PATNA WOMEN'S COLLEGE 1940

IRIS
Journal for Young Scientists
ISSN 2278 – 618X **(Print)**
ISSN 2278 – 6384 **(Online)**

http://www.patnawomenscollege.in/journal

Isolation, Screening and Characterization of Cellulase Producing Bacterial isolates from garbage dumping sites

• **Moni Kumari** • **Muskan Singh** • **Aakriti Lal**
• **Jaya Philip**

Received : November 2017
Accepted : March 2018
Corresponding Author : Jaya Philip

Abstract : *Cellulolytic bacteria were isolated and screened from dumping site of different areas of Patna. Selective media Carboxy methyl cellulose (CMC) agar was used to screen cellulose degrading bacteria from bacterial colonies obtained on nutrient agar (NA) plates. CMC agar medium supplemented with 1% CMC was used. On flooding with Gram's iodine clear zone on CMC agar medium plates were observed showing hydrolysis of cellulose on the plate. Value of total surface area of clear zone is directly proportional to the level of cellulose production. Based upon the morphological, cultural and biochemical characterisation six isolated strain were identified as Bacillus sp. (T1, B1, B3), Pseudomonas sp. (M2), Serratia sp (M1), Streptococcus sp. (M3). A basal medium containing CMC, KH_2PO_4, K_2HPO_4, MgSO4 and $FeSO_4$ at pH 7.0 was used for cellulase production. The assay of cellulase in term of CMCase activity was performed by measuring the release of reducing sugar using DNS method. Physicochemical parameters like incubation time, temperature and pH were optimised for maximum cellulase production. Maximum cellulase was produced by Pseudomonas sp.(M2) and Bacillus sp.(T1) at 37°C and 50°C respectively. All the other isolates showed maximum cellulase production at temperature range of 37-45°C. The pH of initial media affected the crude enzyme production significantly at pH range of 5.5-7.5. All the isolates showed high enzyme activity at pH range of 5.5-7.5, whereas Bacillus sp. (T1) being acidophilic showed its maximum activity at pH 4.0.*

Keywords: *Cellulase; CMCase activity; Filter paper;*

Moni Kumari
B.Sc. III year, Industrial Microbiology (Hons.),
Session : 2015-2018, Patna Women's College,
Patna University, Patna, Bihar, India

Muskan Singh
B.Sc. III year, Industrial Microbiology (Hons.),
Session : 2015-2018, Patna Women's College,
Patna University, Patna, Bihar, India

Aakriti Lal
B.Sc. III year, Industrial Microbiology (Hons.),
Session : 2015-2018, Patna Women's College,
Patna University, Patna, Bihar, India

Jaya Philip
Asst. Prof., Deptt. of Industrial Microbiology,
Patna Women's College, Bailey Road,
Patna – 800 001, Bihar, India.
E-mail : jayaphilipmicrobio@gmail.com

Introduction:

Cellulose is the most abundant biomass on terrestrial and aquatic ecosystem and is the main component of plant biomass (Shankar et al., 2011). It has the greatest potential to resolve both the energetic and environmental demands of bioenergy (Khatiwada et al., 2016). It is dominant waste material from agricultural industry in the form of stalks, stems and husk, there has been great interest in utilizing cellulose as an energy resource and feed (Balachandrababu et al., 2012). The cellulose is polysaccharides that composed 1000-1200 of D-glucose units linked together to form linear chain via ß-1, 4-glycosidic linkages (Salmon and Hudson, 1997). Cellulose is a linear polysaccharide which consists of 1000-1200 glucose residues with â-1,4-glycosidic linkages. Cellulases can effectively hydrolyze cellulose into glucose via synergistic action of three enzymes, Endo-â 1,4-glucanase, cellobiohydrolases, and â-D glucosidase. The process is known as cellulolytic system. The endoglucanase randomly hydrolyzes the â-1,4 bonds in the cellulose molecule, and the exocellobiohydrolases in most cases release a cellobiose unit showing a recurrent reaction from chain extremity. Finally, the cellobiose is converted to glucose by â-glucosidases. 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).

Cellulose is basically the structural component of the primary cell wall of green plants, many forms of algae and Oomycetes. Plants produce 4×10^{9} tons of cellulose annually. It is also 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} tons per annum (Nowak et al., 2005).

Cellulases are inducible enzymes synthesized by a large diversity of microorganisms including both fungi and bacteria during their growth on cellulosic materials. These microorganisms can be aerobic, anaerobic, mesophilic or thermophilic. Filamentous fungi are the major source of cellulases and hemicellulases but the production costs of these enzymes are very high. Bacteria which have high growth rate and short generation time in compared to fungi have good potential to be used in cellulase production. The furthermost potential importance is the ease with which bacteria can be genetically engineered. During the last two decades, the use of cellulases, hemicellulases and pectinases has been increased considerably, especially in textile, food and feed, brewery and wine as well as in pulp and paper industries. However, the most important use of cellulase is in the bioconversion of plant based cellulosic and lignocellulosic waste, which opens the possibility of virtually inexhaustible and unique source of renewable biofuel. Nowadays, these enzymes account for approximately 20% of the world enzyme market used on industrial basis.

Biomass of Dumping site needs to be bioconverted into bioresources through production of value added products such as enzymes. Cellulase can be produced by bacteria using cellulosic dumping site as the raw material. With this view, we here in isolated and screened cellulase producing bacterial isolates from dumping site and optimized some parameters for cellulase production.Cellulases produced from these bacterial isolates were partially characterized and seemed to degrade filter paper under optimum conditions. The production of crude cellulase enzyme was further confirmed by FTIR spectra which resemble standard curve of cellulase enzyme.

Materials and Methods:

Isolation and screening of cellulase producing bacterial isolates we done from the Samples collected from Mithapur vegetable market, Phulwarisharif, Gardanibagh dumping site, pond in Sipara, local garbage site from different areas of Patna, Bihar. Sample was suspended in sterile distilled water (1 ml of soil in 9 ml of distilled water) for preparation of suspension. Six-fold dilution of the suspension (10^{-1} to 10^{-6} times), 200 µl of each dilution was taken and plating was done was on carboxymethyl cellulose (CMC) agar plates (1% CMC, 0.1% KH_2PO_4, 0.1% K_2HPO_4, 0.04% $MgSO_4$, 0.005% NaCl, 0.000125% $FeSO_4$ and 1.8% Agar, pH 7.0) by and incubated at 37°C for 24-48 hrs. The cellulolytic bacterial colonies were selected by formation of clear-zones after application of Gram's iodine solution on CMC agar plates. Bacterial isolates producing significant clear zone on CMC agar were identified based on cultural, morphological and biochemical characteristics as described by Khatiwada et al., 2016 with some modifications.

The production of crude cellulose enzyme was done using Basal media (1% CMC,1% KH_2PO_4, 0.1% K_2HPO_4, 0.04% $MgSO_4$, 0.005% NaCl, and 0.000125% $FeSO_4$, pH 7.0). For seed culture, a fresh isolated colony was inoculated in 5 ml basal media and incubated at 37°C and 120 rpm for 24 hours. The seed culture (5%) was then inoculated in 50 ml production media in a 250 ml conical flask and incubated at the above mention conditions. The cell free supernatant obtained by centrifugation at 5,000 rpm for 20 min at 4°C was used for determining the cellulase activity.

The carboxy methyl cellulase (CMCase) activity was assayed using a method described by Rathore, 2014 with some modifications. 0.5 ml of culture supernatant was added to 0.5 ml of 1% CMC prepared in 50 mM sodium citrate buffer (pH 4.8) in a test tube and incubated at 80°C for 30 min. The reaction was terminated by adding 3.0 ml of dinitrosalicylic acid (DNS) and subsequently placing the reaction tubes in a water bath at 100°C for 15 minutes. One ml of Rochelle salt (Sodium Potassium tartarate) solution (40 gm Rochelle salt in 100 ml distilled water) was then added to stabilize the color. The absorbance/ Optical Density (OD) was recorded at 575 nm wave length against a blank of 50 mM sodium citrate buffer lacking crude sample. One unit of CMCase activity was defined as the amount of enzyme that liberated 1 µmol of reducing sugar (glucose) in 1 min at 37°C and pH 7.0.

To determine the optimum cultivation period for maximum cellulase production, the seed culture was inoculated into the Production media (1% CMC 1% KH_2PO_4, 0.04% $MgSO_4$, 0.005% NaCl, and 0.000125% $FeSO_4$, pH 7.0) and incubated for 96 hours. Culture samples were tested at interval of 24hr for their CMCase activity following Miller (1959) method with some modifications.

To study the optimum temperature for maximum cellulase enzyme production, production medium at pH 7 was inoculated with the seed culture (5% of inoculum fermented in basal media) and incubated at different temperatures (26ºC, 32ºC, 37ºC, 45ºC, and 50°C). Buffer solution of 50mM Potassium phosphate (pH 7.0) were used. Cellulase activity was determined DNS method as described by Miller (1959). The amount of reducing sugar was determined by spectrophotometer.

To investigate the effects of pH on maximum cellulose enzyme activity, 0.5ml of the crude enzyme was added to 500 µL of 1% CMC in buffer of different pH at 37ºC. Cellulase activity was then measured as described above. To study the effects of pH on cellulase activity, different buffers such as

50 mM of sodium citrate (pH 4.0 and 5.0), potassium phosphate (pH 6.0-7.0) and Tris-HCl (pH 8.0-9.0) were used to assay the CMCase activity. To 0.5 ml of 1% CMC prepared in a suitable buffer of a particular pH (pH 4-9), 0.5 ml of crude enzyme was added and further DNS method was used and absorbance was taken at 575nm.(Miller, 1959).

To determine the filter paper degradation capability of the crude cellulase produced, 200 mg filter paper strip was placed in a conical flask containing 50 ml of crude enzyme from each bacterial isolate, and incubated in a shaker incubator at 120 rpm and 37°C for 7 days. The amount of glucose liberated from the filter paper was estimated according to the procedure described by Miller, 1959 with some modifications.

Results and Discussion:

For isolation and screening of cellulase producing bacterial isolates**,** a total of 27 bacterial cultures were isolated from nutrient agar plates. The isolates were screened for cellulase production by plating on CMC and Congo red agar media to observe the zone of cellulose hydrolysis. Among all 27 tested bacterial strains six strains showed cellulase production on CMC and congo red agar. Further these six isolates were used for crude enzyme production. The pure culture produced clear zone when it was flooded with Gram's iodine solution due to hydrolysis of CMC (Figure 1). Gram's iodine forms a bluish-black complex with cellulose but not with hydrolyzed cellulose, giving a sharp and distinct clear zone around the cellulose producing bacterial colonies. The cellulytic isolates such as M1 from Sipara (near a local pond), M2 from Mithapur veg. market, M3 from Mithapur dumping site , B1 from Gardanibagh dumping site, B3 from Phulwarisharif veg. Market and T1 from local garbage site in Patna region.

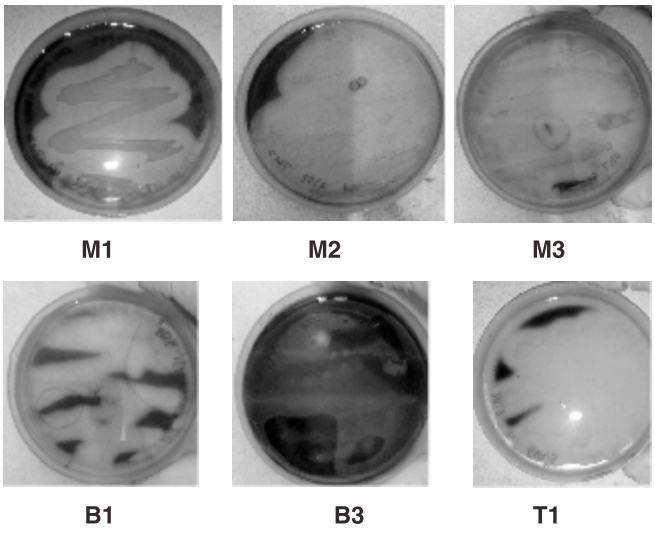

Fig. 1. Visualization of cellulose activity with Gram's iodine solution. Cellulolytic bacteria were screened from the master plate to obtain pure culture. Cellulolytic activity was observed on CMC agar media after incubation at 37°C for 24 h. Typical results of six independent experiments for cellulolytic bacteria on CMC agar media are shown. Zone of CMC hydrolysis of different isolates M1,M2,M3,B1,T1 showed largest clear zone & selected for further identification whereas B3 showed no clear zone and hence it was a non-cellulytic strains.

The bacterial isolates showing cellulase activity was selected for characterization on the basis of morphology and biochemical tests. The parameters investigated were colony morphology, Gram's reactions, motility, carbohydrate fermentation, catalase production, oxidase test, MR-VP reaction, casein hydrolysis. Thus on the basis of cultural, morphological, and biochemical characterizations the isolates have been identified as. ***Serratia*** sp. (M1), ***Pseudomonas*** sp. (M2), ***Bacillus*** sp. (M3, B3, T1) and ***Streptococcus*** sp. (B1). However, for the identification at species level molecular characterization by 16srRNA sequencing should be performed.The results are shown in Fig 2. and Table 1.

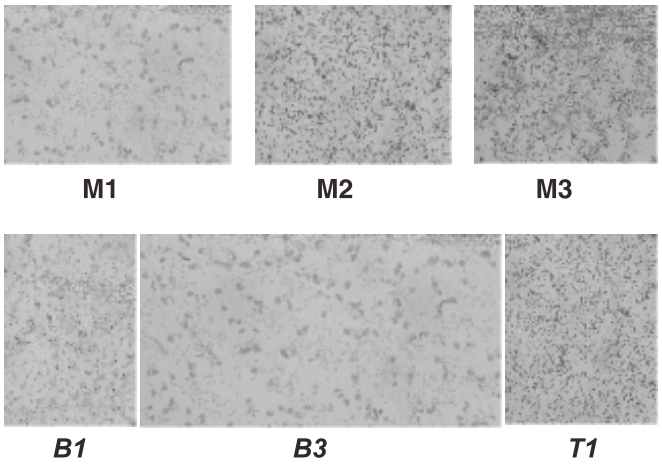

Fig. 2. Gram staining of all the six isolates were done and it was observed that M3, B3 and T1 have rod shape and gram positive whereas M1 and M2 were gram negative rods, isolate B1 was found to be gram positive coccus.

Table 1. Biochemical characterization for cellulolytic bacterial isolates M1, M2, M3, B1, B3 and T1.

Biochemical test	Isolate M1	Isolate M2	Isolate M3	Isolate B1	Isolate B3	Isolate T1
Bacterial shape	Rod	Rod	Rod	Coccus	Rod	Rod
Motility test	Non motile	Non motile	Non motile	Non motile	Non motile	Non motile
Gram staining	-ve	-ve	+ve	+ve	+ve	+ve
Catalase test	-ve	+ve	+ve	+ve	+ve	+ve
Indole test	-ve	-ve	-ve	-ve	-ve	-ve
Lactose fermentation test	-ve	-ve	-ve	+ve	-ve	-ve
Sucrose fermentation test	+ve	-ve	+ve	+ve	+ve	+ve
Dextrose fermentation test	+ve	-ve	+ve	+ve	+ve	+ve
MR	-ve	+ve	+ve	-ve	+ve	+ve
VP	-ve	-ve	-ve	-ve	-ve	-ve
Casein test	+ve	+ve	+ve	+ve	-ve	+ve

To estimate the cellulase production at different incubation time, cultivation temperature, initial pH of the culture media and agitation are the most striking features to influence the production of enzymes. The optimum parameters were determined for cellulase production from the efficient isolates. However for detection of the best isolate which can produce cellulase enzyme at comparatively higher level is done by keeping all the others factor same for six different isolates i.e. incubated at 37°C for fermentation period of 24 hours in the production media of pH 7.0. The enzyme production capacity is determined by DNSase method. Data analysis by spectrometry clearly depicts that highest enzyme production was done by isolate M3 with absorbance of 0.290 nm followed by isolate T1 with absorbance of 0.242 nm.

Endonuclease assay using CMC by CMCase method was performed and CMCase activity of isolate M3 had the highest activity followed by T1, M1, B3, B1 and very less activity was shown by M2 as shown in Fig 3.

According to IUPAC, endoglucanase (CMCase) assay is a fixed conversion method, which requires 0.5mg of absolute glucose released under reacrtion condition. The reducing end concentration is measured by the DNS method. The enzyme dilution series was prepared with at least one dilution must be made of one enzyme sample.

The CMCase activity of the concentrated enzyme solution in terms of IU/ml:CMCase = 0.185/EDR

Where, 5mg glucose=5mg/ (1.8mn/m mol) *0.5ml*30 min =0.185 m mol/min/ml.

EDR values can be obtained by: 0.29*concentration of glucose/0.5 mg/ml.

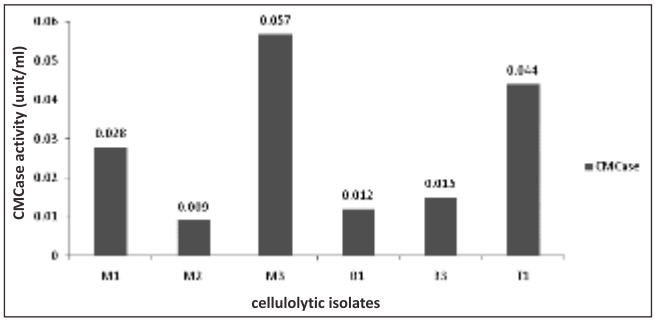

Fig. 3. Graph showing CMCase activity of various isolates where M3 had the highest activity followed by T1, M1, B3, B1 and very less activity of M2

The optimization of various parameters for maximum cellulase production of the selected isolates was done.

After fermentation at the different parameters the crude enzyme was collected for determination of enzyme activity. CMC was taken as cellulose source for Enzyme activity determination by DNase method (Miller, 1959).

Effect of incubation time reveals that Bacillus isolates (M3 and T1) show maximum cellulase production at 24 h of cultivation, whereas Pseudomonas and streptococcus isolates (M2 and B1) show optimal cellulase production at 42 h of cultivation (Figure 4). After 72 hours all the isolate seems to lose its enzymatic activity and their absorbance decreases. The cultivation was done at 37 and pH 7 in shaker incubator for 24hrs, 48 hrs, 72 hrs.

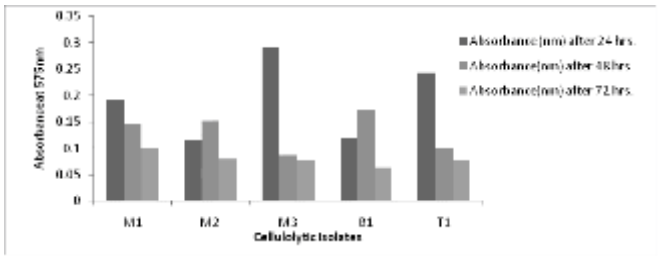

Fig. 4. Bar Graph showing Incubation time and absorbance at 575nm of the isolates

Temperature is one of the most important variables controlling bacterial growth and enzyme production. The effects were examined at various temp on the crude ellulase activity ranging from 26°C to 50°C. Cellulase from bacterial isolates show optimum activity at 37°C. ***Bacillus*** and ***Pseudomonas*** isolates showed production optima at 37°C, while ***Serratia*** sp. showed maximum cellulase production at 37°C (Figure 5.). However ***Bacillus*** sp. (T1) showed maximum activity at 50°C showing its thermophillic nature.

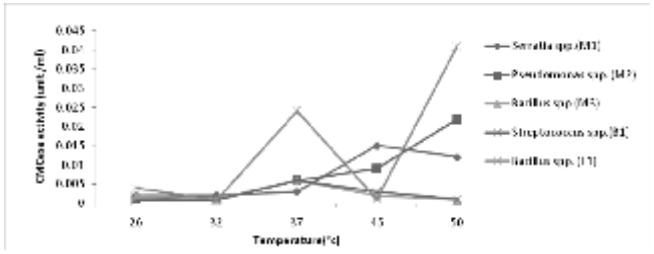

Fig. 5. Effect of temperature on CMCase activity

The effect of pH on the enzyme activity of six isolates showed different absorbance in buffer solution of sodium citrate pH 7, ***Pseudomonas*** sp. (M2) showed highest cellulase activity which was 0.044 ml/mg. at pH 6. Whereas, ***Bacillus*** sp. (T1) showed highest activity pH 4 (Fig. 6).

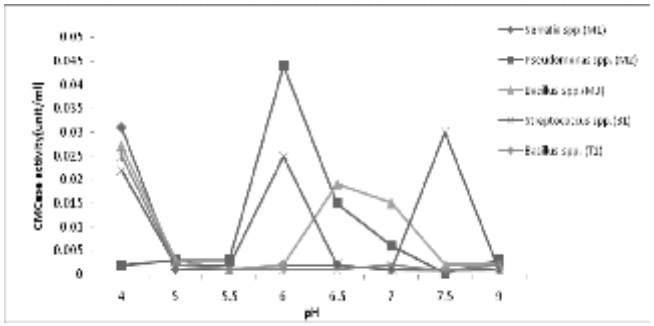

Fig. 6. Effect of pH on CMCase activity

Degradation of filter paper by crude cellulase produced was studied by taking a 200 mg of filter paper was treated in conical flasks containing 50 ml of crude enzyme. After 3 days of incubation at 37°C, it was noted by visible inspection that the crude cellulase degraded the filter paper while the filter paper treated with distilled water remained unchanged. Estimation of reducing sugar released in the solution after one week revealed that the filter paper was degraded by crude enzyme (Fig. 7).

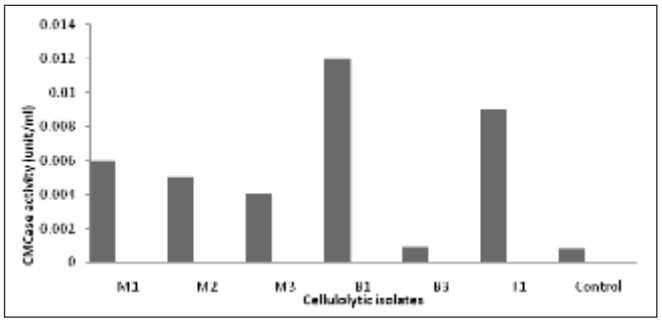

Fig. 7. Graph showing isolates having different CMCase activity where filter paper degradation was maximum by *Streptococcus* sp.(B1) and *Bacillus* sp.(T1). Isolate B3 showed no degradation showing its non cellulolytic properties in favour of no zone formation by flooding with gram's Iodine

Fourier – transform infra-red spectroscopy (FTIR) was used to observe the spectra of crude cellulase enzyme (Fig. 8).

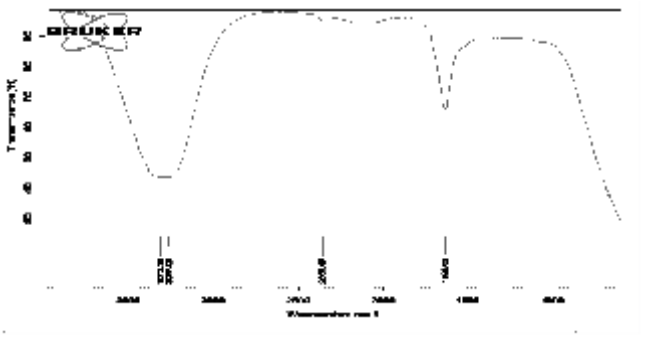

Fig. 8. FTIR spectra of isolated Serratia sp.(M1) showed changes in peak at lower wavelength along with lesser height of peak at higher wavelength.

As a result of screening, several isolates of mesothermic and thermophilic cellulolytivc bacteria growing in the acidic or neutral pH were obtained. The microorganism we isolated are result of selective condition applied during fermentation period. Isolates were identified as *Serratia* sp.(M1), *Pseudomonas* sp. (M2), *Bacillus* sp. (M3,B3,T1) and *Streptococcus* sp.(B1). The cellulolytic isolates such as M1 from Sipara (near a local pond), M2 from Mithapur vegetable market, M3 from Mithapur dumping site , B1 from Gardanibagh dumping site, B3 from Phulwarisharif vegetable Market and T1 from Local garbage site in Patna region.

Isolates *Bacillus* sp.(M3) showed maximum cellulase activity at pH range of 5.5-7.5 and temperature 37°C after 24 hrs of fermentation period, Whereas *Pseudomonas* sp. (M2) and *Streptococcus* sp. (B1) showed maximum enzyme activity after 48 hrs of cultivation period at pH 6.0 at temp of 45°C - 50°C. Our results of isolated strains and their optimised condition of maximum cellulose degrading activity are in favour of the work done by Khatiwada et al. (2016).

The genus *Bacillus* (T1) is morphologically distinctly different from our isolates of *Bacillus* sp. (M3, B3) since it showed its maximum activity at higher temperature(50°C) range and under acidic condition (pH 4.0) whereas, the isolates M3 showed enzyme activity at temperature range of 37°C and isolate B3 showed no or very little cellulose enzyme production even at optimesd conditions. Therefore, we must conclude that the strain (T1) we isolated belongs to thermophilic group of *Bacillus* genus.

Our isolated colonies of thermophilic *Bacilllus* sp.(T1) showed cellulase enzyme activity even at higher temperature range of 45°C-50°C, this is in contradiction of the work of Khatiwada *et al.*, 2016 where no cellulose degrading capacity is found at higher temperature. But this favours the work of Mohagheghi et al. (1986). The majority of isolated bacterial colony was of Bacillus sp. showing its diversity of cellulolytic bacterial strain than other species. This result favour the work done by Mohagheghi et al.(1986).

Filter paper degradation activity is maximum shown by Streptococcus sp.(B1) followed by Bacillus sp. (T1), our results of degradation is both

different and similar from the work of Khatiwada et al.,2016 as they does not obtained any *Streptocoocus* sp. during their study however *Bacillus* sp. degradation match to their work.

The spectra obtained by FTIR spectroscopy is very much similar to the curve of free cellulase obtained by Mishra and Sardar (2015), showing the purity of the our celllulase crude enzyme at significant level.

Conclusion :

Six cellulolytic bacterial isolates were obtained from soils excavated from different areas of Patna. The Gram-positive rods were deduced to be *Bacillus* sp., *Streptococcus* sp., *Serratia* sp. from both morphological and biochemical analysis. The isolate's enzyme activity was found to be high over a range of temperatures i.e. from 37°C to 50°C with the optimum temperature being 37°C. However *Bacillus* sp. (T1) showed maximum activity at 50°C showing its thermophilic nature. Similarly, enzyme activity was found to be high at the range of pH 5.5 to pH 7.5 with an optimum of pH 6.0 suggesting that the isolate *Pseudomonas* sp. (M2) showed more cellulose activity at acidic pH. 4.0. Application of these six isolates in industries may have several advantages such as high growth rate and ability to secrete proteins extracellularly.

More studies are however needed before industrial application of this isolates. These include enzyme activity assays of the purified specific cellulases for comparison with the results in this study and with those that have been purified. Similar studies should be extended to other environments in the country.

Acknowledgement:

We extend our gratitude to our Former Principal, Dr. Sister Marie Jessie A.C. for giving us this opportunity to carry out this research. We are thankful to the Research Committee for all the support provided. We would also like to thank the college staff for their constant assistance.

References :

Balachandrababu M.A., Revathi Masi, Yadav Amit, Sakthivel Natrajan (2012). Purification and characterization of a Thermophilc cellulose from a Novel cellulolytic strain*, Paenibacillus barcinonensis* . *JMB,* 10:4014.

Khatiwada P., Ahmed J., Sohag M.H., Islam K., Azad A.K. (2016). Isolation, screening and characterization of cellulase producing bacterial isolates from municipal solid wastes and rice straw wastes. *J Bioprocess Biotech*, 6:280.

Miller G.L. (1959).Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem.*, 31:426-428.

Mohagheghi A., Grohmann K., Himmel M., Leighton L., Updegraff D.M. (1986). Isolation and Characterization of *Acidothermus cellulolyticus* gen. nov., sp. nov., a new genus of thermophilic, acidophilic, cellulolytic bacteria. *International Journal of Systematic and Evolutionary Microbiology,* 36:435-443.

Nowak J., Florek M., Kwiatek W., Lekki J., Chevallier P., Zeiba E. (2005). Composite structure of wood cells in petrified wood. *Mate Science English*, 25:119-30.

Salmon S., Hundson S.M. (1997). Crystal morphology, biosynthesis and physical assembly of cellulose, chitin and chitison. *Rev Macromol Chem Physics*, 37:199-276.

Shankar T., Mariappan V., Isairasu L. (2011). Screening cellulolytic bacteria from the mid gut of the popular composting earthworm, *Eudrilus eugeniae*. *World Journal of Zoology*, 6:142-148.

Wen Z., Liao W., Chen S. (2005). Production of cellulose by *Trichoderma reesei* from dairy manure. *Technol*, 96:491-499.