Conference Paper

Cloning and Over-expression of *xyn*B Gene of *Bacillus subtilis* subsp. spizizenii W23 into *Escherichia coli* Origami Host Cells

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Abstract

The *xyn*B gene of *Bacillus subtilis* subsp. spizizenii W23 is predicted to encode a xylan 1,4-beta-xylosidase. Application of XynB enzymes in industries is wide. Production of this enzyme in its host cells is naturally restricted by repression process. It will give certain beneficial to over-expressed the enzymes in other host-cells under inducing promoter. This study aimed to clone the *xyn*B gene from *Bacillus subtilis* subsp. spizizenii W23, to pMMB67EH plasmid, and to over-express the *xyn*B gene in *Escherichia coli* Origami as host cells. The *xyn*B gene was successfully amplified by polymerase chain reaction (PCR) technique using a pair of primers flanking the gene sequence and chromosomal DNA of the W23 strain as a template. The *xyn*B gene inserted in recombinant plasmid was confirmed by PCR detection using primers pair's specific for *xyn*B gene and for the vector, then continued by restriction analyses. The result showed that transformants clone 9 and 10 bear the recombinant pMMB-*xyn*B plasmid. The xylanase activity of *xyn*B gene in *Escherichia coli* Origami clone 10 was detected by sodium-dodecyl-sulfate polyacrylamide gel analyses and with addition of isopropyl-β-D-thio-galactoside (IPTG) as an inducer. The protein seem to be over-expressed as intra- and extra-cellular protein detected on SDS-PAGE gel. Result from xylan degrading activity on Luria-Bertani-xylan-IPTG plate with addition of Congo Red, showed that the cells with pMMB-*xyn*B recombinant plasmid have clear zone around the colonies while the transformant bearing an empty plasmid showed no clear zone. It could be concluded that the *xyn*B gene of *Bacillus subtilis* subsp.spizizenii W23 has been successfully been cloned on pMMB67EH plasmid and over-expressed in the *Escherichia coli* Origami cells as intra- and extra-cellular protein, as observed on SDS-PAGE gel analysis. The protein has activity on xylan degradation.

Keywords: *Bacillus subtilis* subsp. spizizenii str. W23; cloning; *Escherichia coli* Origami; over-expression, xylan-beta 1,4-xylosidase (*xyn*B).

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1. Introduction

Xylanases are important enzymes for degradation of xylans [1], the major components of hemicellulosic plant materials. Xylan consists of a backbone of polymer β-1,4-linked-xylose with side chains of xylose, arabinose, acetyl, methylglucuronic, coumaric, hexoses, and uronic acid. Hemicellulose together with cellulose and lignin can be found in plant cell wall in the form of lignocelluloses. Complete degradation of xylans requires the involvement of several enzymes [2]. Xylan 1,4-β-xylosidase (4-β-D-xylan xylohydrolase, EC 3.2.1.37) remove D-xylose residues from the non-reducing termini, and endoxylanases (4-β-D-xylan xylanohydrolase; EC 3.2.1.8) catalyze endohydrolysis of (1→4)-β-D-xylosidic linkages in xylans. All those enzymes degrade the xylan backbone into xyloses whereas others enzymes such as acetyl esterase, ferulic esterase, glucuronosidase or arabinosidase remove the side chains residues [3].

Xylanases are important enzymes for many industries. In pulp and paper industries [4, 5], candy, and syrup industries [6]. Xylo-oligosacharides products have wide applications in industries, such as in juice-processing and textile industries, bread industry [7]. In poultry industries, they are added in feed processing to improve the feed digestions and nutrients uptake [3, 8, 9].

Xylanases have been found in various organisms such as *Thermatoga maritima* [10], *Aspergillus niger* [11], *Clostridium cellulovorans* [12], *Bacillus sp.*CCMI 966 [13], *Cellulomonas uda* [14], *Thermoanaerobacterium* sp. strain [15], *Dictyoglomus thermophilum* [16], *Ustilago maydis* [17] and many others bacteria and fungi [6]. The enzymes properties may be slight different depend on the sources organisms.

Over-expression of genes encoding celluloses and xylanases has many advantages since lignocelluloses are present abundantly in forest and agricultural as wastes. In other hands, these wastes still contain nutrients for fermentation process to produce important stuffs [18, 19]. The lignocelluloses degradation may help the carbon recycle in the earth. Furthermore, the degradation products may be used as nutrients for microorganisms in fermentation processes. Many studies showed that hydrolytic products could increase cell biomass and bioproducts [19, 20] with which no competition for food and feed requirements. Expressions of *xyn* gene in microorganism producers enhanced the xylan degradation and increase the bioproducts, such as ethanol [21].

In order to produce this enzyme in large scale, we intended to clone the gene from *Bacillus subtilis* subsp. spizizenii W23 and introduced into others host cells, such as *Escherichia coli* Origami. Productions of proteins in original cells have advantages over other bacterial cells since *Bacillus* has a protein secretory system. However, native cells commonly have inner repression process which causes restriction in practice. Several study revealed the presence of xyn operon regulation [22], for instance carbon catabolite repression of *Bacillus subtilis* xylanases [23, 24]. Because of that repression,

it will be more advantageous if this *xyn*B gene can be over-expressed not in its native host cells. Further study can be performed to regulate or manipulate the process even in native host cells.

Commonly, xylanases genes were found in genomic library fragments of microorganisms found in their nature habitats, such as done for *Paenibacillus* sp. [25], *Bacillus pumilus* [26]. However, the fragments often contain the whole operon with intact regulator in the fragment [26]. In this study, we isolated the *xyn*B gene by PCR amplification. *Bacillus subtilis* subsp. spizizenii W23 genome have compete genome sequence for this purpose. The difficulty of this step is in the designing of primers for amplification the whole coding sequence. Sequences around xynB gene region are rather complicated for primers designing [27]. In this flanking area, no sequences are available for proper annealed primer. Based on several software analyses, the sequences contain repeated nucleotides, low GC content and several unspecific sequences (NCBI Primer Design Tool [28], Primer 3 [29, 30], Clone Manager Suite Professional softwares). In this study, we managed to design the primers pair for whole coding sequence of *xyn*B gene and amplified it for construction of recombinant plasmid.

The aims of this study are to construct a recombinant plasmid containing *xyn*B gene from *Bacillus subtilis* subspecies spizizenii W23 and to over-express the *xyn*B gene in *Escherichia coli* Origami as host cells under tac promoter.

2. Materials and methods

2.1. Bacteria and plasmid

Escherichia coli Origami and *Escherichia coli* DH5α (Novagen) containing pMMB67EH plasmid [31]. *Bacillus subtilis* subsp.spizizenii W23 is a kindly gift from Northern Regional Research Laboratory, NRRL)

2.2. Amplification of *Bacillus subtilis* subsp.spizizenii W23 *xyn*B gene

The open reading frame (ORF) of *Bacillus subtilis* subsp.spizizenii W23 gene was amplified by polymerase chain reaction (PCR) using primers that were designed based on the genomic sequence of *Bacillus subtilis* subsp.spizizenii W23 strain obtained from Genebank database (www.ncbi.com) [27], using Clone Manager Professional Suite (DNA Star, USA) software. For-xynB_BSUW23: 5'-CGAGCGGCCGAGCTCATGAAGATTA TCAATCCAGTACTTAAAGGATTCAA-3', and Rev-xynB_BSUW23: 5'-CCGCTGGTCTAGAGGCT CCTGTAAAGAACCCTCCTCCC-3'. Both primers were added with a restriction sites at its

5'-ends (underlined), and synthesized by Operon. As a PCR template was chromosomal DNA of Bacillus subtilis subsp.spizizenii W23. PCR was settled as follow, predenaturation at 94 °C for 2 min, denaturation at 94 °C for 30 s, annealing at 60 °C for 1.5 min, elongation at 72 °C for 2.5 min, 30 cycles and post-extension at 72 °C for 10 min.

2.3. Cloning of PCR fragment on pMMB67EH vector

The *xyn*B gene was cloned into pMMB67EH under the control of tac promoter [31]. Before ligation, the PCR product was digested with *Sac*I and *Xba*I enzymes and then was ligated to the similarly digested pMMB67EH. The resulting pMMB-*xyn*B plasmid was used to transform the *Escherichia coli* Origami cells which were made competence by $CaCl_2$ using heat shock method, as described by Sambrook et al. [32].

2.4. Analyses of recombinant plasmid

Screening of transformants was done by plating the transformants on LB plate containing 100 $\mu g \cdot .mL^{-1}$ ampicillin (LB-amp). The transformants grew on LB-amp plates were then selected for the presence of recombinant plasmid in the cells by PCR techniques. For vector detection, PCR was performed using vector-specific primers, SeqForpMMB67 dan SeqRevpMMB67 primers [33]. The amplification product was predicted to be a 2 035 bps band, of which the 1 523 bps was the size of *xyn*B gene sequence and the rest was the vector parts. While for *xyn*B detection, the For-xynB_BSUW23 and Rev-xynB_BSUW23 primers pair was used. The recombinant plasmids generate a single band amplicon with a size of ~1 549 bps. As a control, *Escherichia coli* Origami, with or without plasmids, were used as templates. All of these negative controls have no amplicon.

The restriction analyses were done for recombinant plasmid that gave positive result in PCR detections. The restriction analyses were performed using *Sac*I, *Xba*I and *Eco*RI (Fermentas) restriction enzymes according to the manufacturer's instruction and then visualized on 1 % agarose gel. *Xba*I or *Sac*I will linearized pMMB-xynB to be a linear fragment with a size of 10 334 bps. *Eco*RI will digest proper recombinant plasmid to two fragments of ~1005 and ~9 329 bps, while digestion with *Xba*I and *Sac*I simultaneously gave two fragments, 1 523 bps which confirmed for the size of *xyn*B insert and 8 811 bps for the size of pMMB-vector. Un-cut empty plasmid, pMMB67EH, had a size of ~8 828 bp.

2.5. Over-expression verification by SDS-PAGE analysis

Over-expression of xynB gene in *Escherichia coli* Origami cells was verified on sodium-dodecyl-sulfate polyacrylamide (SDS-PAGE) gel analyses. The cells were grown in LB-amp, at 37 °C, and induce with 1 mM IPTG. The pellet was separated from the supernatant by centrifugation at 13 000 *g* for 1 min [(*g* = Relative Centrifugal Force (RCF)], then sonicated to break the cells membrane. The separation of proteins was performed on SDS-PAGE gel with 3 % stacking gel and 12.5 % separating gel [32]. The proteins bands were visualized by Coommassie blue G-250 staining.

2.6. Xylan degradation activity

The xylan degradation activity was assayed by growing the transformants on LB-amp plate containing 1 % xylan with addition of 1mM IPTG, incubated at 37 °C for 7 d. The *Escherichia coli* Origami transformants harboring xylanase gene showed a clear zone around the colony upon Congo red addition [34]

3. Results and discussion

The *xynB* gene had been amplified successfully using For-*xyn*B_BSUW23 and Rev-*xyn*B_BSUW23 primers. PCR product revealed as a single band with a size of 1 549 bps (Fig. 1). Commonly, isolation of genes encoding xylanases from various bacteria and fungi have been done by digestion of genomic DNA, cloning the fragments and then the target were selected based on its activity [26]. Both primers were predicted as not good primers by the Clone Manager Suite Professional software, however in fact the amplification was succeeded in this study.

The screening of *Escherichia coli* Origami transformants were performed based on ampicillin resistance [33]. There were many transformants colonies grew on LB-amp plates, as many as colonies of *Escherichia coli* Origami transformed using the empty plasmid, pMMB67EH. The pMMB67EH is a vector plasmid contained ampicillin resistance marker. Since ampicillin resistance is quite common in bacterial cells and laboratory plasmids, so further analyses have to be performed.

Ten of *Escherichia coli* Origami transformants grew on LB-amp plates were selected to detect the presence of target plasmid by PCR using *SeqForpMMB67* and *SeqRevp-MMB67* primers. The result showed that nine of *Escherichia coli* Origami transformants had a fragment with proper size of ~2.0 kbs on 1 % agarose gel. Based on this analysis, these nine *Escherichia coli* Origami transformants were confirmed to contain the pMMB-recombinant plasmid.

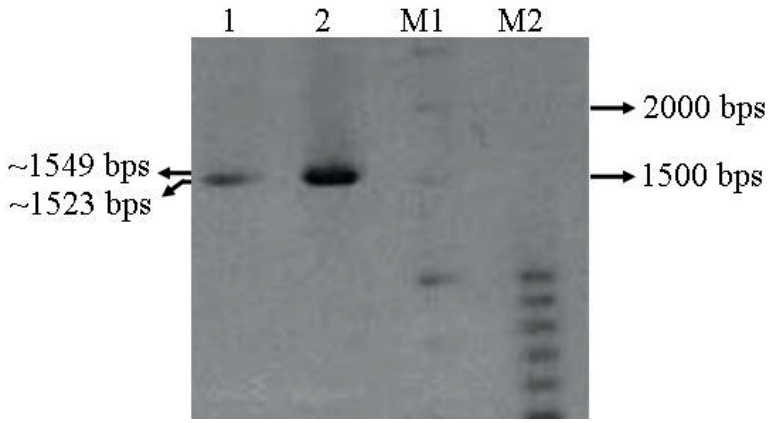

Figure 1: The *xynB* gene was amplified from *Bacillus subtilis* subsp. spizizenii W23 genome. The PCR product had a band of ~1 549 bp (line 2), while digestion with *Sac*I and *Xba*I resulted to three fragments with sizes of approximately 1 523 bps, 14 bps and 12 bps (line 1). The 14 and 12 bps fragments were too small to be existed on this gel under the electrophoresis condition of this experiment. The PCR product and restriction fragments had proper sizes as predicted. M1 and M2: 1 kb and 100 bps DNA *ladder* (Fermentas).

To confirm the presence of *xyn*B in the recombinant plasmids, two of the positive transformants, *Escherichia coli* Origami clones 9 and 10, were chosen for next PCR detection using For-*xyn*B_BSUW23 and Rev-*xyn*B_BSUW23 primers. Before PCR process, the plasmids were isolated from clones 9 and 10 which then were used as templates in PCR amplification. PCR result showed that both transformants have pMMB-*xyn*B plasmids with *xyn*B gene in proper orientation.

For further analysis, restriction analysis was done to confirm the orientation of the *xyn*B gene in the pMMB67EH vector. The pMMB-*xyn*B plasmid was extracted from *Escherichia coli* Origami transformants clone 9 and 10, then were digested using *XbaI*, and/or *SacI*. Restriction analyses results showed that digested recombinants plasmids have proper fragments sizes. All of the analyses results confirmed that the *Escherichia coli* Origami clone 9 and 10 contain the proper pMMB-*xyn*B recombinant plasmid.

To verify that the *xyn*B gene positioned in frame on the recombinant plasmid so that over-expressed properly in transformant cells, the proteins of clone 10 were profiled on SDS-PAGE gel (Fig. 2).

Deducted from the DNA sequence, the protein is predicted to have a molecular mass of ~61 337 Da. SDS-PAGE result showed that this positive clone revealed a thicker protein band in the size of ~61 kDa, both in intra- and extra-cellular fractions. This result gave impression that this protein can be an intra- or extra-cellular protein.

To confirm the presence of active protein and its localization, assay was also performed by Congo Red assay. The activity to degrade xylan by the extra-cellular enzyme resulted to clear red zone surrounding the colonies upon Congo red addition. Clear zone surrounding colony of transformant gave confirmation that the transformant over-expressed the active XynB enzyme and it might be secreted into the medium. No clear zone appeared around the wild type colony (Fig. 3). It meant that the *xyn*B

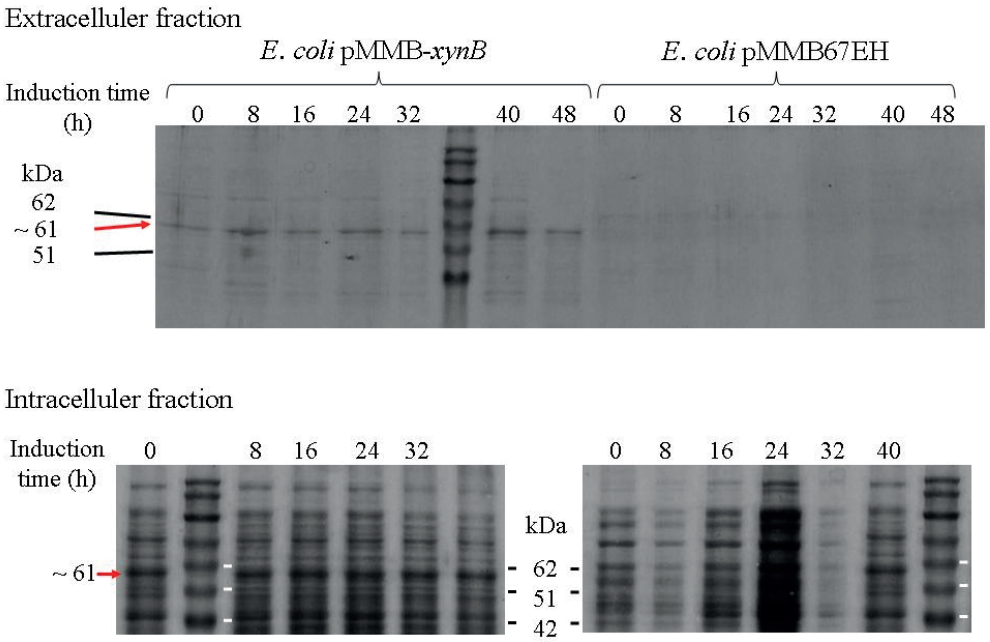

Figure 2: SDS-PAGE proteins profiles of over-expressed *E. coli* Origami pMMB-*xynB* cells grown in LB-amp at 37 °C, shaked at 200 rpm (1 rpm = 1/60 Hz), under 1 mM IPTG induction.

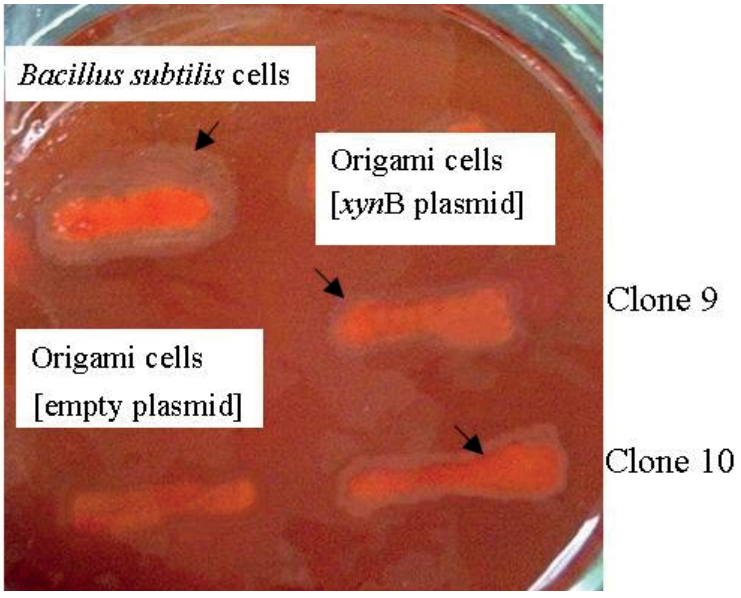

Figure 3: The expression of pMMB-*xyn*B in *Escherichia coli* Origami transformants assayed on LB-amp plate containing 1 % xylan and 1 mM IPTG. The colonies of cells bearing *xyn*B recombinant plasmid showed a clear zone (black arrow, →) around the colonies however no clear zone appeared around transformant bearing empty plasmid. 37 °C, for 7 d.

gene was over-expressed from pMMB-*xyn*B in this positive transformants. The *Bacillus subtilis* subsp.spizizenii W23 cells, as an original gene source, showed the highest xylan degrading activities. This result was corroborated to genomic sequence database, indicates that *Bacillus subtilis* subsp.spizizenii W23 has several genes which were predicted to encode lignocellulases enzymes, including the *xyn*A and *xyn*B genes [27].

Based on protein structure analyses using PROPSEARCH software [35], *xyn*B gene of *Bacillus subtilis* subsp. spizizenii W23 is predicted to encode a β-xylosidase (1,4-β-D-xylan xylohydrolase; EC 3.2.1.37). Compare to *xyn*B from *Bacillus pumilus* [26], the latter is known as intracellular endoxylanases. XynB from *Bacillus pumilus* was detected as an intra-cellular protein by software and by experiment, without signal peptide [26]. Different from the previous protein, this *Bacillus subtilis* W23 XynB protein is predicted by PrediSi [36] dan SignalP software(www.ExPassy.com), as a non-secreted protein, without signal peptide sequences. Based on CELLO Prediction software, the most possible protein location is in cytoplasm, however there is a half possibility to be an extracellular protein [37]. In contrast, according to MTHMM software [38], this XynB protein is predicted to be an extracellular protein although it contains no signal peptide. This result also reveal in ProtComp [39] analyses result. Although predicted ProtComp software as a cytoplasmic protein, a part of the amino acid residues is located in membrane as integral and transmembran protein. This result can be explained by the fact that the *Bacillus subtilis* sequence have only 48 % similarity to *Bacillus pumilus* that the sequence might be unique and have different properties [40]. Other possibility is that this gene might be expressed abundantly in cytoplasm so that secreted out of the cells. It is interesting to find that this gene was secreted by transformants and gave impact on xylan around the colony. However, the localization of *Bacillus subtilis* subsp.spizizenii W23 XynB protein still need to be verified.

4. Conclusion

It could be concluded that the *xyn*B gene of *Bacillus subtilis* subsp.spizizenii W23 has been successfully been cloned on pMMB67EH plasmid and over-expressed in the *Escherichia coli* Origami cells both intra- and extra-cellularly, as observed on SDS-PAGE gel analysis. The transformants bearing the pMMB-*xyn*B recombinant plasmid had activity to degrade xylan on LB-xylan plate while the transformant bearing an empty plasmid showed no activity.

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International Conference on Natural Resources and Life Sciences (NRLS-2016)

The 1st International Conference on Natural Resources and Life Sciences – NRLS 2016 – was organized by the Faculty of Biotechnology, University of Surabaya, Indonesia. The theme of this conference is set on the theme of 'Multidisciplinary Science for Better Achievement'. The conference facilitated the exchange of useful information on life sciences and natural resources' exploration practices for the future human needs. Over 120 researchers participated in the conference from countries and regions such as Germany, Netherland, Nepal, Korea, Thailand, Malaysia, and Indonesia. From over 113 abstracts and presentations, 22 papers are selected to be published in KnE Conference Proceeding, representing the themes of 2016, that is, Food Biotechnology, Plant Biotechnology, Medical Biotechnology & Forensics, and Environmental Biotechnology & Renewable Energy. All 22 manuscripts in KnE Life Sciences, vol. 2017, have been reviewed by the experts from University of Groningen, University of Potsdam, RWTH Aachen University, Kyung Hee University, Universiti Selangor, and University of Surabaya, Indonesia. The reviewing process at KnE involves experts having professional editing background from four countries (Indonesia, Latvia, Malaysia, and Sweden).

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Cloning and Over-expression of xynB Gene of Bacillus subtilis subsp spizizenii W23 into Escherichia coli Origami Host Cells

By: Wahjudi, M (Wahjudi, Mariana)[1]; Catherina (Catherina)[1]; Wangunhardjo, NM (Wangunhardjo, Nita Marcelia)[1]; Suryadjaja, E (Suryadjaja, Ernest)[1]; Daniel, X (Daniel, Xavier)[2]

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Abstract

The xynB gene of Bacillus subtilis subsp. spizizenii W23 is predicted to encode a xylan 1,4-beta-xylosidase. Application of XynB enzymes in industries is wide. Production of this enzyme in its host cells is naturally restricted by repression process. It will give certain beneficial to over-expressed the enzymes in other host-cells under inducing promoter. This study aimed to clone the xynB gene from Bacillus subtilis subsp. spizizenii W23, to pMMB67EH plasmid, and to over-express the xynB gene in Escherichia coli Origami as host cells. The xynB gene was successfully amplified by polymerase chain reaction (PCR) technique using a pair of primers flanking the gene sequence and chromosomal DNA of the W23 strain as a template. The xynB gene inserted in recombinant plasmid was confirmed by PCR detection using primers pair's specific for xynB gene and for the vector, then continued by restriction analyses. The result showed that transformants clone 9 and 10 bear the recombinant pMMB-xynB plasmid. The xylanase activity of xynB gene in Escherichia coli Origami clone 10 was detected by sodium-dodecyl-sulfate polyacrylamide gel analyses and with addition of isopropyl-beta-D-thio-galactoside (IPTG) as an inducer. The protein seem to be over-expressed as intra-and extra-cellular protein detected on SDS-PAGE gel. Result from xylan degrading activity on Luria-Bertani-xylan-IPTG plate with addition of Congo Red, showed that the cells with pMMB-xynB recombinant plasmid have clear zone around the colonies while the transformant bearing an empty plasmid showed no clear zone. It could be concluded that the xynB gene of Bacillus subtilis subsp. spizizenii W23 has been successfully been cloned on pMMB67EH plasmid and over-expressed in the Escherichia coli Origami cells as intra-and extra-cellular protein, as observed on SDS-PAGE gel analysis. The protein has activity on xylan degradation.

Keywords

Author Keywords: Bacillus subtilis subsp spizizenii str. W23; cloning; Escherichia coli Origami; over-expression; xylan-beta 1,4-xylosidase (xynB)

KeyWords Plus: XYLAN-DEGRADING ENZYMES; CATABOLITE REPRESSION; MICROBIAL XYLANASES; PREDICTION; ETHANOL; OPERON; GROWTH; PULP

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Conference Paper

Cloning and Over-expression of *xyn*B Gene of *Bacillus subtilis* subsp. spizizenii W23 into *Escherichia coli* Origami Host Cells

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Abstract

The *xyn*B gene of *Bacillus subtilis* subsp. spizizenii W23 is predicted to encode a xylan 1,4-beta-xylosidase. Application of XynB enzymes in industries is wide. Production of this enzyme in its host cells is naturally restricted by repression process. It will give certain beneficial to over-expressed the enzymes in other host-cells under inducing promoter. This study aimed to clone the *xyn*B gene from *Bacillus subtilis* subsp. spizizenii W23, to pMMB67EH plasmid, and to over-express the *xyn*B gene in *Escherichia coli* Origami as host cells. The *xyn*B gene was successfully amplified by polymerase chain reaction (PCR) technique using a pair of primers flanking the gene sequence and chromosomal DNA of the W23 strain as a template. The *xyn*B gene inserted in recombinant plasmid was confirmed by PCR detection using primers pair's specific for *xyn*B gene and for the vector, then continued by restriction analyses. The result showed that transformants clone 9 and 10 bear the recombinant pMMB-*xyn*B plasmid. The xylanase activity of *xyn*B gene in *Escherichia coli* Origami clone 10 was detected by sodium-dodecyl-sulfate polyacrylamide gel analyses and with addition of isopropyl-β-D-thio-galactoside (IPTG) as an inducer. The protein seem to be over-expressed as intra- and extra-cellular protein detected on SDS-PAGE gel. Result from xylan degrading activity on Luria-Bertani-xylan-IPTG plate with addition of Congo Red, showed that the cells with pMMB-*xyn*B recombinant plasmid have clear zone around the colonies while the transformant bearing an empty plasmid showed no clear zone. It could be concluded that the *xyn*B gene of *Bacillus subtilis* subsp.spizizenii W23 has been successfully been cloned on pMMB67EH plasmid and over-expressed in the *Escherichia coli* Origami cells as intra- and extra-cellular protein, as observed on SDS-PAGE gel analysis. The protein has activity on xylan degradation.

Keywords: *Bacillus subtilis* subsp. spizizenii str. W23; cloning; *Escherichia coli* Origami; over-expression, xylan-beta 1,4-xylosidase (*xyn*B).

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1. Introduction

Xylanases are important enzymes for degradation of xylans [1], the major components of hemicellulosic plant materials. Xylan consists of a backbone of polymer β-1,4-linked-xylose with side chains of xylose, arabinose, acetyl, methylglucuronic, coumaric, hexoses, and uronic acid. Hemicellulose together with cellulose and lignin can be found in plant cell wall in the form of lignocelluloses. Complete degradation of xylans requires the involvement of several enzymes [2]. Xylan 1,4-β-xylosidase (4-β-D-xylan xylohydrolase, EC 3.2.1.37) remove D-xylose residues from the non-reducing termini, and endoxylanases (4-β-D-xylan xylanohydrolase; EC 3.2.1.8) catalyze endohydrolysis of (1→4)-β-D-xylosidic linkages in xylans. All those enzymes degrade the xylan backbone into xyloses whereas others enzymes such as acetyl esterase, ferulic esterase, glucuronosidase or arabinosidase remove the side chains residues [3].

Xylanases are important enzymes for many industries. In pulp and paper industries [4, 5], candy, and syrup industries [6]. Xylo-oligosacharides products have wide applications in industries, such as in juice-processing and textile industries, bread industry [7]. In poultry industries, they are added in feed processing to improve the feed digestions and nutrients uptake [3, 8, 9].

Xylanases have been found in various organisms such as *Thermatoga maritima* [10], *Aspergillus niger* [11], *Clostridium cellulovorans* [12], *Bacillus sp.*CCMI 966 [13], *Cellulomonas uda* [14], *Thermoanaerobacterium* sp. strain [15], *Dictyoglomus thermophilum* [16], *Ustilago maydis* [17] and many others bacteria and fungi [6]. The enzymes properties may be slight different depend on the sources organisms.

Over-expression of genes encoding celluloses and xylanases has many advantages since lignocelluloses are present abundantly in forest and agricultural as wastes. In other hands, these wastes still contain nutrients for fermentation process to produce important stuffs [18, 19]. The lignocelluloses degradation may help the carbon recycle in the earth. Furthermore, the degradation products may be used as nutrients for microorganisms in fermentation processes. Many studies showed that hydrolytic products could increase cell biomass and bioproducts [19, 20] with which no competition for food and feed requirements. Expressions of *xyn* gene in microorganism producers enhanced the xylan degradation and increase the bioproducts, such as ethanol [21].

In order to produce this enzyme in large scale, we intended to clone the gene from *Bacillus subtilis* subsp. spizizenii W23 and introduced into others host cells, such as *Escherichia coli* Origami. Productions of proteins in original cells have advantages over other bacterial cells since *Bacillus* has a protein secretory system. However, native cells commonly have inner repression process which causes restriction in practice. Several study revealed the presence of xyn operon regulation [22], for instance carbon catabolite repression of *Bacillus subtilis* xylanases [23, 24]. Because of that repression,

it will be more advantageous if this *xyn*B gene can be over-expressed not in its native host cells. Further study can be performed to regulate or manipulate the process even in native host cells.

Commonly, xylanases genes were found in genomic library fragments of microorganisms found in their nature habitats, such as done for *Paenibacillus* sp. [25], *Bacillus pumilus* [26]. However, the fragments often contain the whole operon with intact regulator in the fragment [26]. In this study, we isolated the *xyn*B gene by PCR amplification. *Bacillus subtilis* subsp. spizizenii W23 genome have compete genome sequence for this purpose. The difficulty of this step is in the designing of primers for amplification the whole coding sequence. Sequences around xynB gene region are rather complicated for primers designing [27]. In this flanking area, no sequences are available for proper annealed primer. Based on several software analyses, the sequences contain repeated nucleotides, low GC content and several unspecific sequences (NCBI Primer Design Tool [28], Primer 3 [29, 30], Clone Manager Suite Professional softwares). In this study, we managed to design the primers pair for whole coding sequence of *xyn*B gene and amplified it for construction of recombinant plasmid.

The aims of this study are to construct a recombinant plasmid containing *xyn*B gene from *Bacillus subtilis* subspecies spizizenii W23 and to over-express the *xyn*B gene in *Escherichia coli* Origami as host cells under tac promoter.

2. Materials and methods

2.1. Bacteria and plasmid

Escherichia coli Origami and *Escherichia coli* DH5α (Novagen) containing pMMB67EH plasmid [31]. *Bacillus subtilis* subsp.spizizenii W23 is a kindly gift from Northern Regional Research Laboratory, NRRL)

2.2. Amplification of *Bacillus subtilis* subsp.spizizenii W23 *xyn*B gene

The open reading frame (ORF) of *Bacillus subtilis* subsp.spizizenii W23 gene was amplified by polymerase chain reaction (PCR) using primers that were designed based on the genomic sequence of *Bacillus subtilis* subsp.spizizenii W23 strain obtained from Genebank database (www.ncbi.com) [27], using Clone Manager Professional Suite (DNA Star, USA) software. For-xynB_BSUW23: 5′-CGAGCGGCCGAGCTCATGAAGATTA TCAATCCAGTACTTAAAGGATTCAA-3′, and Rev-xynB_BSUW23: 5′-CCGCTGGTCTAGAGGCT CCTGTAAAGAACCCTCCTCCC-3′. Both primers were added with a restriction sites at its

5′-ends (underlined), and synthesized by Operon. As a PCR template was chromosomal DNA of Bacillus subtilis subsp.spizizenii W23. PCR was settled as follow, predenaturation at 94 °C for 2 min, denaturation at 94 °C for 30 s, annealing at 60 °C for 1.5 min, elongation at 72 °C for 2.5 min, 30 cycles and post-extension at 72 °C for 10 min.

2.3. Cloning of PCR fragment on pMMB67EH vector

The *xyn*B gene was cloned into pMMB67EH under the control of tac promoter [31]. Before ligation, the PCR product was digested with *Sac*I and *Xba*I enzymes and then was ligated to the similarly digested pMMB67EH. The resulting pMMB-*xyn*B plasmid was used to transform the *Escherichia coli* Origami cells which were made competence by $CaCl_2$ using heat shock method, as described by Sambrook et al. [32].

2.4. Analyses of recombinant plasmid

Screening of transformants was done by plating the transformants on LB plate containing 100 $\mu g \cdot .mL^{-1}$ ampicillin (LB-amp). The transformants grew on LB-amp plates were then selected for the presence of recombinant plasmid in the cells by PCR techniques. For vector detection, PCR was performed using vector-specific primers, SeqForpMMB67 dan SeqRevpMMB67 primers [33]. The amplification product was predicted to be a 2 035 bps band, of which the 1 523 bps was the size of *xyn*B gene sequence and the rest was the vector parts. While for *xyn*B detection, the For-xynB_BSUW23 and Rev-xynB_BSUW23 primers pair was used. The recombinant plasmids generate a single band amplicon with a size of ~1 549 bps. As a control, *Escherichia coli* Origami, with or without plasmids, were used as templates. All of these negative controls have no amplicon.

The restriction analyses were done for recombinant plasmid that gave positive result in PCR detections. The restriction analyses were performed using *Sac*I, *Xba*I and *Eco*RI (Fermentas) restriction enzymes according to the manufacturer's instruction and then visualized on 1 % agarose gel. *Xba*I or *Sac*I will linearized pMMB-xynB to be a linear fragment with a size of 10 334 bps. *Eco*RI will digest proper recombinant plasmid to two fragments of ~1005 and ~9 329 bps, while digestion with *Xba*I and *Sac*I simultaneously gave two fragments, 1 523 bps which confirmed for the size of *xyn*B insert and 8 811 bps for the size of pMMB-vector. Un-cut empty plasmid, pMMB67EH, had a size of ~8 828 bp.

2.5. Over-expression verification by SDS-PAGE analysis

Over-expression of xynB gene in *Escherichia coli* Origami cells was verified on sodium-dodecyl-sulfate polyacrylamide (SDS-PAGE) gel analyses. The cells were grown in LB-amp, at 37 °C, and induce with 1 mM IPTG. The pellet was separated from the supernatant by centrifugation at 13 000 *g* for 1 min [(*g* = Relative Centrifugal Force (RCF)], then sonicated to break the cells membrane. The separation of proteins was performed on SDS-PAGE gel with 3 % stacking gel and 12.5 % separating gel [32]. The proteins bands were visualized by Coommassie blue G-250 staining.

2.6. Xylan degradation activity

The xylan degradation activity was assayed by growing the transformants on LB-amp plate containing 1 % xylan with addition of 1mM IPTG, incubated at 37 °C for 7 d. The *Escherichia coli* Origami transformants harboring xylanase gene showed a clear zone around the colony upon Congo red addition [34]

3. Results and discussion

The *xynB* gene had been amplified successfully using For-*xyn*B_BSUW23 and Rev-*xyn*B_BSUW23 primers. PCR product revealed as a single band with a size of 1 549 bps (Fig. 1). Commonly, isolation of genes encoding xylanases from various bacteria and fungi have been done by digestion of genomic DNA, cloning the fragments and then the target were selected based on its activity [26]. Both primers were predicted as not good primers by the Clone Manager Suite Professional software, however in fact the amplification was succeeded in this study.

The screening of *Escherichia coli* Origami transformants were performed based on ampicillin resistance [33]. There were many transformants colonies grew on LB-amp plates, as many as colonies of *Escherichia coli* Origami transformed using the empty plasmid, pMMB67EH. The pMMB67EH is a vector plasmid contained ampicillin resistance marker. Since ampicillin resistance is quite common in bacterial cells and laboratory plasmids, so further analyses have to be performed.

Ten of *Escherichia coli* Origami transformants grew on LB-amp plates were selected to detect the presence of target plasmid by PCR using *SeqForpMMB67* and *SeqRevpMMB67* primers. The result showed that nine of *Escherichia coli* Origami transformants had a fragment with proper size of ~2.0 kbs on 1 % agarose gel. Based on this analysis, these nine *Escherichia coli* Origami transformants were confirmed to contain the pMMB-recombinant plasmid.

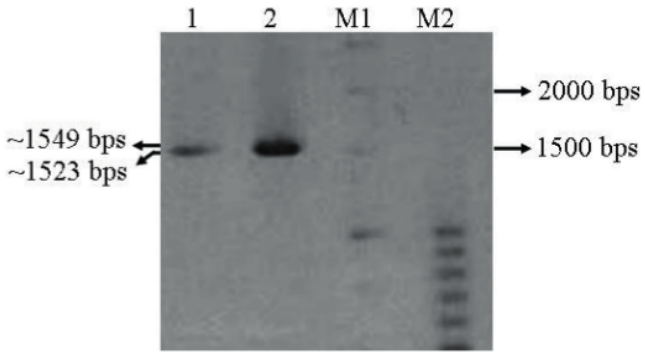

Figure 1: The *xynB* gene was amplified from *Bacillus subtilis* subsp. spizizenii W23 genome. The PCR product had a band of ~1 549 bp (line 2), while digestion with *Sac*I and *Xba*I resulted to three fragments with sizes of approximately 1 523 bps, 14 bps and 12 bps (line 1). The 14 and 12 bps fragments were too small to be existed on this gel under the electrophoresis condition of this experiment. The PCR product and restriction fragments had proper sizes as predicted. M1 and M2: 1 kb and 100 bps DNA *ladder* (Fermentas).

To confirm the presence of *xyn*B in the recombinant plasmids, two of the positive transformants, *Escherichia coli* Origami clones 9 and 10, were chosen for next PCR detection using For-*xyn*B_BSUW23 and Rev-*xyn*B_BSUW23 primers. Before PCR process, the plasmids were isolated from clones 9 and 10 which then were used as templates in PCR amplification. PCR result showed that both transformants have pMMB-*xyn*B plasmids with *xyn*B gene in proper orientation.

For further analysis, restriction analysis was done to confirm the orientation of the *xyn*B gene in the pMMB67EH vector. The pMMB-*xyn*B plasmid was extracted from *Escherichia coli* Origami transformants clone 9 and 10, then were digested using *Xba*I, and/or *Sac*I. Restriction analyses results showed that digested recombinants plasmids have proper fragments sizes. All of the analyses results confirmed that the *Escherichia coli* Origami clone 9 and 10 contain the proper pMMB-*xyn*B recombinant plasmid.

To verify that the *xyn*B gene positioned in frame on the recombinant plasmid so that over-expressed properly in transformant cells, the proteins of clone 10 were profiled on SDS-PAGE gel (Fig. 2).

Deducted from the DNA sequence, the protein is predicted to have a molecular mass of ~61 337 Da. SDS-PAGE result showed that this positive clone revealed a thicker protein band in the size of ~61 kDa, both in intra- and extra-cellular fractions. This result gave impression that this protein can be an intra- or extra-cellular protein.

To confirm the presence of active protein and its localization, assay was also performed by Congo Red assay. The activity to degrade xylan by the extra-cellular enzyme resulted to clear red zone surrounding the colonies upon Congo red addition. Clear zone surrounding colony of transformant gave confirmation that the transformant over-expressed the active XynB enzyme and it might be secreted into the medium. No clear zone appeared around the wild type colony (Fig. 3). It meant that the *xyn*B

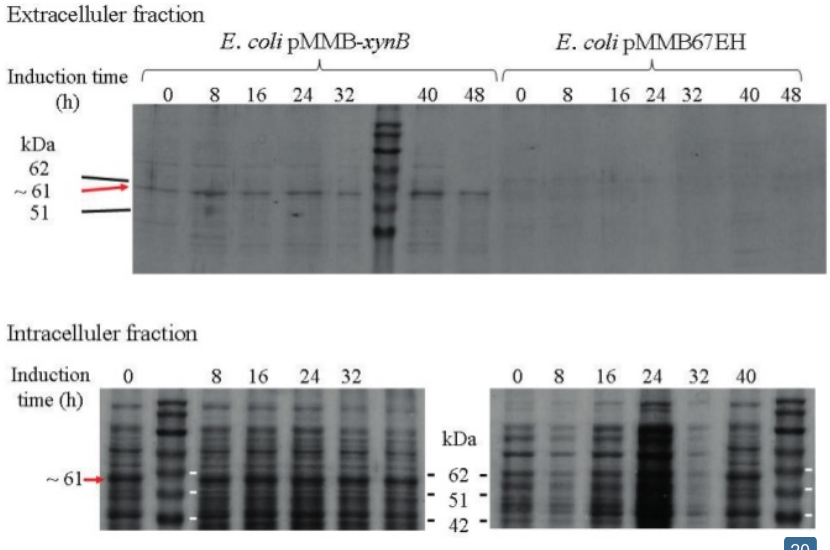

Figure 2: SDS-PAGE proteins profiles of over-expressed *E. coli* Origami pMMB-*xynB* cells grown in LB-amp at 37 °C, shaked at 200 rpm (1 rpm = 1/60 Hz), under 1 mM IPTG induction.

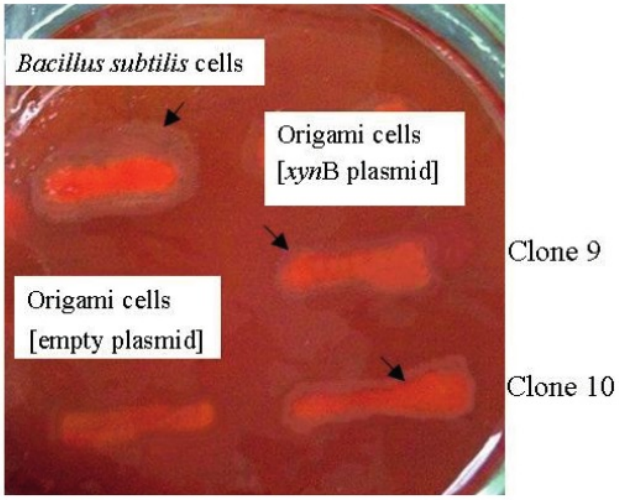

Figure 3: The expression of pMMB-*xyn*B in *Escherichia coli* Origami transformants assayed on LB-amp plate containing 1 % xylan and 1 mM IPTG. The colonies of cells bearing *xyn*B recombinant plasmid showed a clear zone (black arrow, →) around the colonies however no clear zone appeared around transformant bearing empty plasmid. 37 °C, for 7 d.

gene was over-expressed from pMMB-*xyn*B in this positive transformants. The *Bacillus subtilis* subsp.spizizenii W23 cells, as an original gene source, showed the highest xylan degrading activities. This result was corroborated to genomic sequence database, indicates that *Bacillus subtilis* subsp.spizizenii W23 has several genes which were predicted to encode lignocellulases enzymes, including the *xyn*A and *xyn*B genes [27].

Based on protein structure analyses using PROPSEARCH software [35], *xyn*B gene of *Bacillus subtilis* subsp. spizizenii W23 is predicted to encode a β-xylosidase (1,4-β-D-xylan xylohydrolase; EC 3.2.1.37). Compare to *xyn*B from *Bacillus pumilus* [26], the latter is known as intracellular endoxylanases. XynB from *Bacillus pumilus* was detected as an intra-cellular protein by software and by experiment, without signal peptide [26]. Different from the previous protein, this *Bacillus subtilis* W23 XynB protein is predicted by PrediSi [36] dan SignalP software(www.ExPassy.com), as a non-secreted protein, without signal peptide sequences. Based on CELLO Prediction software, the most possible protein location is in cytoplasm, however there is a half possibility to be an extracellular protein [37]. In contrast, according to MTHMM software [38], this XynB protein is predicted to be an extracellular protein although it contains no signal peptide. This result also reveal in ProtComp [39] analyses result. Although predicted ProtComp software as a cytoplasmic protein, a part of the amino acid residues is located in membrane as integral and transmembran protein. This result can be explained by the fact that the *Bacillus subtilis* sequence have only 48 % similarity to *Bacillus pumilus* that the sequence might be unique and have different properties [40]. Other possibility is that this gene might be expressed abundantly in cytoplasm so that secreted out of the cells. It is interesting to find that this gene was secreted by transformants and gave impact on xylan around the colony. However, the localization of *Bacillus subtilis* subsp.spizizenii W23 XynB protein still need to be verified.

4. Conclusion

It could be concluded that the *xyn*B gene of *Bacillus subtilis* subsp.spizizenii W23 has been successfully been cloned on pMMB67EH plasmid and over-expressed in the *Escherichia coli* Origami cells both intra- and extra-cellularly, as observed on SDS-PAGE gel analysis. The transformants bearing the pMMB-*xyn*B recombinant plasmid had activity to degrade xylan on LB-xylan plate while the transformant bearing an empty plasmid showed no activity.

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