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Enhancing the activity of cellulase enzyme using ultrasonic irradiations

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ABSTRACT

The present work investigates the effect of low intensity ultrasonic irradiation on the cellulase activity. The effect on the kinetic and thermodynamic parameters as well as the molecular structure of cellulase enzyme was evaluated with the help of the chemical reaction kinetics model, Arrhenius equation, Eyring transition state theory, Michaelis–Menten equation, fluorescence spectroscopy and circular dichroism (CD) spectroscopy. It has been established that ultrasound had a positive effect on the activity of cellulase enzyme, though the selection of operating conditions played a crucial role in deciding the intensification. The maximum cellulase activity was observed at 17.33 W/cm^2 intensity and ultrasonic treatment time of 30 min, under which the enzyme activity was increased by about 25% over the untreated enzyme. After the ultrasonic treatment, thermodynamic parameters E_a, ΔH, ΔS and ΔG were reduced by 64.7%, 68%, 37.3% and 1.3%, respectively. In addition, fluorescence and CD spectra revealed that the ultrasonic treatment had increased the number of tryptophan on cellulase surface, and changed the molecular structure of cellulase enzyme favourably to provide more access to the active sites.

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1. Introduction

In recent times power ultrasound has been shown to have significant application in food and biotechnology processes [1]. For several years, ultrasound has been used as a method for enzyme inactivation but recently it has been reported that ultrasound does not inactivate all enzymes especially under mild conditions [2]. The ultrasound generated by periodic mechanical motion of an ultrasonic probe transfers energy into the solution and causes alterations in pressure leading to the creation of small rapidly growing bubbles [3]. These bubbles are enlarged during the negative pressure cycle and after undergoing oscillations in size during multiple acoustic cycles, finally collapse violently which generates high pressures, temperatures and shear forces. Even though high ultrasonic intensity or extended sonication time can denature enzymes, it has been shown that use of ultrasonic treatment at appropriate frequencies and intensity levels can lead to enhanced enzyme activity [4]. Ultrasound also results in favourable conformational changes in protein molecules without altering the structural integrity of the enzymes [5].

The ability of ultrasound to increase the activity of enzymes and to reduce the mass transfer resistances in the process makes this treatment a potential option for the conversion of lignocellulosic biomass under mild conditions using cellulase for the production of bioethanol. Cellulase (endo-1,4-β-D-glucanase) refers to a group of enzymes produced chiefly by fungi, bacteria, and protozoans that catalyze the process of hydrolysis of cellulose. Cellulases are widely used in various fields such as pulp and paper industry, textile industry, bioethanol industry, wine and brewery industry, food processing industry, animal feed industry, agricultural industries, etc. [6]. Cellulase breaks down cellulose into smaller polysaccharides or completely into β-glucose units which can be further fermented to bioethanol. Considering the commercial importance of cellulase, it is essential to find an effective method to improve the activity of cellulase.

In spite of the fact that ultrasound shows a potential to modify the enzyme activity, very less publications have targeted to study the effect of power ultrasound on the performance of enzymes. Actually, majority of the papers published in this area evaluated the effectiveness of the ultrasound-aided processes, without focussing on the intensification of catalytic activity using ultrasound. Souza et al. [7] carried out a comprehensive study and examined the activity of a commercial amylase enzyme after ultrasonic treatment in an ultrasonic bath at 40 kHz. It has been reported that ultrasonic treatment can promote enzyme activity. Glucose oxidase enzyme activity under ultrasonic irradiation was evaluated by Guiseppi-Elie et al. [8]. The results proved that the sonicated enzyme at 23 kHz showed a altered composition with reduced α-helix and β-sheet fractions upon extended sonication compared with the

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unsonicated enzyme. Along with the changes in the secondary structure, small subsequent decrease in the enzymatic activity was observed indicating that time and intensity are the important parameters [8]. Effect of ultrasound on the activity of dextranase has been investigated by Bashari et al. [9]. The highest activity of dextranase was observed with ultrasound treatment at 25 kHz and 40 W for 15 min, under which the enzyme activity increased by 13.4% as compared to the untreated enzyme.

Enzymes are typically used at their optimal conditions, where they demonstrate highest activity, thus achieving maximum reaction rate. Therefore it is necessary to understand the influence of the ultrasound on the effectiveness of the enzyme functioning under ideal conditions. Thus there is a necessity of more research in this area to better understand the relationship of the 'sonication–enzyme action'. This would facilitate the development of effective processes in the field of sono-biotechnology. Nguyen and Le [10] have indicated that ultrasound intensity of 12 W/ml had a positive effect on the cellulase with 18% increase in its activity but, the mechanism of ultrasound action on cellulase has not been reported. Therefore, the major focus of this work was to investigate the effect of ultrasonic treatment on the activity of cellulase enzyme. In order to explore the change in enzyme activity, the effects of ultrasound on activity, thermodynamics as well as molecular structure of cellulase were investigated with the help of the Eyring transition state theory, Arrhenius equation, circular dichroism (CD) spectroscopy and fluorescence spectroscopy. A simple kinetic model, based on Michaelis–Menten equation, has also been introduced in order to investigate the variations in the kinetic parameters.

2. Materials and methods

2.1. Materials

Cellulase enzyme was obtained as a gift sample from Advanced Biotechnologies, Mumbai, India. The enzyme activity was 205,000 carboxymethyl cellulose unit per gram (CMCU/g). Carboxymethyl cellulose, citric acid, 3,5-dinitrosalicylic acid (DNSA), phenol, NaOH, and potassium sodium tartrate were procured from S.D. Fine Chemicals and all were of analytical grade. Bovine serum albumin was obtained from Sigma Aldrich.

2.2. Ultrasonic treatment of cellulase

The device used for ultrasonic treatment of enzyme was a probe sonicator obtained from Dakshin Ultrasonics, Mumbai. The ultrasonic irradiation at a frequency of 20 kHz was transferred through a cylindrical horn. The experimental setup is shown in Fig. 1. Cellulase powder was dissolved in citrate buffer of pH 4.8 and made up to the final concentration of 1.0 g/l. 200 ml cellulase sample solution was put into the 250 ml beaker, and the beaker was placed in a water bath maintained at temperature of 50 °C as it is the optimum temperature for cellulase enzyme. The effect of ultrasound at different ultrasonic time was investigated over the range 5 to 70 min, whereas the effect of ultrasound intensity was evaluated over the range 2.88 to 23.10 W/cm^2. The cellulase activities were also investigated at different temperatures over the range of 20 to 50 °C, under ultrasonic treatment time of 30 min and 17.33 W/cm^2 intensity.

2.3. Analysis

2.3.1. Assay of cellulase activity

The activity of cellulase enzyme was determined using DNSA method described by Miller [11]. 0.5 M CMC was used as the substrate for hydrolysis by cellulase. The absorbance was measured at 540 nm with Chemito Spectroscan UV 2700 Double beam UV–Vis spectrophotometer, which was used to calculate the concentration of glucose released from CMC. 1 CMCU is the amount of enzyme, which under standard conditions degrades CMC to reducing carbohydrates with a reduction power corresponding to 1 μmol glucose per minute.

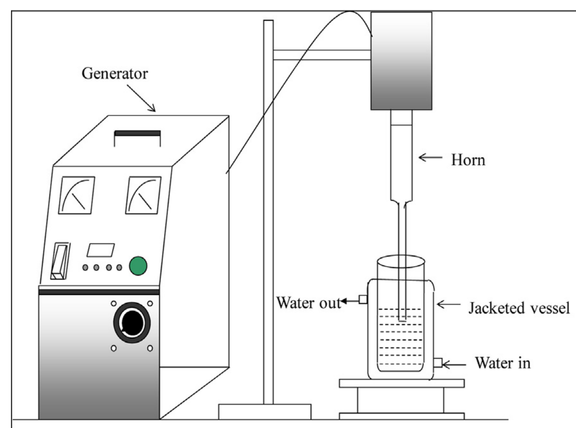

Fig. 1. Experimental setup for ultrasonic treatment of cellulase enzyme.

2.3.2. Soluble protein estimation

The total soluble protein content was determined by Bradford method, using bovine serum albumin as a standard [12]. To 0.25 ml of protein sample, 2.5 ml of Bradford reagent was added and the absorbance was immediately measured at 595 nm. For the calibration purpose, bovine serum albumin (BSA) was used.

2.4. Effect of ultrasound treatment on cellulase kinetics and thermodynamics

2.4.1. Ultrasonic treatment

For the determination of rate constants, 200 ml cellulase solution (1.0 g/l) was treated with ultrasound probe (20 kHz) at 17.33 W/cm^2 under different temperature (20, 30, 40, 50 °C) for 30 min. After that, 1.0 ml of ultrasound treated enzyme solution was added into the 3 ml of 0.5 M CMC solution. All the experiments were carried out at pH 4.8 in citrate buffer. For the determination of thermodynamic parameters, cellulase enzyme was treated with ultrasound probe (20 kHz) at 17.33 W/cm^2 for 30 min, and hydrolysis experiments were conducted at temperature of 20, 30, 40 and 50 °C, respectively.

2.4.2. Determination of kinetic parameters

The chemical kinetic model for cellulase enzyme used in the current work was based on the first-order kinetics as depicted in the following equation [13]:

$$\ln \frac{C}{C_0} = -kt \quad (1)$$

where C is the concentration of CMC at time $t = t$ (μg/ml), C_0 is the initial concentration of CMC, t is time, k is the total reaction rate constant involving the rate constants of ultrasonic action k_{us} and intrinsic activity k_c of cellulase (Eq. (3)). As it is difficult to measure the decrease in the CMC concentration, the reaction rate can be reflected by the increase in the amount of glucose released by CMC as follows [14]

$$\ln(V_\infty - V_t) = -kt + \ln V_\infty \quad (2)$$

where V_t is the concentration of glucose at time $t = t$ (μg/ml), V_∞ is the ultimate concentration of glucose (μg/ml), which is obtained from the hydrolysis experiment conducted under pH 4.8 at 50 °C

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