

Cellulolytic Potential of Actinomycetes Isolated from Different Habitats

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Abstract Twenty actinomycete isolates capable of utilizing lignocellulosic biomass were isolated from different samples of agricultural field soil, compost and decaying vegetation. Qualitative screening of all isolates during plate assay method using Congo red showed 17 of them producing a clear zone of hydrolysis around the colonies indicating cellulose hydrolytic potential of the isolates. Quantitative estimation of filter paper (FPase) and carboxymethylcellulase (CMCase) activities exhibited by the positive isolates showed that highest CMCase was produced by NAA2 (0.366 IU/ml), while highest FPase activity of 0.075 FPU/ml was observed in NAA14. All of the isolates showed FPase activities comparable to that shown by *C. fimi* (0.023 FPU/ml) taken as a standard, confirming the cellulose degradation ability of the isolates.

Keywords Actinomycetes, Cellulolytic, CMCase, FPase, Habitats

1. Introduction

The increasing demand for energy and rising cost of rapidly depleting fossil fuels has necessitated the search for renewable energy sources of energy [1]. Lignocellulosic biomass, including industrial, forest, agricultural and domestic wastes, is an attractive alternative to the current fossil fuels based sources of energy [2]. Lignocellulose is primarily composed of cellulose, hemicellulose and lignin [3]. Cellulose is the most abundant biopolymer present on the earth [4]. The end product of cellulose hydrolysis, i.e., glucose, a fermentable sugar, is the most desired chemical for the synthesis of biofuel ethanol [5]. The degradation of cellulose is a complex process, which can be carried out both chemically as well as enzymatically [6]. Enzymatic hydrolysis is, however, an environmentally benign process, mediated through a complex of enzymes known as cellulases. Cellulase enzyme system comprises three classes of enzymes: (1) 1,4- β -endoglucanase, (2) 1,4- β -exoglucanase, and (3)

β -glucosidase (cellobiase), which act in synergism to hydrolyze cellulose completely to the monomeric glucose [7].

Cellulases enzymes are known to be produced by a wide variety of microorganisms including bacteria, fungi and nematodes [8]. Most of the research work on cellulose degrading microbes has been focused chiefly on the fungi. Actinomycetes are relatively less explored. Recently research studies have gained momentum in search of bacteria, including actinomycetes, for the production of potent cellulose degrading enzymes. Actinomycetes are filamentous, gram positive bacteria, found abundantly in soil and other habitats rich in degrading organic matter. They are known to actively take part in natural lignocellulose degradation, as a result of their capability to synthesize extracellular polymer hydrolases such as cellulases, hemicellulases, xylanases, chitinases, pectinases, amylases, peptidases, proteases and keratinases [9]. Reports have shown cellulase production by different actinomycetes belonging to *Cellulomonas* [10], *Streptomyces* [11], *Micromonospora* [12], *Actinopolyspora*, *Actinoplanes*, *Microbiospora*, *Thermomonospora*, *Rhodococcus*, *Nocardia* and *Thermoactinomyces* genera [8]. The present study focuses on isolation of cellulose degrading actinomycetes from different habitats followed with evaluation of their cellulolytic potential.

2. Material and Methods

2.1. Sample Collection

Samples of soil, compost and decaying vegetation, etc. were collected from different regions of Haryana, Punjab & Himachal Pradesh, India. The samples were stored in sterile containers at 4°C till their processing.

2.2. Enrichment and Isolation of Cellulose Degrading Actinomycetes

For the enrichment of cellulose hydrolyzing

actinomycetes, 1gm of the sample was inoculated in a liquid enrichment medium consisting of minimal salt medium (0.2% KH_2PO_4 , 0.2% K_2HPO_4 , 0.2% NH_4NO_3 , 0.05% NaCl , 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001% $\text{Fe}_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$, 0.001% $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (pH 7.0) supplemented with 1% cellulose powder and 1% pretreated lignocellulosic biomass. This was followed with the incubation at 30°C, at 120rpm for 4 days. Before inoculation, the samples were dried at 60°C for 3 days [13].

The actinomycete enriched samples were serially diluted and appropriate dilutions were spread plated on minimal salt medium containing 1% lignocellulosic biomass (pretreated by soaking in 0.5M NaOH solution at room temperature for 2h.). The plates were incubated at 30°C for 7-8 days till appearance of actinomycete colonies.

2.3. Primary Screening of Cellulose Degrading Actinomycetes

The isolates obtained were screened qualitatively by Congo red assay method. The cellulose hydrolysis ability of the colonies grown on the minimal agar medium supplemented with 1% CMC (carboxymethyl cellulose) was tested by flooding the plates with 0.1% Congo red dye solution followed with destaining using 1M NaCl solution for 15-20min. The appearance of a zone of hydrolysis around the colonies indicates synthesis of extracellular cellulases by the microbes. The cellulolytic potential of the positive isolates was evaluated by measuring cellulolytic index (CI), i.e., ratio of diameter of zone of hydrolysis to the diameter of the colony.

2.4. Secondary Screening of Isolates Positive in Primary Screening

The isolates positive in the primary screening were activated in starch casein agar (SCA) medium (1% starch, 0.2% KNO_3 , 0.2% NaCl , 0.2% K_2HPO_4 , 0.005 % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.002 % CaCO_3 , 0.001% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 1.5% agar agar, pH 7.0) till sporulation. For enzyme production, 1 disc (10mm) of activated culture was inoculated in 25ml of modified Mandel's Medium containing 0.1% peptone, 0.14% $(\text{NH}_4)_2\text{SO}_4$, 0.2% KH_2PO_4 , 0.03% urea, 0.03% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 1ml of trace element solution containing 5mg/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; 1.6 mg/L $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$; 1.4 mg/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ and 2mg/L CoCl_2 . The incubation was done at 30°C at 180rpm for 7 days. The enzyme was extracted by centrifugation of the flask contents at 10,000rpm at 4°C for 15-20min followed with filtration

through Whatman filter paper no.1. The filtrate obtained was used as the crude enzyme for further assays.

2.5. Enzyme Assays

The crude enzyme extracts from all isolates were assayed for CMCase and FPase activities using method by Ghose *et al* [14]. The reaction mixture for determining CMCase activity consisted of 0.5ml of appropriately diluted crude enzyme extract and 0.5 ml of substrate (1% CMC prepared in 100mM sodium acetate buffer, pH 4.8). The reaction mixture for FPase assay consisted of 0.5ml of enzyme and 0.5ml of 100mM sodium acetate buffer (pH 4.8) containing 50mg (1x6cm) of Whatman filter paper no.1. The reaction was carried out by incubating contents at 50°C for 10 min during CMCase and for 30 min in FPase assay. The reactions were terminated by addition of 3ml of dinitrosalicylic acid followed with boiling for 5-10 min for the development of colour [15]. Finally the amount of reducing sugars released was determined spectrophotometrically at 540nm, by comparing the results with a glucose standard. The activity was expressed in U/ml. One unit (IU) of enzyme activity is defined as the amount of enzyme required to release 1 μmol of glucose in the reaction mixture per minute under specified conditions.

3. Results and Discussion

3.1. Isolation & Primary Screening of Actinomycete Isolates

Total 20 isolates were obtained from different samples, i.e., soil samples from waste land rich in decaying litter, gardens, wetland; decaying leaves and vegetation; termite infested wood and soil and compost. Qualitative screening by Congo red plate assay method showed 17 of them showing zone of hydrolysis indicating production of extracellular cellulases by them. The diameter of zone of hydrolysis as well as the CI values (ranging from 2.5 to 9.0) observed for various isolates were different. The results were compared with that found in case of the standard isolate, *Cellulomonas fimi* NCIM 5015. Table 1 shows the results of qualitative screening of the isolates along with the CI values estimated for them. Colony morphologies of all positive isolates and zone of hydrolysis shown by them have been depicted in Figure 1 and 2 respectively.

Table 1. Qualitative Screening of Cellulolytic Actinomycetes & their CI values

S. No.	Actinomycete Isolate	Source of Isolate	CI value
Standard	<i>C. fimi</i>	NCIM 5015	1.4
1	NAA 1	Waste Land Soil, Ambala City, Haryana, India	5.0
2	NAA 2	Waste Land Soil, Ambala City, Haryana, India	6.0
3	NAA 3	Waste Land Soil, Ambala City, Haryana, India	6.3
4	NAA 4	Garden Soil, Ambala City, Haryana, India	5.0
5	NAA 5	Garden Soil, Ambala City, Haryana, India	6.6
6	NAA 6	Wetland Soil, Trilokpur, Himachal Pradesh, India	5.0
7	NAA 7	Termite Infested Soil, Kurukshetra, Haryana, India	4.0
8	NAA 8	Termite Infested Wood, Kurukshetra, Haryana, India	2.5
9	NAA 9	Garden Soil, CPPRI, Uttar Pradesh, India	5.0
10	NAA 10	Garden Soil, CPPRI, Uttar Pradesh, India	4.8
11	NAA 11	Decaying Leaves, Trilokpur, Himachal Pradesh, India	4.9
12	NAA 12	Kitchen garden Soil, Kurukshetra, Haryana, India	3.7
13	NAA 13	Compost, Kurukshetra, Haryana, India	6.8
14	NAA 14	Compost, Kurukshetra, Haryana, India	5.7
15	NAA 15	Compost, Kurukshetra, Haryana, India	9.0
16	NAA 16	Decaying Vegetation, Kurukshetra, Haryana, India	3.0
17	NAA 17	Decaying Vegetation, Kurukshetra, Haryana, India	3.2

All results were recorded in triplicates & CI values were calculated as average of the three results

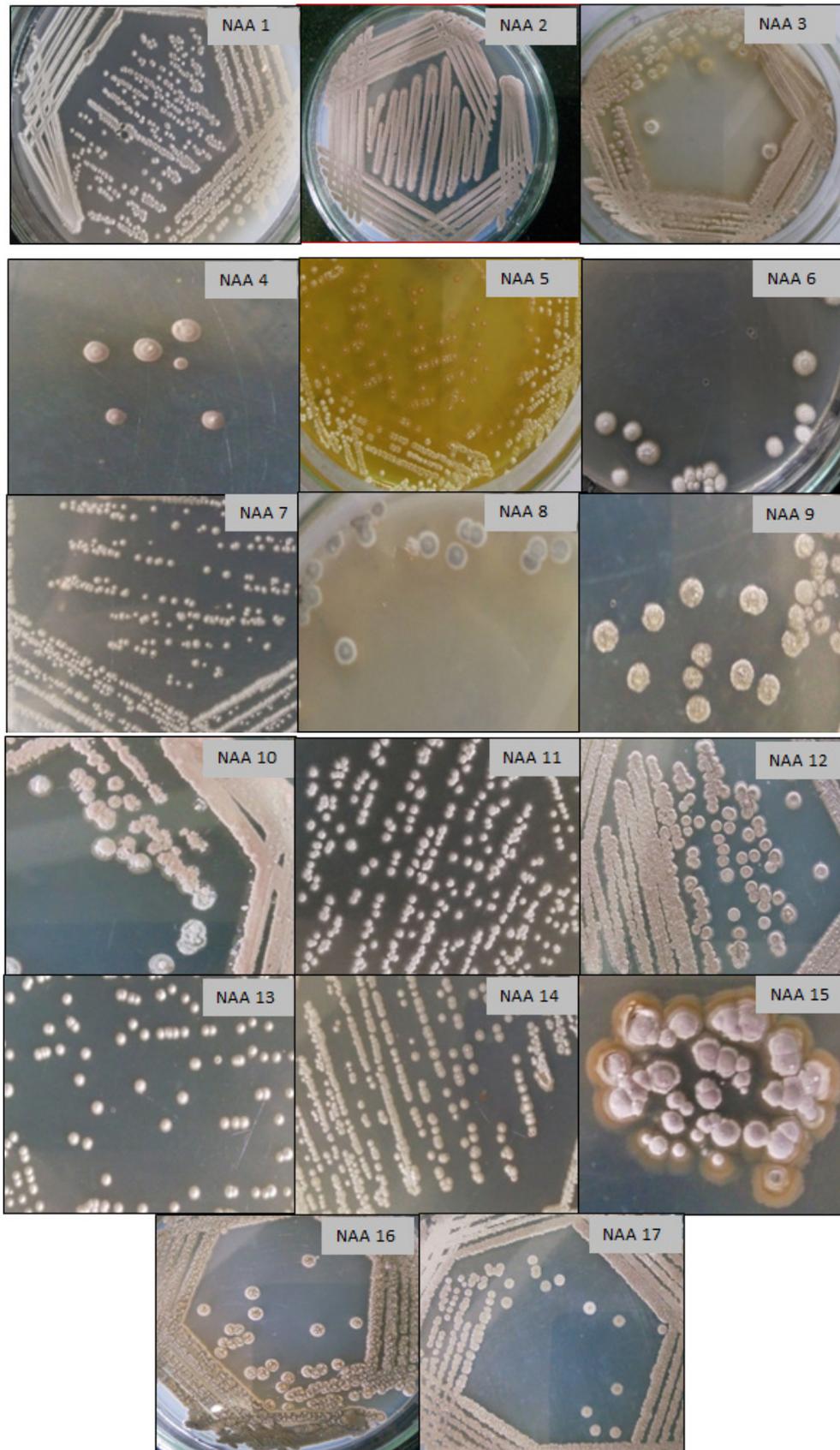


Figure 1. Colony Morphology of Different Actinomycete Isolates (on SCA medium)



Figure 2. Zone of Hydrolysis of Different Actinomycete Isolates (on CMC agar medium)

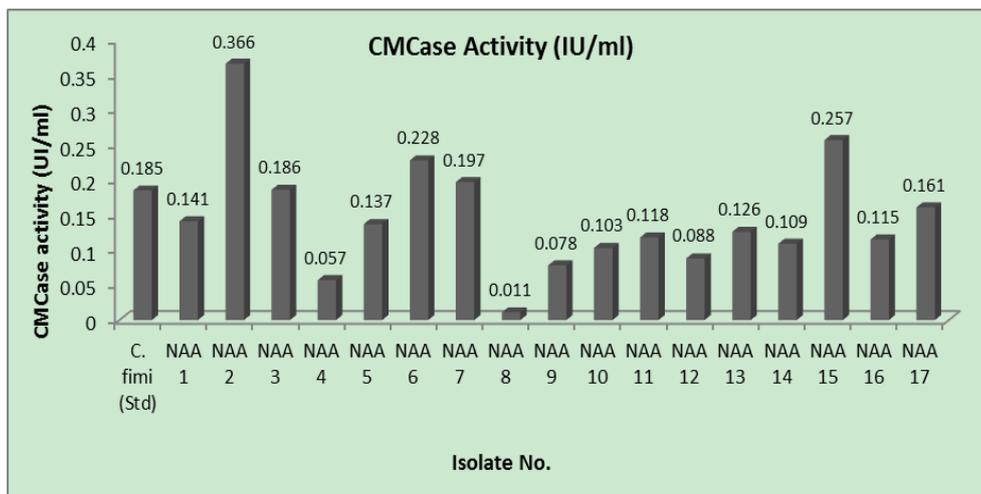


Figure 3. CMCase activity shown by different actinomycete isolates

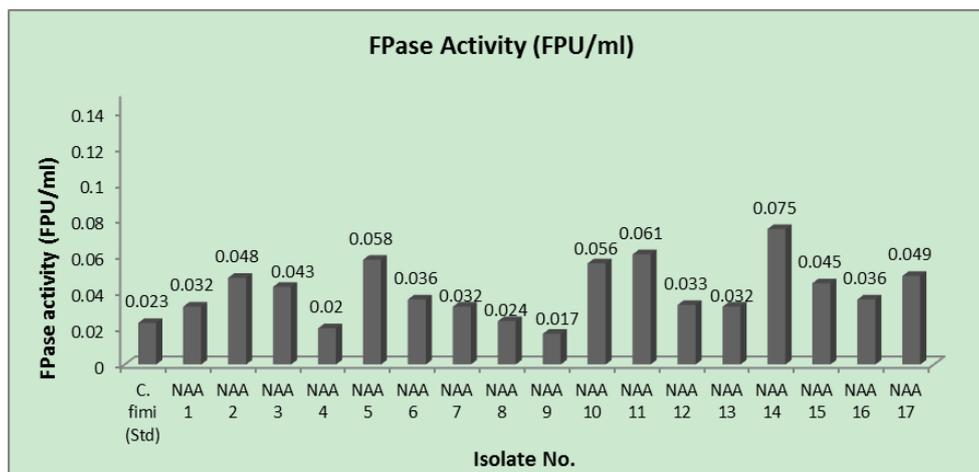


Figure 4. FPase activity shown by different actinomycete isolates

3.2. Secondary Screening of Actinomycete Isolates

Quantitative screening of isolates showed that the isolate NAA2 produced largest amount of endoglucanase (CMCase), i.e., 0.366 U/ml. The standard isolate of *C. fimi* NCIM 5015 produced 0.185 IU/ml of CMCase and 0.023 FPU/ml of FPase. Four other isolates, i.e., NAA3 (0.186 IU/ml), NAA6 (0.228 IU/ml), NAA7 (0.197 IU/ml) and NAA15 (0.257 IU/ml), also showed CMCase values higher than or close to that of the standard culture. Figure 3 represents the data of the CMCase activities shown by various isolates. On the other hand, all of the isolates showed FPase activities higher than or near to that shown by *C. fimi*. Highest FPase activity (0.075 FPU/ml) was observed in NAA14. NAA11, NAA5 and NAA10 produced 0.061, 0.058 and 0.056 FPU/ml of FPase respectively. Figure 4 depicts the FPase activities exhibited by all isolates. From the results, this can be concluded that zone of hydrolysis is not proportional to the synthesis of cellulolytic enzymes in the production medium. Also there's no correlation between FPase and CMCase activities.

Many other reports have revealed the cellulolytic potential of different actinomycetes isolated from various ecological niches. Jeffrey and Azrizal [16] isolated cellulolytic actinomycetes from different soil samples and screened them for the production of halo around colonies indicating cellulose hydrolysis. Identification studies revealed that all isolates belonged to *Streptomyces* group. Bui [17] successfully isolated *Streptomyces* and *Actinomyces* actinomycetes from coffee exocarps showing high cellulolytic potential. Das et al [9] isolated cellulose degrading *Streptomyces griseochromogenes*, *Streptomyces rochei*, *Streptomyces plicatus* and *Streptomyces enissocaesilis* from diverse habitats. In another study by Mohanta [6], cellulolytic actinomycetes were isolated from mangrove forest sediments, which produced large zone of hydrolysis and exhibited FPase and CMCase activities in the range of 0.266±0.001 to 0.734±0.001 IU/mL and 0.501±0.014 to 1.381±0.024 IU/mL respectively.

4. Conclusions

85% of the actinomycetes isolated from different samples showed cellulose degradation potential in the plate assay. Comparison of the endoglucanase and FPase production by the positive isolates with the standard isolate of *C. fimi* confirmed the cellulose hydrolysing capability of the isolates. Quantitative comparisons revealed high potency of the isolates. The present work is, however, a preliminary study which requires further elaboration. Future research can be executed focused on optimization of physicochemical parameters for enhancement of cellulases production by the isolates. The cellulolytic potential of the isolates can especially be beneficial in hydrolysis of the cellulosic materials such as the saccharification of lignocellulosic biomass for ethanol production.

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