Indonesian Journal of
Tropical and Infectious Disease
Vol. 2. No. 1 January–March 2011

EXPRESSION OF β-XYLOSIDASE ENCODING GENE IN PHIS1525/ *Bacillus megaterium* MS941 SYSTEM

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ABSTRACT

β-Xylosidase encoding gene from G. thermoleovorans IT-08 had been expressed in the pHIS1525/ B. megaterium MS941 system. The β-xylosidase gene (xyl) was inserted into plasmid pHIS1525 and propagated in E. coli DH10β. The recombinant plasmid was transformed into B. megaterium MS941 by protoplast transformation. Transformants were selected by growing the recombinant cells on solid LB medium containing tetracycline (10 µg/ ml). The expression of the β-xylosidase gene was assayed by overlaid the recombinant B. megaterium MS941 cell with agar medium containing 0.2% methylumbelliferyl-β-D-xyloside (MUX). This research showed that the β-xylosidase gene was succesfully sub-cloned in pHIS1525 system and expressed by the recombinant B. megaterium MS941. The addition of 0.5% xylose into the culture medium could increase the activity of recombinant β-xylosidase by 2.74 fold. The recombinant B. megaterium MS941 secreted 75.56% of the expressed β-xylosidase into culture medium. The crude extract β-xylosidase showed the optimum activity at 50° C and pH 6. The recombinant β-xylosidase was purified from culture supernatant by affinity chromatographic method using agarose containing Ni-NTA (Nickel-Nitrilotriacetic acid). The pure β-xylosidase showed a specific activity of 10.06 Unit/ mg protein and relative molecular weight ± 58 kDa.

Key words: *Expression, β-xylosidase,* ***Geobacillus thermoleovorans IT-08, pHIS1525, Bacillus megaterium MS941***

INTRODUCTION

β-D-Xylosidases (1,4-β-D-xylan-xylo-hydrolase, EC 3.2.1.37) are hemicellulases that hydrolyze xylooligosaccharides to xylose and are essential for the complete utilization of xylan. The branching and variability of the xylan structure requires the concerted action of several hemicellulolytic enzymes including endo-1, 4-β-xylanases that hydrolyze the xylan backbone, and β-xylosidases that cleave the resulting xylooligomers into xylose monomers.[1]

Plasmid pTP510 is a recombinant plasmid containing xylanolytic enzyme encoding gene from *G. thermoleovorans* IT-08. The xylanolytic enzymes were expressed intracellularly by the recombinant pTP510/ *E. coli* DH5α. The recombinant plasmid containing three genes: exo-xylanase (*exo-xyl*), α-L-arabinofuranosidase (*abfa*), and β-xylosidase (*xyl*) (GenBank Accession Nos. DQ387046, DQ387047, and DQ345777, respectively).

Escherichia coli plays a prominent role as a heterologous protein production host due to extensive research efforts over the last several decades. Albeit well known and used for protein production, several intrinsic problems hamper the system's unrestrained usage. *E. coli* lacks protein export mechanisms therefore the protein produced accumulates intracellularly, mostly in the form of inactive inclusion bodies due to the high concentrations present. The presence of endotoxins and the inability to attach glycosidic residues constrict its application for pharmaceutical proteins. The Gram-positive bacterium *Bacillus megaterium* offers several advantages over *E. coli* as a protein production system. It does not possess endotoxins and has a high secretion capacity. Compared to *Bacillus subtilis* it is distinguished by higher plasmid stability and a lower intrinsic protease activity which is a significant advantage for a secretory protein production system. Important prerequisites like an efficient transformation system, stable and freely replicating plasmids and the ability to integrate heterologous genes into the genome are met.[2]

The use of a Gram-positive bacterium could facilitate protein production due to the lack of an outer membrane allowing direct secretion of proteins into the growth medium. In contrast to *B. subtilis*, *B. megaterium* does not produce alkaline proteases. Another advantage of this bacterium is the high stability of plasmids during growth, which allows a stable gene expression in long term cultivations and bioreactors. *B. megaterium* has been used for the production of several recombinant proteins, e.g. dextransucrase,[3] and lysozyme specific single chain Fv (scFv) fragment D1.3.[4] Recently, a set of free replication vectors and genetically optimized *B. megaterium* strains for the intra- and extracellular production of affinity tagged recombinant proteins were developed. They were successfully employed for the production and purification of dextransucrase,[5] levansucrase,[3] and penicillin amidase.[6]

Plasmid pHIS1525 is a shuttle vector for *E. coli*/ *B. megaterium,* containing a signal peptide for secretion of heterologous protein of interest into the culture medium. The pHIS1525 system is also equipped with a 6x His-tag sequence for purification and detection of the expressed His-tagged target proteins.[2, 3]

In this work, we reported the expression of β-xylosidase encoding gene from *G. thermoleovorans* IT-08 in the pHIS1525/ *B. megaterium* MS941 system.

MATERIALS AND METHODS

Cultures

Escherichia coli DH10β and *Bacillus megaterium* MS941 were grown in LB (Luria Bertani) medium (composition : 1% NaCl, 1% tripton, 0.5% *yeast extract*). Recombinant *E. coli* DH5α, containing xylanolytic enzyme encoding gene (pTP510) from *G. thermoleovorans* IT-08, was grown in LB medium containing ampicillin (100μg/ ml).

Chemicals

All analytical chemicals and media component used were pure grade and available commercially.

Amplification and sub-cloning

β-Xylosidase encoding gene (*xyl*) was amplified from pTP510 using a pair of primers which designed according to the sequence of β-xylosidase of *G. thermoleovorans* IT-08 (GeneBank no. DQ345777) : F_{xyl} 5'-TTATT*GAGC TC*TCGAATATTCTAACCCAG-3' and R_{xyl} : 5′−CAA GA*GCATGC*AATATTTCAGGAATATATTTAAACC-3'. The *xyl* gene was inserted into plasmid pHIS1525 and propagated in *E. coli* DH10β before transforming into the *B. megaterium*. The recombinant plasmids were analyzed by restriction analysis and sequencing. The recombinant plasmid was transformed into *B. megaterium* MS941 by protoplast transformation method in the isotonic medium containing polietilen glikol.[7] Transformants were selected by growing the recombinant *B. megaterium* MS941 on the solid LB medium containing tetracycline (10 μg/ ml).

Assay of β-xylosidase expression

The expression of the β-xylosidase gene was assayed using 0.5% agar containing 0.2% methylumbelliferyl-β-D-xyloside (MUX). Recombinant *B. megaterium* MS941 cells on LB medium (+Tet 10 μg/ml) were overlaid with suspension of phosphate buffer pH 6.0 containing 0.5% agar and 0.2% methylumbelliferyl-β-D-xyloside (MUX). The assayed Petri plates were incubated at 60^0 C over-night. The β-xylosidase expression was monitored on the UV-trasluminator.

Enzyme assay

The enzyme activity was determined by measuring the release of *p*-nitrophenol from *p*-nitrophenyl-β-D-xylopyranoside (*p*NPX). The mixture of 100 μl enzyme sample and 900 μl of 1 mM *p*NPX, was incubated at 50° C for 30 min. The reaction was stopped by adding 0,1 ml of 0,4 M Na_2CO_3 solution. The release of p-nitrophenol was measured using spectrophotometer UV-vis at λ 405 nm. 1 (one) unit of the β-xylosidase activity was defined as the amount of the enzymes releasing 1 μmol p-nitrophenol equivalent per minute under the assay condition.

Effect of xylose on β-xylosidase activity

The effect of xylose on β-xylosidase activity was studied by producing the enzyme in various concentration of xylose as an inducer and time of addition into the culture medium. The enzyme activities were determined toward *p*NPX as a subtrate. The enzyme production was carried out in 100 ml cotton plugged Erlenmeyers which contained 20 ml fresh LB medium and 1% over-night pre-culture of recombinant *B. megaterium* MS941, and cultivated 37° C 250 rpm. The concentration of xylose was varied: 0, 0.25 and 0.50% (w/v), and the time of addition was varied: 0, 1, 2, 3, and 4 hours after cultivation (OD578 of 0–0.2. The enzyme was harvested after 10 h cultivation. The supernatant was collected by centrifugation at 10,000 rpm, 4° C for 20 min. The β-xylosidase activity was determined toward *p*NPX as a substrate.

Production of recombinant β-xylosidase

The enzyme production was carried out in 500 ml cotton-plugged Erlenmeyers containing 100 ml fresh LB medium, and inoculated by 1% of pre-culture of recombinant *B. megaterium* MS941 and cultivated 37° C, 250 rpm. Expression of Xyl was induced by adding 0.5% sterile xylose at an OD578 of 0.1. The enzyme was harvested after 10 h cultivation. The supernatant I (called secreted enzyme) was collected by centrifugation at 10,000 rpm, 4° C for 10 min. The pellet cell was resuspended in citrate-phosphate buffer pH 6.0 and lysed by two passages through a ultrasonicator at 20 kHz for 60 sec. Debris cells was removed by centrifugation at 10,000 rpm, 4° C for 10 min. The supernatant II called non-secreted enzyme. The β-xylosidase activities of enzymes, both secreted and

non-secreted enzyme were determined toward pNPX as a substrate.

Effect of pH and temperature on β-xylosidase activity

The optimum pH of the crude β-xylosidase activity was determined by measuring the enzymes activity toward *p*NPX as a substrat in pH range of 5–8 at 50° C for 30 min. The optimum temperature of the crude β-xylosidase activity was determined by measuring the enzymes activity toward substrat *p*NPX in defferent temperature range 50–70° C.

Purification of recombinant β-xylosidase

The recombinant β-xylosidase (Xyl) was purified by affinity chromatography method using agarose containing Ni-NTA (*Nickel-Nitrilotriacetic Acid*). Protein was precipitated from supernatant by adding 40% saturated $(NH_4)_2SO_4$. The precipitant was collected by centrifugation at 13,000 rpm, 4° C for 10 min. The desalted protein was resuspended with buffer A (50 mM bufer fosfat pH = 8,0 + 250 mM NaCl + 5 mM imidazole + 5 mM β-merkaptoetanol), then loaded onto a column containing 1 ml Ni–NTA agarose (Qiagen) and pre-equilibrated with buffer A and incubated 2 h at cool room. After discarding the flow through, the column was washed with five column volumes of buffer A, and eluted with five column volumes of buffer B (50 mM phosphate pH 8.0, 250 mM NaCl, 100 mMimidazole and 1 mM β-mercaptoethanol). All of the fractions were analysis by SDS-PAGE method.[8]

RESULTS AND DISCUSSION

Amplification and sub-cloning

Based on the structure analysis, β-xylosidase gene was successfully amplified from pTP510 and sub-cloned in plasmid pHIS1525/ *E. coli* DH10β. The recombinant plasmid named pSMX (Fig.1).

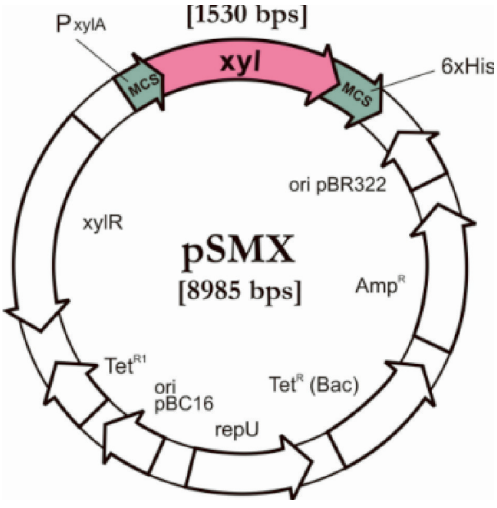

Figure 1. The pSMX map

β-xylosidase expression test

The expression of β-xylosidase in *B. megaterium* MS941 was assayed using methylumbelliferyl-β-D-xyloside (MUX) reagent. This work showed that the recombinant β-xylosidase was successfully expressed by recombinant *B. megaterium* MS941. Among 12 colonies of assayed recombinant *B. megaterium* MS941, there were two colonies (BM1 and BM3) flourescented on the UV transluminator. It was been concluded that the recombinants *B. megaterium* MS941 BM1 and BM3 expressed the β-xylosidase extracellularly. The β-xylosidases catalyse the hydrolysis of glycosidic linkage of MUX, and release xylose and umbelliferon (Fig 2).

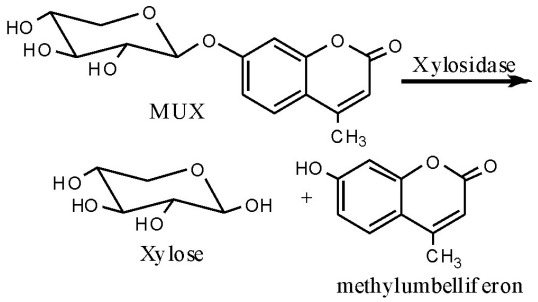

Figure 2. Hydrolysis of MUX by β-xylosidase

The effect of xylose addition

Recombinant plasmid pSMX contains *xylA* promoter (P*xylA*) and *xyl*R repressor. The expression recombinant β-xylosidase in *B. megaterium* MS941 system was xylose-inducible. The addition of 0.5% xylose into ke culture medium at OD_{578} = 0.332 could improve the growing of the recombinant *B. megaterium* MS941.

The influence of xylose concentration and the time of addition into the culture medium toward the activity of β-xylosidase, were studied. The addition of 0.25–0.50% xylose into the culture medium at 2 h cultivation (OD_{578} = ± 0,1) increased the enzyme activity by 2.0–2.74 fold. The enzyme showed an activity of 0.0343 ± 0.0022 Unit/ ml at the absence of xylose. The optimum condition to induce the β-xylosidase activity was the addition of 0.50% xylose at 2 h cultivation (Fig 3.).

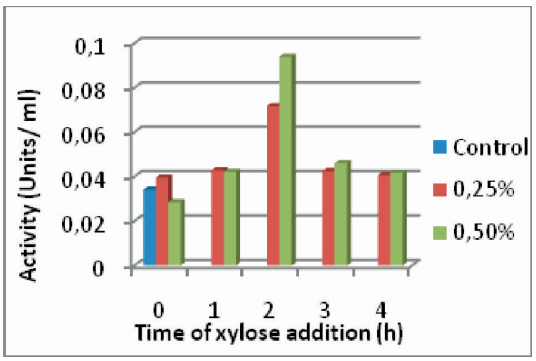

Figure 3. The effect of xylose concentration and time of addition into culture medium toward β-xylosidase activity

Effect of pH and temperature to enzyme activity

The optimum activity of the recombinant enzyme was reached at 50° C and pH 6 (Fig 4.). The crude extracts of β-xylosidase from recombinant *B. megaterium* MS941 the optimum activity at 50° C and pH 6.

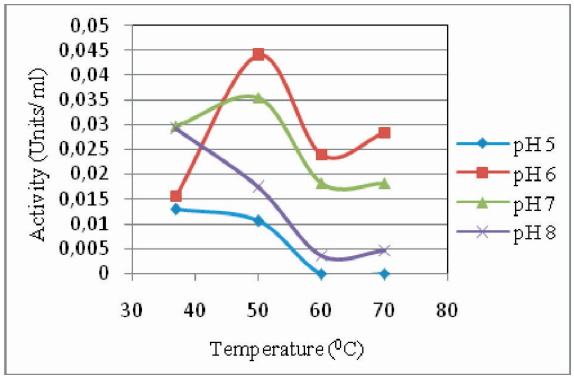

Figure 4. The enzyme activity toward *p*NPX at different pH and temperature

This condition was also reported by Puspaningsih (2005) for recombinant β-xylosidase expressed in pET-xyl/ *E. coli* BL21 DE3 [10]. However, the optimum temperature and pH optimum was different for the recombinant β-xylosidase expressed in pTP510/ *E. coli* DH5α. The enzyme had optimum activity at 70° C dan pH 5. On other hand, the β-xylosidase expressed in the origin strain, *Geobacillus thermoleovorans* IT-08, showed the optimum activity at 70° C and pH 6 This different optimum temperature and pH may be caused by the differential host and surrounding protein. The recombinant pTP510/ *E. coli* DH5α and its origin strain (*Geobacillus thermoleovorans* IT-08) expressed a xylanolytic enzyme, exo-xylanase, α-L-arabinofuranosidase, and β-xylosidase.[9]

Wagschal *et al*. (2008) has synthezed and cloned the *G. thermoleovorans* IT-08 β-xylosidase into pET29b/ *E. coli* BL21 (DE3). The recombinant β-xylosidase (GbtXyl43A) showed the optimum activity at pH 5.0 thermal atability (t1/2) 970 min at 51,2° C.[11]

Enzyme secretion

The analysis of enzyme secretion showed that the recombinant *B. megaterium* MS941 was capable to secrete 75.56% of the expressed β-xylosidase enzyme, into the culture medium. The using of *B. megaterium* MS941 with a detectable extracellular protease deleted showed the improvement the secretion of dextrasucrase [2]. One of the factors in protein secretion is the protein folding. In the production of recombinant penicillin G amidase (PGA) in *B. megaterium*, calcium ion was an important factor in protein folding and maturation. The presence of calcium ion impacted the secretion of PGA protein in *B. megaterium* MS941. The addition of 2.5 mM $CaCl_2$ into the LB medium improved the PGA secretion by 2.6 fold compared to the absence of $CaCl_2$ [6]. The preliminary study, showed that metal ions such as Mg^{++}, Mn^{++}, Zn^{++}, Ca^{++} and Fe^{++} impacted the activity of β-xylosidase from *G. thermoleovorans* IT-08. The addition of 0.5–2.5 mM metal ions improved the β-xylosidase toward substrate *p*NPX.

Purification of recombinant enzyme

The SDS-PAGE analysis showed that the protein purification revealed a pure β-xylosidase protein in fraction B2. The pure enzyme was showed by a single protein band ± 58 kDa on the electrophoregram (Fig 5.)

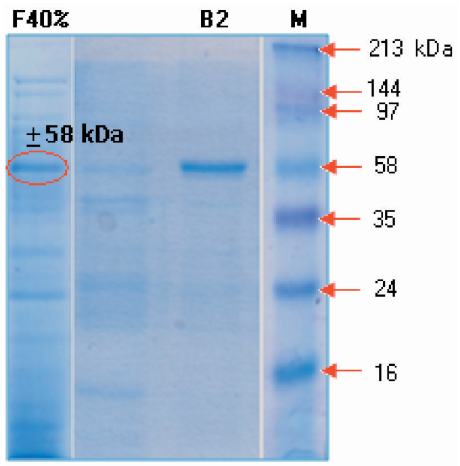

Figure 5. SDS-PAGE analyzed of pure β-xylosidase B2 = fraction 2 of eluted enzyme by buffer B M= protein penanda (16-213 kDa, *Intron Biotechnology*)

The purification of β-xylosidase by affinity chromatography using agarose coloum containing Ni-NTA revealed a pure enzyme with specific activity of 10.06 Units/mg protein, or 24.19 fold compared to the activity of supernatant.

CONCLUSION

β-xylosidase encoding gene from *G. thermoleovorans* IT-08 was succesfully expressed in pHIS1525/ *B. megaterium* MS941.

ACKNOWLEDGEMENTS

This research was supported by Directorat General of Higher Education Departement of National Education indonesia, throught "Hibah Bersaing Project" 2006-2007. The authors would like to thank Prof. Dr. Dieter Jahn and Laboratorium Microbiology, Technical University Braunschweig, Germany for the giving of plasmid pHIS1525 and strains (*Bacillus megaterium* MS941 and *E. coli* DH10β).

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