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KYIV NATIONAL UNIVERSITY OF TECHNOLOGIES AND DESIGN
Faculty of Chemical and Biopharmaceutical Technologies
Department of Biotechnology, Leather and Fur

QUALIFICATION THESIS

on the topic **Construction of genetically engineered bacteria producing L-rhamnose isomerase**

First (Bachelor's) level of higher education
Specialty 162 "Biotechnology and Bioengineering"
Educational and professional program "Biotechnology"

Completed: student of group BEBT-20
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APPROVE
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_____
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ASSIGNMENTS
FOR THE QUALIFICATION THESIS
Wang Peili

1. Thesis topic **Construction of genetically engineered bacteria producing L- rhamnose isomerase**
Scientific supervisor Iryna Voloshyna, Ph.D., As. prof.

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2. Initial data for work: assignments for qualification thesis, scientific literature on the topic of qualification thesis, materials of Pre-graduation practice

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SUMMARY

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Rare sugars are sugars with very little content in nature, which have the characteristics of low calorie and little absorption. With the advancement of individuals' lives, people started to focus more and more on their physical well-being. At present, high-calorie sweeteners widely existing in the food and medicine fields have a trend of being replaced by low-calorie functional sweeteners. D-allose is an important rare sugar. Its physiological functions include anti-inflammation, neuroprotection, inhibition of cancer, adjuvant cancer treatment, immunosuppression, anti-oxidation, cryoprotection and so on. It has a good prospect in food, medicine, clinical and other fields. However, the amount of D-oxoxlose in nature is very small , and it is not easy to extract from natural products. However, the chemical synthesis method has many by-products and is not easy to purify. In this study, L-rhamnose isomerase (L-RhIase) from *Bacillus subtilis* was amplified, and the recombinant plasmid pET-22b-*l-rhi* was constructed. The recombinant plasmid was transformed into *E.coli* BL21star (DE3) to express L-RhIase. To provide theoretical and technical support for the efficient production of D-allose, L-RhIase was used to catalyze D-piscose to D-allose.

Key words: D-allose; L-rhamnose isomerase; Construction of engineering bacteria

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INTRODUCTION

The relevance of the topic is about using L-RhI to catalyse D-piscose to produce D-allose. Rare sugars are a class of sugars or derivatives that occur in small amounts in nature. Rare sugars are widely used as an additive in food, medicine and other fields. They are essential for the food, pharmaceutical and nutrition industries to produce low-calorie sweeteners, microbial growth inhibitors, and leavening agents.

D-allose, a rare sugar found in nature, is a non-nutritive sweetener with the same sweetness as sucrose but very low calorie. It has a variety of health functions at the same time. The physiological functions of D-allose mainly include the following aspects, and it has a bright application prospect in food and medicine fields. However, the current research on the production of D-allose is not comprehensive and systematic. In this study, we constructed a way for the enzymatic synthesis of D-allose by genetically engineered recombinant bacteria, which provides a theoretical and practical basis for the production of D-allose.

The purpose of the study is to develop a two-step enzymatic biocatalytic pathway for the preparation of D-allose. Currently, D-allose can be obtained by chemical synthesis methods, but these methods usually require the use of expensive reagents and complex steps, and have certain limitations in terms of environmental friendliness and yield. The industrialization of biosynthesis of D-allose is relatively low, and further process improvement and economic feasibility studies are still needed.

Therefore, finding an efficient, economical and environmentally friendly biosynthesis path has become the focus of research. Rhamnose-isomerase is an enzyme that can catalyze the conversion of D-piscose to D-allose. Through genetic engineering technology, the L-rhamnose-isomerase gene can be introduced into *E.coli* to make them capable of producing the enzyme. Using these engineered strains, efficient production from the cheap substrate D-piscose to D-allose can be achieved.

The objectives of this paper is to study the heterologous expression of rhamnose isomerase gene, and then establish a series of methods on the construction of L-rhamnose producing isomerase gene engineering bacteria, and determine the enzyme activity of the expressed L-rhamnose-isomerase.

The object of the study is to use common techniques in the field of molecular biology--gene recombination technology to realize following objectives.

L-RhI from Bacillus subtilis was amplified and recombined, then transferred into cloning vector for amplification, and tested by PCR and SDS-PAGE. Finally, it was transferred into expression vector E.coli BL21 (star) for mass expression, and the conversion rate of enzyme was determined by HPLC. With the above series of steps, the biological production and yield measurement of D-allose were preliminarily realized.

Research methods

1.Aiming at the current production status of D-allose domestically and abroad, an engineering bacterium for efficient production of L-RhI was proposed to improve the production path and save the cost.

2.Literature and data retrieval.

3.The recombinant plasmid expressing L-RhI and the gene cloning vector were constructed by using molecular biological methods such as genome extraction, primers design, PCR amplification, receptor cell transformation, recombinant vector screening, and recombinant plasmid PCR verification after transformation.

4.The enzyme convert ratio was determined by HPLC.

The practical significance of the results obtained are as follows.

Through this study, the catalytic mechanism of L-rhamnose-isomerase can be deeply understood, providing theoretical and practical basis for the development of efficient D-allose production methods. This research is of great significance for the development of sweetener, drug synthesis and related industries in the fields of food and biomedicine.

To sum up, the significance of this topic is to deeply understand the catalytic mechanism of L-rhamnose-isomerase in the carbohydrate metabolism pathway, construct efficient genetically engineered recombinant bacteria based on the corresponding coding genes, develop efficient, economic and environmental protection production technology, improve the yield and purity of D-allose, and reduce production costs, which has important theoretical and application value.

CHAPTER 1
LITERATURE REVIEW

1.1 Overview of rare sugars

Rare sugar is a naturally occurring, trace monosaccharide or its derivatives, which is characterized by low calories and is not easy to be absorbed by the human body, so it can be used as a low-calorie functional sweetener, some rare sugar health function is also extremely prominent, such as D-allose sugar, which plays an important function in various fields such as food, medical health; However, rare sugars are scarce in nature, and the extraction process is complex, which restricts the large-scale and commercial application of rare sugars; Therefore, the search for a suitable purification or preparation method is the hot spot currently.

1.2 D-allose overview

1.2.1 Structure of D-allose sugar

D-allose, a rare monosaccharide in nature, belongs to cis-hexalose, which is the aldose isomer of D-piscose and the differential isomer of D-glucose at the C-3 position1. Its relative molecular weight is: 180.16, the molecular formula is: $C_6H_{12}O_6$, and the melting point is: 128℃.

D-allose is a pentahydroxy aldehyde, which often exists in a ring form in solution, and can also exist in a straight chain form. There are two different conformations after the formation of the ring, one is the furan form of the five-membered ring, whose conformation is the hydroxyl condensation of the aldehyde group connected with the carbon atom at position 4, and the other is the furan form, whose conformation is the hydroxyl condensation of the aldehyde group connected with the carbon atom at position 5. After the formation of the semi-acetal hydroxyl group of the ring molecule, it can form the differential isomer of α- and β-.

D-allose is basically β-pyran type existing in natural carbohydrate compounds, D-allose is dissolved in water, and the proportion of β-D-pyran allose accounts for more than half (77.5%)19].

1.2.2 Physicochemical properties of D-allose

(1) Physical properties

D-allose is a chiral molecule, with optical activity, its rotation is [A]20+14° (c=1.00%, H2O), D-allose is non-toxic and tasteless, white crystal at room temperature, its melting point is 128℃, D-allose is easily soluble in water, insoluble in ethanol and other organic solvents.

(2) Chemical properties

D-allose is a reducing sugar, which can undergo the Maillard reaction.

Maillard reactions, also known as carbonyl-amino reactions and non-enzymatic browning reactions, are a series of complex chemical reactions between amino groups (such as proteins and amino acids) and carbonyl groups (such as reducing sugars), resulting in a brown-black substance (melanoid). These reactions affect the quality, appearance, odor and nutritional value of foods.

D-allose is not toxic. In 2010, Iga Y and other researchers conducted subchronic and acute experiments on D-allose in rats and tested that D-allose was non-toxic and harmless to rats2.

1.2.3 Biological function of D-allose

D-allose is a 0 calorie functional sweetener, but also has good health effects, its health effects are mainly reflected in:

(1) Anti-inflammatory effect: improve neurological dysfunction, reduce cerebellar infarct volume

Adding D-allose to the ischemic reperfusion injury model can effectively promote the recovery of nerve function in the injured brain of mice. The researchers speculate that Gal-3 may reduce the level of autophagy and the secretion of

inflammatory factors such as TNF-α and IL-8 by down-regulating its expression. Play a certain neuroprotective role3.

(2) Inhibit cancer and prevent the proliferation of cancer cells

D-allose can inhibit the proliferation and differentiation of a variety of malignant tumor cells in sufficient quantities, but its specific inhibitory mechanism has not been fully understood4, studies have shown that D-allose can inhibit the proliferation of liver cancer, oral cancer and non-small cell lung cancer cells in vitro under cell culture conditions. In addition, it has been shown to inhibit tumor growth in animals6.

(3) Synergies in cancer treatment

D-allose has synergies with traditional anticancer drugs, for example, it can reduce the non-specific cytotoxicity of 5-fluorouracil7.

(4) Immunosuppression

D-allose can inhibit the proliferation of segmented neutrophils in a dose-dependent manner and can decrease platelet counts without causing significant side effects8.

(5) Anti-oxidation

Previous experiments have verified that D-allose can inhibit high salt-induced hypertension and reduce the accumulation of superoxides in the aorta. Studies have shown that D-allose exhibits antioxidant capacity only in the presence of oxidative stress in vivo or in vitro, thereby clearing reactive oxygen species8.

(6) Other effects

D-allose has the function of cell freezing protection, which can be applied to organ transplantation surgery, etc.9.

1.2.4 Metabolic pathway of D-allose

This paper takes *Escherichia coli* as an example to elaborate the metabolic pathway of D-allose.

The cell membrane of *E.coli* contains transporters, through which D-allose enters the cytoplasm. There are three types of transporters responsible for transporting D-allose, namely, ABC transporter protein (AlsA), D-allose binding protein (AlsB) and transport element (AlsC), among which AlsA and AlsC constitute ABC transporters.

The specific transport and metabolic processes are as follows: D-allose binds to AlsB and enters the cytoplasm via ABC transporter. In the cytoplasm, D-allose undergoes three catalytic transformation into d-fructose-6-phosphate1, thus entering the glycolytic pathway; First, D-Alorosaccharide is catalyzed by D-Alorosine kinase (AlsK) to produce D-Alorosaccharide 6-phosphate; Then D-allose-6-phosphate can be produced by D-allose-6-phosphate isomerase (AlsI) under D-allose-6-phosphate; The latter eventually forms D-fructose-6-phosphate (Zhang Min et al.) under the action of D-Alallose-6-phospho3-differential isomerase (AlsE), which enters glycolysis for decomposition capacity.

1.3 L-rhamnose-isomerase

1.3.1 Overview of L-rhamnose-isomerase

L-rhamnose isomerase (EC 5.3.1.14, L-Rhamnose isomerase, L-RhI), is an aldoketo isomerase that shows rich substrate specificity and can catalyze the isomerization of a variety of sugars. Including the conversion of multiple pairs such as L-rhamnose, L-rhamnketose, and D-allose and D-piscose between pentanones and hexulose to produce products known as "rare sugars". L-RhI is becoming increasingly important. Plays a key role in the biosynthesis of uncommon sugars11.

Many studies have shown that the enzymatic reaction of L-RhI can be used to produce D-allose in a promising way. In this paper, the research progress on the structure, catalytic mechanism, microbial origin and enzymatic reaction properties of L-RhI at home and abroadwas reviewed, and the research progress on the production of D-allose and other rare sugars by L-RhI was summarized.

1.3.2 The structure of L-rhamnose-isomerase

In 2000, KORNDORFER and other researchers used the multiple isomorphic replacement method to solve the crystal structure of *E. coli* L-RhI.

The apparent molecular mass of L-RhI is about 188kDa. The enzyme is a single subunit structure composed of 420 amino acids, and its subunits are mainly composed of the N-terminal domain, the central domain and the C-terminal domain.

L-RhI in Escherichia coli is a polymerase, its spatial structure is a $4(\beta/\alpha)_8$-barrel tetracer, the central region is 8 α-helix and 8 β-fold formed $(\beta/\alpha)_8$-barrel central region; The N-terminal and C-terminal are connected by multiple α-helix fragments and disordered folded fragments to form a domain, which is involved in both the interaction between monomers and the formation of active sites12.

In comparison with the structure of other known isomerases, L-RhI was found to be most similar to D-xylose isomerase (D-XI). Comparing the sequences of the two enzymes according to the structure of the two enzymes revealed a 13% sequence identity that had not been detected so far. Suggesting that the two enzymes evolved from a common precursor.

At the same time, KORNDORFER and other researchers determined the complex structure of L-RhI with its inhibitor, L-rhamnol, as well as its natural substrate, L-rhamnose, suggesting that the disordered extended ring in the natural enzyme becomes ordered when the substrate binds, and that it may eliminate large amounts of solvents during catalysis. In the crystal structure of L-RhI (which is not necessarily the same in vivo), the L-RhI "structure" site binds to Zn^{2+}, and after the substrate binds, Mn^{2+} is simultaneously bound near the enzyme's "catalytic" site13.

1.3.3 The catalytic mechanism of L-rhamnose-isomerase

In 2010, Yoshida et al. performed X-ray crystal diffraction analysis of L-RhI in *Pseudomonas stutzeri* and reported its X-ray crystal structure, explaining its extensive substrate specificity.

For the aldehyde-ketone isomerization of L-RhI, a metal-mediated hydride shift mechanism has been previously reported, but the detailed catalytic mechanism is not fully understood14.

In order to elucidate the whole reaction mechanism, Yoshida et al. determined the X-ray structure of *P. stutzeri* L-RhI (S329K and D327N) in substrate/product complexes bound to the co-factor Mn^{2+} form and two inactive mutant forms of *P. stutzeri* L-RhI (S329K and D327N).

The Mn^{2+} bound enzyme structure showed that the catalytic site switches between the two forms as the metal ion shifts to recognize pyranose and furanose ring substrates. By resolving the structure of S329k-substrate conjugate, we can study the metal-mediated hydride displacement mechanism of L-RhI in detail.

Structural analysis and additional modeling of the D327n-substrate conjugate showed that Asp327 is responsible for ring-opening of furanosaccharides and that water molecules with metal ions are involved in ring-opening of pyranyosaccharides 15.

The reaction catalyzed by L-RhI catalyzes the tautomerization of aldehyde-keto groups, with the H of the substrate switching between C1 and C2. The second H is transferred between O1 and O2 11.

The catalytic center of the isomerase contains a metal ion, and L-RhI appears to follow the metal-mediated mechanism of 1, 2-hydride shift, a process of carbocation rearrangement that catalyzes metal ions to promote the displacement of protons from O2 to O1, creating C1's carbocation and hydrogen atoms in hydride form, Which in turn shifts from C2 to C1.

X-ray crystal diffraction analysis of Escherichia coli L-rhamnose-isomerase (ECRI) reveals the presence of two metal ions, one "structural" (M-1) to aid substrate bonding and the other "catalytic" (M-2) to aid hydride transfer, commonly thought of as Zn^{2+} and Mn^{2+}, respectively. The occurrence of aldehyde-ketone isomerism in ECRI is thought to be based on metal-mediated hydride shift, with the O1, O2, O3

atoms of L-rhamnose coordinating with M-1 (Zn^{2+}) and M-2 (Mn^{2+}). The catalytic amino acids Lys236 and Asp302, together with the catalytic metal ions (Mn^{2+}) and the catalytic water molecules, are thought to be synergistically involved in the aldehyde-ketone isomerization through the hydride shift mechanism 11.

1.3.4 Microbial origin and enzymatic properties of L-rhamnose-isomerase

(1) Microbial sources of L-RhI

According to research, *E.coli*, *Lactobacillus plantarum, Pseudomonas stutzeri, Bacillus pallidus* Y25, *Mesorhizobium loti Tono, Bacillus halodurans* ATCCBAA125, *Dictyoglomus turgidum* DSMZ6724, *Bacillus subtilis* ATCC 23857, *B.subtilis* str.168, *Thermobacilluscomposti* KWC4, *Caldicellulosiruptorobsidiansis* OB47 and *Clostridium stercorarium* generally exists in a variety of microorganisms such as L - rhamnose isomerase.

2 Enzymatic properties of L-RhI

1) Optimum temperature

In the process of catalyzing the isomerization of saccharide, the reaction temperature plays a key role. The reaction rate of aldehyde-ketone isomerization can be improved effectively at higher temperature, and the reaction can be shifted to the direction of producing D-allose. This result can increase the yield, reduce the viscosity of the reactant and increase the solubility. However, too high temperature can easily lead to non-enzymatic Browning and produce more by-products, resulting in more expensive costs.

L-RhI among different microorganisms exhibit optimal temperatures at higher temperatures (≥60℃), for example, *T. maritima* ATCC 43589[16], *C.saccharolyticus* ATCC 4349417 and *B. Subtilis* ATCC2385718, the optimal L-RhI temperatures were 60, 85 and 90 ℃, respectively. In 2010, Prabhu determined the optimal L-RhI temperature of *B.halodurans*, and the study showed that in the presence of 1mM Mn^{2+}, BHRI remained stable at 60℃ for more than 10h, and the activity remained

above 90% after 15h. However, at 70℃ and 80℃, the stability of the enzyme decreases sharply, with half-lives of 25min and 5min respectively11.

However, the tolerance of different strains to high temperature stress was significantly different. Previous studies have found that L-RhI obtained from strains such as *T.Aritima* ATCC 43589 has good heat resistance and is a catalytic enzyme for the production of D-allose with potential industrial applications 12.

In 2011, Prabhu reported a kind of L-RhI that could withstand high temperature. The team cloned it by *B.halodurans* gene and overexpressed it in *E.coli*. After isolation and purification, the optimal temperature was determined. It was found that this enzyme (BHRI) had a higher optimal temperature (70℃) than other reported L-RhI. Under the condition of 1mM Mn^{2+}, BHRI could maintain stable enzyme activity at 60℃ for more than 10h, and the activity remained above 90% after 15h. At 70℃ and 80℃, the stability of BHRI dropped sharply. The half-lives were 25min and 5min, respectively.

2) Optimum pH value

At present, the optimal pH of L-RhI from different microbial sources has been found to be biased towards moderate pH or higher pH. For example, the optimal pH of L-RhI from *B. halodurans* ATCC BAA-125, *C. saccharolyticus* ATCC 43494, and *T. saccharolyticum* NTOU1 is 7.0. At pH6.0, the relative enzyme activity of the second strain was reduced to 20%.

L-RhI from *M.loti* Tono and *P.stutzeri* reached 9.0 at optimal pH; In the BHRI reported by Prabhu et al., the activity pH of L-RhI ranged from 6.0 to 10.0, and its activity was greater than 75%. In summary, existing studies have shown that most L-RhI exhibit good catalytic activity under high pH and neutral conditions, but their catalytic activity is relatively weak under low pH conditions11.

In the large-scale biological method of industrial production of D-allose, the common method is enzymatic reaction, which is divided into two parts, that is, the use of D-psicose 3-epimerase (DPE) and L-RhI to catalyze. The optimal pH range of

D-piscose 3-aberration isomerase is between 7.5 and 9.0, while the optimal pH value of L-RhI is mostly 7.0 to 8.5.

Therefore, the pH range of the biological conversion of D-piscose should be the optimal pH range of the two enzymes, between 7.5 and 8.5. Too high pH will improve the non-specific Browning probability of D-piscose. While too low pH will be unfavorable to the H^+ dissociation of the active center in the L-RhI reaction 12.

3) Metal ions

Metal ions can bind to the active site of L-RhI enzyme, which is crucial for maintaining the spatial structure stability of aldehyde ketone isomerase. Previous studies have shown that L-RhI from most bacteria has a strong dependence on metal ions, and mainly Mn^{2+} and Co^{2+}.

4) Kinetic parameters of enzyme reaction

L-RhI in various strains has a common character -- it has a strong affinity with L-rhamnose, and can easily transform L-rhamnose into L-rhamnolose.

The substrate specificity of L-RhI in different bacteria is different. For example, *E.coli* L-RhI can catalyze L-rhamnose, L-lythose, L-mannose, etc., while D-ribose cannot be catalyzed. While L-RhI of *Pseudomonas stelleri* showed a wider substrate adaptability12.

KORNDORFER et al. analyzed the crystal structure of L-RhI and found that *E.coli*'s L-RhI has a hydrophobic pocket, which contains Val53, Ile105, Tyr106, Phe336. These amino acids result in a strict substrate specificity for the enzyme13.

Prabhu et al. performed a kinetic parameter analysis of L-RhI in *B.halodurans*, determining the initial velocity in a standard analytical mixture with a pH of 7. BHRI's turnover (kcat) for L-rhamnoose, L-mannose, L-lythreose, D-gulose and L-taloose were 8971, 5333, 12960, 437 and 254 min^{-1}, respectively. The catalytic efficiencies (kcat/Km) of BHRI for L-rhamnose, L-mannose, L-glucose, L-talose and D-glucose were 17, 45, 16.8, 2.4 and 2.9 min^{-1} mM^{-1}, respectively. For L-rhamnose and L-mannose, the turnover (kcat) of BHRI was comparable to the L-RhI of *P. stutzeri* (10300 and 5380 min^{-1}, respectively) and superior to the L-RhI of *B. pallidus*

(4,080 and 4,610 min^{-1}, respectively). The turnover rate (kcat) of L-lythreose was 2 to 7 times higher than that of *P. stutzeri* (6020 min^{-1}) and *B. pallidus* (1860 min^{-1}).Salt Bridges play important roles in protein structure and function, such as oligomerization, molecular recognition, allosteric regulation, α-helical capping, and stabilization of protein folded conformation. Utilize carboxylic acid oxygen atoms applied to Glu and Asp (i.e. OE in Glu or OD in Asp) and side chain nitrogen atoms of Arg, Lys or His (i.e. NE, NH1 and NH2 in Arg; NZ of Lys; His's ND1 and NE2) interatomic distance severing (3-4 A) to calculate the salt bridge, and analysis of the charged residues involved in the formation of the salt bridge revealed 11 charged residues in L-RhI (BHRI) in *B. halodurans*, 7 in L-RhI (ECRI) in *E. coli*, Five L-RhI (YPRI) in *Y. pestis*, making up 16, 10, and 9 salt Bridges respectively 11.

1.3.5 L-rhamnose-isomerase catalyzes the production of D-allose

The most ideal and economical way to produce D-allose by biological method is to catalyze the D-glucose difference to isomerization, to the C-3 differential isomer: The most ideal and economical way to produce D-piscose is that DPE catalyzes the differential isomerization of D-fructose to produce D-piscose, but the research reports have not yet found this enzyme. And then coupled with L-RhI to catalyze the keto isomerization of D-piscose to produce D-allose. In terms of product purification, it has been reported that CA-based separation resin can separate mixed solution of intermediate product and substrate (D-fructose) and mixed solution of product and intermediate product19.

In 2014, Han Wenjia et al. explored the method of efficient production of rare sugar, and proposed to improve the conversion efficiency of rare sugar by constructing a double enzyme coupled conversion system. *E.coli* BL21 pET-RDPE, a heteroexpressing host bacterium of DPE, and *E.coli* BL21 pET-21a-L-RhI, a heteroexpressing host bacterium of L-RhI, were cultured and expressed in Luria-Bertani (LB) medium, and the optimal transformation conditions were explored: DPE: L-RhI enzyme dosage ratio 1: 10 (the final RDPE concentration is 0.05mg/mL), the

optimal temperature is 60℃, the optimal pH9.0, under the condition that the concentration of D-fructose is 2%, the equilibrium is reached at the concentration ratio of this concentration for 10h, and the final concentration of D-piscose is 5.12g/L, and the concentration of D-allose is 2.04g/L. This study provides a theoretical and research reference for the production of high value-added rare sugar mixtures with low value-added and high yield fructose-rich HFCS. The reaction shows that the multi-stage enzymatic reaction of conversion of rare sugars by biological conversion can effectively convert low value-added monosaccharide substrates into high value-added and functional rare sugars. This study provides the research and application basis for the production of rare sugars such as D-piscose and D-allose by multi-stage enzymatic reaction20.

1.4 The research significance and content of this paper

1.4.1 Research significance

With the development of modern society, the living standard and material basis of the general public have been greatly improved. At the same time, due to the increasing pressure of life, people's sub-health conditions are becoming more and more serious. In recent years, people's attention to health care is increasing day by day, and rare sugar occupies an important position in functional health food. At the first International Sugar Association Congress in 2002, rare sugars were defined as monosaccharides or their derivatives, which are rare in nature21. At present, more than 50 rare sugars have been identified in natural products, such as D-trehalose, D-allose, D-tagose, L-Ribose, etc. Rare sugars are very important in the food, pharmaceutical and nutritional industries, and can be used to make low-calorie sweeteners, growth inhibitors to inhibit microorganisms, and leavening agents.

Rare sugar -- D-allose, has a wide range of biological functions such as anti-tumor, anti-oxidation, immune regulation, especially has a good effect on cancer suppression and adjuvant cancer radiotherapy and chemotherapy. The main

production methods of D-allose are chemical synthesis and biocatalysis, and the production of D-allose by biological enzyme has been widely concerned because of its many advantages. D-allose can be catalyzed by sugar isomerase, such as D-galactose 6-phosphate isomerase, D-ribose-5-phosphate isomerase, and so on.

However, since most enzymes have by-products, the isomerization of D-piscose catalyzed by L-RhI is the most direct way to produce D-allose. We cloned L-rhamnose-isomerase (L-RhI) gene *l-rhi* from *Bacillus subtilis* 168, and explored the construction of genetically engineered strains producing L-RhI and the expression of L-rhamnose-isomerase, which laid the foundation for the biosynthesis of D-allose.

Conclusions to chapter 1

Based on the principle and practice of molecular biology and genetic engineering, *l-rhi* derived from *Bacillus subtilis* 168 was cloned and *E.coli* BL21star(DE3) was used as the expression host to explore the expression of L-RhI. The expressed L-RhI was used as catalyst to further catalyze the production of D-allose. The specific research content can be divided into the following points:

1. Culture of donor strains: Take the preservation solution of *Bacillus subtilis* 168 strains, apply it to LB solid medium without antibiotics, and culture it at room temperature 37℃ overnight; Single colonies with good growth were selected and transferred to LB liquid medium without antibiotics, and cultured overnight in a shaking bed at 37℃. After the bacterial strains propagated overnight, they were purified by plasmid extraction kit and their DNA content was determined.

2. Amplification of *l-rhi*: L-RhI gene sequence *l-rhi* was obtained from NCBI, and the amplification primer pair was designed. Using the genome of Bacilius subtilis 168 as the template, the PCR reaction system was configured to amplify the *l-rhi* fragment.

3. Enzyme digestion reaction: restriction endonuclease NdeI enzyme digestion of expression vector pET-22b and *l-rhi* gene sequences.

4. liquid recovery of enzyme digestion products: about 6h after the enzyme digestion reaction, the liquid recovery of enzyme digestion products was carried out, and its content was detected by micro nucleic acid analyzer, and it was stored in the refrigerator at -20℃.

5. Construction of recombinant plasmid: The recombinant system of Exnase®II enzyme (Vazyme Biotech Co.) was used to reconstruct the gene and carrier fragments. After configuration of the system, the reaction was performed at 37℃ for 30min, and the ice bath was immediately taken for 5min.

6. Recombinant plasmid transformation of *E.coli* DH5α : *E.coli* DH5α cells were added with 10μL of connecting solution in 50μL and placed under ice for 30min; Then the temperature was raised in a water bath at 42℃ for 45s, and then in an ice bath for 2 minutes, 500μL of antibiotic-free LB medium was added, cultured at 37℃ for 1h, and 50μL of the conversion solution was coated on LB-AMP solid state plate at 37℃ overnight.

7. Colony PCR verification of the inverters: About 10 individual colonies were selected and 10μL ddH2O was added. Using 1μL as a template, colony polymerase chain reaction was used to verify the conversion effect; LB solution containing Amp was transfected with positive transformants and was shaken overnight at 37℃ at 200r/min. Finally, the plasmid was sequenced and identified as pET-22b-*l-rhi*.

8. Transformation of recombinant plasmid pET-22b-*l-rhi* to *E.coli* BL21star(DE3) : *E.coli* BL21star(DE3) cells in receptive state were induced by CaCl2 method, and the recombinant vector was transformed into *E.coli* BL21star(DE3).

9. Inducing the expression of the target protein L-RhI: Different experimental groups were designed and incubated in the bacterial solution at different temperatures and IPTG concentrations to express the target protein. SDS-PAGE was configured to test whether the target protein was expressed and its concentration.

CHAPTER 2
OBJECT, PURPOSE, AND METHODS OF THE STUDY

2.1 Introduction

D-allose is an important rare sugar, and in the current study, the known efficient method for the production of D-allose is to use L-rhamnose isomerase as a catalyst and D-allose as a substrate to catalyze the interconversion of aldehydes and ketones to produce D-allose.

In this chapter, the heterologous expression of L-RhI derived from *Bacillus subtilis* 168 was used to obtain a high purity of free enzyme L-RhI, and the convert ratio of L-RhI could be determined to obtain D-allose as a substrate.

2.2 Experimental materials

2.2.1 Strains and plasmids

The strains used in this experiment were: the source strain of the target gene was *Bacillus subtilis* 168, which was derived from the laboratory-preserved strain, the cloned strain was *E.coli* DH5α, which was mainly used for the construction and amplification of plasmids, the expression strain was *E.coli* BL21star (DE3) for the expression of the target gene, and the plasmid vector was pET-22b. The plasmid and strain sources of this experiment are shown in the following table(Tab. 2.1).

Table 2.1-Bacterias and plasmids used in this chapter

Strands or Plasmid	Sources
Strands	
B.subtilis 168	Laboratory preservation
E.coli DH5α	TransGen
E.coli BL21star（DE3）	Laboratory preservation
Plasmid	
pET-22b	Laboratory preservation

2.2.2 The main reagents and culture media

Preparation method of the main reagent

(1) PBS buffer:

specification: pH 8.0, 20mM, 100mL

method: 5.3mL, 20mM NaH2PO4, 94.7mL 20mM Na2HPO4.

(2) 5× Loading Buffer:

Method: 60mM Tris-HCI(pH 6.8), 25%glycerol, 2%SDS, 0.1%Bromophenol blue.

(3) 5×Tris-GlycineRunning buffer:

Method: Dissolve 5g of SDS, 72g of glycine, and 15.1g of chloroform in 1000mL of water.

(6) Coomassie Brilliant Blue Staining Solution:

Preparation method: 1.0g Coomassie Brilliant Blue R-250 1.0 g, 450mL methanol, 450mL single distilled water, stir the above three reagents until dissolved, and add 100mL glacial acetic acid.

Preparation method of culture medium

(1) LB medium (liquid):

Preparation method:10g/L peptone,5g/Lyeast,10g/Lsodium chloride.

After sterilization at 115°C for 30 minutes, 20g/L agar powder was added for sterilization8.

2.2.3 Main instruments and equipment

The instruments and equipment used in this study are as follows (Tab. 2.2).

Table 2.2-The instruments used in this study

Instrument	Manufacturers	instrument model
Incubator	Weifang medical devices	WMK-08
Cryogenic centrifuge	Shanghai Lishen	Neofuge 23R
Combined full-temperature oscillator	Haocheng experimental instrument	HCY-123B
Induction	Midea appliances	C21-ST2118
Refrigerator	Zhongke Meiling	BCD-218ZM2D
Ultrasonic cleaner	Hangzhou Farrant	KQ-50B
Circulating water type multi-purpose vacuum pump	Shanghai Long March	SHB-Ⅲ
Electronic balances	METTLER TOLEDO	AL104
Thermostatic water bath	Shanghai Jinghong	DK-S24
Gel imager	Bio-Rad	Gel Doc XR
Thermal cycler	Eppendorf	22331
Electrophoresis apparatus	Beijing 61	DYY-11B
Nucleic acid quantifier	Eppendorf	Bio Photometer Plus
pH meter	Thermo orion	Thermo orion
Vacuum drying oven	Shanghai Senxin	DZG-6020

2.3 Experimental Methods

2.3.1 Extraction of the genome and amplification of the coding genes

（1）Acquisition of the genome of *Bacillus subtilis*

Coat the glycerol preservation solution of *B. subtilis* 168 on a non-resistant LB solid medium at 37 °C and incubate overnight.

On the second day, the cultured LB solid medium was taken out, the single colonies that grew out were observed, the vigorous single colonies were picked, and they were inoculated into the non-resistant LB liquid medium, and incubated at 37 °C at 200 r/min overnight.

The next day, 1mL of culture medium was taken with a pipette, added to a centrifuge tube, centrifuged at 10000r/min, and centrifuged at 4°C for 5min to collect the bacterial pellet.

Digestion buffer and protease solution were added to the pellet, resuspended until the cells were completely lysed, GD buffer was added to remove protein impurities in the solution, and pure water was added to obtain genomic DNA.

The concentration of the extracted genomic DNA of *Bacillus subtilis* 168 was determined with a micronucleic acid analyzer, and the DNA was preserved in a 20 °C freezer for later use.The specific extraction steps are shown in the Bacterial Genome Extraction Kit.

（2）Amplification of genes encoding *Bacillus subtilis*

Search L-RhI gene sequence of *B.subtilis* 168 by NCBI（Gene ID: 938836），and design PCR forward primer and reverse primer.

The primer sequences are as follows(Tab. 2.3).

Table 2.3-Primers used in this chapter

primer	forward primers（F）	reverse primers（R）
B.S-L-RhI	5'CGGCAGCAGGTATTTCATATGATGACCATAAAAGCCAATTATGACA3'	5'TAAGAAGGAGATATACATATGTTAGACAATCGGAGAAGATGCCT3'

Pay attention to the design principles of the primers：

Look for one or two restriction enzyme sites that cannot be present in the gene of interest.

Upstream primers: primer base + restriction enzyme recognition sequence + 18bp or so sequence of the positive strand of DNA 5' of the target gene, downstream primers: primer base + restriction enzyme recognition sequence + reverse complementary sequence of about 18bp of the positive strand of DNA of the target gene 5'.

The restriction enzyme site is as close to the target gene as possible, and it is best to use different enzymes for the two enzyme sites.

Using the genome of B. subtilis 168 strain as a template, the PCR reaction system was configured, the *l-rhi* fragment was amplified, and the configured PCR system is shown as follows(Tab. 2.3).

Table2.3-PCR system for encoding gene of amplification

reagent	volume
5×PrimeSTAR Buffer(Mg^{2+} plus)	10μL
dNTP Mixture(2.5mM each)	4μL
forward primer(10 μM)	1μL
reverse primer(10 μM)	1μL
Bacilus subtilis 168 genome	1μL
DNA Polymerase	1μL
ddH_2O	32μL
gross volume	50μL

PCR amplification conditions: Pre-denaturation 95℃,3min;Denaturation 98℃,10min;Extinguishing 58℃,30sec;Enlongating 72℃,55sec;30 circulate;72℃ Enlongating 10min;12℃ heat insulation.

2.3.2 Validation of amplification products

After PCR amplification, electrophoresis was performed to determine the amplification of the gene of interest, and finally the gene product of interest was collected with a gene purification kit。

2.3.3 Construction of recombinant vectors

（1）Plasmid-containing strains were activated

The cloned vector strain used in this study was *E.coli* DH5α. First, *E.coli* DH5α containing the cloned plasmid vector was activated.

The DH5α stored in the laboratory was removed and placed at room temperature for a period of time to melt, and then transferred to a super-clean workbench for strain transfer.

After high temperature steam sterilization, the pipette head was allowed to absorb 50μL bacterial solution, and the bacterial solution was injected into the surface of Amp resistant LB solid medium, and the outer flame of the alcohol lamp was used to burn the coaster. After sterilization and cooling, the *E.coli* bacterial solution was inoculated, sealed with sealing strips, and the date and name were

marked, and the DH5α was inverted in a constant temperature incubator at 37℃ and cultured overnight.

Take out the LB-AMP plate cultured overnight, the ultra-clean workbench is sterilized by ultraviolet irradiation, and then the plate is placed on the ultra-clean workbench, and the inoculation ring is burned by the external flame of the alcohol lamp. After cooling, the single colony with better growth state is selected, and the colony is coated on the LB solid medium containing Amp resistance by the plate scribing method, and the sealing strip is sealed. Mark the date and name with a black marker, place it in a constant temperature incubator at 37 ° C, culture it overnight, and continuously carry out the above contents 2-3 times to restore the activity of frozen bacteria.

Culture strain, improve strain activity and concentration: Take the latest activated medium, sterilize the ultra-clean workbench with ultraviolet light, sterilize the outer flame of the inoculation ring alcohol lamp, select the single colony with good growth condition with it or the pipette head after high temperature steam sterilization, inoculate the single colony in 200mL of LB liquid medium containing Amp resistance, and culture overnight in a shaking bed of 200 r/min at 37 ℃.

(2) Plasmids purification

Take out the culture medium of *E.coli* DH5α containing pET-22b cultured in the previous step.

The plasmid was purified by referring to the steps of plasmid small extraction kit. After extraction, the extracted pET-22b of 2μL was taken and placed on the detection table of ultra-micro nucleic acid protein analyzer to detect the content of pET-22b, the data was recorded, and pET-22b was stored at -20℃ for subsequent use.

(3) Enzyme digestion of plasmid

l-rhi gene sequence contains Nde Ⅰ cleavage site, so in this experiment, we used restriction endonuclease Ⅰ single enzyme cleavage vector plasmid pET-22b, and incubated at 37℃ for 6h to ensure complete cleavage(Tab. 2.4).

Table 2.4-Mononuclease reaction system

reagent	volume
Nde Ⅰ	1μL
10×buffer	2μL
pET-22b	≤1μL
ddH_2O	up to 20μL

(4) Recovery of plasmid carrier digestion products

After the end of enzyme digestion, the plasmid and the target gene are usually recovered by a universal DNA recovery kit, and the pET-22b enzyme digestion products extracted in the previous step and the *l-rhi* enzyme digestion products of the target gene are recovered by liquid recovery. But we choose enrichment and recovery by EC columnin to enhance the recovery rate in this study.

In this article, in order to improve the concentration of DNA recovery, we use the EC adsorption column enrichment nucleic acid recovery and purification method, so that the enzyme digestion solution through the adsorption column purification recovery operation treatment, the use of dideoxy distilled water during elution.

For the recovered products, the use of trace nucleic acid analyzer to determine the concentration of DNA, the purified plasmid and the target gene in the -20℃ refrigerator storage, standby, easy to follow the connection.

(5) Construction of recombinant plasmid

The *l-rhi* fragment was recombined with the linearized vector recovered after digestion. In order to prevent the inactivation of the recombinant enzyme, the recombinant system of Exnase®II enzyme (Vazyme Biotech Co.) should be configured in an ice water bath. In order to prevent the self-linkage of the vector and the target gene, the recombinant enzyme should be added at the end. The system in which the reagents were added in turn was placed in a centrifuge for a short period of centrifugation, and the reactants were mixed to make the reaction more complete. Finally, the system was incubated overnight at 22℃ in the PCR instrument for about 16 hours(Tab. 2.5).

Table2.5-Exnase® Ⅱ enzyme recombination system

reagent	volume
5×CE Ⅱ Buffer	4μL
linear vector	50-200ng
insertion element	20-200ng
Exnase® Ⅱ	4μL
ddH2O	Up to 20μL

On the second day, refrigerate the incubated recombinant plasmid linking solution at -20 ℃ in the refrigerator.

2.3.4 Preparation of Escherichia coli receptor state

In this chapter, the receptive cells of Escherichia coli DH5α were constructed by $CaCl_2$ method.

(1) Solid LB medium was prepared.

(2) Remove the *E.coli* from the refrigerator at -20℃, melt it on the ice, and then put it on the ultra-clean work table after ultraviolet sterilization, burn the inoculation ring with alcohol lamp, and inoculate the *E.coli* liquid on the LB solid medium with the method of plate marking, and then seal the sealing paper, and culture it inversely at 37℃ in the constant temperature incubator for 12-16 h.

(3) Configure the liquid LB medium and add 2mL liquid LB medium into the 10mL centrifuge tube.

(4) The cultured Escherichia coli dish was removed from the shaking table, and a single colony was found, inoculated in LB liquid culture medium, and incubated at 37℃ overnight.

(5) The overnight culture medium was taken out and mixed with 50 mLLB liquid at the ratio of 1:50. The culture was carried out in the shaking table at 37℃ 200r/min for about 4h until *E.coli* reached the logarithmic growth stage (OD600=0.3~0.5).

(6) Ultraclean workbench was sterilized by ultraviolet light. 1mL of *E.coli* bacteria liquid in the logarithmic growth stage was absorbed on the ultraclean workbench and injected into 1.5mL EP tube after sterilization. The EP tube was placed on ice for 20min to maintain the permeability of cell membrane.

(7) After the end of resting, put the EP tube into the centrifuge, centrifuge at 5000r/min for 10min, collect the precipitation of bacterial cells, discard the supernetting of LB liquid medium, take the sterile 0.1mol/L CaCl2 into the ice to cool, take 100μL solution to re-suspend the bacterial precipitation, and use the pipette to gently blow to achieve the effect of re-suspension. The re-suspension was placed on the ice for 20min, centrifuged at 5000r/min for 10min, and the centrifuge supernatant was abandoned, that is, the first CaCl2 treatment was completed.

(8) Sterile 0.1mol/L CaCl2 containing 10% glycerol was placed on ice for cooling, and 100μL of suspended bacteria were precipitated, that is, the preparation of Escherichia coli receptive state was completed, and the receptive cells were stored in a -20℃ refrigerator.

2.3.5 Construction and screening of recombinant strains

(1) Plasmid transformation of the susceptible *E.coli* DH5α was performed

Adding the ligating solution: Take the configured recombinant plasmid connecting solution out of the refrigerator at -20℃ in advance, and then place it at room temperature for thawing. When the connecting solution melts, take out the E.OLI receptor cells pre-configured in the previous step from the refrigerator, and quickly thaw them in the ice cube, 5min later, until the massive bacteria melt, and immediately operate on the super-clean work table: Use pipette gun to add 10μL recombinant plasmid connecting solution to *E.coli*, dial the bottom of the centrifuge tube by hand, mix the connecting solution with *E.coli* solution, and stand in ice for 25-30min.

Heat shock and ice bath: After standing, put the bacterial solution into the 42℃ constant temperature water bath for 45s, and then quickly insert it into the ice, ice

bath for 2min. This step should avoid violent shaking to prevent the reduction of conversion efficiency.

Add LB liquid medium and resuscitate: Add 500μL of antibiotic-free LB liquid medium into the centrifuge tube after the ice bath, mix well, and put it into the metal bath shaking table at 37℃ at a rotational speed of 200r/min, and resuscitate for 1h.

Centrifugation and coating of thalli: After the end of recovery, centrifuge under 5000r/min, centrifuge for 1min, sedimentation of bacteria, pipette 500μL superclear discard, mix with oscillator, or use pipette tip gently blow heavy suspended bacteria 2-3 times, the remaining about 100μL bacterial liquid into the ultra-clean table, the coater into the alcohol lamp flame burning, sterilization, plate coating method, The bacterial solution is coated in LB-Amp solid medium.

The inoculated petri dish is sealed with an airtight strip, marked with date and name on a plate with a black marker, inverted, placed in a 37℃ constant temperature incubator and cultured overnight.

(2) Transformant culture and PCR verification of the colony

The target plasmid for PCR amplification: On the second day, the overnight culture medium was taken out, and 6 positive single colonies were selected for observation, and then inoculated into the resistant LB liquid medium overnight. The transformation was verified by PCR on the second day. First, in order to avoid false positive results, we extracted and purified the plasmid first, and re-designed the upstream and downstream primers, wherein the upstream primer template was the upstream sequence of the positive plasmid Nde Ⅰ chain, and the downstream primer template was the reverse complementary sequence of the positive chain of the Nde Ⅰ + target gene. The sequences are as follows (Tab. 2.6).

Table 2.6-Primers used in this section

Primers	Sequence
Forward primer	5'ACTTTAAGAAGGAGATATACATATG3'
Reverse primer	5'CATATGTTAGACAATCGGAGAAGATGCCT3'

Purify the plasmid according to the instructions of the plasmid small extraction kit, take 2μL of the plasmid and put it into the ultramicro nucleic acid protein analyzer, determine the concentration of the mass particle, and make a record.

We amplified the extracted vector and put it into agar-gel for electrophoresis. The molecular weight of the vector was tested here. Compared with the estimated value (6768bp), the error-free plasmid was the desired plasmid pET-22b-*l-rhi*.

(3) Transformation of pET-22b-*l-rhi* into Escherichia Coli BL21star(DE3)

The receptive cells of *E.coli* BL21star(DE3) were prepared by the same method, stored at -20℃, removed when needed, and melted on ice for 5min.pET-22b-*l-rhi* recombinant plasmid was added into the receptive cell suspension, mixed with a light spinning centrifuge tube, and placed in an ice bath for 30min.

Put the EP tube into a 42℃ water bath to heat shock for 60s, and then quickly move the EP tube to the ice bath to cool it for about 2min. During this period, avoid shaking, otherwise the conversion efficiency will be adversely affected.

500μL of antibiotic-free LB medium was added to each centrifuge tube, mixed well, and resuscitated in a shaker at 150r/min at 37℃ for 60 min.

The plates and bacteria solution were placed on ultra-clean work table irradiated by ultraviolet light, and appropriate amount of culture solution was added into LB solid culture dish containing corresponding antibiotics. The bacteria coater or glass column burned with alcohol is evenly coated next to the alcohol lamp.

After all the bacterial solution is absorbed, the plate is turned upside down and cultured at 37 ° C for 12 to 16h.

The rest of the culture solution was placed in the refrigerator at 4℃, and the preservation was determined according to the growth of bacteria on the medium.

2.3.6 Expression and purification of L-rhamnose-isomerase

(1) Induced expression of L-RhI protein

The single colony of positive BL21star (DE3) after transformation of recombinant plasmid was selected and placed in 5mL of LB liquid medium

containing Amp resistance, and cultured overnight in a shaking bed at 150r/min at 37℃.

After the culture was completed, 1mL of bacteria solution was added into 50mL LB liquid medium for expansion culture, and then the culture was shaken in a shaking table at 37℃.

After about 3h culture, 1mL BL21star (DE3) bacterial solution should be taken out every 30min with a pipette and put into a spectrophotometer to detect the optical density value (LB-Amp liquid medium without bacterial solution in the same group was used as a blank control to ensure that its light absorption value was a parameter for correctly measuring the growth of *E.coli*). When the optical density reached about 0.6, BL21star (DE3) reached the logarithmic growth stage, and L-RhI induction could be performed at this time.

IPTG was added to the BL21star (DE3) bacterial solution whose optical density reached the required value, so that the final concentrations of IPTG were 0.1, 0.5 and 1.mM, respectively.

Then the experimental groups with different IPTG concentrations were placed in a shaker at different temperatures (16℃ and 37℃) and cultured for about 6h. In order to judge whether IPTG successfully induced protein expression, a control group test should be set up. 7 ml of bacterial solution was pre-removed from each experimental group, and IPTG was not added as a blank control.

After collecting the bacterial solution, centrifuge it for 5mmin at 5000r/min at 4℃, and then discard the supernatant.

The bacterial sediment was washed with 3 mL of PBS buffer. After the bacterial solution is repeatedly blown to suspension state, it is placed in the centrifuge, the rotational speed is 5000r/min, centrifuge for 5min, and discard the supernatant. This process was repeated 3 times, and finally PBS was added to suspend the bacteria again.

PBS buffer solution (Phosphate buffer saline) is commonly used in biochemical and molecular biology research, and its main components are KCl, NaCl, KH_2PO_4 and Na_2HPO_4.

The latter two have a wide range of pH buffers with secondary dissociation; The first two can increase salt ion concentration.

Place ice from an ice maker in a small beaker and insert the centrifuge tube vertically into the ice. The cell breaking operation was performed for 35min using an ultrasonic cell crusher.

It should be noted that the transformer rod should be inserted into the bacterial solution, about 1cm away from the bottom of the centrifuge tube, and should not touch the wall of the cup.

Pay close attention to the position of the centrifuge tube during the crushing process to prevent ice melting and changes in the position of the centrifuge tube from affecting the crushing effect.

A foam raft can also be used to secure the centrifuge tube in the ice water bath. Bubbles should be prevented during crushing, and the bacterial solution should become clear and transparent after crushing.

Centrifuge the broken bacterial solution for 5min (10000r/min), take the supernatant containing L-RhI with a pipette and inject it into a new EP tube for later use.

(2) SDS-PAGE electrophoretic analysis and verification of target protein

Prepare protein samples: Absorb 16μL of each superserum containing cellular protein obtained in the previous step (blank control group and experimental group), put them into EP tube, add 4μL of 5×SDS Sample Loading Buffer (including SDS, glycerol, TRIS and bromophenol blue). Heat it in a water bath for about 10 minutes until the protein is denatured by boiling; After boiling, it is cooled at room temperature until it is the same as room temperature.

Then it is placed on the centrifuge for a short time to mix the solution and wait for the subsequent electrophoresis of protease.

Sampling: The SDS-PAGE protein gel is preconfigured, and then placed in the electrophoresis tank, adding the protein electrophoresis buffer, so that it is completely over the gel plate. After taking out the comb in the prefabricated gel and exposing the spot tank, the protein electrophoresis operation can be performed.

Draw 10μL Maker and inject into the spot sample tank. When injecting, pay attention to the tip of the gun slightly hanging over the notch to avoid puncturing the notch and causing the solution to flow out. At the same time, the sample tank should be properly positioned to avoid the sample dispersing into the buffer. Then use the pipette gun to absorb the 20μL induction group sample and inject it into the subsequent tank. Finally, 20μL blank control group protein samples were absorbed and injected into the adjacent point sample tank.

Electrophoresis: The voltage was adjusted to 70V for the concentrated electrophoresis of the glue. Until the protein forms a straight line at the junction of the separation glue and concentrate glue, change the voltage to 135V and continue running glue until the bromophenol blue indicator strip reaches the lower edge of the glue.

Bromophenol blue can be used as an electrophoresis indicator. Its color is blue in the solution. When added to the electrophoresis buffer solution, it can move with the electric field, because its relative molecular weight is smaller than most proteins, so the electrophoresis speed is faster. When bromophenol blue electrophoresis reaches the bottom of the tank, it can be regarded as the end of electrophoresis.

For dyeing: Wear gloves and carefully remove the gel from the electrophoresis tank to avoid damaging the gel; Peel back the plastic sheet. Place the egg white gel in the stain dish and add just enough Coomassie bright blue to soak the gel in it well. Shake in the shaker, so that the two substances are fully in contact, and dye for about 1h. After finishing, use a small flask to collect the Coomassie bright blue dye.

For decolorization: Pour a certain amount of decolorization solution, still in the shaker shock condition for about 2h decolorization, and then change the decolorization solution, continue to shock decolorization overnight.

For imaging: Place the decolorized gel in the Bio-Rad gel imager, take pictures, and save the imaging results.

2.3.7 Determination of L-rhamnose-isomerase activity

enzyme activity, also known as enzyme activity, is the ability of an enzyme to catalyze a certain chemical reaction. Under certain conditions, it can be expressed by the reaction velocity of a certain chemical reaction catalyzed by it22.

The unit of enzyme activity is the amount of enzyme required to catalyze 1 μmol D-piscose to produce D-allose per min in the supernatant of 1mL broken enzyme under the optimum enzyme activity conditions.

Substrate Select a solution containing 10g D-allose per liter (pH 9.0), take an appropriate amount of solution, preheat to 70℃, and add an appropriate amount of enzyme solution. Under the condition of 70℃, the reaction was carried out for 10min.

The sample was boiled for 10min for enzyme inactivation treatment, and then the treated sample was centrifuged in a 10000r/min centrifuge for 10min. After centrifugation, the product was filtered and then analyzed by high performance liquid chromatograph (HPLC) for sugar content.

Conclusions to chapter 2

1. In this chapter, the heterologous expression of L-RhI derived from *Bacillus subtilis* 168 was used to obtain a high purity of free enzyme L-RhI, and the convert ratio of L-RhI could be determined to obtain D-allose as a substrate.

2. This chapter lists the strains, plasmids, experimental reagents, experimental equipment and experimental methods used in the experiment.

3. The exquisite steps are as follows: Extraction of the carrier plasmids, amplification of the wanted gene sequence of B. subtilis, purification of the wanted plasmids and gene sequences by EC column enrichment, connection between plasmids and gene sequences by recombinase system, transformation of recombinant plasmids into host bacteria(E. coli DH5α), cultivation and screening of transformed

bacteria, transformation of appropriate plasmid pET-22b-*l-rhi*, expression of L-RhI protein, detection of the convert ratio of D-allose.

CHAPTER 3
EXPERIMENTAL PART

3.1 Genome extraction and target gene amplification

Bacillus subtilis 168 used in this study was kept in the laboratory. The strain was cultured without resistance in solid and liquid to rejuvenate it. After overnight culture, the culture medium was taken out and obvious turbidity was found in the bacterial liquid, which initially indicated that *Bacillus subtilis* had been effectively expanded.

Before extracting the target gene, we first placed the liquid medium containing the target strain at room temperature for 4 hours, so that the bacteria precipitated at the bottom of the conical bottle, and then absorbed the bacterial solution from the bottom of the conical bottle, so that the number of bacteria in the culture medium absorbed by the pipette will be more.

The 1mL bacterial culture liquid was moved to the centrifuge tube for centrifuge precipitation, the bacterial sedimentation coefficient was large, and the rapid sedimentation occurred in the centrifuge field, and then the genome extraction kit was used for bacterial cracking and nucleic acid extraction. In terms of elution, we chose 30μL ddH2O at 65℃ for multiple elution, firstly because ddH2O at 65℃ can improve the elution effect, and secondly because pure water can effectively promote the subsequent enzyme shear and recombination effect.

After extraction, the concentration was determined in the micro nucleic acid measuring instrument, and its absorbance value was determined. The absorbance under $A_{260/280}$, $A_{260/230}$ and A_{260} all belong to the standard indicators, and it can be inferred that the genomic DNA extracted by this step is pure.

Enter the official website of NCBI, retrieve L-RhI sequence of *B.subtilis* 168, design primers accordingly, send them to the company for synthesis, and amplify accordingly. The primer we designed here was a single enzyme cut primer, and its recognition site was Nde Ⅰ: 5’ CATATG 3’.

According to (2.3.2) above, the relative molecular weight of *l-rhi* sequence was verified by 0.8% agar-gel electrophoresis, and the following results were obtained (FIG. 3-1). It can be seen that there is a bright amplification band at about 1300bp in the figure, which is basically consistent with the estimated molecular weight of 1275bp(Fig. 3.1).

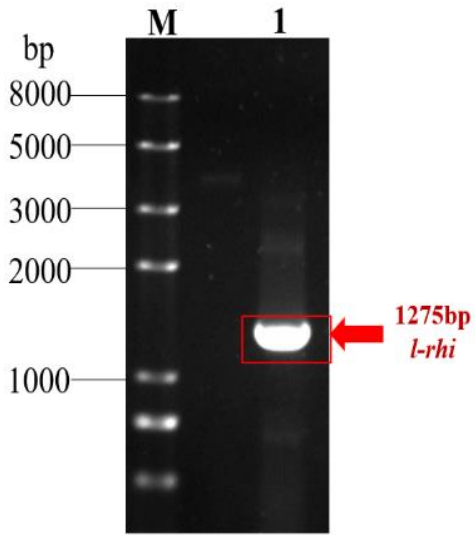

Figure 3.1-PCR electrophoresis plot of the *l-rhi* gene

3.2 Construction of recombinant vectors

The plasmids were purified by standard operation according to the instructions of the small plasmid extraction kit. After extraction, about 2μL of plasmid solution in the tube was aspirated and its concentration was determined. The empty plasmid was digested by restriction enzyme Nde Ⅰ. After digestion, the digested product was stored at -20℃ in freezer.

Purification of nucleic acids was performed using EC adsorption column enrichment. The restriction enzyme products of the plasmid and the target gene were removed and melted at room temperature, and the two were respectively poured into the EC adsorption column for enrichment. During elution, 50μL ddH2O was also used to improve the washing effect and facilitate the subsequent recombination step.

The l-rhi gene was ligated with the vector pET-22b by recombination, and the recombinant double chain loop vector Pet-22b-L-Rhi was finally obtained. Build method as shown in figure 3-2.

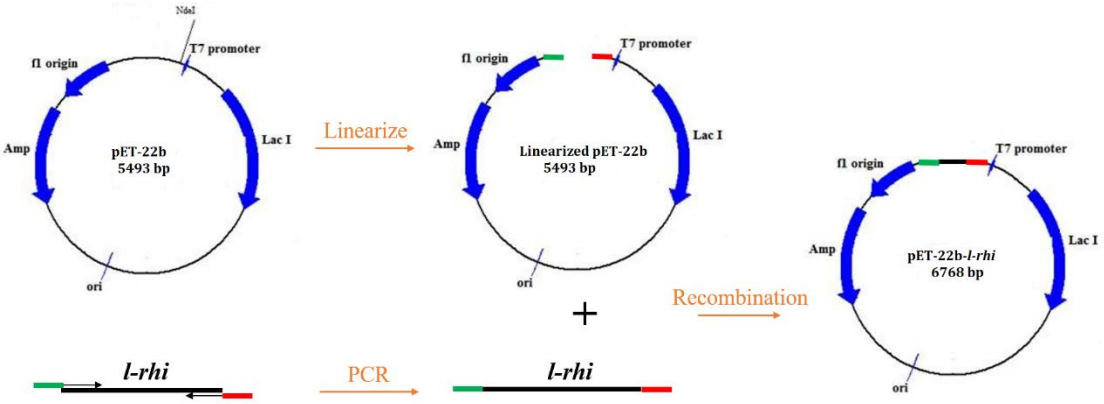

Figure 3.2-Plasmid pET-22b-*l-rhi* structure diagram

3.3 Construction and screening of L-rhamnose-isomerase clone host

Recombinant plasmid was used to transform *E.coli* cloning vector DH5α, and LB-Amp resistant solid medium was used for screening. Single colonies were selected the next day, and 6 positive colonies with good culture conditions were selected, and the single colonies were transferred to LB-Amp liquid medium and cultured overnight in a constant temperature shaking bed at 37℃and 200r/min.

The plasmid was extracted according to the instructions of the plasmid small extraction kit, and the purified plasmid carrier was selected for further polymerase chain reaction, and after agarose electrophoresis

To verify the recombinant effect and transformation results. In order to avoid genome interference amplification results of *E.coli* DH5α, we used the strategy of separately designing upstream and downstream primers from different sources to avoid interfering genes and reduce the probability of electrophoretic false positive. Plasmid genes were used for upstream primers and target gene sequences were used for downstream primers.

The results of gel electrophoresis after amplification were shown as follows (Fig. 3.3).

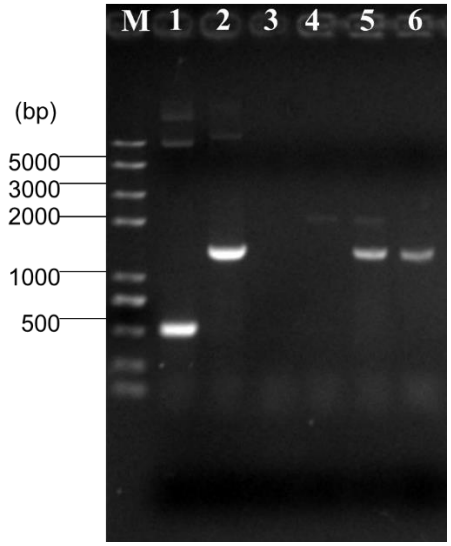

Figure3.3-Results of electrophoresis of recombinant plasmids

3.4 Induced expression and purification of the target protein L-RhI

The recombinant plasmid verified as positive was transferred to the expression strain. The expression vector selected in this study is *E.coli* BL21star(DE3), which has the advantage that the host bacteria contains rnE131 gene mutant (RNaseE) that can enhance the stability of intracellular mRNA. The mutation of these genes can reduce the expression level of intracellular Rnase, thereby improving the stability and content of mRNA. Therefore, the expression level of the target protein can be effectively improved.

BL21star (DE3) was resuscitated, transformed and induced by isopropyl -β-D-thiogalactoside. Finally, bacterial sedimentation, ultrasonic crushing and protein centrifugation were carried out. Finally, protein supernatant was obtained, and protein expression and expression levels were detected by electrophoresis of sodium dodecyl sulphate-polyacrylamide. To obtain clear protein electrophoresis without induction treatment or treated with IPTG at different concentrations and at different temperatures, as shown as follows. As can be seen from the figure, isopropyl-β-D-thiogalactoside has the best induction effect at 16℃, 1.0mM(Fig. 3.4).

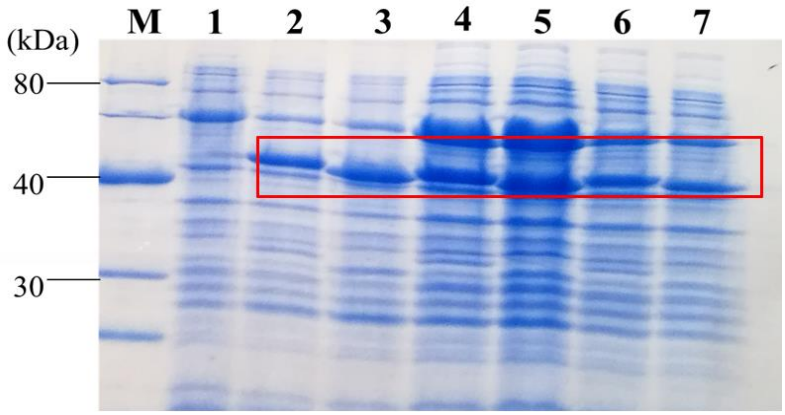

Figure 3.4-electrophoresis plot of L-RhI

3.5 Determination of L-RhI conversion

With 10 g/L D-piscose solution (pH 9.0) as the substrate, 9 mL of substrate solution was preheated at 70 ℃, and 1 mL of enzyme solution was added. After 4 hours of reaction at 70 ℃, the reaction was inactivated by boiling for 10 min, the sample was centrifuged at 10000 rpm for 10 min, and the sugar content was analyzed by HPLC. The conversion of L-RhI isomerized D-allose was determined to be approximately 28%.

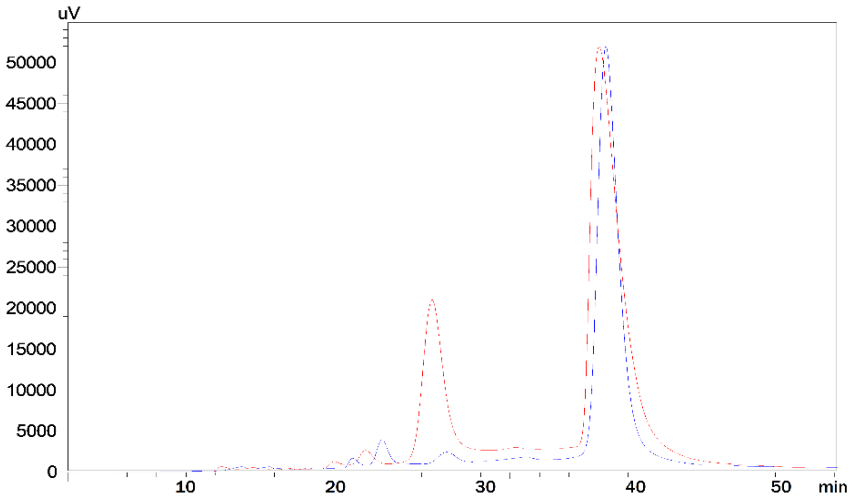

Figure 3.5-HPLC analysis of L-RhI isomerization products

Conclusions to chapter 3

1. In this chapter, we discuss the detailed results of every step. And attach corresponding graphs and tables to illustrate more clearly.

2. As there exists two Nde Ⅰ recognition sites in l-rhi sequence, meanwhile, we adapt Nde Ⅰ to cut the plasmid pET-22b, so we choose to design primers suitable for homologous recombination.

3. Accordingly, we use recombinase system to connect plasmid and target gene. There are lots of advantages of recombinase system, such as sole cutting of carrier plasmid instead of cutting both plasmids and target gene. Therefore, we have more choices of restriction enzyme. Recombinases have more efficiency than ligase as well.

4. In order to avoid *E. coli* DH5α genome interference amplification results, upstream and downstream primers from different template sources were designed separately to avoid interference genes and reduce the probability of false positive electrophoresis. The plasmid sequence was used for the upstream primer template and the target gene sequence was used for the downstream primer template.

CONCLUSIONS

1. In this experiment, the target gene *l-rhi* was successfully extracted from *Bacillus subtilis* 168, and the recombinant plasmid pET-22b-*l-rhi* was successfully constructed, and Escherichia coli BL21(DE3)star was successfully transformed.In this experiment, the recombinant bacterium *E.coli* BL21 (DE3) star-pET-22b-*l-rhi* was cultured until its light absorption value OD600=0.6, then 0.1, 0.5 and 1mM IPTG were added into the groups and induced at 16℃ and 37℃ for 6h to obtain the optimal induction conditions: A large amount of protein L-RhI could be obtained by 1mM IPTG at 16℃. Using L - RhI protein into D-piscose sugar available around 28% of the D-allose sugar.

2. Due to the limitation of experiment time, this experiment needs to be improved in the following aspects, and it is expected to be further studied in the following aspects in the future: In this experiment, recombinant strains expressing L-RhI were preliminarily obtained, and their enzyme activities were detected. However, the conversion rate and repeatability of D-piscose to D-allose reaction catalyzed by free enzymes need to be optimized. Therefore, further enzyme immobilization should be carried out in the future, and appropriate immobilization strategies should be selected. Because Escherichia coli belongs to Gram-negative bacteria, its outer membrane has a double-layer structure, and the protein secreted in the bacteria is poor, and it is easy to form inclusion bodies. Subsequently, strains containing molecular chaperones that promote secretory expression can be selected for the expression of target proteins.

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APPENDIX

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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Cloning and characterization of a rhamnose isomerase from *Bacillus halodurans*

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Abstract Whole-genome sequence analysis of *Bacillus halodurans* ATCC BAA-125 revealed an isomerase gene (*rhaA*) encoding an L-rhamnose isomerase (L-RhI). The identified *L-RhI* gene was cloned from *B. halodurans* and over-expressed in *Escherichia coli*. DNA sequence analysis revealed an open reading frame of 1,257 bp capable of encoding a polypeptide of 418 amino acid residues with a molecular mass of 48,178 Da. The molecular mass of the purified enzyme was estimated to be ~48 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and 121 kDa by gel filtration chromatography, suggesting that the enzyme is a homodimer. The enzyme had an optimal pH and temperature of 7 and 70°C, respectively, with a k_{cat} of 8,971 min^{-1} and a k_{cat}/K_m of 17 min^{-1} mM^{-1} for L-rhamnose. Although L-RhIs have been characterized from several other sources, *B. halodurans* L-RhI is distinguished from other L-RhIs by its high temperature optimum (70°C) with high thermal stability of showing 100% activity for 10 h at 60°C. The half-life of the enzyme was more than 900 min and ~25 min at 60°C and 70°C, respectively, making *B. halodurans* L-RhI a good choice for industrial applications. This work describes one of the most thermostable L-RhI characterized thus far.

Keywords *Bacillus halodurans* · Characterization · Rhamnose isomerase · Thermostability

Ponnandy Prabhu and Thanh Thi Ngoc Doan contributed equally to this work.

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Introduction

L-Rhamnose (6-deoxy-L-mannose) is the most common deoxy hexose sugar and a component of many polysaccharides and glycoproteins in plants. L-Rhamnose is an important component of mycobacterial cell walls (Gottschalk 1986). There are four structural genes involved in L-rhamnose metabolism. They were found to be *rhaA*, *rhaB*, *rhaD*, and *rhaT* encoding rhamnose isomerase, rhamnulose kinase, rhamnulose-1-phosphate aldolase, and rhamnose permease, respectively (Power 1967). Rhamnose isomerase (L-rhamnose ketol isomerase, E.C. 5.3.1.14) converts L-rhamnose to L-rhamnulose (6-deoxy-L-fructose), which is followed by phosphorylation of the primary hydroxyl group mediated by a specific rhamnulose kinase, and finally, by an aldol cleavage into dihydroxyacetone phosphate, and L-lactaldehyde is catalyzed by L-rhamnulose 1-phosphate aldolase (Korndorfer et al. 2000; Moralejo et al. 1993).

Rhamnose isomerase has been found to possess a wide range of substrate specificity, which includes isomerization of several sugars producing so-called "rare sugars". Rare sugars exist in small amounts in nature. Currently, isomerase enzymes are becoming increasingly significant and play a pivotal role in the synthesis of uncommon sugars (Izumori 2002). Rare sugars including D-psicose, L-tagatose, L-fructose, D-tagatose, L-xylulose, and others have been proven to be of significance in food industries, also in pharmaceutical and nutritional industries for the production of reduced calorie sweeteners, inhibitors of microbial growth, and bulking agents (Bautista et al. 2000; Lawson et al. 2002; Levin et al. 1964; Livesey and Brown 1995).

The reaction catalyzed by L-RhI is an aldose–ketose isomerization in which one hydrogen atom is transferred between C1 and C2 of the substrate. The second hydrogen also moves between O1 and O2. Isomerases that contain a metal ion at the catalytic center appear to follow a metal-mediated 1,2-hydride shift mechanism (Whitlow et al. 1991). The 1,2-hydride shift is a carbocation rearrangement, where the catalytic metal ion facilitates the movement of proton from O2 to O1, creating a carbocation at C1 and a hydrogen atom in the form of a hydride, which then shifts from C2 to C1. X-ray crystal structure of *Escherichia coli* L-rhamnose isomerase (ECRI) revealed the presence of two metal ions; one is "structural" (M-1) to help substrate binding and the other is "catalytic" (M-2) to help hydride shift, which are considered as to be Zn2+ and Mn2+, respectively (Korndorfer et al. 2000). Catalysis of the aldose–ketose isomerization of ECRI is proposed to occur by a metal-mediated hydride shift; where O1, O2, and O3 atoms of L-rhamnose coordinate with M-1 (Zn2+) and M-2 (Mn2+). The catalytic amino acids Lys236 and Asp302, together with the catalytic metal ion (Mn2+) and the catalytic water molecule, are thought to assist the aldose–ketose isomerization reaction through a hydride shift mechanism (Korndorfer et al. 2000).

Previously, X-ray crystal structure of L-RhI from *Pseudomonas stutzeri* was reported in complex with L-rhamnose and D-allose, providing insights into the broad substrate specificity of L-RhI (Yoshida et al. 2007). X-ray structures of *P. stutzeri* L-RhI indicated that the parts of the substrate binding site that interact with the substrate at the 1, 2, and 3 positions were equivalent to those in the *E. coli* L-RhI, while other parts that interact with 4, 5, and 6 were similar to D-xylose isomerase, which led to the loose substrate recognition. Residues involved in recognizing substrates with different configurations at C4 and C5 were identified to be His101 and Asp327 (Yoshida et al. 2007).

Apart from L-RhI, an L-rhamnose catabolizing enzyme, L-rhamnose mutarotase has been characterized from *Rhizobium leguminosarum*, providing information of a genetic locus necessary for rhamnose uptake and catabolism (Richardson et al. 2004); its X-ray crystal structure has been reported (Richardson et al. 2008). In *R. leguminosarum*, it appears that catabolism of L-rhamnose is initialized by a kinase, then presumably, an isomerase and a dehydrogenase/aldolase (Richardson et al. 2004). Structural insights into the monosaccharide specificity of L-rhamnose mutarotase from *E. coli* have also been studied (Ryu et al. 2005).

L-RhI from *P. stutzeri* was characterized to have broad substrate specificity, catalyzing the isomerization between L-rhamnose and L-rhamnulose, L-mannose and L-fructose, L-lyxose and L-xylulose, D-ribose and D-ribulose, and D-allose and D-psicose (Leang et al. 2004a). Another gene encoding L-RhI was cloned and characterized from *Bacillus pallidus* (Poonperm et al. 2007). It has been reported that thermo-tolerant enzymes can provide a higher reaction rate and process yield, higher solubility of substrates and products, and also help to reduce contaminations (Mozhaev 1993; Wasserman 1984). Thus, L-RhI with high activity at high temperature would be interesting.

Attempts to explain the stability of thermophilic proteins on the basis of sequence and structure have been reported (Chakravarty and Varadarajan 2000). In proteins, two oppositely charged functional group residues often interact to form salt bridges. Salt bridges play important roles in protein structure and function such as oligomerization, molecular recognition, and domain motions (Perutz 1970; Fersht 1972). It has been consistently observed that salt bridges are more frequent in proteins from thermophiles as compared to those from mesophiles (Yip et al. 1998; Kumar and Nussinov 1999). Experimentally, salt bridges have been shown to stabilize the GCN4 leucine zipper (Spek et al. 1998).

In this study, we report the cloning, heterologous expression, and characterization of a highly thermostable L-RhI (BHRI) from *B. halodurans*. We provide experimental data that BHRI is L-RhI which is highly thermostable and has selected substrate specificity. The characterization of BHRI adds an interesting member to the family of isomerases.

Materials and methods

Materials

Reagents for PCR, Ex-*Taq* DNA polymerase, genomic DNA extraction kit, and pGEM-T easy vector were purchased from Promega (Madison, WI). T4 DNA ligase and restriction enzymes were obtained from New England Biolabs (Beverly, MA). pQE-80L expression vector, plasmid isolation kit, and Ni-NTA super flow column for purification were from Qiagen (Hilden, Germany). Oligo-

nucleotide primers were obtained from Bioneer (Daejeon, South Korea). Electrophoresis reagents were from Bio-Rad (Hercules, CA), and all chemicals for assay were from Sigma–Aldrich (St. Louis, MO).

Bacterial strains and culture condition

B. halodurans ATCC BAA-125 was obtained from Korea Research Institute of Bioscience and Biotechnology (Daejeon, South Korea). *B. halodurans* was grown in tryptic soy broth (pH 6.8) at 37°C. *E. coli* DH5α and *E. coli* BL21 (DE3) were used as hosts for transformation of plasmid and expression, respectively. Both the *E. coli* strains were grown in Luria-Bertani medium supplemented with ampicillin (100 $\mu g ml^{-1}$) at 37°C.

Cloning and expression of L-RhI gene from *B. halodurans*

The *rhaA* gene was amplified by PCR from *B. halodurans* using two oligonucleotide primers, 5′-GCGGATCCATGAGCATGAAAAGTCA-3′ (*Bam*HI restriction site is underlined) and 5′-TTAAGCTTATGGCGCTGGAGCAGC-3′ (*HindIII* restriction site is underlined). *Bam*HI and *Hind*III sites were incorporated into the forward and reverse primer for cloning into the expression vector pQE-80L. The amplified PCR product with flanking restriction sites was at first cloned into pGEM-T easy vector and transformed into *E. coli* DH5α. The cloned *rhaA* gene was confirmed to be free of point mutations by DNA sequencing using the BigDye Terminator sequencing method and an ABI PRISM 3700 sequencer (Applied Biosystems, Foster City, CA). Plasmid DNA (with insert) and the expression vector pQE-80L was digested with restriction enzymes *Bam*HI and *Hind*III to release the insert (*rhaA* gene) and to create a double-strand break in the expression vector, respectively. The insert released from pGEM-T vector was ligated with pQE-80L vector to give pQE-*rhaA*, which is under the control of the T5 promoter and expresses BHRI as a protein fused to the N terminus of a His_6 tag. The recombinant plasmid was then transformed into *E. coli* BL21(DE3), and the expression of recombinant enzyme was performed using 0.4 mM of isopropyl-β-D-thiogalactopyranoside (IPTG). The induced cells were harvested by centrifugation at 4°C for 15 min at 10,000×*g*, rinsed with phosphate-buffered saline, and stored at 20°C.

Purification of BHRI

To purify the recombinant BHRI, cell pellets were resuspended in binding buffer (50 mM NaH_2PO_4, 300 mM NaCl, and pH 8.0). The cell suspension was incubated on ice for 30 min in the presence of 1 mg/ml lysozyme. Cell disruption was carried out by sonication at 4°C for 5 min, and the lysate was centrifuged at 14,000×*g* for 20 min at 4°C to remove the cell debris. The resulting crude extract was retained for purification. The cell-free extract was applied onto a Ni-NTA Super flow column (3.4×13.5 cm, QIAGEN) previously equilibrated with binding buffer (50 mM NaH_2PO_4, 300 mM NaCl, and pH 8.0). Unbounded proteins were washed out from the column with a washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, and pH 8.0). Then, the BHRI protein was eluted from the column with an elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, and pH 8.0). Enzyme fractions were analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by staining with Coomassie blue R250.

Protein quantification and determination of molecular mass

Protein concentrations were determined by the Bradford method using Bovine serum albumin as a standard protein (Bradford 1976). The molecular mass of the native enzyme was determined by gel filtration chromatography. One milliliter of purified enzyme was applied to a Sephacryl S-300 high-resolution column (16/60) (Amersham, UK) and eluted with 50 mM potassium phosphate buffer (pH 7.2) containing 0.15 M NaCl at a flow rate of 1 ml min^{-1}. To determine the molecular mass of BHRI, the column was calibrated with aldolase (MW 168,000), albumin (MW 67,000), ovalbumin (MW 43,000), chymotrypsinogen (MW 25,000), and ribonuclease A (MW 13,700) as reference proteins (Amersham, UK). The subunit molecular weight was examined by SDS-PAGE under denaturing conditions, using the prestained ladder marker (Bio-Rad) as reference proteins.

Enzyme assay

L-RhI activity was measured by determination of the amount of formed L-rhamnulose. In standard conditions, the reaction mixture contained 1 mM $MnCl_2$, ~15 μg of enzyme, 50 mM L-rhamnose (substrate), and malate buffer 50 mM (pH 7) to bring the final volume to 100 μl. The reaction mixture was incubated at 70°C for 10 min followed by cooling the samples on ice to stop the reaction. The generated L-rhamnulose was determined by cysteine carbazole sulfuric acid method, and the absorbance was measured at 560 nm (Dische and Borenfreund 1951). Isomerization of the substrate and accumulation of product in the reaction mixture were determined by colorimetric method and high performance liquid chromatography (DIONEX) using a separation column (NH2P-50 4E, Shodex) at 30°C eluted with 75% acetonitrile at a flow rate of 0.5 $ml min^{-1}$. One unit of L-RhI activity was defined as the amount of enzyme catalyzing the formation of 1 μmol keto sugar per minute under above specified condition.

Physicochemical characterization

The effect of temperature on the activity of BHRI was analyzed by assaying the enzyme samples over the range of 30–100°C for 10 min. The optimum pH of BHRI was determined using the standard assay conditions with three buffer systems, sodium acetate buffer (50 mM, pH 4–5), malate buffer (50 mM, pH 5–7), and Tris-HCl buffer (50 mM, pH 7–10). The thermal stability of the enzyme was investigated by incubating the enzyme in 50 mM malate buffer at various temperatures (50°C, 60°C, 70°C, and 80°C) in the presence of 1 mM Mn^{2+}. At certain time intervals, samples were withdrawn, and residual activity was measured under standard assay conditions.

Effect of metal ions on BHRI activity

Before studying the effect of metal ions on BHRI activity, the purified enzyme was dialyzed against 50 mM malate buffer (pH 7) containing 10 mM EDTA for 24 h at 4°C. Subsequently, the enzyme was dialyzed against 50 mM EDTA-free malate buffer (pH 7). Then, the enzyme was assessed under standard conditions in the presence of several metal ions ($MgCl_2$, $MnCl_2$, $CoCl_2$, $ZnCl_2$, $CaCl_2$, $FeSO_4$, $CuSO_4$, KCl, and $BaCl_2$) with a final concentration of 0.1 mM and 1 mM. The measured activities were compared with the activity of the enzyme without added ions under the same conditions.

Determination of kinetic parameters

Kinetic parameters of BHRI were determined in 50 mM malate buffer (pH 7) containing 1 mM Mn^{2+}. The samples were incubated at 70°C for 10 min. Kinetic parameters such as K_m (mM) and V_{max} (U mg-protein^{-1}) for substrates were obtained by non-linear regression fitting of the Michaelis–Menten equation using Prism 5 (Graphpad software, Inc., CA, USA). The data represent an average of all statistically relevant data with a standard deviation of less than 10%.

Results

Identification and characterization of the *rhaA* gene encoding an L-RhI

The sequence analysis of the whole genome of *B. halodurans* ATCC BAA-125 suggested the presence of an L-RhI. The *orf* (rha*A*) was annotated as a putative L-RhI, suggesting that this *orf* might encode an L-RhI converting L-rhamnose to L-rhamnulose. Thus, we considered BHRI as a candidate L-RhI in *B. halodurans*. The *rhaA* gene encodes a polypeptide of 418 amino acids, with a calculated molecular mass of 48,178 daltons. The overall GC content is about 47.3%, which is similar to that of the chromosomes of *Bacillus* species (35.4–46.2%) (Takami et al. 2000; Veith et al. 2004). The deduced *rhaA* gene product BHRI showed 58%, 57%, 65%, 58%, 57%, 57%, and 16% amino acid identity with L-RhIs from *E. coli*, *Yersinia pestis*, *Geobacillus sp*, *Salmonella enterica*, *Enterobacter sakazakii*, *Klebsiella pneumoniae*, and *P. stutzeri*, respectively.

Heterologous expression of *rhaA* gene and identification of an L-RhI

In order to check its proposed function, *rhaA* was cloned in the T5 RNA polymerase-based plasmid pQE80L to give pQE80L-*rhaA* and heterologously expressed in *E. coli* BL21(DE3). Analyses carried out with the extracts of *E. coli* BL21(DE3) harboring pQE80L-*rhaA* revealed the presence of a high level of L-RhI compared with the control *E. coli* BL21(DE3) cells harboring plasmid pQE80L. To determine if L-rhamnulose was produced from L-rhamnose specifically by BHRI and not by another enzyme induced in the host cell as a consequence of the over-expression of the *rhaA* gene, the BHRI enzyme was purified. Total protein extracts from *E. coli* BL21(DE3) transformed with pQE80L-*rhaA* or with pQE80L as control were analyzed by SDS-PAGE. A 48-kDa protein, which was in agreement with the predicted molecular mass for the BHRI protein, could be identified in total and soluble extracts only from cells harboring pQE80L-*rhaA* and induced by IPTG (Fig. 1). The enzyme was purified to homogeneity of about 15-fold by Ni-NTA affinity chromatography. The purified L-RhI was colorless, and its UV-visible spectrum showed no evidence of chromogenic cofactor. Isomerization of L-rhamnose to L-rhamnulose was monitored by the production of ketose referred to the absorbance at 560 nm. These findings strongly supported the assumption that the L-RhI activity observed in crude extracts of *E. coli* BL21(DE3) harboring pQE80L-*rhaA* corresponded to that of the BHRI protein.

Determination of molecular weight and quaternary structure

In gel filtration chromatography on Sephacryl S-300 high-resolution column (16/60) (Amersham, UK), BHRI eluted as a symmetrical peak between aldolase and bovine albumin, corresponding to a M_r of approximately 121 kDa (Fig. 1a). The subunit molecular weight of the enzyme was ~48 kDa, as determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Fig. 1b). These results indicate that the enzyme migrates as a dimer in gel filtration and thus may also be present and active as a homodimer in solution. However, the microbial L-RhIs

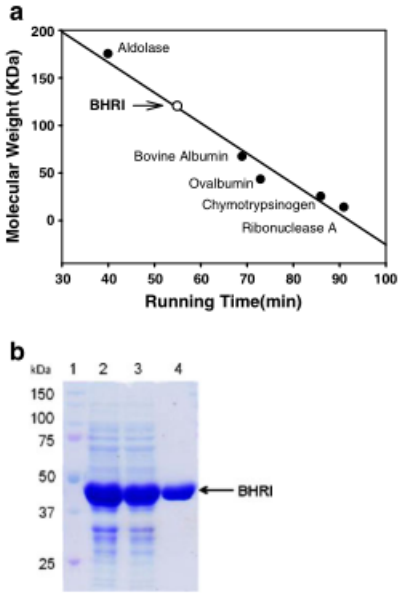

Fig. 1 Determination of the molecular mass of BHRI by SDS-PAGE and gel filtration chromatography. **a** Determination of native molecular mass of BHRI by gel filtration chromatography on a sephacryl S-300 high resolution column from Amersham. The column was calibrated with standard molecular weight proteins such as aldolase (168 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa), and ribonuclease A (13.7 kDa). **b** Determination of subunit molecular mass by SDS-PAGE. *Lane 1* molecular standard marker, *lane 2* crude extract, *lane 3* soluble protein form, and *lane 4* purified BHRI. An *arrow* indicates the purified enzyme eluted from the Ni-NTA superflow column

including *E. coli* L-RhI and *P. stutzeri* L-RhI that have been characterized in some detail occur chiefly as homo-tetramer with a subunit molecular mass of 46–48 kDa (Korndorfer et al. 2000, Yoshida et al. 2007).

Optimum pH, temperature, and thermal stability

The optimum temperature for isomerization of L-rhamnose by purified BHRI was 70°C as shown in Fig. 2a. The optimum pH for isomerization was 7.0, with 75%, 85%, 92%, and 90% of maximum activity at pH 6.0, 8.0, 9.0 and 10.0, respectively (Fig. 2b). The pH range for BHRI activity was large, with greater than 75% of the activity at pH values of 6.0–10.0. The isoelectric point of the BHRI was 5.5 as determined by isoelectric focusing. This value agrees with the theoretical value (5.58) estimated from the amino acid sequence. Maximal L-RhI activity at pH 7 and an alkaline pH optimum for L-rhamnose isomerization are common features of similar enzymes isolated from diverse microbial systems. In the presence of 1 mM Mn^{2+}, BHRI was stable at 60°C for more than 10 h and could retain more than 90% activity after 15 h. However, the stability of the enzyme decreased drastically at 70°C and 80°C with half-life duration of 25 min and 5 min, respectively (Fig. 3).

Effects of metal ions

BHRI activity was measured in the presence of metal ions or with various other compounds. The enzyme was purified as previously described without metal ions followed by extensive dialysis in the presence of 10-mM EDTA. BHRI without any metal ions showed a negligible activity. Metal ions at low concentration of 0.1 mM had no significant effect on the activity of the enzyme. Thus, 1-mM concentration of metal ion was used for study. BHRI activity was not stimulated by Ca^{2+}, Ba^{2+}, Na^{2+}, Fe^{2+}, or Cu^{2+}. However, Mn^{2+}, Co^{2+}, Mg^{2+}, and Zn^{2+} enhanced BHRI activity 100-, 93-, 57- and 20-fold, respectively (Table 1), and appeared to be required for catalytic activity of BHRI.

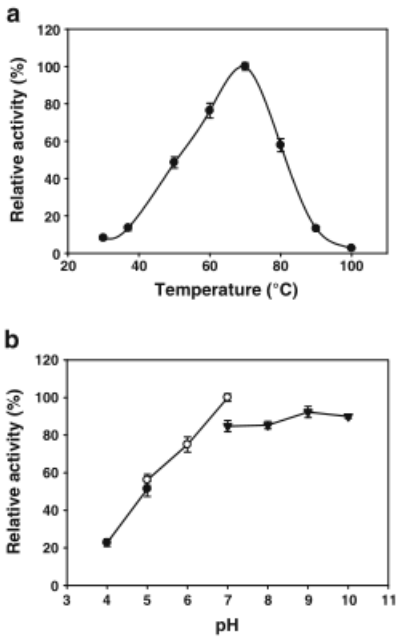

Fig. 2 Effect of temperature (**a**) and pH (**b**) on the activity of BHRI. Assays were carried out under standard conditions in the presence of 50-mM L-rhamnose. Activities at the optimal temperature and pH were defined as 100%. Each value represents the mean of triplicate measurements and varied from the mean by not more than 10%. 50-mM sodium acetate buffer (*black circle*), 50-mM malate buffer (*white circle*), 50-mM Tris-HCl buffer (*black down-pointing triangle*)

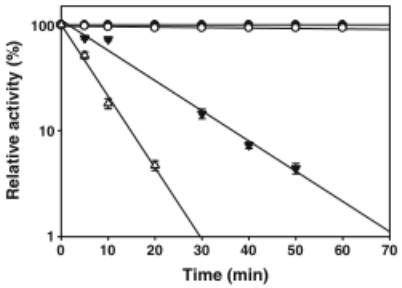

Fig. 3 Thermal stability profiles of the purified BHRI in the presence of 1 mM Mn^{2+} at 50°C (*black circle*), 60°C (*white circle*), 70°C (*black down-pointing triangle*), and 80°C (*white up-pointing triangle*). Residual activity was measured at standard conditions

Substrate specificity

The characterization of BHRI as an L-RhI then allowed for the investigation of its substrate specificity for various aldoses. L-Rhamnose, L-mannose, L-arabinose, L-talose, L-lyxose, L-ribose, L-xylose, D-galactose, D-glucose, D-mannose, D-xylose, D-gulose, D-ribose, and D-allose (50 mM) were used to examine the substrate specificity of BHRI. BHRI had a high preference for L-rhamnose, L-mannose, L-talose, L-lyxose, and D-gulose. Rather, other aldoses did not serve as substrate for BHRI in the presence of Mn^{2+}. The aldose substrates with hydroxyl group oriented in the same direction at C2 and C3 were converted reversibly by the enzyme to the corresponding ketoses. The enzyme preferred aldose substrates with C2 and C3 hydroxyl group in the right-hand side configuration (fischer projection) such as L-rhamnose, L-mannose, L-talose, L-lyxose, and D-gulose (Table 2).

Table 1 Effect of different metal ions on the activity of BHRI

Metal ion	Specific activities (U mg-protein^{-1})	
	0.1mM	1mM
None	1.5 (1)	1.5 (1)
Zn^{2+}	2.0 (1)	22 (20)
K^{+}	0.8 (1)	2.5 (2)
Mn^{2+}	17 (15)	110 (100)
Mg^{2+}	9.0 (8)	63 (57)
Co^{2+}	13 (12)	102 (93)
Ca^{2+}	2.0 (1)	3.5 (3)
Ba^{2+}	0.9 (1)	2.0 (1.5)
Na^{+}	0.8 (1)	2.0 (1.5)
Cu^{2+}	1.9 (1)	3.5 (3)
Fe^{2+}	0.75 (1)	2.5 (2)

The purified enzyme was assayed in the standard assay condition with 0.1 or 1 mM metal ions. Relative activities are represented in parenthesis. Each value represents the mean of triplicate measurements and varied from the mean by not more than 10%

Kinetic parameters

Initial velocities were determined in the standard assay mixture at pH 7. The enzyme represented a turnover rate (k_{cat}) of 8,971, 5,333, 12,960, 437, and 254 min^{-1} for L-rhamnose, L-mannose, L-lyxose, D-gulose and L-talose, respectively. The catalytic efficiency (k_{cat}/K_m) values of BHRI against L-rhamnose, L-mannose, L-lyxose, L-talose, and D-gulose were 17, 45, 16.8, 2.4, and 2.9 min^{-1}mM^{-1}, respectively. For L-rhamnose and L-mannose, BHRI exhibited a relatively similar turnover rate (k_{cat}) compared to *P. stutzeri* L-RhI (10,300 and 5,380 min^{-1}, respectively) whereas, better than L-RhI from *B. pallidus* (4,080 and 4,610 min^{-1}, respectively). For L-lyxose, the turnover (k_{cat}) was twofold to sevenfold higher than *P. stutzeri* (6020 min^{-1}) and *B. pallidus* (1,860 min^{-1}).

Salt bridges play important roles in protein structure and function such as oligomerization, molecular recognition, allosteric regulation, α-helix capping, and stabilization of the folded conformation of proteins (Dragan et al. 2004). Salt bridges were calculated (Barlow and Thornton 1983; Musafia et al. 1995 Costantini et al. 2008) using an interatomic distance cutoff (3–4 Å) applied to the carboxylate oxygen atoms of Glu and Asp (i.e., OE in Glu or OD in Asp) and one side chain nitrogen atom of Arg, Lys, or His (i.e., NE, NH1, and NH2 of Arg; NZ of Lys; and ND1 and NE2 of His) (Kumar and Nussinov 1999). Analysis of the charged residues involved in salt bridge formation revealed the presence of 11 charged residues in BHRI, seven residues in ECRI, and five residues in YPRI (Fig. 4), which account for 16, ten, and nine salt bridges, respectively (Supplementary Table 1).

Discussion

Recombinant L-RhIs have been characterized from *E. coli* (Badia et al. 1991), *P. stutzeri* (Leang et al. 2004a), and *B. pallidus* (Poonperm et al. 2007). The structure of ECRI and

Table 2 Enzyme activities of BHRI for various substrates

Substrate	V_{max} (U mg-protein^{-1})	Relative activity (%)
L-rhamnose	187	100
L-mannose	111	59
L-lyxose	270	144
D-gulose	9.1	5
L-talose	5.3	3

Fig. 4 Multiple sequence alignment of L-rhamnose isomerases from *B. halodurans* (BHRI), *E. coli* (ECRI), and *Y. pestis* (YPRI). The alignment was performed using DS 2.5 program. The secondary structure cartoon shown is based on the Kabsch and Sander method (Kabsch and sander 1983). The secondary structure elements are color coded, with helices in *red*, strands in *blue*, and coils in *beige*. Amino acids strongly conserved in all are displayed as *black letters* displayed in *dark blue*, and weakly conserved residues are displayed in *blue*. Catalytic amino acids are *bold letters* overlined in *red*. Metal binding amino acids are *bold letters* overlined in *yellow*

its relationship with xylose isomerase have been reported (Korndorfer et al. 2000). X-ray crystal structure of L-RhI from *P. stutzeri* was also reported, providing insights into its broad substrate specificity (Yoshida et al. 2007). However, thermal stability of the known recombinant L-RhIs (Tables 3 and 4) is still insufficient for its application to continuous L-rare sugar production in industry. Our study describes a thermostable L-RhI from *B. halodurans*.

Based on analysis of the genome sequence of *B. halodurans* ATCC BAA-125, an L-RhI encoding gene

Table 3 Amino acid profile (%) of L-RhIs selected from various mesophiles and thermophiles

Organism	Polar amino acids				Charged amino acids		
	Gln	Asn	Ser	Thr	Glu	Lys	Arg
Thermophiles							
Thermotoga petrophila	2.36	3.14	3.93	3.14	10.99	7.07	6.81
Dictyoglomus thermophilum	2.27	4.03	4.03	3.53	9.57	9.57	4.79
Thermotoga naphthophila	1.84	3.42	3.95	3.42	10.26	7.89	7.11
Bacillus pallidus	2.67	4.61	6.55	3.88	9.47	7.52	7.04
Oceanobacillus iheyensis	2.40	5.28	5.28	4.80	9.83	6.95	4.32
Average	2.30	4.09	4.74	3.75	10.02	7.8	6.01
Mesophiles							
E. coli	4.30	2.86	7.16	4.06	7.16	3.82	6.68
Salmonella typhimurium	4.53	2.86	6.44	4.53	6.68	4.06	6.92
Yersinia pestis	4.31	3.11	6.46	4.31	5.74	3.11	7.18
Klebsiella pneumonia	4.53	4.30	5.97	4.06	6.44	3.58	6.44
Shigella dysenteriae	4.53	2.63	6.92	4.30	6.92	3.82	6.44
Citrobacter koseri	4.77	3.10	7.16	4.06	6.92	3.82	6.44
Average	4.49	3.14	6.68	4.22	6.64	3.70	6.68
Bacillus halodurans	2.63	2.87	4.78	4.55	9.33	6.94	5.74

Calculations were performed based on the sequence present in the Gene bank

Table 4 Biochemical and kinetic properties of L-rhamnose isomerase from various organisms

Organism	Mr (kDa)	Quaternary structure	k_{cat} (min^{-1})			K_m (mM)			Thermal stability (°C/$t_{1/2}$)	References
			L-rhamnose	L-mannose	L-lyxose	L-rhamnose	L-mannose	L-lyxose		
E. coli	47	Tetramer	NR	NR	NR	2.0	5.0	5.0	50/10 min	Badia et al. 1991
B. pallidus	47	NR	4,080	4,610	1,860	4.89	28.9	16.1	60/60 min	Poonperm et al. 2007
P. stutzeri	47	Tetramer	10,300	5,380	6,020	11.9	55.5	61.7	50/10 min	Leang et al. 2004a
B. halodurnas	48	Dimer	8,971	5,333	12,960	528	119	771	70/25 min 60/<900 min	This study

NR not reported

(*rhaA*) was proposed and identified. The *rhaA* gene was cloned from *B. halodurans* and over-expressed in *E. coli*, and L-RhI activity was confirmed in the gene product BHRI. Isomerization performed at high temperatures offers several advantages such as high conversion yield, faster reaction rate, and reduced contamination. Although *B. halodurans* is a mesophile, BHRI was stable up to the temperatures of 60°C retaining 90% of its activity till 15 h and it has a half-life of 25 min at 70°C (Table 4). BHRI showed the highest thermostability compared with L-RhIs from *P. stutzeri* (Leang et al. 2004a, b), *B. pallidus* (Poonperm et al. 2007), and *E. coli* (Badia et al. 1991). BHRI is considerably appropriate for the use in bioreactor system, as it provides high activity and thermostability compared to L-RhIs from other strains.

This enzyme had higher turnover rate (k_{cat}) for L-lyxose than the inherent substrate L-rhamnose (Table 4), which is different from that found in other L-RhIs from *P. stutzeri* (Leang et al. 2004a) and *E. coli* (Badia et al. 1991). Detailed interpretation of the substrate specificity of BHRI relies on three-dimensional structural studies of BHRI, which is our future goal of study. The reason for its high thermostability was investigated by comparing the amino acid composition of L-RhIs from mesophile and thermophilic organisms (Table 3). Previous reports indicate that there is a decrease in the content of polar amino acids (Q, N, S, or T) and an increase in the charged amino acids (K, E, or R) in the thermophilic and hyperthermophilic enzymes (Sterner and Liebl 2001; Chakravarty and Varadarajan 2000). When compared with the L-RhIs of mesophiles, an increase in the number of charged amino acids (glutamate and lysine) and a decreased number of polar uncharged amino acids (glutamine and serine) are noted in BHRI. The percentage (%) of glutamate and lysine was 9.33 and 6.94 in BHRI, while it was 6.64 and 3.70 in mesophilic L-RhIs (Table 3). The increase in charged amino acid content is likely to enhance the occurrence of salt bridges, which is a unique property of thermophilic protein stability (Xiao and Honig 1999). Thus, an increased proportion of glutamate and lysine in BHRI is consistent with the property of thermophilicity in L-RhIs. In BHRI, the percentage of glutamine and serine was 2.63 and 4.78 compared to 4.49 and 6.68 in mesophilic L-RhIs (Table 3). The decreased content of uncharged polar residues is likely to minimize deamidation and backbone cleavages involving glutamine and serine (Tomazic and Klibanov 1988). The decreased profile of glutamine and serine would also account for the high thermal stability of BHRI. A similar study to understand thermostability by varying amino acid composition was reported in a xylose isomerase of *Bacillus licheniformis* (Vieille et al. 2001). Experimental evidence for the effect of amino acid composition on the thermostability has been proved using *E. coli* Ribonuclease H1 (You et al. 2007) and *Achromobacter xylosoxidans* penicillin G acylase (Cai et al. 2004).

In a folded protein, pairs of neighboring opposite charged residues often interact to form salt bridges (Fersht 1972). When compared with other L-RhIs (ECRI and YPRI), BHRI had a greater number of salt bridges, supporting the high thermostability of BHRI. There were 11 charged residues in BHRI but only seven in ECRI and five in YPRI, which would account for formation of 16, ten, and nine salt bridges in each respective protein. Thus, the BHRI protein structure is likely to be better stabilized by salt bridges. Reports on salt bridge stabilization of protein structure have been published. Hyperthermophilic citrate synthase had a greater number of charged residues and salt bridge networks than did the mesophilic protein (Kumar and Nussinov 2004). Many previous studies (Karshikoff and Ladenstein 2001; Declerck et al. 2000; Fu et al. 2010) are in agreement with our result suggesting that the thermostability was enhanced via an increased number of salt bridges in the protein. Thus, the significant overall differences in the number of potential interactions in BHRI compared to the other L-RhIs suggest that increased electrostatic interaction is one of the major contributors to the high thermostability of BHRI.

In summary, the *rhaA* gene encoding L-RhI has been cloned from *B. halodurans* and over-expressed in soluble form. The purified BHRI protein exhibited L-RhI enzymatic

activity, thereby confirming the identity of the protein. The evidence from enzymology and bioinformatics experiments strongly suggests that the gene is a member of the L-RhI family. Compared to the other known L-RhIs, BHRI exhibits the highest thermostability, demonstrating its potential use in industrial applications. The successful identification and over-expression of BHRI allows us to characterize a novel L-RhI and now sets the stage for more detailed investigations of this enzyme such as X-ray crystallography and protein engineering.

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