Screening and Optimization of Cellulase Production of *Bacillus subtilis* TD6 Isolated from *Takifugu rubripes* Fish

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Abstract

Cellulase enzymes have attracted considerable attention in recent years due to their great biotechnological and industrial potential. Cellulase enzymes provide a key opportunity for achieving tremendous benefits of biomass utilization through the bioconversion of the most abundant cellulosic material into the simplest carbohydrate monomer, glucose. Nowadays, the sources of cellulase-producing bacteria have been broadened into the presence of symbiotic bacteria in herbivorous animal and also from marine. *Takifugu rubripes* or known as Puffer fish is a unique poisonous vertebrate but nevertheless is considered a delicacy in Korea. The diet of the puffer fish includes mostly algae. This dietary habit considers Puffer fish as host of cellulase-producing bacteria, especially on its gut. In the present study an attempt has been made to search for the cellulolytic bacteria in the gut of *Takifugu rubripes.* Fifty five microorganisms have been isolated using 1% (w/v) Carboxymethyl cellulose (CMC) as substrate. Congo red dye test and DNS method were then used for screening the extracellular cellulase activity of the strains. Among them, TD6 strain has shown the highest performance in term of cellulase activity. In order to evaluate the optimum culture condition of the isolate TD6 for cellulase production, the strain was grown at various temperatures, pH, carbon sources, and nitrogen sources. Under optimum condition, the maximum specific activity of 2.13 U/mg protein was achieved after growth the strain with 1.5% CMC at 45ºC pH 6 for 3 days, respectively. Based on 16S rRNA gene analysis it is proposed that the strain was identified as *Bacillus subtilis*.

Key words: cellulase-producing bacteria, *Takifugu rubripes*, *Bacillus subtilis*, Carboxymethyl cellulose, DNS method

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Introduction

Cellulose, a polymer of glucose residues connected by β-1, 4 linkages, being the primary structural material of plant cell wall, is the most abundant carbohydrate in nature (Sangbrita *et al.,* 2006). Therefore, it has become considerable economic interest to develop processes for effective treatment and utilization of cellulosic material as inexpensive carbon sources. Utilization of cellulose as a nutrient source requires the enzyme cellulase that cleaves β-1,4 glycosidic bonds in the polymer to release glucose units (Barr *et al.*, 1996). Cellulase is the enzyme that hydrolyzes the β-1, 4-glycosidic bonds in the polymer to release glucose units (Nishida *et al.,* 2007).

Relatively very few animals are able to utilize this cellulosic material eficiently (Goodenough & Goodenough, 1993). Biodegradation of cellulose and hemicellulose, the recalcitrant components of the plant cell wall by the cellulolytic activity of endosymbionts of digestive tract of diverse taxa including termites, shipworms, sea turtles, herbivorous birds, ruminants and reptiles, has been well studied (Sangbrita *et al.,* 2006). However, the scenario of fermentative nutrition in dominant aquatic herbivores, the hydrobionts, remains poorly understood. As the digestive tract of fish is an open system constantly contacting the surrounding water, the microflora of water plays an important role in formation of microflora of the digestive tract of fish (Strom, 1990; Hansen *et al.*, 1992). Being rich in nutrients, the environment of the digestive tract of fish, in comparison with surrounding water, confers a more

favourable growth environment for microorganisms. Recently a diverse microbial community has been reported fromthe guts of various fish (Bairagi *et al.*, 2002).

Fish are unable to produce cellulase endogenously, but they harbour microbial populations in their digestive tracts which help in the digestion of plant materials (Sangbrita, 2006; Bairagi *et al.*, 2002). However, information on proper identification and characterization of these microorganisms is limited (Ghosh *et al.*, 2002). *Takifugu rubripes* or known as Puffer fish is a unique poisonous vertebrate but nevertheless considered a delicacy in Korea. The diet of the puffer fish includes mostly algae. This dietary habit considers Puffer fish as host of cellulase-producing bacteria, especially on its gut.

In the present study an attempt has been made to search for the cellulolytic bacteria in the gut of *T. rubripes* or known as Puffer fish. The strains were isolated using CMC agar medium and tested for their extracellular cellulase activity. The strains were screened on the basis of their performance in terms of cellulase production. One potent strain was selected and identified on the basis of their cellulase activity. This strain might play a significant role in fermentative degradation of cellulose so that plant ingredients fermented with these strains may be incorporated efficiently to formulate cost effective fish feed.

Materials and Methods

Test Fish. The *T. rubripes* fish was divided into two parts of samples: muscle and gut. These samples were washed with buffer solution (10 mM sodium citrate/0.1 M NaCl pH 7) and homogenized using the same buffer.

Medium used. Carboxymethyl cellulose liquid medium (CMC broth) containing 10 g of CMC, 4 g of K_2HPO_4, 4 g of $Na_2HPO_4.2H_2O$, 0.2 g of $MgSO_4.7H_2O$, 0.001 g of $CaCl_2$, 0.004 g of $FeSO_4.7H_2O$, 2 g of tryptone in 1L of distilled water, and adjusted pH to 7 was used as enrichment medium and test for pH and temperature optimation condition. Tryptone Soya Agar (TSA) medium containing 45 g of TSA in 1L of distilled water pH 7. Basal medium I contain CMC broth medium without CMC. Basal medium 2 contain CMC broth medium without tryptone.

Isolation of Cellulase Producing Bacteria. About 1 mL of homogenized fish sample was inoculated into 100 mL vial with 50 mL of CMC broth and incubated at 37°C for 2 days. A 0.1 mL of the culture broth was then taken and poured aseptically with sterilized TSA and CMC agar medium, in duplicate. These culture plates were incubated at 37°C for 48 h. They were then examined for the development of bacterial colonies. The well-separated colonies with apparently different morphological appearance were streaked separately on TSA and CMC agar plates to obtain pure cultures. Single colonies from the streaked plates were transferred to TSA and CMC agar slants. The intensity of cellulase production by the isolated bacterial strains was analyzed on CMC agar plates. Pure cultures of bacterial colonies were transformed individually on CMC agar plates. After 24 hours of incubation the congo red dye was applied. The plates were thoroughly washed with 1M NaCl solution after 30 min. A clear zone formed around the growing colonies of cellulase positive cultures against the red background was taken as the indication of cellulase activity.

Strain Identification. The potential strain was identified based on its 16S rRNA gene sequence. The isolation of genomic DNA from bacteria was done using method modified by Jeff Newman (1998). Primers used in PCR reaction were Primer Bioneer M 0818 (16S-350F) 5’ CCT ACG GGA GGC AGC AGT and M 0819 (16S-820R) 3’ CGT TTA CGG CGT GGA CTA C (Chiang et al. 2006). PCR reagents (PRIME START Hot Star DNA polymerase), 1 Kbp plus 100 bp Ladder marker EBM-1003, 1 Kbp Ladder marker EBM-1002, Gel extraction method was done using QIAquick gel extraction kit protocol using a microcentrifuge (QIAGEN). For cloning, Top cloner Blunt V2 Vector was used. In further step, plasmid purification using Nucleogen Biotechnology Plus Plasmid Mini Kit (400). Sequence data obtained was aligned by using ClustalX (Thompson *et al.*, 1997). Phylogenetic trees were constructed by using distance matrix and boot-strapped distance matrix methods as implemented in the programs of the PHYLIP and TreeView program packages (Page, 2003).

Optimization Culture Condition for Cellulase Production. Optimum culture conditions, including pH (4, 5, 6, 7, and 8), temperature (25, 30, 37, 45 and 50°C), carbon sources (CMC, avicel and α-cellulose), CMC concentration (0.5 to 2%; w/v), nitrogen sources (peptone, yeast extract, tryptone, and $(NH_4)_2HPO_4$) and tryptone concentrations (0.1 to 1.0%; w/v). After 144 hours incubation, culture broths were then centrifuged at 10,000 rpm for 15 minutes to obtain supernatants which were later measured cellulase activity based on DNS method (Ghose, 1987).

Utilizing agricultural wastes as substrate. The agricultural wastes (pineapple peel, corncobs and rice hulls) were washed thoroughly with water and dried in oven at 70°C for three days. It was ground to powder using blender and sieved through different mesh sizes (US standard screen). In all the experiments, the size of particles was kept 500 µm. The selected strain was cultivated on medium broth containing these agricultural wastes (3%; w/v), on optimum pH and temperature. After 72 hours incubation, culture broths were then centrifuged at 10,000 rpm for 15 minutes to obtain supernatants which were later measured cellulase activity based on DNS method (Ghose, 1987).

Cellulase activity. Cellulase activity was measured by the DNS (3,5-dinitrosalicylic acid) method (Ghose, 1987), through the determination of the amount of reducing sugars liberated from CMC solubilized in 50 mM sodium citrate buffer, pH 4.8. This mixture was incubated for 30 min at 50°C and the reaction was stopped by the addition of DNS solution. The treated samples were boiled for 5 min, cooled in water for color stabilization, and the optical density was measured at 540 nm. The cellulase activity was determined by using a calibration curve for glucose. One unit of enzyme activity was defined as the amount of enzyme that released 1 µmol of glucose per minute.

Protein Determination. For protein determination SMART™ Bicinchoninic acid (BCA) Protein Assay Kit for standard assay was used. The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water soluble complex exhibits a strong absorbance at 562 nm.

Results and Discussion

The information regarding the intestinal microbial flora in fish is less and there is paucity of information in the field of microbial enzyme activity in fish gastrointestinal tracts. A few reports concerning microbial cellulase production in the gastrointestinal tract of fish are available. Generally, bacteria are abundant in the environment in which fish live and it is impossible to avoid them being a component of their diet. The bacteria ingested by the fish along with their diet may adapt themselves to the environment of the gastrointestinal tract and form a symbiotic association (Sangbrita *et al.,* 2006).

A total of 55 bacterial isolates were isolated using CMC agar medium from gut of *T. rubripes*. Screening of bacteria was conducted by using congored dye test as a preliminary study and potentiality of the strain was checked by measuring the clear zone formed around the colony. When applied the congo red test, all isolates showed positive results with different ranging of clear zone ranging. Upon further quantitative determination of cellulose degrading enzyme, all 55 isolates displayed activity of cellulase with the highest enzyme activity lied on isolate TD6 (Figure 1). This isolate was found to have zone formation of about 11 mm (Figure 1). Ariffin *et al.,* (2006) described, about 9 bacterial strains (EB1-EB9) obtained from oil palm empty fruit bunch showed positive result in congo red dye test, of which EB3 showed the highest clear zone diameter. *Salinivibrio* sp. Strain NTU-05, isolated from soil taken from around Szutsau saltern exhibited clear zone around the colony on CMC agar plate, indicating that it secretes cellulose degrading enzyme (Wang *et al.,* 2009).

A total of 500bp of the 16S rRNA gene was sequenced and used for the identification of isolated bacterial strain. The sequence of strain TD6 was compared to sequences in nucleotide databases using a nucleotide-nucleotide BLAST program. A 16S rRNA gene sequence based phylogenetic tree showing the relationships between the test strain TD6 and selected representatives of the genus *Bacillus* is given in Figure 2. It is

evident from phylogenetic analysis of 16S rRNA gene that the isolate TD6 represents a genomic species in the species *Bacillus subtilis*. We identified the strain TD6 as *Bacillus subtilis* TD6.

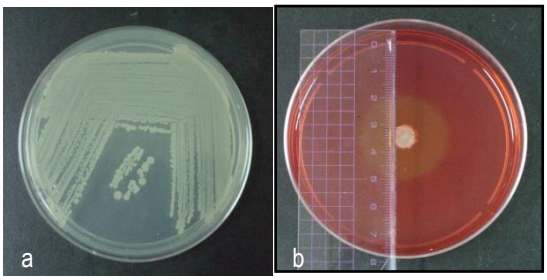

Figure 1. Strain TD6 (a). Samples were incubated at 45°C for 2 days, stained with Congo red and destained with 1M NaCl solution. Clear zone indicated the hydrolysis of CMC as a result of cellulase production (b).

In order to evaluate the optimum condition for cellulase production by *B. subtilis* TD6, the strain was grown at various pH, temperatures, carbon sources, and nitrogen sources. The isolate TD6 could produce highest amount of cellulase when grown in CMC broth pH 6 (4.68 U/mL). The enzyme activity was reduced when grown at lower or higher pHs (Figure 3). On the comparison to other organism, the optimal pH value of other cellulolytic organisms varied from similar optimal pH value such as *Bacillus* strain CH43 and HR68 (pH 5-6.5) (Crispen *et al.*, 2000); an acidic condition such as *Trichoderma reesi* strain QM-9414 (pH 3.5) (Krishna *et al.*, 2000), and *Trichoderma harzianum* (pH 5.5) (Sibtain *et al.*, 2009); a neutral condition such as *Aspergillus niger* and *A. nidulans* (pH 7.0) (Usama & Hala, 2008) to alkaline conditions such as *Bacillus* sp strain KSM-s237 (pH 9.0-12.0) (Hakamada *et al.*, 1997).

The effect of temperature on the cellulase production by strain TD6 was determined at various temperatures ranging from 25°C to 50°C at pH 7.0 (Figure 4) by using CMC broth. The isolated strain in this study was able to survive over a broad range of temperature. The most significant cellulase production was observed between 30°C and 45°C with maximum activity at 45°C (4.1 U/mL). Therefore, the isolated strain was classified as mesophilic strain. It was reported that the optimum temperature for cellulase production by *Pseudomonas fluorescens* was 35°C (Bakare *et al.*, 2005). In the case of *Bacillus* sp CM120-1, maximum production of cellulases occurred at 45°C (Krairitthichai & Thongwai, 2008).

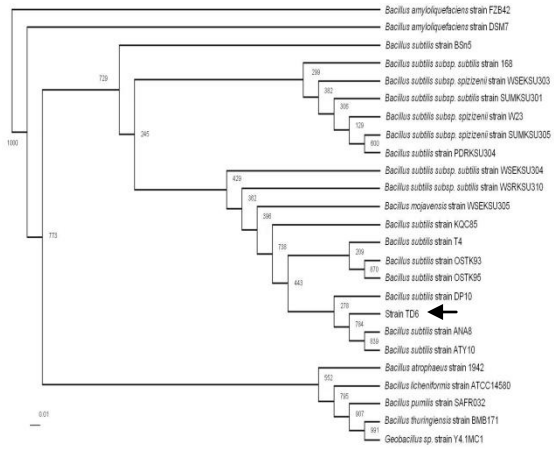

Figure 2. A neighbor-joining tree showing the phylogenetic position of the strain TD6 based on the 16S rRNA gene sequences. Bar, 0.01 subtitution per position. Bootstrap values are shown in 1000 replicates.

Different cellulose types, CMC, micro-crystalline cellulose (avicel), and α-cellulose were added separately to basal medium 1 as a sole carbon source. They resulted in cellulase activity (U/mL) of 4.21, 1.98, and 2.21 respectively. The maximum cellulase yield was obtained with CMC, while the minimum production was found in the presence of avicel, this may be explained on the basis of absorption of the enzymes onto cellulose (Figure 5). CMC was also required for the cellulase production by members of genus *Bacillus* (Femiola *et al.*, 2008). Various concentrations (0.5, 1.0, 1.5 and 2.0%; w/v) of high viscosity CMC were added to the medium. The best results were obtained with 1.5% of medium viscosity of CMC (Figure 6). It was reported that 1% (w/v) CMC resulted in highest cellulase production (0.79 IU/mL) on *Trichoderma harzianum* (Sibtain *et al.*, 2009).

Organic (yeast extract, peptone, tryptone) and inorganic $(NH_4)_2HPO_4$ compounds were added to the basal medium 2 to measure their effect on cellulase activity. They showed that all examined compounds stimulated the growth and cellulase activity. The organic compounds stimulated higher cellulase yields compared with inorganic compound. The highest production, 3.99 U/mL respectively, was obtained with tryptone while the lowest,

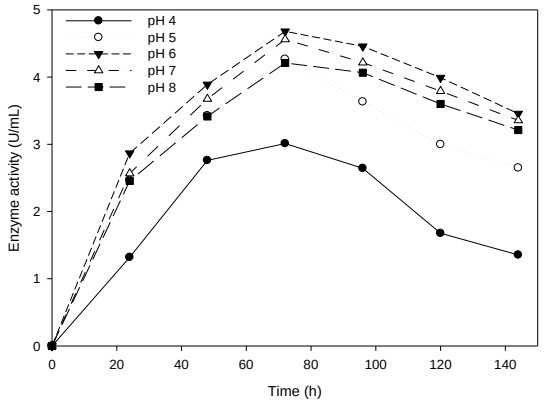

Figure 3. Effect of various pH on cellulase activity of the isolate *B. subtilis* TD6.

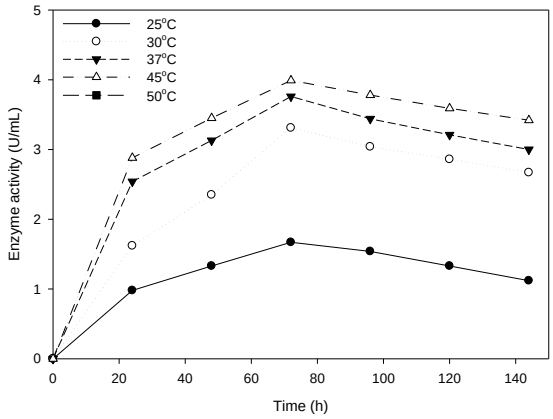

Figure 4. Effect of various temperature on cellulase activity of isolate *B. subtilis* TD6.

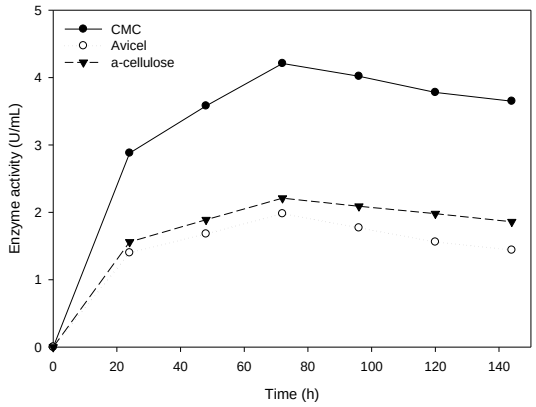

Figure 5. Effect of various carbon sources on celluase activity of the isolate *B. subtilis* TD6.

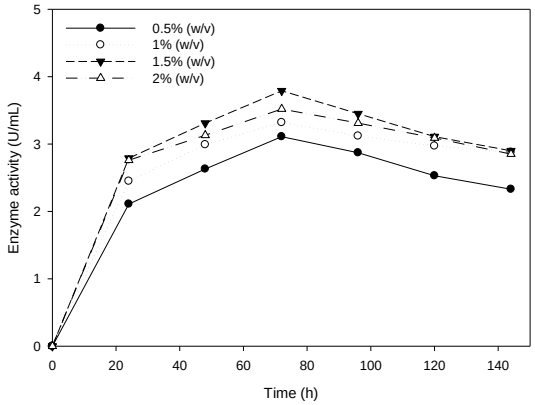

Figure 6. Effect of various CMC concentration on cellulase activity of isolate *B. subtilis* TD6.

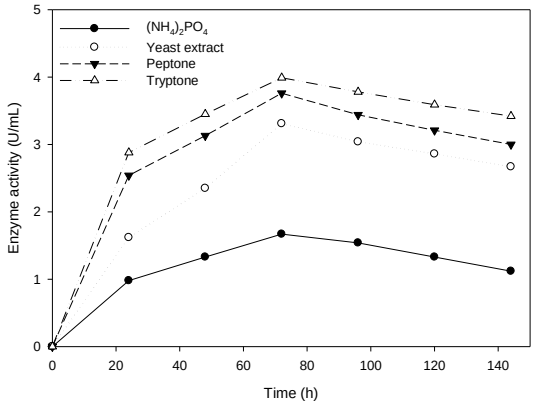

Figure 7. Effect of various nitrogen sources on cellulase activity of the isolate *B. subtilis* TD6.

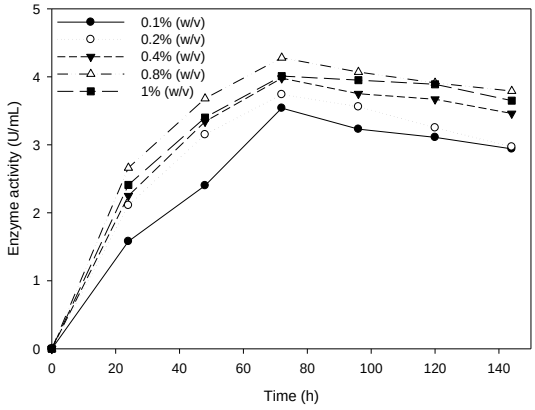

Figure 8. Effect of various tryptone concentration on cellulase activity of isolate *B. subtilis* TD6.

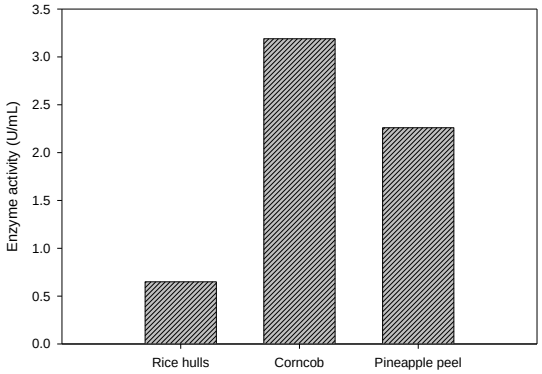

Figure 9. Profile of CMCase production by *B. subtilis* TD6 on agricultural wastes.

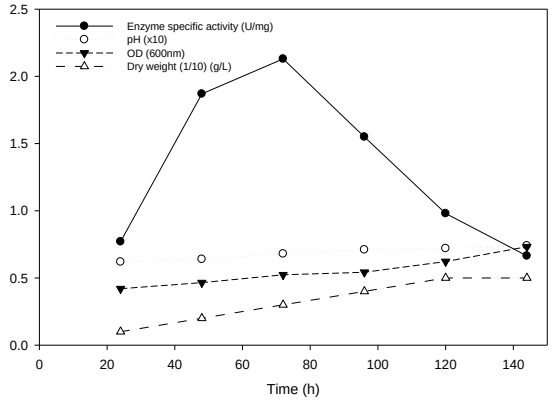

Figure 10. Fermentation profile of *B. subtilis* TD6 under optimum condition.

1.67 U/mL, was resulted by using $(NH_4)_2HPO_4$ (Figure 7). Higher concentrations of tryptone in the culture media were followed by an increase of cellulase activity. The optimum cellulase activity, 4.28 U/mL, was obtained by using 0.8% (w/v) tryptone (Figure 8).

It was found that at optimum condition, pH 6, 45°C, CMC 1.5% (w/v), tryptone 0.8% (w/v) and incubation time for three days, the isolate TD6 had the highest specific activitiy values of 2.13 U/mg protein, respectively. After 96 hours of cultivation, the enzyme activity was decreased (Figure 9). Hydrolysis rate declines with time due to product inhibition and enzyme inactivation (Newman, 2009). On comparison with other sources, *Bacillus thermoalcalilphilus* (Sarkar & Upadhyay, 1993) showed the specific cellulase activity of 0.63 U/mg protein respectively. Heck *et al* (2002) showed the specific cellulase activity of *Bacillus* sp BL16 and *Bacillus subtilis* BL62 0.89 and 1.08 U/mg protein, while Sibtain *et al* (2009) showed the specific cellulase activity of *Trichoderma harzianum* was 2.25 U/mg.

The use of purified cellulosics as substrates is uneconomical for large scale production of cellulase. Therefore cheaply available agricultural lignocellulose wastes were tested to find out whether they could support the production of cellulase by *B. subtilis* TD6 at 3% (w/v) level. The *B. subtili*s TD6 was capable to produce cellulase by using agricultural wastes. The results obtained showed that corncob stimulated higher activity than other agricultural wastes which was 3.19 U/mL. The cellulase activity obtained by using pineapple peel was 2.26 U/mL while for untreated rice hulls, was 0.65 U/mL (Figure 10).

Conclusion

Strain TD6 was isolated from *Takifugu rubripes* gut and identified as *Bacillus subtilis* TD6. The strain produced a cellulolytic enzyme capable of hydrolyzing cellulose into glucose. The enzyme activity was highest in the presence of CMC as carbon source, and tryptone as nitrogen source. After incubation in optimum condition (pH 6, temperature 45°C, CMC 1.5% (w/v) and tryptone 0.8% (w/v)), the highest cellulase specific activity was 2.13 U/mg protein, respectively. *B. subtilis* TD6, also proved to utilize agricultural wastes for cellulase production.

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