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Kinetic parameters and thermodynamic values of β-xylosidase production by *Kluyveromyces marxianus*

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Abstract

Kinetic parameters for production of β-xylosidase by *Kluyveromyces marxianus* were determined in growth media containing glucose, xylose, cellobiose, sucrose and lactose as carbon sources. *K. marxianus* achieved maximum β-xylosidase specific product yield ($Y_{P/X}$) when grown on xylose. Basal level of activity was achieved in cultures grown on glucose. Kinetic parameters of enzyme production and cell mass formation were correlated. Enzyme synthesis was regulated by an induction mechanism and growth-dependent repression mechanism. Thermodynamic analysis revealed that the cell system exerted protection against thermal inactivation. A partially purified enzyme showed good stability when incubated at 60 °C and was quite stable at a pH of 5.0–7.0 and may be exploited for commercial applications.

Keywords: Corn steep liquor; Induction; *Kluyveromyces*; Repression; Thermotolerant; β-Xylosidase

1. Introduction

β-Xylosidase (EC 3.2.1.37) is one of the component enzymes of the hemicellulase complex and is widely distributed in nature. It catalyzes the hydrolysis of alkyl and aryl glycosides as well as that of xylobiose and xylo-oligosaccharides to xylose. In industry, it is employed for hydrolysis of bitter compounds from grape fruit during juice extraction and liberation of aroma from grapes during wine making (Manzanares et al., 1999). β-Xylosidase, in synergistic action with *endo*-β-xylanases (EC 3.2.1.8) and debranching enzymes namely α-glucuronidases, esterases and glycosidases have potential applications in production of biofuels and in the processing of food (La Grange et al., 2001). Cellulase-free xylanases have an important role in reducing consumption of chlorine and chlorine dioxide in paper and pulp industry (Tsujibo et al., 2001).

Kluyveromyces marxianus has been employed for production of biomass, enzymes and ethanol (Belem and Lee, 1998). Recently, production of β-xylosidase by *Kluyveromyces* spp. and other yeasts has been described (Manzanares et al., 1999) but no detailed information is available on its production by *K. marxianus*. Xylose, xylobiose, synthetic β-xylosides and *xylo*-oligosaccharides are inducers of this enzyme in yeasts, but, like other enzymes, other carbon sources may also serve as inducers (Rajoka et al., 1997). This work reports kinetics of enhanced production of β-xylosidase following growth of *K. marxianus* on different carbon sources under optimized fermentation conditions (an initial pH 5.5, an inoculum size of 10%, and yeast extract of 0.3%). Activation enthalpy and entropy of this process were determined to provide insight into the kinetics of the system and understand the phenomena involved in both enzyme production and its thermal inactivation. Among the different approaches available in literature for their estimation (Aiba et al., 1973), the Arrhenius plots (Aiba et al., 1973) were selected preferably because they proved to be the best tool as was also observed for other fermentation systems (Converti and Dominguez, 2001).

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2. Methods

2.1. Organism and growth conditions

A thermotolerant yeast strain, *K. marxianus* var. *marxianus* (Rajoka et al., 2003) was used in these studies and was maintained on malt extract plates and grown in liquid medium containing carbon sources (20 g/L) and KH_2PO_4 (2.0 g/L), $(NH_4)_2SO_4$ (5.0 g/L), $MgSO_4 \cdot 7H_2O$ (1.0 g/L), yeast extract (3.0 g/L) at 35 °C.

2.2. Enzyme production

Growth kinetics of the organism was examined in 1-L Erlenmeyer flasks containing glucose, cellobiose, lactose, sucrose and xylose (20 g/L each) as sole carbon sources, corn steep liquor as nitrogen source in place of ammonium sulphate (unless mentioned otherwise) and other nutrients. The inoculum was prepared in glucose medium at 35 °C on a rotary shaker (150 rpm). The cultures were centrifuged (10,000*g*) for 30 min, washed twice with saline solution, and used at 10% (v/v) in saline solution containing 0.25 mg dry cells/mL. All experiments were carried out at 35 °C (unless otherwise mentioned) in triplicate on a gyratory shaking-incubator for up to 48–72 h. The cells were recovered, sonicated, fractioned as described by Rajoka et al. (1997) and the cell extract was assayed. The cell mass was determined gravimetrically.

2.3. Effect of varying temperature on β-xylosidase production

To calculate the kinetic and thermodynamic values, the data of batch fermentation were gained by performing experiments (three runs) on xylose-corn steep liquor-based media on an orbital shaker (150 rpm) at a temperature in the range of 22–45 °C.

2.4. Enzyme assays

β-Xylosidase was assayed using 1 mM *p*-nitrophenyl-β-D-xylopyranoside as a substrate in 50 mM sodium acetate buffer with a pH of 5.0 (unless otherwise stated). One millilitre of the properly diluted enzyme sample was incubated with 1 mL of the substrate solution at 40 °C for 10 min. The reaction was stopped by the addition of 2 mL of 1 M sodium carbonate solution. The liberated *p*-nitrophenol was measured at 400 nm with a spectrophotometer. One IU of β-xylosidase was defined as the amount of enzyme that released 1 μmol *para*-nitrophenol per min under the assay conditions.

2.5. Protein determination

The proteins were determined by Lowry's method (1976) using bovine serum albumin as the standard.

2.6. Saccharides determination

Saccharides were analyzed by HPLC (Perkin–Elmer, USA) with column HPX-87H (300 × 78 mm, Bio-Richmond, California) maintained at 45 °C in a column oven. Sulphuric acid (0.002 N) served as a mobile phase at 0.6 mL/min. The samples were detected using refractive index detector and quantified using Turbochron4 software of Perkin–Elmer, USA.

2.7. Determination of kinetic parameters

All kinetic parameters were determined as described by Lawford and Rousseau (1993) in shake-flasks (Converti and Dominguez, 2001). Thermodynamics of enzyme production was performed as described by Rajoka et al. (2003).

2.8. pH tolerance

After 40 h of xylose fermentation, cells were harvested and β-xylosidase was extracted according to the procedure described by Rajoka et al. (1997). The enzyme was partially purified by precipitation with 30% and 50% ammonium sulphate, dialyzed, and assayed after 30 min incubation in different buffers (containing phenyl methyl sulphonyl fluoride as protease inhibitor) as described by Siddiqui et al. (1997).

2.9. Temperature tolerance

The partially purified enzyme containing 0.1 mM PMSF was incubated at different temperatures for 30 min (Siddiqui et al., 1997) and remaining β-xylosidase activity was determined.

2.10. Statistical analysis

Treatment effects were compared by the protected least significant difference method and have been presented as two-factor factorial design in the form of probability (*p*) using MstatC software (Snedecor and Cochran, 1980).

2.11. Thermostability

Partially purified β-xylosidase was re-dissolved in 50 mM MES monohydrate buffer (pH 5.5) and assayed for thermostability (Siddiqui et al., 1997).

2.11.1. Activation energy (E_a)

Activation energy was determined using the data obtained for optimum temperature. Arrhenius relationship was used to calculate the activation energy required by the enzyme to hydrolyze soluble starch as described by Siddiqui et al. (1997).