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Production of cold-adapted cellulase by *Verticillium* sp. isolated from Antarctic soils

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Abstract

Background: Cellulose can be converted to ethanol by simultaneous saccharification and fermentation (SSF). The difference between the optimal temperature of cellulase and microbial fermentation, however, has been identified as the critical problem with SSF. In this study, one fungal strain (*AnsX*1) with high cellulase activity at low temperature was isolated from Antarctic soils and identified as *Verticillium* sp. by morphological and molecular analyses.
Results: The biochemical properties of crude *AnsX*1 cellulase samples were studied by filter paper cellulase assay. The maximum cellulase activity was achieved at low temperature in an acidic environment with addition of metal ions. Furthermore, *AnsX*1 cellulase demonstrated 54-63% enzymatic activity at ethanol concentrations of 5-10%. *AnsX*1 cellulase production was influenced by inoculum size, carbon and nitrogen sources, and elicitors. The optimal culture conditions for *AnsX*1 cellulase production were 5% inoculum, wheat bran as carbon source, $(NH_4)_2SO_4$ as nitrogen source, and sorbitol added in the medium.
Conclusions: Our present work has potential to enable the development of an economic and efficient cold-adapted cellulase system for bioconversion of lignocellulosic biomass into biofuels in future.

Keywords: Antarctic soil, cellulose, filter paper cellulase assay, psychrotrophic fungi, *Verticillium* sp.

INTRODUCTION

Plant cell wall is made up of the complex structures of cellulose, hemicellulose and lignin, and stores sustainable renewable energy captured through the process of photosynthesis. This lignocellulosic biomass can be converted into biofuels used as an alternative energy source to alleviate the growing energy demands worldwide, and decrease the impact of fossil fuel emissions on the atmosphere (Henry, 2010). Cellulose is the major structural component of plant cell wall. It is a fibrous, insoluble, and high molecular weight polysaccharide consisting of 2,000-20,000 glucose units linked by β-1,4 glycosidic bonds.

A number of fungi and bacteria express cellulase, enabling them to utilize cellulose as a carbon source. Because of high enzyme activity and production, the current extensively studied cellulases are from fungi species such as *Aspergillus*, *Trichoderma*, *Penicillium*, and *Phanerochaete chrysosporium* (Coral et al. 2002). Fungal cellulases are a consortium of three hydrolytic enzymes comprised of endo-(1,4)-β-D-glucanase (EC 3.2.1.4), exo-(1,4)-β-D-glucanase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21) (Goyal et al. 1991). They act together to hydrolyze cellulose into soluble sugars for cell metabolism. Cellulose can be converted to ethanol by simultaneous saccharification and fermentation (SSF) (Kádár et al. 2004). The critical problem with SSF is the difference between the optimal temperature of

cellulase and microbial fermentation. Fermentation requires an operating temperature of 35ºC; however, the frequently used fungal cellulases have maximum hydrolysis efficiency at 50ºC (Kádár et al. 2004). A possible strategy to address this problem is to identify fungal cellulases capable of hydrolysis at lower temperatures.

A psychrotrophic organism has a capacity to survive at temperatures less than 7ºC and grow at temperatures around 20ºC. Several of these fungal species such as *Cadophora malorum*, *Geomyces pannorum*, *Penicillium herbarum*, and *Thelebolus microspores*, have been isolated from pristine soils of Antarctica (Kerry, 1990; Duncan et al. 2008). These strains may produce cellulase with high cellulytic activity at low and moderate temperatures. In this study, we screened ten fungal isolates from the soils of Antarctica for the presence of cellulase. Among them, the fungal strain with the highest cellulase activity, *AnsX*1, was identified as *Verticillium* sp. by morphological and molecular analyses. The biochemical characteristics of crude *AnsX*1 cellulase samples were determined by filter paper cellulase (FPase) assays. The effects of inoculum size, carbon and nitrogen sources, and the addition of elicitors on cellulase production were evaluated by measuring filter paper units (FPU) during fungal growth.

MATERIALS AND METHODS

Soil materials for the selection of fungal strains

Antarctic soils were collected around the Great Wall Station at geographic location of 62º13'15" S, 58º57'45" W, during the 24th Antarctic scientific expedition of China.

Isolation and selection of fugal strains from Antarctic soils

Ten grams of crude soil sample was extracted with 90 ml of autoclaved Mili-Q water at room temperature for 20 min. Five milliliters of soil extracts were transferred to a 250 ml flask with 45 ml enrichment culture (EC) medium containing 1% peptone, 0.5% yeast extract and 1% NaCl (pH 7.0), and cultured at 15ºC for 24 hrs on a rotary shaker (150 rpm). The gradient dilutions (10^{-4}, 10^{-5}, 10^{-6}) of the above enriched sample were smeared on the selection medium A containing 0.5% carboxylmethyl cellulose sodium salt (CMC-Na), 0.25% peptone, 0.05% yeast extract, 0.05% $MgSO_4$, 0.1% KH_2PO_4, 0.1% Na_2HPO_4 and 1.3% agar (pH 7.0), and cultured at 15ºC for 24 hrs. The separated single colonies were inoculated on selection medium B containing 2% CMC-Na, 0.25% peptone, 0.05% yeast extract, 0.15% KH_2PO_4, 0.25% Na_2HPO_4 and 1.3% agar (pH 7.0), and cultured at 15ºC for 24 hrs. The candidate strains were selected according to the Congo red staining assay.

Morphological and molecular characteristics of selected strain

The colonies selected by the Congo red staining assay were inoculated into 200 ml cellulase production (CP) medium containing 0.5% $(NH_4)_2SO_4$, 0.02% $MgSO_4$ and 2% wheat bran, and cultured at 15ºC for 24 hrs on a rotary shaker (150 rpm). Total cellulase activity was measured by FPase assay as described by the analytical procedure of the National Renewable Energy Laboratory (Adney and Baker, 1996). Briefly, crude cellulase was incubated with 50 mg Whatman No. 1 filter paper strip in 50 mM of citric acid/Na_2HPO_4 buffer for exactly 60 min at 50ºC. The glucose was released from filter paper and separated by centrifugation (4,000 rpm and 30 min). The supernatants were transferred to the new tubes. The glucose was then measured by DNS method (Miller, 1972). Briefly, the above tubes with 1.5 ml appropriate glucose dilutions and 3.0 ml dinitrosalicylic acid (DNS) reagent were boiled for 5.0 min and cooled down in the cold ice-water bath. The colour-developed reaction mixture was added with 2.5 ml Mili-Q water, mixed, and separated by brief centrifugation. The absorbance of the supernatant was measured at 540 nm. One FPU of enzyme activity is defined as the amount of enzyme required to release 1 µmol of glucose per ml under assay condition. The colony with highest enzyme activity was subcultured onto Potato Dextrose Agar (PDA) medium and cultured at 15ºC for 24 hrs to study its morphological characteristics. The changes in the edge and colour of the colony were observed for several days.

The above cultures were centrifuged at 4ºC with 18,000 g for 15 min to separate fungal strains from liquid medium. Genomic DNA was isolated from freeze-dried fungal strain pellets following the modified 2 x CTAB procedure (Doyle and Doyle, 1987). The universal primers were designed from the ribosomal

DNA internal transcribed spacers (ITS). The PCR products were purified and sequenced. The sequencing results were aligned with known ITS homologues from the NCBI database to identify the taxonomy of fungal strain isolated from the soils.

Preparation of crude *AnsX*1 cellulase

*AnsX*1 was a cellulase-excreting fungal stain. To prepare crude *AnsX*1 cellulase, *AnsX*1 was inoculated into 200 ml CP medium and cultured at 10ºC for 4 days on a rotary shaker (150 rpm). The fungal cultures supernatant were harvested by centrifugation (4,000 rpm, 30 min and 4ºC). The supernatants were collected as crude cellulase samples for further studies.

Biochemical properties of crude *AnsX*1 cellulase

In order to study the biochemical properties of *AnsX*1 cellulase, total cellulase activity of crude cellulase samples were analyzed under various conditions, such as different temperatures, pHs, metal ions, and ethanol concentration. To determine the optimal temperature of *AnsX*1 cellulase, crudecellulase samples and 50 mg Whatman No. 1 filter paper strip were incubated in 50 mM of citric acid/Na_2HPO_4 buffer with neutral pH (pH = 5.3) for 1 hr at 23, 33, 35, 38, 40, 43, 50, and 53ºC. To determine the optimal pH of *AnsX*1 cellulase, crudecellulase samples 50 mg Whatman No. 1 filter paper strip were incubated in 50 mM of citric acid/Na_2HPO_4 buffer with different pH values ranging from 3.0 to 9.0 at 38ºC for 1 hr. The effects of reaction temperature and pH value on *AnsX*1 cellulase stability were studied. Crude *AnsX*1 cellulase samples were incubated in 50 mM of citric acid/Na_2HPO_4 buffer (pH 5.3) without filter paper strip at different temperatures for 1 hr, and then measured by FPase assay under the standard conditions. The activity without treatment was taken as 100%, and the relative cellulase activity was measured to determine the stability of *AnsX*1 cellulase at different temperatures. To study effects of pH value on *AnsX*1 cellulase stability, crude *AnsX*1 cellulase samples were incubated in 50 mM of citric acid/Na_2HPO_4 buffer (pH 3.0-9.0) without filter paper strip at 38ºC for 60 min, and then measured by FPase assay under the standard conditions. The relative cellulase activity was measured to determine the stability of *AnsX*1 cellulase at different pH values. To study the effect of metal ions on the activity of *AnsX*1 cellulase, 10 mM of EDTA, Li^+, K^+, Na^+, Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+} or Zn^{2+} was added into 50 mM of citric acid/ Na_2HPO_4 buffer with optimal pH. In addition, the effect of various concentrations of K^+ or Ca^{2+} (1, 10 and 100 mM) was examined at optimal temperature and pH. To study the effect of ethanol on the activity of *AnsX*1 cellulase, enough ethanol was added to yield a final concentration of 5, 10, 15 or 20% in 50 mM of citric acid/ Na_2HPO_4 buffer. The reaction solution including crudecellulase samples 50 mg Whatman No. 1 filter paper strip was incubated at optimal temperature for 60 min. Total cellulase activity was measured by FPase assay.

Effects of different culture conditions on *AnsX*1 cellulase production

To optimize the fungal culture conditions, the effects of inoculum size, carbohydrate and nitrogen sources, and elicitors on cellulase production were studied by measuring total cellulase activity of crude *AnsX*1 samples from 2nd day after inoculation at 1 day interval. To examine optimal inoculum size for cellulase production, seed germination (SG) medium was prepared as described below. Briefly, 200 g of wheat bran was extracted by boiling in 1 l Mili-Q H_2O for 30 min, and filtered through 0.5 mm filter. The total volume of wheat bran extract was brought to 1 l with Mili-Q H_2O. The single colony grown on the selection medium A plate was inoculated into 500 ml SG medium and pre-cultured for 4 days. The 2, 5 or 10% inoculum size of pre-cultured *AnsX*1 was inoculated into the CP medium and cultured at 15ºC for 7 days on a rotary shaker (150 rpm) for cellulase production. To examine the optimal carbohydrate source for cellulase production, carboxymethyl cellulose (CMC), microcrystalline cellulose (MCC), or 2% of starch, instead of wheat bran, was used as the carbohydrate resource in CP medium. To examine the optimal nitrogen source for cellulase production, either 0.5% of NH_4NO_3, urea or yeast nitrogen base (YNB) replaced $(NH_4)_2SO_4$ and was used as the nitrogen resource in CP medium. To study the effect of elicitor on cellulase production, 0.1% of maltose, lactose, or sorbitol was add to the CP medium.

Statistical analysis

Triplicate samples were collected for each treatment. Data were subjected to analysis of variance (ANOVA). The significance of treatments was tested at the $P < 0.05$ level. Standard errors are provided in all figures as appropriate. All the statistical analyses were performed with the SPSS package (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Morphological and molecular characteristics of fugal strains from Antarctic soils

Twenty putative fungal strains were isolated from the crude Antarctic soil extracts. Ten fungal strain colonies with detectable cellulase activity, *AnsX*1-10, were selected by using a rapid and sensitive Congo red staining assay system (Figure 1a). The strains were cultured in liquid CP medium and collected for FPase assay. Among them, *AnsX*1 showed the highest FPU and was selected for further analysis.

To study the morphological characteristics of *AnsX*1, the strains were cultured on the PDA flat plate by using the scratching method. Compared with other isolated fungal strains, *AnsX*1 had the thicker mycelial cushion. It developed a villiform colony with a whitish surface and yellowish back 2 weeks after inoculation (Figure 1b). The molecular characteristics of *AnsX*1 were determined by analyzing the 16S rDNA internal transcribed spacer (ITS) region sequences. Based on blast analysis, the ITS sequences of *AnsX*1 had 97% similarity to those of *Verticillium* sp. and thus was preliminarily identified as *Verticillium* sp.

Effect of reaction temperature and pH on *AnsX*1 cellulase activity

The biochemical properties of cellulases depend on their origin. The temperature and pH of solution affect on the structure and activity of cellulase. The optimum temperature and pH for the activity of crude *AnsX*1 cellulase were examined.

FPase assays indicate that *AnsX*1 cellulase had the highest rate of enzyme activity (0.8 FPU/ml) at 38ºC which was 12ºC lower than that of current used commercial cellulase enzymes, and was stable at temperatures below 40ºC (Figure 2a and 2b). Compared with other fungal cellulases, *AnsX*1 cellulase showed comparable FPU activity to *Trichoderma reesei* Rut C-30 cellulase (Sun et al. 2008), and a lower activity than the commercially available cellulase produced from *Aspergillus niger* and *Trichoderma reesei* (Sigma). Neither the lower (23ºC) nor the higher temperature (53ºC) facilitated a high rate of cellulase activity (Figure 1a).

The effect of pH on the cellulase enzyme activity was studied by measuring the FPU activity at 38ºC. *AnsX*1 cellulase had the highest enzymatic activity at pH 5-6 and lowest at pH > 8 or < 3 (Figure 2c). The predicted optimum pH was 5.3. *AnsX*1 cellulase had comparable stability at pH 5-6 and was able to retain more than 80% of maximum activity even after 1 hr treatment at 38ºC (Figure 2d). A cellulase previously isolated from an alkali-tolerant *Verticillium* strainshowed the high cellulase activities at neutral and alkaline pH values (Solovyeva et al. 1999). Compared with the abovecellulase, *AnsX*1 cellulase had highest enzymatic activity at low temperature (38ºC) in an acidic environment (pH 5.3). It supports our hypothesis that the environmental factors had an important impact on the biochemical properties of cellulase, and the cellulase with capability of hydrolysis at lower temperatures can be identified from the extreme environment.

Effect of metal ions on *AnsX*1 cellulase activity

Metal ions have an effect on the activity and adsorption of cellulase on cellulose. To study the impacts of metal ions on *AnsX1* cellulase activity, we first measured cellulase activity in the presence of EDTA at 38ºC and pH 5.3. Compared with the control, *AnsX*1 cellulase activity was reduced to 30% in the 10 mM concentration of EDTA (Figure 3a). This suggests that the presence of trace metal ions in solution was required to maintain *AnsX*1 cellulase activity (Ferchak and Pye, 1983). Furthermore, we assessed

*AnsX*1 cellulase activity in the presence of various cations. The ions were independently added as their chlorides. The final concentration of each ion was 10 mM. *AnsX*1 cellulase activity assays demonstrate a significant inhibitory effect in the presence of Li^{+}, Fe^{3+} and Zn^{2+}, respectively (Figure 3a). In contrast, a 27% increase in *AnsX*1 cellulase activity was achieved in the presence of K^{+} or Ca^{2+} (Figure 3a). No effects were observed in the presence of Na^{+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+} and Mn^{2+}. These data imply that *AnsX*1 cellulase might use some metal ions as cofactors for activation or stabilization during the interaction with cellulose.

Both K^{+} and Ca^{2+} addition resulted in a 27% increase in *AnsX*1 cellulase activity and were selected for further studies. Compared with the control, the highest cellulase activity was obtained with 10 mM K^{+}. The presence of 100 mM Ca^{2+} resulted in a severe inhibition of cellulase activity (Figure 3b). Similar *AnsX*1 cellulase activity was observed in the presence of various concentrations of K^{+} (Ferchak and Pye, 1983). However, *AnsX*1 cellulase had a tolerance to higher concentrations of K^{+} (Figure 3b).

Effect of ethanol concentration on *AnsX*1 cellulase activity

Cellulases are inhibited by glucose which is the end-product of cellulose hydrolysis. To overcome the feedback inhibitory effects, simultaneous saccharification and fermentation (SSF) has been developed to fulfill the concurrent conversion of sugar into ethanol. Although ethanol has less inhibition on cellulase than cellobiose and glucose, it can denature protein structure and has a negative influence on cellulase activity. In this work, *AnsX*1 cellulase demonstrated 54-63% enzymatic activity at ethanol concentrations of 5-10% (Figure 4). In contrast, the cellulase prepared from a *Trichoderma reesi* retained 36% of its original activity at an ethanol concentration of 6.0% (Wu and Lee, 1997). Similarly, about 50% of *P. decumbens* cellulase activity was reduced at an ethanol level of 7% (Chen and Jin, 2006). Our data suggest that *AnsX*1 cellulase was able to tolerate high ethanol concentration and had potential to use for SSF.

Effect of inoculum size on *AnsX*1 cellulase activity

We studied the effect of inoculum size on *AnsX*1 cellulase activity. The highest cellulase activity was reached on the 4^{th} day after inoculation with 5% inoculum (Figure 5). The increased inoculum size reduced the time required to attain the maximum cellulase activity. For example, the maximum activity was obtained on the 6^{th} day after inoculation with 2% inoculum while it was reached on the 3^{rd} day with 10% inoculum (Figure 5). The larger inoculum size, however, dramatically decreased the production of *AnsX*1 cellulase due to a diffusion barrier in the liquid medium. This suggests that there is an inoculum threshold for *AnsX*1 and it has critical effects on cellulase production (Domingues et al. 2000). Thus, we selected a 5% inoculum for further studies.

Effect of carbon sources on *AnsX*1 cellulase production

Cellulose is hydrolyzed by cellulase and offers the carbon source for fungi cell metabolism in the form of glucose. Cellulase biosynthesis can be induced in the presence of cellulose or cellobiose, but repressed by glucose (Xiao et al. 2004). Thus, the type of carbon sources in the medium influence cell metabolism, cellulase biosynthesis, and play a vital role in the growth of fungi. CMC, MCC, 2% starch, and wheat bran as carbon sources influenced *AnsX*1 cellulase production. Among the various carbon sources, the highest cellulase activity and shortest time required for maximum activity were achieved by using wheat bran as the carbon source (Figure 6a). In contrast, less than 30% cellulase activity was obtained by using either CMC or MCC as the carbon source.

CMC and MCC were the best carbon sources for *A. niger* cellulase production while sucrose was the best for *Trichoderma* sp. (Gautam et al. 2011). The cellulase of *P. chrysogenum* PCL501 was stimulated by cellulose and sawdust in the absence of glucose (Nwodo-Chinedu et al. 2007). In this work, wheat bran was the best carbon source for the production of *AnsX*1 cellulase (Figure 6a). Starch, MCC and CMC used as the sole carbon source had potential catabolite repression effects on fungi cellulase (Berry and Paterson, 1990). Wheat bran is known as a complex carbon source including cellulose and hemicellulose. Hemicellulose can be depolymerized by most microorganisms because it has greater solubility than cellulose. The simple sugars derived from hemicellulose can furnish the *AnsX*1 metabolism but less repression on its cellulase express. Given the low cost of wheat bran together, wheat bran is an attractive carbon source for the production of *AnsX*1 cellulase.

Effect of nitrogen sources on *AnsX*1 cellulase production

Nitrogen is another limiting factor that influences the production of *AnsX*1 cellulase. The effect of various nitrogen sources on *AnsX*1 cellulase activity was examined. The *AnsX*1 strain had access to organic (YNB and urea) or inorganic ($(NH_4)_2SO_4$ and NH_4NO_3) forms of nitrogen (Figure 6b). In the presence of the various nitrogen sources, the cellulase activity of *AnsX*1 increased in the following order: urea (minimum) < NH_4NO_3 < $(NH_4)_2SO_4$ = YNB (maximum). Furthermore, the time required to reach the maximum cellulase activity in the medium with YNB and $(NH_4)_2SO_4$ was less than that with urea and NH_4NO_3 (Figure 6b). Compared with YNB, $(NH_4)_2SO_4$ had a low cost and was selected as an effective nitrogen source for further studies.

Effect of elicitors on *AnsX*1 cellulase production

Small soluble compounds such as cellobiose, sophorose, and lactose can induce cellulase genes expression in fungi (Llmén et al. 1997). The effect of various elicitors on *AnsX*1 cellulase activity was studied.

A significant increase of cellulase activity was also obtained by adding maltose or lactose in the medium. However, the time required to reach the maximum enzymatic activity was delayed in the presence of maltose or lactose (Figure 7). The above results suggest that poor carbon sources (lactose and maltose) could provoke the production of fungi cellulase. The addition of sorbitol resulted in a 200% increase in cellulase activity compared with the control (Figure 7). As reported previously, sorbitol has been recognized as a carbon source which neither promotes nor inhibits cellulase expression (Llmén et al. 1997). However, our results clearly showed that sorbitol had impacts on *AnsX*1 cellulase biosynthesis. It is possible that unknown molecular mechanisms may regulate *AnsX*1 cellulase genes expression. It is also possible that sorbitol plays a role in *AnsX*1 cell metabolism. The above alternatives are supported by the fact that the most carbon sources promoting cellulase expression are also used for growth by the fungi.

In summary, the fungal strain with highest cellulase activity (*AnsX*1) was isolated from Antarctic soils and identified by morphological and molecular analyses. The biochemical properties of crude cellulase demonstrate that *AnsX*1 cellulase was a cold-adapted enzyme in an acidic environment with metal ions and had a comparable tolerance to ethanol. Furthermore, the effects of inoculum size, carbohydrate and nitrogen sources, and elicitors on *AnsX*1 cellulase activity were studied to establish the optimum culture parameters for cellulase production. The optimal culture conditions for *AnsX*1 cellulase production were 5% inoculum, 2.0% wheat bran as carbon source, 0.5% $(NH_4)_2SO_4$ as nitrogen source, and 0.1% sorbitol added in the medium. Together with the development of genetic manipulation of cellulase structure, our present work will enable the development of an economic and efficient cold-adapted cellulase system for the bioconversion of lignocellulosic biomass into biofuels.

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REFERENCES

ADNEY, B. and BAKER, J. (1996). Measurement of cellulase activities. In: Laboratory Analytical Procedure No. 006. Golden, CO: *National Renewable Energy Laboratory*. p. 1-8.

BERRY, D.R. and PATERSON, A. (1990). Enzymes in Food Industry. In: SUCKLING, C.J. ed. *Enzyme Chemistry Impact and applications*. 2nd ed. Chapman and Hall, London, p. 306-351.

CHEN, H. and JIN, S. (2006). Effect of ethanol and yeast on cellulase activity and hydrolysis of crystalline cellulose. *Enzyme and Microbial Technology*, vol. 39, no. 7, p. 1430-1432. **[CrossRef]**

CORAL, G.; ARIKAN, B.; UNALDI, M.N. and GUVENMES, H. (2002). Some properties of crude carboxymethyl cellulase of *Aspergillus niger* Z10 wild-type strain. *Turkish Journal of Biology*, vol. 26, no. 4, p. 209-213.

DOMINGUES, F.C.; QUEIROZ, J.A.; CABRAL, J.M.S. and FONSECA, L.P. (2000). The influence of culture conditions on mycelial structure and cellulase production by *Trichoderma reesei* Rut C-30. *Enzyme and Microbial Technology,* vol. 26, no. 5-6, p. 394-401. **[CrossRef]**

DOYLE, J.J. and DOYLE, J.L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin,* vol. 19, no. 1, p. 11-15.

DUNCAN, S.M.; MINASAKI, R.; FARRELL, R.L.; THWAITES, J.M.; HELD, B.W.; ARENZ, B.E.; JURGENS, J.A. and BLANHETTE, R.A. (2008). Screening fungi isolated from historic Discovery Hut on Ross Island, Antarctica for cellulose degradation. *Antarctic Science,* vol. 20, no. 5, p. 463-470. **[CrossRef]**

FERCHAK, J.D. and PYE, E.K. (1983). Effect of cellobiose, glucose, ethanol, and metal ions on the cellulase enzyme complex of *Thermomonospora fusca*. *Biotechnology and Bioengineering,* vol. 25, no. 12, p. 2865-2872. **[CrossRef]**

GAUTAM, S.P.; BUNDELA, P.S.; PANDEY, A.K.; KHAN, J.; AWASTHI, M.K. and SARSAIYA, S. (2011). Optimization for the production of cellulase enzyme from municipal solid waste residue by two novel cellulolytic fungi. *Biotechnology Research International,* vol. 2011, p. 1-8. **[CrossRef]**

GOYAL, A.; GHOSH, B. and EVELEIGH, D. (1991). Characteristics of fungal cellulases. *Bioresource Technology,* vol. 36, no. 1, p. 37-50. **[CrossRef]**

HENRY, R.J. (2010). Evaluation of plant biomass resources available for replacement of fossil oil. *Plant Biotechnology Journal*, vol. 8, no. 3, p. 288-293. **[CrossRef]**

KÁDÁR, Z.; SZENGYEL, Z. and RÉCZEY, K. (2004). Simultaneous saccharification and fermentation (SSF) of industrial wastes for the production of ethanol. *Industrial Crops and Products*, vol. 20, no. 1, p. 103-110. **[CrossRef]**

KERRY, E. (1990). Effect of temperature on growth rates of fungi from Subantarctic Macquarie Island and Casey, Antarctica. *Polar Biology*, vol. 10, no. 4, p. 293-299. **[CrossRef]**

LLMÉN, M.; SALOHEIMO, A.; ONNELA, M.L. and PENTTILA, M.E. (1997). Regulation of cellulase gene expression in the filamentous fungus *Trichoderma reesei*. *Applied Environmental Microbiology,* vol. 63, no. 3, p. 1298-1306.

MILLER, G.L. (1972). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry,* vol. 31, no. 3, p. 426-428. **[CrossRef]**

NWODO-CHINEDU, S.; OKOCHI, V.I.; SMITH, H.A.; OKAFOR, U.A.; ONYEGEME-OKERENTA, B.M. and OMIDIJI, O. (2007). Effect of carbon sources on cellulase (EC 3.2.1.4) production by *Penicillium chrysogenum* PCL501. *African Journal of Biochemistry Research,* vol. 1, no. 1, p. 6-10.

SOLOVYEVA, I.V.; OKUNEV, O.N.; KRYUKOVA, E.G. and KOCHKINA, G.A. (1999). Occurrence of neutral and alkaline cellulases among alkali-tolerant micromycetes. *Systematic and Applied Microbiology,* vol. 22, no. 4, p. 546-550. **[CrossRef]**

SUN, W.C.; CHENG, C.H. and LEE, W.C. (2008). Protein expression and enzymatic activity of cellulases produced by *Trichoderma reesei* Rut C-30 on rice straw. *Process Biochemistry,* vol. 43, no. 10, p. 1083-1087. **[CrossRef]**

WU, Z. and LEE, Y.Y. (1997). Inhibition of the enzymatic hydrolysis of cellulose by ethanol. *Biotechnology Letters,* vol. 19, no. 10, p. 977-979. **[CrossRef]**

XIAO, Z.Z.; ZHANG, X.; GREGG, D.J. and SADDLER, J.N. (2004). Effects of sugar inhibition on cellulases and β-glucosidase during enzymatic hydrolysis of softwood substrates. *Applied Biochemistry and Biotechnology*, vol. 115, no. 1-3, p. 1115-1126. **[CrossRef]**

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Figures

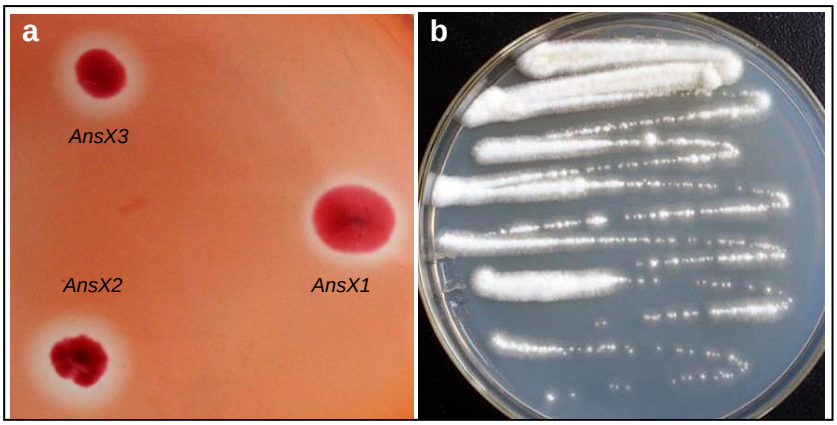

Fig. 1 Morphological characteristics of *AnsX*1. (a) Congo-red staining assay to identify candidate fungal strain from Antarctic soil isolates. (b) Villiform colony with whitish surface grown on cellulase production medium plate 2 weeks after inoculation.

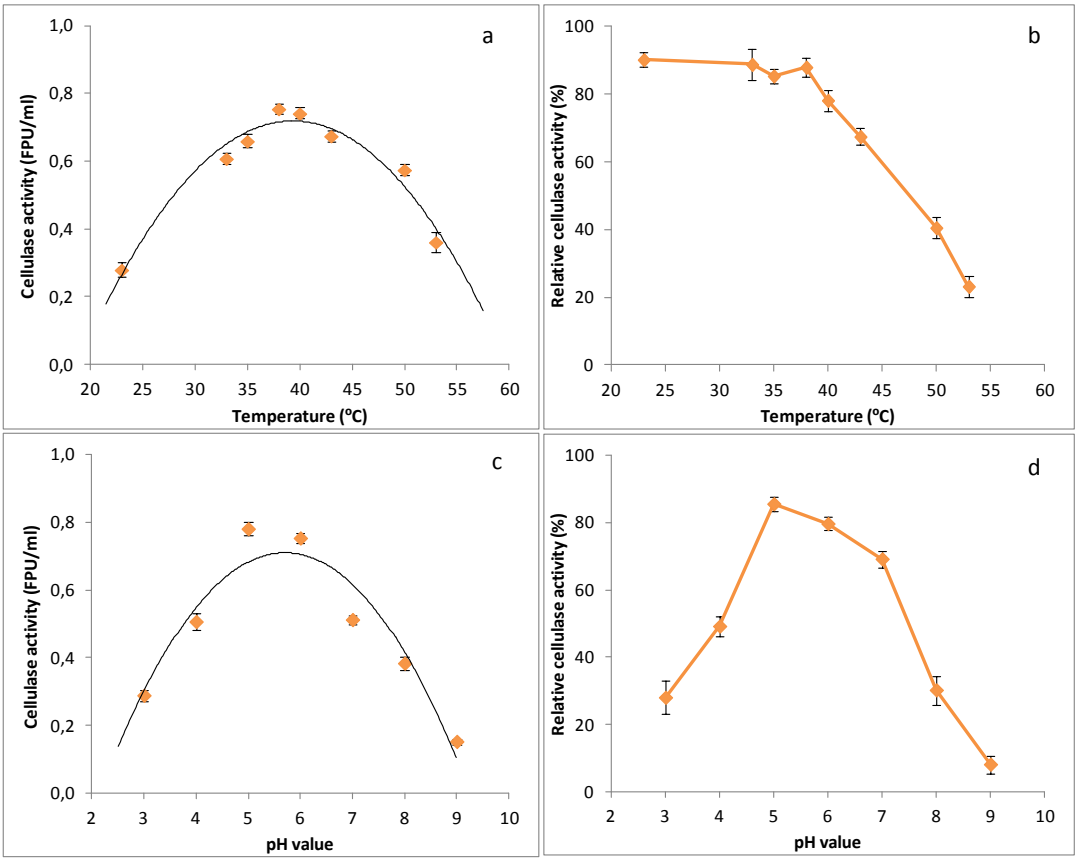

Fig. 2 Effects of reaction temperature and pH on *AnsX*1 cellulase activity and stability. (a) Effects of reaction temperature on *AnsX*1 cellulase activity. (b) Effects of reaction temperature on *AnsX*1 cellulase stability. Crude *AnsX*1 cellulase samples were incubated in 50 mM of citric acid/Na_2HPO_4 buffer (pH 5.3) without filter paper strip at different temperatures for 1 hr, and then measured by FPase assay under the standard conditions. The activity without treatment was taken as 100%. (c) Effects of reaction pH on *AnsX*1 cellulase activity. (d) Effects of pH values on *AnsX*1 cellulase activity. Crude *AnsX*1 cellulase sample was incubated in 50 mM of citric acid/Na_2HPO_4 buffer (pH 3.0-9.0) without filter paper strip at 38ºC for 1 hr, and then measured by FPase assay under the standard conditions. The activity without treatment was taken as 100%. Value are mean ± SE (n = 3).

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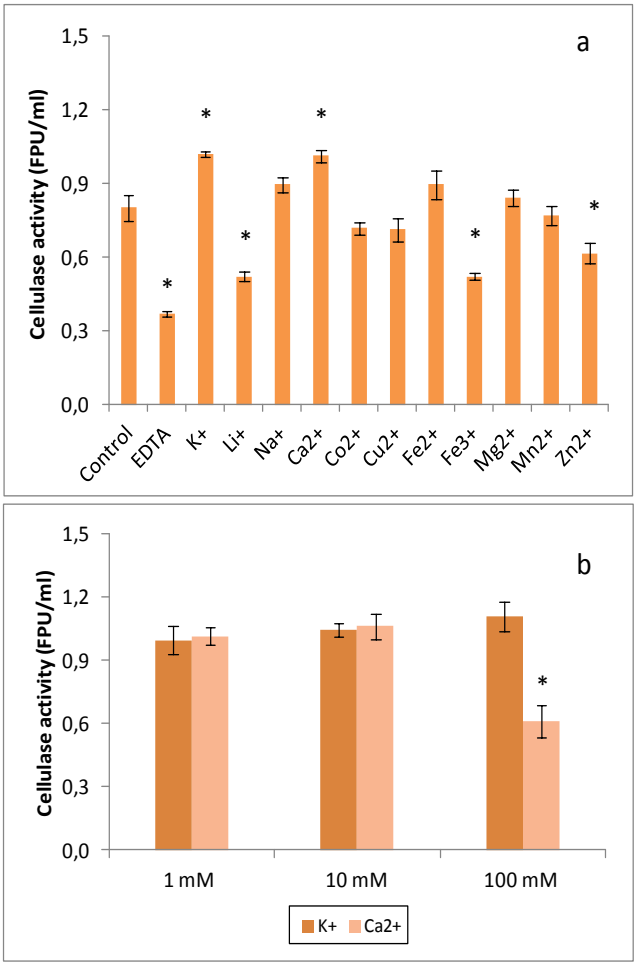

Fig. 3 Effects of metal ions on *AnsX*1 cellulase activity. (a) Crude *AnsX*1 cellulase samples were incubated in 50 mM of citric acid/Na_2HPO_4 buffer with 10 mM of EDTA, Li^+, K^+, Na^+, Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+} or Zn^{2+}. (b) Crude cellulase samples were incubated in 50 mM of citric acid/Na_2HPO_4 buffer with 1, 10, and 100 mM of K^+ and Ca^{2+}. Value are mean ± SE (n = 3). One asterisk indicates significance corresponding to $P < 0.05$ (One way ANOVA, Dunnett's test).

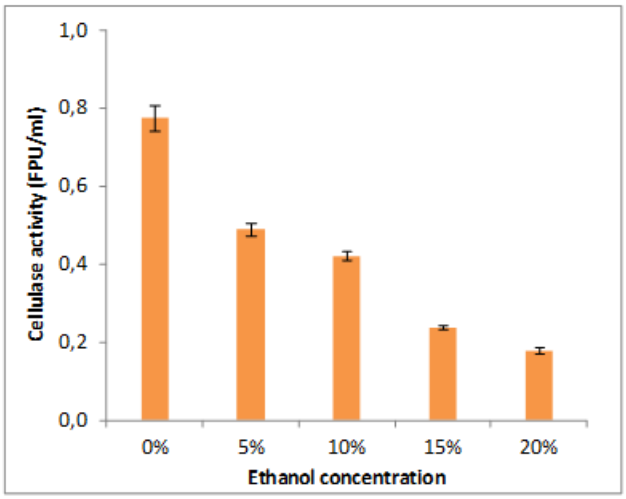

Fig. 4 Effects of ethanol concentration on *AnsX*1 cellulase activity. Value are mean ± SE (n = 3).

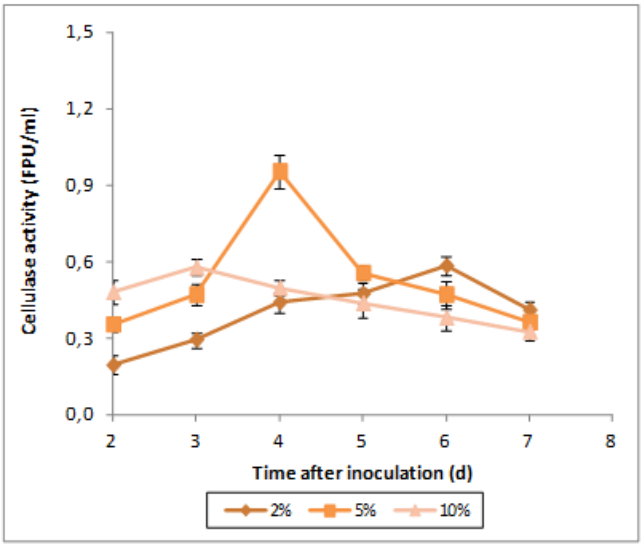

Fig. 5 Effects of inoculum size on *AnsX*1 cellulase production. Time course of cellulase production of *AnsX*1 were detected when cultured with 2% (diamonds), 5% (squares) or 10% (triangles) inoculum size of precultured strains. Value are mean ± SE (n = 3).

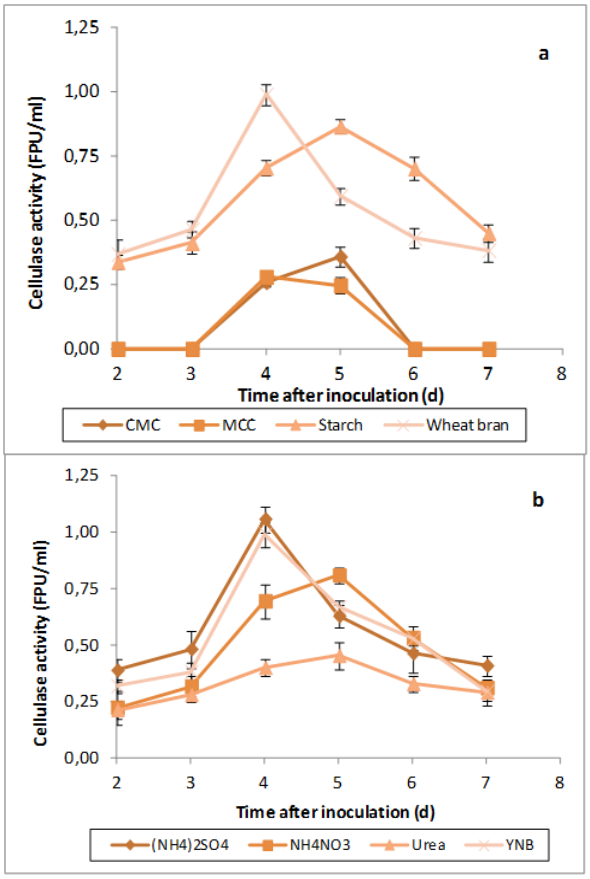

Fig. 6 Effects of carbon and nitrogen sources on *AnsX*1 cellulase production. Time course of cellulase production of *AnsX*1 were detected when cultured in cellulase production medium with CMC (diamonds), MCC (squares), 2% starch (triangles) and wheat bran (circles) as carbon source (a) or with $(NH_4)_2SO_4$ (diamonds), NH_4NO_3 (squares), Urea (triangles) and YNB (circles) as nitrogen source (b). Value are mean ± SE (n = 3).

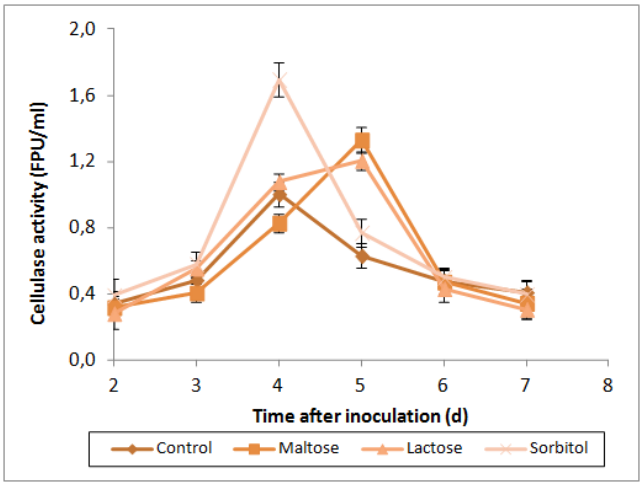

Fig. 7 Effects of elicitors on *AnsX*1 cellulase production. Time course of cellulase production of *AnsX*1 were detected when cultured alone (diamonds) or with maltose (squares), lactose (triangles), and sorbitol (circles) in cellulase production medium. Value are mean ± SE (n = 3).