Cloning and expression of dextransucrase from *Leuconostoc citreum* ABK-1

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Abstract: Dextransucrase is an enzyme in the glycoside hydrolase 70 family that can catalyze sucrose to fructose and glucose. The glucose can be transferred into acceptor saccharides to synthesis dextran in a transglycosylation reaction. Dextransucrase gene was newly discovered in *Leuconostoc citreum* ABK-1. We preliminarily annotated the gene and found that *Lcdexm* was 99.50% identical to the dextransucrase gene in *Leuconostoc citreum* DS. This work aimed to clone and express the dextransucrase from *Leuconostoc citreum* ABK-1. In this report, we successfully cloned the dextransucrase gene of *Leuconostoc citreum* ABK-1, *Lcdexm*. The *Lcdexm*, 4,398 base pairs, was subcloned into pET21b. The recombinant plasmid was then transformed into *Escherichia coli* BL* for protein expression. The computed size of the *Lc*DEXM enzyme was performed on ExPASy and the result showed that its size was 168 kDa with a pI of 5.15. The optimization of protein expression was observed at 20°C for 6 hours after induced with 0.5 mM IPTG. SDS PAGE analysis was used to investigate the expression of the *Lc*DEXM enzyme. We found that the enzyme was an intracellular expression. To additional confirm the protein expression, western blot analysis was performed. The signal of the anti-6×histidine antibody against 6×histidine tagged *Lc*DEXM enzyme was detected.

Keywords: *Leuconostoc citreum* ABK-1; detransucrase; molecular cloning; protein expression

1. Introduction

Exo-polysaccharides (EPS) are essential carbohydrates which widely use in both food and non-food industries [11]. Many reports have shown that EPS was produced by lactic acid bacteria (LAB). EPS are characterized into two groups: homopolysaccharide and heteropolysaccharide. Homosaccharides can be derived into 4 major groups: α-D-glucan, β-D-glucan, fructan, and polygalactans. The features of homosaccharides are differed by the main chain glycosidic bonds. For examples, the α-D-glucan can be clustered as dextran (α-1,6 glycosidic linkage), mutans (α-1,3 linkage), alternan (α-1,6 and α-1,3), and reuteran (α-1,6 and α-1,4) [2]. Interestingly, the dextran products from different LAB's detransucrase are different among species. There are several studies reported the differences of dextrans in LAB species. The *Leuconostoc citreum* NM105's, from Manchurian sauerkraut, produced dextran composed of α-1,2 branch with high water solubility [13]. *Weissella confusa* Cab3's dextran contained linear α-1,6 chain with α-1,3 branching [10]. *Leuconostoc pseudomesenteroides* YF32 and *Leuconostoc pseudomesenteroides* DRP-5's dextran from soybean paste and homemade wine comprised only a linear chain with α-1,6 [4,13]. *Leuconostoc citreum* B-2's dextran from fermented pineapple composed for α-1,6 main chain with α-1,2 and α-1,3 branching [5]. Previous studies found 4 glucansucrase genes in *Leuconostoc citreum* ABK-1 [12]. We preliminarily analyzed dextransucrase gene in glucansucrase gene. Dextran can be synthesized by dextransucrase enzyme which can

catalyze the transfer of D-glucopyranosyl residues from sucrose to form polymers. Hence, this study aims to express the novel dextransucrase from *Leuconoctoc citreum* ABK-1.

2. Materials and Methods

2.1 Cloning of LcDEXM gene.

Dextransucrase gene of *Leuconostoc citreum* ABK-1 gene (*Lcdexm*) was amplified using PrimeStar® DNA polymerase. The PCR conditions using in this experiment were pre-denaturation at 98°C for 30 seconds, denature at 98°C for 10 seconds, annealing at 55°C for 20 seconds, and elongation at 72°C for 4.3 minutes. The stages from denaturation to elongation were set for 25 cycles. The final extension was performed at 72°C for 5 minutes. Two sets of primers that we used in this experiment were NdeI-5′CCAACATATGCAGTTACTGTCTCCAATAATTCGAATAC3′ and XhoI-5′CCTTCTCGAGAGCGACTGAGACAAAGTAACCTTGGTCATCG3′. The PCR product was cloned into the pET21b vector. *Escherichia coli* strain TOP10 was transformed with the recombinant plasmid. The transformants were selected and analyzed by restriction enzyme digestion. Further analysis with 0.7% agarose gel electrophoresis, the positive clones were sent to sequencing (1st BASE) for confirmation [9, 12].

2.2 Overexpression of Lcdextransucrase protein (LcDEXM) in E. coli system.

The recombinant plasmid was cloned into One Shot® BL21 Star™ (DE3) pLysS *E. coli* as an expression host. The transformants were grown in LB broth at 37°C, 250 rpm until OD_{600} reached 0.5. Then adding 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and culture was grown at 20°C, 250 rpm for 6 hours. The culture was harvested by centrifugation at 8,000×g for 10 minutes. The supernatant was discarded, and the pellet was stored at -20°C for further studies. The analyses of the protein expression were confirmed with 8% SDS-PAGE gel and western blot analysis.

2.3 LcDexM enzymatic assay.

The *Lc*DEXM enzyme was tested for an activity with 3,5-dinitrosalicylic acid (DNS assay). The DNS was turned into 3-amino-5-nitrosalicylic acid when it was incubated with reducing sugar, fructose. The enzymatic reaction contained 250 mM sucrose, 1 mM $CaCl_2$, and 50 mM acetate buffer pH 5.5. 50 µl of the enzyme was added to the reaction then incubated at 30°C for 15 minutes. The reaction was terminated by adding 500 µl of DNS and boiled for 10 minutes. The reaction measured the absorption at OD 540 nm. One unit of *Lc*DexM activity meant releasing 1 µM of fructose per minute [3, 6, 12].

3. Results

3.1. Construction of Lcdexm gene

3.1.1. Gene annotation of Lcdexm gene

The blastn results showed the top 20 ranks of sequence identity. These alignments indicated that the *Lcdexm* gene was annotated as in *Leuconostoc* sp. Additionally, the sequence was identical to dextransucrase gene in *Leuconostoc citreum* DS (accession number AB362781.1), *Leuconostoc mesenteroides* B-1299CB4 (accession number DQ497800.1), *Leuconostoc mesenteroides* NRRL B-1355 (accession number AJ250172.1), *and Leuconostoc mesenteroides* B-742CB (accession number AY280636.1), which were 99.50%, 99.22%, 99.22%, and 99.20%, respectively (Table 1). This result could imply that the *Lcdexm* gene could be a dextransucrase gene. Moreover, the blastp results revealed that *Lc*DEXM protein was identical to *Leuconostoc citreum* hydrolase enzyme (accession number HCN55689.1) and dextransucrase enzyme (accession number BAF96719.1) as well as the dextransucrase (accession number AAG38021.1) and glucosyltransferase enzyme (accession number AAB95453.1) of *Leuconostoc mesenteroides*. The identities of those enzymes were 99.20%, 99.59%, 99.14%, and 99.27%, respectively (Table 2).

Table 1. DNA sequence alignment of *Lcdexm* gene.

Bacterial strain	Sequence	Accession number
Leuconostoc citreum ABK-1	*GTAAATGGTACTATGTAACCAGT GATAACACACTTGCTAAGGGGTT GACTACTGTTGACA*	This work
Leuconostoc mesenteroides B-1299CB4	*GTAAATGGTACTATGTAACCAGT GATAACACACTTGCTAAGGGGTT GACTACTGTTGACA*	DQ497800.1
Leuconostoc mesenteroides NRRL B-1355	*GTAAATGGTACTATGTAACCAGT GATAACACACTTGCTAAGGGGTT GACTACTGTTGACA*	AJ250172.1
Leuconostoc mesenteroides B-742CB	*GTAAATGGTACTATGTAACCAGT GATAACACACTTGCTAAGGGGTT GACTACTGTTGACA*	AY280636.1

Table 2. Protein sequence alignment of *Lc*DEXM enzyme.

Bacterial species	Sequence	Accession number
Leuconostoc citreum ABK-1	*IGLKAINGHNYYFDSLGQLKKGFTG VIDGQVRYFDQESGQEVSTTDSQIK EGLTSQTTDY*	This work
Leuconostoc citreum	*IGLKAINGHNYYFDSLGQLKKGFTG VIDGQVRYFDQESGQEVSTTDSQIK EGLTSQTADY*	HCN55689.1
Leuconostoc citreum	*IGLKAINGHNYYFDSLGQLKKGFTG VIDGQVRYFDQESGQEVSTTDSQIK EGLTSQTTDY*	BAF96719.1
Leuconostoc mesenteroides	*IGLKAINGHNYYFDSLGQLKKGFTG VIDGQVRYFDQESGQEVSTTDSQIK EGLTSQTTDY*	AAG38021.1
Leuconostoc mesenteroides	*IGLKAINGHNYYFDSLGQLKKGFTG VIDGQVRYFDQESGQEVSTTDSQIK EGLTSQTTDY*	AAB95453.1

3.1.2 Restriction enzyme analysis of construction of Lcdexm gene in pET21b vector

We constructed the *Lcdexm* gene in the pET21b vector with NdeI and XhoI restriction sites. The size of the *Lcdexm* gene was 4,398 base pairs. The results on 0.7% agarose gel exhibited the construction after we had analyzed by double restriction enzymes (figure 1, A). The gel showed that the insert fragment's size approximated 4 kb comparing to 1kb DNA ladder (New England Biolabs®) which matched to the real size of the gene. We further confirmed the result by DNA sequencing (1St Base).

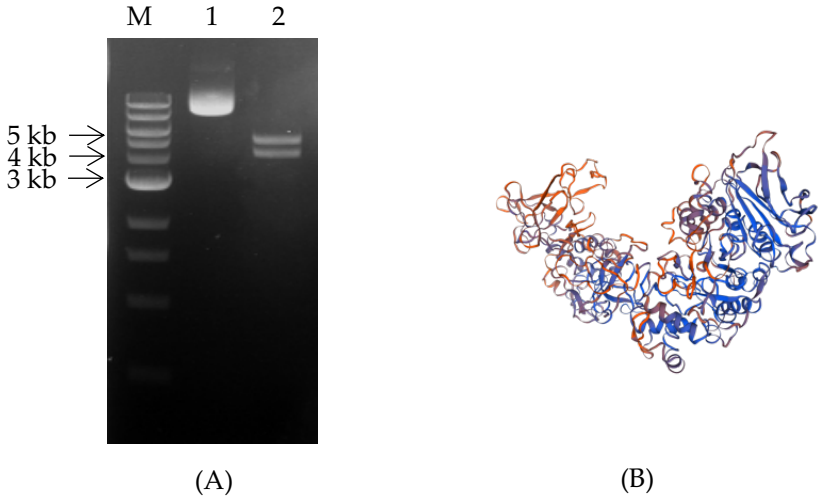

Figure 1. 0.7% of agarose gel electrophoresis comparing between uncut recombinant plasmid (lane 1) and NdeI+XhoI cut recombinant plasmid (lane 2). M was 1kb DNA ladder (New England Biolabs®). Lane 1 was an uncut plasmid, lane 2 was the NdeI+XhoI cut plasmid displayed 2 bands, the pET21b vector with a size of 5.4 kb (upper), and *t*he *Lcdexm* gene with a size of 4,398 base pairs (lower). (B) homology modeling of *Lc*DEXM protein using Swiss-model prediction. The template used to predict the structure was dextransucrase from *Leuconostoc mesenteroides* (PDB:3TTQ).

*3.2 Overexpression of Lc*DEXM *enzyme in E. coli system.*

*3.2.1 Optimization of Lc*DEXM *enzyme expression in E. coli system*

The expression condition was conducted under 3 selected temperatures which were 20°C, 30°C, and 37°C coupled with the induction of 0.5 mM IPTG. The result showed that the optimal temperature of this enzyme was at 20°C which was detected by enzyme activity. At 20°C, the enzyme activity was 15.1 unit/ml enzyme while 30°C and 37°C were 6.52 and 0.768 unit/ml enzyme (figure 2, A), respectively. In addition, we also clarified the effect of IPTG concentrations and the expression times. We investigated the enzyme expression after induction with 0, 0.1, 0.2, 0.4, 0.5, and 0.8 mM IPTG at 3, 6, 9, and 18 hours postinduction. The experiments revealed that the optimal IPTG concentration was at 0.5 mM for 6 hours (figure 2, B). The trendline of IPTG induction at each time point showed the matching that the peaks reached at 0.5 mM IPTG at every time point.

*3.2.2 Western blot analysis of Lc*DEXM *enzyme expression*

The expression of the recombinant *Lc*DEXM enzyme was further analyzed by western blot analysis. The recombinant enzyme contained 6×His tagged resulting from the pET21b vector. The western blots against the anti-histidine antibody revealed the signals of the *Lc*DEXM enzyme to confirm the protein expression. The *Lc*DEXM enzyme, computed the size using ExPASy server, was 168 kDa. The result showed that our protein of interest had a signal against an anti-histidine antibody in lanes 1 and 2 on the membrane (figure 3, A).

3.2.3 Analysis of protein expression with 8% SDS-PAGE

The protein expression was again confirmed with 8% SDS-PAGE gel with the aim of increasing of protein size around 168 kDa. The gel exhibited the increasing of protein expression after induction with 0.5 mM IPTG for 6 hours. This evidence made us clearly

detected the overexpression of *Lc*DEXM enzyme in *E. coli* system. When comparing the result on SDS gel (figure 3, B (lane 1 and 3)), the proteins had been increasing after induced with 0.5 mM IPTG.

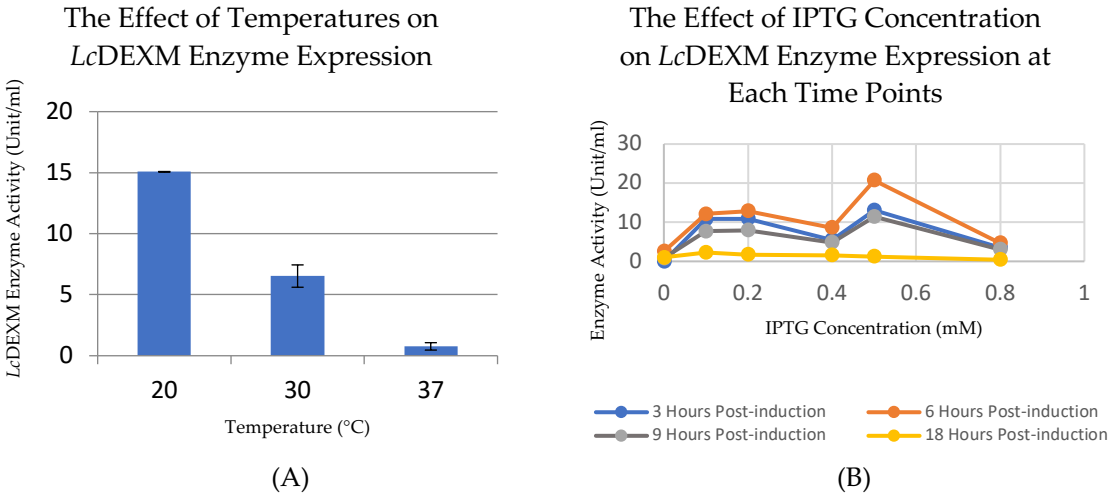

Figure 2. The relationship between temperature and the activity of an enzyme. This graph showed that the optimal temperature was at 20°C. In this experiment, 1 unit of an enzyme referred to the releasing of 1µM of fructose per minute. (B)The optimal IPTG concentration and time coarse analysis of *Lc*DEXM Enzyme expression. The result showed that the optimal condition was at 0.5 mM IPTG and induced for 6 hours.

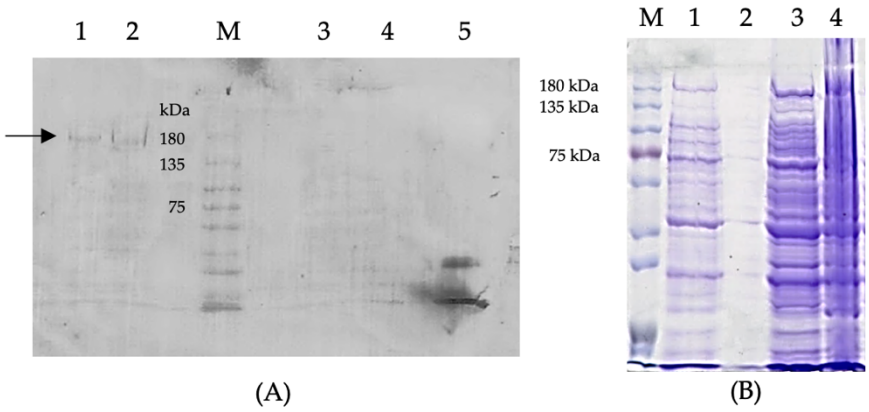

Figure 3. (A) Western blot analysis of *Lc*DEXM enzyme expression. M was BLUEstain 2 protein ladder, lanes 1 and 2 were crude *Lc*DEXM enzymes. Lanes 3 and 4 were negative control being *Lc*DEXM enzyme without 6×His tagged, and lane 5 was a protein with 6×His tagged as a positive control. (B) 8% of SDS-PAGE gel analysis of *Lc*DEXM expression. M was BLUEstain 2 protein ladder, lane 1 was uninduced enzyme collected before induction with 0.5 mM IPTG, lane 2 was extracellular enzyme, lane 3 was an intracellular crude *Lc*DEXM, and lane 4 was inclusion bodies.

4. Discussion

4.1 Construction of *Lcdexm* gene.

The construction of the *Lcdexm* gene was successfully cloned in pET21b vector. We decided to use this vector to facilitate the protein purification with Ni-NTA column [1]. This vector provided C-terminus 6×Histidine tagged. Furthermore, these 6×his tagged could confirm the protein expression through western blot analysis [9]. The gene annotation was analyzed that *Lcdexm* gene was identical to dextransucrase gene in *Leuconostoc* species.

4.2 *Overexpression of Lc*DEXM *enzyme in E. coli system.*

The trend of protein expression showed that the expression level raised from 0 to 0.2 mM IPTG and raised again in 0.5 mM IPTG then dropped in a higher concentration of IPTG. The expression reached the highest expression at 5 mM IPTG at each time point. This phenomenon related to the study of (Nguyen and Schumann 2014) also found that at 1 mM IPTG concentration exhibited the a low level of protein expression [7].

5. Conclusions

This experiment can successfully clone and express the full-length dextransucrase from *Leuconostoc citreum* ABK-1 in *E. coli* system. The expression optimization showed that this enzyme was the highest expression in 0.5 mM IPTG for 6 hours after induction. The further experiment we aim to finish is to characterize this enzyme and its products.

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