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Combined of ultrasound irradiation with high hydrostatic pressure (US/HHP) as a new method to improve immobilization of dextranase onto alginate gel

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ABSTRACT

In this research work, dextranase was immobilized onto calcium alginate beads by the combination of ultrasonic irradiation and high hydrostatic pressure (US/HHP) treatments. Effects of US/HHP treatments on loading efficiency and immobilization yield of dextranase enzyme onto calcium alginate beads were investigated. Furthermore, the activities of immobilized enzymes prepared with and without US/HHP treatments and that prepared with ultrasonic irradiation (US) and high hydrostatic pressure (HHP), as a function of pH, temperature, recyclability and enzyme kinetic parameters, were compared with that for free enzyme. The maximum loading efficiency and the immobilization yield were observed when the immobilized dextranase was prepared with US (40 W at 25 kHz for 15 min) combined with HHP (400 MPa for 15 min), under which the loading efficiency and the immobilization yield increased by 88.92% and 80.86%, respectively, compared to immobilized enzymes prepared without US/HHP treatment. On the other hand, immobilized enzyme prepared with US/HHP treatment showed V_{max}, K_M, catalytic and specificity constants values higher than that for the immobilized enzyme prepared with HHP treatment, indicated that, this new US/HHP method improved the catalytic kinetics activity of immobilized dextranase at all the reaction conditions studied. Compared to immobilized enzyme prepared either with US or HHP, the immobilized enzymes prepared with US/HHP method exhibited a higher: pH optimum, optimal reaction temperature, thermal stability and recyclability, and lower activation energy, which, illustrating the effectiveness of the US/HHP method. These results indicated that, the combination of US and HHP treatments could be an effective method for improving the immobilization of enzymes in polymers.

1. Introduction

Immobilized enzymes are currently the object of considerable interest. This is due to the expected benefits over soluble enzymes or alternative technologies. The number of applications of immobilized enzymes is increasing steadily [1]. Effects of immobilization on activity, stability, and even selectivity of some enzymes have been well documented [2,3]. For example, immobilization of lipase can improve activity by shifting the equilibrium between open and closed form towards the open, more active form and the enantiomeric ratio of lipase products may be changed from 1 to almost 100 by using different immobilization preparations [3]. In addition, chymotrypsin can be stabilized by a factor of 60,000 by immobilization [4]. From the technological point of view, immobilized enzymes can easily be separated from the reaction liquid and make laborious separation steps unnecessary and enables repetitive use of enzymes and hence significant cost savings. Additional benefits arise from stabilization against harsh reaction conditions which are deleterious to soluble enzyme preparations. Due to the wide variation, both, in the properties of the individual enzyme species and requirements of reaction technology for target compounds, it is advisable to exploit fully the potential of immobilization technology. Different methods involved in this technology are comprehensively reviewed by Tischer and Wedekind [5].

In our previous research work, we have established a new ultrasonic method assisted the immobilization of dextranase onto Ca-alginate gel beads [6]. This method has improved the loading efficiency and immobilization yield of the enzyme onto calcium alginate beads as well as catalytic kinetics activity of immobilized dextranase at all reaction conditions studied. Compared with immobilized enzyme prepared without US treatment, the immobilized enzymes prepared with a new US method exhibited a higher:

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pH optimum, optimal reaction temperature, activation energy, and thermal stability, as well as, a higher recyclability, which, illustrating the effectiveness of the ultrasound method [6]. Therefore, we considered to study the effect of combination of other methods with the ultrasonic irradiation on the immobilization of enzymes.

Tyrosinase was covalently immobilized onto amino-functionalized carbon felt surface via glutaraldehyde-coupling under US treatment for 10 min [7]. Authors were succeeded in (1) shortening the enzyme immobilization time from 16 h to 10 min using US treatment, (2) enhancing the sensitivity of *p*-chlorophenol (*p*-CP) as a substrate, and (3) improving the operational stability of *p*-CP. Recently, Meridor and Gedanken have also immobilized α-amylase on polyethylene films using sonochemical irradiation method [8]. These immobilization methods improved the catalytic activity of α-amylase at all the reaction conditions studied. In addition, physical features of ultrasound assisted enzymatic and chemical reactions were studied by different researcher [9,10]. Khanna et al. have attempted to provide an insight into the mechanistic aspects of ultrasound mediated bioconversion of glycerol to ethanol and 1,3-propanediol by immobilized *Clostridium pasteurianum* cells on silica support [11].

The effect of pressure was studied in an enzymatic reaction with an immobilized biocatalyst. Naringinase was immobilized by entrapment in the calcium alginate beads and used to catalyze, at high pressure, the hydrolysis of naringin to naringenin [12]. Recently, Eisenmenger, and Reyes-De-Corcuera, have studied the effects of HHP on the stability and activity of immobilized lipase in hexane. They reported that, HHP reduced thermal inactivation of lipase by up to 152% after 4 h at 80 °C and 400 MPa when compared to incubations at low pressure [13].

This study was, therefore, undertaken to investigate the combined effects of ultrasound and high hydrostatic pressure (US/HHP) treatments on the immobilization of dextranase onto calcium alginate polymer. Loading efficiency and immobilization yield were determined, furthermore, the catalytic kinetics activity and thermodynamics parameters of the immobilized enzyme under US/HHP treatments were compared with that of the free enzyme, and with immobilized enzyme prepared using US and HHP, separately.

2. Materials and methods

2.1. Materials

Dextranase produced by *Chaetomium erraticum*, dextran from *Leuconostoc mesenteroides* (Mw ~ 2000 KDa (T_{2000}), according to Fluka's specification, Product No. 9577), crystalline bovine serum albumin, and sodium alginate from brown algae were obtained from Sigma–Aldrich (Shanghai, China). All other chemicals and solvents used were of analytical grade.

2.2. Ultrasound and high hydrostatic pressure equipments

The experimental ultrasound apparatus used in this work has been described in details in our previous work [14]. The ultrasound generators probe, could deliver a maximum power of 100 W at 25 kHz. The HHP experimental apparatus used for this work was also described in details in our previous work [15]. The pressurization fluid was water:propylene glycol (30:70). The maximum working pressure of the HHP machine was 900 MPa. Polypropylene tubes with a volume of 10 cm^3 were used in HHP experiments.

2.3. Dextranase activity assay

Dextranase activity was measured by determining the rate of hydrolysis of dextran (T_{2000}) at pH 5.0 and 40 °C. After enzymatic degradation of dextran, the reducing sugars released were quantified by two different methods. A spectrophotometric method measured reducing sugars, which react with 3,5 dinitrosalicylic acid to give a yellow–brown color, which was measured at 540 nm [16,17]. To confirm this result, the reducing sugars released were also measured by HPLC system [18]. One dextranase unit (DU/ml) was the amount of enzyme that degrades dextran (T_{2000}) to produce reducing sugar equivalent to 1 mg isomaltose per hour at 40 °C and pH 5.4.

2.4. HPLC analysis and chromatographic conditions

The analysis of samples after dextran (T_{2000}) was hydrolyzed by immobilized dextranase enzyme was carried out according to the method as described by Bashari et al. [14]. The chromatographic system consisted of a Waters 1525 binary pump and differential refractive index detector (RI-150, Japan). Thermo Aps-2 Hypersil column (250 mm 4.6 mm ID; Waters, USA) was used for separation. The column thermostat was set at 30 °C. The aqueous mobile phase was acetonitrile:water (70:30). Sample injection volume was 20 μL and the flow rate was 1 mL/min. Standard calibration curve was constructed by plotting peak areas against concentrations of pure isomaltose standard. Throughout the experimental work, data was collected and integrated using LC solution software (SHIMADZU, Japan). For quantification, the peak areas were determined, and the concentrations calculated with external calibration curve. Linearity of the calibration curve was assessed by determining the coefficient of correlation (R^2) of the points of the curve, which was higher than 0.998.

2.5. Immobilization of dextranase using US

Preparation of alginate beads using US was done by standard ionotropic gelation as described by Won et al. [19], with some modifications, as described in our previous work [6].

2.6. Immobilization of dextranase using HHP

Preparation of alginate beads using HHP treatment was done by standard ionotropic gelation as described by Won et al. [19], with some modifications [6]. An equal volume of 2% sodium alginate solution in water and dextranase (3 mg/ml) were mixed, then, the resulting solution was packaged into 10 mL polypropylene tubes. The tubes were placed into the vessel and treated under HHP ranging 100–600 MPa for different time (5, 10, 15, 20, 30, 35 and 40 min) at 4 °C. After the HHP treatment, the mixture was dropped into 0.2 M $CaCl_2$ prepared in 0.05 M sodium acetate buffer pH 5.4 in continuous shaking at 4 °C. After 20 min of hardening, the beads were separated from the calcium chloride solution by vacuum filtration. They were washed on a filter two times with 0.05 M sodium acetate buffer (pH 5.4). The filtered calcium chloride solution and the two washings were collected for loading efficiency determination. Then the beads were stored in 0.05 M sodium acetate buffer (pH 5.4) containing 0.02% NaN_3 at 4 °C until used. The average diameter of beads was 3 mm. The loading efficiency of immobilized enzyme was calculated according to Eq. (1) as described by Won et al. [19]:

$$\text{Loading efficiency } (\%) = \frac{C_i V_i - C_f V_f}{C_i V_i} \times 100, \quad (1)$$

where, C_i represents the initial protein concentration, V_i is the initial volume of enzyme solution, C_f is the protein concentration in the total filtrate, and V_f is the total volume of the filtrate.

The amount of immobilization enzyme yield in the calcium alginate beads was calculated according to Eq. (2) as described by Won et al. [19]: