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ORIGINAL PAPER

Modeling and optimization of lipase-catalyzed production of succinic acid ester using central composite design analysis

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Abstract Esterification of succinic acid with oleyl alcohol catalyzed by immobilized *Candida antarctica* lipase B (Novozym 435) was investigated in this study. Response surface methodology (RSM) based on a five-level, four-variable central composite design (CCD) was used to model and analyze the reaction. A total of 21 experiments representing different combinations of the four parameters including temperature (35–65°C), time (30–450 min), enzyme amount (20-400 mg), and alcohol:acid molar ratio (1:1-8:1) were generated. A partial cubic equation could accurately model the response surface with a R^2 of 0.9853. The effect and interactions of the variables on the ester synthesis were also studied. Temperature was found to be the most significant parameter that influenced the succinate ester synthesis. At the optimal conditions of 41.1°C, 272.8 min, 20 mg enzyme amount and 7.8:1 alcohol:acid molar ratio, the esterification percentage was 85.0%. The model can present a rapid means for estimating the conversion yield of succinate ester within the selected ranges.

Keywords Succinic acid · Dioleyl succinate · Enzymatic synthesis · Esterification · Modeling · Central composite design

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Introduction

Dicarboxylic acid esters are widely synthesized due to their excellent properties such as good thermal stability, low volatility, high flash point, and low toxicity [5]. Succinate ester is a dicarboxylic acid ester with multiple industrial direct and indirect applications, especially in food, agricultural and pharmaceutical industries. Succinates have potential applications as the starting material for producing bulk chemicals such as 1,4-butanediol (a precursor to biodegradable plastics), ethylene diamine disuccinate (a biodegradable chelator) and diethyl succinate (a "green" solvent replacement for dichloromethane) with a $15 billion market [13]. Traditionally, succinates are produced via petroleum-based chemical processes or esterification reactions using acidic or metal catalysts [20]. Nowadays, enzymatic synthesis as a 'green' alternative to chemical synthesis of esters offers attractive characteristics of biological systems such as substrate selectivity, mild reaction conditions, simpler and less expensive reaction setup, and low energy requirement [15].

Lipases (E.C. 3.1.1.3) are well known and attractive among the most widely used biocatalysts. Their ability to perform synthesis reactions in non-aqueous media have made them extensively used to produce useful esters [17]. Among the lipases, *Candida antarctica* lipase B has been reported to show a high catalytic activity for esterification of dicaroxylic acids [11].

Synthesis of bio-based succinate by fermentation process using natural or engineered microorganisms has been reported by several researchers [13, 21]. Some disadvantages of the method, such as low productivity due to side reactions, low activity of microorganisms in the presence of high concentrations of succinate and the requirement of media that are too expensive for large-scale use, have

caused low bio-based succinate production that is not yet able to support bulk chemical markets [13]. Application of enzymes to produce these high value-added esters may overcome such limitations. *Candida antarctica* lipase B was employed as a catalyst for copolymerization of succinic acid and 1,4-butanediol to produce low molecular weight oligomers [3]. Jiang [8] also reported immobilized *Candida antarctica* lipase B-catalyzed production of di(n-hexadecyl) succinate and its use as a substrate in the synthesis of aliphatic copolyesters. However, so far, neither specific study on the interactive effect of reaction parameters nor any optimal conditions have been reported for these esters.

Considering the high benefits and demands of succinate esters, process optimization plays a very significant role in their economical enzymatic synthesis. Furthermore, for any design of future upscale enzymatic synthesis of esters, finding the optimal reaction conditions is essential. Statistical experimental design approaches have been extensively employed in bioprocess optimization, because these methods can explore the experimental space while studying various variables by requiring a small number of experiments [1]. Response surface methodology (RSM) and central composite design (CCD) are efficient statistical techniques that have been widely applied in the modeling and optimization of various biotechnological processes [7, 10].

The objective of the present work was to model and optimize the lipase-catalyzed esterification reaction between succinic acid and oleyl alcohol to produce dioleyl succinate ester. The effects of four reaction parameters (temperature, time, substrate molar ratio and amount of enzyme) on the conversion of ester were evaluated, and polynomial model equations for the degree of esterification were established by CCD and RSM.

Materials and methods

Materials

Novozym® 435 (specific activity 10000 PLU/g) was purchased from NOVO Nordisk A/S (Bagsvaerd, Denmark) and consists of *Candida antarctica* lipase B (triacylglycerol hydrolase, EC 3.1.1.3) physically adsorbed within the macroporous acrylic resin (poly[methyl methacrylate-co-butyl methacrylate]) [3]. Succinic acid and oleyl alcohol (cis-9-octadecen-1-ol) were purchased from Merck Co. (Darmstadt, Germany). All other chemicals and solvents used in this study were of analytical grade.

Lipase-catalyzed esterification

Different molar ratios of succinic acid and oleyl alcohol were mixed corresponding to the different substrate molar ratios (alcohol:acid 1:1–8:1) generated by experimental design (CCD), in 30-ml screw-capped vials. Five milliliters of hexane was added as solvent. Selection of hexane (log P = 3.5) as a solvent was based on prior solvent screening studies performed on synthesis of dicarboxylic acid esters for several solvents including hexane, heptane, ethyl acetate, buthanol and acetonitrile [2]. Different amounts of lipase (20–400 mg), which were generated by CCD, were subsequently added. The reaction was carried out in a temperature-controlled (accuracy of ± 0.1°C) horizontal water bath shaker at 150 rpm at different temperatures (35–65°C) and for different time periods generated by the experimental design (30–450 min) (Table 1).

Table 1 Range of variables for the central composite design

Variable	Levels				
	−1.682	−1	0	+1	+1.682
Temperature, A (°C)	35.00	41.08	50.00	58.92	65.00
Time, B (min)	30.00	115.13	240.00	364.87	450.00
Enzyme amount, C (mg)	20.00	97.03	210.00	322.97	400.00
Substrate molar ratio, D	1.00	2.42	4.50	6.58	8.00

Analysis and characterization

The reaction was terminated by dilution with 5 ml of ethanol:acetone (50:50 v/v), and the enzyme was removed by filtration. Remaining free acid in the reaction mixture was determined by titration with 0.1 M NaOH using phenolphthalein as the indicator. The moles of acid reacted were calculated from the values obtained for the control (without enzyme) and the test samples. The ester formed was expressed as equivalent to conversion of the acid [16]. Further identifications of ester formation were carried out by FT-IR spectroscopy (Perkin Elmer, model 1650) and thin layer chromatography using a chloroform/hexane (95:5) solvent system. Formation of the diester was also confirmed by gas chromatography/mass spectroscopy (GC/MS) analysis performed on an Agilent (model GC 7890A; model MS 5975C; Agilent Technologies Inc., Palo Alto, Ca) instrument with a HP-5 column (0.32 mm × 30 m, 0.25 μm). The carrier gas was helium, and the total gas flow rate was 74.2 ml min^{-1}. The injector temperature was set at 250°C. The oven temperature was maintained at 50°C for 5 min, elevated to 280°C at a rate of 8°Cmin^{-1} and held for 10 min. The only product with an R_f value of 0.7 (in TLC analysis) and a retention time of 38.574 min (in GC analysis) was confirmed to be dioleyl succinate by mass spectroscopy analysis. The molecular ion of the product (m/z = 618.5) was barely detectable. In fact, the esters of higher alcohols show much weaker molecular ion peaks [14]. However, an ion peak at m/z = 590.5 was

observed due to the formation of $[M + 2H]^+$ after losing two methyl groups, which confirms the formation of dioleyl succinate. Another important ion peak is related to the formation of ion asilium, $[RCO]^+$, due to α-cleavage reactions and loss of the alkoksi group from the ester ($m/z = 351.2$). Other bond cleavage occurred through some pathways and gave fragment ions at m/z 281.0, 207.0, 135.1 and 55.1.

Experimental design and statistical analysis

Response surface methodology (RSM) was applied to model the lipase-catalyzed synthesis of dioleyl succinate ester. To obtain a proper model for optimization, a four-factor, five-level central composite design (CCD) was employed, requiring 21 experiments. The fractional factorial design consisted of eight factorial points, eight axial points and five center points. Each coded variable can describe a cubical region in three-dimensional space representing the design region [12]. The design of experiments employed is presented in Table 2. The experiments were produced in random order and performed in triplicate. A software package of Design Expert® Version 6.0.6 (State-Ease Inc., Statistics Made Easy, Minneapolis, MN) was used in this study. After testing increasingly complex models from linear to partial cubic to model the data, a partial cubic polynomial equation was developed to study the effects of the variables on the conversion of ester. The partial cubic model does not contain pure cubic terms. It includes all the terms in a quadratic model, but also includes terms having cubic interactions [4]. The fit of the model was evaluated by a coefficient of determination (R^2) and analysis of variance (ANOVA).

Results and discussion

Model fitting and analysis of variance (ANOVA)

Fitting of the data to various models and their subsequent ANOVA showed that the reaction of succinic acid and oleyl alcohol was most suitably described with a partial cubic polynomial model. The equation of the model based on the coded values is as follows (Eq. 1):

$$\begin{aligned}\text{Conversion}(\%) = {} & +37.34 - 6.68A - 0.96B - 5.79C \\ & + 2.34D - 5.45A^2 - 0.59B^2 + 10.96C^2 \\ & - 2.90\ D^2 + 3.30AB + 7.81AC \\ & - 5.44AD + 0.90BC - 9.06BD \\ & + 17.00B^2C \end{aligned} \quad (1)$$

Table 2 Composition of various experiments of the central composite design (in term of coded factors), actual and predicted conversions for synthesis of dioleyl succinate

Exp. no.	Variable					
	Temperature (°C)	Time (min)	Enzyme amount (mg)	Molar ratio	Actual conversion (%)	Predicted conversion (%)
1	0.000	0.000	0.000	0.000	37.0	37.3
2	−1.682	0.000	0.000	0.000	31.2	33.1
3	1.000	−1.000	−1.000	1.000	18.9	18.2
4	−1.000	1.000	1.000	1.000	46.8	44.8
5	0.000	1.682	0.000	0.000	32.1	34.0
6	0.000	0.000	0.000	0.000	36.8	37.3
7	−1.000	1.000	−1.000	1.000	36.9	36.2
8	1.682	0.000	0.000	0.000	8.8	10.7
9	0.000	0.000	0.000	0.000	41.2	37.3
10	0.000	0.000	0.000	0.000	40.0	37.3
11	0.000	0.000	0.000	0.000	36.2	37.3
12	0.000	0.000	0.000	1.682	31.2	33.0
13	1.000	1.000	−1.000	−1.000	29.2	27.3
14	−1.000	−1.000	1.000	−1.000	36.7	36.0
15	−1.000	−1.000	−1.000	−1.000	33.0	31.0
16	0.000	0.000	−1.682	0.000	76.2	78.0
17	0.000	0.000	0.000	−1.682	23.3	25.2
18	0.000	−1.682	0.000	0.000	35.4	37.3
19	1.000	−1.000	1.000	1.000	56.4	54.4
20	1.000	1.000	1.000	−1.000	67.8	67.1
21	0.000	0.000	1.682	0.000	56.7	58.6

Table 3 The analysis of variance (ANOVA) for the partial cubic model

Source	Sum of squares	Degree of freedom	Mean square	*F* value	*P*value
Model	4,726.40	14	337.60	28.66	0.0003
A-temperature	252.23	1	252.23	21.41	0.0036
B-time	5.25	1	5.25	0.45	0.5293
C-amount of enzyme	189.54	1	189.54	16.09	0.0070
D-substrate molar ratio	30.97	1	30.97	2.63	0.1561
AB	36.16	1	36.16	3.07	0.1303
AC	488.44	1	488.44	41.46	0.0007
AD	98.23	1	98.23	8.34	0.0278
BC	6.43	1	6.43	0.55	0.4881
BD	271.77	1	271.77	23.07	0.0030
A^2	443.79	1	443.79	37.67	0.0009
B^2	5.23	1	5.23	0.44	0.5300
C^2	1,796.51	1	1,796.51	152.49	<0.0001
D^2	125.25	1	125.25	10.63	0.0172
B^2C	957.63	1	957.63	81.28	0.0001
Residual	70.69	6	11.78		
Lack of fit	51.09	2	25.55	5.21	0.0768
Pure error	19.60	4	4.90		
Cor total	4,797.08	20			

where A is the temperature, B the time, C the amount of enzyme and D the substrate molar ratio.

The ANOVA for the model is provided in Table 3. The *F* value of the model (28.66) with a *P* value 0.0003 implied that the model was significant at the 95% confidence level. The small *P* value (< 0.001) and a suitable coefficient of determination ($R^2 = 0.9853$) also showed the suitability of the model for representing the real relationship among the reaction parameters [6]. The model also showed no lack of fit at a 95% level of significance. Adequate precision measured the signal-to-noise ratio. Ratios greater than 4 indicated adequate model discrimination. The adequate precision for the developed model was 23.231, indicating that the model could be used to navigate the design space. The coefficients of the response surface model are also presented in Table 3. A *P* value greater than 0.05 indicated the term was not significant. In this case A, C, A^2, C^2, D^2, AC, AD, BD and B^2C were significant model terms. Figure 1 shows the actual values of conversion yield versus those predicted by the model. A linear distribution was observed, which was indicative of a well-fitting model. Equation 1 was then used to study the effect of reaction parameters on the ester synthesis.

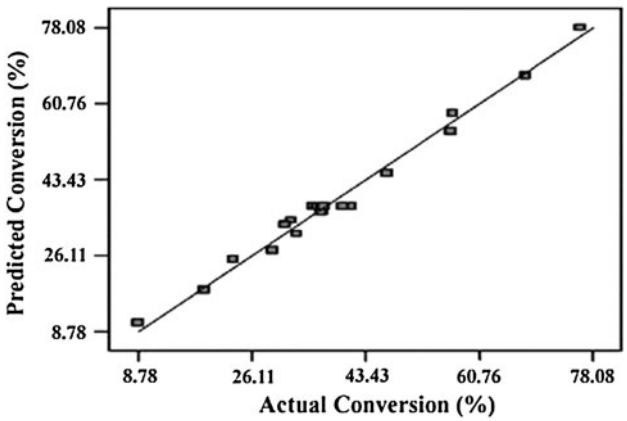

Fig. 1 Correlation of actual conversion yields and values predicted by the response surface model

Effect of parameters and optimum condition

According to ANOVA, temperature is the most significant parameter (*F* value = 21.41) that affects the succinate ester synthesis. The relationships between temperature and other reaction parameters have been studied by planned series of contour plots generated from the predicted model. The plots also indicate the effect of the four reaction parameters on the synthesis of ester. Contour plots in Fig. 2 represent the effect of varying temperature and reaction time on the synthesis of dioleyl succinate at a substrate molar ratio (alcohol:acid) 4.5:1 and 210 mg enzyme. Increasing the temperature promotes collisions between enzyme and substrate molecules and results in enhancement of the reaction rate [16]. The maximum conversion was predicted at 40.2°C. The extent of esterification decreased with an increase in temperature from 40.2 to 65.0°C. Considering the fact that Novozym 435

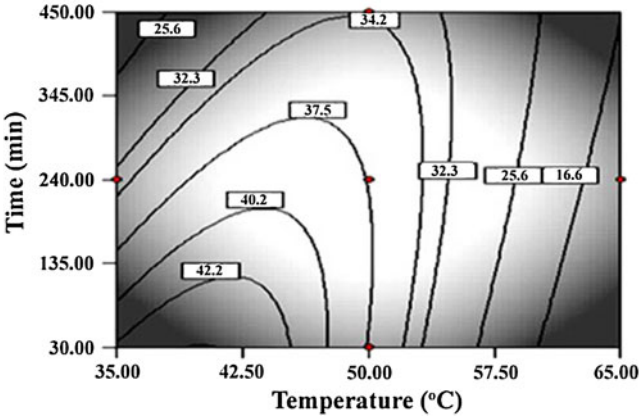

Fig. 2 *Contour plots* showing the interaction between two parameters, time and temperature, in the synthesis of dioleyl succinate. The *numbers* inside the *contour plots* indicate conversion yield (%) of the ester

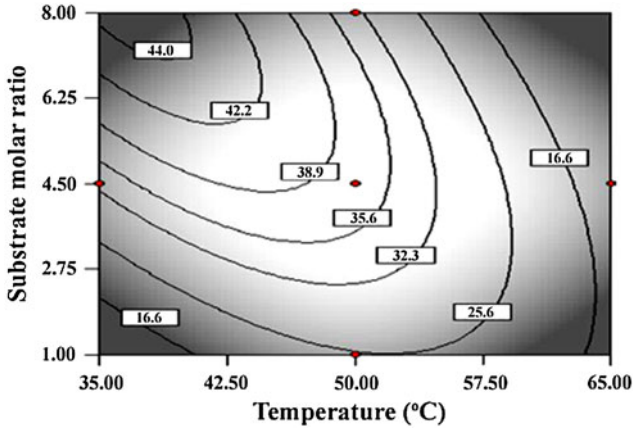

Fig. 3 *Contour plots* showing the interaction between two parameters, substrate molar ratio and temperature, in the synthesis of dioleyl succinate. The *numbers* inside the *contour plots* indicate conversion yield (%) of the ester

possesses high thermostability in organic solvents [3], this result can be related to the decrease in equilibrium constants at higher reaction temperatures. By increasing the time, at any given temperature, a decrease in ester formation was observed. According to ANOVA, time is not a significant parameter that can influence the ester conversion. As can be seen in the contour plot, by prolonging the time from 30 to 450 min at 50°C, only about a 3% decrease in the conversion was observed. It seems that the reaction equilibrium is reached in a short reaction time. By increasing the time, the volume of accumulated water (byproduct of esterification reaction) increases and thus hydrolysis of the ester will happen [19]. Furthermore, the presence of excessive water in the undried catalyst, which might have been slowly released into the reaction mixture during the esterification reaction, can cause the hydrolysis of the ester product.

Figure 3 shows the effect of varying the substrate molar ratio and temperature on the synthesis of succinate ester at 240 min and 210 mg of enzyme. The interactive effect of substrate molar ratio and time is significant on the conversion yield. Again, it is clear that the optimum temperature is in the range of 35.0–42.5°C. Because of the low solubility of succinic acid in the reaction mixture, the maximum conversion is obtained at higher molar ratios of alcohol to acid. Reduction of viscosity and also more availability of the alcohol for the enzyme can also be attained at higher amounts of alcohol. However, increasing the alcohol amount does not affect the conversion at higher temperatures.

The effect of varying enzyme amount and temperature on the ester synthesis is presented in Fig. 4. The substrate molar ratio and reaction time were fixed at their center points. Amount of enzyme showed significant linear and quadratic effects on the synthesis of ester. The interactive

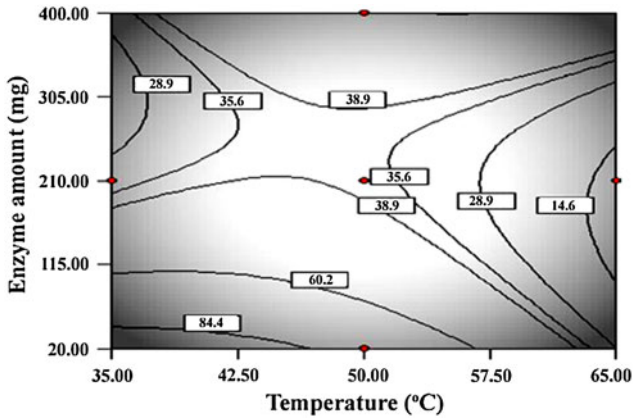

Fig. 4 *Contour plots* showing the interaction between two parameters, enzyme amount and temperature, in synthesis of dioleyl succinate. The *numbers* inside the *contour plots* indicate conversion yield (%) of the ester

effect of temperature and enzyme amount was also highly significant. Generally, by increasing the enzyme molecules an increase in the number of active sites takes place and hence more substrate molecules are converted into products [16]. However, in this study, a decrease in the conversion was observed by increasing the enzyme amount. A higher amount of enzyme may cause diffusional restrictions and mass transfer limitations that can be seen in systems containing immobilized enzyme and poorly soluble compounds [9]. Furthermore, by increasing the enzyme amount, the amount of water present in the undried catalyst increases, which leads to the hydrolysis of the ester.

RSM is able to predict the optimum combination of parameters, on the basis of the ridge maximum analysis and the canonical analysis, to obtain maximum conversion [18]. The maximum conversion (89.0%) for synthesis of dioleyl succinate by using minimum amount of enzyme

was predicted at the condition of 41.1°C, 20 mg enzyme amount, 7.8:1 alcohol:acid molar ratio and 272.8 min. The actual experimental value obtained was 85.0%.

Conclusion

The modeling of immobilized *Candida antarctica* lipase-catalyzed synthesis of dioleyl succinate ester was successfully performed by central composite design and response surface methodology. The R^2 (0.9853) and ANOVA implied that the model satisfactorily represented the real relationship of the four main reaction parameters and the response. A high conversion yield (85%) was obtained at the optimum condition that can be used for future upscaling of the process.

References

1. Abdel-Fattah YR, Saeed HM, Gohar YM, El-Baz MA (2005) Improved production of pseudomonas aeruginosa uricase by optimization of process parameters through statistical esperimental designs. Process Biochem 40:1707–1714
2. Abdul Rahman MB, Zaidan UH, Basri M, Hussein MZ, Rahman RNZA, Salleh AB (2008) Enzymatic synthesis of methyl adipate ester using lipase from *Candida rugosa* immobilized on Mg, Zn and Ni of layered double hydroxides (LDHs). J Mol Catal B Enzym 50:33–39
3. Azim H, Dekhterman A, Jiang Z, Gross RA (2006) *Candida antarctica* lipase B-catalyzed synthesis of poly(butylene succinate): shorter chain building blocks also work. Biomacromolecules 7:3093–3097
4. Bhak G, Song M, Lee S, Hwang S (2005) Response surface analysis of solid state growth of *Pleurotus ostreatus* mycelia utilizing whey permeate. Biotechnol Lett 27:1537–1541
5. Gryglewicz S (2001) Enzyme catalyzed synthesis of some adipic esters. J Mol Catal B Enzym 15:9–13
6. Gunawan ER, Basri M, Abdul Rahman MB, Salleh AB, Rahman RNZA (2005) Study on response surface methodology of lipase catalyzed synthesis of palm-based wax ester. Enzyme Microb Technol 37:739–744
7. Jeong GT, Yang HS, Park DH (2009) Optimization of transesterification of animal fat ester using response surface methodology. Bioresour Technol 100:25–30
8. Jiang Z (2008) Lipase-catalyzed synthesis of aliphatic polyesters *via* copolymerization of lactone, dialkyl diester, and diol. Biomacromolecules 9:3246–3251
9. Lee DC, Park JH, Kim GJ, Kim HS (1999) Modeling, simulation, and kinetic analysis of a heterogeneous reaction system for the enzymatic conversion of poorly soluble substrate. Biotechnol Bioeng 64:272–283
10. Macedo GA, Pastore GM, Rodrigues MI (2004) Optimising the synthesis of isoamyl butyrate using *Rhizopus sp.* lipase with a central composite rotatable design. Process Biochem 39:687–692
11. McCabe RW, Taylor A (2004) An investigation of the acyl-binding site of *Candida antarctica* lipase B. Enzyme Microb Technol 35:393–398
12. McCarron PA, Woolfson AD, Keating SM (1999) Response surface methodology as a predictive tool for determining the effects of preparation conditions on the physicochemical properties of poly(isobutylcyanoacrylate) nanoparticles. Int J Pharm 193:37–47
13. McKinlay JB, Vieille C, Zeikus JG (2007) Prospects for a bio-based succinate industry. Appl Microbiol Biotechnol 76:727–740
14. Pavia DL, Lampman GM, Kriz GS (2009) Introduction to spectroscopy, 4th edn. Brooks/Cole Cengage Learning, Belmont, Australia, pp 477–478
15. Petersson AEV, Gustafsson LM, Nordblad M, Borjesson P, Mattiasson B, Adlercreutz P (2005) Wax esters produced by solvent-free energy-efficient enzymatic synthesis and their applicability as wood coatings. Green Chem 7:837–843
16. Radzi SM, Basri M, Salleh AB, Ariff A, Mohammad R, Abdul Rahman MB, Rahman RNZA (2005) High performance enzymatic synthesis of oleyl oleate using immobilised lipase from *Candida antartica*. Electron J Biotechnol 8:291–298
17. Sun SY, Xu Y (2009) Membrane-bound 'synthetic lipase' specifically cultured under solid-state fermentation and submerged fermentation by *Rhizopus chinensis*: A comparative investigation. Bioresour Technol 100:1336–1342
18. Twu YK, Shih IL, Yen YH, Ling YF, Shieh CJ (2005) Optimization of lipase-catalyzed synthesis of octyl hydroxyphenylpropionate by response surface methodology. J Agric Food Chem 53:1012–1016
19. Villeneuve P, Barea B, Sarrazin P, Davrieux F, Boulanger R, Caro Y, Figueroa-Epinoza MC, Graille J (2003) Synthesis of pyroglutamic acid fatty esters though lipase-catalysed esterification with medium chains alcohols. Enzyme Microb Technol 33:79–84
20. Wang D, Li Q, Li W, Xing J, Su Z (2009) Improvement of succinate production by overexpression of a cyanobacterial carbonic anhydrase in *Escherichia coli*. Enzyme Microb Technol 45:491–497
21. Zeikus JG, Jain MK, Elankovan P (1999) Biotechnology of succinic acid production and markets for derived industrial products. Appl Microbiol Biotechnol 51:545–552