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Production, optimization and characterization of α-amylase and glucose isomerase producing *Bacillus megaterium* BPTK5 from cassava waste

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ABSTRACT

A mesophilic bacterium, Bacillus megaterium BPTK5 was isolated from cassava waste samples collected from starch processing plant (TN, India). The isolate was assayed for its ability to produce α-amylase and glucose isomerase. Experiments were set to observe the effects of incubation time, pH, temperature, and carbon and nitrogen sources on the growth of BPTK5 and its enzyme activities. Optimum biomass and amylolytic activity was achieved after 76 h of incubation, at 35°C, pH 6.0. Among the various carbon and nitrogen sources investigated, starch and casein were found to be the best inducers of α-amylase. An increase in glucose isomerase activity was observed at 48 h of incubation at 35°C with the initial pH of 6in the presence of Xylose and peptone. Thus Bacillus megaterium BPTK5 was proved to be an industrially important microbe involved in HFCS manufacturing.

Key Words: Starch, Casein, CMC column, HFCS, *Bacillus megaterium*.

INTRODUCTION

In most of the food industries, Carbohydrate sweeteners were used for their sweetness when compared to conventional sweeteners. The carbohydrate sweeteners include monosaccharides such as glucose, fructose, and galactose. Of them fructose was found to be sweeter than its structural isomer, glucose and so as many researches were done for the conversion of glucose into fructose. The carbohydrate sweeteners were available in various forms includes cane juice, fruit juice concentrates, corn syrups, etc [1].

High fructose corn syrups (HFCS) are similar to common corn syrups which involves the use of enzymes or chemicals in the conversion of glucose into fructose. The well familiar HFCS includes HFCS 42, which is commonly used in baked goods (42% fructose and 58% glucose) and HFCS 55, used in soft drinks (55% fructose and 45 % glucose) [2,3]. The chemical conversion of glucose and fructose were known as Lobry de Bruyn-Alberda van Ekenstein transformation which requires high pH and temperature; and also the reaction is non-specific which leads most of the industries for the usage of enzymatic production of HFCS.

For the enzymatic production of HFCS two groups of enzymes were mainly employed. The first group of enzymes involved was amylases which help in the conversion of starch and dextrins present in the corn grains into simpler glucose residues. Alpha amylase, which catalyzes the hydrolysis of starch, is commercially produced by fungi [4] and bacteriaof the genus *Bacillus* [5] includes *Bacillus licheniformis, Bacillus stearothermophilus,* and *Bacillus amyloliquefaciens* [6]. These enzymes account for about 30% of the world's total enzyme production [7].

The second group of enzymes includes glucose isomerase (GI) which helps to convert the glucose residues into its isomer fructose which is sweeter than glucose [1]. Glucose isomerase is one of the three high valued enzymes in the world, whereas amylase and proteases were the other two. Various microorganisms were found to produce GI including *Pseudomonas hydrophila, Bacillus stearothermophilus, Bacillus megabacterium, Bacillus coagulans, Bifidobacterium* spp. According to Wiseman [8], the applications of the GI may lead them as the most important of all industrial enzymes of the future.

In the view of advantages offered by both the enzymes, the present study was aimed at the isolation of the bacteria which was able to produce both the enzymes and optimizing the fermentation parameters for the enhanced production of both the enzymes.

MATERIALS AND METHODS

Sample collection

The cassava waste samples were collected from a starch processing industry located in Salem, TN, India. The collected samples were brought to lab in a sterile container and stored aseptically.

Screening of enzyme producers

The cassava waste sample was diluted upto 10^{-7} concentration and the bacterial colonies were enumerated by spreading on Nutrient agar plates. The isolated bacterial strains grown were assayed for the production of both the enzymes.

a. Alpha amylases

The isolated colonies obtained from NA plates were screened for the α-amylase producing ability by streaking them on starch agar plates containing soluble starch 1.0%, yeast extract 0.4, K_2HPO_4 0.1, and $MgSO_4.7H_2O$ 0.15, pH 7.0 at 37°C [9]. The iodine solution was added to the plates after 24 hours incubation in order to observe the development of clear zones around the colonies due to the starch hydrolysis. The α-amylase positive strains were further screened for the production of Glucose isomerase.

b. Glucose isomerase

The glucose isomerase production was assayed by seliwanoff's reaction [10]. For the assay, the amylase positive strains were grown overnight in LB broth, centrifuged and the supernatant containing the crude enzyme were collected. To 0.2 ml of the crude enzyme added 0.3 ml of substrate (0.1M glucose) and boiled for 30 minutes. 1 ml of the seliwanoff's was added and heated for 5 minutes. The development of cherry red colour indicates the presence of fructose which resulted due to the presence of the enzyme glucose isomerase.

The isolates showing positive for both the enzymes were sub cultured on Nutrient agar for maintaining pure culture. The strain with maximum enzyme activity was selected (assigned as BPTK5).

Characterization and identification of bacteria

The isolate BPTK5 was characterized based on Bergey's Manual of Systemic Bacteriology [11] and the further identification was done using 16S rDNA sequence analysis according to Gomaa and Momtaz [12]. The DNA isolated from the strain MNJ 23 was subjected to PCR amplification of 16S rDNA using the primers: Forward: (AGAGTTTGATCCTGGCTCAG) and Reverse: (AAGGAGGTGATCCAGCCGCA). The amplified region of DNA was sequenced and was ascertained to its systematic position using NCBI-BLAST.

Basal mediums for the production α-amylase and Glucose isomerase

For the optimization studies, a basal medium was designed for α-amylase which contains Lactose 1%, Yeast extract 2%, KH_2PO_4 0.05%, $MnCl_2$-$4H_2O$ 0.00015%, $MgSO_4.7H_20$ 0.025%, $CaCl_2.2H_2O$ 0.005%, $FeSO_4.7H_2O$ 0.001%, pH 7.0 [13]. In concurrent, Glucose isomerase also, a basal medium was designed as proposed by Fikret Uyar and

Baysal, [14] (Peptone 1%, Yeast extract-0.5%, K_2HPO_4 -0.3%, $MgSO_4.7H_2O$ -0.1%, Xylose-1%, pH 7.0). The parameters were optimized using the basal medium by one-at-a-time optimization strategy. After the production of enzymes, the culture medium was centrifuged and the supernatant was used as the crude enzyme source for the assay procedures.

Enzyme assays

a. Alpha amylases

The amylase was assayed by using starch as substrate. To 2.0 ml of the crude enzyme, 2.0 ml of 0.1 M acetate buffer and 1% soluble starch were added (pH 7.0). The reaction mixture was incubated at 37°C for 30 min. The amount of reducing sugar released was determined by the addition of 3, 5 dinitrosalicylic acid followed by boiling for 10 min according to **Bernfield [15]**. The color developed was read at 540 nm. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol of reducing sugar in one minute under the assay condition.

b. Glucose Isomerase

Glucose isomerase activity was determined according to the method described by Chen et al [16]. The reaction mixture contained 0.5 ml of 0.2 M sodium phosphate buffer (pH 7.0), 0.2 ml of 1M glucose, and 0.1 ml of 0.1M $MgSO_4.7H_20$, 0.1 ml of 0.01 M $CoCl_2.6H_20$, and 0.2 ml of crude enzyme. 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. One unit of glucose isomerase activity was defined as the amount of the enzyme that produced 1 µmol of fructose per min under the assay conditions.

Biomass determination

The bacterial biomass was determined by measuring the absorbance of the culture medium at 600 nm [17].

Parameter optimization studies

Incubation time:

The role of incubation time on the production of both the enzymes were analyzed by incubation the respective production medium inoculated with the bacterial strain BPTK5 were incubated and the amount of the enzyme produced and biomass were assayed at regular intervals (6, 12, 24, 48, 72, 96 h).

Initial pH and Incubation temperature

The production medium was adjusted at various levels of pH by NaOH solution (4.0, 5.0, 6.0, 7.0, 8.0 and 9.0) and the effect of initial pH on α-amylase and Glucose isomerase production and biomass were studied. To study the effect of incubation temperature on α-amylase and Glucose isomerase production, the flasks with the production medium were inoculated and incubated at various temperatures ranging from 30-60°C and the enzymes produced were assayed.

Carbon and nitrogen source

To study the effect of different carbon sources on enzyme production, Glucose, sucrose, galactose, lactose, xylose and starch were selected as carbon sources. The conventional carbon source of the production medium was replaced with these carbon sources and the prodiction of the enzymes were studied. Organic and inorganic nitrogen sources like Yeast extract, casein, peptone, NH_4Cl, $(NH_4)_2SO_4$ and $NaNO_3$ were amended to the production medium with the isolate for enzyme production and the amount of enzyme produced and the biomass were analyzed.

Mass production and purification of the enzymes

The productions of the enzymes were carried out under the optimized fermentation conditions and were purified separately.

The production medium was centrifuged at 5000 rpm for 10 mins and collected the supernatant. The crude enzyme in the culture supernatant was precipitated using ammonium sulphate precipitation using 70% saturation for α-amylase [18] and 65% saturation for Glucose isomerase [19] and dialysed overnight.

The α-amylase was purified by the method proposed by Krishnan and Chandra [20]. The precipitated α-amylase was dissolved in 0.01 M Sodium phosphate buffer (pH 6.4). The elution was done using CMC column pre-equilibrated with the same buffer under a linear gradient of NaCl. The fractions with maximum amylase activity was pooled, concentrated and stored at 4 °C.

For Glucose isomerase, the dialysed sample was further purified using DEAE-Sepharose column pre-equilibrated with Mops buffer (5mM $MgCl_2$ and 0.5 mM $CoCl_2$). The active fractions with higher enzyme activity were pooled, concentrated and stored [19].

SDS PAGE analysis:
The further protein profile and the presence of enzymes were confirmed by SDS PAGE analysis [21]. The standard marker was used for reference purpose and Coomasie brilliant blue staining was done for band visualization.

RESULTS AND DISCUSSION

Starch industry waste is one of the potential sites which may contain biotechnologically potent microbes. Cassava waste provides a good source for microorganisms to flourish their enzymatic activities. In this study the cassava waste sample collected from starch processing industry was screened for bacteria producing α-amylase and Glucose isomerase by their respective screening media. After 24 h incubation, 16 strains showed clear zones upon the addition of iodine solution was found to be positive for amylase and 6 strains developed cherry red colour on seliwanoff's reaction which indicate the presence of the organisms producing Glucose isomerase enzyme [10]. Among all the screened isolates, a bacterial isolate produced intense zone and colour, the strain BPTK5 was used for further identification.

Based on the preliminary characterization, the isolate BPTK5 was found to be Gram negative, spore forming bacteria. The further confirmation using 16S rDNA sequence of the isolate revealed a close relatedness to *Bacillus megaterium* with 100% similarity. Hence the strain was confirmed as *Bacillus megaterium.*

The optimization of cultural conditions is very important for the production of enzymes. When the isolate was grown in liquid culture medium to determine optimum incubation time, maximum amylase production was obtained at 72 h of incubation whereas Glucose isomerase at 48 h. The biomass yield was found to increase with an increase in the incubation period. Ashwini et al [22] reported the maximum production of amylase was occurred at 96 h of incubation. The optimum incubation time for Glucose isomerase lies in 36 h for the bacteria isolated by Nobel Surya Pandidurai et al, [23].

The pH of the production medium strongly affects many enzymatic processes and transport of compounds across the cell membrane. In this study the effect of pH on α-amylase and glucose isomerase production was investigated with varying pHs (4-9). It was noted that the isolate exhibited maximum α-amylase and glucose isomerase production at pH 6 (Fig. 2). The study falls in line with Utong *et al*. [24] who described the ability of *Bacillus sphericus* to produce α-amylase if grown at pH 6-9 range. The results obtained were in line with the results of Nobel Surya Pandidurai [23] in which the glucose isomerase produced by Enterobacter agglomerans also possess the optimum pH of 6.0. From the result it has been understood that both the enzymes were active at acidic temperature and will be suitable for industrial applications.

Temperature is also one of the important parameter that has to be controlled for any enzyme production [25]. The optimum temperature for α-amylase and glucose isomerase production for the test isolate was found to be 35°C. the result indicated the isolate BPTK5 was falls under mesophillic. The most of the amylases produced so far possess the optimum temperature ranges from 30-50°C [22]. Nobel Surya Pandidurai et al [23] also got the optimum temperature of 37°C for the production of glucose isomerase.

The addition of carbon sources in the form of carbohydrates influences the better production of enzymes [26]. Of the carbon sources tested for inducing enzyme production starch was found to be the better carbon source for the production of α-amylase and xylose for the glucose isomerase. In contrast to our results lactose was found to be the better carbon source for amylase production [22]. Xylose was found to be a good carbon source for glucose isomerase by many bacteria which helps to isomerizes xylose in addition to glucose and hence it is also termed as Xylose isomerase [27].

Next to the carbon source, nitrogen is found to be playing prominent role in the growth and development of the bacteria. Of the nitrogen sources tested for their role in growth and enzyme production, casein and peptone were found to be assisting the enhanced production of α-amylase and glucose isomerase respectively. Aiyer [28] reported ammonium dihydrogen phosphate to be a better nitrogen source for enzyme production by *B. licheniformis* SPT 278

than other tested inorganic nitrogen sources. In addition to the peptone, extracts of beef and yeast were also found to be enhancing Glucose isomerase production [29].

The ultimate purpose of the parameter optimization studies was to enhance the enzyme production. Hence, the mass production of the enzymes was carried out with the optimized fermentation conditions [30]. The enzymes produced were purified with the help of ammonium sulphate precipitation followed by ion exchange chromatography.

The purified enzymes were analyzed for their protein profile using SDS-PAGE analysis. The presence of protein band near the 60 kDa indicates the presence of α-amylase since most of the amylases reported reported were lies between 55-66 kDa [31, 32, 33]. The purified Glucose isomerase migrated as a single band corresponding to molecular mass of 60 kDa. The findings are in accordance with Chen *et al* [16].

CONCLUSION

Glucose and fructose are simple monosaccharides with same molecular formula (C_6H_5OH). Since fructose is sweeter than glucose, and so preferred for the preparation of HFCS. Since the enzymatic production of HFCS requires these two groups of enzymes, it is necessary to produce the enzymes in industrial scale. The isolation of single microorganism for producing both the enzymes will helps in the reduction of production cost and effort. The present study clearly illustrated the isolated *Bacillus megaterium* BPTK5 having the potential to produce both the α-amylase and glucose isomerase and also their fermentation parameters were optimized. Both the enzymes were well proven to have applications in the production of HFCS and further studies have to be done for industrial application.

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