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Solid State Fermentation of Agro-Residues for the Production of Amylase from *Bacillus subtilis* for Industrial Applications

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A B S T R A C T

Bacillus subtilis has been widely used to produce hydrolytic enzymes for various industrial applications. In this study, solid state fermentation was carried out using various agro- industrial wastes for the production of alpha amylase using *B. subtilis*. Among the tested agro-residues, wheat bran supported more enzyme production. The process parameters elucidated were type of substrate, incubation time, pH, moisture content of the medium, carbon source and nitrogen source. Starch (1%, w/w) and yeast extract (0.5%, v/v) were enhanced the enzyme production. Optimization of the physical parameters revealed the optimum incubation period, pH for amylase production by the isolate as 48 h and 7.0 respectively.

Introduction

Amylase is one of the important enzymes widely used in various industries. This enzyme hydrolyses starch and is used commercially for the production of sugar syrups from starch which consist of maltose, glucose and higher oligosaccharides. These enzymes are of potent significance in various biotechnological industries ranging from food, detergent, brewing, pharmaceutical, textile and paper industries (Babu and Sathyanarayana, 1995). To meet the industrial need, low cost production of amylase is highly preferable to decrease the overall production cost. Amylases are produced by fungi, bacteria, plants and animals. Moreover, due to efficient production strategies, microorganisms have potential to contribute to a number of industrial processes. Such industrially important bacteria are mainly found within the genus *Bacillus* because of their fast growth rates that lead to very short fermentation cycles and their capacity to secrete proteins into extra cellular medium (Baysal *et al.*, 2003).

Alpha – amylase cleaves the 1,4-α-D-glycosidic linkages between adjacent glucose unit inside the linear starches, glycogen and oligosaccharides in a random manner. Solid state fermentation (SSF) is widely used for the production of amylases effectively. Agro-residues are generally applied in SSF and have

numerous advantages. The agro-residues such as, rice bran, coconut oil bran and wheat bran have replaced the high cost synthetic media generally used in submerged fermentation (SmF) for amylase preparation because of their low cost, simplicity, availability, lesser water output and better productivity. Additionally SSF process solves the pollution problem occurring due to their disposal in the surrounding (Nigam and Singh, 1995). Agro-wastes contain high starch content (60–70% by weight) which can be utilized as an important nutrient source by microorganisms like fungi and bacteria, for the synthesis of alpha-amylase which is under the control of catabolic repression (Stredansky *et al.,* 1999).

Production of α amylase has been carried out through SmF and SSF. However, the uses of a synthetic culture medium are highly expensive and are not economical. Agricultural by-products are generally considered to be potent substrates for SSF to produce enzymes (Rameshkumar and Sivasudha, 2011). In recent years the technique of SSF process has been used more extensively for the production of enzymes. The important factors that affect synthesis of microbial enzymes in a SSF system include particle size of the substrate, pH of the culture medium, moisture level of the substrate, inoculum concentration, carbon and nitrogen sources. Thus SSF process involves the screening of various agro-industrial wastes for product formation and microbial growth (Sodhi *et al.,* 2005). Availability of the agro-residues and cost are the two important considerations and hence, the selection of suitable solid substrate plays significant role in the development of SSF process. The concentration and composition of medium greatly affect the growth and production of enzymes from bacteria and fungi (Nigam and Singh, 1995). In the present study, the process parameters were optimized to enhance the production of amylase from *B. subtilis* in SSF.

Materials and Methods

Microorganism

The hydrolytic enzyme producing *B. subtilis* was previously isolated from the soil and was used for the optimization of protease production. This organism hydrolyzed starch, hence this it was subjected for optimization of amylase production.

Inoculum

In this study, the selected bacterial isolate was grown on nutrient agar medium for 24 h at 37 °C. A loopful culture of this organism was transferred to nutrient broth medium (0.5% peptone, 1% yeast extract, 0.5% NaCI, pH 7.0) and incubated in rotary shaker at 150 rpm for 18 h at 37 °C.

Substrates

Apple peel, banana peel, rice bran, wheat bran, green gram husk and pine apple peel were obtained locally. These materials were dried for a week under sunlight. All substrates were ground into powder using a mixer grinder.

Production of amylase in SSF

In this study, initially all substrates (apple peel, banana peel, rice bran, wheat bran, green gram husk and pine apple peel) were screened for the production of amylase.

Briefly, SSF was carried out by adding 5.0 g of dried substrate individually in a 100 mL Erlenmeyer flask to which Tris buffer (pH 8.0, 0.05 M) was added to adjust the moisture level. The substrate was mixed and sterilized for 20 min at 121 °C. About 5% (v/w) inoculum was added and all inoculated flasks were kept in incubator at 37 °C for 96 h. About 50 ml double distilled water was added

to the fermented flasks and the contents of the flasks were harvested and enzyme assay was carried out.

Extraction of enzyme

The enzyme from the fermented substrate was extracted twice with double distilled water. The slurry was then squeezed through cheesecloth and the extracts were pooled. It was centrifuged for 10,000 rpm for min at 4 °C to separate small particles of different substrates and cells. The cell free supernatant was used in enzyme assay.

Enzyme assay

Alpha –amylase assay was determined using starch as a substrate. In this assay, the reaction mixture containing 1.0 ml substrate (1%) which was prepared in 0.1 M sodium phosphate buffer (pH 7.0) and 0.50 ml of crude enzyme solution was added. These were incubated for 30 min at 37 °C.

Then 1.0 ml 3,5-dinitrosalicylic acid solution added and place in a boiling water bath for 5 min. The absorbance was measured at 489 nm in a UV-Visible spectrophotometer.

One unit (U) of alpha-amylase activity was defined as the amount of enzyme that releases 1 µmol of reducing sugar as maltose per minute, under assay conditions and expressed as U/g of agro-waste substrate. All the experiments were carried out in triplicates and the average value was calculated.

Optimization of culture medium to enhance enzyme production

Optimization of culture medium components and fermentation process is of primary importance in the production of industrial enzymes. Wheat bran was used as the substrate for optimization of amylase production, which showed maximum production of amylase in initial experiments. The candidate strain was inoculated into the culture medium and enzyme was extracted.

Effect of fermentation period on enzyme production

The amylase activity was determined in the culture medium for every 24 h of fermentation period up to 96 h in order to evaluate the effect of fermentation period. Enzyme was extracted and amylase assay was carried out from the culture medium.

Effect of moisture content on amylase production

To evaluate the effect of the initial moisture content on amylase production, the initial moisture content of the solid substrate was adjusted from 60 to 110% (v/w) using Tris - HCl buffer (pH 8.0). Enzyme was extracted and amylase assay was carried out.

Effect of pH on amylase production

The effect of pH on amylase production was elucidated. Buffer at various pHs was added individually (90%, v/w) with the solid medium.

The pH of the buffer solution was varied from pH 6.0 to 11.0. After fermentation, enzyme was extracted and amylase assay was carried out.

Effect of carbon source on amylase production

To evaluate the effect of carbon sources on amylase production, the carbon sources such as, lactose, sucrose, maltose, xylose and starch were supplemented with the wheat bran medium at 1.0% (w/w) level. Enzyme was extracted and amylase assay was carried out.

Effect of nitrogen source on amylase production

To evaluate the effect of nitrogen sources on amylase production, the nitrogen sources such as yeast extract, beef extract, peptone, urea and ammonium sulphate were supplemented with the solid medium at 0.5% (w/w) level. Then enzyme was extracted and amylase assay was carried out.

Results and Discussion

In this study, amylase production was carried out by optimizing various physical parameters and nutrient sources. *B. subtilis* was selected on the basis of clear halo zone around the colony on the starch agar plate. *Bacillus* sp. we are well known and widely used for the production of amylases by various industries for producing beverages, food, pharmaceutical products and chemicals (Gangadharan *et al.,* 2006). In recent years, α-amylase by *Bacillus* sp. is economically produced using SSF. Moreover, the global demand for α- amylase production is increasing rapidly than its production, implying that economical processes are required to replace or supplement the present processes (Alvarez-Vasquez *et al.,* 2000). Furthermore, SmF cannot be adapted to specific process conditions such as the use of sugar-rich by-products (Wasay *et al.,* 2001). The α-amylase production by bacterial species is significantly influenced by various fermentation parameters. For achieving the production of α-amylase, it is essential that the study of influence of nutrient and physical factors on α-amylase production (Anto *et al.,* 2006).

The important parameters that govern the SSF fermentation process are type of substrates, incubation period, nitrogen sources, inorganic nutrients, pH and incubation temperature. In order to achieve maximum α-amylase production these parameters need to be optimized. In the present study various agro-residues were used as substrates for amylase production and the results are presented in Figure 1. Agro-residues are rich of carbon and nitrogen sources. In this study, production of amylase varied considerably. Among the agro-residues, wheat bran showed more amylase production than other selected substrates. Moreover, all substrates supported bacterial growth and amylase production. The results of the present study were in concurrence with amylases from *Bacillus subtilis* (Baysal *et al.,* 2003) and *Bacillus amyloliquefaciens* (Gangadharan *et al.,* 2006). Wheat bran was the good substrate for α-amylase production when compared to all the other tested agro-residues. This might be due to the presence of various nutrients and their large surface area for microbial growth and product formation (Babu and Satyanarayana, 1995). For further optimization experiments, wheat bran was used as the substrate for α-amylase production.

The present study demonstrated that the maximum production of amylase by *B. subtilis* was obtained at 48 h of incubation period at 37 °C (Fig. 2). A significant reduction in enzyme activity was registered afterwards because of the depletion of essential nutrients in the culture medium. Moisture content is one of the significant factors affecting the production of amylase. The significance of moisture content in SSF and its function on the biosynthesis of enzymes has been attributed to the interference of moisture in the physical properties of solid particles of culture medium. The enzyme production attained a peak, when moistened content of the medium was reached 80%. Enzyme production was decreased with lower moisture level (Fig. 3). This might be due to an insufficient water availability preventing a good diffusion of gas and solutes, thereby resulting in complete arrest or slowdown of cell metabolism (Baysal *et al.,* 2003).

Fig.1 Screening of agro-residues for the production of amylase

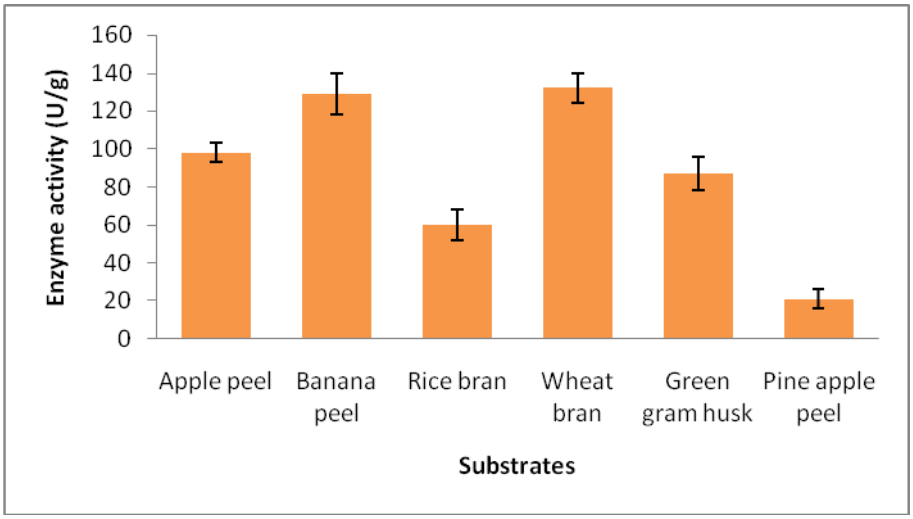


Fig.2 Effect of fermentation period on enzyme production

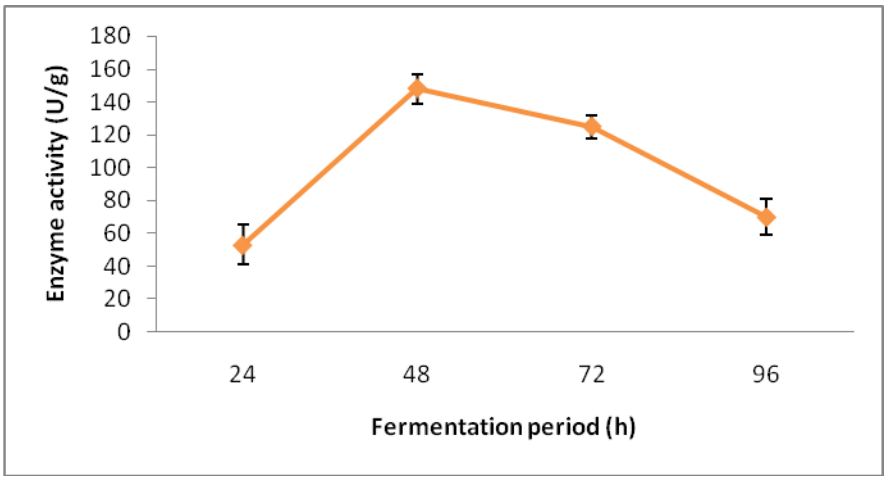


Fig.3 Effect of moisture content on amylase production

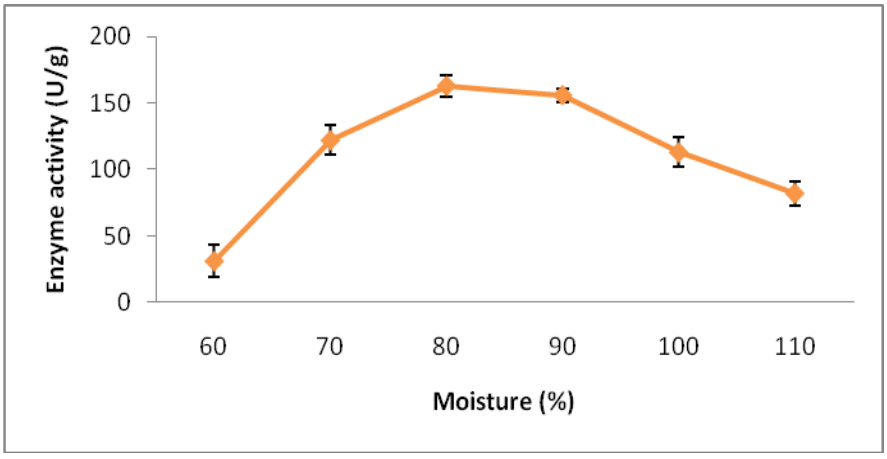


Fig.4 Effect of pH on amylase production

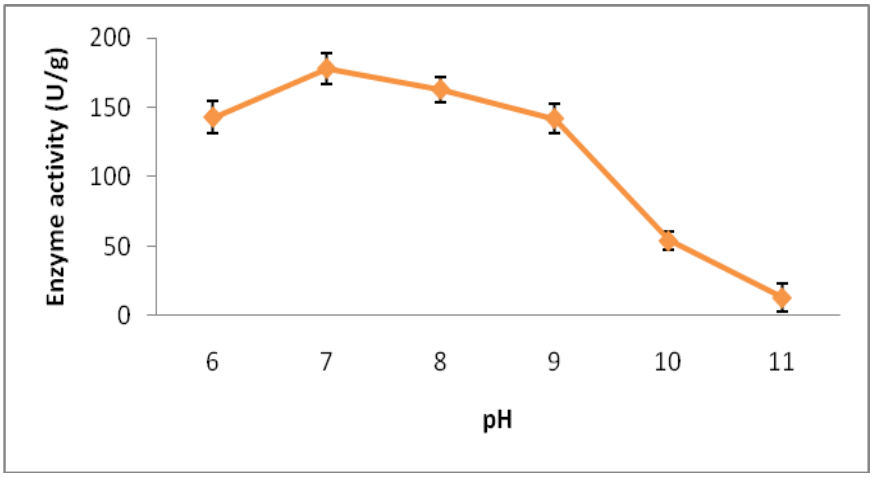


Fig.5 Effect of carbon sources on enzyme production

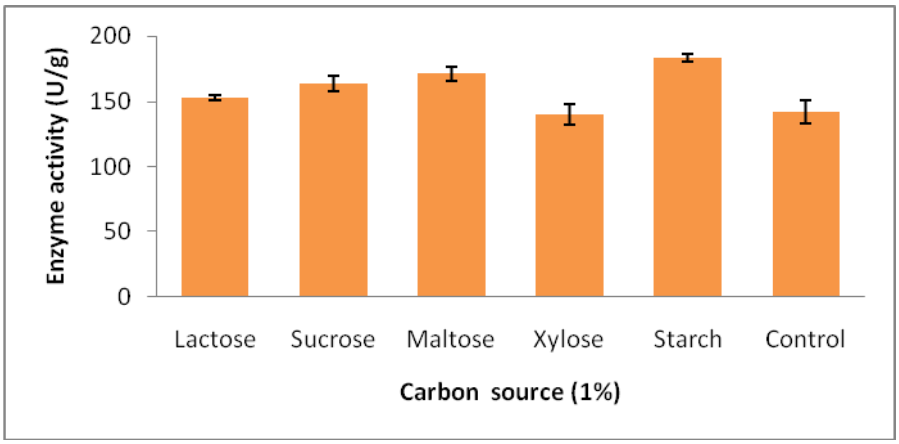
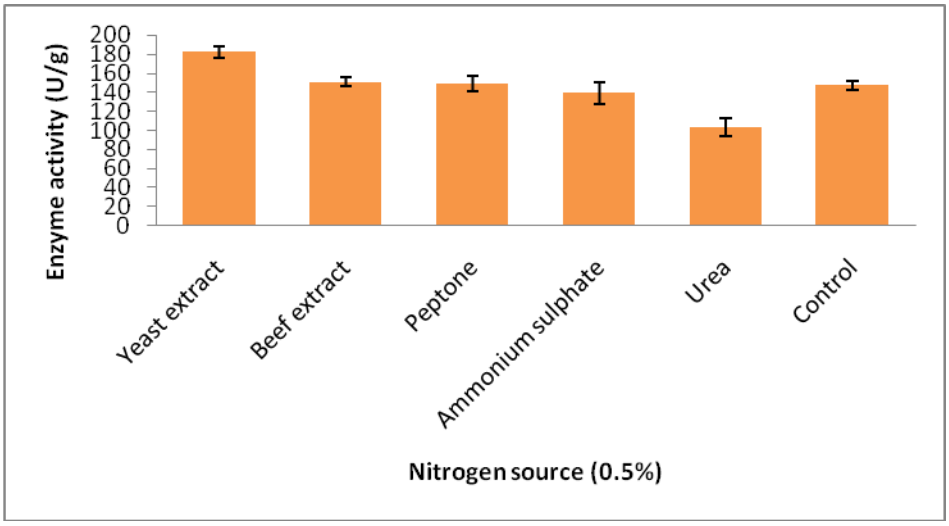


Fig.6 Effect of nitrogen sources on amylase production



It was reported that higher moisture contents decreased porosity, reduced gas volume and exchange, promoted development of stickiness, resulted in static hindrance of the growth of the organisms through reduction in inter particle spaces, changed substrate particle structure and decreased diffusion and impaired oxygen transfer. Low moisture content in the culture medium resulted in poor adsorption of enzyme to the substrate particle (Swain and Ray, 2007).

Amylase production was high at pH 7.0, however the optimum range of amylase production between pH value 6 and 9 (Fig. 4). This study was similar with previous results. Nusrat and Rahman (2007) reported maximum amylase production from *Bacillus* sp. at neutral pH. A change in the pH of the culture medium alters the ionization of nutrients and reduces their availability to the bacteria that results in reduction of their metabolic activity when the pH of the culture medium altered; it results in the reduction of amylase activity in the culture medium mainly due to the denaturation of proteins. In enzyme bioprocess, culture conditions and medium constituents were significantly influenced on extracellular amylase production. According to the present study, starch plays an important role as inducer in the culture medium because starch is a complex carbohydrate and it is metabolized by the bacterial isolates in a slower manner and thus, accumulates maximum amylase in the culture medium. The effect of various carbon sources on of amylase production from *Bacillus* sp. revealed that among the carbon sources, starch (1% w/v) was found to be the best inducer for amylase production (Fig. 5). However, a significant reduction in the activity of amylase was observed when xylose was added with the wheat bran medium as a source of carbon. Carbon is not only an important medium component in the culture medium, but also one of the essential elements for the growth and metabolism of bacteria resulting in stimulation of enzyme production. The optimal carbon source for amylase production by *Bacillus* sp. was registered when starch was used as a carbon source. These results were similar with the findings of Karatas *et al.,* (2013) who demonstrated maximum amylase production in the presence of starch in the culture medium. In this study supplementation of the medium with yeast extract (0.5%, w/w) showed maximum amylase activity. On the other hand, amylase production was very low in the production medium containing urea (0.5% w/w). Among various nitrogen sources tested, yeast extract was the good nitrogen source for the isolate in order to achieve maximum production of amylase (Fig. 6). It was reported that, yeast extract play an important role in enzyme production due to the presence of coenzymes, nitrogenous constituents, growth factors and essential elements. Also, yeast extract was a good inducer for amylase production from *Bacillus* sp. (Salman *et al.,* 2016). In the present investigation, the optimized medium enhanced more than 1.5 fold amylase yield than that of unoptimized medium.

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