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SYSTEMS BIOTECHNOLOGY

Green and efficient production of octyl hydroxyphenylpropionate using an ultrasound-assisted packed-bed bioreactor

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Abstract A solvent-free system to produce octyl hydroxyphenylpropionate (OHPP) from *p*-hydroxyphenylpropionic acid (HPPA) and octanol using immobilized lipase (Novozym® 435) as a catalyst in an ultrasound-assisted packed-bed bioreactor was investigated. Response-surface methodology (RSM) and a three-level-three-factor Box-Behnken design were employed to evaluate the effects of reaction temperature (x_1), flow rate (x_2) and ultrasonic power (x_3) on the percentage of molar production of OHPP. The results indicate that the reaction temperature and flow rate were the most important variables in optimizing the production of OHPP. Based on a ridge max analysis, the optimum conditions for OHPP synthesis were predicted to consist of a reaction temperature of 65°C, a flow rate of 0.05 ml/min and an ultrasonic power of 1.74 W/cm^2 with a yield of 99.25%. A reaction was performed under these optimal conditions, and a yield of 99.33 ± 0.1% was obtained.

Keywords Esterification · Lipase · Packed-bed bioreactor · Solvent-free · Ultrasound

Introduction

Since the first chemical revolution in the late eighteenth century, synthetic chemicals have made human life more convenient and comfortable. Synthetic chemicals are widely used in our everyday life, from commodity materials to ingredients in food and pharmaceutical products. On the other hand, the synthesis of chemicals produces hazardous waste, and some of these compounds or byproducts possess toxic, pathogenic and carcinogenic effects, which cause environmental health concerns. Therefore, there is an urgent need to develop "green" ways to synthesize functional molecules without damaging the environment. Compared with chemical catalysis, the enzymatic synthesis of molecules is more compatible with variations in the quality of raw materials. Enzymatic synthesis requires only simple purification steps and is conducted under moderate reaction conditions [9]. Lipase-catalyzed esterification can be carried out either in organic solvents or in a solvent-free system. The solvent-free system, which is a simple mixture of substrates, offers the advantages of the elimination of toxic solvents, the maximization of substrate concentration, and a reduction in the costs of purification and organic solvents [4, 19].

Phenolic compounds are secondary metabolites of plants and play an important role in defense mechanisms that protect against pathogens, parasites and UV radiation [18]. These compounds possess antioxidant, antibacterial,

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anti-inflammatory and anticancer activities [1, 11]. Due to their phenolic nucleus and an extended side-chain conjugate, phenolic compounds can readily form resonance-stabilized phenoxy radicals, which account for their antioxidant properties. With the increasing demand for natural ingredients and the health concerns associated with the use of synthetic antioxidants, phenolics have the potential to replace synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) [13]. However, the hydrophilic nature of phenolics reduces their solubility and stability in emulsions and oil-based formulas, resulting in low antioxidant activity [14, 20]. Thus, it is important to enhance the lipophilic ability of these compounds in order to improve their utility as natural antioxidants in oil-based products.

One of the most effective ways to increase the lipophilic ability of phenolic compounds is through the esterification of phenolic acids with alkyl alcohol. Caffeic acids, cinnamic acids, ferulic acids, *p*-hydroxybenzoic acids, *p*-hydroxyphenylpropionic acids and their analogues have been successfully esterified with different alkyl alcohols in the presence of solvents or under solvent-free conditions [2, 5, 10, 16, 17, 19, 21]. The relative esterification activities of phenolic acids for lipase are *p*-hydroxylphenylpropionic acid > cinnamic acid > *p*-coumaric acid > ferulic acid [17]. Immobilized lipase B from *Candida antarctica* demonstrates greater activity than other biocatalysts for the esterification of various phenolic acids with medium- and long-chain alkyl alcohol [17, 21]. The conversion efficiency varied depending on the structure of phenolics and alcohols, the solubility of substrates and products, the enzyme selectivity, the reaction temperature and the water activity [8, 20].

Ultrasound, a new technique in green chemistry, has proven to be a useful tool for accelerating enzymatic reactions in a variety of systems [6, 12, 22]. Specifically, the collapse of the cavities created by ultrasound waves can disrupt the phase boundary between compounds and facilitate mixing. Ultrasound improves mass transfer and provides activation energy for the initiation of reactions. To the best of our knowledge, we are the first group to combine the three state-of-the-art green technologies (i.e., enzyme catalysis, solvent-free systems and sonochemistry) with a packed-bed bioreactor to synthesize phenolic esters.

The purpose of this study was to develop an ultrasound-assisted packed-bed bioreactor (which was packed with lipase) to synthesize a phenolic ester (octyl hydroxyphenylpropionate, OHPP) from a phenolic acid (p-hydroxyphenylpropionic acid, HPPA) with octanol (Scheme 1). The reaction was performed at a moderate temperature in the absence of solvents. A statistical experiment design and response surface methodology (RSM) analysis was employed to investigate the relationship between the reaction variables (reaction temperature, flow rate and ultrasound power) and the reaction efficiency (molar conversion %) in order to obtain the optimal conditions for esterification.

Materials and methods

Materials

Immobilized lipase (triacylglycerol hydrolase, EC 3.1.1.3; Novozym® 435) from *C. antarctica*, supported on acrylic resin beads, was purchased from Novo Nordisk Bioindustrials, Inc. (Bagsvaerd, Denmark). According to the commercial production manual, the lipase's catalytic activity was 10,000 PLU/g (propyl laurate units per gram), and it contained 1–2% (w/w) moisture. *p*-Hydroxyphenylpropionic acid (HPPA) and 1-octanol (99.5% pure) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Anhydrous sodium sulfate and a molecular sieve (4 Å) were purchased from Davison Chemical (Baltimore, MD, USA). All chemicals were analytical reagent grade.

The enzymatic synthesis of OHPP by ultrasound assistance was carried out in a 40-kHz ultrasonic bath. Ultrasonic powers were supplied from flat-plate ultrasound transducers in the bath bottom ($d = 4.5$ cm). Ultrasonic power was adjusted using a scaled dial disc on the bath panel and measured for its power using the calorimetric method [7]. When the intensity of ultrasound is reported as W/cm^2, the area of the transducer needs to be known to calculate the power delivered to a given volume of the inner bath.

Experimental design

A three-level-three-factor Box-Behnken design, with three replicates at the center, was employed. The 15 runs were performed in a random order. The variables and their levels that were selected for the study of OHPP synthesis were reaction temperature (45–65°C), flow rate (0.1–0.05 ml/min) and ultrasonic power (1.46–1.98 W/cm^2). Table 1 displays the independent factors (x_i), levels and experimental design in terms of coded and uncoded variables.

Synthesis of OHPP in the ultrasound-assisted packed-bed bioreactor

Octanol was dehydrated with molecular sieves (4 Å) for 24 h. HPPA (100 mM) and octanol were mixed thoroughly in a feeding flask. The esterification reaction was carried out in a packed-bed reactor consisting of a 25-cm-long stainless steel tube with a 0.46 cm inner diameter. The diagram of the apparatus is shown in Fig. 1.

The system was placed in the temperature-controlled ultrasonic bath. The HPPA-octanol mixture was pumped through the continuous reactor (a packed-bed column with

Scheme 1 Lipase-catalyzed synthesis of OHPP from HPPA and octanol

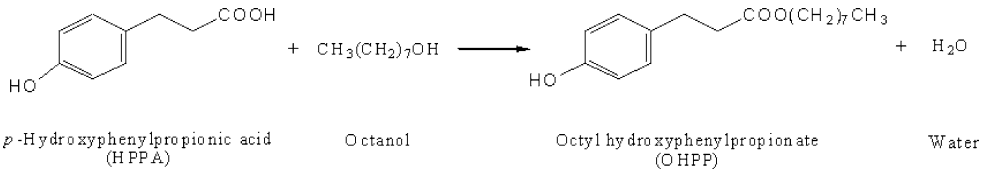

Table 1 The 15 experiments and their results, analyzed via three-level-three-factor Box-Behnken design

Treatment[a] no.	Factors			
	Reaction temperature (°C) x_1	Flow rate (ml/min) x_2	Ultrasonic power (W/cm^2) x_3	Molar conversion (%) $Y \pm$ SD
1	1[b](65)	1(0.1)	0(1.72)	62.60 ± 0.63
2	1(65)	−1(0.05)	0(1.72)	88.69 ± 0.72
3	−1(45)	1(0.1)	0(1.72)	38.41 ± 0.14
4	−1(45)	−1(0.05)	0(1.72)	51.47 ± 0.52
5	1(65)	0(0.075)	1(1.98)	72.70 ± 0.88
6	1(65)	0(0.075)	−1(1.46)	70.97 ± 0.59
7	−1(45)	0(0.075)	1(1.98)	40.25 ± 1.39
8	−1(45)	0(0.075)	−1(1.46)	26.36 ± 0.77
9	0(55)	1(0.1)	1(1.98)	23.99 ± 1.04
10	0(55)	1(0.1)	−1(1.46)	46.38 ± 1.07
11	0(55)	−1(0.05)	1(1.98)	74.32 ± 1.60
12	0(55)	−1(0.05)	−1(1.46)	73.75 ± 0.46
13	0(55)	0(0.075)	0(1.72)	47.69 ± 0.35
14	0(55)	0(0.075)	0(1.72)	53.93 ± 0.62
15	0(55)	0(0.075)	0(1.72)	47.78 ± 0.79

[a] The treatments were run in random order

[b] The values (−1), (0) and (1) are coded levels

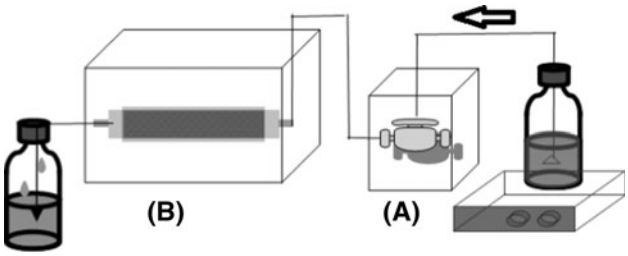

Fig. 1 The diagram of the ultrasound-assisted packed-bed bioreactor. **a** Pump; **b** temperature-controlled ultrasonic bath

1 g Novozym® 435 lipase; particle size 0.3–0.9 mm) at the designed conditions.

The mechanism of action of ultrasound in enhancing the efficiency of Novozym® 435-catalyzed reactions is the central theme of this article. The high-energy ultrasonic waves would cause cavitations in the liquid solution. While subsequent collapses of the cavitation bubbles may cause a thorough mixing and stirring of the liquid solution, the energy thus released could accelerate the chemical and/or enzymatic reactions that occur in the solution.

Determination of OHPP

The OHPP formation was determined by injecting a 1-μl aliquot of the reaction product in splitless mode into a gas chromatograph (Hewlett Packard 7890, Avondale, PA, USA) equipped with a flame-ionization detector (FID) and a MXT-65TG fused silica capillary column (30 m × 0.25 mm i.d.; film thickness 1 μm; Restek, Bellefonte, PA, USA). The injector and FID temperatures were set at 280 and 300°C, respectively. The oven temperature was maintained at 200°C for 1 min, increased to 250°C at 100°C/min, kept at 250°C for 2.5 min and then increased to 300°C for 2 min at 50°C/min. Nitrogen was used as a carrier gas, and the flow rate was 5.6 ml/min. The percent of molar conversion was defined by Eq. 1:

$$\text{Molar conversion}\,(\%) = \frac{A_{100\ \text{mM HPPA}} - A_{\text{remnant HPPA}}}{A_{100\ \text{mM HPPA}}} \times 100\% \quad (1)$$

where $A_{100\ \text{mM HPPA}}$ and $A_{\text{remnant HPPA}}$ represent the peak area of 100 mM HPPA and HPPA after esterification, respectively.

Statistical analysis

The experimental data (Table 1) were analyzed by response surface regression (RSREG) procedures with SAS software to fit the following second-order polynomial Eq. 2:

$$Y = b_0 + \sum_{i=1}^{3} b_i x_i + \sum_{i=1}^{2} \sum_{j=i+1}^{3} b_{ij} x_i x_j \quad (2)$$

where Y is the response (percent of molar conversion); b_0 is a constant, b_i, b_{ii} and b_{ij} are coefficients; and x_i and x_j are the uncoded independent variables. The ridge max option was used to compute the estimated ridge of maximum response for increasing the radii from the center of the original design.

Results and discussion

Primary experiment

The flow rate of the substrate is an important parameter in a packed-bed bioreactor. Figure 2 shows that the highest conversion (95%) of OHPP was obtained at a flow rate of 0.01–0.05 ml/min. The molar conversion decreased from increasing the flow rate from 0.05 to 0.1 ml/min. The increase in substrate flow rate caused a reduction in the residence time of substrate in the packed-bed reactor, which resulted in poor interaction between lipases and substrates. The results indicate that higher molar conversions were obtained with slower flow rates. A similar conclusion can be found in the results of Chang et al. [3], who used isopropanol and soybean oil in a molar ratio of 1:4.14, a reaction temperature of 51.5°C and a flow rate of 0.1 ml/min with Novozym® 435 in a packed-bed reactor. Their results indicated that the molar conversion increased with decreasing flow rates, with the highest conversion (76%) obtained at a flow rate of 0.1 ml/min.

To investigate the relationship between reaction conditions (reaction temperature, flow rate and ultrasonic power) and the molar production of the OHPP, RSM, combined with a three-level-three-factor Box-Behnken design, was employed in this study. The optimal conditions of OHPP synthesis can be found inside the experimental region (Table 1) through the analysis of statistics and contour plots.

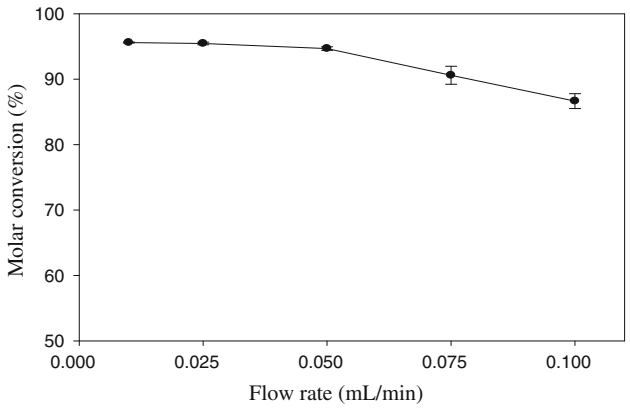

Fig. 2 Effect of flow rate on lipase-catalyzed synthesis of OHPP. Reaction conditions: temperature of 55°C; ultrasonic power of 1.98 W/cm^2

Development of an empirical model

The RSREG procedure from SAS was employed to fit the second-order polynomial Eq. 2 to the experimental data (Table 1). Among the various treatments, the highest molar production of the OHPP (88.69%) was treatment no. 2 (reaction temperature of 65°C; flow rate of 0.05 ml/min; ultrasonic power of 1.72 W/cm^2), and the lowest molar production of the OHPP (23.99%) was treatment no. 9 (reaction temperature of 55°C; flow rate of 0.1 ml/min; ultrasonic power of 1.98 W/cm^2). The second-order polynomial Eq. 2 obtained was as follows:

$$\begin{aligned} Y(\%) = & -154.22 + 0.07x_1 + 147.39x_2 + 201.66x_3 \\ & + 0.04x_1^2 - 13.03x_1x_2 + 10,026x_2^2 - 1.169x_1x_3 \\ & - 883.08x_2x_3 - 21.54x_3^2 \end{aligned} \quad (3)$$

The analysis of variance (ANOVA) data (Table 2) indicated that the second-order polynomial model was an adequate representation of the actual relationship between the response and the significant variables (P-value = 0.0309; $R^2 = 0.916$).

Obtaining optimal synthesis conditions

The optimal condition for the synthesis of OHPP was determined by ridge max analysis, which approximates the estimated maximum response for increasing the radii from the center of the original design [15]. Table 3 indicates that the maximum molar production of the OHPP was 99.25% at a flow rate of 0.05 ml/min, a reaction temperature of 65°C and an ultrasonic power of 1.74 W/cm^2.

Table 2 ANOVA for synthetic variables pertaining to the response of molar conversion

Source	*df*	Sum of squares	Prob > F[a]
Linear	3	4,108.288	0.0051
Quadratic	3	215.217	0.5192
Crossproduct	3	211.202	0.5259
Total model	9	4,534.707	0.0309
Lack of fit	3	391.597	0.0906
Pure error	2	25.589	
Total error	5	417.187	
R^2	0.916		

[a] Prob > F: level of significance

Mutual effect of parameters

Figure 3a shows the effect of reaction temperature, flow rate and their mutual interaction on OHPP synthesis at an ultrasonic power of 1.98 W/cm^2. At the lowest reaction temperature (45°C) with the highest flow rate (0.1 ml/min), the molar production of the OHPP was 27%. A reaction with the highest temperature (65°C) and a flow rate of 0.05 ml/min led to 95.94% conversion of OHPP. An increased temperature and a decreased flow rate resulted in an increased molar production of the OHPP. The effect of varying the reaction temperature and ultrasonic power on esterification at a flow rate of 0.05 ml/min is shown in Fig. 3b. At any given ultrasonic power from 1.46 to 1.98 W/cm^2, an increase of temperature led to a higher molar production of the OHPP.

Verification of the empirical model

The validity of the predicted model was examined by additional independent experiments at the suggested optimal synthesis conditions. The predicted value was 99.25% molar conversion, and the actual experimental value was 99.33 ± 0.1%. A chi-square test (P-value = 0.99, degrees of freedom = 3) indicated that the observed values were statistically the same as the predicted values and that the generated model adequately predicted the percent molar production of the OHPP. In a previous study by our group, the synthesis of OHPP with 95.9% molar conversion required a reaction of 58.2 h at a temperature of 52.9°C and an enzyme amount of 37.8% in an orbital shaking water bath (180 rpm) [19]. However, the residence time was only 83 min (column volume/flow rate), and 99.33% of molar conversion was achieved in a packed-bed bioreactor with ultrasound assistance. It is possible that the increasing collision effects between the two substrates and the enzyme decrease the required reaction time. Xiao et al. [23] also demonstrated that the use of ultrasound accelerated the enzymatic synthesis of sugar ester. The authors suggested that the acceleration was likely due to an increase in collisions between the substrates and the enzyme. The result confirms that, at a high enzyme concentration (e.g., packed-bed reactor), ultrasound leads to a higher reaction rate and conversion yield.

Table 3 Estimated ridge of maximum response for variable molar conversion

Coded radius	Estimated response (% corporation)	Standard Error	Uncoded factor values		
			X_1 (°C)	X_2 (ml/min)	X_3 (W/cm^2)
0.00	49.80	5.27	55.00	0.08	1.72
0.50	62.81	4.96	58.65	0.07	1.72
1.00	79.26	5.27	61.99	0.06	1.73
1.50	99.25	8.76	65.05	0.05	1.74

Conclusions

The use of ultrasound assistance in the Novozym® 435-catalyzed synthesis of OHPP from HPPA and octanol with a solvent-free system in a packed-bed bioreactor was developed successfully. A second-order model depicting the relationship between the response and the three parameters was established. The reaction temperature and flow rate significantly affected the percent yield of OHPP. With ultrasound assistance, the collision effects enhance

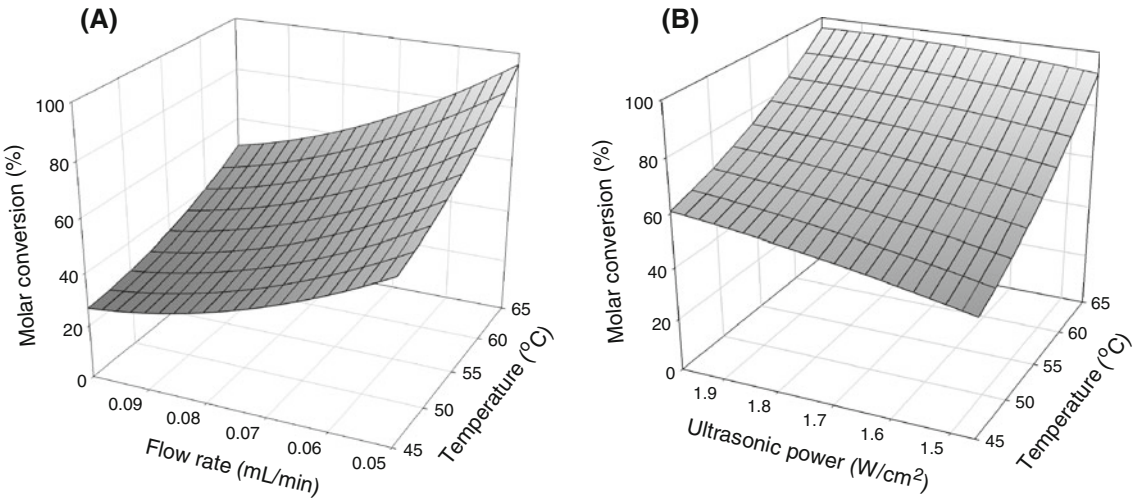

Fig. 3 Response-surface plot showing the effects of **a** reaction temperature, flow rate and their mutual interaction on OHPP synthesis; **b** reaction temperature, ultrasonic power and their mutual interaction on OHPP synthesis

the reaction rate, leading to a higher conversion yield of OHPP. The ultrasound-assistance packed-bed bioreactor not only decreases the reaction time, but also increases the synthesis efficiency. In addition, the solvent-free synthesis of OHPP is suitable for the cosmetics and food-production industries. From an economic viewpoint, this approach is considered a potential alternative for continuous production OHPP for industrial application.

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