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Production of extracellular alpha amylase from *Aspergillus oryzae* by solid state fermentation utilizing agricultural wastes

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Extracellular starch degrading enzyme alpha amylases was produced under solid state fermentation by *Aspergillus oryzae* utilizing the agricultural wastes as fermention medium and characterized along with optimization of various environmental conditions for its production. In our study the production of alpha amylases was maximum at 30°C of 5th day of fermentation with 1 : 1 ratio of wastes to water at stationary condition when the C : N ratio was maintained at 17 : 1 with an adequate substrate (waste) concentration and particle size. The enzyme showed a maximum activity of 18007.4331U/gds at 60°C with a pH of 7.0. Studies were also carried out to determine the specific activity, Km and Vmax value of the enzyme produced.

Key words : Alpha amylase, solid state fermentation, agricultural wastes, *Aspergillus oryzae*

INTRODUCTION

The processing of fruits and vegetable leads amount of organic residues which are a kind of agricultural wastes. This wastes are one of the cause of environmental pollution. In general most of this agricultural wastes are used as cattle feed or converted to biogas or compost. However greater environmental and economic benefit could result from the conversion of these by-products of higher value. Bio-conversion of this wastes not only reduces disposal problem but also environmental pollution along with production of value added products as reported by Bose *et al.,* (2004, 2006). Solid state fermentation (SSF) holds tremendous potential for the production of enzyme. It can be of special interest in those processes where the crude fermented product may be used directly as the enzyme sources as reported by Tengerdy, (1998). In addition to the conventional applications in food and fermentation industries, microbial enzymes have attained significant role in biotransformation involving organic solvent media, mainly for bioactive compounds. This system offers numerous advantages over submerged fermentation (SmF) system, including high volumetric productivity, relatively higher concentration of the products, less effluent generation, requirement for simple fermentation equipment, etc as reported by Pandey, (1991, 1992, 1994) and Doelle *et al.*, (1992). Agro-industrial residues are generally considered the best substrate for SSF processes, and use of SSF for production of enzyme is no exception to that. Sugar cane bagasses, wheat bran, rice bran, banana waste, tea wastes, etc have been used as substrate material in SSF as reported by Mitra *et al* (1994), Selvakumar, *et al.* (1994), Babu *et al* (1994), Nigam *et al* (1994), Pandey *et al* (1993, 1995), Selvakumar *et al* (1998) and Tengerdy (1996). Enzymes are among the most important products obtained for human needs through microbial sources as reported by Pandey *et al* (1999). Microbial amylases could be potentially useful in the phramaceutical and fine-chemical industries if enzymes with suitable properties could be prepared as reported by Pandey *et al* (2000). Alpha-amylases (1,4-alpha-D-glucan glucanhydrolases, EC 3.2.1.1) is a widely distributed secretary enzyme which is one of the most popular and important from of industrial amylases according to Gupta *et al* (2003). Comparative studies were made on alpha-amylase production using different substrates as reported by DeAlmedia *et al* (2003). Comparative studies were made on alpha amylase production using different substrates as reported by DeAlmeida *et al* (1997), Shankaranand *et al* (1992) and Shah *et al* (1991). *Thermomyces lanugionse* a thermophilic fungus was reported to be an efficient producer of alpha amylse by Jensen B et al (1992) & Amesen S et al (1998). Studies on acid stable alpha amylase also carried out using *A. kawachii*

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IFO 4308 by Sudo S et all (1994). Other microorganisms like *Saccharomycopis capsularia & acagulans* have also been reported by Soni S K et al (1996) & Babu K R et al (1995) as good alpha-amylase producers.

Considering the fact that agricultural wastes can be used as media in solid state fermentation process for producing alpha-amylase enzyme using *Aspergillus sp.* the present study was undertaken to evaluate the effect of various enviornmental conditions on production of enzyme alpha-amylase by SSF using *Aspergillus oryzae.* Studies were also carried out to determine the specific activity and Kinetics of the enzyme produced.

MATERIALS AND METHODS

Microorganism

Aspergillus oryzae (NCIM No. 645) collected from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune (India) was maintained on Czapek Dox agar medium consisting of Glucose 5%, $NaNO_3$ 0.2%, KCl 0.05%, $MgSO_4.7H_2O$ 0.05%, $FeSO_4.7H_2O$ 0.001%, KH_2PO_4 0.1%, Agar 3% with a pH of 5 and stored under refrigerated condition at 4°C.

Utilization of a agricultural wastes for SSF

House hold agro-wastes (i.e., especially vegetable wastes) in Kolkata Municipal area were used as substrate in this study. These agro-wastes were dried at 60°C in Tray drier for 4 hrs. and made to powder in a mixer grinder. These agro-wastes powder was used as medium for SSF through out the study.

Scanning Electron Microscope (SEM)

Scanning electron microscope (JEOL, JSM 5200, Tokyo, Japan) were used at an accelerating voltage of 20kV to view the substrate (waste) without the growth of *Aspergillus oryzae* in three dimensions and to determine the absence of microbial growth on it. Agro-waste particle sample used as control were mounted stubs with adhesive tape and sputters coated gold approx. 19 A° thick for 2.5 min at 10 mA before observation with SEM. A micrograph was taken for the sample at magnification X 750. Same prodcedure were followed for another sample of substrate with the growth of *Aspergillus oryzae.*

Production of alpha amylase by SSF

Production of Alpha amylase by *Aspergillus oryaze* was carried out using 20 gm. agrowaste material in standard size Roux bottle. A set of bottles were taken and plugged with cotton wool. The fermentation was carried out under stationary condition at 30°C. Alpha amylase secreted into the spent medium was monitored at regular interval of time. After an approximate time of incubation, bottles were removed and enzyme was extracted with distilled water by shaking for 4 hr. at 30°C. The ratio of waste to water was 1 : 2.5 w/v.

Solid were removed by filtration followed by centrifugation at 10,000 rpm (C-24, REMI, India) for 20 min. Clear supermatant was used for alpha amylase activity measurement.

Enzyme assay

Activity of enzyme produced was measured in Units (U). 1 unit of alpha amylase activity is defined as the amount of enzyme that releases 1 μ mole (micromole) of reducing sugar per minute from soluble starch at pH 7.0 and 30°C. The assay method were carried out according to the methods followed by Shaw J-F, Lin F-P, Chen S-C, CHen H-C (1995) and Miller G L (1959). The enzyme activity was expressed in U/gds (i.e., gram dry solid) according to the method descrbed by Ramachandran *et al* (2004).

Effect of various environmental conditions on production of alpha amylase by SSF using A. oryzae

The optimum time of production of enzyme was evaluated by determining the enzyme activity upto 7 days at 24 hrs. interval of time.

The hydration (initial moisture level) on enzyme production was evaluated by varying the ratio (w/v) of waste to water as 1 : 0.5, 1 : 1, 1 : 1.5.

The temperature to get maximum production of enzyme was obtained by carrying out the fermentation at 25°C, 30°C and 35°C and the enzyme activity was mesured at particular interval of time.

Fermentation was also carried out at stationary, 50 rpm and 100 rpm agitated condition. Fermentation was also carried out with samples of different particle sizes as 0.48 mm, 0.25 mm and 0.03 mm respectively and enzyme activity was meaured at particular time interval. The particle size was measured by passing samples through respective mesh sizes.

Ratio of carbon to nitrogen is an essential factor in production of microbial enzyme by fermentation. Thus, study was carried out to evaluate the effect of C : N on enzyme production by taking substrates with C : N of 16.1, 17.1 & 18.1. Carbon content of the wastes were measured by Walky and Black's method as reported by Jakson M L (1967) & total nitrogen were meausred by Kjeldal micro digestion method as reported by Piper de J (1967).

Effect of pH and temperature on activity of alpha amylase

Activity of enzyme produced by SSF was carried out at various pH ranging from 6.8-7.4 and temperature ranging 30°C – 80°C to evaluate the effect.

Enzyme Kinetics

Measurement of Specific activity and Kinetics of enzyme produced. Specific activity of alpha amylase produced by SSF was determined by taking maximum activity of enzyme and cellular protein of *A. oryzae* as described by Lowry *et al.* (1951).

K_m and V_{max} values of enzyme produced was calculated at 60°C and pH 7.0 by plotting Lineweaver-Burk plot as mentioned by Lehninger *et al.* (1993).

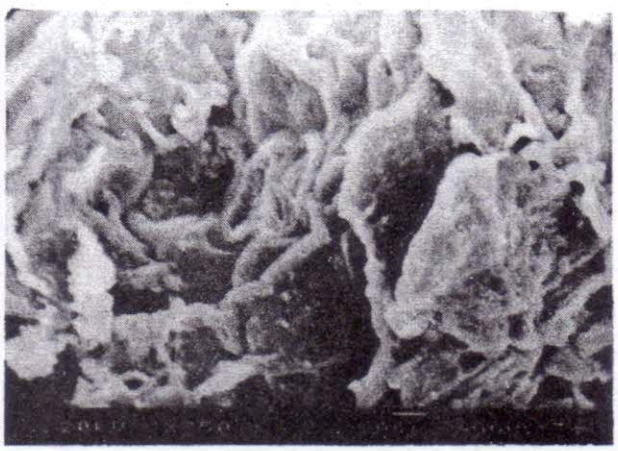
RESULTS AND DISCUSSIONS

SEM for conformation of growth

The comparison of micrographs taken at magnification X 750 for both substrate and substrate with the growth of *Aspergillus oryzae* confirms the presence of the mould as inculum in the fermentation media (Fig. 1).

Optimization of time for SSF

Time for fermentation is an essential factor which determines the optimum production of enzyme. In this study production of alpha amylase was maximum on 5th day which showed as activity of 2851. 1796 U/gds (Fig. 2).



(A) = Agro-waste without growth of *Aspergillus oryzae*. Magnification X 750.

(B) = Agro-waste with growth of *Aspergillus oryzae*. Magnification X 750.

Fig. 1 : Scanning Electron Microscopy for growth of *Aspergillus oryzae* on agro-wastes at magnification X 750

Effect of hydration

Water content of substrate (media) for growth of mould is an important factor. Excess or less water in media can not only affect the growth of mould, but also reduces the production of enzyme. In our study maximum production of enzyme took place with an activity of 3001.2410 U/gds at a ratio of 1 : 1 (w/v) of waste to water (Fig. 3).

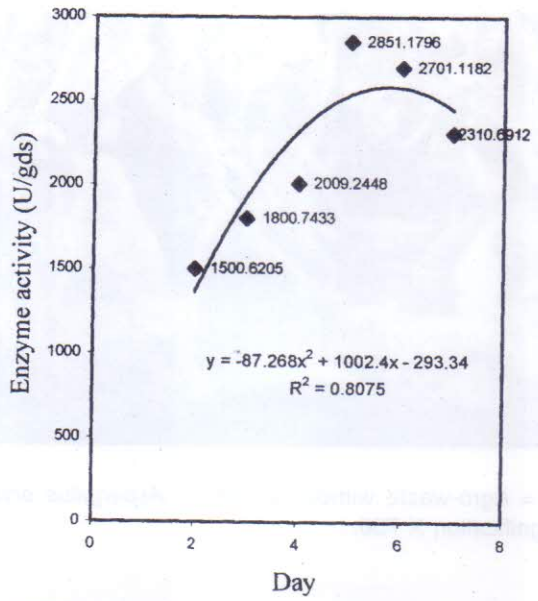


Fig. 2 : Effect of fermentation time on production of alpha-amylase enzyme by solid state fermentation utilizing agro-wates as fermention medium

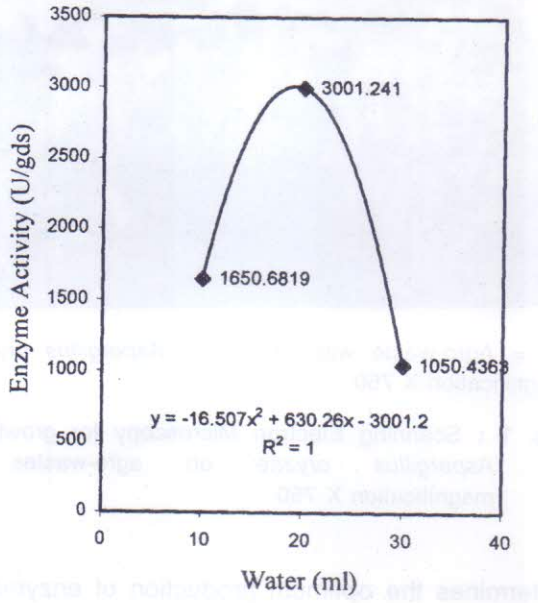


Fig. 3 : Effect of hydration of production of alpha-amylase enzyme by solid state fermentation utilizing agro-wastes as fermentation medium.

Effect of temperature

Fermentation is affected with change in temperature. Change in temperature reduces the mould growth along with reduction in enzyme produced. In this study maximum production of enzyme occurred when the fermentation was carried out at 30°C with an activity of 3601.4866 U/gds (Fig. 4).

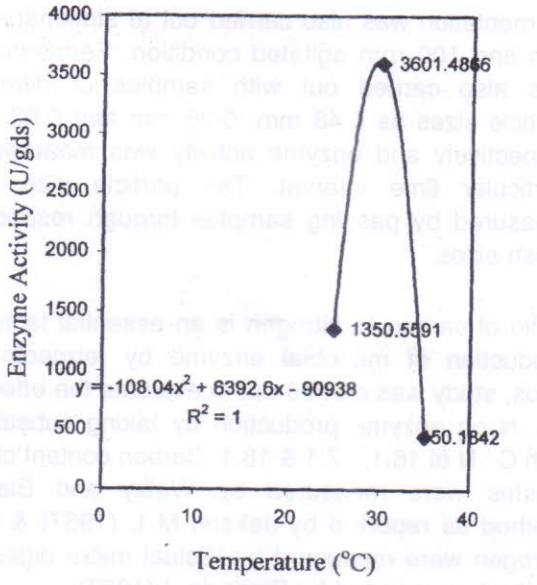


Fig. 4 : Effect of temperature on production of alpha-amylase enzyme by solid state fermentation utilizing agro-wastes as fermentation medium.

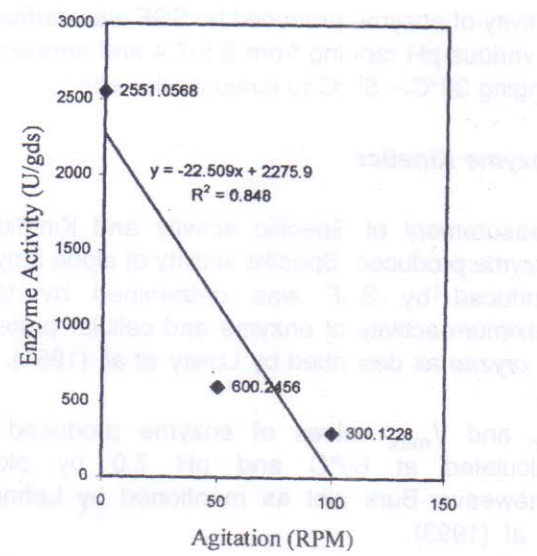


Fig. 5 : Effect of agitation on production of alpha-amylase by solid state fermentation utilizing agro-wastes as fermentation medium.

Effect of agitation

Study on production of alpha amylase from agricultural wastes by SSF revealed that enzyme production was maximum with an activity of 2551.0568 U/gds in stationary condition compared to agitating condition (Fig. 5).

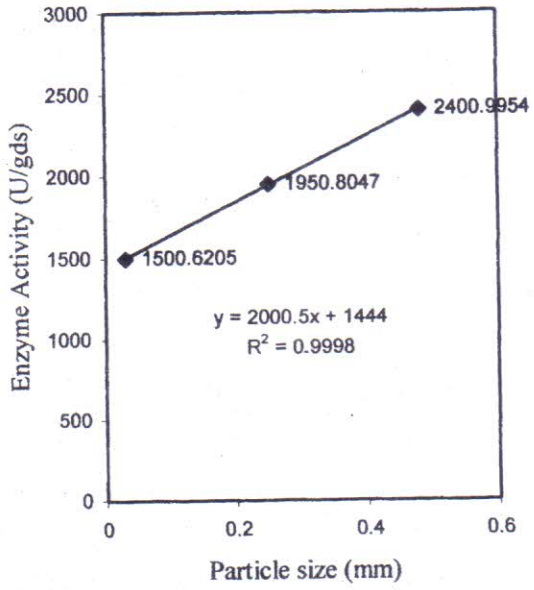


Fig. 6 : Effect of particle size of agro-wastes utilized as fermentation medium on production of alpha-amylase by solid state fermentation.

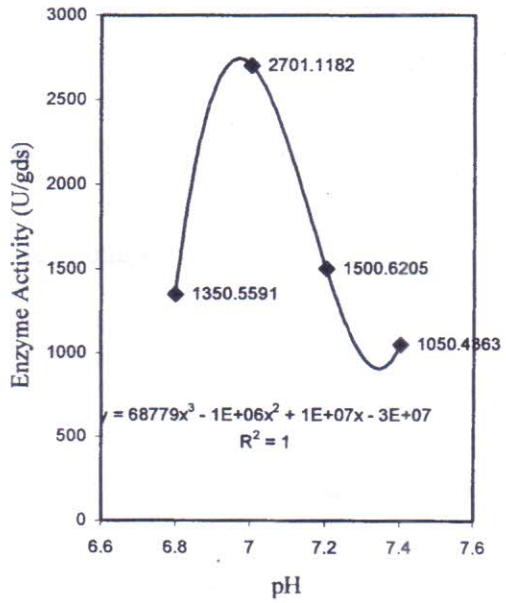


Fig. 8 : Effect of pH on activity of enzyme alpha-amylase.

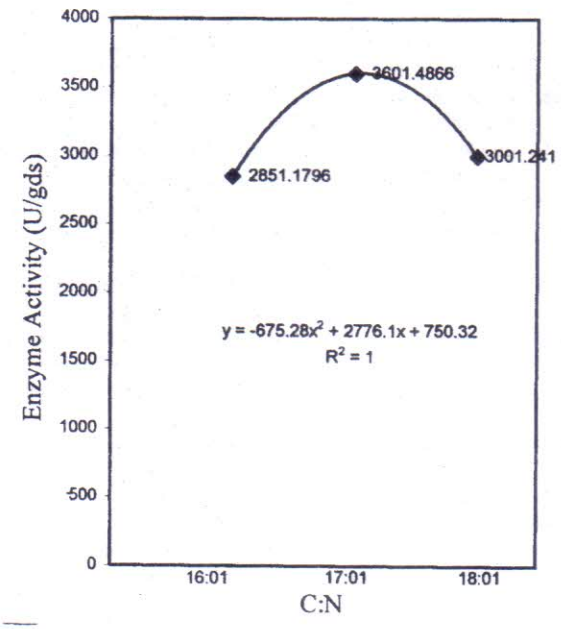


Fig. 7 : Effect of C : N of agro-wastes utilized as fermentation medium on production of alpha-amylase by solid state fermentation

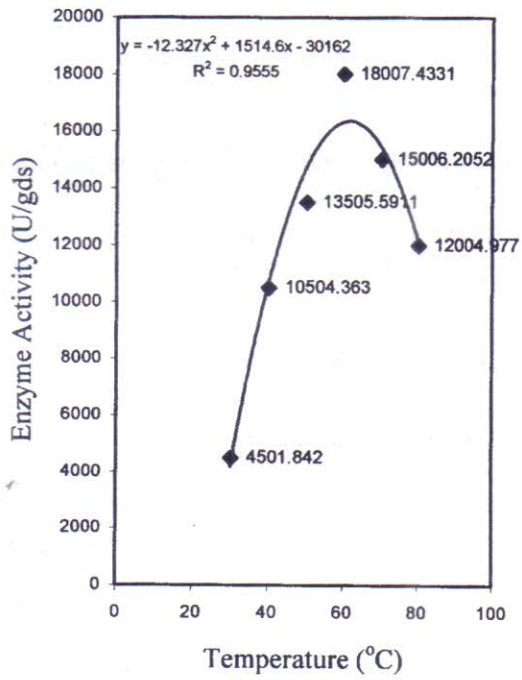


Fig. 9 : Effect of temperature on activity of enzyme alpha-amylase.

Effect of particle size of substrate (media)

The particle sizes of substrate used as media in fermentation process for the growth of microorganism have a great effect on enzyme produced. In our study maximum production of enzyme occurred at a particle size of 0.48 mm with an activity of 2400.9954 U/gds (Fig. 6). This study also indicates that particle size is directly proportional to the enzyme produced.

Effect of C : N substrate (fermentation media)

C : N is another essential factor for the growth of microbes. Production of microbial enzyme varies with the variation of C : N of fermentation media. In

our study maximum production of enzyme occurred at C : N of 17 : 1 with an activity of 3601.4866 U/gds (Fig. 7).

Effect of pH on activity of enzyme alpha amylase produced

Activity of enzyme greatly depends upon the pH of buffer maintained during incubation for assay. Change in pH results in variation on activity of enzyme. In this study maximum activity (2701.1182) U/gds) of alpha amylase was obtained at pH 7.0 (Fig. 8).

Effect of temperature on activity of enzyme produced

Studies were made on the effect of temperature on activity of alpha amylase produced. It was found that the enzyme produced showed maximum activity (18007.4331 U/gds) at 60°C (Fig. 9). This study also indicates that the enzyme produced is a kind of thermostable enzyme.

Specific activity & Kinetis of enzyme produced

Specific activity of enzyme produced was measured by taking the amount of cellular protein of *A. oryzae* (Lowry's method) and the maximum activity of enzyme at 60°C. This enzyme showed a specific activity of 342.99 U/mg.

Studies were also made to determine the K_m and V_{max} value of the alpha amylase produced by SSF. This enzyme showed at K_m of 0.25 mg of starch per ml and a V_{max} of 5263. 1579 μ mole of glucose per minute per ml at 60°C, pH 7.0.

CONCLUSION

The results obtained in this study indicates that agricultural wastes could be utilized for production of extracellular alpha amylase enzyme by solid state fermention (SSF) using *Aspergillus oryzae.* The enzyme produced was maximum on 5th day of fermentation at stationary condition with 1 : 1 ratio of water to agricultural waste at 30°C. Alpha amylase obtained in our study showed a maximum activity of 18007.4331 U/gds at 60°C with a pH of 7.0 when compared with other incubation temperatures and pH of the assay method.

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