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AMYLASE PRODUCTION AND GROWTH PATTERN OF TWO INDIGENOUSLY ISOLATED *ASPERGILLI* UNDER SUBMERGED FERMENTATION: INFLUENCE OF PHYSICO-CHEMICAL PARAMETERS

SAIRA YAHYA[1,2]*, FAIZ MUHAMMAD[1,3], MUHAMMAD SOHAIL[1] AND SHAKEEL AHMED KHAN[1,4]

[1]*Department of Microbiology, University of Karachi, Karachi, Pakistan*
[2]*Department of Biosciences, Shaheed Zulfikar Ali Bhutto Institute of Science and Technology, Karachi, Pakistan*
[3]*Department of Microbiology, Balochistan University of Information Technology, Engineering and Management Sciences, Quetta, Pakistan*
[4]*Department of Biosciences, Barrett Hodgson University, Karachi, Pakistan*
**Corresponding author's email: saira.yahya@yahoo.com*

Abstract

Amylases are the industrial enzymes that are produced on large scale using *Bacillus* or *Aspergillus* species. Keeping in view the significance of Aspergilli in fermentation industries, amylase production and growth pattern of two indigenous Aspergilli, *A. tubingensis* SY 1 and *A. niger* MS 101, was determined under submerged fermentation (SmF) conditions. Enzyme production was improved by optimizing different physico-chemical parameters. Highest amylase production was achieved at 30ºC and pH 5.9. Maltose, starch and glucose were found to be good inducers of amylase in addition to nitrogen source, peptone. Amylase production and fungal growth kinetic studies under optimum conditions revealed volumetric amylase production (IU/L.h) of 271.67 and 70.62 for *A. tubingensis* SY 1 and *A. niger* MS 101, respectively. Amylase production was found to be significantly correlated with the fungal growth, protein secretion and utilization of starch by the fungi and pH changes in the medium.

Key words: *Aspergillus tubingensis*, *Aspergillus niger*, Growth kinetics.

Introduction

Starch industries world-wide exploit a vital collection of enzymes called amylases. The amylolytic enzymes have diverse characteristics and hydrolyze α-glucosidic linkages in starch either by endo- or exo- action. This group of enzymes include α-, β, γ- amylases, as well as, pullulanase and isoamylase. There are plentiful biotechnological uses of amylases such as in the production of dextrins, oligosachharide syrups, bioethanol and also in the yard goods and paper industries (Sivaramakrishan *et al*., 2006; De Souza & De Oliveira, 2010). The characteristics of amylases are important for their aptness to a particular process (Van der Maarel *et al*., 2002).

Amylases are extensively distributed among microorganisms and these microbes are generally applied for the industrial amylase production. Among the microorganisms, fungi are able to produce variety of amylases in considerable amounts compared to bacteria. The genus *Aspergillus* is thought to have an enormous potential for commercial amylase production. *Aspergillus niger, A. oryzae, A. awamori* are the most common industrial Aspergilli (Sangeeta & Rintu, 2009; Sundarram & Murthy, 2014).

The emergent economic importance of Aspergilli requires an explicit classification of an *Aspergillus* isolate. Molecular methods have shown distinctive and significant advances in identification of fungi and are preferred over conventional techniques because they are specific, sensitive and rapid. Among these techniques, the eukaryotic rDNA (rRNA cistron) is the major target for the evaluation of mycological community (Möhlenhoff *et al*., 2001; Kowalchuk *et al*., 2006; Begerow *et al*., 2010). Thus rDNA PCR amplification is carried out for the identification of fungal species.

The amylase production and fungal growth is greatly affected by various environmental and nutritional conditions (Gerlach *et al*., 1998; Wang *et al*., 2003). Growth morphologies of Aspergilli under submerged fermentation varies from filamentous to pelleted growth forms that affects the general cell performance (Johansen *et al*., 1998). Also, the amylase yield and volumetric productivity can be decidedly controlled by the growth conditions provided and this can have a substantial impact on the overall process economics. During any fermentation, in addition to pH and temperature, the most important parameter is the substrate consumption, which is also coupled to the cell growth and enzyme production.

To date, in Pakistan, industries that use amylases generally import them which cause a loss of foreign reserves. Moreover, considering abundant natural resources and a diverse microbial population, as enzyme producer that the country harbors, it can easily develop fermentation industries indigenously, including amylase production (Sohail *et al*., 2009). Thus, it is imperative to improve methods for enzyme production to obtain substantial amounts of amylases from indigenous microbial population to limit our dependency on imported enzymes.

In the present work, amylase production has been studied under different environmental conditions using diverse nitrogen and carbon sources as inducers/ repressors of amylase production. At different time intervals, enzyme production and cell growth by the Aspergilli were measured. The relationship of mold growth to the kinetics of product formation was also determined.

Materials and Methods

Fungal strains, growth medium and fermentation: Two indigenously isolated Aspergilli were used in this study. Fungal spores from SDA plates (96 h old) were transferred to tubes containing sterile saline. Spore suspension (0.1 ml; ~2 X 10^4 spores/ml) was transferred to Erlenmeyer flasks having 10 ml fermentation medium (g/L): starch (5.0); NH_4NO_3 (3.0); $MgSO_4.7H_2O$ (0.5); KCl (0.5); $FeSO_4.7H_2O$ (0.01); KH_2PO_4 (1.0); peptone (10.0) and incubated in a shakobator (150 rpm) at 30ºC for 96 h. The effects of cultivation time, temperature (25-40ºC), initial medium pH (3.0- 10), carbon sources (0.25-2.0%) and nitrogen sources (0.1-0.9%) on amylase production were determined. After fermentation, the contents were filtered through membrane filters (0.45 µm) using a filtration pump. These filters (having the fungal mass) were then dried to a constant weight at 80ºC. Filtrates were analyzed for amylase activity, glucose, protein, starch and final pH of the medium.

Fungal DNA Extraction, amplification and sequencing: The fungi were grown in Sabouraud's dextrose broth (at 30ºC and 150 rpm) for 2 days. The harvested mycelium froze in liquid nitrogen were placed in a mortar, ground to powder, suspended in 650 µl of lysis buffer (Na- EDTA 50mM pH-8, Tris-HCl 100mM pH-8, 1% SDS and 10mg/ml RNase A) and transferred to an Eppendorf containing glass beads (0.5 g, dia. 0.5mm). The mixture was then homogenized (15 min), centrifuged (13000 rpm) for 3 min and the supernatant (0.5 ml) was transferred to another Eppendorf having 0.1 ml potassium acetate buffer (3M, pH 5.5). The contents in the tube were vortexed and centrifuged (13000 rpm) for 3 min, supernatant (0.5 ml) was transferred into another tube containing isopropanol (0.5 ml), contents mixed and centrifuged (13000 rpm) for 2 min. The supernatant was then discarded and pellet washed with ethanol (70%; 0.75 ml). After centrifugation (30 s), ethanol was discarded and pellet (containing DNA) air-dried. The DNA was suspended in 0.05 ml distilled water and was stored (-20ºC) till use (Feng *et al*., 2010).

The 5.8S, 28S rDNA and ITS region were subjected to PCR amplification using general primers ITS4 5' TCCTCCGCTTATTGATATGC 3' and ITS3 5'-GCATCGATGAAGAACGCAGC-3'. The amplification was carried out as described by Anderson & Cairney (2004) in 25 µl reaction volume using template DNA (20-100 ng) and each primer (20 µM) using PCR Master Mix (Bio Basic Inc). The thermal cycler settings were:

1- First denaturation at 95ºC (5 min.),
2- 40 cycles of denaturation (30 sec., 95ºC), annealing (1 min., 48 - 58ºC), extension (1 min., 72ºC)
3- Final extension at 72ºC (5 min.).

The molecular size of amplicons was determined using standard DNA Ladder of 100 bp (Invitrogen, USA).

The PCR amplicons were sequenced (Centralized science lab, University of Karachi) by a Genetic analyzer (ABI Prism® 3130). Sequences were viewed by BioEdit Sequence Alignment Editor Software (Ibis Biosciences. USA), interrogated using the BLAST algorithm (Altschul *et al*., 1990) and submitted to the National Center for Biotechnology Information website.

Repression and/or Induction of amylase production: Fungal isolates were allowed to grow at 30ºC in a shakobator (150 rpm) for 96 h in the presence of either starch, glucose or maltose as a sole carbon source (Sohail *et al*., 2005). Following incubation, fungal growth was separated by centrifugation and washing was carried out by saline (twice) and distilled water (once). Washed microbial mass from medium having glucose was shifted to the fermentation medium containing either maltose or starch. Whereas, washed fungal mass from the medium having starch was transferred to the culture medium supplemented with either maltose or glucose, as a sole source of carbon. Similarly, the cell mass from medium supplemented with maltose was shifted to either glucose or starch containing medium for 96 h at 30ºC. The final cell-free culture supernatant was used for amylase titer determination.

Analytical methods

Amylase assay: Cell-free culture supernatant (25µl) and soluble starch (25µl; 0.5 % w/v) were mixed and incubated at 60ºC. The reaction was stopped by the addition of 1% DNS (150 µl) and boiling (5 min.). The reaction was then cooled on ice (5 min,), distilled water (720 µl) added and A_{550} recorded using a UV / Vis spectrophotometer (Beckman Coulter, DU 730). One unit of enzyme activity is the µmoles of glucose produced by 1 ml of the enzyme in 1 min and calculated using a glucose standard curve (Miller, 1959).

Protein assay: Protein titer was assessed by BioRad Quickstart Kit (BioRad, USA) using BSA as a standard. Cell-free supernatant (50 µl) was added in the reagent (50 µl) and A_{595} read using a 96- well plate reader (Bradford, 1976).

Glucose detection: Cell-free supernatant (10 µl) was added in 1ml of the glucose-oxidase reagent (Randox Laboratories limited, UK) and incubated at 25ºC for 25 min. A_{600} of the glucose standard and the samples was recorded against the reagent within 1 h.

Starch detection: Cell-free supernatant (25 µl) and 25 µl Na- acetate buffer (50 mM; pH 5.6) were mixed, Acetic acid (50 µl; 1 M) was added and volume made up with distilled water (up to 2.35 ml). Then 50 µl iodine reagent (1% I^-: 10% KI: d/w mixed in a ratio of 1:1:3) was added and A_{660} recorded. By using starch as a standard, the absorbance readings from samples were quantitatively converted to their corresponding starch concentrations.

Statistical analysis

The experiments were performed in triplicate and their mean and standard deviations were calculated using MS Excel 365. Pearson's coefficient of correlation (r) was determined for the fungal growth parameters using IBM SPSS statistic 20 (significant at p<0.05).

Results and Discussions

Aspergilli are important microbial agents used in fermentation industries for enzyme production. The most commonly used *Aspergillus* species in microbial fermentations are *A. terreus, A. awamori* and *A. niger* (Sohail *et al*., 2009). In recent years, rather a new species, *A. tubingensis* has also been found its potential in the industrial sector for commercial production of enzymes such as inulinase and tannase (Trivedi *et al*., 2012; Xiao *et al*., 2015), however, the literature on *A. tubingensis* amylases are limited. Keeping this in mind, current study was initiated by identification and cultivation of two fungal strains under different fermentation conditions for amylase production.

Fungal identification: Aspergilli can be characterized by microscopy and colonial characteristics but for taxonomical purposes, to explain evolutionary links among closely associated species and their applications in biotechnological processes, molecular methods are widely used. Therefore, the fungal strains used in this study were characterized by molecular methods to identify them accurately.

The results specify that the amplicons of the isolates MS 101 and SY 1 were of ~350 bp (Fig. 1). According to the BLAST analysis of the nucleotide sequences, SY 1 and MS 101 were identified as *A. tubingensis* and *A. niger*, respectively with GeneBank accession numbers KX243269 (MS 101) and KX243270 (SY 1). Both these *Aspergillus* strains belong to the section Nigri of Aspergilli (Parenicová *et al*., 2001). Fungal strain identification using ITS3/ITS4 primer set, is in line with the literature. De Aguirre *et al*., (2004) identified nine strains of Aspergilli using the fungus specific prime pair ITS4 and ITS3. Parallel results of fungal characterization using these primers were observed by White *et al*., (1990), Chen *et al*., (2000) and Shin-ichi Fujita *et al*., (2001).

Phylogenetic relationship among the known Aspergilli and the isolated strains was determined by generating a cladogram. The predictable phylogenetic position of the strains is shown in Fig. 2. The closest phylogenetic neighbor of *Aspergillus tubingensis* SY 1 was found to be *Aspergillus tubingensis* isolate F4-04 while the closest phylogenetic neighbor of *Aspergillus niger* MS 101 was the *Aspergillus niger* strain SCAU-F-99 (Fig. 2).

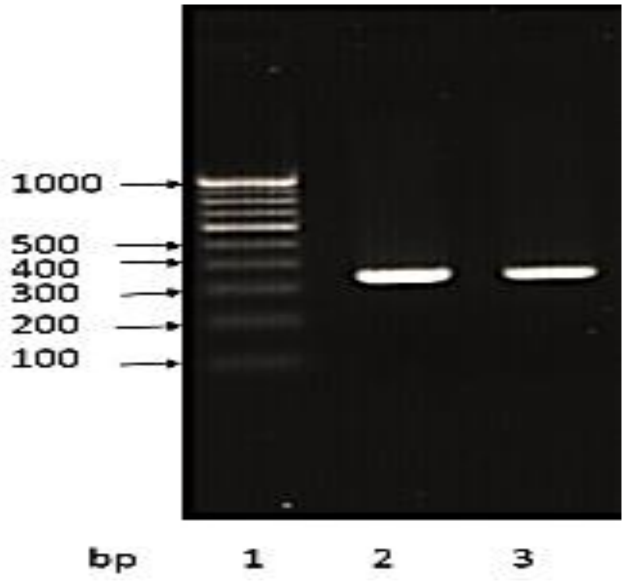


Fig. 1. PCR amplicons obtained from the fungal and yeast DNA using ITS primers. Lane 1: 1Kb ladder; Lane 2-3: PCR products using primer pair ITS3/ITS4 from fungal strains MS 101 and SY 1.

Physico-chemical parameter optimization for amylase production: Production of microbial enzymes is dependent on a variety of chemical and physical parameters, such as nitrogen and carbon sources, pH, inoculum-size, agitation and temperature (Pedersen & Nielson, 2000; Shariq & Sohail, 2020). In order to attain maximum titers of amylases from the selected strains, amylase production was optimized by using one-factor at a time approach under submerged fermentation settings.

Among the physical conditions, the most important parameter to maintain and control maximum enzyme production is temperature (Sundarram & Murthy, 2014). Temperature shifts during fermentation affects substrate degradation. According to Gupta *et al*., (2003), most of the amylolytic fungal strains produce amylase optimally within range of a temperature (25ºC-40ºC). The results of *A. tubingensis* SY 1 and *A. niger* MS 101 revealed that 30ºC was the optimum temperature for enzyme production from both Aspergilli (Fig. 3), indicating that amylase production is temperature dependent.

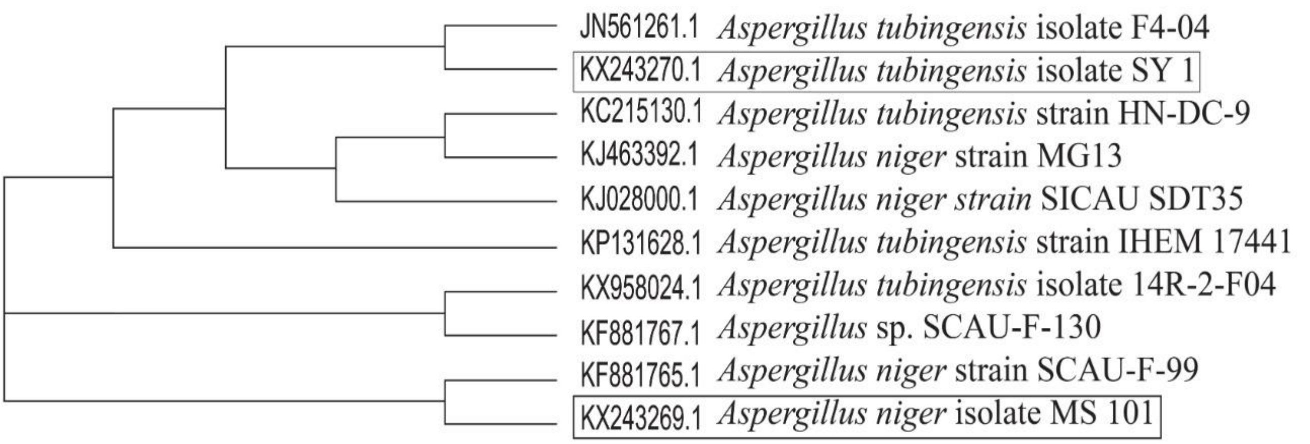


Fig. 2. Phylogenetic cladogram for *Aspergillus tubingensis* SY 1 and *Aspergillus niger* MS 101 based on 5.8S rRNA gene partial sequence; internal transcribed spacer 2 (ITS 2) complete sequence; and 28S rRNA gene partial sequence.

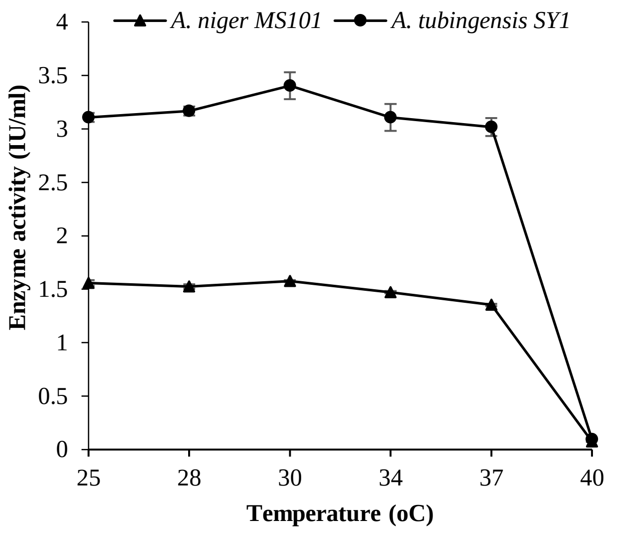


Fig. 3. The effect of temperature on amylase production when fungi were cultivated on medium containing starch as a sole carbon source.

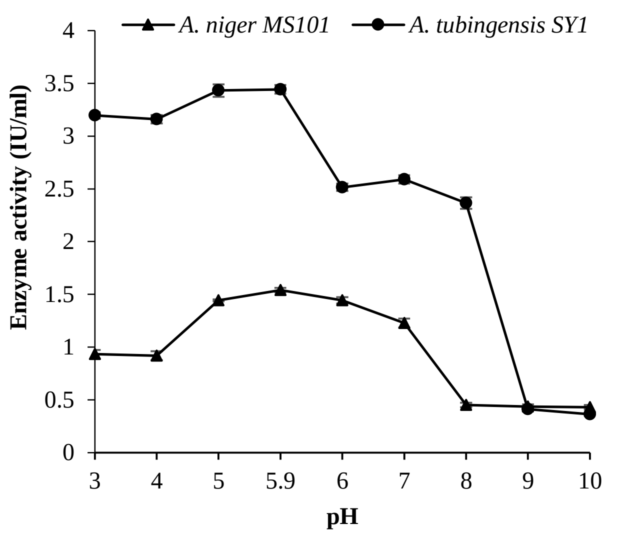


Fig. 4. The effect of pH on amylase production after growing fungi on medium containing starch as a sole carbon source.

Another factor that strongly influences the enzyme production is the initial pH of the production medium (Prakasham *et al*., 2006). Due to unfavorable pH of the medium, the accessibility of nutrients is reduced, resulting in lower enzyme yields, morphological changes in microbes as well as it impacts the stability of the product in culture media (Gupta *et al*., 2003). Therefore, the pH dependent amylase expression was investigated by varying the initial pH of starch supplemented media from 3-10. The results indicated that pH 5.9 was optimum for enzyme production by the fungal strains (Fig. 4). A pH value towards acidic side has also been reported for *A. ochraceus* (Nahas & Waldermarin, 2002) and *A. niger* (Hernandez *et al*., 2006) in other studies.

To enhance the amylase production by the fungi, different nitrogen sources (inorganic and organic) were selected and studied. Among them, 0.5% peptone and KNO_3 showed highest amylase titers by *A. niger* MS 101 while 0.9 % peptone was suitable for maximum amylase production by *A. tubingensis* SY 1 (Fig. 5). Low-levels of amylase were observed when NH_4NO_3 and NH_4Cl were supplemented to the fermentation medium, as sole N-source. Marlida *et al*., (2000) and Hernandez *et al*., (2006) also identified peptone as superior nitrogen source for amylase production by *Acremonimum* sp. and *A. niger*. Contrary to this, Pandey (2005) obtained more titers of amylase by *A. niger* in the presence of inorganic nitrogen. Peptone contains amino acids, inorganic salts, peptides, vitamins, sugars and lipids. Numerous studies have displayed that cell-growth, volumetric and specific productivities can be enhanced by peptone in a variety of expression systems by affecting the protein profile that results in changes in a cell's metabolic behavior (Franek *et al*., 2003; Mendonca *et al*., 2007; Davami *et al*., 2015).

Amylase expression can be repressed or induced by diverse carbon sources (Saito & Yamamoto, 1975). In this study, among the carbon sources used, minimum starch concentrations that supported maximum enzyme secretion in *A. tubingensis* SY 1 and *A. niger* MS 101 were 0.5% and 0.25%, respectively (Fig. 6). The results also revealed that glucose (2%) and maltose (2%) were better sources of carbon for amylase production by *A. tubingensis* SY 1 and *A. niger* MS 101, followed by sucrose (Fig. 7). Lactose was a poor source for extracellular amylase production. It was observed that either 2% maltose or glucose in the growth medium enhances the amylase production (0.5 - 1.0 folds) when compared to starch in both strains indicating that amylase production is an inducible process.

To confirm whether glucose and maltose really induce the production of amylase, a set of experiments were performed (section 2.3). The data verifies that shifting of starch grown cells of fungal strains to maltose containing medium resulted in an increase by 2.7- and 4.1-folds in amylase production by *A. tubingesis* SY 1 and *A. niger* MS 101, respectively. Followed by maltose, glucose was observed as an inducer of amylase production. However, after transferring the cells from glucose or maltose supplemented medium to a medium containing starch, a repression in the enzymatic activity was noted in both the fungal strains (Fig. 8). This can be attributed to the less growth that is generally obtained when microorganisms are transferred from simple carbon source to a complex carbon source.

Although maltose has been used in amylase production, the use of starch is nonetheless universal (Liu & Xu, 2008; Sharma & Satyanarayana, 2011). A study by Eratt *et al*., (1984) showed that starch and maltose acted as inducers in *A. oryzae*. The expression of amylolytic genes is generally repressed by glucose in a CreA-dependent manner (Felenbok & Kelly, 1996) but some reports have shown that glucose induces alpha amylase synthesis (Carlsen & Nielsen, 2001). Yuriko *et al*., (2012) revealed that in a CreA-deficient *A. nidulans* strain, glucose induced the amylase activity. The transcriptional activator responsible for amylolytic gene induction in *Aspergillus* sp. is *Amy*R (vanKuyk *et al*., 2012; Zhang *et al*., 2016). It can be presumed that the Aspergilli used in this study may be *Cre*A- deficient and additional ratification is required.

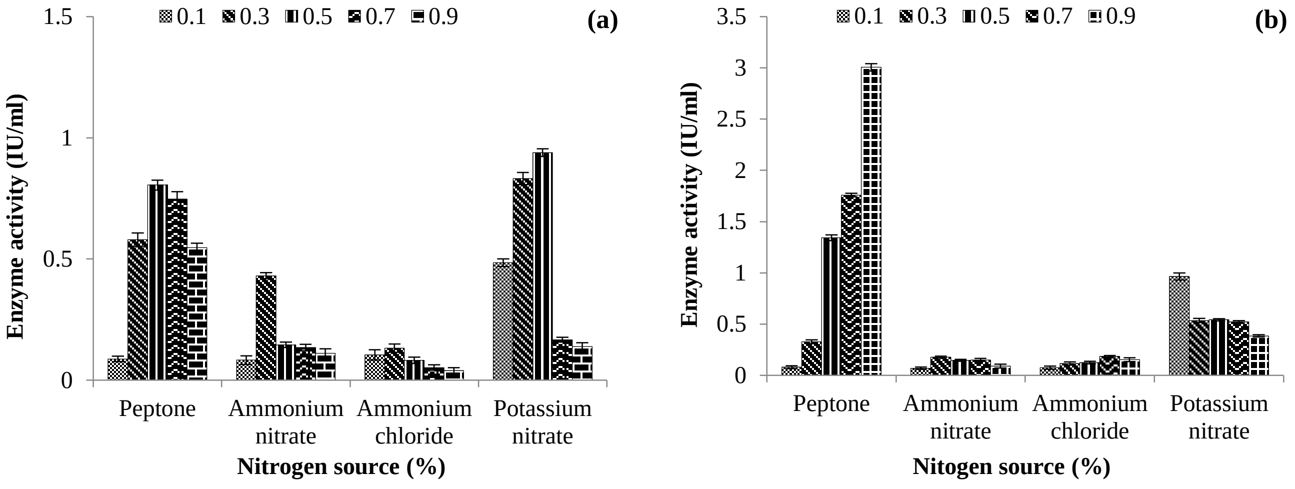


Fig. 5. Effect of different nitrogen sources in production medium on extracellular amylase production from (a) *A. niger* MS 101 and (b) *A. tubingensis* SY 1.

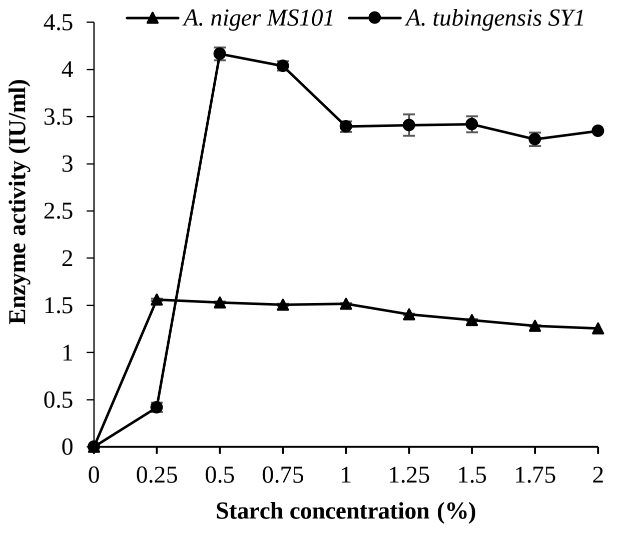


Fig. 6. The effect of starch concentration in amylase production medium.

Amylase production and fungal growth kinetics: Amylase production and fungal growth kinetic experiments were performed out for the correlation of enzyme production with the phases of fungal growth. The study showed that the molds propagated quickly and maximal mycelial mass obtained after 48 h and 56 h of incubation of *A. tubingensis* SY 1 and *A. niger* MS 101, respectively (Fig. 9), after that, a gradual drop in mycelial mass was witnessed, representative of the commencement of the late growth phase that is characterized by hyphal breakdown and exhaustion of certain nutrients. Enzyme activities in the culture supernatants increased sharply after 48 h as shown in Fig. 9. Reports confirmed that enzyme secretion usually occurs maximally during the late exponential and early stationary phases (Lampen, 1965; Stinson & Merrick, 1974). According to Gupta *et al*., (2003), enzyme secretion during the late growth phases occurs maximally because of reduction in catabolic-repression as the nutrients in the medium depletes.

During the course of fermentation, starch was rapidly utilized by the fungi within 32 h of incubation, after which there was a rise in fungal mass and amylase titer. A pH increase from 5.9 to 6.7 was observed after 24 h growth of *A. tubingensis* SY 1 and to 7.05 after 32 h growth of *A. niger* MS 101 which later shifted towards pH 4 (Fig. 8). Growth kinetic results revealed that after 48 h of fermentation, pH dropped to ~4.5. These pH variations indicate that the starch and nutrient consumption by the fungi led to acidification of the medium. Swift *et al*., (1998) and Whitaker & Long, (1973) found that pH not only affected the regulation of protein expression but also affected fungal growth significantly.

The prominent features of kinetic studies are summarized in Table 1. The data indicates a short lag-phase of 8 h in both strains. The generation time of *A. tubingensis* SY 1 was shorter (17 h) than *A. niger* MS 101 (~25 h). Volumetric biomass and amylase production of *A. tubingensis* SY 1 were higher than that of *A. niger* MS 101, which also influenced the specific productivity. The higher biomass production and shorter generation time coupled to enhanced volumetric amylase production indicate that the enzyme expression is growth-linked.

Amylase synthesis was statistically linked with fungal growth as shown in Table 2. Positive correlation was observed between fungal biomass and amylase production from *A. tubingensis* SY 1 (r = 0.845) and *A. niger* MS 101 (r = 0.538). Such kind of linear correlation has previously been reported for *Bacillus* sp. (Cordeiro *et al*., 2002) and for *Rhodothermus marinus* (Gomes *et al*., 2003). There were significantly positive correlations among total extracellular protein, amylase production and time for both Aspergilli ($p<0.05$). While amylase production from the fungal strains presented negative correlations with the final pH and starch content of the medium. The results also specify the dependence of amylase production on the variations that occur during the course of fermentation along with the external factors such as temperature.

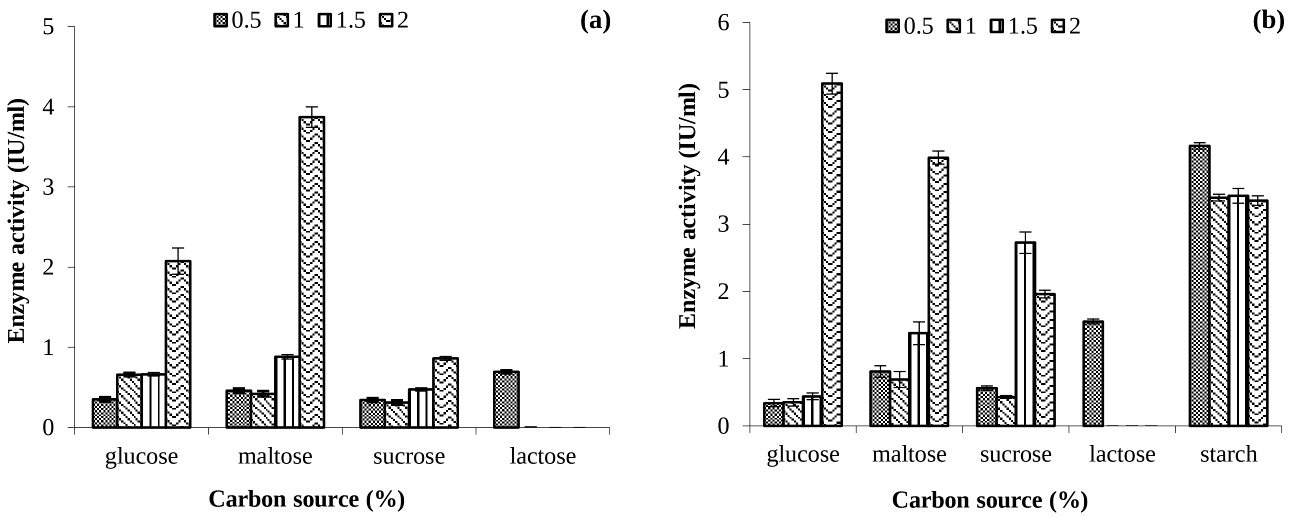
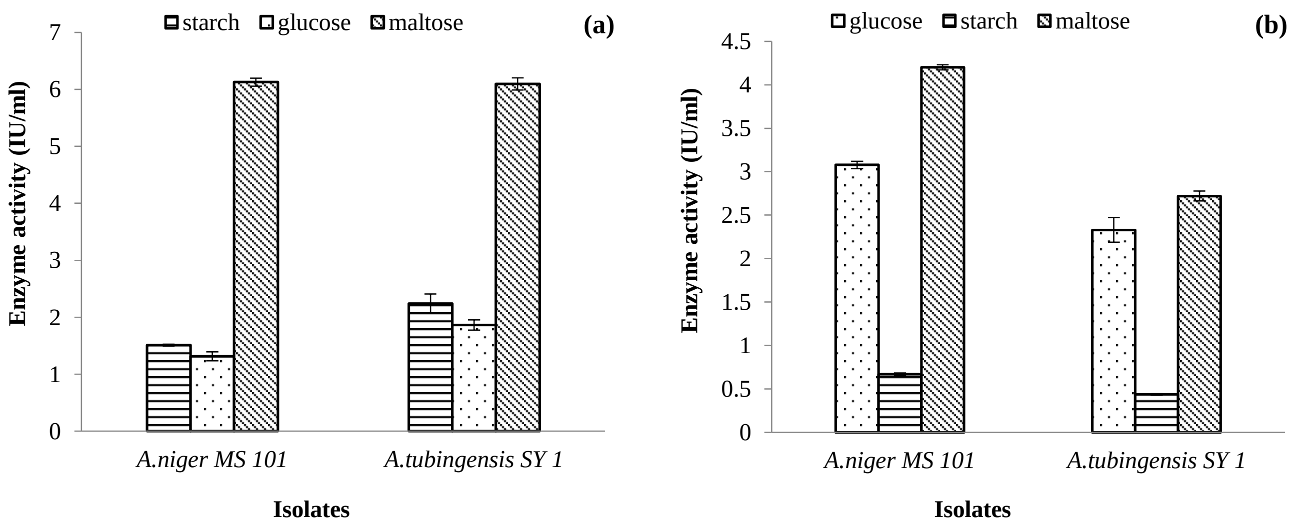


Fig. 7. Effect of different carbon sources in production medium on extracellular amylase production from (a) *A. niger* MS 101 and (b) *A. tubingensis* SY 1.



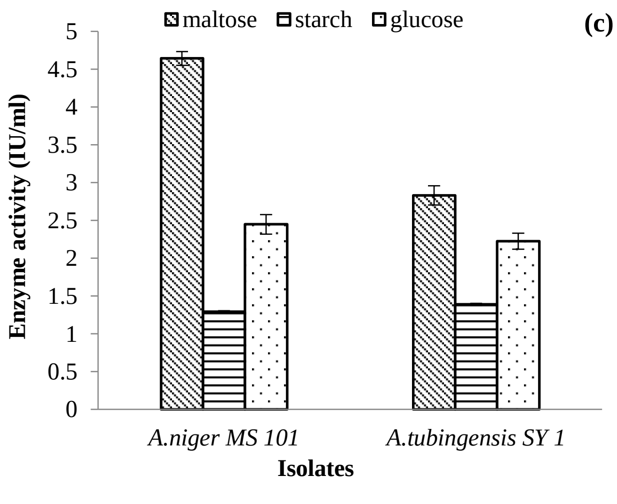


Fig. 8. Induction of amylase production. Fungi grown in (a) starch containing medium and then shifted to medium containing glucose or maltose; (b) glucose containing medium and then shifted to medium containing starch or maltose containing medium and (c) maltose containing medium and then shifted to medium containing starch or maltose containing medium. A concentration of 2% was used in case of maltose and glucose while the concentration of starch was 0.25% for *A. niger* MS 101 and 0.5% for *A. tubingenis* SY 1 respectively.

Table 1. Summary of growth kinetics indicating lag- and log- phase, generation time (g), volumetric amylase production (Q_p), volumetric biomass production (Q_x) and specific productivity ($Y_{p/x}$), when selected fungal strains were grown in enzyme production medium.

Strain	Substrate	Duration (h)		g	Q_p	Q_x	$Y_{p/x}$
		Lag-phase	Log-phase	(h)	(IU/L/h)	(mg/L/h)	(IU/mg)
Aspergillus niger MS 101		8	48	24.84	70.62	80.20	0.88
Aspergillus tubingensis SY 1	Starch	8	40	17.42	271.67	122.5	2.19

Table 2. Correlation analysis between amylase production and final pH of the medium, biomass production, starch content total and total extracellular protein secretion in two strains of *Aspergilli*.

Strain	Time (h)	Final pH	Biomass production (mg/10ml)	Total extracellular protein (µg/ml)	Starch content (g/L)
Aspergillus niger MS 101	0.862 [a]	- 0.835[a]	0.538[a]	0.844[a]	- 0.861[a]
	0.000[b]	0.001[b]	0.071[b]	0.001[b]	0.000[b]
Aspergillus tubingensis SY 1	0.794 [a]	- 0.871[a]	0.845[a]	0.771[a]	- 0.920[a]
	0.002[b]	0.000[b]	0.001[b]	0.003[b]	0.000[b]

[a] Pearson's coefficient of Correlation; [b] probability is significant at 0.05 level

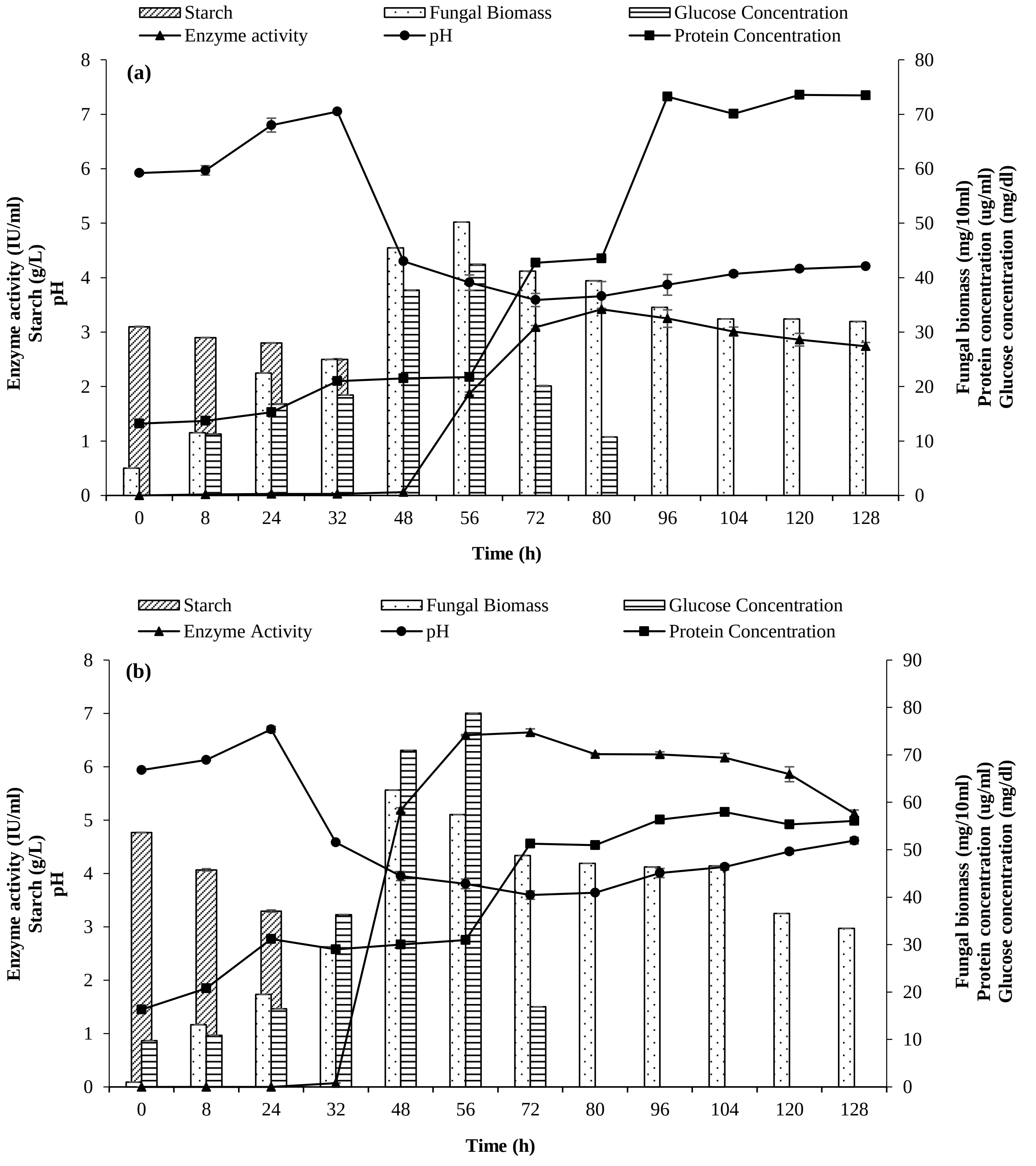


Fig. 9. Time course of amylase production, fungal growth and medium pH by (a) *A. niger* MS 101 and (b) *A. tubingensis* SY 1 using starch as a sole carbon source.

Conclusions

It was concluded that both cultural and nutritional conditions were imperative for amylase production and growth of *A. tubingensis* SY 1 and *A. niger* MS 101. The Aspergilli are capable of amylase production in ~72 h with ample volumetric productivities. These fungal strains can be utilized in achieving commercially relevant quantities of amylases.

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