IOSR Journal of Applied Chemistry (IOSR-JAC)
e-ISSN: 2278-5736. Volume 3, Issue 5 (Jan. – Feb. 2013), PP 31-43
www.iosrjournals.org

High Performance Enzyme-Catalyzed Synthesis and Characterization of a Nonionic Surfactant

Atena Adnani*[1], Naz Chaibakhsh[2], Hossein Abbastabar Ahangar[1], Mahiran Basri[3] , Raja Noor Zaliha Raja Abdul Rahman[4], Abu Bakar Salleh[4]
[1]*Faculty of Science, Islamic Azad University-Najafabad Branch, Najafabad, Esfahan, Iran*
[2]*Department of Chemistry, Faculty of Science, University of Guilan, Rasht, 4199613769 Guilan, Iran*
[3]*Faculty of Science, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia*
[4]*Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Malaysia*

Abstract: *Sugar alcohol esters have a high potential for widespread application in various industries because of their surface active properties. In this work, fatty acid ester of a sugar alcohol was produced through Novozym 435-catalyzed esterification of xylitol and capric acid in nonaqueous media. Taguchi orthogonal array method based on three-level-six-variables (L_{27}) and artificial neural network with Levenberg–Marquardt algorithm were applied to evaluate the effects of synthesis parameters and to optimize the reaction conditions. Both developed models have shown good quality predictions in terms of the conversion of xylitol caprate with a high R^2 (>0.9) and a low mean square error (MSE). The maximum conversion of ester achieved was 88% requiring a small amount of enzyme and molecular sieve. Furthermore, the properties of the produced ester show that it is a suitable emulsifier for industrial application.*

Keywords- *Lipase, Xylitol ester, Surfactant, Optimization, Taguchi method, Artificial neural network*

I. Introduction

Fatty acid esters of sugar alcohols have several interesting and potentially useful properties such as surface and emulsifying activity, antitumor activity and antibacterial effects. Because of their amphiphilic nature, nontoxicity and biodegrability, sugar alcohol esters are widely used as surfactants in food, cosmetics, detergent, pharmaceutical and biomedical industries [1]. The classical chemical method of synthesis of these esters faces several major drawbacks. The conventional approach using chemical catalysts requires high temperature which causes product coloration, high energy consumption and the recovery of considerable amount of side products [2]. However, the use of biological catalysts has a great potential for synthesis due to the direct use of unmodified substrates, mild reaction conditions, low energy requirement and high regioselectivity of the biocatalyst [3].

Modeling and optimization of an esterification process plays an important role in the economical manufacturing of the ester on the basis of quality, cost, and the process performance. The classical method of optimization involves varying one variable at a time (OVAT) and keeping the others constant. The OVAT approach is not only time-consuming but also cannot depict the complete effects of the parameters on the process and ignores the combined interactions between the parameters [4]. In contrast, Taguchi orthogonal array method is an effective statistical technique. It is very helpful in optimizing experimental conditions and can be employed to investigate the experimental space using a small number of experiments [5]. Taguchi method can also be important in studying the influence of several factors on the process performance to find out which factor has more effect on the response. Robustness is a necessity in nearly all modeling and optimization methodologies. The variation in the quality of a product may result from environmental and manufacturing variables as well as the noise factors, which cannot be easily controlled. The Taguchi method uses signal to noise (S/N) ratio as a measure in determining the robustness of a process. Therefore, optimization of parameters using this method not only makes the product quality close to target value, but also minimizes the variation in quality [6].

Artificial neural network (ANN) is another modelling approach that has been applied successfully in modelling of biological systems [7]. Essentially, ANNs are interconnected network structures designed in order to process information and acquire knowledge in a similar way the human brain does [8]. ANNs can deal with multiple independent (input) and dependent (output) variables simultaneously while having no prior information about their functional relationship [8]. The most important advantage of ANN application in biotechnology is that the nonlinear dynamic characteristics of complex biological systems can be easily captured without the requirement for identifying defined rules by the researcher. The future of ANN application in biotechnology is very promising and seems to expand in the near future.

In the present study, Taguchi orthogonal array and ANN analyses of enzymatic synthesis of xylitol caprate has been carried out using the commercial immobilized lipase, Novozym 435. The effects of six reaction parameters (temperature, time, substrate molar ratio, amount of enzyme, amount of molecular sieve and amount of solvent) on the substrate conversion were evaluated and the process conditions were optimized. Some physicochemical characteristics of the produced surfactant were also determined for its potential industrial application.

II. Materials and Methods

2.1. Materials

Novozym 435 (*Candida antarctica* lipase B immobilized on macroporous resin, specific activity of 10,000 PLU/g) was purchased from Novo Nordisk A/S (Bagsvaered, Denmark). The acyl donor was capric acid (C10) (Merck, Germany). The acyl acceptor was xylitol (Aldrich, USA). Hexane, tert-butanol (t-BuOH), ethanol (EtOH), acetone, sulfuric acid and sodium hydroxide were purchased from Merck, Germany. Molecular sieve (3Å, bead, 4-8 mesh, K_nNa_{12}-n $[(AlO_2)_{12}\ (SiO_2)_{12}].xH_2O$) was used as water adsorbent (Aldrich, USA). Chloroform, methanol, acetic acid and n-decan were obtained from Merck, Germany. α–Naphtol was obtained from Aldrich, USA. Kieselgel 60 TLC plastic sheets were purchased from Merck, Germany.

2.2. Esterification Reaction

Different molar ratios of capric acid to xylitol and various amounts of molecular sieve were mixed in 50 ml flasks. Specified amounts of hexane were added as the solvent. Different amounts of enzyme were subsequently added. The reaction was performed on the reflux system at 200 rpm agitation speed on a magnetic stirrer (C-MAG HS7) at different time periods and temperatures. The selection of hexane as solvent was based on the prior research [9] in which using lipase resulted in a high reaction yield in the synthesis of sugar polyol esters. Novozym 435 was selected as the catalyst after preliminarily screening of three commercial immobilized enzymes including Novozym 435, Lipozyme RMIM and Lipozyme TLIM, whereby employing Novozym 435 led to the highest percentage conversion.

2.3. Analysis and Characterization

The esterification reaction was terminated by adding 5 ml of t-BuOH: EtOH: acetone (5:5:5 v/v/v) and filtration of the enzyme. The remaining free acid in the reaction mixture was determined by titration with 0.1 M NaOH in the presence of phenolphthalein and using the pH meter model EUTECH (end point of capric acid = 9.8). Control for each reaction mixture (without enzyme) was treated the same way as the reaction samples. All experiments were performed in triplicate. The amount of free acid in the reaction mixture and control was specified and the percentage of conversion was defined as the ratio of converted acid in the reaction mixture to total amount of acid used in the reaction (Equation 1) [10].

$$\% \text{ Conversion} = (1 - A/B) \times 100 \qquad (1)$$

A= moles of the acid in the reaction mixture

B = initial moles of the acid

Xylitol ester formation was confirmed by thin layer chromatography using chloroform/methanol/acetic acid/water (80:15:8:2 v/v/v/v) solvent system. Compounds were revealed by spraying the silicagel plate with α–naphtol solution (1.5 g of α–naphtol was dissolved in 51 ml of EtOH and then 4 mL of water and 6.5 mL of sulfuric acid 18 M was added). The products (black spots) were obtained by carbonization at 105 °C [11]. Further identification of ester was carried out by FT-IR (Perkin Elmer, model 1650) and gas chromatography/mass spectroscopy (GC-MS) on a Shimadzu (model GC 17A; model MS QP5050A, Tokyo, Japan) instrument equipped with a non polar column (fused silica capillary column SGE BPXS, 30 m×0.25 mm ID×0.25 μm thickness).

2.4. Purification of the Product

For purification of the diester (the major product of esterification), after termination of the reaction, the enzyme, molecular sieve and unreacted xylitol were filtered. The filtrate consisting of capric acid and xylitol esters was extracted by aqueous NaOH (0.1M) and hexane in a 100 ml separatory funnel. The organic layer was washed five times with aqueous NaOH and hexane and then dried over Na_2SO_4. The solvent was evaporated thereafter and the residue was chromatographed on Silica gel 60 eluted by a mixture of chloroform and methanol (20:1 v/v). The structure of the purified diester was characterized using ^{1}H-NMR and ^{13}C-NMR spectroscopy. NMR spectra were recorded on JEOL 400MHz FTNMR Spectrometer in $CDCl_3$ using tetramethylsilane (TMS) as the internal standard.

2.5. Taguchi Orthogonal Array Experimental Design

Taguchi has created orthogonal arrays (OAs) to decrease experimental error and to increase the effectiveness and reproducibility of the experiments [5]. By this method, complete parameter space can be studied with minimum number of experiments. The analysis of variance (ANOVA) was applied to determine which parameter is statistically significant. The effective factors are recognized with the statistically significant influence and important effects. A software package of Design Expert, version 6.07 (State-Ease Inc., Statistic made Easy Minneapolis, MN, USA) was used for regression and graphical analysis of the obtained data. A six-factor, three-level orthogonal array design was considered for the synthesis of xylitol ester in this study, leading to a set of 27 experiments (L_{27}). The variables and their levels for the xylitol ester synthesis were reaction time (7-24 h), temperature (30-60 ^{o}C), substrate molar ratio, xylitol: fatty acid, (0.33-1.00), enzyme amount (0.05-0.30 g), molecular sieve amount (1-4 g), amount of solvent (10-30 ml) obtained based on the preliminary experiments by the conventional one-variable optimization approach. This six strategy factors are shown by letters (A-F). Each parameter is represented by a column in the orthogonal array, whereby 6 parameter combinations are available, thus only 27 experiments are required to study the entire experimental space using L_{27} orthogonal array.

The signal to noise ratio (S/N) is a measure of the sensitivity of quality related to the uncontrollable factors (error) in the experiment. Higher S/N ratio is always desirable that will result in smaller product variance around the target value. By using Taguchi's S/N ratio, the response mean and variation are assessed simultaneously. The S/N ratio is calculated from the following equation [12]:

$$S/_N = -10\ log\ (\frac{1}{n}\sum_{i=1}^{n}\frac{1}{y_i^2}) \qquad (2)$$

where n is the total number of experiments in the orthogonal array and y is the observed data (conversion). Table 1 shows the design matrix with the actual and predicted conversions and average S/N ratio for 27 trial conditions. With the ANOVA and S/N analysis, optimal arrangement of the process parameters would be predicted by combining the factors levels that have the highest main effect value. Finally, a confirmation experiment was carried out to verify the optimal conditions predicted by Taguchi analysis.

Table 1. Composition of various runs in the Taguchi orthogonal array of L_{27}, actual and predicted values of xylitol caprate conversion

Run no.	A	B	C	D	E	F	Actual conversion (%)	Predicted conversion (%)	S/N ratio (actual)
1	2	2	3	1	1	2	69.33	72.36	36.82
2	3	2	1	2	1	2	87.50	95.06	38.84
3	2	3	1	1	2	1	58.27	58.54	35.31
4	3	2	1	1	3	1	70.19	66.15	36.93
5	2	2	3	2	2	3	86.85	84.82	38.77
6	1	3	3	3	2	1	85.31	90.68	38.62
7	3	2	1	3	2	3	77.18	75.42	37.75
8	1	3	3	1	3	2	40.22	41.69	32.09
9	3	3	2	3	3	2	80.82	77.36	38.15
10	1	2	2	2	3	1	80.62	82.39	38.13
11	3	1	3	2	3	3	77.71	78.16	38.00
12	3	1	3	3	1	1	79.12	83.56	37.97
13	3	1	3	1	2	2	59.92	57.68	35.55
14	1	1	1	2	2	2	19.86	20.32	26.00
15	2	1	2	3	2	2	33.03	31.81	30.38
16	2	3	1	2	3	2	88.00	87.60	38.89
17	2	1	2	2	1	1	44.03	44.67	32.87
18	2	3	1	3	1	3	82.43	82.41	38.32
19	1	2	2	3	1	2	54.38	51.52	34.71
20	1	2	2	1	2	3	25.88	26.76	28.26
21	3	3	2	1	1	3	75.01	78.07	37.50
22	1	3	3	2	1	3	61.00	55.40	35.71
23	3	3	2	2	2	1	84.19	84.63	38.50
24	2	2	3	3	3	1	74.05	72.61	37.39
25	2	1	2	1	3	3	26.36	26.98	28.42
26	1	2	1	1	1	2	31.62	29.14	30.00
27	1	1	1	3	3	3	14.69	15.64	23.34

A= Reaction time (h), B= Temperature (^{o}C), C= Amount of enzyme (g), D= Amount of molecular sieve (g), E= Substrate molar ratio, F= Amount of solvent (ml)

2.6. Artificial Neural Network (ANN)

The MATLAB neural network toolbox version 7.8 was employed in this study to develop the ANN model. The six parameters employed in Taguchi design was used as the inputs to the network. The output was the xylitol ester conversion. A set of data including 30 experiments were used for training the network. Most training data were obtained from Taguchi orthogonal array design. According to Hung *et al.*, application of orthogonal arrays for data collection can reduce the number of data required for training the neural network, and increase the accuracy [13]. The experimental data used for training the ANN are presented in Table 2. In order to ensure robustness of the network and avoid "overfitting" phenomenon, a second test dataset was used (Table 2). The data were normalized within the range of [−1, 1] to increase the ability of the network to learn the relation between inputs and output [14]. A multilayer perceptron based feed-forward neural network which uses Levenberg–Marquardt algorithm (LMA) was applied for modeling the enzymatic reaction. LMA, which is a combination of steepest descent and the Gauss-Newton method, is usually used as a fast and efficient training algorithm for moderate-sized feedforward ANNs. Different neural networks were trained and the optimum number of hidden layers and neurons and the type of transfer function were determined. The performance of the ANN model was measured by mean square error (MSE) and coefficient of determination (R^2) between the values predicted by the model and the actual values (Equations 3 and 4):

$$R^2 = 1 - \frac{\sum_{i=1}^{n}(y^*_{kp} - y_{kp})^2}{\sum_{i=1}^{n}(y_{kp} - \bar{y})^2} \quad (3)$$

$$MSE = \frac{\sum_{i=1}^{n}(y^*_{kp} - y_{kp})^2}{n} \quad (4)$$

where y is the average of y over the n samples, and y_{kp} and y^*_{kp} are the actual and predicted outputs, respectively.

2.7. Determination of the Hydrophile-Lipophile Balance (HLB) Value

The HLB is a valuable parameter for the classification of nonionic surfactants. The HLB values were calculated from the partial weights of a hydrophilic group and a hydrophobic group in the whole compound according to the following equation [15]:

$$HLB_{value} = \left(\frac{W_P}{W_S}\right) \times 20 = \frac{M\ Hydrophilic}{M\ Hydrophilic + M\ Lipophilic} \times 20 \quad \text{(Equation 5)}$$

where M is molecular weight of the portion of the molecule, W_p is partial weight of a hydrophilic group and W_s is weight of total molecule.

HLB number ranges from 0 to 20, corresponding to the most lipophilic and the most hydrophilic property. The HLB value of each fraction was calculated accordingly and the HLB value for the product mixture consisting of mono-, di-, tri- and tetra xylitol esters was calculated as follows:

(6)

where x_i is percentage abundance of each ester.

$$HLB_{mixture} = \sum_{i=1}^{n} x_i \times HLB_i$$

Table 2. Experimental training and testing data of ANN for the synthesis of xylitol ester

A	B	C	D	E	F	Actual conversion (%)
Training data						
18	60	0.12	2.5	0.33	10	87.20
7	50	0.12	1.0	0.50	30	25.88
18	60	0.30	4.0	2.00	20	83.00
24	60	0.05	1.0	0.50	10	76.00
24	50	0.05	1.0	1.00	10	70.00
18	50	0.30	1.0	0.33	20	69.33
7	30	0.05	2.5	0.50	20	19.86
24	60	0.12	4.0	1.00	20	80.81
7	30	0.05	4.0	1.00	30	14.69
18	60	0.30	4.5	1.00	20	79.00
18	50	0.30	2.5	0.50	30	86.85
7	60	0.30	2.5	0.50	10	83.00

18	30	0.12	1.0	1.00	30	26.36
18	30	0.12	4.0	0.50	20	33.04
24	30	0.30	4.0	0.33	10	79.12
7	60	0.30	4.0	0.50	10	85.31
18	60	0.05	1.0	0.50	10	58.27
7	30	0.05	1.0	0.33	10	31.62
7	50	0.12	4.0	0.33	20	54.38
24	60	0.12	2.5	0.50	10	84.18
18	50	0.05	2.5	1.00	20	86.00
18	60	0.05	2.5	0.33	10	87.00
18	70	0.30	4.0	1.00	20	40.00
18	30	0.12	2.5	0.33	10	44.03
24	30	0.30	1.0	0.50	20	59.92
24	30	0.12	2.5	0.50	30	45.00
24	60	0.12	2.5	1.00	10	85.00
24	60	0.12	1.0	0.33	30	75.01
24	50	0.30	1.0	0.33	30	78.00
18	50	0.30	4.00	1.00	10	74.00
Testing data						
24	50	0.05	2.5	0.33	20	87.50
18	60	0.05	2.5	1.00	20	88.00
7	60	0.30	2.5	0.33	30	61.00
24	30	0.30	2.5	1.00	30	77.18
24	50	0.05	4.0	0.50	30	77.18
18	60	0.05	4.0	0.33	30	82.43
7	60	0.30	1.0	1.00	20	40.22
7	50	0.12	2.5	1.00	10	80.62

A= Reaction time (h), B= Temperature (^oC), C= Amount of enzyme (g), D= Amount of molecular sieve (g), E= Substrate molar ratio, F= Amount of solvent (ml)

2.8. Physicochemical Characterization of Xylitol Caprate Surfactant

Some physicochemical properties of xylitol ester namely, melting point, refractive index and saponification values were analyzed according to AOCS Test Methods Cd 1d-92(97), Cc 3-25, Ca-40(97) and Cd 3-25 [16]. The melting point of the product mixture was determined by the capillary method using digital melting point apparatus (model Electrothermal IA9000). The refractive index of the product was measured by Refracto 30GS (Mettler Toledo, US) instrument.

The emulsifying activity of xylitol ester was determined by a modified version of the method described by Cooper and Goldenberg [17]. Equal volumes of the different solutions (0.5% (w/v)) in distilled water and *n*-decan were added to glass tubes. The mixtures were shaken vigorously by a vortex and allowed to stand for 24 h. Emulsification index (E_{24}) was expressed as the percentage of the total height occupied by the emulsion. Span 20 and Tween 80 were used as the control surfactants.

2.9. Irritancy Test

Dermal irritancy of xylitol ester was measured by using the *In Vitro* International's Irritection Assay System (In Vitro International, Irvine, CA) in order to predict their potential to cause irritation on human skin. Standard volume dependent dose-response studies based on cosmetic protocols were performed with the Dermal Irritection Assay test methods [18]. The assay is based on the fact that dermal irritating compounds cause alterations or denaturalization of collagen, keratin and other dermal protein structures. Samples of 50, 75, 100 and 125 mg were weighed and applied onto a synthetic membrane disc biobarriers. Protein reagents (oligometric protein, glycoprotein, lipids constituents and globulins) and blanking buffers were added to the 24-well assay plate. The synthetic discs which contained various amounts of ester samples were inserted into the corresponding blank and test sample wells of the plates and incubated at 25 °C for 24 hours. The optical density (OD) of the protein blanking buffer and test samples was then recorded by MRX microplate reader at a wavelength of 450 nm [19].

III. Results and Discussion

3.1. Taguchi Orthogonal Array Design and Analysis of Variance

Experimental design along with the observed and predicted responses and average S/N ratio are shown in Table 1.

The predicted values were seen to be sufficiently correlated with the observed values. Table 3 shows the analysis of variance (ANOVA). The model F-value of 37.36 with prob> F-values <0.0001 implies the model is significant. There is only 0.01% chance that a model F-value this large could occur due to noise. Typically, a R^2 value of 0.7500 implies the model is adequate for representing the real relationship among the factors while a R^2 of > 0.9000 indicates that the model describes the real situation well [20].

Table 3. Analysis of main selected factorial terms and ANOVA of xylitol caprate synthesis with orthogonal array model

Terms	Sum of squares	Degree of freedom	Mean square	F value	Prob> F
A	153.46	2	76.73	113.22	< 0.0001
B	171.04	2	85.52	126.18	<0.0001
C	45.57	2	22.79	33.62	< 0.0005
D	35.11	2	17.56	25.90	<0.0011
E	11.83	2	5.92	8.73	0.0167
F	22.92	2	11.46	16.91	0.0034
CE	20.94	4	5.24	7.73	0.0151
DE	45.58	4	11.40	16.81	0.0021

ANOVA

Source	Sum of squares	Degree of freedom	Mean square	F-value	Prob>F
Model	506.46	20	25.32	37.36	0.0001
Residual	4.07	6	0.68		
Corrected total	510.53	26			

Regression statistics

Standard deviation	0.82	R^2	0.9920
Mean	34.93	Adjusted R^2	0.9655
CV	2.36	Predicted R^2	0.8387
PRESS	82.35	Adequate precision	21.578

In this research, the model obtained from ANOVA indicates that the coefficient of determination (R^2) was 0.9920. In addition, the model has an adequate precision of 21.578. The adequate precision value is an index of signal to noise and a value > 4 is a necessity for a model with a good fit. The model also shows standard deviation, mean, coefficient of variation (CV), predicted residual sum of square (PRESS), adjusted R^2 and predicted R^2. For calculation of the standard deviation of a population, it is necessary to calculate the population's variance. Numerically, the standard deviation is the square root of the residual mean square. The standard deviation of the model was 0.82. Mean is overall average of all the response data. The mean value is 34.93. The coefficient of variation (CV) is calculated from the average and standard derivation. In this orthogonal array model, CV is 2.36. Furthermore, PRESS is a measure of how the model fits each point in the design. The PRESS is computed by first predicting where each point should be from a model that contains all other points except the one in question. The amount of PRESS with this method is 82.35. Adjusted R^2 is the measure of the amount of variation around the mean explained by the model adjusted for the number of terms in the model. The adjusted R^2 in this model is 0.9655. Predicted R^2 is a measure of the amount of variation in new data explained by the model that is 0.8387.

Analysis of the main factors in xylitol caprate synthesis is shown in Table 3. Values of probe>F less than 0.05 indicated that model terms were significant. The non-significant terms that make the model insignificant were eliminated on the basis of probe>F-values larger than 0.05. Therefore, A (reaction time), B (temperature), C (enzyme amount), D (molecular sieve amount), E (molar ratio), F (effect of solvent), CE (interaction between enzyme amount and molar ratio), and DE (interaction between molecular sieve and molar ratio) are significant model terms in xylitol caprate synthesis.

3.2. Effect of Significant Parameters on the Synthesis of Xylitol Ester

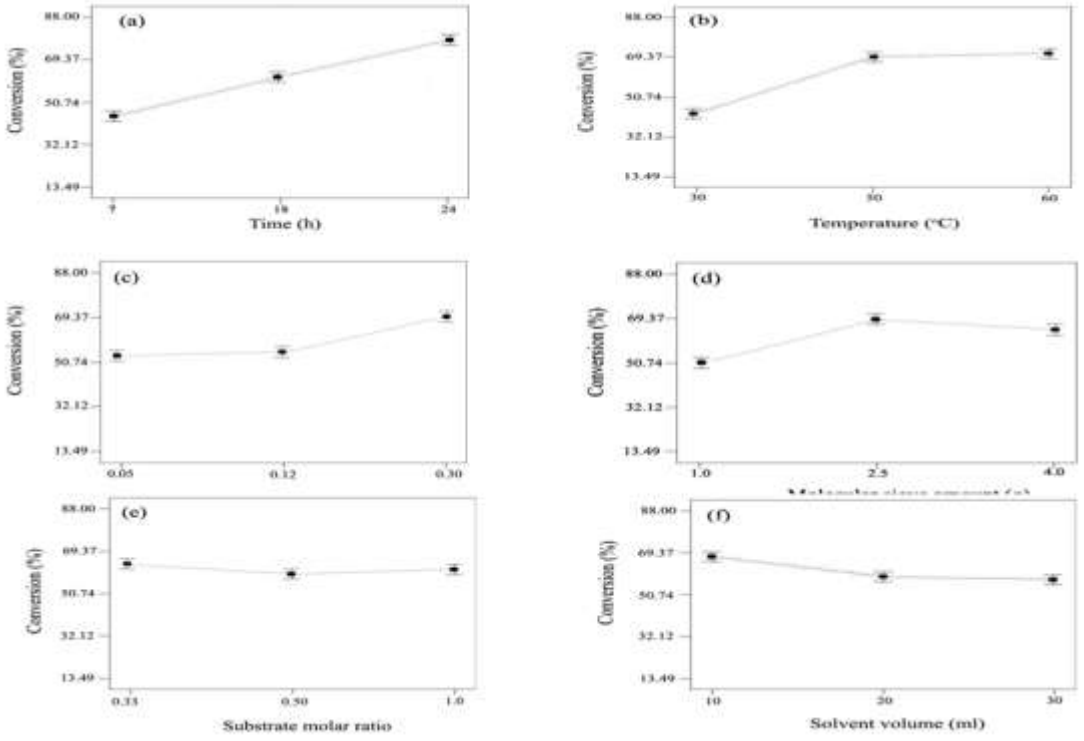
The main effect plots of the model clearly depict the optimum level of each significant factor in xylitol caprate synthesis. The effect of parameters (time, temperature, enzyme amount, molecular sieve amount and amount of solvent) was predicted by 27 trial experiments that were obtained using orthogonal array method. In addition, according to the conventional OVAT, three levels which produced a better response were selected for the orthogonal array design. The effect of parameters on S/N ratio which increased the accuracy of the model was quite similar to what could be seen for the conversion of ester.

Effect of time on esterification reaction using orthogonal array prediction is shown in Fig.1(a). The model predicted that a period of 24 h was adequate for reaction of xylitol with capric acid. The conversion and S/N ratio increased from 7 to 24 h. Prolonging the reaction time after 24 h could lead to increase in the production of water molecules in the reaction medium.

In this condition, the amount of molecular sieve used was not enough for removing the water which was produced in the reverse hydrolysis process, apart from the reaction which has attained the equilibrium state. As the reaction proceeded, the substrate concentration decreased which led to a drop in degree of saturation of the enzyme with substrate [21].

Fig.1(b) shows one factor orthogonal array plot as a function of temperature. The percentage conversion and S/N increased steeply from 30 to 50°C, but from 50 to 60°C, the conversion and S/N increased gradually. The use of moderate temperature (50-60°C) is beneficial since power costs can be reduced and enzyme stability can be preserved during prolonged operation. Increasing temperature promotes collisions between acyl-enzyme complex and alcohol molecules to result in an accelerated rate of reaction. On the other hand, temperatures higher than 60°C tended to induce enzyme inactivation due to denaturation process and caused decrease in the conversion of ester. With increase in temperature, binding equilibria of substrates is reduced while acid dissociation and solubility is increased that result in unfavorable esterification conditions [22].

Fig.1(c) depicts the prediction of Taguchi orthogonal array for the effect of varying amount of enzyme on esterification reaction between xylitol and capric acid. The conversion was slightly increased when the enzyme loadings were increased. However, maximum average conversion was obtained when the enzyme amount was at 0.3 g. Additional active sites of enzyme can cause increase in the formation of acyl-enzyme complex and subsequently the products [23]. Further increase in enzyme to substrate ratio did not significantly increase the conversion. This could be due to the fact that, in the presence of a high amount of enzyme, the active site could not be exposed to the substrates and the molecules of the enzyme aggregated together [24]. Furthermore, very high amount of enzyme may lead to a more concentrated mixture and contributed to the decreasing reaction rate. Effects of water removal using varying amount of molecular sieve are presented in Fig.1(d). Response graph shows that the percentage of conversion increased with increasing the amount of molecular sieve up to 2.5 g.



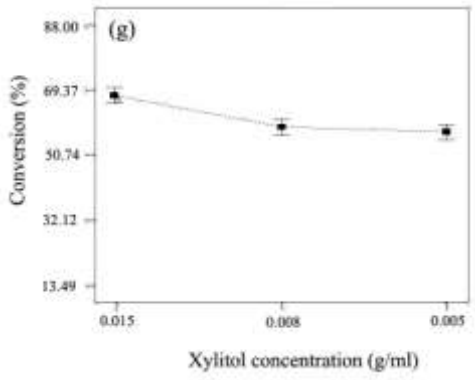


Figure 1. Average values of the conversion at three levels for the effect of (a) reaction time, (b) temperature, (c) enzyme amount, (d) molecular sieve amount, (e) substrate molar ratio, (f) solvent volume, and (g) xylitol concentration.

A certain amount of water is required for keeping the enzyme in its active conformation and also favors the shift in equilibrium of the reaction towards synthesis [25]. However, higher amounts of desiccant eliminate too much water causing excessive dehydration of enzyme and reaction medium [26]. The adverse phenomenon of stripping of essential water from an enzyme into a nonaqueous medium facilitates enzyme aggregation, which attributed to decrease in enzyme activity. Therefore, the amount of the molecular sieve must be controlled carefully since optimization of the reaction system needs a suitable balance between the opposite effects.

The effect of varying molar ratio of xylitol: fatty acid on the conversion is illustrated in Fig.1(e). The percentage of conversion was relatively constant. According to the F-value of the parameters effect, substrate molar ratio is not as significant as other parameters in the reaction. A little increase in the conversion at substrate molar ratio of 0.33 (xylitol:fatty acids1:3) may be attributed to greater availability of the acid to enzyme [23].

Two important properties of a solvent to be used in biocatalysis are its effect on substrate/product solubility (subsequent partitioning) as well as its effects on the reaction and hydration state of the biocatalyst [27]. In addition, it has influence on enzyme activity and stability. Solvent also alters the enzyme specificity, substrate specificity, enantioselectivity, prochiral selectivity, regioselectivity and chemoselectivity [28]. Fig.1(f) shows the main factor plot of solvent amount. By increasing the volume of hexane, the conversion was decreased, which may be attributed to inhibition of enzyme. If solvent amount was considered in terms of xylitol concentration (Fig.1 (g)), increasing the concentration of xylitol increased the percentage conversion since more substrate was available to bind the catalytic site of the enzyme and underwent reaction. Furthermore, higher concentrations of substrates caused more collisions between enzyme and substrate molecules. Taguchi plots of predicted responses for the interaction of molar ratio and molecular sieve amount is illustrated in Fig.2.

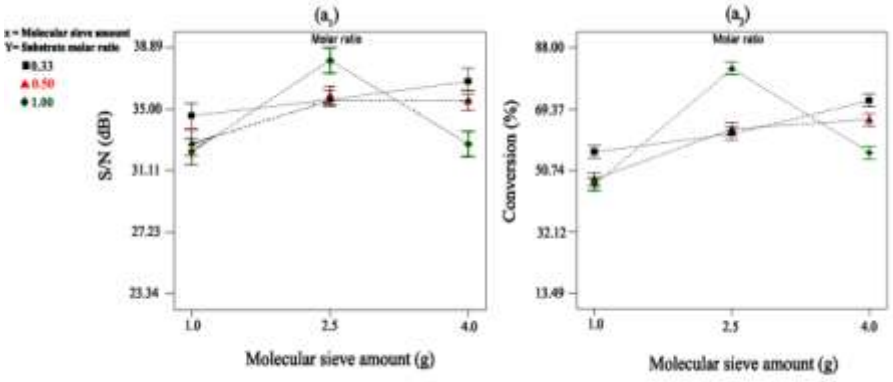


Figure 2. Interaction graphs, average values of S/N ratio and conversion at level 1-3 between the amount of molecular sieve (D) and molar ratio (E), (a_1) S/N ratio (a_2) conversion.

The interaction between molar ratio of 1 and molecular sieve of 2.5 g showed the highest S/N and average conversion. On the other hand, negative effect of increasing molecular sieve amounts was observed at molar ratio of 1. Usually water requirement of enzyme changes with substrate concentrations [25]. The chemical nature of the employed substrates, one hydrophobic and the other hydrophilic, may significantly alter the water distribution in the system, affecting enzymatic activity, stability and relative rates of hydrolysis and synthesis [29]. Generally, proportionally suitable amount of the desiccant is required with definite amount of substrates

molar ratio. However, amounts of desiccant exceeding its optimal level may lead to excessive dehydration of the reaction medium. Moreover, generally very high substrate concentrations more than specific amount for reaction, increase viscosity of reaction medium and together with large amounts of molecular sieve, may impose additional mass transfer limitations on the system [30]. Fig.3 shows the predicted responses for the enzyme amount and substrate molar ratio interaction. While the positive effect of increasing biocatalyst amount was clearly evident at molar ratio of 0.5 (xylitol:fatty acids, 1:2), a less significant increase of S/N and conversion was observed at 0.33 (xylitol:fatty acids, 1:3). The results indicated that high conversions were possible with small amounts of enzyme when high amount of substrate (fatty acid) was used. This is beneficial from the economical aspect since the cost of enzyme is usually higher than that of substrates.

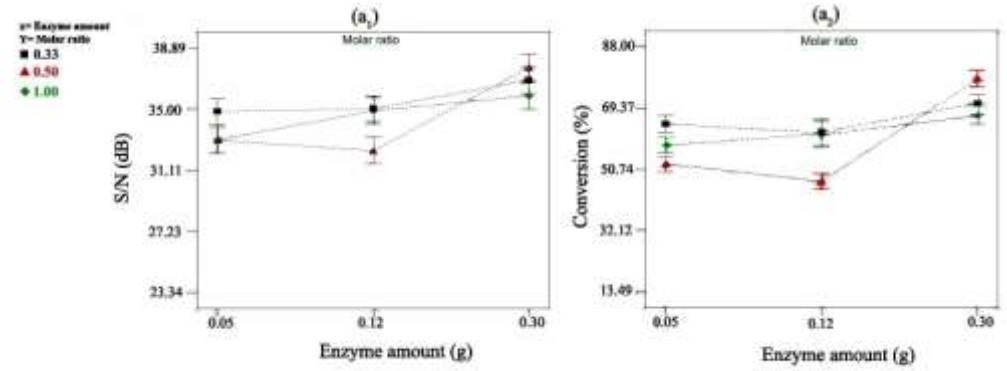


Figure 3. Interaction graphs, average values of S/N ratio and conversion at level 1-3 between the amount of enzyme (C) and molar ratio (E), (a_1) S/N ratio, (a_2) conversion.

3.3. Optimization of Reaction Conditions and Model Validation

The Taguchi method can indicate the optimal combination of parameters to obtain the highest percentage conversion. By using the optimization function of the Design Expert Software, five other experiments with desirability value of 1 were used to predict the optimal conditions for Novozym 435-catalyzed synthesis of xylitol ester. The desirability method makes use of an objective function, D, called the desirability function according to Equation 7:

$$D=(d_1 \times d_2 \times \ldots \times d_n)^{1/n} = \left(\prod_{i=1}^{n} d_i\right)^{1/n} \quad (7)$$

where n is the number of responses in the measure and d_i is the desirable ranges for each response.

The optimum conditions are presented in Table 4. Experiments were then carried out under recommended conditions and resulting responses were compared to the predicted values. The maximum conversion obtained was 88.00% with S/N 38.85 with reaction time of 18 h at 60°C, minimum enzyme amount 0.05 g, molecular sieve amount 2.5 g, molar ratio 1 and 20 ml solvent. At minimum amount of molecular sieve, maximum amount of enzyme (0.30 g) is required to result in a relatively high conversion. If reaction time is considered important for the industry, at minimum time (7 h), 80.62% conversion can be achieved. The results show that actual values are in good agreement with the predicted ones. Therefore, it has been demonstrated that Taguchi method can be applied effectively to optimize the enzymatic synthesis of xylitol ester.

Table 4. Optimum condition solutions for xylitol caprate synthesis

Optimum Conditions							Conversion (%)			
Exp	**A (h)**	**B (°C)**	**C (g)**	**D (g)**	**E**	**F (ml)**	**Predicted S/N**	**Predicted**	**Actual**	**Error (%)**
1	18	60	0.05	2.5	1.00	20	38.85	87.60	88.00	0.45
3	24	60	0.30	1.0	1.00	20	38.33	82.51	81.66	-1.03
4	7	50	0.12	2.5	1.00	10	38.43	83.50	80.62	-3.44

A= Reaction time, B= Temperature, C= Amount of enzyme, D= Amount of molecular sieve,
E= Molar ratio, F= Amount of solvent

3.4. Artificial Neural Network Analysis

In this research, for prediction of xylitol ester conversion, various feedforward neural networks (FFNs) with different configurations were trained using the Levenberg-Marquardt algorithm (LMA). The training data was utilized to compute the network parameters. Moreover, in order to find the optimal number of hidden layers and hidden neurons, training was also performed for various 6-x-y-z-1 architectures. After 8 iteration step (a step in the training process that the network is presented with a new pattern and different set of weights), the target was achieved and the network training was completed.

The number of neurons in hidden layers is very important as it influences the learning time and generalization property of the network. There is no general rule for choosing the number of neurons in a hidden layer and it depends on the complexity of the system. The optimal number of neurons can be selected through a trial and error process as it has been performed in this study. The best network was used to compare the actual and predicted outputs for the training and testing data.

The goal of network training is to minimize the mean square error (MSE) between the measured value and the predicted output. The network with two hidden layers including seven and five neurons in the first and second hidden layers exhibited the best performance in predicting the conversion of the ester based on the mean square error (MSE). Fig. 4 shows the coefficient of determination for the network with the optimum number of hidden layer. An excellent correlation between experimental and predicted values for the training set with R^2 equal to 1 was obtained.

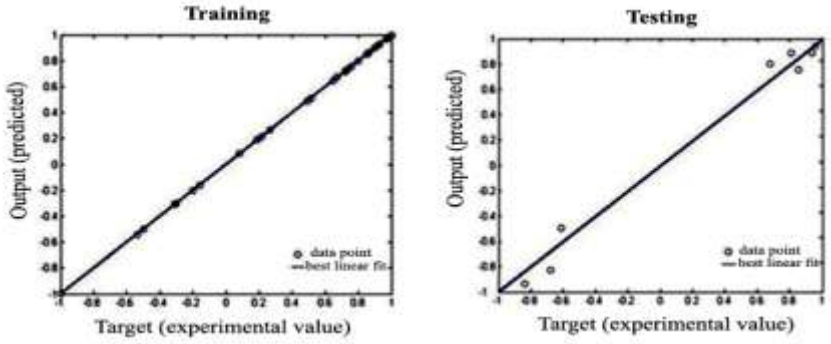


Figure 4. Correlation between the actual and predicted values of the ANN models with respect to training and testing data for the best hidden layer (two hidden layers).

In order to check the accuracy and generalization of the model, the network performance was also investigated with respect to the testing dataset. The modeling errors of training and testing data are shown in Table 5. As it can been seen in the Table, the network with two hidden layers shows the best performance. The best network was used in the further part of the study for prediction of the conversion with various operational parameters.

Table 5. ANN Modeling error with respect to training and testing data

Number of hidden layers	MSE		R^2	
	Training	Testing	Training	Testing
1	4.1725e-22	0.55669	1	0.95322
2	2.3413e-29	0.03751	1	0.97739
3	7.2669e-24	0.05215	1	0.97483

3.5. Comparison of Taguchi and ANN Prediction

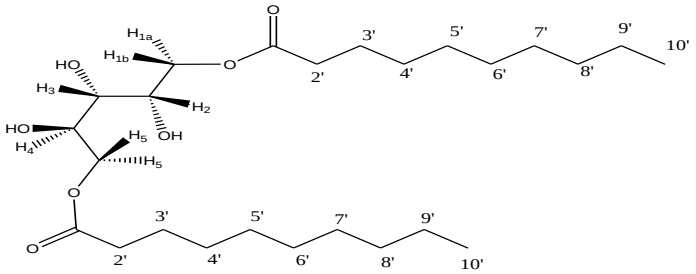
The performance of the Taguchi OA and ANN in the estimation of ester conversion in Novozym 435-catalyzed synthesis of xylitol ester was compared. The predicted responses achieved from Taguchi and ANN were compared with the observed values. R^2 and MSE between experimental and predicted values for a set of testing data including optimum conditions were 0.997413 and 2.91588 for Taguchi OA, and 0.999246 and 0.85000 for the ANN, respectively.

Although both models offered good quality predictions of the percentage conversion, ANN was slightly better than Taguchi in data fitting and estimation capability. In fact, neural networks are more accurate modeling techniques due to their ability to learn and handle nonlinear and dynamic situations typical of biological processes [7]. However, ANNs have the disadvantage of requiring large amounts of training data in comparison to Taguchi method. In the present study, to overcome this ANN problem, the Taguchi OA design was applied to decrease the number of experiments. The combination of Taguchi parameter design and ANN can generate a very efficient tool for modeling of the process and the optimal settings of operational condition.

3.6. Characterization of Xylitol Caprate Ester

Production of xylitor ester was monitored by TLC. The ester was observed with a retention factor of 0.66. FTIR analysis has shown a strong absorption band at 1733 cm^{-1} that belongs to C=O stretching of the ester and a broad band at 1259 cm^{-1} corresponded to C-O stretching vibration of the ester. GCMS analysis showed that formation of xylitol diester (retention time: 15.972 min) was predominant over mono (retention time: 15.604 min), tri (retention time: 16.074 min) and tertra (retention time: 17.198 min) esters with 68.81, 17.00, 9.59 and 4.60% conversion, respectively. According to Gebhardt *et al*, *Candida antarctica* lipase B possesses a confirmed regioselectivity for the acylation of primary OH groups of sugar alcohol molecules [31]. The structure of the diester purified (96% purity) by column chromatography was characterized using ^{1}H-NMR and ^{13}C-NMR spectroscopy as follows (Scheme 1):

^{1}H-NMR (400MHz, $CDCl_3$, δ): 4. 09 (dd, $J_{H1a\text{-}H1b}$= 11.0 Hz, $J_{H1(a,b)\text{-}H2}$= 6.4Hz, 4H); 3.88 (m, 2H, H_2); 3.81 (m, 1H, H_3); 2.26 (t, $J_{2'\text{-}3'}$= 7.4Hz, 4H, $H_{2'}$); 1.29 (m, 30H, $H_{3'}$-$H_{9'}$); 0.88, (t, $J_{H9'\text{-}H10'}$= 7.4 Hz, 7H, $H_{10'}$); ^{13}C-NMR (100 MHZ, $CDCl_3$, δ): 173 (C=O), C3 (72), C2, C4 (68), C1, C5 (62), C2' (34), C3' (32), C4'-C7' (29-30), C8' (24), C9' (22), C10' (14).



Scheme 1. Xylitol dicaprate

3.7. The HLB Value of the Produced Surfactant

The results show that the nonionic surfactant derived from xylitol and capric acid in solvent-based system has an HLB value of 8.7 which is close to Span 20 (sorbitan monolaurate) with HLB value of 8.6. The HLB value of xylitol caprate indicates that the surfactant prepared is lipophilic and can be utilized as an emulsifier for water in oil emulsions and as a wetting and spreading agent.

3.8. Physicochemical Characteristics of Xylitol Caprate

The physicochemical properties of the produced ester are important in determining its application. The properties of xylitol caprate ester are presented in Table 6. The corresponding physicochemical value of the lipophilic commercial surfactant (Span 20) is also included in the Table for comparison.

Table 6. Physicochemical properties of resultant surfactant

Characteristics	Xylitol caprate	Span 20
Physical form	Light yellow Semisolid	Oily liquid
Refractive index	1.443	1.474
HLB value	8.7	8.6
Melting point (°C)	46	-
Emulsification index (E_{24}) (%)	33.3	40.0
Saponification value (mg KOH/g)	240-314	158-170
Solubility in		
Water	insoluble	insoluble
Mineral oil	Soluble	slightly soluble

Refractive index is usually used as a rapid method to monitor the chemical property. Refractive index values of xylitol caprate (1.443) were observed to be slightly higher than capric acid with refractive index of 1.417. According to Shahidi [32], the refractive index increases with increase in length of hydrocarbon chain.

A large amount of KOH (240-314 mg KOH/g) was needed to saponify xylitol diester, the main product of the esterification, compared to Span 20 comprising mono ester (sorbitan monolaurate) as the main ester in the

compound. Similar findings were observed by Abdul Rahman and Herawan [33]. The physicochemical study shows that xylitol ester has good physicochemical characteristics and is suitable for use in food, detergent, cosmetics and pharmaceutical industries.

The emulsification index (E_{24}) for xylitol ester was relatively high (33.3%) compared to conventional surfactants, Span 20 with E_{24} of 40.0 and Tween 80 with E_{24} of 32.0%.

3.9. Irritancy Test

Nonionic surfactants like Spans have low irritancy compared to other surfactants. The results of irritancy test showed that the samples are slightly-irritant with Human Irritancy Equivalent (HIE) score of 0.90-1.20, which is the slightly-irritant level. Although surfactants usually have irritation, in formulation with other compounds like oil or water, the irritation is reduced or disappeared.

IV. Conclusion

Optimization of reaction parameters in lipase-catalyzed production of xylitol ester was successfully performed by Taguchi OA and ANN techniques. Experimental comparison of the two optimization methods indicated that though both developed models provided good quality predictions, the ANN showed slight superiority over Taguchi approach for showing nonlinear relationships. A high substrate conversion (88%) and a high purity ester (96%) were obtained at the optimum conditions. Physicochemical characterization of the produced xylitol dicaprate showed good emulsifying properties and its suitability for industrial application. Findings from this research would be useful for several industries which look for more sustainable processes and products.

References

[1]. I.S. Yoo, S.J Park, and H.H. Yoon, Enzymatic synthesis of sugar fatty acid esters, *Journal of Industrial Engineering Chemistry, 13,* 2007, 1-6.

[2]. C. Blecker, S. Danthine, M. Pétré**,** G. Lognay, B. Moreau**,** E.L. Vander, M. Paquot, and C. Deroanne, Enzymatically prepared *n*-alkyl esters of glucuronic acid: The effect of freeze-drying conditions and hydrophobic chain length on thermal behavior, *Journal of Colloid and Interface Science, 321*, 2008, 154-158.

[3]. S. Tarahomjoo, and I. Alemzadeh, Surfactant production by an enzymatic method. *Enzyme and Microbial Technology 33,* 2003, 33-37.

[4]. M. Basri, R.N.Z.R.A. Rahman, A. Ebrahimpour, A.B. Salleh, E.R. Gunawan, and M.B. Abdul Rahman, Comparison of estimation capabilities of response surface methodology (RSM) with artificial neural network (ANN) in lipase-catalyzed synthesis of palm-based wax ester, *BMC Biotechnology*, *7*, 2007, 1-14.

[5]. Y.R. Abdel-Fattah, H.M. Saeed, Y.M. Gohar, and M.A. El-Baz, Improved production of *Pseudomonas aeruginosa uricase* by optimization of process parameters through statistical experimental designs, *Process Biochemistry, 40*, 2005, 1707-1714.

[6]. J.Y. Houng, H.F. Hsu, Y.H. Liu, and J.Y. Wu, Applying the Taguchi robust design to the optimization of the asymmetric reduction of ethyl 4-chloro acetoacetate by baker's yeast. *Journal of Biotechnology***,** *100,* 2003, 239-250.

[7]. D. Bas, and I.H. Boyaci, Modeling and optimization II: comparison of estimation capabilities of surface response methodology with artificial neural networks in biochemical reaction, *Journal of Food Engineering*, *78*, 2007, 846-854.

[8]. D. Bas, F.C. Dudak, and I.H. Boyaci, Modeling and optimization III: Reaction rate estimation using artificial neural network (ANN) without a kinetic model. *Journal of Food Engineering, 79*, 2007, 622-628.

[9]. R. Gulati, P. Arya, B. Malhotra, A.K. Prasad, R.K. Saxena, J. Kumar, A.C. Watterson, and V.S. Parmar, Novel biocatalytic esterification reactions on fatty acids: synthesis of sorbitol 1(6)-monostearate. *Arkivoc*, 3, 2003, 159-170.

[10]. S. Mat Radzi, M. Basri, A.B. Salleh, , A. Ariff, R. Mohammad, M.B. Abdul Rahman, and R.N.Z.R.A. Rahman, High performance enzymatic synthesis of oleyl oleate using immobilised lipase from *Candida antarctica*, *Electronic Journal of Biotechnology*,*8,* 2005, 299-298.

[11]. S. Soultani, J.M. Engasser, and M. Ghoul, Effect of acyl donor chain length and sugar/acyl donor molar ratio on enzymatic synthesis of fatty acid fructose esters, *Journal of Molecular Catalysis B: Enzyme, 11*, 2001, 725-731.

[12]. M. Nalbant, H. Gokkaga, and G. Sar, Application of Taguchi method in the optimization of cutting parameters for surface roughness in turning, *Materials and Design*, *28*, 2007, 1379-1385.

[13]. Y.C. Hung, M.G. Her, and C.Y. Chen, Application of neural networks augmenting Taguchi's orthogonal arrays for simulating kinematics problems of robotics, *International Journal of Computer Applications in Technology, 20*, 2004, 180-187.

[14]. M.Y. Rafiq, G. Bugmann, and D.J. Eastebrook, Neural network design for engineering applications, *Computers & Structures, 79*, 2001, 1541-1552.

[15]. W.C. Griffin, Calculation of HLB values of non-ionic surfactants, *Journal of the Society of Cosmetic Chemists, 5*, 1954, 235-249.

[16]. D. Firestone, *Official Methods and Recommended Practices of the AOCS*; The American Oil Chemists' Society, AOCS Press: Champaign, IL, USA, 1998; Method: Cd 1d-92(97), Cc 3-25, Ca-40(97) and Cd 3-25.

[17]. D.G. Cooper, and B.G. Goldenberg, Surface-active agents from two *Bacillus* species, *Applied and Environmental Microbiology, 53*, 1987, 224-229.

[18]. Z.A. Abu Hassan, R. Ismail, and S. Ahmad, Safety evaluation for dermal and ocular irritation of palm dihydroxystearic acid as a cosmetics ingredient, *Journal of Oil Palm Research, 17*, 2005, 160-167.

[19]. P.S. Keng, M. Basri, M.B. Abdul Rahman, A.B. Salleh, R.N.Z.A. Rahman, and A. Ariff, Optimization of palm based fat esters production using statistical experimental designs, *Journal of Oleo Science, 54*, 2005, 519-528.

[20]. P.D. Halland, *Experimental Design in Biotechnology* (Marcel Dekker Inc, NY: USA, 1989).

[21]. M.B. Abdul Rahman, U.H. Zaidan, M. Basri, M.Z. Hussein, R.N.Z.R. Abdul Rahman, and AB. Salleh, Enzymatic synthesis of methyl adipate ester using lipase from *Candida rugosa* immobilized on Mg, Zn and Ni of layered double hydroxide (LDHs), *Journal of Molecular Catalysis B: Enzyme, 50*, 2008, 33-39.

[22]. S. Hari Krishna, S. Divakar, S.G. Prapulla, and N.G. Karanth, Enzymatic synthesis of isoamyl acetate using immobilized lipase from *Rhizomucor miehei*, *Journal of Biotechnology*, *87*, 2001, 193-201.
[23]. E.L. Soo, A.B. Salleh, M. Basri, R.N.Z.A. Rahman, and K. Kamaruddin, Response surface methodological study on lipase-catalysed synthesis of amino acid surfactants, *Process. Biochemistry*, *39*, 2004, 1511-1518.
[24]. A. Kumari, P. Mahapatra, V.K. Garlapati, and R. Banerjee, Enzymatic transesterification of Jatropha oil, *Biotechnology for Biofuels*, 2, 2009, 1-7.
[25]. D. Bezbradica, I. Karalazi, N. Gnjanovi, D. Mijin, S.S. Arinkovi, and Z. Knezevic, Studies on the specificity of *Candida rugosa* lipase catalyzed esterification reactions in organic media, *Journal of the Serbian Chemical* Soc*iety*, *71*, 2006, 31-41.
[26]. S. Gayot, X. Santarelli, and D. Coulon, Modification of flavonoid using lipase in non-conventional media: effect of the water content, *Journal of Biotechnology*, *101*, 2003, 29-36.
[27]. M. Nordblad, and P. Adlercreutz, Effects of acid concentration and solvent choice on enzymatic acrylation by *Candida antarctica* lipase B, *Journal of Biotechnology*, *133*, 2008, 127-133.
[28]. E. Garcia-Urdiales, I. Alfonso, and V. Gotor, Enantioselective enzymatic desymmetrizations in organic synthesis, *Chemical Rev*iews, *105*, 2005, 313-354.
[29]. Y.C. Cheng, and S.W. Tsai, Effects of water activity and alcohol concentration on the kinetic resolution of lipase-catalyzed acyl transfer in organic solvents, *Enzyme and Microbial Technology*, *32*, 2003, 362-368.
[30]. C. Torres, and C. Otero, Part III. Direct enzymatic esterification of lactic acid with fatty acids, *Enzyme and Microbial Technology*, 29, 2001, 3-12.
[31]. S. Gebhardt, S. Bihler, M. Schubert-Zsilavecz, S. Riva, D. Monti, L. Falcone, and B. Danieli, Biocatalytic generation of molecular diversity: Modification of Ginsenoside Rb_1 by β-1,4-Galactosyltransferase and *Candida antarctica* lipase, Part 4, *Helvetica Chimica Acta*, *85*, 2002, 1943-1959.
[32]. F. Shahidi, *Bailey's Industrial Oil and Fat Products*, (John Wiley and Sons Inc, NY: USA, 2006).
[33]. R. Abdul Rahman, and T. Herawan, Properties of biosurfactant enzymatically prepared from fructose and palm fatty acid, *Journal of Oil Palm Research*, *12*, 2000, 117-122.