A Novel Strain of *Planomicrobium* Isolated from Paper Mill and its Capacity of Cellulose Degradation

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A novel strain, designated WH2-56, was isolated from a slime sample collected from a paper company along the Yangtze River during March, 2018. Phylogenetic analyses based on 16S rRNA gene sequences revealed that strain WH2-56 was related to members of the genus *Planomicrobium*. Cellulolytic activity of the sample was screened and confirmed by Congo red-polysaccharide interactions and examined by broth culture using filter paper (FP) with no starch as the sole carbon source. Field emission scanning electron microscopy (FE-SEM) was used to confirm the delicate morphological changes of FP during bio-degradation. Different cellulosic materials were used to measure biodegradation effects and optimum incubation conditions. The activity of FPase and carboxymethyl cellulase (CMCase) were checked by 3,5-dinitrosalicylic acid (DNS agents) with different carbon sources, which showed a peak at 0.62 U/mL of CMCase on day 4, and at 0.38 U/mL of FPase on day 5.

Keywords: Cellulose degradation; Carbon metabolism; Planomicrobium; Cellulase activity

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INTRODUCTION

Planomicrobium were isolated and proposed 100 years ago, variously from the Korean traditional fermented seafood, Antarctic Sea ice, coastal sediment, frozen food, cold desert, glacier, diesel oil, and sulfurous spring (Van and Kazuo 1985; Junge *et al*. 1998; Yoon *et al*. 2001; Romano *et al.* 2003; Dai 2005; Mayilraj *et al.* 2005; Zhang *et al.* 2009; Das and Tiwary 2014). These are extreme, hypersaline, and humid environments, indicating the high tolerance of the strain toward its environment. In different growth phases of the genus, its shape transforms into cocci, rods, or a mixture of both. In addition to the remarkable activity of lipase, protease, and the stable protection for plants from heavy metals and organic contaminants (Liu *et al*. 2013; Das and Tiwary 2014; Jin *et al.* 2016; Teymouri *et al.* 2016), its biodegradation potential on cellulose can also highlight the importance of the functions of ecosystem services that this genus provides. There has been a lack of attention to such issues in the published literature.

Paper mills are an appropriate and adaptable environment for providing bacteria and fungus on development, reproduction, and nutrition (Pokhrel and Viraraghavan 2005; Roest K *et al.* 2005; Zwieten *et al.* 2010; Shi *et al.* 2018; Su *et al.* 2018; Zainith *et al.* 2019). The present work represents the first report of *Planomicrobium* isolated from a paper machine along the Yangtze River in China, and it was expected to have

biodegradation ability. There are diverse protocols to efficiently isolate and characterize cellulolytic microorganisms. One common method employs carboxymethyl cellulose with dye (CMC-Congo red) and filter paper, used as sole carbon source, which is degraded by cellulolytic microorganisms (Pointing 1999; Kameshwar and Qin 2018).

Cellulose, a linear polymer of 500 to 15000 glucose units that are linked by β-1,4 bonds, constitutes up to 45% of the dry weight of biomass (Pérez *et al.* 2002; Young *et al.* 2014; Mathews *et al.* 2015); it comprises a large proportion of agricultural waste (Lynd *et al.* 2002). For environmental protection, the use of renewable resources as substitutes for nonrenewable ones is of strategic significance (Fan *et al.* 2017), especially the reutilization of cellulose-rich agriculture wastes, which have massive annual production (Guo *et al.* 2010). Its linear chains and microcrystalline structure make cellulose a resistant material (Hamid *et al.* 2015). As the interest in developing cellulose-based material has increased, research exploring cellulolytic microorganisms has expanded; these microbes play a key role in maintaining all significant nutrient cycles (Chen 2014), especially the global carbon cycle (McDonald *et al.* 2012). Moreover, cellulose biodegradation is perceived as an indispensable process to replenish the carbon content of soil (Feng *et al.* 2007; Maki *et al.* 2009; Stursova *et al.* 2012).

Biodegradation of the complex polymer is facilitated by diverse cellulase enzyme polymer produced by microorganisms. For example, bacteria use diverse cellulolytic enzymes to achieve the degradation of cellulose for using it as the primary source of their main biochemical cycles, and in general the carbon cycles. Microbiological and enzymatic treatment is expected to be safe, green, and environmentally friendly (Bilal *et al.* 2017), but it is influenced by the strain type, conditions of cultivation, and carbon sources in the medium (Niranjane *et al.* 2007; Gao *et al.* 2008). Even bacteria with low cellulolytic capacity can be activated in the presence of an easily metabolized carbon source (Amore *et al.* 2013; Yamakawa *et al.* 2013; Raulo *et al.* 2016), which is due to the gene expression patterns of their enzymes.

In this study, three pure cellulosic materials—filter paper (FP), microcrystalline cellulose (MC), and 1% hardwood pulp (HP)—and two agricultural wastes with a different content of components—wheat straw (WS) and corn stalk (CS)—were selected to be the sole carbon source to evaluate the utilization and enzyme activities.

EXPERIMENTAL

Slime Sample and Isolation Procedure

Microbes diversity can have subtle differences with different longitude and latitude during different seasons (Tirado *et al.* 2016). To collect samples as completely as possible, slime samples were collected from four pulp mills of different geographic position along the Yangtze River and different end-product including cultural, living, and wrapping paper. Also, the sampling time were selected in March, June, July, and December of 2018. Samples were immediately placed on ice and later stored at 4 °C.

Congo Red Isolation, Purification, and Screening

Congo red dye can be use for a quick and clear method to screen cellulolytic microbes (Taha *et al.* 2015). Congo red medium was composed as follows (Teather and Wood 1982; Kasana *et al.* 2008; Gupta *et al.* 2012): sodium carboxymethyl cellulose, 5.0 g; ammonium sulfate, 2.0 g; potassium dihydrogen phosphate, 1 g; heptahydrate

magnesium sulfate, 0.5 g; sodium chloride, 0.5 g; sodium nitrate, 1 g; peptone, 0.5 g ; yeast extract, 0.5 g; Congo red, 0.2 g; agar, 13 g, distilled water 1 L; pH 7.

The isolation procedure was performed using cultures from the paper slime samples that showed Congo red-decolorizing activity. The cultures in which decolorization were observed were subsequently isolated and transferred to fresh medium. For purification, the transfer was repeated several times until the isolates were pure in morphology. Isolates were inoculated onto three spots to the Congo red dye medium, within a certain distance per plate, and then incubated at 37 °C for three days. Cellulose degradation was identified *via* a legible transparent zone around the colony on a red background (Lu *et al.* 2004; Sangma and Thakuria 2018). The hydrolysis capacity (HC) was proportional to the value of the ratio between the diameter of the zone and colony, which can screen better-performance ones. A higher ratio indicates higher potential hydrolysis capacity.

Morphologies of FP

Filter paper (FP) was used to make a further examination. The medium recipe was similar to the medium above, which utilize FP to replace CMC-Na as the sole carbon source, with the dye and agar removed. Erlenmeyer flasks (250 mL) containing 100 mL of autoclaved (16 min, 120 °C) medium were inoculated with 5% (v/v) pregrown culture (0.74 mg protein/mL) in log phase. The flasks were incubated at 37 °C on a rotary shaker (150 rpm) for a week. The surface morphological transformations of FP inoculated by the strains for 3 and 6 days were checked by field emission scanning electron microscopy (FE-SEM, JEOL, Tokyo, Japan) to identify the structural changes of cellulose during the cultivation, while the uninoculated FP group was used as control.

Biodegrading Efficiency by WH2-56

Metabolic and cellulase activity

Isolates were pregrown to an optical density at 600 nm of 1.0, then washed in M9 buffer twice before being inoculated into the fermentation broth (100 mL in 250 mL conical flasks and orbital shaking at 180 r/min). The fermentation broths were the same as Congo red medium, with no dye or agar. Crude enzymes were obtained every 24 h by taking samples from the cultures and centrifuging at 6000 r/min for 15 min at 4 °C to remove suspended solids. The supernatants were filtered through a sterile 0.22 μm pore polyvinylidene fluoride membrane and then used as the base to monitor the activity of total cellulase activity (FPase) (King *et al.* 2009; Oszust *et al.* 2017) and carboxymethyl cellulase (CMCase) (Miller *et al.* 1960; Zhao *et al.* 2008) by 3,5-dinitrosalicylic acid (DNS agents) with different substrates, which showed the cellulose-degrading capacities of the microbes. The standard curve was constructed *via* 1g/L glucose, and the absorption subsequently determined at 550 nm. The tube with no crude enzymes was used as blank. All tests were replicated three times. Activities of the enzyme were expressed by international units (U), which were defined as the amount of cellulose needed per minute to produce 1 μmol glucose. All assays were carried out in triplicate.

The metabolic activity of microorganisms was measured through the ratio of the respiration (OD 490 nm) and mycelium growth (OD 750 nm), which compares the biomass development and the metabolic activity (Pinzari *et al.* 2014, 2016). The cultures were taken off for measuring the optical density of OD 490 nm and OD 750 nm every 24 h during the incubation period.

Weight loss ratio

Three different cellulosic material, hardwood pulp (HP), microcrystalline cellulose (MC), and FP were prepared to a final concentration of 1% in the broth medium. Pre-grown WH2-56 was inoculated 2% (v/v) to the different cellulosic media. In consideration of the influence of environmental parameters, each conical flask was prepared at pH 7.0 and incubated at different temperatures (28, 37, and 45 °C), adjusted to different pH (6, 7, 8), and incubated at 37 °C for a week. All broth media were taken out every 24 h for measurement of pH, enzyme activities, and the concentration of total protein, which was measured by ultraviolet spectrophotometry at 280 nm. After one week of incubation, all cultures were filtered, and the residual cellulose was washed with diluted hydrochloric acid and heated at 60 °C until it reached a constant weight. The weight loss ratio (*V*) was calculated as follows,

$$V = \frac{w^* - w}{w^*} \quad (1)$$

where *w* is weight of residue cellulose after a two-week incubation and *w** is the original addition of cellulosic material.

Using different biomass material as carbon source

To evaluate different carbon sources utilization and enzyme activities, agricultural wastes with different cellulose contents were added to the carbon source choices in subsequent operations. As the FP medium recipe mentioned before, wheat straw (WB), corn stalk (CS), and glucose (used as a control, CTRL) were used as the sole carbon source (1%w/v) to measure the cellulolytic ability of WH2-56. Wheat straw and corn stalk were washed, dried, and cut to 2 to 3 cm length before using the Wiley mill. Particles of sizes of 40- to 60-mesh were collected in sealed plastic bags at room temperature. WH2-56 were inoculated to the broth medium with 5% inoculum, and uninoculated samples were used as controls. The analyses were performed in analytical triplicate for each of the biological duplicate experiments (n = 6). After a two-week incubation, the components (cellulose, klason lignin, acid solution, and ash) of the biomass were determined as described previously (Taniguchi *et al.* 1982; Zhu *et al.* 2009), and the enzyme activities were measured every 24 h.

16S rDNA Sequence Analysis

The DNA extraction was achieved by kit (Bioteke, Nanjing, China), and then stored at -20 °C for further analysis. The 16S rDNA fragment was amplified by polymerase chain reaction (PCR) with a pair of universal primers, 27f and 1392r. The PCR mixture was composed of 5 µL of 10* Ex Taq buffer, 4 µL of dNTP, 3 µL of DNA extract as a template, 2 µL of each primer (10 µM), and 0.5 µL of rTaq, and water up to a volume of 50 µL. The amplification protocol was as follows: initial denaturation at 94 °C for 5 min; 33 cycles of denaturation at 94 °C for 30 s, primer annealing at 55 °C for 30 s, and extension at 72 °C for 1.5 min; and final extension at 72 °C for 7 min. The PCR product was subjected to electrophoresis on a 1% agarose gel, and the 1200 bp band was purified by a PCR purification kit (Tiangen, Beijing). The sequencing procedure was achieved by GenScript Biotech Corp., Nanjing, China, and the data was compared to the NCBI database in BLAST (http://www.ncbi.nlm.nih.gov/BLAST).

RESULTS AND DISCUSSION

Isolation, Purification and Screening

After inoculating to the Congo red-CMCNa plate for 3 days, isolates WH2-56 showed a great performance with a high value of the diameter ratio, which means a higher degradation capacity of cellulose. An automated colony-counter was used to take high-definition photos (Fig. 1a) of the plates, which made the legible transparent zone around the colony easy to observe. WH2-56 had a smaller colony and larger transparent zone than other strains; its high ratio (average 16.79 as) identified it as a potential cellulolytic genus. Single colonies of WH2-56 inoculated to LB plate (Fig. 1b) were circular, smooth, slight convex compared to the edge and pale orange in color.

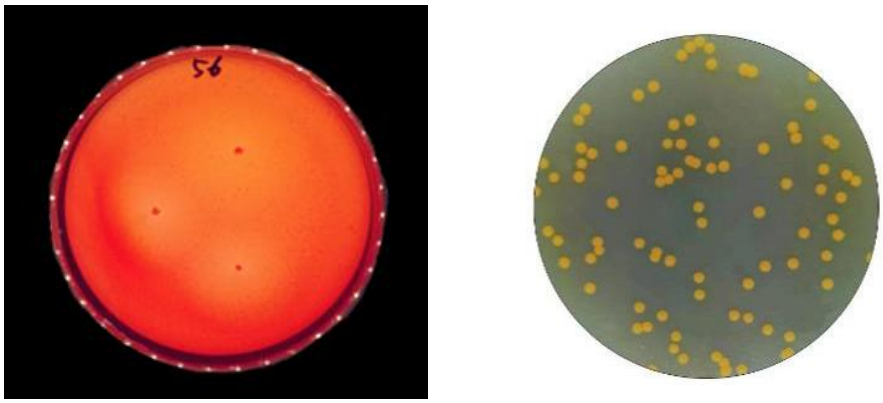

Fig. 1. Photos of WH2-56 (a) after 3 days incubation in Congo red plates by automated colony-counter; colonies cultured on LB plate (b)

To avoid the ambiguity caused by visual interpretation of FP surface (Pointing 1999), FE-SEM was employed for a precise distinction result. FE-SEM images of the control fiber showed a surface with flat and close edge, while the experimental ones showed an embossed, serrated, and cracked state, especially on the sixth day, which means the growth of WH2-56 opened up the space of pores between the fibers of FP (Fig. 2). Because FP was the sole carbon source in the medium, the activities of cellulase enzyme were better activated and much easier to observe through FP degradation. This phenomenon verified the fact that isolate WH-56 broke the surface and structure of cellulose, which confirmed its ability to degrade cellulose.

Biodegradation of Cellulose by Wh2-56

When comparing the different carbon sources, the microcrystalline cellulose group performed a higher degradation effect among the three cellulosic materials, while the filter paper group showed weaker degradation. This result may reflect the contact area between isolate and cellulose fibers, and the content of cellulose of different materials. Environmental parameters influence the degradation efficiency and the growth of cultures. With initial concentration of 10.0 g/L of carbon source, there was an increased removal ratio of microcrystalline cellulose up to 52.1% at pH 6.0. The optimum pH value was around 6.0 at a constant incubation temperature (Fig. 3a). In line with the pH value, the best temperature for biodegradation was 37 °C, which was related to the growth of WH2-56.

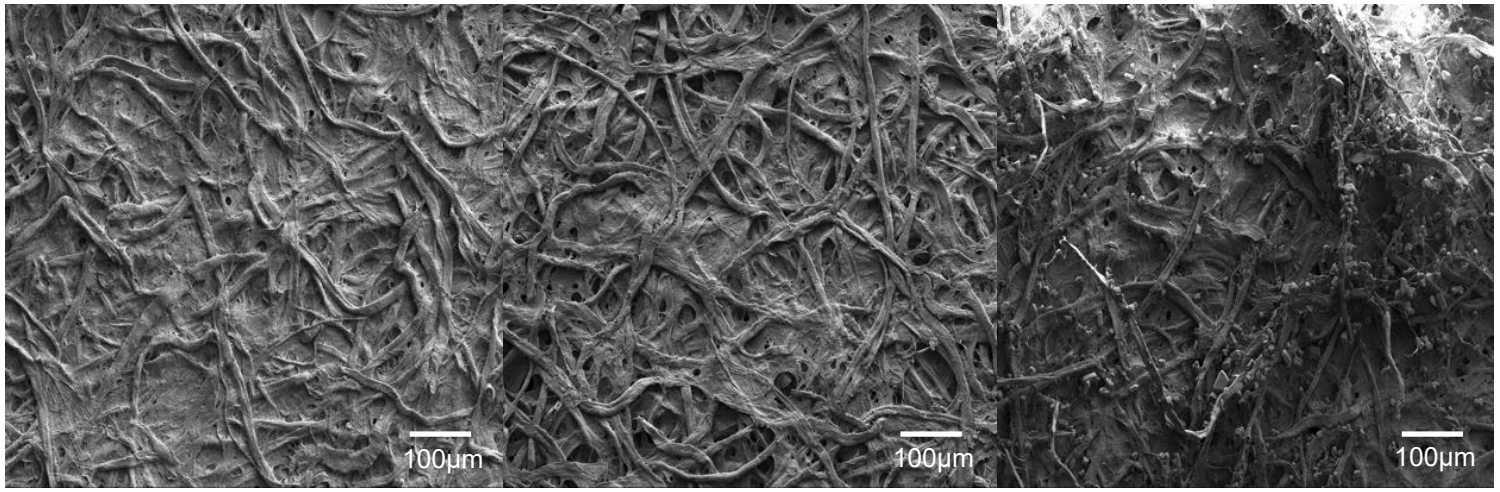

Fig. 2. FE-SEM images of FP samples of the control (a), inoculated by WH-56 after 3 days (b) and 6 days (c). The magnification was 100 times.

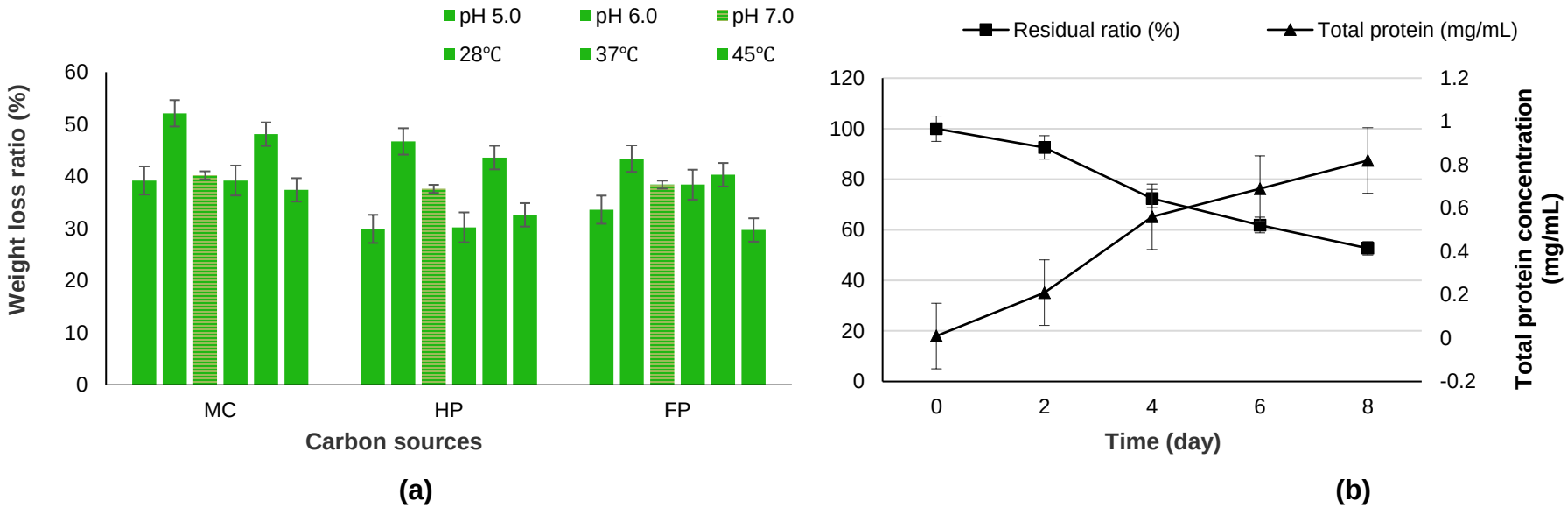

Fig. 3. (a) the weight loss ratio of three carbon sources in different incubation condition of pH and temperature; (b) time course of biodegradation of microcrystalline cellulose and protein concentration under optimized incubation condition (37 °C, pH 6.0). Error bars indicate 95% confidence intervals

The residual ratio of cellulose and total protein concentration in the optimum conditions (37 ℃, pH 6.0) are shown in Fig. 3b. As time went by, the degradation ratio was in proportion to the protein content of the culture, which reflected to the effect of enzyme efficiency. Maximum changing rates were occurred on day 3, both the protein concentration and degradation ratio exhibited a sharp increase, which may reflect the enzymatic and metabolic activities. Residual ratio of cellulose reached 52.7% on day 8, while total protein concentration increased to 0.82mg/mL.

As for the selected agricultural wastes, native corn stalk was composed of cellulose (39.3%), hemicellulose (24.8%), Klason lignin (20.9%), ash and others (10.2%), and acid-soluble lignin (4.8%), while wheat straw was 37.1%, 27.8%, 14.6%, 11.2%, and 9.3%, respectively. After incubation, all the main components of agriculture fuel had partially degraded (Table 1), which resulted from the growth of WH2-56 and carbon source utilization. In corn stalk groups, 18.4% cellulose, 13.3% hemicellulose, and 7.3% Klason lignin were degraded, indicating that WH2-56 can promote the degradation of other components in agricultural wastes.

Table 1. Residual Amounts of Components of Carbon Source after Fortnight's Incubation

Carbon Source	Residual Amounts (%)			
	Hemicellulose	Cellulose	KL	AL
wheat straw	90.5	88.2	95.4	87.0
corn stalk	86.7	81.6	92.7	84.1

*The initial agriculture waste content was 10 g L^{-1}. KL, Klason lignin; AL, acid soluble lignin.

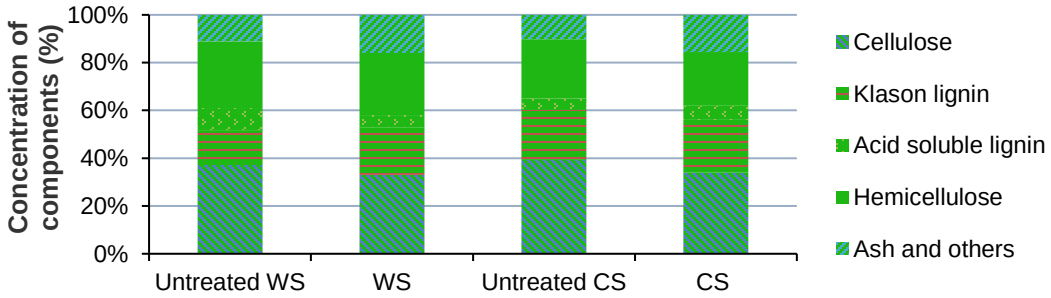

Fig. 4. Percentage changes of composition of component of agricultural waste after two-week incubation

The changes in the component content of WS (wheat straw) and CS (corn stalk) were clear. Compared with initial state, cellulose percentages of the total weight of WS and CS decreased 4.1% and 5.3% respectively, while ash increased 4.8% and 5.3% accordingly, which revealed the biodegradation by WH2-56 (Fig. 4). Cellulose content of the two agriculture wastes decreased twice as much as the hemicellulose, which indicated that the biodegradation of WH2-56 is selective and potential.

Metabolic and Cellulase Activity

FPase and CMCase were detected by DNS agent with FP-buffer and CMCNa solution as carbon source, respectively. Analyzing each enzymatic activity separately, the total cellulase enzyme (FPase) achieved a more steady fluctuation. The low FPase activity may related to the species-specific and carbon source-difference characteristics of cellulase

(Wen *et al.* 2005); wheat bran as carbon source acquired positive results on FPase activity (Brijwani *et al.* 2010; Oberoi *et al.* 2010; Dhillon *et al.* 2011). The reaction between mineral substances and mediums, the nature of carbon source which added to the medium and the energy-consuming process of enzymatic products may lead to different degrees on inhibition in expression of relevant enzymatic genes (Amore *et al.* 2013).

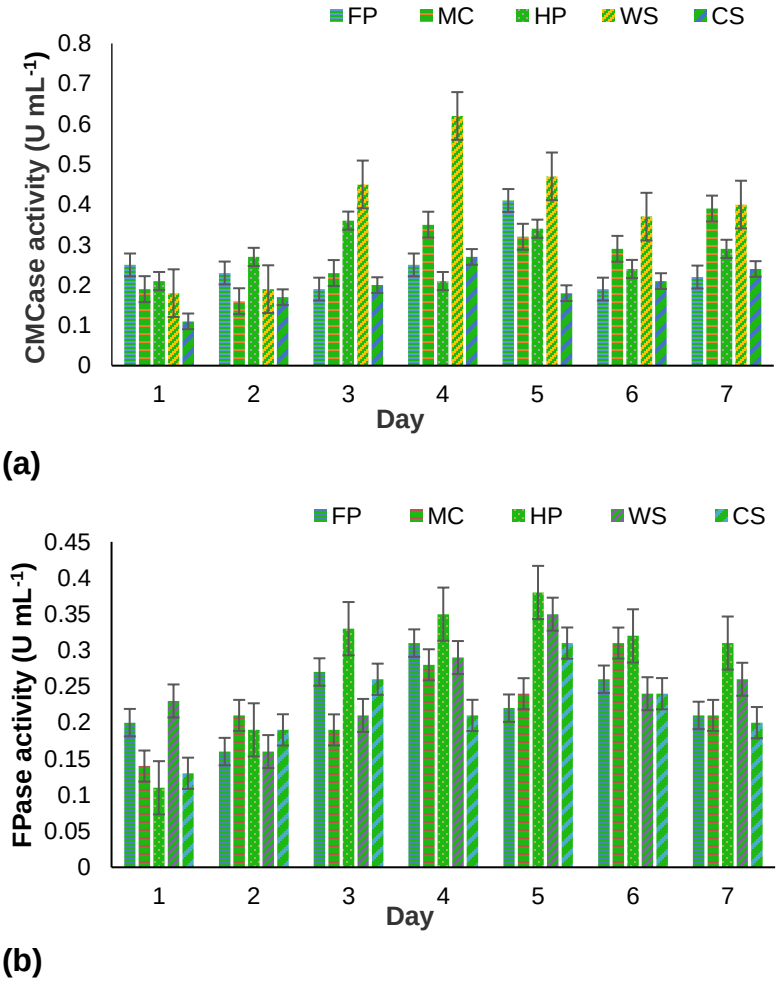

Fig. 5. Time course of CMCase activity (a) and FPase (b) under five different carbon source, with the modified incubating condition mentioned above. Error bar means ± SD(standard deviation), n = 3.

The results showed subtle differences between five carbon sources, except for the HP sample, which peaked at 0.47 U/mL on day 5 (Fig. 5, a). By comparison with the drop on day 5 of CMCase, FPase still performed a stable promotion, which revealed the synergistic effects of other cellulases secreted by WH2-56. Activities of CMCase appeared clear discrimination among different carbon sources, with the peak at 0.62 U/mL of WS on day 4 (Fig.5, left) and 0.41 U/mL of FP on day 5, which were higher than other carbon sources.

Analysing each carbon source separately, at the beginning of incubation, the WS medium presented a lower stage on CMCase, and then on day 3 it increased sharply to over twice times than the former day, which lasted until the end of culture period. Similarly, the FPase of HP medium performed a low value at the first two days and increased steadily to maintain over 0.3 U/mL during the whole period. Similar results occurred during the first

three days of other carbon source mediums, which may relate to the decrease of pH value (around 6) of the fermentation broth (data not shown). Both the CMCase and FPase activity of CS medium observed were around 0.2 U/mL constantly, seemed not to be an easily metabolized carbon source compared with others in activating the expression of enzyme genes.

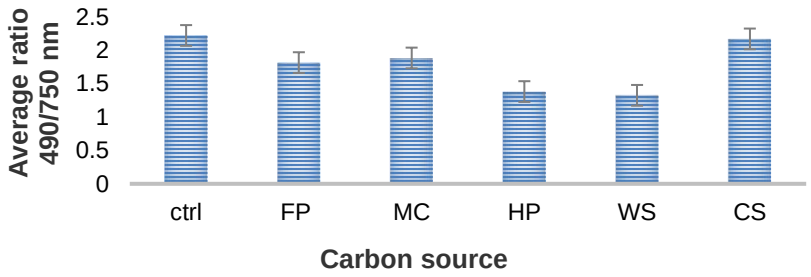

Fig. 6. Average ratio 490/750 nm of WH2-56 cultivated in five different carbon sources. A lower ratio indicates more efficient metabolism, which means the mycelium growth is higher than the metabolic activity

During incubation, metabolic activities were examined by the ratio 490/750 nm (Fig. 6) of samples taken every 24 h. WH2-56 inoculated in 5 different carbon sources all had a better performance on metabolic efficiency than the control, while the results of CS were similar to the control. Compared with other carbon sources, the WS medium showed the most efficient metabolism, while the HP medium came second, which kept in line with the results of enzymatic activities to some extent.

16S rDNA Sequencing

Compared with NCBI database sequences, the closest phylogenetic relatives were *Planomicrobium koreense* JG07T, *Planomicrobium* sp. SCU63, and *Planomicrobium* sp. Y50 with 99.8 %, 99.6 %, 99.6% 16S rRNA gene sequence similarity, respectively. Because 99% similarity is sufficient to acquire a credible result regarding genus and species (Janda and Abbott 2009), phylogenetic analyses based on 16S rRNA gene sequences revealed that strain WH2-56 was related to members of the genus *Planomicrobium*. Since domestic research on this species has been insufficient, also in view of the sequence complexity, the identification of the strain cannot be determined conclusively by similarity, thus, we identified it as a novel strain of the member of *Planomicrobium*. The nucleotide sequences were submitted to the NCBI GenBank Databases under accession number MN475274.

CONCLUSIONS

1. The present work examined the potential biodegradation capacity on cellulose and also other components of agricultural wastes of WH2-56, which were related to the *Planomicrobium* genus and isolated from a paper mill along the Yangtze River.
2. The experiments were focused on the changing shapes during different growth stage, activities of lipase and proteinase of *Planomicrobium* strains, and the cellulose-biodegrading potential.

3. The *Planomicrobium* sp. performed various levels of degradation and utilization effects on different carbon sources, especially the cellulose-based materials like agricultural wastes. This behavior of metabolism and utilization can be deemed as an indispensable and potential process to accelerate the carbon circle, degrade agricultural wastes, and replenish the carbon content of soil.

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