*Journal of Experimental Sciences Vol. 1, Issue 11, Pages 25-29 [2010]*
*www.jexpsciences.com*

**Regular Article**

# Optimization of Culture Conditions for the Production of Extracellular Cellulase from *Enterococcus pseudoavium*

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**ABSTRACT**: Bacteria that inhabit decaying vegetables were isolated and screened for the production of the enzyme, amylase. One species identified as *Enterococcus pseudoavium* exhibited relatively higher amylase activity of the bacterial species tested. The bacterium produced the highest extracellular amylase at 72 h in a medium containing starch (1% w/v), galactose (0.5% w/v), and peptone (0.5% w/v), at pH 7.0. The extracellular enzyme was partially purified and its starch hydrolyzing potential was evaluated. The organism when grown with paper pulp deinked the pulp completely just after four days of growth. The bacterial cells immobilized in sodium alginate beads when cultured with paper pulp could decolorize it within 4 days. The extracellular amylase produced by *Enterococcus pseudoavium* effectively deinked and decolorized paper pulp within 4 days after incubation. The enzyme efficiently removed the starch present in fabric and thus it could be very well used as an ingredient in commercial detergent.

**Key words:** *Enterococcus pseudoavium*; Amylase; Deinking; Decolorization

## Introduction

α - amylase (α-1, 4-glucan-glucanhydrolase, EC 3.2.1.1) is an extracellular enzyme. This enzyme degrades α -1, 4-glucosidic linkage of starch and related products in an exo fashion and releases oligosaccharides. The mode of action, properties and product of hydrolysis of this enzyme differ significantly depending on the source of enzyme. Two types of enzymes have been recognized, termed liquefying and saccharifying. The main difference between them is that the saccharifying enzyme produces a higher yield of reducing sugar than the liquefying enzyme. One of the largest industrial users of the enzyme is the starch processing industry. Starch is a plentiful carbohydrate source consisting of two types of molecules, amylose (Normally 20-30%) and amylopectin (Normally 70-80%). Both consist of polymers of α-D-glucose units in the $^4C_1$ conformation. In amylose these are linked (1→4)-, with the ring oxygen atoms all on the same side, whereas in amylopectin about one residue in every twenty or so is also linked - (1→6) - forming branch-points. The relative proportions of amylose to amylopectin and - (1→6) - branch-points both depend on the source of the starch, for example, amylomaizes contain over 50% amylose whereas 'waxy' maize has almost none (~3%). Amylases constitute a class of industrial enzymes, which alone form approximately 25% of the total enzyme market covering many industrial processes such as sugar, textile, paper, brewing, distilling industries and pharmaceuticals (Mamo, 1999; Oudjeriouat, 2003; Pandey, 2000). Bacteria and fungi secrete amylases to the outside of the cells to carry out extra cellular digestion. When they have broken down the soluble starch, the soluble end products such as glucose or maltose are absorbed into their cells. Ellaiah *et al.* (2002) identified amylolytic activity from several fungal species isolated from Indian soils and found *Aspergillus sp.* to possess the highest amylase activity (73 U/mL$^{-1}$). Ugru *et al.*, (1997) produced extracellular amylase using yam peel as carbon source in shake flask cultures of a hemophilic strain of *Aspergillus niger.*

An attempt has been made in the present study to isolate, identify and optimize the culture conditions for amylase producing bacteria isolated from decaying vegetables. Attempt has also been made to evaluate the potentiality of the extracellular enzyme for industrial application. The results obtained have been presented in this paper.

## Materials and Methods

### Isolation and preparation of pure cultures of bacteria

Decaying vegetables were collected from nearby vegetable markets and the decayed portions were excised and inoculated on nutrient agar plates. The agar plates were incubated at room temperature (~27ºC). Pure cultures were prepared by streak plate method and they were maintained on agar plate by sub culturing once in a week.

### Screening bacteria for amylase activity

The isolated bacteria were grown on starch agar medium containing starch (10g/L), Peptone (5 g/L), NaCl (5 g/L), Beef extract (3 g/L) and agar (20 g/L). pH of the medium was adjusted to 7.0. Individual bacterium was inoculated on the starch agar medium and the plates were incubated at 37°C for 24 h. After the incubation, the agar medium was flooded with an aqueous solution of iodine (1% w/v) for 15 min. The amylase produced by individual bacterial species or strains hydrolyze the starch incorporated in the medium. The aqueous iodine solution turns the intact starch incorporated in the medium into blue while the hydrolyzed zone appears transparent. The ratio of the diameter of clear zone to colony diameter was measured in order to select the highest amylase producing bacterium. The largest ratio was assumed to contain the highest activity.

### Identification of highest cellulase producing bacterium

The bacterium which showed the highest cellulase activity was identified by using the book of **classification of bacteria form bergeys manual of determination bacteriology (**Bergey**, 1984).**

### Optimization of culture conditions for the highest amylase productionEffect of incubation time on the amylase production

The bacterium *Enterococcus pseudoavium* was incubated in the production medium and was incubated in a shaker at room temperature (~27ºC) for different period of time i.e., 24, 48, 72 and 96 h after incubation. The total protein content and amylase activity were estimated as per the method described below.

### Medium for amylase production

The medium containing peptone (4g/L), potassium chloride (0.5g/L), magnesium sulphate (0.5g/L), galactose (5.0g/L), and starch (10.g/L) was used as the production medium. Different carbon and nitrogen sources, pH and substrate concentrations were used to determine the level of amylase produced. The bacterium was incubated in the medium for 72 h and cell free supernatant was collected by centrifugation at 7000 rpm for 15 min. The clear culture supernatant served as the enzyme source. The total protein content in the culture supernatant was determined by the method of Bradford (1976). Amylase activity in the culture supernatant was estimated following the method of Miller *et al.*, (1959). To 1mL culture fluid 1mL of starch solution (1% w/v) was added and incubated at 27$^0$C for 15 min.The reactions was stopped by the addition of 2 mL DNS reagent and boiled for 5 min in water bath. The tubes were then cooled under running tap water. One milliliter

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ISSN: 2218-1768

of Rochelle's salt solution was added to each tube and the absorbance was read at 540 nm in a spectrophotometer (Miltonroy). Controls were maintained with boiled culture fluid with the substrate. The enzyme activity was calculated using maltose standard graph. One unit of amylase activity was defined as the amount of enzyme that hydrolyzes 1 µmole of starch into glucose under the assay conditions. The specific activity was expressed as unit activity per milligram protein/ min.

**Effect of carbon and nitrogen sources on amylase production**

Different carbon sources such as glucose, galactose, maltose and lactose and nitrogen sources such as peptone, yeast extract, sodium nitrate and ammonium chloride were incorporated in the production medium as described above at a final concentration of 0.5g/L. The bacterium was grown in the medium with different carbon and nitrogen sources for 72 h at room temperature. Culture supernatant was collected and used for the estimation of total protein and amylase activity.

**Effect of pH on amylase production**

Prior to inoculation, the pH of the production medium was adjusted to 6, 6.5, 7, 7.5 and 8.5. The bacterium, *E. pseudoavium* was inoculated in the production medium with different pH's and incubated for 72 h at room temperature. The total protein content and amylase activity were measured as described earlier.

**Effect of concentration of starch on amylase production**

Three different concentrations of starch i.e., 0.5, 1.0 and 1.5% were added in to the production medium and the amylase activity was estimated using the method described earlier.

**Partial purification of amylase**

After determining the suitable duration of growth, carbon, nitrogen sources, pH, and the concentration of starch. The bacterium was grown in the production medium with the above optimal parameters and incubated for 72 h at room temperature. The cell free culture supernatant was collected by centrifugation and the clarified supernatant was used for precipitation of proteins with ammonium sulphate with different saturation levels such as 50, 60, and 70% (w/v) saturations. The proteins thus precipitated were dissolved in sodium acetate buffer (100 mM; pH 5.5) and dialyzed against the same buffer (10 mM; pH 5.5). The dialyzed proteins were lyophilized and stored at -20°C until further use.

**Qualitative assay for amylase after ammonium sulphate precipitation**

The starch agar medium was poured into petridishes and after solidification, wells (10 mm diameter) were formed with the help of cork borer under aseptic condition. The wells were filled with the clarified culture supernatant and incubated at 37°C for 2 days. The well filled with the buffer alone served as the negative control.

**SDS-PAGE**

Electrophoresis of proteins on polyacrylamide gel was carried out following the method of LaemmLi (1970). The ammonium sulfate precipitated protein was separated on 12% (w/v) gel using a mini gel electrophoresis system. The separated proteins on the gel were stained with Silver nitrate. The molecular mass of the partially purified amylase enzyme was determined by comparing the relative mobilities of the known protein molecular weight standards (Genei, Bangalore, India) run concurrently on the gel.

**Evaluation of *E. pseudoavium* amylase for industrial applications**

**Deinking potential using bacterial cells**

Printed white papers with ink on it were pulped by soaking in hot water for 2 h, macerated in a domestic mixer. The macerated pulp was oven-dried at $50^0$C overnight and stored in sterile container under refrigeration until further use. LB broth (50 mL) with 0.1% starch was prepared and 1.5 g of the sterile paper pulp was mixed and inoculated with cells of *E. pseudoavium*. The culture was incubated for 4 days at room temperature. Uninoculated LB broth with 1.5 g paper pulp was maintained as negative control. The deinking of the paper pulp was qualitatively assessed visually.

**Deinking potential using cell-free culture filtrate**

The bacterial cultures were grown in LB broth with starch (0.1% w/v) for 96 h at room temperature. The culture was filtered through 0.45 µM filter (Nalgene, USA). The sterile culture filterate was mixed with 1.5 grams of paper pulp and incubated at room temperature for 4 days. Uninoculated LB broth with 1.5 gram paper pulp was maintained as control.

**Deinking potential using immobilized bacterial cells**

50 milliliters of exponentially grown cells of *E. pseudoavium* was mixed with 50 mL of 2% sodium alginate solution. The mixture was added drop wise in 0.2M $CaCl_2$ and the beads formed were inoculated with 1.5 grams of paper pulp in a fresh medium containing starch (0.1% w/v). The pulp with the beads was incubated for 96 h at room temperature. After this the pulp was collected by filtration through filter paper and air dried for 24 h. The pulp treated with uninoculated medium served as the control

**De-sizing**

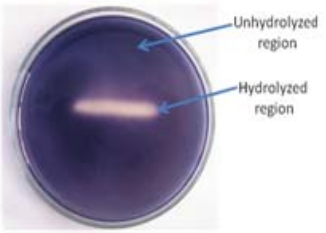
Cotton fabric sized with starch was taken and tested for the presence of starch using iodine solution. Appearance of blue colour indicates the presence of starch. 1 sq. inch of fabric cut and incubated in cell free supernatant for two days. A similar piece of fabric was incubated in sodium acetate buffer (100 mM; pH 5.5) was maintained as control. After the incubation period, the fabric was tested for the presence of starch with iodine solution.

## Results

**Isolation and screening of amylase producing bacteria**

As many as 14 different bacterial strains were isolated from decaying vegetables. Pure cultures of all the fourteen strains were prepared on agar plates. All the strains were tested for amylase activity on a medium containing starch as the substrate. As shown in Fig.1 the amylase produced by the bacterium hydrolyzed the substrate and released hydrolysate out of the medium. Upon reaction of the medium with aqueous iodine solution, the hydrolyzed regions appeared transparent while the unhydrolyzed regions appeared intense blue. After 24 h of incubation, all the 14 strains of bacteria showed signs of growth on starch agar but only one strain hydrolyzed the starch very efficiently. That strain to the bergey's manual of determinative of bacteriology. Since the sole carbon source in the medium was starch the strong hydrolytic activity shown by the bacterium is a clear evidence for the production of amylase.

Figure: 1 Qualitative assay for the production of amylase by bacterial strains isolated from decaying vegetables using iodine solution

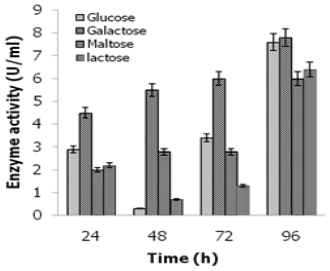



**Optimization of culture conditions for amylase production**

**Effect of carbon sources on amylase production**

Carbon sources such as glucose, galactose, maltose and lactose at a final concentration of 0.5% (w/v) was incorporated in the medium and the bacterium was grown for different time periods like 24, 48, 72 and 96 h. The bacterium *E. pseudoavium* produced the highest amount (6.05 U/mL) of amylase enzyme in the medium incorporated with galactose at 96 h after incubation (Fig.2) followed by medium incorporated with glucose, lactose and maltose. The least amount of enzyme activity was recorded at 72 h in medium incorporated with glucose (3.31 U/mL), maltose (3.06 U/mL) and lactose ((1.25 U/mL).

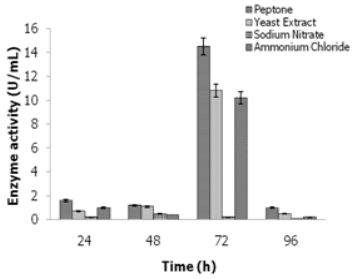
Figure: 2 Effect of carbon sources on the production of amylase by *Enterococcus pseudoavium*




**Effect of nitrogen sources on amylase production**

Different nitrogen sources such as peptone, yeast extract, sodium nitrate and ammonium chloride were incorporated in the medium at a final concentration of 0.5% and the bacterium was incubated for 24, 48, 72 and 96 h. As shown in Fig. 3 the bacterium produced the highest level of amylase (14.47 U/mL) in the medium incorporated with peptone at 72 h. Yeast extract (10.87 U/mL) and ammonium chloride (10.38 U/mL) also induced the production of amylase at significant level at the same time. The bacterium had produced low level of amylase in the medium incorporated with sodium nitrate (0.144 U/mL).

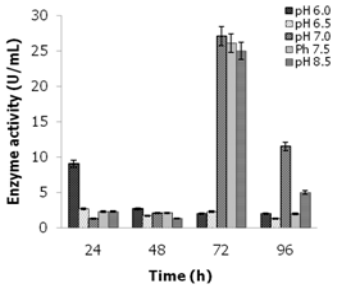
Figure: 3 Effect of nitrogen sources on amylase production by *Enterococcus pseudoavium*




**Effect of pH on amylase production**

As shown in fig.4 the bacterium produced highest amount of amylase in a medium maintained with pH of 7.0 (27 U/mL) at 72 h followed by 7.5 (26.5 U/mL) and 8.5 (25.2 U/mL). There was a drastic decrease in the enzyme activity when the organism was grown in medium whose pH was adjusted to either 6.0 (1.87 U/mL) or 6.5 (2.08 U/mL). The organism did not produce significant amount of enzyme when the incubation period is kept below and above 72 h.

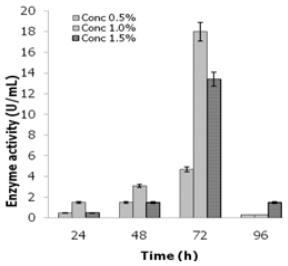
Figure: 4 Effect of pH on amylase production by *Enterococcus pseudoavium*




**Effect of substrate concentration on amylase production**

The bacterium *E. pseudoavium* was grown in medium containing different concentrations of starch i.e., 0.5, 1.0 and 1.5% (w/v) for 24, 48, 72, and 96 h. After the specified incubation time, amylase activity was measured from the culture supernatant. As shown in Fig.5, the highest amylase activity was observed in medium incorporated with 1% (w/v) (18.14 U/mL) at 72 h followed by 1.5 (w/v) (13.1 U/mL) and 0.5% (w/v) (4.60 U/mL). Cultures grown below and above 72 h showed very little activity (Fig.5).

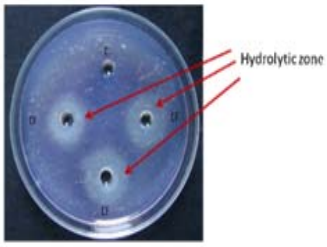
Figure: 5 Effect of substrate concentration on amylase production by *Enterococcus pseudoavium*




**Qualitative assay for the detection of amylase activity**

The cell free culture supernatants were tested for amylase activity qualitatively using a starch agar plate. Just after solidification of the medium, wells (10 mm dia) were formed with the help of a cork borer. The wells were filled with culture supernatants and incubated for 48 h at room temperature. Iodine solution (1% w/v) was over layered on the agar and observed for the formation of hydrolytic zone around the well. As shown in Fig. 6, the wells filled with culture supernatant hydrolyzed the starch around the wells (arrows) whereas the well filled with uninoculated medium which served as the control did not show hydrolytic activity around the well.

Figure: 6 Qualitative assay for amylase activity

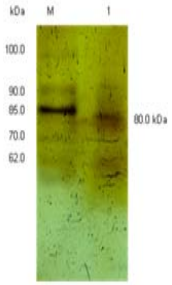



C-Control; CF-culture filtrate

**Electrophoresis analysis of partially purified amylase on SDS-PAGE**

The proteins were precipitated from the culture supernatant with ammonium sulfate, dialyzed and lyophilized. The proteins were electrophoresed on 12% (w/v) polyacrylamide gel. As shown in Fig. 7, there was a prominent protein band observed in the test sample. The molecular mass of the protein was determined as 80 kDa by comparing the relative mobility of the standard protein markers run along with the test sample.

Figure: 7 SDS- PAGE analysis of amylase enzymic protein



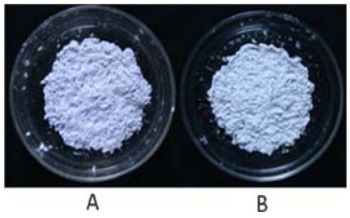

Lane M: Protein marker; Lane 1- Partially purified amylase

**Analysis of *E. pseudoavium* amylase for industrial applications**

**Deinking potential using bacterial cells**

Macerated paper pulp was incubated with growing cells in LB broth for 96 h and the decolourization of the paper pulp was examined. As shown in Fig. 8, the pulp incubated with growing cells decolorized the ink to certain extent while the pulp incubated with uninoculated medium did not decolorize (Fig.8).

Figure: 8 Analysis of de-inking potential of Enterococcus pseudoavium growing cells with pulp

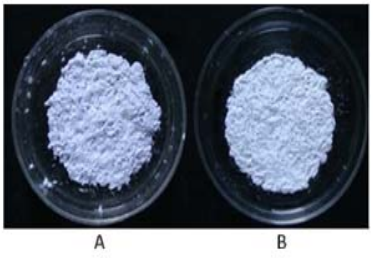



A-control, B-paper pulp treated with bacterial cells

**Deinking trials using cell-free bacterial culture filtrate**

Paper pulp when incubated with cell-free culture supernatant was deinked (Fig.9). However, the culture supernatants from the bacterial culture supplemented with starch were effective in deinking and decolorization of the pulp.

Figure: 9 Analysis of de-inking potential of Enterococcus pseudoavium culture supernatant with paper pulp

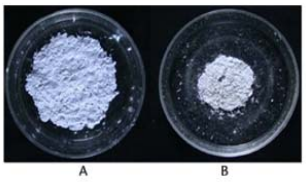



A-control (pulp treated with uninoculated medium)
B-paper pulp treated with culture supernatant

**Deinking trials using immobilized bacterial cells**

In order to analyze the decolourizing potentials of *E. pseudoavium,* the cells were immobilized in sodium alginate beads and incubated with paper pulp (1.5% w/v) in LB medium for 96 h. As shown in Fig. 10, the immobilized cells decolorized the pulp to a greater extent.

Figure: 10 De-inking potential of immobilized cells of Enterococcus pseudoavium with paper pulp

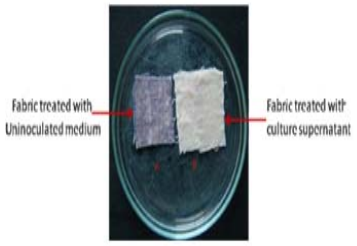



A-control, B – pulp treated with immobilized cells

**De-sizing potential of amylase of *Enterococcus pseudoavium***

The sized cotton fabric was incubated with cell-free supernatant for 48 h and analyzed for de-sizing. After 48 h treatment, the fabric was treated with 1% iodine solution which acts as an indicator for the presence of starch. Blue colouration indicates the presence of starch. No colour formation indicates that the fabric was de-sized. As shown in Fig. 11, the fabric treated with the culture supernatant completely removed the starch.

Figure: 11 Analysis of Enterococcus pseudoavium culture supernatant for de-sizing of fabric




## Discussion

An attempt has been made in the present study to isolate amylase producing bacteria from decaying vegetables, optimize the culture conditions for the optimal production of amylase and evaluate its potential for industrial application for the removal of inks and dyes and de-sizing. The principal reason why the bacterial strains were isolated from vegetables is that they have considerable amount of starch in them and naturally inhabit starch degrading bacteria. An aqueous iodine based method was used to screen for the starch degrading bacteria. Initially, decaying vegetable samples were inoculated on a medium containing 0.1% (w/v) starch. The plates were incubated at room temperature for 48 h. After this, the plates were flooded with aqueous iodine solution. If a bacterium produces amylase, it will hydrolyze the substrate (starch) incorporated in the medium surrounding the bacterial growth and therefore the iodine solution cannot stain those regions. The hydrolyzed regions appear transparent (Fig.1). While those areas where the starch is intact, the iodine solution stain it and turn into blue. Based on whether hydrolytic zone appear or not around the growth of a particular bacterium, the starch hydrolyzing bacteria were isolated. A total number of 14 bacterial strains were isolated from decaying vegetables. Of the 14 isolates only one strain was able to hydrolyze starch incorporated in the medium very efficiently (Fig.1&6). Therefore, this particular strain was chosen for identification. The strain was identified as *E. pseudoavium* by Bergey's manual of determinative bacteriology and maintained in agar slants.

The next objective was to optimize the culture conditions for *E. pseudoavium* for maximum amylase production. Different carbon and nitrogen sources, different pH ranges and different concentrations of starch were tested for amylase production in the medium as described in materials and methods. Among the different carbon sources tested, the highest amylase activity was observed in the medium incorporated with galactose irrespective of the duration of incubation (Fig.2). However the amylase activity increased gradually when the duration was increased up to 96 h. Amylase activity in the medium incorporated with glucose also elicited amylase activity similar to galactose but it occurred only at 96h. Maltose and lactose induced amylase activity to a lesser extent (Fig.2). There are reports that bacteria such as *Bifidobacterium adolescentis, Geobacillus thermodinitrificans, Bacillus subtilis, Bacillus sp* produced significant levels of amylase when these organisms were grown in the medium containing starch (Lee, 1997; Thaddeus, 2005; Swain, 2006; Sudharhsan, 2007; Mishra, 2008).

Among the different nitrogen sources tested for amylase activity, peptone induced the highest amylase production followed by yeast extract and ammonium chloride (Fig.3). Induction of amylase by peptone was more pronounced at 72 h when compared to the production at 24, 48 and 96 h. Sodium nitrate did not induce amylase production to an appreciable level at all the incubation times (Fig.3). Similar induction of amylase by peptone has been previously observed in *Bifidobacterium adolescentis* (Lee, 1997), *Bacillus subtilis* and *Bacillus amyloliquifaciens* (Sarikaya, 1999; Gangadharan, 2006), *Bacillus lichneformis*, sp121 (Aiyer, 2004), *Geobacillus thermodinitrificans* (Thaddeus, 2005), and *Bacillus sp* (Mishra, 2008). It is interesting to note that this organism did not produce amylase up to 48 h and the induction occurred only at 72 h. It is also interesting that the production ceases after 72 h. It would be interesting to study the factor which contributes to the sudden decrease of this enzyme after 72 h.

The amylase produced by *E. pseudoavium* appears to be requiring either neutral of slightly basic pH for its optimal activity because the bacterium produced the highest level of amylase when the pH of the medium was adjusted to 7-8.5 at 72 h. There was a drastic decrease in the production of amylase when the pH is maintained below 7.0. The bacterium produced amylase in the culture medium only at 72 h and not at 24, 48 and 96 h (Fig.4). It is clear that the organism in addition to carbon and nitrogen sources requires a minimum period of 72 h of incubation for maximum amylase production. *Geobacillus thermodinitrificans* and *Bacillus sp* preferred an optimum pH of 7.0. However the enzyme production occurred at 24 h. produced highest amount (Thaddeus, 2005; Sudharhsan, 2007), *Bacillus lichneformis*, sp121 and *Bacillus subtilis* produced maximum amylase at alkaline pH 9.0 (Aiyer, 2004; Swain, 2006). On the contrary, *Aspergillus niger* AM07 produced the highest amount of amylase at a pH of 4.0 (Omemu, 2004). In the present study the bacterium *Enterococcus pseudoavium* produced maximum amylase at 72 h irrespective of carbon and nitrogen and pH used. Similar such observations have been already made with amylases produced by *Aspergillus niger* and *Bacillus* sp (Omemu, 2004; Mishra, 2008).

Among the three different substrate concentrations of starch, 1.0% (w/v) proved to be the optimal concentration (Fig.5). The amylase activity decreased drastically when the concentration of the substrate is either increased or decreased from 1.0%. Partial purification of amylase from the culture supernatant was attempted by selectively precipitating the enzymic proteins with ammonium sulphate. Among the three different saturations used, the proteins precipitated with 70% (w/v) showed relatively higher amylase activity. Electrophoretic analysis of the partially purified protein on SDS-PAGE indicated a prominent protein band and whose molecular mass was determined as 80 kDa by comparing the mobilities of the standard molecular markers (Fig.7).

Attempts were also made to evaluate the de-inking and sizing potentials of amylase produced by *E. pseudoavium* through the conditions standardized as above. Printed white papers were macerated and the pulp obtained was incubated with the intact cells as well as with clarified culture supernatant. In both the cases, removal of the dye from the pulp occurred very efficiently which suggests that this enzyme could be used for commercial deinking processes (Fig.8 & 9). Attempt to find out the potentiality of the amylase in deinking and sizing processes, intact cells were immobilized and the macerated paper pulp and cotton fabrics were incubated with the immobilized cells. The immobilized cells could remove the dye from the pulp and starch from the fabric completely (Fig.10 & 11) which gives a strong pointer for its potential use in deinking and sizing processes.

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