Pak. J. Bot., 53(2): 715-721, 2021. DOI: http://dx.doi.org/10.30848/PJB2021-2(17)

DYNAMICS OF AMYLASES PRODUCTION POTENTIAL BY *ASPERGILLUS NIGER* ISOLATES FROM SOIL

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Abstract

Amylases production potential of non-toxigenic starch hydrolyzing indigenous *Aspergillus niger* was determined. *A. niger* soil isolates (06) identified based on macroscopic and microscopic characters were screened for amylases production on starch agar. Three isolates (ANS03, ANS06 and ANS10) with higher starch hydrolyzing index (0.24, 0.25 and 0.20 respectively) were selected for optimization of physical and chemical conditions for higher enzyme production. Physicochemical factors including pH (4.5, 6, and 7.5), temperature (22, 28, and 37°C), substrates (maize, rice husk and wheat bran) and substrate concentration (1, 3, and 5%) were evaluated for dynamics in amylases production potential by one time one factor method. Post incubation of seven days with standardized inoculums (10^6 spores / mL) of selected isolates, filtrates were obtained. Qualitatively, extracellular enzymes in filtrate were detected by iodine test and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). All isolates showed starch hydrolysis to some extent by iodine test. Electrophoresis analysis revealed three proteins with 50.12, 48.98 and 37.15kDa molecular weights. Amylases were quantified from filtrates by dinitrosalicylic acid (DNS) method. Among three isolates, ANS06 is the best producer with 150.61±3.26 IU amylase activity at 37°C, pH 6 and wheat bran (5%) and lowest amylase activity was found (.039±.03) at same temperature and pH but with maize (1%). ANS03 showed highest amylase activity (40.04IU±.37) at 37°C, pH 6 using 5 percent maize as substrate and lowest activity was (0.56±.08). The maximum enzyme production of ANS10 was 86.23±1.31IU at 37°C, pH 6 and 5 percent wheat bran and lowest 0.04±0.01 IU. Statistically, all isolates showed significant difference in amylase activity at all selected physical and chemical conditions. It was concluded that starch hydrolyzing potential of indigenous *A. niger* isolate can be used for mass production.

Key words: Amylase, *Aspergillus niger*, Starch, Optimization, Electrophoresis.

Introduction

Amylases are group of enzymes which cause hydrolysis of starch and convert it into glucose, maltose and short chain saccharide units. Major amylases involved in starch hydrolysis include: α-amylase, β-amylase and δ-amylase (Gurung *et al*., 2013). The first enzyme produced on industrial scale for the treatment of digestive disorder in 1894 from fungi was an amylase (Mojsov, 2012). The amylases contribute major share (25%) to enzyme market in the world (Dehkordi & Javan, 2012). Amylases are used for hydrolyses of insoluble starch as well as aqueous suspension of starch granules. These are used in starch liquefaction (Chai *et al*., 2012), paper industry, textile industry, food industry, pharmaceutical industry, fermentation, brewing, detergents and baking (Silva *et al*., 2013). Alpha amylase is produced in gastrointestinal tract of birds in low quantity (Noy & Sklan, 1995; Yuan *et al.*, 2017). To improve the digestibility of starch-based diets in birds, α amylase can be used as feed supplement (Onderci *et al*., 2006). It is also used in fruit juices to remove haziness, for conversion of glucose to fructose (starch syrups) and in making cakes as anti-stalling agent (Kiran & Chandra, 2008).

Amylases are produced by animals, plants and microorganisms (Monga *et al.*, 2011). Amylases from microbial sources are cost effective to meet the industrial demands. Microorganisms which can produce amylases are bacteria, fungi and actinomycetes (Gupta *et al.*, 2003; Abdulaal, 2018). Amylases from microorganism especially from fungi (*Aspergillus species*) are more attractive because of ubiquitous nature of fungi and high rate of production. Moreover, these can be manipulated on genetic level for enzyme production (Kathiresan & Manivannan, 2006). *Aspergillus niger* is the most important specie capable of producing α amylase so far (Monga *et al*., 2011).

Substrates based on agriculture industry are generally used as cheaper source for amylase production (Mukesh kumar *et al*., 2012). Optimization of culture conditions is of utmost importance for better amylase production and paves the way for effective and fruitful commercialization (Roheena Abdullah *et al*., 2014). *A. niger* isolates from soil of livestock farms were screened for starch hydrolysis and optimized under physical and chemical conditions to determine production potential.

Materials and Methods

Aspergillus niger isolated from soil samples (n=145) collected from livestock farms located in and around Lahore district were screened for starch hydrolysis. Selected isolates of *A. niger* were evaluated for dynamics of amylases production potential under physical and chemical conditions.

Identification of *Aspergillus niger*: *A. niger* isolates were identified by macroscopic and microscopic characters. Macroscopic characters of colonies included were texture, growth pattern and pigmentation both on obverse and reverse sides of cultures. Microscopic characters observed were type of hyphae, spores, vesicles and metullae.

Screening for amylases production: *A. niger* isolates were initially screened for amylase production potential using starch agar (Meat extract 3g, peptic digest of animal tissue 5g, soluble starch 2g, agar 3%) using standard conditions (Ominyi *et al.*, 2013). Isolates were spotted on starch agar and incubated at 25ºC±3 under humidity for three days. Iodine solution was used to observe starch hydrolysis.

Optimization for amylases production: Spore suspensions were prepared by mixing a loop full of spores from pure culture of *A. niger* isolates in one mL of sterile normal saline. Spores were counted using neubar chamber and adjusted at 10^6 spores per mL (Morris & Nicholls, 1978). Effect of temperature, pH, substrate and substrate concentrations was observed by one factor one-time method. Selected isolates were inoculated in basal medium ($NaNO_3$ 1g, K_2 HPO_4 1g, $MgSO_4$ $7H_2$ O 0.5g, $FeSO_4$ 0.01g) having pH 6 and 1% of each substrate (maize, wheat bran and rice husk) with standardized inoculums (10^6 spores). These were incubated at three different temperatures 22, 28 and 37°C for 7 days (Khan & Yadav, 2011). Similarly, for pH optimization, basal medium with three different pH levels (4.5, 6 and 7.5) and 1% of maize, wheat bran and rice husk concentration were prepared and incubated at same temperature (37°C) for 7 days post inoculation of fungi (Varalakshmi *et al.,* 2009). At constant values of temperature (37°C) and pH (6), selected isolates were optimized for substrate concentration using 1, 3 and 5% maize, wheat bran and rice husk with constant inoculum and incubation period (Tripathy *et al.*, 2011). Post incubation of 7 days cultures were filtered using whatmann filter paper. The filtrate was used as a source of crude for qualitative and quantitative analysis of amylases (Lawal *et al.*, 2014).

Qualitative detection of amylases: Qualitative detection of amylases was carried out by iodine test and sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE). Iodine test was performed by the standard protocol. Filtrate (1mL) was reacted with 20 µL of iodine solution in a sterile glass test tube. The mixture was gently shaken, and color change observed (Yoo *et al*., 1987).

Molecular weights of starch hydrolyzing enzymes were determined by SDS-PAGE. Enzymes were partially purified by ammonium sulphate and re-dissolved in phosphate buffer saline. The enzymes were resolved on 12.5 percent separating polyacrylamide gel and visualized by commassie brilliant blue staining. Molecular weights of enzymes were calculated using standard curve plotted between relative flow values of protein ladder and their respective log molecular weights (Laemmli *et al*., 1970).

Quantification of amylases: Enzyme assay was performed by dinitrosalicylic method. One mL of filtrate (crude enzyme) and one mL of 1% soluble starch solution prepared in 500mM phosphate buffer having pH 6 were incubated at 37ºC. Post incubation of 30 minutes 3mL of DNS (3-5, Dinitro salicylic acid) reagent was added. This was incubated at boiling temperature for ten minutes. Absorbance was recorded at 570nm. Unit activity was calculated. One-unit activity was defined as amount of enzyme which liberated one µmol of reducing sugars from substrate at 37ºC in 30 minutes (Brindha *et al.*, 2011).

Statistical analysis

Data were analyzed by one-way ANNOVA followed by Duncan's multiple range posthoc test using statistical package for social sciences (SPSS version 16).

Results

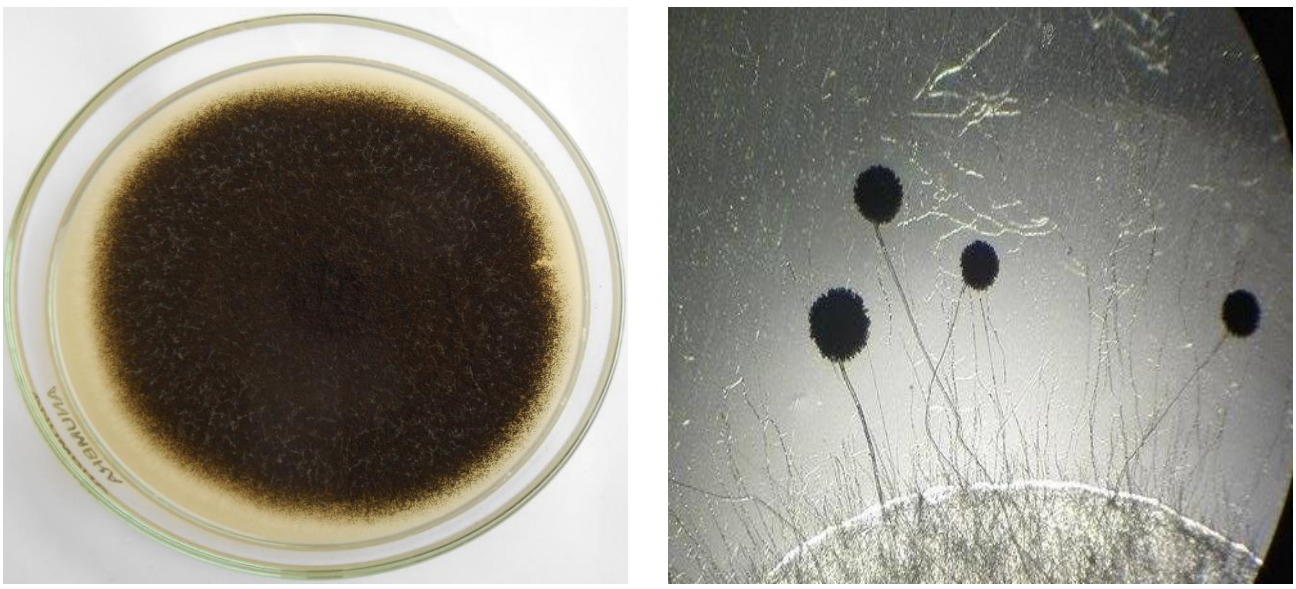
Aspergillus niger isolates from soil samples were screened for starch hydrolysis and optimized under different physical and chemical conditions for better production of amylases.

Identification of *A. niger*: The macroscopic characters observed on sabouraud dextrose agar were cottony texture, colonies with blackish brown and white margins on obverse side (Fig. 1a) without pigment production on reverse side. Microscopic characters observed at 400X magnification were vesicle having phialospores with conidiophore originating from foot cell and hyaline septate hyphae (Fig. 1b). Out of 145 soil samples, 6 isolates were identified as *A. niger*.

Screening for amylases production: Starch hydrolysis was detected by zone of hydrolysis (absence of blue color around colony) by pouring iodine solution on *A. niger* cultures (Fig. 2a). Out of (6) cultured on starch agar, three isolates ANS03, ANS06 and ANS10 showed higher hydrolyzing index (Diameter of clear zone/ Fungal colony diameter). The ratio of zone of hydrolysis to colony diameter of six isolates is presented in Table 1.

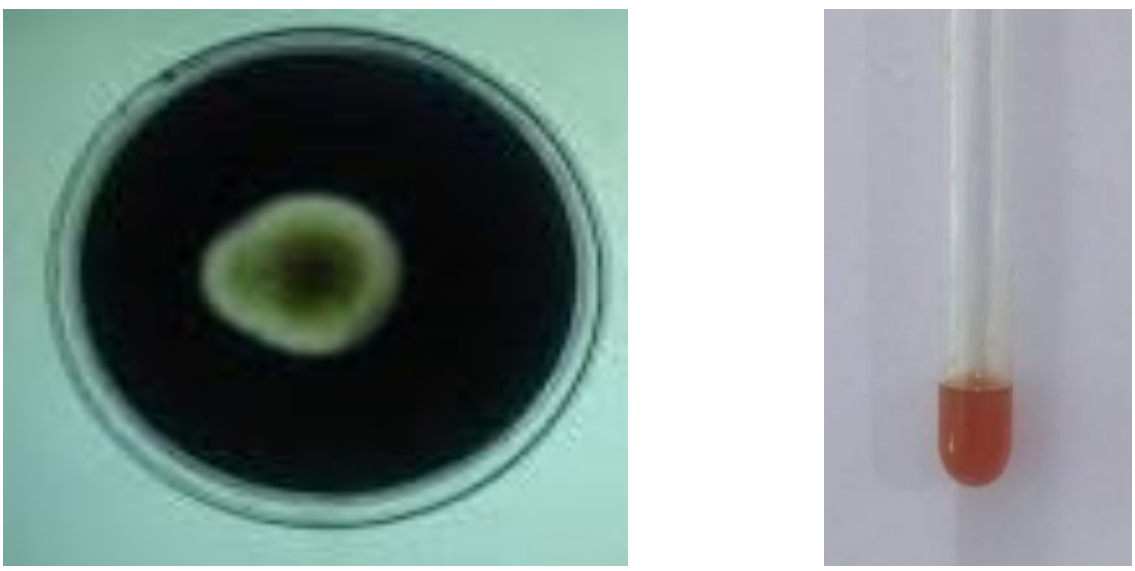
Qualitative analysis of amylases: In iodine test three isolates exhibited varying degree of starch hydrolysis from colorless (complete hydrolysis), to yellow (good hydrolysis), brown (moderate hydrolysis) and minute hydrolysis indicated by appearance of blue color on fixing filtrate (source of crude enzyme) and iodine solution (Fig. 2b). All isolates showed three major proteins on 12.5% resolving gel with molecular weights as 50.12kDa, 48.98kDa and 37.15kDa (Fig. 3).

Quantification of amylases: The amylase production potential of *A. niger* isolates was determined using 1% concentrations of three substrates at pH 6 at three different temperatures (Table 2). In case of maize, ANS03 showed highest amylase activity (13.13±.29) at 22°C, whereas at 28°C ANS06 was highest producer of amylases (15.24± .73). At 37°C ANS10 produced highest quantity of enzyme (7.47±.16). On wheat bran substrate the highest enzyme activity observed was of ANS10 at 37°C (15.47±.88) and 22°C (10.60±.18). Whereas at 28°C highest amylase activity (5.70±.00) observed was of ANS06. Similarly, highest enzyme activity was of ANS10 at 22°C (6.66±.15) and 28°C (6.13±.05) using rice husk substrate. Whereas at 37°C ANS06 (6.55±.08) was highest producer. On the whole the highest enzyme activity was found 15.47±.88 using wheat bran by ANS10 at 37°C and lowest 0.04±.01 by same isolate from rice husk. Statistical analysis revealed significant difference (p>0.05) among amylases production potential of three isolates at different temperatures.



(a) **(b)**

Fig. 1. Macroscopic and microscopic characteristics of *Aspergillus niger* isolate
(a): Macroscopic view and (b): Microscopic view



(a) **(b)**

Fig. 2. Screening of *Aspergillus niger* isolates for amylase production by iodine
(a): On starch agar and (b): Using filtrate of broth culture.

Table 1. Zone of hydrolysis to colony diameter ratio of *Aspergillus niger* isolates.

S. No.	Isolate No.	Zone of hydrolysis (cm)	Colony diameter(cm)	Starch hydrolysis index
1.	ANS01	0.5	2.7	0.19
2.	ANS02	0.3	3.0	0.10
3.	ANS03	0.6	2.5	0.24
4.	ANS06	0.5	2.0	0.25
5.	ANS08	0.1	3.2	0.03
6.	ANS10	0.7	3.5	0.20

Among the three selected isolates using one percent maize, the highest enzyme producer was ANS10 with 4.27±.09 IU activity at 37°C and 4.5 pH. At pH 6.0 ANS10 and at 7.5 ANS06 were best enzyme producers with 7.47±.16 and 5.97±.11 IU activity, respectively. Similarly, using wheat bran as substrate at pH levels 4.5 and 6 ANS10 was the highest producer with 3.92±.12 and 15.47±.88 enzyme activities. The highest enzyme producer was ANS06 with 7.05±.08 activity at pH 7.5. Using rice husk (1%) the highest amylase producers were ANS03 (3.92±.11), ANS06 (6.55±.08) and ANS03 (5.98±.13) at pH 4.5, 6 and 7.5 respectively (Table 3). Statistically significant difference (p<0.05) observed among amylase production potential at three different pH values.

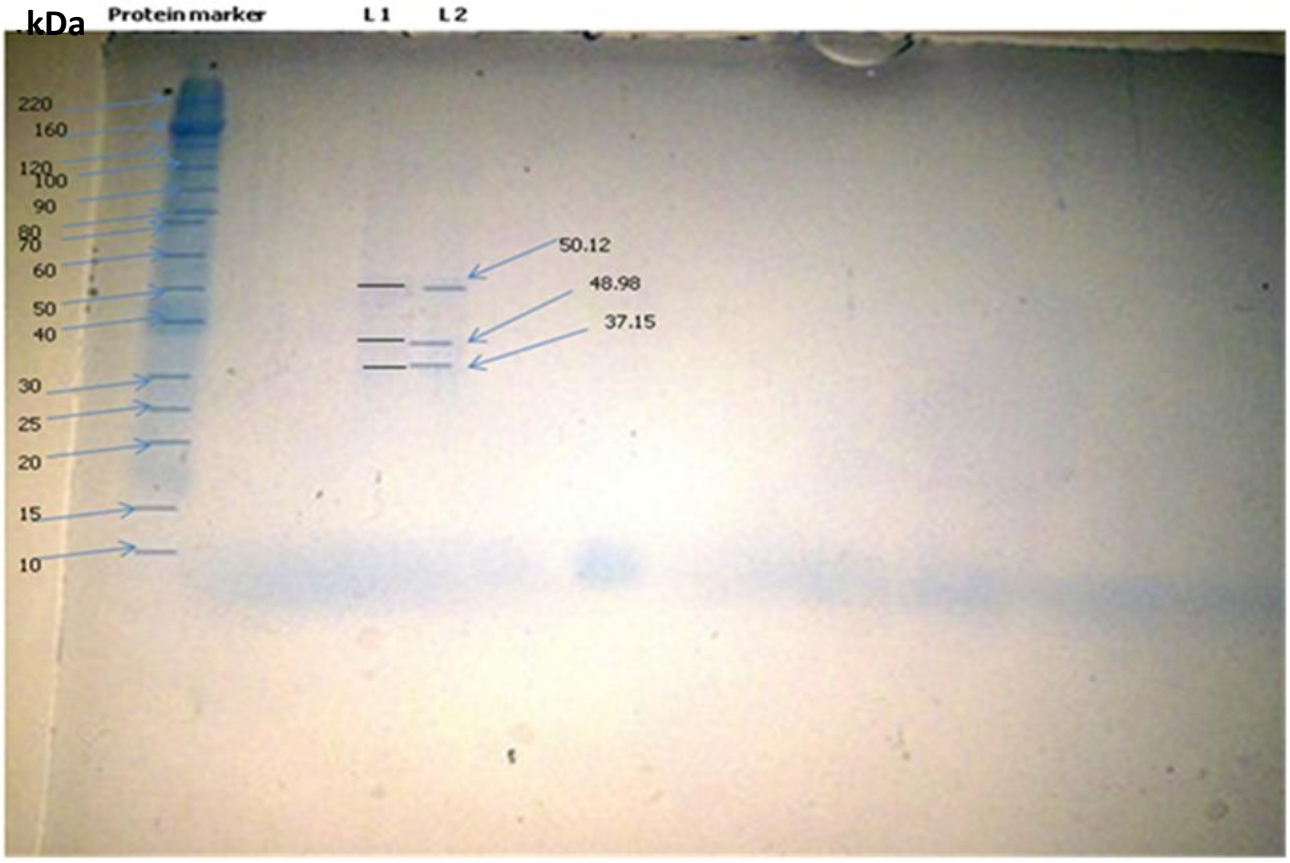


Fig. 3. Qualitative detection of amylases by sodium dodecyl sulphate polyacrylamide gel electrophoresis
kDa: Kilodalton and L1, L2: Filtrate of *Aspergillus niger* isolates.

Table 2. Dynamics in amylases production at different temperatures using one percent substrate and pH 6.

S. No.	Substrate	Isolate No.	22˚C	28 ˚C	37 ˚C
1.	Maize	ANS03	13.13 ± 0.29^{c}	11.92 ± 0.37^{b}	4.95 ± 0.08^{a}
		ANS06	5.17 ± 0.028^{c}	15.24 ± 0.73^{b}	38 ± 0.005^{a}
		ANS10	6.64 ± 0.011^{a}	15.20 ± 0.52^{c}	7.47 ± 0.16^{b}
2.	Wheat bran	ANS03	6.27 ± 0.09^{c}	4.65 ± 0.09^{b}	2.92 ± 0.16^{a}
		ANS06	6.49 ± 0.00^{c}	5.70 ± 0.00^{a}	6.12 ± 0.01^{b}
		ANS10	10.60 ± 0.18^{b}	4.95 ± 0.00^{a}	15.47 ± 0.88^{c}
3.	Rice husk	ANS03	5.76 ± 0.21^{c}	3.79 ± 0.11^{b}	0.56 ± 0.08^{a}
		ANS06	6.27 ± 0.08^{b}	4.02 ± 0.11^{a}	6.55 ± 0.08^{c}
		ANS10	6.66 ± 0.15^{c}	6.13 ± 0.05^{b}	0.04 ± 0.01^{a}

Means with same superscripts differ non-significantly and with different differ significantly

Table 3. Dynamics in amylases production at different pH using 1% substrate and temperature 37ºC.

S. No.	Substrate	Isolate No.	4.5	6	7.5
1.	Maize	ANS03	3.54 ± 0.04^{a}	4.95 ± 0.08^{c}	4.45 ± 0.08^{b}
		ANS06	3.68 ± 0.24^{b}	039 ± 0.0003^{a}	5.97 ± 0.11^{c}
		ANS10	4.27 ± 0.09^{a}	7.47 ± 0.16^{b}	4.38 ± 0.15^{a}
2.	Wheat bran	ANS03	3.83 ± 0.08^{b}	2.92 ± 0.16^{a}	3.97 ± 0.20^{b}
		ANS06	3.81 ± 0.08^{a}	6.12 ± 0.01^{b}	7.05 ± 0.08^{c}
		ANS10	3.92 ± 0.12^{a}	15.47 ± 0.88^{c}	6.92 ± 0.09^{b}
3.	Rice husk	ANS03	3.92 ± 0.11^{b}	0.56 ± 0.08^{a}	5.98 ± 0.13^{c}
		ANS06	3.80 ± 0.22^{a}	6.55 ± 0.08^{c}	4.21 ± 0.15^{b}
		ANS10	3.33 ± 0.08^{b}	0.04 ± 0.01^{a}	5.07 ± 0.10^{c}

Means with same superscripts differ non-significantly and with different differ significantly

Table 4. Dynamics in amylases production at different substrate concentrations at pH 6 and temperature 37 ˚C.

S. No.	Substrate	Isolate No.	1%	3%	5%
1.	Maize	ANS03	4.95 ± 0.08[a]	21.10 ± 0.8[b]	40.04 ± 0.37[c]
		ANS06	10.28 ± 0.09[a]	24.67 ± 0.64[b]	63.20 ± 0.37[c]
		ANS10	7.47 ± 0.16[a]	30.87 ± 0.32[b]	49.37 ± 0.68[c]
2.	Wheat bran	ANS03	2.92 ± 0.16[a]	21.53 ± 1.04[c]	17.68 ± 0.72[c]
		ANS06	6.12 ± 0.01[a]	29.00 ± 0.37[b]	150.61 ± 3.26[c]
		ANS10	15.47 ± 0.88[a]	29.63 ± 0.35[b]	86.23 ± 1.31[c]
3.	Rice husk	ANS03	0.56 ± 0.08[a]	21.37 ± 0.24[c]	20.78 ± 0.42[b]
		ANS06	6.55 ± 0.08941[a]	15.45 ± 0.78[b]	16.77 ± 0.09[c]
		ANS10	0.04± 0.01[a]	57.53 ± 0.65[c]	17.77 ± 0.52[b]

Means with same superscripts differ non-significantly and with different differ significantly

Using maize as substrate ANS06 (63.20±.37) was the highest amylase producer at 5 percent concentration. Similarly using wheat bran ANS06 (150.61±3.26) was the highest enzyme at 5 percent concentration. Using rice husk ANS03 (20.78±.42) was the highest producers of amylases (Table 4). Amylase production potential of selected isolates vary significantly (p<0.05) by statistical analysis except ANS03, in case of wheat bran where mean amylase activity at 1% concentration vary significantly from 3 and 5%. However, at 3 and 5% amylase production potential of ANS03 using wheat bran was statistically non-significant (p>0.05).

Discussion

Amylases have a lot of application in various industries. To meet the industrial demands in economical ways, efforts are being done to find cheaper substrates. For this purpose, agro-industrial wastes are being analyzed (Casarotti *et al.*, 2017). Using cheaper substrates physical and chemical parameters for optimization of production potential were determined. The conditions can be set on industrial scale to get higher yield of enzyme. Screening for starch hydrolysis was done on starch agar. Starch hydrolyzing index of all isolates of *A. niger* is less than 1. Starch hydrolyzing index around one had been reported by *A. niger* isolates which is in contrast with present (Janda *et al.*, 2009; Khokhar *et al.*, 2011). However, the main purpose of this experiment was screening. It was not for the quantification of enzyme.

Qualitative analysis for amylases was done by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins which were separated on gel had molecular weights of 50.12kDa, 48.98kDa and 37.15kDa in agreement to the molecular weight range (40 to 70kDa) were reported previously (El-Fallal *et al*., 2012). In another experiment molecular weight of alpha amylase from *Aspergillus niger* determined was 43kDa (Varalakshmi *et al*., 2009). Molecular weight of amylase produced by *A. oryzea* was 68kDa (Ramachandran *et al*., 2004). Protein responsible for starch hydrolysis had 56kDa molecular weight which was purified from *Aspergillus flavus* (Sidkey *et al.*, 2011). So, these proteins were suspected as amylases.

Temperature is one of the key factors influencing the amylase production potential and fungal growth. Temperature used in most of the studies conducted for amylase production and optimization was in the range of 25°C to 37°C (Francis *et al.*, 2003). In present study one *A. niger* isolate (ANS03) showed best enzyme activity at 22°C. The highest enzyme activities of other two isolates ANS06 and ANS10 were at 28°C. *A. niger* exhibited highest enzyme activity at 35°C (Rajasekar & Dhamodharan, 2013). Better production of enzymes had been observed at 40°C for other isolates as well (Abdalwahab *et al*., 2012). Maximum enzyme activity at 37°C was observed using groundnut as substrate, in case of coconut oil it was 40°C, in case of rice bran, the maximum activity was found at 45°C (Suganthi *et al*., 2011). Optimum temperatures for higher yield of amylases had been observed to be 28°C, 22°C and 32.2°C by different *A. niger* isolates (Roses & Guerra, 2009). Results of present study were strengthened by previous reports (Varalakshmi *et al*., 2009; Khan & Yadav, 2011). It can be concluded that temperature have an effect on production potential of amylases by *A. niger*.

The pH of medium affects fungal growth and amylase production. Different pH levels (5, 6.5 and 9) to optimize amylase production by fungal isolates including *A. niger* (Pathak & Narula, 2013). In case of *A. niger* maximum enzyme activity was obtained at pH 9. In another experiment 4.5, 5.5 and 6.5 pH levels were used for amylase producing *Aspergilli.* The optimum enzyme activity was observed at 4.5 (Shafique *et al*., 2009). *Aspergilli* including *A. niger* were cultured at 4, 5, 6, 7 and 8 pH values to optimize enzyme production (Abdalwahab *et al*., 2012). Comparable to present study optimum pH 6 had been documented for highest amylase production (Rajasekar & Dhamodharan, 2013). Optimization experiments were conducted of *A. niger* at 4.5,5, 6.5, 7, 7.5,8 and 9. Optimum pH was different for different substrates. In case of rice husk and wheat bran optimal pH values were 5.5 and 6.5, respectively. There was increase in enzyme activity with increase in pH up to 8, while at 9 enzyme activities decreased (Suganthi *et al*., 2011). In other experiments highest enzyme production observed was at pH 6.2 and 7.5 (Varalakshmi *et al*., 2009; Khan & Yadav, 2011). In agreement pH 6 had been recorded and results of present study were strengthened (Roses & Guerra, 2009). Results of present study were in agreement with these findings in relation to pH value for enzyme production.

Source of starch affect amylase production potential of *A. niger*. Rice flour as a substrate was used in optimization of *Aspergilli* for amylase production (Abdalwahab *et al.*, 2012). In another experiment, rice bran was used as growth medium for optimization of *A. niger* for amylase production (Rajasekar & Dhamodharan, 2013). Other substrates such as wheat bran, coconut oil cake, ground nut cake and black gram bran had been used and black gram bran proved best (Suganthi *et al.*, 2011). Khan and Yadav (2011) optimized the *A. niger* using wheat bran, rice husk and vegetable peels as a substrate and found the optimum condition for highest enzyme activity. Varalakshmi *et al*., (2009) used wheat bran as substrate for *A. niger* amylase optimization experiments. Adejuwon (2010) isolated *A. niger* and optimized growth conditions using starch as substrate. In present study maize, wheat bran and rice husk were used as a source of starch maize was the best.

Along with substrate, its concentration is very important in optimization experiments. Abdalwahab *et al*., (2012) found that there was maximum enzyme activity at 2% rice flour substrate. Adejuwon (2010) observed increase in activity of enzyme with increased concentration of starch. In present study, direct relation was found between substrate concentration and enzyme activity.

Conclusion

Aspergillus niger (ANS06) was the highest producer of amylases at pH (6) and 37ºC using 5% wheat bran as substrate. This isolate can be used for amylase production on industrial scale.

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(Received for publication 22 April 2019)