Production Optimization, Purification and Characterization of Glucoamylase from *Arachniotus sp.*

[1]S. SHAUKAT, [2]M. A. BAJWA, [1]M. A. WAQAR* AND [3]T. SOHAIL
[1]*Dr. Panjwani Center for Molecular Medicine and Drug Research, University of Karachi, Karachi, Pakistan*
[2]*Department of Chemistry, University of Agriculture, Faisalabad*
[3]*PCSIR Labs. Complex, Karachi. Pakistan*

(Received 27th February, 2006, revised 29th March, 2006)

Summary: A fermentation process for the production of glucoamylase was optimized to enhance enzyme production by *Arachniotus sp.* by using the waste bread as substrate. Supplementation of the fermentation medium by low concentrations of corn steep liquor, yeast extract and cane molasses enhanced glucoamylase production. Maximum yield of glucoamylase was 14.44 U/ml noted after 03 days of fermentation with 1.5 % corn steep liquor, 0.1 % yeast extract and 1.0 % cane molasses under pre-optimized culture conditions. Glucoamylase produced was then purified and characterized. Glucoamylase was purified by ammonium sulfate prescriptation and column chromatography on Sephadex G-150 and Sepharose. The enzyme exhibits maximum activity at pH 5 and 60°C. The enzyme was found to be activated by Ca^{2+} up to the concentration of 5mM whereas 2% inhibition was observed at 2.5 M urea. Addition of calcium also increased the thermostability of the enzyme. These properties make it applicable to other biotechnological properties and can be used in industries for the management of waste and glucose production.

Introduction

A large quantity of waste bread has the potential of being used industrially and, thereby, to improve bread economics and eliminate the large environmental problem presented by waste bread.

Amylases are capable of hydrolyzing starch and related saccharides. The best known are alpha-amylase, beta-amylase and glucoamylase. They belong to glucoside hydrolyases in the system of which they form their own families including 13, 14 and 15, respectively in the enzyme classification system [1]. Glucoamylase use the alpha-inverting reaction mechanism [1]. The availability of high activity of glucoamylase is an essential element in successful process for enzymatic conversion of different starches derived from maize, wheat, rye and potato into glucose by removing single glucose residue from a soluble oligosaccharide at the non reducing end until all the oligosaccharides are converted into glucose.

Glucoamylase [α-1,4-glucan glucohydrolase EC 3.2.1.3] is used in food industries in the production of different antibiotics, amino acid, ethanol and organic edible acid production processes [2]. The enzyme is also used in the dextrose production, in baking industry and in the brewing of low calorie beer and whole grain hydrolysis of alcohol industry [3]. The primary commercial application of glucoamyase is the production of glucose syrup from starch.

Generally fungi secrete glucoamylase and other saccrifying enzymes depending upon the composition of growth medium and fermentation conditions [4]. Over the past decade, the white rot fungi have been studied for their ability to degrade recalcitrant environmental pollutants [5]. *Arachniotus sp.* is one of the member of this family and has been used for the economic utilization of many waste products.

In our previous study [6], it has been found that the strain *Arachniotus sp.* which produces consistent and reproducible glucoamylase and can be obtained under non-limiting nitrogen and carbon sources. Hence, the present project was designed to utilize waste bread for the production of glucose, to optimize the production conditions of glucoamylase by providing the additional nutritional sources in the fermentation system and to characterize the enzyme produced after its purification. Thus, it will assist further in determining the suitability of the enzyme for industrial application.

*To whom all correspondence should be addressed.

Results and Discussion

The study was carried out for the production of glucoamylase by *Arachniotus sp.* using waste bread as a substrate in order to manage and utilize the waste material into useful products. In line with one of the author's previous study [6], where the glucoamylase produced effectively under non-limiting carbon and nitrogen sources, corn steep liquor, yeast extract and cane molasses were used for additional carbon, nitrogen, vitamins, minerals and energy sources under pre-optimized condition. No activity of glucoamylase could be detected when the fungus was grown in the basal medium without starch. To find out the enzyme characteristics produced under these optimized conditions, the enzyme was purified to rule out any interference and subjected to some kinetic parameters.

Optimization of culture conditions

Glucoamylase production by *Arachniotus sp.* was enhanced by the stepwise addition of corn steep liquor, yeast extract and cane molasses and peaked with 1.5% corn steep liquor (12.50 U/ml), 0.1% yeast extract (13.50 U/ml) and 1.0% cane molasses (14.44 U/ml). Further addition of these nutrients led to a steady decline in growth performance and gluco-amylase formation (Fig. 1). A significant increase in the enzyme production occurred P value = 0.02 between the maximum activity after the addition of additional nutritional sources and LSD values are more than 5% for all the treatment means.

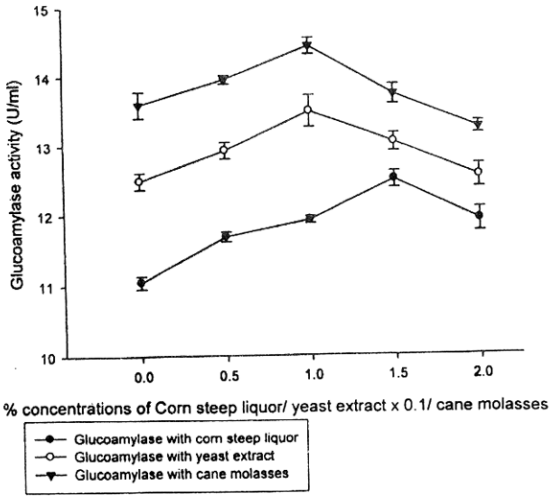

Fig. 1: Effect of corn steep liquor, yeast extract and cane molasses on glucoamylase production by *Arachniotus sp.*

Corn steep liquor provides glucose and other nutrients which help to promote the growth of micro-organisms [7]. Supplement of culture medium with corn steep liquor upto 1.5% level, supplied additional energy and nutrient source and promoted the growth of fungus which also increased the secretion of glucoamylase. Beyond this optimum concentration, the production was decreased due to the catabolic repression [8]. Panday *et al* [9] also reported the increase in the production of glucoamylase by *Aspergillis niger* on rice bran using corn steep liquor as additional nutritional source. Enayati *et al.*, [10] reported that in low glucose containing medium, there is sufficient cAMP to fully charge the CAP protein and the level of cAMP/CAP is insufficient to allow transcription of catabolic sensitive operons. But as the level of glucose increases, the enzyme in the medium which is sensitive to catabolic effect is dephosphorylated and gives its phosphate group to glucose which becomes active and cause catabolic repression.

Yeast extract promote the growth of micro-organisms because it contains a mixture of B-complex vitamins and some essential aminoacids. Adinarayana *et al.*, [11] reported the enhanced production of glucoamylase by the addition of vitamins and aminoacid sources. Hewitt *et al.* [12] reported that *Aspergillus niger* produced maximum amylase in optimum growth medium containing 0.1% yeast extract. The difference in the optimum concentration of yeast extract reported by different researchers may be due to the fact that culture conditions vary with the requirements of the different organisms in growth media of different substrates.

Molasses is a mixture of soluble sugars and also contain some mineral nutrients and heavy metal ions [13]. Supplementation of culture medium with molasses up to 1.0% level supplied additional source in the form of soluble sugars and minerals which can promote the growth of fungus and the secretion of glucoamylase. But beyond this concentration, the production is decreased. It may be due to the effect of catabolic repression [10]. Excess mineral and heavy metal toxicity on the other hand, resulted in decreased glucoamylase production because fungi are unable to withstand extra burden of minerals and heavy metals [13]. Other workers [14-16] also reported an increase in amylases production by the addition of soluble sugars and a respective effect of their increase concentration in the fermentation media.

Enzyme Purification

The enzyme was purified to rule out the possibility of any synergistic effect of other amylases in crude preparation. The purified enzyme, which appears as a single band (64 kDa) on SDS-PAGE, was used for further studies (Fig. 2). Table-1 shows the purification profile of the glucoamylase.

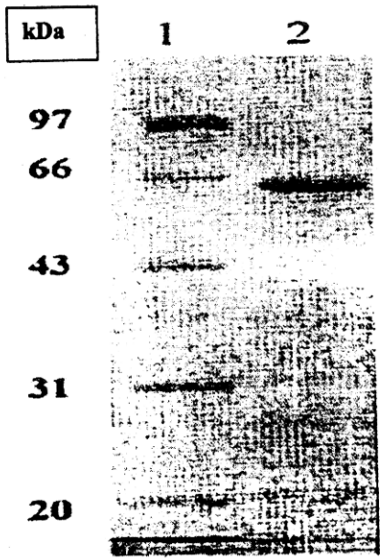

Fig. 2: SDS-PAGE pattern of the purified glucoamylase produced by *Arachniotus sp*

Table-1: Purification profile of glucoamylase produced by *Arachniotus sp.*

Purification Steps	Total protein (mg)	Total enzyme (U)	Specific activity (U/mg)	Purification (-fold)	Yield (%)
Supernatant	478	1358	2.84	1.0	100
Ammonium sulfate precipitaton	144.8	1190	8.22	2.89	87.63
Gel filtration	49.8	786	15.78	5.55	57.9
Q-sepharose	10.6	326	30.75	10.82	24

Determination of pH and temperature optimum

The optimum pH of glucoamylase hydrolysis was determined at 35°C (Fig. 3). The optimum pH (5.0) was found to be similar to those reported by others [17]. Some varying results are also reported with some different cultures. Some of which are *Aspergillus niger* pH 4.5 [18]; *Aspergillus tamarli* pH 4.5-6.0 [19]; *Monascus purpureus* pH 4.2 [20] at the end respectively. This variation may be due to the

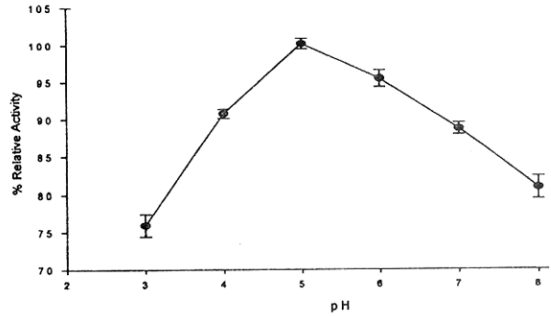

Fig. 3: Effect of pH on *Arachniotus sp.* glucoamylase activity.

variation in culture conditions or variation in the aminoacid sequence of glucoamylase; however, the range seems to be pH 4.0-6.0.

The optimal temperature of purified glucoamylase was observed in the interval between 40 to 90°C at pH 5 (Fig. 4). Values of optimum temperature of glucoamylase hydrolysis vary according to the producing microorganism. The glucoamylase produced by different culture conditions and organisms had different temperature optima. *Aspergillus tamari* presented 50-60°C [19]; *Aspergillus niger* 60-65°C [18]; *Aspergillus niger* 45°C [21]; *Monascus purpureus* 55°C [20] and *Scytalidium thermophilum* 60°C [22]. However, 50-60°C seems to be the range of optimum temperature of glucoamylases.

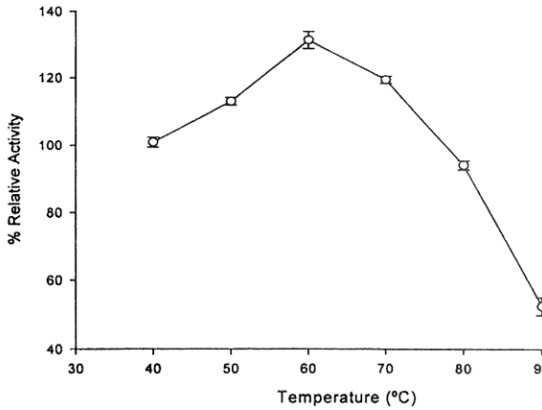

Fig. 4: Effect of temperature on glucoamylase activity.

Effect of calcium and urea

The enzyme activity was analyzed in the presence of 1-6 mM concentrations of calcium at

temperatures ranged from 40-70°C and 1.25-5 M concentrations of urea at optimum temperature. The enzyme shows its maximum activity at 5mM concentration of calcium ($CaCl_2$) and showed thermostability with the addition of calcium (Fig. 5). The activity at 70°C with 5mM concentration of calcium is approximately the same to that at 60°C without calcium. Although the enzyme's thermostability is its intrinsic property dictated by its primary structure, many external factors like ions influence the activity of the enzyme [23]. Lin *et al.*, [24] reported that the thermostability of glucoamylase was enhanced in the presence of calcium. Chen *et al.*, [25] also reported the enhanced activity of glucoamylase in the presence of 1mM calcium but did not study the effects of further increased concentration of calcium. The observations recorded in this investigation are consistent with those of Kumar *et al* [23] that the activity of the enzyme is increased in the presence of calcium ions.

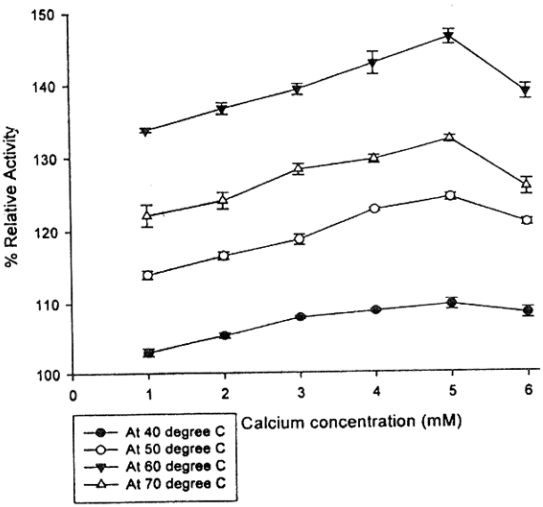

Fig. 5: Effect of varied concentrations of calcium at different temperatures on glucoamylase activity and its thermostability.

Urea works as an inhibitor for the enzyme's activity by destabilizing the protein structures. Urea weakens the hydrophobic interaction between the substrate and the tryptophan residue in the subsite of glucoamylase [26]. Glucoamylase produced by *Arachniotus sp.* showed 2% inhibition up to the concentration of 2.5M (Fig. 6). Glucoamylase produced by different fungi showed varied decrease in the activity in response to the addition of different

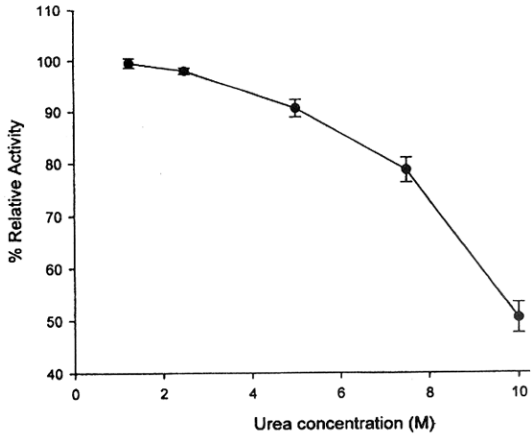

Fig. 6: Effect of urea on glucoamylase activity.

concentrations of urea [27]. This activity variation, among the glucoamylase produced by different strain of fungi, may be due to the difference in amino acid sequences of tryptophan residues at the subsite.

In conclusion, the glucoamylase from *Arachniotus sp.* is a novel biocatalyst that may find potential application in the starch processing as it allows starch hydrolysis without gelatinization and thermo stable in the presence of calcium. Furthermore this study adds a step in the efforts for the waste management in order to clean the environment and its reuse in the formation of valuable products.

Experimental

All the experiments were run in triplicate and all the data values are given as mean ± S.E.

Microbial culture and inoculum

Pure culture of a filamentous fungus *Arachniotus sp.* was produced from plant pathology department, University of Agriculture, Faisalabad. The fungal culture was raised on potato dextrose agar (PDA) slants. For the preparation of inoculum, the fungus was grown in a liquid medium containing (g/100ml): glucose, 2.0; Urea, 0.3; $MgSO_4.\ 7H_2O$, 0.05; KCl, 0.015; KH_2PO_4, 0.008 and $ZnSO_4.7\ H_2O$, 0.001. The pH of the medium was adjusted at 4 and the temperature was 30°C [6]. After inoculation, incubation was done at 37°C (150 rev/min) for three days to get a homogenous conidial suspension.

Substrate and growth conditions

One of the objectives of this study was to utilize the waste material for the production of glucoamylase. For this purpose dried waste bread was crushed into small pieces and further dried at 60°C to 0% (w/w) moisture. Prior to use the dried slices were ground in a Wiley Mill to 2 mm particle size. The basal medium used in this study was (g/100ml): $(NH_4)_2SO_4$, 0.4; $MgSO_4.7H_2O$, 0.04; $CaCl_2.2H_2O$, 0.04 and KH_2PO_4, 0.4. Erlenmeyer flasks (500 ml) containing 2g waste bread as substrate in 100ml growth medium (pH 4) were sterilized (121°C). The media was inoculated 5ml containing 10^7-10^8 spores/ ml and the flasks were incubated at 30°C for 72 hours [28].

Optimization of production

In the first step of experiment the enzyme was produced in the pre-optimized culture condition [6]. In the next step of investigation, the waste bread medium was supplemented with varying concentrations of corn steep liquor, yeast extract and cane molasses as additional nutritional source to enhance glucoamylase production under pre-optimized process conditions. The traditional step wise strategy involved the study of effect of varying a parameter in the presence of pre-optimized process conditions.

Enzymatic assay and protein determination

The fermented biomass samples were filtered through Whatman filter paper No. 1. The filtrates were centrifuged (5,000 x g) at -10°C for 15 minutes and the supernatants were subjected to assay. Glucoamylase activity was determined through the rate at which glucose is liberated from the starch and was measured by its ability to reduce 3,5 dinitrosalicylic acid [29]. The assay conditions were 1% (w/v) starch in 16 mM sodium acetate buffer of pH 4.8 at 35°C for 10 minutes and the reaction was stopped by placing tubes in boiling water bath for 10 minutes, and then immediately cooled in ice. One unit of enzyme activity was defined as the quantity of enzyme that released 1µmol of glucose per minute. The specific activity was expressed as unit of activity per mg of protein. The concentration of protein was determined by Lowry's method modified by Hartree [30], using bovine serum albumin as standard.

Enzyme Purification

Ammonium sulfate (65% final concentration) was added to the supernatant. After stirring it for 4 hours at 4 °C, the precipitate was collected by centrifugation at 12,000 xg. The pellet was dissolved in 10ml of 50mM Tris-HCL buffer (pH 8) and was loaded on a Sephadex G-150 column (Sigma). Fractions of 2ml were collected and assayed for enzymatic activity. The fractions having activities were pooled and loaded on to a Q-Sepharose column (Sigma). The column was washed with Tris-HCl buffer. The bound protein was eluted stepwise with 50 mM Tris HCl buffer containing 0.15 M NaCl (two column volumes) followed by 0.25 M NaCl (three column volumes). The fractions having enzyme activity were pooled, dialyzed and concentrated. Protein concentration was estimated after each purification step by the method described above.

SDS Polyacrylamide gel electrophoresis

The 10 µg sample of protein with glucoamylase activity was submitted to 12% SDS-PAGE, according to Laemmli [31], using the Amersham Biosciences molecular mass pattern, consisting of phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (43 KDa), carbonic anhydrase (31 kDa) and trypsin inhibitor (20 kDa) as markers.

Effect of pH

The effect of pH on glucoamylase activity was determined by assaying the enzyme as mentioned before with the difference that the activity was determined at different pH ranging from pH 3 to 8 by using McIlvaine buffers at 35°C.

Effect of temperature

Glucoamylase was incubated at different temperatures ranging from 40-90°C at pH 5.0 for1 hour and assayed as described before.

Effect of urea and calcium

In order to determine the effect of glucoamylase activity in the presence of denaturing agent, the enzyme was incubated in the presence an increasing concentrations of urea (1.25 M to 5 M) for 30 minutes.

The enzyme was also assayed at different temperatures with constant concentrations of calcium to look at the activity and thermostability.

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