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The Utilization of Auto-Inducible *Plyb* Promoter and Media Optimization for Cell Density-Dependent Expression of Recombinant Thermoalkalophilic Xylanase in *Bacillus subtilis* DB104

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Strong promoters are one of the fundamental aspects to increase the level of gene expression, and one of approach to improve the recombinant enzyme productivity so that the efficiency of production cost for enzyme production in industrial scale can be reached. Here we assessed the application of a cell density-dependent promoter and media optimation to promote cell growth and protein expression of *Bacillus subtilis* without excess usage of inducers. An auto-inducible P*ylb* promoter that is potential to provide inducer-free enzyme production was cloned and introduced into xylanase recombinant system in *B. subtilis* DB104 by PCR cloning and protoplast transformation. A 200 bp target gene was successfully inserted in between *xynCM1* ORF -coding for *B. halodurans* CM1 xylanase- and its native promoter sequence at the upstream region. The disruption of the native promoter was intended to replace the native promoter with P*ylb*. Recombinant xylanase gene under *Pylb* was successfully expressed in *B. subtilis* DB104 and the enzyme was produced at stationary phase. Different media with various concentrations of glucose and nitrogen were used to optimize recombinant xylanase expression. It achieved a higher level of xylanase expression compared to wild-type *Bacillus* and recombinant xylanase with native promoter expressed in *B. subtilis* in media containing a 2-fold recipe of LB media thus leads to increase cell density and xylanase expression (81.461 U mL^{-1}).

Key words: auto-inducible, *Bacillus subtilis* DB104, P*ylb*, xylanase

Promotor yang kuat adalah salah satu aspek mendasar untuk meningkatkan tingkat ekspresi gen, dan salah satu pendekatan untuk meningkatkan produktivitas enzim rekombinan sehingga efisiensi biaya produksi enzim pada skala industri dapat tercapai. Di dalam studi ini aplikasi promotor yang bergantung pada kepadatan sel tanpa penggunaan induser secara berlebihan dari induser dan optimasi media untuk mendorong pertumbuhan sel dan ekspresi enzim xilanase di *Bacillus subtilis* telah dilakukan. Promotor P*ylb* yang *auto inducible* berpotensi untuk menyediakan produksi enzim bebas-induktor telah dikloning dan dimasukkan ke dalam sistem rekombinan xilanase di *B. subtilis* DB104 *via* kloning PCR dan transformasi protoplas. Gen target sepanjang 200 bp berhasil disisipkan di antara xynCM1 ORF –yang mengkodekan *B. halodurans* CM1 xylanase- dan promotor asli. Gangguan promotor asli dimaksudkan untuk menggantikan promotor asli dengan promoter P*ylb*. Gen xilanase rekombinan di bawah P*ylb* berhasil diekspresikan dalam *B. subtilis* DB104 dan enzim diproduksi pada fase stasioner. Media yang berbeda dengan berbagai konsentrasi glukosa dan nitrogen digunakan untuk mengoptimalkan ekspresi xilanase rekombinan. Tingkat ekspresi xilanase yang lebih tinggi daripada *B. subtilis* non rekombinan ataupun *B. subtilis* rekombinan yang mengandung gen xilanase alkalotermofilik dengan promoter asli ditemukan di dalam media yang mengandung resep LB 2 kali lipat dari media LB sehingga mengarah pada peningkatan kepadatan sel dan ekspresi xilanase (81,461 U mL^{-1}).

Kata kunci: *auto-inducible*, *Bacillus subtilis* DB104, P*ylb*, xilanase

In gene expression, promoters are known as one of the key factors to have an important role in defining where the gene transcription starts. It is in the upstream on an open reading frame near the transcription site starts and comprises a set of sequence that has recognizable patterns. The strength of the promoters would likely affect RNA polymerase binding and therefore can contribute to the enhancement of target gene expression (Mekler *et al.* 2012; Einav and Philipp 2019). It is suggested that choosing the promoters that are suitable for our target genes should be done wisely to obtain the desired level of expression (Schumann 2007; Wenzel *et al.* 2011; Einav and Philipp 2019).

There are many types of promoters used in heterologous protein expression system of recombinant host from *Bacillus* genus, that is constitutive promoters, inducer-specific promoters, and auto-inducible promoters (Schumann 2007; Yu *et al.* 2015), among which induced-promoters are the most commonly used to date. However, these inducible promoters are not cost-efficient in the context of

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industrial application because of the requirement of certain chemicals and compounds to be added in the process. For example P*xyl*, one of the most used in industry, needs xylose as its inducers (Yu *et al.* 2015; Meyers *et al.* 2019). Native inducible-promoter of genes encoding enzymes also needs its substrate to produce high-level protein production, such as xylan for xylanase (Gupta *et al.* 2008; Ulfah *et al.* 2011; Helianti *et al.* 2018) and skim milk for protease (Ulfah *et al.* 2011; Cu *et al.* 2015). Since the usage of constitutive promoter can generate toxic protein that can be lethal for the host, this leads us to consider the advantage of auto-inducible promoters which can ideally facilitate efficient production process at low cost with an optimum condition (Yu *et al.* 2015; Meyers *et al.* 2019; Trung *et al.* 2019).

Several highly-efficient non-inducible promoters in *Bacillus subtilis* had been identified by Yu *et al.* (2015), among them was P*ylb* which strongly promoted β-galactosidase expression and showed significant superiority from the rest of selected promoters. This promoter was not only actively expressing reporter gene *bgaB* during stationary phase but also boosting a higher activity of β-galactosidase up to 5000 times. Hence, this P*ylb* promoter is potential to be used for overexpressing other recombinant gene product with no inducer needed (Yu *et al.* 2015) especially one which utilizes *B. subtilis* as cell factory.

B. subtilis DB104 is a Gram-positive bacterium that is generally recognized as a safe (GRAS) non-toxic organism (Westers *et al.* 2004; Watzlawick and Altenbuchner 2011). Along with members of *Bacillus* genera, its ability to secrete protein into extracellular sphere and grow in mesophilic condition at 37 ˚C in minimum media with various carbon and nitrogen source (Wenzel *et al.* 2011; Mageshwaran *et al.* 2014] has attracted scientists to use them as recombinant host in large-scale production as they cover more than one-third of industrial enzyme (Meissner *et al.* 2015; Watzlawick and Altenbuchner 2019]. On top of that, *B. subtilis* DB104 has alkaline protease gene deleted (Δ*aprA3*), allowing them to secrete less protease and therefore the target protein will have lower denaturation risk (Kawamura and Doi 1984) and it also might be more suitable to express genes from the same Gram-positive bacteria than *E. coli*.

Our recent work has been focused on utilize native promoter for expression of xynCM1 gene in *B. subtilis* DB104. Whilst it could enhance the enzyme production in the host compared to the native *B. subtilis* DB104 (Haniyya *et al.* 2019), the productivity of recombinant clones was still almost 4 times lower than the wild-type *B. halodurans* CM1. The expenses of using xylan or raw corncob as inducers also rise concerns both in terms of cost-efficiency and convenience of upstream and downstream processes.

The utilizing an auto-inducible promoter from *B. subtilis* is very much necessary to upgrade the recombinant protein production system in *B. subtilis* DB104. Hence, the current study aimed to clone and utilize P*ylb* promoter for cell density-dependent expression of thermoalkalophic xylanase originally from *B. halodurans* and the strategy with optimized media. Media optimization was also a highlight in our study since it was very critical for cell growth and therefore played as an important factor in determining P*ylb* promoter efficiency.

MATERIALS AND METHODS

Strains, Cultivation, and DNA Extraction. *Eschericia coli* DH5α was used only as cloning host and *B. subtilis* DB104 was engineered for subcloning and recombinant protein production. The *wild-type* strains were grown in default condition at 37 ˚C, 150 rpm for overnight in LB media (0.5% yeast extract, 1% NaCl, and 1% peptone). The recombinant strains were also cultivated in stated condition using LB media with respective antibiotics (100 µg mL^{-1} ampicillin and 5 µg mL^{-1} erythromycin) unless stated otherwise.

Whole genomic DNA of *B. subtilis* DB104 was extracted using extraction kit from Thermo Fisher Scientific. According to its protocol for Gram-positive bacteria, an overnight culture was collected and treated with 180 µL Gram-positive bacteria lysis buffer which consists of 20 mM Tris-HCl pH 8.0, 2 mM EDTA, and 20 mg mL^{-1} of lysozyme. After incubation at 37 °C for 30 min, 200 µL of Lysis Solution and 20 µL of Proteinase K were added to the suspension to perform cell lysis and protein removal. The sample was incubated at 56 °C while vortexing to ensure a uniform suspension until the cells were completely lysed (about 30 min). To obtain purified genomic DNA, 20 µL RNAse was added to the suspension and followed with the addition of 400 µL 50% ethanol. The prepared lysate was then transferred to the filtered column and washed by Wash Buffer I and Wash Buffer II of each contained pure ethanol. Before DNA elution was performed, the column was spun at maximum speed (12,000 ×g) for 3 min to dry. Genomic DNA was obtained after a 5-min incubation step with 200 µL Elution Buffer. All centrifugation was done at

6,000—8,000 ×g except for the drying process.

Isolation of *Pylb* Promoter. The P*ylb* promoter was amplified from gDNA of *B. subtilis* DB104 by a set of primers based on work of Yu *et al*. (2015) but modified for restriction-free cloning by the addition of backbone sequences in 5' or 3' overhangs in both primer F1 and R1 (Table 1). Genomic DNA was isolated using GeneJET Genomic DNA Purification Kit [#K072, Thermo Fisher Scientific, Waltham, USA]. The P*ylb* gene was amplified by PCR in a 50 µL reaction mixture composed of 10 µL 5× HF Buffer, 1 µL 10 mM dNTPs, 2.5 µL of 10 µM forward and reverse primers, 1.5 µL 100% DMSO, 5 µL template gDNA, 0.625 µL Phusion HiFi DNA Polymerase [Thermo Fisher Scientific, Waltham, USA], and 26.875 µL nuclease-free water to bring the total volume of 50 µL. After a swift initial denaturation of 98 ˚C for 30 s, amplification was performed in 30 cycles of 10 s at 98 ˚C, 15 s at 72 ˚C, and 30 s at 72 ˚C, followed by a final extension at 72 ˚C for 5 min to ensure the fragment elongation. The 256 bp fragment of P*ylb* gene was then purified using GeneAid Gel/PCR DNA Fragments Kit [DF100, Geneaid Biotech, New Taipei City, Taiwan] by mixing it with 250 µL DF Buffer prior to sample transfer into DF column by centrifugation at 14,000 ×g for 30 s. The wash step with the addition and 1-minute incubation of 600 µL Wash Buffer followed and the purified DNA was eluted at last with 50 µL Elution Buffer.

Construction of Plasmid for Xylanase Gene Expression with Mega Primer Approach. The recombinant plasmid was constructed through restriction-free cloning by PCR insertion of P*ylb* gene into pSKE194.*natprom-alkxynCM1-inlip* as template plasmid. The insertion was occurred between native promoter and *xynCM1* gene to replace upstream region as gene promoter. There were two megaprimers used in the experiment (Fig 1), the purified P*ylb* as the targeted gene and *xynCM1* gene to assist the annealing process of targeted gene into the template (Ulrich *et al.* 2012; Mathieu *et al.* 2014). The assisting-gene were amplified by the method described in previous study (Helianti *et al.* 2018).

PCR was performed in a 50 µL reaction mixture composed of 10 µL 5× HF Buffer, 1 µL 10 mM dNTPs, 1.5 µL 100% DMSO, 25 ng plasmid template, 100 ng purified P*ylb* target gene, 100 ng purified *xynCM1* assisting-gene, 0.5 µL Phusion HiFi DNA Polymerase [Thermo Fisher Scientific, Waltham, USA], and 34 µL nuclease-free water to bring the total volume of 50 µL. A quick initial denaturation was performed at 98 ˚C for 30 s and followed by 30 cycles gene amplification

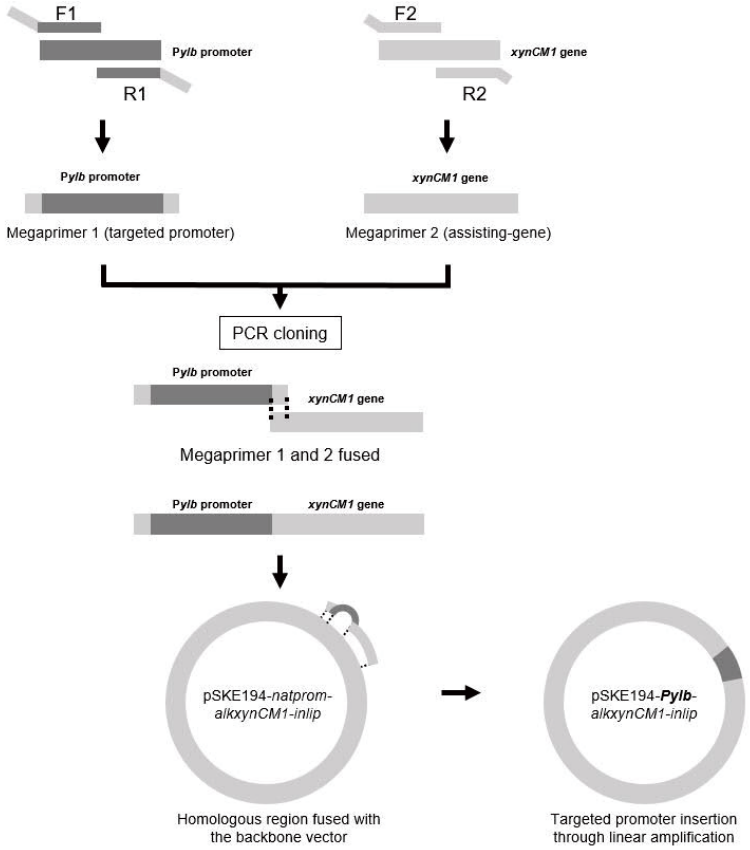

Fig 1 The process of megaprimer-assisted cloning.

Table 1 List of primers used in the study

Primer	Sequence	Usage
F1	5'-GCATTTTACTTTGCTACGAAAGGAGAATTTGTGAAAAGACCAACGGAGCCT CCG-3'	P*ylb* cloning
R1	5'-GGTTTTCTAAACAGTGTAATCATACAAATCTCCCCCTTTGTTGTTTC -3'	P*ylb* cloning
F2	5'-ATGATTACACTGTTTAGAAAACCTTTTG -3'	*xynCM1* isolation
R2	5'- GTATCGATAATTCTCCAGTAAGCAGGTTTC -3'	*xynCM1* isolation
F3	5'-CCAAGCTTATTTCAATGAGTATTG -3'	Recombinant ver ification
R3	5'-GTTGACTTGGCTGCTGTACAGAAG -3'	Recombinant verification

consisted of 10 s at 98 ˚C, 30 s at 54 ˚C, and 5.5 min at 72 ˚C. At last, a final extension was wrapped at 72 ˚C for 10 min to ensure the fragment elongation. A recombinant DNA was obtained and purified by GeneAid Gel/PCR DNA Fragments Kit [DF100, Geneaid Biotech, New Taipei City, Taiwan] with the method described previously prior to recombinant verification.

The verification was again performed by PCR using F3 and R3 primers (Table 1). The reaction mix in a total volume of 20 µL was simply composed by 4 µL 5× MyTaq Reaction Buffer, 1 µL 10 µM F3 and R3 primers, 1.5 µL purified recombinant DNA, 0.25 µL MyTaq HS DNA Polymerase [Bioline, London, UK], and 12.25 µL ddH_2O. A 30-cycles PCR was done under the condition: initial denaturation at 95 ˚C for 1 min, denaturation at 95 ˚C for 15 s, annealing at 50 ˚C for 15 s, extension at 72 ˚C for 15 s, and final extension at 72 ˚C for 10 min.

After it was verified, in-vitro ligation was proceeded by the incubation of 2 µL 10× T4 DNA Ligase, 16 µL PCR product, and 1 µL T4 Polynucleotide Kinase [Promega, Madison, USA] at 37 ˚C for 30 min. After the first incubation finished, 1 µL T4 DNA Ligase [Thermo Fisher, Waltham, USA] was added and the incubation was prolonged at room temperature for 30 min. To eliminate non-recombinant parental plasmid, 0.5 U μL^{-1} *Dpn*I was added before the reaction was transformed into *E. coli* DH5α competent cells. Verification of recombinant strains was done using both PCR (which the reaction described previously) and plasmid digestion with *Mfe*I.

Subcloning to *B. subtilis* DB104 and Recombinant Verification. Plasmid transformation in *Bacillus* was conducted using protoplasting method proposed by Chang and Cohen (1979). To make *Bacillus* competent cells, the wild-type *B. subtilis* DB104 strain was grown in LB media at 37 ˚C, 150 rpm, for overnight as a starter culture. It was inoculated into 20 ml of Pennasay broth [Difco, Detroit, USA] until the OD_{600} reached 0.4—0.6. The cell was harvested using centrifugation at 3800 rpm, 4 ˚C, for 15 min [Gyrozen Centrifuge, 50 ml rotor]. The protoplasts were formed after 2-h incubation at 37 ˚C in SMMP solution (0.5 M sucrose, 0.02 M maleic acid, 0.02 M MgCl2.6H_2O, and 3.5% Pennasay powder) containing freshly-added 2 mg mL^{-1} lysozyme [Sigma-Aldrich, St. Louis, USA]. It went under several steps of washing before a final 1.5 mL of SMMP solution was gently added to resuspend cell pellets for plasmid transformation.

The DNA plasmid was prepared using a double-strength SMM solution (1 M sucrose, 0.04 M maleic acid, and 0.04 M $MgCl_2$.6H_2O) about 1:1 ratio of DNA and solution volume in a total of 50 µl. All of DNA mixture was added to 0.5 ml protoplast suspension before the addition of 1.5 ml 40% PEG6000 in SMM Solution (0.5 M sucrose, 0.02 M maleic acid, and 0.02 M $MgCl_2$.6H_2O). The suspension was incubated for 2 min at room temperature. About 5 ml of SMMP Solution was added prior to cell separation by centrifugation at 2900 rpm, 4 ˚C, for 10 min. The cell pellet was resuspended by 1 ml of SMMP Solution once again and incubated at 37 ˚C for 1.5 h with 100 rpm agitation. About 500 µL of cell suspension was spread on DM3 agar (0.5 M sodium succinate, 0.5% casamino acid, 0.5% yeast extract, 0.35% K_2HPO_4, 0.15% KH_2PO_4, 0.5% glucose, 0.02 M $MgCl_2$.6H_2O, 0.01% BSA, and 1% agar) containing 5 µg/ml erythromycin and the growth of recombinant colonies could be seen after 2 days incubation at 37 ˚C. We then selected the positive recombinant colonies by performing colony PCR and plasmid extraction for *Bacillus* as described by Voskuil and Chambliss (1995). Recombinant xylanase activity was checked qualitatively by measuring the ratio of clear zone diameter to bacterial colony diameter (in cm) after 24 h of incubation on LB agar pH 7 and pH 9 in the presence

of 2% (w/v) beechwood xylan. Non-recombinant *B. subtilis* DB104 was used as negative control.

Plasmid Extraction of Recombinant *B. subtilis* DB104. The erythromycin-resistant transformant *B. subtilis* DB104 were selected and cultured in LB media containing erythromycin (5 μg mL^{-1}) under 37 °C at 150 rpm agitation. Plasmid isolation performed with SET buffer (Voskuil and Chambliss 1993) after centrifugation at 12,000 rpm at 4 °C for 5 min to obtain cell pellet. The cell pellet resuspended in 200 μL in SET buffer (25% sucrose, 0.05 M EDTA, 0.05 M Tris-HCl pH 8) containing 5 mg mL^{-1} lysozyme and incubated at 37 °C for 10 min. 0.2 N NaOH and 1% SDS was added to the suspension and flipped until clear. 5 M of cold KCOOH was added and homogenized, then centrifuged. 650 μL of cold phenol:chloroform:isoamyl-alcohol (25:24:1) was added to 750 μL of supernatant and homogenized by vortexing at full speed. Aqueous phase from the suspension was obtained by centrifugation. 620 μL of aqueous phase was obtained and 620 μL of chloroform:isoamyl-alcohol (24:1) was added to suspension and homogenized by vortexing at full speed. The suspension was then centrifuged and 550 μL of aqueous phase was moved to new microtube. 550 μL of cold isopropanol was added, homogenized, and centrifuged. 1 mL of alcohol 70% was added to the pellet and centrifuged at 12000 rpm at 4 °C for 5 min. The pellet DNA was then resuspended in ddH2O with RNAse (20 μg mL^{-1}).

Growth Curve Observation and Media Formulation. Recombinant *B. subtilis* DB104 harboring pSKE194-*Pylb-alkxynCM1-inlip* was cultured overnight in 10 mL of LB media in the presence of 5 μg mL^{-1} erythromycin under 37 °C at 150 rpm agitation. Two percent of the culture was then used to inoculate 100 mL of fresh LB media containing the same antibiotic under 37 °C at 150 rpm agitation for 24 h. Samples were taken every 2 h to observe bacterial cell density by measuring OD_{600} then plotted against time to obtain bacterial growth curve.

Media optimization strategy used to achieve high-cell density with modification in yeast extract and glucose concentration. Several media used were based on LB and SOC media. LB media (1% tryptone, 0.5% yeast extract, 1% NaCl) and 2X LB media with 2-fold concentration recipe containing 5 μg mL^{-1} erythromycin [Sigma-Aldrich, St. Louis, USA] were used. Soybean flour media used as an alternative source of nitrogen containing 1.89% (w/v) soybean flour and 1% NaCl. For glucose optimization, SOC media-based were used containing 1% and 5% (w/v) glucose for each variation.

Xylanase Assay. Xylanase activity was assayed using dinitrosalicylic acid (DNS) to measure reducing sugar produced from xylan hydrolysis (Bailey *et al.* 1992; Miller 1959) in triplicate with some modifications. Crude enzyme was obtained from culture supernatant after centrifugation at 12000 rpm at 4 °C for 5 min. 50 μL of supernatant was added to 450 μL xylan substrate containing 0.5% (w/v) beechwood xylan in 0.5 M Tris-HCl buffer pH 9. The reaction takes place at 70 °C for 5 min at 300 rpm using a thermoshaker. After incubation, 750 μL of DNS reagent (1% dinitrosalicylic acid, 0.2% phenol, 0.05% sodium sulfite, 1% sodium hydroxide, and 20% (w/v) potassium sodium tartrate) was added immediately. As for blank, 50 μL of sample was added after DNS addition. All the samples and blanks were boiled for 5 min and let cool to room temperature. 250 μL of water was added, homogenized, and reducing sugar released was measured at 540 nm. Standard curve obtained from xylose suspension ranging from 0; 0.2; 0.4; 0.6; 0.8; and 1 mg mL^{-1} using the same protocol. Xylanase activity stated in Unit (U) defined as the amount of enzyme releasing 1 μmol of reducing sugar per minute under appropriate assayed condition.

RESULTS

P*ylb* Cloning and Verification. DNA fragment of 256 bp coding for *Pylb* promoter was integrated into pSKE194-*natprom-alkxynCM1-inlip* by PCR cloning resulting an 8757 bp recombinant plasmid (Fig 2A). The insertion can occur because of the presence of 56 bp of overlapping region in both ends of P*ylb* fragments so that they annealed into the backbone and promoting the formation of new recombinant plasmid in the next cycle of amplification. As depicted in the figure, the gene cassette was constructed of three genes with the position of P*ylb* promoter between the native promoter and *xynCM1* gene. The flanking regions of lipase locus at both 5' and 3' ends were intended for further experiments with chromosomal integration and could be used for the verification of positive recombinants (Fig 2B).

The positive recombinants of *E. coli* DH5α and *B. subtilis* DB104 were confirmed by PCR using F3 and R3 primer set and proved to be harboring the targeted gene P*ylb* (200 bp) within a 2061 bp gene cassette (Fig 3C). This result was also supported by *Mfe*I digestion which cut the plasmid into two fragments of 7456 bp

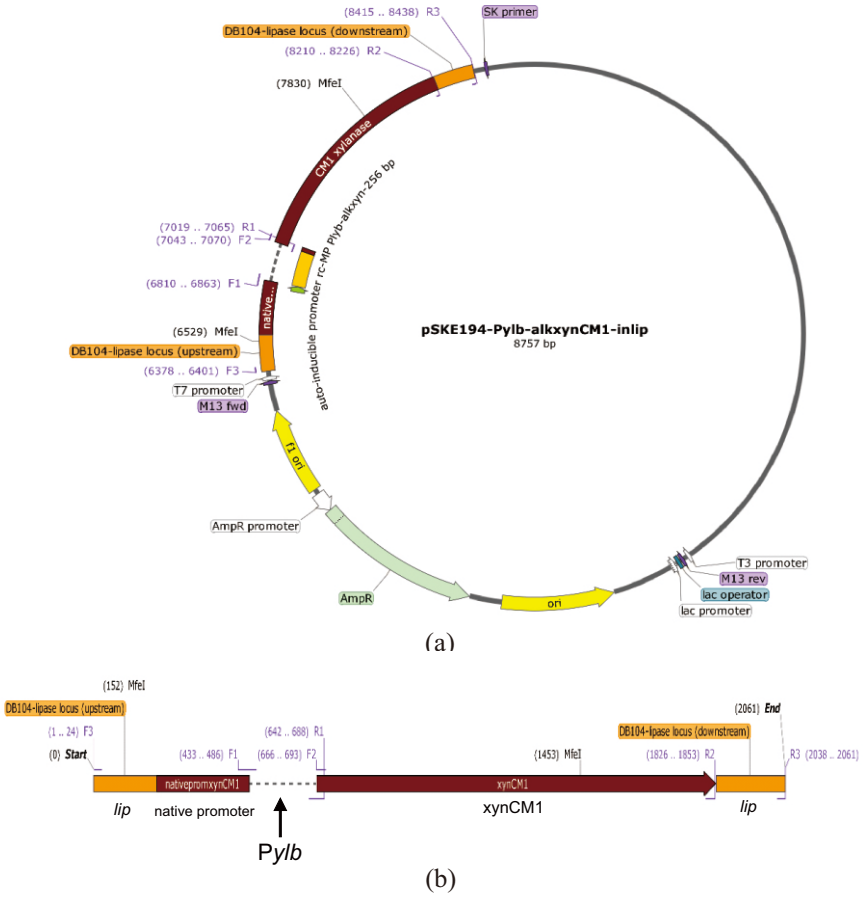

Fig 2 The constructed plasmid and gene cassette of P*ylb*. (a) The whole construct of recombinant plasmid pSKE194-*Pylb-alkxynCM1-inlip* (8757 bp) and its features; (b) A 2061 gene cassette containing 200 bp P*ylb* fragment.

and 1301 bp after 1-hour incubation at 37 ˚C that were notably delineated from the negative one. According to the vector map, *Mfe*I digestion of recombinant plasmid generated 2 fragments of 1.3 and 7.5 kb, while control with lack of target gene formed smaller bands of 1.1 and 7.5 kb corresponding to the result (Fig 3D). Three out of four potential *B. subtilis* DB104 transformants were identified as positive clones, however, only one clone (R3) was carried out for further experiments of protein expression and media formulation.

Qualitative Assay of Xylanase. Qualitative assay of xylanase produced by recombinant *B. subtilis* performed in LB xylan media pH 7 and pH 9 to detect alkaline xylanase and compared to *B. subtilis* DB104 wild-type. Clear zone ratio 2.00 surrounding recombinant *B. subtilis* colony in LB media pH 9 (Fig 4D) showed alkaline xylanase activity and no distinct clear zone observed in LB media pH 7 (Fig 4C). As control, *B. subtilis* DB104 wild-type grown on LB media pH 7 (Fig 4A) showed distinct xylanase activity around the colony with clear zone ratio 1.40, while no clear zone detected in LB media pH 9 (Fig 4B).

Growth Curve and Media Optimization to Achieve High Cell-Density. The growth curve of recombinant xylanase-producing *B. subtilis* was investigated to determine when the enzyme was highly-produced. Growth curve observed in LB media for 24 h with *B. subtilis* DB104 wild-type as control bacteria (Fig 5). Both wild-type and recombinant *B. subtilis* entered the log phase after 2 h of cultivation and the recombinant *B. subtilis* reached the cell density peak after 9 h, while wild-type strain entered the stationary phase after 11 h. *B. subtilis* wild-type reached higher cell density than recombinant *B. subtilis*. Based on the growth curve, samples were taken at 5 h (log phase), 9 h (late log phase), 14 h (stationary phase), and 24 h (late stationary phase) after inoculation to determine xylanase activity.

All samples taken from recombinant and wild-type *B. subtilis* exhibit xylanase activity measured by DNS method. Both supernatant from recombinant and wild-type bacteria shows higher level of enzyme expression taken from 24-h supernatant culture (Table 2). Moreover, recombinant xylanase (R3) performed 2.5-fold higher level of enzyme expression (23.874 U mL^{-1})

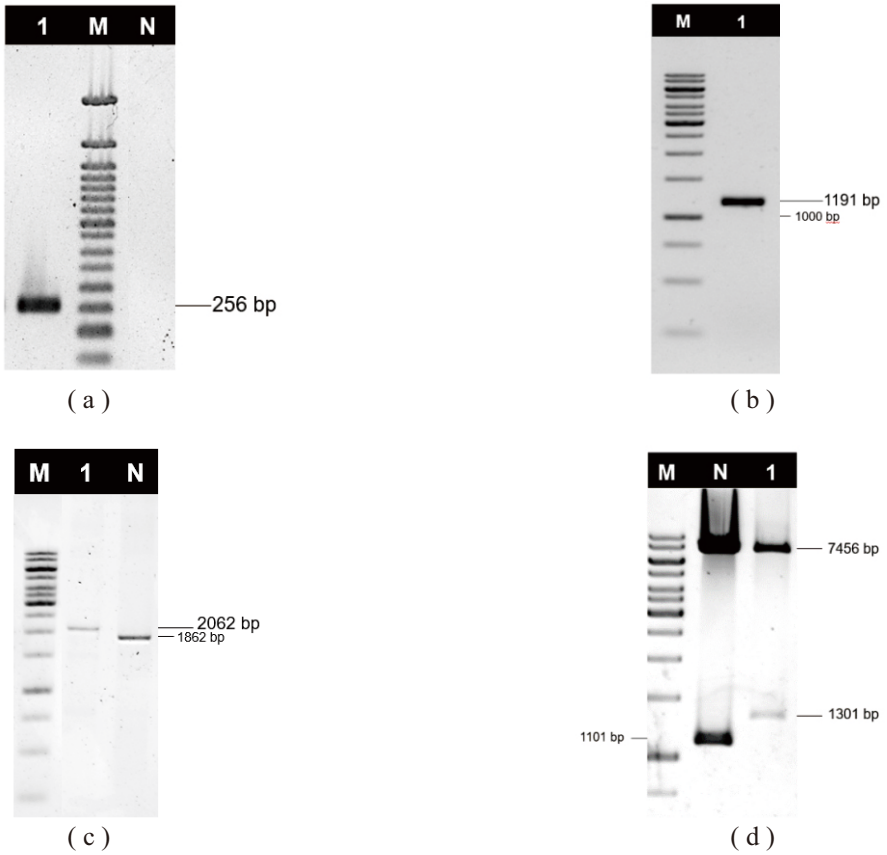

Fig 3 The insertion and verification of P*ylb* gene. (a) PCR amplification of 1) 256 bp P*ylb* gene and its N) negative control from *B. subtilis* DB104; (b) PCR amplification of 1) 1191 bp assisting-fragments *xynCM1* from gDNA of *B. halodurans* CM1; (c) Colony PCR verification of positive clones harboring pSKE194-*Plyb-alkxynCM1-inlip* with its N) negative control of pSKE194-*natprom-alkxynCM1-inlip* (no inserted P*ylb* gene); and (d) *Mfe*I digestion of 1) positive recombinant plasmid and its N) negative control after 1-hour incubation at 37 ˚C.

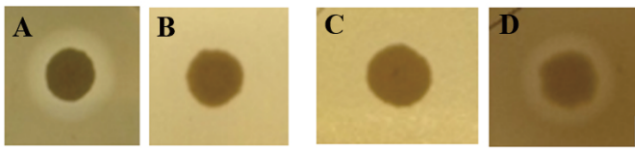

Fig 4 Qualitative assay of xylanase from *B. subtilis* DB104 wild-type grown on LB in the presence of 2% (w/v) of beechwood xylan pH 7 (A); LB-xylan pH 9 (B); recombinant *B. subtilis* harboring pSKE194-*Plyb-alkxynCM1-inlip* grown on LB-xylan pH 7 (C) and LB-xylan pH 9 (D).

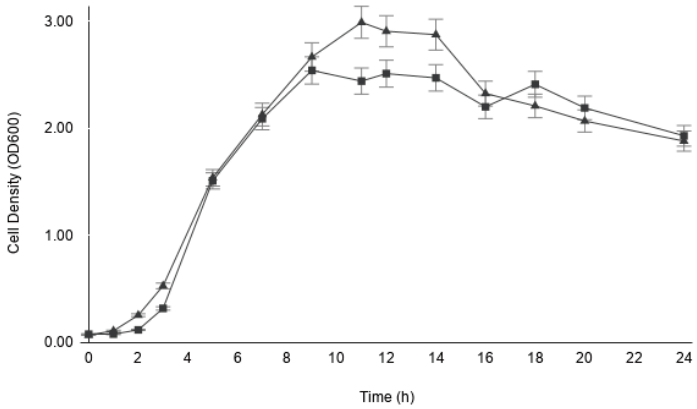

Fig 5 *B. subtilis* DB104 growth curve in LB media at 37 °C. Wild-type *B. subtilis* (▲) showed higher peak cell density than P*ylb* promoter-dependent recombinant xylanase (■).

Table 2 Xylanase activity measured by DNS method from supernatant culture of wild-type and recombinant *B. subtilis* DB104

Time (-hour)	Xylanase activity (U mL^{-1})	
	R3	wild-type
5	6.664	4.911
9	10.499	6.328
14	18.080	8.769
24	23.874	9.435

Table 3 Cell density (OD_{600}) and xylanase activity (U mL^{-1}) of recombinant *B. subtilis* from different media compared to controls

Media	wild-type		xynCM1 with native promoter		xynCM1 with P*ylb* promoter	
	OD_{600}	Activity (U mL^{-1})	OD_{600}	Activity (U mL^{-1})	OD_{600}	Activity (U mL^{-1})
LB	1.905	2.871	2.205	26.944	2.710	21.598
2X LB	5.050	20.600	5.680	73.192	5.025	81.461
Soybean flour media	-	3.978	-	13.995	-	31.299
SOC	7.040	29.436	6.250	22.504	5.720	60.893
SOC (with 1% glucose)	3.645	15.207	2.335	10.949	2.930	19.304
SOC (with 5% glucose)	3.995	61.551	3.980	28.937	4.365	47.937
SOB (with starter SOC)	5.965	3.294	4.525	24.508	2.200	24.766

than wild-type xylanase from *B. subtilis* (9.435 U mL^{-1}). It also reached almost 4 times higher than log-phase recombinant xylanase (6.664 U mL^{-1}).

To determine the effect of both carbon and nitrogen supplementation in media, yeast extract and glucose were used for *B. subtilis* growth with LB media and SOC media as the base media (Table 3). Not only xylanase activity, cell density was also measured by spectrophotometer at 600 nm (OD_{600}). The expression of recombinant xylanase produced by clone R3 harbouring P*ylb* promoter was compared with wild-type *B. subtilis* and recombinant *B. subtilis* carrying the same xylanase gene but with the native promoter as controls (Table 3).

LB media with a 2-fold recipe (2X LB) used as the first step to determine the effect of higher content of yeast extract addition on the *B. subtilis* growth. Cell density at stationary phase was increased 2-fold higher than other bacteria grown on LB media, with OD_{600} 5.025 for recombinant *B.subtilis* harbouring P*ylb* promoter. Recombinant xylanase under P*ylb* promoter control reached the highest result in enzyme activity (81.461 U mL^{-1}), almost 4-fold higher than grown on LB media. It is also higher than xylanase produced by wild-type *B. subtilis* xylanase (20.600 U mL^{-1}) and recombinant xynCM1 with native promoter (73.192 U mL^{-1}). Soybean flour media as an alternative source to supply nitrogen requirement in *B. subtilis* growth gave no better result in maintaining high activity of recombinant xylanase, even though recombinant xylanase under P*ylb* promoter control remains the highest activity among others.

SOC media were used with different concentrations of glucose (1% and 5%) and compared to original SOC media (20 mM of glucose). Biomass yield from high glucose content in SOC media measured by cell density (OD_{600}) revealed a decreasing cell density compared to original SOC media. Meanwhile, wild-type and recombinant *B. subtilis* grown in SOB media with SOC media as former media starter exhibited higher cell density compared to excess glucose media, even though it did not surpass the cell density from original SOC media. Recombinant xylanase under P*ylb* promoter control produced in SOC media with original concentration of glucose (20 mM) showed highest xylanase activity also among other variety of SOC media.

DISCUSSION

The P*ylb* gene (256 bp) from *B. subtilis* was successfully inserted into the plasmid by the assistance of megaprimer of the same 1191 bp xylanase gene that was already incorporated in it. The cloning method this study used was slightly different from the original RF cloning in which only one megaprimer (as targeted gene) needed (Mathieu *et al.* 2014). In our experiment we used two megaprimers, one was our P*ylb* gene which possessed flanking regions of homology with the assisting-gene so that in the first cycle of PCR both

could anneal as one joint-fragment (total size of 1447 bp). This leads to relatively easier insertion of joint-fragment into the plasmid even in low annealing temperature because the assisting-gene has extended homologous region with the vector at 5' end. No additional pair of primers was used in the reaction for cloning, resulting linear amplification not exponential (Ulrich *et al.* 2012; Mathieu *et al.* 2014).

P*ylb* promoter is an auto-inducible promoter which promotes gene expression from late log phase to stationary phase with no requirements of inducer (Yu *et al.* 2015). Insertion of this promoter is necessary to upgrade recombinant xylanase production from recombinant *B. subtilis* DB104. This bacteria itself (wild-type) performed xylanase activity from encoded gene in its chromosome (Helianti *et al.* 2016) and observed in pH 7, while recombinant xylanase from *B. subtilis* harbouring pSKE194-*Pylb-alkxynCM1-inlip* performed alkaline xylanase as the gene originated from alkalothermophilic *B. halodurans* CM1 (Wibowo *et al.* 2016). This was confirmed by qualitative assay of recombinant xylanase which shown xylanase activity from recombinant bacteria in xylan plate media pH 9. In addition, recombinant xylanase were produced in increasing activity corresponding to bacterial growth curve and highly produced in stationary phase (24 h after inoculation).

As stated above, gene expression under P*ylb* promoter highly expressed along with increasing cell density (Yu *et al.* 2015). In order to obtain a higher cell density, media composition needs to be optimized. *B. subtilis* biomass obtained at the end of the logarithmic phase increased following the increasing quantities of yeast extract added to the media. Specific growth rate of *B. subtilis* supplemented with 0.8% (w/v) of yeast extract increased up to 6-fold and no further significant change above that quantity (Romero-Garcia *et al.* 2009). In this research, 2X LB media contained 1% (w/v) of yeast extract and only produced a 2-fold increase in cell density. The presence of yeast extract was confirmed to affect *B. subtilis* growth and increase xylanase activity if a certain amount of yeast extract were added. Furthermore, recombinant xylanase under P*ylb* promoter control constantly expressed at a higher level compared to other xylanases even if the media were switched to an alternative source of nitrogen.

Glucose supplementation on batch fermentation of *B. subtilis* obtained higher cell dry weight, with optimum glucose concentration identified as 3.07% (w/v) (Zhong *et al.* 2014). According to the results in this experiment, excess carbon source in modified media did not enhance biomass yield and xylanase expressed from recombinant *B. subtilis* was not higher than in original SOC media with 20 mM glucose. It turned out that excess energy source in media (usually carbon source) exhibited a high rate of carbon consumption and low energetic growth efficiency. High carbon source in the growth of microorganisms generates uncoupling of anabolism and catabolism thus lead to variety of energy spilling reaction and metabolic shifting in electron pathway to less efficient called overflow metabolism (Dauner *et al.* 2001). In contrast, energy limitation generates catabolism coupling to anabolism so higher biomass is achieved (Russel and Cook. 1995). From this research, maintaining glucose concentration at certain amount while increasing nitrogen content might be able to express a higher level of recombinant xylanase with P*ylb* promoter control in *B. subtilis* DB104.

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