

CHARACTERISATION AND STABILISATION OF RECOMBINANT *Humicola insolens* ENDOGLUCANASE PRODUCED IN *Pichia pastoris*

ABDUL FATTAH, S.S.¹, MOHAMED, R.¹, WAN SEMAN, W.M.K.¹, JAHIM, M.J.², ILLIAS, R.M.³,
ABU BAKAR, F.D.¹ and MURAD, A.M.A.^{1*}

¹School of Biosciences and Biotechnology, Faculty of Science and Technology,
Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia

²Department of Chemical and Process Engineering, Faculty of Engineering and Built Environment,
Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia

³Department of Bioprocess Engineering, Faculty of Chemical and Natural Resources Engineering,
Universiti Teknologi Malaysia, 81310 Skudai, Johor, Malaysia

*E-mail: munir@ukm.edu.my

ABSTRACT

Cellulases are industrially important hydrolytic enzymes that are applicable in the bioconversion of cellulosic biomass to simple sugars. In this work, *Pichia pastoris* carrying an endoglucanase cDNA (CMC3) from a thermophilic fungus, *Humicola insolens*, was grown in a 30 L bioreactor to produce recombinant CMC3 in a fed-batch cultivation mode. After optimisation of the cultivation conditions, a total of 5.3 gL⁻¹ proteins were obtained in a 20 L working volume after a 40 h induction with methanol. CMC3 expresses a β -1,4-endoglucanase with a specific activity of 62.83 U mg⁻¹, demonstrating its specificity for hydrolysing carboxymethyl cellulose as a substrate. No detectable hydrolysis on SigmaCell[®] cellulose, Avicel or beechwood xylan was observed. The recombinant CMC3 displayed moderate thermostability, being stable at up to 50°C for more than 72 h. Metal ions such as Mn²⁺ and Co²⁺ enhanced the CMC3 activity, while Ni⁺, Zn²⁺ and Cu²⁺ inhibited the enzyme activity. The CMC3 produced in *P. pastoris* was stable under long-term storage, retaining 84% and 75% of its initial activity after 4 months of storage at 4°C and 25°C, respectively. The addition of stabilisers further improved the enzyme stability by 7% and 5% at 4°C and 25°C, respectively.

Key words: Production, characterisation, stabilisation, endoglucanase, *Humicola insolens*

INTRODUCTION

The annual worldwide production of cellulose biomass is estimated to be approximately 1.5 trillion tonnes; approximately 70% of this waste is lignocellulosic materials, making it an essentially inexhaustible source of raw materials for environmentally friendly and biocompatible products (Kim and Yun, 2006). Lignocellulosic material consists primarily of three different types of polymers: cellulose, hemicellulose and lignin. Cellulose consists of glucose chains linked by β -1,4-glycosidic bonds. Cellulose degradation requires three types of cellulases: endo- β -glucanase (EC 3.1.2.4), exo- β -glucanase (EC 3.1.2.91), and β -glucosidase (EC 3.2.1.21) (Sharada *et al.*, 2013). Endo- β -glucanase randomly attacks internal O-glycosidic bonds, resulting in glucan chains of different lengths; exo- β -glucanase acts on the ends

of the cellulose chain and releases β -cellobiose as the end product; and β -glycosidases act specifically on β -cellobiose to produce glucose (Maheshwari *et al.*, 2000).

Cellulases have potential value in the alternative fuel and chemical industries because they efficiently use fermentable carbohydrates from existing agri-industrial plant wastes (Vishnu and Mala, 2012). Lignocellulosic substrates have a promising future as a potential chemical feedstock for replacing the petroleum feedstocks used for the manufacture of ethanol, acetone and butanol. Most commercial cellulases (including β -glucosidase) are produced by *Trichoderma* species and *Aspergillus* species (Kirk *et al.*, 2002). The genus *Humicola* has been known to produce thermostable endoglucanases (Maheshwari *et al.*, 2000). In this work, endoglucanase cDNA (CMC3) from the thermophilic fungus *Humicola insolens* was expressed in a *Pichia pastoris* expression system using a 30 L bioreactor and the properties of the

* To whom correspondence should be addressed.

recombinant enzymes were characterised. We also explored the usage of stabilisers to stabilise the enzyme for prolonged storage.

MATERIALS AND METHODS

Expression of recombinant CMC3 in *P. pastoris*

P. pastoris carrying CMC3 cDNA from the thermophilic fungus *H. insolens* under the regulation of the AOX1 promoter was a kind gift from Mohd Yusof Nor Rahim from the Universiti Kebangsaan Malaysia. *P. pastoris* was grown in a 30 L bioreactor with a 20 L working volume under optimised conditions (pH 5.0, 20°C during a 40 h methanol induction phase) to produce the recombinant CMC3 in fed-batch cultivation mode. The secreted proteins were analysed using SDS-PAGE.

Enzyme assay

The CMC3 activity was measured quantitatively by a colorimetric 3,5-dinitrosalicylic acid (DNS) assay with carboxymethyl cellulose as the substrate. The total reaction mixture of 1 mL contained 8 µg of enzyme and 1% (w/v) of substrate solution in 50 mM acetate buffer, pH 5.0, and was incubated at 50°C for 10 min. Aliquots of 500 µL of the reaction mixture were mixed with 500 µL of DNS reagent, followed by boiling at 95°C for 5 min. Each enzyme assay was performed in triplicate. The absorption of the reaction mixture was measured at 550 nm using a UV spectrophotometer. One unit (U) of enzyme activity is defined as the amount of enzyme required to produce 1 µmol of reducing sugar in one minute under the assay conditions.

Substrate specificity and thermostability of CMC3

The substrate specificity of CMC3 was determined by measuring the enzyme activity towards various substrates, such as carboxymethyl cellulose, Beechwood xylan, Sigmacell® cellulose and Avicel. The thermostability was investigated by measuring the residual activities under standard assay conditions after pre-incubation of the enzyme in the absence of substrate at temperatures from 40°C to 60°C for fixed time intervals (1, 2, 3, 4, 5, 12, 16, 24, 48 and 72 h).

Effect of metal ions, chemical reagents, and stabilisers on CMC3

To analyse the effects of various metal ions and chemical reagents on the activity of CMC3, 10 mM (final concentrations) of Mn²⁺, Co²⁺, Ca²⁺, NH₄⁺, K⁺, Na⁺, Fe²⁺, Li⁺, Ni⁺, Zn²⁺ and Cu²⁺ was added individually during the activity measurement. The effects of 10 mM ethylenediaminetetraacetic acid (EDTA) and urea were also determined. The effects of stabilisers such as 0.01% (w/v) sodium azide, 1%

(v/v) glycerol, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM EDTA and 1 mM dithiothreitol (DTT) on the activity of CMC3 were investigated by measuring the residual activities under standard assay conditions after pre-incubation of the enzyme in the presence of an individual stabiliser or a mixture of stabilisers at 4°C and 25°C for fixed time intervals (1, 2, 3, 4, 6, 8, 10, 12 and 16 weeks).

RESULTS AND DISCUSSION

The endoglucanase (CMC3) from the thermophilic fungus *H. insolens* was produced as an active recombinant protein in *P. pastoris* that was cultivated in a 30 L bioreactor. Based on the SDS-PAGE profile, CMC3 was expressed as the dominant protein in the *P. pastoris* culture filtrate, with a molecular weight of ~52 kDa (Fig. 1). This result is in agreement with the calculated mass of CMC3 predicted based on amino acid sequence information. An average of 5 gL⁻¹ of crude CMC3 was consistently produced by *P. pastoris* from the 20 L growing medium.

The catalytic activity of CMC3 was assayed under optimum conditions at pH 5.0 and 50°C. CMC3 hydrolysed soluble carboxymethyl cellulose effectively, with a specific activity of 62.83 Umg⁻¹. This activity is comparable to or higher than the reported activity of fungal recombinant endoglucanases, such as EglI from *Trichoderma*

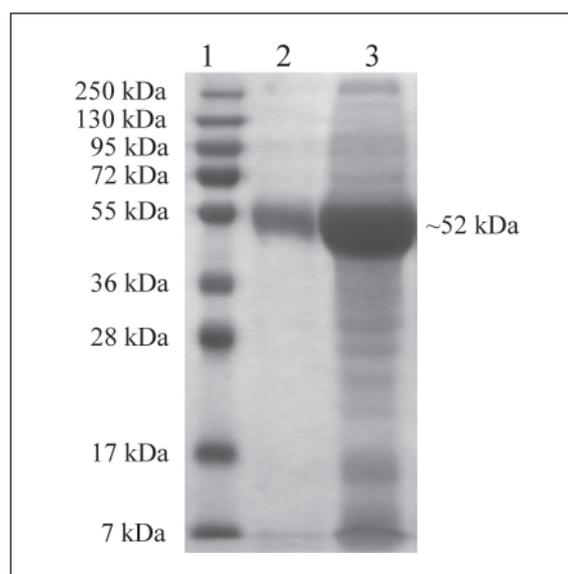


Fig. 1. SDS-PAGE of recombinant CMC3 from the thermophilic fungus *H. insolens*. Lane 1: protein marker; lane 2: crude recombinant CMC3 protein; lane 3: concentrated crude recombinant CMC3 protein.

reesei (49 U_{mg}⁻¹) and EglA from *Aspergillus niger* (9.47 U_{mg}⁻¹) (Nakazawa *et al.*, 2008; Quay *et al.*, 2011). No detectable hydrolysis on crystalline cellulose, Sigmacell[®] cellulose or Avicel was observed. The recombinant CMC3 had moderate thermostability and was stable at 50°C for more than 72 h (Fig. 2). These properties make the enzyme suitable for use in hydrolysis processes that require moderate temperatures but longer incubation periods.

Metal ions can influence the enzyme catalytic activity by acting as enzyme cofactors or inhibitors. The effects of several metal ions and chemical reagents on CMC3 activity are shown in Fig. 3. The addition of metal ions, such as Mn²⁺ and Co²⁺, increased the CMC3 activity by approximately 2.58 and 1.3-fold, respectively, compared with the

control assay. On the other hand, Zn²⁺ inhibited the enzyme activity. An increase in endoglucanase activity with the addition of Mn²⁺ has also been reported for endoglucanase from *A. niger* (Quay *et al.*, 2011) and *Penicillium chrysogenum* (Chinedu *et al.*, 2008).

The effect of storage on CMC3 activity was determined because storage stability is an important parameter for the industrial application of enzymes. During long-term storage, proteins tend to lose their activity as a result of proteolysis, aggregation or microbial contamination. In this work, the CMC3 produced in *P. pastoris* was highly stable even after prolonged storage. The enzyme maintained 84% and 75% of its original activity after 4 months of storage at 4°C and 25°C, respectively (Fig. 4). To further improve the enzyme stability during storage,

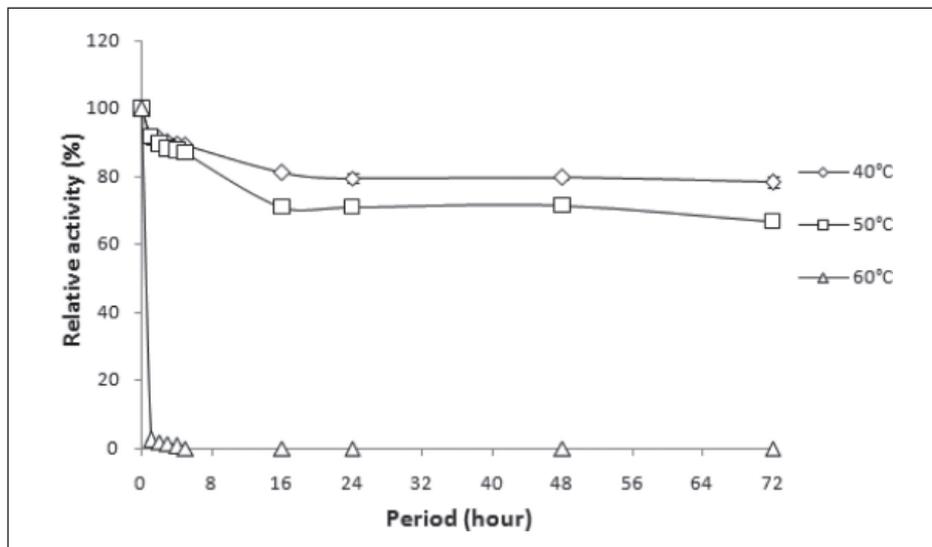


Fig. 2. Thermostability of CMC3 after a 72 h incubation at different temperatures.

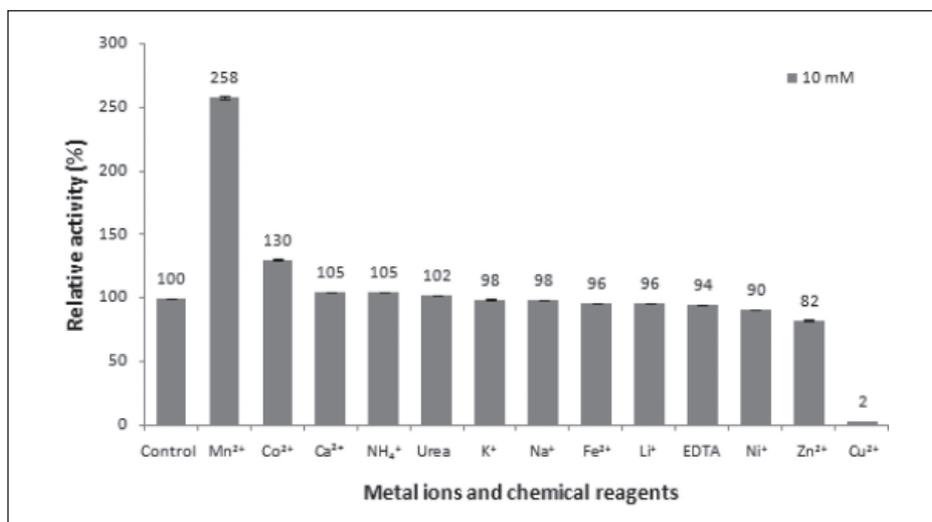


Fig. 3. Effects of metal ions and chemical reagents on CMC3 activity.

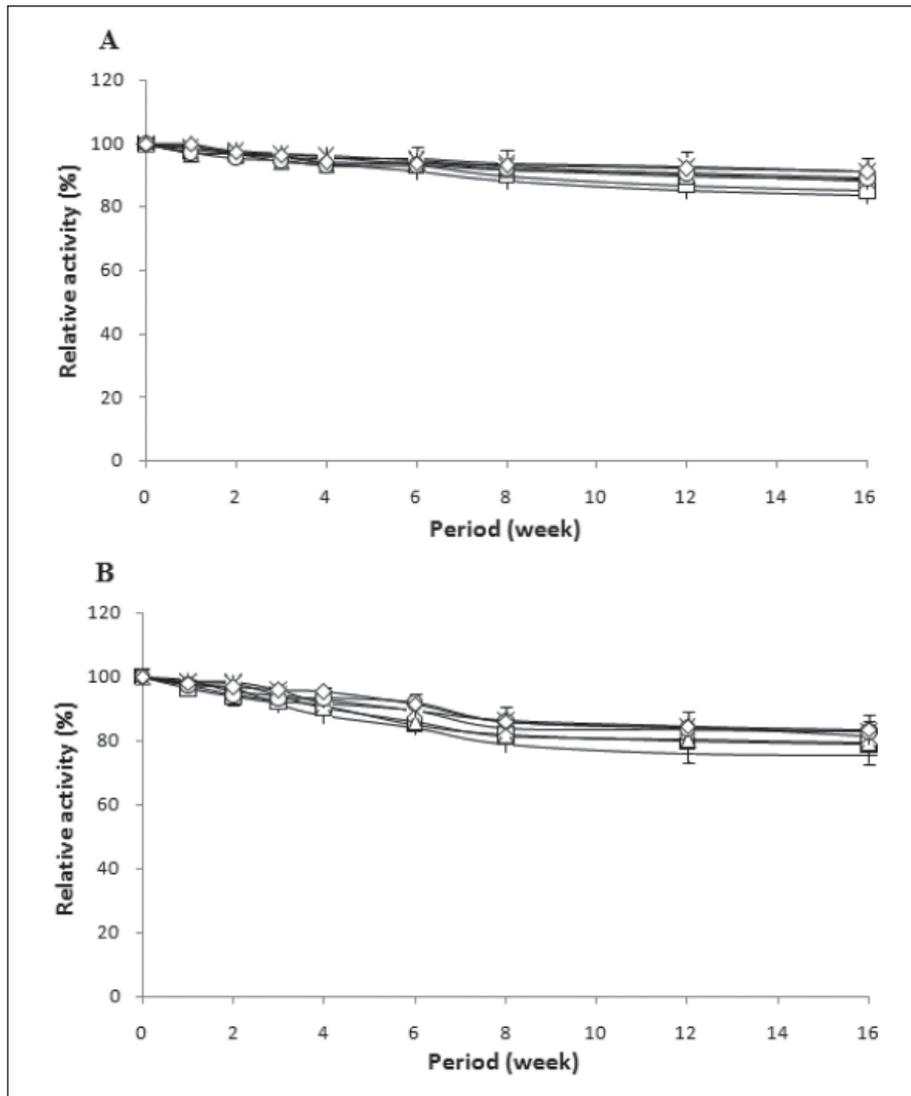


Fig. 4. Effects of stabilisers on CMC3 stability at various storage temperatures for a period of 16 weeks. (A) Storage of CMC3 at 4°C in the presence of various stabilisers, (B) storage of CMC3 at 25°C in the presence of various stabilisers. —|— without stabiliser, —□— 0.01% sodium azide, —△— 0.01% sodium azide + 1% glycerol, —×— 0.01% sodium azide + 1 mM PMSF, —*— 0.01% sodium azide + 1 mM EDTA, —○— 0.01% sodium azide + 1 mM DTT, —◇— 0.01% sodium azide + 1% glycerol + 1 mM PMSF + 1 mM EDTA + 1 mM DTT.

several known enzyme stabilisers, such as sodium azide, glycerol, PMSF, EDTA and DTT, individually or as a mixture, were added to CMC3. Sodium azide is an anti-microbial agent that is able to inhibit microbial growth, while the protease inhibitor PMSF helps to prevent proteolytic cleavage of the protein. On the other hand, metal chelators (EDTA) and reducing agents (DTT) maintain the protein in a reduced state. We observed that the addition of a stabiliser mixture containing 0.01% (w/v) sodium azide, 1% (v/v) glycerol, 1 mM PMSF, 1 mM EDTA and 1 mM DTT improved the CMC3 activity after prolonged storage at 4°C and 25°C by 7% and 5%, respectively, compared with the control assay (Fig. 4a & 4b).

In conclusion, recombinant CMC3 from the thermophilic fungus *H. insolens* has been produced as an active protein in *P. pastoris*. The enzyme showed good activity towards soluble cellulose and was stable during prolonged storage at 4°C and 25°C. The addition of stabilisers enhanced the stability of the enzyme during long-term storage.

ACKNOWLEDGEMENTS

This research was funded by the Ministry of Agriculture (MOA), Malaysia under the Technofund grant TF0310F86 and by the Universiti Kebangsaan Malaysia under research grant UKM-DLP-2012-019.

REFERENCES

- Chinedu, S.N., Nwinyi, C.O. & Okochi, V.I. 2008. Properties of endoglucanase of *Penicillium chrysogenum* PCL501. *Australian Journal of Basic and Applied Sciences*, **2(3)**: 738-746.
- Kim, J. & Yun, S. 2006. Discovery of cellulose as a smart material. *Macromolecules*, **39**: 4202-4206.
- Kirk, O., Borchert, T.V. & Fuglsang, C.C. 2002. Industrial enzyme applications. *Current Opinion in Biotechnology*, **13**: 345-351.
- Maheshwari, R., Bharadwaj, G. & Bhat, M.K. 2000. Thermophilic fungi: their physiology and enzymes. *Microbiology and Molecular Biology Reviews*, **64**: 461-488.
- Nakazawa, H., Okada, K., Kobayashi, R., Kubota, T., Onodera, T., Ochiai, N., Omata, N., Ogasawara, W., Okada, H. & Morikawa, Y. 2008. Characterization of the catalytic domains of *Trichoderma reesei* endoglucanase I, II, and III, expressed in *Escherichia coli*. *Applied Microbiology and Biotechnology*, **81**: 681-689.
- Quay, D.H.X., Bakar, F.D.A., Rabu, A., Said, M., Illias, R.M., Mahadi, N.M., Hassan, O. & Murad, A.M.A. 2011. Overexpression, purification and characterization of the *Aspergillus niger* endoglucanase, EglA, in *Pichia pastoris*. *African Journal of Biotechnology*, **10**: 2101-2111.
- Sharada, R., Venkateswarlu, G., Venkateshwar, S. & Rao, M.A. 2013. Production of cellulase – a review. *International Journal of Pharmaceutical, Chemical and Biological Sciences*, **3(4)**: 1070-1090.
- Vishnu, M. & Mala, R. 2012. Trends in bioconversion of lignocellulose: Biofuels, platform chemicals & biorefinery concept. *Progress in Energy and Combustion Science*, **38(4)**: 522-550.

