September 2010 ISSN : 0974 - 7435 Volume 4 Issue 3

Trade Science Inc.

BioTechnology

An Indian Journal

FULL PAPER

BTAIJ, 4(3), 2010 [156-159]

Production of extracellular α-amylase enzyme by *Bacillus amyloliquefaciens* using coffee pulp waste as a substrate

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Received: 31st March, 2010 ; Accepted: 10th April, 2010

ABSTRACT

Solid state fermentation (SSF) process was used to produce extra cellular amylase enzyme from *Bacillus amyloliquefaciens* by using coffee pulp waste as a substrate. This substrate gave maximum yield of amylase enzyme (750U/gm) at 120hrs of incubation under optimum conditions. The maximum relative enzyme activities of crude and purified enzymes were shown at pH 8.0-9.0 and at 45-55°C respectively.

KEYWORDS

Coffee pulp;
α-amylase;
Bacillus amyloliquefaciens;
SSF;
Enzyme activity;
Ammonium salt precipitation.

INTRODUCTION

Coffee pulp as a waste is usually disposed off without any treatment and left to degrade naturally in heaps, with the uncontrolled liberation of noxious odor and nutrient load leaches out as a consequence. It may take six to eight months to achieve stabilization of the organic matter and moreover the nitrogen in the residue does not contain more than 2% of the dry weight[9]. Coffee is the one of the most important agricultural commodity in the world. *Coffea arabica* and *Coffea robesta* are the two principal verities of the genus cultivated all over the world for commercial production.

Coffee has traditionally been grown under the canopy of the flowering forest or inter-cropped fruit trees.

In industrial processing of coffee cherries is done to isolate coffee seeds by removing shell and mucilaginous part from the cherries. There are two methods of processing, i.e., wet or dry process, the solid residues (sub-product) obtained are termed as pulp or husk. In India, the coffee cherries are generally processed by the wet method resulting in the coffee pulp, which are rich in organic nature and nutrients.

Solid-state fermentation (SSF) has been considered as a useful tool for biomass energy conservation, solid waste treatment and production of added value molecules such as enzymes, organic acids and biologically active secondary metabolites[13].

The abundant use and cost effective agricultural by products, such as cereal grains, wheat bran, etc., are commonly use to produce α-amylase in solid-state fermentation (SSF) by the growth of microorganisms on moist substrates in the absence of free flowing water; where water is present in an absorbed or in complex form with the solid matrix and substrates[5]. Thus, SSF

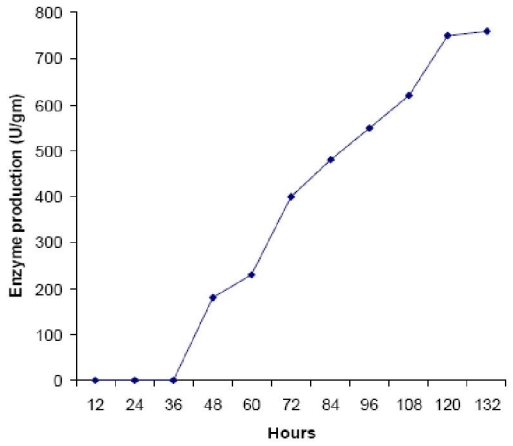

Figure 1 : Production of α-amylase amylase at different time interval in SSF

is an alternative attractive process to produce microbial enzymes due to its lower capital investment and lower operating cost[6].

Alpha amylases are the endo acting enzymes which randomly hydrolyze α-1,4-glycosidic bonds between adjacent glucose units in the starch polymer leading to the formation of linear and branched oligosaccharides. These starch hydrolytic enzymes co 30% of the world's enzyme consumption. Thus, α-amylases are used extensively in sugar, textile, alcohol and paper industries. They are also used in food industry such as baking, brewing, preparation of digestive aids, production of cakes, fruit juices and starch syrups.

Many reports are available on the production of α-amylase enzyme in SSF by fungal species but there are few reports on the production of α-amylase by bacteria in SSF. Among these, α-amylase production is of very low yield by *Bacillus* sp. on various substrates. In the present study, the high level of α-amylase was produced by *Bacillus amyloliquefaciens* on coffee pulp waste under the solid state fermentation process. The properties of the enzyme activity on degradation of starch at different pH and temperature profiles revealed that coffee pulp is a novel substrate for the production of commercially valuable α-amylase enzyme.

MATERIALS AND METHODS

Collection and process of coffee pulp waste

The coffee pulp waste of *Coffea arabica* was collected from the coffee seed processing industry at Sirumallai Hills, India and air dried in laboratory.

TABLE 1 : Summary of α-amylase produced from coffee pulp waste

Enzyme processing	Total protein (mg)	Total activity (U/ml)	Specific activity	Yield (%)	Purification fold
Crude enzyme	350	1470	4.2	100	1.0
Ammonium sulphate precipitation	270	1444	5.3	98.2	1.2

Seed culture preparation

The *Bacillus amyloliquefaciens* (MTCC 610) obtained from the IMTECH, Chandihargh. was grown on agar plates containing Nutrient Peptone 0.5%, Soluble starch 1%, Agar 2%. The culture had stored at 4°C and sub-cultured monthly. The seed medium used for overnight culture was a modified form of medium described by Anderson et al.[3] used for seed culture preparation. Seed medium was g/l: 10 D. Glucose; 5 Peptone; 2 Yeast extract; 1.5 NaCl; 0.5KH_2PO_4; 0.5 $MgSO_4.7H_2O$; 0.1 $CaCl_2$. The pH of the medium was adjusted to 7 by 1M NaOH and 1M HCl before sterilization. The D-glucose was sterilized by using (0.45μm) filtration process.

Solid state fermentation

The experiment was conducted in 1000ml Erlenmeyer flasks containing 100g of sterilized coffee pulp as substrate and 10 ml of salt solution containing the following (g/l) in distilled water: K_2HPO_4, 5; $MgSO_4.7H_2O$, 0.5; NH_4NO_3, 0.2M; $CaCl_2$, 0.0275M. Distilled water was added to achieve final substrate moisture content of 80%. After sterilization, the flasks were cooled and inoculated at 37°C for 168 hrs. During incubation, the fermented substrates from the flasks were harvested and assayed at 24hrs intervals.

Assay of amylase enzyme

At the end of the fermentation, the solid fermented substrate was mixed with 150 ml of distilled water and agitated thoroughly on a magnetic stirrer for 30 minutes. Then the entire contents were filtered through the muslin cloth and residue was treated with 100 ml of distilled water and the process repeated. The filtrates were collected, centrifuged at 10,000rpm for 10 minutes and the clear supernatant used as the enzyme source.

α-amylase activity had determined by the method of Okolo et al.[12]. The reaction mixture consisted of 1.25ml of 1% soluble starch, 0.5ml; 0.1ml 0.1M ac-

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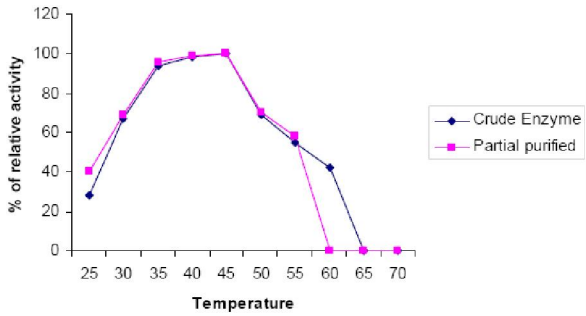

Figure 2 : Amylase enzyme activity with temperature

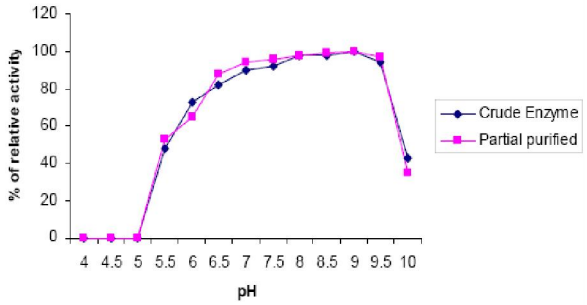

Figure 3 : Amylase enzyme activities with pH

etate buffer (pH 7.0) and 0.25ml of crude enzyme extract. After 30min of incubation at 37°C the liberated reducing sugars (glucose equivalent) were estimated by the Dinitrosalicylic acid (DNS) method[11]. The color developed was read at 575nm using spectrophotometer. Glucose was used as the standard and the blank contained 0.75ml of 0.1M acetate buffer (pH 7.0) and 1.25ml 1% starch solution. One unit (IU) of α-amylase is defined as the amount of enzyme releasing 1μmol of glucose equivalent per minute under assay condition. Amylase activity was also determined at different pH (4-10) and temperature (25-65°C) by added with 1 % (w/v) soluble starch as substrate[4]. The residual protein in the sample was estimated with crystalline serum albumin as standard by the method of Lowry, et al.[10] and the percentage of the yield (PE) also calculated by the following equation: PE (%) = [(B-A)/B]×100, where A indicates the residual protein after adsorption and B represents the protein concentration in the original enzyme solution.

Enzyme purification

The crude enzyme was collected after fermentation and partial purification of enzyme by ammonium salt precipitation. The enzyme was subjected to 70% of ammonium sulfate and the precipitates were collected by centrifuged at 10000 rpm for ten minutes. The enzyme precipitates were suspended in acetate buffer 0.1M at pH 7. The partially purified enzyme extract used to determine enzyme activity by DNS method and concentrated by lyophilization process.

RESULT AND DISCUSSION

Amylase enzyme production in SSF

The coffee pulp used as the substrate for α-amylase enzyme production under solid state fermentation. This substrate offered better substrate for the growth of *Bacillus amyloliquefaciens* culture. The higher amount of the enzyme production 550U/g was obtained after 120 hours of the incubation (Figure 1) is in stark contrast to enzyme yield of 261U/g from agro industrial waste substrates such as wheat bran, millet cereal, etc.[1,8].

Enzyme activity at different temperature and pH

The temperature and pH are the most important factors, which directly influence enzyme activity. The maximum enzyme activity was recorded at 35-45°C of both purified and crude enzymes and any further increase in temperature decrease in the activity of amylase (Figure 2); the enzyme showed good stability upto 50°C. The similar result reported by Elliah et al.[8]. Tallies with the present findings that the enzyme has considerable thermostability favorable to brewing and food processing industries[14]. Figure 3 represents maximum enzyme activity maximum obtained at pH 8-9 both purified and crude enzymes. Most bacterial α-amylases are optimally active till the pH 9.0[7]. This enzyme activity at various pH reported here concurs with reports of fungal amylase produced by solid state fermentation on different substrates such as coconut oil cake, ground nut oil cake and rice bran[2]. This result shows the wide range of pH is of much use in processes of textile and food industries.

Enzyme activity of crude and partially purified enzyme

The maximum enzyme activity of partially purified enzyme at the 70% of fraction with the high yield of 92.5% and specific activity of 5.4 was shown on TABLE 1.

This result is supported by Dhanya Gangadharan. This property of the higher yield of purified enzyme for

degrading starch indicates its potential for use in industries on a large scale.

CONCLUSIONS

From these results, we conclude that the higher yield of commercial amylase enzyme production was achieved by using coffee pulp waste as a substrate under solid state fermentation. As this substrate is easily available and cheap, it is process can be further improved for large scale production of this industrially valuable enzyme by solid state fermentation.

ACKNOWLEDGEMENT

The authors express their gratitude to the Management, Mahendra Educational Institutions, Kalippatti, Namakkal for providing facilities and encouragement.

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