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Screening Isolation and Characterization of Cellulase Producing Micro-Organisms from Soil.

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***ABSTRACT**: Cellulose may be hydrolyzed using enzymes to produce glucose, which can be used for the production of ethanol, organic acids and other chemicals. Cellulases are a group of hydrolytic enzymes capable of hydrolyzing the most abundant organic polymer i.e. cellulose to smaller sugar components including glucose subunits. Cellulase is expensive and contributes only 50% to the overall cost of hydrolysis due to the low specific activity. This enzyme has enormous potential in industries and is used in food, beverages, textile, laundry, paper and pulp industries etc. Therefore, there has been much research aimed at obtaining new microorganisms producing cellulose enzymes with higher specific activities and greater efficiency. Presently, work is aimed at screening and isolating cellulolytic fungi from the soil samples collected from 2 different areas of Himachal. Total 21 fungal isolates were isolated from these soil samples, out of which four isolates were showing the cellulase activity. The fungal isolate designated as PISS-3 isolated from Paper Industry soil sample from HRA Mill Village Tibhi, Indora, Kangra H.P was noticed to show maximum zone of hydrolysis of carboxy-methyl cellulose. The cellulase activity was assayed by Carboxymethyl cellulase "CMCase" (endoglucanase) assay.*

***KEYWORDS:** Cellulase, Fungi, CMC, DNS, Glucose, Protein estimation.*

I. INTRODUCTION

Cellulose is the most common abundant, renewable biopolymer on earth and domestic waste materials from agriculture representing about 1.5 x 10^{12} tons of the total annual biomass production through photosynthesis in the topics **(Klemm *et al.*, 2002; Bhat, 2000)**. Cellulose is considered as one of the most important sources of carbon on this planet and its annual biosynthesis by both land plants and marine occurs at a rate of 0.85×10 11 tonnes per annum **(Nowak *et al.*, 2005).** Cellulase degradation and its subsequent utilisations are important for global carbon sources. The value of cellulose as a renewable source of energy has made cellulose hydrolysis the subject of intense research and industrial interest **(Bhat *et al.*, 2000).** There has been much research aimed at obtaining new microorganisms producing cellulase enzymes with higher specific activities and greater efficiency **(Subramaniyan and Prema, 2000)**.

Over the years, a number of organisms, in particular fungi, possessing cellulose-degrading enzymes have been isolated and studied extensively **(Bhat *et al.*, 1997**). Cellulolytic enzymes play an important role in natural biodegradation processes in which plant lignocellulosic materials are efficiently degraded by cellulolytic fungi, bacteria, actinomycetes and protozoa. In industry, these enzymes have found novel applications in the production of fermentable sugars and ethanol, organic acids, detergents and other chemicals. Cellulases provide a key opportunity for achieving tremendous benefits of biomass utilization **(Wen *et al.*, 2005).**Cellulolytic enzymes are synthesized by a number of microorganisms. Fungi and bacteria are the main natural agents of cellulose degradation **(Lederberg, 1992).** The cellulose utilizing population includes aerobic and anaerobic mesophilic bacteria, filamentous fungi, thermophilic and alkaliphilic bacteria, actinomycetes and certain protozoa **(Alexander, 1961).** However, fungi are well known agents of decomposition of organic matter, in general, and of cellulosic substrate in particular **(Lynd *et al.*, 2002).** Cellulose is the major component of plant biomass. Plants produce 4×10^{9}tons of cellulose annually.

It is a polymer of β-1,4 linked glucose units. Its crystalline structure and insoluble nature represents a big challenge for enzymatic hydrolysis. Microorganisms are important in conversion of lignocelluloses wastes into valuable products like biofuels produced by fermentation. Successful bioconversion of cellulosic materials mainly depends on the nature of cellulose, sources of cellulolytic enzyme and optimal conditions for catalytic activity and production of enzymes. For many years, cellulose degrading bacteria have been isolated and characterized for obtaining more effective cellulases from variety of sources such as soil, decayed plant

materials, hot springs, organic matters, feces of ruminants and composts. Researchers keep on working to isolate microorganisms with higher cellulase activity. Cellulose is the structural component of the primary cell wall of green plants, many forms of algae and the oomycetes. Some species of bacteria secrete it to form biofilms. Cellulolysis is the process of breaking down cellulose into smaller polysaccharides called cellodextrins or completely into glucose units, this is a hydrolysis reaction. Biotechnology of cellulases and hemicellulases began in early 1980s, first in animal feed followed by food applications **(Chesson, 1987; Thomke *et al*., 1980; Voragen, 1992; Voragen *et al*., 1980, 1986).** The major industrial applications of cellulases are in textile industry for 'bio-polishing' of fabrics and producing stonewashed look of denims, as well as in household laundry detergents for improving fabric softness and brightness. Besides, they are used in animal feeds for improving the nutritional quality and digestibility, in processing of fruit juices, in baking etc. Utilisation in de-inking of paper is yet another emerging application **(Tolan and Foody, 1999).** The cellulases that are used so far for the above-mentioned industrial applications are those from fungal sources **(Tolan and Foody, 1999).**

II. MATERIAL AND METHODS

Soil samples were collected from two different locations i.e. Paper Industry Soil Sample (PISS) taken from HRA Mill Village Tibhi Indora Kangra H.P. and Bami Soil Sample (BSS) collected from Mandi H.P. from a depth of 1-15 inches from the top and sieved through a 2 mm sieve constituted the soil sample. The samples were dispensed into bags and were brought to the laboratory. Fungal isolates were isolated by serial dilution method. 1 g of soil was transferred to 10 ml of distilled water in test tubes. Dilutions were made up to 10-6 and 0.1 ml of soil suspension was spread on to the sterilized Sabraud dextrose agar media (SDA) which contain 1% CMC. pH of the medium was adjusted to 7. After autoclaving at 121 °C and 15 lbs pressure, 20 ml of sterile medium were transferred to sterile Petri plates and allowed for solidification. After solidification of the medium 0.1 ml of soil suspension was spread with the help of spreader and incubated at 28 °C for 7 days. The fungal cultures grown on the medium were subcultured for repeated times. Pure culture was transferred on to the Sabraud dextrose agar slants and maintained at 4 °C for further studies.

Morphological Identification of isolates: Fungal isolates were identified on the basis of morphological characteristics. For routine work of fungal identification the commonly used stain is called mounting fluid i.e. lactophenol plus cotton blue.

Screening of cellulolytic fungi: The isolated fungal cultures were screened for their ability to produce cellulases complex following the method of Teather & Wood (1982). Czapek-Dox medium used in this method contained (g/l): sucrose – 30, NaNO3 – 2, K2HPO4 - 1, MgSO4 – 0.05, Kcl – 0.5, FeSO4 – 0.01, carboxy-methyl cellulose – 1%, Agar agar - 20. pH of the medium was adjusted to 5. After autoclaving at 121°C and 15 lbs. pressure, the medium was poured into Petri plates and allowed to solidify. Cavities of 6 mm size were made in the solidified medium and inoculated with 0.1 ml of fungal suspension prepared from 7 day old slants. The plates were incubated at room temperature (28 ± 2 °C) for three days to allow fungal growth, then again incubated for 18 h at 50 °C which is the optimum temperature for cellulases activity. After incubation, 10 ml of 1% Congo - Red staining solution was added to the plates that were shaken at 50 rev/ min for 15 min. The Congo - Red staining solution was then discarded, 10 ml of 1 N NaOH was added to the plates and shaken again at 50 rev/min for 15 minutes. Finally 1 N NaOH was also discarded and the staining of the plates was analyzed by noticing the formation of clear or yellowish zones around the fungal spore inoculated wells.

Production of Cellulases from Fungal Isolates.

The isolated fungal cultures were used to know their potential for cellulase production and activities. A volume of 100 ml of Czapek-Dox broth medium amended with 1% cellulose was distributed into separate 250 ml conical flasks. The pH of the medium was adjusted to 5. After autoclaving at 121°C and 15 lb. pressure, the fungal spore suspensions were inoculated into the conical flasks. The flasks were incubated at 32 °C on a rotary shaker at 120 rpm for 3 days. After 3 days, culture filtrate was collected, centrifuged at 6000 rpm for 15 min and supernatant was used to the estimation extracellular protein content . Not only this, it also used crude cellulase source.

Extracellular protein content: - After 7 days of incubation, the contents of the flasks were aseptically passed through Whatman No.1 filter paper to separate mycelial mat from culture filtrates. An aliquot of this culture filtrates was used for estimation of extra cellular protein content according to the method of Lowry *et al.* (1951). Bovine serum albumin was used as protein standard. Suitable aliquots of filtrates were mixed with 5 ml of alkaline solution. After 30 min, 0.5 ml of appropriately diluted Folin-Ciocalteau reagent was added. The color developed was read at 550 nm by using the spectrophotometer.Activity of Cellulase in the culture filtrates was determined and quantified by carboxy-methyl cellulase method (Ghosh 1987). The reaction mixture with 1.0 ml of 1% carboxymethyl cellulose in 0.2 M acetate buffer (pH 5.0) was pre-incubated at 50°C in a water bath for

20 minutes. An aliquot of 0.5 ml of culture filtrate with appropriate dilution was added to the reaction mixture and incubated at 50 °C in water bath for one h. Appropriate control without enzyme was simultaneously run. The reducing sugar produced in the reaction mixture was determined by dinitro- salicylic acid (DNS) method (Miller 1959). 3, 5-dinitro-salicylic acid reagent was added to aliquots of the reaction mixture and the color developed was read at wavelength 540 nm.

III. RESULTS

The soil sample contained considerable population of fungi Total 21 fungal isolates were isolated from the two different soil samples i.e. 12 isolates from Paper Industry Soil Sample and 9 isolates from Bami Soil Sample (fig:1, table-1). The Fungi grown on the selective media supported the growth of the fungi by using cellulose as the carbon source (Khalid *et al.*, 2006). The isolated strains were carefully identified by morphological Characteristics include color of the colony and growth pattern studies. Some of the microscopic characteristics examined under the microscope include spore formation and color. Efficient cellulase producing fungi isolates were finally selected based on the zone of the clearing around the fungi on carboxyl methyl cellulase agar (CMC agar) plates (Bakare *et al.*, 2005; Immanuel *et al.*, 2006). The appearance of the clear zone around the colony when the Congo red solution was added (Wood and Bhat, 1988) was strong evidence that the fungi produced cellulase in order to degrade cellulose. Out of 21 fungal isolates, only 4 isolate produced zones of hydrolysis in CMC agar plates within 3 days and results were represented (fig.2, table.2).

Sr. No.	Nature of Sample	No. of fungal populations
1.	Paper Industrial Soil Sample(PISS)	12
2.	Bami Soil Sample(BSS)	9

Table-1: showing the fungal isolates on to the medium.

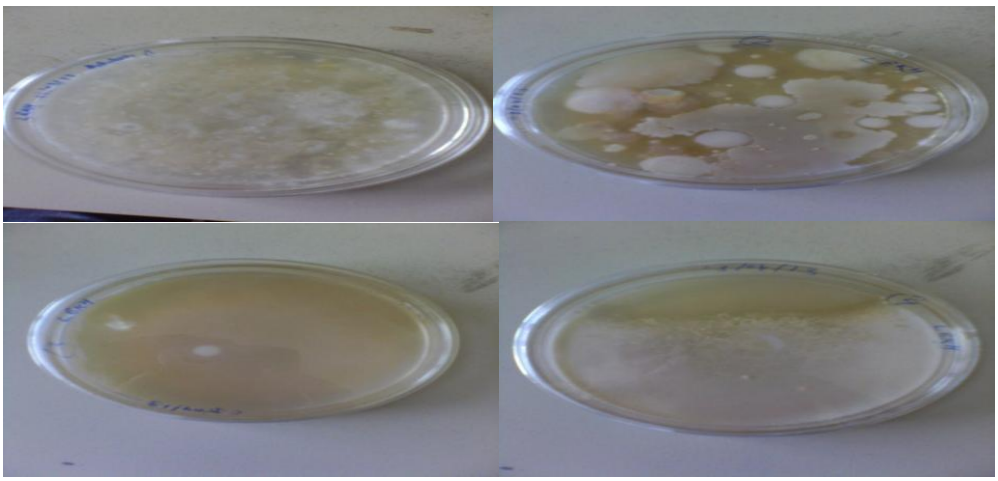

Fig.1: Fungal isolates.

The fungi were isolated and screened from samples collected from paper industry and bami sample for cellulase production by Congo red assay. Isolated fungal strains were subjected to screening. The diameter of clear zone (fig: 2, table: 2)**.** Isolate **PISS-3** showed highest zone of clearance as 7 mm of colony diameter.

Sr. No.	Culture Strains	Zone of clearance(mm)
1.	PISS-1	2
2.	PISS-2	5
3.	PISS-3	7
4.	BSS-1	3

Table-2: zone of clearance around the fungal culture on screening.

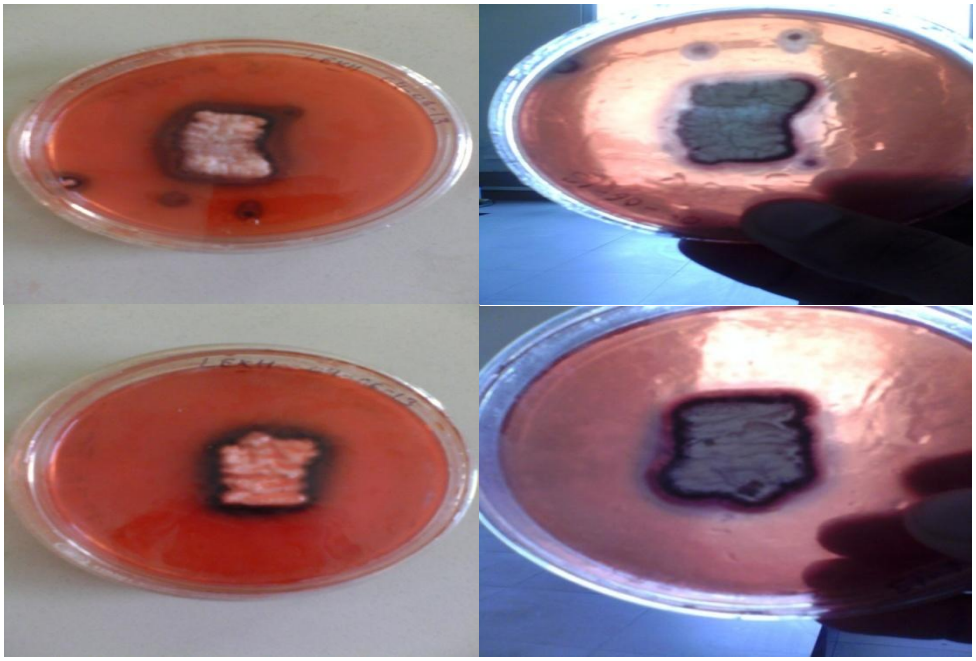

Fig.2: Fungal culture showing cellulase activity.

Protein Estimation.

Protein content of the isolated fungi was estimated by using the Folin Lowry's method and optical density of the strain was compared with the BSA standard curve (fig:3) to calculate the amount of protein (mg/ml) present in the supernatant used in cellulase assay.

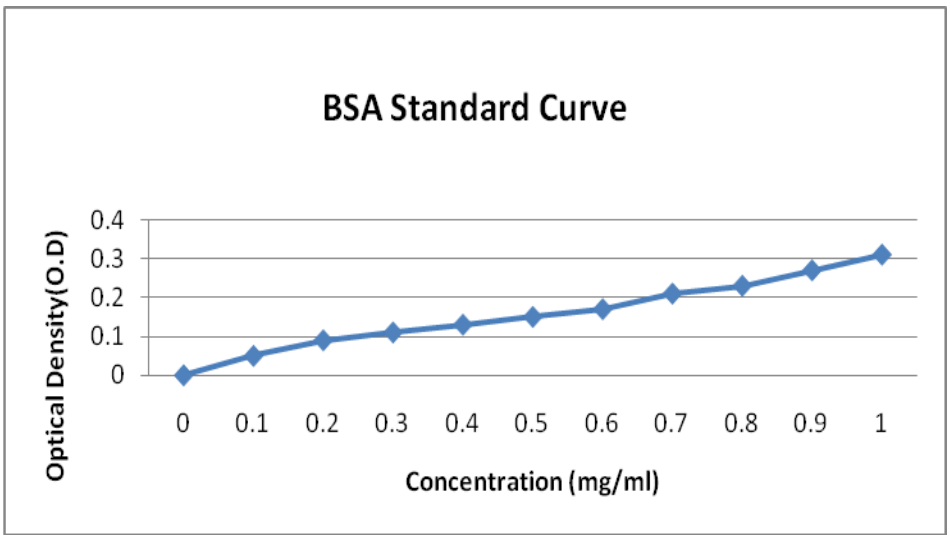

Fig.3: showing the BSA standard curve.

Table-3: Table showing the protein content present in PISS-3 fungal isolate by using BSA standard curve.

Sr. No.	**Fungal isolates**	**Optical Density**	**Protein(mg/ml)**
1.	PISS-3	0.050	0.1

The amount of protein present in the sample was found to be **0.1mg/ml.**

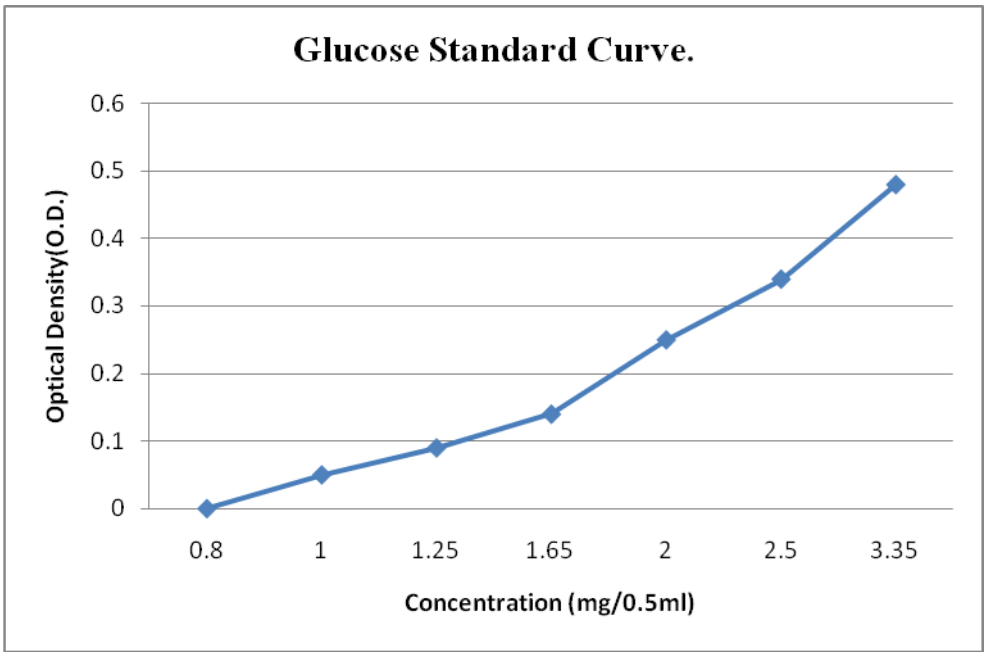

Fig.4: showing Glucose standard curve.

Table-4: Table showing the release of glucose mg/0.5ml in the fungal isolate on comparison with the Glucose standard curve.

Sr. No.	Fungal isolates	Optical Density	glucose(mg/0.5ml)
1.	PISS-3	0.055	1.0

The amount of glucose released by the sample was found to be **1.0mg/0.5ml.**

Cellulase activity.

Cellulase activity was assayed using dinitrosalisic acid (DNS) reagent by estimation of reducing sugars released from the sample. Sugars liberated were determined by measuring absorbance at 540 nm. Cellulase production was estimated by using glucose standard curve. The activities of enzyme cellulases were determined by using the following formula:

Enzyme activity = amount of glucose liberated/mg protein/30minutes (Ghose T.K. *et al* 1987).
Enzyme activity of the PISS-3 strain is found to be **20mg glucose librated/mg protein/30min** at 32°C.

IV. DISCUSSION

As cellulose can be regarded as the most abundant and biologically renewable resource for bioconversion, its exploitation can be maximized on hydrolysis to glucose and other soluble sugars which can be further fermented into ethanol for use as liquid fuel. Cellulases are the enzymes responsible for the cleavage of the β–1, 4–glycosidic linkages in cellulose. They are members of the glycoside hydrolase families of enzymes that hydrolyze oligosaccharides and/or polysaccharides. The major goals for future Cellulase research would be: (1) reduction in the cost of cellulase production and (2) improving the power of cellulases to make them more effective, so that less enzyme is needed **(Akpan I. *et al.* 1999).** Degradation of cellulosic materials is a complex process and requires participation of microbial cellulolytic enzymes. Habitats where these substrates are present are the best sources for isolation of cellulolytic microorganisms. About one fifth of fresh water and soil samples yield cellulose degrading fungi after enrichment but some samples did not bear such kind of fungi. This is due to existence of microenvironments where different growth conditions for cellulose degrading microorganisms are present. Several microorganisms have been discovered for decades which have capacity to convert cellulose into simple sugars but the need for newly isolated cellulose degrading microorganism still continues.

In the present study, soil samples were collected from different places of Himachal Pradesh. Two different types of soil samples taken from the different locations of Himachal Pradesh i.e. Paper Industry Soil Sample taken from HRA Mill Village-Tibhi, Indora Kangra H.P. and Bami Soil Sample of soil from Mandi H.P. Current study focuses on to the fungal isolates. The soil sample contained considerable population of the cellulase producing fungi. The Fungi grown on the selective media supported the growth of the fungi by using cellulose as the carbon source, for example Sabraud Dextrose Agar (SDA) with 1% of CMC. Total 21 fungal cultures were isolated from soil samples. The isolated fungi was purified by repeated sub-culturing on the Sabraud Dextrose Agar medium at regular intervals and incubating at 29°C. Morphological identification of the fungal isolates were performed by fungal staining of the cultures. Efficient cellulase producing fungi isolates were finally selected based on the zone of the clearance around the fungi. The appearance of the clear zone around the colony when the Congo red solution was added was a strong evidence that the fungi produced cellulase inorder to degrade cellulose. Among all these 21 fungal isolates only four fungal isolates (PISS-1, PISS-2, PISS-3, BSS-1) were showing the cellulase activity on screening with congo red. Out these four fungal isolate PISS-3 have greater cellulase producing capability as it shows maximum zone i.e 7mm of clear zone around the fungal culture.

The cellulase activity of fungal isolate PISS-3 was analyzed by evaluating the cellulase liberated in CMC solution through DNS method. The cellulase activity of fungal isolate was mathematically calculated by using the data of protein content (mg/ml) present in the supernatant of isolates which was estimated by Folin Lowrey's method. BSA Standard curve was used for determining the amount of protein present in the culture sample. The O.D. of the sample was compared with the standard curve of the BSA protein. The amount of protein present in the sample PISS-3 strain was estimated to be 0.1mg/ml. Cellulase activity of the enzyme was measured by cellulase assay. Cellulase Assay was done by DNS method (3, 5-dinitrosalicylic acid) and the activity of the enzyme was expressed in mg/ml/min. It was calculated by the following formula:Enzyme activity = amount of glucose liberated/mg protein/30minutes.In the previous studies made on cellulase by **Lone M.A. *et al.* (2012),** the cellulase activity of *A. niger mtc872* was 59.25mg glucose liberated/mg protein/30minutes. But in our work the enzyme activity of the PISS-3 isolate was found to be 20mg glucose librated/mg protein/30min at 32°C. Future work of the present work includes increases the cellulase production by modifying the substrate and production conduction, purification of enzyme in order to gain higher specific activity of cellulase by the help of sophisticated purification procedures including Salt precipitation, Dialysis & Ion Exchange chromatography.

V. CONCLUSION

Cellulases are one of the most widely used enzymes required for the preparation of fermented foods. Apart from food and starch industries, in which demand for them is increasing continuously, they are also used in various other industries such as paper, pulp and textile etc. with increase in its application spectrum, the demand is for the enzyme with specificity. Present research is focused on to the isolation characterization of fungal isolates from the soil sample. In the present study, soil samples were collected from the different locations of Himachal Pradesh, India. Two different type of soil samples taken from the different locations of Himachal Pradesh i.e Paper Industry Soil Sample taken from HRA Mill Village Tibhi Indora Kangra H.P. and Bami Soil Sample from Mandi H.P. There were 21 fungal isolates were isolated from these two soil samples. Out of these 21 isolates of fungus only 3 isolates were showing the positive result for cellulase activity. Testing of cellulase isolates for cellulase activity was performed by performing Congo red staining of the isolates of the fungus. Morphological identification of the fungal isolates were done by fungal staining. The fungal isolate showing greater zone of clearance on screening was used further for production process. Production of the Cellulase by inoculating the isolate on to the Czepak Dox Broth for 72 hours. BSA standard curve was used for the estimation of the protein in the culture sample. Different dilutions of the BSA were used for the preparation of BSA standard curve. By comparing the O.D. of the culture sample with the standard graph of the BSA the concentration of Cellulase mg per ml of the sample was calculated. The amount of protein present in the sample PISS-3 strain was estimated to be 0.1mg/ml. The standard curve for glucose was prepared by using O.D. of the different dilution of glucose with citrate buffer. The amount of release of glucose mg per ml of the sample was calculated by DNS method. The amount of glucose present in the sample was found to be 1mg/ml. Enzyme activity of the PISS-3 strain was found to be 20mg glucose librated/mg protein/30min at 32°C. At last we conclude the PISS-3 fungal isolate is important for industrial use.

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REFERENCES

[1] Akpan I, Bankole M.O., Adesemowo A.M. and Latunde-Dada G.O., *Trop. Sci.*, 1999, 39, 77-79.

[2] Bakare. M.K., Adewale I.O., Ajayi A., and Shonukan O.O., purification and characterization of a thermostable endoglucanase from *Aspergillus niger*, African Journal of Biotechnology, 2005, 9, pp. 898.

[3] Bhat, M.K., Cellulases and related enzymes in biotechnology. In Biotechnology Advances vol.18,2000 pp. 355-383.

[4] Bhat, M.K. and Bhat, S., Cellulose degrading enzymes and their potential industrial applications. Biotechnol Adv., 1997, 15: 583–620.

[5] Chesson A., Supplementary enzymes to improve the utilization of pigs and poultry diets. In: Haresign W Cole DJA editors Recent advances in animal nutrition London: Butterworths, 1987, 71–89.

[6] Ghose TK., Pure Apple. Chem., 1987, 59, 257-268.

[7] Immanuel, G., Dhanusa, Prema, P., and Palavesam, A., "Effect of different growth parameters on endoglucanase enzyme activity by bacteria isolated from coir retting effluents of estuarine environment," *International Journal Environment Science Technology*.2006, 3(1), 25-34.

[8] Khalid, Yang, W.J., Kishwar, N., Rajput, Z.I. & Arijo, A.G., Study of cellulolytic soil fungi and two nova species and new medium. *J. Zhejiang Univ. Sci. B*, 2006, 7(6): 459-466.

[9] Klemm D, Schmauder H.P., Heinze T., Biopolymers, vol VI, edited by E.Vandamme, S De Beats and A Steinb chel (Wiley-VCH, Weinheim), 2002, pp. 290 – 292.

[10] Lederberg J., Cellulases. In: Encyclopaedia of Microbiology (Vol. 1; A-C). Academic Press, Inc. 1992.

[11] Lynd LR, Weimer PJ, Van Zyl WH & Pretorious IS, Microbial cellulose utilization: Fundamentals and Biotechnology, Microbiology. Microbial Mol Bio Rev. 2002, 66: 506-577.

[12] Lone MA, Wani MR, Bhat NA, and Resmi MA., Evaluation of cellulase enzyme secreted by some common and stirring Rhizosphere fungi of *Juglans Regia* L. by DNS method. In Journal of Enzyme Research. 2012,Vol 3, issue 1, 18-22.

[13] Nowak, J., Florek, M., Kwiatek, W., Lekki, J., Chevallier, P. and Zieba E, Composite structure of wood cells in petrified wood. Mater Sci Eng, 2005, C25:119–30.

[14] Subramniyan, S. and Prema, P., Cellulase-free xylanases from Bacillus and other microorganisms. FEMS Microbiol. Lett. 2000, 183: 1-7.

[15] Thomke S., Rundgreen M., Hesselman K. The effect of feeding high-viscosity barley to pigs. In: Proceedings of the 31st meeting of the European Association of Animal Production, Commission on Animal Production, Munich, Germany. 1980, 5.

[16] Tolan, J.S and Foody, B., Cellulase from submerged fermentation. In: Advances in Biochemical Engineering: Biotechnology Vol 65. Recent Progress in Bioconversion of Lignocellulosics. (Tsao, G.T, Ed.), SpringerVerlag, Berlin, 1999 pp. 41–67.

[17] Teather, R. M., and P. J. Wood. Use of Congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. Appl. Environ. Microbiol.1982, **43:**777-780.

[18] Voragen AGJ, Heutink R, Pilinik W, Solubilization of apple cell walls with polysaccharide degrading enzymes. J Appl Biochem. 1980, 2:452–68.

[19] Voragen AGJ, Wolters H, Verdonschot-Kroef T, Rombouts FM, Pilnik W, Effect of juice-releasing enzymes on juice quality. In: International Fruit Juice Symposium, The Hague (NL), May 1986. Zurich: Juris Druck Verlag, 1986, 453–62.

[20] Wen , Z., Liao, W. and Chen, S., Production of cellulase by Trichoderma reesei from dairy manure. Bioresour. Technol. 2005, 96: 491- 499.

[21] Wood, T.M. and Bhat, K.M., Methods of measuring cellulase activities. *Methods in Ezymology*.1988, 160: 87-117.