Volume : 2 | Issue : 9 | September 2013 • ISSN No 2277 - 8179

Research Paper

Screening, Characterization and Optimization of Cellulase Producing Bacteria Isolated from Paper Sludge

Environment

KEYWORDS : Cellulase, paper sludge, pH, temperature, textile industry.

Dr. M. Pandimadevi — Department of Biotechnology, School of Bioengineering, SRM University, Kattankulathur

Faridha Begum .I — Department of Biotechnology, School of Bioengineering, SRM University, Kattankulathur

ABSTRACT

Production of microbial enzymes at the commercial level is most actively grown area of research now a day. Screening of potential strains from new sources and there by optimizing production conditions for industrial use. Cellulase is one of the several commercial enzymes which have been used in various industries like paper and pulp, textile, bio-fuel production, detergents, feed and food industry and brewing etc. Recently application of cellulase in biofuel production and de-inking of paper has further driven the attention of research towards cellulase. Hence research is required to screen different diverse habitats for potential cellulase producing microorganism to lower down the cost of production. This research is focused on the isolation and characterization of cellulase producing bacteria from paper mill waste as a new source of possible substrate for cellulase production and further optimizing the different production parameter to improve the cellulase production. Paper sludge is chosen as a source because it is a waste and its disposal is a cause of environmental concern. This work also involves characterization of isolated enzyme from bacteria present in the paper sludge by checking its stability at varying pH and temperatures. The characterization of enzyme is required to develop efficient application of enzyme in the textile industry.

1. INTRODUCTION

Cellulose is the most abundant biomass; it is renewable and inexpensive resource for the bioconversion to biofuels and bioproducts. There are many sources to derive cellulosic biomass from such as municipal waste, agricultural residues, forestry or pulp and energy crops (Greene, 2004).

In India, most of the enzymes are imported at huge costs which warrant absolute need for its commercial indigenous production to reduce the market price (Sukumaran *et al*., 2005). Cellulose is commonly degraded by an enzyme called cellulase. Cellulase refers to a class of enzymes produced chiefly by fungi, bacteria and protozoans that catalyze the cellulolysis of cellulose (Immanuel *et al*., 2006).

Cellulases have attracted much interest because of the diversity of their application. The major industrial applications of cellulases are in textile industry for 'bio-polishing' of fabrics, household laundry detergents for improving fabrics softness and brightness (Cavaco – Paulo, 1998).

The major obstacle facing in the development of lignocellulosic biofuels is the cellulose hydrolysis stage. There is a lack of microorganisms which can produce sufficient amounts of all three types of cellulases to efficiently breakdown crystalline cellulose to glucose. The bio-refining process remains economically unfeasible due to a lack of biocatalysts that can overcome costly hurdles such as cooling from high temperature, pumping of oxygen / stirring and neutralization from acidic or basic pH. The extreme environmental resistance of bacteria permits screening and isolation of novel cellulases to help overcome these challenges.

In this study focused efficient aerobic cellulase producing microorganisms were isolated from paper sludge waste. The purpose of this work was to identify and characterize these isolates displaying the greatest cellulose activity for the possible use in large scale bio-stoning industry.

2. MATERIALS AND METHODS

2.1 Sample Collection

Paper sludge sample was collected from Srinivasa Paper mills located in Red Hills, Chennai using sterilize technique. The sample was collected in a semisolid form and was ensured that the consortium of bacteria present in the sample was not denatured.

2.2 Isolation of bacteria from Paper Sludge

The bacterial isolates were isolated from paper sludge by serial dilution agar plating method and the sample was serially diluted up to 10^{-6} dilution and spread on a sterile nutrient agar plates using the spread plate method. Plates were incubated at 37ºC. Mixed culture obtained were differentiated based on colony morphology and named as PF1, PF2 and PF3.

2.3 Screening for cellulase producing bacteria

All the three isolates were obtained in the previous step were subjected to cellulose screening by streaking them on agar plates containing 1% Carboxyl methyl cellulose (CMC).

2.4 Zone of hydrolysis – Congo Red Assay

The CMC agar plates were incubated at 37ºC for 5 days to allow the secretion of cellulase. After incubation, the hydrolysis zone was visualized and then the hydrolysis zone on the agar medium was flooded with 1% Congo red aqueous solution for 15 minutes. The Congo red solution was then poured off, and the plates were further treated by flooding with 1 M NaCl for 15 minutes. The cellulase activities of the microorganisms were observed and diameter of clear zones around colonies of PF1 and PF2 isolates were compared.

2.5 Identification of cellulase producing bacteria

The isolates were further studied and characterized for biochemical and sugar fermentation test (Buchanan and Gibbons, 1974). Identification of the isolates was confirmed by 16S rRNA sequence analysis (Altschul *et al*., 1990).

2.6 Growth Curve

In order to study the duration at which stationary phase (known for maximum secondary metabolite production) is reached by the isolate, it was inoculated in sterile CMC broth media and the growth of isolate was tracked by reading the absorbance of the inoculated media at 540 nm against uninoculated blank.

3. Enzyme Activity Assay

3.1 Preparation of crude enzyme

After incubation, the cultures were centrifuged and supernatants were used as source of crude enzyme (Kotchoni, *et al*., 2003). The crude enzyme was utilized for determination of enzyme activity.

3.2 Dinitrosalicylic acid (DNS) Method

Cellulase activity was measured by the DNS (3,5 –dinitrosalicylic acid method) (Miller, G.L., 1959), through the determination of the amount of reducing sugars liberated from carboxyl methyl cellulose (CMC) solubilized in 50 mM Tris-HCl buffer, pH 7.0. This mixture was incubated for 20 min at 70°C. For crystalline cellulose substrates, incubation times were extended to 2

Volume : 2 | Issue : 9 | September 2013 • ISSN No 2277 - 8179

hour and the reaction was stopped by the addition of DNS solution. The treated samples were boiled for 10 minutes, cooled in water for color stabilization, and the optical density was measured at 550 nm. The cellulose activity was determined by using a calibration curve for glucose. One unit of enzyme activity was defined as the amount of enzyme that released 1 µmol of glucose per minute.

4. Partial purification of Cellulase

4.1 Ammonium sulphate precipitation method

About 20 ml of the crude enzyme was brought to 80% saturation with solid ammonium sulphate (Lee *et al*., 2006). The mixture was left overnight at 4°C in a magnetic stirrer. Centrifuge the mixture and the pellet was dissolved in 10 ml of 50 mM sodium acetate buffer (pH 5.5) for further purification.

5. Optimization of production of cellulase enzyme

Cellulase production depends upon the composition of the fermentation medium. Medium optimization for over production of the enzyme is an important step and involves a number of physico-chemical parameters such as the incubation period, pH, temperature and supplemented substrate in submerged fermentation. For the initial optimization of the medium, the traditional method of "one variable at a time" approach was used by changing one component at a time while keeping the others at their original level. The selected cellulolytic strains were grown in selected media consisting of selected substrates for enzyme production. Studies were performed in shake flasks to optimize different fermentation conditions for hyper cellulose production.

5.1 Carbon Source

The basal medium supplemented with different volumes of carbon substrates name as sucrose, maltose, lactose, glucose, starch and CMC were studied for the enzyme production. The broth was distributed into different flasks and 1% of each carbon source was then added before inoculation. Using different carbon sources, media was prepared keeping pH, temperature and incubation period as constants. Enzymatic assay was performed using cellulose assay after 120 hours to find the optimum carbon source for enzyme production.

5.2 Nitrogen Source

The basal medium supplemented with various nitrogen compounds namely beef extract, yeast extract, gelatin, glycine and peptone was studied for the enzyme production. The broth was distributed into different flasks and 0.2% of each nitrogen sources were then added before inoculation. Using different nitrogen sources, media was prepared keeping temperature, pH and incubation period as constants. Enzymatic assay was performed using cellulase assay after 120 hours to find the optimum nitrogen source for enzyme production.

5.3 Effect of temperature, pH and incubation period

To select the suitable temperature, pH and incubation period for fermentative production of the enzyme the selected bacterial isolate was cultivated with varying temperatures of 27°C and 37°C, pH range 6-8, incubation period range of 24 to 120 hours, by keeping all other parameters constant for hours.

6. RESULTS AND DISCUSSION

6.1 Isolation of bacteria from paper sludge

Mixed cultures obtained were differentiated based on colony morphology and named tentatively as PF-1, PF-2 and PF-3 based on the initials of the group members.

Colony morphology

Table 1: Colony morphology of the isolates

Properties	Isolate PF-1	Isolate PF-2	Isolate PF-3
Shape	Circular	Irregular	Circular
Size	Small	Medium	Medium
Elevation	Elevated	Convex	Elevated
Surface	Smooth	Smooth	Rough
Edges	Entire	Undulate	Entire
Color	White	Yellow	Yellow
Structure	Opaque	Translucent	Opaque
Degree of growth	Moderate	Scanty	Scanty
Nature	Spreading	Confluent	Discrete

6.2 Screening for cellulase producing bacteria

The three isolates were streaked on CMC media and incubated for 24 hours. The growth was seen on the PF-1 and PF-2 isolates. The CMC medium was found to be a reliable for screening cellulase producing bacteria (Emerson *et al*., 1963). From the Fig.4.1 it can be observed that PF-1 and PF-2 isolates show growth when cultured in a CMC containing medium and the cellulase producing bacteria.

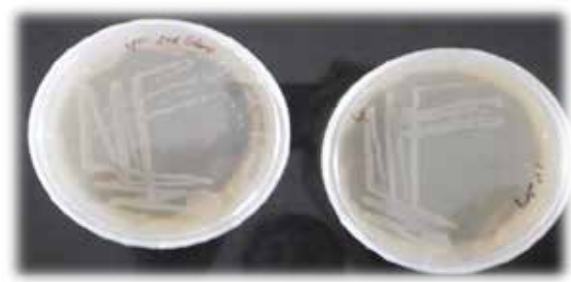


Fig. 1: Colonies of PF-1 PF-2 isolates

6.3 Screening Method (Zone of Hydrolysis)

Using Congo red dye the zone of hydrolysis was observed. Isolate PF-1 showed bigger diameter when compared to PF-2. So it was selected for biochemical characterization. It indicates that the bacteria is producing cellulase enzyme for the conversion of cellulose into glucose units for its metabolic activities. Thus Congo red dye is unable to stain the area around the bacterial colonies.

Table 2: Biochemical Tests

S.No..	Biochemical Tests	Results obtained
1	Gram Staining	Negative
2	Methyl Red	Negative
3	Voges Proskauer	Positive
4	Indole	Negative
5	Citrate Utilization	Positive
6	Gelatinase	Negative
7	Triple sugar iron agar	Lactose fermenter
8	Oxidase	Negative
9	Catalase	Positive
10	Nitrate reduction	Positive
11	Urease	Negative

6.4 16S rRNA sequencing

The following sequence was obtained:

>PF2-16SF-<TGCaAGTCGaCGGTAGCACAGAGAGCTTGCTCTCGGGTGACGAGTGGCGGACGGgTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAATGTCGCAAGACTGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAgactactACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGTTGCGGTTAATAACCGCAGCAATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAG-

GCTGGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGacAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGcAAACAGGATTAGATACCtGGTAGcCC>

The BLAST result showed that it had the maximum similarity with:Enterobacter cloacae

Scientific Classification
Kingdom : Bacteria
Phylum : Proteobacteria
Class : Gamma Proteobacteria
Order : Enterobacteriales
Family : Enterobacteriaceae
Genus : *Enterobacter*
Species: *cloacae*

6.5 Growth Curve
From the Fig.2, it can be seen that cellulase, a secondary metabolite formed in the stationary phase is produced after ten hours of incubation. It can be concluded that the bacteria takes time to utilize the nutrients from the medium and then starts producing its metabolites.

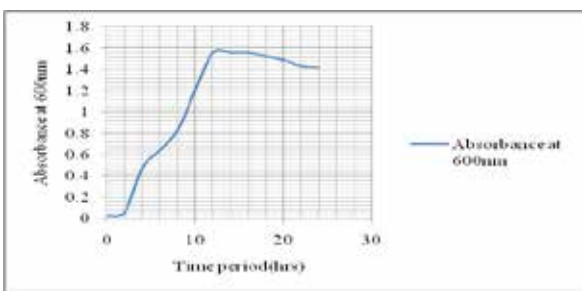


Fig.2: Growth curve

6.6 Study of enzyme activity
The values are used as a standard to find the glucose concentration present in the test samples when the substrate cellulose is degraded into glucose units by the cellulase enzyme present in the sample. The slope obtained from the Fig.3 is 0.108. It is used to find the unknown glucose concentration.

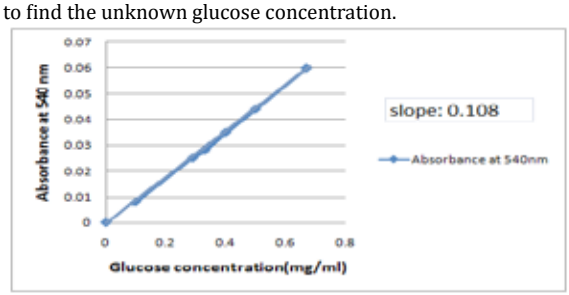


Fig.3 : Standard graph of Absorbance (540nm) Vs Glucose concentration (mg/ml)

Effect of enzyme activity at 27°C (a) pH 6

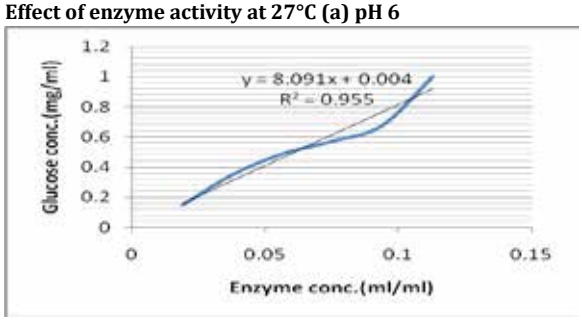


Fig. 4: Glucose concentration Vs Enzyme concentration (a) pH 6

Effect of enzyme activity at 27°C (b) pH 7

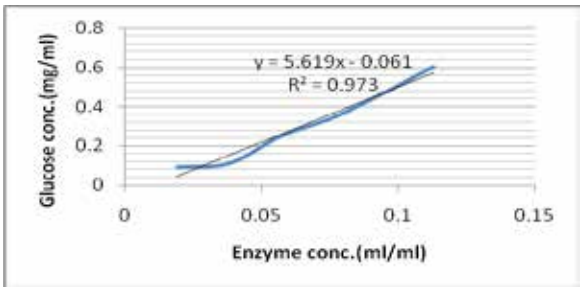


Fig.5: Glucose concentration Vs Enzyme concentration (b) pH 7

Effect of enzyme activity at 27°C (c) pH 8

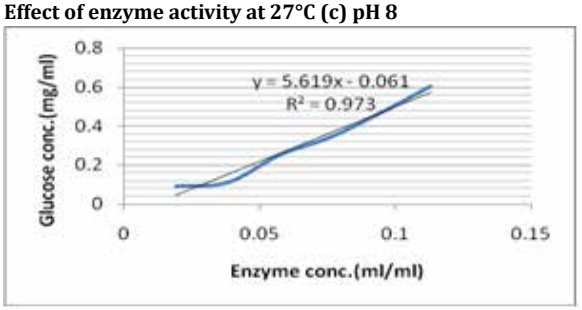


Fig. 6: Glucose concentration Vs Enzyme concentration 27°C (c) pH 8

Comparison of enzyme activity values on different pH at 27°C

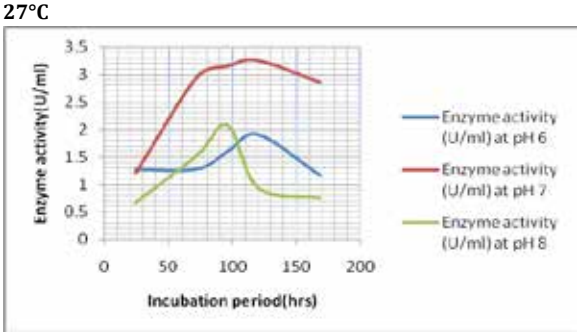


Fig. 7: Enzyme activity Vs incubation period (Hours) at 27°C

From the Fig.7 is seen that at a temperature of 27ºC, when the medium has a pH of 7, the enzyme activity reaches a value of 3.25U/ml. This value decreases to 2.08 U/ml when the pH is increased to 8, the enzyme activity is decreased to 1.908U/ml. These values show that at 27ºC, the maximum enzyme activity can be obtained at pH 7.

Effect of enzyme activity at 37°C (a) pH 6

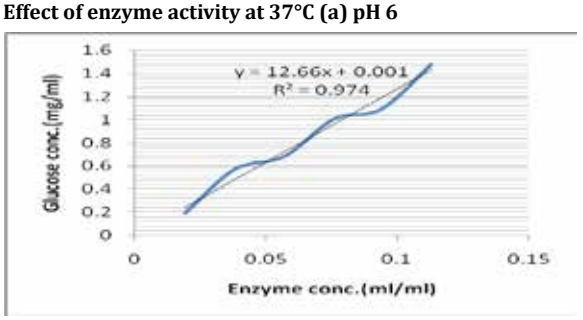


Fig. 8: Glucose concentration Vs enzyme concentration at 37°C

Volume : 2 | Issue : 9 | September 2013 • ISSN No 2277 - 8179

(a) pH 6

Effect of enzyme activity at 37°C (b) pH 7

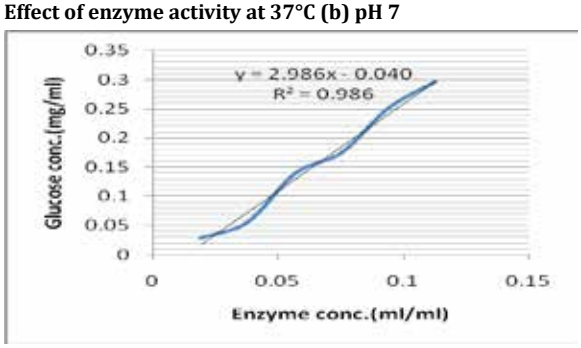


Fig. 9: Glucose concentration Vs enzyme concentration at 37°C (b) pH 7

Effect of enzyme activity at 37°C (c) pH 8

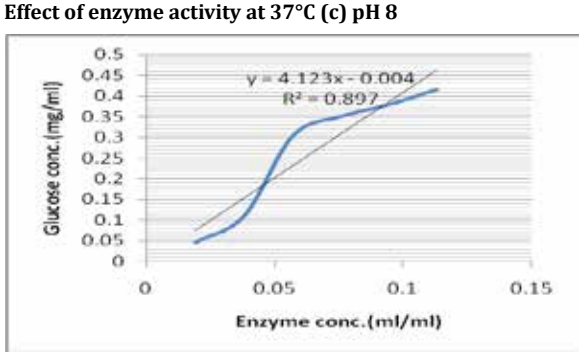


Fig.10: Glucose concentration Vs enzyme concentration at 37°C (c) pH 8

Comparison of enzyme activity on different pH at 37°C

From Fig.11 was noted that at a temperature of 37ºC, when the medium has a pH of 6, the enzyme activity reaches a value of 4.5 U/ml. This value decreases to 1.526 U/ml when the pH is increased to 8 and decreases the enzyme activity of 1.004 U/ml at pH 7. These values show that at 37ºC, the maximum enzyme activity can be obtained at pH

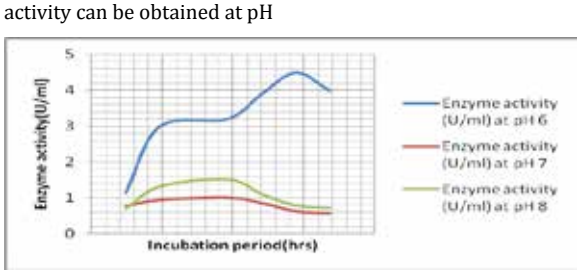


Fig.11: Enzyme activity Vs incubation period

Temperature and pH on microbial growth and enzyme production has a great influence. If the culture temperature is too high, micro-organisms grows faster, but on aging, the enzyme production is decreased, and if the temperature is too less, the microbial growth is also slow and resulting in long production cycle. From the Fig. 4.11 and 4.15, a comparison is made. At 27ºC and at pH 7, the maximum enzyme activity was found to be 3.25 U/ml. But at 37ºC and pH 7, the maximum enzyme activity was found to be 4.5 U/ml.

Thus the Optimum conditions giving the maximum enzyme activity were as follows:

- Optimum temperature is 37ºC, optimum pH is 6 and followed by the Incubation period of 120 hours.
- The pH optimization showed the acidophilic nature of the isolated strain.

Effect of carbon sources on enzyme activity

Table 3: Effect of carbon sources on enzyme activity

Carbon Sources	Enzyme activity U/ml
Sucrose	0.1494
Maltose	3.43
Lactose	5.8
Glucose	2.165
Starch	0.494
CMC	4.35

The components present in a medium play a crucial role in the growth of the bacteria as well as on its metabolism. The activity of the enzyme produced by bacteria depends on the utilization of the components present in the medium. From the Fig.12 was seen that a maximum enzyme activity of 5.18 U/ml is obtained when lactose is used as a carbon source.

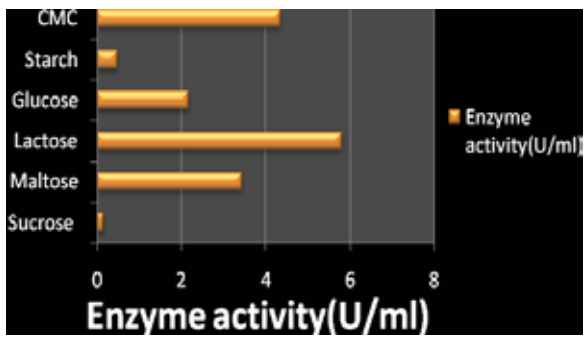


Fig. 12: Effect of carbon sources on enzyme activity

Effect of nitrogen sources on enzyme activity

Table 4: Effect of nitrogen sources on enzyme activity

Nitrogen Sources	Enzyme activity (U/ml)
Beef extract	3.56
Yeast extract	3.22
Gelatin	1.43
Glycine	2.25
Peptone	4.26

From the fig.13 was found that when peptone is used as a nitrogen source in the medium the enzyme activity is found to be the highest. So peptone is the best nitrogen source.

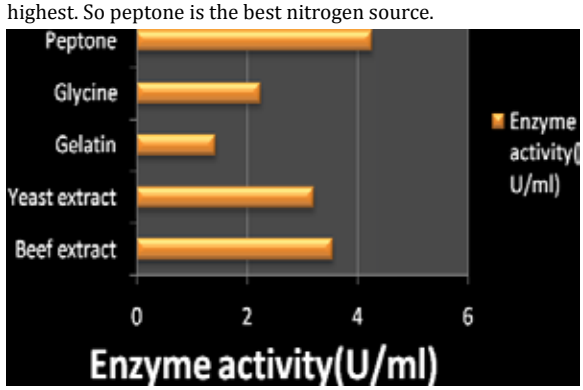


Fig.13: Effect of Enzyme activity on different nitrogen sources

7. Biostoning

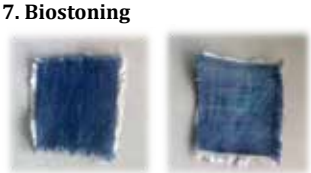


Fig. 14.1 Control Fig.14.2 Experiment

The Fig.14.1 and 14.2 shows the effect of the cellulase enzyme on the denim fabric giving it a faded appearance. The Fig 14.1 is darker and the Fig 14.2 is showing a lighter shade. The enzyme acts on the denim fabric and degrades the cellulose fibres that cause fading.

8. CONCLUSION

In this study, microorganisms were isolated from Paper mill waste as a possible substrate for cellulase production by submerged fermentation. The extracted crude enzyme was partially purified by ammonium sulphate precipitation method at 85% saturation. The enzyme activity of the crude enzyme was found to be 4.513 U/ml and after partial purification it was increased to 6.25 U/ml, the enzyme activity was found to be highest at pH 6, predicting its acidophilic nature and at a temperature of 37ºC. Various carbon and nitrogen sources were used and their effect on the enzyme activity was studied. Lactose supplemented medium showed an increase in the enzyme activity with a value of 5.8 U/ml and peptone was found to be the best nitrogen source giving an enzyme activity of 4.26 U/ml. Thus lactose was considered to be the best carbon source for the high enzyme activity. The medium optimization was performed using Response Surface Methodology (RSM). The predicted cellulase enzyme activity was 7.104 U/ml. Validation was performed using RSM and an experimental value of 6.8 U/ml was obtained. Finally, the cellulase activity was checked on denim fabric and a fading effect was observed. From this finding, it indicates clearly that cellulose obtained from the paper sludge waste may be used as a biostoning agent in textile industry since it possesses tremendous potential for making jeans smooth via an environmental friendly technique.

REFERENCE

1. Altschul, S. F., Jenkins, A., Kristiansen, B. E., Froholum, C. O. 1990. Basic Local Alignment Search Tool. Journal of Molecular Biology, 215, 403-410. | 2. Buchanan, R.E., Gibbons, N.E. 1974. Bergey's of determinative bacteriology. America: United States of America, 529-563. | 3. Cavaco-Paulo, A. 1998. Mechanism of cellulose action in textile processes. Carbohydra. Polym. 37, 273-277. | 4. Emerson, J. E., and Weiser, O. L. 1963. Detecting Cellulose digesting bacteria. Journal of Bacteriology, 86,891. | 5. Greene, N. 2004. How biofuels can help end America's oil dependence. Nat. Res. Def. Council Rep.,1-86. | 6. Immanuael, G.,Dhanusha, R., Prema, P., Palavvesam, A. 2006. Efffect of different growth parameters on endoglucanase enzyme activity by bacteria isolated from coir retting effluents of estuarine environment. International Journal of Environmental Science and Technology, 3, 25-34. | 7. Kotchoni, O.S., Shonukan, O.O., Gachomo, W.E. 2003. Bacillus pumillus, Bp CRI 6, a promising candidate for cellulose production under conditions of catabolite repression. African Journal of Biotechnology, 2(6):140-146. | 8. Lee, Y.J., Kim, B.K., Lee, B.H., Jo KI, Lee, N.K. 2006. Purification and characterization of cellulose produced by Bacillus amyoliquefaciens DL-3 utilizing rice hull.College of Natural Resources and Life Science, 840, 604-714. | 9. Miller, G.L. 1959. Use of dinitrosalicylic acid reagent for determination of sugar. Analytical Chemistry, 31, 426-428. | 10. Sukumaran, R.K., R.R. Singhania and A. Pandey. 2005. Microbial cellulose production, applications and challenges. Journal of Secientific industrial Research, 64, 832844. |