

RESEARCH ARTICLE

Optimization of Agroresidues for α -Amylase Production by *Bacillus subtilis* PS03 and its Application in Detergent Industry

S. Jagadeeswari¹ and R. Santhi^{2*}

¹Assistant Professor, D.G. Vaishnav College, Chennai, TN, India

²Principal, Tagore College of Arts and Science, Chennai, TN, India
shanmkands@gmail.com*; +91 9444054515

Abstract

Several agroindustrial residues were screened as the substrate for the production of α -amylase by *Bacillus subtilis* strain PS03. Among the different cheap substrates tested, whey wastewater served as promising substrate favoring the better production of α -amylase by the test isolate. pH 8 and temperature 35°C were found to be optimum for maximum α -amylase production using whey wastewater as substrate. α -amylase enzyme was partially purified by ammonium sulphate precipitation and dialysis. The addition of the partially purified α -amylase enzyme to commercial detergent improved its performance by destaining the blood stains. Considering its destaining properties, α -amylase produced by *Bacillus subtilis* strain PS03 may be considered as detergent additive in detergent industries.

Keywords: Agroresidues, *Bacillus subtilis*, α -amylase, purification, detergent.

Introduction

Amylases are the enzymes with second largest market worldwide. The cost of the enzyme is determined by the cost of raw materials used for their production. Hence, the reduction of production cost by using cheap raw materials will reduce the price of the enzyme as well (Pandey *et al.*, 2000a,b). Amylases hydrolyze insoluble starch into soluble glucose units. Starch is the major storage unit of plants. Starch serves as the major part of the human diet for most of the people in the world. α -amylases find their application in diverse industrial processes such as food, pharmaceutical industries, etc. α -amylase is produced by bacteria, fungi, plants, animals and humans (Sidhu *et al.*, 1997; Serin *et al.*, 2012). The major bacteria belong to *Bacillus* species and fungi such as *Aspergillus niger*, *Penicillium* sp., *Cephalosporium* and *Rhizopus* are the major α -amylase producing microorganisms (Suganthi *et al.*, 2011). The application of starch in textile industries includes desizing and removal of stains. The industrial scale production of α -amylase was done using submerged fermentation with the help of microorganisms (Konsoula and Liakopoulou, 2004; 2007; Lin *et al.*, 1997).

The major drawback of using synthetic medium is due to their expensive cost which needs to be replaced with viable and economically available cheap resources. A very good source of nutrients is still being left as the agricultural and other industrial byproducts which can serve as a good source of nutrients for different microbial metabolite production (Kunamneni *et al.*, 2005). The market cost of the enzymes depends on the cost of substrates and media used for the production of respective enzymes.

The increase in the cost of substrates reflects in the increase of market cost of the enzyme (Rao *et al.*, 1998; Mukherjee *et al.*, 2008). Most of the agroindustrial wastes such as wheat bran, sugarcane bagasse, paddy bran, and whey wastewater remain unutilized and are discarded without knowing their true value. The market demand of enzymes increases every year so as their price which necessitates the use of cheap and easily available substrates for managing their market cost (Khushboo *et al.*, 2016). The present study focuses on the production of α -amylase enzyme by *Bacillus subtilis* PS03 using these agro-industrial wastes. After optimization, the α -amylase produced by *Bacillus subtilis* was used for the destaining carbohydrate waste in the cotton fabric.

Materials and methods

Test organism: The bacterial strain *Bacillus subtilis* PS03 (Genbank Accession No. KT160016) isolated from tannery effluent collected near Chennai, TN, India was used for the present study (Santhi and Jagadeeswari, 2015).

Screening for α -amylase production: *Bacillus subtilis* strain PS03 was screened for α -amylase production as described by Bertrand *et al.* (2004). The strain was streaked on starch agar plates (g/L: Soluble starch, 10, yeast extract, 2; peptone, 5; MgSO₄, 0.5; NaCl, 0.5; CaCl₂, 0.15; agar, 20) and incubated at 37°C for 24 h. After incubation, 1% iodine solution was poured on agar plates and the plates were observed for the formation of clear zone around the colony.

Optimization of agro-residues for α -amylase production: Cheap substrates such as wheat bran, paddy bran, whey wastewater and sugarcane bagasse were procured from local market, Chennai. All the cheap substrates except whey wastewater were pretreated by incubating with 2% NaOH at room temperature for 24 h and washed with hot distilled water wash till the pH reached 5.5. The preparation was further incubated at 30°C for 1 h at 200 rpm, filtered, autoclaved and used for further studies (Ashfaque *et al.*, 2014). The bacterial inoculum was prepared by inoculating 1 mL of overnight grown culture of *Bacillus subtilis* PS03 into 100 mL of production medium (g/L: soluble Starch, 20; yeast Extract, 4; Peptone, 10; MgSO₄·7H₂O, 0.5; NaCl, 0.5; CaCl₂, 0.2) incubated for 24 h at 37°C. Different substrates such as wheat bran, paddy bran, whey wastewater and sugarcane bagasse were used as substrates for α -amylase production at 10 g/L concentration. The Erlenmeyer flasks were incubated in a shaker at 240 rpm for 24 h and the culture supernatants were used for assaying of α -amylase activity. The cheap substrates which showed the maximum α -amylase activity were further used for optimization studies.

Optimization of process parameters: The effect of different process parameters on α -amylase production with whey wastewater was assessed by growing *Bacillus subtilis* PS03 in the production medium (g/L) with 1% whey wastewater, yeast extract, 4; peptone, 10; MgSO₄·7H₂O, 0.5; NaCl, 0.5; CaCl₂, 0.2. For the investigation of optimal incubation time for α -amylase production, the bacterial culture was inoculated in the production medium and kept for 96 h. Samples were withdrawn aseptically every 6 h interval and assayed for α -amylase activity. Effect of pH and temperature on α -amylase activity was studied by inoculating the bacterial strain in medium with different pHs varying from 4 to 11 and incubated each medium at different temperatures varying from 25-55°C separately.

α -amylase assay: The α -amylase activity was assayed based on DNSA (3, 5 dinitro salicylic acid) method as described by Saxena and Singh (2011). The reaction mixture composed of 1 mL 1% starch incubated with supernatant containing different dilutions of enzyme along with 1 mL of citrate phosphate buffer (pH 6). The reaction mixture was incubated for 30 min at 50°C and the reaction was stopped by adding 2 mL of DNS. The reaction was then kept in a boiling water bath for 10 min and absorbance value was read at 540 nm using a Spectrophotometer against glucose as the standard. One unit of enzyme activity can be defined as the amount of enzyme that releases 1 μ M of reducing sugar as glucose per min under the standard assay conditions (U/mL/min). The total protein concentration in the enzyme mixture was determined using Bradford method (Bradford, 1976) with bovine serum albumin as standard.

Partial purification of α -amylase: α -amylase enzyme produced under optimized conditions was partially purified by using ammonium sulphate precipitation and dialysis as described by Saxena and Singh (2011). Briefly, the supernatant was mixed with known weighed (NH₄)₂SO₄ with constant stirring at room temperature. Then the precipitate was obtained by centrifugation at 12000 x g for 20 min at 4°C and dissolved in 0.1 M phosphate buffer (pH 6). The precipitate was then dialyzed using 0.1 M phosphate buffer for 48 h by changing the buffer at every 24 h and this final crude enzyme was utilized for further studies.

Preparation of cotton fabric stained with carbohydrate stain: The cotton fabrics were cut into small pieces (10 X 10 cm), washed with distilled water and stained with blood. About 10 mg/mL of partially purified α -amylase enzyme was added to the test tube containing commercial detergent (Ariel®) prepared at the concentration of 7 mg/mL. The blood stained cotton fabrics were incubated in the test tube for 30 min and dried. The commercial detergent served as positive control and distilled water served as negative control. The cotton fabrics were observed before and after wash procedure for stain removal (Harshada and Narayan, 2015).

Results and discussion

The *Bacillus subtilis* strain PS03 was isolated from the tannery effluent and was identified using 16S rRNA sequence analysis. The amplified 16S rRNA sequence was submitted to the Genbank under the accession number KT160016 (data not shown). While screening for α -amylase, the strain showed a large zone of clearance in starch amended plates which revealed the ability of the bacteria to produce α -amylase enzyme (Fig. 1). It is necessary to screen the bacteria for producing enzyme in large quantity for the industrial application (Kokab *et al.*, 2003; Konsula and Liakopoulou, 2004).

Fig. 1. Amylolytic activity of *Bacillus subtilis* PS03.



Several agriculture related industries produce huge quantities of agroresidues which poses serious threat to the environments. These residues can be utilized as nutrient sources for production of different valuable compounds such as enzymes and different secondary metabolites (Soccol and Vandenberghe, 2003). The selected agroindustrial residues were screened for their potential as substrate for α -amylase production by *B. subtilis* PS03. The results showed that the α -amylase production was higher in the medium amended with whey wastewater (107 U/mL) when compared to other agroindustrial residues (Fig. 2). Whey wastewater contains carbohydrate at increased concentration than the other substrates tested, hence served as a suitable substrate for α -amylase production. Similarly, wheat bran was found to be better substrate for α -amylase production by *Bacillus licheniformis* (Ikram-ul-Haq *et al.*, 2003).

The result also indicated that the α -amylase production by *Bacillus subtilis* PS03 depends on the type of substrate used. Several factors are needed to be considered during the selection of agroindustrial residues such as, availability, particle size, inoculum concentration and cost of the substrate (Pandey *et al.*, 2000). Similar reports were also available to produce enzymes using agrowaste as substrate by *Halomonas* sp. (Vijayaraghavan and Vincent, 2012). Rajshree and Rajni (2011) also examined the role of several agroresidues and found oil seed cake as suitable substrate for amylase production by *Bacillus* sp. The production of α -amylase by *Bacillus subtilis* PS03 was also influenced by a variety of parameters such as pH, temperature, incubation period and nutrient components. For achieving the maximum enzyme production, it is necessary to perform a detailed study on the influence of both physical and chemical parameters as reported by several researchers (Hashemi *et al.*, 2010; Kriaa and Kammoun, 2015; Sahnoun *et al.*, 2015; Selvam *et al.*, 2016). The effect of incubation period on the amylase production is shown in Fig. 3. *Bacillus subtilis* PS03 had the ability to produce maximum amylase in the period of 24-48 h. Maximum production of amylase (119 U/mL) was observed during 48 h incubation. Tanyildizi *et al.* (2005) and Gangadharan *et al.* (2008) have reported that maximum amylase production was achieved at 72-96 h incubation period. *Bacillus subtilis* PS03 has shorter period of incubation for the production of α -amylase when compared to earlier reports. *Bacillus subtilis* PS03 could grow and produce α -amylase over a wide range of pH (4-11). Maximum α -amylase production was observed at pH 8 (Fig. 4). Similarly, Tanyildizi *et al.* (2007) observed pH 7 as optimum for amylase production by *Bacillus amyloliquefaciens*. For the amylase production, most of the *Bacillus* sp. reported to have optimum pH between 7-10 (Goyal *et al.*, 2005; Sodhi *et al.*, 2005; Saxena *et al.*, 2007). Temperature also plays a very important role in the amylase production by the *B. subtilis* PS03.

Fig. 2. Agroresidues as substrates on amylase production.

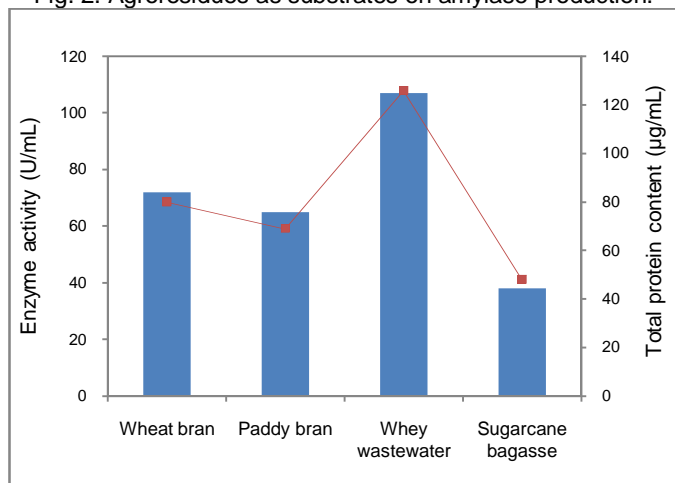


Fig. 3. Effect of incubation time on amylase production.

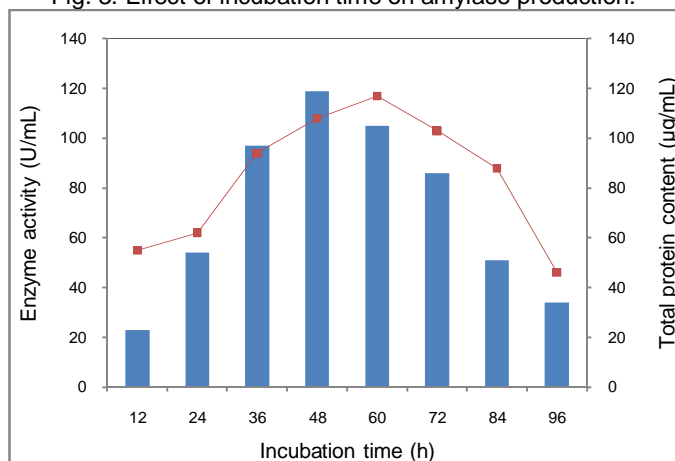


Fig. 4. Effect of pH on amylase production.

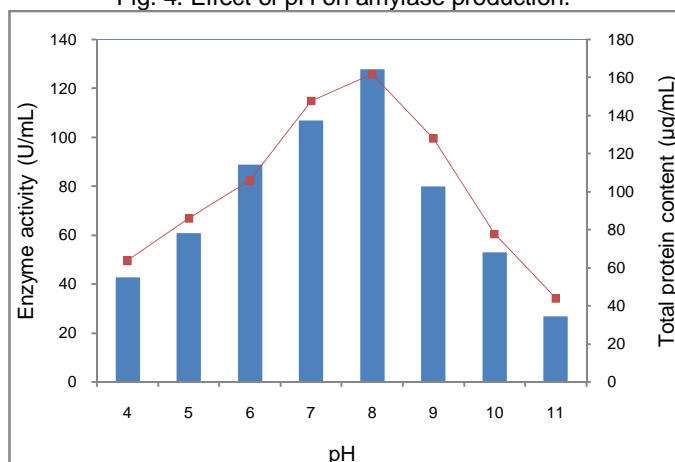


Figure 5 shows the amylase activity of the isolate at different temperatures. Maximum enzyme activity of 142 U/mL was observed by the isolate at 35°C. The finding of this present study supports the finding of Asgher *et al.* (2007) who found that amylase produced by *Bacillus subtilis* JS2004 gave the best activity at 37°C. Similarly, Rajagopalan and Krishnan (2009) investigated α -amylase production using *Bacillus subtilis* KCC103 with different low-cost agro-residues by shake flask method. Maximum α -amylase production when the medium supplemented with wheat bran and sunflower oil cake at 1:1 ratio showed a productivity of 90 IU/mL during 36 h of incubation. They also enhanced the yield by 14 fold by optimizing various parameters such as pH, temperature and incubation time resulting with productivity of 1258 IU/mL in a bioreactor. The amylase enzyme produced was precipitated using 70% saturation of ammonium sulfate, dissolved in Tris-HCl buffer and dialyzed against the same buffer.

The application of partially purified α -amylase enzyme as detergent additive was tested by wash experiment in distilled water solutions containing detergent powders and partially purified enzyme. The distilled water solution containing detergent powder partially removed the blood stain after incubation for 30 min. The complete removal of blood stain was observed in the solution amended with partially purified amylase enzyme. The addition of amylase enzyme to the detergent solution significantly improved their blood stain cleansing potential (Fig. 6). The results evidenced the stain removal efficiency of amylase enzyme produced by *Bacillus subtilis* PS03. Several reports were available regarding the removal of blood stains from cloths by the enzymes produced by *Bacillus* sp. (El-Hadj *et al.*, 2007; Pathak and Deshmukh, 2012). Since most of the fabric stains were either proteinaceous or carbohydrate in nature, enzymes like amylases and proteases can be used as a detergent additive. Most of the bacterial enzymes isolated from *Bacillus* sp. possess ability to survive at extreme pH and temperature. Based on the results obtained, amylase enzyme produced by *Bacillus subtilis* PS03 could be considered as a potential additive in the detergents for the efficient removal of the stains.

Conclusion

Evaluation of wheat bran, paddy bran, whey wastewater and sugarcane bagasse as a substrate for α -amylase production was investigated. Among the cheap sources tested, whey wastewater was chosen as the substrate for the effect of fermentation period, temperature and pH of the medium for the production of α -amylase by *Bacillus subtilis* PS03. With whey wastewater as substrate, pH 8 was found to be optimum for maximum amylase production at 35°C. The produced α -amylase from the present study was tested as detergent additive for removing blood stains and results were also promising suggesting their application as an additive in detergent formulation.

Fig. 5. Effect of temperature on amylase production.

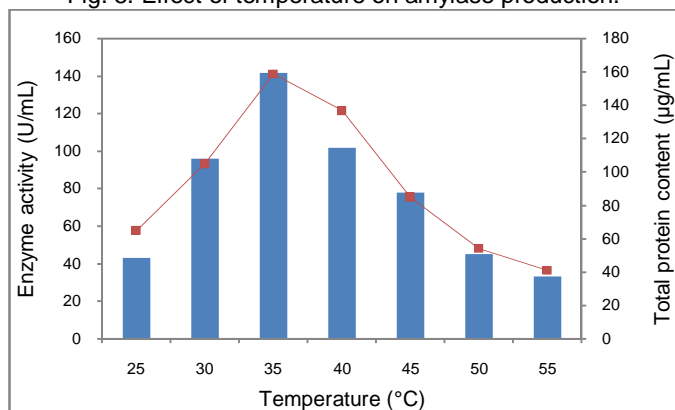
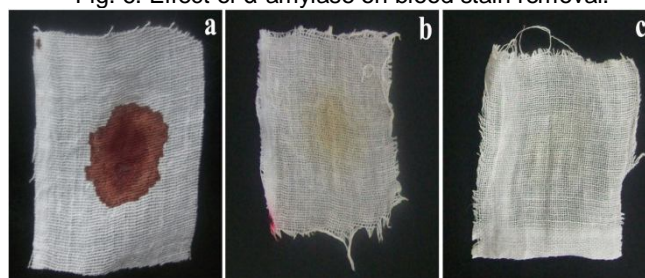


Fig. 6. Effect of α -amylase on blood stain removal.



a) Negative control—blood stained cotton fabrics treated with distilled water; b) Positive control—blood stained cotton fabrics treated with commercial detergent; c) Test-blood stained cotton fabrics treated with partially purified α -amylase enzyme and commercial detergent.

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