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

QUALIFICATION THESIS

on the topic **Expression of high-temperature resistant β-glucosidase in *Pichia pastoris***

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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Kyiv 2024

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APPROVE
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«___»_____2024

ASSIGNMENTS
FOR THE QUALIFICATION THESIS
Liu Xiao

1. Thesis topic **Expression of high-temperature resistant β-glucosidase in *Pichia pastoris***
Scientific supervisor Iryna Voloshyna, Ph.D., As. prof.

approved by the order of KNUTD "_____" _____2024, №_____

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

3. Content of the thesis (list of questions to be developed): literature review; object, purpose, and methods of the study; experimental part; conclusions

4. Date of issuance of the assignments_____

EXECUTION SCHEDULE

№	The name of the stages of the qualification thesis	Terms of performance of stage	Note on performance
1	Introduction	From 01 April 2024 to 11 April 2024	
2	Chapter 1. Literature review	From 06 April 2024 to 20 April 2024	
3	Chapter 2. Object, purpose, and methods of the study	From 21 April 2024 to 30 April 2024	
4	Chapter 3. Experimental part	From 01 May 2024 to 10 May 2024	
5	Conclusions	From 07 May 2024 to 12 May 2024	
6	Draw up a bachelor's thesis (final version)	From 20 May 2024 to 25 May 2024	
7	Submission of qualification work to the supervisor for feedback	From 25 May 2024 to 27 May 2024	
8	Submission of bachelor's thesis to the department for review (14 days before the defense)	27 May 2024	
9	Checking the bachelor's thesis for signs of plagiarism (10 days before the defense)	31 May 2024	
10	Submission of bachelor's thesis for approval by the head of the department (from 7 days before the defense)	03 June 2024	

I am familiar with the task:

Student _____ Liu XIAO

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SUMMARY

Liu Xiao. Expression of high-temperature resistant β-glucosidase in *Pichia pastoris* – Manuscript.

Qualification thesis on the specialty 162 «Biotechnology and Bioengineering». – Kyiv National University of Technologies and Design, Kyiv, 2024.

β-Glucosidase is involved in the degradation of carbohydrates, phenolic compounds, some natural products and antibiotics, and the decomposition of cellulose, lignin and other polysaccharides in the plant cell wall, which is important for the release and recycling of nutrients, and is widely used in the fields of food, pharmaceuticals, skincare and cosmetics, etc. Thermal stability of β-glucosidase is one of the key factors determining the cost-effectiveness of biorefinery. Utilizing microbial molecular biology technology, microbial technology, and enzyme engineering technology to create high-temperature-resistant β-glucosidase is of great scientific significance and great market application prospect.

In this study, the β-glucosidase gene was cloned from a strain of *Pyrococcus furiosus* and molecularly modified to obtain the β-glucosidase mutant gene *371-β-glucosidase* (*T371A*). The mutant gene was transformed into *Pichia pastoris* yeast strain GS115 by polyethylene glycol-mediated transformation, and SDS-PAGE was used to demonstrate that the *T371A* gene had been integrated into the *Pichia pastoris* yeast genome and was successfully expressed, which provided a reliable basis for the subsequent evaluation of the high-temperature tolerance of β-glucosidase and the preparation of its enzyme preparations.

The results showed that the mutant β-glucosidase exhibited significant thermophilicity and thermal stability with increasing temperatures, a feature consistent with the *Pyrococcus furiosus* strain from which the gene was derived. The data showed that the enzyme activities of *T371A* β-glucosidase and enzyme powder were increased by 44.30% to 257.17% and 40.93% to 161.27%, respectively, compared with those of the wild-type β-glucosidase under high temperature

conditions. By putting the transgenic Picrasinol yeast into a 50L fermenter for scale-up culture and high-temperature spray-drying treatment to become enzyme powder, the enzyme powder with a yield of 4.17 g/L and a water content of 9.9% was obtained, and its enzyme activity was only lost by 17.23%, which possessed the same catalytic properties as that of *T371A*, and the *T371A* enzyme powder also showed the advantages of a longer storage period and a higher quality of storage. It was further found that the enzyme activity of *T371A* and its enzyme powder continued to increase with the increase of reaction time under the high temperature condition of 100 ℃, which indicated that the enzyme did not reach the maximum reaction rate at 100 ℃ and the optimal temperature was much higher than 100 ℃, which demonstrated significant heat resistance and thermal stability.

In summary, β-glucosidase, as an enzyme with a wide range of application prospects, its heat resistance is of great significance and application prospects in industrial and research fields. This study lays the foundation for expanding its application in a wider temperature range by exploring its heat resistance.

Key words: β-glucosidase; High temperature resistance; High temperature spray drying; Enzyme powder

TABLE OF CONTENTS

INTRODUCTION

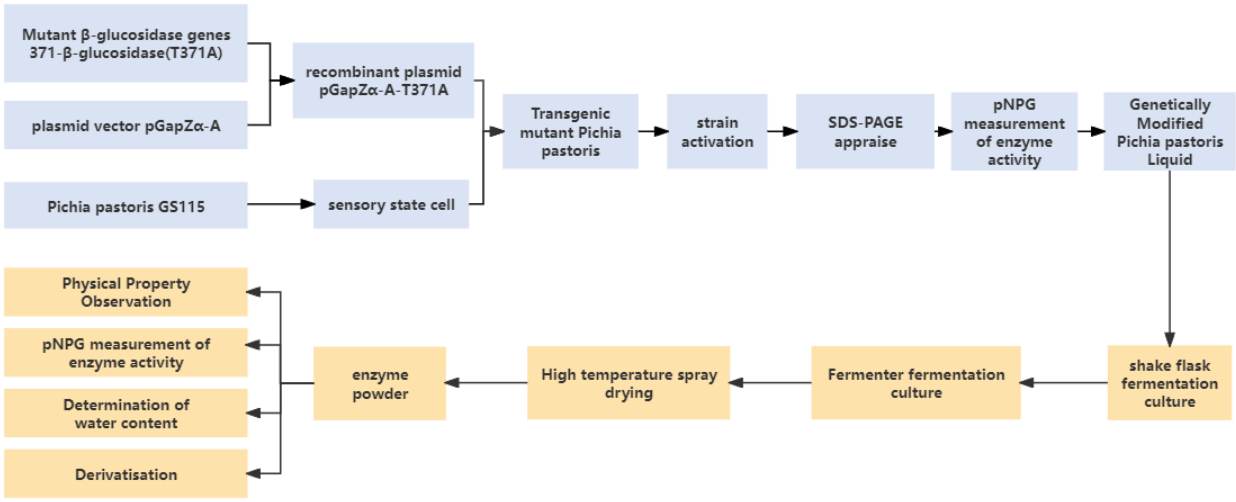
β-glucosidase is involved in the degradation of carbohydrates, phenolic compounds, some natural products and antibiotics, and the decomposition of cellulose, lignin and other polysaccharides in the plant cell wall, which is important for the release and recycling of nutrients, and is widely used in the fields of food, pharmaceuticals, skincare and cosmetics, etc. β-glucosidase is widely distributed in nature in many organisms, including plants, microorganisms and animals. β-glucosidase is widely distributed in nature in many organisms, including plants, microorganisms and animals. β-glucosidases of microbial origin are widely used in industrial production, biotechnology and scientific research due to their advantages and features such as high purity and stability, large-scale production capacity, easy optimisation and modification, wide substrate adaptability, environmental friendliness and ease of operation.

Its scientific novelty lies in the following elements. Thermal stability of β-glucosidase is one of the key factors determining the cost-effectiveness of biorefineries [1] and their applicability in industries such as beverages, pulp, paper and petroleum [2, 3]. The use of enzymes to hydrolyse biomass at elevated temperatures increases reaction rates, enhances substrate solubility, and reduces microbial contamination [1] . In addition, enzymes with higher thermal stability usually remain active in solution for a longer period of time (as indicated by the half-life (t50)), which reduces the operating costs of biorelevant industries.

The optimal reaction temperature of β-glucosidases varies depending on the source, species, and specific enzyme, with the vast majority of enzymes exhibiting the highest catalytic activity and efficiency at their optimal temperature. β-glucosidases typically exhibit better activity in the moderate to high temperature range, with most β-glucosidases having optimal reaction temperatures between 40°C and 60°C [4] . However, conventional β-glucosidases are often not adapted to the high temperatures required by biorelevant industries, and high-temperature-tolerant β-glucosidases derived from hyperthermophilic organisms have great potential for application.

In summary, the creation of high-temperature-resistant β-glucosidase using microbial molecular biology technology, microbiological technology, and enzyme engineering technology is of great scientific significance and has great market application prospects.

The experimental methodology is shown in the Figure below.



The subjects of the study were obtained from Pyrococcus furiosus after molecular modification of the mutant gene 371-β-glucosidase (T371A).

In this study, we cloned the β-glucosidase gene from *Pyrococcus furiosus* and obtained the mutant β-glucosidase gene *T371A* after molecular modification, transformed the mutant gene into Pichia pastoris, and evaluated the high-temperature tolerance of *T371A*-expressed β-glucosidase, as well as compared it with wild β-glucosidase. Further, we placed the Pichia pastoris GS115 strain expressing *T371A* into a fermenter for scale-up and dried it to become enzyme powder, and again evaluated the high-temperature tolerance of its expressed β-glucosidase. The purpose of this study was to identify and evaluate the high-temperature tolerance of the mutant β-glucosidase, and to expand the application of β-glucosidase in a wider temperature range by increasing its optimal reaction temperature.

Subject of study: β-glucosidase.

β-glucosidase is involved in the degradation of carbohydrates, phenolic compounds, some natural products and antibiotics, and the decomposition of cellulose, lignin and other polysaccharides in the plant cell wall, which is important

for nutrient release and recycling, and is widely distributed in eukaryotes, archaea, bacteria and viruses.

β-glucosidase is widely used in the fields of food, pharmaceuticals, skin care and cosmetics, biotechnology and scientific research due to its advantages and features such as high purity and stability, large-scale production capacity, ease of optimisation and modification, wide adaptability of substrates, environmental friendliness and ease of operation

Object of study Mutant: β-glucosidases, he name of the mutant β-glucosidase gene obtained after molecular modification by cloning the β-glucosidase gene was*371-β-glucosidase(T371A)*

Purpose of the study: High temperature resistant industrial value

High product yield and low pollution rate

The use of enzymes to hydrolyse biomass at elevated temperatures increases reaction rates, enhances substrate solubility and reduces microbial contamination, reducing the potential for complex reactions due to contamination.

Low production costs

The high temperature reaction reduces the energy consumption for heating and holding, thus reducing the operation and energy of biologically related industries, and Pyrococcus furiosus proteins are highly resistant to chemical denaturation and protease degradation and are active for a longer period of time.

Wide range of applications

In high-temperature industrial production or applications, conventional β-glucosidase cannot adapt to the high-temperature conditions they require, and mutant enzymes that are resistant to high temperatures can expand applications over a wider temperature range.

CHAPTER 1
LITRATURE REVVIEW

1.1 β-glucosidase

1.1.1 synopsis

β-glucosidase (EC 3.2.1.21), also known as β-D-glucoside hydrolase, is a glycoside hydrolase that specifically hydrolyses the β-D-glucose bond with other ligands to produce a ligand such as β-D-glucose as a collective term for a class of glucoside hydrolases (GH)[5] . It is also known as bitter amygdalase because it was first discovered by Liebig and Wohler in 1837 in bitter almonds, and because it also has significant activity against sugars such as cellobiose and gentiobiosaccharides, so it is also known as cellobiase (CB, β-G) or gentiobiosidase. In cellulose degradation, β-glucosidase plays a key role as a rate-limiting enzyme [6], catalysing β-glycosidic bonds mainly to the non-reducing ends of disaccharides, flavonoid glycosides, oligosaccharides, aryl glucosides and alkyl glucosides [7]. In addition, β-glucosidases exhibit transglycosidic activity, which allows the formation of β-1,6-glycosidic bonds, as well as the transfer of ligands and products generated during hydrolysis of glucose molecules to other substrates.

1.1.2 Physical and chemical properties

Generally speaking, β-glucosidases can be divided into two major categories, intracellular and extracellular enzymes, in which most microorganisms express only one of the enzymes, but there are also some special cases in which certain microorganisms express both categories of enzymes. In this experiment, intracellular β-glucosidases are mainly discussed.

There are significant differences between different sources of β-glucosidases in terms of their physicochemical properties (including molecular weight, isoelectric point, optimum temperature, optimum pH, thermal stability, etc.) [8] . The optimum temperature of β-glucosidases from different sources in enzymatic reactions varies widely. Usually, its optimum temperature ranges from 40-110°C, but most of the β-

glucosidases from thermophilic microorganisms have an optimum temperature of 40-70°C [9] . The β-glucosidase isolated from *Pyrococcus furiosus* in this experiment may have an optimum temperature of more than 100°C. Typically, β-glucosidases from fungal and bacterial sources have lower optimum temperatures than β-glucosidases from archaeal sources. For example, β-glucosidases from the archaeal fungus *Pyrococcus furiosus* have an optimum temperature of up to 102-105 °C in enzymatic reactions [10], β-glucosidases from the fungus *Trichoderma reesei* have an optimum temperature of only 70 °C in enzymatic reactions [11] , and β-glucosidases from the bacterium *Bacillus circulars subsp. Alkalophilus* has an optimum temperature of 37°C for β-glucosidase [12] . In addition, β-glucosidases from a variety of different sources vary equally in their thermal stability. By studying the protein conformation and conformation of different families of β-glucosidases and comparing β-glucosidases from thermophilic microorganisms and common microorganisms, it was found that the active centre of β-glucosidases remained basically unchanged during the long-term evolution of β-glucosidases. However, during the reaction process of degrading the substrate, it is possible to cause minor structural changes in some parts to adapt to the higher reaction temperature [13] .

The isoelectric points of most β-glucosidases are in the acidic range, i.e., between 3.5 and 5.5 [14] . In general, enzymes obtained from microorganisms from extreme environments may possess corresponding extreme enzymatic properties, such as tolerance to high/low temperatures or strong acids/bases, and such extreme enzymes are usually derived from archaea. Large differences in relative molecular weights exist between β-glucosidases of different origin, composition and spatial structure. In general, the relative molecular weights of β-glucosidases are mainly distributed between 40-340 kDa [15] . For example, the β-glucosidase with high thermal stability obtained by Kengen et al. from the archaeon *Pyrococcus furiosus* has a molecular weight of about 230 kDa and is a tetramer consisting of four subunits with a relative molecular weight of about 58 kDa [10] .Takashima et al. obtained a β-glucosidase with high thermal stability from the fungi *Humicola grisea* and

Trichoderma reesei isolated β-glucosidases Bgl4 and Bgl2 with molecular weights of about 54 and 52 kDa [16] . Different β-glucosidases originate from different protein families, and their relative molecules and their structures are significantly different, with the molecular weights of the GH1 family sources focusing on the range of 40-60 kDa, and usually having a (β/α) 8-barrel structure, which is conserved to a certain extent, while the relative molecular weights of the GH3 family sources are in the range of 60-140 kDa, and they are usually of relatively complex structures.

1.1.3 Classification

Henrissat and Bairoch proposed hydrophobic cluster analysis (HCA), a nucleotide sequence identity scheme based on nucleotide sequence and folding similarities of enzymes. Based on the HCA results, it was shown that the α-helix and β-fold are similarly positioned in the folded conformation of many β-glucosidases and that the positions of their active centre sites and highly conserved amino acids are usually adjacent. Sequence identity classification methods can reveal structural features of enzymes as well as catalytic mechanisms of action [17] . In the CAZY database, β-glucosidases are classified into the families GH1, GH3, GH5, GH9, GH30 and GH116. Conserved motifs of the same family usually play an important role in the structure or catalytic mechanism [18]. Although classification based on sequence identity can be described from a structural point of view, substrate specificity is still predominantly used in classifying β-glucosidases. When the structural information of the enzyme is lacking, the classification of glucosidases can generally only be carried out in a substrate-specific manner. β-Glucosidases are mainly derived from the GH3 family and the GH1 family of glycosyl hydrolases, but also partly from the GH5, GH9, and GH30 families [19] . the GH1 family is mainly derived from animals, plants, and archaea, and it encompasses about 60 species of β-glucosidases, and it also includes 6-phosphoglucosidases and thioglucosidases [20] . Many of the enzymes in the GH1 family also catabolise β-galactosides. the GH3 family encompasses about 44 β-glucosidases and hexosaminidases from bacteria,

yeasts and moulds as the main sources [21] . Most of the β-glucosidases isolated from fungi belong to the GH3 family [22] .

1.1.4 Catalytic mechanisms

Most of the β-glucosidases in the GH1, GH3, GH5, GH30 and GH116 families belong to the conserved enzymes that use a heterocapsule-carbon-retentive mechanism for hydrolysis of substrates [23] . The catalytic hydrolysis process can be divided into two steps, glycosylation and deglycosylation, where the most important catalytic sites are two glutamate residues, one of which acts as a nucleophile close to the N-terminus and the other glutamate residue acts as a proton donor close to the C-terminus. The glutamate residues are attacked by the acid-catalytic mechanism on the heterocapital carbon to produce glycosylase intermediates (E-S) during the glycosylation reaction [24] . During glycosylation, water molecules attack the heterohead carbon by a base-catalysed mechanism, resulting in the production of glycosylated products and leading to the regression of β-glucosidase to the protonated state [25]. β-Glucosidase's catalytically active site determines the substrate specificity, which varies according to the substrate, allowing certain enzymes to hydrolyse multiple substrates. At the same time, β-glucosidases must remain conformationally unchanged in order to participate in the catalytic reaction. For example, Bauer et al [26] showed that β-glucosides isolated from the thermophilic bacterium *Pyrococcus furiosus* and the non-thermophilic bacterium Agrobacterium follow a two-substitution reaction mechanism in hydrolysing glycosidic bonding action, following the same mechanism for the catalytic reaction [27] .

1.1.5 Applications

β-glucosidases are present in many plants and animals as well as microorganisms [28] , and they are also distributed in archaea [29] , bacteria [30] , eukaryotes [31] , viruses [32] , and some undefined species [33] . In the microbial degradation of cellulose, β-glucosidase plays a key role. In plants, β-glucosidase is closely related to cell wall differentiation and maturation, metabolic pigments and

fruit ripening [34] . In mammals, β-glucosidase, on the other hand, is involved in the hydrolysis of glucose ceramide. It is important for the release and recycling of nutrients and is widely used in food, pharmaceuticals, skin care cosmetics, etc. β-Glucosidase can be used as a food additive for the improvement of food taste and aroma, and in some processed foods it can degrade bitter substances and improve the overall quality of the food [35] . In fruit juice production, β-glucosidase is used to degrade pectin and improve the clarity and taste of fruit juice [36] . In beer and wine brewing, β-glucosidase can be used to remove or degrade some bitter substances and improve the flavour of wine [37] ; in the field of skincare and cosmetics, β-glucosidase can help to exfoliate the cuticle on the surface of the skin, hydrolyse the keratin in the cuticle, promote the shedding of aged cuticle and facilitate the metabolism, so as to improve the texture and complexion of the skin, and reduce the wrinkles and fine lines [38] . β- glucosidase also has a moisturising effect, absorbing and retaining water to keep the skin moist. β-Glucosidase can promote the renewal of epidermal cells, which helps to remove surface pigmentation, thus achieving the effect of whitening [39] .In the process of drug manufacturing, β-glucosidase can be used to synthesise certain drugs or organic molecules, and its catalytic effect is important for the synthesis of some glycosides. β-glucosidase can be used to prepare some natural products or compounds with biological activities, including the synthesis of plant secondary metabolites [40] . In addition, some drugs contain β-glucosidase for helping patients to digest and absorb polysaccharides from food [38]. The above applications demonstrate the versatility and importance of β-glucosidases in the food, skin care cosmetics and pharmaceutical industries.

1.2 Pyrococcus furiosus

Pyrococcus furiosus (P. furiosus) is a coccobacillus, usually occurring singly or in pairs, with a diameter of about 0.8-1.5 μm, Gram-negative staining, a single-ended tufted flagellum, no complex organelles, and the largest and most protein-rich

ribosomes in the archaeal domain. Its organism is covered with a layer of glycoprotein (cell wall), which can be distinguished from bacteria. It is an absolute anaerobe, using S as the acceptor of electrons rather than O2. The bacterium is a hyperthermophile discovered by G. Fiala and K. O. Stetter in 1986, which can grow normally at extreme temperatures, and is an extreme species in the archaeal domain, with an optimal growth temperature of 100 ° C. It uses organic matter as a carbon and energy source, and is a chemoenergetic species. and energy source, belonging to chemo-energetic heterotrophic microorganisms [41] . Because *Pyrococcus furiosus* has been exposed to high temperatures for a long time, the enzymes in the body of the bacterium have gradually evolved high-temperature stability, which enables it to maintain its activity in environments far beyond the normal temperature range and participate in a variety of physiological and biochemical reactions in the body of the bacterium.

Extremophiles are a group of microorganisms that can survive, grow and even thrive in extreme environments. Based on the conditions under which they grow under extreme physical parameters, they have been classified as (hyper)thermophilic (high temperature), cryophilic (low temperature), acidophilic (low pH), alkaliphilic (high pH), pressophilic (high pressure), and salinophilic (high salinity) [42] . As a result, their biomolecules, such as proteins, nucleic acids and lipids, have evolved to participate normally in life activities under these harsh conditions. Due to these special properties, enzymes from extreme microorganisms (extremophiles) have great potential not only in basic research but also in biotechnological applications. Extreme enzymes are able to extend the range of reaction conditions suitable for biocatalysis and have high value for applications in sugar chemistry, detergent production, lipid and oleochemistry, and food processing [43, 44] . Thermotolerant enzymes can be obtained from extreme thermophilic bacteria that grow optimally above 60° C or from hyperthermophilic bacteria that grow optimally above 90° C. The species found at the highest temperatures (103-113° C) are exclusively archaea. Studies of extremophilic enzymes in hyperthermophilic archaea have focused on *Pyrococcus*

furiosus and Thermococcus strains [45]. Heat-stable extremophiles are not only good models for understanding protein stability, but also have important potential for biotechnological applications. In addition, reactions at higher temperatures reduce the possibility of complex reactions due to contamination. Characterisation of proteins from hyperthermophilic bacteria has shown that they are extremely thermally stable and may have an optimal catalytic temperature that exceeds the maximum growth temperature of their hosts [10, 46] . In addition to their remarkable thermal stability, proteins from hyperthermophilic bacteria are often found to be highly resistant to chemical denaturation and protease degradation [47]. One of the most stable enzymes identified to date is β -glucosidase from *Pyrococcus furiosus*, which has a half-life of 85 h at 100° C [48] . During growth on fibrous disaccharides, β -glucosidase can produce up to 5% of total *Pyrococcus furiosus* cellular protein and is involved in the hydrolysis of the β -1,4-glycosidic bond between the two glucose portions of the disaccharide [10]. In addition, β -glucosidase has been extensively studied in members of three domains of life: eukaryotic, bacterial and archaeal [49-51] . β -glucosidase from *Pyrococcus furiosus* is a suitable model enzyme for characterising the structure-function relationship of superheat-stable enzymes.

1.3 Pichia pastoris

Pichia pastoris is a methylotrophic yeast. Its advantages as a protein-producing

host organism include microbiological safety, rapid growth to high cell densities, and ease of genetic manipulation. Pichia pastoris supports efficient protein secretion, allows complex folding processes and facilitates downstream purification [52, 53].

Pichia pastoris is an important protein-producing host organism and the establishment of an efficient gene editing system is essential for its metabolic modification [54] . The bacterium lacks natural plasmids in vivo; therefore, in order to achieve exogenous gene expression, expression vectors must be homologously recombined with the host chromosome to integrate the exogenous gene expression framework into the chromosome. These expression vectors usually include elements

such as promoters, exogenous gene cloning sites, termination sequences and screening markers. Typically, these vectors are shuttle plasmids, which are first amplified and replicated in E. coli before being introduced into the host Pichia pastoris cells. To ensure that the product can be secreted extracellularly, the expression vector must also contain a signal peptide sequence [55, 56].

Two of the more common Pichia pastoris host bacteria, GS115 and KM71, carry the HIS4 nutrient-deficient marker. strain GS115 is a Mut+ type, i.e., normal methanol utiliser, which has the AOX1 gene, while strain KM71 has the ARG4 gene inserted at the AOX1 locus with the phenotype Muts, i.e., slow methanol utiliser. Both strains can be used in general yeast transformation methods [57, 58] .

1.4 Status of research

High-temperature-resistant β-glucosidase is used to produce dairy-free milk that can be autoclaved for lactose intolerant patients. As milk is the main source of calcium and vitamin D, patients with lactose intolerance often do not receive adequate amounts of the nutrient, which can lead to poor health outcomes. The production of dairy-free milk could provide a solution to this problem by adding β-glucosidase directly to milk, providing a way to maintain good health while avoiding symptoms of lactose intolerance. The use of heat-resistant β-glucosidase to produce lactose-free milk during the pasteurisation process avoids the risk of reducing microbial contamination by adding the enzyme after pasteurisation [59] .

High-temperature resistant β-glucosidase improves the potency of the medicinal herb Panax notoginseng triterpenes (PN), whose main constituent is San Qi saponins (NGs), and high-temperature resistant β-glucosidase hydrolyzes low-potency NG-R1 and hydrolyzes only the external glucose of NG-R1 to produce the desiccated, high-potency NG-R2, which results in the large-scale biotransformation of NG-R1 to NG-R2 [60] .

In addition to the above research areas, high-temperature-resistant β-glucosidase has important application scenarios in many industrial and research fields. For example, biomass degradation and biofuel production, food processing and brewing industry, biocatalytic reactions, environmental protection and pollution control, pharmaceutical and biomedical research.

Conclusions to chapter 1

β-glucosidase is involved in the degradation of carbohydrates, phenolic compounds, some natural products and antibiotics, and the decomposition of cellulose, lignin and other polysaccharides in the plant cell wall, which is important for the release and recycling of nutrients, and is widely used in the fields of food, pharmaceuticals, skincare and cosmetics, etc. β-glucosidase is widely distributed in nature in many organisms, including plants, microorganisms and animals. β-glucosidase is widely distributed in nature in many organisms, including plants, microorganisms and animals. β-glucosidases of microbial origin are widely used in industrial production, biotechnology and scientific research due to their advantages and features such as high purity and stability, large-scale production capacity, easy optimisation and modification, wide substrate adaptability, environmental friendliness and ease of operation.

Thermal stability of β-glucosidase is one of the key factors determining the cost-effectiveness of biorefineries and their applicability in industries such as beverages, pulp, paper and petroleum [58, 59]. The use of enzymes to hydrolyse biomass at elevated temperatures increases reaction rates, enhances substrate solubility, and reduces microbial contamination [57]. In addition, enzymes with higher thermal stability usually remain active in solution for a longer period of time (as indicated by the half-life (t50)), which reduces the operating costs of biorelevant industries.

The optimal reaction temperature of β-glucosidases varies depending on the source, species, and specific enzyme, with the vast majority of enzymes exhibiting the highest catalytic activity and efficiency at their optimal temperatures. β-glucosidases typically exhibit better activity in the moderate to high temperature range, with most β-glucosidases having optimal reaction temperatures in the range of 40° C to 60° C.[60 However, conventional β-glucosidases are often However, conventional β-glucosidases are often unable to adapt to the high temperatures required by biorelevant industries, and high-temperature-resistant β-glucosidases derived from hyperthermophilic organisms have great potential for application.

In summary, the creation of high-temperature-resistant β-glucosidase using microbial molecular biology technology, microbiological technology, and enzyme engineering technology is of great scientific significance and has great prospects for market application.

CHAPTER 2
OBJECT, PURPOSE AND METHODS OF THE STUDY

2.1 Experimental Materials and Instruments

2.1.1 Experimental strains and plasmids

(1) Strain: Pichia pastoris GS115 was purchased from Thermo Fisher Scientific.

(2) Plasmid:

① pGapZα-A plasmid vector was purchased from Thermo Fisher Scientific.

② *371-β-glucosidase* (*T371A*) gene was kept in our laboratory.

2.1.2 Instrumental enzymes and reagents

(1) Tool enzyme: Linearisers AVR II was purchased from Thermo Fisher Scientific.

(2) Reagents

① Reagent kit K1730-01 (solution I, solution II, solution III, as shown in Table 2-1) was purchased from Thermo Inc.

Table 2-1 Contents of Kit K1730-01

ingredient	Product Description
Solution I	Sorbitol solution containing ethylene glycol and DMSO for preparation of recipient cells
Solution II	Transformation of sensory cells with PEG solution
Solution III	Salt solutions for washing and plating transformed cells

② Zeocin (zeocin)

③ 10% ammonium persulfate (APS)

④ 10% sodium carbonate solution (Na2CO3)

⑤ Tetramethylethylenediamine (TEMED)

⑥ pH4.8 Buffer (acetic acid configuration)

⑦ 10% sodium dodecyl sulfate (SDS), stored at room temperature.

⑧ 4-Nitrophenyl-β-D-glucopyranoside (pNPG)

⑨ Decolouring solution (1L): 100ml of methanol, 100ml of glacial acetic acid, and fixed to 1000mL

⑩ Kaomas Brilliant Blue R-250 staining solution (1L): Kaomas Brilliant Blue R250 1g, add methanol 450ml, glacial acetic acid 100ml

⑪ 30% gel storage solution: acrylamide (Acr) 29.2 g, methylenebisacrylamide (Bis) 0.8 g, add distilled water to 100 mL, 4 ℃ refrigerator storage.

⑫ Separation Gel Buffer (1.5 mol/L): Tris (18.17 g), dissolved in distilled water, adjusted to pH=8.8 with 6 mol/L HCI, volume adjusted to 100 mL, stored in refrigerator at 4℃.

⑬ Concentrated Gel Buffer (0.5 mol/L): Tris 6.06 g, dissolved with distilled water, adjusted to pH=8 with 6 mol/L HCI, and fixed to 100 mL, stored in the refrigerator at 4℃.

⑭ Electrophoresis buffer (pH 8.3): sodium dodecyl sulfate (SDS) l g, tris(hydroxymethyl)aminomethane (Tris) 3 g, glycine (Gly) 14.4 g, dissolved with double-distilled water and fixed volume to 1000mL, stored in the refrigerator at 4℃.

⑮ Sampling buffer: 5 mol/L tris-hydroxymethylaminomethane hydrochloride (Tris-HCl) (pH=6.8) 1.25 mL, glycerol 2 mL, 10% sodium dodecyl sulphate (SDS) 2 mL, β-mercaptoethanol 1 mL, 0.1% bromophenol blue (BPB) 0.5 mL, add distilled water to volume to 10 mL.

2.1.3 Culture medium

YPD medium: 10 g/L Yeast Extract, 20 g/L Peptone, 20 g/L glucose (solid medium containing 20 g/L agar), sterilised at 115 ℃ for 20 min.

2.1.4 Experimental apparatus

The main experimental apparatus is shown in Table 2-2.

Table 2-2 Main experimental instrumentation and manufacturers

Instrument name	model number	manufacturer (of a product)
Small Vertical Electrophoresis Tank	Mini-	Bio-Rad
Enzyme Marker	EPOCH-2	BioTek
Ultra High Speed Centrifuge	Centrifuge 5840R	Eppendorf
Freezer Cabinet （4℃、-20℃）	BCD-539WT	Haier
Gel Imager	JY04S-3C	Beijing JUNYI
Universal Electrophoresis	PowerPacUniversal	Bio-Rad
Clean Bench	SW-CJ-1FD	AIRTECH
Watertight Constant Temperature Incubator	GHP-9160	Shanghai YIHENG
Horizontal Full-temperature Oscillation Incubator	WS-HR	Zhejiang HUAYUAN

2.2 Experimental Methods

2.2.1 Origin of mutant β-glucosidase gene *371-β-glucosidase (T371A)*

The β-glucosidase gene was cloned from *Pyrococcus furiosus* and molecularly modified by replacing adenine with guanine at 1111 bp of its gene, resulting in a mutation of amino acid 371 from threonine to alanine in the amino acid sequence. The mutant genotype name is *371-β-glucosidase* (*T371A*).

2.2.2 Preparation of recombinant plasmids

The construction of recombinant plasmid pGapZ α-A-*T371A* is shown in Figure 2-1.

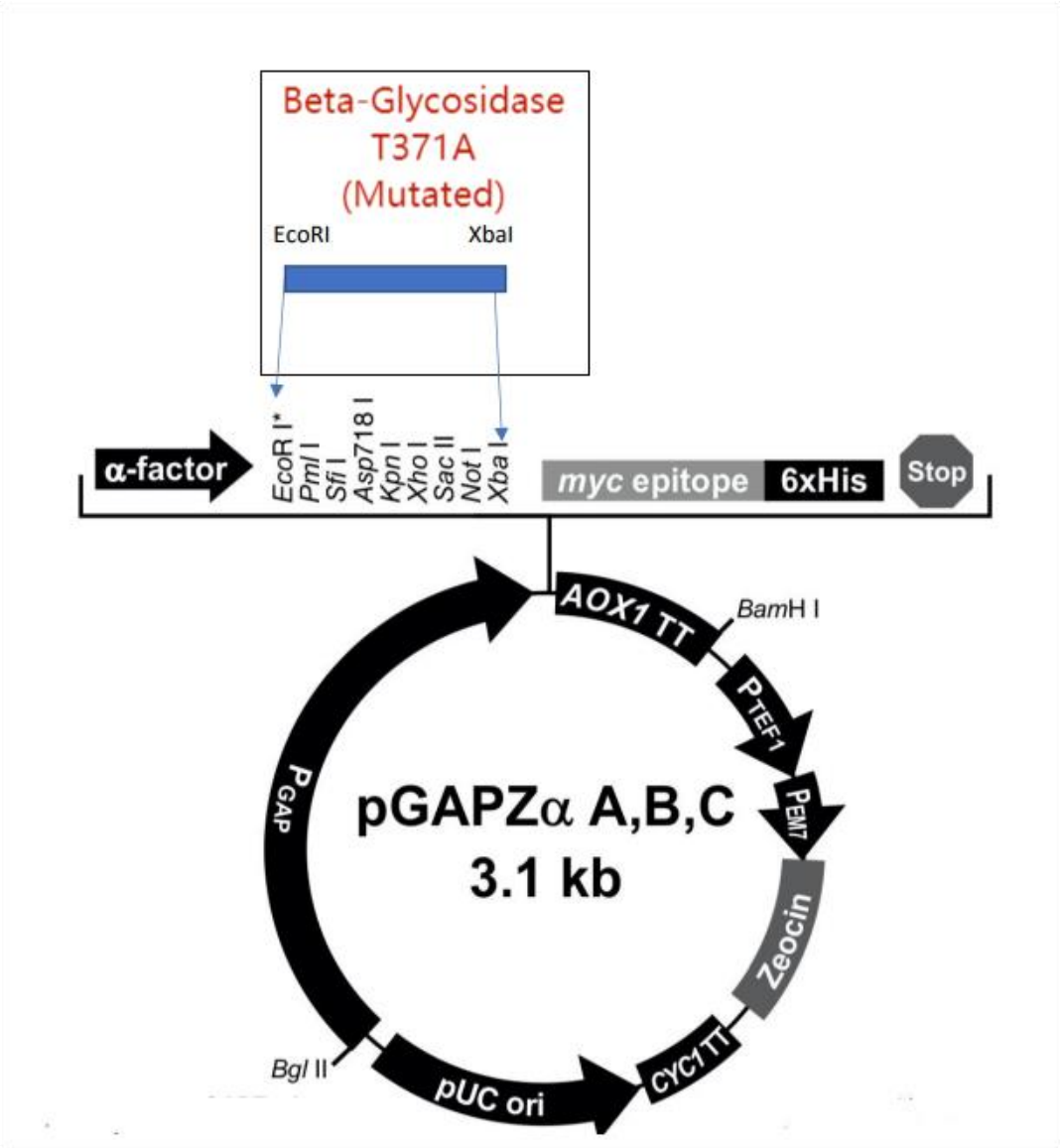


Figure 2-1 Recombinant plasmid pGapZ α -A-*T371A*

2.2.3 Preparation of receptor cells

Receptor cells of Pichia pastoris GS115 strain were prepared using kit K1730-01 (purchased from Thermo).

(1) Pichia pastoris GS115 strain was inoculated into 10 ml YPD liquid medium and cultured overnight at 28-30° C in an oscillating incubator (250-300 rpm).

(2) Dilute the OD600 value of cells cultured overnight on 10 ml YPD liquid medium to 0.1-0.2. then incubate in a shaking incubator at 28-30° C until the OD600 value reaches 0.6-1.0 (about 4-6 hours).

(3) Centrifuge the cells at 500 × g for 5 min at room temperature to pellet the cells and discard the supernatant.

(4) Resuspend the cell pellet in 10 ml of solution I.

(5) Repeat the process of (3) and (4) once.

(6) Place 50-200 μl of receptor cells into labelled 1.5 ml sterile screw-capped microcentrifuge tubes.

(7) At this point, the cells can be stored at room temperature and used directly for transformation or frozen for future use. If the cells are to be frozen, the tubes can be placed in a polystyrene foam box or wrapped in several layers of tissue paper and placed in a refrigerator at -80° C. Care should be taken not to freeze the cells rapidly in liquid nitrogen.

2.2.4 Genetic transformation

Genetic transformation was performed using the polyethylene glycol-mediated method:

(1) Linearise the recombinant plasmid pGapZ α-A-*T371A* with the linearising enzyme AVR II

(2) For each transformation, one tube of sensory cells was thawed at room temperature and a 50μl aliquot of sensory cells was placed into a sterile microcentrifuge tube.

(3) Add 3μg of linearised Pichia pastoris expression plasmid vector pGapZ α-A-*T371A* to the sensory cells.

(4) Add 1 ml of Solution II to the DNA/cell mixture and vortex mix by vortex mixer.

(5) Incubate the transformation reaction in a water bath or incubator at 30° C for 1 hour. Mix the transformation reaction by vortexing every 15 minutes (failure to mix the transformation reaction every 15 minutes will result in a decrease in transformation efficiency).

(6) The cells were heat shocked in a 42° C water bath for 10 min.

(7) The recombinant plasmid containing Zeocin (Zeocin) resistance gene was transformed, and the cells were divided into 2 microcentrifuge tubes (about 525 μl each), 1 ml of YPD medium was added to each tube, and cultured in an incubator at 30° C for 1 hour.

(8) Centrifuge the cells at 3000 × g for 5 min at room temperature to pellet the cells and discard the supernatant.

(9) Resuspend the cell pellet in 1 ml of solution III.

(10) Centrifuge the cells at 3000 × g for 5 min at room temperature to pellet the cells and discard the supernatant.

(11) Resuspend the cell pellet in 100~150μl of solution III in solution.

(12) Apply the whole transformation on a suitable selection plate using a sterile applicator and incubate in an incubator at 30C for 2-4 days. Each transformation produced about 50 colonies.

2.2.5 Strain activation

(1) Make solid medium for YPD, culture single colonies, and store them at 4° C.

(2) Use the tip of a sterile gun to gently stick at a single colony each time, inoculate it into 2-5 mL of YPD liquid medium, add 1/1000 of Zeocin, and incubate it for 15-20 hours at 30℃, 220rpm on a shaker.

(3) Transfer the bacterial solution to about 200-500 mL of YPD liquid medium and continue to incubate for 20 hours, (Zeocin can be added at 1/2000).

(4) Transfer to 2L-20L YPD liquid medium and continue to incubate for another 20 hours, no Zeocin will be added until the end of this step. (During the step-by-step amplification process, 1 mL of yeast can be amplified about every 20 hours to fill up 50 mL-100 mL of YPD liquid medium, so it should be avoided that the incubation time is too long).

(5) Take 20 uL of bacterial liquid, apply it to the YPD solid medium containing zeocin, and incubate it in the incubator at 30 ℃ for 36 hours.

(6) Pick large single colonies, each time using the tip of a sterile gun to gently stick on a single colony, inoculate in 3 mL of YPD medium with 1/1000 of zeocin. incubate overnight at 30 ℃, 220 rpm on a shaker.

(7) Transfer 3 mL of colony solution to 30 mL of YPD medium, add 1/2000 Zeocin. 30 ℃, 220 rpm shaking bed incubation overnight.

(8) Transfer 30 mL of the strain to 200 mL of YPD medium. 30 ℃, 220 rpm, shaking bed culture for 72 hours.

(9) Store the YPD liquid medium with completed strain activation at 4 ℃ for subsequent use.

2.2.6 SDS-PAGE identification

(1) Take 1 mL of the bacterial solution, centrifuge at 5000 × g for 5 min, discard the bacterial body and keep the supernatant.

(2) Take two clean glass plates (one short plate - with grooves and one long plate), align the two plates, place them on the main body of the gel maker, and then clamp them tightly with a wedge plate, aligned on both sides. Then put it on the glue maker and clamp it, then use a pipette to add water between the two sides of the glass plate and leave it for 10min to see if it leaks night. If there is no leakage, pour out the water, if leakage, reassemble and continue to leakage check.

(3) Preparation of separation glue:

Firstly, fix the glass plate for glue making on the glue making rack, make sure to fix it tightly to avoid liquid leakage. Configure 12% of the separation glue (add in the order in Table 2-3), mix thoroughly and then fill the glue, and then add the water sealing glue. When the glue is solidified, pour off the upper layer of water and dry it with absorbent paper. The height of the separating gel is usually located 1cm below the comb; the higher the temperature, the faster the gelation speed, usually the gelation process is about 30min.

Table 2-3 12% Separator Adhesive Formulation Table

Reagent Name	12 % Separating Gel (8 parts, 35 ml)
30%Gel Stock Solution	14 ml
Separation Gel Buffer	8.75 ml
Distilled Water	12.06 ml
10%APS	175 μl
TEMED	15 μl

(4) Preparation of concentrated glue:

Configure 5.1 per cent concentrated glue (add in the order shown in Table 2-4), mix thoroughly and fill with glue, followed by immediate insertion of the comb. After the glue solidifies, slowly pull out the comb. Avoid the appearance of air bubbles within the glue board.

Table 2-4 5.1% Separator Adhesive Formulation Table

Reagent Name	5.1 % Concentrate Gel (8 portions, 12 ml)
30%Gel Stock Solution	2 ml
Gum Buffer Concentrate	3 ml
Distilled Water	6.89 ml
10%APS	100 μl
TEMED	10 μl

(5) Treatment of protein samples:

Treat the samples to be tested with up-sampling buffer, sample: up-sampling buffer = 8:2 or 9:1, mixed, boiling water bath for 5-10 minutes.

(6) Sampling:

After the samples to be tested are treated with the sample buffer, add them sequentially to the gel wells with a micropipette gun, usually with a sample volume of 20 μ l. Protein Marker is a pre-stained protein of known molecular weight, which does not need to be treated with the sample buffer, and is added directly to the gel wells, usually with a sample volume of 5 μ l. During the sampling process, to avoid the sample from overflowing or entering into the other lanes, the empty lanes can be

added to the 5 μl sample buffer, to prevent the diffusion of neighbouring lanes into the empty lanes. The empty lane can be added with 5 μl of sample buffer to prevent the adjacent lanes from diffusing into the empty lane.

(7) Electrophoresis:

Put the gel rack into the electrophoresis tank and add electrophoresis solution not over the gel plate. Concentrated gel voltage selected at 80 ~ 110V, the bromophenol blue indicator in the sample reaches the separation gel, the separation gel voltage is selected at 160 ~ 200V, the electrophoresis process should keep the voltage stable. Until the bromophenol blue front indicator line to the lower end of the electrophoresis tank 1-2 cm to stop.

(8) Staining:

After the end of electrophoresis, remove the glue plate and gently put it into the

staining tank for staining with Coomassie Brilliant Blue, staining for about 20-30 min. reuse of the staining solution needs to extend the staining time.

(9) Decolourisation:

After the completion of staining, remove the gel from the stain and recover the staining solution, rinse the gel with distilled water for 2-3 times, and then put it into the decolouration tank, add the decolouration solution for diffusion decolouration, and decolourize it for several times until the background is clear and there is no background colour.

(10) Result processing:

The gel showing protein bands was photographed and analysed.

2.2.7 Determination of β-glucosidase enzyme activity

β-Glucosidase can hydrolyse p-nitrophenol-β-D-glucopyranoside (pNPG) to p-nitrophenol (pNP), the aqueous solution of the latter is yellow in colour with an absorption peak at 420 nm. As the amount of p-nitrophenol (pNP) produced increases, the absorbance value at 420 nm will change accordingly. Therefore its enzyme activity can be determined by this principle.

(1) Plotting pNP standard curve:

Fix 1 g of pNP to 100 ml with Buffer (pH=4.8) and add 100 ul 10% Na2CO3 respectively, prepare a series of pNP solutions with gradient concentration according to Table 2-5, mix them, and then measure the absorbance value of the solution at 420 nm. The absorbance value was taken as the vertical coordinate, and the number of micromoles of 4-nitrophenol was taken as the horizontal coordinate, and a standard curve was made to find the linear regression equation.

2-5 Table 2-5 pNPG standard curve components configuration table

Component Concentration	0	0.05	0.1	0.15	0.2	0.25	0.3
pNP	0 ul	10 ul	20 ul	30 ul	40 ul	50 ul	60 ul
Buffer	200 ul	190 ul	180 ul	170 ul	160 ul	150 ul	140 ul
Na2CO3	100 ul	100 ul	100 ul	100 ul	100 ul	100 ul	100 ul

(2) Take the supernatant of the medium, centrifuge it at 4° C for 10 min, and take 9 portions of 100 μl supernatant.

(3) Take 2 portions of 100 μl Buffer as control.

(4) Add 50 μl of pNPG solution (0.1 g pNPG with 10 ml Buffer) to the samples to be tested and the control samples respectively. The enzyme activity (i.e. mass of substrate broken down per minute per ml of supernatant) is determined by using β-glucosidase to break down pNPG into pNP (p-nitrophenol) and glucose, and pNP is coloured under alkaline conditions.

(5) The samples to be tested were placed in a water bath at 50℃, 60℃, 70℃, 80℃, 90℃ and 100℃ for 30 min, and the control samples were placed in a water bath at 50℃ for 30 min, and 50μl of 10% sodium carbonate was added to the samples after removing them, and the enzyme activity was detected by using an enzyme counter after the colour development reaction appeared.

(6) The samples to be tested and the control group samples were placed in 100℃ environment with water bath heating for 30min, 60min and 90min, and then 50ul 15% sodium carbonate was added after taking them out, and the enzyme activity was detected by using enzyme marker after the appearance of the colour reaction in order to detect the enzyme tolerance under the condition of 100℃.

Conclusions to chapter 2

This section describes the methods of cloning the β-glucosidase gene from *Pyrococcus furiosus* and obtaining the mutant β-glucosidase gene T371A after molecular modification, transformed the mutant gene into *Pichia pastoris*, and evaluated the resistance to high temperatures of the β-glucosidase expressed by T371A. Also shown are the steps for producing the enzyme using *Pichia pastoris* GS115 expressing T371A in a fermenter.

CHAPTER 3

EXPERIMENTAL PART

3.1 Comparison results

3.3.1 Results of *T371A* gene sequence and amino acid sequence comparison

The *Pyrococcus furiosus* wild-type β-glucosidase gene sequence was compared with the mutant β-glucosidase (*T371A*) gene sequence by SnapGene software (shown in Figure 2-2), and the amino acid sequence comparison was further generated by the software (shown in Figure 2-3).

Figure 2-2 Base pairwise comparison of mutant β-glucosidase (*T371A*) with *Pyrococcus furiosus* wild-type β-glucosidase (PF gene)

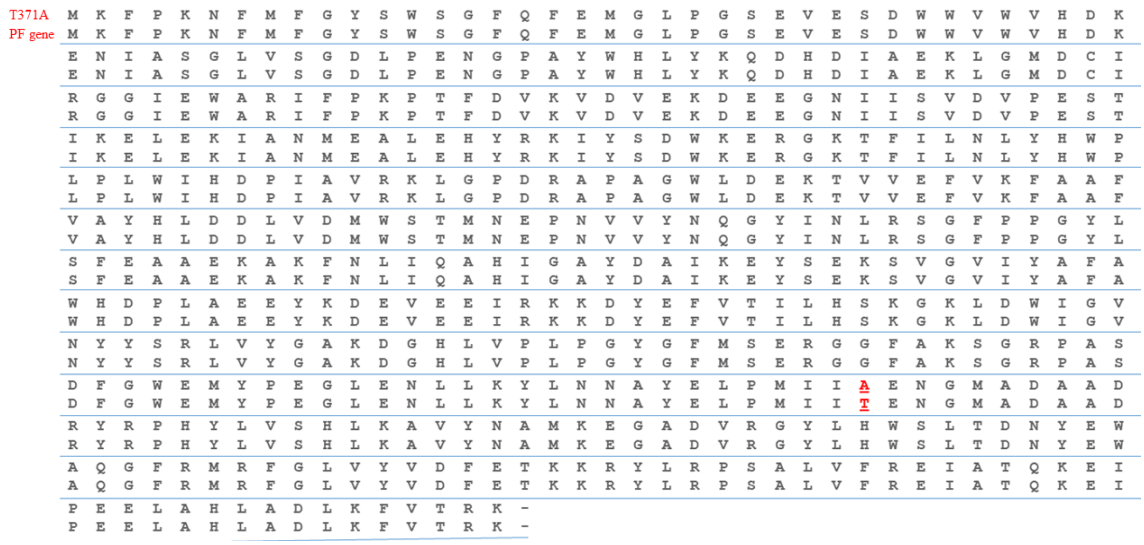


Figure 2-3 Amino acid comparison of mutant β-glucosidase (*T371A*) and *Pyrococcus furiosus* wild type β-glucosidase (PF gene**)**

As can be seen in Figure 2.2, adenine (A) was replaced with guanine (G) at 1111 bp in the gene sequence of the mutant β-glucosidase (*T371A*) as compared to the wild-type β-glucosidase gene sequence of *Pyrococcus furiosus*; and, as can be seen in Figure 2.3, the change in DNA sequence resulted in a change in the *Pyrococcus furiosus* Amino acid 371 in the amino acid sequence of wild-type β-glucosidase was mutated from threonine to alanine.

3.3.2 SDS-PAGE identification

As shown in Figure 2-4 below, M is the DNA standard marker, the blank group is in lane ④, the purpose band of *T371A* protein is in lane ⑤, and the band of non-transgenic Pichia pastoris β-glucosidase protein is in lane ⑥, and the band of wild-type β-glucosidase protein can also be observed. The corresponding band can be clearly observed in lane ⑤, which is darker in colour. According to the experimental results, *T371A* gene was successfully expressed in Pichia pastoris.

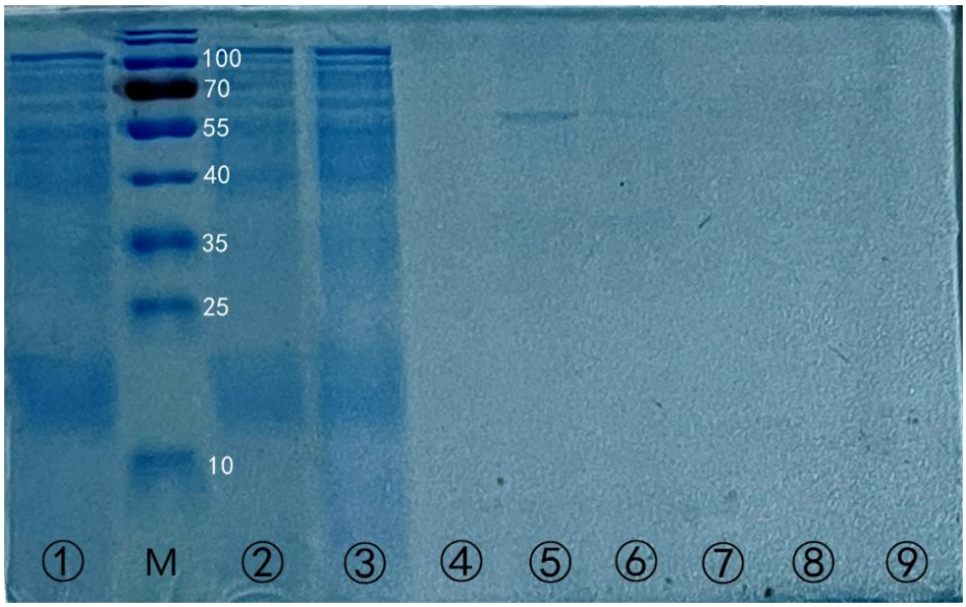


Figure 2-4 SDS-PAGE identification of mutant β -glucosidase expression in Pichia pastoris

3.3.3 Determination of β-glucosidase enzyme activity

(1) Preparation of pNP standard curve

The absorbance value of the solution was measured at the wavelength of 420 nm by enzyme labelling instrument, as shown in Figure 2-5, and the pNP standard curve was plotted with the absorbance value as the vertical coordinate and the number of micromoles of 4-nitrophenol (pNP) as the horizontal coordinate, and a linear regression equation was obtained as y=0.2635x-0.00008, with a correlation coefficient of R2=0.9982. 99.82% explanatory power for the enzyme activity of the solution, so this linear regression overall model can be more accurate to derive the enzyme activity of the solution.

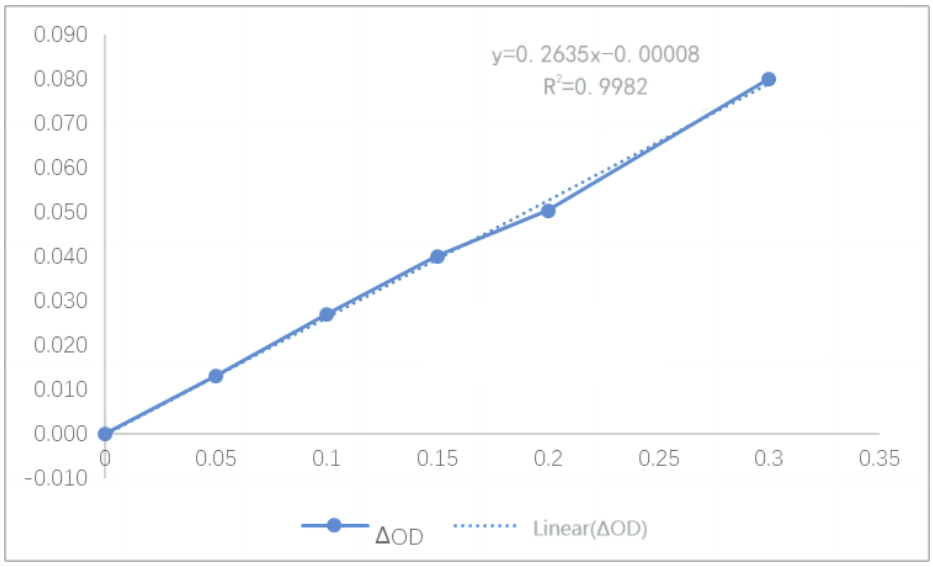


Figure 2-5 pNP standard curve diagram

(2) Enzyme activity after 30 min reaction at different temperature conditions

The activity of mutant β-glucosidase was detected under different temperature conditions and compared with wild-type β-glucosidase. From Table 2-6 and Figure 2-6, it can be seen that the enzyme activity of the mutant β-glucosidase expressed by transgenic Pichia pastoris increased linearly with the increase of temperature, and the enzyme was able to maintain a significant increase in enzyme activity at temperatures much higher than the common optimal enzyme activity temperature, which showed its significant thermophilicity.

Mutant β-glucosidase and wild-type enzyme activities showed a linear increase with increasing temperature, and the enzyme activity of mutant β-glucosidase was about 257.17% higher than that of wild-type β-glucosidase below 70° C, indicating that the enzyme catalytic capacity of mutant β-glucosidase was much higher than that of wild-type enzyme at 50-70° C. With the increase of temperature, the enzyme activity of mutant β-glucosidase was about 44.30% higher than that of wild-type β-glucosidase, indicating that mutant β-glucosidase had better enzyme catalytic ability and thermal stability at high temperatures compared with wild-type β-glucosidase.

Temperature Items

Table 2-6 Data table of enzyme activities of wild type and mutant β-glucosidase at different temperatures

Temperature Items	Wild-type β-glucosidase enzyme activity（ug/ml/min）	Mutant β-glucosidase enzyme activity（ug/ml/min）
50℃	33.56 ± 5	134.31 ± 5
65℃	52.28 ± 5	164.23 ± 5
80℃	120.08 ± 5	214.83 ± 5
90℃	190.05 ± 5	233.93 ± 5
100℃	198.21 ± 5	259.47 ± 5

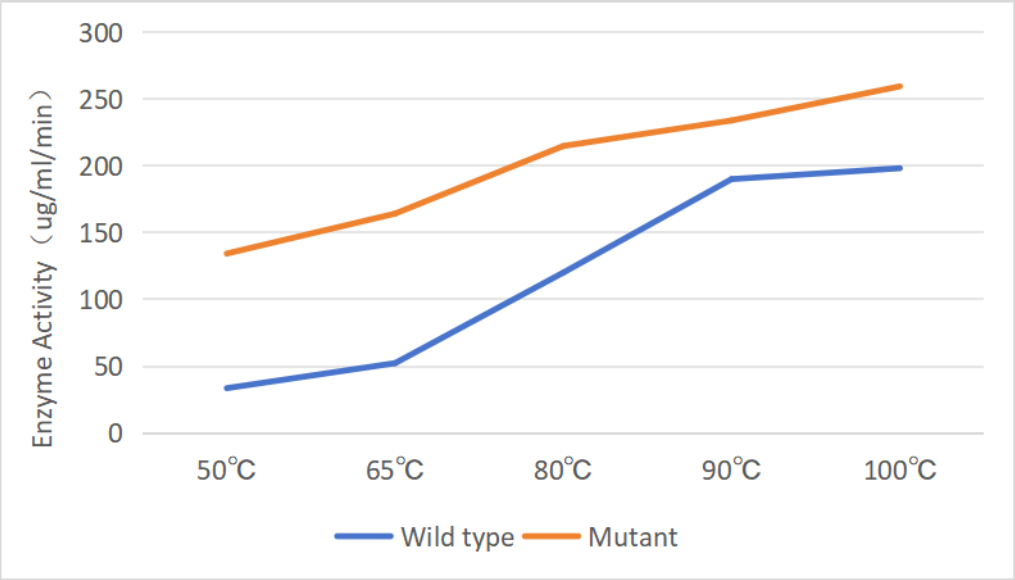


Figure 2-6 Trend of enzyme activity of wild-type and mutant β-glucosidase at different temperatures

(3) Enzyme activity at different times under 100° C conditions

The activity of mutant β-glucosidase was detected at different times under 100℃ reaction conditions. From Table 2-7 and Figure 2-7, it can be seen that the enzyme activity of the mutant β-glucosidase expressed by transgenic Pichia pastoris increased linearly with the increase of time under the 100℃ reaction condition, showing its remarkable thermophilicity and high temperature resistance. It indicates that the enzyme of mutant β-glucosidase did not reach Vm at 100° C and the

optimum temperature was much higher than 100° C, demonstrating remarkable thermal stability.

Table 2-7. Enzyme activity data of mutant β-glucosidase at 100° C for different times of reaction.

Response Time	Enzyme Activity（ug/ml/min）
30min	259.47 ± 5
60min	303.66 ± 5
90min	360.08 ± 5

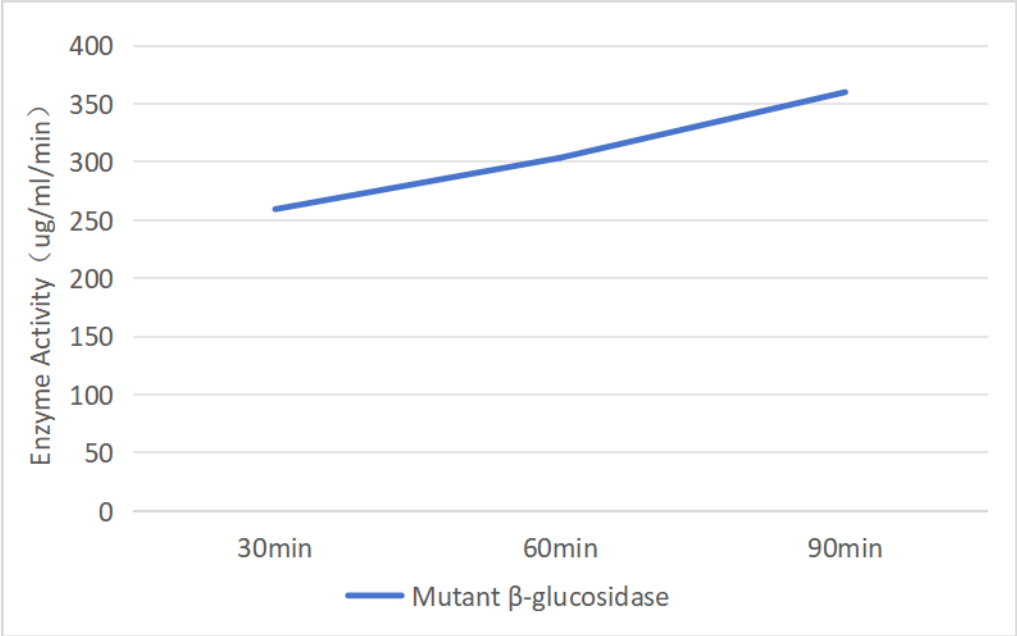


Table 2-7. Trends of enzyme activity of mutant β-glucosidase at 100° C for different times of reaction

Preparation and assay of mutant β-glucosidase enzyme powder

3.1 Experimental materials and apparatus

3.1.1 Experimental strain

The engineering strain was the transgenic Pichia pastoris GS115 strain obtained from the above experiments (kept in the laboratory of A504, Food and Engineering Building, Qilu University of Technology).

3.1.2 Experimental reagents

① Alkaline buffer (NaOH, pH=8.8)

② 4-Nitrophenyl- β -D-glucopyranoside (pNPG)

③ pH4.8 Buffer (acetic acid configuration)

④ 10% sodium carbonate solution (Na2CO3)

3.1.3 Culture medium

YPD medium: 10 g/L Yeast Extract, 20 g/L Peptone, 20 g/L glucose, (solid medium containing 20 g/L agar), sterilised at 115 ℃ for 20 min.

3.1.4 Experimental apparatus

Main experimental apparatus: as shown in Table 3-1.

Table 3-1 Main experimental instrumentation and manufacturers

Instrument name	model number	manufacturer (of a product)
Enzyme Marker	EPOCH-2	BioTek
Ultra High Speed Centrifuge	Centrifuge 5840R	Eppendorf
Freezer Cabinet (4℃、-20℃)	BCD-539WT	Haier
Watertight Constant Temperature Incubator	GHP-9160	Shanghai Yiheng
Horizontal Full-temperature Oscillation Incubator	WS-HR	Zhejiang Huayuan
Experimental High Temperature Spray Dryer	BILON-6000Y	Shanghai BiLon
Fully Automatic Electrically Heated Steam Generator	TY12-0.7-D	Tonyi Boiler
50L Stainless Steel Automatic Fermenter	BLBI0-50SJA-UIP	Bailun Bio
Electrothermal Constant Temperature Blast Drying Oven	DHG-9240A	Shanghai JINGHONG

3.2 Experimental Methods

3.2.1 Strain activation

The transgenic Pichia pastoris stored at 4 ℃ was inoculated in YPD solid medium using plate streaking method, and incubated at 37 ℃ for 16 hours to obtain the activated single colony.

3.2.2 Shaking bed fermentation culture

Pick single colonies inoculated in YPD liquid medium, 37 ℃, 200 r / min shaking bed culture 20-24 hours, measure the OD600 value of the bacterial liquid. When the OD600 is 1.0, end the shaker culture and obtain the seed liquid.

3.2.3 Fermentation culture in fermenter

(1) Add the ingredients according to Table 3-2 (added after the seed liquid is sterilised) into a 50L microbial fermenter (effective volume 35L).

Table 3-2 **Fermenter Ingredients Table**

Dosage	Ingredient（35L）
Seed Liquid (added after sterilisation is complete)	1.75 L（5%）
Peptone	700 g（2%）
Glucose	700 g（2%）
Yeast Paste	350 g（1%）
Distilled Water	28 L（80%）
Condensate (from sterilisation process)	3.5 L（10%）

(2) Turn on the steam boiler, open the inlet valve, add water, open the steam valve and close the drain valve.

(3) Load the alkaline buffer (pH=8.8) into the pH refill bottle, connect the refill bottle and the refill needle with the silicone tube and clamp the silicone tube with the water-stop clamp, the other end of the refill bottle is connected to the fermenter through the filter.

(4) Test the air tightness of the fermenter, turn on the air compressor, adjust the air pressure reducing valve so that the air pressure into the tank is maintained at 0.20Mpa, open the regulating valve in front of the air flow meter into the air, check the fermenter, filters, pipelines, valves, etc., airtightness is good, there is no leakage, to ensure that there is no change in air pressure within 30 minutes.

(5) Turn on the fermenter solid elimination, sterilise the ingredients in the fermenter:

① the first stage of warming (room temperature →90 ℃): close the circulation valve, fully open the exhaust valve, half open the exhaust valve, micro-open jacket steam valve, speed to 150-200 rpm, if the temperature is slow, you should open a large jacket steam valve, shut down the small exhaust valve, control a small amount of steam can be, and thereafter, by adjusting the opening of the jacket steam valve, to maintain the jacket pressure of less than 0.2 Mpa.

② the second stage of heating (90 ℃→115 ℃): when the tank temperature reaches 98 ℃, the speed to 50 rpm, ready to steam boiler steam into the tank, open the bottom valve, close the valve into the tank, micro-open all the filters sewage valve to ensure that the air valve is closed, open the drain valve below the filter, slowly open the filter before the steam valve into the steam exhaust despite the steam condensate in the channel, open the air diaphragm after the filter into the tank After the filter into the tank air diaphragm valve, through the air distributor to the tank into the steam temperature at the same time the air pipeline and air filter sterilization, maintain the filter pressure at 0.1-0.15Mpa. 3 minutes after observing a large amount of steam coming out of the exhaust port, open all the inlet valves, open the steam valve, micro-closing the exhaust valve. Make the irrigation pressure rises, the temperature will rise, and so the temperature reaches 115 ℃, at this time the irrigation pressure should be in 0.11-0.13Mpa, this jacket, bottom valve and air piping three-way intake to ensure the quality of sterilisation.

③ Maintenance stage (115 ℃): when the temperature rises to 115 ℃, the tank pressure rises to 0.12 Mpa, start timing the sterilisation for 20 minutes. In the process,

by controlling the opening of the bottom valve and exhaust valve to control the temperature and air pressure inside the tank smoothly, over-temperature or over-pressure, reduce the amount of steam into the volume or open the exhaust volume; low temperature or low pressure, increase the amount of steam or reduce the exhaust volume. The sampling valve should be slightly opened to control a small amount of steam coming out for sampling line sterilisation.

④ The first stage of cooling (115 ℃→110 ℃): natural cooling stage, this time is to rely on the air in and out of the jacket convection for cooling. Should ensure that the filter drain valve slightly open, properly adjust the rotor flow meter, close the steam valve and bottom valve, open the air inlet valve, stop the steam boiler. At this time, the air into the filter into the tank, pay attention to the filter pressure should be less than 0.2Mpa. In the first stage of cooling, need to cooperate with the opening of the circulation valve, through the drain valve to remove the pressure in the jacket, you can close the drain valve.

⑤ The second stage of cooling (110 ℃→ room temperature): when the temperature drops below 110 ℃, open the water valve, adjust the speed to 200 rpm, the programme will automatically inject cooling water into the jacket and eventually automatically control the temperature to the temperature required by the fermentation process. When the temperature drops to 100 ℃, close the filtering valve, it should be noted that with the drop in tank temperature, the pressure inside the tank will also drop, so you need to adjust the rotor flowmeter or exhaust valve at any time to prevent the pressure from dropping to less than 0 Mpa, to maintain the tank pressure at about 0.05 Mpa. When the temperature in the tank reaches the temperature required for the fermentation process, close the water valve, end the cooling, and select the automatic temperature control to be ready for use.

(6) Shaking bottle flame pouring seed:

① Before inoculation should be closed near the fermenter doors and windows to ensure that there is no strong air circulation near the inoculation port, and spray 75% alcohol on the inoculation port near the disinfection and sterilisation, prepared in

advance: alcohol cotton, pliers, tweezers, press the seed ring, asbestos mesh gloves, wrenches.

② Use cotton balls containing 75% alcohol to clean the arm, and then the inlet diaphragm valve slightly off, while opening the exhaust valve to maintain the tank pressure at about 0.02 MPa, pre-screw the inoculation port, ready for the inoculation of the preparatory work.

③ Fill the groove of the inoculation ring with cotton balls containing 75% alcohol, place the inoculation ring at the inoculation port and light the flame, use a spanner to open the inoculation port and open the inoculation vial under the protection of the flame, and after the mouth of the vial is burnt in the flame, pour the strains into the vial quickly (at this time, the pressure of the vial should be close to zero, but should not be zero).

④ Sterilise the inoculation cap over the flame and then tighten it, followed by increasing the air intake to raise the pressure in the tank.

(7) Set the fermentation parameters temperature at 37℃, adjust the rotational speed to 200 rpm, incubate for 20 h, control the pH at 6.5-7.5, and the fermentation tank pressure at 0.06 MPa to start fermentation.

(8) After the end of fermentation, the discharge is to use the tank pressure to discharge the fermentation liquid from the discharge pipe, the tank pressure can be controlled at 0.05-0.10 Mpa.

3.2.4 High temperature spray drying

(1) high-temperature spray dryer for spray gun test, take water as a test solution, the spray gun out of the spray gun mounting holes, the spray gun vertical square to the ground, start the peristaltic pump and air pump, inhale the water spray, observe the atomisation whether there is a significant offset, atomised droplets are too large, can be adjusted appropriately airflow nozzle, to improve the atomisation effect.

(2) Take the fermentation bacterial liquid placed in the feed pipe, set the process parameters for the inlet air temperature of 100 ℃, fan speed of 80%, peristaltic pump speed of 20%, through the needle 5S, start drying.

(3) Take the material and add material every 2 hours, and keep repeating until the high temperature spray drying is finished.

3.2.5 Measurement of enzyme powder yield

The obtained enzyme powder was weighed and calculated in g/L according to Equation 3-1.

Yield (g/L) =(actual amount of product/actual amount of raw material)

×100% (3-1)

3.2.6 Measurement of enzyme activity of enzyme powder

(1) Plotting pNP standard curve:

Prepare a series of pNP solutions with gradient concentration according to Table 2-5 by fixing 1 g of pNP. to 100 ml with Buffer (pH=4.8) and adding 100 ul of 10% Na2CO3 respectively, and after mixing, measure the absorbance value of the solution at the wavelength of 420 nm. Take the absorbance value as the vertical coordinate and the number of micromoles of 4-nitrophenol as the horizontal coordinate, make a standard curve and find the linear regression equation.

(2) Take 6 portions, each portion of 0.1 g enzyme powder dissolved in 1 ml Buffer, centrifuged at 4 ℃ for 10 min, 100 μl supernatant.

(3) Take 2 portions of 100 μl Buffer as control.

(4) Add 50 μl of pNPG solution (0.1 g pNPG with 10 ml Buffer) to the samples to be tested and the control samples respectively. The enzyme activity (i.e. mass of substrate broken down per minute per ml of supernatant) is determined by using β-glucosidase to break down pNPG into pNP (p-nitrophenol) and glucose, and pNP is coloured under alkaline conditions.

(5) The samples to be tested were placed in a water bath at 50℃, 60℃, 70℃, 80℃, 90℃ and 100℃ for 30 min, and the control samples were placed in a water bath at 50℃ for 30 min, and 50μl of 10% sodium carbonate was added after removal, and the enzyme activity was detected by using an enzyme marker after a colour development reaction appeared.

(6) The samples to be tested and the control samples were heated in water bath at 100℃ for 30min, 60min and 90min, and then 50ul of 15% sodium carbonate was added after removing them, and the enzyme activity was detected by using enzyme marker after the colour reaction appeared to detect the enzyme tolerance at 100℃.

3.2.7 Measurement of water content of enzyme powder

It was carried out according to the Determination of Moisture in Food of Food Safety National Standard GB 5009.3-2016.

20 g of enzyme powder was taken and placed in a drying oven and dried under standard atmospheric pressure (101.3 kPa) at a temperature of 100 ° C for 1 h. The weight lost on drying in the sample was determined using the volatilisation method, including hygroscopic water, some crystalline water and substances that can be volatilised under these conditions [61] , and then the moisture content was calculated from the weighed values before and after drying, and the calculations were made according to Eqn. 3-2, with the unit of %.

Moisture content (%) = (original weight - dried weight) / dried weight × 100 %

= Water weight / Drying weight ×100 % （3-2）

3.3 Characteristics of the finished product of β-glucosidase enzyme powder

3.3.1 Finished product of β-glucosidase enzyme powder

Figure 3-1 Mutant β-glucosidase enzyme powder finished product

The finished product of β-glucosidase enzyme powder obtained by high-temperature spray drying is shown in Figure 3-1, which is in the form of powder, flower-white, without obvious odour and easily soluble in water.

3.3.2 Enzyme powder yield

In this study, 146.055 g of enzyme powder was successfully obtained from 35 L of fermentation broth using high-temperature spray drying technique, with a yield of 4.17 g/L. This result demonstrated the high efficiency and feasibility of high-temperature spray drying technique in the preparation of enzyme powder, and also reflected the high yield of enzyme powder. The high temperature spray drying gave the enzyme powder a longer storage period, higher storage quality, and more convenient storage method.

3.3.3 Enzyme powder β-glucosidase enzyme activity

(1) pNP standard curve

The pNP standard curve was plotted as y=0.2635x-0.00008, R2=0.9982, see 3.2.3 pNPG enzyme activity determination section for details.

(2) Enzyme activity after 30 min under different temperature conditions:

The enzyme activity of the mutant β-glucosidase enzyme powder obtained by fermentation in a 50L fermenter and spray drying at high temperature was detected at different temperatures and compared with the enzyme activity of mutant β-glucosidase and wild-type β-glucosidase cultured in shake flasks. From Table 3-3 and Figure 3-2, it can be seen that the enzyme activity of the mutant β-glucosidase powder obtained by fermentation and high temperature spray drying had the same trend as that of the mutant β-glucosidase cultured in shaking flasks, which both increased linearly with the increase of temperature, and the enzyme was able to maintain a significant increase in enzyme activity at temperatures much higher than that of the common optimal enzyme activity, which showed its significant thermophilicity.

The enzyme activity of all three of them showed a linear increase with the increase of temperature, and the enzyme activity of the mutant β-glucosidase enzyme powder obtained by fermentation and high-temperature spray-drying was only 17.23% lower than that of the mutant β-glucosidase enzyme cultured in shaking flasks, which indicated that the amount of enzyme activity lost after high-temperature spray-drying was less, but it greatly prolonged the enzyme's storage time and the quality of storage.The enzyme activity of mutant β-glucosidase enzyme powder was much higher than the wild-type β-glucosidase enzyme at the temperature below 70° C. The enzyme activity of the enzyme powder at the temperature below 70° C was much higher than that of the wild-type β-glucosidase enzyme. The enzyme activity of the enzyme powder was about 161.27% higher than that of the wild-type β-glucosidase, indicating that the enzyme catalytic capacity of mutant β-glucosidase was much higher than that of the wild-type enzyme at 50-70 ° C. With the increase of temperature, the enzyme activity of mutant β-glucosidase was about 40.93% higher than that of wild-type β-glucosidase, indicating that although mutant β-glucosidase enzyme powder lost a small portion of its enzyme activity after high temperature spray-drying, it was more better enzyme catalytic ability and thermal stability at high temperature compared with wild-type β-glucosidase.

Table 3-3 Data table of enzyme activity of wild type, mutant β-glucosidase and mutant β-glucosidase enzyme powder at different temperatures

Temperature Items	Wild-type β-glucosidase enzyme activity（ug/ml/min）	Mutant β-glucosidase (shake flask culture) enzyme activity（ug/ml/min）	Mutant β-glucosidase (fermenter/spray-dried) enzyme powder enzyme activity（ug/ml/min）

50℃	33.56 ± 5	134.31 ± 5	80.78 ± 5
65℃	52.28 ± 5	164.23 ± 5	147.34 ± 5
80℃	120.08 ± 5	214.83 ± 5	215.42 ± 5
90℃	190.05 ± 5	233.93 ± 5	228.85 ± 5
100℃	198.21 ± 5	259.47 ± 5	243.74 ± 5

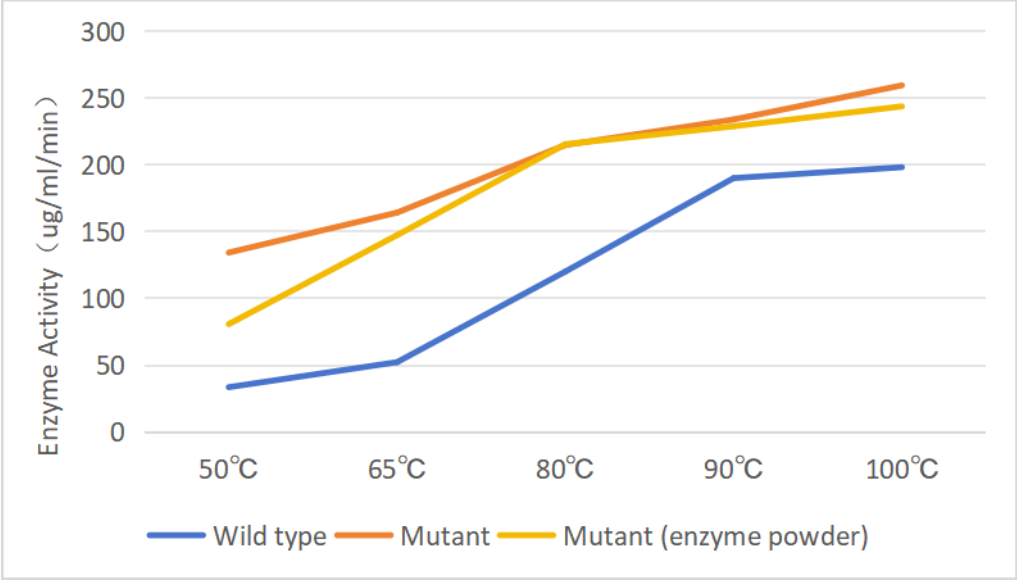


Figure 3-2 Trend of enzyme activity of wild-type, mutant β-glucosidase and mutant β-glucosidase enzyme powder at different temperatures

(3) Enzyme activity at different times under 100° C conditions:

The activity of mutant β-glucosidase enzyme powder was detected and compared with mutant β-glucosidase enzyme activity at different times under 100° C reaction conditions. From Table 3-4 and Figure 3-3, it can be seen that the enzyme activity of mutant β-glucosidase enzyme powder obtained by fermentation and high-temperature spray drying had the same trend as that of mutant β-glucosidase under the reaction condition of 100° C, and increased linearly with the increase of time, showing its remarkable thermophilicity, high temperature resistance and thermal stability. The enzyme activity of mutant β-glucosidase powder obtained by fermentation and high-temperature spray drying was only 9.70% lower than that of mutant β-glucosidase, which indicated that the amount of enzyme activity lost after

high-temperature spray drying was less, but the storage time and quality of the enzyme were greatly prolonged.

Table 3-4. Enzyme activity of mutant β-glucosidase enzyme powder at 100° C for different times of reaction conditions

Time Project	Mutant β-glucosidase enzyme activity (ug/ml/min)	Mutant β-glucosidase enzyme powder enzyme activity (ug/ml/min)
30min	259.47 ± 5	243.74 ± 5
60min	303.66 ± 5	275.62 ± 5
90min	360.08 ± 5	320.12 ± 5

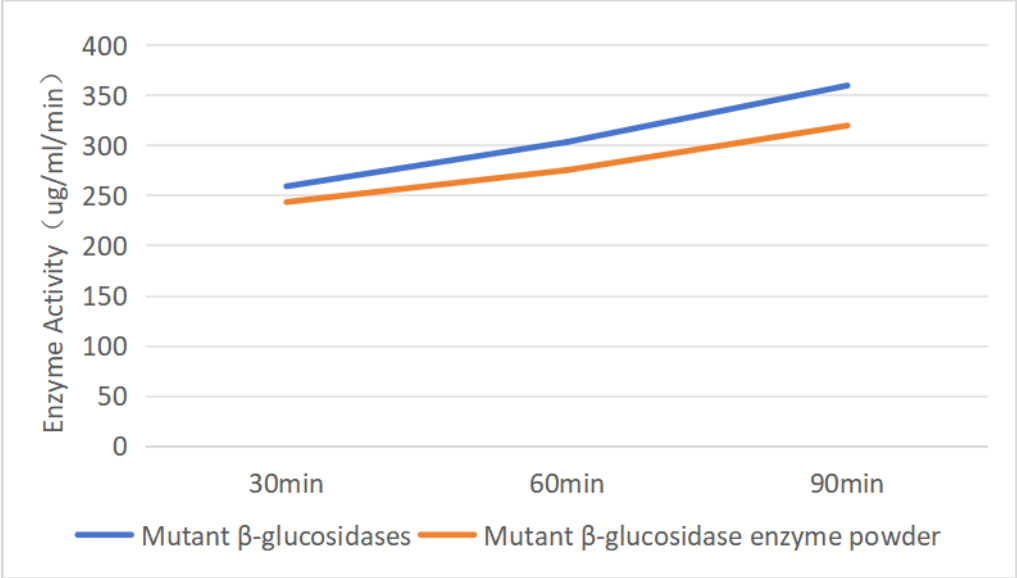


Figure 3-3. Trend of enzyme activity of mutant β-glucosidase and enzyme powder at different times under reaction conditions at 100° C

Conclusions to chapter 3

The wild-type β-glucosidase gene sequence of *Pyrococcus furiosus* was compared with the mutant β-glucosidase (T371A) gene sequence using SnapGene software

As can be seen, adenine (A) was replaced by guanine (G) at 1111 bp in the sequence of the mutant β-glucosidase gene (T371A) compared to the sequence of the wild-type β-glucosidase gene of Pyrococcus furiosus; a change in the DNA sequence

resulted in a change in amino acid 371 *Pyrococcus furiosus* in the amino acid sequence of wild-type β-glucosidase was changed from threonine to alanine.

The linear regression equation was obtained as y=0.2635x-0.00008, with a correlation coefficient of R2=0.9982. The explanatory power of solution enzyme activity is 99.82%, so this general linear regression model may be more accurate to determine solution enzyme activity.

As the temperature increased, the enzymatic activity of the mutant β-glucosidase was about 44.30% higher than that of the wild-type β-glucosidase, indicating that the mutant β-glucosidase had better enzymatic catalytic ability and thermal stability at high temperatures compared with the wild-type . β-glucosidase type.

CONCLUSIONS

In this study, the mutant β-glucosidase gene *371-β-glucosidase* (*T371A*), which was cloned and molecularly modified from *Pyrococcus furiosus*, was transferred into Pichia pastoris by the polyethylene glycol-mediated method, and the SDS-PAGE proved that the *T371A* gene had been integrated into Pichia pastoris genome and was successfully expressed. SDS-PAGE demonstrated that the *T371A* gene had been integrated into the genome of *Pichia pastoris* and successfully expressed, which provided a reliable material for the evaluation of the high temperature tolerance of β-glucosidase expressed by the gene and the preparation of enzyme powder.

It was found that the β-glucosidase expressed by *T371A* gene has the characteristics of maintaining high activity and stability under high temperature environment. With the increase of temperature the transgenic enzyme showed significant thermophilicity and thermal stability, a feature that was consistent with the *Pyrococcus furiosus* strain from which the transgene originated. The results of the study showed that the enzyme activity of the mutant β-glucosidase was about 257.17% higher than that of the wild β-glucosidase at 50-70°C, and with increasing temperature, the enzyme activity of the mutant β-glucosidase was about 44.30% higher than that of the wild β-glucosidase. The transgenic Pichia pastoris strain was put into a fermenter for large-scale cultivation, and spray-dried at high temperature to become enzyme powder, and the enzyme powder with a yield of 4.17 g/L and a water content of 9.9% was obtained, which had an enzyme activity loss of only 17.23% and the same catalytic properties as that of *T371A*, with an enzyme activity of about 161.27% higher than that of the wild β-glucosidase enzyme activity under the condition of 50-70 ℃. Its enzyme activity was about 161.27% higher than that of wild β-glucosidase at 50-70 ℃, and about 40.93% higher than that of wild β-glucosidase at 100 ℃. Moreover, the mutant β-glucosidase enzyme powder obtained longer storage period, better storage quality and more convenient storage method. It was further found that the enzyme activity of mutant β-glucosidase increased continuously with the increase of reaction time at 100 ℃, indicating that the enzyme

activity of mutant β-glucosidase did not reach Vm at 100 ℃, and the optimal temperature was higher than 100 ℃, which demonstrated the remarkable heat resistance and thermal stability.

In this study, β-glucosidase powder was successfully prepared by high-temperature spray drying method. This enzyme powder had a water content lower than the national standard, had a negligible loss of activity, was highly resistant to high temperatures, and demonstrated excellent storage stability. The experimental results indicate that high-temperature spray drying improves the extraction efficiency of the enzyme powder, which makes it more convenient and efficient to prepare the enzyme powder from large-scale liquid cultures. β-glucosidase optimal reaction temperatures vary depending on the source, species, and specific enzyme, and the vast majority of enzymes exhibit the highest catalytic activities and efficiencies at their optimal temperatures. β-glucosidase usually exhibits better activities in the moderate to high temperature range. Most β-glucosidases have an optimum reaction temperature between 40°C and 60°C. However, in some industrial processes it is often the case that the reaction temperature of the β-glucosidase is higher than that of the β-glucosidase. However, higher reaction temperatures are often required in some industrial processes, where high temperatures may help to increase the catalytic activity of the enzyme, and increasing the optimum reaction temperature of β-glucosidase may increase the reaction rate and productivity. Meanwhile, in some industrial processes, increasing the reaction temperature of the β-glucosidase may allow the reaction to be carried out at a higher temperature, thereby reducing energy consumption for heating and holding and saving energy costs. In addition, in some special application environments, such as industrial production under high temperature conditions or specific biotechnological applications, which require the enzyme to have high thermal stability and activity, increasing the optimal reaction temperature of the β-glucosidase can make it more adaptable to these specific application environments. Some industrial processes may require higher temperatures and conventional enzymes may not be suitable for these high temperature conditions.

By increasing the optimal reaction temperature of β-glucosidase, its application in a wider temperature range can be expanded.

In summary, the creation of high-temperature-resistant β-glucosidase using microbial molecular biology technology, microbiological technology, and enzyme engineering technology in this study is of great scientific significance and has great prospects for market application.

Prospect

Limited to time and other factors, this study still has some limitations, such as the failure to comprehensively assess the performance of mutant enzymes in various high-temperature environments and the study of the optimum temperature, as well as the further optimisation of high-temperature spray drying process parameters. Therefore, future studies can be expanded and deepened in the following aspects:

(1) Further modification and optimisation of the β-glucosidase gene: although we have successfully modified the β-glucosidase gene and improved its high-temperature tolerance, it is still possible to enhance its performance by further genetic modification. For example, other heat tolerance or stability-related gene fragments can be introduced, or more precise modifications can be made by computer-aided design to obtain more desirable mutant enzymes.

(2) Basic biological studies: In order to gain a deeper understanding of the nature and function of β-glucosidase, a more comprehensive assessment of the stability and activity of the mutant enzyme under different high-temperature conditions as well as its optimal temperature to further reveal its application potentials and constraints, more in-depth basic biological studies are needed. For example, the molecular structure of the enzyme, its mechanism of action, and the mechanism compatible with the high-temperature environment can be studied.

(3) Process optimisation and cost reduction: Although the high-temperature spray drying method was used to prepare the enzyme powder in this study and achieved better results, it is still possible to further improve the yield and quality of the enzyme powder, as well as to reduce the production cost and improve the

efficiency of extraction through process optimisation. For example, the effect of different drying conditions on the performance of enzyme powder can be studied, or other more efficient extraction and purification methods can be explored.

(4) Expanding application fields: at present, high-temperature-resistant β-glucosidase is mainly applied in specific industrial fields, but with the improvement of its performance, it can be considered to be applied in a wider range of fields, such as bio-energy, pharmaceuticals, food and so on. This can not only promote the development of related industries, but also provide more opportunities and challenges for the research of β-glucosidase.

(1) Biomass degradation and biofuel production: high-temperature resistant β-glucosidase plays a key role in biomass degradation and biofuel production. Biomass such as cellulose and lignin are major components of plant tissues, and high-temperature-resistant β-glucosidase can efficiently degrade these complex biomasses at high temperatures, releasing carbon sources such as glucose, which can provide feedstock for biofuel production.

(2) Food processing and brewing industry: In the food processing and brewing industry, high-temperature-resistant β-glucosidase can be used to improve the taste, texture and quality of products. For example, in the beer brewing process, high temperature resistant β-glucosidase can be used to remove bitter substances and improve the taste of beer.

(3) Biocatalytic reactions: High temperature resistant β-glucosidase also plays an important role in various biocatalytic reactions. It can catalyse various glucoside bond reactions under high temperature conditions, such as enzymatic synthesis, conversion and synthesis of chemicals.

(4) Environmental Protection and Pollution Control: High-temperature resistant β-glucosidase can be used in the field of environmental protection and pollution control, such as for biological treatment of organic wastewater and organic pollutants in wastewater, as well as playing a role in bio-composting and organic waste treatment.

(5) Pharmaceutical and biomedical research: high-temperature-resistant β-glucosidase also has potential applications in pharmaceutical and biomedical research, for example, for the synthesis and preparation of biomedical imaging markers, drug synthesis and enzyme-catalysed reactions in the process of chemical synthesis.

LIST REFERENCES

[1] PATEL A K, SINGHANIA R R, SIM S J, et al. Thermostable cellulases: Current status and perspectives [J]. Bioresour Technol, 2019, 279: 385-92.

[2] HAKI G D, RAKSHIT S K. Developments in industrially important thermostable enzymes: a review [J]. Bioresour Technol, 2003, 89(1): 17-34.

[3] YOAV S, STERN J, SALAMA-ALBER O, et al. Directed Evolution of Clostridium thermocellum β-Glucosidase A Towards Enhanced Thermostability [J]. International Journal of Molecular Sciences, 2019, 20(19): 4701.

[4] KANNAN P, SHAFREEN M M, ACHUDHAN A B, et al. A review on applications of beta-glucosidase in food, brewery, pharmaceutical and cosmetic industries [J]. Carbohydr Res, 2023, 530: 108855.

[5] 夏伟. 嗜热真菌第 3 家族 β-葡萄糖苷酶的多样性和分子改造 [D]. 浙江大学; 浙江大学, 2016.

[6] BHATIA Y, MISHRA S, BISARIA V S. Microbial beta-glucosidases: cloning, properties, and applications [J]. Crit Rev Biotechnol, 2002, 22(4): 375-407.

[7] SINGHANIA R R, PATEL A K, SUKUMARAN R K, et al. Role and significance of beta-glucosidases in the hydrolysis of cellulose for bioethanol production [J]. Bioresource Technology, 2013, 127: 500-7.

[8] 潘利华, 罗建平. β-葡萄糖苷酶的研究及应用进展 [J]. 食品科学, 2006, 27(12): 5.

[9] BOHLIN C, OLSEN S N, MORANT M D, et al. A comparative study of activity and apparent inhibition of fungal β- glucosidases [J]. Biotechnology and Bioengineering, 2010, 107.

[10] KENGEN S W, LUESINK E J, STAMS A J, et al. Purification and characterization of an extremely thermostable beta-glucosidase from the hyperthermophilic archaeon Pyrococcus furiosus [J]. Eur J Biochem, 1993, 213(1): 305-12.

[11] SCHMID G, WANDREY C. Purification and partial characterization of a cellodextrin glucohydrolase (beta-glucosidase) from Trichoderma reesei strain QM 9414 [J]. Biotechnol Bioeng, 1987, 30(4): 571-85.

[12] PAAVILAINEN S, HELLMAN J, KORPELA T. Purification, characterization, gene cloning, and sequencing of a new beta-glucosidase from Bacillus circulans subsp. alkalophilus [J]. Appl Environ Microbiol, 1993, 59(3): 927-32.

[13] 王欢. 嗜热毛壳菌 β-葡萄糖苷酶 BGL3 基因的克隆、表达和分子改造 [D]. 山东大学; 山东大学, 2018.

[14] 牛瑜. 6-磷酸-β-葡萄糖苷酶 Pbgl25-217 的克隆、表达与酶学性质研究 [D]. 山东大学; 山东大学, 2011.

[15] 缪婷婷. 植物乳杆菌 WU14 和嗜热古菌来源的 GH1 家族的 β-葡萄糖苷酶的克隆表达及酶学性质研究 [D]. 江西农业大学; 江西农业大学, 2021.

[16] TAKASHIMA S, NAKAMURA A, HIDAKA M, et al. Molecular cloning and expression of the novel fungal beta-glucosidase genes from Humicola grisea and Trichoderma reesei [J]. Journal of biochemistry, 1999, 125 4: 728-36.

[17] HENRISSAT B, BAIROCH A. Updating the sequence-based classification of glycosyl hydrolases [J]. Biochem J, 1996, 316 (Pt 2)(Pt 2): 695-6.

[18] OPASSIRI R, POMTHONG B, AKIYAMA T, et al. A stress-induced rice (Oryza sativa L.) beta-glucosidase represents a new subfamily of glycosyl hydrolase family 5 containing a fascin-like domain [J]. Biochem J, 2007, 408(2): 241-9.

[19] CANTAREL B, COUTINHO P, RANCUREL C, et al. The carbohydrate-active ENZYMES database (CAZy): an expert resource for glycogenomics. Nucleic Acids Res 37:D233-D238 [J]. Nucleic acids research, 2008, 37: D233-8.

[20] TIWARI R, KUMAR K, SINGH S, et al. Molecular Detection and Environment-Specific Diversity of Glycosyl Hydrolase Family 1 beta-Glucosidase in Different Habitats [J]. Front Microbiol, 2016, 7: 1597.

[21] ZHANG C, ZHANG L, WANG D, et al. Evolutionary History of the Glycoside Hydrolase 3 (GH3) Family Based on the Sequenced Genomes of 48 Plants and Identification of Jasmonic Acid-Related GH3 Proteins in Solanum tuberosum [J]. Int J Mol Sci, 2018, 19(7).

[22] SUZUKI K, SUMITANI J-I, NAM Y-W, et al. Crystal structures of glycoside hydrolase family 3 β-glucosidase 1 from Aspergillus aculeatus [J]. Biochemical Journal, 2013, 452(2): 211-21.

[23] GEBLER J, GILKES N R, CLAEYSSENS M, et al. Stereoselective hydrolysis catalyzed by related beta-1,4-glucanases and beta-1,4-xylanases [J]. Journal of Biological Chemistry, 1992, 267(18): 12559-61.

[24] QI M, JUN H-S, FORSBERG CECIL W. Cel9D, an Atypical 1,4-β-d-Glucan Glucohydrolase from Fibrobacter succinogenes: Characteristics, Catalytic Residues, and Synergistic Interactions with Other Cellulases [J]. Journal of Bacteriology, 2008, 190(6): 1976-84.

[25] LITZINGER S, FISCHER S, POLZER P, et al. Structural and Kinetic Analysis of Bacillus subtilis N-Acetylglucosaminidase Reveals a Unique Asp-His Dyad Mechanism* [J]. Journal of Biological Chemistry, 2010, 285(46): 35675-84.

[26] BAUER M W, KELLY R M. The family 1 beta-glucosidases from Pyrococcus furiosus and Agrobacterium faecalis share a common catalytic mechanism [J]. Biochemistry, 1998, 37(49): 17170-8.

[27] ZECHEL D L, WITHERS S G. Glycosidase Mechanisms: Anatomy of a Finely Tuned Catalyst [J]. Accounts of Chemical Research, 2000, 33(1): 11-8.

[28] KETUDAT CAIRNS J R, ESEN A. β-Glucosidases [J]. Cell Mol Life Sci, 2010, 67(20): 3389-405.

[29] 胡丹. 橡胶籽中 β-葡萄糖苷酶的热稳定性研究 [D]; 海南大学, 2016.

[30] BARABOTE R D, XIE G, LEU D H, et al. Complete genome of the cellulolytic thermophile Acidothermus cellulolyticus 11B provides insights into its ecophysiological and evolutionary adaptations [J]. Genome Res, 2009, 19(6): 1033-43.

[31] MAHESHWARI R, BHARADWAJ G, BHAT M K. Thermophilic fungi: their physiology and enzymes [J]. Microbiol Mol Biol Rev, 2000, 64(3): 461-88.

[32] MALBOOBI M A, LEFEBVRE D D. Isolation of cDNA clones of genes with altered expression levels in phosphate-starved Brassica nigra suspension cells [J]. Plant Molecular Biology, 1995, 28(5): 859-70.

[33] ANTWERPEN M H, GEORGI E, ZOELLER L, et al. Whole-genome sequencing of a pandoravirus isolated from keratitis-inducing acanthamoeba [J]. Genome Announc, 2015, 3(2).

[34] NYYSSöNEN M, TRAN H M, KARAOZ U, et al. Coupled high-throughput functional screening and next generation sequencing for identification of plant polymer decomposing enzymes in metagenomic libraries [J]. Front Microbiol, 2013, 4: 282.

[35] 张媛媛, 苏敏, 朴春红, et al. 微生物来源的β-葡萄糖苷酶在食品工业中应用进展 [J]. 食品工业科技, 2019, 40(16): 329-35.

[36] 宛晓春. 水果风味及风味酶的研究 [D]. 江南大学; 江南大学, 1992.

[37] 李平, 宛晓春, 陶文沂, et al. 黑曲霉 β-葡萄糖苷酶的食品增香应用 [J]. 食品与发酵工业, 2000, 26(2): 3.

[38] KANNAN P, SHAFREEN M M, ACHUDHAN A B, et al. A review on applications of β-glucosidase in food, brewery, pharmaceutical and cosmetic industries [J]. Carbohydrate Research, 2023, 530: 108855.

[39] 张琪, 钟葵, 周素梅. 裂褶多糖保湿功效评价 [J]. 中国食品学报, 2015.

[40] MAI Z, SU H, ZHANG S. Characterization of a Metagenome-Derived β-Glucosidase and Its Application in Conversion of Polydatin to Resveratrol [J]. Catalysts, 2016, 6(3): 35.

[41] STRAUB C T, SCHUT G, OTTEN J K, et al. Modification of the glycolytic pathway in Pyrococcus furiosus and the implications for metabolic engineering [J]. Extremophiles, 2020, 24(4): 511-8.

[42] FENDRIHAN S, LEGAT A, PFAFFENHUEMER M, et al. Extremely halophilic archaea and the issue of long-term microbial survival [J]. Reviews in Environmental Science and Bio/Technology, 2006, 5(2): 203-18.

[43] DEMIRJIAN D C, MORíS-VARAS F, CASSIDY C S. Enzymes from extremophiles [J]. Current Opinion in Chemical Biology, 2001, 5(2): 144-51.

[44] VAN DEN BURG B. Extremophiles as a source for novel enzymes [J]. Current Opinion in Microbiology, 2003, 6(3): 213-8.

[45] ADAMS M W. Enzymes and proteins from organisms that grow near and above 100 degrees C [J]. Annu Rev Microbiol, 1993, 47: 627-58.

[46] KATES M, KUSHNER D J, MATHESON A T. The Biochemistry of archaea (archaebacteria), F, 1993 [C].

[47] FONTANA A. How Nature Engineers Protein (Thermo) Stability; proceedings of the Life Under Extreme Conditions, Berlin, Heidelberg, F 1991//, 1991 [C]. Springer Berlin Heidelberg.

[48] KENGEN S W, DE BOK F A, VAN LOO N D, et al. Evidence for the operation of a novel Embden-Meyerhof pathway that involves ADP-dependent kinases during sugar fermentation by Pyrococcus furiosus [J]. J Biol Chem, 1994, 269(26): 17537-41.

[49] HENRISSAT B. A classification of glycosyl hydrolases based on amino acid sequence similarities [J]. Biochem J, 1991, 280 (Pt 2)(Pt 2): 309-16.

[50] HENRISSAT B, BAIROCH A. New families in the classification of glycosyl hydrolases based on amino acid sequence similarities [J]. Biochem J, 1993, 293 (Pt 3)(Pt 3): 781-8.

[51] SVENSSON B, SøGAARD M. Mutational analysis of glycosylase function [J]. Journal of Biotechnology, 1993, 29(1): 1-37.

[52] DELIC M, VALLI M, GRAF A B, et al. The secretory pathway: exploring yeast diversity [J]. FEMS Microbiology Reviews, 2013, 37(6): 872-914.

[53] ZANG J, ZHU Y, ZHOU Y, et al. Yeast-produced RBD-based recombinant protein vaccines elicit broadly neutralizing antibodies and durable protective immunity against SARS-CoV-2 infection [J]. Cell Discov, 2021, 7(1): 71.

[54] SINZINGER K, OBST U, GüNER S, et al. The Pichia pastoris enzyme production platform: From combinatorial library screening to bench-top fermentation on residual cyanobacterial biomass [J]. Journal of Bioresources and Bioproducts, 2024, 9(1): 43-57.

[55] 宋磊. 重组人血白蛋白-白介素-2(125ser)融合蛋白在毕赤酵母中的表达,纯化和检测 [D]; 山东大学, 2010.

[56] 李兵. 内切纤维素酶 EGⅢ在毕赤氏酵母中的表达及其吸附催化动力学研究 [D]; 山东大学, 2005.

[57] 白雪, 马瑜, 陈百宝, et al. 酵母 Elp3 在毕赤酵母中的分泌表达及活性测定 [J]. 河南师范大学学报：自然科学版, 2012, 40(4): 5.

[58] 黄岩山, 马国昌, 杨志愉, et al. 毕赤酵母表达重组人白介素 11 的生产方法 [Z]. CN

[59] LI B, WANG Z, LI S, et al. Preparation of lactose-free pasteurized milk with a recombinant thermostable β-glucosidase from Pyrococcus furiosus [J]. BMC Biotechnology, 2013, 13: 73 -

[60] ZHONG P, XIU Y, ZHOU K, et al. Characterization of a novel thermophilic beta-glucosidase from Thermotoga sp. and its application in the transformation of notoginsenoside R1 [J]. 3 Biotech, 2022, 12(11): 289.

[61] 食品安全国家标准 食品中水分的测定 [Z]. 中华人民共和国国家卫生和计划生育委员会. 2016: 12