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Full Length Research Paper

Production of extracellular amylase from agricultural residues by a newly isolated *Aspergillus* species in solid state fermentation

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The production of extracellular amylases by solid state fermentation (SSF) was investigated employing our laboratory isolate *Aspergillus* sp.MK07. Various agricultural residual substrates like wheat bran, rice bran and green gram husk were studied for enzyme production. Highest enzyme production was obtained with wheat bran as a substrate. Effects of process variables, namely: incubation period, temperature, initial moisture content, pH, supplementary carbon, nitrogen source and inoculum level on production of amylase have been studied and accordingly, optimum conditions have been determined. It was found that amylase production was highest at 120 h of incubation period at 30°C, 70% initial moisture content, 5.0 pH and 5% inoculum level. Supplementation of carbon (starch) and nitrogen source (peptone) showed an increase in amylase production and the highest amount of amylase production obtained under all optimized conditions was 164 U/g.

Key words: Solid state fermentation, optimization, *Aspergillus*, fermentation, amylases.

INTRODUCTION

Amylases are a group of important enzymes which are mainly employed in the starch processing industries for the hydrolysis of polysaccharides like starch into simple sugars (Akpan et al., 1999; Mitchell and Lonsane, 1990; Damien et al., 2010). Amylases accounts for about 30% of the world's enzyme production (Vander et al., 2002; Rita et al., 2009). Due to wide range of application of amylases in various sectors like confectionary, baking, paper, textile, detergent and pharmaceutical, many are gaining the attention of researchers (Sivaramakrishnan et al., 2006; Dhanya et al., 2009). Amylases are produced on a large scale normally by submerged fermentation (SmF) but in recent years, the attention towards solid state fermentation (SSF) is increasing due to some additional advantages like lower capital expenditure, cheaper fermentation media, superior productivity, reduced energy requirements and absence of rigorous control of fermentation parameters (Ellaiah et al., 2002; Marian et al., 2010).

In SSF, the usage of water is also less compared to SmF and produces lower waste water; relatively the downstream processing cost is also cheap (Pandey et al., 1990; Sangeeta and Rintu, 2010). Many researchers have done enormous amount of work with various bacterial strains using SSF and the advantages of SSF over SmF are widely discussed in literature (Lonsane and Ramesh, 1990; Ajay pal et al., 2010). Generally, in SSF, the products are formed at or near the surface of the solid material with low moisture content (Pandey et al., 1990). Many researchers have studied amylases production with a wide variety of substrates and microorganisms like bacteria, yeast and fungus (Sivaramakrishnan et al., 2006: Ashis et al., 2009). Due to the ever increasing demand of this enzyme, people are still trying to increase the productivity of amylases by a variety of approaches like selection of a high enzyme producing strain, process optimization, usage of cheaper substrates, effective downstream processing, etc (Lalit et al., 2010). Among the various types of amylases produced, commercially

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Abbreviations: SSF, Solid state fermentation; **SmF,** sub merged fermentation.

thermostable amylases are gaining much more advantages in comparison to other types (Popovic et al., 2009).

Many researchers have done good amount of work on isolation of some of the thermo tolerant strains (Audinarayana et al., 2005). Several reports have been published by many researchers showing amylase production with *Aspergillus* sp. (Ellaiah et al., 2002; Ahmad et al., 2010). However, it would be economically competitive if the isolated strain shows some advantages over existing products and every strain has its own unique microbial and biochemical properties (Prakasam et al., 2006; Imran et al., 2010). The global market for enzymes is expected to increase by 3.3% annually (Sivaramakrishnan et al., 2006). The present investtigation is aimed to isolate an *Aspergillus* sp. locally which can grow on inexpensive agricultural residual substrates and produce amylase at a competitive rate.

MATERIALS AND METHODS

Fungal strain

Isolation of the fungal strains was carried out with various soil samples collected from local starch plant near Hyderabad. *Aspergillus* species were identified by standard blotter method (Abdul and Anderson, 1973). They were identified on the basis of morphological characteristics and certain standard confirmatory tests.

Isolation of the strain

The soil suspension was diluted 10^{-3} to 10^{-6} times and 0.5 ml of each diluted suspension was then transferred by spread plate method with a sterile glass spreader on petri plates containing Czapex Dox starch agar medium .The petri plates were incubated at 30°C for 4 - 5 days. Based on the zone of clearance on the starch agar plates, 13 colonies were picked and individual amylase activity of selected colonies were carried out; strain MK 07 was used for further studies. The young colonies of fungal cultures were aseptically picked and transferred to Czapex Dox starch agar slants with 1% starch. These slants were then incubated at 30°C for 4 days and after sufficient growth, they were stored at 4°C in the refrigerator.

Inoculum preparation

Actively growing and heavily speculating ten days old Czapex Dox starch agar slant culture was added to 10 ml sterile 0.85% sodium chloride salt solution. The spores were gently scraped off with the help of a sterile needle and contents were passed through glass wool so as to obtain spore inoculums free of mycelial bits. A volume of 1 ml of spore suspension contained more than 2.6×10^6 spores.

Substrates

Different agro industrial residual substrates like wheat bran, rice bran and green gram husk were collected from the local market and processed using USA standard sieve set of numbers 10 and 18 to obtain mean particle size of 2.0 - 1.4 and1.4 - 1.0 mm and stored till further use.

Solid state fermentation

Different substrates were taken in 250 ml Erlenmeyer flasks and to each flask, a predetermined quantity of water was added to adjust the moisture content, mixed thoroughly and autoclaved at 121°C for 15 min at 1 kgF/cm^2. After cooling the flasks to room temperature, they were inoculated with varying concentrations of fungal spores. To investigate the optimum inoculum level, the inoculum concentration was increased accordingly. Initial enzyme concentration was checked with three substrates and further optimization was carried out using wheat bran as substrate for SSF and all further experiments were carried out systematically in such a way that the parameter optimized in one experiment was maintained at its optimum level in further experiments.

Enzyme extraction

Alpha amylase was extracted from SSF medium by a simple contact method (Lonsane and Ramesh, 1990). After specified incubation time in each case, 100 ml of sodium phosphate buffer pH 6.9 was added to each flask. The flasks were shaken at 150 rpm for half an hour and the material was filtered through whatman filter paper no 1, the filtrate was centrifuged at 1000 rpm for 10 min at -10°C. The supernatant was carefully collected and used as crude enzyme extract.

Measurement of amylase activity

Amylase activity was determined by the spectrophotometric method described by Bernfield (1955). In an assay mixture containing enzyme extract, starch as substrate and DNS as coupling reagent, one unit of amylase activity is defined as the amount of enzyme that releases one micromole of reducing sugar as glucose per minute under the assay conditions. Enzyme activity is expressed as units per gram dry substrates (U/g).

RESULTS

Evaluation of different agricultural residues as substrates for amylase production

The results in the present study indicated that amylase production pattern varied with the type of raw material used. Maximum enzyme production obtained with wheat bran as substrate was 128 U/g. To carry out further experiments, wheat bran was used as the substrate. These results are shown in Figure 1.

Influence of varying inoculum levels

Varying concentrations of inoculum levels were studied in this experiment and the various range of inoculum (% spores) selected in this experiment ranged from 3 to 15%. Enzyme production varied with percentage of inoculum and the maximum enzyme production was 106 U/g with 5% inoculum. Increase of inoculum level from 5 to 10 or 15% showed a marginal decrease in amylase production. The results are shown in Figure 2.

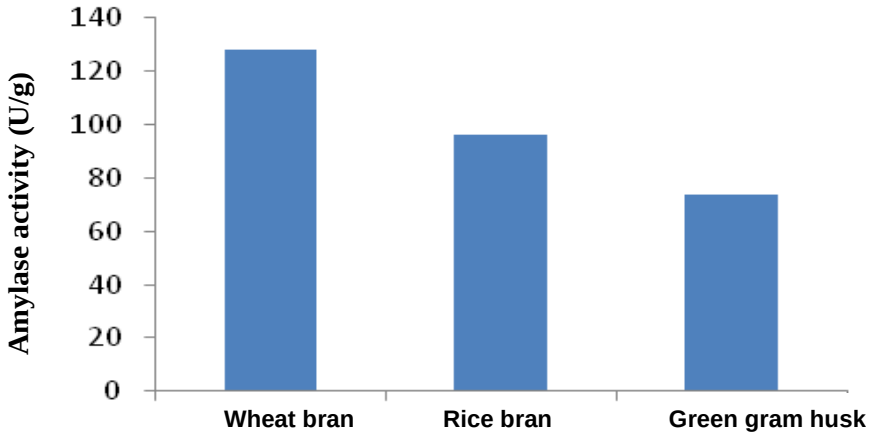

Figure 1. Amylase activity with different agricultural residual substrates.

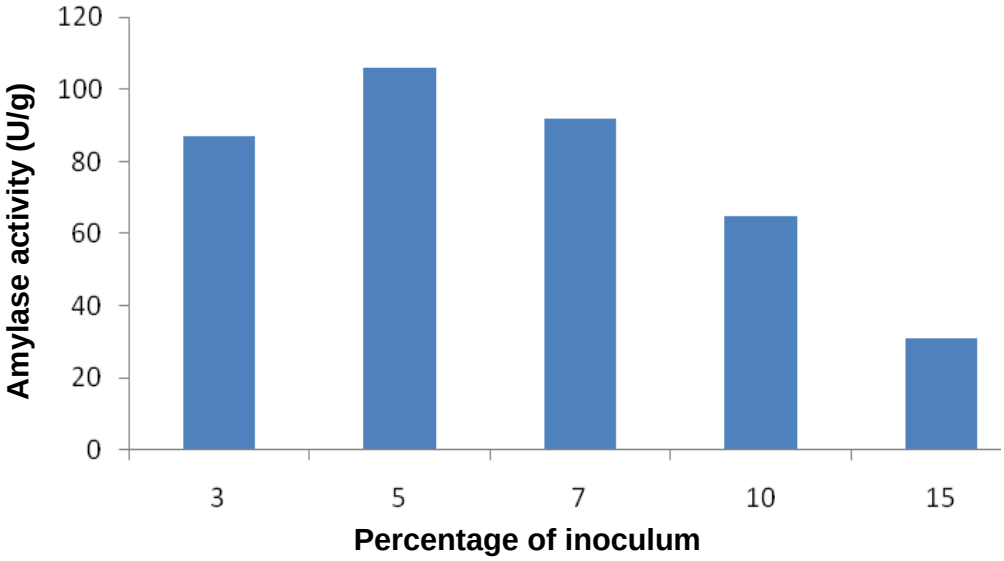

Figure 2. Amylase activity at varying percentages of inoculum levels.

Role of incubation time

The results of the present study showed that amylase production increased with increase in incubation time linearly till 120 h and on further incubation, there was a decrease in the amylase production (Figure 3). Maximum amylase production was obtained at 120 h (132 U/g).

Influence of initial moisture content

In the present study, maximum enzyme production was obtained with an initial moisture content of 70% and the maximum enzyme production obtained was 116 U/g. Up to 70%, there was a linear increase in enzyme production and upon further increase, there was a marginal decrease in enzyme production (Figure 4).

Influence of pH

The results in the present experiment revealed that the strain isolated had an optimum pH of 5.0 with a maximum enzyme activity of 119 U/g. With increase in pH value from strong acidic phase to a neutral phase enzyme activity increased up to a pH of 5.0 and upon further increase in pH, enzymatic activity decreased as shown in Figure 5.

Influence of temperature

The isolated *Aspergillus* strain was tested in a wide range of temperatures ranging from 20 to 40°C. Maximum amylase production was obtained at a temperature of 30°C (114 U/g) as shown in Figure 6. In the present

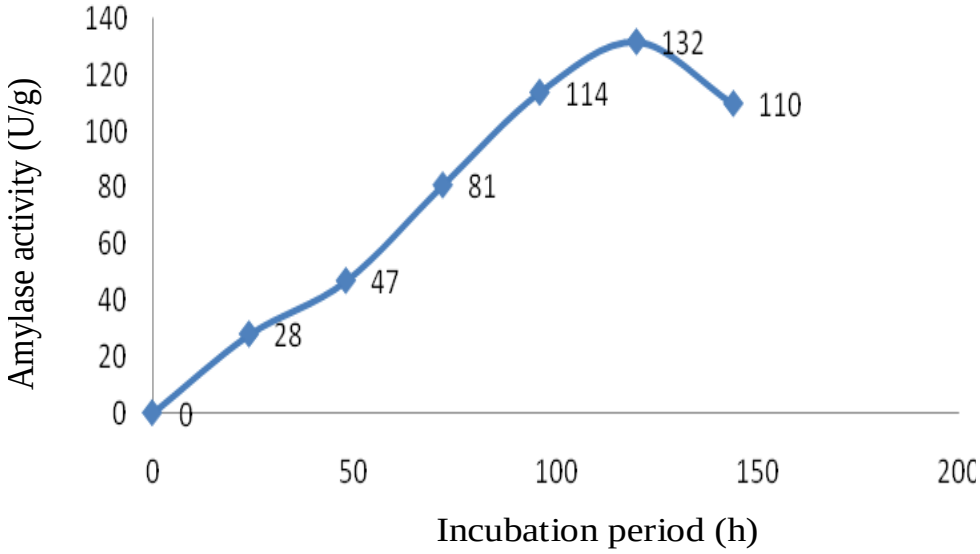

Figure 3. Amylase activity at different hours of Incubation.

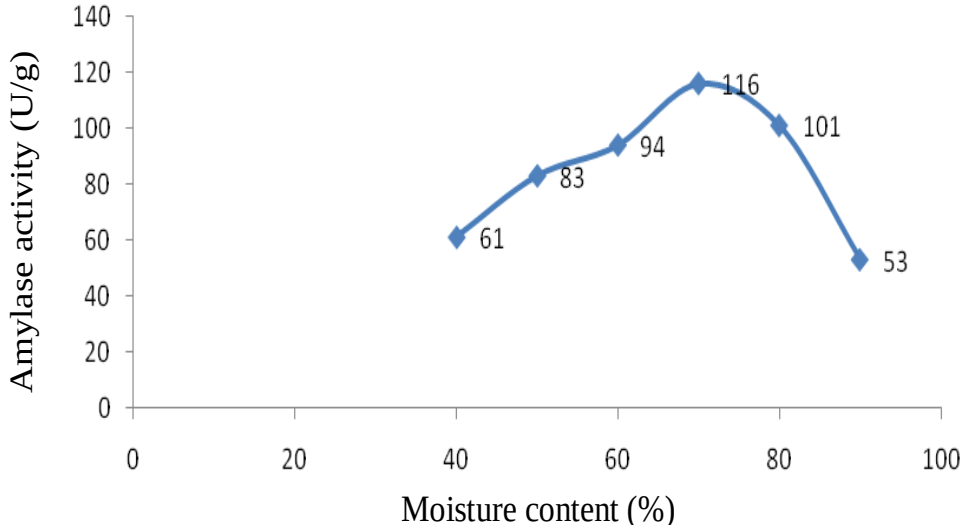

Figure 4. Amylase activity at varying concentrations of initial moisture content.

experiment with increase in temperature, enzyme production increased up to a certain level and upon further increase of temperature, production decreased.

Effect of additional carbon supplementation on the substrate

Several carbon substrates like glucose, starch, maltose, lactose and sucrose were tested along with control to evaluate the enzyme production by SSF. On supplementation of various carbon substrates, maximum enzyme production was exhibited by starch (1% w/w). Results showed different impact on enzyme production with different substrates (Figure 7). The maximum enzyme production obtained was 139 U/g with 1% w/w starch. Supplementation of sucrose also enhanced enzyme production.

Effect of additional nitrogen source supplementation on the substrate

The effect of additional nitrogen sources like peptone, urea, sodium nitrite, yeast extract and ammonium nitrite at 1% supplementation on the medium was tested and the results were further analyzed. A control flask was maintained without additional nitrogen supplementation.

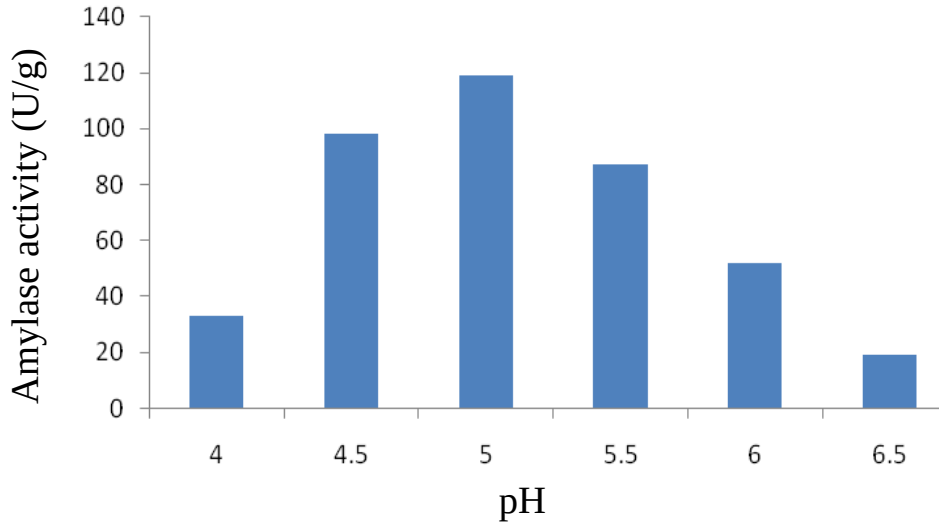

Figure 5. Amylase activity at different pH.

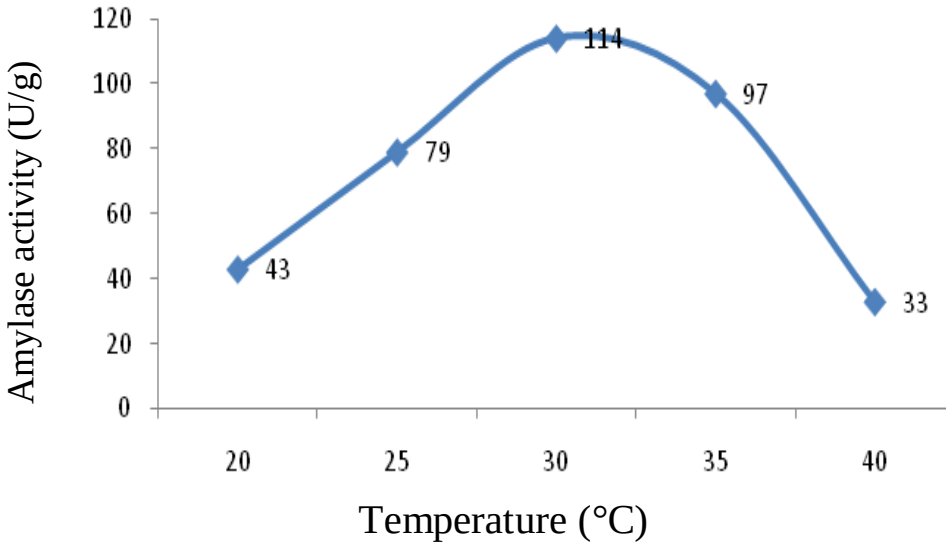

Figure 6. Amylase activity at different incubation temperatures.

Among all the nitrogen sources tested, the maximum enzyme production was obtained with peptone (1% w/w) (Figure 8). Maximum enzyme production obtained was 137 U/g. Urea also showed a considerable amount of increase in amylase production compared with the control.

DISCUSSION

The selection of a suitable agricultural residue as a substrate for SSF is one of the most critical factor to be considered. Several substrates have been screened for high enzyme production through SSF (Ashis et al., 2009). The availability and the cost of the raw material are two important parameters that have to be considered while selecting a raw material in SSF (Pandey et al., 2000). The substrate selected should allow the maximum growth of the organism and also should ease in high product formation. In the present study, three agricultural residual substrates like rice bran, wheat bran and green gram husk were evaluated for their maximum enzyme production. Wheat bran supported the highest enzyme production compared to the other two substrates and these results were similar to the observations made by Ellaiah et al. (2002).

Maximum enzyme production could be obtained only after a certain incubation time which allows the culture to

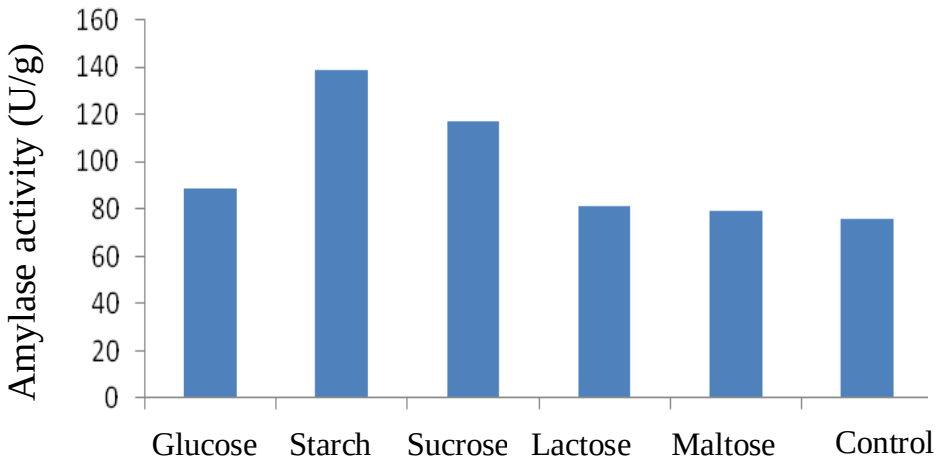

Figure 7. Amylase activity with additional supplementation of various carbon substrates.

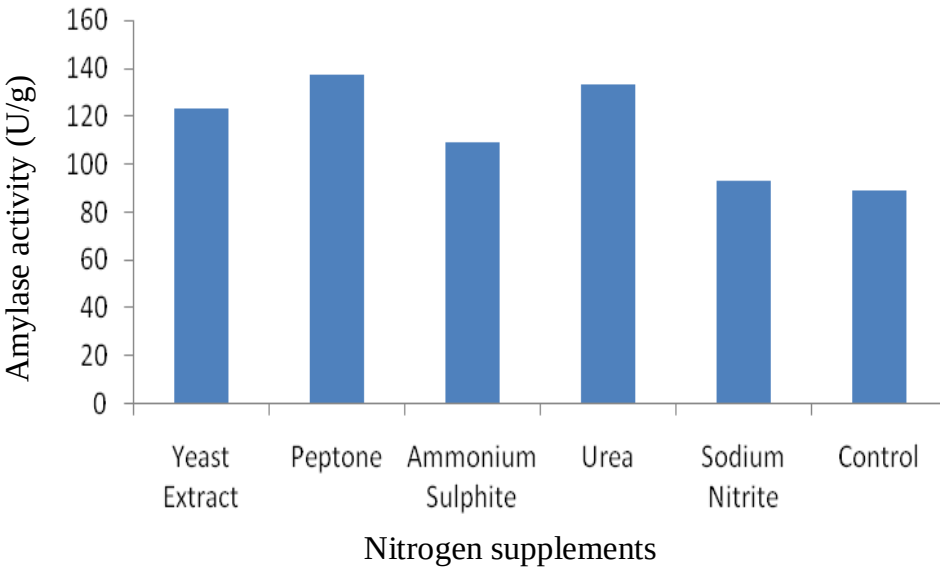

Figure 8. Amylase activity with additional supplementation of various nitrogen substrates.

grow at a study state. Enzyme production of each strain is based on the specific growth rate of the strain. Growth rate and enzyme synthesis of the culture are the two main characteristics which are mainly influenced by incubation time (Ellaiah et al., 2002). The results obtained in the present experiment are in accordance with the results expressed by Audinarayana et al. (2005). Similar observations were also made by Olama et al. (1989) with *Aspergillus flavus* and *Penicillium purpurescence.*

Initial moisture content plays an important role in enzyme production during SSF. In SSF, most of the microbial growth and product formation takes place at or near the surface of the solid substrate. Thus, it is very crucial to provide optimized water level that controls the water activity of the fermenting substrate for achieving maximum product (Pandey et al., 2000; Reeta et al., 2009). Ellaiah et al. (2002) reported that 80% of initial moisture content was optimum for the *Aspergillus* strain. Kunamneni et al. (2005) reported that 90% of the initial moisture content is optimum for amylase production by *Thermomyces lanuginosus.*

Growth and metabolism along with enzyme production is governed by an important factor called pH (Sivaramakrishnan et al., 2006). Amylase production by microbial strains strongly depends on the extracellular pH, as culture pH strongly influences many enzymatic reactions and also for the transport of various components across the cell membrane (Nahas and Waldermarin, 2002; Ellaiah et al., 2000). Different organisms have different pH optima and any modification in

their pH optima could result in a decrease in their enzyme activity (Lehninger et al., 2008; Radhounane et al, 2009). Hema et al (2006) observed pH 6.5 as optimum for *Bacillus sp*. Our results were also in accordance with the observations made by Nahan and Waldermarin (2002), stating that pH 5.0 was optima for *Aspergillus* sp. Olama et al. (1989) stated that pH 7.0 was optima for *A. flavus*.

Temperature is one of the most important parameter to be optimized for maximum enzyme production. Optimum temperature for maximum enzyme production depends on the characteristics of the strain (Ahmad et al., 2010). In SSF, temperature plays a very important role in enzymatic synthesis (Lonsane and Ramesh, 1990; Imran et al., 2010). Mukherjee and Majomdar, (1993) also reported 30°C as optimum temperature for *A. flavus*. Sadhukhan et al. (1990) reported that 45°C was optimum for *Myceliophthora thermophila*. The present investigation reports were comparable to the results expressed by Ellaiah et al. (2000). Olama et al. (1989) reported maximum enzyme activity by *A. flavus* at 45°C. Hayashida et al. (1998) also expressed similar results stating 30°C as optimum for *A. flavus*. Some thermophilic strains like *Thermotoga maritima* also showed maximum enzyme production at 30°C, as stated by Vieilli et al. (2001).

Many researchers have studied the effect of additional carbon supplementation on the substrates (Karima et al., 2009). Ellaiah et al. (2002) reported maximum enzyme activity on supplementation with fructose. Anto et al. (2006) reported maximum enzyme production on supplementation with sucrose. Sadhukhan et al. (1990) results were also in accordance with the results of our experiment stating that starch was the best supplement for *M. thermophila*.

The present study showed some similarity with the observations made by Pandey, (1990) which reported that there was high enzyme production on supplementation of sodium nitrate as nitrogen source for *A. niger*. Anto et al. (2006) reported maximum enzyme production on supplementation of yeast extract to the medium for fungal culture. Ellaiah et al. (2002) obtained maximum enzyme production on supplementation of urea. Cherry et al. (2004) obtained maximum enzyme production upon supplementation of ammonium phosphate as a nitrogen source. Shaista et al. (2003) reported maximum enzyme activity upon supplementation with 0.2% peptone as a nitrogen source for *Bacillus* species.

Conclusion

The results obtained in the present study indicated that *Aspergillus* sp. could be a potential strain for amylase production by SSF with wheat bran as the substrate. The enzyme production was influenced by various physiological and chemical nature of the substrate as well as the conditions during SSF. Among the various parameters screened for SSF, pH, temperature, supplementation of carbon and nitrogen sources along with incubation period played an important role for higher amount of amylase production. After optimization of individual parameters separately, when further experi-ment was carried out with all optimized parameters, amylase production increased to a maximum of 164 U/g.

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