

Article

Enhanced Extracellular Production and Characterization of Sucrose Isomerase in *Bacillus subtilis* with Optimized Signal Peptides

Dan Guo [†], Mingyu Li [†], Mengtong Jiang, Guilong Cong, Yuxin Liu, Conggang Wang ^{*} and Xianzhen Li

School of Biological Engineering, Dalian Polytechnic University, Dalian 116034, China

^{*} Correspondence: wangcg@dlpu.edu.cn; Tel.: +86-411-86318692[†] These authors contributed equally to this work.

Abstract: Sucrose isomerase (SIase) catalyzes the hydrolysis and isomerization of sucrose into isomaltulose, which is an important functional sugar widely used in the food industry. However, the lack of safe and efficient expression systems for recombinant SIase has impeded its production and application. In this study, enhanced expression of a SIase from *Klebsiella* sp. LX3 (referred to as KsLX3-SIase) was achieved in *Bacillus subtilis* WB800N, by optimizing the signal peptides. First, 13 candidate signal peptides were selected using a semi-rational approach, and their effects on KsLX3-SIase secretion were compared. The signal peptide WapA was most efficient in directing the secretion of KsLX3-SIase into the culture medium, producing a specific activity of 23.0 U/mL, as demonstrated by shake flask culture. Using a fed-batch strategy, the activity of KsLX3-SIase in the culture medium was increased to 125.0 U/mL in a 5-L fermentor. Finally, the expressed KsLX3-SIase was purified and was found to have maximum activity at 45 °C and pH 5.5. Its K_m for sucrose was 267.6 ± 18.6 mmol/L, and its k_{cat}/K_m was 10.1 ± 0.2 s⁻¹mM⁻¹. These findings demonstrated an efficient expression of SIase in *B. subtilis*, and this is thought to be the highest level of SIase produced in a food-grade bacteria to date.

Keywords: isomaltulose; sucrose isomerase; cloning and expression; biochemical characterization; signal peptides



Citation: Guo, D.; Li, M.; Jiang, M.; Cong, G.; Liu, Y.; Wang, C.; Li, X. Enhanced Extracellular Production and Characterization of Sucrose Isomerase in *Bacillus subtilis* with Optimized Signal Peptides. *Foods* **2022**, *11*, 2468. <https://doi.org/10.3390/foods11162468>

Academic Editor: Xianghui Qi

Received: 21 July 2022

Accepted: 12 August 2022

Published: 16 August 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Isomaltulose is a sucrose isomer with similar physical properties and flavor to sucrose [1,2]. Isomaltulose has been recognized as a functional sugar with superior properties, including high acid stability, a low glycemic index, and non-cariogenicity [2]. It is also beneficial for the growth of bifidobacteria in the human intestine [3]. In addition, isomaltulose is non-toxic and non-mutagenic according to Ame's test [4]. Currently, isomaltulose is increasingly used as a substitute for sucrose in food processing and as a raw material for the production of surfactants [3,4]. Isomaltulose occurs naturally in trace quantities in honey and sugar cane. Its chemical synthesis is complex. Currently, isomaltulose is produced commercially from sucrose using a bacterial strain that naturally expresses sucrose isomerase (SIase) [5]. SIase uses sucrose as a substrate and converts it mainly to isomaltulose, together with a small proportion of trehalulose, and trace byproducts, including glucose and fructose [5,6].

SIase has been discovered in several microbial strains, including *Erwinia rhapontici*, *Serratia plymuthica*, *Klebsiella* sp, *Protaminobacter rubrum*, and *Pantoea dispersa* [5]. The low yield of SIase and a lack of food-safe genetic background have impeded the industrial production of SIase using natural strains. Therefore, the industrial production of isomaltulose would benefit from a strain capable of expressing SIase efficiently using a food-grade heterologous expression system. *Escherichia coli* (*E.coli*) is the most commonly used host for heterologous

protein expression, because of its rapid growth rate, high-level of expression of the recombinant protein, and ease of genetic manipulation. SIases from various microbes (including *Protaminobacter rubrum* CBS 547.77, *Pantoea dispersa* UQ68J, and *Klebsiella* sp. LX3) have been expressed in *E.coli* as an intracellular enzyme and the expression of SIase in *E.coli* has been enhanced using response surface methodology [7–10]. However, the soluble form of SIase in *E.coli* is typically found in very low quantities, and numerous forms of misfolded enzyme have been detected in inclusion bodies [8,11]. In addition, the expressed SIase in *E.coli* is localized in the cytoplasm, necessitating a costly and time-consuming purification process [9,12]. Furthermore, *E.coli* is unsuitable for the production of SIase in the food industry because of safety concerns raised by the synthesis of endotoxins. Therefore, heterologous expression of SIase, achieved using food-grade strains, would be more desirable. SIase is currently expressed in food-grade strains such as *Lactococcus lactis*, *Yarrowia lipolytica*, and *Bacillus subtilis* [5]. SIase from *Enterobacter* sp. FMB-1 has been expressed and secreted in *Lactococcus lactis* MG1363, with an activity of less than 3.0 U/mL [13]. SIase from *Erwinia rhapontici* NX-5 has been expressed as an intracellular form in *Bacillus subtilis* WB800 using the plasmid pHA01, and a 5.2 U/mL whole-cell activity was achieved after 35 h of fermentation performed in a 7.5-L fermentor [14]. SIase from *Pantoea dispersa* UQ68J has been expressed in *Yarrowia lipolytica*, and an activity level of 6.2 U/mL was detected in the culture supernatant [15]. To date, the heterologous expression of SIase in food-grade strains has still yielded a low level of the enzyme [5].

Bacillus subtilis, a well-known generally recognized as safe (GRAS) bacterium, which is a designation by the United States Food and Drug Administration (US-FDA) that a substance added to food is considered safe by experts under the conditions of its intended use. *Bacillus subtilis* has been utilized extensively in commercial enzyme production. *Bacillus subtilis* possesses a number of advantages, including nonpathogenicity, suitability for large-scale fermentation, and a diverse toolkit for genetic manipulation [16–20]. In addition, various signal peptides and promoters can be used to achieve effective heterologous protein expression in *Bacillus subtilis* [16,21,22]. Signal peptides have been shown to have a great influence on the secretion of enzymes in *Bacillus subtilis*. The optimization of the signal peptide of a specific enzyme was generally carried out using high-throughput screening of the signal peptide library or semi-rational design based on the signal peptide composition [23,24]. Using an optimized CitH signal peptide identified in a signal peptide library, the activity of a thermostable glucosidase was increased from 1.00 to 5.20 U/mL in the culture supernatant, by expressing the enzyme as a secretory form in *B. subtilis* [25]. An increase in the titer of an alkaline polygalacturonate lyase produced in *B. subtilis* was achieved using a semi-rational strategy with six signal peptides [24]. However, a single signal peptide cannot be optimal for all heterologous proteins. Therefore, it is necessary to screen for a suitable signal peptide for a specific target protein.

To overcome the bottlenecks resulting from inadequate SIase expression and secretion in food-grade strains, we optimized the regulatory element of the signal peptide in the expression plasmid, to improve the expression of SIase from *Klebsiella* sp.LX3 (referred to as KsLX3-SIase) in *Bacillus subtilis*. Using a semi-rational strategy, the candidate signal peptides were selected and screened, yielding an optimized expression cassette. Using a fed-batch fermentation, the expression of KsLX3-SIase was further enhanced. The recombinant KsLX3-SIase was subsequently purified and characterized. The findings of this study could provide a highly efficient and safe system for the expression of SIase, facilitating large-scale production and utilization of SIase.

2. Materials and Methods

2.1. Strains, Vectors, and Reagents

The gene encoding SIase from *Klebsiella* sp.LX3 (GenBank accession number AY040843) was cloned and stored in our laboratory. The protease-defective derivative of strain 168, *Bacillus subtilis* WB800N, and the expression vector pHT254 were obtained from AtaGenix Laboratories Co., Ltd. (Wuhan, China). PrimeSTAR HS DNA polymerase, DNA, and protein molecular weight markers were obtained from Takara (Dalian, China). A Plas-

mid Extraction Kit and DNA Gel Purification Kit were purchased from Sangon Biotech (Shanghai, China). Ni-NTA affinity chromatography resin was purchased from Sangon Biotech (Shanghai, China). HPLC grade acetonitrile was purchased from Merck (Darmstadt, Germany). Hypersil APS-2 NH₂ column (4.6 × 250 mm, 5 μm) was purchased from ThermoFisher Scientific (Bellefonte, PA, USA). All other chemical reagents used in this study were of analytical grade, unless otherwise stated. MilliQ water was used to prepare buffers and all of the buffers were filtered through a 0.22-μm syringe filter, before use. Luria–Bertani (LB) medium (tryptone 10.0 g/L, yeast extract 5.0 g/L, and NaCl 10.0 g/L) were used to culture *E.coli* and *Bacillus subtilis* strains. For protein expression, *B. subtilis* was cultured in 2 × Yeast Extract and Tryptone (YT) medium (tryptone 16.0 g/L, yeast extract 10.0 g/L, and NaCl 5.0 g/L). The fermentation medium consisted of 8 g/L glycerol, 8.5 g/L soybean peptone, 8.5 g/L beef paste, and 5 μg/mL chloramphenicol. The feeding solution contained 150 g/L glucose, 100 g/L soybean peptone, and 50 g/L beef paste.

2.2. Plasmid Construction

Different signal peptides were fused to the N-terminus of Slase. These signal peptides included WapA, Bpr, LipA, Yjfa, Mpr, SacB, YvgO, AmyQ, YfhK, AmyE, WprA, Vpr, and AprE. The gene encoding each signal peptide–Slase fusion protein was synthesized and inserted into the pHT254 vector at the *Bam*HI/*Aat*II sites, to generate different pHT254-*Pgrac100*-signal peptide–Slase plasmids, which were constructed by Synbio Technologies (Suzhou, China). All DNA manipulations and molecular cloning experiments were performed following the conventional protocols [26]. *B. subtilis* WB800N was transformed with these plasmids to obtain a recombinant strain capable of producing Slase fused to a signal peptide. *B. subtilis* competent cells were prepared and transformed with the plasmids, as previously described [27]. The transformants harboring different pHT254 vectors were selected on LB-agar plates supplemented with 5 μg/mL chloramphenicol as a selection marker. Positive colonies were confirmed using colony PCR and DNA sequencing (Beijing Genomics Institution, Beijing, China).

2.3. Expression of KsLX3-Slase in *Bacillus subtilis*

To screen the signal peptides for efficient expression of KsLX3-Slase, single colonies harboring various pHT254-*Pgrac100*-signal peptides–Slase were inoculated into LB medium supplemented with 5 μg/mL chloramphenicol and incubated for 12 h at 37 °C. A 0.4-mL sample of the seed culture was then transferred into a 100-mL Erlenmeyer flask containing 20 mL of 2 × YT medium supplemented with 5 μg/mL chloramphenicol and incubated at 37 °C. For inducible expression under the control of the *Pgrac100* promoter, IPTG was added to the culture to a final concentration of 1 mM when the OD₆₀₀ reached 0.8–1.0. After the addition of IPTG, the culture was further incubated at 30 °C for 24 h, to induce the expression of Slase.

2.4. Fed-Batch Fermentation

Fed-batch fermentation was conducted at 37 °C in a 5-L bioreactor. The seed cultures were prepared by inoculating a single colony of the engineered *B. subtilis* strain into 5 mL of 2 × YT medium supplemented with 5 μg/mL chloramphenicol, followed by 8 h of incubation at 37 °C. A 2-mL sample of the resulting seed culture was transferred to 200 mL of 2 × YT medium containing 5 μg/mL chloramphenicol and incubated at 37 °C for 8 h. The resulting culture was added to 1.8 L of fermentation medium in the fermenter, to bring the total volume of the cultures to 2 L. Ammonia solution and phosphoric acid were used to maintain the pH of the culture at 7.0. The dissolved oxygen (DO) concentration was maintained at 30%. A feeding solution was added to the fermentation culture when the dissolved oxygen concentration rose rapidly. Slase expression was initiated by the addition of IPTG to a final concentration of 1 mM when the OD₆₀₀ of the culture reached 10–12, and the fermentation temperature was adjusted to 30 °C for the entire duration of Slase expression. Samples of the culture were taken at specific time points and centrifuged at

10,000× *g* for 15 min, and the supernatant was analyzed for SIase activity, to assess the expression level of SIase.

2.5. Purification of KsLX3-SIase

The culture supernatant was filtered through a 0.45- μ m filter, concentrated, and then subjected to immobilized metal ion affinity chromatography (IMAC) using a nickel-nitrilotriacetic acid (Ni-NTA) column coupled to an AKTA purifier 100 system (GE Healthcare, Umeå, Sweden). The column was loaded with the sample and then washed with wash buffer (50 mM PBS, 50 mM imidazole, pH 8.0), to remove nonspecific bound proteins. The flow rate was maintained at a constant 1 mL/min. The column was then eluted with elution buffer (50 mM PBS, 100 mM imidazole, pH 8.0) to remove the bound SIase, which was then dialyzed against 20 mM pH 8.0 Tris-HCl buffer using a 10 kDa cut-off filter and then concentrated using ultrafiltration. The purified SIase was subjected to SDS-PAGE using 12% gel and its protein concentration was determined using the BCA method, with bovine serum albumin (BSA) as a standard.

2.6. Characterization of KsLX3-SIase

2.6.1. Assay of the SIase Enzyme Activity

SIase activity was assayed by measuring the conversion of sucrose to isomaltulose. In this assay, 400 μ L of 50 mM citric acid-sodium phosphate buffer (pH 6.0) solution containing 40 g/L sucrose was mixed with 100 μ L of diluted SIase and incubated at 45 °C for 15 min. The reaction was terminated by boiling the sample for 15 min. After that, the sample was centrifuged at 10,000× *g* for 15 min and the resulting supernatant was filtered through a 0.22- μ m membrane filter. The amount of isomaltulose in the filtrate was then determined by HPLC analysis using a Hypersil APS-2 column coupled to an Agilent 1260 system equipped with a refractive index detector (Agilent, Waldbronn, Germany). The column was eluted with a mobile phase, consisting of acetonitrile and water (80:20 *v/v*), at a flow rate of 1.0 mL/min. One unit of SIase activity was defined as the amount of enzyme that produced 1 μ mol isomaltulose per min under the specified conditions.

2.6.2. Effects of Temperature and pH on SIase Activity

The optimum temperature of SIase was determined by measuring its activity at a temperature ranging from 20 to 60 °C. To determine the thermostability of SIase, the enzyme was pre-incubated at various temperatures, ranging from 35 °C to 60 °C, for 30 min, and the residual SIase activity was then measured under the optimal conditions established. To determine the optimum pH for SIase activity, the assay was performed in 50 mM citric acid-sodium phosphate buffer, with pH ranging from 4.0 to 8.0, at 45 °C for 15 min. To test the stability of SIase at different pH values, the purified enzyme was pre-incubated at 25 °C in a pH ranging from 4.0 to 8.0 for 24 h, and the residual activity was then measured under optimal conditions.

2.6.3. Determination of Kinetic Parameters

To determine the kinetic parameters, the purified SIase was incubated at 45 °C for 15 min in a 50 mM citric acid-sodium phosphate buffer (pH 5.5) containing sucrose ranging from 8 to 640 mM. The K_m , V_{max} , and k_{cat} were determined from Michaelis-Menten plots using (GraphPad Prism 9, GraphPad Software, San Diego, USA).

2.6.4. Storage Stability of the SIase

To determine the storage stability of SIase, the enzyme was kept at 4 °C for 14 days. The SIase activity of samples taken at specific time intervals was determined under optimal conditions. Residual activity was calculated with reference to the initial activity.

2.6.5. Time Course of Production of Isomaltulose

The time course reaction was conducted in a biotransformation mimic system (10 mL capacity), using a 50 mM citric acid-sodium phosphate buffer (pH 5.5) solution containing 80 g/L, 120 g/L, and 160 g/L sucrose. Slase concentration was set at 8 µg/mL. The reaction was conducted at 25 °C for 6 h, with shaking at 200 rpm. At specified intervals, aliquots of the reaction mixture were taken, and the isomaltulose produced was quantified by HPLC, with the same parameters as described in detail in Section 2.6.1.

3. Results and Discussion

3.1. Optimization of Signal Peptides for Slase Expression

Insufficient Slase secretion by food-grade strains has impeded the industrial production of isomaltulose. The signal peptide is crucial for the expression and secretion of the target protein. Recent research has demonstrated that an optimal signal peptide for a protein can be determined by a semi-rational design, based on signal peptide composition [24]. Signal peptides with a more positively charged N-domain, a more hydrophobic H-domain, and a more conserved C-domain can better facilitate the secretion of the target protein [24,28,29]. Based on the literature [28], thirteen signal peptides were selected as candidates for this study (Table 1). These signal peptides were placed upstream of the KsLX3-Slase coding sequence, which was under the control of a strong IPTG-inducible promoter Pgrac100 [30], and the resulting recombinant plasmids were separately introduced into *Bacillus subtilis* WB800N for the expression of KsLX3-Slase. The signal peptide WapA was found to yield the highest expression of KsLX3-Slase, as detected by measuring its activity (23.0 U/mL) in the culture supernatant (Figure 1). In addition, each of the selected signal peptides was able to direct the secretion of KsLX3-Slase to the outside of the cell. WapA is a native twin-arginine signal peptide of *B. subtilis*, suggesting that KsLX3-Slase could be secreted through both the Tat and Sec pathways. The result indicated that screening for an optimal signal peptide is necessary, to ensure the efficient secretion and high yield of a specific protein expressed in *B. subtilis*. Therefore, all subsequent experiments aiming to optimize the expression of KsLX3-Slase were performed with the WapA signal peptide.

Table 1. Comparison of various signal peptide sequences used for KsLX3-Slase expression.

Signal Peptide	Sequence	Secretory Pathway	Charged N-Region	Hydrophobic Amino Acid
AmyE	MFAKRFKTSLPLFAGFLLLHLVLAGPAAASA	Sec	3	20
AprE	MRSKKLWISLLFALTLIFTMAFSNMSVQA	Sec	3	15
AmyQ	MIQKRKRTVSFRLVLMCTLLFVSLPITK TSA	Sec	4	12
Bpr	MRKKTKNRLISSVLSTVVISSLLFPGAAGA	Sec	5	13
Mpr	MKLVPRFRKQWFAYLTVLCLALAAV SFGVPAKA	Sec	4	17
Vpr	MKKGII RFLVSVLFFALSTGITGVQAAPA	Sec	3	16
YjfA	MKRLFMKASLVFAVVFVFAVKGAPAKA	Sec	3	16
YvgO	MKRIRIPMTLALGAALTIAPLSFASA	Sec	3	15
SacB	MNIKKFAKQATVLTFTTALLAGGATQAF A	Sec	3	11
YfhK	MKKKQVMLALTAAGLGTALHSAPAAKA	Sec	3	17
LipA	MKFVKRRRIALVTILMLSVTSLFALQPSAKAA	Tat	2	15
WapA	MKRRKRRNFKRFIAAFLVLALMISLVPADVLA	Tat	6	16
WprA	MKRRKFSSVVA AVLIFALIFSLFSPGTKAAA	Tat	4	16

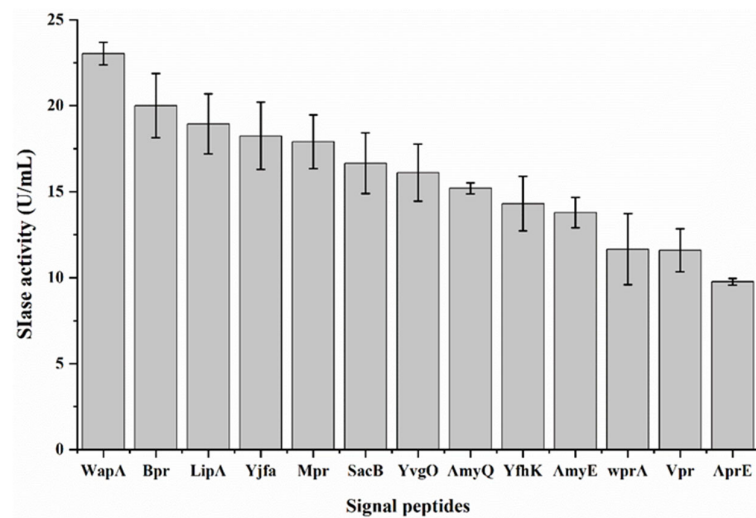


Figure 1. Effects of different signal peptides on the secretion of KsLX3-Slase expressed in *B. subtilis* WB800N. Extracellular Slase activity was assayed with sucrose as the substrate. The production of isomaltulose from sucrose was determined by HPLC. Data are the means \pm SD from triplicate samples.

3.2. Production of Slase Using a Fed-Batch Strategy

Using a food-grade expression system to produce Slase would facilitate its widespread application in the food industry. Using fed-batch fermentation carried out in a 5 L fermenter, the production capacity of the recombinant strain WB800N/pHT254-*Pgrac100-WapA-Slase* was determined. Carbon and nitrogen resources were supplemented, so that cell growth and Slase expression could be effectively controlled. During the fermentation process, the rotatory speed was coupled to the DO, to maintain a DO level of 30%. The biomass (OD_{600}) and Slase activity in the extracellular medium were examined. As shown in Figure 2, the extracellular Slase activity peaked at 125.0 U/mL after 38 h of fermentation. The results of SDS-PAGE confirmed that the amount of secreted Slase continuously increased during the fermentation process (Figure 3).

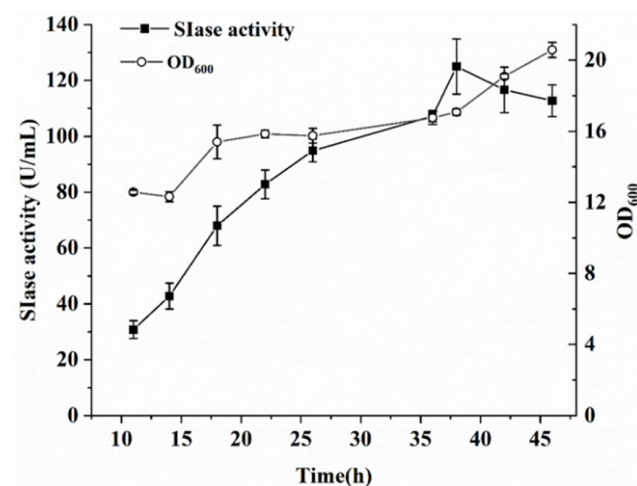


Figure 2. Fed-batch fermentation of the recombinant WB800N/pHT254-*Pgrac100-WapA-Slase* in a 5-L fermenter. Extracellular Slase activity (filled square), biomass (open cycle). Data are the means \pm SD from triplicate samples.

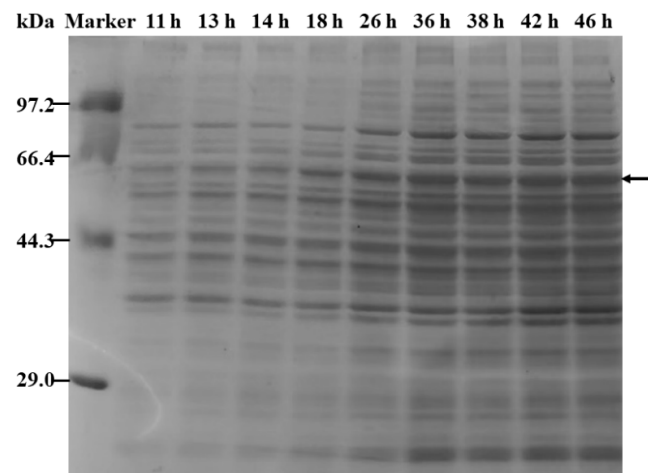


Figure 3. SDS-PAGE analysis of Slase in the culture supernatant of the fed-batch fermentation.

The results seemed to validate the production of Slase using the food-grade strain. In *Lactococcus lactis* MG1363, Slase consisting of heterogenous forms was secreted with an activity lower than 3 U/mL [13]. A food-grade yeast strain, *Yarrowia lipolytica*, has also been used to express Slase, achieving a Slase activity of 49.3 U/mL in the culture supernatant [12]. However, the cultivation of yeast is rather lengthy (more than 72 h) and costly. Compared with these strains, *B. subtilis* offers several significant advantages, including a high efficiency of secretion for the target protein within a shorter fermentation time. In a previous study, when Slase from *Erwinia rhapontici* NX-5 was expressed in *B. subtilis* WB800 under the control of the P_{grac} promoter, the enzyme was mainly found in intracellular form, with total Slase activity in the cell extract reaching 5.2 U/mL of culture in a 7.5-L fermenter [14]. In our study, using the optimal signal peptide WapA, the activity of KsLX3-Slase in the culture medium was determined to be 125.0 U/mL, which is the highest level of Slase activity produced in a food-grade bacteria to date. The increased level of KsLX3-Slase in our study demonstrated the necessity of optimizing a signal peptide for efficient expression of a specific protein. In addition, the extracellular secretion of Slase in this study could simplify the purification process, which would be desirable for industrial production of Slase. The fermentation process could be enhanced by systematically optimizing the feeding strategies [16].

3.3. Enzymatic Properties of the Purified KsLX3-Slase

To investigate the enzymatic properties of KsLX3-Slase expressed in *B. subtilis*, the recombinant KsLX3-Slase was purified and characterized. The expressed KsLX3-Slase was purified by affinity chromatography using Ni-NTA. SDS-PAGE analysis of the purified KsLX3-Slase revealed a single band with a molecular mass of approximately 66 kDa (Figure 4). Previously, the expression of the recombinant Slase has mainly been carried out in *E. coli*, and only a low level of Slase was expressed, as an intracellular protein. Using *B. subtilis* as a host could, therefore, greatly facilitate the secretion of the Slase into the medium of the culture, simplifying its purification.

The effect of temperature on the activity of the purified KsLX3-Slase was measured at temperatures ranging from 20 to 60 °C. KsLX3-Slase exhibited a maximum activity at 45 °C, with more than 90% of its maximal activity occurring at 35 °C to 50 °C, but its activity decreased sharply above 50 °C (Figure 5A). The optimal temperature of KsLX3-Slase was therefore slightly higher than that of most other Slases, such as those of *Erwinia rhapontici* NX-5, *Klebsiella pneumoniae* NK33, and *Pantoea dispersa* UQ68J, which have an optimal activity at 30–35 °C [8,31,32]. The thermostability of KsLX3-Slase was evaluated at temperatures ranging from 35 °C to 60 °C. KsLX3-Slase was found to retain more than 97% of its maximum activity after incubation for 30 min at temperatures between 35 °C

and 50 °C, indicating that the enzyme exhibited good thermostability at these temperatures (Figure 5B). The KsLX3-Slase began to denature at temperatures above 50 °C.

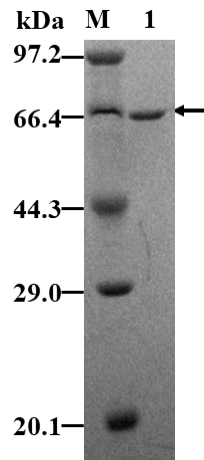


Figure 4. SDS-PAGE analysis of KsLX3-Slase expressed in *B. subtilis* and purified using Ni-NTA chromatography. M: protein marker, 1: purified Slase.

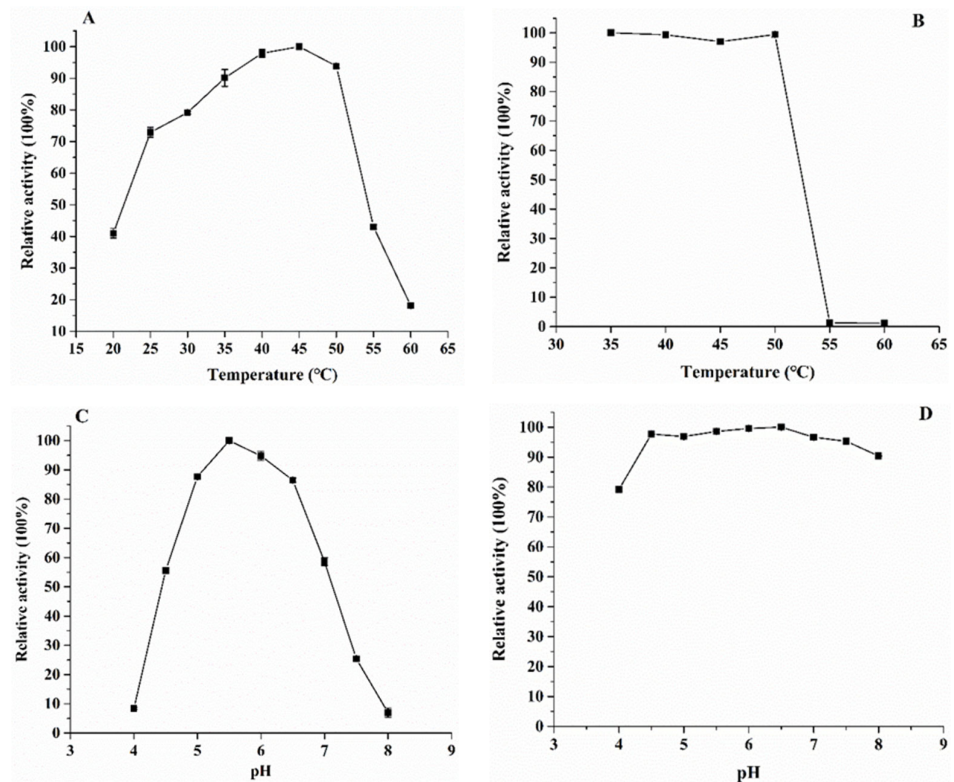


Figure 5. Effects of temperature and pH on the properties of KsLX3-Slase. Effect of temperature on enzyme activity (A) and stability (B). Effect of pH on enzyme activity (C) and stability (D). Data are the means \pm SD from triplicate samples.

The effect of pH on the activity of KsLX3-Slase was determined at a pH ranging from 4.0 to 8.0. The enzyme was found to have optimal activity at pH 5.5, but it exhibited good activity in the pH range of 5.0 to 6.5 (Figure 5C). The activity decreased rapidly at a pH greater than 6.5 or less than 5.0. Most recombinant Slases tend to have an optimal pH between 5.0 and 6.0 [5], which is similar to what was determined for KsLX3-Slase. The results of the pH stability analysis indicated that KsLX3-Slase was stable over a wide range

of pHs at 25 °C (Figure 5D). After incubation at pHs between 4.5 and 8.0 for 24 h, the enzyme retained more than 90% of its maximum activity.

The specific activity of purified KsLX3-SIase under optimal reaction conditions was 594 U/mg. Using a Michaelis–Menten plot (Figure S1), the K_m and k_{cat}/K_m of KsLX3-SIase were determined to be 267.6 ± 18.6 mM and 10.1 ± 0.2 s⁻¹mM⁻¹, respectively. The K_m value was comparable to that of SIase from *Erwinia rhapontici* NX-5 [31]. In addition, the k_{cat}/K_m of KsLX3-SIase was higher than that of *K.planticola* UQ14 S, *Pantoea dispersa* UQ68J, and *Erwinia* sp. Ejp617 [5,33], indicating its high catalytic efficiency, which is advantageous for practical applications. The storage stability of KsLX3-SIase was examined by measuring the residual enzyme activity upon storage at 4 °C for 14 days. KsLX3-SIase maintained 82% of its initial activity after 14 days, demonstrating its superior stability over a long storage period (Figure 6).

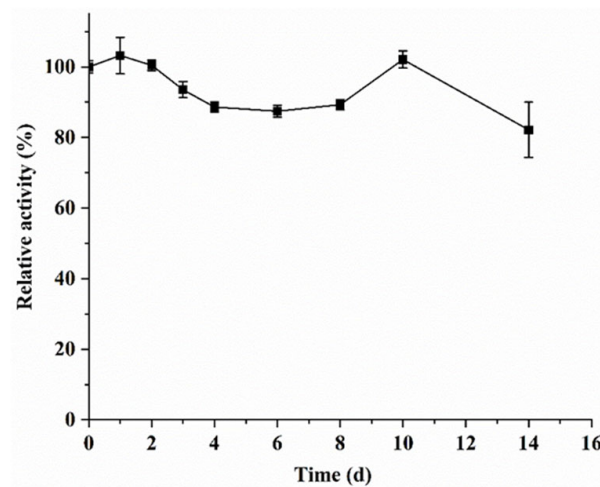


Figure 6. Storage stability of KsLX3-SIase. Data are the means \pm SD from triplicate samples.

The time-course reaction catalyzed by KsLX3-SIase produced in *B. subtilis* was investigated by employing various sucrose concentrations. As depicted in Figure 7, the maximum yields of isomaltulose were $84.2 \pm 0.3\%$, $84.2 \pm 1.4\%$, and $87.8 \pm 0.3\%$ when the sucrose concentrations used were 80 g/L, 120 g/L, and 160 g/L, respectively, demonstrating that a high yield of isomaltulose could be obtained with this enzyme in the presence of a high sucrose concentration. In addition, the rate of isomaltulose yield was rapid during the initial 60 min but then decreased with further culturing time, for all three concentrations of sucrose. This phenomenon might be explained by the inhibitory effect of the by-products, glucose and fructose, or the reduced stability of SIase in the high viscosity of the reaction mixture. A high yield of isomaltulose is necessary for its cost-effective industrial production. The yield we obtained for KsLX3-SIase was greater than that reported for the SIase from *Enterobacter* sp. The yields of isomaltulose achieved with FMB-1 expressed in *Lactococcus lactis* MG1363 and *Saccharomyces cerevisiae* EBY100 were 72% and 7.4%, respectively [13,34]. In addition, the yield of isomaltulose produced by KsLX3-SIase expressed in *B. subtilis* was comparable to the yields of isomaltulose obtained with other SIases expressed in several different organisms (Table 2). For example, the SIase from *Erwinia rhapontici* NX-5, which was expressed in *Bacillus subtilis* spores gave a yield of 92%, whereas the SIase from *Pantoea dispersa* UQ68J expressed in *Yarrowia lipolytica* gave a yield of 93% [35,36].

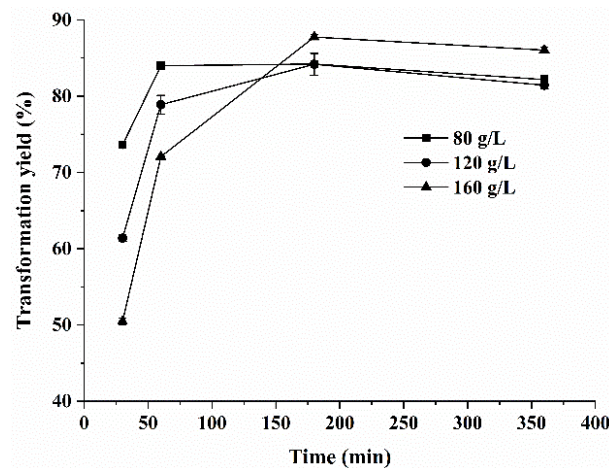


Figure 7. Time course of isomaltulose production catalyzed by KsLX3-Slase. Data are means \pm SD from triplicate samples.

Table 2. Conversion of sucrose to isomaltulose using Slases from various microbes with different expression systems.

Slase Source	Expression System	Conversion Conditions	Yield of Isomaltulose (%)	Reference
Enterobacter sp. FMB-1	Lactococcus lactis MG1363	pH 6.0, 30 °C	72	[13]
Enterobacter sp. FMB-1	Saccharomyces cerevisiae EBY100	pH 6.0, 45 °C	7.4	[34]
Erwinia rhapontici NX-5	Bacillus subtilis 168	pH 6.0, 30 °C	92	[35]
Pantoea dispersa UQ68J	Yarrowia lipolytica	pH 6.0, 30 °C	93	[36]
Klebsiella sp.LX3	Bacillus subtilis WB800N	pH 5.5, 25 °C	87.8	This study

4. Conclusions

In this study, high-level extracellular production of a Slase from *Klebsiella* sp.LX3 in *Bacillus subtilis* was achieved by optimizing the signal peptides. The signal peptide WapA had the highest secretion efficiency for KsLX3-Slase and resulted in an activity level of 23.0 U/mL in the culture medium. Using a fed-batch strategy, the extracellular KsLX3-Slase activity in the culture was increased to 125.0 U/mL, which is the highest level of Slase activity expressed in a food-grade bacteria to date. In addition, KsLX3-Slase exhibited an optimal activity at 45 °C and pH 5.5, as well as a good catalytic efficiency and a high yield of isomaltulose under various sucrose concentrations. These results demonstrated an efficient expression of Slase in *B. subtilis*, laying the foundation for scaled-up production and industrial application of Slase.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/foods11162468/s1>, Figure S1: Michaelis–Menten plot for purified KsLX3-Slase with sucrose as a substrate.

Author Contributions: Investigation, data curation, writing—original draft, D.G. and M.L.; Investigation, data curation, M.J., G.C., and Y.L.; Investigation, data curation, supervision, conceptualization, writing—review and editing, funding acquisition, C.W.; writing—review and editing, X.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Natural Science Foundation of Liaoning Province of China (grant number 20180550668), and the Startup Foundation of Dalian Polytechnic University (grant number 6102072022).

Data Availability Statement: The data that support the findings of the present study are available from the corresponding author on reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Sawale, P.D.; Shendurse, A.M.; Mohan, M.S.; Patil, G.R. Isomaltulose (Palatinose)-an emerging carbohydrate. *Food Biosci.* **2017**, *18*, 46–52. [[CrossRef](#)]
2. Tian, Y.; Deng, Y.; Zhang, W.; Mu, W. Sucrose isomers as alternative sweeteners: Properties, production, and applications. *Appl. Microbiol. Biotechnol.* **2019**, *103*, 8677–8687. [[CrossRef](#)] [[PubMed](#)]
3. Rhimi, M.; Haser, R.; Aghajari, N. Bacterial sucrose isomerases: Properties and structural studies. *Biologia* **2008**, *63*, 1020–1027. [[CrossRef](#)]
4. Sokolowska, E.; Sadowska, A.; Sawicka, D.; Kotulska-Bablinska, I.; Car, H. A head-to-head comparison review of biological and toxicological studies of isomaltulose, d-tagatose, and trehalose on glycemic control. *Crit. Rev. Food Sci. Nutr.* **2021**, *62*, 5679–5704. [[CrossRef](#)]
5. Liu, L.; Bilal, M.; Luo, H.; Zhao, Y.; Duan, X. Studies on biological production of isomaltulose using sucrose isomerase: Current status and future perspectives. *Catal. Lett.* **2021**, *151*, 1868–1881. [[CrossRef](#)]
6. Pilak, P.; Schiefner, A.; Seiboth, J.; Oehrlein, J.; Skerra, A. Engineering a highly active sucrose isomerase for enhanced product specificity by using a “battleship” strategy. *Chembiochem* **2020**, *21*, 2161–2169. [[CrossRef](#)]
7. Lee, H.C.; Kim, J.H.; Kim, S.Y.; Lee, J.K. Isomaltulose Production by Modification of the Fructose-Binding Site on the Basis of the Predicted Structure of Sucrose Isomerase from “*Protaminobacter rubrum*”. *Appl. Environ. Microb.* **2008**, *74*, 5183–5194. [[CrossRef](#)]
8. Wu, L.; Birch, R.G. Characterization of the highly efficient sucrose isomerase from *Pantoea dispersa* UQ68J and cloning of the sucrose isomerase gene. *Appl. Environ. Microbiol.* **2005**, *71*, 1581–1590. [[CrossRef](#)]
9. Zhang, D.; Li, X.; Zhang, L.H. Isomaltulose synthase from *Klebsiella* sp. Strain LX3: Gene cloning and characterization and engineering of thermostability. *Appl. Environ. Microbiol.* **2002**, *68*, 2676–2682. [[CrossRef](#)]
10. Wang, C.; Li, S.; Xu, H.; Wei, Y.; Cai, H. Optimization of fermentation medium of sucrose isomerase by recombinant *Escherichia coli* through response surface method. *Chin. Biotechnol.* **2011**, *31*, 92–97.
11. Zhang, F.; Cheng, F.; Jia, D.X.; Gu, Y.H.; Liu, Z.Q.; Zheng, Y.G. Characterization of a recombinant sucrose isomerase and its application to enzymatic production of isomaltulose. *Biotechnol. Lett.* **2021**, *43*, 261–269. [[CrossRef](#)]
12. Zhang, P.; Wang, Z.; Liu, S.; Wang, Y.; Zhang, Z.; Liu, X.; Du, Y.; Yuan, X. Overexpression of secreted sucrose isomerase in *Yarrowia lipolytica* and its application in isomaltulose production after immobilization. *Int. J. Biol. Macromol.* **2019**, *121*, 97–103. [[CrossRef](#)]
13. Park, J.; Jung, J.; Seo, D.; Ha, S.; Yoon, J.; Kim, Y.; Shim, J.; Park, C. Microbial production of palatinose through extracellular expression of a sucrose isomerase from *Enterobacter* sp. FMB-1 in *Lactococcus lactis* MG1363. *Bioresour. Technol.* **2010**, *101*, 8828–8833. [[CrossRef](#)]
14. Wu, L.; Wu, S.; Qiu, J.; Xu, C.; Li, S.; Xu, H. Green synthesis of isomaltulose from cane molasses by *Bacillus subtilis* WB800-pHA01-pall in a biologic membrane reactor. *Food Chem.* **2017**, *229*, 761–768. [[CrossRef](#)]
15. Zhang, P.; Wang, Z.P.; Sheng, J.; Zheng, Y.; Ji, X.F.; Zhou, H.X.; Liu, X.Y.; Chi, Z.M. High and efficient isomaltulose production using an engineered *Yarrowia lipolytica* strain. *Bioresour. Technol.* **2018**, *265*, 577–580. [[CrossRef](#)]
16. Yang, H.; Qu, J.; Zou, W.; Shen, W.; Chen, X. An overview and future prospects of recombinant protein production in *Bacillus subtilis*. *Appl. Microbiol. Biot.* **2021**, *105*, 6607–6626. [[CrossRef](#)] [[PubMed](#)]
17. Zocca, V.; Correa, G.G.; Lins, M.; de Jesus, V.N.; Tavares, L.F.; Amorim, L.; Kundlatsch, G.E.; Pedrolli, D.B. The CRISPR toolbox for the gram-positive model bacterium *Bacillus subtilis*. *Crit. Rev. Biotechnol.* **2022**, *42*, 813–826. [[CrossRef](#)] [[PubMed](#)]
18. Wang, Z.; Gong, J.; Qin, J.; Li, H.; Lu, Z.; Shi, J.; Xu, Z. Improving the intensity of integrated expression for microbial production. *ACS Synth. Biol.* **2021**, *10*, 2796–2807. [[CrossRef](#)] [[PubMed](#)]
19. Duan, X.; Dai, Y.; Zhang, T. Characterization of Feruloyl Esterase from *Bacillus pumilus* SK52.001 and its Application in Ferulic Acid Production from De-Starched Wheat Bran. *Foods* **2021**, *10*, 1229. [[CrossRef](#)]
20. Zhang, H.; Li, X.; Liu, Q.; Sun, J.; Secundo, F.; Mao, X. Construction of a Super-Folder fluorescent Protein-Guided secretory expression system for the production of phospholipase d in *Bacillus subtilis*. *J. Agric. Food Chem.* **2021**, *69*, 6842–6849. [[CrossRef](#)]
21. Zhang, K.; Su, L.; Duan, X.; Liu, L.; Wu, J. High-level extracellular protein production in *Bacillus subtilis* using an optimized dual-promoter expression system. *Microb. Cell Fact.* **2017**, *16*, 1–15. [[CrossRef](#)] [[PubMed](#)]
22. Li, L.; Dong, F.; Lin, L.; He, D.; Chen, J.; Wei, W.; Wei, D. Biochemical characterization of a novel thermostable type I pullulanase produced recombinantly in *Bacillus subtilis*. *Starch-Stärke* **2018**, *70*, 1700179. [[CrossRef](#)]
23. Zhang, K.; Su, L.; Wu, J. Recent Advances in Recombinant Protein Production by *Bacillus subtilis*. *Annu. Rev. Food Sci. Technol.* **2020**, *11*, 295–318. [[CrossRef](#)]
24. Zhang, J.; Kang, Z.; Ling, Z.; Cao, W.; Liu, L.; Wang, M.; Du, G.; Chen, J. High-level extracellular production of alkaline polygalacturonate lyase in *Bacillus subtilis* with optimized regulatory elements. *Bioresour. Technol.* **2013**, *146*, 543–548. [[CrossRef](#)]
25. Khadye, V.S.; Sawant, S.; Shaikh, K.; Srivastava, R.; Chandrayan, S.; Odaneth, A.A. Optimal secretion of thermostable Beta-glucosidase in *Bacillus subtilis* by signal peptide optimization. *Protein Express. Purif.* **2021**, *182*, 105843. [[CrossRef](#)]
26. Russell, D.W.; Sambrook, J. *Molecular Cloning. A Laboratory Manual*, 3rd ed.; Cold Spring Harbor Laboratory Press: New York, NY, USA, 2001.
27. Xue, G.; Johnson, J.S.; Dalrymple, B.P. High osmolarity improves the electro-transformation efficiency of the gram-positive bacteria *Bacillus subtilis* and *Bacillus licheniformis*. *J. Microbiol. Meth.* **1999**, *34*, 183–191. [[CrossRef](#)]
28. Tjalsma, H.; Bolhuis, A.; Jongbloed, J.D.; Bron, S.; van Dijk, J.M. Signal peptide-dependent protein transport in *Bacillus subtilis*: A genome-based survey of the secretome. *Microbiol. Mol. Biol. Rev.* **2000**, *64*, 515–547. [[CrossRef](#)]

29. Zanen, G.; Houben, E.N.; Meima, R.; Tjalsma, H.; Jongbloed, J.D.; Westers, H.; Oudega, B.; Luirink, J.; van Dijk, J.M.; Quax, W.J. Signal peptide hydrophobicity is critical for early stages in protein export by *Bacillus subtilis*. *FEBS J.* **2005**, *272*, 4617–4630. [[CrossRef](#)] [[PubMed](#)]
30. Phan, T.T.; Tran, L.T.; Schumann, W.; Nguyen, H.D. Development of Pgrac100-based expression vectors allowing high protein production levels in *Bacillus subtilis* and relatively low basal expression in *Escherichia coli*. *Microb. Cell Fact.* **2015**, *14*, 72. [[CrossRef](#)] [[PubMed](#)]
31. Li, S.; Cai, H.; Qing, Y.; Ren, B.; Xu, H.; Zhu, H.; Yao, J. Cloning and characterization of a sucrose isomerase from *Erwinia rhapsontici* NX-5 for isomaltulose hyperproduction. *Appl. Biochem. Biotechnol.* **2011**, *163*, 52–63. [[CrossRef](#)]
32. Aroonnuan, A.; Nihira, T.; Seki, T.; Panbangred, W. Role of several key residues in the catalytic activity of sucrose isomerase from *Klebsiella pneumoniae* NK33-98-8. *Enzyme Microb. Technol.* **2007**, *40*, 1221–1227. [[CrossRef](#)]
33. Zhang, F.; Cheng, F.; Jia, D.; Liu, Q.; Liu, Z.; Zheng, Y. Tuning the catalytic performances of a sucrose isomerase for production of isomaltulose with high concentration. *Appl. Microbiol. Biot.* **2022**, *106*, 2493–2501. [[CrossRef](#)] [[PubMed](#)]
34. Lee, G.; Jung, J.; Seo, D.; Hansin, J.; Ha, S.; Cha, J.; Kim, Y.; Park, C. Isomaltulose production via yeast surface display of sucrose isomerase from *Enterobacter* sp. FMB-1 on *Saccharomyces cerevisiae*. *Bioresour. Technol.* **2011**, *102*, 9179–9184. [[CrossRef](#)] [[PubMed](#)]
35. Zhan, Y.; Zhu, P.; Liang, J.; Xu, Z.; Feng, X.; Liu, Y.; Xu, H.; Li, S. Economical production of isomaltulose from agricultural residues in a system with sucrose isomerase displayed on *Bacillus subtilis* spores. *Bioproc. Biosyst. Eng.* **2020**, *43*, 75–84. [[CrossRef](#)] [[PubMed](#)]
36. Li, L.; Wang, H.; Cheng, H.; Deng, Z. Isomaltulose production by yeast surface display of sucrose isomerase from *Pantoea dispersa* on *Yarrowia lipolytica*. *J. Funct. Foods* **2017**, *32*, 208–217. [[CrossRef](#)]