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Microorganisms have the ability to secrete enzymes when they are grown in the presence of certain substrates. Amylases are among the most important industrial enzymes and are of great significance in biotechnological studies. Amylases have potential application in a wide number of industrial processes such as food, fermentation, textile, paper, detergent and pharmaceutical industries. Starch is an important storage product of many economically important crops such as rice, wheat, maize, tapioca and potato. In the present study, α-amylase was produced from rice waste water by *Aspergillus niger* NCIM 1342 using submerged fermentation technology. Utilization of rice waste water as the substrate for amylase production reduces the production cost and disposal problem of organic wastes. Result showed that in submerged condition maximum α-amylase was produced when only rice waste water and distilled water was used in the ratio 3:1 after 3 days of incubation at 30^0C.

Key words: α-amylase, *Aspergillus niger*, submerged fermentation, rice waste water

INTRODUCTION

Amylases are a group of hydrolases which can specifically cleave glycosidic bonds in starch. There are two important groups of amylases which includes glucoamylase and α-amylase. Glucoamylase (exo-1,4-á-D-glucan glucanohydrolase, E.C. 3.2.1.3) that hydrolyze single glucose units from the non-reducing ends of amylase and amylopectin (Anto *et al*. 2006) and α-amylases (endo-1, 4-α-D-glucan glucohydrolase. E.C. 3.2.1.1) are extracellular enzymes that can randomly cleave 1, 4-α-D-glucosidic linkages between adjacent glucose units inside the linear amylase chain (Castro *et al*. 2010; Anto *et al*. 2006; Pandey *et al*. 2005). Spectrum of applications of alpha-amylase has widened in many sectors such as clinical, medicinal and analytical chemistry. Besides their use in starch saccharification, they also find applications in baking, brewing, detergent, textile, paper and distilling industry (Ramachandran *et al*. 2004).

Industrial enzymes have been produced from plant, animal and microorganisms. The concentration of enzyme in plant source is generally low but starch processing industries requires large quantities of enzyme. On the other hand if the enzyme is from animal source it is generally obtained from the by-product of meat industry and so its supply is limited. However the α-amylase from microbial source can be produced in abundant quantities. Amylase has been derived from several fungi, yeasts, bacteria and actinomycetes, however, enzymes from fungal and bacterial sources have dominated applications in industrial sectors. Major advantage of using fungi for the amylase production is the economical bulk production capacity (Shah *et al*. 2014). Many species of *Aspergillus* and *Rhizopus* are used as a fungal source of α-amylase (Pandey *et al*. 2005). Usually production of amylase from fungi has been carried out using well defined chemical media by submerged fermentation (SmF) and solid state fermentation (SSF) (Miranda *et al.*1999), although traditionally these have been obtained from submerged cultures because of ease of handling and greater control of environmental factors such as temperature and pH. Due to the increasing demand for this enzyme in various industries, there is enormous interest in developing enzymes with better properties such as raw starch degrading

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amylases suitable for industrial applications and their cost effective production techniques (Shivaramakrishnan *et al*. 2006).

For amylase production starchy substrate is required. The submerged productions of α-amylase using synthetic media have been reported by many workers (Tigue *et al*. 1995; Haq *et al*. 1998; Hamilton *et al*. 1999). The contents of synthetic media such as nutrient broth, soluble starch as well as other contents might be replaced with low cost starchy substrates. Pure substrate is too costly for enzyme production ([El-Naggar and Abdelwahed, 2012) and food industry have problem with disposal of high organic waste which have direct concern with environmental pollution when discharge to estuaries. One of such high starchy waste material is rice waste water which is a sticking water obtained after boiling rice. Rice water is the suspension of starch obtained by draining boiled rice (*Oryza sative*) or by boiling rice. Rice water is also a milky liquid which contain vitamin B, E and mineral. Rice water is relatively containing good source of carbohydrate, calcium, iron, and vitamin. The protein content is about 36-58% and presence fat is 16-25% (Mathew *et al*. 2016). The present study is designed to evaluate the production of α-amylase by using *Aspergillus niger* NCIM 1342 through submerged fermentation using rice waste water as the substrate and optimization of different process parameters for maximum production of α-amylase.

MATERIALS AND METHODS

Organism used

Aspergillus niger NCIM 1342 was grown on Czapek Dox agar medium and subcultured monthly and stored at 4^0C.

Amylase production by submerged state fermentation using rice waste water (Medium I) Submerged fermentation was carried out in 100 ml Erlenmeyer flask by taking 20 ml of rice waste water (used as a carbon source) and distilled water mixed in the ratio 1:1. The medium was sterilized by autoclaving followed by cooling to room temperature and inoculated with one 8mm diameter's disc of *Aspergillus niger* NCIM 1342 from Czapek Dox media plates using sterile cork borer. After inoculation medium containing flasks were incubated at 30^0 C on orbital shaker at 120 rpm for fermentation.

Amylase production by submerged state fermentation using rice waste water supplemented with various mineral salts (Medium II)

Submerged fermentation was carried out in 100 ml Erlenmeyer flask by taking 20 ml of rice waste water (used as a carbon source) and distilled water mixed in the ratio 1:1 and supplemented with different mineral salts at 0.1% concentration [$MgSO_4$, $(NH_4)_2SO_4$, KH_2PO_4, K_2HPO_4 and Na-citrate] (Ekperigin, 2007). The medium was sterilized by autoclaving followed by cooling to room temperature and inoculated with one 8mm diameter's disc of *Aspergillus niger* NCIM 1342 from Czapek Dox media plates using sterile cork borer. After inoculation medium containing flasks were incubated at 30^0 C on orbital shaker at 120 rpm for fermentation.

Extraction of enzyme

After fermentation the enzyme was extracted for alpha-amylase activity assay. Fermented broth was filtered through Musleen cloth to remove mycelia of *Aspergillus niger* NCIM 1342. This filtrate was centrifuged at 10,000 rpm for 10mins at 4^0C and clear supernatant was used as a source of the enzymes (Harikrishna *et al*. 2000).

Enzyme assay

A reactive mixture contained 0.5 ml of 1% (w/v) starch in 0.05 M citrate buffer (pH 4.8) and 0.5 ml of culture supernatant. The mixture was incubated at 50^0C for 30 min (Ghose, 1886). The reducing sugar released was measured using 3, 5-dinitrosalicyclic acid (DNSA). Resulting reducing sugar was measured at 540 nm (Miller, 1959). One international unit (IU) was defined as the amount of enzyme that releases one milligram of reducing sugar (glucose equivalents) per minute under standard assay conditions.

Incubation time

The fermentation was carried out for up to 7 days for medium I and 11 days for medium II. Enzyme assay was done after every 24 hrs interval for both the cases to get maximum enzyme activity.

Dilution ratio

To determine effect of dilution ratios of rice waste water and distilled water on the enzyme production, the media was prepared using different ratios of rice water and distilled water, i.e. 20ml rice water + 0ml distilled water, 15ml rice water +5 ml distilled

water, 10ml rice water +10ml distilled water, and 5ml rice water + 15ml distilled water.

Inoculum size

The inoculum size was optimized by preparing the inoculum on Czapek Dox agar plates, using sterile cork borer of 8 mm size. In different flasks, containing fermentation media, inoculated with1,2,3,4 and 5 numbers of discs of *Aspergillus niger* NCIM. Discs were transferred aspeptically and the flasks were incubated at 30^{0}C on orbital shaker at 120 rpm.

Inoculum age

This experiment was carried out by taking 1 disc of 8mm size of different age of culture of *Aspergillus niger* NCIM from Czapek Dox agar media plates, of different ages, i.e. 3days, 4 days, 5 days, 6 days, 7 days. Media was inoculated by using sterile cork borer under aseptic conditions. After inoculation all flasks were kept in orbital shaker at 120 rpm for 3 days time interval. After incubation enzymatic assay were carried out at regular intervals.

Temperature

To determine the effect of various temperatures on α-amylase production, the flasks were incubated at temperature, 25^0, 30^0, 35^0 and 40^{0}C. After incubation enzymatic assay were carried out at regular intervals.

Agitation

To determine the effects of agitation on the fermentation process, the flasks containing fermentation medium were incubated in orbital shaker set at different r.p.m. values, i.e. 50, 80, 120 and 150 rpm, at 30^{0}C temperature. Enzymatic assay were performed for all the flasks at regular time interval.

All the experiments were done in triplicate and the mean values with standard errors are reported.

RESULTS AND DISCUSSION

Optimization of different process parameters for α-amylase production

The optimization of cultural conditions was studied based on the stepwise modifications of different process parameters such as incubation time, inoculum size, inoculum age, dilution ratio, temperature and aeration.

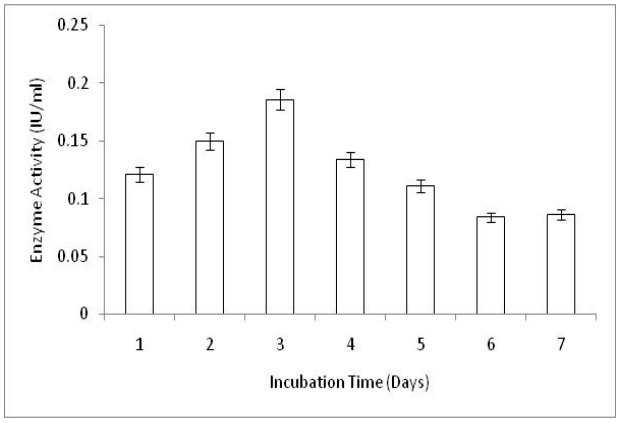

Fig. 1: Optimization of incubation time on production of á-amylase in Medium I (containing only rice waste water)

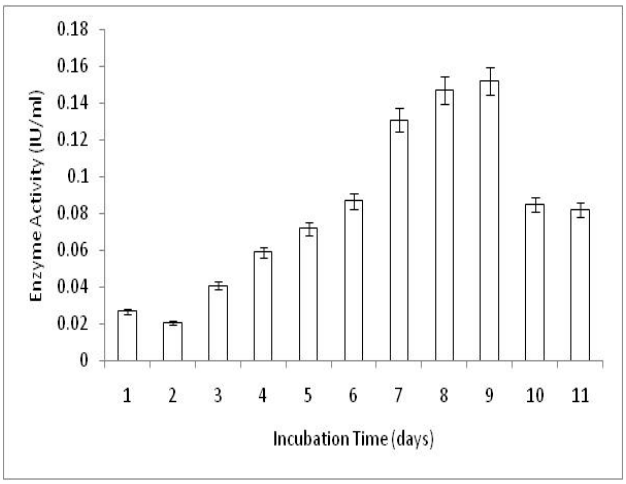

Fig. 2: Optimization of incubation time on the production of á-amylase in Medium II (containing rice waste water supplemented with various metal salts)

Time course play a very crucial role in fungal metabolic activity and growth. The incubation time necessary for optimal biosynthesis varied between different enzymes produced from one substrate as well as same enzyme produced from different substrates (Smith *et al*. 1996). Maximum α-amylase production was obtained on day 4 when medium I was used which contained only rice waste water (Fig. 1) while day 9 was the optimal fermentation period for medium II which contained rice waste water supplemented with different metal salts (Fig. 2). The total yield was also high in medium I (0.186 IU/ml) compared to medium II (0.152). So, all the further process parameters were optimized using only medium I. The results indicated that the enzyme was secreted early in active growth phase

and reached maximum towards the end of exponential growth phase in case of medium I but for medium II enzyme production reached its maximum in the stationary phase of growth. The various metal salts present in medium II might had some inhibitory effect on the production time and yield of the enzyme which resulted in shifting of production time from exponential phase to

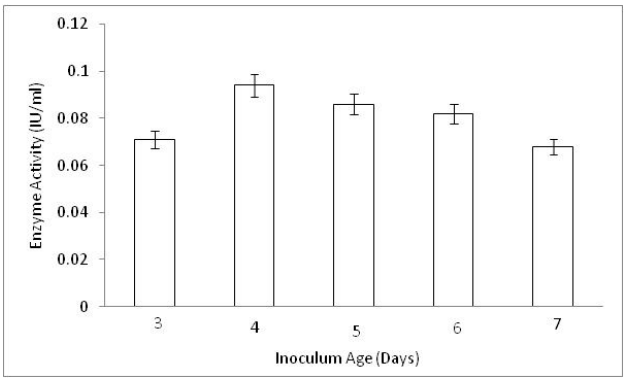

Fig. 3: Optimization of inoculum age on the production of á-amylase

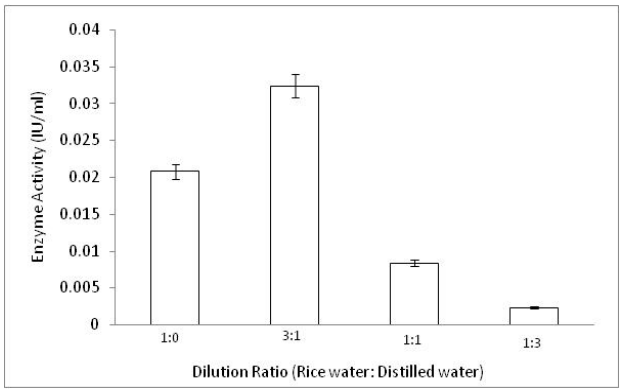

Fig. 4: Optimization of dilution ratio of the substrate on the production of á-amylase

stationary phase of growth. Shah *et al*.(2014) reported 72 hrs to be the optimum production time for α-amylase production by *Aspergillus oryzae* using starchy waste water.

Different age of *Aspergillus niger* NCIM 1342 from day 3 to day 7 was selected for the production of enzyme. Results indicate that maximum amylase production was observed when the age of *A.niger* NCIM 1342 was 4 days (Fig.3). Inoculum age plays a very critical role in fungal metabolic activity and growth. The inoculum age necessary for optimal biosynthesis varied between different enzymes produced from one substrate (Smith *et al*. 1996). This type of observation due to, the substances are initially more susceptible, making a rapid rise in biosynthesis of enzymes. But with prolongation of cultural time, the susceptible portions are completely hydrolyzed by microorganisms which inhibit the enzyme secretion pathways (Haq *et al*. 2006). The inoculum age was 120 hrs when *A. oryzae* was used for the production of α-amylase (Shah *et al*. 2014).

The nature and amount of carbon source in culture media is important for the growth of the organism as well as for the production of enzymes. By varying the ratio of rice waste water and distilled water optimum production (0.0324 IU/ml) was recorded when the ratio was fixed at 3:1 (Fig. 4). Too much concentrated as well as diluted substrate adversely affects the production of the enzyme.

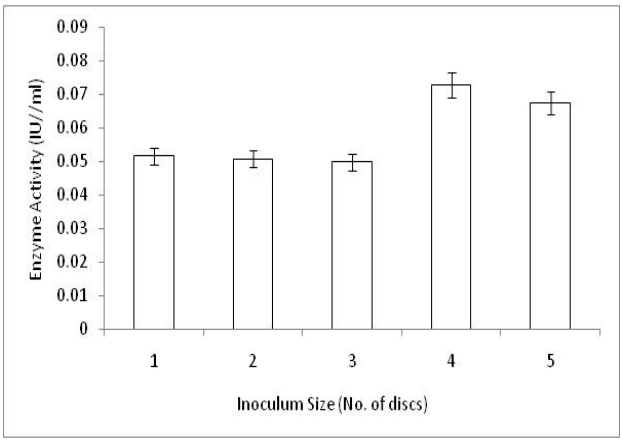

Fig. 5: Effect of inoculum size on the production of á-amylase

It is one of the most important parameter which affects the enzyme activity by affecting substrate utilization rate. To determine the optimum inoculum size for enzyme biosynthesis, it was examined with addition of different inoculums size from 1-5 spore discs of 8 mm size of *A. niger* NCIM 1342 into the culture medium. Maximum amylase production was (0.0728 IU//ml) found when inoculum size was 4 discs (Fig. 5). Further increase or decrease in inoculum size decreases amylase production. Initial microbial load also affects the growth and primary metabolite production. The smaller inoculums size may extend the lag phase of fungal growth (Sharma *et al*. 1996).An increase in inoculums size generally improves the growth and growth related activities of the fungal culture up to certain level, but then there could be a reduction in microbial activity due to nutrient limitation. This requires a longer time to grow to yield optimum number to

utilize the substrate and to form desired product. Shah *et al*. reported that 5 discs of *A.oryzae* was optimum for amylase production (Shah *et al*. 2014).

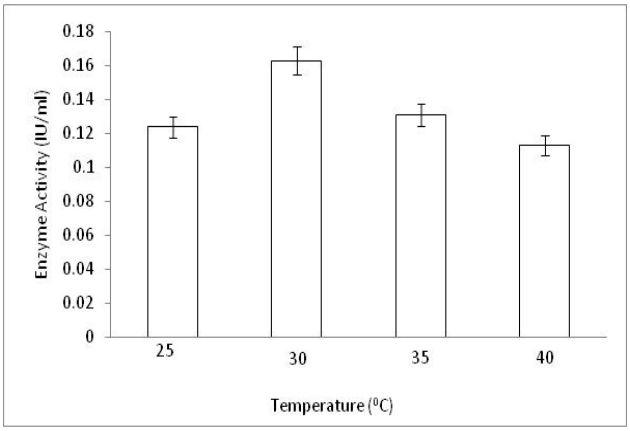

Fig. 6: Effect of temperature on the production of á-amylase

The effect of varying incubation temperature, 25^0, 30^0, 35^0 and 40^{0}C was checked on the production of α-amylase using *A.niger* NCIM 1342. Result presented in Fig. 6 shows that maximum amylase production was observed at 30^{0}C. Probably the most important factor among all the physical variables affecting the performance is the incubation temperature because both cell growth and the production of enzymes and other metabolites are usually sensitive to temperature

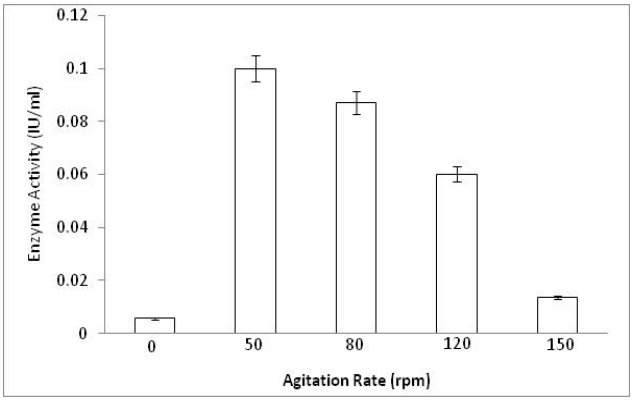

Fig. 7: Effect of aeration/agitation on the production of á-amylase

(Krishna, 2005). á-amylase production by fungi is related to the growth which sequentially depends upon the incubation temperature (Muhammad *et al*. 2012). Hence, the optimum temperature depends on whether the culture is mesophilic or thermophilic (Sivaramakrishnan *et al*. 2006). The decrease in enzyme activity was observed at higher temperature because of change in membrane composition and cause protein catabolism as well as inhibition of fungal growth. Also the deleterious effect of high and low temperature on spore germination, cell growth, product formation, sporulation consequently effects the overall productivity of the fermentation process(Moreaux, 1980).

Microorganisms vary in their oxygen requirement. In particular, O_2 acts as a terminal electron acceptor for oxidative reactions to provide energy for cellular activities. The variation in the agitation speed has been found to influence the extent of mixing in the shake flasks and also affect the nutrient availability (Ascimento and Martins, 2004). Oxygen must be supplied to all aerobic cultures to satisfy the request for growth and enzyme production. This is mainly obtained by correct set up of aeration and agitation leading to the transfer of a sufficient oxygen amount to each cell. At low aeration and agitation, no sufficient oxygen was probably available for the fungal growth and consequent enzyme production (Fenice *et al*. 2012). Enzyme-producing activity of *A.niger* NCIM 1342 was examined under various agitation intensities. Results presented in Fig 7 show that an agitation rate of 50 rpm was optimum for the enzyme production. At this speed, aeration of the culture medium was increased which could lead to sufficient supply of dissolved oxygen in the media (Kumar and Takagi, 1999). Nutrient uptake by fungus might have also increased (Beg *et al*. 2003) resulting in increased amylase production. From 80 rpm onwards amylase activity was found to be reduced. This was perhaps due to denaturation of enzymes caused by high agitation speed (Geok *et al*. 2003). High agitation rates could also damage mould filaments, so that reduction of amylase producers will result in decreased amylase production.

Production of α-amylase was tested using rice water and rice waste water supplemented with various salts and it was found that media containing only rice water gave a much higher yield at less incubation time than rice waste water supplemented with mineral salts media. After parametric optimization maximum amylase production was observed at 30^{0}C and 50rpm agitation rate. Optimum production was also obtained when the inoculum was 4days old and inoculum size was 4 discs. A dilution ratio of 3:1 (rice water: distilled water) was also found to be optimum for the enzyme production. From these results it can be concluded that rice waste water can be used for large scale production of α-amylase which will reduce the production coat of

α-amylase and will also solve the disposal and pollution problem.

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