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Biochemical Engineering Journal 31 (2006) 25–30

Biochemical Engineering Journal

www.elsevier.com/locate/bej

Improved cavitational cell disruption following pH pretreatment for the extraction of β-galactosidase from *Kluveromyces lactis*

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Received 8 June 2005; received in revised form 20 March 2006; accepted 28 May 2006

Abstract

The efficient release of intracellular β-galactosidase by ultrasonic disruption of cells treated with aqueous solutions of different pH has been investigated to improve the cavitational efficacy of the cell disruption process. Pretreatment of the cells at different pHs for various time intervals was studied. The maximum yield (95 ± 3 U/ml) was obtained by pretreatment of the cells at pH 4.4 for 6 h and subsequent ultrasonic cell disruption for 40 min. The maximum yield on ultrasonic disruption without pretreatment was 7.2 ± 0.6 U/ml. The energy efficiency of the disruption process for releasing β-galactosidase using different pre-treatments has been calculated and compared.

Keywords: Cell disruption; pH treatment; Cavitation; Enzyme release

1. Introduction

An optimal extraction process is required for the purification of intracellular proteins. Cell disruption is the most prominent unit operation in the intracellular product recovery protocol and the most energy intensive. Several methods have been reported for the cell disruption [1–9]. Physical methods are often favoured for disruption due to the cost and operational limitations of other methods. However, the yields of mechanical and physical methods per unit energy consumed are low due to their energy intensive nature and low product concentrations. Pretreatment of the cells prior to their disruption has been postulated as a desirable step to improve the energy efficiency of the mechanical disruption methods. Among the methods available for the cell disruption at the laboratory scale, sonication is one of the most commonly employed [10–12].

The combined use of heat and mechanical stress for the selective release of the cytoplasmic β-galactosidase from *Kluveromyces lactis* cells has been reported in our earlier work [13]. Umakoshi et al. [14] illustrated the cumulative increase in the release of β-galactosidase on the simultaneous combined use of heat and chemical stress for its selective recovery, while their sequential application resulted in an order of magnitude increase. Similarly, the increased release of β-galactosidase from *E. coli* was observed on heat treatment in the presence of glycine with the interacting effect of these causative agents being largest at low energy inputs [15]. Further, the combination of heat and enzymatic pretreatment, with mechanical cell disruption has been reported [16,17]. High pressure homogenization of *Candida utilis* following pretreatment with the enzyme Zymolase resulted in 95% disruption following four passes compared to 65% disruption under the same homogenization conditions in the absence of the pre-treatment [16]. Vogels and Kula [17] demonstrated the beneficial cumulative release on treatment of *Bacillus cereus* with any combinations of heat (45–65°C for 30 min), the enzyme Cellosyl and mechanical disruption by high pressure homogenisation and bead mills.

The number of passes required through a high pressure homogenizer was shown to be reduced by the pretreatment of the recombinant *E. coli* with guanidine hydrochloride and Triton X-100 for the release of human growth hormone [18]. Here, the required release of inclusion bodies was obtained on 1 pass through the homogeniser at 41 MPa following chemical pretreatment, compared with 1 pass at 62 MPa in the absence of pre-treatment. Improved efficiency of cell disruption on the combined use of chemical and mechanical processes for the disruption of the Gram-negative bacteria *Ralstonia eutrophus* and *E. coli* have also been reported [19,20]. Chemical treatments used

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doi:10.1016/j.bej.2006.05.015

included pH shock, monovalent cation concentration, temperature and osmotic shock. The sequential and differential release of different products from the same cells has been studied by Haung et al. [21]. A method for the selective recovery of recombinant proteins from the periplasm of *E. coli* has been reported [22]. The protein release from *E. coli* cells permeabilised with guanidine-HCl and Triton X-100 has also been studied [23].

In the present study, for the efficient recovery of the intracellular enzyme β-galactosidase from *Kluveromyces lactis*, the effect of pH pretreatment on the subsequent release of the enzyme by ultrasonication has been investigated systematically.

2. Materials and methods

2.1. Microorganism

The microorganism *Kluveromyces lactis* NCIM 3566, obtained from National Chemical Laboratory, Pune, India was used as a source of β-galactosidase.

2.2. Cultivation of microorganisms

The stock culture was maintained at 4 °C on the YMPD agar containing yeast extract 0.3%, malt extract 0.3%, peptone 0.5% and dextrose 1%. A 48 h culture was used to inoculate the seed culture medium (YMDP medium) in a 100 ml conical flask with a working volume of 20 ml and incubated at 30 ± 2 °C for 24 h. A 5 ml aliquot of the seed culture was used to inoculate 100 ml medium in a 250 ml conical flask. Growth was carried out at 30 ± 2 °C on a rotary shaker (175 rpm) for 18 h. The growth medium reported by Flores et al. [24] was used. The resultant biomass was separated from the medium using Remi Research centrifuge operating at 8000 rpm (corresponding to 15,000 × *g*) for 20 min at room temperature. The biomass was washed with phosphate buffer (pH 6.6, 0.05 M) before pretreatment in buffer solutions of different pH.

2.3. Procedure for pH treatment

Harvested cells were suspended in buffer solutions of different pH. Treatment was carried out for 2 to 8 h in a 100 ml conical flask using a 50 ml working volume. A 2% (wet w/v) cell suspension was agitated on a rotary shaker at 175 rpm at room temperature (30 ± 2 °C). The suspension was centrifuged to remove the treatment buffer and the cells resuspended in 50 ml phosphate buffer (0.05 M) at pH 6.6 to avoid the enzyme denaturation during its release by subsequent ultrasonication. Since the assay procedure always needed a pH of 6.6, this pH was also included as one of the liquid pH options for treatment.

2.4. Cell disruption by ultrasonication

Cell disruption was carried out using an ultrasonic bath (M/s Dakshin Ltd., Mumbai, India) with an operating frequency of 22.5 kHz and a power rating of 120 W. The calorimetrically measured efficiency of this system was found to be 40% i.e. it actually dissipated 48 W of energy directly into the system. The rest of the electrical energy was lost in the energy transfer cycle i.e. electrical energy to mechanical energy to cavitational energy dissipation etc. The cell disruption was carried out in 250 ml beaker (6 cm in diameter and 11.5 cm in height) using a working volume of 50 ml of cell suspension. The beaker was kept at the central position at a height of 7.5 cm from the bottom of the bath. The bath was filled with water to a level of 14 cm. The temperature of the water was maintained at 10 ± 2 °C throughout the disruption process by its periodic replacement. The disrupted cell suspension was sampled at regular intervals and centrifuged at room temperature in a Remi Research centrifuge using the Eppendorf tube rotor operating at 8000 rpm (corresponding to 15,000 × *g*) for 20 min to remove the cell debris. The clear supernatant was collected and analyzed for the activity of the enzyme β-galactosidase and the total soluble protein concentration. The results presented are averages of three to four experiments at each of the operating conditions.

2.5. Analytical methods

β-Galactosidase activity was measured by the hydrolysis of substrate *o*-nitrophenyl-β-D-galactopyranoside (ONPG) to *o*-nitrophenol at 30 °C and pH 6.6 [25]. The total soluble protein estimation was carried out by the modified Folin Lowry method with bovine serum albumin (BSA) as a standard [26].

3. Result and discussion

3.1. Effect of pH pretreatment on extracellular release of enzyme and protein

From Table 1, it is observed that the extracellular enzyme activity was higher where the pretreatment pH was in the alkaline range, compared to acidic pH. Protein release at alkaline pH (>pH 9, and preferably pH 11.0–12.5) has been reported

Table 1
Extracellular enzyme activity and total soluble protein concentration following pretreatment for 2 h at varying pH

pH (0.05 M PO_4 buffer)	Enzyme activity (A) (U/ml)	Protein concentration (P_T) (mg/ml)	Specific activity ($S_A = A/P_T$) (U/mg)
4.4	0.0025	0.216	0.0115
6.6	0.283	0.238	1.189
8.8	0.948	0.119	7.966
9.25	0.85	0.098	8.67

A: enzyme activity; P_T: protein concentration; S_A: specific activity; formula used for calculation: $S_A = A/P_T$.