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PRODUCTION OF GLUCOAMYLASE BY *RHIZOPUS* SP. IN LIQUID CULTURE

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Abstract

Seventeen *Rhizopus* isolates were used for the production of glucoamylase. An extensive screening program was carried out to select the potential isolates. Among those, *Rhizopus*-RFF showed the 100% relative activities and farther investigations were carried out by the *Rhizopus*-RFF. To optimize the suitable environmental condition for the production of maximum activities of glucoamylase, some of their physicochemical parameters such as temperature, range of pH, incubation period, and nitrogen sources were separately tested. 10% potato starch was used as substrate for fermentation process. The highest percentage of enzyme activity was observed at 45ºC with pH 4.5. Combined use of 0.3% polypeptone and 0.3% yeast extract was yielded 8.33 μ/gm the highest activities of enzyme.

Introduction

Glucoamylase (GA) is a hydrolyzing enzyme. It can degrade both amylose and amylopectin by hydrolyzing both α-1,4 and α-1,6 glucosidic links of starch and produce glucose (Ono *et al*.,1964; Elegado & Fujio, 1993). Hence glucoamylase can convert starch completely to glucose. Now a days, glucoamylase is one of the most important enzymes in food industries (Cook, 1982; Beuchat, 1987; Soccol *et al*., 1992; Soccol, 1992), as it is used for the production of glucose and fructose syrup from liquefied starch (Dale & Langlois, 1940; Nigam & Singh, 1995; Nguyen *et al*., 2002). It is also employed in baking, juice, beverage pharmaceuticals, and many fermented foodstuffs industries for commercial production (Hesseltine, 1965; Raimbault, 1981), in some cases textile, leather and detergents industries (Whister *et al*., 1984; Reed & Rhim, 1987). Due to its increasing demand, the production technique of glucoamylase and α amylase has been studied in detail.

The enzyme was reported to produce by many fungi like *Aspergillus awamori, A. saitoi, A. oryzae, Rhizopus* sp, *Mucor* sp, *Penicillium* sp., and Yeast (Tsujisaka *et al.*, 1958; Sen and Chakarabarty, 1984). Among these, *Rhizopus* spp. are considered good producers of amylolytic enzyme (Takahashi, 1994; Jin *et al*., 1999). On the basis of the importance of glucoamylase, the present study has been taken to optimize the glucoamylase production by *Rhizopus* sp. in liquid culture.

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Materials and Methods

Fungal isolates: 17 *Rhizopus isolates* were collected from the stock culture of Industrial Microbiology Section of IFST, BCSIR, Dhaka. Pure cultures were maintained in PDA media at 4˚C and were sub cultured at 30 days interval. For enzyme production mycelial suspension was used as inoculum.

Screening of glucoamylase: 17 isolates of *Rhizopus sp.* were used for the production of enzyme. The enzyme activity was measured by the method of Stiles *et al*., (1926). Extensive screening was carried out by measuring residual glucose and glucoamylase activity. Among 17 isolates, *Rhizopus*-RFF shows relatively highest glucoamylase activity then other isolates.

Culture condition and glucoamylase production: For the production of glucoamylase liquid culture was performed. To select the optimum growth condition for maximum enzyme production, following cultural parameters was studied stepwise using potato starch powder, Yeast extract, Polypeptone, and $(NH_4)_2SO_4$. Fungus *Rhizopus*-RFF was inoculated in PDA in test-tube and these inoculums were inoculated at 30˚C for 7 days to produce enough mature spore. The fungal spores from 7days old culture were suspended in 10 ml of sterile water. The fungal suspension was then shaken gently to make homogeneous suspension. From this suspension 1 ml (containing about 20 X 10^{10} spores) was used as inoculum and was inoculated in the 20 ml liquid medium in 250 ml of Erlenmeyer flask. The incubated flasks were shaken at 150rmp.

To determine the incubation period the liquid culture media with inoculum were incubated up to 7 days. For determining the effect of temperature on enzyme production, various temperatures ranging from 30 to 55 ºC were used. The pH values of initial cultures were adjusted to 3, 3.5, 4, 4.5, and 5 to find its optimum value. Yeast extract, polypeptone, $(NH_4)_2\ SO_4$ and yeast extract with polypeptone, were used as the nitrogen sources to determine the better nitrogen source.

Crude enzymes were extracted and assayed after 24 hours of inoculation starting from the 2^{nd} day up to the 7the day of incubation. Each of the parameters described above was made separately with three replicates.

Extraction of culture filtrate: Cultivated broth (liquid media with enough mycelia and spores) was centrifuged at 10,000 rpm for 20 minutes at 4˚C. The supernatant was used as the source of extra cellular enzyme. The culture filtrate was dialyzed over night with dialyzing tubes in running water. The reducing sugar of the extra cellular enzyme was determined according to Stiles *et al*. (1926).

Enzyme assay: Glucoamylase was measured as follows: 5ml of 1% soluble starch. 1 ml of 0.2 M acetate buffer at pH 4.5, 1 ml of distilled water and 1 ml enzyme solution were incubated at 40˚C in water bath for 10 minutes and the amount of reducing sugar was estimated by the method of Stiles *et al*. (1926). One unite of Glucoamylase was defined as 1µ mol reducing sugar liberated per minute under assay condition (Alazard & Raimbault, 1981).

Studies of saccharification: 2 ml of 2% starch (soluble) and 2 ml of 50 time's diluted previously prepared enzyme were taken in a test tube and then incubate at different temperature such as 40˚C, 50˚C and 60˚C. Sample were taken out at every hour and immersed immediately in boiling water for 3 minutes to stop the enzyme activity. The accumulation of reducing sugar was measured by the Stiles *et al.,* 1926's method. One blank was performed for each experiment.

Results and Discussion

For selecting the potential glucoamylase producing *Rhizopus* sp. 17 isolates of *Rhizopus s*pp. were used and *Rhizopus*-RFF was identified as the potential fungi for glucoamylase production. Therefore different cultural parameters were studied stepwise by using this potential isolate *Rhizopus*-RFF. Glucoamylase degrades different starch substrate at various ranges (Coutinho & Reilly, 1997). In present study among 17 *Rhizopus* spp. 100% relative glucoamylase activity was found for *Rhizopus* RFF (Table 1). On the basis of the screening program, *Rhizopus*-RFF was selected for optimizing the glucoamylase activates (Jahangeer *et al*., 2005; Ikram-ul-Haq and Ali 2007; Saadia *et al.,* 2008).

Table1: Screening of glucoamylase activity by using17 species of *Rhizopus*

Fungal Isolates	Conc. of glucose (mg/ml)	Glucoamylase activity (µml)	Relative activity (%)
R0	0.067	2.97	34.21
R1	0	0	0
R2	0	0	0
R3	0.142	6.31	72.69
R5	0.105	4.66	53.68
R6	0	0	0
R7	0.105	4.66	53.68
R8	0	0	0
R9	0	0	0
R1	0	0	0
R11	0	0	0
R12	0	0	0
R13	0	0	0
R14	0.067	5.95	68.54
RFF	0.173	8.68	100
RM	0.157	6.97	80.29
Ro	0	0	0

(0) indicates that the glucose concentration has no significant value according to the Stiles table. (The table values started from 0.3 cc thiosulphate of 0.005N but the results from mentioned species were found less then 0.3cc.)

It is essential to know the suitable cultural parameters of the cultural conditions in which maximum enzyme production occurs. From Figure-1, it is clear that the temperature has greatly influenced by the production of glucoamylase by *Rhizopus* sp**.** Maximum 7.37 µml enzyme activity was found at 45ºC (Fig.1). Poorer enzyme activities were observed above or bellow this temperature. Previous reports depict that 35ºC temperature shows maximum enzyme activity by *Rhizopus delemar* (Soccol *et al*., 1994) and *Aspergillus niger* (Feroza *et al.*, 1998).

To study the effect of pH on the production of the enzyme, different buffers were used to provide a pH range from 3 to 6. The optimum pH range of glucoamylase varies from 4.5 to 5.5 with stability at pH 7.0 (Taylor *et al*., 1978). According to James and Lee (1997), the range of glucoamylase pH is between 3.7 and 7.4; *Rhizopus*- RFF showed maximum enzyme activity (8.33 µml) at pH 4.5 (Fig. 1). It can therefore be concluded that the pH has a significant control over the enzyme production.

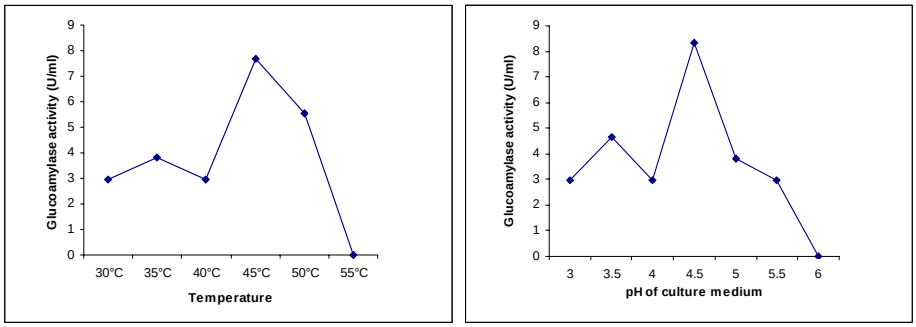

Fig. 1: Effect of temperature and pH on the production of glucoamylase

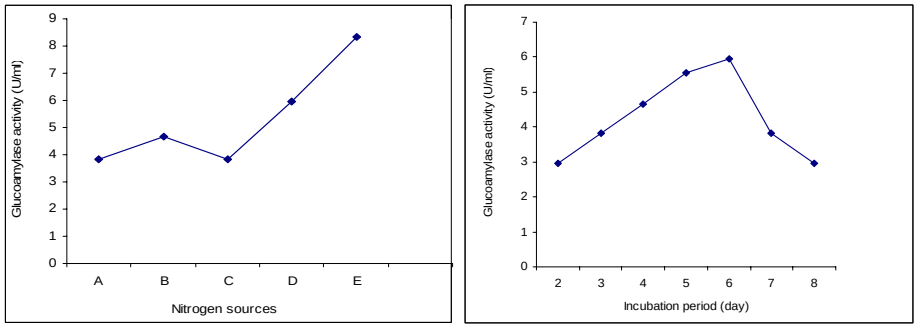

Fig. 2: Effect of nitrogen sources [(A): $(NH_4)_2SO_4$; (B): peptone; (C): yeast extract; (D): urea; (E): peptone & yeast extract] and incubation period on the production of glucoamylase

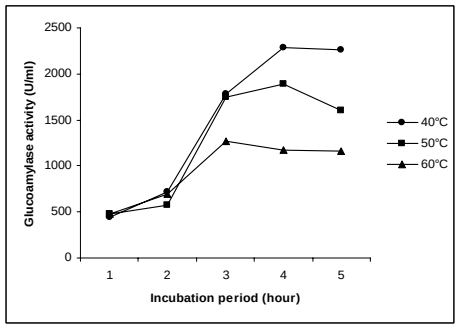

Fig. 3: Saccharification of 2% starch with crude extracted glucoamylase at different temperature

Nitrogen sources have a great effect for microbial growth and in the production of extra cellular enzymes. The results of the different combinations of organic nitrogen source are presented in Fig. 2. The maximum enzyme activity was found by using the mixture of yeast extract and polypepton. Lower enzyme activity was found by using them separately.

To determine the optimum incubation period for the highest enzyme production, *Rhizopus* RFF was incubated at 30ºC and enzyme was extracted at every 24 hours starting from 2nd day of the incubation till 8th day. The highest percentage of enzyme activity was found at 6th day of incubation. It is probably due to the secretion of toxic inhibitory substances and pH changes of the medium or decrease of growth supplement in the medium (Fig. 2). In addition, saccharification of raw potato starch with crude enzyme shows that, four hours is required for the complete hydrolysis of starch at 40ºC (Fig. 3).

Conclusion: The results of this study show that highest glucoamylase activity was found at 45ºC with pH 4.5 in liquid cultures. The optimum fungal growth as well as glucoamylase production was found in the mixture of yeast extract and polypepton as nitrogen source at the 6th day of incubation where other nitrogen sources were not given significant results. Complete hydrolysis of starch with glucoamylase required 4 hours at 40ºC temperature. Present findings indicate that the glucoamylase produced by fungi Rhizopus RFF has a potential activity of hydrolyzing starch.

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