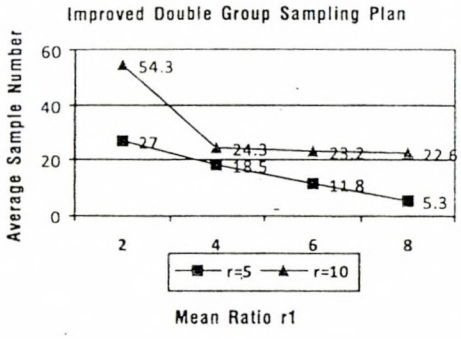

Figure 2

From the Figure 2 one can observe that the ASN value of Exponential distribution r=5 is minimum as compared with r=10.

Conclusion

One can observe that as mean ratio increases there is decrease in number of groups and acceptance number, if the life time of the item follows Exponential distribution and at the same time there is increase in probability of acceptance when the test termination time multiplier decreases. In proposed double group sampling plan for Exponential distribution, ASN value of r=5 is minimum as compared with r=10.

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BIOCHEMICAL CHARACTERIZATION OF AMYLASE FROM *ASPERGILLUS NIGER* AND ITS APPLICATION IN DESIZING

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Abstract

Reduction in pollution and improvement in the fabric quality can be achieved using enzymatic treatment. *Aspergillus niger* isolated from distillery effluent spilled soil was selected for the study due to its maximum amylase activity. The optimum amylase activity was observed at the pH nine in glycine sodium hydroxide buffer at 60°C for 60 minutes and the enzyme was stable for the first two hours of incubation. *A.niger* utilized one per cent of starch for maximum amylase activity in medium supplemented with 5g NaCl. Calcium ions were found to activate the enzyme activity whereas mercury ions inhibited the activity to higher extent when compared to other ions. The enzyme was found to desize the fabric effectively. The visual inspection proved all sample to have good general appearance and better texture after enzyme treatment. Luster and whiteness did not show significant change after treatment. All the samples showed reduction in stiffness after desizing with enzymes. Hence, amylases can be explored in textile sector that requires a wide range of optimal conditions.

Keywords: Amylase, *Aspergillus niger*, Enzymatic desizing, Biochemical characterization

Introduction

Textile industry is one of the major polluters by discharging dyes and chemical waste into terrestrial and aquatic systems. A large number of chemicals of diverse nature are involved in textile processing and the effluents pose a serious threat to the environment. With the globalization of textile industry and increase in the awareness towards the environment, times are changing very fast for the industry which has to remain updated continuously. The most exciting area of textile research is enzymatic processing which have started playing a consistent role in substituting a waste water treatment plant (Muthu and Prasad, 2005). Currently, people have become more eco-conscious. In these circumstances, textile wet processors are looking for eco-friendly alternatives for chemicals/auxiliaries and dyes used in textile industry. Today, the concept of clean or green chemistry has triggered the use of enzymes in all areas of chemical technology

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(Prabhu and Arputharaj, 2003). Enzymes have been applied, or at least tried, in every step of textile wet processing. Recently it has been observed that enzymes are safe to use, easy to control and biodegradable. Enzymatic processes commonly work at lower temperatures than conventional chemicals and can often offer reduced cycle times and water savings (Adeniran, 2008).

Microbial culture becomes more important in the textile industry due to two major advantages; they allow an economical technology with low energy consumption ("Chemical energy") and low emission ("green") processes. Researches are carried out to isolate enzymes from waste material and effluent discharged soil at effective cost which would be a boon for the application of textile industries and other fields. Microbial enzymes have proved to be of industrial importance because of their increased applications (Prabhu and Arputharaj, 2003). Enzyme assisted processes are envisaged environmentally as an alternative to the conventional methods. They have become an integral part of the textile industry, replacing existing chemical process. One of the chemical processing steps in textile industry is scouring where there is a wide scope for application of enzymes.

Enzymatic desizing using amylases is an established process that has been in use for many years (Pai and Kahndual, 2003). Amylases are among the most important industrial enzymes which are of great Amylase is a hydrolytic enzyme which catalyses the breakdown of dietary starch to short chain sugars, dextrins and maltose. In the textile industry amylases are used to remove starch based size for improved and uniform wet processing and it selectively hydrolyses starch to desize the fabric completely.

With this background the present study was formulated to isolate, identify, optimize and characterize amylase producing fungi from distillery effluent spilled soil and to check its role in desizing of fabrics.

Materials and Methods

Isolation, Screening and Identification of Amylolytic Fungi

Selection of the Source

Distillery effluent is found to possess high sugar content and it is usually washed off into drains, nearby streams together with waste water or into the land as fertilizer, thus contributing to pollution load, or deposited in the factory's compound, which can lead to serious environmental problems. Hence the present study was carried out to assess the potential utilization of the effluent spilled soil as a source for isolating amylase producing fungi.

Collection of the Sample

The soil from the effluent discharged area (Mohanur, Namakkal District) was collected, dried and crushed by using a porcelain mortar and pestle and stored in

Isolation of Amylolytic Fungi

One gram of the soil sample was weighed and diluted serially from 10^{-1} to 10^{-6}. The different dilutions were plated on rose bengal chloramphenicol agar medium using spread plate technique (Jenson, 1968). The plates were incubated at room temperature for 5 days and the well grown colonies were picked and further purified by streaking. Subsequent subculturing was done for every 30 days.

Screening and Identification of Isolated Fungi for Amylase Production

The fungal isolates were screened for their zone of clearance (Ratio between the clearing zone diameter and colony diameter of fungi). The fungal isolate which exhibited maximum zone of clearance was selected and identified based on lactophenol cotton blue staining. The identified fungal isolate was assessed for amylase activity by DNS method.

Extraction of Amylase

The isolates which showed maximum zone of clearance was grown in Sabouraud's dextrose broth (glucose - 2g, peptone - 1 g, chloramphenicol - 4 mg and distilled water–100 ml) at room temperature for 5 days. After 5 days of incubation the cells were separated by centrifugation at 10,000 rpm at 4° C for 20 minutes. The cell free supernatant served as the crude

Biochemical Characterisation of Amylase

Effect of pH on amylase activity

The optimum pH of the enzyme was determined by varying the pH using the following buffers: 0.2 M citrate phosphate buffer [pH 3–4], 0.2 M sodium acetate buffer [pH 5 – 6], Tris HCl buffer [pH 7–8] and glycine sodium hydroxide buffer [pH 9 – 10].

Effect of pH on Amylase Stability

To determine the pH stability of amylase, the enzyme was pre incubated in glycine sodium hydroxide buffer [pH 9] for different time intervals [1, 2, 3 and 4 hours]. The residual amylase activity was determined at every one hour interval.

Effect of Temperature on Amylase Activity

The optimum temperature of the enzyme was evaluated by measuring its activity at different temperatures [30, 35, 40, 45, 50, 55, 60, 65 and 70° C] in 0.2 M glycine sodium hydroxide buffer for one hour and the enzyme activity was determined.

Effect of Temperature on Amylase Stability

Thermostability was determined by incubation of crude enzyme in 0.2 M glycine sodium hydroxide buffer [pH 9] at optimum temperature for four hours. At every one hour time interval the enzyme activity was determined.

Effect of Incubation Time on Amylase Activity

To ascertain the effect of incubation

substrate reaction mixture was incubated for different incubation periods (10, 20, 30, 40, 50, 60, 70 and 80 minutes) at optimum temperature and the enzyme activity was assayed.

Effect of Substrate Concentration on Amylase Activity

The effect of substrate concentration on enzyme activity was measured at different concentrations of starch in the reaction mixture (0.25, 0.50, 0.75, 1.0, 1.25, 1.5, 1.75 and 2%) at optimum temperature for one hour. The contents were analysed for enzyme activity.

Effect of NaCl on Amylase Activity

The enzyme was incubated in glycine sodium hydroxide buffer [pH 9] containing various concentrations of NaCl [1, 2, 3, 4, 5, 6, 7, 8 and 9] at optimum temperature for 60 min and the enzyme activity was determined.

Effect of Metal Ions on Enzyme Activity

For determining the effect of metal ions on amylase activity, enzyme assay was performed after pre incubation of the enzyme with various metal ions, each at a concentration of 2mM at optimum temperature for 60 minutes. The metal ions used for the assay are $CaCl_2$, H_2O, $MgSO_4$, $7H_2O$, $FeSO_4$, KCl, $MnSO_4$, $4H_2O$, $ZnSO_4$, $7H_2O$, $BaCl_2$, $HgCl_2$ and $CuSO_4$.

Hydrolysis of Raw Starch

A reaction mixture containing 50 mg of raw starch, 2 ml of water, 2 ml of enzyme solution and 4 ml of 0.2 mM glycine sodium hydroxide buffer [pH 9] were incubated in test tubes at 60°C for 5 days under shaking condition. After incubation the mixtures were centrifuged at 10,000 X g for 10 minutes at 4°C and the sugar in the supernatant was quantified using DNS method. The extent of hydrolysis of raw starch (R_h) was defined by the following formula,

$$R_h(\%) = (A_1 / A_0) \times 100$$

where A_1 was the amount of sugar in the supernatant after the reaction and A_0 was the amount of raw starch before the reaction (Fukuda *et al.*, 2005).

Analysis of Hydrolysis Products

Sugar products were identified using paper chromatography through Whatman No. 1 filter paper. Paper chromatography was developed using the ascending chromatography technique in a solvent system of acetic acid / n – butanol / water (4:1:3, v/v) at room temperature. The sugars were visualized by dipping the paper into 0.2 per cent potassium permanganate dissolved in 100 ml of sodium carbonate. A yellow color spot behind pink background indicates the presence of hydrolysed products (sugar).

Role of Amylase in Desizing

Selection of Material

Cotton is only fabric which has found a unique place in the world of textiles. Cotton is well known for its good strength, durability and its hydrophilic nature, which makes it easy for wet process. Considering

these reasons, the cotton based fabric selected for the study was ghada (unbleached).

Enzymatic Desizing

Enzymatic desizing in any case can efficiently remove the starch without damaging cotton fabrics.

One gram of the material was weighed in the electronic balance. Based upon the weight of the fabric (1 g) the enzyme was added to this in different ratio (1:1,1:2,1:3,1:4,1:5 and crude enzyme) containing 0.2g of NaCl (Material : liquid ratio = 1g : 30 ml). The ghada cloth was soaked in different ratio of diluted enzymes for varying time intervals (1 to 6 hours) at 60°C. Then the samples were removed and washed in cold water and dried. After drying, the samples were soaked in water and the temperature was raised to 100°C for deactivation of enzymes for 10 minutes. The set up was allowed to cool and a drop of 0.005 N iodine solution was added and the colour after 30 seconds was noted.

With starch, iodine gives deep blue–black colour, which falls to blue and then brownish yellow, and yellow as the starch is broken down. A pale yellow brown colour indicates the complete break down of starch. The test is extremely sensitive and it will be positive even for traces of starch residue.

The desized sample was placed over clean white chart and a drop of 0.005 N iodine solution was allowed on the fabric and the colour after 30 seconds was naturally evaluated. A light blue colour of the fabric is indicative that 80 – 90 per cent conversion of starch to glucose has been achieved.

Evaluation of Treated Fabrics (Subjective evaluation)

The desized samples were evaluated visually by 16 post graduate students mastering Life Sciences and graded by a panel of judges. General appearance, texture and stiffness were the main factors taken into consideration for evaluation.

Results and Discussion

Screening and Identification of Amylolytic fungi

About 20 fungal isolates were screened from the effluent spilled soil sample. Based on the ratio between the fungal clearing zone diameter and colony diameter, 5 fungal isolates exhibited maximum zone of clearance among the 20 strains and they were screened for amylase activity.

Table 1 shows the amylolytic activity of the selected 5 fungal isolates namely S_1, S_2, S_3, S_4 and S_5 in which isolate S_4 showed maximum activity and selected for further study and identified.

Table 1. Amylase Activity of the Selected Fungal Isolates

No. of strains	Amylolytic activity (U / ml / min)
S_1	1.21
S_2	1.06
S_3	1.10
S_4	**1.75**
S_5	1.33

Identification of Fungi

Based on the morphology the organism was found to be *Aspergillus niger*. The surface of the colonies on rose bengal chloramphenicol agar medium was found to be black in colour. Immature colonies were found to be covered with white fluffy aerial mycelium and the mature colonies were black which had salt peppery effect. The reverse side of the plate was buff coloured. The vesicles were small and globose shaped. Sterigmata were found to be arranged in two series. The conidiophores were short and had thick smooth walls.

Biochemical Characterisation of Amylase from *A.niger*

Effect of pH on Amylase Activity

The results on the effect of pH on enzyme activity at different pH (3-11) were tested and depicted in Table 2.

Table 2. Effect of pH on Amylase Activity Using Different Buffers

Buffers	pH	OD value	Enzyme activity (U/ml/min)
Citrate phosphate	3	0.08	20
	4	0.12	30
Sodium acetate	5	0.14	35
	6	0.30	75
Tris HCl	7	0.35	87.5
	8	0.61	152.5
Glycine sodium hydroxide	9	0.76	190
	10	0.60	150
	11	0.55	137.5

pH is the most important factor that markedly influence the enzyme was pH activity. The amylase activity was measured at various pH in different buffers. The results in Table 2 showed that the maximum activity was observed at pH 9 (Glycine sodium hydroxide). The results indicated that the increase in H^+ concentration beyond the optimum pH will destroy the enzymes, because of their proteinaceous nature and hence lowers the enzyme action (Lehninger *et al.*, 1993).

In fermented cassava waste water amylase activity was optimum at pH 9 [glycine sodium hydroxide buffer] treated with *A. flavus, A. niger* and *Rhizopus oryzae* which supports the findings of the present study.

Effect of pH on amylase stability

The influence of pH stability on amylase activity was depicted in Table 3.

Table 3. Effect of pH on amylase stability at different time intervals

Incubation time (Hours)	OD value	Enzyme activity U/ml/min
1	0.74	185
2	0.74	185
3	0.70	175
4	0.63	157.5

The pH stability studies on amylase indicated that there was a decrease in the activity of amylase after two hours of incubation at pH 9. It is observed that the enzyme activity was stable at first two hours of incubation (60, 120 min).

Effect of Temperature on Amylase Activity

The effect of amylase activity was studied at different temperatures ranging from 30 – 70° C at optimum pH [9] was recorded and presented in Table 4.

Table 4. Amylase Activity of Different Temperature

Temperature (°C)	OD value	Enzyme activity U/ml/min
30	0.62	155
35	0.72	180
40	0.76	190
45	0.78	195
50	0.79	197.5
55	0.82	205
60	0.89	222.5
65	0.58	145
70	0.50	125

Enzyme activity increased with temperature within the range of 30 – 60° C. A reduction in enzyme activity was observed at temperatures above 60° C, which indicates the complete inactivation of enzyme. Above the optimum temperature, the enzymes being proteinaceous in nature undergo denaturation with loss of activity which in turn is due to loss of secondary and tertiary structure of the protein moiety. As the enzyme gets inactivated after the optimum temperature the reaction which it catalyses slows down and ultimately stops (Lehninger *et al.,* 1993). Kundus and Das (1970), reported that the optimal temperature for amylase isolated from *A. oryzae* was at 60°C and after 60°C there was inactivation of the enzyme.

Effect of Temperature on Amylase Stability

The effect of temperature on amylase stability incubated at different time intervals is tabulated in Table 5.

Table 5. Effect of temperature on amylase stability at different time intervals

Incubation time (Hours)	OD value	Enzyme activity U/ml/min
1	0.15	37.5
2	0.15	37.5
3	0.12	30.0
4	0.11	27.5

The residual activity of crude enzyme incubated at different intervals of time [1, 2, 3 and 4 hour] was determined. The temperature stability study indicates that there was a general decrease in the stability of the enzyme with increase in time

[180 – 240 minutes]. This decrease in stability may be attributed to the formation of incorrect conformation due to process such as hydrolysis of the peptide chain, destruction of amino acid and aggregation (Sohokker and Boekel, 1999).

Effect of Incubation Time on Amylase Activity

To determine the incubation time on amylase activity the enzyme assay mixture was incubated at 60°C for different minutes. After every 10 minutes time interval amylase activity was determined and the results are depicted in Table 6.

Table 6. Amylase Activity at Different Incubation Time

Incubation time (Minutes)	OD value	Enzyme activity U/ml/min
10	0.50	125.0
20	0.53	132.5
30	0.62	155.0
40	0.65	162.5
50	0.68	170.0
60	0.72	180.0
70	0.58	145.0
80	0.53	132.5

The filtrates of the fungal isolate exhibited highest amylase activity when the enzyme substrate reaction mixtures were incubated for 60 min. After 60 minutes of incubation period there was a gradual decrease in the enzyme activity. Anupama *et al.* (2007) reported that the amylase extracted from *Aspergillus* species IGI 121 activity when the enzyme substrate reaction mixture was incubated at 5 minutes.

Effect of Substrate Concentration on Amylase Activity

The effect of various concentrations of starch on the activity of amylase is shown in Table 7.

Table 7. Amylase Activity at Different Levels of Substrate Concentration

Starch (%)	OD value	Enzyme activity U/ml/min
0.25	0.30	75.0
0.50	0.36	90.0
0.75	0.37	92.5
1.00	0.75	187.5
1.25	0.70	175.0
1.50	0.65	162.5
1.75	0.58	148.0
2.00	0.58	148

There was a drastic increase in the enzyme activity with increase in starch concentration from 0.25 – 1 per cent. Whereas there was a slight decline in the activity of the enzyme from 1.25 – 2 per cent starch and subsequently the activity remained constant. The result of the present study was in agreement with the findings of Alli *et al.* (1998) who reported that the effect of substrate concentration from 0.25 to 1 per cent led to progressive increase in amylase activity in *A. flavus, A. niger, R. oryzae* and *M. pusillus,* grown in cassava waste water.

Treatmen[illegible] starch at an elevated

followed by liquefaction which is done by chemicals or by enzymes. Enzymatic liquefaction has an edge over chemical treatment process. Microbes are in general the source of commercial enzymes and *Aspergillus* species was found to be the most active amylase producers. Genetic studies revealed that starch is a suitable inducer in case of fungi (Sohai *et al.,* 2005).

Effect of NaCl on Amylase Activity

The effect of various concentrations of NaCl on the activity of amylase is shown in Table 8.

Table 8. Amylase Activity at Different Concentrations of NaCl

NaCl (g)	OD value	Enzyme activity U/ml/min
1	0.33	82.5
2	0.35	87.5
3	0.38	95.0
4	0.40	100.0
5	0.53	132.5
6	0.50	125.0
7	0.49	122.5
8	0.34	85.0
9	0.32	80.0

It is observed that NaCl concentration on amylase activity was found to be activated at 1 -5 g and as the concentration of salt increases above 5 there was a decline in the amylase activity. Mohapatra *et al.* (1998) have reported that metallic chlorides are usually potent activators of amylases.

Wakim *et al.* (1969) reported that activity but they are not mandatory for the activity of the enzyme but could be a contribution from the chloride ion and the cations (Obeh and Ajele, 1997).

Effect of Metal Ions on Amylase Activity

The effect of addition of various metal ions to the enzyme assay mixture was examined and the results are shown in Table 9.

Table 9. Effect of Metal Ions on Amylase Activity

Metal ions	OD value	Enzyme activity U/ml/min
$CuSO_4$	0.07	17.5
$CaCl_2 . H_2O$	0.20	50
$MgSO_4 . 7H_2O$	0.06	15
$FeSO_4$	0.05	20
KCl	0.06	15
$MnSO_4 . 4H_2O$	0.6	15
$ZnSO_4 . 7H_2O$	0.05	12.5
BaCl2	0.05	12.5
$HgCl_2$	0.03	7.5

Amylases are usually inhibited or activated by metal ions (Zeid, 1997). The amylase from *A. niger* was activated by 2 mM $CaCl_2.H_2O$, but inhibited by other metal ions to a variable extent. Results suggest that amylase did not require any ions for catalytic activity except calcium ions. Amylases contain atleast one Ca^{2+} ion and the affinity of calcium is much stronger than that of the other ions (Gupta *et al.,* 2003).

A stronger inhibitory effect was

$BaCl_2$, $ZnSO_4$, KCl, $MnSO_4$ and $MgSO_4$. Iefuji *et al.* (1996) reported that the enzyme activity was greatly inhibited in the presence of Hg^{2+}, Fe^{2+} and Cu^{2+}. Gupta *et al.* (2003) reported that the inhibition of mercuric ions may indicate the importance of indole amino residues in enzyme function, as has been demonstrated for other microbial α – amylases.

The inactivation by Cu^{2+} and Hg^{2+} is indicative of the presence of carboxyl groups or histidine residue in the enzyme molecule (Dixon *et al.,* 1964). The reversal of metal ion inhibition by histidine and absence of inhibition of enzyme activity by these chemicals are suggestive for the presence of serine and imidazole group (Barnard and Stain, 1959).

Hydrolysis of Raw Starch

Raw starch was resistant to enzymatic hydrolysis because of the larger size of granules (Dettori *et al.,* 1992). The potential application of starch digesting amylase from *A. niger* was evaluated by studying the extent of hydrolysis of starch granules. It is observed that the enzyme preparation could substantially hydrolyze the raw starch in a short duration of time at 60° C indicating the thermostability of enzymes.

The relative extent of amylase adsorption of starch by amylase from *A. niger* was found to be high when compared with control (without enzyme). This indicated that the extent of hydrolysis of raw starch (R_h) was 162.5 per cent.

The enzyme was incubated with starch to establish the specificity of amylase produced by *A. niger*. The enzyme hydrolyzed that starch and the hydrolysis product from the digestion of starch namely maltose and glucose were observed on paper chromatogram. This shows that amylases attack the α (1,4) glycosidic linkages of starch in a random endoamylolytic fashion (Gupta *et al.,* 2003) and have been reported in many fungi, including yeasts (Prieto *et al.,* 1995 and Kelly *et al.,* 1985).

Desizing of the Fabric

In the present study the amylase extracted from *A. niger* was used for desizing, since commercial enzymes are found to be cost effective, time consuming and requires large quantity of chemicals, energy and water. A pilot study was carried out to overcome this with different dilutions (1:1, 1:2, 1:3, 1:4 and 1:5) of enzymes and the effect of crude enzyme on the fabric ghada (unbleached) was studied and evaluated. The results showed that all the dilutions were found to effectively desize the fabric within 1– 6 hrs. When the water with the fabric was treated with a drop of iodine solution, the water remains white which indicated the action of amylase on starch. Also when iodine was added to fabric after drying the cloth blue colour was not observed on the fabric which indicated the

cleavage of starch by amylase. The desized samples were visually examined by a panel of 16 students belonging to the department of Life Sciences.

Visual Examination

The results of the visual examination of the fabric using crude enzyme and diluted enzyme are presented in Table 10.

Table 10. Visual Inspection of the Desized Samples

Samples	General appearance			Texture			Stiffness		
	G	F	P	S	C	R	L	M	H
1:1	70	44	-	96	4	-	86	80	-
1:2	90	20	-	100	-	70	30	60	-
1:3	80	20	-	90	10	70	30	40	-
1:4	70	30	-	90	10	80	20	50	-
1:5	90	10	-	90	10	80	20	90	-
Crude enzyme	90	10	-	100	0	90	10	98	-

G – GOOD F – FAIR P – POOR S – SOFT C – COURSE
L – LOW M - MEDIUM H - HIGH R - ROUGH

The desized samples were evaluated and it was found that the fabric texture was enzymatically scoured and fabric was graded as soft by the judges. The softness in the bioscoured fabric may be attributed to the residual wax on the fabric material (Paul and Naik, 1997). The fabric was found to be good and soft in appearance when compared with control. The stiffness of fabric was reported as low and medium by few judges, which indicates the action of amylase on starch present in the fabric.

Conclusion

From the above study it can be concluded that the use of microbial enzymes can be expected to expand into many areas of the textile industry replacing the existing chemicals or mechanical processes.

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