Polish Journal of Microbiology
2009, Vol. 58, No 3, 269–273

ORIGINAL PAPER

Isolation and Identification of a New Fungal Strain for Amylase Biosynthesis

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Received 7 March 2009, revised 2 July 2009, accepted 7 July 2009

Abstract

Fungi are well known for their ability to excrete enzymes into the environment. The fungal isolate FSS60 was the best amylase producer among one hundred and thirty-six isolates obtained from Syrian soils and tested for amylase production. According to the sequence of the internal transcribed spacer (ITS) rDNA gene, the isolate was identified as *Aspergillus flavus*. Optimal initial pH for amylase production was found to be 9.0. The enzyme was optimally active at 50°C and pH 5.0.

Key words: *Aspergillus flavus*; amylase; ITS; submerged culture

Introduction

α-amylase (EC3.2.1.1, 1,4-α-D-glucan-glucanohydrolase) is an extracellular enzyme, which is involved in the starch processing industries where it breaks starch down into simple sugar constituents (Akpan *et al*., 1999; Haq *et al*., 2002). The amylase enzymes have also potential applications in a number of industrial processes including brewing, baking, textiles and detergents (Gupta *et al*., 2003; Pandey *et al*., 2000). Today, the new potential of using microorganisms as biotechnological sources of industrially relevant enzymes has stimulated renewed interest in the exploration of extracellular enzymatic activity in several microorganisms (Akpan *et al*., 1999; Buzzini and Martini, 2002; Gupta *et al*., 2003). These enzymes are found in animals, plants, bacteria and fungi. Sources of amylases in yeast, bacteria and other fungi have been reported and their properties described by (Chi *et al*., 2007, Liu and Xu, 2008, Gupta *et al*., 2008). Due to their diversity, fungi have been recognized as a source of new enzymes with useful and/or novel characteristics. Amylases constitute a class of industrial enzymes accounting for approximately 25% of the enzyme market (Rao *et al*., 1998).

PCR amplification using universal primers targeted to conserved regions within the rRNA complex, followed by DNA sequencing of the internal transcribed spacer (ITS) regions, shows promise to identify a broad range of fungi to the species level (Chen *et al*., 2001; Henry *et al*., 2000; Iwen *et al*., 2002; Pryce *et al*., 2003; Li *et al*., 2007).

Considering the industrial importance of amylase, in this present study, fungi were isolated from soil and screened for amylase production. The investigation led to the identification of a high amylase producing isolate, FSS60.

Experimental

Materials and Methods

Fungi isolation. Soil samples were collected from different areas of Syria, cereal fields, olive fields, forests and gardens. One gram of soil sample was dissolved in 100 ml of sterilized distilled water, and then diluted up to $1/10^4$ times, from which, 0.5 ml volumes were pipetted onto potato dextrose agar (PDA) and incubated at 30°C for three days. Fungi

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were isolated from each plate and subcultured on PDA. Subculturing was continued until a pure isolate was obtained. Stock cultures were maintained on potato dextrose agar at 4°C.

Fungus screening for amylase production. One hundred and thirty-six fungal isolates were screened for amylase production in Erlenmeyer flasks (100 ml) containing 25 ml of basal culture medium (g/l); 10.0 – wheat bran, 5.0 – yeast extract, 10.0 – $Na_2HPO_4 \times 2H_2O$, 0.5 – KCl, 0.15, $MgSO_4 \times 7H_2O$. The pH was adjusted to 6.5 before sterilization. The flasks were sterilized at 120°C for 20 min. Fresh fungal spores have been used as inocula and the flasks were incubated at 30°C for 5 days in a rotary shaker (120 rpm).

Biomass. Mycelial dry weight was determined by filtering the culture medium through pre-weighed Whatman filter paper No 44, dried to a constant weigh at 80°C and reweighed. The difference in weight denoted the mycelial growth of fungus.

Soluble protein. Soluble proteins were analyzed according to the method of Lowry *et al.* (1951) after preliminary precipitation with 50% trichloroacetic acid (TCA). Bovine serum albumin (BSA) was used as a standard.

Amylase assay. α-amylase activity was determined as described by Okolo *et al.* (1995). The reaction mixture consisted of 1.25 ml of 1% soluble starch, 0.25 ml of 0.1 M acetate buffer (pH 5.0), 0.25 ml of distilled water, and 0.25 ml of crude enzyme extract. After 10 min of incubation at 50°C, the liberated reducing sugars (glucose equivalents) were estimated by the dinitrosalicylic acid (DNS) method of Miller (1959). The blank contained 0.5 ml of 0.1 M acetate buffer (pH 5.0), 1.25 ml of 1% starch solution and 0.25 ml of distilled water. One unit (IU) of α-amylase is defined as the amount of enzyme releasing 1 μmol glucose equivalent per minute under the assay conditions.

Genomic DNA isolation. Total genomic DNA of selected isolates was extracted from 48 h growing cultures medium (2% glucose, 1% yeast extract and 1% peptone). 1.5 ml of cultured cells was collected by centrifugation at 13 000 rpm for 5 minutes. Cells were washed with distilled water and digested in 750 μl enzymatic lysis solution (10 μl of proteinase k 20 mg/ml, 2% SDS, 1% 2-mercaptoethanol, 1% CTAB and 10 mM EDTA in 50 mM Tris pH 8 buffer) and incubated for 30 minutes at 60°C. The lysate was extracted with phenol: chloroform: isoamyl alcool (25:24:1) three times. The extract was purified by adding 1/10 of the volume of sodium acetate 3 M and 1 ml ethanol. The mix was vortexed and placed for 15 minutes on ice. Sediment high molecular weight DNA was obtained after 5 minutes of centrifugation at 13 000 rpm washed with 70% ethanol and air-dried. The final DNA pellet was dissolved in 50 μl hydration solution and stored at –20°C. DNA concentration was estimated by measuring the absorbance at 260 nm. The quality of the isolated genomic DNA was calculated by the ratio OD_{260nm}/OD_{280nm}.

PCR amplification of the 5.8 S rDNA. PCR was used to amplify 5.8 S rDNA gene of a-amylase producing isolate. Primers used for PCR and DNA sequencing are ITS1 (5'-TCC GTA GGT GAA CCTGCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TATGC-3'). The standardized PCR conditions were as follows: one cycle of denaturation at 95°C for 5 minutes followed by 25 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 2 minutes, and one cycle of extension at 72°C for 10 minutes. PCR products were visualized by electrophoresis in 1% (w/v) agarose gel stained by ethidium bromide.

5.8 S DNA sequencing. PCR amplicons were purified using Microcon Y-100 filters (Millipore) and sequenced using ABI Prism® Big Dye® terminator v3.1 cycle sequencing kit (Applied Biosystems) according to manufacturer's instructions. The sequencing products were purified by ethanol precipitation. Sequencing reactions were carried out on an ABI PRISMA™ DNA Sequencer (Perkin-Elmer, Gembloux, Belgium). The sequences obtained (Length, approximately 500 bp) were then assembled in silico (Vector NTI) using overlapping to form contiguous sequence.

Phylogenetic analysis. Phylogenetic analysis was realized by an alignment of consensus sequences of 5.8S genes collected in an international database (Genebank). The resultants were then expressed in percentage of homology between the submitted sequence and the most relevant sequences from the database.

Results and Discussion

Screening of amylase producing fungus. One hundred and thirty-six fungal isolates from soil samples collected from different areas of Syria, cereal fields, olive fields, forests and gardens were evaluated for amylase production. Table I shows the amylase activity for the best ten fungi isolates. From this group, the highest producing amylase isolate, FSS60 was selected for further studies.

Identification of isolate FSS60. Generally, filamentous fungi are identified mainly using morphological characteristics. However, these methods of identification are often problematic, as thing can be different morpho/biotypes within a single species. They are also time consuming, and require a great deal of skill and expertise. DNA sequence analysis methods are an objective, reproducible, and rapid means of identification, therefore, they have been

Table I
Extracellular amylase activity from filamentous fungus isolates

Isolate No	Dry wt. (mg/ml)	Protein (µg/ml)	Amylase (IU/ml)
FSS3	5.74	365.54	5.62
FSS9	6.21	482.64	7.01
FSS24	6.62	870.65	4.49
FSS40	6.68	255.92	3.92
FSS53	7.24	122.08	3.72
FSS60	7.22	1059.08	11.13
FSS80	8.16	418.62	3.93
FSS118	7.12	509.85	4.18
FSS123	7.24	742.15	6.74
FSS132	7.90	1109.85	4.35

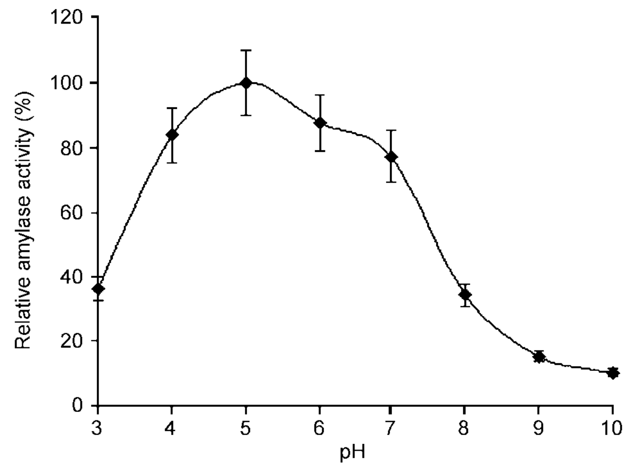

Fig. 2. Influence of pH on activity of amylase produced from *Aspegillus flavus* FSS60 Relative activity was determined at 50°C.

widely used (Li *et al*., 2007). Identification of the isolate FSS60 was done using 5.8S DNA gene sequences. The nucleotide BLAST similarity search analysis, based 5.8S DNA gene sequence revealed that isolate FSS60 belong to the genus *Aspergillus*. The closest phylogenetic neighbour according to the 5.8S DNA gene sequence data for FSS60 isolate was Aspergillus *flavus* with 100% of homology.

Influence of initial pH on amylase production by *Aspergillus flavus* FSS60. Amylase is a pH sensitive enzyme. Therefore, the selection of optimal pH is essential for the production of α-amylase (McMohan *et al*., 1999). α-amylase production by this isolate was observed in a range of pH (4.0–10.0). The production was found to be best at pH 9.0. Below and above this pH α-amylase production was lower (Fig. 1). Moreira *et al*. (1999) found that the amylase production by *Aspergillus tamarii* was higher at pH 6 while Nahas and Waldemarin (2002) observed the maximum amylase production by *Aspergillus ochraceus* at initial pH 5.0. Tiwari *et al.* (2007), however, reported maximum amylase production by *Penicillium rugulosum* at pH 7. In the present study, the maximum production was recorded at pH 9 which indicates that the selected isolate prefers alkaline conditions for better enzyme production.

Effect of pH on amylase activity. The optimum pH was determined by measuring the activity at 40°C over a range of pH from 4 to 10 using the following buffer: Citrate-phosphate buffer (pH 4–6), Na-phosphate buffer (pH 7–8), and glycine – NaOH (pH 9–10). The favorable pH range for amylase activity of *Aspergillus flavus* FSS60 was between 4.0–6.0, with an optimum at pH 5.0 (Fig. 2). A significant drop in enzyme activity was observed below pH 5.0 and above pH 6.0. The enzyme behaviour clearly indicates that it is more suitable for any application in the pH range of 4.0–6.0. Similar pH optimum was reported for other filamentous fungus (Ramachandran *et al*., 2004).

Effect of temperature on amylase activity and stability. The influence of temperature on amylase activity of the crude enzyme showed that enzyme

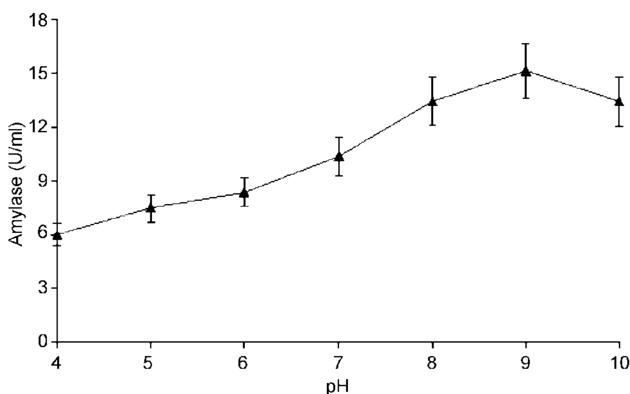

Fig. 1. Influence of pH of the medium on amylase production by *Aspegillus flavus* FSS60 at 30°C under submerged fermentation.

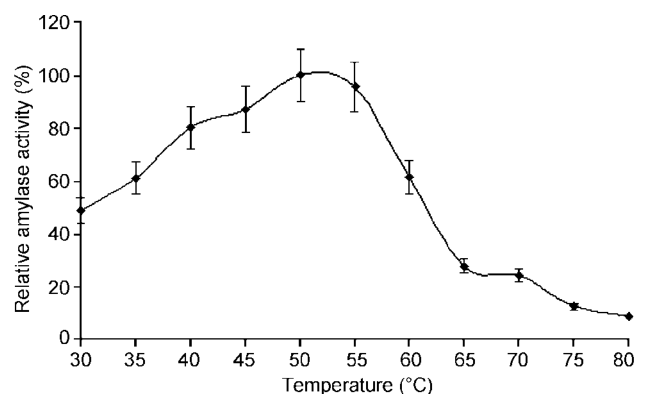

Fig. 3. Optimum temperature of amylase produced by *Aspergillus flavus* FSS60 in submerged culture. Relative activity was determined at pH 5.

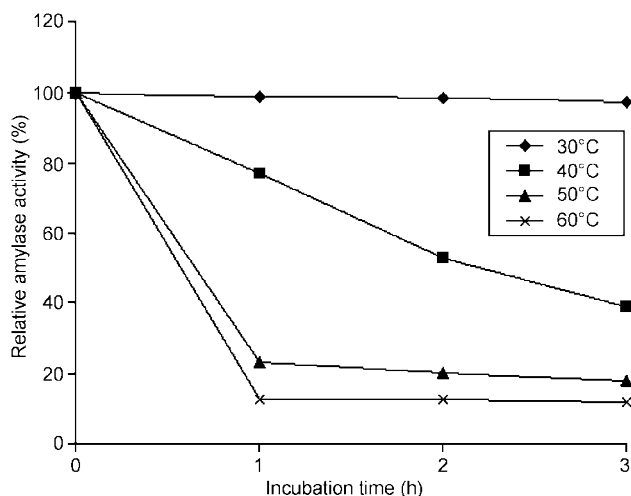

Fig. 4. Thermostability of amylase from *Aspergillus flavus* FSS60.

activity increased progressively with increase in temperature from 30°C to 50°C (Fig. 3). A sharp decrease in amylase activity was observed between 55°C (95.69%) and 65°C (27.59%). The retained activity at 80°C was only (8.77%). Similar results were reported by Ramachandran *et al.* (2004) and Kunamneni *et al.* (2005), the optimum temperature was 50°C for maximum activity of amylase produced by *Aspergillus oryzae and Thermomyces lanuginosus*. In other investigations, 35°C and 40°C were reported as optimum temperatures for amylase production by *Aspergillus oryzae* and *Penicillium griseofulvum* respectively (Ragunathan and Swaminathan, 2005; Ertan *et al.*, 2006).

Thermal stability tests were carried out by preincubating amylase up to 3 h in a range of temperatures from 30–65°C (Fig. 4). The enzyme remained stable after 3h of incubation at 30°C, while at 40°C the residual amylase activity was 77.37%, after 1h and 38.83% after 3 h. The enzyme was sensitive at 50°C, retaining 23.18% activity after 1 h exposure and only 18.02 % activity after 3 h. At 60°C the residual xylanase activity was only 12.86% after 1 h and 11.83% activity was observed after 3 h. These results indicated that the suitable temperature range for industrial application for amylase from *Aspergillus flavus* FSS60 was 30–40°C.

Conclusion. In conclusion, homology analysis of 5.8S gene provides suitable phylogenetic data that can be used to determine both close and very distant relationships. In the present study, this approach has allowed the identification of industrially important amylase producing organism, FSS60. The closest phylogenetic neighbour according to 5.8S gene sequence data for FSS60 isolate was *Aspergillus flavus*. The results indicated that optimum amylase activity was at pH 5.0 and 50°C. Amylase production by *Aspergillus flavus* FSS6 should be optimized for biotechnological applications.

Acknowledgement

We are grateful to Atomic Energy Commission of Syria (AECS) who financially supported this research which was realized in both, the Centre Wallon de Biologie Industrielle (CWBI), Gembloux Agricultural University, Belgium and the Atomic Energy Commission of Syria (AECS), Department of Molecular Biology and Biotechnology, Damascus, Syria.

Literature

Akpan I., M.O. Bankole and A.M. Adesemowo. 1999. Production of alpha amylase by *Aspergillus niger* in a cheap solid medium using rice bran and agricultural material. *Trop. Sci*. 39: 77–79.

Buzzini P. and A. Martini. 2002. Extracellular enzymatic activity profiles in yeast and yeast like strains isolated from tropical environments. *J. Appl. Microbiol*. 93: 1020–1025.

Chen Y.C., J.D. Eisner, M.M. Kattar, S.L. Rassoulian-Barrett, K. Lafe, U. Bui, A.P. Limaye and B.T. Cookson. 2001. Polymorphic internal transcribed spacer region 1 DNA sequences identify medically important yeasts. *J. Clin. Microbiol*. 39: 4042–4051.

Chi H.L.Z., X. Wang, X. Duan, L. Ma and L. Gao. 2007. Purification and characterization of extracellular amylase from the marine yeast *Aureobasidium pullulans* N13d and its raw potato starch digestion. *Enzyme Microb. Technol*. 40: 1006–1012.

Gupta R., P. Giras, H. Mohapatra, Y.K. Gaswami and B. Chauhan. 2003. Microbial α-amylase: a biotechnological perspective. *Process Biochem*. 38: 1599–1616.

Gupta A., V.K. Gupta, D.R. Modi and L.P. Yadava. 2008. Production and characterization of a-amylase from *Aspergillus niger*. *Biotechnol*. 1: 1–6.

Ertan F., H. Yagar and B. Balkan. 2006. Some properties of free and immobilized a-amylase from *Penicillium griseofulvum* by solid state fermentation. *Prep. Biochem. Biotechnol.* 36: 81–91.

Haq I., H. Ashraf, R. Abdullah and A.H. Shah. 2002. Isolation and screening of fungi for the biosynthesis of alpha amylase. *Biotechnol*. 2: 61–66.

Henry T., P.C. Iwen and S.H. Hinrichs. 2000. Identification of *Aspergillus* species using internal transcribed spacer regions 1 and 2. *J. Clin. Microbiol*. 38: 1510–1515.

Iwen P.C., S.H. Hinrichs and M.E. Rupp. 2002. Utilization of the internal transcribed spacer regions as molecular targets to detect and identify human fungal pathogens. *Med. Mycol*. 40: 87–109.

Kunamneni A., K. Permaul and S. Singh. 2005. Amylase production in solid state fermentation by the thermophilic fungus *Thermomyces lanuginosus*. *J. Biosci. Bioeng*. 100: 168–171.

Li Y., Z. Liu, F. Cui, Y. Xu and H. Zhao. 2007. Production of xylanase from a newly isolated *Penicillium* sp. ZH-30. *World J. Microbiol. Biotechnol*. 23: 837–843.

Liu X.D. and Y. Xu. 2008. A novel raw starch digesting α-amylase from a newly isolated *Bacillus* sp. YX-1: Purification and characterization. *Biores. Technol*. 99: 4315–4320.

Lowry O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem*. 139: 265–275.

McMohan E.M., C.T. Ketty and W.M. Forgarty. 1999. High maltose producing amylotic system of a *Streptomyces* sp. *Biotechnol. Lett*. 21: 23–26.

Miller G.L.1959. Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Ann. Chem.* 31: 426–428.

Moreira F.G., F.A. Lima, S.R.F. Pedrinho, V. Lenartoviez, C.G.M. Souza and R.M. Peralta. 1999. Production of amylases by *Aspergillus tamarii*. *Rev. Microbiol*. 30: 157–162.

Nahas E. and M.M. Waldemarin. 2002. Control of amylase production and growth characteristics of *Aspergillus ochraceus*. *Rev. Latin. Microbiol*. 44: 5–10.

Okolo B.N., L.I. Ezeogu and C.N. Mba. 1995. Production of raw starch digestive amylase by *Aspergillus niger* grown on native starch source. *J. Sci. Food and Agricul*. 69: 109–115.

Pandey A., P. Nigam, C.R. Soccol, V.T. Soccol, D. Sing and R. Mohan. 2000. Advances in microbial amylases. *Biotechnol. Appl. Biochem*. 31: 135–152.

Pryce T.M, S. Palladino, I.D. Kay and G.W. Coombs. 2003. Rapid identification of fungi by sequencing the ITS1 and ITS2 regions using an automated capillary electrophoresis system. *Med. Mycol*. 41: 369–381.

Ragunathan R. and K. Swaminathan. 2005. Growth and amylase production by *Aspergillus oryzae* during solid state fermentation using banana waste as substrate. *J. Environ. Biol.* 26: 653–656.

Ramachndran S., A.K. Patel, K.M. Nampoothiri, S. Chandran, G. Szakacs, C.R. Soccol and A. Pandey. 2004. Alpha amylase from a fungal culture grown on oil cakes and its properties. *Braz. Arch. Biol. Technol*. 47: 309–317.

Rao M.B., A.M. Tanksale, M.S. Gathe and V.V. Deshpande. 1998**.** Molecular and biotechnological aspects of microbial proteases. *Microbiol. Mol. Biol. Rev.* 62: 597–635.

Tiwari K.L., S.K. Jadhav and A. Fatima. 2007. Culture condition for the production of thermostable amylase by *Penicillium rugulosum*. *Glob. J. Biotechnol. Biochem*. 2: 21–24.