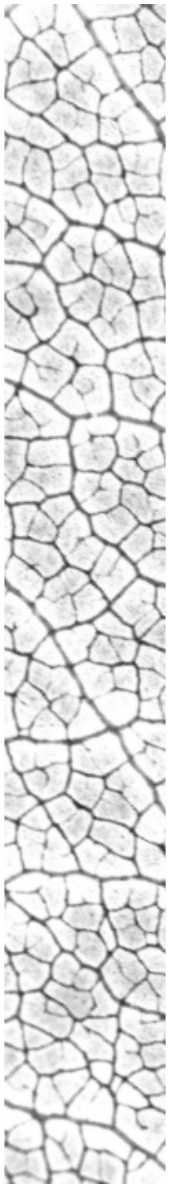

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Molecular cloning and expression of levansucrase gene from *Bacillus licheniformis* BK1 isolated from Bledug Kuwu Mud Crater

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Abstract
Halophilic bacteria are halotolerant microorganisms commonly found in natural environments containing high NaCl concentration. *Bacillus licheniformis* BK1 is a moderate halophilic bacterium isolated from Bledug Kuwu mud crater, Central Java, Indonesia. This bacterium optimally grows in LB medium containing 15% (w/v) NaCl, and known to produce levansucrase. The levansucrase gene from this bacterium has been successfully isolated and sequence (accession number MF774877.1). The obtained pET-*lsbl-bk1* recombinant clone was expressed in *E. coli* BL21 (DE3) and overexpressed by IPTG induction. The obtained Lsbl-bk1 levansucrase recombinant in the supernatant has a specific activity of 545.678 U/mg protein, in which the unit is defined as µmol of glucose released per minute in sucrose containing medium. This activity is 74% higher compared to those from *B. licheniformis* BK1 wild type. On the other hand, the cell lysate only showed an increase of 46%. The nucleotide sequence of *lsbl-bk1* gene indicated that the open reading frame consists of 1,452 bases encoding 483 amino acid residues of Lsbl-bk1 protein with a cleaved signal peptide between Ala^{29} and Lys^{30}. This protein is predicted as a member of glycoside hydrolase family, a typical for levansucrase. In addition, three conserved residues of the predicted catalytic triad were identified to be Asp^{93}, Asp^{256}, Glu^{352}.

Keywords: levansucrase, *Bacillus licheniformis* BK1, halophilic, glycoside hydrolase

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INTRODUCTION

Levan is a natural homopolysaccharide obtained in plants and microorganisms (Zhang *et al*. 2014). The structure of the polyfructan levan is predominantly composed of D-fructofuranosyl residues, which are joined together by β-(2,6)-glycosidic bonds in the main chain and by β-(2,1)-glycosidic linkage in the branch chains (Nakapong *et al.* 2013). There are various naturally occurring levans, with different molecular weights and numbers of residues in its side chains, depending upon the source and growth conditions of the producing organisms (Abdel-Fattah *et al*. 2012).

Levans have a wide industrial applications, such as in food, medicine, and cosmetics (Kim *et al*. 2005, Nakapong *et al*. 2013, Srikanth *et al*. 2015), since levan is biocompatible, biodegradable, biofloculatted, highly soluble in oil, and possessing low viscosity (Dahech *et al*. 2011, Li *et al*. 2013, Sarilmiser and Oner 2014). Low molecular weight levans, in the range 720–1296 D with 5–9 fructose molecules, commonly known as fructooligosaccharides (FOS) are a non-digestible food fibers commercially produced as food additives (Jalan *et al*. 2013). Currently, levan is also being developed as nanoparticle material for drug delivery, magnetic carriers, nanoreactors, and as a nucleating agent (Maciel *et al*. 2012, Nakapong *et al*. 2013, Pektaş et al. 2015, Sezer 2011, Srikanth *et al*. 2015).

Naturally occurring fructooligosaccharides were synthesized by the catalytic action of fructosyltransferases (FtFase), which catalyzed the hydrolysis of sucrose to produce glucose and fructose, and concurrently perform transfructosylation to attach fructose into an acceptor molecule and forming levan. (Antosova and Polakovic 2001, Belghith *et al*. 2012, Navid et al. 2014). Fructosyltransferases (FTFs) (E.C.2.4.1.9), are a levansucrase that belong to the glycoside hydrolase family (GH68). The active site of the GH68 family consists of a catalytic triad functioning as a catalytic nucleophile, general acid-base catalyst, and transition state stabilizer (Dahech *et al*. 2012, Naumoff

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2011, Ozimek *et al*. 2004). In microorganisms, levansucrase is accumulated in the periplasm which is then excreted into the medium for catalysis (Donot *et al*. 2012). A wide range of bacteria produce different types of levansucrase, catalyzing D-fructose binding formation to sucrose and producing poly- and oligomers of levan (Naumoff 1999). Several isolated levansucrase producing bacteria include *Bacillus subtilis (*Esawy *et al.* 2011), *Bacillus licheniformis* (Ghaly *et al*. 2007), *Zymomonas mobilis* (Silbir *et al*. 2014), H*alomonas* sp. (Poli *et al*. 2009), *Erwinia herbicola* (Cote 1998), and *Pseudomonas syringae* (El-Gilany et al. 2014, Srivastava *et al*. 2012).

Levan can be synthesized *in vitro*, in a medium containing sucrose and levansucrase (Esawy *et al*. 2013). Since 1902, levansucrase with optimal characteristics has been commercially produced from various microorganisms (Zhang *et al*. 2014). Lately, research on levansucrase production by recombinant DNA technology has been increasing, employing *E.coli* as host cell. In 1998, the recombinant clone and the nucleotide sequence of a levansucrase gene from *P. syringae* has been successfully expressed in *E. coli* using vector-based P_{lac} promoter (Hettwer *et al*. 1998). This paper reports cloning and expression of levansucrase gene from halophilic *B. licheniformis* BK1.

MATERIALS AND METHODS

Bacterial Strain, Vector, and Chemicals

B. licheniformis BK1 strain was isolated from the brine liquid of Bledug Kuwu mud crater, Central Java, Indonesia (Permatasari *et al*., 2016). The pGEM-T Easy and pET-30a(+) cloning vectors were obtained from Promega. The *E. coli* TOP10 and *E. coli* BL21 (DE3)pLysS were available in Biochemistry Laboratory of ITB.

KAPA *Taq* PCR kit, oligonucleotide primers, and Geneaid mini plasmid kit were ordered from Genetika Science. Restriction endonucleases *Eco*RI and *Bam*HI, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), isopropyl-β-D-thiogalactopyranoside (IPTG), and Bradford reagent were purchased from Thermo Fisher Scientific. *T4 DNA ligase* was purchased from Promega. Ampicillin, tetracycline, and kanamycin were purchased from Biobasic. Other chemicals were purchased from Sigma, Merck, Biorad, or Amresco.

Primer Design and PCR

Specific primers for amplification of *lsbl-bk1* gene were designed based on levansucrase gene sequences from ten strain of *B. licheniformis* available in GenBank. Within the primer, restriction sites of *Bam*HI and *Eco*RI were added in forward and reverse primers respectively for correct cloning orientation into pET-30a(+) expression vector.

The amplification was performed by PCR using 15 µL solution of Kapa Taq PCR kit in which template and primer composition was according to company protocol. Predenaturation was carried out at 95^0C for 3 min, followed by 34 cycles of denaturation at 95^0C for 30 s, annealing at 58^0C for 30 s, extension at 72^0C for 1.5 min, and final extension at 72^0C for 5 min. The PCR product was confirmed by electrophoresis on 1% (w/v) agarose gel.

Cloning of Levansucrase Gene from *B. licheniformis* BK1

The PCR product was ligated into pGEM-T linear using *T4 DNA ligase*. The ligation product was transformed into competent *E.coli* TOP10 cell using the heat shock method (Şahpaz *et al*., 2016). The transformants were plated onto LB agar medium containing 100 µg Ampicilin, 0.1 mM IPTG, 20 µg/mL X-Gal for blue-white screening. The recombinant pGEM-*lsbl-bk1* plasmid in white colonies was isolated using a modified alkaline lysis method (Geneaid) and then confirmed by size screening, re-PCR, and sequencing analysis. The recombinant pGEM-*lsbl-bk1* was also confirmed by restriction analysis using *Bam*HI and *Eco*RI.

The putative *lsbl-bk1* gene was subcloned into linearized pET-30a by ligating the digested pGEM-*lsbl-bk1*. The ligation product was transformed into competent *E. coli* BL21 (DE3) by the heat shock method and plated out on LB agar medium containing 50 µg kanamycin. The recombinant pET-*lsbl-bk1* plasmid was isolated and confirmed by size screening, re-PCR, restriction analysis, and sequencing.

Sequence Analysis and Protein Structure Prediction

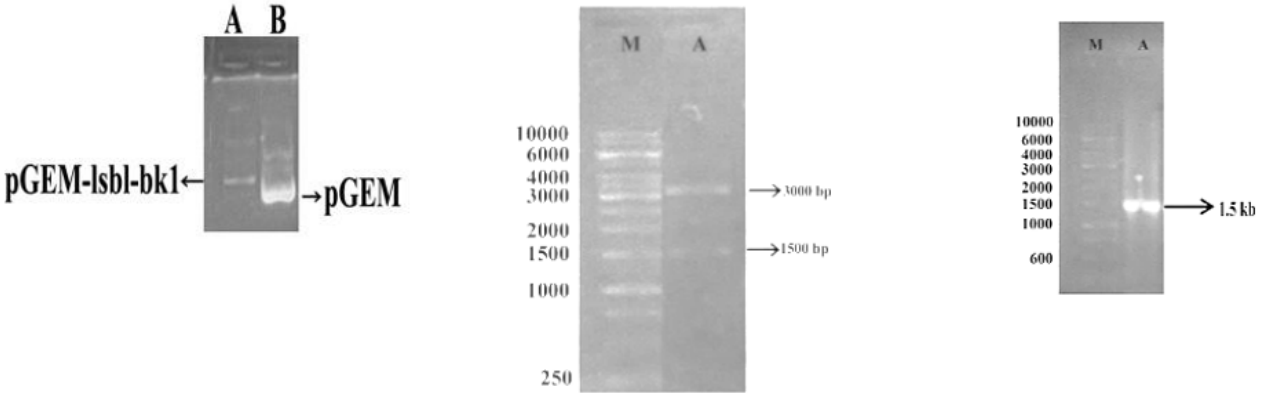
BLAST analysis was performed to the obtained recombinant levansucrase using the public NCBI online database. SignalP 4.1 online software was used to predict the signal peptide in Lsbl-bk1. Multiple sequence alignments were done using ClustalX. The molecular mass and isoelectric point (pI) was predicted with ExPASy ProtParam tool (http://web.expasy.org/cgibin/protparam/protparam). Conserved domains were analyzed with the conserved domain database (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). SAS is used to analyze the catalytic site in the protein sequence (https://www.ebi.ac.uk/thornton-srv/databases/sas/). SOPMA is a server tool to predict the secondary structure with a number of conformational states (https://npsa-prabi.ibcp.fr/cgi-n/npsa_automat.pl?page=/NPSA/npsa_sopma.html). Structural modeling for Lsbl-bk1 levansucrase were obtained using the Swiss model web tool (https://swissmodel.expasy.org).

Expression of pET-*lsbl-bk1* in *E. coli* BL21 (DE3)

The correct transformant *E. coli* BL21 (DE3)pLysS carrying pET-*lsbl-bk1* was inoculated into 100 mL LB medium containing 50 µg kanamycin and incubated at 37°C. Enzyme expression was induced by adding 1 mM

Table 1. Primers for amplification of *lsbl-bk1* gene

Primer	Nucleotide sequences (5'-3')	Length	Tm (°C)	%GC
Forward	5'-GGATCCATGAACATCAAAAACAT(C/T)GCT-3'	27	65.2	40.7
Reverse	5'-GAATTCTTATTT(A/G)TTTACCGTTA(G/A)TTG-3'	27	60.7	29.6



(a) (b) (c)

Fig. 1. Electrophoregram of size screening (a), *Bam*HI-*Eco*RI double digest of pGEM-*lsbl-bk1* (b), and re-PCR of pGEM-*lsbl-bk1* (c). (M= Benchtop 1 kb DNA ladder, A=pGEM-*lsbl-bk1*)

IPTG when the optical cell density (OD_{600}) in the culture reached 0.6-1.0. A total of 10 mL of the culture was then drawn in every hour for four consecutive hours to analyze the presence of the enzyme by SDS-PAGE, both in the supernatant and the cell lysate. The molecular mass of levansucrase separated by SDS PAGE was identified by comparing the band with a protein marker standard (Promega). The presence of levansucrase from the SDS-PAGE results is further confirmed by zymogram analysis. After SDS-PAGE, the gel was rinsed with deionized water briefly and removed by soaking in 1% (v/v) Triton X-100 in 50 mM phosphate buffer (pH 6.0) for 2h at 4°C. The excess Triton X of the gel was washed with deionized water and then soaked overnight in 20% (w/v) sucrose in 50 mM buffer (pH 6.0).

Levansucrase Assays

Levansucrase activity in the crude extract was measured by the dinitrosalicylic acid (DNS) method. The reaction was performed in 50 mM phosphate buffer (pH 6.0) containing 20% (w/v) sucrose at 50°C for 10 min. The reaction was stopped by adding 600 µL DNS and heated for 10 min in boiling water. The glucose formed was measured by spectrophotometry at 510 nm. One unit of levansucrase spesific activity is defined as the quantity of reducing sugar liberated by 1 µg of levansucrase (as protein equivalence) in 1 min. The concentrations of protein were measured according to the Bradford method using BSA as the standard (Bradford 1976, Davooabadi and Shahsavari 2014).

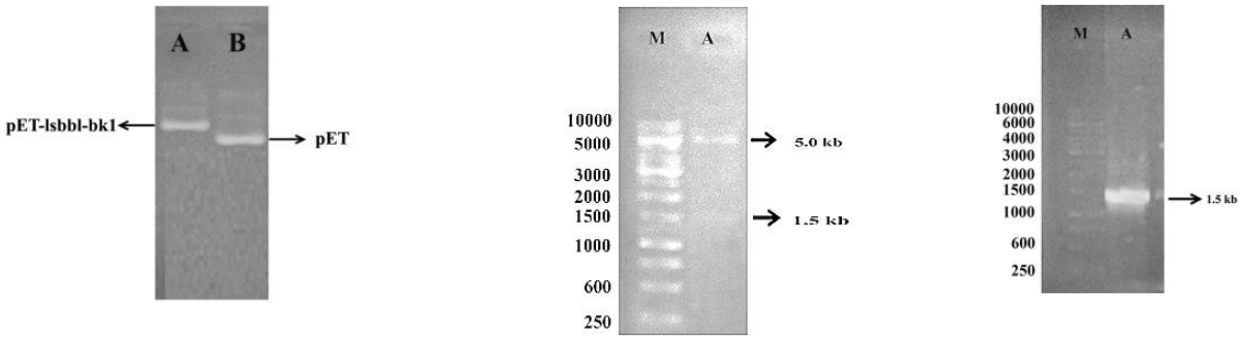
RESULTS AND DISCUSSION

Primer Design

Primer design was started by comparing levansucrase gene sequences from ten *B. licheniformis* strains available in the GenBank (https://www.ncbi.nlm.nih.gov/ genbank/). The average length of levansucrase gene fragments were about 1450 kb, from the ATG start codon to the TTA stop codon. It was observed that a quite large region at the beginning and at the end of the gene was conserved. Therefore, 21 nucleotides from the start codon and another twenty-one nucleotides at the end of the gene were chosen as primers. Several different bases were designed as degenerates. Degenerates primer position at the 13th and 24th of reverse primers, furthermore presence of a degenerated position at the 3' end of forward primers. Additional *Bam*HI and *Eco*RI restriction sites were respectively added to the forward and reverse primer for correct cloning orientation. These restriction sites are neither present in a levansucrase gene nor in pET-30a(+). The designed primers are presented in **Table 1**. *Bam*HI and *Eco*RI sites are underlined.

Amplification and Cloning of the *lsbl-bk1* Gene

The designed primers were used in PCR experiment using *B.licheniformis* BK1 genomic DNA as a template. The obtained amplicon, as a putative *lsbl-bk1* gene, were cloned into pGEM-T Easy vectors and transformed into *E. coli* TOP10. The supposed correct clone was identified through blue-white and size screening, restriction analysis and confirmed by re-PCR. The pGEM-T Easy vector was used in the first step of cloning because it is convenient to clone the PCR product. The result is shown in **Fig. 1**. It could be seen that the size of recombinant plasmid (isolated from white transformant) was larger compared to pGEM-T (isolated from blue transformant). Restriction analysis using *Bam*HI and *Eco*RI confirmed the presence of a 1500 bp insert



(a) (b) (c)

Fig. 2. Confirmation of recombinant pET-*lsbl-bk1* in *E.coli* BL21 (DE3). (a) size screening of pET-*lsbl-bk1*, (b) double digest on pET-*lsbl-bk1*with *Bam*HI and *Eco*RI, and (c) re-PCR of pET-*lsbl-bk1*. (M= Benchtop 1 kb DNA ladder, A=pET-*lsbl-bk1*, B=pET-30a(+))

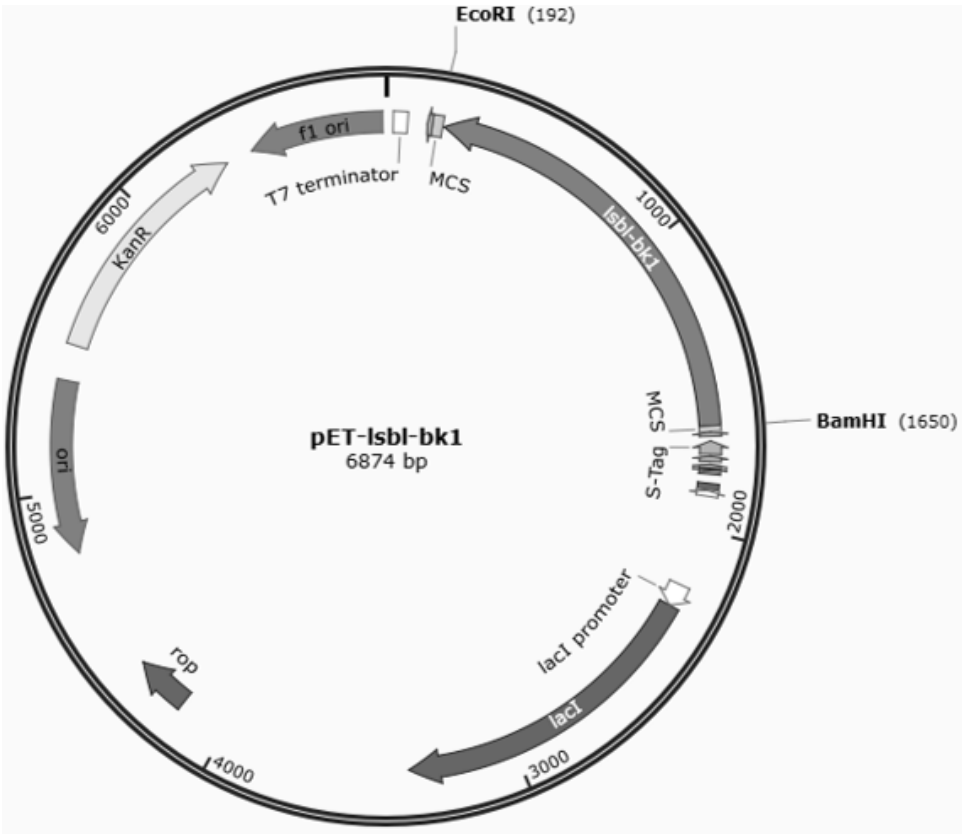


Fig. 3. The constructed map of recombinant pET-*lsbl-bk1*

(putative *lsbl-bk1* gene) in pGEM-T. The re-PCR confirmed this result.

Subcloning *lsbl-bk1* Fragments into pET-30a(+) Expression Vector

The pET-30a(+) vector and recombinant pGEM-*lsbl-bk1* were both double digested by *Bam*HI and *Eco*RI in two separate tubes, heat inactivated, ligated by *T4 DNA ligase*, and then transformed into competent *E. coli* BL21 (DE3). The obtained transformants on kanamycin medium would harbor either pET-30a(+) or pET-*lsbl-bk1* recombinant clone. Therefore, size screening was performed, which was further confirmed by restriction analysis and re-PCR (**Fig. 2**). It could be seen that the 1.5 kb fragment (putative levansucrase gene) was visible in restriction analysis and re-PCR. The whole sequence has been deposited to the NCBI with the accession number of MF774877.1. The *lsbl-bk1* correct orientation in the recombinant pET-*lsbl-bk1* was confirmed by its nucleotide sequence, in which the putative levansucrase gene was inserted in correct orientation with ATG start codon downstream to T7 promoter. The constructed map of the recombinant pET-*lsbl-bk1* is presented in **Fig. 3**.

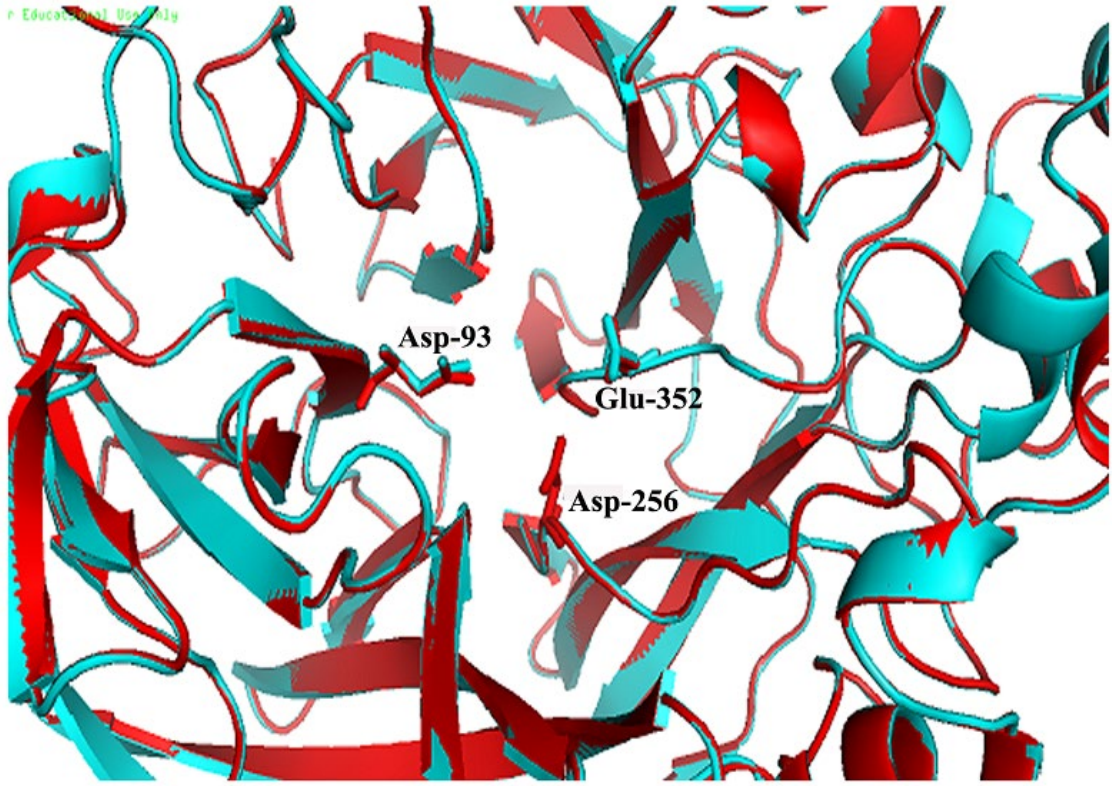


Fig. 4. Three dimension structural alignment comparing levansucrase from *B. licheniformis* Lsbl-bk1 (cyan) and *B. megaterium* 3OM7 (red)

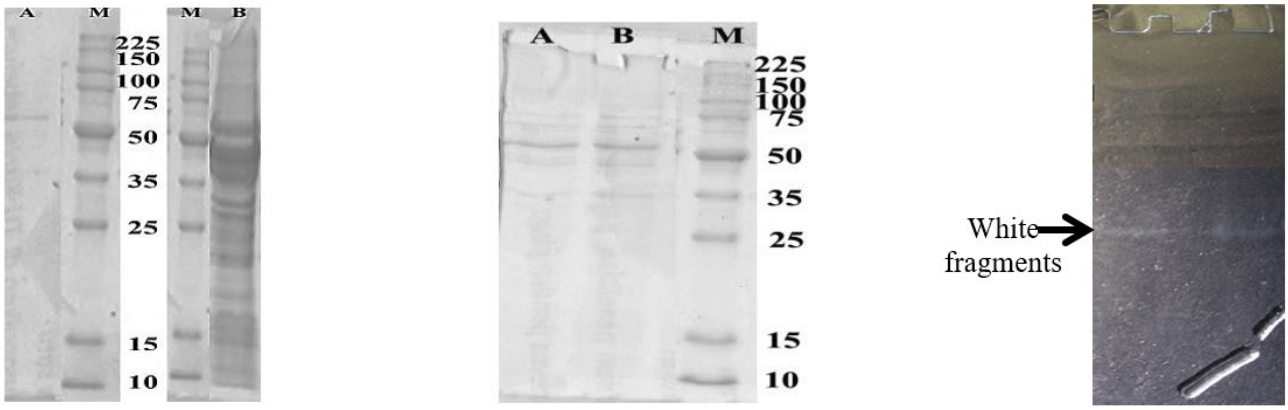
Sequence Analysis and Protein Structure Prediction

The determined nucleotide sequence of *lsbl-bk1* showed an open reading frame (ORF) of 1452 nucleotides with 99% similarity to levansucrase gene from ten *B. licheniformis* strains available in NCBI. The protein translated from this sequence is predicted to have a molecular weight of 53.7 kDa with an isoelectric point of 8.95. BLASTp to NCBI indicated that this protein has 96% similarity to other levansucrase of the mention ten *B.licheniformis* strains. These results confirmed that the isolated *lsbl-bk1* DNA fragment is coding for levansucrase.

Further analysis revealed the presence of a putative signal peptide containing 483 residues which cleaved between Ala^{29} and Lys^{30}. Based on conserved domain searches using NCBI's Conserved Domain Database (CDD), Lsbl-bk1 protein domain are a member of the glycosyl hydrolase, which is typical for levansucrase. Three conserved residues of the predicted catalytic triad were identified to be Asp^{93}, Asp^{256}, Glu^{352}. Members of this clan catalyzes the hydrolysis in two steps in which aspartate is taking place close to N-terminus and acts as the catalytic nucleophile. On the other hand, glutamate acts as the general acid or base.[5] Swiss-model analysis showed that the Lsbl-bk1 had an identity of 74.06% to the similar crystalized protein-levansucrase from *B. megaterium* (3OM7) as shown in **Fig. 4**. SOPMA analysis suggested that the Lsbl-bk1 protein secondary structure was mainly composed of random coils, beta turn, helices, and extended strands (folds) accounted for 43.06%, 11.80%, 16.56%, and 28.57%, respectively.

Expression of Lsbl-bk1

The Lsbl-bk1 was expressed under the control of T7 promoter in pET-*lsbl-bk1*, hence could be induced by isopropyl-beta-D-thiogalactopyranoside (IPTG) for overexpression in the *E.coli* BL21 (DE3) host. The results of expression experiments with our pET-*lsbl-bk1* are presented in **Fig. 5**. It could be seen that the supernatant and the lysate did contain Lsbl-bk1 around 50 kDa, consistent with the molecular weight predicted by the Protparam tool. The present of levansucrase in the supernatant suggests that the Lsbl-bk1 from *B. licheniformis* contained a signal peptide. The obtained data is also consistent with in silico avalysis of the deduced amino acid sequence using SignalIP 4.1 software, in which Lsbl-bk1 has a signal peptide in N-terminal region. This is identical to a recombinant levansucrase from *B. licheniformis* 8-37-0-1 (Lu *et al*. 2014). This result is also in line with the result of Henzel (2004), which showed that the secreted proteins and the majority of cell-surface protein in *E. coli* possessed an N-terminal signal peptide, which is typically between 15 and 40 amino acids, and subsequently cleaved from the mature protein. Yamabhai (2007) also reported that various signal peptides of *Bacillus* can be recognized by



(a) (b) (c)

Fig. 5. (a) SDS-PAGE analysis of Lsbl-bk1 crude extract in supernatant (lane A) and lysate (lane B). (b) SDS PAGE analysis of solubilized Lsbl-bk1 crude extract on cell pellet (lane A) and cell lysate (lane B). (c) Zymogram analysis of Lsbl-bk1 in supernatant. Lane M=protein molecular weight markers

Table 2. Levansucrase activity from the recombinant clone compare to its wild type

Crude enzyme	Total volume (mL)	Activity of levansucrase (U/mL)	Total activity (Units)	Protein (mg)	Spesicific activity (U/mg)
B. licheniformis BK1 (supernatant)	45	2.465	110.917	0.478	232.170
B. licheniformis BK1 (lysate)	10	22.571	225.710	2.192	102.960
Recombinant Lsbl-bk1 (supernatant)	45	4.298	193.417	0.354	545.678
Recombinant Lsbl-bk1 (lysate)	10	22.664	226.626	1.710	132.561

E.coli secretion machinery. This explained the presence of Lsbl-bk1 in the medium.

SDS-PAGE analysis as shown in **Fig. 5a** showed that the content of Lsbl-bk1 in the cell lysate (lane B) is higher compared to that in the supernatant (lane A). This was further confirmed by zymogram analysis (**Fig. 4c**). The levansucrase in the cell pellet and in the lysate appeared to have the same concentration (**Fig. 4b**) indicated that this Lsbl-bk1 is a soluble protein.

Further evaluation of signal peptide on the expression and secretion of levansucrase Lsbl-bk1 in *E.coli* was performed by DNS method to compare enzyme activities with wild type enzyme. DNS quantification after IPTG induction to recombinant clone were shown in **Table 2.** It could be seen that levansucrase produced by the recombinant clone possessed higher activity compared to its wild type. Levansucrase Lsbl-bk1 in the medium and in cell lysate increase by 74% and 0.41% respectively, indicating that the *E. coli* host was able to properly secrete functional levansucrase.

CONCLUSION

The recombinant levansucrase from *B. licheniformis* BK1 has been successfully isolated by PCR approach. The whole sequence has been deposited to the NCBI with the accession number of MF774877.1. A high level expression of the Lsbl-bk1 levansucrase was detected in *E. coli* BL21 (DE) host under the control of T7 promoter inducible by IPTG. The specific activity of levansucrase in Lsbl-bk1 recombinant was 545.678 U/mg protein, which is 74% higher compared to its wild type produce by *B. licheniformis* BK1. Sequence analysis of *lsbl-bk1* showed the open reading frame of 1,452 bases encoding for 483 amino acid residues with a signal peptide cleaved between Ala^{29} and Lys^{30}. Three conserved residues of the predicted catalytic triad were identified to be Asp^{93}, Asp^{256}, Glu^{352}.

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