three levels that evaluated variables: substrate molar ratio (A), time (B) and temperature (C) on the response, namely, to achieve high percentage yield of butyl butyrate while establishing the optimized conditions of the reaction. As seen from Table 2, the predicted values obtained with a model fitting technique were observed to be sufficiently correlated with the observed values. In this study, the best fitting model was established by a regression analysis. Fitting of the data to various models that include linear, two factorial, quadratic and cubic models. Based on the data collected, the subsequent ANOVA revealed that the esterification of butanoland butyric acid to produce butyl butyrate was appropriately described with a quadratic polynomial model as depicted in Equation 2:

$$\text{Conversion} = + 79.04 + 5.55A + 25.50B + 2.30C + 0.75AB + 2.15AC + 3.25BC - 11.70A^2 - 5.60\ B^2 - 12.69C^2 \qquad \text{(Equation 2)}$$

Statistical testing of the model was done using the Fisher's statistical test for ANOVA and the results are tabulated in Table 3. It is pertinent to highlight here the significance of a model is represented by a P-value < 0.05. According to the computed F-value (300.15) of the model, it is considerably higher than the tabular $F_{0.05\ (9,7)}$= 3.68, indicatingthe degree of freedom relative to residual obtained for the model was significant at the 5% confidence level. The small *P*-value (<0.0001) indicated the model was highly significant and a suitable coefficient of (R^2= 0.9974) implies that the quadratic polynomial model was sufficient to explain the actual relationship between the response and the variables. The obtained R^2suggests that the total variation consisted more than 99% for the acid conversion to the independent variables. Onlyless than 1% of the total variation that could not be explained by the model.

It has been described in literature that the experimental and predicted values are well correlated when the value of R (multiple correlation coefficients) approaches the value of 1 (Pujari*et al*., 2000). The ANOVA (Table 3) also revealed that all of the independent variables (A: molar ratio, B: time; C: temperature) were statistically significant (P-value < 0.0001) for the Novozyme 435 catalyzed esterification of butanol and butyric acid to

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produce butyl butyrate.All terms that represented the mutual interaction between variables were found to be significant (AC: molar ratio x temperature; BC: time x temperature) for the exception of AB (molar ratio x time). However, the insignificant term was retained in the model in order to preserve the required hierarchy of the model (Piexoto, 1990).

Table 2: Experimental and results of the Box-Behnken design

Run	Substrate molar ratio	Time (h)	Temperature (°C)	Yield conversion %	Predicted Conversion (%)
1	3	6	50	79.5	79.04
2	3	2	35	38.0	36.20
3	3	6	50	79.5	79.04
4	5	6	35	55.0	55.75
5	1	6	35	48.6	48.95
6	1	2	50	30.0	31.45
7	3	6	50	80.0	79.04
8	1	10	50	82.0	80.95
9	3	10	35	80.0	80.70
10	5	10	50	95.0	93.55
11	5	2	50	40.0	41.05
12	3	10	65	90.0	91.80
13	5	6	65	65.0	64.65
14	3	6	50	78.1	79.04
15	3	6	50	78.1	79.04
16	3	2	65	35.0	34.30
17	1	6	65	50.0	49.25

Table 3: Analysis of variance and model coefficients

Source	Sum of squares	Degree of freedom	Mean Square	F-value	P-value	
Model	7082.24	9	786.92	300.15	< 0.0001	significant
A-molar ratio	246.42	1	246.42	93.99	< 0.0001	
B-time	5202	1	5202	1984.2	< 0.0001	
C-temperature	42.32	1	42.32	16.14	0.0051	
AB	2.25	1	2.25	0.86	0.3851	
AC	18.49	1	18.49	7.05	0.0327	
BC	42.25	1	42.25	16.12	0.0051	
A2	575.89	1	575.89	219.66	< 0.0001	
B2	131.81	1	131.81	50.27	0.0002	
C2	678.58	1	678.58	258.83	< 0.0001	
Residual	18.35	7	2.62			
Lack of Fit	15.24	3	5.08	6.53	0.0508	not significant
Pure Error	3.11	4	0.78			
Cor Total	7100.6	16				

Figure 1 illustrates the comparison between predicted and actual butyl butyrate yield (%). The graph represents the values of the experimental and predicted yields which correlated well ($R^2 = 0.9974$). The adjusted R^2 value (0.9941) was found to be close to the R^2value which confirms that the quadratic polynomial model is adequate to explain the actual relationship between the response and the significant variables. The high value of adequate precision (50.006) strongly indicates that the signal to noise ratio obtained for this model is satisfactory. According to the ANOVA factors (Table 3) the F-value for the lack of fit was 6.53, is lower than the tabulated value of $F_{0.05\ (3,\ 4)} = 6.59$, implying that the lack of fit is not significant relative to pure error. This indicates the data in the experimental domain are well represented by the model (Nuthalapati*et al*., 1999). The lack of fit measures the failure of the model to represent the data in the experimental domain at points which are not included in the regression. The non-significance of the lack of fit shown here signifies that model equation is sufficient for predicting the percentage conversion of butyl butyrate under any combination of values of the variables.

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Effect of process variables on the percentage yield of butyl butyrate

Figure 2 illustrates the interaction between percentage yield of butyl butyrate and parameters evaluated: molar ratio (A), time (B) and temperature (C). The percentage yield that corresponded to A increased steadily with the increment of substrate molar ratio acid to alcohol. However, the conversion slightly decline when the molar ratio of butanol:butyric acid approached molar ratio acid:alcohol of 1:4. Lower conversion of butyl butyrate is observed at molar ratio of 1:1 and conversely, the highest conversion was attained when the molar ratio was increased to 1:3. The outcome shows that Novozyme435catalyzed esterification of butanol with butyric acid to produce butyl butyrate was more favourable at higher alcohol to acid molar ratio, consistent with previous reports by Salihu and co-workers (2013).

Meanwhile, for B, the percentage yield of butyl butyrate revealed a drastic increment with the increase in incubation time. It was observed that the yield ofbutyl butyrate increased to attain the highest yield of the ester at 10 h. The rather steep ascending trend demonstrated by B is reflected by a very large *F*-value (1984.2) as compared to A (93.99) and C (16.14), hence, confirming time is the most important variable in the esterification reaction. The variable, C: temperature, illustrated a steady upward trend of percentage conversion of ester, consistent with reports of the slow unravelling of the lipase structure when the reaction temperature is increased (Chaibakhsh*et al*., 2009). As seen in Figure 2, the optimum temperature for this study was approximately 50°C to afford the highest percentage conversion of butyl butyrate but concentration of the ester decline when the temperature exceeded ~50 °C.

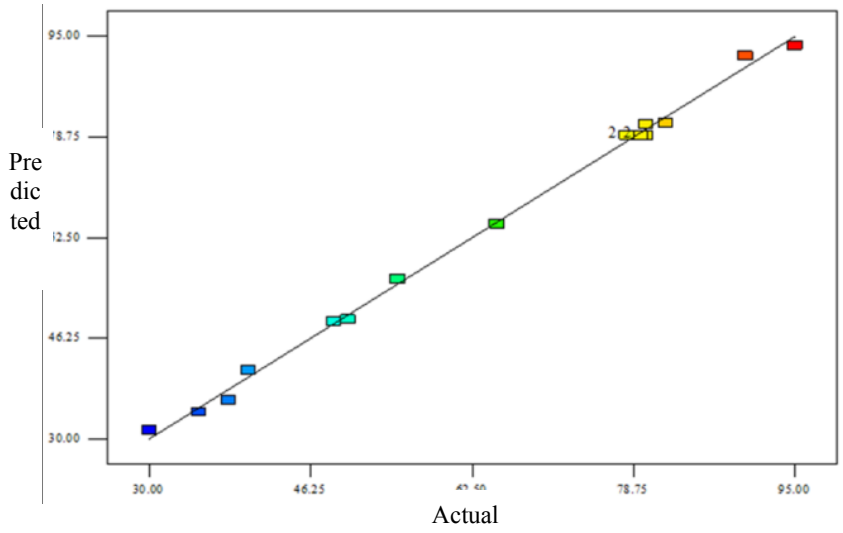

Figure 1: Comparison between the predicted and actual values for the percentage yield of butyl butyrate in the esterification reaction catalyzed by Novozyme 435.

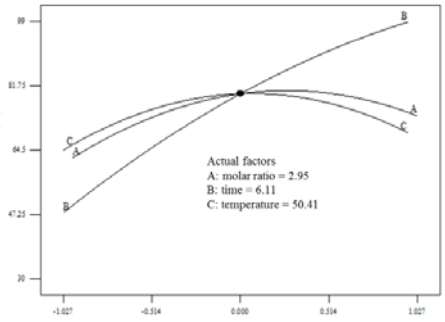

Figure 2: The deviation of the reference point for the percentage conversion of butyl butyrate for the effect of A) molar ratio, B) time and C) temperature.

Effect of molar ratio and temperature

It was observed that the interactions between the two reaction variables were significant because of the small P-value (0.0327). According to the ANOVA of factors (Table 3), the F-value indicates that the effect of substrate molar ratio (93.99) is more significant compared to the temperature (16.14). It was clear that the mutual interaction between the independent variables (A: molar ratio; B: temperature) exhibited a strong and perfect interaction as implied by the obtained elliptical contours (Figure 3). A maximum predicted yield is acquired and represented by the surface confined by the smallest ellipse in the contour plot. The interaction between temperature and molar ratio are relatively significant and appeared to be strong, however, their mutual interaction was less favorable (*F*-value = 18.49) when compared to interactions between time and temperature (*F*-value = 42.25) (Table 3). It can be seen that a percentage of conversion of butyl butyrate as high as approximately 80 % could be attained when the molar ratio of acid:alcohol is between 1:3 to 1:4 and reaction temperature in the range of 47 to 55 °C. The production of the ester increases as the temperature increases can be due to the increase in the kinetic energy of the system. Such increase promotes collision between enzymes and substrate molecules, hence inducing more effective interaction between enzyme and substrates. These changes are likely to acceleratethe production rate of the ester product (Abdul Rahman*et al*., 2011, Wahab*et al*., 2014).

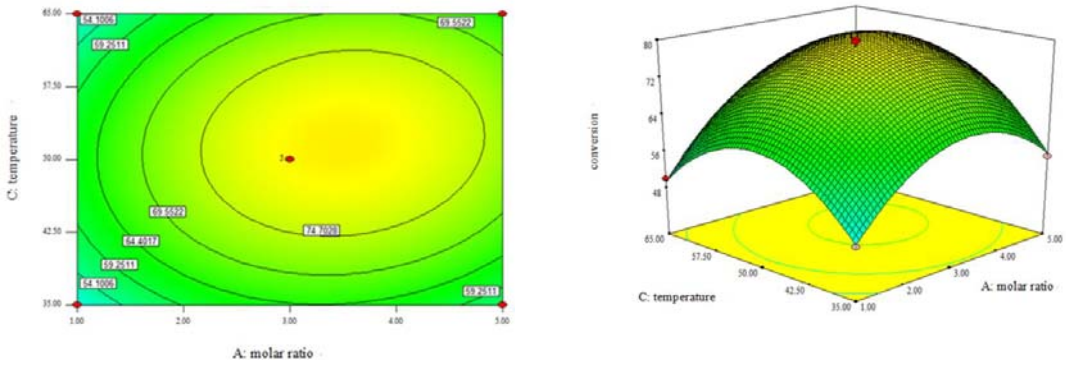

Figure 3: Contour and surface response plots showing the mutual interaction of substrate molar ratio (A) and temperature (C) at constant time.

Effect of time and temperature

Figure 4 illustrates the response surfaceand contour plot for the effect of time (B) and temperature (C), and their mutual interaction on the enzymatic synthesis of butyl butyrate at constant molar ratio of 1:3 (acid to alcohol). It is observed that interactions of the two reaction parameters are significant because of the small *P*-value (0.0051). The effect of time on the yield of butyl butyrate is very significant as compared to the temperature of the reaction. This is evident from the F-value that revealed the effect of time (1984.20) is considerably larger compared to the temperature (16.14), indicating that temperature played a crucial part in governing the yields of the ester. The figure also showed a fascinating interaction between the two process variables. Both variables had to be increased to their maximum values in order to achieve high yields of the ester, hence signifying the direct proportionality between time and temperature of the reaction.

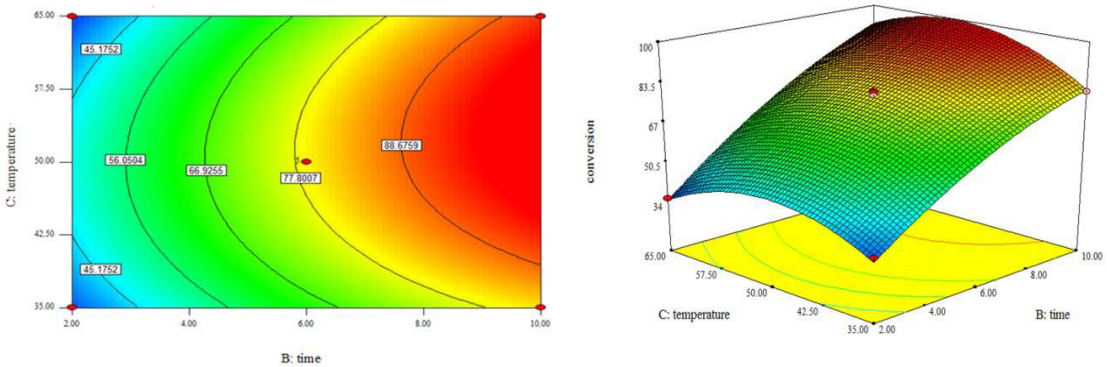

Figure 4: Contour and surface response plots showing the mutual interaction of time (B) and temperature (C) at constant

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molar ratio of 1:3 (butyric acid: butanol).

From the investigation, it was apparent fromthe contour and surface plots (Figure 4) that a yield of butyl butyrate as high as 90% was attainable at between 8 to 10 h and at any value of temperature between 50 to 57.5 °C. On the other hand, it also revealed when both variables were set at their lowest values, the percentage conversion of butyl butyrate was the lowest (45.18 %). The results implied two things: (1) time is one of the key parameters that may decide on the extent of the conversion of butyl butyrate and (2) enzyme activity can be improved by increasing both time and temperature of reaction. Prolonging the contact time between the enzyme and substrates was seen to considerably improve the yield of butyl butyrate. The ascending trend in percentage conversion of the ester, with the increase of reaction time could be associated with the increased contact time between the enzymes and substrates in the reaction, thereby and enhancing propensity of effective collisions (Liu *et al*., 2014). The results seen here evidently show reaction time has a positive effect on the esterification reaction that is also corroborated by a positive coefficient for the factor of time in the predictive equation (Equation 1).

Attaining optimum conditions of the model

Response surface methodology can be employed to determine the optimal combination of parameters in the way of obtaining the highest percentage yield (Salihu*et al*., 2014). To check adequacy of the model equation, some sets of confirmation experiments were carried out within and outside of the design space based on the established optimum conditions (Salihu*et al*., 2014) by the BBD. From the various experimental runs, the highest percentage yield obtained was at 95% which corresponded to substrate molar ratio acid:alcohol (1:5) at 50°C and 10 h of incubation time. UsingtheDesign Expert 7.1.6 optimization functions, the experiments with the highest desirabilityand percentage conversionwereemployed to obtain the optimalconditions for the Novozyme 435 catayzed esterification.Two sets of optimum conditions (Table 4) were selected and afforded the highest conversions of 99.62 % and 99.55 % using (i) molar ratio butyric acid:butanol 1:3.93, 9.93 h at 56.09 °C and (ii) molar ratio butyric acid:butanol 1:3.35, 9.79 h at 53.90°C, respectively.

Table 4: The attained optimum conditions for the Novozyme 435 catalyzed enzymatic synthesis of butyl butyrate

	molar ratio	Time (h)	Temperature(°C)	Conversion Yield (%)	Desirability
30	3.93	9.92	56.09	99.62	1
34	3.35	9.79	53.90	99.55	1

Analysis of butyrate

The peak to confirm the formation of butyl butyrate (after 10 h incubation) wasobserved as intense absorption in the spectrum (Figure 5) at 1736.58 cm-1. The peak for aliphatic ester C–O stretch was observed at 1181.44 cm^{-1} (Stuart, 2005).

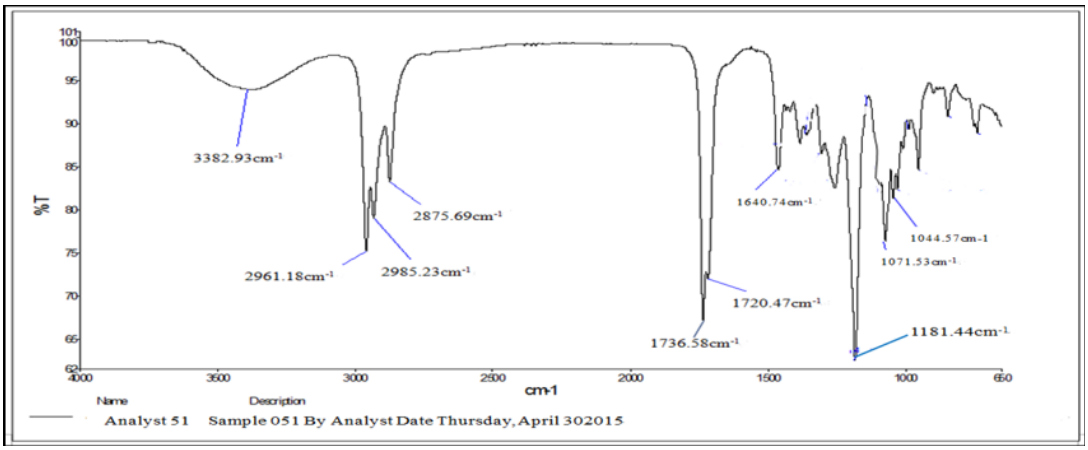

Figure 5:: FTIR spectrum of reaction mixture acid alcohol (1: 3) catalyzed with Novozyme 435 in 10 h.

Based on Figure 6, in comparison, there were significant differences for the peak butanol and butyric acid (Figure 6a) and butyl butyrate (Figure 6b) in both chromatograms corresponding to 0 h and 10 h reaction time. Both of the chromatograms illustrate hexane peaks appeared first at the lowest retention time due to its low boiling point. This pattern of peaks follows suchpatterns which were in Figure 6a, the peak of butanol appeared at retention time of 3.077 min followed by butyric acid at of 5.244 min. However, in Figure 6b, the peaks corresponding tobutanol and butyric acid were clearly not observed for the exception of a peak that belonged to butyl butyrateobservable at 5.300 min.

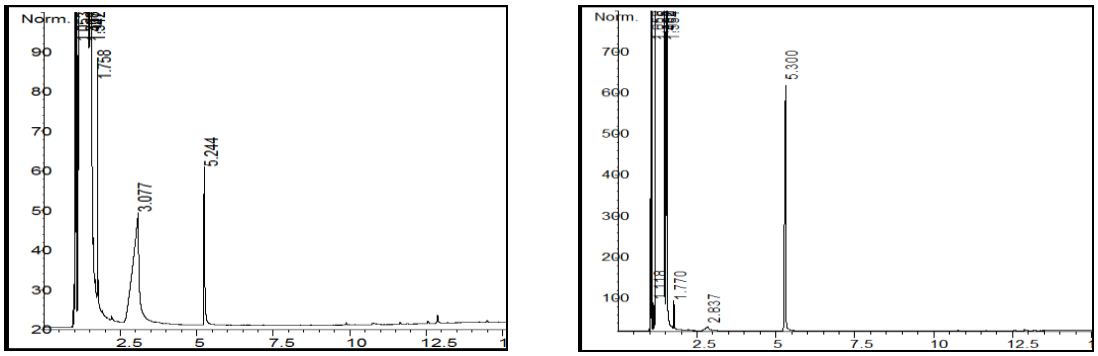

Figure 6: GC FIDChromatogram of the reaction mixture for synthesis of butyl butyrate catalyzed by Novozyme 435, at (a) 0 h and (b) 10 h.

CONCLUSION

The present study successfully evaluated the optimized conditions suggested by the response surface methodological technique i.e. BBD utilizing Novozyme 435 as biocatalysts for producing butyl butyrate *via* a solvent-less reaction system. The outcome strongly suggests that Novozyme 435 was able to produce high yield of the ester. Results of the FTIR and GC analyses further proved the presence of high quantities of butyl butyrate with the complete absence of butyric acid (limiting reagent) and limited amount of the excess butanol. Percentage conversion of butyl butyrate was achieved by using statistical approach utilizing Response surface methodology by the Box-benkhen Design (BBD) using three variables namely as substrate molar ratio, time and temperature. Experimentally, it was found that the highest yield of conversion of 95.0 % was obtained at optimized condition of 50°C, 1:5 (butyric acid:butanol), enzyme content 5 mg/mlby substrate volume at 10 h reaction. By using Design Expert 7.1.6 optimization functions, the model predicted highest percentage conversion at two optimized condition (i) 99.62 % in optimized conditions of butyric acid: butanol molar ratio of 1: 3.93, 9.93 h at temperature of 56.09°C and (ii) 99.55% in optimized conditions of butyric acid: butanol molar ratio of 1: 3.35, 9.79 h at temperature of 53.90°C.

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