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A-AMYLASE FROM *BACILLUS SUBTILIS*: A POTENT DESIZING AGENT

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ABSTRACT

Reason behind the study: The textile industry is indispensable for human civilization and hence it attracts several ideas for the improvement of its quality and durability. In this line, desizing by amylase has aroused a great deal of concern in the current textile industry. The objective of this study was production and optimization of amylase by *Bacillus subtilis* in order to exploit the potential of amylase in textile industries.

Main findings: This work involves optimization of production of amylase using *Bacillus subtilis* and testing of their utility for cotton desizing. An Incubation period of 72 h, initial diluent pH of 7.0, 30°C incubation temperature, inoculum size of 1% (v/v) were optimum for the maximum amylase production yielding enzyme titer of 249 IU/ml. Supplementation of different carbon (1%), nitrogen (0.5%) and metal ions (1Mm) and detergents (1%) to the fermentation media suppressed amylase activity. Moreover, the desizing potential of α-amylase was assessed *via* different standard assays. The crude enzyme showed efficient desizing of cotton fabric at pH 6.0, 65°C within 40 minutes with Tegewa rating 9, properties potential for desizing in textile industries.

Conclusion: The purpose of this study is to produce amylase enzyme by *Bacillus subtilis* in submerged fermentation and optimizing its culture conditions to attain higher yield of amylase enzyme. The amylase obtained was further used to test its potential in desizing the cotton fabrics as that of the commercial enzyme.

Keywords: Amylase, *Bacillus subtilis*, Optimization, Desizing, Cotton fabrics

INTRODUCTION

α-Amylases (E.C.3.2.1.1) are pervasive enzymes produced by plants, animals, and microbes where they play a prevailing part in carbohydrate metabolism. However, the microbial source is a preferred choice for the production of amylase because of their stability, availability and various other beneficial properties more diverse than other enzymes derived from plants and animals. Bacterial amylases are generally regarded as a preferred choice for starch processing. Bacillus species such as *B. stearothermophilus, B. subtilis*, *B. megaterium*, *B. macerans* and *B. amyloliquefaciens* are reported to be the excellent producers of thermostable a-amylase using submerged fermentation and has been commercially used for the production of amylase for diverse applications **[1, 2]**.

Amylases have great implication in industrial processes such as textiles, food, leather, paper, sugar, and detergents **[3]**. As amylases are used in various industries, any step that involves low-cost production, enhanced activity and stability can lead to better technological advances.

During the pre-processing of fabric, in textile industries, to avert the yarn from breaking, a removable defensive layer is applied to the threads (sizing). Starch is a very prevalent sizing agent as it is inexpensive, effective and is easily removable **[4]**. For further processing of the fabric, such as dyeing and finishing, it requires removal of sizing agent which is called desizing. For this desizing purpose, instead of using harmful chemicals, *α*-amylase enzyme can be used **[5]**. Owing to current environmental conditions, it's a necessity to use processes that involve microbes and enzymes and substitute harsh chemicals. In the present work, we investigated production and optimization of amylase enzyme which can be used as a potential desizer on fabrics in textile industries.

MATERIALS AND METHODS

Culture: *Bacillus subtilis* (GenBank Accession Number JN032305) a potent producer of amylase, isolated from the rhizospheric soil **[25]**. The bacterium was grown in basal media containing (g L^{-1}) $NaNO_3$-5; yeast extract-10; NaCl-5, and the pH was 7.0 ± 0.1.

Inoculum preparation and submerged fermentation: Inoculum with a CFU of 14.64× 10^8 cells mL^{-1} was used (1%) to inoculate the media and incubated at 37^0C for 72 h on an orbital shaker at 130 rpm for submerged fermentation. The cells were later

centrifuged at 10,000 *g* for 20 minutes at 4^0C, and the resulting supernatant was collected and used as crude amylase enzyme. Determination of amylase activity was measured by di-nitro salicylic acid (DNSA) method **[6]**. One unit of amylase activity was defined as the amount of enzyme that released 1 µmol glucose per mL per minute.

Optimization of production conditions: The effect of various physical and nutritional parameters was studied through one factor at a time approach, as given in **Table 1**.

Table 1: Physical and nutritional parameters and their ranges tested for the production of amylase from *B.Subtilis*

Physical Parameters	Range tested	Nutritional Parameters	% (w/v)
Time course (h)	24-120	Carbon: Glucose, Lactose, Maltose, Mannitol, Sucrose	1
pH	5-9	Nitrogen: Beef extract, Yeast extract, Tryptone, Peptone, Ammonium chloride, Ammonium sulfate, Ammonium nitrate, Potassium nitrate, Casein, Urea	0.5
Temperature (^{O}C)	20-40	Metal ions: Ca^{2+}, Cd^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, $Cu2^{+}$, Fe^{2+}, Hg^{2+}, and Zn^{2+}	1mM
Inoculum size (% v/v)	0.5-3.0	Detergents: SDS, Tween 20, EDTA, Glycerol	1

Evaluation of desizing of cotton fabrics by amylase: The potential of α-amylase to desize the cotton fabrics was assessed by using the sized, 100% cotton gray fabric 1 × 1 cm^2. The competence of amylase for desizing was detected by weight loss assay, reducing sugar test, TEGEWA test and drop absorbency test. Fourier transform infrared (FTIR) spectroscopy analysis and scanning electron microscopic (SEM) analysis were also done to find the associated functional groups and observe the surface morphology of the fabric, respectively.

Determination of weight loss (%): This experiment was conducted as per Sahinbaskan and Kahraman **[7]**.

Reducing sugar assay: The reducing sugars that were liberated from the fabric due to enzyme treatment were estimated by the DNSA method with glucose as the standard at OD 540 nm and expressed in µg mL−1.

Drop absorbency test: A drop absorbency test was performed by AATCC Test method 79-2000 **[8]**.

TEGEWA test: This experiment was conducted as per Sahinbaskan and Kahraman **[7]**.

SEM and FTIR Analysis: The SEM analysis was done to assess the removal of sizing agent (starch) by perceiving the changes in surface morphology of fabric **[7]**. The fabrics were coated with gold and observed using a Jeol JSM-6510LV microscope.

Also, the fabric samples were subjected to FTIR analysis to determine the associated functional groups. Scans were carried out for fabric sample, and the spectra were obtained

between the wave numbers 4,000 and 400 cm−11 **[9]**.

Statistical analysis: All assays corresponding to production, optimization, and desizing were carried out in triplicates and values were expressed as mean ± SD. The statistical analyses were performed using the SPSS software program. Duncan and one-way analysis of variance tests were used for comparative study.

RESULTS & DISCUSSIONS

Effect of incubation time, inoculum size, pH and temperature: The optimum incubation time for enzyme production was 72 h (243.7 IU/ml), beyond which the enzyme production began to decline gradually **(Table 1)**. The reason enzyme depletion can be due to the denaturation of the enzyme caused by the interaction with other components in the medium **[10]** and probably due to depletion of nutrients available to microbes. This result is in agreement with studies of Nurullah Akcan **[11]**, where high production of amylase by *B.subtilis* was noted at 72 h, when he screened from 12 to. 120 h at regular intervals. Highest α -amylase (245.1 IU/ml) was produced at pH 7.0. With further increase in pH, it was observed a drastic decrease in α-amylase production. Behal *et al.,* **[12]** studied thermostable amylase producing *Bacillus* sp. that revealed an optimum enzyme production at pH 8.0 whereas in other species the optimum production was at pH 7.0 **[13]**. Highest α -amylase (247 IU/ml) was produced at 30°C. Further increase in temperature resulted in decrease in α -amylase production. The inactivation at high temperature can be possible because of amino acid destruction or hydrolysis of the peptide chain. Inoculum size plays a very significant role in enzyme fermentation **[14]**. 1% (w/v) of inoculum yielded maximum enzyme production (248 IU mL−1) **(Table 1)**, beyond which there was a noticeable decline in activity. Possibly because as the inoculum in the medium increased, the bacteria would have grown rapidly which lead to nutrient depletion in the initial stage which consecutively would have resulted in the accumulation of other by products. Whereas, at lower concentration of inoculum, enzyme production was very less because bacteria would have grown slowly, and the rate at which bacteria reaches its stationary phase would have increased **[15]**.

Effect of carbon and nitrogen sources: Of all the carbon sources tested, the basal media which had yeast extract as the carbon source in it gave highest amylase activity, 249.5 IU mL^{-1} **(Table 3)**, whereas supplementation of other carbon sources did not show any

profound increase in amylase activity. Other than being a carbon source, yeast extract also provides nitrogenous compounds, sulphur, trace nutrients, vitamin B complex and other important growth factors, which are essential for the growth of microorganisms. Even in the case of nitrogen, the basal media which already had Yeast extract and sodium nitrate in combination as its nitrogen source in it yielded maximum enzyme activity, 249.5 IU mL^{-1} **(Table 3)**, which was in agreement with the study done by Hamilton *et al,* **[16]** who reported that organic nitrogen sources are preferred for amylase production. However, other nitrogen sources repressed amylase activity. In another study, a significant increase of enzyme yield was reported **[17]** when sodium nitrate was supplemented in the production media to produce amylase by *Aspergillus oryzae*.

Effect of metal ions and detergents on amylase production: Inhibitory effects were recorded on addition of various metal ions (0.1 mM) and detergents (1%) in the basal medium **(Figure 1a & 1b)**. This could be because metals tend to bring pH changes in the medium. In the study conducted by Deshpande SS *et al*., **[18]** it was found Ca^{2+} had an effect on enzyme activity as well as in stabilization in the defense against protease.

Desizing of cotton fabric by amylase

Amylase produced in optimized condition was taken for desizing analysis by certain parameters as shown in **Table 4**. As per the report by Saravanan *et al.,* **[9]**, it was found there is no connection between desizing and reducing sugars released. Whereas in the present study, liberated reducing sugars and weight loss correlated with each other. On the surface of the fiber, there are hydrophobic components, such as dried starch residues present which furnish poor absorbency. Henceforth, removal of these impurities will help in fast absorption of water. In the present exploration, fabrics displayed the lowest absorbency time as ~4 Sec upon treatment of amylase, confirming better absorbency than the untreated fabric. Prompt absorption of the water droplet was recorded in the enzymatic desized fabric, possibly because amylase tends to break the waxy layer on the surface aiding in absorbency of water. Our finding is in agreement with the result of Saravanan *et al.*, 2011 **[9]**, who also reported ~2 Sec as absorbency time.

The SEM images **(Figure 2)** evidently displayed the desizing efficiency of *α*-amylase enzymes. The surface of the control fabric sample was seen with starch coating over the fabric, whereas in fabrics treated with *α*-amylase, the coating of starch was

removed and consequently arrangements of yarn in the fabrics were distinctly visible.

The FTIR analysis **(Figure 3)** exposed a substantial difference in the peak absorbance values of control and test fabric. In the control fabric, the sharp peaks at 3000-3500 indicate the presence of pectin substance of cotton wax, whereas there is a notable reduction in the absorbance values in the enzyme treated sample.

Table 2: Optimized physical parameters for the production of amylase from *B.Subtilis*

Physical Parameters	Range tested	Optimum	Amylase Activity (IU/ml)
Time course (h)	24-120	72	243.7
pH	5-9	7	245.1
Temperature (^{O}C)	20-40	30	247
Inoculum size (% v/v)	0.5-3.0	1.0	248

Table 3: Effect of different carbon and nitrogen sources on amylase from *B. subtilis*

Carbon Sources (1%(w/v))	Amylase activity (IU/ml)	Nitrogen Sources (0.5%(w/v)	Amylase activity (IU/ml)
Sucrose	47.85	Beef extract	53.2
Glucose	47.85	Tryptone	56.05
Maltose	204.6	Ammonium chloride	20.9
Mannitol	82.5	Ammonium sulfate	20.9
Lactose	44.5	Ammonium nitrate	21.85
Starch	120	Potassium nitrate	34.2
Basal media	249.5	Yeast extract	14.25
		Peptone	119.5
		Casein	86.45
		Urea	43.7
		Basal media	249.5

Table 4: Comparative analysis of desizing potential of α-amylase

Parameters	Negative Control	Positive Control	Test amylase
Weight loss (%)	1.00	66.66	39
Reducing sugar (IU/ml)	73.7	237.85	130.65
Drop absorbency time (Sec)	300	2	4
Tegewa Scale Rating	2	9	8

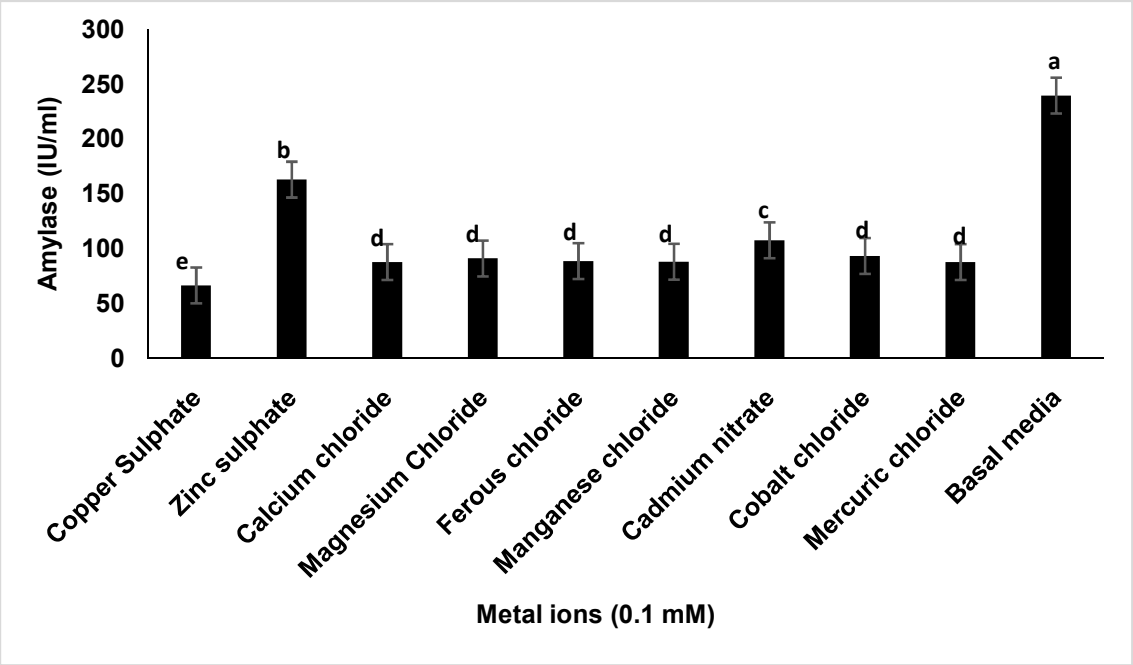

Figure 1 (a): Effect of different metal ions on amylase production by *B.subtilis*

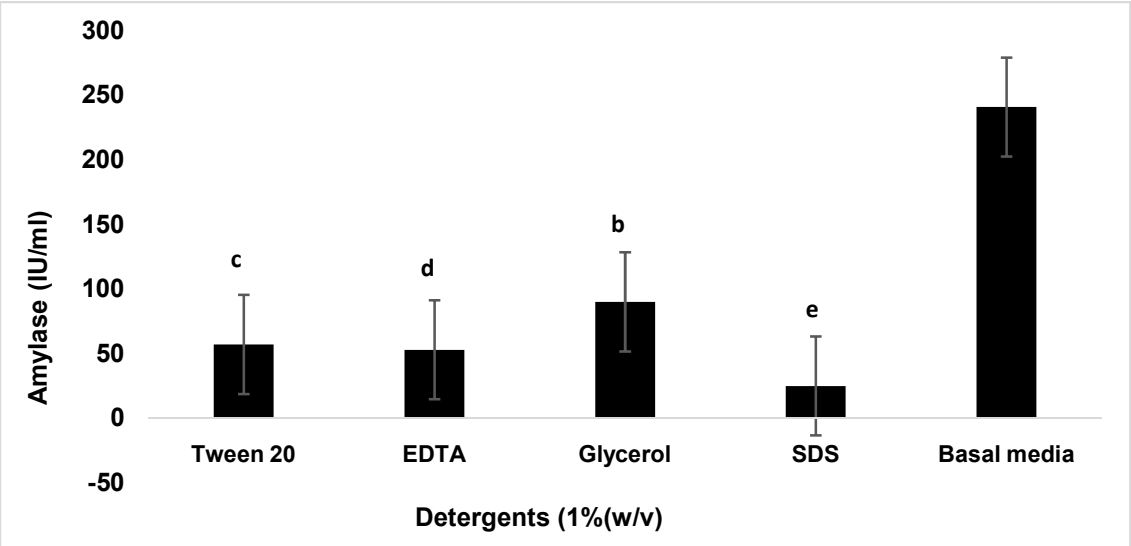

Figure 1 (b): Effect of different detergents (1%) on amylase production by *B.subtilis*

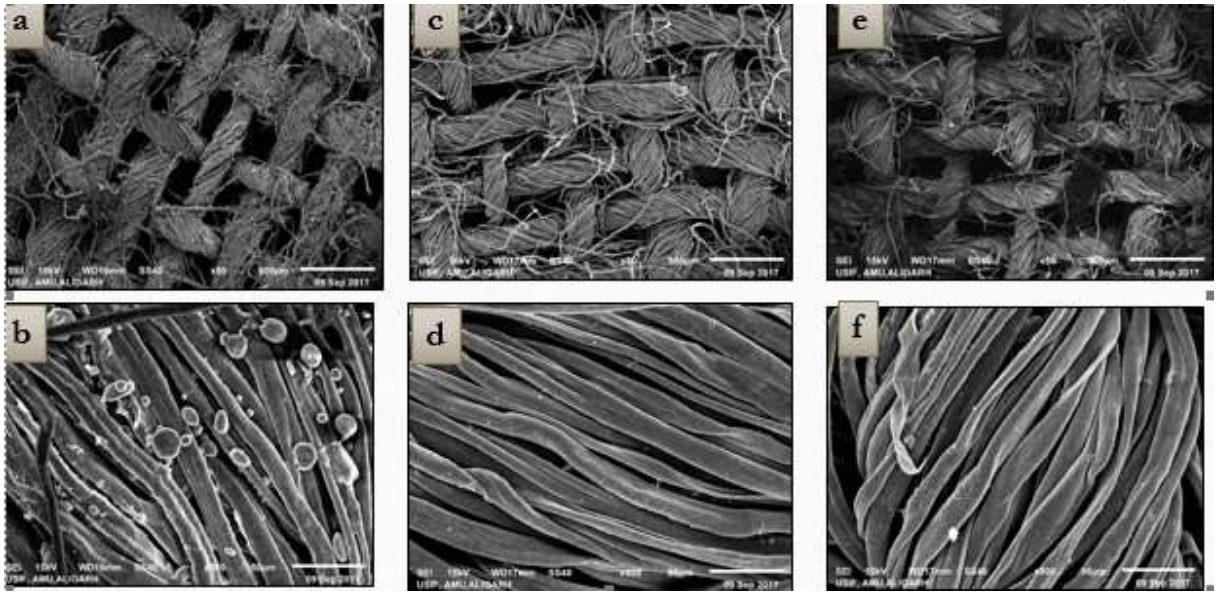

Figure 2: SEM images: (a) untreated cotton fabric a & b, (b) cotton fabric treated with positive control c & d and (c)cotton fabric treated with test amylase e & f

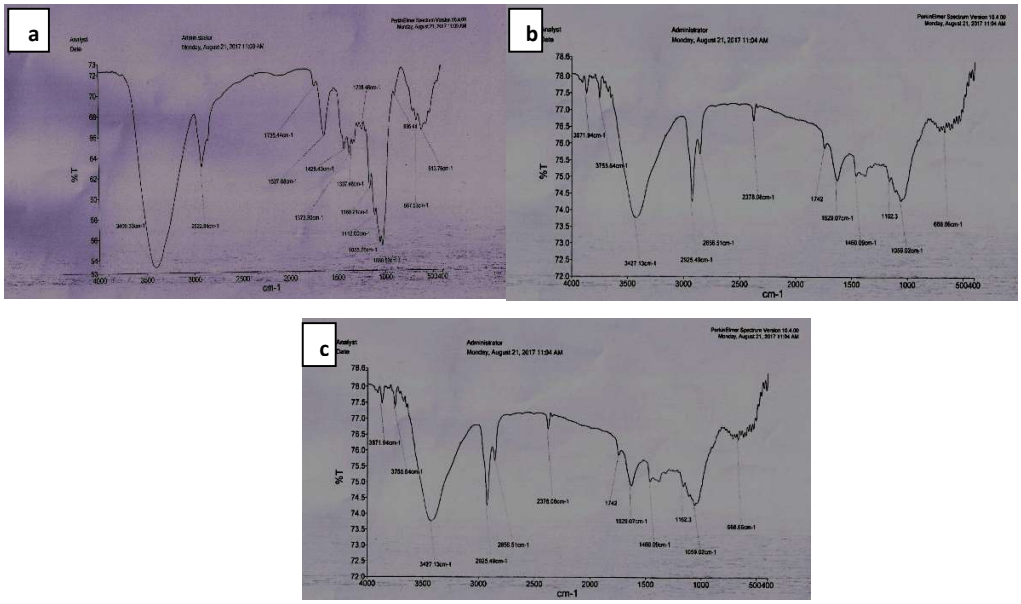

Figure 3: (a) FTIR Spectra of cotton fabric before desizing; (b) FTIR Spectra of cotton fabric – Desizing done with positive control; (c) FTIR Spectra of cotton fabric –Desizing done with test amylase

CONCLUSION

The study demonstrates a single step production of amylase using *Bacillus subtilis*. The productivity of amylase obtained at optimized fermentation parameters, using single factor at a time approach, is 249 IU/ml. Optimum concentrations of factors for production of amylase such as substrate concentration 1.0 % (w/v), inoculum concentration 1% (v/v), and pH 7.0 at 30°C

incubation temperature were determined for incubation period of 72 h. Although, few reports on the desizing application of *α*-amylase enzyme in recent years has already been reported Chand *et al.,***[19]** and Saravanan *et al*.,**[9]** the present study focuses on the desizing activity of amylase in comparison with commercial *α*-amylase, and the results observed was in agreement with the commercial amylase. A continued research on optimization of amylase production and testing its desizing potential is essential to meet the growing demand for amylase in today's market. Consequently, this crude *α*-amylase is here by shown to be a potential desizing agent of cotton fabrics.

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