Process Biochemistry 61 (2017) 80–87

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Improvement of pectinase, xylanase and cellulase activities by ultrasound: Effects on enzymes and substrates, kinetics and thermodynamic parameters

Luíza M.G. Dalagnol[a], Vitória C.C. Silveira[a], Halisson Baron da Silva[a], Vitor Manfroi[b], Rafael C. Rodrigues[a,*,1]

[a] Biotechnology, Bioprocess and Biocatalysis Group, Institute of Food Science and Technology, Federal University of Rio Grande do Sul, Av. Bento Gonçalves, 9500, P.O. Box 15090, ZC 91501-970, Porto Alegre, RS, Brazil
[b] Beverages Technology Lab, Institute of Food Science and Technology, Federal University of Rio Grande do Sul, Brazil

ARTICLE INFO

Keywords:
Enzyme activity
Ultrasound
Thermodynamics
Kinetics

ABSTRACT

Ultrasound effects were investigated on pectinase (PE), xylanase (XLN) and cellulase (CE) activities at different pH, temperatures, and by sonication pre-treatment, comparing the reaction at ultrasound bath (US) and at a mechanical stirring (MS). In general, US increased the activity of the enzymes by 5% for PE, 30 % for XLN and 25% for CE compared to MS. US provided a higher activity at extremes pH (pH 3 and 7), mainly for XLN and CE. The substrate and enzyme pre-sonication enhanced the activities. The previous sonication of xylan increased the xylanase activity in almost 30% under US and almost 20% under MS. On the other hand, cellulase pre-sonication increased the activity in 50% under US and 40% under MS. The catalytic efficiency (V_{max}/K_M) increase 25% for PE and 17% for higher XLN and CE under US. US affected the PE activity at low temperature improving 10% the PE activity, while its effect was more representative at high temperatures, where the enzymatic activities of XLN and CE were 33% and 15% higher. Our results demonstrated that ultrasound can affect enzymes and substrates, making it a powerful tool for enzymatic-catalyzed reactions.

1. Introduction

Ultrasound (US) is an emerging technology defined as sound waves with vibrational energy at frequencies that exceeds the hearing limit of the human ear (20–100 kHz) [1,2]. It is considered a "green" technology due its ecofriendly properties with high efficiency, and have been frequently used in several fields, including food industry; promoting a high reproducibility of the process, selective extraction, sterilization benefits and reduces on processing time and costs [3–7].

The main effects of US in a liquid medium are attributed to the physical and chemical phenomena, which can be generic resumed as the implode collapse of gas micro bubbles dissolved in the liquid (cavitation), and the free radical formation, respectively [8,9]. Cavitation can cause alterations on cell molecular structures, improving the mass transfer, minimizing the processing time, enhancing the quality and ensuring safety of food products by protein and pathogens denaturation [7–9]. However, these phenomena may also cause alterations in enzymes functionality, since them act in an aqueous medium [10,11].

The US effects on enzyme structure and activity have been the subject of some researches. US has been used as a method for enzyme inactivation [10,12–14], however, some studies have shown that US may activate and improve the enzymes activities [11,15,16]. Sonication may change the biocatalyst mainly through three mechanisms: thermal effect, raising the temperature due to cavitation; free radical formation, generated by sonication of water molecules, such as ·OH and H·; and molecular alterations, caused by the mechanical and shear forces from cavitation, resulting in microstreaming and bubbles collapse [4,11,13,17]. However, the interference of these effects are strongly dependent of US intensity and duration [18]. Low-intensity US helps to disintegrate the enzyme molecular aggregates into smaller fragments, exposing more active sites, contributing to the enzymes activity increase [13,19,20]. Meanwhile, high-intensity US combined with high temperature and time can inactivate the enzymes by exposing them to extreme conditions and leading to degradation by hydrogen peroxide and other free radicals reactions [10,14].

In this sense, the knowledge of US effects on enzymes activity is extreme important. Although some effects are known for certain enzymes, it can be different for others due to differences in the amino acid sequence and enzyme structure [13]. Thus, in this work, the impact of ultrasound treatment on different parameters of pectinase (EC

* Corresponding author.
E-mail address: rafaelcrodrigues@ufrgs.br (R.C. Rodrigues).
[1] www.ufrgs.br/bbb.

http://dx.doi.org/10.1016/j.procbio.2017.06.029
Received 8 June 2017; Received in revised form 28 June 2017; Accepted 30 June 2017
Available online 01 July 2017
1359-5113/

3.2.1.15), xylanase (EC 3.2.1.8) and cellulase (EC 3.2.1.4) activities was studied. These enzymes are responsible for the degradation of structural polymers of plant cell walls, as: pectin, a class of complex colloidal acid polysaccharides, predominantly consisted of 1,4-linked α-D-galacturonic acid residues; xylan, a hemicellulosic polysaccharide containing a backbone of xylose residues linked by β-1,4-glycosidic bonds; and cellulose, a homopolymer of β-(1,4)-linked cellobiose residues [21–23], and are widely used on textile and paper industries, but especially on the food sector, in juice, wine and animal feed industries [25–29].

In this work, for the first time, to the best of our knowledge, the three main enzymatic complexes (PE, XLN and CE) for juice and wine industry, from the same enzymatic preparation, were evaluated under sonication or in a mechanical stirring bath (MS), at different pH and temperature. Additionally, it was measured the kinetic and thermodynamic parameters for each enzyme, with or without US treatment. Besides, we performed the pre-sonication treatment of enzymes and substrates solutions before the enzymatic reaction that allows to better understand the effects of ultrasound on enzyme activity and to observe a synergistic effect between ultrasound and enzymes in the substrate hydrolysis. Statistical analysis was perfomed in all experiments to ensure the significance of our data.

2. Material and methods

2.1. Materials and chemicals

Zimopec PX5® is an enzymatic preparation containing pectinase, xylanase and cellulase activities from *Aspergillus niger*, and was obtained from Vêneto Mercantil (Brazil). In all experiments the powder enzymatic preparation was diluted 400 x (2.5 mg mL^{-1}) in the suitable buffer to obtain a liquid enzymatic mixture containing around 0.1 mg mL^{-1} of protein. Pectin from apple (ID 76282, 70–75% of esterification degree), Whatman no. 1 filter paper and xylan from beechwood were purchased from Sigma Aldrich (St. Louis, MO). All other chemicals were of analytical or HPLC grade.

2.2. Enzymatic activities

The amount of reducing groups released for all enzymatic activities was determined using the 3,5-dinitrosalicyclic acid (DNS) method proposed by Miller [28]. All experiments were performed in triplicate on US or MS, and the results are presented in terms of mean and standard deviation. Moreover, for all enzymatic activities blank tube controls were performed without enzyme or substrate. Otherwise stated, temperature and pH for each enzyme activity are described in each method.

2.2.1. Determination of pectinase activity

Pectinase activity was estimated using pectin from apple (1.0 mg mL^{-1}) as substrate at 37 °C for 1 min, (total volume 1 mL; citrate buffer pH 4.8, 0.05 M), according to a method described by Dal Magro et al. [29]. One unit of pectinase was defined as the amount of enzyme required to produce 1 μmol of galacturonic acid per minute under reaction conditions.

2.2.2. Determination of xylanase activity

Xylanase activity was determined following the method proposed by Bailey et al. [30] using xylan (10 mg mL^{-1}) as substrate. The reaction was carried out for 5 min at 50 °C, (total volume 5 mL; sodium acetate buffer pH 5.0; 0.05 M). One unit of xylanase was defined as the amount of enzyme required to produce 1 μmol of xylose per minute under reaction conditions.

2.2.3. Determination of cellulase activity

Cellulase activity was measured using Whatman n° 1 filter paper (50 mg mL^{-1}) as substrate, at 50 °C for 5 min, (total volume 5 mL; citrate buffer pH 4.8, 0.05 M), according to a method described by Dal Magro et al. [29]. One unit of cellulase was defined as the amount of enzyme required to produce 1 μmol of glucose per minute under reaction conditions.

2.3. Equipment

The equipment used to all experiments was an ultrasonic bath (Unique Inc., model USC 2800A, Brazil), with temperature control and 9.5 L of working volume presenting these dimensions 300 × 240 × 150 mm (length × width × height), equipped with two disc transducers placed at the bottom of the reactor at ultrasonic frequency of 40 kHz and the total ultrasonic power 220 W. For control assays a shaking thermostatic water bath (MS) was used (MA-093, Marconi, Piracicaba, Brazil).

2.4. Effect of pH on enzymes activity under US

The enzymes activities were measured varying the pH from 3 to 7 (acetate or citrate buffer (0.05 M) for pH from 3 to 5, and phosphate buffer (0.05 M) for pH from 6 to 7) and the enzymatic reactions were conducted as described in Section 2.2, under US or MS. The results were reported as relative activity considering the maximum enzyme activity in each experimental group as 100%, and the average and the standard deviation of three assays.

2.5. Effect of temperature on enzymes activity under US

The enzymes activities were measured varying the temperature from 30 to 60 °C, and the enzymatic reactions were conducted as described in Section 2.2, under US or MS. The results were reported as relative activity considering the maximum enzyme activity in each experimental group as 100%, and the average and the standard deviation of three assays.

2.6. Effect of sonication on enzyme and substrate solution

In order to verify the effect of sonication on enzymatic activities, the enzyme and substrates solutions were sonicated individually for different times (0, 1, 5, 10, 15 and 30 min) and then used to measure the enzyme activity.

Initially, the enzyme solution (2.5 mg mL^{-1} in 0.05 M sodium citrate buffer, pH 4.8, total volume 5 mL) was sonicated for 1, 5, 10, 15 and 30 min, and an aliquot was taken to measure the enzyme activity on US or MS. In the same way, substrate solution (pectin 1 g mL^{-1}; filter paper 50 mg mL^{-1}; xylan 10 mg mL^{-1} diluted in 0.05 M sodium citrate buffer, pH 4.8, total volume 5 mL) was also sonicated for 1, 5, 10, 15 and 30 min, and the enzymes activities were measured on US and MS. All experiments were conducted in triplicate considering the average and the standard deviation, and the results are presented in terms of relative activity.

2.7. Enzymatic kinetics

Different concentrations of substrate (pectin, 0.1–10 mg mL^{-1}; filter paper, 2.5–100 mg mL^{-1}; and xylan, 1–25 mg mL^{-1}) were employed to determine the kinetics parameters. All the assays were conducted based on the description of Section 2.2 under US or MS.

The reaction rates for the three enzymes at different substrate concentrations were measured and the values of the Michaelis–Menten constant (K_M) and maximum reaction rate (V_{max}) were calculated fitting the initial reaction rates (v) for each substrate concentration (S) to the Michaelis–Menten Eq. (1):