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Effect of metal salts on production of fungal alpha amylase and amyloglucosidase by solid state fermentation utilizing agricultural wastes as medium

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Extracellular alpha amylase and amyloglucosidase were produced from *Aspergillus oryzae* NCIM 645 under solid state fermentation. House hold agro-wastes such as mixture of potato peels, wastes potatoes, peas peals, non edible parts of spinach, cabbag and cauliflower were used as medium in our present study. These are considered as major pollutants due to unfavorable gas production via natural fermentation beside creating disposal problem. Investigations were carried out to evaluate the effect of different chemical compounds of various metals (metal salts) such as $FeSO_4.7H_2O$, $CuSO_4.5H_2O$, $MnCl_2.4H_2O$, $MgSO_4.7H_2O$, $KH_2PO_4.7H_2O$ and $ZnSO_4.7H_2O$ with various concentrations on production of enzymes alpha amylase and amyloglucosidase obtained from *Aspergillus oryzae* by solid state fermentation utilizing above agricultural wastes as the fermentation medium. Results indicated that maximum activity of alpha amylase was obtained with 10 ppm $ZnSO_4.7H_2O$ solution and maximum activity of amyloglucosidase was obtained with 10 ppm $MgSO_4.7H_2O$ solution.

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

INTRODUCTION

Large amount of residues from fruit and vegetable processing units, also known as agricultural wastes, are one for causing 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 can be obtained from the conversion of these by-products. 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 environmental pollution along with production of value added products (Bose *et al.,* 2004).

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 procedures are less costly. Some of the advantages of SSF over conventional submerged cultures involving fungi include simplicity in equipment and prevention of bacterial contamination (Pandey, 1992a). 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 particulate structure and by the large surface area of contact which facilitate mass transfer between gas and liquid phases (Raimbault, 1998).

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The filamentous fungi are the most important group of micro-organisms 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 bio-conversion of solid substrates (Raimbault, 1998). The *Aspergillus* spp. have a long history of use as producers of secreted proteins which include amyloglucosidase, amylase, etc (Gwynee and Devchand 1992). *Aspergillus niger* has been used for the production of amyloglucosidase in submerged culture (Janz *et al.*, 1977 and Kennedy, 1987). Strains of *Aspergillus niger* have been used for production of glucoamylase in solid cultures (Selvakumar *et al.*, 1994; Pandey and Radhakrishan 1993, 1995a; Selvakumar *et al.*, 1998; Pandey., 1990, 1991, 1992b, 1992c; Ashakumary *et al.*, 1994; Pandey *et al.*, 1995b, 1996). Studies have also been made on thermostable glucoamylase from *Rhizopus niveus* (Moriyama *et al.*, 1977).

Many microorganisms can hydrolyze starch, specially fungi which are then suitable for SSF application involving starchy substrates. Glucoamylase, α-amylase, β-amylase, pullulanase and isoamylase are involved in the processes of starch degradation. Mainly α-amylase and gluco-amylase 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 (Raimbault, 1998), Amyloglucosidase (synonym glucoamylase; exo-1, 4-α-D-glucan glucano-hydrolase, EC-3.2.1.3) is an important industrial enzyme used for the production of glucose syrup (Moriyama *et al.*, 1977). Various fungal amyloglu-cosidase have been employed in the production of sugar syrup with different dextrose equivalents (DE) (Forgarty, 1983; Nissen, 1987).

Enzymes are among the most important products obtained for human needs through microbial sources (Pandey *et al.*, 1999). Microbial amylases could be potentially useful in the pharmaceutical and fine-chemical industries if enzymes with suitable properties could be prepared (Pandey *et al.*, 2000). Alpha-amylases (1, 4-alpha-D-glucan glucanhy-drolase, EC 3.2.1.1) is a widely distributed secretary enzyme which is one of the most popular and important form of industrial amylases (Gupta *et al.*, 2003). Comparative studies were also made on alpha-amylase production using different substrates (De Almeida *et al.*, 1997; Shankaranand *et al.*, 1992a; Shah *et al.*, 1991). *Thermomyces lanuginose* a thermophilic fungus was reported to be an efficient producer of alpha-amylase (Jensen *et al.*, 1992; Amesen *et al.*, 1998). Studies on acid stable alpha amylase were also carried out using *A. kawachii* IFO 4308 (Sudo *et al.*, 1994). Other microorganisms like *Saccharomycopis capsularia, B. coagulans* etc have also been reported as good alpha-amylase producers (Soni *et al.*, 1996; Babu and Satyanarayana, 1995).

Metal salt plays very important role in metabolic activity of microorganisms. It can initiate or inhibit the production rate of various enzymes & organic acids required in our day to day life. Metal salt acts as the source for metal ions (eg. cataion/anaion) and regulates the metabolic activity of the organism. Various metal salts such as $FeSO_4.7H_2O$, $CuSO_4.5H_2O$, $ZnSO_4.7H_2O$ etc. have been used in evaluating the production of metabolites and enzymes from various fungal sources (Shankaranand and Lonsane., 1992b; Nandakumar *et al.*, 1999) and plants sources (Oboh, 2005; Rao *et al.*, 2005).

Considering the above facts, studies have been carried out to evaluate the effect of various metal salts e.g. $FeSO_4.7H_2O$, $CuSO_4.5H_2O$, $MnCl_2.4H_2O$, $MgSO_4.7H_2O$, $KH_2PO_4.7H_2O$ and $ZnSO_4.7H_2O$ with different concentrations (which were selected randomly) on the production of fungal alpha amylase and amyloglucosidase (glucoamylase) utilizing agricultural wastes as the fermentation medium.

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. A suspension of the mold i.e. one loopfull in 5 ml of sterile water was

used as the inoculum for each Roux bottle in our present study. A constant ratio of 4 : 1 (w/v) of waste to inoculum was maintained through out the study.

Utilization of agricultural wastes for SSF

House hold agro-wastes (i.e. mainly vegetable wastes, a mixture of potatoes, peas peels, non-edible part of spinach, non-edible part of cabbage, non-edible parts of cauliflower etc.) of Kolkata Municipla area were used as substrate in this study. These agro-wastes were sun dried for about 2 days and then dried at 60°C in Tray drier (ICT, India) for 4 hrs. and made to powder using a Mixer grinder (jx 5, Bajaj Electronics Ltd, India). These agro-waste powder was used as medium for SSF throughout the study. The mixed wastes in our present study showed an initial moisture of 10%.

Proparation of metal salt solutions

Production of enzyme alpha amylase and amyloglucosidase by *Aspergillus* spp. varies with the addition of different Metal salts with different concentration. Metal salt solutions were prepared in double distilled water & the concentrations were maintained at ppm level. Each metal salt solutions were prepared in 2 sets with concentration of. 0.1 ppm, 1.0 ppm, 10 ppm, 50 ppm and 100 ppm. The metal salts used are as follows : (i) $FeSO_4.7H_2O$ (as a source of Fe^{2+}); (ii) $CuSO_4.5H_2O$ (as a source of Cu^{2+}); (iii) $MnCl_2.4H_2O$ (as a source of Mn^{2+}); (iv) $MgSO_4.7H_2O$ (as a source of Mg^{2+}); (v) $KH_2PO_4.7H_2O$ (as a source of K^{2+}); and (vi) $ZnSO_4.7H_2O$ (as a source of Zn^{2+}).

Production of alpha amylase and amylo-glucosidase by SSF

Production of alpha amylase and amyloglucosidase by *Aspergillus oryzae* was carried out using 20 g of agro-waste material (particle size of 0.48 mm for alpha amylase and 0.03 mm for amyloglucosidase repectively) and 20 ml of metal salts solutions 1 : 1 (w?v) with desired concentration in standard size Roux bottle (1 liter capacity). Two sets of standard size Roux bottles for each metal salts were taken and plugged with cotton wool. The fermentation was carried out under stationary condition at 30°C. Alpha amylase and amyloglucosidase secreted into the spent medium was monitored at regular interval of time. After 4th day of incubation for amyloglucosidase and 5th day of incubation for alpha amylase, Roux bottles for each enzyme were removed and enzymes were extracted with double distilled water by shaking for 4 hrs. at 30°C. The ratio of waste to water was 1 : 2.5 (w/v). Solids were removed by filtration followed by centrifugation at 10,000 rpm (C-24, REMI, India) for 20 minutes. Clear supernatants were used for measurement of alpha amylase activity and amyloglucosidase activity.

Required conditions for fermentation were optimized previously for both the above mentioned enzymes. A control was maintained by adding double distilled water instead of above mentioned salt solutions.

Enzymes assay

Activity of enzymes produced was measured in Units (U). One unit of alpha amylase activity and amyloglucosidase activity are defined as the amount of enzymes that releases 1 μ mole of reducing sugar per minute from soluble starch at 30°C and pH 7.0 and pH 4.8 respectively. The assay method were carried out according to the methods followed by Shaw *et al.* (1995), Tanuja *et al.* (1997) and Miller (1959). The enzyme activity was expressed in U/gds (i.e. gram dry solid) according to the method described by Ramachandran *et al.* (2004).

RESULTS AND DISCUSSION

Activity of different enzymes depend on presence of different metal ions. Thus, investigations were carried out to study the effect of metal ions for production of amyloglucosidase and alpha amylase enzymes by using metal salt solutions with the agricultural wastes used in our present study as fermentation medium.

From the result it is clear that, alpha amylase showed a maximum activity of 4589.2263 U/gds at 0.1 ppm concentration of $FeSO_4.7H_2O$ (Fig. 1), 3830.1012 U/gds at 1 ppm concentration of $CuSO_4.5H_2O$ (Fig. 2), 9488.9894 U/gds at 1 ppm concentration of $MnCl_2.4H_2O$ (Fig. 3), 7763.7186 U/gds at 1 ppm concentration of $MgSO_4.7H_2O$ (Fig. 4), 7246.1374 U/gds at 10 ppm concentration of $KH_2PO_4.7H_2O$ (Fig. 5) and 9834.0436 U/gds at 10 ppm concentration of $ZnSO_4.7H_2O$ (Fig. 6) with a control (without metal salt solution) showing an

activity of 3450.5416 U/gds. A possible reason for this type of result may be due to the necessity of some metals in developing a highly active enzyme by acting as their prosthetic groups in specific concentrations.

From the result it is clear that maximum yield of amyloglucosidase enzyme obtained 151.8971 U/gds at 1 ppm concentration of $FeSO_4.7H_2O$ (Fig. 7), 276.1767 U/gds at 100 ppm concentration of $CuSO_4.5H_2O$ (Fig. 8), 402.4329 U/gds at 50 ppm concentration of $MnCl_2.4H_2O$ (Fig. 9), 627.3226 U/gds at 10 ppm concentration of $MgSO_4.7H_2O$ (Fig. 10), 112.8157 U/gds at 10 ppm concentration of $KH_2PO_4.7H_2O$ (Fig. 11) and 138.5415 U/gds at 10 ppm concentration of $ZnSO_4.7H_2O$ (Fig. 12) with a control (without metal salt solution) showing an activity of 165.7077 U/gds. This type of result indicates that certain metals can serve as activators by acting as a proper prosthetic group for the enzyme in specific concentrations, whereas others can act as inhibitors for the enzyme.

Except $FeSO_4.7H_2O$, $KH_2PO_4.7H_2O$ and $ZnSO_4.7H_2O$ all other metal salts used in our experiment gave an increased activity of enzyme amyloglucosidase compared to the activity shown by the control, whereas for production of alpha amylase

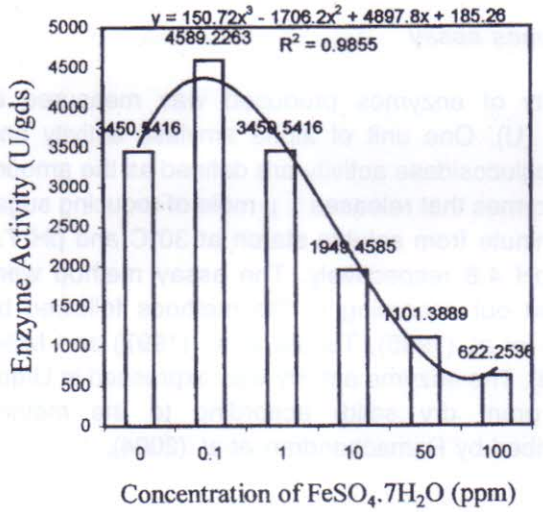

Fig. 1 : Effect of $FeSO_4.7H_2O$ on production of alpha amylase from *Aspergillus oryzae* utilizing agricultural wastes.

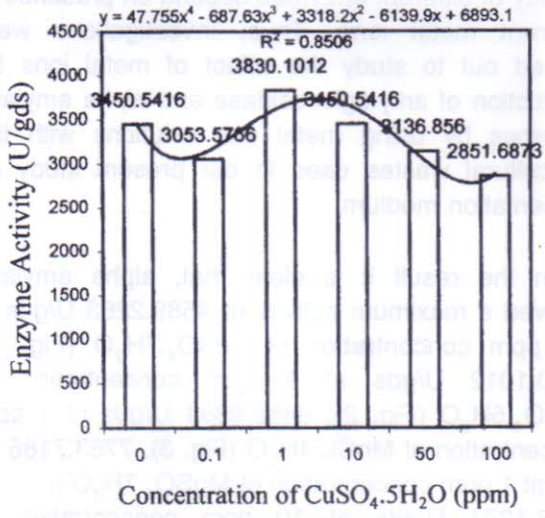

Fig. 2 : Effect of $CuSO_4.5H_2O$ on production of alpha amylase from *Aspergillus oryzae* utilizing agricultural wastes.

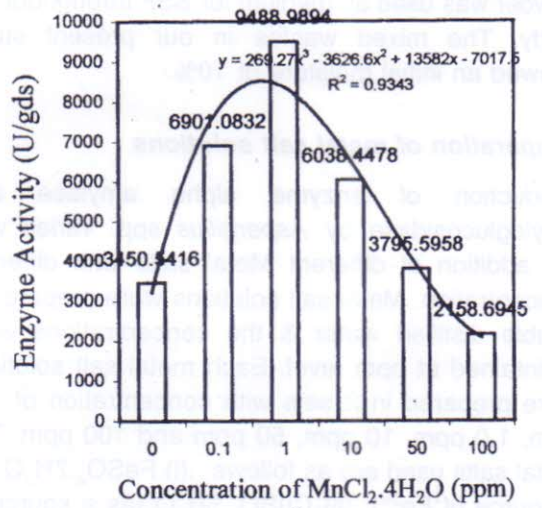

Fig. 3 : Effect of $MnCl_2.4H_2O$ on production of alpha amylase from *Aspergillus oryzae* utilizing agricultural wastes.

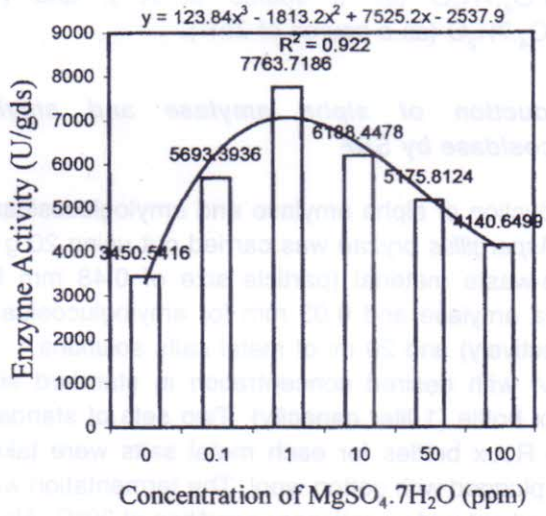

Fig. 4 : Effect of $MgSO_4.7H_2O$ on production of alpha amylase from *Aspergillus oryzae* utilizing agricultural wastes.

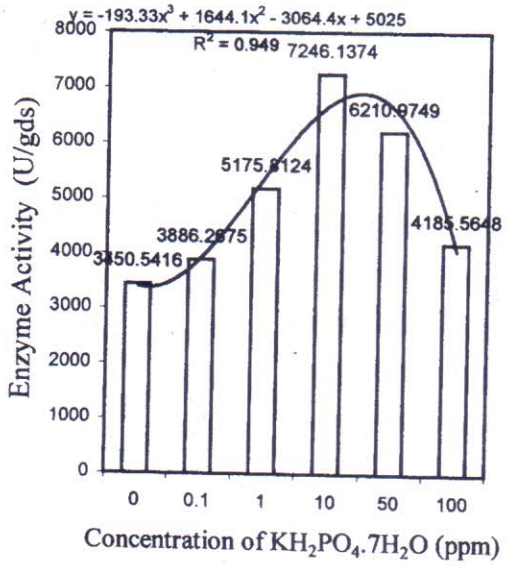

Fig. 5 : Effect of $KH_2PO_4.7H_2O$ on production of alpha amylase from *Aspergillus oryzae* utilizing agricultural wastes.

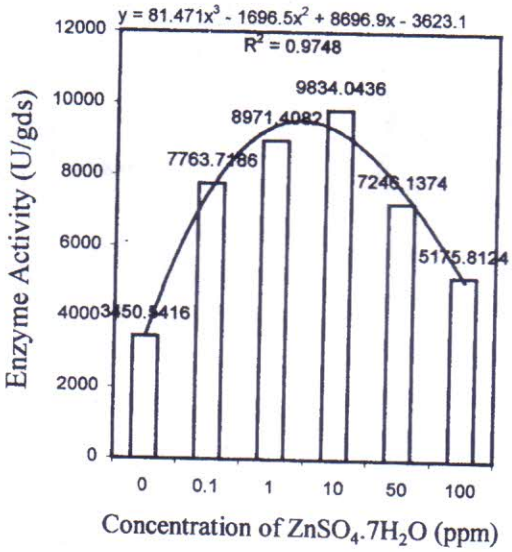

Fig. 6 : Effect of $ZnSO_4.7H_2O$ on production of alpha amylase from *Aspergillus oryzae* utilizing agricultural wastes.

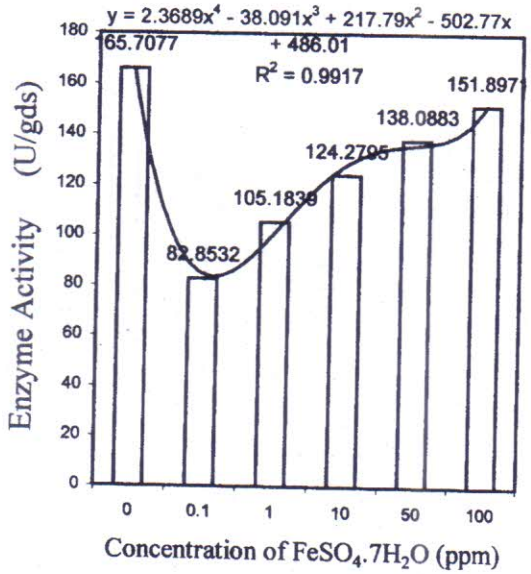

Fig. 7 : Effect of $FeSO_4.7H_2O$ on production of alpha amyloglucosidase from *Aspergillus oryzae* utilizing agricultural wastes.

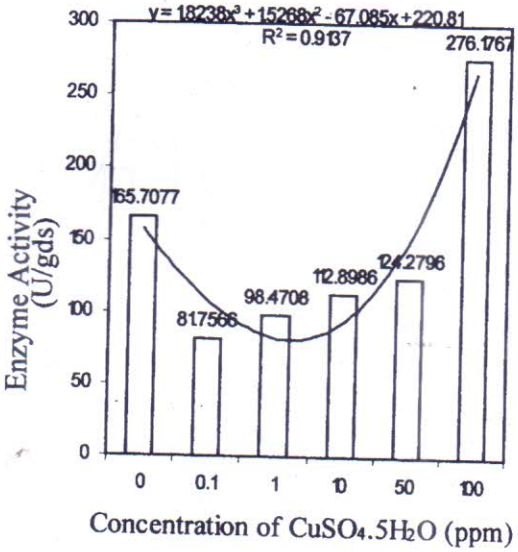

Fig. 8 : Effect of $CuSO_4.5H_2O$ on production of alpha amyloglucosidase from *Aspergillus oryzae* utilizing agricultural wastes

the above metal salts used in our present study gave increased activity compared to control.

The maximum activity of alpha amylase was obtained when $ZnSO_4.7H_2O$ solution was used in medium at a concentration of 10 ppm. Thus, Zn^{2+} ion increased the production of alpha amylase obtained from *Aspergillus oryzae* compared to other metal ions of the metal salts used in our present study when mixed with agricultural wastes acting as the medium for solid state fermentation. The maximum activity of amyloglucosidase was obtained when $MgSO_4.7H_2O$ solution was used in medium at a concentration of 10 ppm. Thus, Mg^{2+} ion increases the production of amyloglucosidase obtained from *Aspergillus oryzae* compared to other metal ions of the metal salts used in our present study when mixed with agricultural wastes acting as the medium for solid state fermentation.

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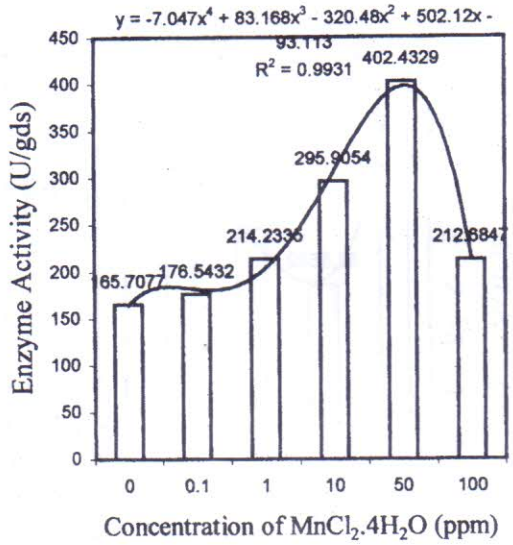

Fig. 9 : Effect of $MnCl_2.4H_2O$ on production of alpha amyloglucosidase from *Aspergillus oryzae* utilizing agricultural wastes.

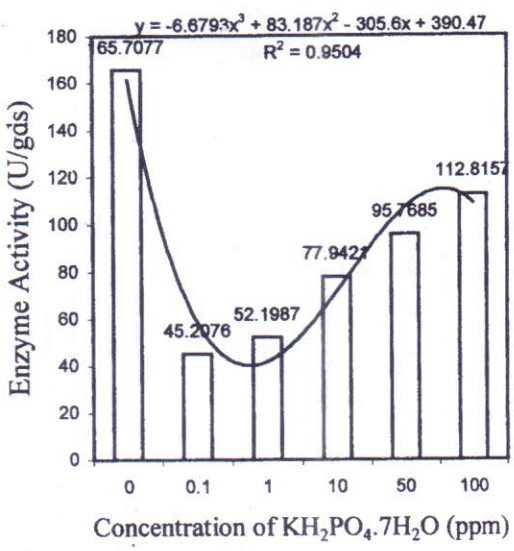

Fig. 11 : Effect of $KH_2PO_4.7H_2O$ on production of alpha amyloglucosidase from *Aspergillus oryzae* utilizing agricultural wastes.

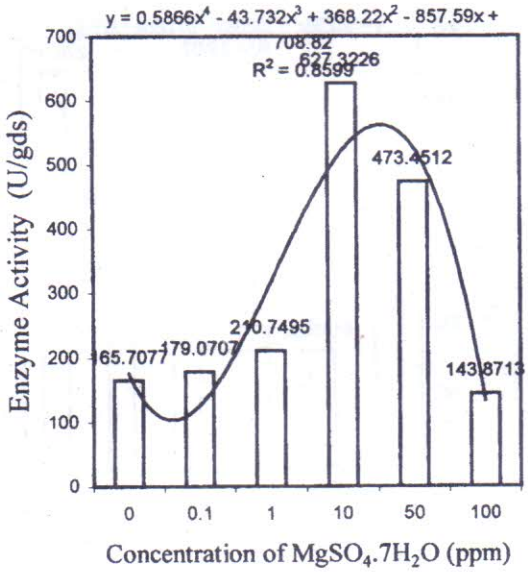

Fig. 10 : Effect of $MgSO_4.7H_2O$ on production of alpha amyloglucosidase from *Aspergillus oryzae* utilizing agricultural wastes.

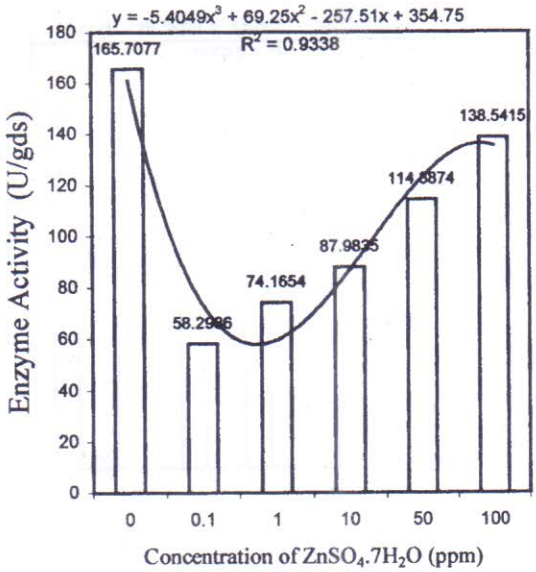

Fig. 12 : Effect of $ZnSO_4.7H_2O$ on production of alpha amyloglucosidase from *Aspergillus oryzae* utilizing agricultural wastes.

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