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Molecular cloning of *kman* coding for mannanase from *Klebsiella oxytoca* KUB-CW2-3 and its hybrid mannanase characters

Nawapan Pongsapipatana[a,b,d], Piyanat Damrongteerapap[a], Sudathip Chantorn[c], Wilawan Sintuprapa[a], Suttipun Keawsompong[a,b,d], Sunee Nitisinprasert[a,b,d,*]

[a] *Specialized Research Unit: Prebiotics and Probiotics for Health, Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Bangkok, Thailand*
[b] *Center for Advanced Studies for Agriculture and Food, KU Institute for Advanced Studies, Kasetsart University (CASAF, NRU-KU), Bangkok 10900, Thailand*
[c] *Department of Biotechnology, Faculty of Science and Technology, Thammasat University, Thailand*
[d] *Center of Excellence on Agricultural Biotechnology: (AG-BIO/PERD O-CHE), Bangkok 10900, Thailand*

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ABSTRACT

Gene encoding for β-mannanase (E.C 3.2.1.78) from *Klebsiella oxytoca* KUB-CW2-3 was cloned and expressed by an *E. coli* system resulting in 400 times higher mannanase activities than the wild type. A 3314 bp DNA fragment obtained revealed an open reading frame of 1164 bp, namely *kman-2*, which encoded for 387 amino acids with an estimated molecular weight of 43.2 kDa. It belonged to the glycosyl hydrolase family 26 (GH26) exhibited low similarity of 50–71% to β-mannanase produced by other microbial sources. Interestingly, the enzyme had a broad range of substrate specificity of homopolymer of ivory nut mannan (6%), carboxymethyl cellulose (30.6%) and avicel (5%), and heteropolymer of konjac glucomannan (100%), locust bean gum (92.6%) and copra meal (non-defatted 5.3% and defatted 7%) which would be necessary for *in vivo* feed digestion. The optimum temperature and pH were 30–50 °C and 4–6, respectively. The enzyme was still highly active over a low temperature range of 10–40 °C and over a wide pH range of 4–10. The hydrolysates of konjac glucomannan (H-KGM), locust bean gum (H-LBG) and defatted copra meal (H-DCM) composed of compounds which were different in their molecular weight range from mannobiose to mannohexaose and unknown oligosaccharides indicating the endo action of mannanase. Both H-DCM and H-LBG enhanced the growth of lactic acid bacteria and some pathogens except *Escherichia coli* E010 with a specific growth rate of 0.36–0.83 h^{-1}. H-LBG was more specific to 3 species of *Weissella confusa* JCM 1093, *Lactobacillus reuteri* KUB-AC5, *Lb salivarius* KL-D4 and *E. coli* E010 while both H-KGM and H-DCM were to *Lb. reuteri* KUB-AC5 and *Lb. johnsonii* KUNN19-2. Based on the nucleotide sequence of *kman-2* containing two open reading frames of 1 and 2 at 5′ end of the +1 and +43, respectively, removal of the first open reading frame provided the recombinant clone *E. coli* KMAN-3 resulting in the mature protein of mannanase composing of 345 amino acid residues confirmed by 3D structure analysis and amino acid sequence at N-terminal namely KMAN (GenBank accession number KM100456). It exhibited 10 times higher extracellular and periplasmic total activities of 17,600 and 14,800 units than *E. coli* KMAN-2. With its low similarity to mannanases previously proposed, wide range of homo- and hetero-polysaccharide specificity, negative effect to *E. coli* and most importance of high production, it would be proposed as a novel mannanase source for application in the future.

1. Introduction

β-Mannanase (EC 3.2.1.78), an *endo*-acting type enzyme, is one of the mannan degrading enzymes and is capable of digesting D-1,4-mannopyranosidic linkages randomly within a backbone of either mannan or heteromannan (galactomannan, glucomannan and galactoglucomannan) resulting in manno-oligosaccharide [1]. It has been used in the feed industry as a feed enzyme incorporated with other enzymes to improve feed quality by degrading the mannan and heteromannan components in feed ingredients such as palm kernel meal and copra meal into simple sugars used as an energy source and prebiotic manno-oligosaccharide (MOS) [1,2]. These degraded mannan and heteromannan in the feed ingredients also resulted in the elimination of anti-nutritional substances,

* Corresponding author at: Specialized Research Unit: Probiotics and Prebiotics for Health, Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University 50 Ngam Wong Wan Rd, Lat Yao Chatuchak Bangkok 10900, Thailand.
E-mail address: fagisnn@ku.ac.th (S. Nitisinprasert).

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improvement in the feed digestibility and benefit to beneficial microorganisms in the animal gut [3]. MOS also has an enhancing effect in stress environment toleration of heat, stimulating gastric and intestinal juice [4].

β-mannanase has been produced by various sources. There are numerous studies on the production of mannanase including the cloning and expression of the mannanase gene from many sources of fungi and bacteria; for example, *Aspergillus niger* [5,6], *Penicillium freii* [7], *Penicillium pinophilum* [8], *Bacillus* species [9–12], *Paenibacillus* sp. [13,14], *Erwinia carotovora* CXJZ95-198 [15], Bifidobacterium adolescentis [16] and *Sphingomonas* sp. JB13 [17]. Recently, Olaniyi et al. [18] successfully isolated 8 bacterial strains belonging to *Bacillus subtilis*, *Paenibacillus polymyxa* and Klebsiella edwardsii from agricultural waste. They were able to produce mannanase with activities in the range 87.958–103.200 U/ml and these were expected to apply to β-mannanase production in solid state fermentation.

Klebsiella is a Gram-negative bacterium which has been found in clinical habitats (hospital environment) as an opportunistic pathogen and in non-clinical habitats such as the human intestinal tract, animals, water, soils and plants where *Klebsiella oxytoca* associates with most of the habitats examined [19]. It is also involved in plants as one of the nitrogen-fixing microbes; for example, the mechanism of *K.* oxytoca VN13 in wheat roots is to enter plant tissue and to degrade the plant cell wall by degrading enzyme, and colonizing [20]. Mannanase producing *K. oxytoca* KUB-CW2-3 isolated from fermented coconut waste [21] showed an interesting characteristic in the hydrolysis of copra meal, which is an agricultural waste, and its hydrolysate showed growth-promoting properties of Lactobacilli and growth inhibition of *Salmonella* Enteritidis [22] indicating potential prebiotic properties. However, it produced only a low mannanase yield of approximately 0.5 U/ml of mannanase. Heterologous expression of protein in an *E. coli* system has been successfully used to increase the yield from various bacterial sources. Songsiririthigul et al. [23] reported an expression of mannanase gene of *Bacillus licheniformis* strain DSM13 in an *E. coli* system. The total mannanase activity of approximately 50,000 units could be obtained from 1 l of culture of recombinant *E. coli*. Also, Summpunn et al. [24] reported that heterologous expression in an *E. coli* system of mannanase gene from *B. subtilis* BCC41051 increased production yield 38 folds compared to *B. subtilis* BCC41051. In this study, we propose the cloning and expression of mannanase in *E. coli* system to increase the production of mannanase including its characterization.

2. Materials and methods

2.1. Bacterial strains and culture conditions

K. oxytoca KUB-CW2-3 was used as a source of the mannanase gene and cultured in nutrient broth (Merck, Germany) at 37 °C for 18 h. *E. coli* DH5α and TOP10, used as a cloning and expression host, respectively, were cultured in Luria-Bertani broth (1% trytone, 0.5% yeast extract and 1% NaCl) and incubated at 37 °C on a rotary shaker at 200 rpm for 18 h. Lactic acid bacteria and pathogenic bacteria used for mannan hydrolysate evaluation and their cultivation conditions are shown in Table 1.

2.2. Vectors

Plasmid vector pUC19 and pFlag-CTS obtained from the School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology were used as a cloning and expression vector, respectively. *E. coli* DH5α harboring plasmid pUC19 and pFlag-CTS were cultured in Luria-Bertani broth containing 100 μg/ml of ampicillin (Amresco, USA) and incubated at 37 °C with shaking at 200 rpm for 18 h. The plasmids were extracted by using QIAprep® Spin Miniprep Kit (Qiagen, USA).

2.3. Construction of genomic library

Genomic DNA of K. oxytoca KUB-CW2-3 was extracted using the modified method of Sambrook and Russell [25] and partially digested by 0.5 units of Sau3AI for 20 min to obtain 3–7 kb DNA fragments. The DNA fragments were ligated into plasmid pUC19 at the BamHI restriction site and transformed into *E. coli* DH5α using a heat-shock technique. The transformants were screened on Luria-Bertani agar containing 100 μg/ml ampicillin, 40 μg/ml X-gal and 0.1 mM IPTG. The white colonies were further screened on Luria-Bertani agar containing 0.5% (w/v) locust bean gum and 100 μg/ml ampicillin to determine their mannanase activities as a halo zone.

2.4. Construction of overexpression system

The expression plasmid pFlag-CTS obtained from the School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology was genetically modified by including C-terminal histidine polymer prior to FLAG peptide used for further purification [23]. The ampicillin resistance gene, ompA coding signal peptide in *E. coli* for periplasmic space secretion and tac promoter using IPTG as an inducer, were also included and used for mannanase gene expression. Two primers targeted mannanase gene were designed as two forward primers of No298-ExF (5′TACAgaattcATGTG CATTTATACAGTAG TTTGC3′) and EX298-SF (5′ATCAgaattcATGACTTCTTCAA CTGCAGTACC 3′) starting at the first and second ATG, respectively, and reverse primer No298-ExR (5′TATAggtaccCCAGCTGATGCT GATGTCGC3′) without stop codon of *kman* to amplify mannanase gene with the linker of EcoRI and KpnI, respectively. The PCR reaction containing 50 ng of recombinant DNA kman-1, 5 μl of 10X buffer, 5 μl of dNTP mix (2.5 mM each), 1 μl of 0.2 μmol of each primers 0.5 μl of Pfu DNA polymerase (1.25 Units) (Fermentas, USA) and dH_2O added to obtain the final volume of 50 μl PCR reactions were subjected to initial denaturation at 95 °C for 3 min, 30 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 3 min followed by final extension at 72 °C for 5 min. The amplified PCR product was ligated to EcoRI and the KpnI site of vector pFlag-CTS and transformed into *E. coli* TOP10 to obtain the recombinant clone producing the mannanase KMAN-2 and KMAN-3.

2.5. Nucleotide sequencing and analysis

The mannanase gene was sequenced at First BASE Laboratories Sdn Bhd, Malaysia. The sequence was analyzed using the NCBI BLAST programs (Rockville, MD, USA; http://blast.ncbi.nlm.nih.gov/Blast.cgi) [26]. The open reading frame of the gene was analyzed using the ORF Finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The conserved domain was analyzed by Pfam (http://pfam.xfam.org/) [27] and NCBI Conserved Domain (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) [28]. The three dimensional (3D) structure was analyzed using the SWISS-MODEL program (http://swissmodel.expasy.org/) [29,30] and the RaptorX program (http://raptorx.uchicago.edu/) [31].

2.6. Purification of β-mannanase

The recombinant *E. coli* KMAN-2 and *E. coli* KMAN-3 were cultured in Luria-Bertani broth containing 100 μg/ml ampicillin and incubated at 37 °C for 18 h. The periplasmic enzyme extraction was performed using the modified method of Yamabhai et al. [32]. Briefly, one percent of overnight culture was transferred to 1000 ml