A Novel Sucrose Isomerase Producing Isomaltulose from Raoultella terrigena

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Abstract: Isomaltulose is widely used in the food industry as a substitute for sucrose owing to its good processing characteristics and physicochemical properties, which is usually synthesized by sucrose isomerase (SIase) with sucrose as substrate. In this study, a gene pal-2 from Raoultella terrigena was predicted to produce SIase, which was subcloned into pET-28a (+) and transformed to the E. coli system. The purified recombinant SIase Pal-2 was characterized in detail. The enzyme is a monomeric protein with a molecular weight of approximately 70 kDa, showing an optimal temperature of 40 °C and optimal pH value of 5.5. The Michaelis constant (Km) and maximum reaction rate (Vmax) are 62.9 mmol/L and 286.4 U/mg, respectively. The conversion rate of isomaltulose reached the maximum of 81.7% after 6 h with 400 g/L sucrose as the substrate and 25 U/mg sucrose of SIase. Moreover, eight site-directed variants were designed and generated. Compared with the wild-type enzyme, the enzyme activities of two mutants N498P and Q275R were increased by 89.2% and 42.2%, respectively, and the isomaltulose conversion rates of three mutants (Y246L, H287R, and H481P) were improved to 89.1%, 90.7%, and 92.4%, respectively. The work identified a novel SIase from the Raoultella genus and its mutants showed a potential to be used for the production of isomaltulose in the industry.

Keywords: sucrose isomerase; isomaltulose; enzymology properties; molecular modification

1. Introduction

According to the World Health Organization, obesity has become a global health problem because of the excessive intake of sucrose and high-fat foods, which leads to metabolic diseases such as type-1 diabetes and cardio-cerebrovascular disease [1,2] that seriously affects people’s health. Thus, many functional sugars have been developed to replace sucrose or function as food additives and this attracts extensive attention for its many promising applications [3].

Isomaltulose (α-D-glucopyranosyl-1,6-D-fructofuranose), which is an isomer of sucrose, is considered an ideal substitute for sucrose [4]. It is a reducing disaccharide that naturally presents in honey and beets in minimal quantities [5] and, thus, is readily accepted by the public [6]. Isomaltulose has a broad market application prospect in the food industry because of good acid stability, low hygroscopicity [7], and high safety [8]. As a new sweetener, isomaltulose is thought to be a potential prebiotic [9] and has many advantages such as low sweetness (around 50% of sucrose) [10], being non-cariogenic [11], fat reducing [12], and low insulin reaction [13].

Isomaltulose exists in small amounts in nature and is usually obtained by using chemical or biological approaches. However, isomaltulose is difficult to be synthesized by
chemical methods and so biochemical conversion is the common means for isomaltulose production [14]. The critical factor in bioconversion is sucrose isomerase (SIase) (EC 5.4.99.11) which is capable in isomerizing sucrose into isomaltulose and trehalulose, but often results in a small quantity of hydrolysis products including glucose and fructose [15]. SIase is found in many microorganisms, such as Protaminobacter rubrum sp. [16], Erwinia rhabontici sp. [17], Serratia plymuthica sp. [18], Enterobacter sp. [19], Klebsiella sp. [20], Pantoea dispersa sp. [21], Pseudomonas mesoacidophila sp. [22], and Agrocbacterium radiobacter sp. [23]. The isomaltulose produced by these SIases accounted for 60–90% of all products. Therefore, it is of great significance to identify novel SIases with potential applications.

The enzyme activity of SIase obtained from wild bacteria is generally low and the highest is only 30 U/mL, which is difficult to meet the demand of industrial production. Thus, a heterogenous expression approach was investigated. SIase genes from different microorganisms have been successfully cloned and expressed in the E. coli system. The expression level of SIase is between 15–654 U/mL.

The generation of by-products and low enzyme activity are two adverse factors in isomaltulose production. For instance, the SIases from Erwinia sp. D12 [24] had 19.8 U/mg of enzymatic activity and 65.7% of conversion rate of isomaltulose. In addition, the SIases from S. plymuthica ATCC159282 [25] had 120 U/mg of enzymatic activity and 72.6% of conversion rate of isomaltulose. Therefore, protein engineering approaches based on structural information can be used to improve the application performance of these SIases.

To date, there has been no SIase explored from the Raoultella genus. Therefore, to expand the microorganism sources and find a promising SIase, this work identified a putative SIase Pal-2 from Raoultella terrigena and expressed this enzyme in the E. coli system. The enzymatic properties of Pal-2 and its potential application for the isomaltulose production were also investigated. Simultaneously, the mutants with high enzyme performance were obtained by the mutagenesis method, which provided a feasible approach with application in the production of isomaltulose.

2. Materials and Methods

2.1. Materials

Restriction endonucleases (BamHI and Xhol), PrimeSTAR® Max DNA Polymerase, Protein molecular weight marker, and DpnI were purchased from Takara (Wuxi, China). Kanamycin, isopropyl-β-D-thiogalactoside (IPTG), Ni-TED sefinose (TM) resin, PCR primers, and competent cell preparation kit were obtained from Sangon Biotech Co., Ltd. (Shanghai, China). Trehalulose and isomaltulose were purchased from Sigma (St. Louis, MO, USA). Other chemical reagents of analytical reagent grade were purchased from the China National Pharmaceutical Group Corporation. Luria–Bertani (LB) culture medium (tryptone 10 g/L, yeast extract 5 g/L, and NaCl 5 g/L) was used to cultivate the recombinant strain and to express enzyme.

2.2. Gene Sequence, Plasmids, and Strains

The whole genome sequence of Raoultella terrigena NCTC 9189 (this strain was modified from genus Klebsiella to genus Raoultella and the name was later changed from Raoultella sp. NCTC 9189 to Raoultella terrigena in July 2020) was resolved by Sanger Institute (United Kingdom) with a genbank accession number of GCA-902109485.1 and a gene pal-2 (genbank ID: VUC84579.1) was predicted as a Slase in this genome. The pal-2 gene was optimized based on codon preference in the E. coli system and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The pal-2 gene was fused with a 6 × histidine-tag sequence at the C- terminus and linked with BamHI and Xhol restriction sites at the 5′-terminals and 3′-terminals, respectively. The expression system E. coli BL21(DE3) and the vector pET-28a (+) were purchased from Sangon Biotech Co., Ltd. (Shanghai, China).
2.3. Cloning, Expression and Purification of Pal-2

The synthesized pal-2 gene and the vector pET-28a (+) were digested by restriction enzymes BamHI and XhoI, respectively. The digested products were ligated by T4 DNA ligase, resulting in the recombinant plasmid pET-28a (+)-pal-2. Then, pET-28a (+)-pal-2 was transformed into E. coli BL21(DE3) by the heat shock method and a colony exhibiting positive Kanamycin resistance was selected.

A colony was inoculated to 10 mL of LB culture medium supplemented with 30 µg/mL Kanamycin to avoid contamination by other bacteria for preincubation at 37 °C and 200 r/min. When the absorbance value of the culture medium at 600 nm reached 0.6, the 10 mL seed fermentation broth was transformed into a 200 mL fermentation LB medium containing 30 µg/mL Kanamycin and the culture was shaken at 37 °C and 200 r/min. When the OD$_{600}$ attained 0.6–0.8, 0.5 mM IPTG was added to induce the expression of the recombinant Pal-2 at 28 °C and 200 r/min for 6 h.

The cells were collected by centrifugation at 12,000 $\times$ g for 5 min and washed twice with a lysis buffer (50 mM Tris–HCl, 100 mM NaCl, and pH 7.0). The cells were resuspended in 20 mL of lysis buffer and then disrupted on ice by sonication (pulse on: 5 s; pulse off: 5 s; 360 W) for 10 min. After removing the cellular debris by centrifugation at 12,000 $\times$ g for 10 min, the supernatant containing the objective protein was filtered through a 0.22 µm syringe filter. The supernatant containing crude recombinant Pal-2 was purified using a Ni-NTA affinity chromatography column at 4 °C according to the instruction of the manufacturer. Briefly, the column was firstly equilibrated with a binding buffer (50 mmol/L Tris-HCl, 500 mM NaCl, and pH 5.5). Then, the supernatant containing crude recombinant Pal-2 was loaded onto the column through a constant flow pump with a flow rate of 1 mL/min and the miscellaneous proteins that did not bind with the Ni-NTA affinity chromatography column were discharged with a washing buffer (50 mmol/L Tris-HCl, 500 mM NaCl, 20 mM imidazole, and pH 5.5). Finally, the recombinant Pal-2 was eluted with an elution buffer (50 mmol/L Tris-HCl, 500 mM NaCl, 200 mM imidazole, and pH 5.5) and was collected according to the peak time of the UV detector. The purified enzyme was dialyzed for 6 h with a dialysis buffer solution without EDTA (50 mmol/L Tris-HCl, and pH 5.5) to remove EDTA.

2.4. Identification of Purified Pal-2

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the molecular weight of recombinant protein. The acrylamide concentration of the stacking gel was 5% and the separating gel was 12%. After mixing 40 µL of pure enzyme solution and 10 µL of 5 $\times$ sample loading buffer, the samples were boiled for 5 min. The protein was stained with Coomassie brilliant blue R250 for 30 min after electrophoresis and the gel was decolorized until the background was transparent. A gel imager system (Tanon Tech Co., Ltd., Shanghai, China) compared the positions of target protein bands and marker bands to determine their molecular weight.

2.5. Protein Concentration Determination

The protein concentration was determined by the BCA method [26]. Bovine serum albumin was used as the standard protein and the standard curve of protein concentration was prepared. In this study, a BCA protein assay kit (Sangon Biotech Co., Ltd., Shanghai, China) was used and the detailed experimental procedures were implemented according to manufacturer’s instruction.

2.6. Enzyme Assay

Recombinant Pal-2 activity was measured in a 1 mL reaction system containing 20% (w/v) sucrose and 50 mM disodium hydrogen phosphate-citrate buffer (pH value of 5.5) for 15 min. The assay mixture was pre-incubated at a given temperature for 5 min. Then,
the reaction was induced by adding the enzyme and stopped by heating in boiling water for 10 min. Finally, the reaction mixture was centrifuged at 12,000× g for 5 min and the supernatant filtrated through a 0.22 µm syringe filter. The concentrations of glucose, fructose, sucrose, isomaltulose, and trehalulose were detected by high-performance liquid chromatography (HPLC) method. Waters e2695 system (Waters Corporation, MA, USA), Waters 2414 RI detector, and a HILICpak VG-50 4E column (4.6 mm I.D. × 250 mm, Shodex, Japan) were used to analyze samples. The samples were eluted using 88% acetonitrile as a mobile phase at the constant flow rate of 1 mL/min. The HILICpak VG-50 4E column temperature and the RI detector temperature were fixed at 60 °C and 40 °C, respectively. The retention times of glucose, fructose, sucrose, isomaltulose, and trehalulose were 8.6, 11.6, 21.2, 22.3, and 23.5 min, respectively. One-unit activity was defined as the amount of enzyme that released 1 µmol of isomaltulose per minute under the temperature of 40 °C and a pH value of 5.5.

2.7. Characterization of Pal-2

2.7.1. Optimal Temperature and Thermostability

The optimal temperature was examined by incubating samples at various temperatures within a range of 20–60 °C at intervals of 5 °C. The reactions were performed at pH 5.5 for 15 min. The group with the highest enzyme activity in the result was defined as 100% of relative enzyme activity.

The purified Pal-2 was pre-incubated at temperatures of 45 °C and 50 °C to evaluate thermal stability. The reaction mixture was measured at intervals of 20 min. Enzyme activity without preincubation was defined as 100% of relative enzyme activity.

2.7.2. Optimal pH and pH Stability

The optimal pH was examined by incubating samples at various pH within a range of 3–9. The reactions were performed at 40 °C for 15 min. Relative activity was normalized to the maximum enzyme activity (100%).

The purified Pal-2 was pre-incubated at various pH within a range of 3–9 for 2 h to evaluate pH stability. The activity of enzyme without preincubation was defined as 100% of relative enzyme activity.

2.7.3. Effect of Metal Ions and Chemical Reagents

The purified Pal-2 was pre-incubated with 10 mM of various metal ions (Mg²⁺, Mn²⁺, Ca²⁺, Ba²⁺, Cu²⁺, Fe²⁺, Fe³⁺, Al³⁺, Sn²⁺, and Zn²⁺) or chemical reagents (DMSO, Tween-20, and Triton X-100) for 2 h. The enzymatic activity in the absence of supplemental metal ions and chemical reagents was regarded as 100%.

2.7.4. Determination of Kinetic Parameters

The kinetic parameters of Pal-2 were determined at different sucrose concentrations (5–250 mM) under the optimal conditions. The Michaelis–Menten constant (Km) and the maximum reaction rate (Vmax) were calculated from the Lineweaver–Burk plot after linear fitting to the data.

2.8. Production of Isomaltulose

Different dosages of Pal-2 within a range of 10–35 U/g sucrose were added to the reaction system containing 200 g/L sucrose at pH 5.5. The reaction was performed at 40 °C for 10 h.

Various concentrations of sucrose (100–500 g/L) were used to produce isomaltulose. The reaction mixtures were incubated at 40 °C and pH 5.5 for 10 h with the optimal enzyme dosage.

In order to determine the relationship between the reaction time and the amount of isomaltulose, the reaction was performed under optimized conditions for 10 h and the reaction mixture was extracted at intervals of 2 h.
2.9. Molecular Modification

2.9.1. Homology Modeling and Molecular Docking

The homology modeling was performed on the website of SWISS-MODEL (https://swissmodel.expasy.org/) (accessed on 15 May 2021) [27] and the constructed model was checked and analyzed on the website of Procheck (https://saves.mbi.ucla.edu/) (accessed on 15 May 2021) [28]. AutoDock software [29] was used for docking to predict the binding site under the default docking parameters.

2.9.2. Site-Directed Mutagenesis

Hotspot Wizard (https://loschmidt.chemi.muni.cz/hotspotwizard/) (accessed on 15 May 2021) [30] was used to identify hot spots based on position and substitution scores from multiple sequence alignment. Based on the mutational landscape in Hotspot Wizard, sites that would generate neutral or beneficial mutations were screened out.

The structure display software PyMOL was used to analyze the SIase mutation site and nearby amino acids.

2.9.3. Construction of Mutants

The plasmid pET-28a (+)-pal-2 constructed in the laboratory was used as the template for cloning and the PCR instrument’s amplification. Primers used for different mutation sites are listed in Table 1.

The PCR amplification system was composed of DNA polymerase Prime Star Max (25 µL), forward primer (10 µM, 1 µL), reverse primer (10 µM, 1 µL), template (100 ng/µL, 1 µL), and ddwater (22 µL). The PCR amplification conditions were 98 °C (3 min) for the initial denaturation, 98 °C (10 s) for denaturation, 55 °C (15 s) for annealing, 72 °C (6 min) for extension, and 72 °C (10 min) for final extension. The target gene fragment obtained by PCR amplification was verified by DNA electrophoresis. Then, 1 µL Dpn I was added to the reaction mixture and the mixture was incubated at 37 °C for 1.5 h to remove the template. The constructed plasmids of mutants were sequenced by Sangon Biotech Co., Ltd. (Shanghai, China) and transformed into E. coli expression system. The purification and identification procedures were the same with those of wild-enzyme Pal-2.

Table 1. The designed primers for mutation site.

| Primer | Sequence (5'-3') |
|--------|-----------------|
| N498P-F | CGTGAACGTGACCAGAACCCAAATCTGTGCTGAAC |
| N498P-R | GTTACGACACAGATTTCGGTTCCGTCAGTTCAG |
| Q275R-F | GGTCGCCAACATCCACCGTATATCCAGGAATGAAAC |
| Q275R-R | GTTACATTTCTGGATATACCGGTTCGATGGAGCC |
| V355A-F | CAAAATGGATACCCCGCCTCAATCAGCTTGGAAAC |
| V355A-R | GTTCCAACCGTATTCACCCCGGCGTGGATATCCCC |
| A492E-F | CTACGTACAGATTAACCGGACACCCTGAACTACGCAAC |
| A492E-R | GTTTTCGGTCAGTTCAGTTCGCGTGAATCTT |
| Y246L-F | GTTTGCATACCCGTCCGACCCTTGTCACATAACCCAGTTTC |
| Y246L-R | GAAAACCTGGATTTTGGACAGGGTCCAGAACGTAACGGAAAC |
| H481P-F | GTAAACCGTGGTCCAGTAAACCCGAACTAC |
| H481P-R | GTAGTTCGAGTTTACCCCGGAACCCAGTTAAC |
| R310P-F | TCAGTTCGAGTTTACCCCGGAACCCAGTTAAC |
| R310P-R | CAGTTATAGCCGGCGGCTCAAGAAAACCT |
| H287R-F | GTGAAGTGCTGTCCCTGGAGCAGACGACGACGACGACGACGAC |
| H287R-R | GTGGCCACGTCAATACCCAGCAGCAGCAGCAGCAGCAGCAG |

Note: The mutant sites were underlined.

3. Results and Discussion

3.1. Sequence Analysis of Pal-2

The Pal-2 from Raoultella terrigena was predicted to encode 599 amino acids and has a theoretical isoelectric point of 5.88 and a molecular mass of 69.8 kDa. The phylogenetic
A phylogenetic tree of SIases from various microorganisms was constructed, including *Gluconobacter thailandicus* sp. NBRC 3257, *Pectobacterium atrosepticum*, *Rhizobium* sp. MX-45, *Pantoea dispersa*, *Enterobacter* sp. FMB-1, *Klebsiella pneumoniae*, *Erwinia rhapontici*, and *Serratia plymuthica* sp. A30. As shown in Table 2, all of these SIases shared more than 45% of identity with Pal-2, where Pal-2 is closer to SIases from *Klebsiella* sp. LX3 and *Klebsiella pneumoniae*. Amino acid sequence alignments of SIases from different microorganisms in Figure 1 shows that most amino acids are strictly conserved in SIase. Similar to the glycoside hydrolase family 13 (GH13) enzymes, SIases also contained a catalytic triad (Asp 199, Glu 255, and Asp 329) and two conserved histidine residues (His 145 and His 368), which are located in the catalytic pocket and involved in the catalysis of the substrate [31]. Most SIase amino acid sequences [32] harbor a 325RLDRD329 motif that determines product specificity and this motif also exists in Pal-2.

![Figure 1. Amino acid sequence alignments of SIases: the strictly conserved areas are shown with a red background and the highly conserved residues are shown using red words surrounded by a blue box.](image-url)
Table 2. Comparison of the amino acid sequences of identified SIases from different microorganisms.

| Percentage Identity |
|----------------------|
| Gluha | Pecto | Rhizo | Pandi | Ente | Rate | KleLX3 | Klepn | Erwi | Serra |
| Gluha | 100   | 49.65 | 51.09 | 51.39 | 51.13 | 51.31 | 50.71 | 50.17 | 51.3  | 50.88 |
| Pecto | 49.65 | 100   | 68.04 | 64.72 | 65.33 | 64.66 | 67.61 | 65.27 | 65.11 | 67.52 |
| Rhizo | 51.09 | 68.04 | 100   | 67.15 | 68.47 | 70.09 | 69.49 | 69.49 | 67.5  | 70.05 |
| Pandi | 51.39 | 64.72 | 67.15 | 100   | 67.28 | 70.47 | 71.25 | 68.74 | 68.73 | 71.38 |
| Ente  | 51.13 | 65.33 | 68.47 | 67.28 | 100   | 79.63 | 82.63 | 81.27 | 71.86 | 71.86 |
| Rate  | 51.31 | 64.66 | 70.09 | 70.47 | 79.63 | 100   | 88.25 | 87.12 | 70.95 | 73.22 |
| KleLX3| 50.71 | 67.61 | 69.49 | 71.25 | 82.63 | 100   | 99.3  | 71.93 | 71.82 | 74.2  |
| Klepn | 51.17 | 65.27 | 69.49 | 68.74 | 81.27 | 87.12 | 99.3  | 100   | 69.9  | 71.82 |
| Erwi  | 51.3  | 65.11 | 67.5  | 68.73 | 69.62 | 70.95 | 71.93 | 69.9  | 100   | 78.66 |
| Serra | 50.88 | 67.52 | 70.05 | 71.38 | 71.86 | 73.22 | 74.2  | 71.82 | 78.66 | 100   |

Note: The symbols Rate, Ente, Erwi, Gluha, KleLX3, Klepn, Pandi, Pecto, Rhizo, and Serra represent microorganisms of respectively.

3.2. Construction, Expression, and Purification of Recombinant Pal-2

The Pal-2 gene was inserted between the polylinker sites BamHI and XhoI of the pET-28a (+) expression vector (Figure 2a). As shown in Figure 2b, two bands appeared after double enzyme digestion of the recombinant plasmid which are about 1800 bp and 5300 bp. The results were consistent with the theoretical value and it indicates that the recombinant plasmid was successfully constructed.

![Figure 2](image-url)

After recombinant plasmid was transformed into E. coli BL21(DE3), the recombinant Pal-2 was overexpressed by IPTG induction and purified. As shown in Figure 2c, an obvious band appeared on the gel at approximately 70 kDa that is consistent with the theoretical molecular mass of Pal-2.

3.3. Characterization of Pal-2

3.3.1. Optimal Temperature and Thermostability

Temperature is one of the crucial factors affecting the catalytic efficiency. Higher temperature usually enhances the substrate solubility to promote the reaction rate. However, higher temperatures can also accelerate enzyme denaturation, thus reducing the catalytic efficiency [33]. The optimum temperature of Pal-2 was assayed at temperatures ranging from 20 °C to 60 °C. As shown in Figure 3a, recombinant Pal-2 had maximal activity at approximately 40 °C, which is similar to that of Erwinia sp. D12 [34]. At 35–50 °C, enzyme activity of recombinant Pal-2 was maintained above 80% and enzyme activity decreased rapidly when the temperature was higher than 50 °C. As shown in Table 3, it can exacerbate
the occurrence of hydrolysis reaction of Slase when the temperature exceeds 50 °C; thus, the ideal temperature for isomaltulose production is 40–50 °C.

Figure 3. Characterization of Pal-2. Values are the means of three replicates ± the standard deviation. (a) The effect of temperature on the activity of recombinant Pal-2. (b) Thermostability of recombinant Pal-2 at different temperatures: 45 °C (●) and 50 °C (■). (c) The effect of pH on the activity of recombinant Pal-2. (d) pH stability of recombinant Pal-2. (e) The effect of metal ions and other reagents on the activity of recombinant Pal-2. (f) Lineweaver–Burk plot of purified recombinant Pal-2 using sucrose as a substrate.

Table 3. Effect of temperature on product ratio.

| Temperature (°C) | Isomaltulose | Trehalulose | Monosaccharide |
|-----------------|--------------|-------------|----------------|
| 20              | 74.60%       | 24.20%      | 1.20%          |
| 30              | 76.90%       | 21.40%      | 2.70%          |
| 40              | 78.30%       | 17.80%      | 3.90%          |
| 50              | 75.50%       | 16.70%      | 7.80%          |
| 60              | 73.60%       | 11.80%      | 14.60%         |
As shown in Figure 3b, only 39.2% of the residual activity of Pal-2 was retained after incubation for 2 h at 45 °C. The thermostability remarkably decreased when the incubation temperature was 50 °C. Pal-2 displayed a thermostability with a half-life duration of 103 min at 45 °C and 48 min at 50 °C. Previous reports have shown that Slase is thermostable. Slase from *Klebsiella pneumonia* sp. was completely deactivated after incubation at 50 °C for 40 min [35] and the half-life of the Slase from *Erwinia* sp. D12 was 30 min at 45 °C. However, in industrial production, Slase is required to have good thermostability to maintain catalytic efficiency and to reduce consumption of enzyme. Therefore, the thermostability of Pal-2 should be improved.

### 3.3.2. Optimal pH and pH Stability

Usually, pH affects the dissociation state of amino acids in the active center as well as the affinity of the enzyme for the substrate [36]. Based on Figure 3c, Pal-2 exhibited maximum activity under weakly acidic conditions of pH 5.5. The enzyme activity of Pal-2 was retained more than 80% at pH 4.5–6.5 and decreased rapidly once the was pH higher than 7.0. As shown in Table 4, the optimal pH of most Slases is weakly acidic ranging from 5.0–6.0, with the exception that the optimal pH of Slase from *Erwinia rhapsontici* NCPPB 1578 is 7.0 [37]. In the production process of functional sugar, mildly acidic reaction condition is usually favorable to inhibit the Maillard reaction and, thereby, reducing the generation of by-products [38].

The pure enzyme solution was incubated at different pH for 2 h to observe the loss of enzyme activity. As shown in Figure 3d, Pal-2 is relatively stable under acidic conditions (pH 4.0–7.0). The residual enzyme activity is 97% at a pH of 5.5 and 83% at a pH of 6.5.

| Slase Source                  | Optimal Temperature (°C) | Optimal pH | Km (mM) | Ref. |
|-------------------------------|--------------------------|------------|---------|------|
| *Erwinia rhapsontici* NX-5    | 30                       | 5.0        | 257     | [17] |
| *Erwinia rhapsontici* D12     | 40                       | 6.0        | 138     | [24] |
| *Klebsiella* sp. LX3          | 35                       | 6.0        | 54.6    | [31] |
| *Klebsiella pneumonia* NK33-98-8 | 30                     | 6.0        | 42.7    | [35] |
| Pantoea dispersa UQ68j        | 35                       | 6.0        | 39.9    | [39] |
| Enterobacter sp. FMB-1        | 50                       | 5.0–6.0    | 49      | [40] |
| Serratia plymuthica AS9       | 30                       | 6.2        | 65      | [41] |
| *Erwinia rhapsontici* NCPPB 1578 | 30                     | 7.0        | 280     | [42] |

### 3.3.3. Effect of Metal Ions and Chemical Reagents

According to the relevant literature, metal ions are related to the stability of some carbohydrases. For example, the addition of Co²⁺ improved the thermal and structural stability of D-allocose 3-heterotropic isomer [43] and L-rhamnose isomerase [44]. As shown in Figure 3e, Mg²⁺, Mn²⁺, Ca²⁺, and Al³⁺ could significantly promote the enzyme activity, while Fe³⁺ and Sn²⁺ could inhibit the enzyme activity. By using domain analysis, it was found that Ca²⁺ binding sites probably exist in Pal-2 and changes of the microenvironment near the Ca²⁺ binding sites might affect the substrate binding effect.

The enzyme activity was increased by 32.7% with 5% Triton X-100. This may be due to the presence of many hydroxide radicals in Triton X-100, which can form hydrogen bonds and further increases the stability of Pal-2 [45].

### 3.3.4. Determination of Enzyme Kinetics

Michaelis–Menten constant (Kₘ) and maximum reaction rate (Vₘₐₓ) of Pal-2 were calculated using the Lineweaver-Burk double reciprocal method. Although most Slase share a high degree of similarity in amino acid sequences, Slases from different sources differ significantly in substrate binding affinity which ranges from 19.2 mmol/L to
280 mmol/L [46]. As shown in Figure 3f, $V_{\text{max}}$ and $K_m$ of Pal-2 using sucrose as the substrate were 286.4 L min/mmol and 62.9 mmol/L, respectively.

3.4. Optimization of Isomaltulose Production

3.4.1. Optimization of Enzyme Dosage for Isomaltulose Biosynthesis

Enzyme dosage usually affects the production of isomaltulose directly, which was investigated in this work. As shown in Figure 4a, with the dosages of 10–25 U/g sucrose, isomaltulose production was enhanced with the increase in Pal-2 dosage and reached the maximum at 25 U/g of enzyme. However, with further increases in dosage, the isomaltulose conversion rate exhibited a stable tendency. Therefore, 25 U/g is the optimal amount of enzyme for the enzymatic synthesis of isomaltulose. The result is similar to that of $P$. dispersa UQ68J [39] and $S$. plymuthica AS9 [41].

![Figure 4](image)

**Figure 4.** Optimization of isomaltulose production. (a) Effect of enzyme dosage on the isomaltulose yield. (b) Effect of sucrose concentration on the isomaltulose yield. (c) Effect of time on isomaltulose yield.

3.4.2. Optimization of Sucrose Concentration for Isomaltulose Biosynthesis

The effect of sucrose concentration on the yield of isomaltulose was studied using various concentrations of sucrose (Figure 4b). The production of isomaltulose increased with increasing sucrose concentration until the sucrose concentration was 400 g/L and then it decreased slightly. It is probable that too high a concentration of sucrose affects its dissolvability and also leads to product inhibition, which are often observed in sugar production processes [47]. Thus, sucrose concentration was chosen at 400 g/L for the further study of Pal-2 producing isomaltulose.

3.4.3. Optimization of Reaction Time for Isomaltulose Biosynthesis

As shown in Figure 4c, the conversion rate of isomaltulose increased significantly within 6 h. After 6 h, the substrate was utterly transformed and the conversion rate of isomaltulose kept stable. Therefore, the reaction time was set as 6 h for the further study. The yield reached 81.7% and the monosaccharide content was less than 2% after 6 h reaction. However, the proportion of monosaccharides in the catalytic products carried out by Slase from $S$. plymuthica ATCC 15928 [25] and $K$. planticola UQ14S [39] was 18% and 19%. The high content of monosaccharides makes it difficult to separate products and reduces economic benefits.

3.5. Molecular Modification of Recombinant Pal-2

3.5.1. Homology Modeling, Molecular Docking, and Design of Mutant Sites

Given the 88% amino acid sequence homology between Pal-2 and Pal-1 derived from $Klebsiella$ sp. LX3, the crystal structure of $Klebsiella$ sp. LX3 (PDB: 1M53, resolution: 2.2 Å) was used as a template for structural modeling with sufficient reliability. The constructed model of Pal-2 was evaluated by the PROCHECK online website. Consequently, 88.9% of the amino acid residues were in the entirely permissible region, indicating that the quality is sufficient (Figure 5b). The tertiary structure of Pal-2 is similar to that of known Slase and...
is composed of three domains (N-terminal domain, C-terminal domain, and sub-domain). The sucrose molecule was docked into the substrate-binding pocket of Pal-2 by using the AutoDock software. The predicted binding sites are H145, R239, R333, H368, and R456 forming a pocket to hold a sucrose molecule (Figure 5a). This model was used for the further investigation for the design of mutants.

**Figure 5.** Construction and expression of mutants. (a) The binding sites of Pal-2 and sucrose. (b) Ramachandran plot of Pal-2 modeling structure. (c) SDS-PAGE of the Pal-2 mutants. Lane M, represents molecular weight marker, Lane 1–8 represents N498P, Q275R, V355A, A492E, Y246L, H481P, R310P, and H287R, respectively.

In order to obtain enzymes harboring higher performance, including enzyme activity and conversion rate, molecular modification was executed in this study. Hotspot Wizard is usually used to generate mutants with higher enzyme stability. However, some reports revealed that the enzymatic activity and conversion can also be improved when the stability was changed. That means the goal is to improve enzymatic stability, which was designed by using Hotspot Wizard, but the unexpected results of enzymatic activity and conversion rate were achieved. Therefore, in this study reverse process was employed; that is, the research goal in this study is to improve conversion rate and enzymatic activity, but mutants were designed using Hotspot Wizard that targets stability.

The model of Pal-2 was analyzed by Hotspot Wizard and hot spots were identified based on position and substitution scores from multiple sequence alignment. Eight amino acid sites were selected to be mutated based on the mutational landscape in Hotspot Wizard. Site-directed mutagenesis of these eight sites was complemented to produce mutants (N498P, Q275R, V355A, A492E, Y246L, H481P, R310P, and H287R). The plasmids of these eight mutants were assayed by sequencing and the correct plasmids were used for the further experiments.

3.5.2. Expression and Purification of Mutants

The successfully constructed plasmids of mutants were transformed into *E. coli* BL21(DE3) to express Pal-2 mutants. As shown in Figure 5c, the mutants were purified and reached electrophoresis pure level with the molecular mass significantly unchanged (approximately 70 kDa), which indicates that these mutants can be used for further assay.
3.5.3. Enzyme Activity of Pal-2 mutants

The enzyme activity of mutants was determined as described in Section 2.6 of this paper. As shown in Table 5, the activity of wild-type Pal-2 was defined as 100% of the relative enzyme activity. Most mutants showed higher enzyme activity compared with wild-type Pal-2. Among them, the activities of mutants N498P and Q275R were significantly enhanced by 89.2% and 42.2%, respectively.

Table 5. Relative activities and the conversion rate of isomaltulose.

| Enzyme      | Relative Activities (%) | Conversion Rate of Isomaltulose |
|-------------|-------------------------|---------------------------------|
| Pal-2       | 100 ± 3.3               | 81.7 ± 3.2                      |
| N498P       | 189.2 ± 3.5             | 84.8 ± 2.8                      |
| Q275R       | 142.4 ± 4.7             | 81.1 ± 3.3                      |
| V355A       | 122.6 ± 3.8             | 82.6 ± 2.7                      |
| A492E       | 103.3 ± 3.2             | 83.7 ± 3.5                      |
| Y246L       | 127.5 ± 3.9             | 89.1 ± 2.9                      |
| H481P       | 115.2 ± 2.7             | 92.4 ± 3.1                      |
| R310P       | 87.2 ± 2.9              | 80.5 ± 3.3                      |
| H287R       | 111.1 ± 3.6             | 90.7 ± 3.9                      |

The amino acid at position 498 is located in the loop region composed of 497–501 amino acid at the N-terminal domain. This loop region formed a salt bridge with the C-terminal domain and is vital for stabilizing the conformation of the active center. The mutation of Asn into Pro caused the shrinking of the catalytic pocket by 1.08 Å, which may alter how the substrate binds to the enzyme. Moreover, Asn498 had a high temperature factor and proline substitution enhanced spatial structure rigidity of Pal-2, which may also be beneficial for the enhancement of enzymatic activity.

The amino acid at position 275 is located in the loop region composed of 497–501 amino acid at the N-terminal domain. This loop region formed a salt bridge with the C-terminal domain and is vital for stabilizing the conformation of the active center. The mutation of Gln to Arg is more favorable than Glu for the enzymatic catalysis of sucrose. After mutation, the structure of the active center of Pal-2 became more compact such that the substrate cannot be easily separated from the active center and may positively affect the kinetic parameters of the enzyme.

3.5.4. Isomaltulose Conversion Rate of Pal-2 Mutants

The maximum conversion rate of isomaltulose was measured under the conditions of pH 5.5, 40 °C, 25 U/g sucrose, and 400 g/L sucrose for 6 h. As shown in Table 5, in comparison with the wild-type enzyme, the conversion rate of isomaltulose can be enhanced by 9.06%, 13.10%, and 11.02%; by using Y246L, H481P, and H287R. Leu is a hydrophobic amino acid. The increased hydrophobicity of pal-2 upon the mutation caused the enzyme molecule to become more stable. In addition, the hydrogen bond between 246 and 241 was broken after mutation, which may alter the spatial conformation of the catalytic pocket, leading to the increased product specificity.

After mutation, Arg287 formed hydrogen bonds with Arg282 and Glu283, which may affect the conformation of the aromatic clamp in the β-folded region and, thereby facilitates substrate recognition and improves the product specificity of the enzyme.

4. Conclusions

This study provided a novel enzyme candidate for industrial production of isomaltulose. Pal-2 showed excellent catalytic ability and sucrose was transformed completely. The conversion rate of isomaltulose reached 81.7% with only 2% of monosaccharide by-products. The molecular modification can realize the significant enhancement of conversion
rate and activity, which provides the basis for the further study of the enzymatic production of isomaltulose. In order to save the production cost, immobilization and key sites exploration of SLase may be the focus of future studies.

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