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Screening and Characterization of Cellulose Degrading Bacterial Isolates of Waste disposal Site

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ABSTRACT

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Cellulose is an abundant natural biopolymer on earth and most dominating agricultural waste. Cellulosic biomass is a renewable and abundant energy source with great potential for bioconversion to value-added bio-products. Cellulose can be degraded by cellulase producing bacteria which is most important biocatalysts for the industrial process. Under favorable environmental conditions, such enzymes facilitate the degradation of cellulosic material and stored carbon in the form of carbon. These microorganisms are diverse in nature and occur in a different habitat. In present investigation isolates were investigated for cellulose degrading potential. Four isolates V8, C25, V1, V4 were *Bacillus sp.*, *Pseudomonas sp.*, *Pseudomonas sp.* and *Acinetobacter sp.* screened for cellulose degradation. Carboxymethyl cellulose solution was used to analyze the cellulose degradation. Each isolates were examined for cellulose degradation potential with endo β -1, 4 glucanase activity and exo- β -1, 4 glucanase activities. All four tested isolates were showed good positive for both endo and exo β -1, 4 glucanase activity and cellulose degradation potential with 33.33 %, 8.53%, 3.84%, 3.12% weight loss, by isolates V1, V8, V4, C 2 5 respectively.

Introduction

Polymer material like cellulose is most common organic compound existing on earth and plants are major source of renewable carbon and energy. About 200 gigatons of CO₂ are fixed of earth every year and the equivalent amount of organic material has to be degraded approximately 30 % by plants and animals to 70 % by microorganisms. On average, cellulose accounts as 50% of the dry weight of plant biomass. Cellulose is the renewable biopolymer with dominating agriculture

waste [Shanmugapriya *et al.*, 2012; Patagundi *et al.*, 2014; Pandimadevi & Begum, 2013]. Cellulose is one of the most abundant raw materials for microorganisms as food and energy source and these microorganisms play a critical role to degrade cellulose is used to produce energy-yielding product eg. ethanol. Degradation of cellulosic material is depends on the availability of hydrolytic enzymes and microorganisms are one of the prominent sources for the hydrolytic enzyme. These

microorganisms exploited commercially for the production of polymer-degrading enzymes [Coughlan, 1985; Shanmugapriya *et al.*, 2012; Patagundi *et al.*, 2014; Nwagu *et al.*, 2012].

Microbes are very important for human being due to their symbiotic nature, it can serve as food, feed, medicine etc. because of their uncountable use in the different area. It is known as nature's 'master chemists'. Nowadays numbers of microbial species were used to produce beneficial products [Anne and Ann, 2007]. The diverse nature of microorganisms appear in the environment with their special properties i.e. adaptation in adverse environmental conditions, such as extreme temperature, pH and salt concentrations. The distinctive feature of the natural environment is that it offers as an inimitable source of microorganisms. The propensity of existing in adverse climatic conditions comes from biodiversity and adaptation nature of microorganisms. These conditions accelerate the biochemical changes in microorganisms and enhance the enzyme stability and survival rates of microorganisms in adverse environmental conditions. Extreme environmental conditions force the microorganisms to produce new proteins, enzymes that also widely use in industrial methods [Shanmugapriya *et al.*, 2012; Patagundi *et al.*, 2014; Shu *et al.*, 2013; Lone *et al.*, 2014].

Cellulose is the largely occurring natural polymer in the environment. It is mostly found in plants biomass (cell walls) as the structural polysaccharide. Cellulose contributes 35 to 50 percent of plant dry weight. Cellulose is made up of structural units of glucose molecules. These molecules link to each other via β -1, 4 glucoside linkage [Lynd *et al.*, 1999; Zhang and Lynd, 2004]. Degradation of cellulose also

important for glucose production point of view. Glucose is the well known simplest carbohydrate sugar and exploited as the alternative energy source for industrial processes. [Lone *et al.*, 2014] Instead biodegradation and enzymatic hydrolysis the other ways of cellulose hydrolysis is a tedious approach, by mass production and recovery of products. In acid hydrolysis, by-products generate hindrance during the recovery process [Cooney *et al.*, 1978; Fennington *et al.*, 1982; Shanmugapriya *et al.*, 2012].

Microbial degradation of cellulose is contributing much amount of carbon flux to the atmosphere and also important for composting and anaerobic digestion. [Lee *et al.*, 2002] Microbial (bacteria and fungi) originated hydrolytic enzymes are broadly used in industrial processes. Cellulolytic bacteria are well known in natural environment belonging from genus *Bacillus*, *Pseudomonas*, *Acidothermus*, *Cellulomonas*, *Streptomyces*, *Micromonospora*, *Caldibacillus*, *Acetivibrio*, *Clostridium*, *Ruminococcus*, *Spirochaeta*, *Anaerocellum* etc. [Patagundi *et al.*, 2014; Kim, 1987; Bergquist *et al.*, 1999; Bagnara *et al.*, 1987; Bagnara *et al.*, 1985; Wachinger *et al.*, 1989; Gallagher *et al.*, 1996; Godden *et al.*, 1984; Khan *et al.*, 1984; Ljungdahl *et al.*, 1984; Svetlichny *et al.*, 1990; Shanmugapriya *et al.*, 2012; Lin *et al.*, 2012] and microorganisms also reported for the degradation of modified cellulose (CMC), such as *Polyporus arcularius* [Manavalan *et al.*, 2015]. Cellulase one of the prominent family of enzymes that use for cellulose degradation. [Maki *et al.*, 2011] Absolute degradation of cellulose is accomplished *in vitro* by an especial group of enzyme. The group of enzyme involves in hydrolysis of crystalline are (1) Exo- β -1, 4 glucanase (2) Endo- β -1, 4 glucanase (3) β -glucosidase [Bhat, 2000; Sadasivam and

Manickam, 1991]. Exo- β -1, 4 glucanase is the enzyme that initiates the hydrolysis. Exo- β -1, 4 glucanase generating cellobiose molecules by cleaving the bound from non-reducing end of cellulose. β -glycosidase play key role, it hydrolyzes the cellobiose. Microorganisms able to producing exo- β -1, 4 glucanase are capable to hydrolyzing native cellulose (filter paper, cotton etc.). [Shi *et al.*, 2011; Shu *et al.*, 2013; Sadasivam and Manickam, 1991] Carboxymethyl cellulose (CMC) serves as modified celluloses. Carboxymethyl cellulose exploited as substrate to analyses cellulase. [El Ahwany & Mohamed, 2008] Microorganisms have great potential to degrade cellulosic material and such approach needs to the intense research on isolation, characterization of new strains of microorganisms. [Demain and Solomon, 1985] Present research focus on characterization of cellulose-degrading microorganisms. The cost of enzymes also depends on its productivity, if there is suitable strains are available then the productivity is high and cost of enzyme production is economic [Nwagu *et al.*, 2012].

Materials and Methods

Chemicals and Media

Carboxymethyl cellulose (CMC) powders were used (purchased from Merck Specialities Private Limited, Mumbai) in present study.

Screening of Bacterial Isolates for Cellulose utilization

A total 10 bacterial strains were selected for screening, these bacterial strains got from departmental culture collection, which were isolated from waste disposable sites. Selected bacterial isolates were examined

for cellulose consumption as a primary carbon source. These isolates were incubated in nutrient broth for 24 hours at 37 °C to prepared active culture. [Soni, *et al.*, 2009] Overnight grown culture was transfer to the agar plate containing 1 % CMC. Plates were incubated for 5 days to allow bacterial growth and enzymatic activity. After the incubation, plates were visualized to hydrolysis of CMC agar medium. CMC agar plates were flooded 1 % Congo red aqueous solution for 15 minutes at room temperature. Plates were poured off and further treated with 1 M NaCl for 15 minutes. The cellulase activities of bacterial strains were observed by zone hydrolysis [Nwagu *et al.*, 2012; Lone *et al.*, 2014; Pandimadevi and Begum, 2013; Duza and Mastan, 2015; Shanmugapriya *et al.*, 2012].

Biochemical and Molecular Characterization

Out of ten only four isolates V8, C 2 5, V1 and V4 were screened based on experiment. Biochemical characterization was done according to Bergey's manual by using biochemical testing kits (HiCarbo™ Kit KB009, KB003 purchased from Himedia laboratories Pvt. Ltd. India). Molecular characterization was done by using 16S rDNA sequence. PCR (polymerase chain reaction) amplification of 16S rRNA gene was done by using 27f (5' CCAGAGTTTGATCMTGGCTCAG 3') and 1492r (5'TACGGYTACCTTG TTAC GACTT 3') set of bacterial primer. Amplified DNA products was sequenced and blast search on NCBI GenBank. [Patagundi *et al.*, 2014; Soni, *et al.*, 2009]

Measurement of Cellulase Activity

The crude enzyme was extracted from the culture. The samples were taken from incubated broth and centrifuged at 14000 g

for 10 minutes at 4 °C, supernatant was collected for further examination of crude enzyme. This assay conducted to analyze exo β -1, 4 glucanase and endo β -1, 4 glucanase activity. [Shu *et. al.*, 2013; Decker *et al.*, 2003; Nwagu *et al.*, 2012; Shi *et al.*, 2011]

Assay for endo β -1, 4 glucanase

The viscometric method was used to determine the endo β -1, 4 glucanase activities by analyzing the viscosity reduction in CMC solution. The 3 ml of 0.5 % carboxymethyl cellulose solutions was mixed with 1 ml of 0.1 M citrate phosphate buffer solution and 1 ml of crude enzyme extract added with 0.1 ml antibiotic (chloramphenicol cyclohexamide) solution that prevents the sample from microbial contamination. After mixing of all solution with the enzyme, it incubated for 16 hours at 37 °C. Control was prepared by adding denatured enzyme (crude enzyme denatured by boiling) extracts. Incubated samples boiled in a water bath for 2-4 minutes and centrifuged at 8000 g for 20 minutes. Supernatant was tested, measuring and viscosity was measured [Sadasivam and Manickam, 1991].

Viscosity reduction measured by formula

Percentage loss in viscosity (V) = $(T_0 - T / T_0 - T_{H_2O}) \times 100$. Where T_0 is the flow time before incubation, T is the flow time after incubation and T_{H_2O} is the flow time of water [Duza and Mastan, 2015; Sadasivam and Manickam, 1991; Shu *et. al.*, 2013].

Assay for exo- β -1, 4 glucanase

The production of reducing sugar by the exo- β -1, 4 glucanase activities and were determined by 3, 5-dinitro salicylic acid (DNS) method. The 50 μ l enzyme extract

was added to 450 μ l of 1 % carboxymethyl cellulose solutions (1% CMC solution prepared in 0.1 M sodium citrate buffer) and incubated for 15 minutes at 55 °C in water bath. Incubation samples were removed from and 500 μ l DNS reagent were added and boiled for 5 minutes. After boiling 1 ml potassium sodium buffer added on samples. Samples were placed for measuring absorbance at 540 nm by spectrophotometer (Systronics 2201) and compared the absorbance to standard glucose curve. Glucose molecule released per unit mg protein expressed the enzyme activity. [Duza and Mastan, 2015; Sadasivam and Manickam, 1991; Shu *et. al.*, 2013]

Estimation of glucose DNS method

The glucose reducing the 3, 5-dinitro salicylic acid (DNS) and reddish color was produced and its intensity measured at 540 nm by spectrophotometer (Systronics 2201). DNS reagent prepared by dissolving 1 gm 3, 5-dinitro salicylic acid, 200 mg crystalline phenol and 50 mg sodium sulphite in 100 ml of 1 % NaOH. Glucose solution prepared with different concentration (0.01mg/ml to 0.1mg/ml). 1 ml of glucose solution added with 3 ml DNS reagent and placed on the water bath, boil for 20 minutes and purple color developed [Sadasivam and Manickam, 1991].

***In vitro* Biodegradation**

Biodegradation assay by weight loss

A volume of 100 ml of Minima broth Davis w/o dextrose (purchased from Himedia laboratories Pvt. Ltd. India) media amended with pre-weight pieces of Whatman filter paper (pH 7.0 \pm 0.2) into 250 ml Erlenmeyer flask. Flasks were inoculated with 100 μ l aliquots of active culture and incubated at 37 °C degraded samples were removed at

stationary phage and compared the weight loss of control (without bacterial culture). [Soni, *et al.*, 2009; Pandimadevi and Begum, 2013; Nwagu *et al.*, 2012; Lone *et al.*, 2014]

Paper Chromatography

Biodegraded compounds were determined by paper chromatography. Paper chromatography was done by following chloroform:acetic solvent system in the ratio of 7:3. For 30 minutes.

Result and Discussion

The total 10 bacterial cultures were examined and out of ten only four isolates were found to suitable for cellulose degradation. All bacterial strains were originally isolated from polymer contaminated waste disposal sites. The previous researcher reported that in addition of some chemical (eg. glucose, acetic acid, maleic anhydride, ethanol etc.) increases the degradation rate of the natural polymer, synthetic polymer and municipal solid waste. [Singh, B., and Sharma, 2008; Raut *et al.*, 2008; Soni, *et al.*, 2009; Nanda and Sahu, 2010; Gabhane, *et al.*, 2012] In Gram's staining determination isolated V8 was Gram positive while other three (C 2 5, V1 and V4) were Gram negative. All four isolates were characterized biochemically and further by molecular identification. Molecular identification was done by 16S rDNA sequencing and sequence similarity was checked in data base of NCBI-Gene bank. And isolates were identified as *Bacillus* sp. strain V8 (accession numbers- KT860420), *Pseudomonas* sp. strain C 2 5 (accession numbers- KT860422), *Pseudomonas* sp. strain V1 (accession numbers- KT860423), *Acinetobacter* sp. strain V4 (accession numbers- KT860424). Apparently, microbes are divers in nature with different type enzymatic activity

reported for polymer (natural and synthetic) degradation.[Patagundi *et al.*, 2014; Singh, B., and Sharma, 2008; Raut *et al.*, 2008; Soni, *et al.*, 2009; Nanda and Sahu, 2010; Gabhane, *et al.*, 2012; Duza and Mastan, 2015; Pandimadevi and Begum, 2013; Lone *et al.*, 2014] Exploitation of these natural occurring microorganisms is suitable approach to cellulose degradation.

Assay of Enzymatic Activity

Measurement of enzymatic activity was conducted for selected isolates. Hydrolytic activity of endo β -1, 4 glucanase was measured by calculating reduction in viscosity of carboxymethyl cellulose solutions. Bacterial strain V4 showed highest hydrolytic activity in compared to other shown in table 1. Percentage reduction in CMC viscosity by bacterial isolates shown in figure 1. Estimation of endo β -1, 4 glucanase was measured by DNS method. Glucose standard curve was prepared by DNS method shown in figure 2. Production of glucose with chromogenic agents in enzymatic hydrolysis and absorbance was measured at 540 nm by the spectrophotometer. The enzyme activity express as mg glucose released/minute/mg protein and maximum enzyme activity were found with bacterial isolate V1 shown in figure 3 [Sadasivam and Manickam, 1991; Shu *et al.*, 2013].

Biodegradation Assays

Cellulose biodegradation assays were performed by screened isolates with cellulose paper pieces. Pre-weighted paper pieces were added to minimal broth with bacterial isolates. After the one month of incubation, incubation paper pieces were recovered at stationary phases and measured the degradation by calculating the percentage weight loss. The results of

percentage weight loss were shown in table 2. Highest bacterial growth was found at stationary phases. Bacterial isolate V1 showed maximum biodegradation with

33.33 % weight loss. Biodegradation of polymers was further determined by CO₂ emission [Nanda *et al.*, 2010].

Table.1 Determination of viscosity reduction

S.N.	Bacterial Isolates	T ₀	T	Viscosity reduction in CMC solution (%)
1	V8	19.48	2.16	90.87
2	C 2 5	11.06	3.34	72.25
3	V1	9.10	1.06	92.62
4	V4	14.17	1.10	95.05

Table.2 Polymer (cellulose) degradation by bacterial isolates

S.N.	Microbial degradation (weight loss percent)	
	Isolates	Degradation percentage of polymer [%]
1	Control	0
2	V8	8.53
3	C 2 5	3.12
4	V1	33.33
5	V4	3.84

Fig.1 Percentage reduction in CMC viscosity by bacterial isolates.

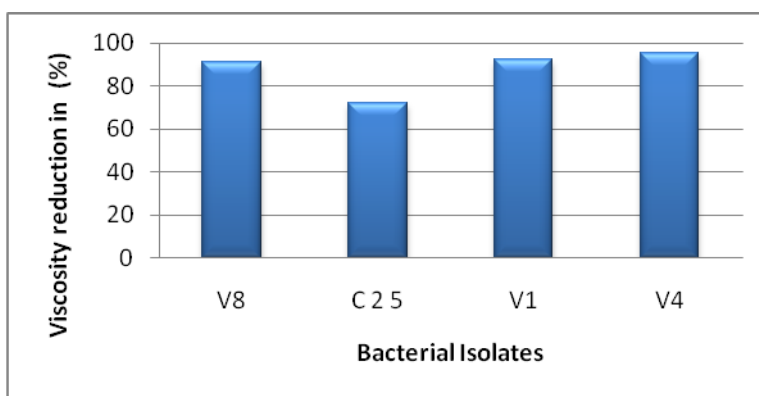


Fig.2 Glucose standard curve

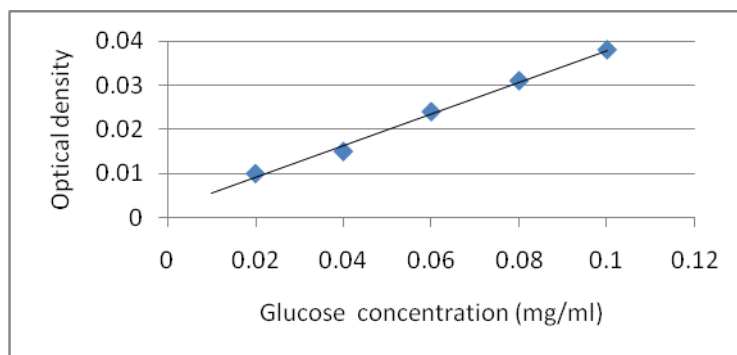
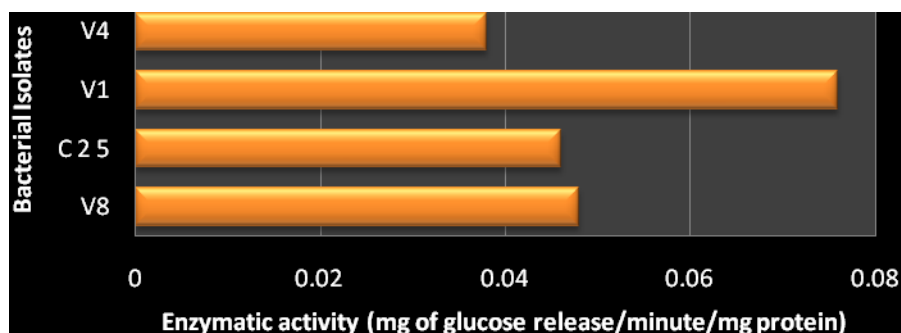


Fig.3 Enzymatic activity (mg of glucose release/minute/mg protein) of bacterial isolates



Paper Chromatography

Paper chromatography was done by chloroform:acetic acid solvent system with the ration 7:3. The R_f value of control with 0.42 and R_f values with 0.68, 0.47, 0.46, 0.45 for treat samples by V1, V8, V4 and C 2 5 respectively.

In conclusion, the present work was carried out for cellulose degradation, characterized screened biochemically and identified by molecular analysis and determined their cellulose degradation efficiency. Out of 10 isolates, 4 bacteria strains were screened and selected to determine their cellulose degradation potential. All four isolates showed exo and endo β -1, 4 glucanase activity and V1 isolated showed maximum degradation. Present studied showed significant results and these bacterial isolates use in future as the promising

approach for cellulose degradation and also for glucose production. Optimization of growth conditions enhances the productivity rate of the enzyme.

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