International Food Research Journal 19 (3): 1261-1266 (2012)

Culture condition forthe production of glucoamylase enzyme by different isolates of *Aspergillus* spp.

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Abstract: Optimization of the growth conditions for maximum production of the glucoamylase enzyme by different isolates of *Aspergillus* spp. isolated from various sources were carried out in liquid culture. Screening of amylase producers was carried out on PDA agar plates supplemented with 2% soluble starch and were detected by the index of amylolytic activity. High thermostable amyloglucosidase yielding isolates designated as, *Aspergillus awamori* (A1and A7) *A. niger* (A10), *A. terreus* (At) and *A. tamarii* (Aw) used in this study. Maximum enzyme production was recorded at pH 6.0 and 2.0% rice bran as a carbon source for all isolates except *Aspergillus terreus* (At) which gave the highest enzyme yield at pH 4.0. The enhancement of the nitrogen supplements appear differently among the different isolates, for *Aspergillus tamarii* (Aw) highest production was obtained in medium supplemented with peptone, whereas *A. niger* (A10) with sodium nitrate, A. awamori (A1and A7) accomplished this with ammonium chloride and *A. terreus* (At) recorded the highest production with ammonium sulphate. These isolates are good producers of extracellular thermostable glucoamylases which could be suitable for application in starch processing and food industries.

Key words: ***Aspergillus isolates,*** **glucoamylases, thermostable enzymes, optimized conditions, Sudanese soil**

Introduction

Glucoamylase (GA) is a hydrolyzing enzyme, consecutively hydrolyzes a-1,4 glycosidic bonds from the non-reducing ends of starch, resulting in the production of glucose. To a lesser extent, it also has the ability to hydrolyze a-1,6 linkages, also resulting in glucose as the end-product (Mertens and Skory, 2006). The enzyme is produced by a variety of microorganisms, and *Aspergillus niger, Aspergillus awamori* and *Rhizopus oryzae* have been considered the most important for industrial application (Coutinho and Reilly, 1997). Hence glucoamylase can convert starch completely to glucose. Nowadays, glucoamylase is one of the most important enzymes in food industries (Soccol, 1992), as it is used for the production of glucose and fructose syrup from liquefied starch (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 (Whistler *et al*., 1984; Reed and Rhem, 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 (Sen and Chakarabarty, 1984). Among these, *Rhizopus* spp. are considered good producers of amylolytic enzyme (Takahashi *et al.*, 1994; Jin *et al.,* 1999). On the basis of the importance of glucoamylase, the present study has been taken to optimize the growth conditions for maximal production of the glucoamylase enzyme by *Aspergillus* spp. in liquid culture.

Materials and Methods

Fungal isolates

Fungi were isolated from different Sudanese soils, cereal grains, rotten fruits and food materials and were also obtained from previous collections. All general chemicals were of Analar grade or equivalent. The Aspergillus cultures were isolated from the soil by the serial dilution method of Clark *et al.* (1988). One gram soil sample was dissolved in 100 ml sterilized distilled water. The soil suspension was diluted up to 10^{-3} and 0.5 ml of diluted suspension was applied into sterilized Petri-dishes and 20 ml of warm melted PDA. For isolation of fungi from other sources, cereal grains and pieces of rotten fruits were surface disinfected with 4% Sodium hypochlorite for 2 min (King *et al*., 1986). The surface disinfected grains and pieces of rotten fruits and also the untreated bread

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pieces were plated on sterilized glass Petri dishes each with 3 layers of moistened filter papers. Then they were incubated at 25ºC for seven days.

Amylase activity of *Aspergilllus* isolates producing the starch digesting amylase was detected on plates by the index of amylolytic activity according to the method described by Hankin and Anagnostakis (1975). A. niger colonies producing large clear zone were picked up and purified by streaking on PDA. Pure cultures were maintained in PDA media at 4°C and were subcultured at 30 days interval. Identification of *Aspergillus* isolates was based on cell and colony morphology characteristics as per the method described by Raper and Fennel (1965).

Screening of amylase production

Aspergillus spp. isolates were selected for the production of the enzyme on the basis of producing larger clear zone. The growth medium contained (%w/v) 0.5 g of rice flour, 100 ml of distilled water in 500 ml Erlenmeyer flasks and then was sterilized by autoclaving at 121ºC and 15 lb/square inch for 15 min. A loop full of spores of the chosen Aspergillus isolates (from a seven-day-old culture grown in PDA slant) was inoculated separately in 250 ml flasks containing 45 ml of the growth medium. These cultures propagated in a rotary incubator shaker (100 rev. / min.) for 24 h at different temperatures 30ºC / 35ºC / 40ºC / 45ºC /50.

Samples were taken from cultures for each temperature at different time intervals 24, 48, 72 and 96 hours. The samples were then centrifuged at 5000 rpm to remove mycelia. The supernatant fluids were collected and used for assaying of amylase activity. Based on the result of this experiment, six strains were selected for subsequent studies.

Enzyme assay

The enzyme assayed by the method of Bernfeld (1951) as described by Pestana and Castillo (1985) using starch as substrate. One unit of enzyme activities was expressed as one mg of glucose liberated per one ml. of culture supernatant at pH 7.5, 40ºC and in one minute. Extensive screening was carried out by measuring residual glucose and glucoamylase activity. The protein content was determined by the method of Bradford (1976) with bovine serum albumin as the protein standard. The specific activity of amylolytic enzyme was taken as units/mg protein. Among 14 isolates, 5 isolate show relatively highest enzyme activity and were selected for subsequent studies.

Culture condition and glucoamylase production

Growth and incubation period was quantified along with the effect of carbon, nitrogen source, phosphorus salts on production of the enzyme. To determine the incubation period the liquid culture media with inoculum were incubated up to 4 days. For determining the effect of temperature on enzyme production, various temperatures ranging from 30 to 50ºC were used. The pH values of initial cultures were adjusted to 4, 5, 6, 7 and 8 to find its optimum value. Ammonium sulphate $(NH_4)_2SO_4$ / ammonium chloride NH_4Cl / sodium nitrate $NaNO_3$ / peptone / beef extract/ yeast extract / urea, were used as the nitrogen sources to determine the better nitrogen source. Diammonium hydrogen phosphate $(NH_4)_2HPO_4$ / sodium dihydrogen phosphate NaH2PO4 /disodium hydrogen phosphate Na_2HPO_4 /potassium dihydrogen phosphate KH_2PO_4 /dipotassium hydrogen phosphate K_2HPO_4 were used as phosphorus salts to determine the effect of different phosphorus salts. Crude enzymes were extracted and assayed after 24 hours of inoculation starting from the 2nd day up to the 4th day of incubation. Each of the parameters described above was made separately with three replicates.

Thin layer chromatography (TLC)

The products of starch hydrolysis by the crude enzyme, as described in the assay method, were identified by thin layer chromatography(TLC) using n-butanol: ethanol: water in a ratio of 4: 2.2: 2 as a solvent and maltose and glucose as standard sugars. Sugars and oligosaccharides were detected as described by Trevelyan *et al*. (1950). The sugar spots appeared as dark brown spots. Identification of the sugars was done by comparing the relative fraction (Rf) values of the samples with that of the standards.

Results and Discussion

Species isolated from soil sample are screened for amylase in starch agar plate. Based on the index of amylolytic activity (Hankin and Anagnostakis, 1975) 14 *Aspergillus* isolates were chosen for further studies.

Effect of temperature and time intervals on enzyme production

Assay of enzyme production was carried out at various temperature ranging from 25 to 50°C. Samples for the determination of the amylase activity were taken for each temperature at different time intervals of 24, 48, 72 and 96 h. It was found that A. awamori (A1 and A7), A. niger (A10), A. tamari (Aw), and A. terreus (At) which showed maximum amylolytic enzyme production at 40 and 45ºC. Accordingly,

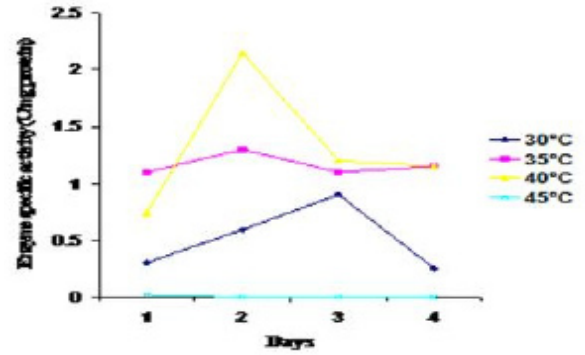

Figure 1. Effect of temperature and time intervals on amylase production by A1

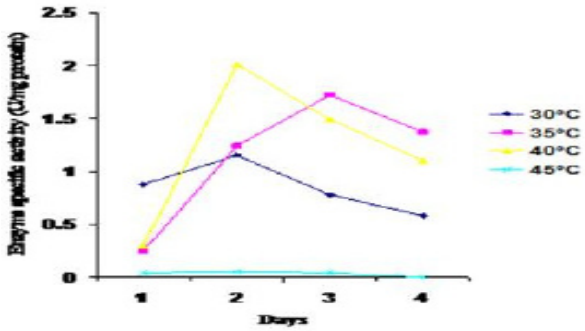

Figure 2. Effect of temperature and time intervals on amylase production by A7

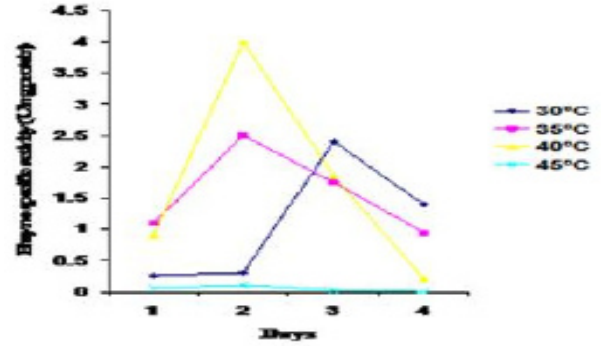

Figure 3. Effect of temperature on time interval on amylase production by A10

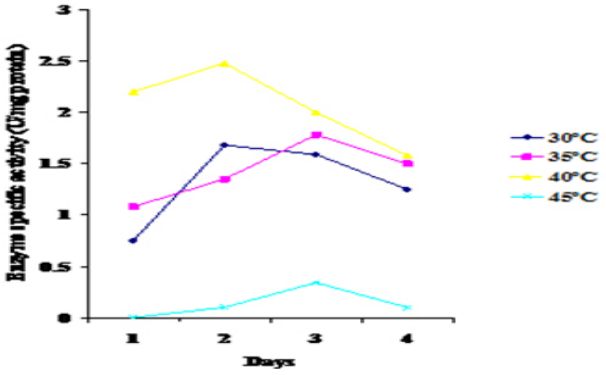

Figure 4. Effect of temperature on time interval on amylase production by AW

these isolates were then chosen for further studies. *A. awamori* (A1 and A7), *A. niger* (A10) and *A. tamarii* (Aw) have shown maximum enzyme production at 40ºC after 48 h with specific activities 2.15, 2.01, 4.06 and 2.5 U/ mg. protein in order (Figures 1, 2, 3 and 4). *Aspergillus terreus* (At) showed maximum performance of enzyme production at 45ºC after 72 h with specific activities of 2.24 U/ mg protein (Figure 5). In fact this result for *A. awamori* (A1 and A7), *A. niger* (A10), *A. tamari* (Aw), and *A. terreus* (At) which showed maximum amylolytic enzyme production at 40 and 45ºC could be of importance industrially as it is always cheaper not to cool the production.

Similar results for production of glucoamylase by *Rhizopus* sp. at 45ºC was reported by Nahar *et al.* (2008). Previous reports depict that 35ºC temperature shows maximum enzyme activity by *Rhizopus delemar* (Soccol *et al*., 1994) and *Aspergillus niger* (Feroza *et al.*, 1998). Increase in the incubation period resulted in a decrease in the production of amylase by culture of *Aspergillus* spp. It may be due to the fact that after maximum production of amylase enzyme (maximum incubation time), the production of other by products result in the depletion of nutrients. These byproducts inhibited the growth of fungi and hence enzyme formation (Feroza *et al*., 1998).

Effect of pH on amylase production

Optimum pH for maximum enzyme production was found to be 6.0 for all the isolates except for *A. terreus* (At) which gave maximum production at pH 4.0 (Figure 6). According to James and Lee (1997) the range of glucoamylase pH is between 3.7 and 7.4 (20) and reported that Rhizopus- RFF showed maximum enzyme activity at pH 4.5. Maximum enzyme production of enzyme occurred at pH 4 to 6, very little growth was observed without enzyme production in medium at initial pH 3 to 4 (Hankin and Anagnostakis, 1975).

Effect of substrate concentration on amylase production

It is clear that 2% rice flour concentration seems to be the concentration that gave maximum production of amylase (Figure 7). Above 2%, there is little increase in enzyme production. These results are mostly obvious in the case of *Aspergillus niger* (A10) and *A. awamori* (A1) with specific activities 4.9 and 4.2 U/ mg protein, respectively. The other four species gave lower increase in production with specific activities 2.7, 2.7, and 2.4 U/mg protein for *A. awamori* (A7), *A. tamarii* (Aw), and *A. terreus* (At), respectively. This indicates that the enzyme is inducible. Ali *et al.* (1989) working on *A. terreus*, Raimbault (1981)

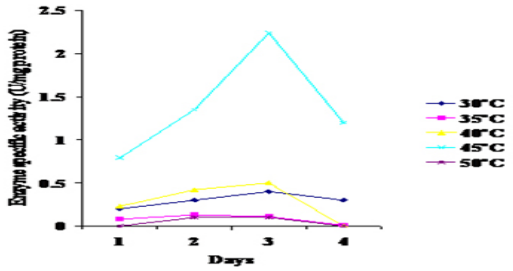

Figure 5. Effect of temperature on time interval on amylase production by At

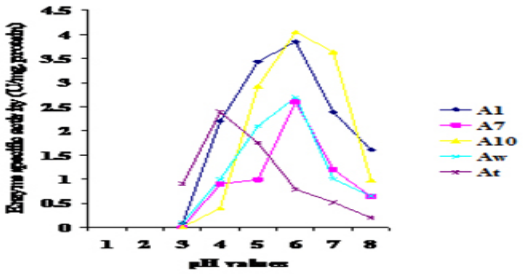

Figure 6. Effect of pH on amylase production by different isolates

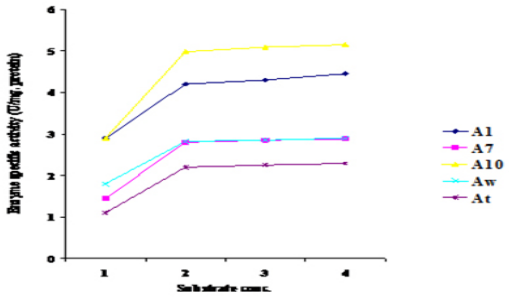

Figure 7. Effect of substrate concentration on amylase production by different isolates

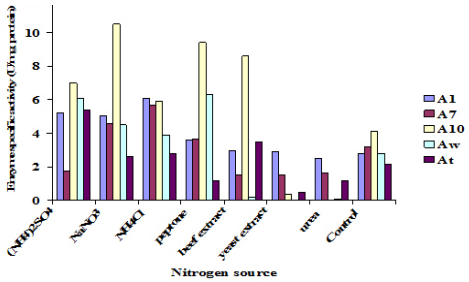

Figure 8. Effect of substrate concentration on amylase production by different isolates

working on *A. awamori* and Mohamed *et al.* (2007) working on *A. foetidus* reached the same conclusion.

Effect of different inorganic and organic nitrogen sources on amylase production

Figure 8 showed that urea and beef extract have negative effect on the production of amylase in all isolates. Also, yeast extract affect negatively enzyme production by *Aspergillus tamarii* (Aw), *A. awamori* (A1) and *A. awamori* (A7) while with *A. niger* (A10) and *A. terreus* (At) it stimulate the production . On the other hand, salts which gave positive significant effect include peptone in the case of *Aspergillus tamarii* (Aw) and *A. niger* (A10), while ammonium sulfate also showed significantly positive effect on *A. tamarii* (Aw), *A. niger* (A10), *A. awamori* (A1) and specially *A. terreus* (At). It has an inhibitory effect on amylase production by *Aspergillus awamori* (A7). Sodium nitrate and ammonium chloride have shown varying positive effects on production of amylase on all *Aspergillus* species. Of highly significant effect are those of sodium nitrate on *A. tamarii* (Aw), *A. niger* (A10) and *A. awamori* (A7) and to a lesser extend *A. awamori* (A1) and *A. terreus* (At). Also of highly significant effects of ammonium chloride are those on *A. awamori* (A1 and A7). Positive effects, also of ammonium chloride are those on *A. tamarii* (Aw), *A. niger* (A10) and *A. terreus* (At).

Nahar *et al.* (2008) reported that among the nitrogen sources used to increase production of glucoamylase enzyme by candida famata, yeast extract was the best organic one followed by meat extract, peptone and tryptone, on the other hand, urea was the best inorganic nitrogen source followed by $(NH_4)_2PO_4$. Goto *et al*. (1998) reported that the production of glucoamylase by *Aspergillus fumigatus* has increased by addition of ammonium nitrate to the basal medium. Ali *et al.* (1989) reported that addition of ammonium sulfate, peptone, yeast extract or beef extract to the culture medium stimulated glucoamylase production by *A. terreus*. Cherry *et al*. (2004) who reported that the fungus *A. fumigatus* produces high amylase activity with yeast extract. Goto *et al.* (1998) reported an increase of glucoamylase production by *A. niger* when the basal culture medium was supplemented with ammonium sulphate. Meat extract was reported by Nishise *et al.* (1988) to increase glucoamylase production by *Rhizopus* sp., while peptone enhanced its production by *A. niger* (Raimbault, 1981). Mohamed *et al.* (2007) reported that ammonium sulphate and ammonium chloride reduced the final yield of amylase enzyme produced by *A. foetidus*, while sodium nitrate stimulated the enzyme production. Chiquetto *et al.* (1992) reported the increase of glucoamylase production by A. awamori NRRL by addition of urea to the culture medium, while other authors reported that very low levels of glucoamylase production was obtained when the nitrogen source was urea (Barton *et al*., 1969; Reed and Rhem, 1987)

Effect of different phosphorus salts on amylase production

As shown in Figure 9 it is clear that disodium hydrogen phosphate has clear significant and

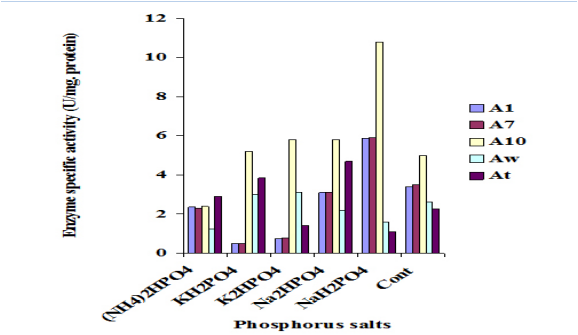

Figure 9. Effect of phosphorus salts on amylase production by different isolates

enhancing effect on glucoamylase production by *Aspergillus niger* (A10), *A. awamori* (A1) and *A. awamori* (A7). All other salts have no effect on glucoamylase production by *A. awamori* (A1) and *A. awamori* (A7). *A. niger* (A10) was positively affected by both di and mono sodium and potassium phosphorus salts in this order. *A. tamarii* (Aw) was also affected positively and significantly by only dipotassium salt. The species *A. terreus* (At) responded to the addition of monosodium, monopotassium, and diammonium phosphate salts. All three salts increase the production to varying degrees. Mohamed *et al.* (2007) working on *Aspergillus foetidus*, Goto *et al*. (1998) working on *A. niger* and Ali *et al.* (1989) working on *A. terreus* reported the repression of glucoamylase synthesis by disodium and potassium phosphate salts while monosodium and potassium phosphate increased its production by these fungi. However, Ramachandran *et al.* (1979) reported that phosphorus salts did not affect glucoamylase production by *A. awamori.*

Thin layer chromatography (TLC)

Products of starch hydrolysis by the enzymes from the five isolates and as identified by thin layer chromatography is seen to be only glucose by comparison with the authentic markers. Accordingly, it is concluded that all the five amylases are glucoamylases. These results are similar to those published by Ali *et al*. (1989), Ramachandran *et al.* (1979) and Dharmsthiti *et al*. (1986) using *A. terreus, A. awamori* and *A. flavus var. columnaris,* respectively.

Conclusion

The results of this study show that highest glucoamylase activity was found in shake flasks provided by a medium containing 2% rice flour concentration, at temperature 40ºC for *Aspergillus awamori* (A1 and A7), *A. niger* (A10), and *A. tamarii* (Aw) and 45ºC for *A. terreus* (At), and initial pH value of 4.0 for *A. terreus* (At) and of 6.0 for all the remaining isolates. Rice flour suspension can be utilized for growth of microorganisms which were used in this work and production of enzyme, although supplementation with nitrogen sources and phosphorus salts is needed in order to increase enzyme level.

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