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ISOLATION, SCREENING AND CHARACTERIZATION OF CELLULOLYTIC BACTERIA FROM COTTON GINNING INDUSTRY EFFLUENT POLLUTED SOIL SAMPLES

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ABSTRACT

Release of industrial liquid waste on to the cultivated lands causes a significant change in biogeochemical cycling and primal matter processing. The present study, cotton ginning industry liquid waste discharged soil (Test) and unexploded soil (Control) were taken from the neighbouring area of the cotton ginning mill, the test and control soil samples were used for the analysis of physico-chemical and biological properties of the soil sample. Further, the test soil samples were used as a source of inoculum for cellulolytic bacteria by using serial dilution and agar plating method. For the isolation and screening of cellulolytic bacteria, selective media that is CMC agar was used. All the isolates were analyzed morphological, physiological, and biochemical characterization, based on analysis, the isolates resembled to *Bacillus subtilis* and *Bacillus cereus.*

KEYWORDS: Polluted soil samples, physico-chemical and biological properties, CMC agar, Cellulolytic Bacteria.

INTRODUCTION

Soil is one of the good natural resources for the growth and development of various types of bacteria. Cellulose is the highly available biomass on the earth surface, produced due to the decaying of plant parts inside the soil. Cellulose is a vastly available carbohydrate in nature, present in the primary structural element in plants. Some cellulolytic bacteria grow abundantly in this soil. Cellulose is degraded by an enzyme i.e., Cellulase, produced from various

microorganisms such as bacteria, fungi, and actinomycetes. Cellulolytic bacteria having a high-rate of cellulase enzyme production comparative to fungi. Cellulase is extensively used in various applications in industries i.e., textile, leather, paper, detergents, and food.[1]The demand for cellulase producing bacteria identification of high stability at high pH and temperature. Cellulase is widely used in fermentation biology and pharmaceutical in the regeneration of biofuels from bio-waste. In the present study, cotton ginning industry polluted soil samples used as a source of inoculum for the isolation of cellulolytic bacteria.

MATERIAL AND METHODS

Soil sample collection

Soil samples were taken from cotton ginning mill effluents polluted area, served as a test soil sample. Soil sample lacking of liquid discharges served as control was taken from the adjacent site of cotton ginning mill. Both soil samples test and control were used for the determination of Physico-chemical and biological activities. Prior to testing, the soil was prepared for analysis as per *APHA 2012 guidelines*.[2]

Physico-chemical and biological properties of soil samples

Physico-chemical properties of soil samples: Both soil samples were analyzedPhysico-chemical and biological properties by using a standard procedure. Soil p^H was determined using an electrode and a 1:1 soil/water mixture[3], electrical conductivity was estimated by the addition of 100ml of water to 1 gr of the soil sample in the Elico conductivity meter. The method described by *Johnson and Ulrich1960*[4], was employed for estimation of 60% water holding capacity, organic carbon & total nitrogen content using the Walkley-black method[5] and Microkjeldhal method[6], soil phosphorous, and potassium[7], respectively.

Biological parameters: The serial dilution and agar plate method were used to enumerate microflora such as bacterial, fungal and actinomycetes population of both the test and control soil samples. One gram of each soil sample was diluted up to 10^{-10}, diluted suspensions of 0.1 ml samples were plated & spread with a sterile spreader on the Nutrient Agar medium (p^H7.4), Martin-Rose Bengal Agar medium (p^H 5.5), and Glycerol Asparagine Agar medium (p^H 5.0), for growth of bacteria, fungi, and actinomycetes respectively. Nutrient Agar plates were incubated at 37°C for 24 hours, Whereas Martin-Rose Bengal Agar and Glycerol Asparagine Agar at 28°C for 5-7 days. After the incubation period, colonies appeared on the agar surface were counted by colony counter.

Isolation and screening of cellulolytic microbes

Soil samples were taken from the polluted area of the cotton ginning mill effluents. These soil samples were used as a source of inoculum. Traditional serial dilution & agar plating method was used for cellulolytic bacteria isolation. Media contains CMC 1.88 g, sodium citrate 0.5 g, K_2HPO_4 7.0 g, KH_2PO_4 2.0 g,$(NH_4)_2SO_4$ 1.0 g, $MgS0_4.7H_20$ 0.1 g, Agar 15 g, Congo red 0.20 g, pH 7.0, Distilled water 1 lit.[8] The plates were incubated at 37°C for 2-3 days and examined across the colony for a clear region. The plates were filled with an aqueous solution of 10% Gram's iodine for 15 min to visualize the hydrolysis zone created by positive cellulase strains; the plates were extracted using 1M NaCl solution.[9] For identification and development of cellulase, the bacterial colonies having a clear zone were chosen.

Identification of cellulolytic bacteria

For the identification of cultural characteristics of the strain of our interest, morphological and biochemical tests were conducted and identified based on characters as stated in Bergey's manual of systematic bacteriology.[10] Indole test, Methyl red test, Voges Proskauer test, Citrate utilization test, Catalase test, Oxidase test, Gelatine test, Motility test, Amylase test, Nitrate reduction test, Carbohydrate fermentation test by standard methods were investigated. The different media were stored in sterile distilled water accordingly, the p^H was maintained.

RESULTS AND DISCUSSION

Physico-chemical and biological properties of soil sample

Physico-chemical properties of soil sample: The analytical results of both test and control soil samples were represented in Table 1. In contrast to, test soil samples underwent changes in all the parameters of Physico-chemical properties compared to control soil sample. The pH of the test soil sample was increased to 7.2 to 8.4, upon the release of effluents. Soil texture in terms of percentages of sand, silt and clay were 20, 50, and 30 in the test, 48, 30, and 22 in control soils, respectively. Water holding capacity, electrical conductivity, organic matter, total nitrogen, potassium and phosphorous were higher in the test sample over the control samples and were 0.32 ml/g, 1.94 µMhos/cm 7.2 %, 0.36g/kg, 322 kg/ha and 478 kg/ha of the test against 0.18 ml/g, 0.32 µMhos/cm, 3.8%, 0.18 g/kg, 164 kg/ha, 286 kg/ha of the control, respectively.

Similarly, discharge of effluent from various industries like, sugar[11], dairy factory[12], and petrochemical industry[13] influenced the physico-chemical properties of soil; this is due to

organic waste that may contribute to maintaining or increasing the organic matter and nutrient content in the soil.[14]

Table 1: Physico-chemical properties of soil as affected by cotton ginning industry effluents.

Properties	Control[a]	Test[b]
Colour	Grey	Thick black
Odour	Normal	Unpleasant/ bad
p^H	7.2	8.4
Electrical Conductivity (µMhos/cm)	0.32	1.94
Soil Texture Sand (%) Silt (%) Clay (%)	 48 30 22	 20 50 30
Water Holding Capacity(ml/g)	0.18	0.32
Organic Matter (%)	3.8	7.2
Total Nitrogen (g/kg)	0.18	0.36
Available Potassium(k_20) [kg/ha]	164	322
Available Phosphorous (P_20_5) [kg/hg]	286	478

Control[a]: soil without cotton ginning industry effluents

Test[b]: soil polluted with cotton ginning industry effluents

Biological properties of soil sample: Microorganisms play a vital role in nutrient cycling and organic matter processing. The micro flora such as bacteria, fungi, and actinomycetes of both soil samples were enumerated and listed in Table-2, two to three-fold higher bacterial, fungal, and actinomycetes population were observed in the test soil over the control.

Table 2: Biological properties of soil as affected by cotton ginning industry effluents.

Microorganisms	Control[a]	Test[b]
Bacteria	53×10^6	$120\times10^{6\ c}$
Fungi	24×10^4	42×10^4
Actinomycetes	15×10^4	35×10^4

Control[a]: soil without cotton ginning industry effluents

Test[b]: soil polluted with cotton ginning industry effluents

[C]: microbial population in terms of colony-forming unitsg^{-1} of soil.

Isolation and screening of cellulolytic microbes

Cellulose is the principal plant building blocks and as a significant fraction of organic carbon in the soil. Microorganisms that live in the soil are responsible for the environmental recycling of this organic carbon.[15] Cellulosic material degradation is a complex process and involves

microbial enzymes. Habitats, where substrates occur, are the best sources of cellulolytic microorganism isolation.[16] Several microorganisms have been discovered for decades that can convert cellulose into simple sugars, but the need for newly isolated microorganisms that degrade cellulose persists.[17][18] The bacteria-producing cellulase was isolated from different samples using serial dilution and agar plating on CMC agar. CMC agar is a selective medium and supports the growth of cellulolytic organisms because organisms producing cellulase can only use cellulose as the source of carbon. The screening of the cellulolytic bacterial isolates was performed on the CMC Agar medium based on the clearing zone around the colony. After the addition of Gram's iodine, the presence of a clear zone around the colony was strong evidence that the bacteria developed cellulase to degrade cellulose. To prevent fungal growth in CMC agar media antifungal agent was added (Fluconazole).

Identification of cellulolytic bacteria

Two strains of cellulolytic bacteria were isolated from various polluted soil samples of the cotton industry. All bacterial isolates were gram-positive, Cocco and rod-shaped, motile, and endo-spore forming bacteria shown in Table-3. Positive reactions for amylase, urease, lipase, oxidase, gelatinase, amylase, indole, methyl red test, and Negative to Voges Proskauer, citrate utilization tests were shown in Table-4. Two isolates ferment the glucose and starch but not fructose and lactose showed in Table -4.

Based on morphological and biochemical characterization the two isolates resembled to *Bacillus subtilis* and *Bacillus cereus.*

Table 3: Morphological characteristic of cellulolytic bacteria.

S.No	Character & Tested	Isolate-I	Isolate-II
1.	Colony morphology	Whitish round, non-slime	Round with creamy colored, slime
2.	Cell morphology	Cocco bacilli	Rod
3.	Gram stain	Positive	Positive
4.	KOH test	Negative	Negative
5.	Endospore stain	Positive	Positive
6.	Sporulation	Positive	Positive
7.	Motility test	Positive	Positive

Table 4: Biochemical characteristic of cellulolytic bacteria.

S.No	Name of test	Isolate-I	Isolate-II
1.	Amylase test	Positive	Positive
2.	Gelatine test	Positive	Positive
3.	Catalase test	Positive	Positive
4.	Urease test	Positive	Positive
5.	Nitrate reduction test	Positive	Positive
6.	Casein hydrolysis	Positive	Positive
7.	Lipase test	Positive	Negative
8.	Indole production test	Positive	Positive
9.	Methyl red test	Positive	Positive
10.	Voges-Proskauer test	Negative	Negative
11.	Simmons citrate test	Negative	Negative
12.	Carbohydrate fermentation test		
	(a) Starch	Positive	Positive
	(b) Glucose	Positive	Positive
	(c) Fructose	Negative	Negative
	(d) Lactose	Negative	Negative

CONCLUSION

Isolation, screening, and identification methods were quick and efficient for the isolation of several good cellulases producing bacteria from a wide variety of samples. In the field of bio refining, it is important to find naturally occurring cellulase producing bacteria from the environment to help overcome costly hurdles in the bio refining process. All isolates, an essential part of future research in developing good cellulases or effective cellulase-producing systems such as microbial consortia used for industry. Isolation, screening, and identification are a good sign for identifying beneficial enzymes of this kind.

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