Isolation and Screening of Cellulolytic Bacteria From Soil And Evaluation of Production And Optimization of Cellulase

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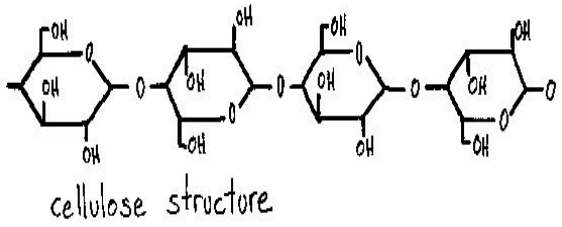
Abstract- *The cellulase producing bacteria were isolated from different soil sample. Detection of extracellular cellulase production was done by way of plate assay. In this method, plates were flooded with congo-red so, the cellulase degrading ability was screened by observing zone around the cellulase-producing microbial colonies. There are total 20 isolates showed positive results. All isolates were evaluated their cellulase activity, among them the isolate S2 and Ms1 displayed the highest enzyme activity in time interval of 72 hours, the highest enzyme activity is 0.6771 mg/ml and 0.4827mg/ml. According to morphological and biochemical studies, the isolates were primarily identified as Clostridium spp. and Bacillus spp. respectably. The optimization of media was done by checking the effect of different source like different carbon source, incubation time, pH, Nacl concentration. Enzyme profiling was done to check; isolate have ability to produce another enzyme like amylase, protease, lipase, pectinase.*

Keywords- Cellulase, Soil sample, Biochemical, Submerged fermentation, Optimization

I. INTRODUCTION

Cellulose is the most abundant biological compound on terrestrial and aquatic ecosystem and is main component of plant biomass [1]. It is the dominant waste material from agricultural industry. Cellulose is a linear polysaccharide residue with beta-1, 4 glycosidic linkages.



Ordinally, the beta-1, 4 glycosidic bonds themselves are not too difficult to break. However, because of these hydrogen bonds, cellulose can form very tightly packed crystallines. These crystals are sometimes so tight that neither water nor enzyme can penetrate them; only exoglucanase, a subgroup of cellulase that attacks the terminal glucosidic bond, is effective in degrading it. Abundant avaibility of cellulose makes it an attractive raw material for producing many industrially important commodity products [2]. Accumulation of municipal solid waste is becoming a serious problem in all developing countries. In most developing countries, the inadequate treatment of municipal solid waste causes a serious problem. With the help of cellulolytic system, cellulose can be converted to glucose either by acid or enzyme hydrolysis which is a multiutility product, in a much cheaper and biologically favorable process [3]. Cellulolytic enzymes play an important role in natural biodegradation process. In industry, these enzymes have found novel application in the production of fermentable sugars and ethanol, organic acid, detergents and other chemicals [11]. Cellulose is commonly degraded by cellulase.

II. MATERIAL AND METHOD

2.1 Sample collection

The soil sample was collected for the isolation of cellulose degrading microbes. The sample includes municipal solid waste soil, agricultural field soil, sugarcane soil sample, sugarcane sample, banana plant soil. Serial dilution 10^{-1} to 10^{-6} was prepared in sterilized distilled water and 0.1 ml of that diluted sample was spread on carboxymethylcellulose medium [11].

Table1: Sampling sites for samples

Serial no	Sample code	Compost site
1	MSP	Soli of municipal solid waste at Punagam
2	SW	Soil of sugarcane waste at parvat pati
3	MSU	Soil of Municipal solid waste At utran
4	AG	Soil of agriculture field soil at sardar Market
5	BS	Soil of banana tree at saroligam
6	LG	Lake garden soil from Motavarachha

2.2 Isolation of celluloytic bacteria

Cellulolytic bacteial isolates were isolated from soil by using serial dilutions and pour and spread plate technique. The medium used for isolation of cellulolytic bacteria contains 1.0% (CMC) Carboxymethylcellulose, 0.1 % k_2HPO_4, 0.02 % $MgSO_4.7H_2O$, 0.1 % NH_4NO_3, 3 % agar for 48 hour of incubation at 37^0c. Bacterial colonies were purified by repeated streaking. The purified colonies were preserved at 4^0c for further identification and screening for cellulase production [5].

2.3 Screening of celluloytic bacteria

Pure cultures of bacterial isolates were individually transferred in CMC agar plates. After incubation for 48 hours, CMC agar plates were flooded with 1% congo red and allowed to stand for 15 minute at room temperature. One molar NaCl was thoroughly used for counterstaining the plates. Clear zones were appeared around growing bacterial colonies indicating cellulose hydrolysis. The Bacterial colonies having the largest clear zone were selected for Identification and cellulase production in submerged system [11].

2.4 Phenotypic Identification

The morphological and colony characteristics were studied using nutrient agar plate. The physiological characteristics of all the obtained isolates were studied the biochemical characteristics (Indole, catalase, oxidase, triple sugar iron test, methyl red, voges-proskauer, citrate, hydrogen sulphide production, nitrate reduction, gelatin hydrolysis, urease, casein hydrolysis, carbohydrate utilization) and sugar fermentation were also carried out using standard reference biochemical tests for identification of medical bacteria by jean E. Mac Faddin.

2.5 Cellulase enzyme production

Newly isolates were screened for cellulase enzyme production in submerged fermentation process. Fermentation medium was Prepared using 1% CMC, 0.2% k_2HPO_4, 0.03% $MgSO_4.7H_2O$, 1% peptone, 0.25% $(NH_4)_2SO_4$ and autoclaved at 121^0c for 15 min. After sterilization, the medium was allowed to cool at room temperature [12]. The medium was inoculated with 1ml of selected Isolates and incubated in a shaker at 37^0c for 72 hour of fermentation period with agitation. After termination of the fermentation period the fermented broth was centrifuged at 10,000 rpm for 10min at 4^0c to remove the unwanted material. The clear supernatant thus obtained after centrifugation served as crude enzyme source [2].

2.6 Estimation of cellulase activity

Cellulase activity was assayed using Dinitrosalicylic acid (DNSa) reagent by estimation of reducing sugar released from CMC solubilized in 0.05 M phosphate buffer at pH 8. The crude enzyme was added to 0.5 ml of 1 % CMC in 0.05 M phosphate buffer and incubated at 37^0c for 30min. After incubation, reaction was stopped by the addition of 2 ml DNSa reagent and boiled at 100^0c in waterbath for 10min. Then addition of 1ml 40% Rochell salt Solution. Librated sugar can be determined by measuring absorbance at 540nm [6].

International Unit (IU)

One unit (U) of enzyme activity is expressed as the quantity of enzyme, which is required to release 1µmol of glucose per minute under standard assay conditions.

Calculation

$$\frac{IU}{ml} = \frac{mg/ml \times 1000}{\text{incubation time} \times \text{volume of substrate} \times \text{Mol.wt.of glucose}}$$

2.7 Extracellular enzyme profiling

Amylase

Starch hydrolysis test was done to examine the activity of amylase. The cellulolytic bacterium was streaked on starch agar plates and incubated at 37°C temp for 24 hour. Hydrolysis of starch was detected by flooding plates with iodine solution and the plates were observed for the presence of clear zones surrounding the colonies.

Pectinase

The production of the enzyme pectinase by the cellulolytic bacterium was tested using pectin agar medium (Appendix). The cellulolytic bacterium was aseptically inoculated on plates by streaking and the plates were incubated at 37^{o}C for 24 hours. All the plates were flooded with 1% aqueous solution of hexadecyltrimethyl ammonium bromide. The formation of a clear zone around the colonies indicated positive result for pectinase.

Protease

Milk agar contains skim milk, peptone, and agar. The cellulolytic bacterium was aseptically inoculated on plates by streaking and the plates were incubated at 37^{o}C for 24 hours. Many organisms can grow on this medium. This medium is used to detect the production of protease that digests casein to soluble peptides. This results in a clear zone. Soluble peptides can then be absorbed by the cell. Casein is responsible for the white color of milk. When digested by exoenzymes, the white agar turns clear and colorless.

Lipase

The production of the enzyme Lipase by the cellulolytic bacterium was tested using tributyrin agar medium (Appendix). The cellulolytic bacterium was aseptically inoculated on plates by streaking and the plates were incubated at 37^{o}C for 72 hours. The formation of a clear zone around the colonies indicated positive result for lipase.

Asparaginase

The production of the enzyme asparaginase by the cellulolytic bacterium was tested using Glucose asparagine medium (Appendix). The cellulolytic bacterium was aseptically inoculated on plates by streaking and the plates were incubated at 37^{o}C for 24 hours. The formation of a clear zone around the colonies indicated positive result for asparaginase.

2.8 Characterization of cellulase production

Isolate S2 was further evaluated for maximum cellulase production. Different factors such as pH, temperature, salt concentration, carbon source, nitrogen source was used to characterization of cellulase producers [11].

Effect of salt on growth and cellulase production in S2:

To study the growth of S2 in various salt concentrations, CMC broth medium was prepared with range of 1% to 15%. Broth having various salt concentrations was inoculated with 0.1 ml of activated culture and incubated under shaker conditions (100rpm) at 37°C. Culture aliquots were withdrawn at 72 hr and observed the growth. Culture aliquots were centrifuged at 10,000rpm for 10 min at 4°C cell free supernatant was used as crude enzyme preparation [7].

Effect of pH on growth and cellulase production in S2:

To study the growth of S2 in various pH range, CMC broth medium was prepared with range of 5 to 11.Broth having various pH was inoculated with 0.1 ml of activated culture and incubated under shaker conditions (100rpm) at 37°C. Culture aliquots were withdrawn at 72 hr and observed the growth. Culture aliquots were centrifuged at 10,000rpm for 10 min at 4°C cell free supernatant was used as crude enzyme preparation [8].

Effect of carbon source on growth and cellulase production in S2:

To study the growth of S2 in various carbon sources, the different carbon source are filter paper strip (FPS), ground nut, wheat bran, cotton. Broth having various carbon sources was inoculated with 0.1 ml of activated culture and incubated under shaker conditions (100rpm) at 37°C. Culture aliquots were withdrawn at 72 hr and observed the growth. Culture aliquots were centrifuged at 10,000rpm for 10 min at 4°C cell free supernatant was used as crude enzyme preparation [2].

Effect of nitrogen source on growth and cellulase production in S2:

To study the growth of S2 in various nitrogen sources, the different nitrogen source are yeast extract, beef extract, ammonium nitrate, sodium nitrates. Broth having various nitrogen sources was inoculated with 0.1 ml of activated culture and incubated under shaker conditions (100rpm) at 37°C. Culture aliquots were withdrawn at 72 hr and observed the growth. Culture aliquots were centrifuged at 10,000rpm for 10 min at 4°C cell free supernatant was used as crude enzyme preparation [9].

Effect of incubation time on growth and cellulase production in S2:

To study the growth of S2 in various incubation times, the broth medium was inoculated with culture then withdrawn the sample from the CMC broth after every 24 hour and check the time gave the highest activity.

2.9 Estimation of degradation of finely grated vegetable peels by the isolate

10 ml of stanier's Basal broth with 100 mg of different cellulosic substrates as sole carbon source viz. vegetable peels were prepared separately. Finely grated vegetable peels were weighed on the electronic balance. The broths were autoclaved and inoculated with the selected strain. The tubes were incubated in the shaker at 37°C for 72 hours. After the incubation period, the culture broths were filtered through previously weighed filter paper. The filter papers with the residues were dried in the hot air oven for 15 min and re-weighed. The differences between the initial and the final weights gave a number of cellulosic substrates degraded by the isolate and also check the enzyme activity which still present in broth [10].

III. RESULT

Based on the primary screening by serial dilution technique on CMC agar plate 34 isolates were found. Among them total 20 isolates were found which show clear zone by congo-red plate assay (qualitative assay).

Fig [A] Negative result

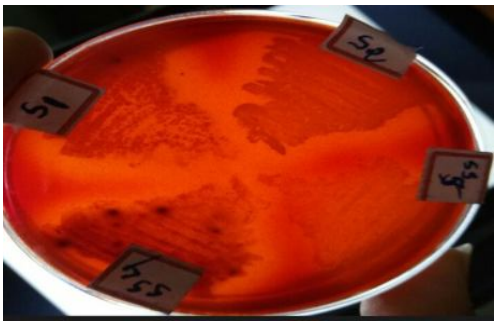


Fig [B] Positive result

Figure 2: Fig [A] and Fig [B] shows the difference between positive and negative result of cellulase producers

The total number of isolates proceeded for their morphological character and motility test. The colony characteristics of the obtained isolates were studied on CMC agar plate, which give circular, white, small/large colonies with entire margin after 24hr incubation. Among them all were gram positive, among them 9 were cocci and 11 were large rod. Following Table.2 shows results of various biochemical characteristics of the isolates.

Table 2: Biochemical characterization of the isolates

Isolates	Oxidase	Catalase	Indole	MR	VP	Citrate	H2s	Urease	TSI	Nitrate	Gelatine	Casein	Glucose	Mannitol	Sucrose	Lactose	Probable identity
Ms1	+	+	+	-	-	-	-	+	A/A	-	-	+	+	-	-	+	*Cellulomonas*
Ms2	-	+	-	+	+	+	-	-	A/A	-	-	-	+	+	+	+	*Staphylococcus spp.*
S1	+	-	+	+	-	+	-	-	A/Alk	+	-	+	+	+	+	+	*Clostridium spp.*
S2	+	-	+	+	+	+	-	-	A/Alk	+	-	-	+	+	+	-	*Clostridium spp.*
Ss1	+	+	+	+	+	+	-	-	Alk/alk	+	-	+	+	+	+	+	*Corynobacter spp.*
Ss2	-	+	-	-	+	+	-	-	Alk/alk	+	-	-	+	+	+	+	*Bacillus spp.*
Ss3	+	+	-	-	+	+	-	-	Alk/alk	+	-	-	+	+	+	-	*Bacillus spp.*
Ss4	+	-	+	+	+	+	-	-	Alk/alk	-	+	-	+	-	+	-	*Clostridium spp.*
Ss5	-	+	-	+	+	+	-	-	Alk/alk	-	-	+	+	-	+	-	*Bacillus spp.*
Ag1	-	+	-	-	+	+	-	-	Alk/alk	-	-	-	+	+	+	+	*Propionibacterium spp.*
Ag2	+	+	+	+	+	+	-	-	Alk/alk	-	-	+	+	+	+	+	*Clostridium spp.*
Ag3	+	+	-	-	+	+	-	-	Alk/alk	+	-	+	+	-	+	-	*Bacillus spp.*
Ag4	-	+	+	-	+	+	-	-	Alk/alk	+	-	-	+	+	+	+	*Staphylococcus spp.*
Ag5	-	+	-	-	+	+	-	-	Alk/alk	+	-	+	+	+	+	+	*Staphylococcus spp.*
Ag6	-	+	-	-	+	+	-	-	A/Alk	-	-	+	+	+	+	+	*Staphylococcus spp.*
Ag7	-	+	-	-	+	+	-	-	Alk/alk	-	-	-	+	+	+	+	*Propionibacterium spp.*
Ag8	+	+	+	-	+	+	+	-	Alk/alk	-	-	+	+	+	+	+	*Clostridium spp.*
Bs1	-	+	-	+	+	+	-	-	Alk/alk	+	-	+	+	+	+	+	*Arcanobacterium spp*
Bs2	+	-	+	-	-	+	-	-	Alk/alk	+	-	+	+	+	-	-	*Bacillus spp.*
Bs3	+	+	+	+	+	-	-	-	A/Alk	-	-	+	+	+	+	+	*Bacillus spp.*

('+': positive, '-': negative, 'A': for acidic gave yellow color and 'Alk': for basic gave pink color on TSI slant)

By studying morphological and biochemical characteristics cellulase producing various species were obtained such as, *Staphylococcus spp., Bacillus spp., Arcanobacterium spp., Propionibacterium spp., Corynobacter spp, Cellulomonas spp.*

3.1 Cellulase enzyme production and estimation of cellulase activity

The optimum parameters were determined for cellulase production from the efficient isolates. After fermentation at the different parameters the crude enzyme product was collected for determination of enzyme activity. Enzyme activity was determined by DNSa method. The activity was checked of all 20 isolates among them the two isolates, which have highest activity, are S2 and Ms1 with enzyme activity 0.2257 and 0.1609 at 540nm absorbance respectively are selected for further analysis. This activity was attained after 72 hr by isolates. From this two the isolate S2 gave the highest activity, so further analysis done by using isolate S2. Here, the graphical representation as bellow.

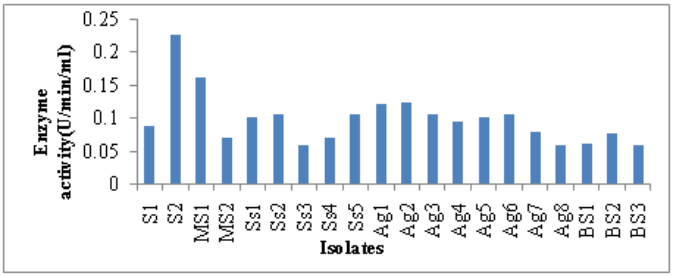


Figure 3: Quantitative analysis

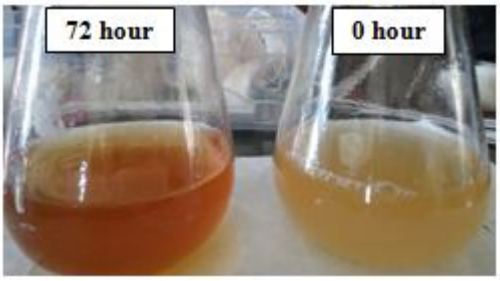


Figure 4: production media for cellulase producer

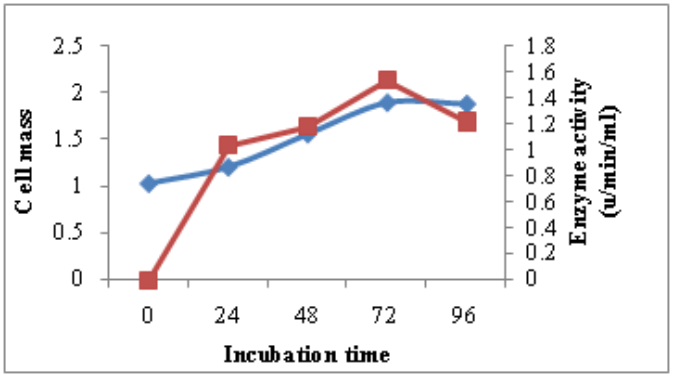


Figure 5: Growth kinetics of isolate S2

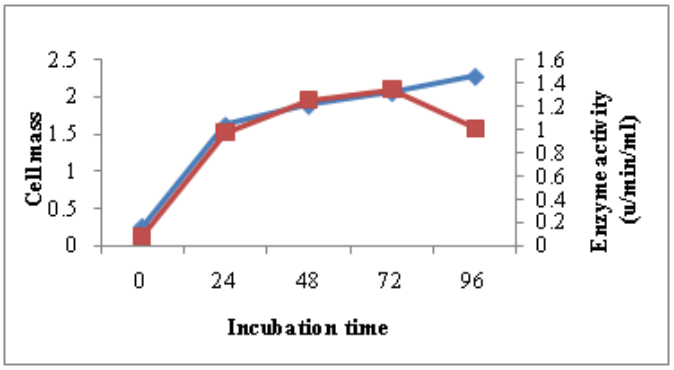
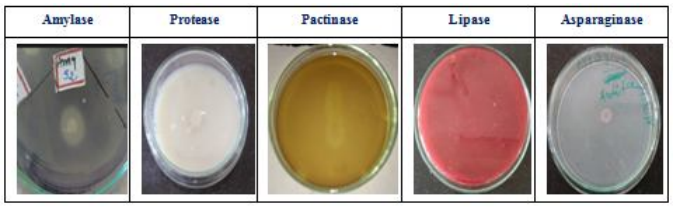


Figure 6: Growth kinetics of isolate Ms1

3.2 Extracellular enzyme profiling

Table 3: enzyme profiling of isolate S2



Enzyme profiling of highest activity isolate, S2 was streaked on starch agar plates for amylase, tributyrin agar medium for lipase, milk agar medium for protease, pectin agar medium for pectinase, glucose asaparagine medium for asparaginase incubated at 37°C temp for 24 hour. After 24 hour with help of different reagent checked the ability. Isolate S2 was able to produce the enzyme amylase, asparaginase and pectinase. While not produce the enzyme by protease, lipase.

3.3 Characterization of cellulase production

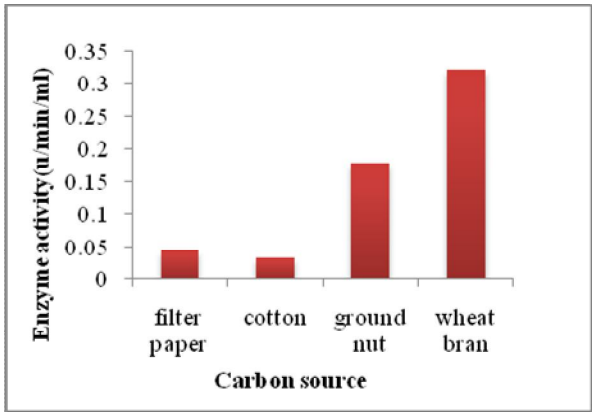


Figure 7: Effect of carbon source in S2

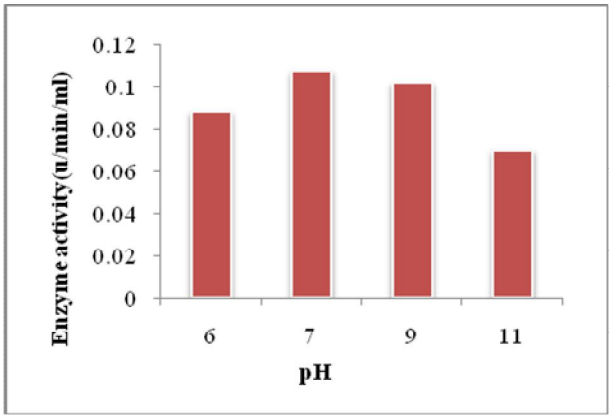


Figure 8: Effect of pH in S2

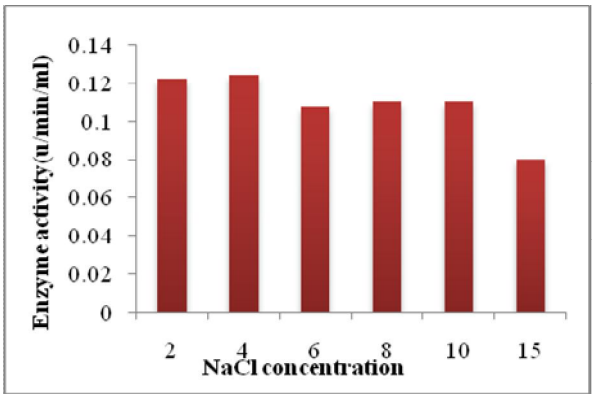


Figure 9: Effect of salt concentration in S2

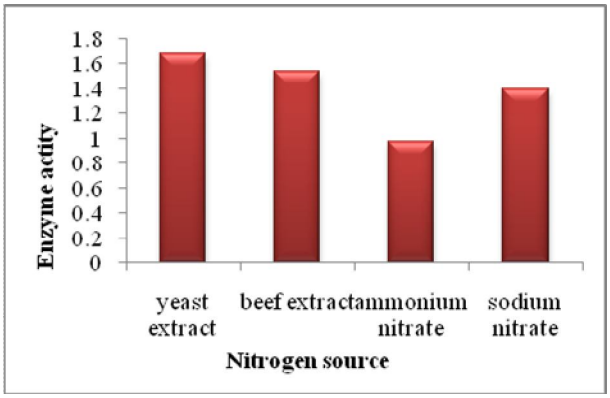


Figure 10: Effect of nitrogen source in S2

3.4 Estimation of degradation of finely grated vegetable peels by the isolate

Table 4: degradation of vegetable peels

	Uninoculated	Inoculated bacterial strain S2
Filter paper weigh	1.987	2.438
Filter paper weigh + vegetables(1gm/100ml)	2.754	2.622
Degradation	0.233	0.816

IV. DISCUSSION

The study highlights the screening, Identification and Production of cellulase producing microorganisms. A total 20 isolates were obtained from different soil sample. Isolates were identify based on their morphological and biochemical characteristics by macfaddin and bergey's manual. The activity was checked of all 20 isolates among them the two isolates, which have highest activity, are S2 and Ms1 with enzyme activity 0.2257 and 0.1609 at 540nm absorbance respectively are selected for further analysis.

V. CONCLUSION

Cellulase is one of the most widely used enzymes in various industries. The present study focused on isolation, screening, and characterization of cellulolytic bacteria and determination of their cellulolytic potential. Different samples which were rich in cellulose biomass were selected as sources for isolation. Isolate S2 from sugarcane sample was found as potent cellulase producer. These S2 was characterized on the basis of morphological, cultural and biochemical analysis and identified as *Clostridium spp.*, with the help of Bergey's Manual of Determinative Bacteriology. Isolate coded S2 was able to grow at neutral pH range and 37 °C. While enzyme production was also maximum at near to neutral pH. Hence it was found that crude cellulase active near to neutral range of pH. Such cellulase producers can be further used to degrade the cellulosic waste and make environment clean. Isolated *Clostridium spp.* was also effective producer of other extracellular enzymes example, amylase, catalase, asparaginase and pectinase. Results obtained from present study are significant for possible industrial and environmental applications of cellulase. Further improvement in cellulase performance can be imparted using various techniques for industrial applications.

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