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Analytical Methods

Optimisation of immobilisation conditions for chick pea β-galactosidase (*Cp*GAL) to alkylamine glass using response surface methodology and its applications in lactose hydrolysis

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ABSTRACT

Response surface methodology was advantageously used to optimally immobilise a β-galactosidase from chick pea onto alkylamine glass using Box–Behnken experimental design, resulting in an overall 91% immobilisation efficiency. Analysis of variance was performed to determine the adequacy and significance of the quadratic model. Immobilised enzyme showed a shift in the optimum pH; however, optimum temperature remained unaffected. Thermal denaturation kinetics demonstrated significant improvement in thermal stability of the enzyme after immobilisation. Galactose competitively inhibits the enzyme in both soluble and immobilised conditions. Lactose in milk whey was hydrolysed at comparatively higher rate than that of milk. Immobilised enzyme showed excellent reusability with retention of more than 82% enzymatic activity after 15 uses. The immobilised enzyme was found to be fairly stable in both dry and wet conditions for three months with retention of more than 80% residual activity.

1. Introduction

Lactose, a disaccharide that comprises the monosaccharides glucose and galactose, is the primary carbohydrate found exclusively in mammalian milk. Milk contains about 3–8% (w/v) lactose and 70–80% of the solid component in cheese whey is lactose (Speer, 1998). β-Galactosidase hydrolyses this lactose into its monosaccharides, which are more soluble, more sweet and more digestible than lactose. Many adults worldwide do not produce adequate amount of lactase and so are unable to digest lactose. Lactose intolerance is a clinical syndrome which includes abdominal pain, nausea, flatulence and bloating, after ingestion of lactose-containing food substances. For most people with lactase deficiency, milk is a discomfort. NIDDK (the National Institute of Diabetes and Digestive and Kidney diseases) estimates that up to 75% of all adult African Americans and Native Americans and 90% of Asian Americans are lactose intolerant.

The lactose content of milk often influences the final decision about the use or continuation of milk in the diet. In addition, lactose is an indispensible component of more than 20% of prescription drugs and about 6% of over-the-counter medicines (source: NIDDK, NIH). Unnecessary avoidance of dairy products due to lactose intolerance may initiate many nutritional compromises. Dairy's role in reducing the risk of osteoporosis and in strengthening bones has long been established and supported by the nutrition and science community worldwide. The US Food and Drug Administration (FDA) has determined that hydrolysed lactose products must contain at least 70% less lactose than the non-hydrolysed product for consumption by the lactose intolerant. Therefore, lactose-reduced or lactose-free dairy products are desired by lactose intolerant individuals.

Milk processing for lactose hydrolysis in the dairy industries has been carried out using β-galactosidase obtained from yeasts strains such as *Kluyveromyces fragilis*, *Kluyveromyces lactis* and *Candida pseudotropicalis*. Plant β-galactosidase could be a better substitute for yeast β-galactosidase, in terms of cost-effectiveness, easy availability, better stability and acceptability by GRAS [Generally Recognized As Safe by FDA] (Dey & Delcampillo, 1984).

Industrial biocatalysts are inherently labile and required to perform in an environment quite different from their natural habitats, and they seldom have the features suitable for industrial catalysts. Biocatalyst stabilisation under operational conditions is a key issue of bioconversion technology; partly this problem can be solved by immobilisation (Reddy & Kayastha, 2006). Biocatalyst stability is also a major concern in almost all bioprocesses, because it influences the overall expenditure of the process. The high efficiency of the immobilisation techniques can be partly attributed to the mild conditions, which minimise enzyme denaturation. Enhanced thermostability has been attributed to the stabilising effects of the support matrix, which prevents the extensive conformational

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changes typical of thermal denaturation. The ability to retain enzyme activity at high temperature expands the range of conditions suitable for enzyme function and provides a number of processing advantages, such as reduced risk of microbial contamination, lower viscosity, improved transfer rates and improved substrate solubility (Luckarift, Spain, Naik, & Stone, 2004). A stable, immobilised enzyme system is suitable for a number of applications that would not be feasible with a soluble enzyme system. One such application is a flow-through mini-reactor system whereby the immobilised enzyme can be continually reused over an extended period of time.

In the last few years, improvements in carrier and immobilisation techniques have opened new options for process development. Glass has been used as an effective immobilisation support matrix with a variety of enzymes and other proteins (Azevedo, Vojinovic, Cabral, Gibson, & Fonseca, 2004; Reddy & Kayastha, 2006).

Response surface methodology (RSM) has been widely adopted for optimisation of various parameters in biochemical processes. RSM is a collection of statistical and mathematical techniques useful for developing, improving, and optimising processes in which a response of interest is influenced by multiple variables and the objective is to optimise this response. It defines the effects of independent variables in combination and generates a mathematical model to analyse complex biochemical processes.

Recently, RSM has been extensively used for optimisation of biochemical processes, which include transesterification of animal fats (Jeong, Yang, & Park, 2009), enzymatic synthesis of fructooligosaccharides (Vega & Zuniga-Hansen, 2011), production of cutinase from *Fusarium oxysporum* (Pio & Macedo, 2007), production of biodiesel from *Madhuca indica* oil (Ghadge & Raheman, 2006), hydrolysis of pectic substrates (Naidu & Panda, 1999), determination of reaction parameters for damaged starch assay (Boyacl, Williams, & Köksel, 2004), optimisation of lactose nano-probe (Dwevedi, Singh, Singh, Srivastava, & Kayastha, 2009) and keratinase immobilisation upon magnetic nanoparticles (Karak, Konwarh, Rai, & Mukherjee, 2009).

This investigation is aimed to optimally immobilise a plant β-galactosidase (purified from seeds of *Cicer arietinum*) onto alkylamine glass. Various parameters (amount of alkylamine, glutaraldehyde concentration, amount of protein and pH) for optimal immobilisation were evaluated by using RSM.

Mode of glutaraldehyde activated coupling with alkylamines:

$$\text{Glass-R}\cdots\text{NH}_2 + \text{Glutaraldehyde} \xrightarrow{27\,^\circ\text{C}} \text{glass-CHO} \xrightarrow{\beta\text{-galactosidase-NH}_2} \text{glass-CH}_2\text{NH-}\beta\text{-galactosidase}$$

2. Materials and methods

Dry seeds of *C. arietinum* were purchased from a local market. All chemicals, buffers and other reagents were of analytical grade. All chemicals were purchased from Sigma–Aldrich (St. Louis, MO). Milli Q (Millipore, Bedford, MA) water with a resistance of higher than 18 MΩ cm was used throughout the experiments.

2.1. Enzyme preparation

*Cp*GAL was purified from soaked seeds of chick pea with a combination of various solvent extraction and chromatographic techniques (Kishore & Kayastha, 2012). Purification protocol is summarised below.

All purification steps were carried out at 4 °C and centrifugation was performed at 8420*g* for 20 min, unless stated otherwise. Dry seeds (70 g) were soaked in extraction buffer (25 mM sodium phosphate buffer, pH 6.8), for 24 h at 4 °C. Soaked seeds were homogenised using a laboratory blender in 140 mL chilled extraction buffer, then squeezed through two layers of muslin cloth and the extract was centrifuged. The pellet containing cell debris was discarded and supernatant was collected. The pH of supernatant was lowered to 4.0, by drop-wise addition of chilled 0.2 M HCl with continuous stirring and incubated for 4 h at 4 °C, causing the precipitation of inactive proteins, which were removed by centrifugation. The clear supernatant was collected and its pH bought back to 6.8 using chilled 0.2 M NaOH. The supernatant was selectively precipitated at 40–60% ammonium sulfate saturation. Protein precipitate was collected by centrifugation and dissolved in 25 mM sodium phosphate buffer, pH 6.1. The enzyme so obtained was dialysed in the same buffer containing 2 M $(NH_4)_2SO_4$ and loaded onto an Octyl Sepharose CL-4B column (10 × 3 cm; Sigma–Aldrich), pre-equilibrated with 200 mL (5 bed volumes) of 25 mM sodium phosphate buffer, pH 6.1 containing 2 M $(NH_4)_2SO_4$. The column was washed with 300 mL of the same buffer containing 1.4 M $(NH_4)_2SO_4$. The bound enzyme was eluted with the same buffer containing 0.75 M $(NH_4)_2SO_4$. The enzyme obtained was extensively dialysed to remove ammonium sulfate against 25 mM sodium phosphate buffer, pH 7.0 at 4 °C. Enzyme thus obtained was applied to a DEAE-Sephacel column (8 × 3 cm), pre-equilibrated with 100 mL of 25 mM sodium phosphate buffer, pH 7.0. The enzyme eluted in flow through volume without any adsorption. The active fractions with high specific activity were pooled and concentrated. Enzyme so obtained was dialysed against 25 mM sodium acetate buffer pH 4.0, for 4 h at 4 °C. The dialysed enzyme obtained from the previous step was loaded onto an SP-Sephadex C-50 column (5 × 3 cm), pre-equilibrated with four column volumes of 25 mM sodium acetate buffer, pH 4.0. Column washing was done with 25 mM sodium acetate buffer, pH 4.0, containing 80 mM NaCl, followed by elution with 160 mM NaCl, in the same buffer. Active fractions were collected, concentrated and dialysed to remove NaCl. This enzyme was loaded onto a Sephadex G-150 column (25 × 1 cm), pre-equilibrated with 25 mM sodium acetate buffer pH 4.0 containing 50 mM NaCl. Active fractions were pooled, dialysed and concentrated for immobilisation purposes.

2.2. Immobilisation onto alkylamine

To each 10 to 30 mg of alkylamine glass was added 1 mL of 1–5% solution of glutaraldehyde ($C_5H_8O_2$) in 25 mM sodium phosphate buffer, pH ranging from 5.0 to 7.0, according to the design of experiment. The mixture was allowed to stand for 4 h in the dark at room temperature with intermittent shaking, followed by washing twice with respective phosphate buffer. Latter, varying amount of purified enzyme preparation (50–100 μg) was added to the beads and the coupling was continued for the next 24 h at 4 °C. The unbound enzyme was removed by washing the beads slowly with ice cold phosphate buffer of appropriate pH. The process was repeated three times to completely wash off any unbound enzyme. Washed fractions were kept for estimation of protein and enzyme activity.

2.3. Protein assay

Protein estimation was done by the Bradford method (1976), using crystalline bovine serum albumin as standard protein.

2.4. Enzyme assays

2.4.1. Soluble

The reaction mixture for activity measurement against ONPG contained, in a final volume of 500 μL, 50 mM glycine–HCl (pH 2.8), 20 mM ONPG substrate and 10 μL of appropriately diluted enzyme. The reactions were carried out at 37 °C for 10 min. Liberated