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Optimized production and characterization of thermostable invertase from *Aspergillus niger* IBK1, using pineapple peel as alternate substrate

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ABSTRACT

Production of thermostable invertase from *Aspergillus niger* IBK1 cultivated under submerged fermentation, using pineapple peel as a low-cost substrate, was investigated. Effects of some physical and nutritional factors such as pH, temperature, carbon sources and nitrogen sources on invertase production were studied. The enzyme was purified and characterized to evaluate its potentials for industrial applications. Maximum yield of invertase (24.20 ± 0.38 U/mL) was at 120 h fermentation period at pH 5.0 and 35 °C. Sucrose, glutamic acid and peptone all enhanced enzyme production. The invertase was partially purified by sephadex G-100 gel filteration chromatography, after which a 34.57-fold increase in specific activity and a yield of 8.78% was achieved. Native molecular weight of the enzyme was 67.7 ± 0.21 kDa using gel permeation chromatography on Sephadex G-100. The optimal pH and temperature of activity were 4.5 and 60 °C, respectively. The enzyme was highly stable at temperature 35–65 °C and at pH 3.0–6.0. K_m and V_{max} values for sucrose were 21.93 ± 3.72 mM and 35.71 ± 2.02 U/min/mL respectively. Na^+, K^+, Ca^{2+} and EDTA all enhanced the activity of *A. niger* IBK1 invertase while Mg^{2+} showed inhibitory effect. This study reveals invertase from *A. niger* IBK1 has potentials for industrial and biotechnological applications

1. Introduction

The global industrial enzymes market worth 3.3 billion and 4.2 billion US dollars in 2010 and 2014, respectively, has continued to grow (Singh et al., 2016). However, the major challenge in comprehensive application of enzymes in industry has been their high production cost (Jana et al., 2013). A significant proportion of this is attributed to growth substrate (Prasad et al., 2011). The utilization of renewable resources such as agro-industrial residues as cheap and readily available substrates for enzyme production has therefore been a subject of intense research. This is with a view to having an alternative way of cost-effective enzyme production and waste management. Agro-industrial wastes are generated in large amounts every year and their reuse in processes is of particular interest due to their renewability, low-cost and characteristics that allow production of different value added products (Borghi et al., 2009). Pineapple peel is a by-product of the pineapple juice processing industry. Its production in large quantities results in serious waste disposal problems which lead to environmental pollution hazards, if not utilized (Rani and Nand, 2004). Interestingly the peel contains a considerable amount of soluble sugars which may be used a substrates in microbial fermentation for production of value added products such as enzymes (Siti Roha et al., 2013).

Invertases [β-D-fructofuranosidase (E. C. 3.2.1.26)] catalyze the hydrolysis of α-1,4-glycosidic bonds of sucrose and release equimolar mixtures of monosaccharides D-glucose and D-fructose called invert sugar (Mobini-Dehkordi et al., 2008). The enzyme attacks the non-reducing fructofuranoside terminal residues of β-fructofuranosides such as sucrose and raffinose (Veana et al., 2011). Invertases exist widely in the biosphere especially in plants and microorganisms, where they occur both intracellularly and extracellularly (Rashad et al., 2006; Hussain et al., 2009). By providing plants with fuel for respiration and carbon and energy for the synthesis of different compounds, invertases play important roles in plant growth and development (Kulshrestha et al., 2013). Invertase is used in the inversion of sucrose in the preparation of invert sugar and high fructose syrups (HFS). Invert syrup is approximately 1.5 times sweeter than sucrose with wide applications in the food and pharmaceutical industries because of its functionally more desirable properties like high solubility and hygroscopic nature (De Almeida et al., 2005; Aranda et al., 2006). It is therefore used as humectants in the manufacture of chocolate-coated

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soft-centred sweeteners, candy products, fondants and after-dinner mints. Invertases are used in the pharmaceutical industry as digestive aid tablets, powder milk for infants' foods, as calf feed preparation, assimilation of alcohol in fortified wines and in the manufacture of inverted sugars as food for honey bees (Rashad and Nooman, 2008). Microbial invertases may also catalyze the synthesis of short chain fructooligosaccharides in which one to three fructosyl moieties are linked to the sucrose skeleton by different glycosidic bonds depending on the source of the enzyme (Sangeetha et al., 2005; Linde et al., 2009). Fructooligosaccharides are one of the promising ingredients for functional foods since they act as prebiotics and exert beneficial effects on human health (Kurakake et al., 2009; Coman et al., 2012; Rolim, 2015).

In this study, we explore the cost effective production of invertase by *Aspergillus niger* IBK1, under submerged fermentation, using pineapple peel as an alternate substrate. Subsequent purification and characterization of the enzyme were then carried out.

2. Material and methods

2.1. Preparation of pineapple peels

The agro-industrial waste pineapple peel (*Ananas comosus*) was obtained locally from the fruit market. It was prepared by exhaustive washing with distilled water, dried at 70 °C for 48 h in an oven and milled to 35 mesh sizes.

2.2. Isolation and maintenance of fungal strain

The fungal strain used in this study was isolated from deteriorating orange fruits and was characterized by colonial morphology and microscopical examinations using lactophenol cotton blue solution mount according to Benson (1990). It was identified as a strain of *Aspergillus niger* IBK1 and was maintained on potato dextrose agar (Fluka Sigma, St. Louis, Mo, USA) slants at 4 °C.

2.3. Submerged fermentation for enzyme production

Invertase was produced by submerged fermentation (SmF) in Erlenmeyer flasks (250 mL) containing 100 mL of medium composed of KH_2PO_4 (1.0 g), K2HPO4 (6.27 g), MgSO4 (0.25 g), peptone (5.0 g), biotin (0.0005 mg), thiamine (0.005 mg), CaSO4 (0.005 mg), FeSO4 (0.5 mg), MnSO4 (0.26 mg), ZnSO4 (0.1 mg), CaSO4 (0.5 mg) all dissolved in 1 L distilled water. Pineapple peels (10.0 g) was added as the substrate. Initial pH of the medium was adjusted to 6.0 and the culture medium was inoculated with 1×10^6 spores/mL and incubated at 30 °C for 5 days. After incubation, the cultures were filtered through glass fibre filter paper (Whatman GF/A) and the cell-free supernatants were used for the estimation of invertase activities. Fermentations were carried out in triplicates.

2.4. Determination of invertase activity and protein concentration

Invertase activity was determined by estimating the amount of reducing sugars released in a reaction mixture containing 0.02 mL of 1.0%w/v sucrose in sodium acetate buffer (0.05 M, pH 4.5) and 0.01 mL of the enzyme extract according to Bergmeyer and Bernt (1974). Reaction was for 60 min at 35 °C. Reducing sugars were quantified by addition of 3.0 mL of glucose oxidase-peroxidase reagent (Sigma-Aldrich, St. Louis, Mo, USA) and further incubation for 5 min at 35 °C. The absorbance was read at 540 nm. One unit of enzyme activity was defined as the amount of enzyme necessary to liberate 1.0 μmol glucose per milliliter per minute under the assay conditions. Protein content was quantified according to Bradford (1976), using bovine serum albumin (Sigma-Aldrich, St. Louis, Mo, USA) as standard.

2.5. Effect of incubation period on fungal growth and invertase production

The time course (24–192 h) of invertase production by fungus was determined and compared with fungal growth. At twenty four hourly intervals, the invertase activities were assessed. Also, the mycelia dry weights of the fungus, obtained after culture filtration and drying at 70 °C, were determined.

2.6. Effects of fermentation conditions and nutritional parameters on invertase production

2.6.1. Effects of pH on production of invertase from A. niger IBK1

The effect pH on production of invertase from *A. niger* IBK was determined by adjusting the pH of fermentation media to different levels 3.0–8.0. Each of the nedium, adjusted to different level, was inoculated with standardised spore suspension (1×10^6 spore/mL) and incubated for 120 h at 35 °C. Cultures were then filtered through glass fibre filter paper and supernatant was recovered as crude enzyme. Production of invertase was quantified according to standard assay procedure earlier described.

2.6.2. Effects of temperature on production of invertase from A. niger IBK1

The influence of temperature on production of invertase from *A. niger* IBK was studied by varying the incubation temperature of fermentation culture from 25 to 60 °C. After incubation for 120 h, cell-free supernatants were obtained by filteration and production of invertase determined according to standard assay procedure.

2.6.3. Effects of carbon sources on production of invertase from A. niger IBK1

The effect of some carbon sources on production of invertase from *A. niger* IBK was evaluated by supplementing the fermentation medium with different carbon sources. These were sucrose, glucose, fructose, rhamnose, maltose, starch, mannose and pineapple peel (1.0% w/v). Incubation was for 120 h at 35 °C. Cultures were then filtered through glass fibre filter paper and supernatants obtained as crude enzyme. Production of invertase was quantified according to standard assay procedure.

2.6.4. Effects of nitrogen sources on production of invertase from A. niger IBK1

The effect of some nitrogen sources on production of invertase from *A. niger* IBK was evaluated by supplementing the fermentation medium with different carbon sources. These were nitrogen sources $(NH_4)_2SO_4$, NH_4Cl, KNO_3, peptone, yeast extract, urea, $NaNO_3$, $Ca(NO_3)_2$. Incubation was for 120 h at 35 °C. Cultures were then filtered through glass fibre filter paper and invertase activities present in supernatant was determined according to standard assay procedure.

2.6.5. Effects of amino acids on production of invertase from A. niger IBK1

The effect of amino acids on production of invertase from *A. niger* IBK was evaluated by supplementing the fermentation medium with some amino acids which included alanine, cysteine, glutamine, glycine, threonine, leucine, glutamic acid and valine (0.5% w/v). Incubation was for 120 h at 35 °C. The fermentation media were filtered through glass fibre filter and cell-free supernatants were recovered as crude enzyme preparations which were assessed for production of invertase according to standard assay procedure.

2.6.6. Effects of pineapple peel concentrations on production of invertase from A. niger IBK1

The effect of different concentrations of pineapple peel (0.2%, 0.4%, 0.6%, 0.8%, 1.0% and 1.2% w/v) on production of invertase from *A.*