International Journal of
Life Science
International Peer Reviewed Open Access Refereed Journal

Int. J. of Life Science, 2019; 7 (3):544-550
ISSN:2320-7817(p) | 2320-964X(o)

Original Article Open Access

Isolation, optimization, characterization of cellulose enzyme production from *Bacillus subtilis*

S. Prabhakar[1], C. Kandeepan[2] and R. Charulatha[3]

[1]Research Scholar, Manonmaniam Sundaranar University, Tirunelveli-627012, TamilNadu, India, sprabhakarrajppa@gmail.com
[2]Assistant Professor and Head, PG and Research Department of Zoology, A.P.A. College of Arts and Culture, Palani-624601, TamilNadu, India
[3]Assistant Professor, Department of Microbiology, Karpagam Academy of Higher Education, Coimbatore- 641021, TamilNadu, India.

Manuscript details:

Received: 22.06.2019
Accepted: 17.09.2019
Published: 30.09.2019

Editor: Dr. Arvind Chavhan

Cite this article as:
S. Prabhakar, C. Kandeepan and R. Charulatha (2019) Isolation, optimization, characterization of cellulose enzyme production from *Bacillus subtilis*, *Int. J. of. Life Science*, Volume 7(3): 544-550.

Available online on
http://www.ijlsci.in
ISSN: 2320-964X (Online)
ISSN: 2320-7817 (Print)

ABSTRACT

Cellulose is considered to be one of the most abundant biopolymers on Earth. Cellulose degrading organism play a vital role in the biosphere by recycling cellulose, the most abundant carbohydrate produce by plant. In this text the present study was conducted with an objective of isolation of cellulolytic organisms from various fields of vellore. The cellulose degrading organisms were screened and characterised. Based on degradation of cellulose the potential organism *Bacillus subtili* sp. was selected for further studies. The strain *Bacillus subtili* sp. was identified as *Bacillus subtilis* based on the physical, biochemical and 16srRNA sequencing. Carboxy methyl cellulose medium (CMC) was the best medium for production of cellulase. The maximum production of cellulase was observed at temperature 30^{0}C and pH 7.5of 137.22 and 137.20 U/L respectively. Overall this study indicates that *Bacillus subtilis* was a potential in degradation of cellulose, which could be used commercially and/or industrially.

Key words: Cellulase, *Bacillus subtilis*, Carboxy methyl cellulose (CMC), Biomass.

INTRODUCTION

Enzymes are delicate protein molecules necessary for life. Cellulose is the most abundant biomass on the earth (Venkata *et al*., 2013) Plant biomass contains cellulose as the major component. Cellulose accounts for 50% of the dry weight of plant biomass and approximately 50% of the dry weight of secondary sources of biomass such as agricultural wastes (Haruta *et al*., 2003). Presently huge amount of agricultural and industrial cellulosic wastes have been accumulating in environment. Cellulose has attracted worldwide attention as a renewable resource

that can be converted into bio- based products and bioenergy (Xing-hua *et al*., 2009). Celluloses are observed as the most important renewable resource for bioconversion. It has been become the economic interest to develop an effective method to hydrolyze the cellulosic biomass (Saraswati *et al*., 2012). Cellulose is commonly degraded by an enzyme called cellulase. This enzyme is produced by several microorganisms, commonly by bacteria and fungi (Immanuel et al., 2006). Cellulase is an important and essential kind of enzyme for carrying out the depolymerization of cellulose into fermentable sugar (Xing-hua *et al*., 2009).

Cellulose occurs in almost pure form in cotton fiber and in combination with other materials, such as lignin and hemicelluloses, in wood, plant leaves and stalks, etc. It has already been used in processing of coffee, in textile industry and in laundry detergents. Cellulose is a long chain polymer, made up of repeating units of glucose, a simple sugar, joined together with β-1,4 glycosidic linkages. Cellulases cause hydrolysis of the individual cellulose fibers to break it into smaller sugars units & finally producing glucose molecules.(Vipul verma *et al*., 2012). Cellulose is the most common organic compound on Earth. It is well known that plants are the most common source of renewable carbon and energy on the earth. Cellulose has no taste, is odourless & is hydrophilic (Ghosal *et al,* 2011). Cellulose is derived from D-glucose units, which condense through β(1→4)-glycosidic bonds (Yakubu *et al*., 2011). Cellulose is the structural component of the primary cell wall of green plants, many forms of algae and the oomycetes. Some species of bacteria secrete it to form biofilms. Cellulolysis is the process of breaking down cellulose into smaller polysaccharides called cellodextrins or completely into glucose units, this is a hydrolysis reaction. Because cellulose molecules bind strongly to each other, cellulolysis is relatively difficult compared to the breakdown of other polysaccharides. Some ruminants like cows and sheep contain certain symbiotic anaerobic bacteria in their normal micro flora, and these bacteria produce enzymes called cellulases that help the microorganism to break down cellulose, the breakdown products are then used by the bacteria for growth. The biological degradation of cellulose has been studied for many years, and a number of cellulolytic enzymes, especially cellulases produced by fungi and bacteria, have been isolated and characterized (Tomme *et al*., 1995).

Cellulose is converted into fermentable sugars by the enzyme cellulase, and cellulase based bio-refinery technologies are versatile and flexible because they utilize cheaper substrates for enzyme synthesis (Mane *et al*., 2007). The ability to degrade cellulose is a character distributed among a wide variety of aerobic, facultative aerobic, anaerobic bacteria. Efforts are going on throughout the world to enhance the production and purity of bacterial cellulases (Sreeja *et al*., 2013). Studying on cellulolytic activity has isolated various bacteria from different environmental sources. (Hatami *et al*., 2008).

Cellulose-degrading enzyme can be used, for example, in the formation of washing powders, extraction of fruit and vegetable juices, and starch processing (Camassola and Dillon, 2007). Cellulase is produced by a large number of microorganisms. They are either cell bound or extracellular. Although a large number of microorganisms can degrade cellulose, only a few of them produce significant quantities of free enzymes capable of completely hydrolysing crystalline cellulose Koomnok, 2005). Cellulases are used in the textile industry for cotton softening and denim finishing; in laundry detergents for colour care, cleaning; in the food industry for mashing; in the pulp and paper industries for drainage improvement and fibre modification, and they are even used for pharmaceutical applications (Cherry, 2013).

MATERIALS AND METHODS:

Isolation of Bacteria

Bacteria were isolated from the soil sample collected different field, Tamilnadu, India. Traditional serial dilution agar plating method was used for the isolation of cellulolytic bacteria. The medium used for cellulolytic bacteria contains 1.0 % peptone, 1.0 % carboxymethylcellulose (CMC), 0.2 % K2HPO4, 1 % agar, 0.03 % MgSO4.7H2O, 0.25 % (NH4)2SO4 and 0.2 % gelatin at pH 7. The Plates were incubated for 48 hours at 30 C.

Microscopic Studies

Gram staining

A loopful of overnight broth culture was subjected to Gram staining procedures and the experts were recorded. Gram staining of freshly grown (incubated for 16 ± 2 h at 37°C) native isolates was carried out as per the standard staining procedure (Cappuccino and Sherman 2002) using the following three processes:

staining with a water-soluble dye called crystal violet, decolorization and counterstaining with safranin.

Biochemical Studies

Catalase production

A loopful of freshly grown (incubated for 16 ± 2 h) culture was smeared on to clean glass slide, to which a drop of 3% H_2O_2 was added and allowed to react for 30 seconds (Cappuccino and Sherman 2002). The presence and absence of effervescence was recorded as catalase positive and negative, respectively.

Oxidase test

A small amount of culture was streaked smoothly on an oxidase disc (*tetra* methyl *para* phenylene diamino dihydrochloride) with an applicator stick. The reaction was assessed by an intense deep purple colour appearing with in 10-60 seconds.

Indole test

The colony from the nutrient agar was inoculated into the indole medium and incubated at 37°C for 24 hours. The formation of red ring upon the addition of 1 ml of indole reagent indicates positive reaction.

Methyl red test

The colony was inoculated into the MR-VP broth tubes and incubated at 37°C for 24-48 hours. Formation of red colour due to the addition of methyl red indicator indicates positive reaction.

Voges Proskauer test

The colony was inoculated into the MR-VP broth tubes and incubated for 48 h (Cappuccino and Sherman 2002). After incubation, Barritt's reagent consisting of a mixture of alcoholic α-naphthol and 40% potassium hydroxide solution was added to the tubes and shaken. The tubes were allowed to stand for 15 min. Development of a deep rose color in the culture 15 min following the addition of Barritt's reagent indicated the presence of acetoin and represented a positive result. The absence of rose color was documented as a negative result.

Citrate utilization test

Aseptically bacterial culture was streaked on Simmons citrate agar slants and incubated for 48 h (Beishir 1991). Positive results were scored for bacterial cultures which showed growth on slant and as well as changing the medium from green to blue (pH indicator). No color change and no growth on the slant was an indication of negative result.

Gelatin hydrolysis

The colony to be tested was was stab inoculated into LB gelatin agar tubes and incubated at 37ºC for 24-48 hours (Cappuccino and Sherman 2002). An uninoculated medium served as a control.The incubated tubes were examined for liquefaction and failure to re-solidify when kept in lower temperature at 4ºC indicates gelatin hydrolysis positive.

Nitrate Reduction test

6 mL of Sterile Nitrate broth was inoculated with the test culture and incubated at 37ºC for 24 h. After incubation alpha-naphthylamine and sulphanilic acid reagents were added. The change in colour of the broth was recorded.

Amylase activity test

Brain heart medium (BHM) amended with starch was used for amylase activity determination.

Starch hydrolysis

Freshly grown test bacterial cultures were streak inoculated on LB agar incorporated with 1% of starch and incubated at 37ºC for 24-48 h. After incubation, the plates were flooded with few drops of Gram's iodine and observed for clear zones around the colony (Cappuccino and Sherman 2002). Presence of clear zone around the colony was scored as positive for starch hydrolysis.

Triple Sugar Iron (TSI) test

Triple Sugar Iron (TSI) slants (M021I, HiMedia) containing three sugars, namely, glucose, lactose, and sucrose, were used for acid and H_2S production test. Acid production after carbohydrate fermentation was detected by the visible change in color from red to yellow.

Physiological tests

Growth at different ph

Aliquots of LB broth with different pH (2.0, 4.0, 6.0, 8.0 and 10.0) were prepared by adjusting the pH of the medium by using 0.1 N NaOH/HCl. After sterilization by autoclaving, the aliquots of sterile LB broth (5 ml) with different pH values were inoculated with 50 µl (10^5-10^6 CFU/ml) of freshly grown (16 ± 2 h) native isolates. The aliquots were then incubated at 37ºC for

24 to 48 h and observed for growth which was indicated by turbidity change.

Growth at different temperature

Aliquots of LB broth (5 ml) were inoculated with 50 µl (10^5-10^6 CFU/ml) of freshly grown (16 ± 2 h) native isolates and incubated at different temperatures (4, 15, 25, 35, 45 and 55°C) for 24-48 h. After incubation the tubes were observed for growth which was indicated by turbidity change.

Growth at different salt concentrations

LB broth tubes prepared with different salt (NaCl) concentrations (4%, 6.5% and 8%) were inoculated with 50 µl (105 -106 CFU/ml) of freshly grown (16 ± 2 h) native isolates and incubated at 37 C for 24 to 48 h. After incubation, growth observation was made based on the turbidity change.

Enzyme production medium

Production medium contained (g/L) glucose 0.5 gm, peptone 0.75 gm, $FeSO_4$ 0.01 gm, KH_2PO_4 0.5 gm, and $MgSO_4$ 0.5 gm. Ten millilitres of medium were taken in a 100 mL conical flask. The flasks were sterilized in autoclave at 121°C for 15 min, and after cooling, the flask was inoculated with overnight grown bacterial culture. The inoculated medium was incubated at 37°C in shaker incubator for 24 h. At the end of the fermentation period, the culture medium was centrifuged at 5000 rpm for 15 min to obtain the crude extract, which served as enzyme source.

Confirmation of cellulose

Confirmation of cellulose-degrading ability of bacterial isolates was performed by streaking on the Carboxy methyl cellulose (CMC) media. Congored(1mg/ml d/w) was used as staining solution and NaCl (0.1M) as a de staining solution. The use of Congo-Red as an indicator for cellulose degradation in an agar medium provides the basis for a rapid and sensitive screening test for cellulolytic bacteria. Colonies showing discoloration of Congo-Red was taken as positive cellulose-degrading bacterial colonies (Wang et al., 2004)

Enzyme Assay

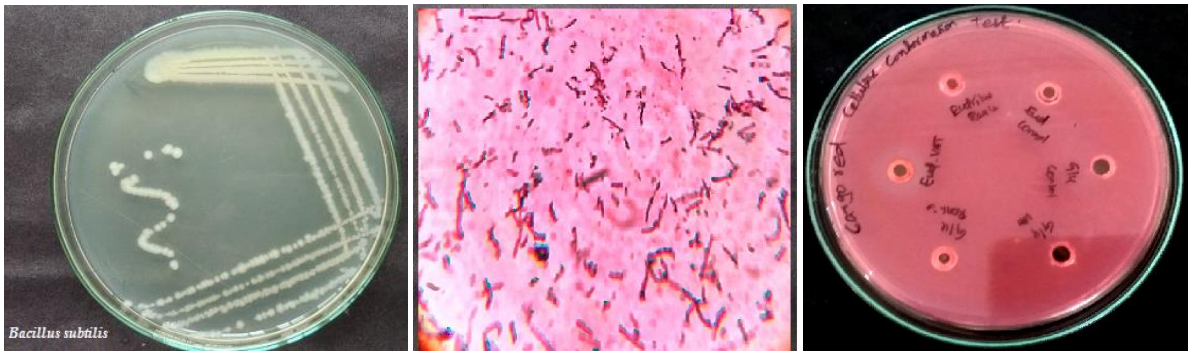
Cellulase activity was measured following the method of (Miller., 1959). Briefly, a reaction mixture composed of 0.2 mL of crude enzyme solution plus 1.8 mL of 0.5% carboxymethyl cellulose (CMC) in 50 mM sodium phosphate buffer (pH 7) was incubated at 37°C in a shaking water bath for 30 min. The reaction was terminated by adding 3 mL of DNS reagent. The colour was then developed by boiling the mixture for 5 min. OD of samples was measured at 575 nm against a blank containing all the reagents minus the crude enzyme.

RESULTS AND DISCUSSION

Biochemical studies

The biochemical tests are given in table-1. the results shows that the isolates are positive for Catalase test, Oxidase test, Voges Proskauer test, Citrate utilization test, Gelatin hydrolysis test, Nitrate Reduction test, Amylase activity test and Starch hydrolysis tests and negative for Indole test and Methyl red test. While for Tripple Sugar Iron (TSI) test, it was acid slant and acid butt without gas and H_2S formation. These results confirm that the isolate is *Bacillus subtilis***.**

The morphological and biochemical characterization revealed the presence of *Bacillus* spp in the samples. All the *Bacillus* isolates were Gram-positive, rod-shaped, spore formers and hydrolyzers of cellulose. The activity of cellulase produced increased from 0 to 48 h with a reducing sugar content of 1.25 mg/m.(Vipul Verma et al., 2012).



;Fig. 1: *Bacillus subtilis*. Microscopic view Cellulose production

Table 1: Biochemical studies of the *Bacillus subtilis*

S.No.	**Characterization Test(s)**	**Result(s)**
01	Catalase production	Positive
02	Oxidase test	Positive
03	Indole test	Negative
04	Methyl red test	Negative
05	Voges Proskauer test	Positive
06	Citrate utilization test	Positive
07	Gelatin hydrolysis test	Positive
08	Nitrate Reduction test	Positive
09	Amylase activity test	Positive
10	Starch hydrolysis test	Positive
11	TSI test	A/A No gas & No black colouration

Table 2: *Effect of different temperature on cellulase production*

S. No.	**Temperature (^oC)**	**Cellulase enyme activity(U/L)**
01	30	137.20
02	40	137.22
03	50	137.16
04	60	90.87
05	70	87.68

Table 3: *Effect of timeprofile on cellulase production*

S.No.	**Time period (Hours)**	**Cellulase Enzyme Activity (U/L)**
01	12	0.14
02	24	10.10
03	36	48.95
04	48	87.64
05	60	102.48
06	72	137.22
07	84	137.01
08	96	130.66
09	108	126.78
10	120	101.34

Table 4: Effect of various salts on the enzyme activity of the isolate *Bacillus subtilis*.

S.No.	**Salts**	**Cellulase Enzyme Activity (U/L)**
01	MgSO4	137.12
02	ZnSO4	137.10
03	MnSO4	137.00
04	FeCl2	136.90
05	CuSO4	137.22

Effect of different temperature on cellulase production

The test tubes containing broth were inoculated with the new isolate *Bacillus subtilis* were incubated at different temperature zones from 30^0 C to 70^0 C and the enzyme activity was assayed. The enzyme activity was maximum at the temperature of 40 to 70^0 C for all the enzymes. The peak activities were 137.20, 137.22, 137.16, 90.87 and 87.68 U/L for the Cellulase respectively. The results are depicted in Table-2.

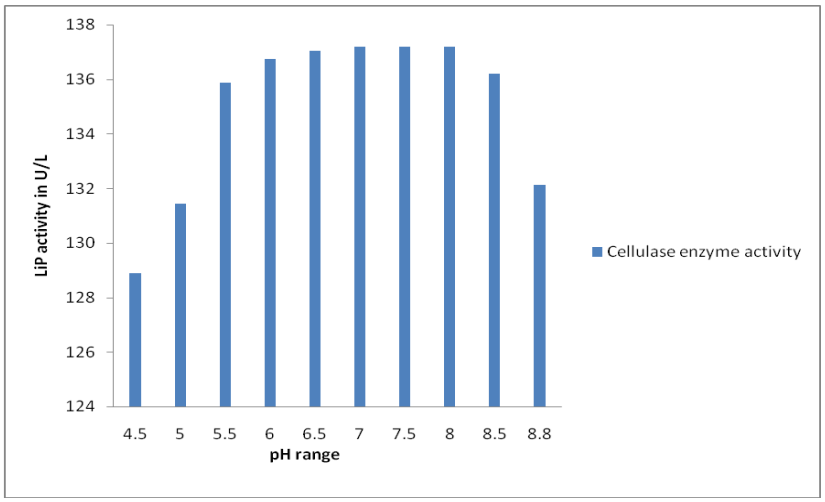


Figure 1: ***Effect of pH on cellulase production***

Effect of timeprofile on cellulase production

The time profile of the newly isolated *Bacillus subtilis*. showed the Cellulase enzyme activity as given in the table 3. The enzyme production varied with respect to time for different enzymes. The maximum Cellulase activity was seen at 72nd hour (137.22U/L).

Effect of various salts on the enzyme activity of the isolate Bacillus subtilis.

The effect of different salts on the growth and enzyme production by the isolate *Bacillus subtilis*. is given in table 4. The results shows that maximum enzyme activity was seen with $MnSO_4$, ZnSO4, MnSO4, FeCl2 and $CuSo_4$ for Cellulase (137.22U/L).

Effect of pH on cellulase production

The organism was grown over wide range of pH values from 4.5 to 8.8. The enzyme maximum activity of 137.20 U/L was observed at the pH of 7.0 and 7.5 for Cellulase enzyme. All the results are shown in fig-1.

DISCUSSION

The optimum pH for endoglucanase activity was found to be 7.5 (0.451± 0.011 IU/ml/min) and stable at pH 6.5 to 9.5. Increasing or decreasing pH beyond this resulted in decline in enzyme activity (Muhammad Irfan *et al*.,2012). Any change in pH caused changes in the enzyme active site. Yin et al. [8] isolated *Cellulomonas* sp. YJ5 showing its optimum pH of 7 and pH stability range of 7.5-10.5. Cellulase enzyme of *B. subtilis* A-54 has optimum pH of 6.5 and stable in pH range of 6.5-8 (Yin *et al*., 2010). According to previous studies, cellulases are active at the pH range of 6.0 to 7.0 from *A. Niger*(Akiba et al., 1995), 5.0 to 7.0 from *Lysobacter* sp. (Ogura *et al*., 2006) and 5.0 to 6.5 from *Bacillus* strains(Mawadza *et al*., 2000). Present fin- dings were significant from *M. circinelloides* (pH 4-7) (Saha *et al*., 2004) and *B. circulans* (4.5-7.0) (Kim *et al*., 1995). This range of pH is important for this enzyme, which can be used, in alka- line environments such as in processing of paper pulp.

The temperature stability result of cellulase obtained from *Bacillus subtilis* and depicted in the table.2 revealed that the enzyme remained stable at 45°C. The enzyme stability declined at temperatures above 50°C. The maximum activity was displayed at 45°C (Saheb *et al*., 2010) with enzymatic activity 15 IU/ml. The optimum pH of the enzyme comes out to be ranging between 6.5 & 7.5(Li-Jung Yin *et al*., 2010) with enzyme activites of 11.5 IU/ml & 12 IU/ml. The optimum substrate concentration for enzyme production came out to be 1.5% with enzyme activity of 20 IU/ml(Vipul Verma *et al*., 2012).

CONCLUSION

For any environment if there is a mixture of these bacteria then there will be great degradation of lignocellulose, which could be used commercially.

Acknowledgement

The authors express their sincere gratitute to the staff members of Abbes Biotech, Coimbatore, Tamilnadu for providing working environment.

Conflict of interest:

The Authors declare no conflict of interest.

REFERENCES

Akiba S, Kimura Y, Yamamoto K, Kumagai H (1995) Purification and characterization of a protease-resistant cellulase from *Aspergil- lus niger*. *J Ferment Bioeng*; 79 : 125–130.

Ghosal *et al.,* 2011) *Der Pharmacia Sinica*, 2 (2): 152-168.

Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar," Analytical Chemistry, vol. 31, no. 3, pp. 426–428. lnte

Hatami S, Alikhani HA, Besharati H, Salehrastin N, Afrousheh M, Yazdani ZJ (2008) Investigation on Aerobic Cellulolytic Bacteria in Some of North Forest and Farming Soils. American- Eurasian J Agric & Environ Sci; 3(5):713- 716.

International Scholarly Research Network. (1-9)29, 2012.

Kim CH (1995) Characterization and substrate specificity of an endo-β- 1,4-D-glucanase I (Avicelase I) from an extracellular multienzyme complex of *Bacillus circulans*. *Appl Environ Mic- robiol*; 61: 959–965.

Li-Jung Yin, Hsin-Hung Lin, and Zheng-Rong Xiao (2010) *Journal of Marine Science and Technology*, 2010, Vol. 18, No. 3, pp. 466-471.

Mane VP, Patil SS, Syed AA, Baig MM (2007) Bioconversion of low quality lignocellulosic agricultural waste into edible protein by Pleurotus sajor-caju (Fr.) Singer. J Zhejiang Univ Sci B 8: 745- 751.

Mawadza C, Hatti-Kaul R, Zvauya R, Mattiasson B (2000) Purification and characterization of cellulases produced by two *Bacillus* strains. J Biotechnol; 83 : 177–187.

Muhammad Irfan, Asma Safdar, Quratulain Syed, Muhammad Nadeem (2012) Isolation and screening of cellulolytic bacteria from soil and optimization of cellulase production and activity. Turkish Journal of Biochemistry–Turk J Biochem. 2012; 37 (3) ; 287–293.

Ogura J, Toyoda A, Kurosawa T, Chong AL, Chohnan S, *et al*. (2006) Purification, characterization, and gene analysis of cellulase (Cel8A) from Lysobacter sp. IB-9374. Biosci Biotechnol Bioc- hem; 70 : 2420–2428.

Saha BC (2004) Production, purification and properties of endogluca- nase from a newly isolated strain of *Mucor circinelloides*. Pro- cess Biochem 2004; 39: 1871–1876.

Saheb *et. al,* (2010) *World Applied Science Journal*, 8(1): 35-42.

Sreeja S.J., Jeba Malar P.W., Sharmila Joseph F.R., Steffi Tiburcius, Immanuel G. and Palavesam A, (2013) Optimization of cellulase production by Bacillus altitudinis APS MSU and Bacillus licheniformis APS2 MSU, gut isolates of fish Etroplus suratensis, Int. J. Adv. Res. & Tech, 2(4):401-406.

Tomme, P, Warren, R. A, and Gilkes, NR (1995) *Advances in Microbial Physiology*, Vol. 37, No. 1, pp. 1-81.

Venkata Naga Raju E, Goli Divakar, T. Rajesh, Akram Ghazi, Asra Pourgharashi (2013) Screening and isolation of cellulase producing Bacteria from dump yards of vegetable wastes. *World journal of pharmacy and pharmaceutical res.:(*1); 428-435.

Vipul Verma, Alpika Verma and Akhilesh Kushwaha. Isolation & production of cellulose enzyme from bacteria isolated from agricultural fields in district Hardoi, Uttar Pradesh, India.

Xing-hua Li, Hua-jun Yang, Bhaskar Roy, Dan Wang, Wan-fuYue, Li-jun Jiang,Enoch Y.Lee S.M and Koo Y.M,The Most Stirring Technology in Future: Cellulase Enzyme and Biomass Utilization. J.Microbiol. Biotechnol, 2009:1(1); 229-233.

Yakubu *et al,* (2011) *Advances in Applied Science Research,* , 2 (6):532-540.

Yin LJ, Huang PS, Lin HH (2010) Isolation of Cellulase-Producing Bacteria and Characterization of the Cellulase from the Isola- ted Bacterium *Cellulomonas* Sp. YJ5. J Agric Food Chem; 58:9833–9837.

Yu-Kyoung Kim, Shin-Chan Lee, Young-Yun Cho, Hyun-Jeong Oh and Young Hwan Ko. Isolation of Cellulolytic *Bacillus subtilis* Strains from Agricultural Environments.

.