J. Gen. Appl. Microbiol., **64**, 293–298 (2018)
doi 10.2323/jgam.2018.02.004

jGAM

Full Paper

Culturable fungal diversity and cellulase production by mixed culture *Aspergillus* fungi from Sanya mangrove

(Received October 26, 2017; Accepted February 15, 2018; J-STAGE Advance publication date: August 29, 2018)

Siquan Zeng,[1,2] Juan Ling,[1] Manzoor Ahmad,[1,2] Liyun Lin,[1,2] Yanying Zhang,[1,3,4] Cong Wang,[1,2] and Junde Dong[1,3,4,*]

[1] *Key Laboratory of Tropical Marine Bio-resources and Ecology, South China Sea Institute of Oceanology, Chinese Academy of Sciences; Guangdong Provincial Key Laboratory of Applied Marine Biology, Guangdong, Guangzhou 510301, China*
[2] *University of Chinese Academy of Sciences, Beijing 100049, China*
[3] *Tropical Marine Biological Research Station, Chinese Academy of Sciences, Sanya 572000, China*
[4] *Institute of Deep-sea Science and Engineering, Chinese Academy of Sciences, Sanya 572000, China*

Fungi are the most suitable cellulase producers attributing to its ability to produce a complete cellulase system. 33 Genus, 175 Species fungi were isolated from Sanya mangrove, Hainan, China. Using congo red cellulose (CMC) medium, five fungi of cellulose-degrading were selected for further study. Molecular biology and morphological identification showed that all of these five fungi belong to *Aspergillus* fungi. The cellulase produced by these fungi were monitored during liquid state fermentation. The optimum conditions study for enzyme production illustrated that the highest activities appeared at pH 3.0, 35°C after fermentation for 3 days. Beyond that, the enzyme activity of mixed fungi is 11–26% higher than pure. The study demonstrated that mixed culture improved the hydrolysis of fungi cellulase.

Key Words: *Aspergillus*; cellulase; fungi; mangrove; mixed culture

Introduction

Cellulose is macromolecular polysaccharide composed of glucose, which is found in animals, plants and microorganisms. The amount of cellulose synthesized globally is 10^{11}–10^{12} tons/year according to Gupta et al. (2016). Cellulase, a group of enzymes that catalyze the hydrolysis of cellulose and related oligosaccharide derivatives is considered a vast application prospect in energy, textile and other fields (Sharada et al., 2013). Fungi, bacteria and actinomycetes are important sources of cellulose. To date, the most efficient cellulase was found in *Caldicellulosiruptor bescii* CelA, which was isolate from hot spring (Brunecky et al., 2013). Most of the fungi having complete cellulase system as compared to bacteria. Numerous biochemical experiments shed light on the nature of cellulase enzyme, especially from the model fungus *T. reesei* (Payne et al., 2015). Recently study showed that solid-state fermentation (SSF) is viable in producing high concentration of cellulase with a lower production cost compared to submerged fermentation. The constraints that affect SSF of cellulase are fermentation medium, duration, pH, temperature and moisture content are highlighted (Yoon et al., 2014).

Mangrove ecosystem is one of the four most productive marine ecosystem, considered as a best source of industrial enzymes. Current studies on mangrove Fungal Cellulases focused on isolating fungi and investigating the production of cellulolytic enzymes from mangrove environments around the world (Hussain et al., 2014). Arfi et al. (2013) found over 400 lignocellulolytic enzymes using de-novo transcriptomic assembly in mangrove fungus, *Pestalotiopsis* sp. NCi6, from *Rhizophora stylosa* trees in Saint Vincent Bay, New Caledonia. The significant results strongly highlight the potential of mangroves as a habitat of efficient halophytes saccharifying fungal-enzymes. In this study, we are interested in exploring the efficiency of mixed culture in cellulose degradation compare to pure culture. Here, five *Aspergillus* fungi with high activity of cellulase were identified and fermentation conditions were further optimized. Our results showed that the mixed cul-

*Corresponding author: Junde Dong, South China Sea Institute of Oceanology, Chinese Academy of Sciences, 164 West Xingang Road, Guangzhou 510301, P.R. China.
Tel and Fax: 0086-202-89107830 E-mail: dongjunde@vip.163.com

None of the authors of this manuscript has any financial or personal relationship with other people or organizations that could inappropriately influence their work.

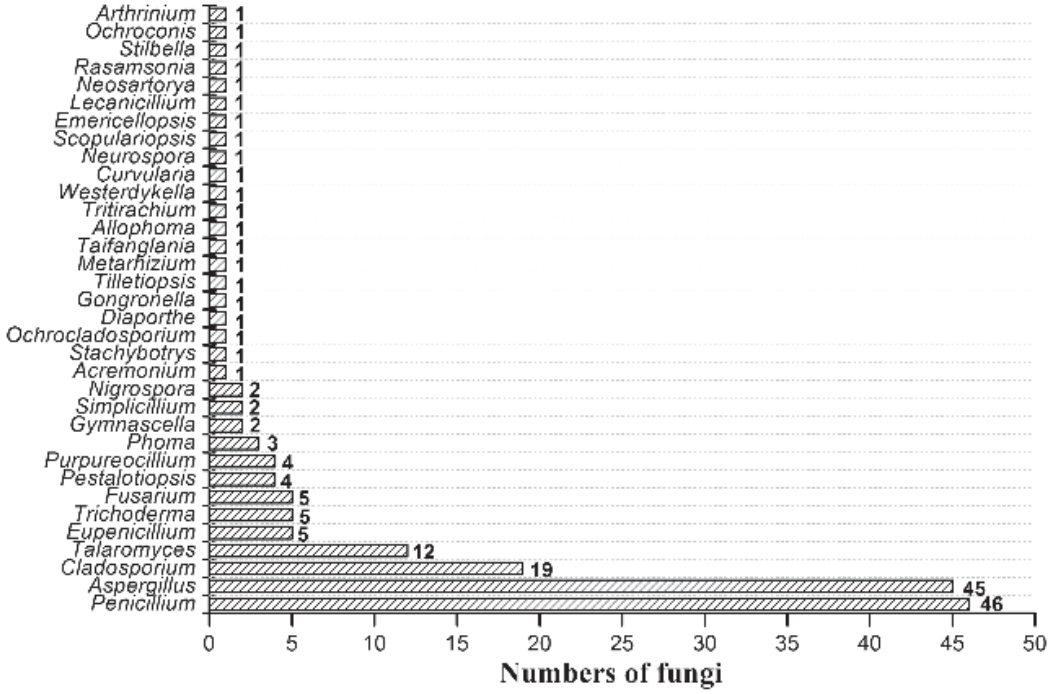

Fig. 1. Distribution of fungal isolates.

Using ITS sequence BLAST to identify the species of isolated fungi.

ture hold more efficient cellulase activity than pure culture under the optimum condition (pH 3.0, 35°C, fermentation 3 days).

Material and Methods

Sample collection. Samples were collected from mangrove, in Hongsha, Yalongwan and Bailu park, Sanya city, Hainan province, China (18.2629N, 109.5772E; 18.1353N, 109.3707E; 18.9455N, 109.3048E). In the 10 × 10 m area, five *Rhizophora stylosa* were chosen by five-spot-sampling method. Soil samples were collected from the depth of 0–30 cm of the root from plant root, the samples were mixed in a sterile polyethylene bag and stored at 4°C.

Isolation of fungi. Flask having 5 g of sample was incubated at 30°C in shaking incubator at 150 rpm for 15 min and then diluted to 10^{-3}–10^{-5}. The samples were poured via spread plate method on modified PDA plate (200 mL Potato extracts l^{-1}, 50 mL Soil extracts l^{-1}, 15 g Agar l^{-1}, 750 mL Aged seawater) supplemented with 100 μg ampicillin and streptomycin sulfate l^{-1}. After incubation at 30°C for 3–5 days, single fungus was transfer to PDA plate to obtain pure culture.

Identification and phylogenetic analysis. All the isolated fungi were characterized on the basis of macroscopic (color, texture, appearance and diameter of colonies) and microscopic (microstructures) characteristics. Molecular identification was performed by ITS amplification following standard protocols. Using ITS sequence alignment data on NCBI, the phylogenetic tree of the ITS gene was established by using the neighbor joining method (Neighbor-Joining) in MEGA 5.0 (Kumar et al., 2004).

Screening of cellulase activity. To test the cellulase activity of pure culture, the fungus was cultured on CMC-Na medium (10 g CMC-Na, 1 g KNO_3, 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 1.5 g NaCl, 0.2 g Congo red, 15 g Agar, 1,000 mL Distilled water) and the area of hydrolysis was measured around the colonies. The ratio between the diameter of fungal colony to its hydrolytic circle represents the cellulase activity.

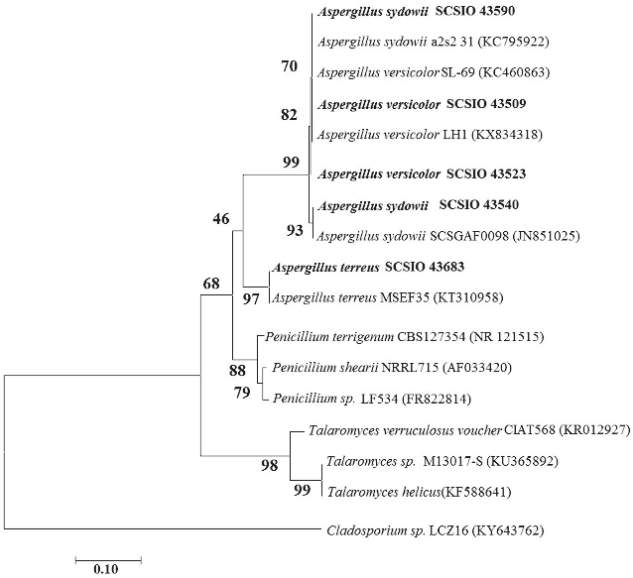

Fig. 2. Phylogenetic tree of five isolated fungi with high cellulose degradation.

Phylogenetic tree were constructed by Mega 5.0 using Neighbor-Joining method.

Fermentation condition analysis. Fungi were grown on PDA medium for 72 h at 30°C to obtain pure culture. For

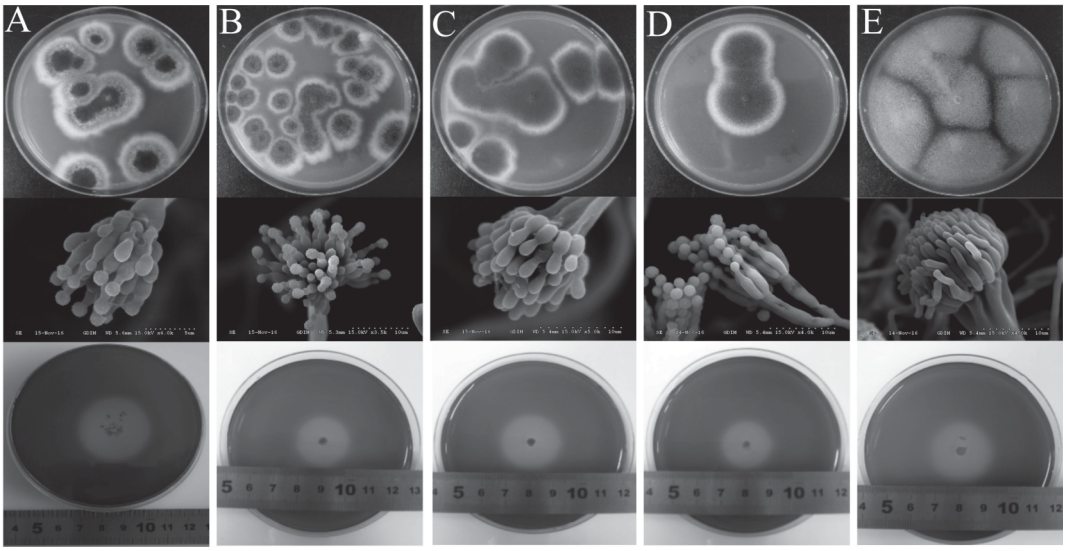

Fig. 3. Morphological characteristics and cellulose degradation activity of fungi.
A. SCSIO43509; B. SCSIO43523; C. SCSIO43540; D. SCSIO43590; E. SCSIO43683. Bar 5 μm.

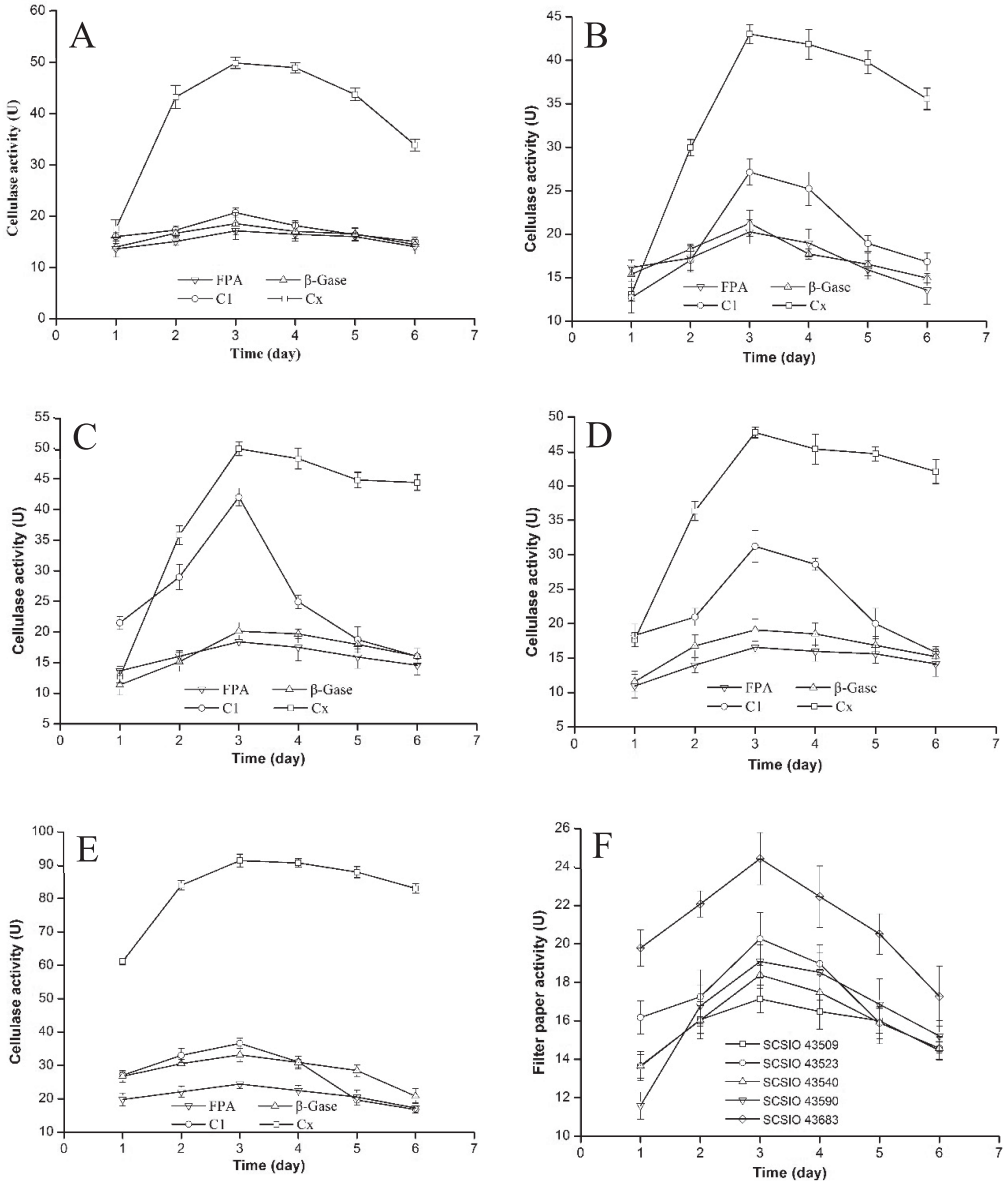

Fig. 4. Effect of pure culture time on cellulase production.
A. SCSIO43509, B. SCSIO43523, C. SCSIO43540, D. SCSIO43590, E. SCSIO43683, F. the relative FPA of five isolates.

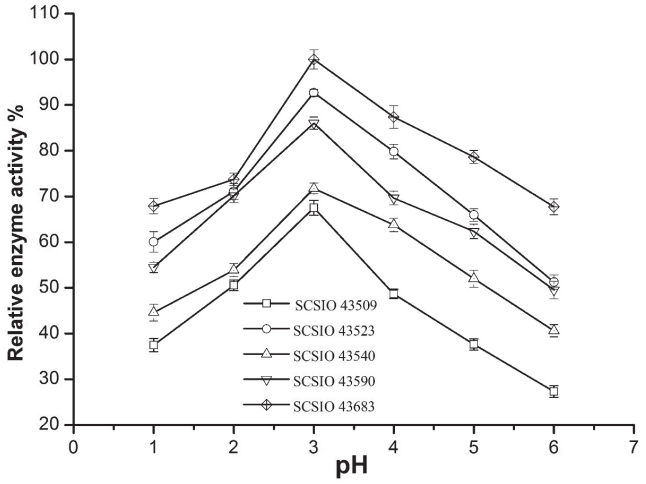

Fig. 5. Effect of pH on enzyme production.

The rate of Cellulase production was monitored at 30°C from pH 1.0–6.0 after 3 d of incubation.

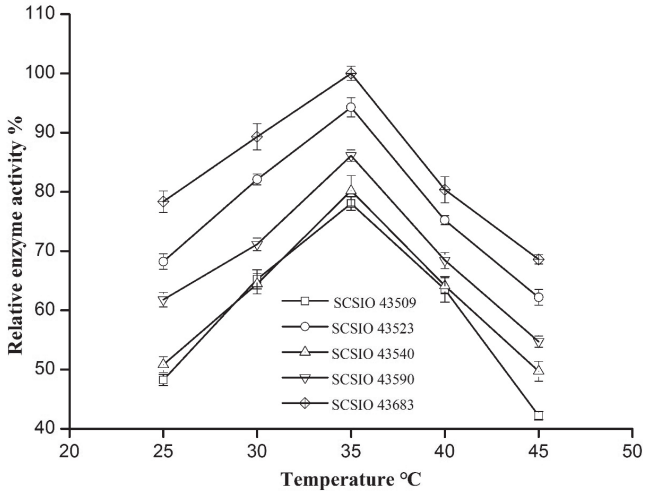

Fig. 6. Effect of temperature on enzyme production.

Cellulase activity was determined at pH 3.0 incubated at different temperature (25–45°C) after 3 d of incubation.

fermentation, 10 colonies were collected with sterilized tips and grown in 200 mL liquid fermentation medium (5 g CMC-Na L^{-1}, 1 g Peptone L^{-1}, 1 g Yeast extract L^{-1}, 1 g Sucrose L^{-1}, 1 g KNO_3 L^{-1}, 0.5 g K_2HPO_4 L^{-1}, 0.5 g $MgSO_4 \cdot 7H_2O$ L^{-1}, 1.5 g NaCl L^{-1}). Flasks were incubated at 30°C in shaking incubator with 180 rpm. To measure the cellulase activity, a reaction mixture consist of 0.5 ml fermentation supernatant and 1.5 mL citric acid/sodium citrate buffer (pH 6.0) containing substrate was incubated at 50°C for 30 min. CMC-Na, microcrystalline cellulose, salicin and filter paper were taken as substrate for the activity determination of endo-1,4-β-D-glucanase (Cx), exo-1,4-β-D-glucanase (C1), β-glucosidase (β-Gase) and filter paper activity (FPA). The reaction was terminated with 2 mL 3,5-dinitrosalicylic (DNS). Reducing sugars were determined by DNS method using D-glucose as standard. One unit (U) of cellulase activity was defined as the amount of enzyme releasing 1 μmol reducing sugar per min.

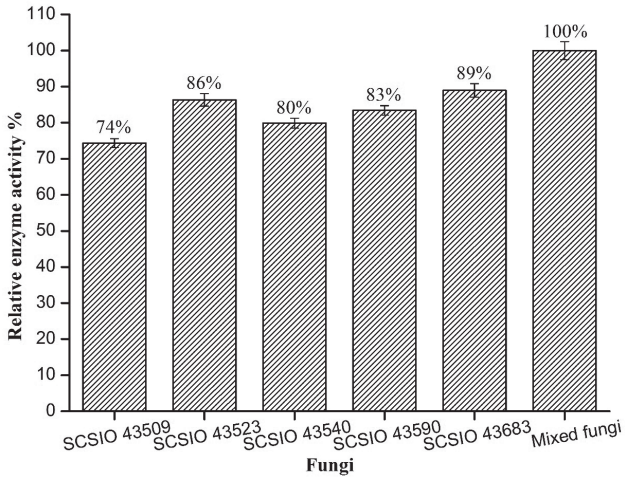

Fig. 7. Comparison of cellulase production bewteen mixed culture and individual culture.

Cellulase production rate was determined at pH 3.0, 35°C after 3 d fermentation.

Results

Isolation and screening of cellulolytic fungi

Over 200 fungi were isolated from rhizosphere sediment on modified PDA medium at 30°C after 3–5 days of incubation. ITS sequence BLAST showed that the fungi can be divided into 33 Genus with 175 Species as shown in (Fig. 1). Most of them belong to Ascomycetes. *Aspergillus* and *Penicillium* were dominant isolates, which account for 25.7% and 26.3%, followed by *Cladosporium* and *Talaromyces*. Among them, 138 fungi showed cellulolytic activity when grown on CMC-Na medium. Five *Aspergillus* spp. with maximum hydrolysis zones were selected for further study.

Identification of potent Aspergillus spp.

Five fungi with high cellulytic activity were named SCSIO43509, SCSIO43523, SCSIO43540, SCSIO43590 and SCSIO43683. The phylogenetic tree indicated that SCSIO43509, SCSIO43523, SCSIO43540, SCSIO43590 and SCSIO43683 had the greatest similarity with the *Aspergillus versicolor* LH1 (KX834318), *Aspergillus versicolor* SL-69 (KC460863), *Aspergillus sydowii* SCSGAF0098 (JN851025), *Aspergillus sydowii* a2s2_31 (KC795922), *Aspergillus terreus* MSEF35 (KT310958), respectively, and the percent identity were 99–100% (Fig. 2). Morphological analysis showed that the color of fungi is olive-green to light yellow, the tip of conidiophores became expanded and the spore head was radial. All the selected strains produce a hydrolysis zone of about 25–30 cm (Fig. 3). Based on morphological and molecular analysis, these five fungi belong to genus *Aspergillus*.

Effects of fermentation time, pH and temperature on cellulase

To identify the effect of fermentation time on enzyme, cellulase complex activity of pure culture was tested after 1 to 6 days of fermentation and the results showed that the enzymatic activity in all isolates ranging from high to low was Cx, C1, β-Gase and FPA. The optimum activity

in all enzymes were achieved after 72 h of fermentation. FPA comparison between fungi showed that the highest enzymatic activity was observed for isolate SCSIO43683, followed by SCSIO43523 and SCSIO43590 respectively, while the lowest activity among these were recorded in SCSIO43540 and SCSIO43509 (Fig. 4). Besides, the optimum pH and temperature of fermentation on FPA for these five fungi is pH 3.0 and 35°C, respectively (Figs. 5 and 6).

Effect of mixed fermentation on cellulase

At the optimal condition (pH 3.0, temperature 35°C, incubation time for 72 h), cellulase activity of mixed and pure culture were carried out (Fig. 7). The result indicated that the enzyme activity of mixed culture was 11–26% higher than the pure culture, and the mixed culture improved the hydrolysis of cellulase.

Discussion

Until now, mangrove ecosystem play an important role in marine fungi research, and it is the second largest source of marine fungi (Sridhar, 2004). In Malaysia, 82 fungi were collected with most dominant group of Ascomycetes (56 species) (Jones and Kuthubutheen, 1989). Schmit and Shearer (2003) listed 625 fungi associated with mangrove forests which contains 278 ascomycetes, 277 mitosporic fungi, 30 basidiomycetes and 14 oomycetes. The fungi from mangrove plant have long been studied but there are few reports on fungi from mangrove sediment of Hainan Island, China. In this study, more than 200 fungi were collected from *Rhizophora stylosa* sediment in Sanya mangrove, Hainan, China. Molecular identification indicated that these fungi belongs to 33 Genus with 175 species and the Ascomycetes being the dominant group (45 *Aspergillus* and 46 *Penicillium*). Majority (138) of these isolates showed cellulose degradation ability.

Fungi have a dominant role in the natural cellulose cycle, and have good adaptability of cellulose degradation. They produces a large amount of various enzymes with complementary activities compare to bacteria, and fungal cellulases are commonly secreted out of the cell (Gupta et al., 2016). Ascomycete fungus, *T. reesei* is the most studied cellulose degrading fungi and used to be the cellulolytic model system in many biotechnological industries (Kubicek, 1992). Currently, an increasing amount of research on cellulase of *Aspergillus* fungi have been carried out and *Aspergillus* fungi displayed good cellulose degradation activity (Xue et al., 2017). In our study, 5 potent fungi with increased cellulolytic activity were found. Morphological and molecular analysis indicated that these five fungi belong to *Aspergillus*, and the similarity index of related species were 99–100%. All strains could produce a hydrolysis circle with diameter of 25–30 cm when cultured on CMC-Na medium. It clearly demonstrated that the cellulase extracted by these *Aspergillus* fungi was efficient.

Cellulase from *Aspergillus* spp. in the current study has a large range of optimal reaction pH and temperature. It displayed similar pH and temperature characteristics to a cellulase production from *Aspergillus niger* MS82 (Sohail et al., 2009). Celluase produced by the mixed fungi culture contains a range of enzymes, which was more efficient than pure culture. Wen et al. (2005) found that the mixed culture of *T. reesei* and *A. phoenicis* produce significantly higher cellulase compare to pure culture *T. reesei*. Similarly, using mixed-culture solid-state fermentation, a system of enzymes with balanced activities was produced that can efficiently hydrolyze lignocellulosic biomass (Brijwani et al., 2010). Our work also found that mixed culture of *Aspergillus* fungi produced more efficient cellulase which activity is 11–26% higher than pure culture. The work demonstrated a possible way to develop an efficient cellulolytic system for biomass degradation.

Acknowledgments

This study was supported by the Strategic Priority Research Program of the Chinese Academy of Sciences (XDA13020300), Guangdong Province Public Welfare Research and Capacity Building Project (2015A020216016), Science and Technology Planning Project of Guangdong Province, China (2014B030301064), Hainan Province Marine Science and Technology Special Project (ZH201415).

References

Arfi, Y., Chevret, D., Henrissat, B., Berrin, J. G., Levasseur, A. et al. (2013) Characterization of saltadapted secreted lignocellulolytic enzymes from the mangrove fungus *Pestalotiopsis* sp. *Nat. Commun.*, e1038.

Brijwani, K., Oberoi, H. S., and Vadlani, P. V. (2010) Production of a cellulolytic enzyme system in mixed-culture solid-state fermentation of soybean hulls supplemented with wheat bran. *Process Biochem.*, **45**, 120–128.

Brunecky, R., Alahuhta, M., Xu, Q., Donohoe, B. S., Crowley, M. F. et al. (2013) Revealing nature's cellulase diversity: The digestion mechanism of *Caldicellulosiruptor bescii* CelA. *Science*, **342**, 1513–1515.

Gupta, V. K., Kubicek, C. P., Berrin, J. G., Wilson, D. W., Couturier, M. et al. (2016) Fungal enzymes for bio-products from sustainable and waste biomass. *Trends Biochem. Sci.*, **41**, 663–645.

Hussain, A., Altenaiji, E., and Yousef, L. (2014) Fungal cellulases from mangrove forests—a short review. *J. Biochem. Technol.*, **5**, 765–774.

Jones, E. B. G. and Kuthubutheen, A. J. (1989) Malaysian mangrove fungi. *Sydowia*, **41**, 160–169.

Kubicek, C. (1992) The cellulase proteins of *Trichoderma reesei*: Structure, multiplicity, mode of action and regulation of formation. *In* Enzymes and Products from Bacteria Fungi and Plant Cells, *Adv. Biochem. Eng./Biotechnol.*, **45**, 1–27.

Kumar, S., Tamura, K., and Nei, M. (2004) MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Briefings Bioinf.*, **5**, 150–163.

Payne, C. M., Knott, B. C., Mayes, H. B., Hansson, H., Himmel, M. E. et al. (2015) Fungal cellulases. *Chem. Rev.*, **115**, 1308–1448.

Schmit, J. P. and Shearer, C. A. (2003) A checklist of mangrove-associated fungi, their geography and known host plants. *Mycotaxon*, **80**, 423–477.

Sharada, R., Venkateswarlu, G., Venkateshwar, S., and Rao, M. (2013) Production of cellulase—a review. *IJPCBS*, **3**, 1070–1090.

Sohail, M., Siddiqi, R., Ahmad, A., and Khan, S. A. (2009) Cellulase production from *Aspergillus niger* MS82: effect of temperature and pH. *New Biotechnol.*, **25**, 437–441.

Sridhar, K. R. (2004) Mangrove fungi in India. *Curr. Sci.*, **86**, 1586–1587.

Wen, Z. Y., Liao, W., and Chen, S. L. (2005) Production of cellulase/b-glucosidase by the mixed fungi culture *Trichoderma reesei* and *Aspergillus phoenicis* on dairy manure. *Process Biochem.*, **40**, 3087–3094.

Xue, D. S., Lin, D. J., Gong, C. H., Peng, C. L., and Yao, S. J. (2017) Expression of a bifunctional cellulase with exoglucanase and

endoglucanase activities to enhance the hydrolysis ability of cellulase from a marine *Aspergillus niger*. *Process Biochem.*, **52**, 115–122.

Yoon, L. W., Ang, T. N., Ngoh, G. C., and Chua, A. S. M. (2014) Fungal solid-state fermentation and various methods of enhancement in cellulase production. *Biomass Bioenergy*, **67**, 319–338.