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Purification and characterization of β-galactosidase from probiotic *Pediococcus acidilactici* and its use in milk lactose hydrolysis and galactooligosaccharide synthesis

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ABSTRACT

β-galactosidase is a commercially important enzyme that was purified from probiotic *Pediococcus acidilactici*. The enzyme was extracted from cells using sonication and subsequently purified using ammonium sulphate fractionation and successive chromatographies on Sephadex G-100 and Q-Sepharose. The enzyme was purified 3.06-fold up to electrophoretic homogeneity with specific activity of 0.883 U/mg and yield of 28.26%. Molecular mass of β-galactosidase as estimated by SDS-PAGE and MALDI-TOF was 39.07 kDa. The enzyme is a heterodimer with subunit mass of 15.55 and 19.58 kDa. The purified enzyme was optimally active at pH 6.0 and stable in a pH range of 5.8–7.0 with more than 97% activity. Purified β-galactosidase was optimally active at 50 °C. Kinetic parameters K_m and V_{max} for purified enzyme were 400 μM and 1.22×10^{-1} U respectively. Its inactivation by PMSF confirmed the presence of serine at the active site. The metal ions had different effects on enzyme. Ca^{2+}, Mg^{2+} and Mn^{2+} slightly activated the enzyme whereas NH_4^+, Co^{2+} and Fe^{3+} slightly decreased the enzyme activity. Thermodynamic parameters were calculated that suggested that β-galactosidase is less stable at higher temperature (60 °C). Purified enzyme effectively hydrolysed milk lactose with lactose hydrolysing rate of 0.047 min^{-1} and $t_{1/2}$ of 14.74 min. This is better than other studied β-galactosidases. Both sonicated *Pediococcus acidilactici* cells and purified β-galactosidase synthesized galactooligosaccharides (GOSs) as studied by TLC at 30% and 50% of lactose concentration at 47.5 °C. These findings indicate the use of β-galactosidase from probiotic bacteria for producing delactosed milk for lactose intolerant population and prebiotic synthesis. pH and temperature optima and its activation by Ca^{2+} shows that it is suitable for milk processing.

1. Introduction

β-galactosidase (EC. 3.2.1.23) is an exoglycosidase that has health benefits and industrial applications. It catalyses the hydrolysis of lactose and structurally related galactosides and also transglycosylation reactions [1]. Study of β-galactosidase production from microorganisms is not new but many of them were not approved because they were not obtained from food grade microorganisms. Microbes are preferred source and yeasts are major commercial source of this enzyme. Moreover, the yield from studied microorganisms is also low that limits their use for commercial purpose. Therefore, β-galactosidase from food grade probiotic microorganisms are safe for human use. Probiotic bacteria producing high level of β-galactosidase is very significant.

Lactic acid bacteria (LAB) are considered as good source of enzyme because of their GRAS status. *Pediococcus acidilactici* is a LAB that possessed all the probiotic attributes [2] and also effectively tenderised meat [3]. It possesses a spectrum of enzyme activities [4] and β-galactosidase activity is 22 times higher than other studied strains even in the absence of inducer [2]. *Pediococcus acidilactici* is also a good source of commercial β-galactosidase because dairy environment is its natural habitat.

β-galactosidase finds prominent place in pharmaceutical industry such as in development of digestive supplements (prebiotics) and treatment of disorders (lactose intolerance). Prebiotics are non-digestible food ingredients that have gained interest because they affect the host beneficially by selectively stimulating growth of indigenous microflora. β-galactosidases are currently being used for galactooligosaccharide (GOS) synthesis i.e. prebiotics. Lactose intolerance is reported in 70% of world population. β-galactosidase is also used in dairy industry for avoiding crystallization of lactose in concentrated frozen dairy products such as

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condensed milk, and ice creams, and in solving the problem of whey disposal by converting whey into lactic acid [5]. This strain under study produced lactic acid using different waste resources [6].

Lactose intolerance, relatively low solubility and sweetness of lactose and GOS synthesis has led to increased demand and development of non-immunogenic and thermostable β-galactosidases of commercial importance. This study reports the purification and characterization of β-galactosidase from probiotic *P. acidilactici* which exhibited high yield, high rate of milk lactose hydrolysis and also synthesised GOS. This is expected that it will reduce the operating cost. Thermal and kinetic properties of the purified enzyme are also discussed.

2. Materials and methods

2.1. Source of study

P. acidilactici earlier purchased from NCDC (National collection of Dairy Cultures), National Dairy Research Institute (NDRI), Karnal is being maintained in our lab.

2.2. Collection of biomass

P. acidilactici was cultured in MRS medium for 24 h at 32 °C under shaking conditions at 250 rpm and then harvested by centrifugation at 9000 rpm for 25 min.

2.3. Materials

o-nitrophenyl-β-d-galactopyranoside (ONPG), MRS media and other chemicals used were from Hi media, Mumbai, India. Chromatographic slurries were from Sigma Aldrich. The reagents used were of analytical grade unless stated otherwise.

2.4. β-galactosidase assay

2.4.1. β-galactosidase assay of whole cells

Bacterial cell pellets were harvested, washed twice and suspended in same volume of Z buffer (One litre contains: 16.1 g $Na_2HPO_4{\cdot}7H_2O$ (0.06 M), 5.5 g $NaH_2PO_4{\cdot}H_2O$ (0.04 M), 0.75 g KCl (0.01 M), 0.246 g $MgSO_4{\cdot}7H_2O$ (0.001 M), 2.7 ml β-mercaptoethanol (0.05 M), pH 7.0). Absorbance of cell suspension was measured at 600 nm against Z buffer. For each reaction mixture 0.1 ml cells were diluted to 1 ml with Z buffer. Diluted cells were permeabilized by adding 100 μl chloroform and 50 μl 0.1% SDS. The tubes were vortexed for 30 s and equilibrated for 5 min in water bath at 28 °C. The reaction was started by adding 0.2 ml ONPG (4 mg/ml) substrate followed by incubation at 28 °C for 10 min. The reaction was stopped by adding 0.5 ml of 1 M Na_2CO_3 and contents were centrifuged to remove debris and chloroform. OD was recorded at 420 nm and 550 nm. Miller units for β-galactosidase were calculated using following formula.

$$\text{Miller units} = 1000 \times [(OD_{420} - 1.75 \times OD_{550})]/(T \times V \times OD600)$$

where OD_{420} and OD_{550} are read from reaction mixture, OD_{600} is cell density in washed cell suspension, T-reaction time (min), V-culture volume (ml) used in assay.

2.4.2. β-galactosidase assay of cell free enzyme

β-galactosidase activity was determined according to Bhomik and Marth [7]. Cell free extract (0.2 ml) was added to 1.6 ml Z buffer. The reaction was started by adding 0.2 ml of 10 mM o-nitrophe nyl-β-d-galactopyranoside (ONPG). Reaction mixture was incubated at 37 °C for 10 min and then reaction was stopped by adding 1 ml of 1 M Na_2CO_3. The absorbance was read against a suitable blank at 420 nm. One unit of enzyme activity was defined as amount of enzyme that liberated 1 μmole of ONP from the substrate per minute under assay conditions.

2.5. Effect of carbohydrate source on β-galactosidase production

Sterilized modified MRS media without any carbon source was prepared. Different carbon source i.e. maltose, sucrose, fructose, lactose, galactose and glucose were added separately at a concentration of 2% each. The media without any carbon source was used as control. Then each media was inoculated with *P. acidilactici* and incubated at 32 °C for 24 h. Cells were harvested at 9000 g and enzyme activity was determined in Miller units. Highest activity was taken as 100%.

2.6. Extraction of β-galactosidase

P. acidilactici cells were incubated for 24 h and then harvested by centrifugation at 9000 rpm for 25 min. Intracellular β-galactosidase was extracted using following different protocols to optimize the extraction:

2.6.1. Sonication

Bacterial cells were sonicated according to Feliu et al. [8] with slight modifications including standardization of time of sonication using 1–10 min of sonication period. Cell suspension (in 50 mM Na-Pohsphate buffer pH 7.0) was sonicated for 1–10 min in ice bath. The extract was then centrifuged at 15,000*g* for 10 min and assayed for enzyme activity.

2.6.2. Lysozyme-EDTA treatment

Lysozyme solution was prepared by dissolving 50 mg of lysozyme in 1.5 ml Tris EDTA buffer pH 8.0. Seventy-five microlitre of this was added to one ml of cell suspension, then mixture was incubated for 30 min at room temperature and centrifuged at 15,000*g* for 10 min. β-galactosidase activity was determined in supernatant.

2.6.3. SDS-chloroform treatment

One hundred microlitre of chloroform and 50 μl of 0.1% SDS were added to 10 ml of cell suspension and incubated for 30 min at room temperature under vortexing conditions. Then it was centrifuged at 15,000*g* for 10 min and assayed for enzyme activity.

2.6.4. Enzyme extraction by lysozyme

Cell pellet was suspended in 5.0 ml of 0.05 M Na-phosphate buffer (pH 6.8) followed by vigorous vortexing. Lysozyme (10 mg ml^{-1}) was added to it and incubated at 37 °C for 15 min. Then 0.5 ml of 4 M NaCl was added and sample was again incubated at 37 °C for another 50 min. It was centrifuged at 10,000*g* for 15 min and enzyme was assayed in supernatant

2.7. Protein content

Protein content was determined by Lowry's method [9] using bovine serum albumin as standard.

2.8. Purification

Purification was achieved in three steps viz. ammonium sulphate fractionation and successive chromatographies on gel filtration and anion exchanger.

Ammonium sulphate precipitation: The extracted proteins were concentrated by saturating the crude extract up to 80% by ammonium sulphate. The precipitates were re-dissolved in 4 ml of 50