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

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Extracellular starch degrading enzyme amyloglucosidase was produced under solid state fermentation by *Aspergillus oryzae* from municipal agricultural wastes and characterized along with optimization of various environmental conditions for its production. In our study the production of amyloglucosidase was maximum at 30°C on 4th day of fermentation with 1:1 ratio of waste to water at stationary condition when the C:N ration was maintained at 16:1 with an adequate substarate (waste) concentration and particle size. The enzyme showed a maximum activity at 60°C with a pH of 4.8. Studies were also carried out to determine the specific activity, Km and Vmax value of the enzyme produced.

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

INTRODUCTION

Industrially important enzymes have traditionally been obtained from submerged culture fermentation because of easy handling and greater control of environmental factors, such as temperature and pH. Solid state fermentation (SSF) constitute an interesting alternative since metabolites so obtained are more concentrated and purification procedure are less costly. Some of the advantages of SSF over conventional submerged cultures involving fungi include simplicity in equipment and prevents bacterial contamination as reported by Pandey (1992). SSF is a well-adapted process for cultivation of fungi on vegetable materials which are broken down by excreted hydrolytic enzymes. In contrast with LSF (Liquid substrate fermentation) where water is in large excess, water activity is a limiting factor in SSF. On the other hand, oxygen is a limiting factor in LSF but not in SSF, where aeration is promoted by the porous and particular structure and by the large surface area of contact which facilitate mass transfer between gas and liquid phases as reported by Maurice (1998).

The filamentous fungi are the most important group of microorganisms used in SSF process owing to their physiological, enzymological and biochemical properties. The hyphal mode of fungal growth and their good tolerance to low water activity (Aw) and high osmotic pressure conditions make fungi efficient and competitive in natural microflora for bioconversion of solid substrates as reported by Maurice (1998). The *Aspergillus* sp. have a long history of use as producers of secreted protein which include amyloglucosidase, amylase, etc. as reported by Gwynee *et al.* (1992). *Aspergillus niger* has been used for the production of amyloglucosidase in submerged culture as reported by Janz *et al.* (1977) and Kennedy (1987). Strains of *Aspergillus niger* was used for production of glucoamylase in solid culturesas reported by Selvakumar *et al.* (1994), Pandey *et al.* (1993, 1995), Selvakumar *et al.* (1998), Pandey (1991, 1990, 1992), Pandey *et al.* (1992, 1996), Ashakumary *et al.* (1994) and Pandey *et al.* (1995). Studies were also made on thermostable glucoamylase from *Rhizopus niveus* as reported by Moriyama *et al.* (1977).

Many microorganisms can hydrolyse starch, specially fungi which are then suitable for SSF

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application involving starchy substrates. Glucoamylase, α-amylase, β-amylase, pullulanase and isoamylase are involved in the processes of starch degradation. Mainly α-amylase and glucoamylase are of importance for SSF. Glucoamylase occurs almost exclusively in fungi including *Aspergillus* and *Rhizopus* groups. This exo amylase produces glucose units from amylose and amylopectin chains as repoted by Maurice (1998). Amyloglucosidase (Synonym Glucoamylase; exo-1, 4-a-D-glucan glucanohydrolase, EC-3.2.1.3) is an important industrial enzyme used for the production of glucose syrup as reported by Forgarty (1983). Various fungal amyloglucosidase have been employed in the production of sugar syrup with different dextrose equivalents (DE) as reported by Forgarty (1983) and Nissen (1987). The large amount of residues from fruit and vegetable processing units which are also known as agricultural wastes are one of the cause of environmental pollution. In general most of this "wastes" may be used as cattle feed or converted to biogas or compost. But, greater environmental and economic benefits could result from the conversion of these by-products of higher value.This can be achieved either by using such materials as multifunctional food ingredient or in order to other processes within the concept of low-residue food production. Thus bio-conversion of these wastes not only reduces disposal problem but also environ-mental pollution along with production of value added products as reported by Bose *et al.* (2004). Considering the fact that agricultural wastes can be used as media in solid state fermentation process for producing amyloglucosidase enzyme using *Aspergillus* sp. the present study has been undertaken to evaluate the effect of various environmental conditions on production of enzyme amyloglucosidase by SSF using *Aspergillus oryzae*. Studies have also been carried out to determine the specific activity and Kinetics of the enzyme produced.

MATERIALS AND METHODS

Collection of data related to deposition of garbages (including agro-wastes) in Kolkata

Data related to deposition of garbage (i.e. food wastes including agro-wastes) in Kolkata were collected from Kolkata Municipal Corporation (KMC) and its processing plant (Eastern Organic Fertilizers Pvt. Ltd.).

Microorganism

Aspergillus oryzae (NCIM No. 645) was collected from National Collection of Industrial Micro-organisms (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 agricultural wastes for SSF

House hold agro-wastes (i.e. vegetable and fruit 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 hr and made to powder in a mixer grinder. These agro-waste powder was used as medium for SSF throughout the study.

Proximate composition of the agricultural waste

Different analysis were carried out for the agricultural wastes used in the solid state fermentation as medium for the growth of microorganisms to determine its proximate composition.

Production of amyloglucosidase by SSF

Production of amyloglucosidase by *Aspergillus oryzae* was carried out using 20 g. agro-waste 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. Amyloglucosidase 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 supernatant was used for amylo-glucosidase activity measurement.

Enzyme assay

Activity of enzyme produced was measured in Units (I.U). One unit of amyloglucosidase activity is defined as the amount of enzyme that releases 1 mole picomole of reducing sugar per minute from soluble starch at pH 4.8 and 30°C. The assay

method were carried out according to the methods followed by Tanuja *et al.* (1997) and Miller (1959).

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

The optimum time of production of enzyme was evaluated by determining the enzyme activity upto 6 days at 24 hr 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 37°C and the enzyme activity was measured 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 measured 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 9:1, 16:1 and 17:1. Carbon content of the wastes were measured by Walky and Black's method as reported by Jakson (1967) and total nitrogen were measured by Kjeldal micro digestion method as reported by Piper (1967).

Effect of pH and temperature on activity of amyloglucosidase

Activity of enzyme produced by SSF was carried out at various pH ranging from 4.4-5.2 and temperature ranging 30°C-70°C to evaluate the effect.

Enzyme Kinetics

Specific activity of amyloglucosidase produced by SSF was determined by taking maximum activity of enzyme and cellular protein of *A. oryzae* as described by Lowry *et al.* (1951). Km and Vmax values of enzyme produced was calculated at 60°C and pH 4.8 by plotting Lineweaver-Burk plot as mentioned by Lehninger *et al.* (1993).

RESULTS AND DISCUSSIONS

Data collection on garbage deposition in Kolkata

The data in Table 1 represented the information on garbage (agro-wastes) collected from Kolkata Municipal Corporation (KMC), Kolkata.

Table 1 : Quantity of Garbage collected by Kolkata Municipal Corporation (KMC) and Private Organization (2002, 2003 and 2004)

Season	Garbage collected by KMC (in an average) in Metric Tons.	Garbage collected by Private organization (in an average) in Metric Tons.
Winter	34,098.40	35,235.65
Summer	34,910.85	33,473.35
Rainy	29,745.95	34,838.80

Table 2 : Composition of agro-wastes used for solid state fermentation in production of amyloglucosidase enzyme

% Acidity (expressed as citric acid)	% Ash		% Carbon	% Nitrogen
0.091	10.5		7.62	0.527
	Water soluble	Water insoluble		
	1.32	98.68		

Determination of proximate composition of agricultural waste

Composition of agricultural wastes used as medium in fermentation process is an essential factor in production of enzyme. In this study the agricultural wastes used as medium for microbial growth showed a composition with 0.091 % acidity, 7.62 % carbon, 0.527 % nitrogen and 10.5 % ash (Table 2).

Optimization of time for SSF

Time for fermentation is an essential factor which determines the optimum production of enzyme. In this study production of amyloglucosidase was maximum on 4th day which showed an activity of 33255253.33 I.U/ml (Fig. 1).

Effect of hydration

Water content of substrate (media) for growth of mold is an important factor. Excess or less water in

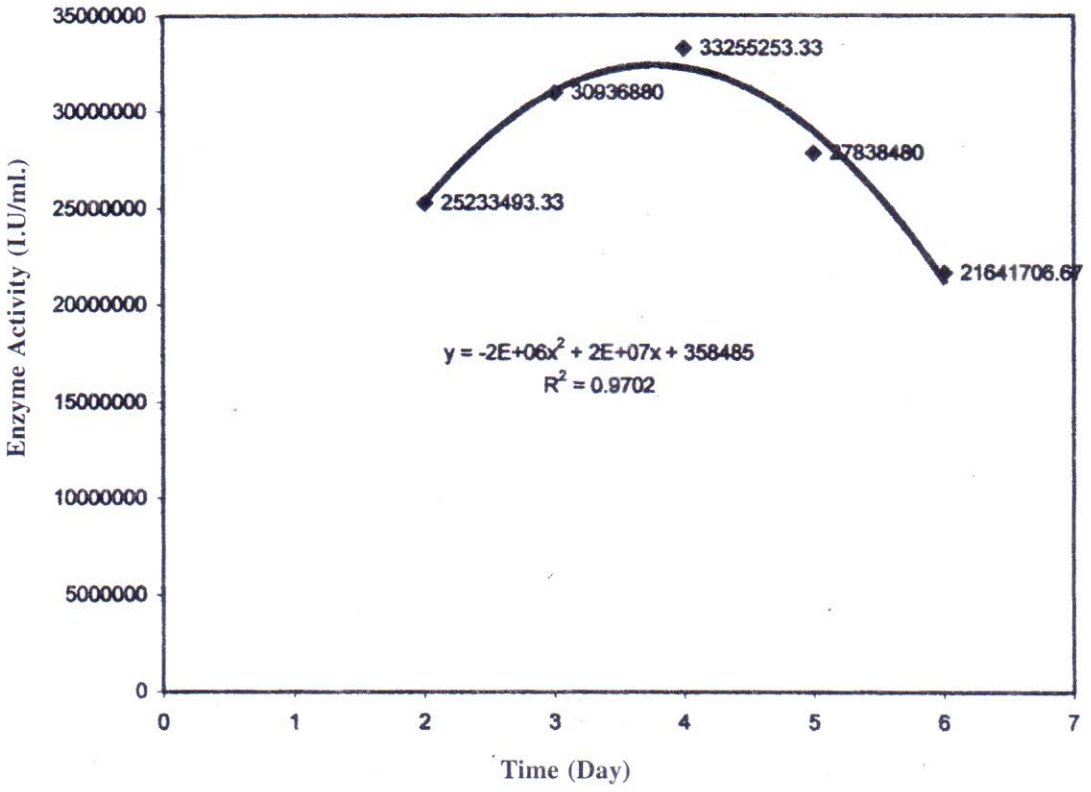

Fig. 1 : Optimization of time for fermentation for production of amyloglucosidase by SSF utilizing agro-wastes.

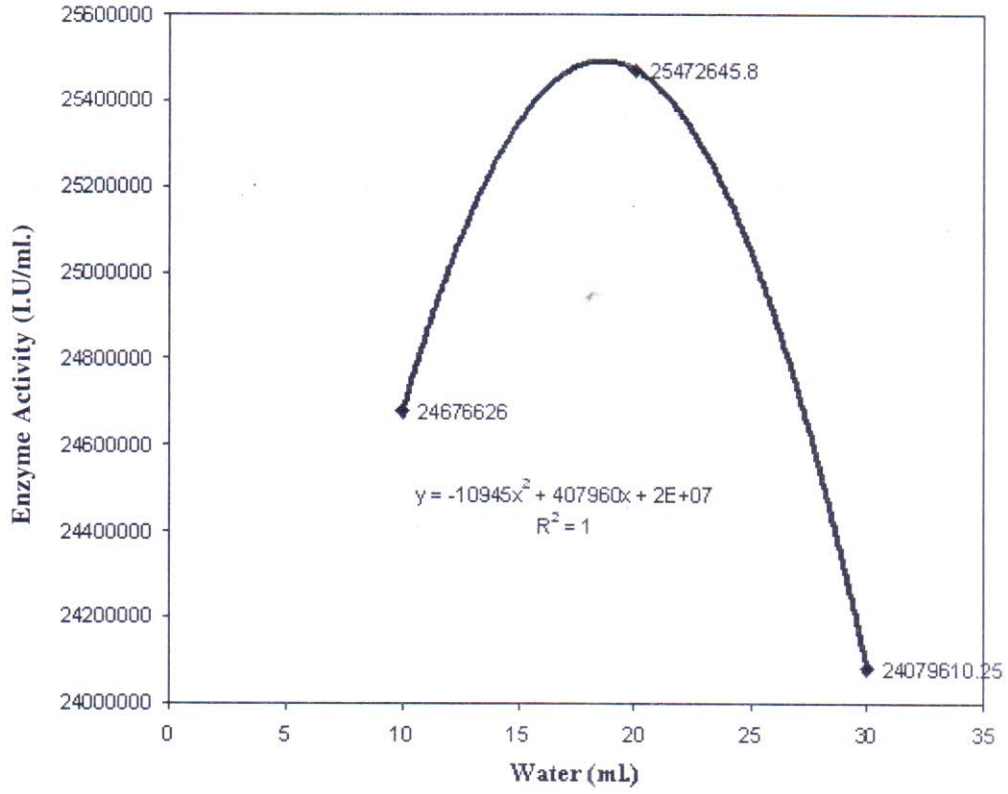

Fig. 2 : Effect of Hydration on production of amyloglucosidase produced by SSF utilizing agro-wastes.

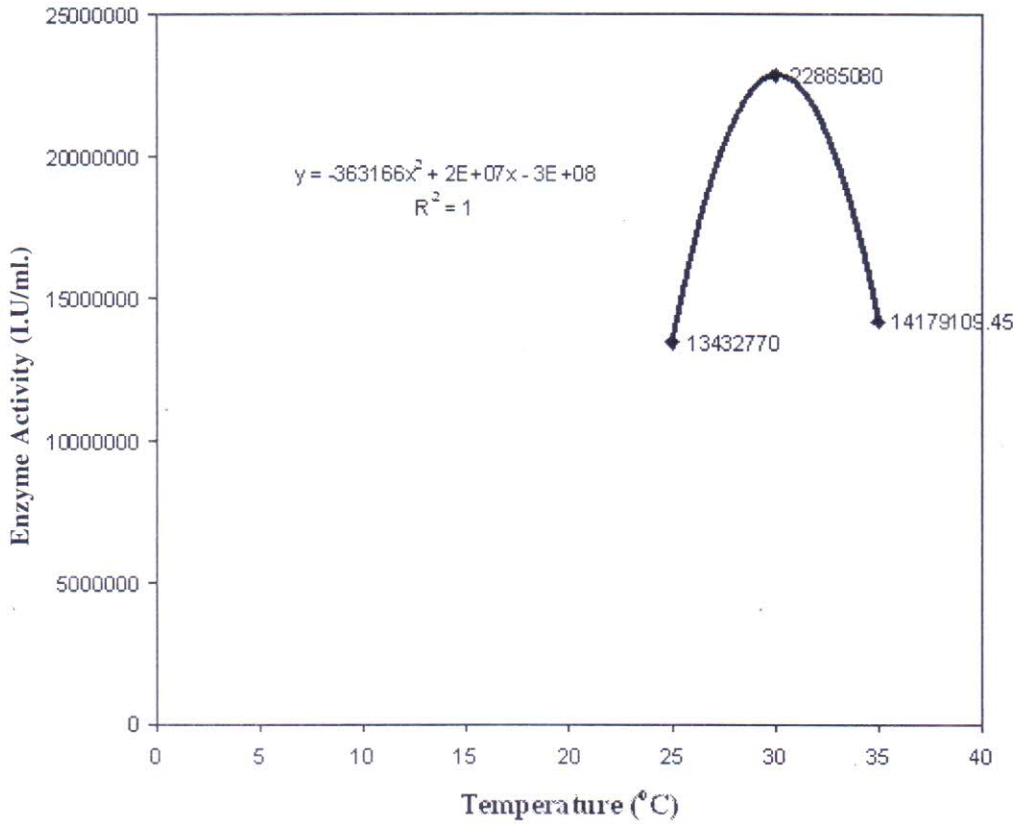

Fig. 3 : Effect of fermentation temperature on production of amyloglucosidase produced by SSF utilizing agro-wastes.

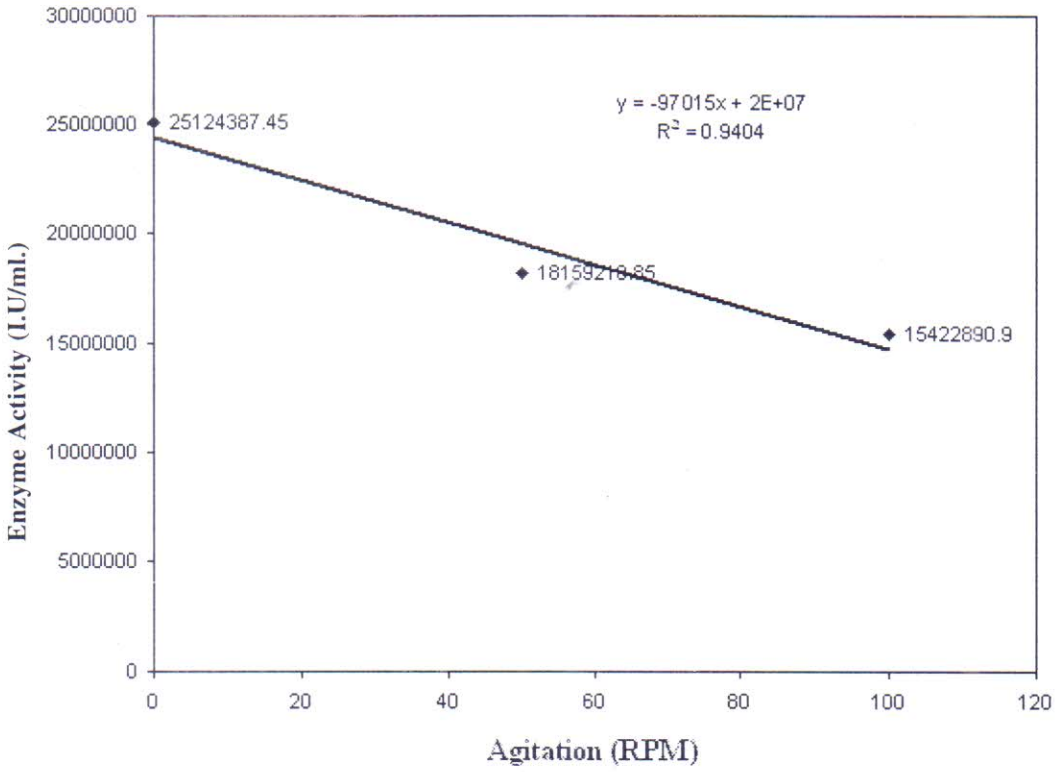

Fig. 4 : Effect of Agitation on production of amyloglucosidase by SSF utilizing agro-wastes.

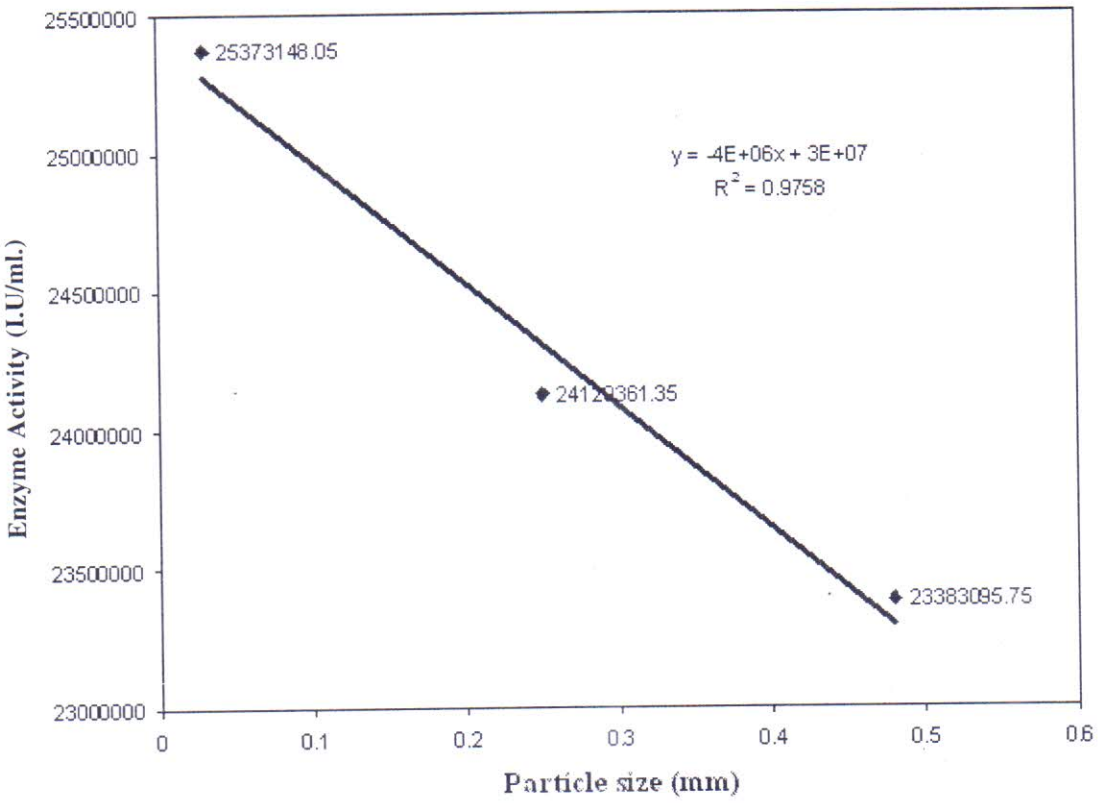

Fig. 5 : Effect of Particle size of the agro-wastes used as medium in SSF on production of amyloglucosidase.

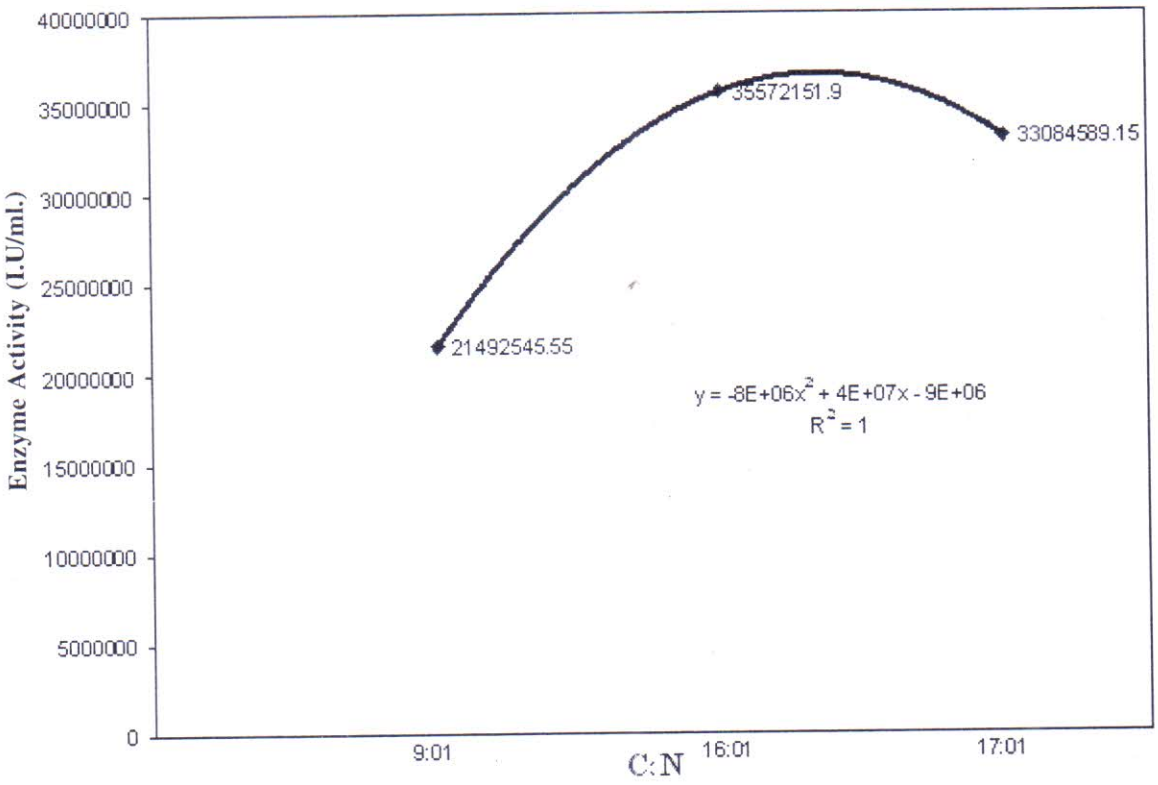

Fig. 6 : Effect of C:N on production of amyloglucosidase by SSF utilizing agro-wastes.

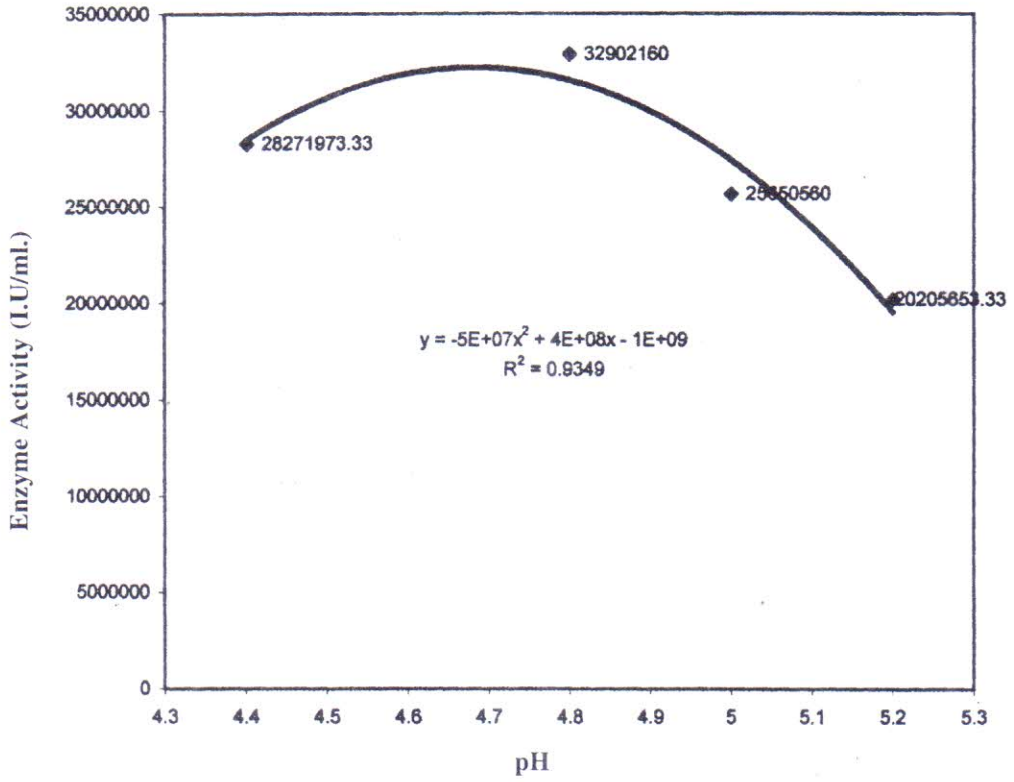

Fig. 7 : Effect of pH on activity of amyloglucosidase.

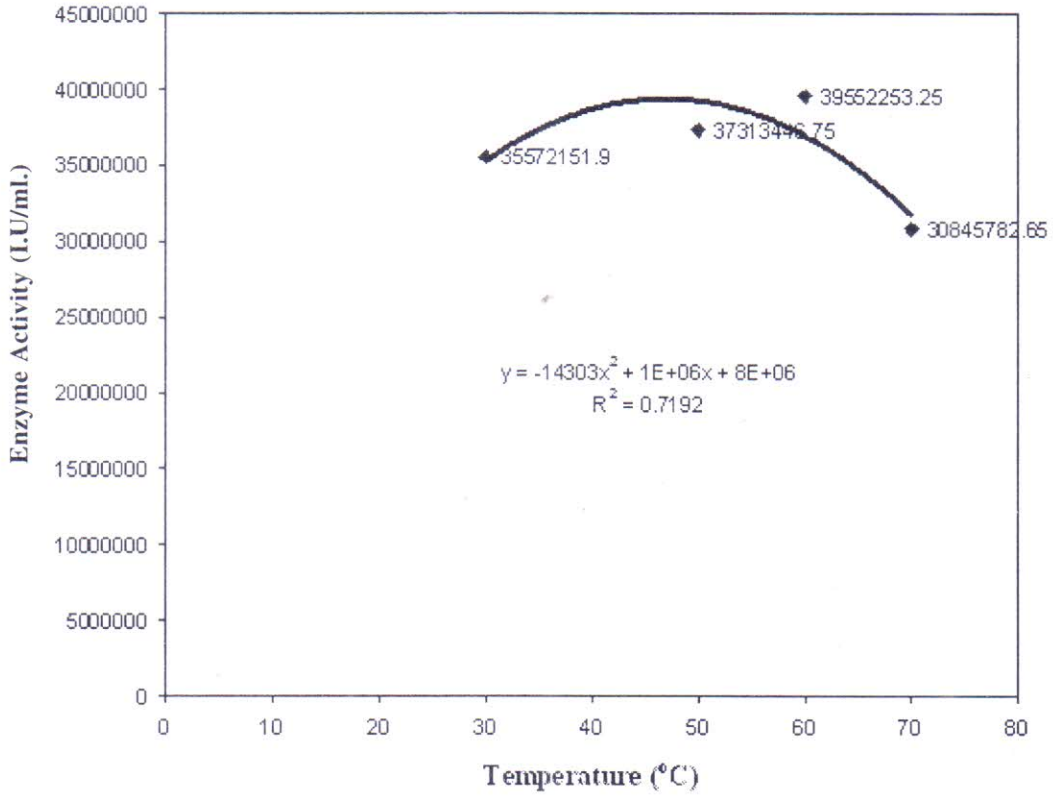

Fig. 8 : Effect of temperature on activity of amyloglucosidase.

media can not only affect the growth of mold, but also reduces the production of enzyme.In our study maximum production of enzyme took place with an activity of 25472645.80 I.U/ml at a ratio of 1:1 (w/v) of waste to water (Fig. 2).

Effect of temperature

Fermentation is affected with change in temperature. Change in temperature reduces the mold 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 22885080.0 I.U/ml (Fig.3).

Effect of agitation

Study on production of amyloglucosidase from agricultural wastes by SSF revealed that enzyme production was maximum with an activity of 25124387.45 I.U/ml in stationary condition compared to agitating condition (Fig.4).

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 this study maximum production of enzyme occurred at a particle size of 0.03 mm with an activity of 25373148.05 I.U/ml (Fig.5). This study also indicated that particle size is inversely proportional to the enzyme produced.

Effect of C:N of 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 this study maximum production of enzyme occurred at C:N of 16:1 with an activity of 35572151.90 I.U/ml (Fig. 6).

Effect of pH on activity of enzyme amyloglucosidase 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 (32902160.00 I.U/ml) of amyloglucosidase was obtained at pH 4.8 (Fig. 7).

Effect of temperature on activity of enzyme produced

Studies were made on the effect of temperature on activity of amyloglucosidase produced. It was found that the enzyme produced showed maximum activity (39552253.25 I.U/ml) at 60°C (Fig. 8).This study also indicates that the enzyme produced is a kind of thermostable enzyme.

Specific activity and Kinetics 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 2996382.82 I.U/ml. Studies were also made to determine the Km and Vmax value of the amyloglucosidase produced by SSF. This enzyme showed a Km of 0.67 mg of starch per ml and a Vmax of 38461538-46 picomole of glucose per minute per ml at 60°C, pH 4.8.

The results obtained in this study indicates that agricultural wastes can be utilized for production of extracellular amylogluosidase enzyme by solid state fermentation (SSF) using *Aspergillus oryzae*. The enzyme produced was maximum on 4th day of fermentation at stationary condition with 1:1 ratio of water to agricultural waste at 30°C. Amyloglucosidase obtained in this study showed a maximum activity of 39552253.25 I.U/ml at 60°C with a pH of 4.8.

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