



## Isolation and Screening of Cellulolytic Microflora from Soil Polluted with Cotton

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### Abstract:

Cotton the white gold of our country has its high yield in the regions of AP. The Industrial wastes of these cotton industries are of high solid powder content and are rich in cellulose, which is often dumped as such into the soil. This soil containing cotton wastes is a good source for a variety of cellulolytic bacteria. The current study uses this cellulose containing soil as a source to isolate the cellulolytic bacteria. Initial isolation was performed using CMC congo red agar medium. Out of the 15 colonies observed one of the most prominent colonies was collected and purified by streak plate method to give single organism isolates. Degradation zones were identified on the CMC medium and were denoted as R-1. According to morphological and biochemical tests the organism is identified as *Bacillus* *sp.*, which was grown on different cellulostic media viz., CMC, Cellulose powder and Cellobiose for different incubation times. Effect of incubation time for the above mentioned substrates on cellulolytic activity was studied by comparing diameters of lysis zones, which are considered as a measure of cellulolytic efficiency. Maximum cellulolytic activity was observed in cellobiose substrate at 0.2% conc., on 12<sup>th</sup> day of incubation.

### Introduction:

Cellulose is a major carbohydrate polymer that consists of a long unbranched chain of glucose units. Cellulose is the most abundant component of plant biomass is found in nature exclusively in plant cell walls, although it is produced by some animals (e.g. tunicates) and a few bacteria (Lynd et al., 2002). In the most cases, cellulose fibrils present in plant cell walls are embedded in a matrix of hemicelluloses and lignin.

Cotton waste is a good source of cellulose and can be utilized for cellulosic ethanol production. "cotton waste" includes the droppings, strippings, fly and other waste products of a cotton mill or of a cotton ginning factory or cotton pressing factory but does not include yarn waste. The cellulolytic organisms

whose major source of food is cellulose would invade on the cellulose and persist in the waste.

Cellulolytic microorganisms are abundant in nature and play important role in the carbon cycle by recycling CO<sub>2</sub> fixed by photosynthesis. It is likely that they are evolved with the appearance of cellulose in plants. Indeed, it is conceivable that some cellulose genes were actually borrowed from plants, in which they appear to play a role in morphogenesis and developmental processes (e.g., in the ripening of fruits such as avocado).

Cellulose degradation occurs in a variety of ecological niches where plant residues accumulate. Thus, cellulolytic microorganisms include a variety of aerobes and anaerobes, mesophiles, and thermophiles. As a general rule, cellulolytic microorganisms found in natural habitats constitute mixed populations, including several cellulolytic and non cellulolytic species, which interact synergistically. These interactions, lead to efficient cellulose degradation with formation of CO<sub>2</sub> and H<sub>2</sub>O in aerobiosis or CO<sub>2</sub>, CH<sub>4</sub>, and H<sub>2</sub>O in aerobiosis.

The current work involves the use of cotton industrial effluents as a major source for the isolation of those organisms that are capable of the cellulose degradation. Once the organisms are identified, they can be later employed for various commercial purposes.

### Materials and Method:

The experimental design was divided into three stages:

- Determination of physicochemical and biological properties of soil.
- Isolation & Charecterization of cellulolytic bacteria.
- Evaluation of cellulolytic activity on different substrates.

Soil Samples of black cotton soil were collected from different locations where effluents had been discharged. Samples of the same type of soil without effluent discharges were collected from the farm which served as control.

**Isolation of Cellulolytic Bacteria from the collected soil samples:**

The soil samples collected were subjected for serial dilution so as to get the visible isolated colonies on the master plate. The growth is restricted to the cellulolytic group with the use of specific media for the isolation.. The media used for the isolation of the bacteria is **cellulose-Congo red agar medium**. The composition of the medium is:

**Cellulose-Congo red agar medium:**

Carboxy methyl cellulose	1.88g
Sodium citrate	0.5g
K <sub>2</sub> HPO <sub>4</sub>	7.0g
KH <sub>2</sub> PO <sub>4</sub>	2.0g
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	1.0g
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.1g
pH	7.0g
Agar	10g
Congo red	0.20g
Distilled water	1000ml

**Preparation of Pure cultures of Cellulose degrading Bacteria by streak plate technique:**

The CCRA plates were observed for the growth of prominent isolated plates so as to produce the pure plates of the cultures. The secondary plating was done by means of streaking onto the fresh CCRA media plates. The growth of the cultures is also followed by the appearance of the zone of clearances around the culture due to the degradation of the cellulose substrate used in the media. The sub culturing procedure was repeated for seven times so as to obtain the pure culture of a single bacterium.

**Screening for the Cellulolytic activity of the selected cultures:**

The pure cultures obtained were screened so as to test their ability to degrade the cellulose in the medium. In this method the bacterial colonies were grown on cellulosic medium without Congo red for two days at 28±2°C and then the medium was flooded with an aqueous solution of Congo red (1mg/ml) for 15 min. The excess Congo red solution was then poured off, and plates were further flooded with 1M NaCl for 15

min. the cellulose hydrolysis zones were visualized. These plates were further stabilized at least for 2 weeks by flooding with 1M HCl, which changes the dye color red into blue and inhibited further enzyme activity. From this screening technique only those bacterial cultures which were cellulolytic have been selected.

**Maintenance of the selected cultures:**

Bacterial isolates selected by the above method were maintained on nutrient agar plates for routine use and stored at 4° C. Bacterial isolates were transferred into fresh plats after every 15 days interval.

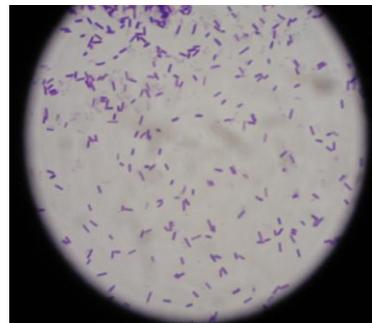
**Physiological characterization based on Gram's test, KOH test and Spore Formation test:**

To identify the physiological characters of the culture these testes have been performed. Gram's test would identify the gram's nature of the culture based on the coloration and decolonization of the bacterial cell and would distinguish the bacteria as gram positive or negative. The Gram's nature is further confirmed by the KOH test were the use of KOH would cause the cell lysis and DNA release in case of gram negative bacteria, but the gram positive cells will remain intact in the effect of KOH. The ability of the bacteria to form spores under unfavorable conditions is detected by Spore Formation test. The use of malachite green dye would help in the detection of the endospores formed within the bacterial cells.

**Biochemical Characterization:**

Biochemical test was conducted to characterize the isolated cellulolytic bacteria based on their reaction towards certain chemical tests and reagent. Principle behind these Biochemical Tests is the enzymatic activities of bacteria that differentiate one from the other. Table 1 shows the list of biochemical tests has been performed to identify the bacteria.

**Figure 1: Showing the Microscopic field view of the selected bacteria**



Gram staining (cocci, shape, R-1) Gram positive (purple color)

**Table 1: showing the Biochemical testes performed along with their purpose**

Biochemical tests	Purpose
<b>Amylase Test</b>	To determine the ability of bacteria to degrade starch by producing amylase.
<b>Gelatin Hydrolysis Test</b>	To determine the ability of bacteria to degrade gelatin by producing gelatinase.
<b>Catalase Test</b>	To identify bacteria producing catalase
<b>Nitrate Reduction Test</b>	To determine the ability of an organism to reduce nitrate (NO <sub>3</sub> ) to nitrite (NO <sub>2</sub> ), or Ammonia or molecule nitrogen
<b>Indole Test</b>	To determine the ability of bacteria to breakdown tryptophan to Indole
<b>Simmon's Citrate Test</b>	To determine the ability of a bacterium to utilize citrate as its only source of carbon
<b>Carbohydrate Fermentation Test</b>	To determine the ability of bacteria to ferment various carbohydrates
<b>Urease Test</b>	To determine the ability of an organism to split Urea, through the production of urease.
<b>Oxidase Test</b>	To determine bacteria producing cytochrome C oxidase
<b>MethylRed Test</b>	To determine the ability of the bacteria to produce organic acids from glucose
<b>Lipase Test</b>	To determine the ability of the bacteria to produce the enzyme lipase
<b>H-L test</b>	To determine weather the bacteria is aerobic or anaerobic
<b>Casein Test</b>	To determine the ability of the bacteria to digest the protein Casein

**Analysis of cellulose degrading activity of bacteria on day by day basis:**

The cellulase enzymatic activity of selected bacteria is analyzed by inoculating the culture on the cellulose agar medium (Hendrick et al., 1995). These plates were incubated at 28±2°C. Observations for growth & degrading zone formation were taken at various time intervals i.e., 3<sup>rd</sup> day, 6<sup>th</sup> day, 9<sup>th</sup> day and 12<sup>th</sup> day. Visual bacterial growth was recorded by flooding the culture plates with congo red solution (1mg/ml) as mentioned earlier to observe the degrading zone formation. The selected isolate was further screened for various cellulosic substrates viz., Carboxymethylcellulose (CMC), Cellobiose and

Cellulose powder at 0.2% w/v conc. on day basis as explained above.

**Results and Discussion:**

The selected bacteria from the pure plates was subjected to physiological and biochemical characterization. The results are summarized as follows:

**Table 2: Showing the results for the Biochemical characterization of the Bacteria**

S.NO	NAME OF THE TEST	R-1(BAC)
1	Amylase production	+
2	Gelatin hydrolysis	+
3	Catalase hydrolysis	+
4	Nitrate reduction test	+
5	Indole test	-
6	Methyl red test	-
7	V-P test	+
8	Simmon's Citrate utilization test	+
9	Carbohydrate fermentation test	-
10	Urease test	-
11	Casein hydrolysis	+
12	H-L test	+
13	Lipase activity	+

**Fig 2: Showing the growth of the bacterium in Congo red agar medium with the cellulolytic zone**



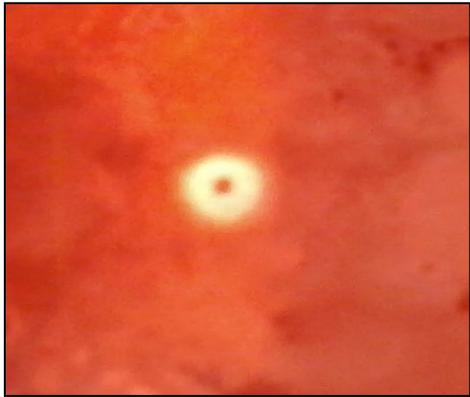
**Cellulolytic activity of isolated strains on various cellulosic substrates:**

The selected cellulolytic bacterium (R-1) is tested for its cellulolytic activity on different substrates viz., carboxymethylcellulose (CMC) cellulose powder and cellobiose.

**Growth and cellulose production on the medium containing carboxymethylcellulose (CMC) substrate:**

In the medium containing 0.2% (w/v) CMC R-1 showed good growth with good cellulolytic zones around the colonies at 12<sup>th</sup> day incubation period.

**Figure 3: Showing the cellulolytic zones around the bacteria**



**Growth and cellulase production in the medium containing Cellobiose substrate:**

In the medium containing 0.2% (w/v) Cellobiose, R-1 showed good growth with good cellulolytic zones around the colonies at 12<sup>th</sup> day incubation period.

**Growth and cellulase production in the medium containing cellulose powder Substrate:**

In the medium containing 0.2% (w/v) Cellulose powder, R-1 showed good growth with good cellulolytic zones around the colonies at 9<sup>th</sup> day incubation period.

**Table 3: Growth and clearing zones on different cellulosic substrates (visual score):**

Bacterial isolate	CMC	Cellobiose	Cellulose powder
R-1	++++	++++	++++

- + Slight growth without clearing zone
- ++ Good growth with less clearing zone.
- +++ Good growth with average clearing zone.
- ++++ Good growth with good clearing zone.

**Table 4: Incubation time (in days) and diameters of lysis zones in cm obtained for the bacteria**

Substrate (0.2%)	3Days	6Days	9Days	12Days
CMC	0.2	0.4	0.4	0.6
CB	0.3	0.4	0.6	0.8
CP	0.2	0.3	0.6	0.6

CP= Cellulose powder, CB= Cellobiose, CMC=Carboxymethylcellulose.

From the above table 4 it can be inferred that Maximum growth and cellulolytic activity (0.8cm) was observed when organism R-1 was grown on Cellobiose Agar medium on 12<sup>th</sup> day of incubation

**Conclusion:**

Cotton industrial effluents soil is rich in cellulosic materials that favored the growth and diversity of cellulolytic micro flora as per properties of tested samples. In the present study cellulolytic bacteria was isolated from cotton industry effluent soil samples. Biochemical tests revealed that the isolated cellulolytic strains belong to Bacillus sps according to Bergy’s manual of systematic bacteriology. As per the colony size and hydrolytic zone diameters the isolates were primarily observed as efficient cellulolytic bacteria. Further study is needed to evaluate the cellulolytic potential by growing in various production media on large scale in addition to biochemical characterization of extracellular cellulase enzymes produced by the isolated bacterial strain.

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