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Selection of cellulase-producing *Trichoderma* sp. and optimization of cultivation conditions

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The aim of this study was to select the wild microorganism with the capacity to produce cellulases. Microbial sources were rotten grass, leaves and straws. Natural lignocellulose as the sole carbohydrate was used to enrich microorganisms. After isolation, the strains were inoculated into liquid media. Filter paper activity (FPA) was used as an index for selection. Sequencing of 26S rDNA D1/D2 region was used to identify the microorganism. Lactose, lactobionic acid, Tween 80 and yeast extract were added into cultivation system to improve FPA. Optimum temperature and pH of cellulase were investigated. After enrichment, isolation and selection according to FPA, one strain was selected. *Trichoderma* sp. A6 was named according to the sequencing results. When lactose (10 g/L) was added into the cultivation system, the FPA was improved from 0.16 to 0.56 IU. Lactose addition could improve FPA significantly. After addition, the FPA at two days amounted to the highest value. However, the addition of lactobionic acid did not increase FPA. Tween 80 addition only improved the FPA at four days to small extent. The 15 g/L yeast extract could increase the FPA at 2 days from 0.60 to 0.92 IU. The combination of Tween 80 and yeast extract did not improve the FPA compared with the treatment of Tween 80 addition. The optimum range of temperature and pH were 37-60 and 4.5-5.5°C, respectively.

Key words: Cellulase, *Trichoderma*, selection, cultivation condition, optimization.

INTRODUCTION

Energy security, petroleum depletion, and global warming have become the main driving forces for developing renewable fuels that can replace petroleum-derived fuels. The renewable fuel expected to be widely used around globe is bioethanol, which is largely produced by fermenting starch- or sugar-containing feedstocks. However, the supply of these crops is relatively limited and many of them can be considered human food resources. Non-food crops will be more advantageous for bioethanol production (Alriksson et al., 2009; Sticklen, 2008).

Lignocellulose is considered as one of the most important carbon sources on Earth and a less-expensive raw material with a great potential to produce energy. Lignocellulosic biomass used for producing ethanol has been a major focus (Lynd et al., 2008; Niranjane et al., 2007). It is known that the general process for converting lignocellulosic biomass into ethanol mainly includes feedstock pretreatment, enzymatic hydrolysis, sugars fermentation, separation of lignin residue, recovery and purifying the ethanol to meet fuel specifications. Currently, there are technological and economic limitations in each step in the conversion process (Alvira et al., 2010). Cost effective pretreatment processes,

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cheaper hydrolytic enzymes and fermentation of pentose sugars have been always common research goals (Keshwani and Cheng, 2009).

Utility cost of enzymatic hydrolysis is low as compared to acid or alkaline hydrolysis, because enzymatic hydrolysis is usually conducted at mild conditions (pH 4.8) and temperature (45-50°C) and does not have a corrosion problem (Singh et al., 2009) However, at present, hydrolysis enzymes are expensively produced in microbial bioreactors (Sticklen, 2008). It is still important that selecting microorganisms with a high capacity to produce enzymes and improving cellulase-producing characteristics (Wang et al., 2010).

Filamentous fungi are widely used in producing cellulases currently. *Trichoderma*, *Aspergillus* and *Penicillium* are recognized with high potentials in the cellulase production (Li and Hou, 2010). In this study, we obtained one strain with cellulase-producing capacity using plate isolation and streaking method. Initial identification and optimization of culture conditions were carried out. This strain could be considered as a wild-type strain for the further physical and chemical mutagenesis. The optimization results of cultivation conditions will provide the instructions for the possible industrialization application of this strain.

MATERIALS AND METHODS

Cultivation and selection

Rotten weeds, leaves and straws were used as microbial sources. The modified Czapek medium (MCD) was used for enrichment media (Gharieb et al., 1999; Li and Hou, 2010). The medium (1 L) contained : $NaNO_3$, 2 g; KH_2PO_4, 1 g; $MgSO_4$. $7H_2O$, 1 g; KCl, 0.5 g; $FeSO_4$, 0.01 g; agar ,15 g. The flask (250 mL) contained 100 mL medium. All media were autoclaved at 121ºC for 15 min and cooled. After solidification, 1 g of sterile straws (10 cm in length) was placed on the agar, as the carbon source. Powders (0.5 g) of decaying weeds, leaves and straws were accessed to the MCD agar (100 mL), respectively. After a 14-day cultivation at 30°C, about 0.05 g straws covered with microorganisms were transferred into another MCD agar (Yang et al., 2011). After five-time repeats, the samples which could grow stably were used for further isolation. Plate dilution method was used to isolate microorganisms according the clolony morphology. The medium was PDA (Wu et al., 2012).

The strains isolated were used to inoculate into the liquid Mandels medium (Wang et al., 2011) (15 g/L microcrystalline cellulose as carbon source). After shaking for seven days, the filter paper activities (FPAs) of the cultivation system were measured. The method of enzyme assay was shown as section 1.2. The strain with the highest enzyme activity was selected for identification and optimization of culture conditions. The *Trichoderma reesei* RUT-C30 were purchased from China Center of Industrial Culture Collection (CICC).

Determination of enzyme activity

Culture supernatants obtained after centrifugation at 5800 × *g* for 10 min at 4°C were used to determine the FPA. Whatman No.1 filter paper (Whatman, England) was used as the substrate to determine enzyme activity. A filter paper strip (1.0 × 6.0 cm, ≈50 mg) was added into 1mL 50 mM citric-phosphate buffer (pH 4.8). After pre-warming at 50°C for 5 min, 0.5 mL crude enzyme solution was added. Then the mixture was incubated at 50°C for 60 min. The release of reducing sugar was measured using the 3, 5-dinitrosalycylic acid method (Ghose, 1987). Glucose was used as the standard for FPA measurements. The color formed by cellulase bound to the substrate was subtracted from that of the standard and the sample tube. Therefore, the amount of cellulase bound to the substrate is not neglibible. One unit (IU) of enzyme activity was defined as the amount of enzyme releasing 1 µmol reducing sugar in 1 min reaction.

Microbial identification

Genomic DNA of pure culture strain was extracted using the EZNA mini DNA kit (OMEGA, USA) according to manufacture's instruction. The 50 µL PCR mixtures contained 15 ng of template DNA, 1× PCR buffer (Mg^{2+} free), 0.16 mM of each dNTP, 1.5 mM $MgCl_2$, 0.45 µM of each primer, and 1 U of Takara rTaq DNA polymerase (Takara, Japan). The primers for amplification of the D1/D2 region of the fungal 26S rRNA gene were NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCG TGTTTCAAGACGG-3') (Baleiras Couto et al., 2005). The thermocycle program consisted of initial DNA denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 52°C for 45 s, and elongation at 72°C for 1 min 30 s, and ending with a final elongation step at 72°C for 6 min. The amplified fragment was confirmed by agarose gel electrophoresis, and then sent to Sangon Biotech (China) for sequencing. The sequences generated in this study were compared with those in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A neighbor-joining tree was constructed using the MEGA 5.0 software (Tamura et al., 2011).

Optimization of culture conditions

Mandels medium was used as the basic medium. A total amount of carbon source of 1 L culture system was 15 g. The addition of lactobionic acid and lactose was 0~15 g, respectively. Accordingly, the addition of microcrystalline cellulose was 15~0 g. The medium (100 ml) was poured into 250 mL flask. All media were autoclaved at 121°C for 15 min. All determinations are equipped with three replications. After optimization of carbon source, yeast extract and Tween 80 were used. The addition of yeast extract was 0~20 g, and Tween 80 was 0-2.0 mL. Each addition level is equipped with three replications. Each replication was measured three times. Therefore, each FPA value in this study is means of nine measurements. Enzyme activities at two, four and seven days of cultivation were measured to determine final cultivation conditions.

Data processing

Data were subjected to ANOVA using Duncan's test of the Statistic Analysis System (Version 8.2, SAS Inst. Inc., Cary, NC). Significance level was 5%.

RESULTS

Screening of strains

After a series of screening, a fungal strain was obtained due to the highest enzyme activity. The growth status of the strain cultivated on PDA medium for three days was

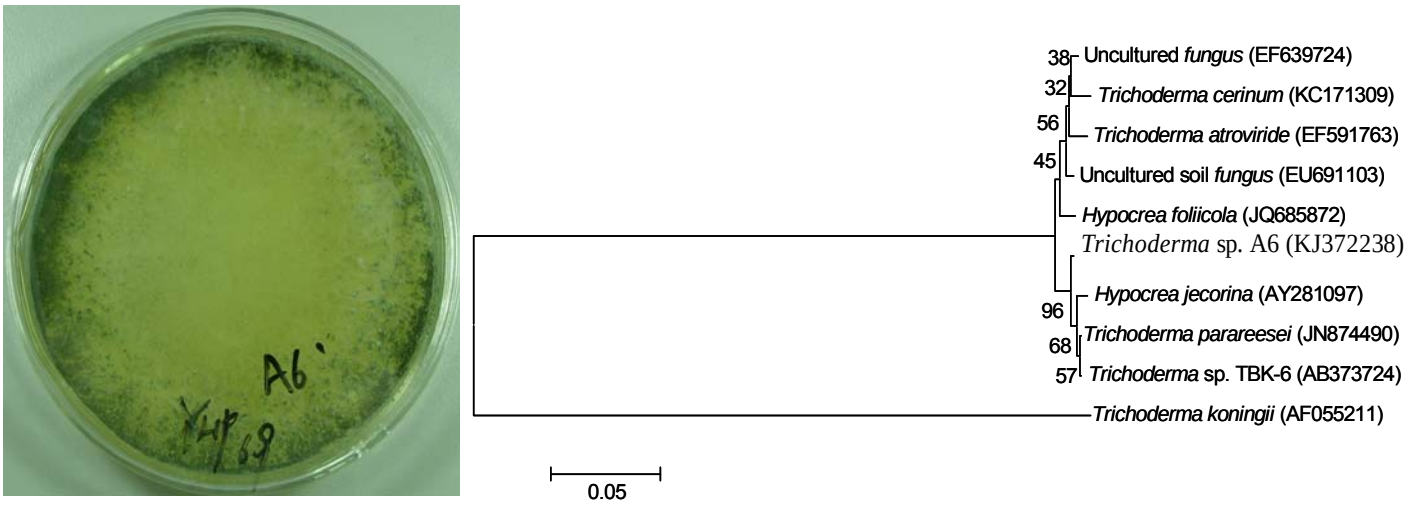

Figure 1. Isolated strain and phylogenetic analysis the fungal 26S rDNA D1/D2 region sequences by means of the neighbor-joining method. The bar represents 5% sequence divergence. The number in parentheses shows the GenBank accession number.

shown in Figure 1. After extraction of genomic DNA, PCR amplification of 26S rDNA D1/D2 region was carried out. After sequencing, phylogenetic tree was constructed and shown in Figure 1. The closest microorganism was *Trichoderma* sp. TBK-6. With 99% sequence similarity, it was identified as *Trichoderma* sp. and named as *Trichoderma* sp. A6.

Optimization of culture conditions

To further enhance A6 enzyme activity, optimization experiments of culture conditions (carbon source, nitrogen source and unknown growth factor) were carried out.

Addition of lactose and lactobionic acid

The previous report showed that the combination of cellulose and lactose as the carbon source could improve the cellulase-producing capacity of *Trichoderma reesei* (Muthuvelayudham et al., 2006). Janas reported that lactose and lactobionic acid could induce cellulase production of Trichoderma *reesei* (Janas, 2002). In this study, lactose and lactobionic acid were respectively added into the medium containing microcrystalline cellulose. The result was shown in Figures 2 and 3.

The FPAs at two days of cultivation were significantly higher with the increase of lactose addition (Figure 2). The FPAs at four days of cultivation were increased significantly in the treatments of 5 and 10 g/L lactose addition. At seven days of cultivation, the FPAs did not significantly change with 5-15 g/L addition of lactose. In all treatments of lactose addition, the FPAs expressed the downward trend from two days of cultivation. This result showed that the improvement of FPAs due to lactose addition occurred at early stage of cultivation. The addition of 10 g/L lactose is the most optimized content.

The result of lactobionic acid addition was shown in Figure 3. The addition of lactobionic acid did not serve to improve enzyme activity. Compared the treatment without any addition, all FPAs in various addition treatments decreased. This result indicated that addition of lactobionic acid did not provide positive effect for enzyme production of *Trichoderma* sp. A6

Addition of Tween 80

Reese and Maguire found that Tween 80 can improve enzyme activity of *Trichoderma* that grew in the medium. However, the mechanism was unclear (Reese and Maguire, 1969). Domingures et al. (2000) reported that the addition of Tween 80 may increase cell permeability and affect cell morphology. As a result, the enzyme activity was improved. In this study, on the basis of adding 10 g/L lactose, the effects of Tween 80 on enzyme production were investigated. The addition gradient was set from 0 to 2 mL/L. The FPAs was shown as Figure 4. The addition of Tween 80 did not affect enzymatic activities at two and seven days of cultivation. At four days of cultivation, the FPAs increased with the addition of Tween 80 in the range of 0.5 to 1.0 mL/L. Finally, the addition concentration of Tween 80 was 1.0 mL/L for the following experiment.

Addition of yeast extract

The main components of yeast extract were peptides, amino acids, flavor nucleotides, B vitamins and trace

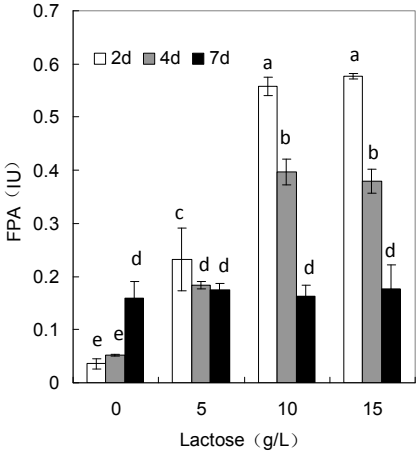

Figure 2. Effects of lactose on filter paper activity in the cultivation system. FPA: filter paper activity. The addition level of lactose was set as 0, 5, 10, and 15 g/L. The corresponding microcrystalline cellulose was 15, 10, 5 and 0 g/L. 2, 4 and 7 days expressed days of cultivation. Different letters on the column indicated significant difference (P<0.05).

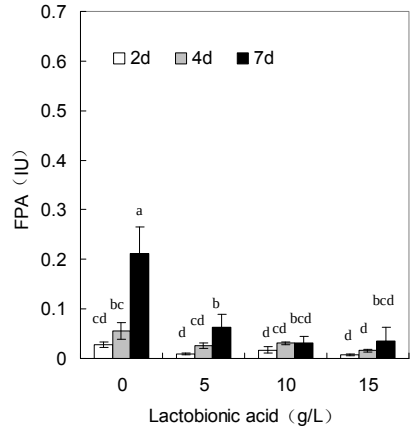

Figure 3. Effects of lactobionic acid on filter paper activity in the cultivation system. FPA: filter paper activity. The addition level of lactobionic acid was set as 0, 5, 10, and 15 g/L. The corresponding microcrystalline cellulose was 15, 10, 5 and 0 g/L. 2, 4 and 7 days expressed days of cultivation. Different letters on the column indicated significant difference (P<0.05).

elements. Some reports showed that yeast extract could stimulate cell growing and cellulase production of *T. reesei* (Domingues et al., 2000; Ryu and Mandels, 1980). In this study, the concentrations of yeast extract were set as 5, 10, 15 and 20 g/L. The results were shown as Figure 5.

The FPAs increased with yeast extract addition in the range of 5 - 15 g/L at 4 days of cultivation. The yeast

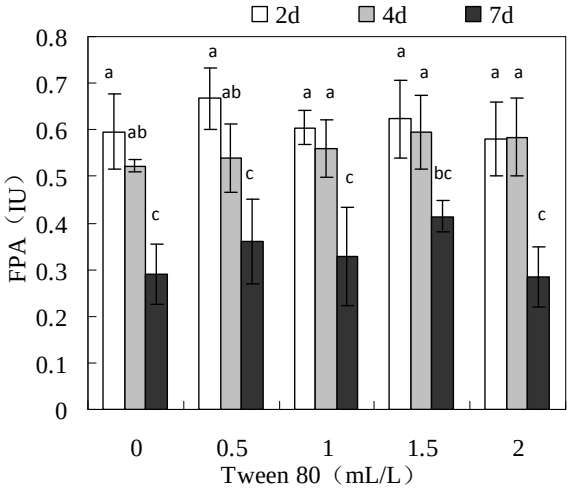

Figure 4. Effects of Tween 80 on filter paper activity in the cultivation system. Tween 80 was set as 0, 0.5, 1.0, 1.5 and 2.0 mL. FPA: filter paper activity. 2d, 4d and 7 days expressed days of cultivation. Different letters on the column indicated significant difference (P<0.05).

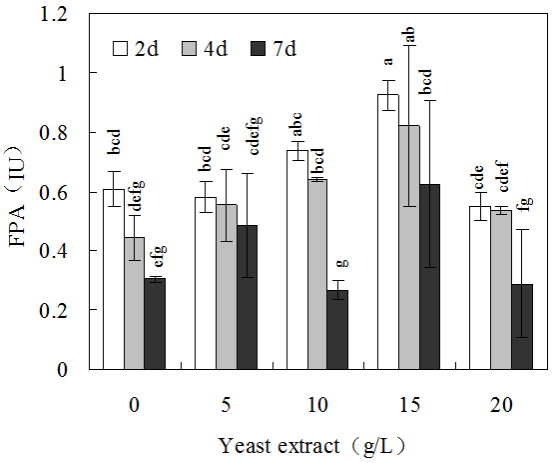

Figure 5. Effects of Yeast extract on filter paper activity in the cultivation system. The addition level of yeast extract was set as 0, 5, 10, 15 and 20 g/L. FPA: filter paper activity. 2, 4 and 7 days expressed days of cultivation. Different letters on the column indicated significant difference (P<0.05).

extract with 20 g/L addition had no significant effect on the increase of enzyme activity. This result indicated that addition of yeast extract could increase enzyme activity at some extent.

Combination of Tween 80 and yeast extract

Tween 80 and/or yeast extract were used to investigate whether their combination was better. As shown as Figure 6, the treatments with yeast extract addition and combination addition had no significant differences. The enzyme activities of with Tween 80 addition were lower than those of the other treatments.

Enzyme activity of A6 and RUT-C30 during the cultivation

After optimization of cultivation media, the enzyme

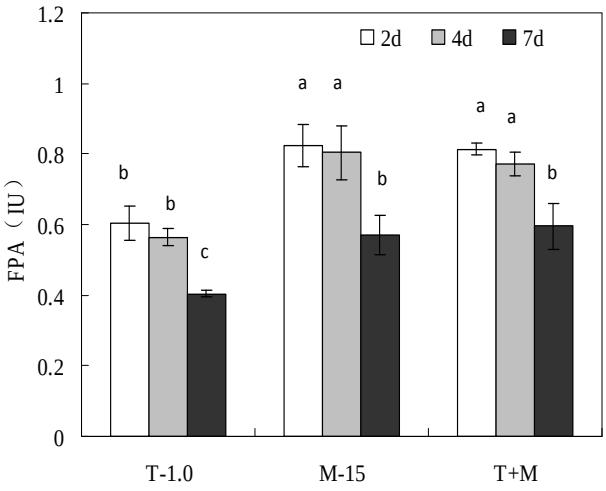

Figure 6. Effects of Tween 80 and yeast extract on filter paper activity in the cultivation system. T-1.0, Tween 80 1.0 mL/L; M-15 yeast extract 15 g/L; T+M, Tween 80 1.0 mL/L plus yeast extract 15 g/L; FPA: filter paper activity. 2, 4 and 7 days expressed days of cultivation. Different letters on the column indicated significant difference (P<0.05).

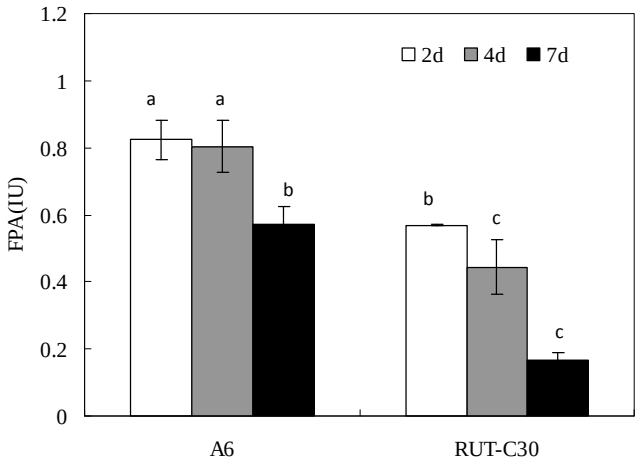

Figure 7. Enzyme activities of A6 and Rut-C30 during the cultivation. The media was Mandels medium (5 g/L microcrystalline cellulose plus 10 g/L lactose as carbon source. Yeast extract 15 g/L was added.). Different letters on the column indicated significant difference (P<0.05).

activities of *Trichoderma* A6 and *Trichocerma reesei* RUT-C30 during the cultivation were compared. From Figure 7, the FPAs of A6 were significantly higher than those of RUT-C30 during the cultivation.

Characteristics of crude enzyme

Fermentation liquid at 4 days of cultivation was taken to determine characteristics of crude enzyme. The temperature and pH of enzyme were tested. As shown in Figure 8, the relative activity of enzyme maintained above 85% in the range of 37 - 60°C. From 4.5 to 5.5 for pH, the relative enzyme activity maintained above 95%.

DISCUSSION

Breeding good microorganism is a crucial factor to improve the cellulase activity. At present, the microbes

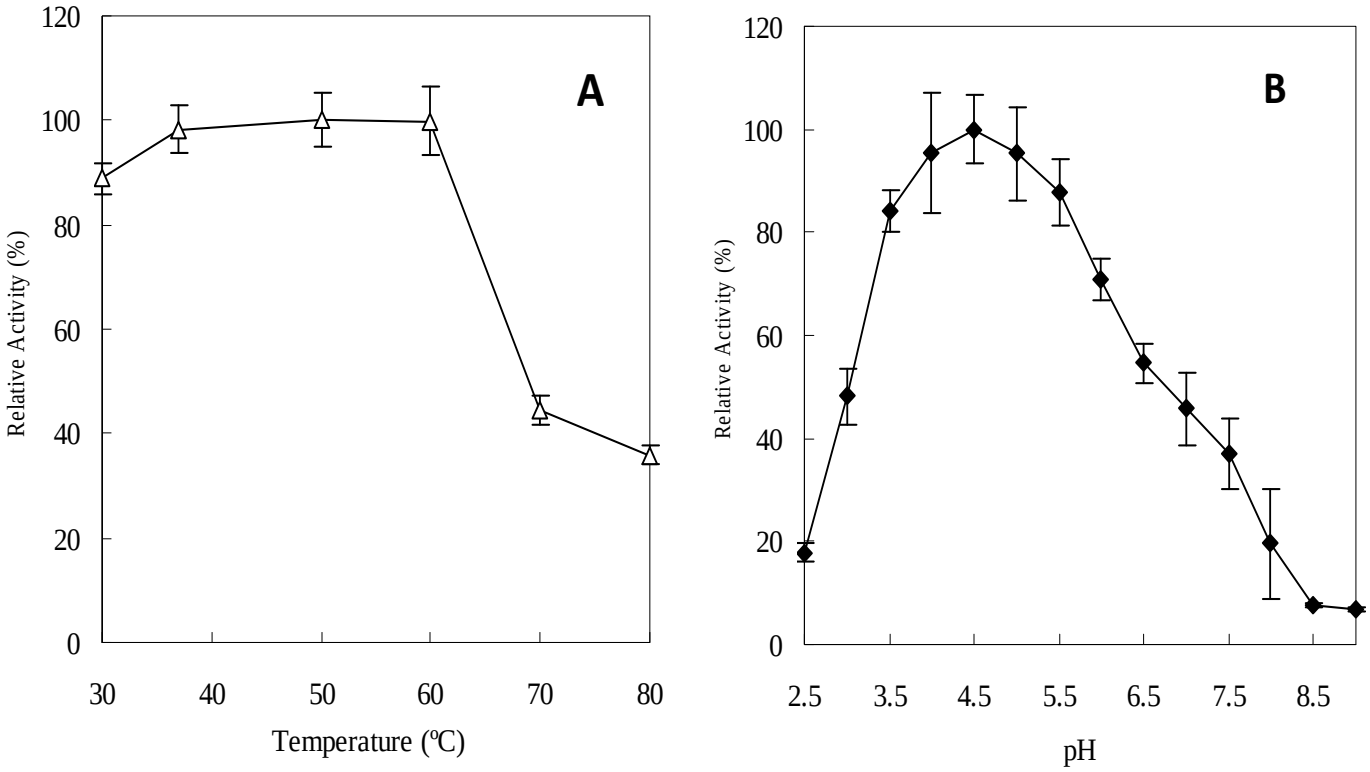

Figure 8. Relative enzyme activity at different temperatures (A) and pH levels (B).

used for cellulase production mostly belong to fungi. *Trichoderma*, *Aspergillus* and *Penicillium* are common microorganisms (Zhang et al., 2009). Currently, methods to improve the cellulase activity of strains are mainly physical, chemical mutagenesis and genetic engineering. Firstly, the parental strains should be with a high capability of cellulase production. Therefore, selection of the parental strains is important and necessary.

In this study, microorganism was derived from natural cellulose-degrading environment. During selection, natural lignocellulose was used as the sole carbon source. FPA was used as the index of cellulase production. Finally, *Trichoderma* sp. A6 was obtained. Previous reports showed that addition of lactose and lactobionic acid in the medium could increase enzyme production (Janas, 2002; Muthuvelayudham et al., 2006). In this study, lactose and lactobionic acid were added to the media, respectively. The results showed that lactose played a significant role in promoting cellulase production of the strain. Lactobionic acid was negative for enzyme production (Figure 3). A possible reason was that the addition of lactobionic acid changed the pH of cultivation system at a large extent. On the basis of lactose addition, the effects of Tween 80 and yeast extract were investigated. Results showed that addition of Tween 80 could increase cellulase, but not obvious (Figure 4). Yeast extract could significantly improve the enzyme activity of the cultivation system (Figure 5). The effect of two-components-combined addition treatment showed no significant difference with the treatment addition of yeast extract (Figure 6). These results indicated that lactose and yeast extract play key roles in cellulase production of *Trichoderma* sp. A6. Compared with *Trichoderma reesei* RUT-C30, A6 owned higher FPAs while cultivated with the optimized media in this study.

Generally, the optimum temperature of cellulase was 45 - 65°C (Akila and Chandra, 2003). In the study, optimum temperature of the A6 strain screened was 37 ~ 60°C. As shown as Figure 7, when the temperatures were 37 and 60°C respectively, the enzyme activity reached 98.5 and 99.5% of the highest activity. In the cellulase application process, the reaction could be effectively progressed at lower temperature. The enzyme activity could be well maintained. At the same time, the energy and costs during the reaction will be reduced (Lv et al., 2007). Therefore, the enzyme from the A6 strain could be considered applied at a relatively low temperature environment.

Conclusion

A wild strain, *Trichoderma* sp. A6, was selected due to a high cellulase production capacity. Lactose and yeast extract could significantly improve the FPA in the liquid cultivation system. Addition proportions of lactose and

yeast extract were 10 and 15 g/L, respectively. The proper temperature range of celluase was 37 - 60°C. The suitable pH range was 4.5 to 5.5.

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