Bangladesh J Microbiol, Volume 25, Number 2, December 2008, pp 99-103

Original Article

Production and Partial Characterization of Extracellular α-Amylase by *Trichoderma viride*

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[Received 20 October 2008; Accepted 07 November 2009]

The production of extracellular α-amylase by the mesophilic fungus *Trichoderma viride* was optimized in shake-flask cultivation. Overall, the data imply that α-amylase from fungus was induced by a variety of starchy substrate but maximum enzyme production was stimulated by corn starch at a concentration of 4.0%. Enzyme synthesis was affected by nitrogen sources, and maximal activity was attained with inorganic than organic nitrogen sources. Effect of different salts in the culture medium was evaluated and it was found that Mg^{2+}, Fe^{2+}, K^{+} and, to some extent, Ca^{2+} and Na^{+} play an important role for optimum production of α-amylase by the fungus. Maximum enzyme production was obtained after 3 days of incubation in a fermentation medium with initial pH 5.0 at 30° under continuous agitation at 180 rpm. These properties make the enzyme suitable for industrial uses.

Key words: *Trichoderma viride*, α-Amylase, Enzyme production optimization, Shake-flask culture

Introduction

Enzymes involved in starch conversion technology are of major importance and considerable interest exists in obtaining new enzymes having improved properties and application[1-3]. An extensive interest has employed people in obtaining new enzymes through continuous research[2-4]. New developments have taken place in the area of starch-degrading enzymes. Enzyme allows the production of sugar syrups with well-defined physical and chemical properties yet again the milder enzymatic hydrolysis results in few side reactions and less browning[1]. The enzymatic hydrolysis of starch is performed by different enzymes grouped in the á-amylase family[5]. Because their rapid break down of the macromolecular structure, fermentable sugars (*e.g.*, glucose, maltose and maltobiose) appear gradually.

The vast amount of starch and other carbohydrates made by photosynthesis become the ultimate energy and carbon sources for non-photosynthetic cells of the animal, plant and microbial worlds[6]. Amylases have got potential industrial applications in the field of fermentation, baking, fruit juice, pharmaceutical, textile and flavoring[7]. Most agricultural biomass (corn/maize, wheat, oats, rice, potato, cassava etc.) containing starch can be used as a potential substrate for the production of gaseous or liquid fuels, vinegar, fructose, feed proteins and chemicals by microbial processes.

α-Amylases are found in various kinds of microorganisms, including bacteria and fungi as well as in higher plants, insects and mammalian tissues[8]. However, industrial α-amylases are produced through fermentation using bacteria and fungi[4]. Because of the commercial and industrial uses, α-amylases from many bacterial and fungal sources have been studied in great detail. Fungi like *Trichoderma harzianum, Aspergillus flavus, Aspergillus oryzae* and *Thermomyces lanuginosus* have been implicated in the production of á-amylase by several investigators[9-12]. This paper reports on á-amylase production by a previously identified *Trichoderma viride* under various fermentation conditions and partial characterization of the enzyme.

Materials and Methods

Microorganisms

Trichoderma viride was obtained from Bangladesh Jute Research Institute, Dhaka, which was previously isolated by Dr. Isidore Gomes[13] and identified by Centraalbureau voor Schimmelcultures (CBS), Baarn, The Netherlands. Another *Trichoderma* isolate was recently isolated from soil sample. Both fungal isolates grew well on common mycological media. In the laboratory the fungi were maintained on potato dextrose agar (PDA) at 30ºC and stock cultures were transferred to fresh medium every 14 days.

Plate assay method

The fungal isolates were tested for amylase activity by employing zone clearing technique[14-15] using starch agar medium. The inoculated plates were incubated at 37°C for 3 days. After incubation, the zone of hydrolysis of starch was detected by flooding the plates with iodine solution. The development of blue colour indicated the presence of starch, while the areas around the hydrolytic organisms appeared clear.

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Enzyme production in shake-flask cultures

The fungi were cultivated in 250-ml Erlenmeyer flasks containing 50 ml of medium with an initial pH 6.0 unless otherwise stated. The shake-flask cultures were carried out at 200 rpm in an orbital shaker incubator (Gallenkamp, Germany) at 30°C for at least 72 h. The culture medium contained 2.0% soluble starch as carbon source, 1.0% NH_4NO_3, 0.14% K_2HPO_4, 0.05% KCl, 0.01% $MgSO_4$ and 0.001% $FeSO_4.7H_2O$. The medium was inoculated with a piece (1 cm^2) of freshly prepared 3-days-old fungal culture on PDA. After incubation, the mycelia were removed by centrifugation and the clear supernatant was used to determine the enzymatic activity. The production of α-amylase was determined at various pH values of the growth medium, incubation temperatures, rotation speeds and incubation periods.

Enzyme assay and analytical methods

α-Amylase was determined by using 1.0% (w/v) soluble starch (Sigma, USA) as substrate in 0.05 *M* phosphate buffer (pH 6.5) essentially according to Gomes *et al.*[16]. The reaction mixture containing 1.8 ml substrate solution and 0.2 ml suitably diluted enzyme solution was incubated at 50°C for 10 min. The reaction was stopped by adding 3 ml dinitrosalicylic acid (DNS) reagent. The reducing sugar released was determined by the method of Miller[17]. One unit (U) of enzyme activity was defined in all cases as the amount of enzyme that liberates 1 μmol of glucose or glucose equivalents from the substrate per min under the assay conditions.

Effect of temperature and pH on enzyme activity

Effect of pH on α-amylase activity was determined by incubating the reaction mixture at pH values ranging from 4.0 to 10.0 using citrate buffer (0.05 *M*, pH 4.0-6.0), phosphate buffer (0.05 *M*, pH 6.5-8.5) and tris-HCl buffer (0.2 *M*, pH 9.0-10.0). Optimum temperature for enzyme activity was determined by conducting the assay at different temperatures ranging from 30 to 80ºC.

Results and Discussion

A newly isolated *Trichoderma* sp. and the laboratory strain of *T. viride* were able to hydrolyze starch showing distinct zone of hydrolysis around the colonies on agar medium supplemented with soluble starch (Table 1). The average zone of hydrolysis and the zone diameter : colony diameter ratio was higher in case of the laboratory strain as compared to the soil isolate. Therefore, further studies on enzyme production in shake-flask cultures were carried out using only *T. viride* isolate.

Table 1. *Demonstration of starch hydrolysis ability by the wild Trichoderma species isolate and Trichoderma viride*

Organism	Incubation time (h)	Diameter of zone on hydrolysis (cm)	Diameter of colony (cm)	Ratio
Trichoderma viride	24	2.7	1.2	2.25
	48	4.2	1.4	3.00
	72	5.5	2.1	2.61
Trichoderma sp.	24	2.3	1.1	2.09
(Soil isolate)	48	3.5	1.6	2.18
	72	4.8	2.3	2.08

The nature and amount of carbon source in culture media is important for the growth and production of extracellular α-amylase in microrganisms. Carbon source greatly influence α-amylase production and the most commonly used substrate is starch[7]. In this study, the effect of different carbon sources like potato, soluble starch, wheat and corn starch on α-amylase production was studied (Figure 1). Among the carbon sources test, *T. viride* produced maximum amount of the enzyme (0.20 U/ml) on corn starch. Though the soluble starch is water-soluble dextrin produced by the partial acid hydrolysis of starch it was not a better inducer of α-amylase as compared to corn starch. It is emphatically known that higher yields of amylase can be obtained in media with complex raw materials containing starch from maize, barley, wheat and malt[18].

It was reported earlier that substrate concentration beyond 1% in fermentation medium did not increase the enzyme production[19] but our strain showed that the increase in concentration of substrate at least up to 4% in medium can also increases enzyme production (Figure 1). However, further increase (5%) of corn starch concentration resulted in decreased enzyme production. Qader *et al.*[20] reported that increase in starch concentration up to 2% in medium can encourage a-amylase by a *Bacillus* sp. while 3% starch in the medium decreased the enzyme production.

The influence of organic and inorganic nitrogen sources on α-amylase production was determined (Figure 1). It has been reported that more α-amylase was produced when organic nitrogen compounds were used. Maximum enzyme production was found with peptone as the nitrogen source[7,21]. It has also been reported that the optimum production of á-amylase for *Bacillus* sp. was found when yeast extract was used[19]. In contrast, our results suggested that inorganic as well as organic nitrogen sources can support good growth and extracellular α-amylase production by the fungus. Growth and the enzyme production for *T. viride* were excellent when inorganic nitrogen sources like NH_4NO_3 and $NaNO_3$ and organic nitrogen sources like beef extract were yeast extract were used in the fermentation medium. However, highest amount of the enzyme was obtained the medium containing 1.0% NH_4NO_3 as the sole source of nitrogen. This result is in agreement with that reported by Nahas and Waldemarin[22], who found that amylase synthesis by *Aspergillus ochraceus* is affected by nitrogen sources, and maximal activity is shown attained with inorganic than organic nitrogen sources. The medium containing both inorganic and organic (1:1 ratio) did not improve the enzyme productivity. This finding is not in agreement with other workers[7,20] who found that inorganic sources inhibit α-amylase synthesis.

The effect of minerals and trace elements on α-amylase production by *T. viride* was studied. The basal medium used contained sources of K^+, Mg^{2+} and Fe^{2+} in the form of 0.14% K_2HPO_4, 0.05% KCl, 0.01% $MgSO_4$ and 0.001% $FeSO_4.7H_2O$. In order to widen the sources of minerals various mono- and divalent salts were incorporated in the fermentation medium (Figure 2). Enzyme

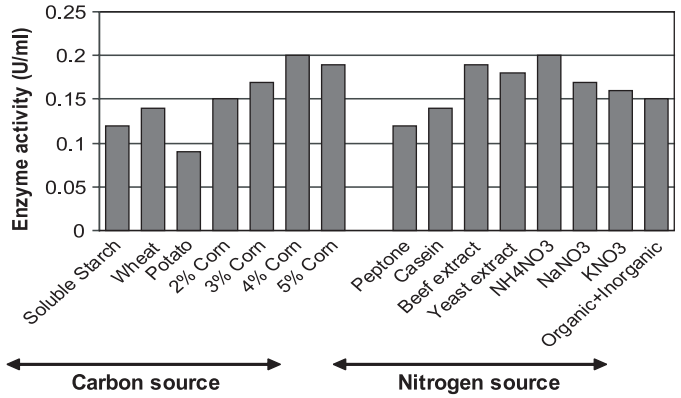

Figure 1. *Effect of supplementation of the culture medium with different carbon (2.0%, unless mentioned) and nitrogen (1.0%) sources on extracellular α-amylase production by Trichoderma viride in shake-flask culture.*

production by the fungus was considerably influenced by the some minerals or trace elements including Mg^{2+} ion in the form of $MgCO_3$ and $MgSO_4$, Fe^{2+} ion in the form of $FeSO_4$ and $Fe(NH_4)_2SO_4.6H_2O$ and K^+ ion in the form of KCl and K_2CO_3. The enzyme production was moderately enhanced in the presence of Ca^{2+} (as $CaSO_4$) and Na^+ (Na_2SO_4) ions. It has been reported that amylase production is enhanced by incorporation of some divalent cations like Ca^{2+}, Sr^{2+}, Mg^{2+} and Ba^{2+} in the culture medium[23].

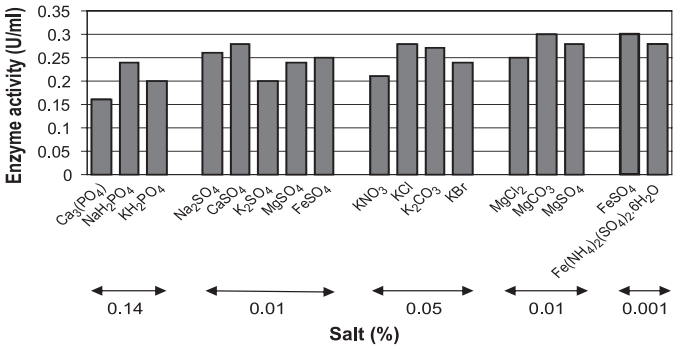

Figure 2. *Effect of supplementation of the culture medium with various salts on the production of α-amylase by Trichoderma viride in shake-flask culture.*

Qader *et al.*[20] reported that the production of α-amylase by *Bacillus* sp. is Ca^{2+} dependent. This may be due to the fact that calcium ion was the best binder, stabilizer and activator of α-amylase[20]. Allan *et al.*[24] reported that in case of *Bacillus licheniformis* supplementation of calcium salt in the medium increased the α-amylase production. Their finding is in accordance with the work reported by Suisheng *et al.*[25] in case of *Bacillus subtilis*. These results may also be due to the increasing availability of the Ca^{2+}, since the enzyme is known to be a calcium metalloenzyme. These results are similar to the findings of Hewitt and Solomons[26] who worked with the culture of *Bacillus amyloliquefaciens*. It has also been reported that stability of α-amylase is calcium dependent[27]. In contrast, inhibition of amylase production and activity by the excess of some mineral elements has been previously reported[28-29]. Abu *et al.*[29] reported that mineral supplementation significantly inhibited amylase production in both monoculture and mixed culture media when complex substrate like sorghum pomace is used as the main substrate.

Figure 3 shows the effect of various factors on extracellular a-amylase production in shake-flask culture by *T. viride*. Production of the enzyme was very dependant on environmental factors. It was found that increase in volume of culture medium in the flask caused reduction of the enzyme production. The incubation period varies with enzyme productions[30]. The 72-h culture of *T. viride* exhibited maximum a-amylase activity of 0.30 U/ml, while further incubation caused decreased in the enzyme production. Short incubation period offers potential for inexpensive production of enzymes[31].

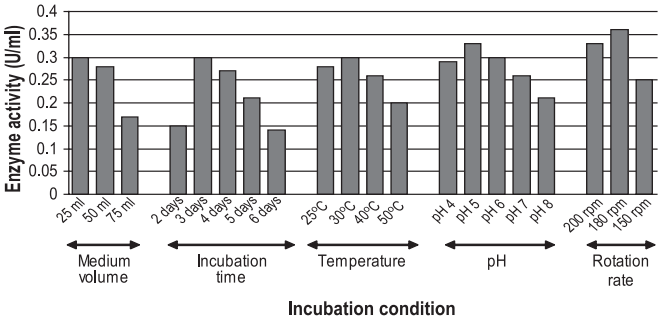

Figure 3. *Effect of various environmental conditions on the production of α-amylase by Trichoderma viride in shake-flask culture*

Temperature optimum for α-amylase production was found to be in a range between 25 and 37°C for the mesophilic fungi[32-34] and the present study recorded 30°C as optimal, which agrees with earlier findings (Figure 3). This could be due to the mesophilic nature of the species. The influence of temperature on α-amylase production is related to the growth of microbes[35]. As per earlier report of Aiba *et al.*[36] high temperature may inactivate the expression of gene responsible for the starch degrading enzyme.

Among physical parameters, pH of the growth medium plays an important role by inducing morphological changes in microbes and in enzyme secretion[35]. The pH change observed during the growth of microbes also affects product stability in the medium[34]. Most of the earlier studies revealed the optimum pH range between 6.0 and 7.0 for the growth of bacterial strains and enzyme production[32,34,37]. This is also true of strain of *T. viride* used in the present study. The enzyme synthesis and the fungal growth were observed between pH 4.0 to 8.0 (Figure 3). The results suggest that there is a stimulation of enzyme synthesis at acidic pH of 5.0 and the higher enzyme production at this pH was concluded as the result of increased mycelial growth and the fungi required acidic pH for the production of α-amylase. Increasing the initial pH of the medium up to pH 7.0-8.0 resulted in a decrease of the amylase production. These results are in

agreement to that reported by Alva *et al.* for a mesophilic *Aspergillus* sp.[38].

Agitation rates have been shown to affect enzyme production in various microorganisms[39-41]. In the present investigation, *T. viride* was grown in shake-flask cultures at different agitation speeds and the maximum α-amylase activity was found at 180 rpm. At this speed, aeration of the culture medium was increased, which could lead to sufficient supply of dissolved oxygen in the media[42]. Nutrient uptake by the fungus also will be increased[43] resulting in increased enzyme production. At 200 rpm, α-amylase activity was found to be reduced. This was perhaps due to denaturation of enzymes caused by high agitation speed[44-45]. Moreover, at agitation speed excessive aeration and agitation could occur which led to cell lysis and increased cell permeability due to abrasion by shear forces[46]. At 150 rpm, insufficient aeration and nutrient uptake perhaps caused the inability of fungus to grow efficiently.

Temperature and pH are the most important factors, which markedly influence enzyme activity. The effect of temperature on the activity of crude enzyme preparation from *T. viride* was determined over a temperature range between 30 and 80ºC for 10 min. The maximum activity (0.51 U/ml) was displayed at 50°C (Figure 4). Further increase in temperature resulted in decrease in the activity of α-amylase. The optimum activity temperature of the fungal amylase is comparable to that of some known fungus α-amylases such as *Aspergillus niger*, *Aspergillus oryzae* and *Mucor pusillus*[47]. The effect of pH on amylase activity was characterised by stability from pH 4.0 to pH 10.0 after 10 min of incubation at 50° C. The optimum pH activity was 6.0 (Figure 5). This result agreed with the optimal pH for certain fungal α-amylase activities *Aspergillus flavus* and *Mucor pusillus*[48]. The culture filtrates of the fungal isolate exhibited highest α-amylase activity when the enzyme-substrate reaction mixtures were incubated for 15 min. As time of incubation increased there was a gradual decrease in the enzyme activity.

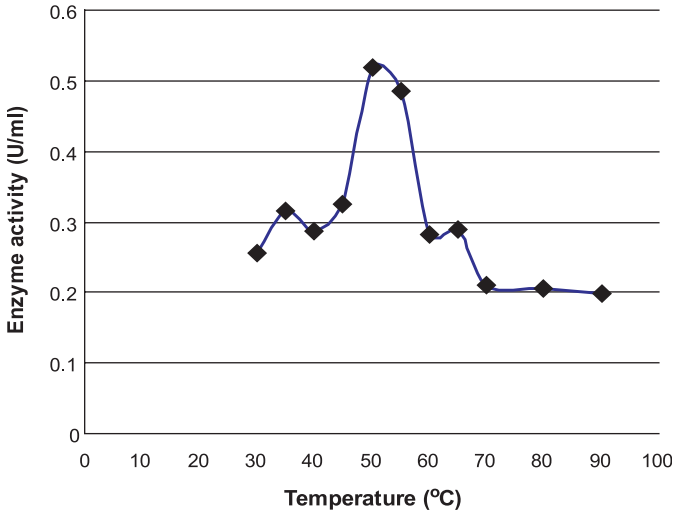

Figure 4. *Effects of growth temperature on α-amylase production by Trichoderma viride.*

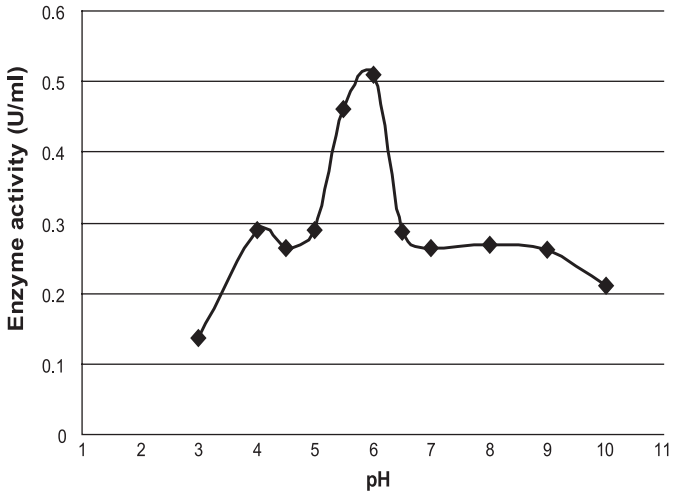

Figure 5. *Effects of media pH on α-amylase production by Trichoderma viride.*

The nature of culture conditions and composition of media for optimal production of α-amylase by *T. viride* has been developed in this study. Enzyme synthesis was affected by carbon, nitrogen and mineral sources, and maximal activity was shown attained with inorganic than organic nitrogen sources. Fungal growth and amylase production as well as activity were found only within a narrow temperature and pH range. The optimum growth of the presently reported fungal isolate at 30°C, whereas maximum enzyme activity was observed at 50°C and making the *T. viride* isolate more suitable for future use in various industries.

It can be concluded that *T. viride* isolate can be industrially exploited for the synthesis of α-amylase, and strain improvement studies can be carried out to enhance enzyme production.

Acknowledgement

The authors are thankful to the authorities of Biotechnology Research Centre, University of Dhaka, Dhaka for financial support.

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