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Effect of different carbohydrates and amino acids on production of fungal amyloglucosidase by solid state fermentation utilizing agricultural wastes

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Extracellular amyloglucosidase was 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 part of spinach, non edible part of cabbage, non edible part of cauliflower etc. were used as medium in our present study. Results indicated that maximum activity of amyloglucosidase (154.21 U/gds) was obtained at 1% concentration of lactose solution when compared to controls (119.26 U/gds) and other carbohydrates used. Maximum activity of amyloglucosidase (589.80 U/gds) was also obtained at 1% concentration of leucine solution when compared to controls (124.10 U/gds) and other amino acids used in our present study.

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

INTRODUCTION

Solid-state fermentation (SSF) is defined as a fermentation process in which microorganisms grow on solid materials without the presence of free liquid. Solid state fermentation(SSF) is the cultivation of microorganisms on moist solid raw materials, such as grains, beans or wheat bran. These include nonaseptic conditions, use of raw materials as substrates, use of a wide variety of matrices (which vary in composition, size, mechanical resistance, porosity, and water holding capacity), low capital cost, low energy expenditure, less expensive downstream processing (in case, if extraction of the product is necessary, it requires less solvent and lower recovery cost than SmF) as reported by Sato *et al.*(1999).It also includes less water usage and lower wastewater output, potential higher volumetric productivity, higher concentration of the products, high reproducibility, lesser fermentation space (the volumetric loading of the substrate is much higher in SSF than in SmF because the moisture level of the SSF is lower, resulting in compact fermentor or fermentation facility), easier control of contamination, and generally simpler fermentation media as studied by Sato and Sudo. (1999), Gowthaman *et al.* (2001), Durand *et al.* (1983) and Durand (2003). This, however, does not mean that SSF can be taken as a foolproof. SSF has been gaining more and more attention in recent years, even in western countries, due to the possibility of using cheap and abundant agro-industrial waste as substrate (Sato and Sudo, 1999; Crowthaman *et al.*; 2001; Durand, 2003).

Agricultural waste is composed of organic wastes (animal excreta in the form of slurries and farmyard manures, spent mushroom compost, soiled water and silage effluent). 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

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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 environmental pollution along with production of value added products as reported by Bose *et al.* (2004).

Amylases are among the most important enzymes in present-day biotechnology. Amylases find potential application in a number of industrial processes such as in the food, fermentation, textiles and paper industries. A wide variety of microorganisms produce, and in most cases secrete extracellularly, amylases with some interesting properties as studied by Dimitrov *et al.*(1997), Ivanova *et al.*(1993), Stefanova *et al.*(1992), Tonkova *et al.*(1993), Uzunova *et al.*(2000), Nahas *et al.*(2002), van der Laan *et al.*(1995) and Boyer *et al.*(1972) . Amylases are enzymes capable of degrading starch and are universally distributed throughout animal, plant and microbial kingdoms. These enzymes which also act on glycogen and related polysaccharides, catalyze hydrolysis of α-1,4- and/ or α-l,6-glucosidic linkages.

Amyloglucosidase (Glucoamylase, α-l,4-glucan glucohydrolase, EC 3.2.1.3) is an exoamylase like β-amylase but removes a single terminal glucose unit from the non-reducing end of the substrates with the inversion of configuration. It hydrolyses α-1,4-, α-1, 6- and also α-1,3-glucosidic bonds as studied by Shenoy *et al.*(1985). Glucoamylases have been reported to occur in microorganisms, animals and plants, especially in *Aspergillus* and *Rhizopus.*

Filamentous fungi apparently constitute the major source of amyloglucosidase among all microbes. Enzymes used in the food and fermentation industries for starch saccharification, brewing and distilling, are produced by fungal SSF in many Asian countries as well as in North America. Many new research findings are being reported, one being the production of this enzyme by *A. niger* in SSF, SmF, and an aqueous, two-phase system of polyethylene glycol (PEG) and salt as reported by Gowthaman *et al.*(2001). These studies included screening of a number of agro industrial residues, and efficacy established individually and in various combinations.

Carbohydrates such as maltose, starch, cellobiose, lactose, glucose, fructose and galactose favour induction of amyloglucosidase (glucoamylase) as reported by Ali *et al.*(1989). Experimental studies have revealed that, different carbon sources such as glucose, sucrose, maltose, lactose and starch at 1% (w/w) concentration when mixed with substrate, show an increased production of the alpha amylase enzyme, especially in cases with lactose and maltose, even with different strains of *Aspergillus oryzae* as reported by Ramachandran *et al.* (2004). Starch and fructose may also serve as potential activators in case of amylase synthesis as studied by Aiyer (2004).

Amino acids such alanine, arginine, glycine, leucine, phenylalanine, proline, and cystine can act as stimulator for the production of alpha amylase from microorganisms as reported by Aguloglu *et al.* (2000). Whereas, glycine, lysine, isoleucine and histidine, have been proved to be vital for glucoamylase synthesis from *Aspergillus* sp. using rice bran as fermentation medium as reported by Alie *et al.*(1989).

Considering the above experiments have been carried out to evaluate the effect of various carbohydrates e.g. glucose, maltose, lactose, sucrose and soluble starch and heat sterilizable (autoclaveable) amino acids e.g. glycine, histidine, proline, leucine and isoleucine (BIONET, website) with different concentrations on the production of fungal 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%, $NaN0_3$ 0.2%, KC1 0.05%, $MgS0_4.7H_20$ 0.05%, $FeS0_4.7H_20$ 0.001%, KH_2P0_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 blank 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 throughout the study.

Utilization of agricultural wastes for SSF

House hold agro-wastes (i.e. mainly vegetable wastes, a mixture of potato peels, wastes potatoes, pea peels, non-edible part of spinach, non-edible part of cabbage, non-edible parts of cauliflower etc.) in Kolkata Municipal 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 hr. and made to powder in a Mixer grinder (jx 5, Bajaj Electronics Ltd, India). These agro-waste powder was used as medium for SSF through out the study. The mixed wastes in our present study showed an initial moisture of 10%.

Preparation of carbohydrate solutions

Carbohydrate solutions were prepared in double distilled water and each carbohydrate solutions was prepared with concentration of. 0.1 %, 0.2%, 0.25%, 0.5% and 1%.The carbon sources were glucose. maltose, lactose sucrose and soluble starch.

Preparation of amino acid solutions

Amino acid solutions were prepared in double distilled water and each amino acid solution was prepared with concentration of. 0.25%, 0.5%, 0.75% and 1%. The amino acids sources were glycine, histidine, proline, leucine and isoleucine.

Production of amyloglucosidase by SSF

Production of amyloglucosidase by *Aspergillus oryzae* was carried out in two sets of standard size Roux bottle. In one set 20 g of agro-waste material (particle size of 0.03 mm) and 20 ml of carbohydrate solutions 1:1 (w/v) with desired ooncentration was taken. In another set 20 g of agro-waste material (particle size of 0.03 mm) and 20 ml of amino acid solutions 1:1 (w/v) with desired concentration was taken. Finally, after aseptic inoculation with *Aspergillus oryzae*, 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 4th day of incubation for amyloglucosidase, both sets of Roux bottles were removed. The enzyme was extracted with double distilled water by shaking for 4 hrs. at 30°C for the medium treated with carbohydrate and amino acid respectvely.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 minutes. Clear supernatants were used for measurement of amyloglucosidase activity.

Required environmental conditions for fermentation were optimized previously for amyloglucosidase as reported by Bose *et al.*(2008). A control was maintained for each set by adding only double distilled water instead of above mentioned carbohydrate and amino acid solutions.

Enzyme assay

Activity of enzyme produced was measured in Units (U) for amyloglucosidase. One(1) unit of amyloglucosidase activity is defined as the amount of enzyme that releases 1 μ mole (micromole) of reducing sugar per minute from soluble starch at pH 4.8 at 30°C. Amyloglucosidase activity was determined at 30°C by mixing 0.1 ml of diluted (adequate dilution with double distilled water) enzyme solution with 0.9 ml of 4% soluble starch dissolved in 0.2 M acetate buffer, pH 4.8. This reaction mixture was incubated for 1 h. After incubation the reaction was stopped by adding 3 ml of dinitrosalicylic acid (DNS) solution . The mixture prepared was heated in boiling water bath for 3 min. Absorbance at 540 nm wavelength (Spectrophotometer, U-2000, Hitachi, Japan) was measured after cooling the DNS-sample mixture at room temperature. For each sample an enzyme blank and a substrate blank was maintained. The above procedure was followed as per a modified method of Tanuja *et al.*(1997) and Miller *et al.*(1959).

The enzyme activity for amyloglucosidase was expressed in U/gds (i.e. gram dry solid) following the method described by Ramachandran *et al.* (2004).

RESULTS AND DISCUSSIONS

From the result it is clear that maximum yield of amyloglucosidase enzyme obtained 75.89 U/gds at 0.1% concentration of glucose (Fig. 1) with an exponential decrease in enzymatic activity, 94.73 U/gds at 1% concentration of maltose (Fig. 2), 154.21 U/gds at 1% concentration of lactose (Fig. 3), 54.21 U/gds at 1% concentration of sucrose (Fig. 4) and 140.37 U/gds at 1% concentration of soluble starch (Fig. 5) with a control (without carbohydrate solution) showing an activity of 119.26 U/gds. This type of

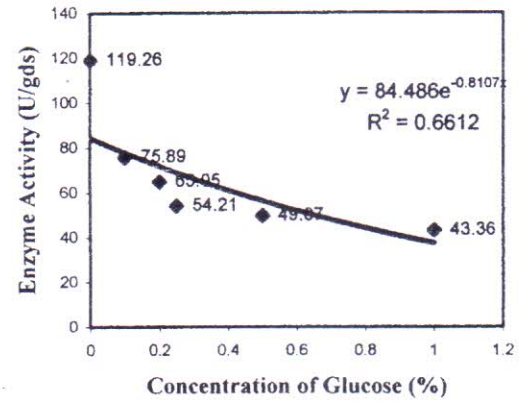

Fig 1. Effect of glucose on production of amyloglucosidase from *Aspergillus oryzae* utilizing agro-wastes.

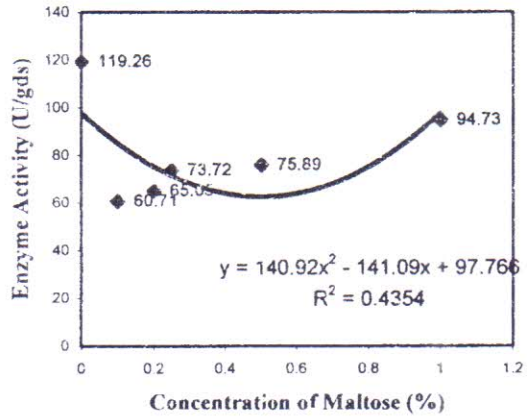

Fig 2. Effect of moltose on production of amyloglucosidase from *Aspergillus oryzae* utilizing agro-wastes.

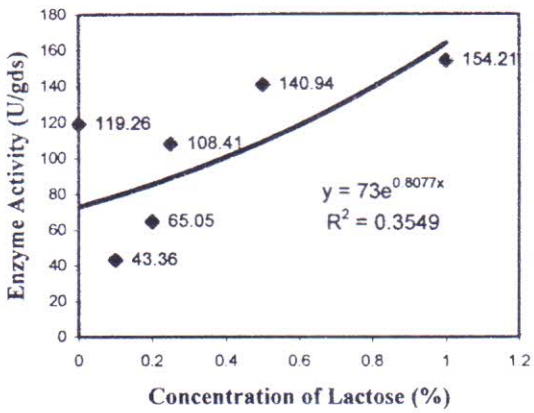

Fig 3. Effect of lactose on production of amyloglucosidase from *Aspergillus oryzae* utilizing agro-wastes.

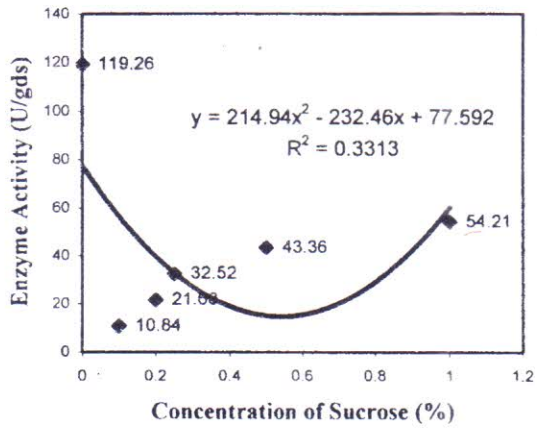

Fig 4. Effect of sucrose on production of amyloglucosidase from *Aspergillus oryzae* utilizing agro-wastes.

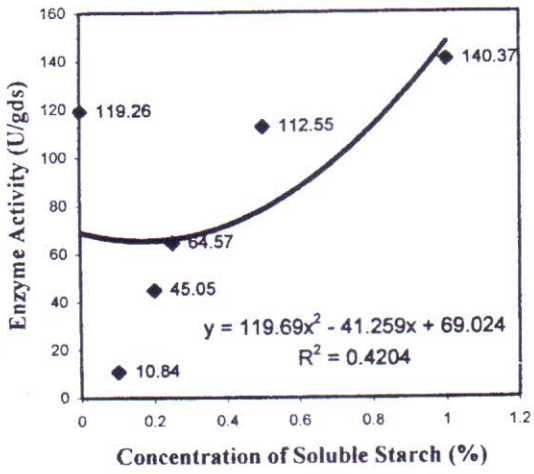

Fig 5. Effect of soluble starch on production of amyloglucosidase from *Aspergillus oryzae* utilizing agro-wastes.

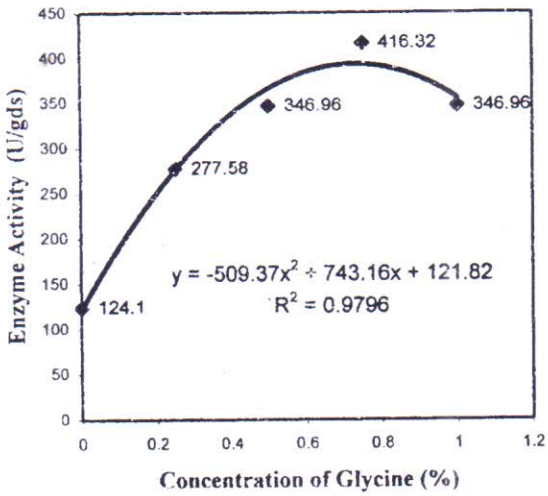

Fig 6. Effect of glycine on production of amyloglucosidase from *Aspergillus oryzae* utilizing agro-wastes.

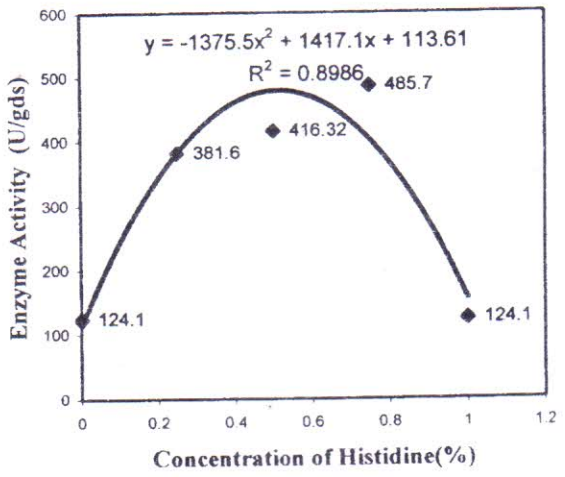

Fig 7. Effect of histidine on production of amyloglucosidase from *Aspergillus oryzae* utilizing agro-wastes.

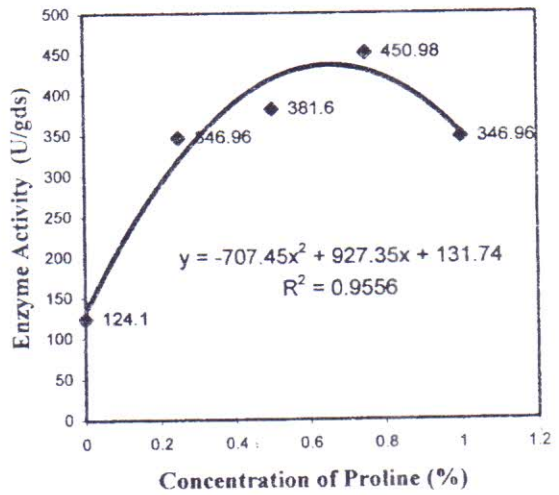

Fig 8. Effect of proline on production of amyloglucosidase from *Aspergillus oryzae* utilizing agro-wastes.

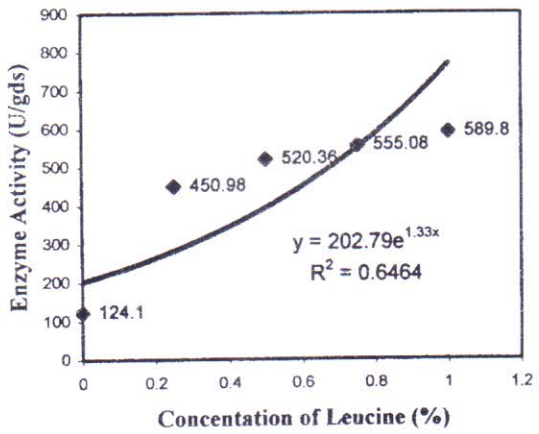

Fig 9. Effect of leucine on production of amyloglucosidase from *Aspergillus oryzae* utilizing agro-wastes.

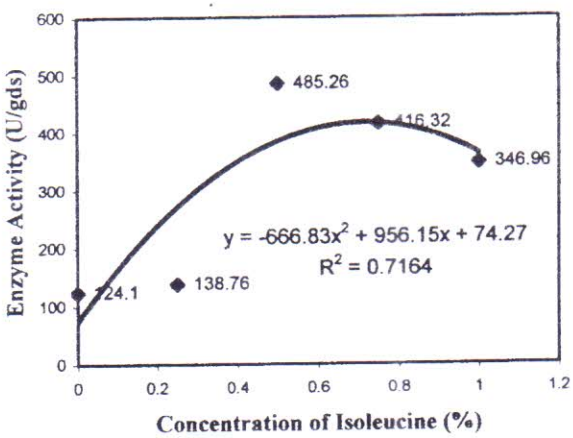

Fig 10. Effect of isoleucine on production of amyloglucosidase from *Aspergillus oryzae* utilizing agro-wastes.

result indicated that, certain carbohydrate sources can serve as activators in specific concentrations by initiating the metabolic activity, which in turn enhanced the need of enzymes to be produced, whereas others can act as suppressors for the above said enzyme in specific concentrations. An enzyme activating effect at higher concentration and inhibitory effect at lower concentration of lactose and soluble starch (Fig.3 & Fig.5) were observed, which may open some new ideas and need further investigations at molecular level to demonstrate a proper reason.

From the result it is clear that maximum yield of amyloglucosidase enzyme obtained 416.32 U/gds at 0.75% concentration of glycine (Fig. 6), 485.70 U/gds at 0.75% concentration of histidine (Fig. 7), 450.98 U/gds at 0.75% concentration of proline (Fig. 8), 589.80 U/gds at 1% concentration of leucine (Fig. 9) and 485.26 U/gds at 0.5% concentration of isoleucine (Fig. 10) with a control (without amino acid solution) showing an activity of 124.10 U/gds. This type of result indicated that, certain amino acids can serve as activators in specific concentrations by initiating the growth and metabolic activity of the microorganism, which in turn enhanced the need of enzymes to be produced. In case of Fig. 9 an exponential pattern had been observed, it may be due to direct proportionality between concentration of leucine and enzyme activity.

The maximum activity of amyloglucosidase was obtained when solution of lactose was used in medium at a concentration of 1%. Thus, lactose as a source of carbon increased the production of amyloglucosidase from *Aspergillus oryzae*

compared to other carbohydrates used in our present study. The maximum activity of amyloglucosidase was obtained when solution of leucine was used in medium at a concentration of 1 %. Thus, leucine acting as a source of nitrogen increased the production of amyloglucosidase from *Aspergillus oryzae* compared to other amino acids used in our present study. This type of results some or other way supports the studies reported by Ali *et al.*(1989), Ramachandran *et al.* (2004), Aiyer (2004) and Aguloglu *et al.* (2000). This also signifies the novelty of the research work where the agricultural wastes which are considered as unwanted and discarded materials of our society, are utilized to produce higher amount of commercially valuable product (enzymes) by making changes in the nutritional requirement (carbohydrates and amino acids) for the mold exploited in an eco-friendly fermentation process.

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