Front. Biol. China 2008, 3(1): 26–31
DOI 10.1007/s11515-008-0002-5

RESEARCH ARTICLE

Heterologous expression and characterization of *man* gene from *Bacillus Subtilis* in *Pichia Pastoris*

Yu QIAO, Xiaobing CHEN, Hongbiao DING (✉), Ming YUE

Feed Research Institute, Chinese Academy of Agricultural Sciences, Beijing 100081, China

Abstract *β*-Mannanase catalyzes endo-wise hydrolysis of the backbone of mannan and heteromannan, which are abundant in the cell wall structure of ungerminated leguminous seeds. The mature *β*-mannanase originated from *Bacillus subtilis* was expressed in *Pichia pastoris*, a methylotrophic yeast, using the leader peptide sequence of *Saccharomyces cerevisiae* α-factor. The cultivation of *β*-mannanase expressing *Pichia pastoris* yields up to 1.8 g/L protein. In the supernatant the activity of the 40 kDa—total mannanase attained a level of 1102.0 IU/mL. The properties of the *β*-mannanase were characterized. Optimum pH and temperature for the recombinant enzyme were 5.5 and 50°C respectively. The enzyme was stable at pH 5.0–10.0 and maintained over 30% original activity after incubating at 70°C for 30 min.

Keywords *β*-mannanase, *Pichia pastoris*, expression

1 Introduction

The *β*-Mannan is the second most abundant hemicellulose in nature, and its highly viscous polysaccharide presents in various feedstuffs and protein concentrates such as guar gum, palm kernel meal, soybean meal and copra meal. The mannans contained in corn-soybean meal broiler diets reduced the feed conversion ratio by more than seven points, causing low feed intake and weight gain, and causing environmental problems (Slominski et al., 1994). Endo-*β*-mannanases (EC 3.2.1.78) catalyze the random hydrolysis of the *β*-1,4-mannosidic backbone of the main mannan chain. Addition of *β*-mannanase was known to improve productive performance by 4.4%–10.3% when added to poultry and swine diets, and significantly enhanced performance and reduced lesion scores in chicks challenged with *Eimerla* sp. and *Clostridium perfringens* (Jackson et al., 2003). Domestic animals do not have secret mannanase themselves, and in general, mannanase constitutes only a small percentage of the proteins secreted by hemicellulose degrading microorganisms. This problem could be solved by cloning and heterologous expression of mannanase encoding genes.

The *β*-Mannanase was first found in plants and germinating seeds, further it was isolated and identified in fungi, bacteria, even blue mussel (Shimahara et al., 1975; Araki, 1982; Akino et al., 1988; Johnson, 1990; Oda et al., 1993; Stålbrand et al., 1993; Xu et al., 2002a; Ootsuka et al., 2006). Because the data were available from several difficult sources on purification and characterization of *β*-1,4-mannanases, the recombinant enzymes were still poorly produced and expressed, and the expression level was relatively low (Stålbrand et al., 1993; Mendoza et al., 1995; Tang et al., 2001; Xu et al., 2002b; Mingardon et al., 2005). The *β*-1, 4-mannanases production strain currently used in animal husbandry now is a *Bacillus lentus* patented by Chemgen Company, the production yield of which is relative high. Indeed expenses for the feeding industry are still high. The *β*-mannanase gene of *B. subtilis* origin has been expressed in the *E. coli* system in denatured form with low yield of enzyme activities, but has not yet been reported expressed by means of the yeast expression system at acceptable level. The *Pichia pastoris* expression system was employed for large-scale production of *β*-mannanase in bench-top bioreactor systems in this report.

2 Materials and methods

2.1 Materials

Restriction endonucleases, T4 DNA ligase, Pyrobest DNA polymersase were retrieved from Takara (Tokyo, Japan). Isopropyl-*β*-D-thiogalactopyranoside (IPTG) and locust bean gum (LBG) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Yeast nitrogen base, D-biotin were

---

Translated from *China Biotechnology*, 2005, 26(7): 52–56 [译自: 中国生物工程杂志]

E-mail: dinghongbiao@mail.caas.net.cn

obtained from Amresco (Solon, Ohio, USA). Yeast extract and tryptone were gained from OXOID Ltd. (Basingstoke, Hampshire, England). Bovine serum albumin was retrieved from Roche (Mannheim, Germany). EasyCompTM Kit for Pichia transformation was purchased from Invitrogen Corporation (Invitrogen Corporation, Calif, Germany). PGEM-T Vector was purchased from Promega (Madison, WI, USA). The polymerase chain reaction (PCR) products purification kit was purchased from Qiagen (Valencia, Calif, Germany). The other chemicals used were regent grade from Beijing Chemical Regents Company.

2.2 Construction of the expression plasmid *pPIC9k-man* for use in *P. pastoris* host cell

*B.subtilis*22, an extracellular *β*-Mannanase producer, was isolated from soil (data are not reported). The chromosomal DNA of *B.subtilis* was prepared as described by Ausubel et al. (1987). The PCR amplification of chromosomal DNA was performed using basal metabolic rate (BMR) (containing coding mature *β*-mannanase) and basal metabolic rate (BMR) (right before the stop codon) according to the reported *β*-mannanase gene sequence from *B.subtilis* (Mendoza et al., 1995). Two specific restriction sites (*Eco*RI at the 5′ end and *Not*I at the 3′ end of the coding sequence, in bold) were incorporated into the PCR primers. The primers used for the PCR are described as below.

BMF: 5′-CG**GAATTC**CACACCGTTTATCCCG TCAAC-3′.
BMR: 5′-TT**GCGGCCGC**AAACCGATTTCAAAAGAA-3′.

A 50-μL PCR mixture contained 500 ng of DNA, 2 mM of each dNTP, 30 pM of each primer and 5 mM of additional magnesium chloride, 2.5 U Pyrobest DNA polymersase. The reaction mixture was denatured for 5 min at 94°C followed by 35 cycles under the conditions of 94°C, 1 min; 57°C, 1 min; 72°C, 1 min and then completed at 72°C for 10 min.

The PCR products were characterized by agarose gel electrophoresis and extracted. Purified products were ligated into pGEM-T vector according to the manufacturer's instructions and transformed into high efficiency competent cells DH-5α. The plasmid was digested with the restriction enzymes *Eco*RI and *Not*I and purified using Gel Purification Kit. Meanwhile, the expression vector pPIC9k was also digested by the same restriction enzymes and purified. The engineered, mature-*β*-mannanase-coding sequence was directionally cloned into the *E.coli*/*Pichia* shuttle vector. The ligation product was transformed into *E.coli* competent cells DH5α. The *E.coli* transformants were selected on plates containing the antibiotic, ampicillin at a concentration of 50 μg/mL. The verification of inserting the PCR fragment into the correct translational reading frame was confirmed by DNA sequencing. Sequencing primers (5′AOX1 and 3′AOX1) were obtained from Invitrogen.

P. pastoris GS115 strain was selected to be a host strain. The *pPIC9k-man* plasmid DNA was purified from *E.coli* cells and linearized with the restriction enzyme BglII to allow integration of the vector DNA into the chromosomal DNA. Pichia transformation was performed using the EasyCompTM Kit, and the transformants were selected on the regeneration dextrose base (RDB) plates. The presence of the *man* gene in the transformants was confirmed by PCR using yeast genomic DNA as the template.

2.3 Expression of *β*-mannanase from transformed *P. pastoris*

For the screening of strains and for the optimization of culture conditions in shaking flasks, buffered complex medium containing glycerol (BMGY) and buffered complex medium containing methanol (BMMY) were used for the growth of the cells and for the induction, respectively, according to Invitrogen's instructions. *β*-Mannanase-producing *P. pastoris* cells were grown in 25 mL BMGY at 30°C to a cell density of 4.5. Transferring the cells into 25 mL BMMY at the desired temperature initiated induction. Methanol was added every 24 h to maintain the induction. The induction was interrupted when the enzyme concentration in the medium leveled off, usually after a period of 96 h. A *β*-mannanase-containing cell free culture supernatant was obtained by centrifugation. Several induction conditions were investigated: pH values of 4, 5, 6 and 7, methanol concentrations of 0.4%, 0.5%, 1%, 2% and 3% (adding every 24 h) and temperatures of 20°C and 30°C.

After optimization, the transformed strain was grown in fermentation basal salts (FBS) medium (containing $CaSO_4$, K_2SO_4, $MgSO_4$, H_3PO_4 and glucose) in a fermenter with the volume of 5 L at 30°C, and pH was controlled and kept around 5.0. Glucose was fed to increase the cell density. After the consumption of glucose, the temperature was lowered to 20°C and induction by 0.4% methanol containing trace elements was started. After an induction period of 96 h the production rate leveled off, the fermentation was interrupted. The concentration of secreted total supernatant proteins was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and expressed *β*-mannanase activity in the culture supernatants was also determined using the standard curve analysis of *β*-mannanase at every 12 h of induction phase.

2.4 Determination of protein content and SDS-PAGE

The protein concentrations of the culture supernatants were determined by method of Coomassie Brilliant Blue G-250 using bovine serum albumin as a standard (Thomas, 1978). The SDS-PAGE was done according to the method of Laemmli (Laemmli, 1970). Gels were stained for protein with Coomassie Brilliant Blue R-250.

2.5 Measurement of enzymic activity and other properties

The *β*-Mannanase activity in culture supernatants was determined by adapting the dinitrosalicylic acid method for reducing sugar analysis according to Stålbrand et al. (1993). As LBG was used as the substrate, the main component of which is galactomannan with a backbone chain of β-1, 4-linked mannosyl substituted with α-1, 6-linked galactosyl side-groups. Enzyme assay was carried out by mixing 50 L of appropriately diluted enzyme samples with 450 L of 50 mM sodium citrate buffer, pH 5.5 containing 0.5% LBG. The mixture was incubated at 50°C for 10 min and the reaction was stopped by adding 1 mL of the dinitrosalicylic acid reagent. After 10 min in a boiling water bath and quickly cooling to room temperature, the degree of enzymatic hydrolysis of the LBG was determined spectrophotometrically by measuring the absorbance at 540 nM.

The following properties of the recombinant β-mannanase were investigated: pH and temperature optimum, pH and temperature stability was determined according to methods reported previously (Chesson, 1987).

2.6 Analysis of sugar

Sugar released from LBG was analyzed by Ion Chromatography under the following conditions: column, Carbopac™ 4 × 250 mm (DIONEX-2500); mobile phase, NaOH and NaAc; column temperature, 60°C; flow rate, 1.0 mL/min; and detector, ED50- Electrochemical Detector.

3 Results

3.1 Construction of expression plasmid

Based on the β-mannanase sequence reported from *B.subtilis* by Ausubel et al. (1987), a pair of PCR primers was designed to amplifying the *man* gene. The *man* gene fragment consisted of 987 bases encoding a 329 amino acid peptide. The amplified clones were sequenced in its entirety and found to be identical with β-mannanase gene sequence in the National Institutes of Health (NIH) genetic sequence database GenBank. The DNA fragment encoding the mature β-mannanase was digested with *Eco*RI and *Not*I from pGEM-T vector and cloned into the same enzyme-digested vector pPIC9k downstream of the alcohol oxidase I (AOXI) promoter. The resultant construct harbored a single open reading frame encoding an 85 amino acid translation product consisting of the α-factor secretion leader peptide. Before the secretion of the peptide into the culture medium, the signal peptide should be cleaved off by the KEX2 gene products at the site (Glu-Lys-Arg-X). The integrity of the recombinant plasmid was confirmed by direct DNA sequencing. This constructed vector was linearized with *Bgl*II and transformed into the competent cells of *P.pastoris* GS115. The transformants were selected on RDB plates and confirmed by colony PCR. Eighty-one colonies presenting strong amplification products were used for small-scaled expression trials.

3.2 Expression of *B.subtilis* β-mannanase by *P. pastoris*

After screening, the recombinant strain that expresses the secretory protein at high level was obtained. The activity of the β-mannanase reached 263.0 IU/mL in flask culture. Among the expression parameters investigated using shasking flask cultures, the induction temperature was most important. At 20°C the level of expression β-mannanase was four times higher than at 30°C. In ferment culture β-mannanase expression was initiated by changing carbon source from glucose to methanol. Induction of β-mannanase was initiated at about 28 h after inoculation, as the biological mass reached 150–170 g/L, by adding 100% methanol containing 12 mL of PTM1 trace salts/L. Sample analysis at 12 h intervals was also performed to show the increasing amounts of β-mannanase presented in the culture medium until the 96 h. Figure 1 shows the time course of β-mannanase activity in a fermenter with the volume of 5 L. The ferment culture supernatants were also analyzed by SDS-PAGE, and the expressed products showed bands of molecular mass of about 40 kDa (Fig. 2). The secreted total supernatant protein concentration in the induction phase was traced and observed to attain the high-level expression of more than 2 g/L after 96 h of induction, The scanning result showed that the secreted β-mannanase protein band (in lane 1–7 of Fig. 3) achieved about 90% of the total supernatant proteins, and the product of β-mannanase was estimated about 1.8 g/L.

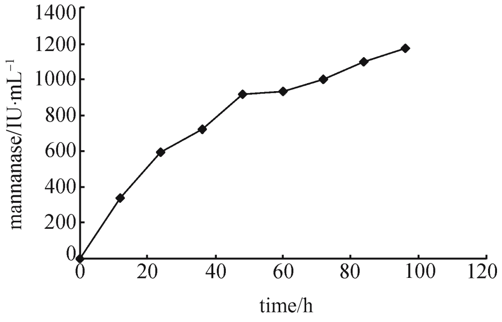

Fig. 1 Time course of β-mannanase activity in a fermenter with the volume of 5 L. Note: induction of β-mannanase was initiated by adding 0.4% methanol containing 12 mL of PTM1 trace salts/L; sample analysis at 12 h intervals was performed; β-Mannanase activity in culture supernatants was determined by adapting the dinitrosalicylic acid method for reducing sugar analysis using LBG as substrate according to Stålbrand et al. (1993).

3.3 Effects of pH and temperature on activity and stability

The β-mannanase activity was measured at various pHs under the stated condition. The optimum pH could be detected at about 5.5 (Fig. 3), and stable pH in the incubation at 50°C for 30 min was investigated at 5–10 (Fig. 3). Optimum temperature could be detected at around 50°C (Fig. 4). The enzyme activity was relatively stable below 70°C in incubation for

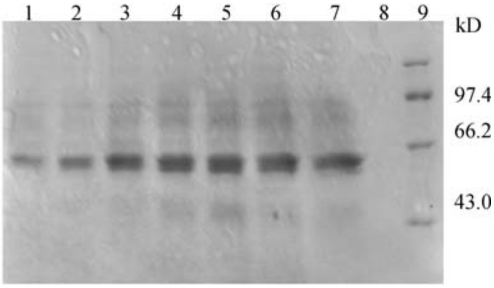

Fig. 2 SDS-PAGE analysis of expressed β-mannanase in a fermenter with the volume of 5 L at different induction times. Note: proteins were stained with Coomassie Brilliant Blue R-250; target protein is indicated by an arrow; lane 1, a sample of the supernatant after 12 h of initial 0.4% methanol containing 12 mL of PTM1 trace salts/L induction; lane 2–7, after 24, 36, 48, 60, 72 and 84 h initial 0.4% methanol induction; lane 8, a sample of the supernatant before methanol induction; lane 9, molecular mass standards indicated in kDa.

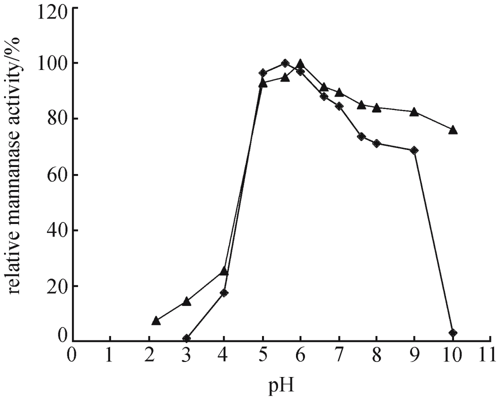

Fig. 3 Effects of pH on β-mannanase activity (◆) and stability (▲). Note: the pH optimum, expressed in percent of maximum, was determined by measuring the activity under standard conditions using buffers of different pH buffers, 50 mM phosphate-citrate (pH 2.2–8) and 50 mM glycine-NaCl-NaOH (pH 9–10); the pH stability was determined by incubating the enzyme at different pH values for 30 min at 50°C; the residual activity is shown as percent of the original activity.

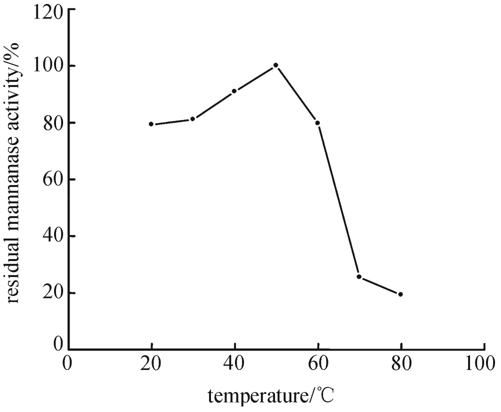

Fig. 4 Effects of temperature on β-mannanase activity. Note: the temperature optimum, expressed in percent of maximum, was determined by measuring the activity at various temperatures in phosphate-citrate buffer, pH 5.5.

30 min, and the investigated residual activity of β-mannanase was over 30% (Fig. 5).

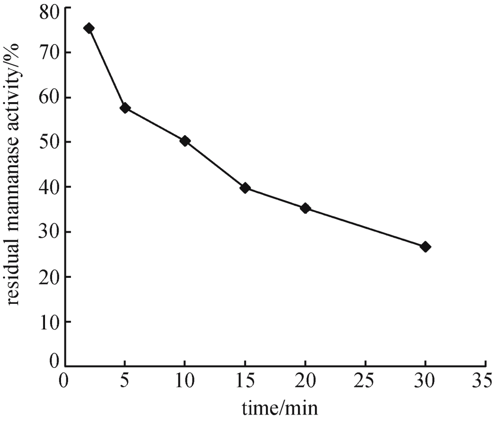

Fig. 5 Effects of temperature on β-mannanase stability. Note: the enzyme incubated at different times at 70°C and β-mannanase activity were determined by measuring the activity under standard conditions; the residual activity is shown as percent of the original activity.

3.4 Reactivity for substrate by β-mannanase

The β-mannanase was incubated with 1% locust bean gum at pH 5.5 and 50°C for 24 h. In locust bean gum, mannose-oligosacchrides were mainly released by β-mannanase, including 40.1% mannose, 35.9% mannobiose, 23.6% mannatriose and 14.5% mannopentaose, respectively (Fig. 6).

4 Discussion

The β-Mannanase was tested and its effectiveness in chick diets was confirmed as early as in 1982. In the early 1990s, ChemGen developed a *Bacillus lentus* β-mannanase into Hemicell® Feed Enzyme that was the first patented feed enzyme. Afterwards, Hemicell® Feed Enzyme was distributed and applied worldwide. It improves energy and protein utilization and body weight uniformity, adds matrix values in hens, broilers, and pigs, and brings economical and environmental benefits to animal farmers.

In order to enhance the application of β-mannanase, it is essential to improve the production levels of the enzymes. In addition to the traditional screening and breeding methods, the β-mannanase genes from different origins such as *Caldocellum saccharolyticum* (Luthi et al., 1991), *Bacillus subtilis* (Ausubel et al., 1987), *Thermoanaerobacterium polysaccharolyticum* (Cann et al., 1999), *Clostridium cellulolyticum* (Tamaru and Doi, 2000) and *Trichoderma reesei* (Stålbrand et al., 1995) were cloned and heterologously expressed in *Escherichia coli*, *Saccharomyces cerevisiae* and *Clostridium acetobutylicum*, but the improvement was trivial. *P. pastoris* was also employed by Tang et al. (2001) and Xu

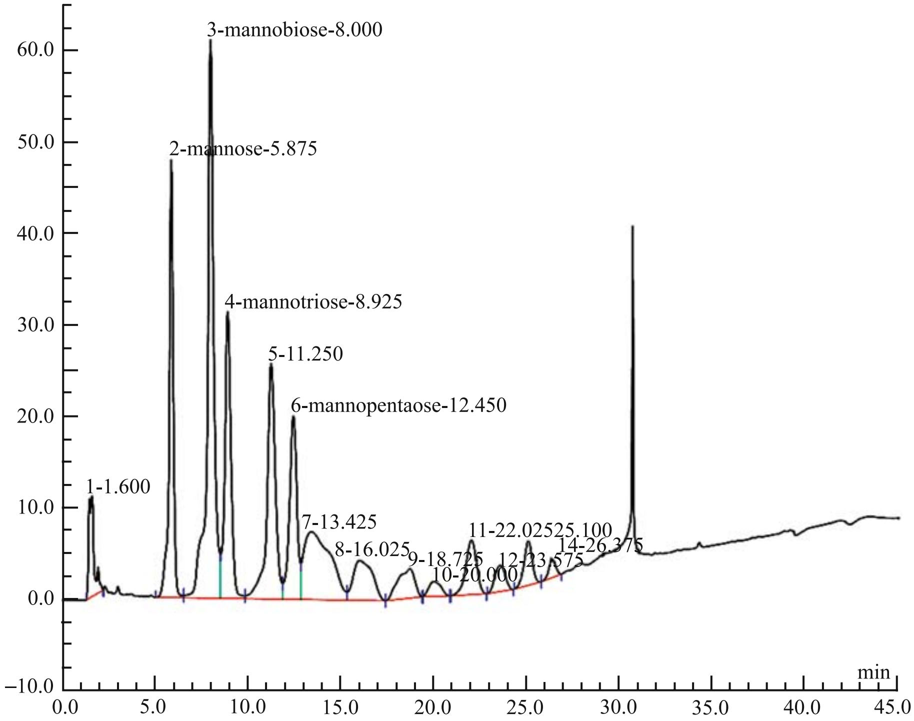

Fig. 6 Ion Chromatography chart of hydrolysis products from locust bean gum by *β*-mannnanase. Note: locust bean gum (1%) was hydrolyzed by *β*-mannanase at pH 5.5 and 50°C for 24 h; IC: column, CarbopacTM 4 × 250 mm; carrier, NaOH, NaAc; column temperature, 60°C.

et al., (2002b) to express *β*-mannanase genes from *Agaricus bisporus* and *Mytilus edulis*, which yielded 3.3 and 41.04 U/mL specific enzyme activity. In the present experiment, the *β*-mannanase expressed in *P. pastoris* reached a crude enzyme activity of 1102.0 IU/mL, which was the highest level reported to date. The enzyme was secreted into the medium, which facilitates its separation from the host yeast. The pH and temperature optima of the recombinant enzyme were close to that of the wide type.

It was reported that *β*-mannanase was toxic to the host as it was capable of degrading the cell walls of *P. pastoris*. This prevented the accumulation and production of *β*-mannanase. In this study it was found that reducing the induction temperature can effectively improve the production level and partially solve the problem. Although the concentration of total protein in the cell culture supernatant varied only slightly with the induction temperature, when the induction temperature was reduced from 30°C to 20°C, there was a significant increase in the concentration of *β*-mannanase activity from 398.0 to 1102.0 IU/mL when expression was allowed to occur at lower temperature. The possible reason is that at low temperatures the expression rate is reduced allowing more time for correct folding of the *β*-mannanase to take place. Similar results were obtained for an antifreeze protein expressed in *P. pastoris* (Li et al., 2001) when an induction temperature of 23°C was used. Low temperatures have been shown to improve the solubility of heterologous proteins in *E.coli* (Broeze et al., 1978; Vasina et al., 1997).

Acknowledgements This research was supported by International Foundation for Science (Grant No. B/3873-1), the National High Technology Research and Development Program of China (Grant Nos. 2003AA241160 and 2005AA246010), and International S&T Cooperation Program of China (Grant No. 20050048).

References

Akino T, Nakamura N, Horikoshi K (1988). Characterization of three mannanases of an alkalophilic *Bacillus sp*. Agric Biol Chem, 52: 773–779

Araki T (1982). Purification and characterization of a novel exo-beta-mannanase from *Aeromonas sp.* F-25. J Biochem, 91: 1181–1186

Ausubel F M, Brent R, Kingston R E, Moore D D, Seidman J G, Smith J A, Struhl K (1987). Current protocol in molecular biology, John Wiley £ Sons, ISBN0-471-63248-1, 241–245

Broeze R J, Solomon C J, Pope D H (1978). Effects of low temperature on in vivo and in vitro protein synthesis in *Escherichia coli* and *Pseudomonas fluorescens*. J Bacteriol, 134: 861–874

Cann I K, Kocherginskaya S, King M R, White B A, Mackie R I (1999). Molecular cloning, sequencing, and expression of a novel multidomain mannanase gene from *Thermoanaerobacterium polysaccharolyticum*. J Bacteriol, 181: 1643–1651

Chesson A (1987). Supplementary enzymes to improve the utilization of pig and poultry diets. In Recent Advances in Animal Nutrition, 71–89

Jackson M E, Anderson D M, Hsiao H Y, Mathis G F, Fodge D W (2003). Beneficial effect of beta-mannanase feed enzyme on performance of chicks challenged with *Eimerla sp*. and *Clostridium perfringens*. Avian Dis, 47: 759–763

Johnson K G (1990). Exocellurar mannanases from hemicellulolytic fungi. World J Microbiol Biotech, 6: 209–217

Laemmli U K (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680–685

Li Z, Xiong F, Lin Q, d'Anjou M, Daugulis A J, Yang S D C, Hew C L (2001). Low-temperature increases the yield of biologically active herring antifreeze protein in *Pichia pastoris*. Protein Expr Purif, 21: 438–445

Luthi E, Jasmat N B, Grayling R A, Love D R, Bergquist P L (1991). Cloning, sequence analysis, and expression in *Escherichia coli* of a gene coding for a beta-mannanase from the extremely thermophilic bacterium "*Caldocellum saccharolyticum*". Appl Environ Microbiol, 57: 694–700

Mendoza N S, Arai M, Sugimoto K, Ueda M, Kawaguchi T, Joson L M (1995). Cloning and sequencing of b-mannanase gene from *Bacillus subtilis* NM-39. Biochimica Biophysica Aata, 1243: 552–554

Mingardon F, Perret S, Belaich A, Tardif C, Belaich J P, Fierobe H P (2005). Heterologous production, assembly, and secretion of a minicellulosome by *Clostridium acetobutylicum* ATCC 824. Appl Environ Microbiol, 71: 1215–1222

Oda Y, Komaki T Tonomura K (1993). Purification and properties of extracellular mannanases produced by *Enterococcus casseliflavus* FL2121 isolated from decayed Konjac. J Ferment Bioeng, 76: 14–18

Ootsuka S, Saga N, Suzuki K I, Inoue A, Ojima T (2006). Isolation and cloning of an endo-beta-1, 4-mannanase from Pacific abalone *Haliotis discus hannai*. J Biotechnol, 125: 269–280

Shimahara H, Suzuki H, Sugiyama N, Nisizawa K (1975). Partial purification of mannanase from the konjac tubers and their substrate specificity in relation to the structure of konjac glucomannan. Agric Biol Chem, 39: 301–312

Slominski B A, Campbell L D, Guenter W (1994). Oligosaccharides in canola meal and their effect on nonstarch polysaccharide digestibility and true metabolizable energy in poultry. Poult Sci, 73: 156–162

Stålbrand H, Saloheimo A, Vehmaanpera J, Henrissat B, Penttila M (1995). Cloning and expression in *Saccharomyces cerevisiae* of a *Trichoderma reesei* beta-mannanase gene containing a cellulose binding domain. Appl Environ Microbiol, 61: 1090–1097

Stålbrand S, Siika-aho M, Tenkanen M, Viikari L (1993). Purification and characterization of two mannanases from *Trichoderma reesei*. J Biotechnol, 29: 229–242

Tamaru Y, Doi R H (2000). The engL gene cluster of *Clostridium cellulovorans* contains a gene for cellulosomal ManA. J Bacteriol, 182: 244–247

Tang C M, Waterman L D, Smith M H, Thurston C F (2001). The cel4 gene of *Agaricus bisporus* encodes a *β*-mannanase. Appl Environ Microbiol, 67: 2298–2303

Thomas S (1978). Refimement of the Coomassie Blue method of protein quantification. Anal Bichem, 86: 142–146

Vasina J A, Baneyx F (1997). Expression of aggregation-prone recombinant proteins at low temperatures: A comparative study of the *Escherichia coli* cspA and tac promoter systems. Protein Expr Purif, 9: 211–218

Xu B, Hägglund P, Stålbrand H, Janson J C (2002a). Endo-1, 4-mannanases from blue mussel, *Mytilus edulis*: Purification, characterization, and mode of action. J Biotechnol, 92: 267–277

Xu B, Sellos D, Janson J C (2002b). Cloning and expression in *Pichia pastoris* of a blue mussel (*Mytilus edulis*) mannanase gene. Eur J Biochem, 269: 1753–1760