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Production and partial characterization of extracellular glucose isomerase using thermophilic Bacillus sp. isolated from agricultural land

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ABSTRACT

In this investigation, glucose isomerase was extracted from Bacillus sp., isolated from agricultural soil sample and purified further by ammonium sulfate fractionation followed by gel filtration chromatography using Sephadex G-25. The purified enzyme over crude was further subjected to partial characterization which includes the assessment of influence of pH, temperature, incubation time and metal ions on the stability and activity of glucose isomerase was observed. As a result, it was found that, glucose isomerase isolated from the thermophilic Bacillus sp. enumerated from the agricultural soil showed maximal stability at pH 8.0 and at temperature 70 °C within 35 min. It was also found that, at the minimum concentration of 5 mM Mg^{2+}, Co^{2+} and Mn^{2+} showed 41%–59% of enzyme activity.
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1. Introduction

Glucose isomerase is one of the three highest tonnage value enzymes, amylases and proteases being the other two (Bhosale et al., 1996). This enzyme can be seen in a number of bacteria which can use xylose as a carbon substrate for growth (Bhosale et al., 1996; Chen, 1980a, b). This enzyme isomerizes D-xylose into D-xylulose by in-vivo and D-glucose into D-fructose by vitro (Chen, 1980c). This activity of the enzyme is used in industry for production of high-fructose corn sirup from corn starch which is a multi-step isomerization reaction catalyzed by glucose isomerase (Lee and Zeikus, 1991). Heat treated and immobilized glucose isomerase is highly stable and can be reused under a continuous operation (Huitron and Limon-Lason, 1978). Thermophilic microorganisms produce intrinsically thermostable enzymes, which have evolved and adapted to the extreme environment of their natural habitats (Amelunxen and Murdock, 1978). The thermophiles and their enzymes are highly needed by industries for the commercial productions since their growth rate is high and the chances of contamination are low. Xylose isomerase catalyzes the reversible isomerization of D-xylose to D-xylulose and has potential application in the conversion of biomass to ethanol (Jeifries, 1983). The enzyme is usually intracellular and is produced by various microorganisms (Kitada et al., 1989). Studies of extracellular production of glucose isomerases have been rare, and only a few reports of production from Streptomycetes and Chainia sp.. are available (Khire et al., 1990).

The aim of the present investigation is to isolate the bacillus species producing extracellular glucose isomerase from the agricultural land and subsequent purification and characterization of the enzyme.

2. Materials and methods

2.1. Microorganism

The thermophilic Bacillus sp. producing glucose isomerase was isolated from the agricultural lands in and around Thirukkalukundram, Kanchipuram District, Tamilnadu, South India (Dey et al., 1992; Hinge et al., 1989).

2.2. Chemicals and microorganisms:

Solvent and other chemicals which were used during this study were purchased from Himedia, Merck and s.d. Fine-Chemicals, Mumbai, India.

3. Methods

3.1. Enzyme production

The thermophilic Bacillus sp. was grown on basal medium containing 0.5% yeast extract, 0.5% peptone, 0.1% K_2HPO_4, 1%

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Na_2CO_3, 0.01% $MnCl_2 \cdot 4H_20$, and 0.005% $CoS0_4 \cdot 7H20$, and D-xylose (1%), xylan (1%), glucose (1%), sorbitol (1%), or wheat bran (5%) was added to the basal medium. The pH was adjusted to 8 with sterile sodium carbonate, added separately (Kwon et al., 1987). Bacteria for xylose isomerase isolation were grown in a 250 ml flask with 50 ml of medium at 50 °C and pH 10 for 16 h on a rotator shaker (250 rpm). The inoculum (10%) was grown for 6 h in the same medium. During the fermentation, subsamples were removed at various time intervals and the bacteria were checked for lysis by phase-contrast microscopy and were also stained for the detection of endospores (Dorner, 1926).

3.2. Enzyme preparation

Cells were separated from the 50 ml culture broth by centrifugation (10,000 × × g for 10 min), and the supernatant fluid was treated as extracellular crude enzyme. Cells (0.2 g [dry weight]) were washed twice with 50 mM potassium phosphate buffer, pH 7.0, suspended in 5 ml of the same buffer, and disrupted by sonication for 2.5 min (five times for 30 s each time) at 4 °C. The clear supernatant fluid was obtained by centrifugation at 13,000 × × g for 15 min and is referred to as intracellular extract.

3.3. Enzyme assay

Glucose isomerase activity was determined according to the modified method of Dische and Borenfreund (1951). The enzyme reaction mixture contained 0.5 ml of 0.2 M sodium phosphate buffer (pH 7.0), 0.2 ml of 1 M D-glucose, 0.1 ml of 0.1 M $MgSO_4 \cdot 7H_20$, 0.1 ml of 0.01 M $CoCl_2 \cdot 6H_2O$ and 0.2 ml of enzyme solution. The enzyme solution was prepared by breaking the bacterial cells with a homogenizer for 1 min and centrifuging the suspension at 12,000 × × g for 10 min. The supernatant was collected and used as enzyme solution. The final volume of the enzyme assay mixture was made up to 2 ml with distilled water. The mixture was incubated at 70 °C for 1 h, and the reaction was stopped by adding 2 ml of 0.5 M perchloric acid. The amount of the product, fructose, was determined by the Seliwanoff's method (Chen et al., 1979).

3.4. Protein determination

Protein content in the supernatant was determined by Bradford (1976) assay.

3.5. Enzyme purification

Ammonium sulfate was added to the crude enzyme extract to 45% saturation, incubated for an hour at 4 °C with gentle mixing. The precipitate was collected by centrifugation at 10,000 rpm for 20 min at 4 °C and dissolved in 0.1 M phosphate buffer (pH 7.0) containing 5 mM MgSO4, 0.5 mM $CoCl_2$. The ammonium sulfate concentration was increased stepwise to 60%, 75% and finally to 90% saturation and the precipitates were harvested accordingly. The fraction containing glucose isomerase activity was pooled and dialyzed overnight against 0.1 M phosphate buffer (pH 7.0) (Liu et al., 1996). Then, a Sephadex G-25 column (3.2 × 38.5 cm) was prepared and equilibrated with 0.05 M phosphate buffer containing 0.15 M NaCl. The dialyzed enzyme was applied to the column and eluted with the phosphate buffer. Fraction containing glucose isomerase activity was collected, concentrated with ammonium sulfate and dialyzed against 0.1 M phosphate buffer (pH 7.0) (Liu et al., 1996).

3.6. Effect of pH on enzyme

The optimum pH for xylose isomerase was determined by using acetate buffer (pH 4–5), potassium phosphate buffer (pH 6–8) and glycine sodium hydroxide buffer (pH 9–12). The enzyme was incubated at various pH values at 50 °C for 30 min in the absence of substrate and the optical density was measured at 280 nm using spectrophotometer.

3.7. Effect of temperature on enzyme

Thermostability was examined by incubating the enzyme solution at pH 7.0 in the presence of 1 mM $CoCl_2$ at various temperatures for 30 min and measuring the optical density at 280 nm using spectrophotometer.

3.8. Effect of metal ions on enzyme

At the beginning, glucose isomerase enzyme purified from Bacillus sp. was treated with 5 mM-EDTA at 60 °C for 1 h, and then washed with double-distilled water at 4 °C to obtain ion-free enzyme preparation. The influence of metal ions on enzyme activity was determined by measuring residual glucose isomerase activity under optimum assay conditions after 15 min preincubation at 80 °C in the presence of various metal ions in varying concentrations (5, 10 and 15 mM). The effect of metal ions on enzyme activity was determined.

3.9. Effect of incubation time on enzyme

The influence of incubation time on the maximal activity of glucose isomerase was also analyzed. After the analysis of effect of pH, temperature and metal ion concentration needed for the optimal activity, a standard reaction mixture was prepared with all the necessary ingredients with optimal pH and temperature. Then standard glucose isomerase assay was performed and the % of enzymatic activity was calculated in every 5 min to know the influence of incubation time on the activity of the enzyme.

3.10. Effect of carbon sources

To understand the role and the necessity of various carbon sources in the production media for glucose isomerase was also studied by adding different carbon sources like glucose, sucrose, galactose, maltose and soluble starch (each 1% separately) along with the basal medium used for the study.

4. Results

4.1. Enzyme purification by chromatography

The enzyme was purified from culture supernatant by ammonium sulfate fractionation step followed by gel filtration chromatography as described in Section 2. The maximum enzyme activity of broth was obtained after 96 h (4 days) of cultivation in media. Fraction collected during 90% saturation of ammonium sulfate showed significant glucose isomerase activity and was further purified by gel filtration chromatography using sephadex G-25 (Graph 1).

Totally 90 fractions (each contains 3 ml) were eluted and observed for optical density at 280 nm. From the graph, it was observed that, three different peaks were obtained from the fractions 17–26, 41–64 and 73–82. By performing standard glucose isomerase assay (Dische and Borenfreund, 1951), it was found that

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