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Optimization of biocatalytic synthesis of Chitosan Ester using response surface methodology

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ABSTRACT
Esterification of chitosan with adipic acid catalyzed by immobilized *Candida antarctica* lipase B was carried out in this study. Response surface methodology (RSM) based on a four-factor-five-level small central composite design (SCCD) was employed to model and analyze the reaction. A total of 21 experiments representing different combinations of the four reaction parameters including chitosan concentration (0.01-0.10 g/mL), temperature (35-65 °C), time (15-240 min), and enzyme amount (5-20 % w/w of total substrate) were generated. A partial cubic equation was fitted to the data with a R^2 of 0.9738 showing a high correlation between predicted and experimental values. The enzyme was capable of esterifying chitosan with a maximum yield of 45.3%. Chitosan concentration was found to be the most significant parameter which influenced the ester synthesis. At the optimal conditions of a short reaction time 15 min, 65°C, 0.10 g/mL of chitosan and 18.7% of enzyme, the esterification percentage was 43.5%.

Keywords: Chitosan; Enzymatic synthesis; Esterification; modeling; Response surface methodology

1. INTRODUCTION

Chitosan (poly[-(1,4)-2-amino-2-deo-xy-D-glucopyranose] (Fig.1) is a natural polysaccharide derived from the chitin of crustaceans, some insects and certain fungi. Chitosan is chemically inert, biocompatible, nontoxic, bio-degradable, eco-friendly and widely available. It has also shown anti-microbial and antifungal activities. These properties as well as the extent of deacetylation and the average molecular weight of this polymer makes it very useful in pharmaceutical formulations and as drug delivery carriers [1]. Chitosan has been applied in preparing beads, films, intragastric floating tablets, microspheres and nanoparticles in the pharmaceutical industry. Chitosan is also being used as thickening agent in cosmetics formu-

lations, flocculent in water purification plants and filling agent in the paper industry [2]. The chemical modification of chitosan for producing a wide range of derivatives with different properties is common [3]. Chitosan esters have been used successfully as potential matrices in many formulations such as in colon-specific oral delivery of sodium diclofenac [4, 5]. By converting the polymer to an ester, the solubility profile is changed significantly. The esteric forms are insoluble under acidic conditions and provide sustained release of the encapsulated agent under basic conditions. Iron cross-linked chitosan succinate has been synthesized to be used as a matrix for oral theophylline beads [6]. Chitosan esters have also shown good potentials for removal of basic dyes from colored textile solutions [7]. They have been used as antimicrobial agents in textile industry for producing cotton fabrics with antimicrobial properties and wrinkle recovery [8]. The esters could overcome the drawbacks of chitosan, such as limited water solubility and poor laundering durability when applied on cotton fabrics [9]. The process of esterification of chitosan is usually performed in acidic medium [6, 8]. There is no report on the yield of reaction. However, high temperature, long reaction time, corrosion of equipments, considerable amount of side products, and the necessity for treatment of wastes are disadvantages of this method. Enzymes are highly efficient bio-catalysts which can catalyze esterification reactions in organic media. Compared to the chemical synthesis, the use of enzymes as catalyst for the reaction offers significant advantages due to mild reaction conditions, short reaction time, high selectivity, and the ease of product separation and biocatalyst reuse [10]. So far, there is no report on the enzymatic synthesis of chitosan esters. Optimization plays an important role in the commercial success of the industry. The conventional method of optimization requires conducting a large number of experiments and investment of lots of time and resources [11]. Furthermore, the effects of interactions between parameters are not considered. However, experimental design approaches provide an efficient way for optimizing the process variables to increase productivity [12]. Optimum condition for the reaction can be obtained by response surface methodology (RSM) approach. RSM is a useful optimization technique comprising multiple regression and correlation analyses to evaluate the effects of several independent factors on the dependent variables [13]. RSM has been successfully employed for the optimization of various enzymatic esterification reactions [14, 15]. The objective of the present work is to investigate the possibility of the enzymatic synthesis of chitosan ester. The study also helps to find out the optimum conditions of ester synthesis and to understand the relationships between the reaction parameters and the conversion yield.

2. EXPERIMENTAL

2.1 Materials

Lipase B from *Candida antarctica*, immobilized on macroporous acrylic resin (Novozym® 435), with specific activity of 10000 propyl laurate unit (PLU), was purchased from Novo Nordisk A/S Company (Bagsvaerd, Denmark). Chitosan (degree of deacetylation ≈ 10%) was obtained from a local supplier and used without further purification. Adipic acid, hexane, ethanol and acetone were purchased from Merck Co. (Darmstadt, Germany). All other reagents were of analytical grade.

2.2 Synthesis of chitosan ester

Synthesis of chitosan ester was carried out in 30 mL tightly sealed vials. The reaction system consisted of adipic acid (0.2 g) and various amounts of chitosan (generated by experimental design) in 6 mL of hexane. Different amounts of Novozym 435 were added to the mixture [16, 17]. Novozym 435 was selected as the biocatalyst for the reaction after preliminarily screening of three commercial immobilized enzymes including Novozym 435, Lipozyme TLIM and Lipozyme RMIM, whereby applying Novozym 435 resulted in the highest conversion. The reaction was performed in a horizontal water bath at 150 rpm for different time periods at different temperature according to the experimental design (Table 1). Reactions were done in triplicate of control (no enzyme added) and samples.

2.3. Characterization and analysis of the product

The reaction was terminated by adding 3.0 mL of acetone/ethanol (1:1, v/v) and the immobilized enzyme was separated by filtration. The remaining unreacted acid was titrated with 0.1 M NaOH using phenolphthalein and the percentage of conversion was calculated [11]. Identification of the ester was performed by FTIR spectrophotometer (Perkin Elmer, model 1650). Further characterization of the product was carried out by gas chromatography/mass spectroscopy (GC/MS) on a Shimadzu (model GC 17A; model MS QP5050A; Shimadzu Corp, Tokyo, Japan) instrument with a BP-20 column (0.25 mm × 30 m, 25 micron). The carrier gas was helium, and the total gas flow rate was 50 mL min^{-1}. The initial temperature of the injector was 230.0°C.

2.4. Design of experiments and statistical analysis

A four-factor-five-level small central composite design (SCCD) (with α = 1.682) was used with total number of 21 experiments. The SCCD comprised of 8 factorial points, 8 axial points and 5 center points. The parameters and their ranges selected were: chitosan concentration (0.01-0.10 g/mL), temperature (35-65 °C), enzyme amount (5-20 % w/w of total substrate) and time (15-240 min). The upper and lower limits of the variables were coded as +1 and -1, respectively. The center points of the variables (coded as 0) were 0.06, 50, 12.5 and 127.5 for chitosan concentration, temperature, enzyme amount and time, respectively. A software package of Design Expert® Version 6.0.6 (State-Ease Inc., Statistics Made Easy and Minneapolis, MN) was used to fit the data obtained to polynomial regression models using the following equation [18]:

$$y = b_0 + \sum_{i=1}^{4} b_i x_i + \sum_{i=1}^{4} b_{ii} x_i^2 + \sum_{i=j}^{3} \sum_{j=i+1}^{4} b_{ij} x_{ij} + e \quad (1)$$

where y is the dependent variable (percentage of conversion), x_i and x_j are the independent variables (factors), b_0, b_i, b_{ii} and b_{ij} are the regression coefficients of model and e is the error of model. The fitness of the model was evaluated by R^2 (coefficient of determination) and analysis of variance (ANOVA). The optimum condition for the reaction was obtained by optimization function of the software.

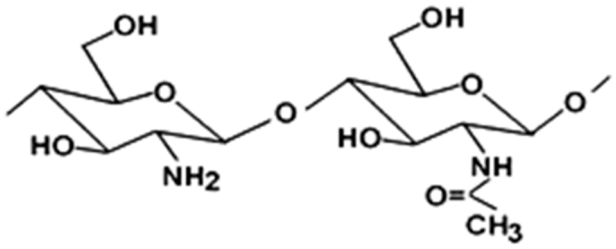

Fig.1. The chemical structure of chitosan.

Table 1. Composition of various experiments used for the synthesis of chitosan adipate

Experiment no.	Variables				
	Chitosan concentration (g/mL)	Temperature (°C)	Enzyme amount (%w/w of total substrates)	Time (min)	Actual conversion (%)
1	0.08	41.1	16.96	194.4	16.2
2	0.06	50.0	20.00	127.5	0.0
3	0.06	50.0	5.00	127.5	0.0
4	0.06	50.0	12.50	127.5	10.0
5	0.06	50.0	12.50	240.0	8.9
6	0.06	35.0	12.50	127.5	12.5
7	0.06	50.0	12.50	15.0	11.6
8	0.03	41.1	16.96	60.6	11.5
9	0.03	58.9	16.96	194.4	11.0
10	0.08	41.1	8.04	194.4	10.1
11	0.03	58.9	8.04	194.4	3.4
12	0.06	50.0	12.50	127.5	12.5
13	0.06	50.0	12.50	127.5	10.2
14	0.06	50.0	12.50	127.5	11.6
15	0.08	58.9	8.04	60.6	9.1
16	0.01	50.0	12.50	127.5	4.3
17	0.08	58.9	16.96	60.6	17.4
18	0.06	65.0	12.50	127.5	15.7
19	0.03	41.1	8.04	60.6	0.0
20	0.10	50.0	12.50	127.5	0.0
21	0.06	50.0	12.50	127.5	11.3

3. RESULTS AND DISCUSSION

3.1. Identification of ester product

The FTIR spectrum of ester product shows the absorption band of C=O bond of ester at1735 cm^{-1} and C–O stretching vibrations at 1033–1200cm^{-1}. Figure 2 shows the IR spectrum of chitosan, adipic acid and chitosan ester, respectively, which indicates that the new ester bond has developed. GC–MS analysis shows the presence of chitosan ester at a retention time of 31 min. The mass spectrum of the product showed molecular ion peak at *m/z* 441, $[M-3H]^{3-}$, corresponding to esterification of chitosan with two molecules of adipic acid ($C_{20}H_{27}NO_{10}$). Other ionic fragments were observed at *m/z* 385, 308, 147 and 57. Figure 3 shows gas chromatogram and mass spectrum of chitosan adipate.

3.2. Model fitting and ANOVA

Fitting of the data to various models and the ANOVA and F-test showed that the enzymatic reaction could be described with a partial cubic polynomial model. The equation of the best fitted model in terms of coded factors is as follows (Equation 2):

$$\text{Conversion yield (\%)} = +10.87 - 1.28A + 0.62B - 0.13D - 3.08A^2 + 1.15B^2 - 3.84C^2 - 0.59AC - 4.64BD + 4.19A^2C - 4.75ABD \quad (2)$$

where A is chitosan concentration, B is temperature, C is enzyme amount, and D is time.

The ANOVA of the model is presented in Table 2. The F-value and Prob (F) test the significance of the regression model. They test the null hypothesis that all of the regression coefficients are equal to zero. The F- value of the model (30.47) with a Prob (F) < 0.0001 showed that the model was significant at 95% confidence level. There is only 0.01% chance that a model F-value of this large scale could be arisen from noises. A suitable coefficient of determination (R^2=0.9738) also showed that the model is capable of representing the real relationship among the variables [19]. The lack of fitness of the model (0.24) was not significant at 95% confidence level. Chitosan concentration is the most significant parameter that affects the production of ester. Although the other parameters are not statistically significant, their dependents (quadratic effects and the interactions) are significant. Figure 4 depicts the actual values of conversion versus those predicted by the model. The linear distribution is indicative of a well-fitted model. The normal probability plot of the model has been also shown in Figure 4. The plot compares the data set with the normal distribution. The straight line shows that the residuals (the error) are actually normally distributed.

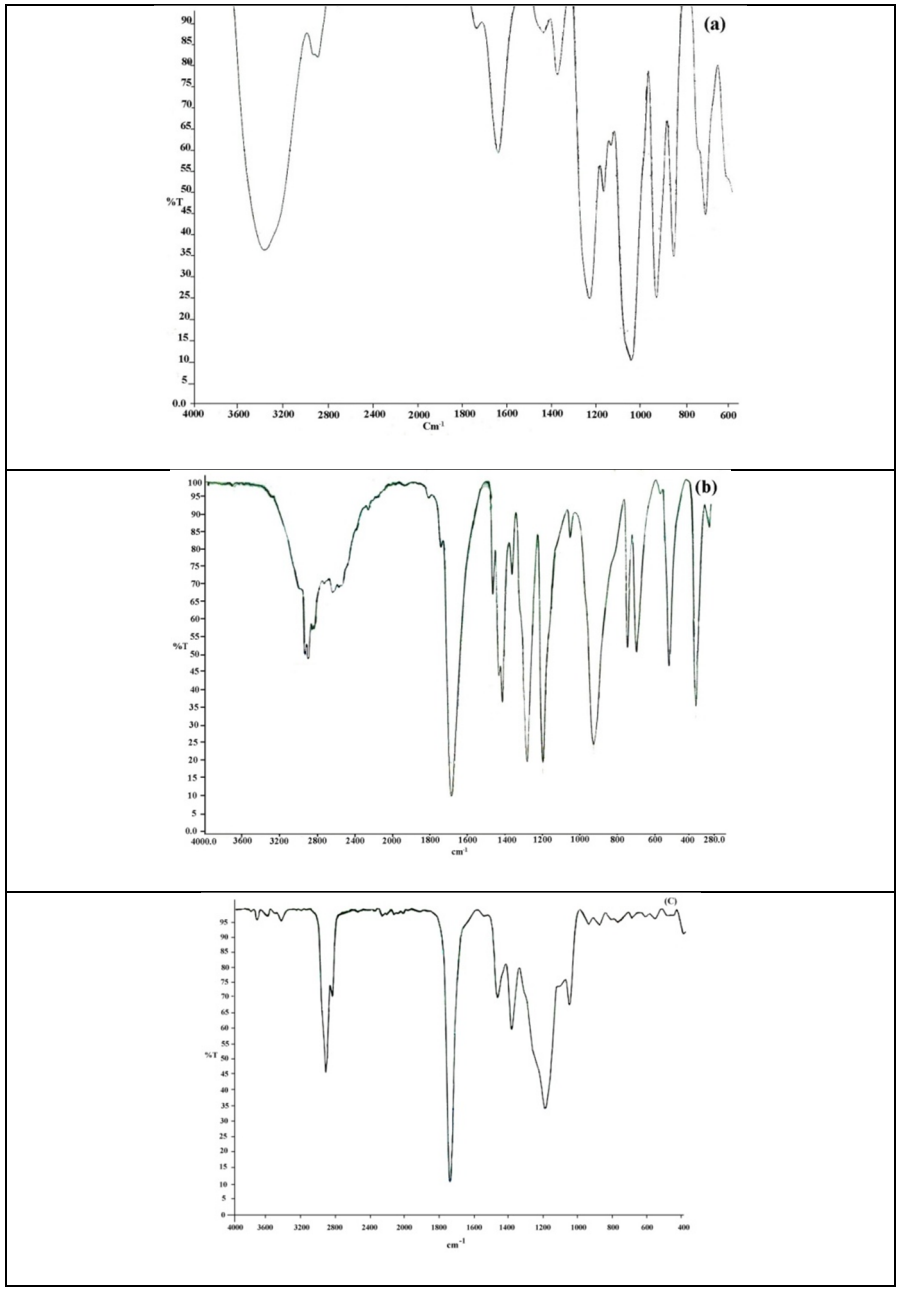

Fig. 2. FTIR spectrum of chitosan (a), adipic acid (b) and chitosan adipate (c).

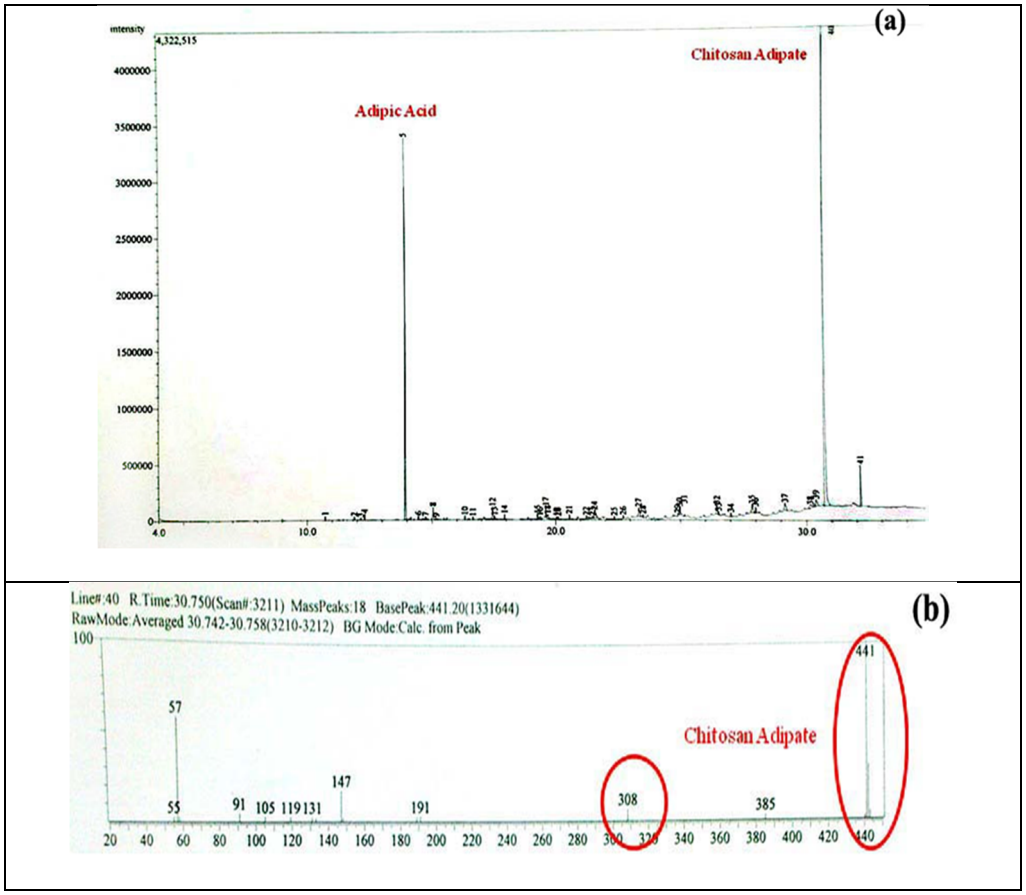

Fig. 3. Gas chromatogram (a) and mass spectrum (b) of chitosan adipate.

Table 2. Analysis of Variance (ANOVA)

Source	Sum of squares	Degree of freedom	Mean square	*F*-Value	*P*-Value
Model	586.36	11	53.31	30.47	< 0.0001
A-Chitosan concentration	9.25	1	9.25	5.28	0.0471
B-Temperature	5.31	1	5.31	3.03	0.1155
C-Enzyme amount	0.000	1	0.000	0.000	1.0000
D-Time	0.25	1	0.25	0.14	0.7152
A^2	118.32	1	118.32	67.63	< 0.0001
B^2	16.32	1	16.32	9.33	0.0137
C^2	183.85	1	183.85	105.08	< 0.0001
AC	2.76	1	2.76	1.58	0.2406
BD	71.37	1	71.37	40.79	0.0001
A^2C	58.11	1	58.11	33.21	0.0003
ABD	72.02	1	72.02	41.17	0.0001
Residual	15.75	9	1.75		
Lack of Fit	11.48	5	2.30	2.15	0.2389
Pure Error	4.27	4	1.07		
Corrected Total	602.10	20			

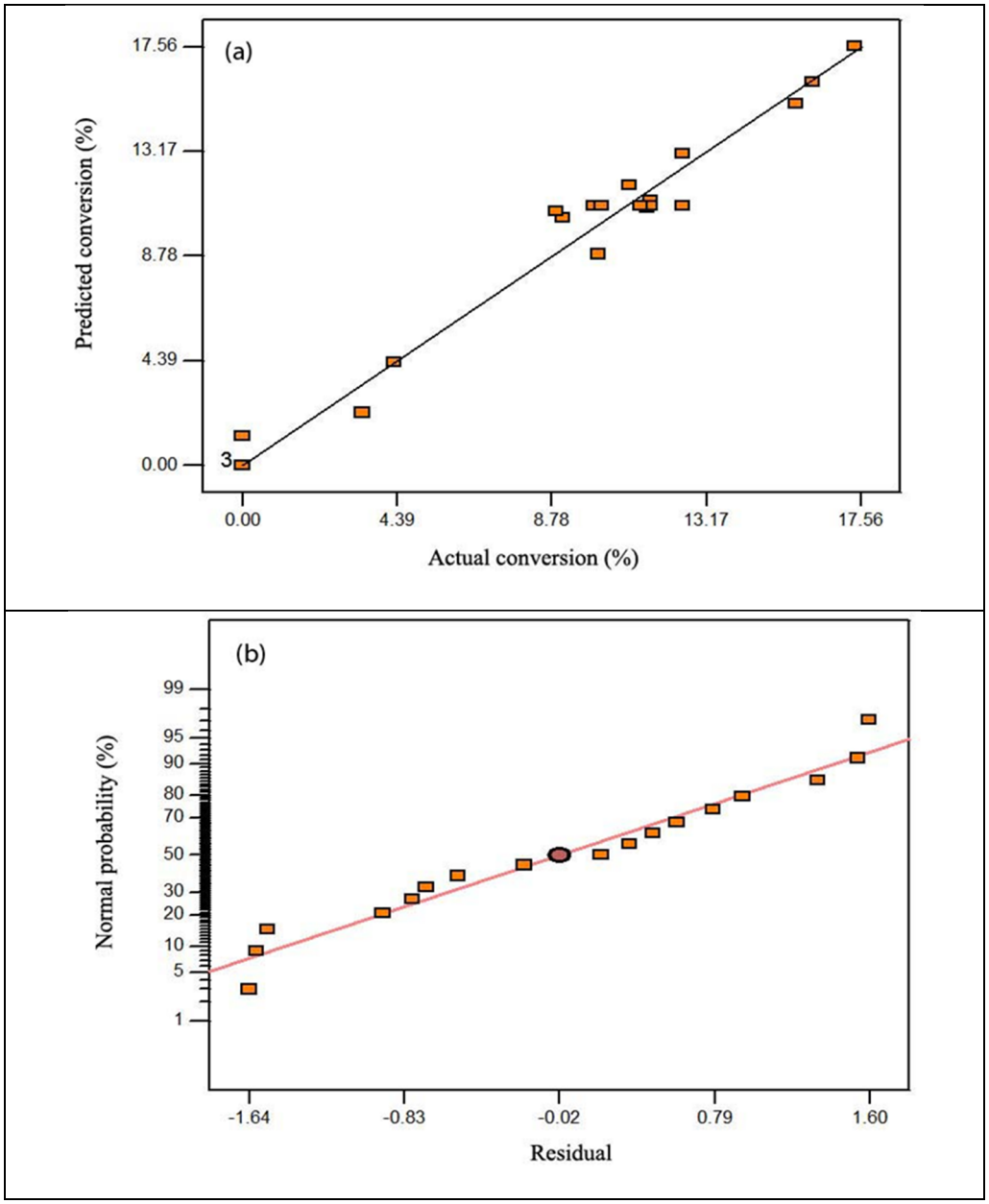

Fig. 4. Plots showing the correlation between actual and predicted conversions (a) and normal probability of residuals (b).

3.3. Effect of parameters and optimum condition

Figure 5 shows the effect of chitosan concentration on the production of ester at 50°C, 12.5% of enzyme and 127.5 min. Chitosan concentration showed highly significant linear and quadratic effects on the esterification. The conversion yield increased by increasing chitosan concentration up to an optimum amount, 0.05 g/mL. The increase in the conversion can be attributed to the more availability of the substrate for the enzyme and thus probability of more substrate and enzyme collision. Furthermore, addition of substrate shifts the reaction equilibrium to the product side. At chitosan concentrations more than 0.05 g/mL, a decrease in the yield was observed. High substrate concentrations more than a specific amount, increase viscosity of reaction medium and impose additional mass transfer limitations on the system [20].

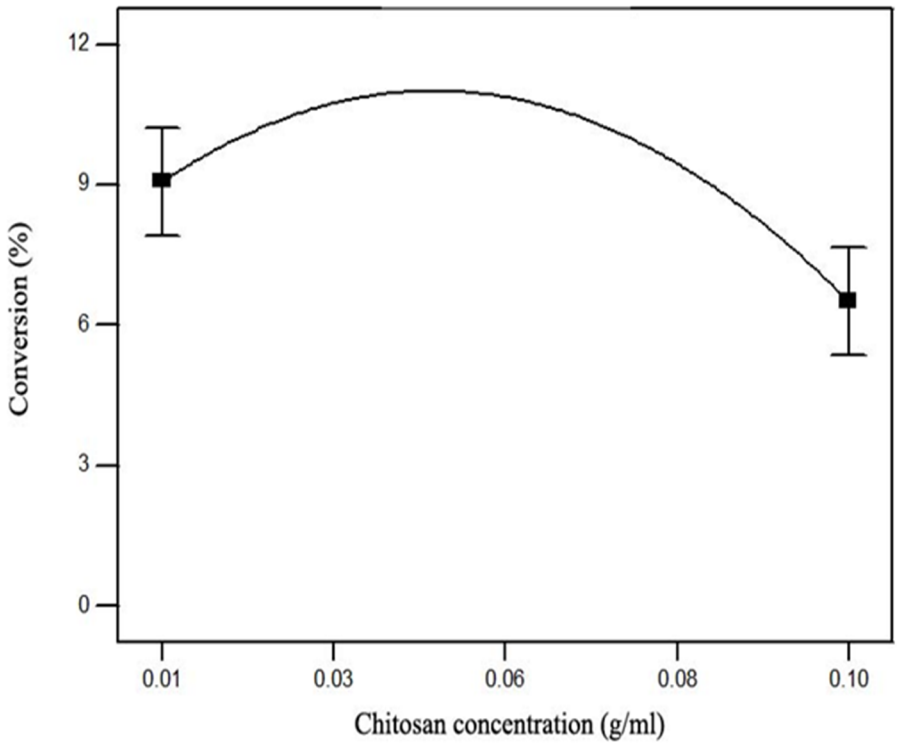

Fig. 5. Effect of various concentrations of chitosan on the synthesis of chitosan ester. Other parameters are kept constant at their center points.

According to ANOVA, the interaction effect of chitosan concentration and amount of enzyme were a significant term in the model. The interaction of parameters has been studied by planned series of contour plots generated from the predicted model. The interaction of chitosan concentration and enzyme amount is shown in Figure 6. Temperature and time were kept constant at their center points. As can be seen in the Figure, maximum conversion is obtained at chitosan concentration of 0.05 g/mL and 12.5% of enzyme. At this concentration of chitosan, increasing the amount of enzyme leads to a decrease in the conversion. This can be due to the diffusional restrictions and mass transfer limitations, which is usually observed in the systems containing immobilized enzyme and poorly soluble compounds [21]. A high conversion yield is also obtained at the low concentration of chitosan (0.01 g/mL) and higher amounts of enzyme. Higher amounts of enzyme provide more catalytic sites for the acyl-enzyme complex formation and enhance the probability of enzyme-substrate collision, hence more substrate molecules are converted into products [22].

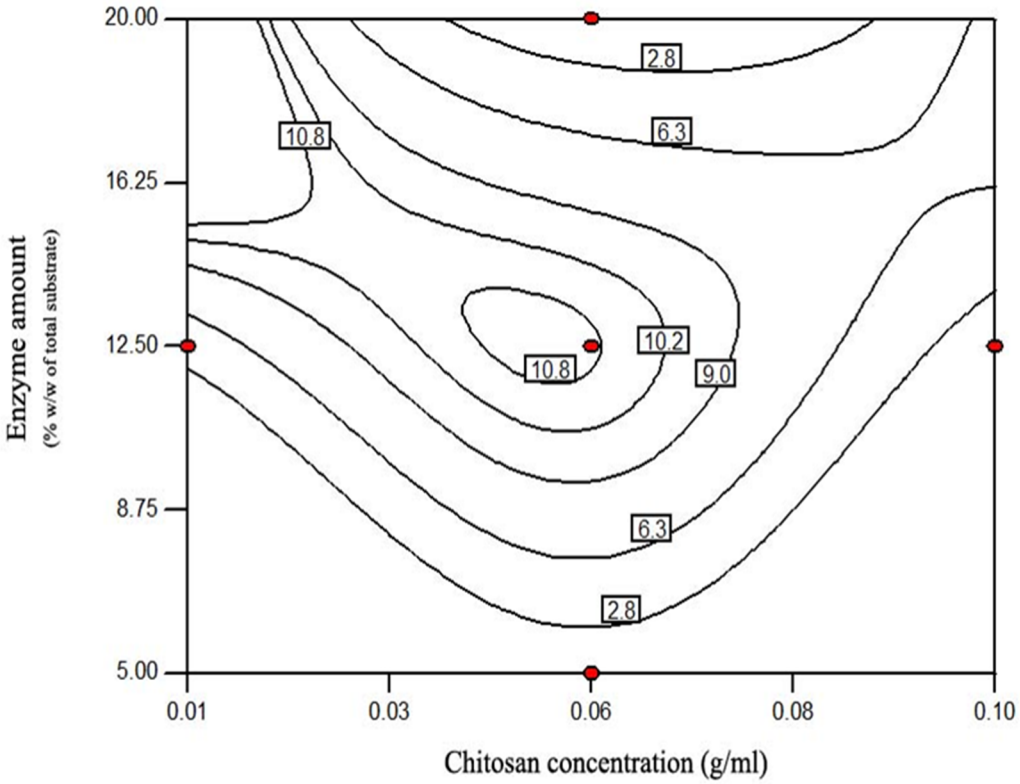

Fig. 6. Contour plots showing the interaction between two parameters, enzyme amount and chitosan concentration, in the synthesis of chitosan adipate. Other variables are constant at their center points. The numbers inside the contour plots indicate conversion yield (%) of the ester.

As can be seen in Table 2, the interaction effect of time and temperature is also a significant term. This interaction is presented in Figure 7. The enzyme amount and chitosan concentration were fixed at their center points. The highest conversions can be obtained at high temperature and short reaction time or low temperature and long time. A rise in temperature increases the kinetic energy of the molecules and causes more collisions between the enzyme and substrates. Therefore, at high temperatures, the reaction equilibrium is reached in a short time. By increasing the time, the volume of accumulated water (byproduct of esterification) increases which leads to hydrolysis of the ester. In addition, the water that exists in the undried catalyst may slowly release into the reaction mixture during the esterification and cause the hydrolysis of the product [23]. At lower temperatures, the rate of collisions between enzyme and substrate molecules and hence the reaction rate would be slow. Therefore, longer reaction time is required for obtaining higher conversions of ester.

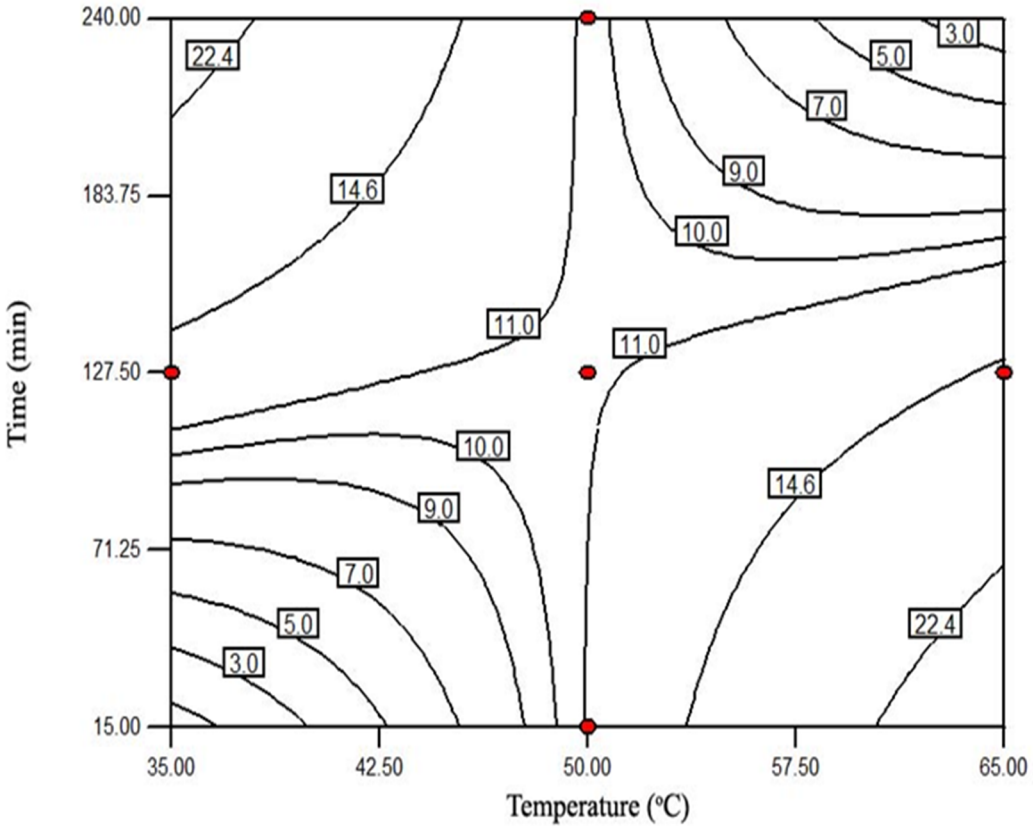

Fig. 7. Contour plots showing the interaction between two parameters, temperature and time, in the synthesis of chitosan adipate. Other variables are constant at their center points. The numbers inside the contour plots indicate conversion yield (%) of the ester.

By using the desirability function (Equation 3), the experiment with the desirability value of 1 was used to predict the optimal condition for the synthesis of ester.

$$\boldsymbol{D = (d_1 \times d_2 \times \ldots . d_n)^{\frac{1}{n}} = (\prod_{i=1}^{n} d_i)^{1/n}} \quad \textbf{(3)}$$

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

Maximum yield (45.3%) was predicted at 240 min and 35°C using 0.10 g/mL of chitosan and 19.1% of enzyme. The actual experimental value obtained was 44.9%. A nearly similar result (43.5%) was also obtained at a short reaction time (15 min) and 65°C using 0.10 g/mL of chitosan and 18.7% of enzyme. The conversion yield predicted by the model was 41.4%. Although the conversion yield is not very high, the short reaction time makes the enzyme suitable as catalyst for the synthesis of chitosan ester.

4. CONCLUSION

Immobilized *Candida antarctica* lipase B-catalyzed synthesis of chitosan adipate was successfully performed. The reaction was modeled and analyzed by response surface methodology. The R^2 (0.9738) and ANOVA implied that the model satisfactorily represented the real relationship of the four main reaction parameters and the response. Chitosan concentration was the only significant parameter that affected the synthesis of chitosan ester. Maximum conversion yield obtained at a reaction time as short as 15 min was 43.5% which matched well with the predicted value of 41.4%.

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بهینه سازی سنتز بیوکاتالیتیکی استر کیتوزان با استفاده از روش سطح پاسخ

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چکیده:

در این تحقیق استریفیکاسیون کیتوزان و آدیپیک اسید در حضور کاتالیست آنزیمی لیپاز تثبیت شده کاندیدا آنتارکتیکا صورت گرفت. برای مدلسازی و آنالیز داده های واکنش از روش سطح پاسخ (RSM) بر اساس طرح مرکب مرکزی کوچک (SCCD) با چهار فاکتور- پنج سطح استفاده شد. مجموعا ۲۱ آزمایش با ترکیب مختلف از چهار فاکتور موثر بر واکنش شامل غلظت کیتوزان (۰/۰۱-۰/۱۰ گرم بر میلی لیتر)، دما (۳۵-۶۵ درجه سلسیوس)، زمان (۱۵-۲۴۰ دقیقه) و مقدار آنزیم (۵-۲۰ درصد وزنی سوبسترا) انجام گرفت. نتایج نشان می دهد داده ها منطبق بر یک مدل درجه سوم جزئی با ضریب تعیین (R^2) ۰/۹۷۳۸هستند که نشان دهنده میزان بالای همبستگی بین مقادیر تجربی و پیشگویی شده توسط مدل است. آنزیم قابلیت کاتالیز واکنش استری کردن کیتوزان را با حداکثر راندمان ۴۵/۳ درصد داشته است. غلظت کیتوزان موثرترین پارامتر موثر بر راندمان واکنش است. در شرایط بهینه با زمان واکنش کوتاه ۱۵ دقیقه، دمای ۶۵ درجه سلسیوس، کیتوزان با غلظت ۰/۱۰گرم بر میلی لیتر و ۱۸/۷ درصد وزنی آنزیم، راندمان استری شدن ۴۳/۵ درصد است.

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