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High Molecular Weight α-Galactosidase from the Novel Strain *Aspergillus* sp. D-23 and Its Hydrolysis Performance

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Abstract: *Aspergillus* sp. D-23 was obtained by ultraviolet-diethyl sulfate (UV-DES) compound mutagenesis from *Aspergillus* sp. C18 that the α-galactosidase was purified from. According to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and non-denaturing polyacrylamide gel electrophoresis (Native-PAGE), the purified enzyme demonstrated apparent homogeneity. The monomeric α-galactosidase's native molecular weight was 125 kDa. The optimal temperature of α-galactosidase was 65 °C, and 75% of the initial enzyme activity could be maintained between 45 and 55 °C. Its optimal pH was 5.0 with good pH stability. After incubating for 2 h at pH 3.0–8.0, it could retain more than 80% of its original activity. Different concentrations of metal ions had different effects on the α-galactosidase activity. High concentrations of Cu^{2+} could strongly inhibit enzyme activity and low concentrations of Fe^{2+} could promote enzyme activity. Additionally, as shown by thin layer chromatography and high-performance liquid chromatography, the enzyme also had good hydrolysis ability, which could efficiently hydrolyze melibiose and raffinose by more than 95%. Therefore, these excellent characteristics could make α-galactosidase a good candidate for the food and feed industries.

Keywords: α-galactosidase; enzymatic characterization; *Aspergillus* sp.; enzymatic hydrolysis; higher molecular weight

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1. Introduction

α-Galactosidase (E.C.3.2.1.22) is classified as an exoglycosidase. It possesses a strong hydrolysis ability that can catalyze the hydrolysis of α-linked galactose moieties from the nonreducing ends of α-linked galacto-oligosaccharides, glycoside proteins, and glycoside lipids [1]. The enzyme is also known as melibiase due to its ability to cleave the a-1,6 link between galactose and glucose in melibiose. Based on the similarities in their amino acid sequence, the α-galactosidases are grouped into glycoside hydrolase (GH) families 4, 27, 32, 36, 57, 97, and 110 (http://www.cazy.org/Glycoside-Hydrolases.html (accessed on 25 August 2022)) [2]. To date, a number of α-galactosidases have been found and identified from a wide variety of animals, plants, and microorganisms [3]. These enzymes have significant potential for use in a variety of sectors including the food and feed industry [4], paper industry [5], sugar production [6], treatment of Fabry's disease [7], and other industries [8].

Soy is rich in proteins and has health benefits for humans and animals. Raffinose oligosaccharides (RFOs), which are present in significant amounts in soybean, are indigestible carbohydrates that are recognized as antinutritional factors and are not broken down by α-galactosidase [9]. As a result, it is simple for both humans and animals to induce flatulence. α-Galactosidases are frequently introduced to the food and feed industries as an exogenous enzyme that hydrolyzes α-1,6-galactoside bonds to moderate the properties that cause flatulence and increase the quantity of food and feed [10].

The food and feed industries choose α-galactosidases from fungal because the fungus has a high level of extracellular secretion and is simple to culture on low-cost media [11].

However, only a small percentage of fungal α-galactosidases are effective at the hydrolyzation of RFOs. For instance, α-galactosidase from *Neosartorya fischeri* P1 [12] and *Paecilomyces thermophila* [13] required 3 h and 6 h, respectively, to almost completely hydrolyze the RFOs. Therefore, there is a huge need for fungal α-galactosidases that are more efficient at destroying RFOs in soybeans. In practical applications such as soybean processing, enzyme stability is essential, and good stability is more beneficial in removing RFOs. When used as a food and feed supplement, α-galactosidase quickly inactivates the ingested enzyme in gastric acid with a low pH value [14]. Thus, α-galactosidases with extensive pH stability are thought to offer huge potential as food and feed additives [10]. Currently, the majority of fungal α-galactosidases have an appropriate pH of acid [2]. For instance, α-galactosidase from *Penicillium* sp. F63 [15] and *Penicillium janczewskii zaleski* [16] was maintained stably at pH 5.5–6.5 and 4.0–6.8, respectively. A few α-galactosidases such as the α-galactosidase from *Thermonyces lanuginosus* [17] in the pH range of 6.4 to 8.3 are stable under alkaline conditions. However, these fungal-derived α-galactosidases are stable only in a partially narrow pH-stable range. In order to broaden industrial applications, it is crucial to find an enzyme with a broad pH range that is active in both acidic and alkaline conditions.

Aspergillus sp. C18, previously isolated at the Laboratory of Applied Microbiology and Enzyme Engineering at Changzhou University, can produce high α-galactosidase activity. For this investigation, we obtained a fungus *Aspergillus* sp. D-23 that was mutagenized by *Aspergillus* sp. C18, from which we identified an α-galactosidase with wide pH stability and high hydrolytic capacity. We investigated the hydrolytic ability of melibiose and raffinose by α-galactosidase as this enzyme is used in the food and feed sectors because of its enzymatic characteristics.

2. Materials and Methods

2.1. Materials

Aspergillus sp. C18 was stored at the Lab of Applied Microbiology and Enzyme Engineering, Changzhou University, and used as the original strain for ultraviolet-diethyl sulfate (UV-DES) compound mutagenesis. *Aspergillus* sp. D-23 was mutated from *Aspergillus* sp. C18, which was deposited with China General Microbiological under CGMCC No. 40330. Q Beads 6FF was obtained from Changzhou Smart-Lifesciences Biotechnology Co. Ltd. The melibiose, raffinose, and *p*-nitrophenyl α-D-galactopyranoside (*p*-NPG) were obtained from Shanghai Aladdin Biochemical Technology Co. Ltd (Shanghai, China).

2.2. Medium Formula

The primary screening medium was prepared with the following: $C_6H_{12}O_6$ 2%, KNO_3 0.3%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, KCl 0.05%, $FeSO_4 \cdot 7H_2O$ 0.001%, agar-based 2%, pH 6.5, 5-bromo-4-chloro-3-indoxyl-α-D-galactopyranoside (X-α-D-galactoside; X-α-Gal) 100 μL; X-α-Gal solution was prepared as follows: 4 mg X- α- Gal was dissolved in 1 mL *N*, *N*-dimethylformamide (DMF); X-α-Gal solution was used as follows: 100 μL X-α-Gal solution was filtered through a 0.22 μm Millipore (Burlington, MA, USA) filter, then coated on the primary screening plate. α-Galactosidase hydrolyzed the colorless X-α-Gal substrate and finally produced a blue product, which was displayed as a blue single colony on the plate.

The solid-state fermentation medium was prepared with the following: wheat bran 32 g, peptone 2 g, $(NH_4)_2SO_4$ 2.8 g, $FeSO_4 \cdot 7H_2O$ 0.05 g, pH 6.0. Then, a volume of 60 mL was reached by adding distilled water. All culture media were sterilized at 121 °C for 30 min.

2.3. UV-DES Mutagenesis Procedure

Spore suspension (1×10^6 CFU mL^{-1}) of *Aspergillus* sp. C18 was prepared and scattered in the Petri dish with a diameter of 9 cm. The UV mutagenesis method procedure is as follows: the spore suspension was irradiated by a UV lamp for 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 min, respectively. The spore suspension in 1 mL was transferred to a screening

medium cultured at 28 °C for 48 h. The mortality was determined in accordance with the quantity of colonies on the Petri dish. The most effective UV-irradiation time used was irradiation with an 80% mortality rate. The formula for calculating the mortality rate was according to Equation (1).

$$Fatality\ rate = \frac{A - B}{A} \times 100\% \quad (1)$$

where A denotes the quantity of colonies (CFU mL^{-1}) growing on the plate prior to mutagenesis, and B denotes the quantity of colonies (CFU mL^{-1}) growing on the agar plate following mutagenesis. These tests were carried out three times.

After separating and purifying the blue single colony in the primary screening plate with a mortality rate of 80%, we selected single colonies from it for solid-state fermentation. A spore suspension (1×10^6 CFU mL^{-1}) of a UV-mutant strain with the highest enzyme activity was prepared and distributed in a 9 cm diameter Petri dish. The DES mutagenesis procedure is as follows: The DES solution and spore suspension were mixed at a volume ratio of 1% in sterile centrifuge tubes and the DES concentration in the centrifuge tubes was 0.4%. After shaking at 28 °C and 160 rpm/min for 20, 40, 60, 90, 120, 150, and 210 min, 2 mL of the 0.5 mol/L sodium thiosulfate solution was added to terminate the reaction. A total of 1 mL of the spore suspension was transferred to a screening medium cultured at 28 °C for 48 h. The mortality rate of mutation was determined in accordance with the quantity of colonies on the Petri dish. These tests were carried out three times. The mutant strains were cultivated by solid-state fermentation.

2.4. α-Galactosidase Assay and Protein Concentration Assay

The α-galactosidase activity assay was referenced to Katrolia, P. [18] with a few minor adjustments. A total of 5 mL of 8 mmol/L *p*-NPG in the Na_2HPO_4–citric acid buffer (pH 4.5) and 1 mL of properly diluted enzyme solution made up the reaction mixture. The reaction was stopped by adding 2 mL of 0.5 mol/L Na_2CO_3 after the mixture was incubated at 50 °C for 10 min. The quality of *p*-nitrophenol released from *p*-NPG was then calculated by measuring the absorbance at 420 nm with an Ultraviolet–Visible Spectrophotometer. The quality of α-galactosidase that released 1 μmol reducing *p*-nitrophenol from the 8 mmol/L substrate solution (*p*-NPG) every minute at pH 4.5 and 50 °C was considered one unit of enzyme activity. The quantity of α-galactosidase that released 1 μmol reducing *p*-nitrophenol from 8 mmol/L substrate solution (*p*-NPG) every minute at pH 4.5 and 50 °C was considered as one unit of enzyme activity.

Using bovine serum albumin as the standard, the Bradford technique was used to calculate the protein content [19].

2.5. Purification of α-Galactosidase

The fermentation product was extracted in the Na_2HPO_4–citric acid buffer (20 mmol/L, pH 7.0) for 3 h and filtered to collect the filtrate, which was the crude enzyme. The collected crude enzyme was filtered through a 0.22 μm Millipore filter. Then, the crude enzyme was loaded onto a pre-equilibrated ion exchange chromatography system that was packed in Q Beads 6FF and was sequential with an increasing gradient of 50 mmol/L, 100 mmol/L, 200 mmol/L, 300 mmol/L, and 1 mol/L NaCl prepared in equilibration buffer (Na_2HPO_4-citric acid buffer). The dialysis bag was used to collect, pool, and concentrate the fractions that had α-galactosidase activity.

The obtained fractions were submitted to protein content analysis using the Bradford method, α-galactosidase activity detection using the *p*-NPG method, and purity analysis using SDS-PAGE. The purification fold and yield were subsequently determined. The calculation methods were as follows:

Yield (%): (The total activity of each step/the total activity of the first step) × 100%.

Purification fold: Specific activity of each step/specific activity of the first step.

2.6. Electrophoretic Analysis

2.6.1. SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis)

The protein homogeneity of the purified α-galactosidase from *Aspergillus* sp. D-23 was performed by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with the Coomassie Brilliant Blue (CBB) method to visualize the resolved protein bands. Then, the relative mobilities were calculated with protein standards to determine the molecular masses of the protein.

2.6.2. Native-PAGE and Activity Staining

Non-denaturing polyacrylamide gel electrophoresis (Native-PAGE) was carried out by using 8% polyacrylamide gel and loading buffers without sodium dodecyl sulfate. None of the samples were boiled. Then, when the electrophoresis was completed, the gel was immersed in a reaction buffer containing 0.02 mg/mL 5-bromo-4-chloro-3-indolyl α-D-galactopyranoside (X-α-Gal) dissolved in 0.02 mol/L Na_2HPO_4–citric acid buffer (pH4.5) and incubated at 30 °C for 4 h. X-α-Gal is the chromogenic substrate of α-galactosidase, which can produce blue products under the catalysis of α-galactosidase. As the incubation time increased, the blue band gradually appeared, which was proven to be α-galactosidase.

2.7. α-Galactosidase Characterization

The enzyme activities were evaluated in the Na_2HPO_4–citric acid buffer (pH4.5) at 30, 35, 40, 45, 50, 55, 60, 65, 70, and 75 °C in order to find the optimal temperature for α-galactosidase. The residual α-galactosidase activity was assessed under standard conditions when the enzyme was incubated at 45, 50, 55, 60, 65, 70, and 75 °C for 0, 30, 60, 90, and 120 min, respectively, to determine its thermal stability. The enzyme activity before incubation was regarded as 100% activity.

The enzyme and the substrate were reacted in the Na_2HPO_4–citric acid buffers with a pH range of 3.0–8.0 in order to investigate the optimal pH of α-galactosidase. The relative activities were assessed at the optimal temperature and various pH levels as previously mentioned. By incubating α-galactosidase at a pH ranging from 3.0 to 8.0 for 60 min and 120 min, respectively, at room temperature, the pH stability was ascertained. Then, the remaining activity was assessed under standard conditions. The enzyme activity before incubation was regarded as 100% activity.

Different metal ions such as Cu^{2+}, Na^{+}, Mg^{2+}, K^{+}, Mn^{2+}, Fe^{2+}, Zn^{+}, and Ca^{2+} were added to the purified enzyme at final concentrations of 1 mmol/L, 5 mmol/L, and 10 mmol/L to test their effects on α-galactosidase activity. The combination underwent an hour-long preincubation at 4 °C. Under optimal conditions, the enzyme's residual activity was assessed. As a control, the reaction solution devoid of metal ions was employed.

2.8. Kinetic Parameters and Substrate Specificity

By incubating the purified enzyme with the substrate at concentrations ranging from 1 to 10 mmol/L and conducting the previously indicated enzyme assay, it was possible to determine the impact of *p*-nitrophenyl α-D-galactopyranoside concentrations on the rate of hydrolysis of α-galactosidase. Then, according to the Lineweaver–Burk plot, the kinetic parameters were established. This method was based on the reciprocal form of the Michaelis–Menten equation, which was plotted with $1/[V]$ as the vertical coordinate and the reciprocal of the substrate concentration, $1/[S]$, as the horizontal coordinate. The formula to calculate the kinetic parameters (K_m and V_{max}) is according to Equation (2).

$$\frac{1}{[V]} = \frac{1}{[S]} \times \frac{K_m}{V_{max}} + \frac{1}{V_{max}} \quad (2)$$

p-NPG hydrolysis activity was assessed utilizing the aforementioned standard α-galactosidase activity test. For natural compounds such as melibiose (50 mmol/L) and raffinose (50 mmol/L), the enzyme activity was measured by the 3,5-dinitro salicylic acid reagent method (DNSA) [20]. The solution in the tube was the combined 100 µL diluted

enzyme solution with 100 µL substrate, which was incubated at 50 °C for 10 min and 1 mL DNS was immediately added to terminate the reaction. Finally, the sample was boiled and detected with an Ultraviolet–Visible Spectrophotometer at 540 nm. The quantity of α-galactosidase that released 1 µmol of reducing sugar each minute was used to define one unit of enzyme activity.

2.9. Enzymatic Hydrolysis of Melibiose and Raffinose

The purified α-galactosidase (5, 10, 20 U/mL) and raffinose (4 mg/mL) or melibiose (4 mg/mL) in 0.02 mol/L Na_2HPO_4–citric acid buffer (pH 4.5) were mixed and incubated for various time (2, 4, 6, 8, and 10 h) periods at 50 °C with a ratio of 1:1. Every two hours, aliquots (1 mL) of the reaction mixtures were taken and measured. The hydrolysates were investigated using thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). For TLC analysis, 3 µL of the hydrolysates was spotted onto 10 cm by 10 cm silica gel G plates and developed in a solvent mixture of n-propanol, acetic acid, and water at a ratio of 6:4:3 (*v*/*v*/*v*). The plate was sprayed with a chromogenic reagent including diphenylamine 1 g, aniline 1 mL, 80% phosphoric acid 5 mL, and acetone 50 mL, and then the plate was heated in an oven at 105 °C for 15 min to detect the saccharides [21]. In addition, HPLC (Agilent 1260, Santa Clara, CA, USA) fitted with a model RCX-30 column (250 mm × 4.6 mm, 7µm) and a refractive index detector was used to measure the saccharides in the hydrolysates [22]. At a flow rate of 1.0 mL/min, a 200 mmol/L NaOH solution was used to elute the column with the column temperature box kept at 35 °C. Then, the 3,5-dinitro salicylic acid reagent method (DNSA), as reported by Miller, G.L. [20] with modifications, was used to assess how much reducing sugar was created following hydrolysis.

3. Results and Discussion

3.1. Compound Mutagenesis of Ultraviolet and Diethyl Sulfate

The strain showed good mutagenic and fatal impacts because of UV and diethyl sulfate. The fatal rate of the strain during the process of mutagenesis was around 80% in order to provide the necessary mutagenic impact. Finally, the best mutagenic condition was 7 min of UV irradiation and 120 min of 0.4% DES treatment. The mutagenic strain and the original strain were subject to solid-state fermentation. The mutagenic strain with the highest enzyme activity was the target strain. After several compound mutations in the current investigation, the α-galactosidase activity of D-23 was higher than that of C18, reaching 273.61 U/g, which was 268% more than that of C18 (101.97 U/g) (Figure 1). The mutant, named as *Aspergillus* sp. D-23, was shown to have a high yield α-galactosidase capability. The enzyme activity of D-23 was higher when compared to the enzyme activity identified from *Aspergillus awamori* (109.27 U/g) [23]. This may be due to the change in the DNA structure caused by mutation breeding, thus improving the enzyme activity.

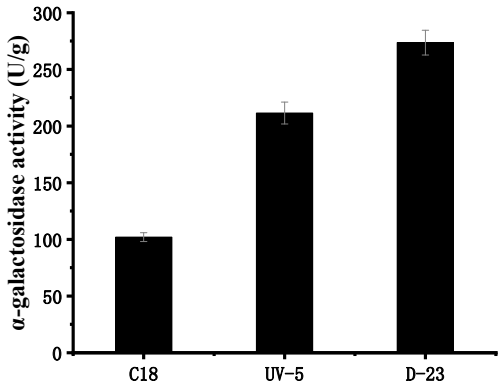

Figure 1. The α-galactosidase activity of various mutagenic strains (C18: Initial strain; UV-5: Ultraviolet mutagenic strain; D-23: Chemical mutagenic strain).

3.2. Purification of α-Galactosidase

The enzyme purification scheme is displayed in Table 1. From the crude enzyme extract, α-galactosidase was purified 7.67-fold with a yield of 64.46%.

Table 1. The purification efficiency of each step.

Procedures	Total Activity (U)	Protein (mg)	Specific Activity (U/mg)	Yield (%)	Purification Fold
Crude enzyme	390.00	1.78	219.45	100.00	1.00
Q	251.34	0.14	1682.70	64.46	7.67

Q is the active fragment in the Q Beads 6FF.

3.3. Homogeneity and Molecular Mass of α-Galactosidase

On SDS-PAGE, the final purified protein sample showed up as a single band (Figure 2a). At the same time, in native-PAGE (Figure 3b,c), the result of staining with 5-bromo-4-chloro-3-indolyl α-D-galactopyranoside (the blue band in Figure 2c) also confirmed the homogeneity of the enzyme. According to the SDS-PAGE estimates, the enzyme's apparent molecular mass was about 125 kDa. Therefore, it is a monomer protein similar to other fungal α-galactosidases, belonging to the molecular weight of most fungal α-galactosidases [24]. This novel α-galactosidase from *Aspergillus* sp. D-23 showed a high molecular mass similar to the molecular weight of this enzyme from *Aspergillus awamori* [23] and the molecular mass was 118 kDa. According to earlier reports, the molecular weight of α-galactosidase from *Aspergillus parasiticus* [25], *Irpex lacteus* [10], and *Thermomyces lanuginosus* [17] was 67.5 kDa, 64 kDa, and 93 kDa, respectively. Despite being classified as monomeric proteins, the α-galactosidases above-mentioned had a lower molecular weight than the α-galactosidase purified from *Aspergillus* sp. D-23.

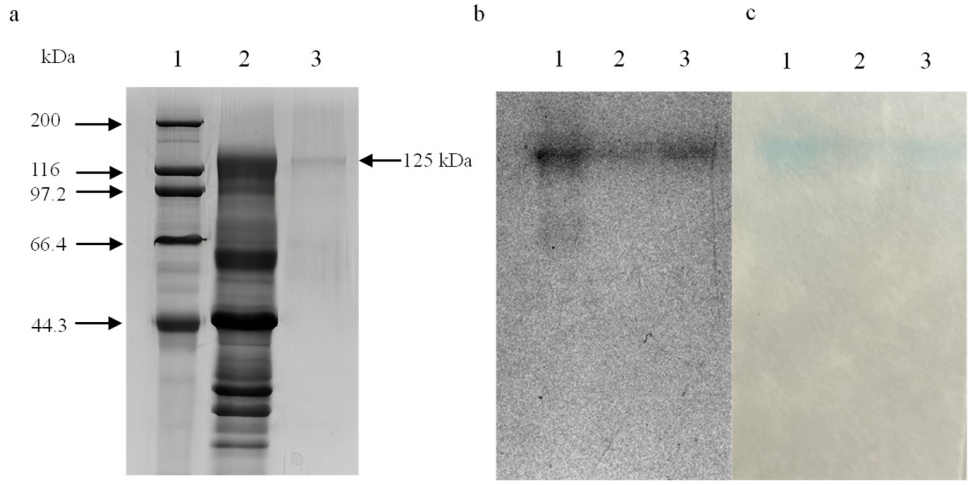

Figure 2. Electrophoretic analysis of α-galactosidase (**a**) 8% SDS-PAGE: Lane 1: Protein marker; Lane 2: Crude enzyme; and Lane 3: Purified enzyme; (**b**) 8% Native-PAGE: Lane 1: Crude enzyme; Lane 2: Purified enzyme; and Lane 3: Purified enzyme after concentration; (**c**) 8% Native-PAGE (similar to (**b**)).

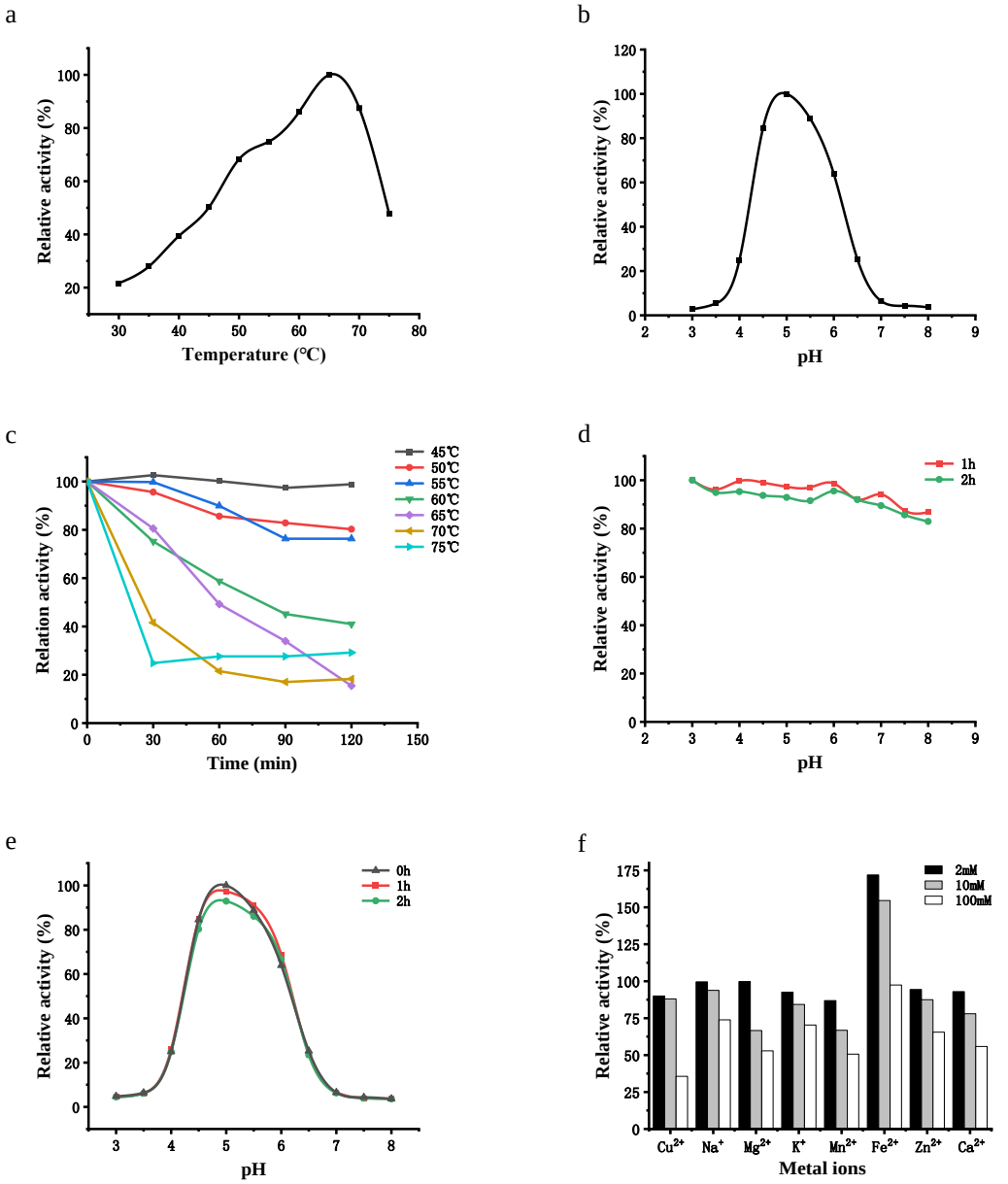

Figure 3. Biochemical characterization of α-galactosidase. (**a**) Effects of temperature on the activity of α-galactosidase. (**b**) Effects of pH on the activity of α-galactosidase. (**c**) Effects of temperature on the stability of α-galactosidase. (**d**,**e**) Effects of pH on the stability of α-galactosidase. (**f**) Effects of metal ions.

3.4. Biochemical Characterization of α-Galactosidase

3.4.1. Temperature and pH Optima and Stability

In the current research, the activity of α-galactosidase increased during the temperature range of 30–65 °C and progressively declined after the temperature exceeded 65 °C (Figure 3a). Therefore, the optimal temperature of α-galactosidase was found to be 65 °C, which is similar to previously reported α-galactosidases from *Thermomyces lanuginosus* [17], *Lichtheimia ramosa* [26], higher (40 °C) than other α-galactosidases from *Penicillium purpurogenum* [15], and lower than the α-galactosidase from *Talaromyces leycettanus* (70 °C) [27]. Furthermore, the thermal stability of the α-galactosidase was also determined. The α-galactosidase maintained 100% relative activity at 45 °C and showed more than 80% relative activity at 50 and 55 °C. After 30 min of incubation, α-galactosidase showed 75.21%, 80.56%, 41.57%, and 24.84% at 60 °C, 65 °C, 70 °C, and 75 °C, respectively. As the incubation increased, the activity of α-galactosidase gradually decreased. After 120 min of incubation,

α-galactosidase exhibited a total decline in its activity (Figure 3c). The results indicated that this α-galactosidase was thermophilic. The thermostability of this enzyme is consistent with that found in *Rhizopus* sp. [28], which also produces a thermostable α-galactosidase. However, its temperature stability also plays a significant role in applications in the food and industrial sectors.

The α-galactosidase optimal pH was pH 5.0 (Figure 3b), which is consistent with the majority of other fungal α-galactosidases reported to have a pH range between 4.0 and 5.5 [18,27]. This indicates that this enzyme is an acidic α-galactosidase. Acidic α-galactosidases are crucial for the metabolism of RFOs [29,30]. At the same time, the pH stability of the enzyme was also studied. After incubation for 2 h in the pH range of 3–7, the activity could still maintain more than 90% (Figure 3d,e), and could remain at more than 80% activity after 2 h of incubation at pH 7–8. On one hand, many fungal α-galactosidases such as from *Penicillium purpurogenum* (4.8–6.5) [15] and *Hericium erinaceus* (2.2–7.0) [21] are active only in the acidic-neutral pH range; on the other hand, some α-galactosidases such as from *Thermonyces lanuginosus* (6.4–8.3) [17] remain stable in a neutral–alkaline pH range. However, the enzyme in this study could remain stable under most acidic and weak alkaline conditions. This capacity allowed the enzyme to adapt to the acidic environment of the gastrointestinal tract, which facilitated the application of food and feed additives. α-Galactosidase may also be employed in various sectors in the future such as eliminating antinutritional factors from soybeans as a result of finding the heat stability and pH stability.

3.4.2. Effect of Metal Ions

The addition of metal ions [Cu^{2+}, Na^{+}, Mg^{2+}, K^{+}, Mn^{2+}, Fe^{2+}, Zn^{2+}, and Ca^{2+} (2, 10, and 100 mmol/L)] had different effects on the enzyme activity (Figure 3f). When the concentration of metal ions was low, the effect of most metal ions on the enzyme was not obvious. With the increase in metal ion concentration, the inhibition of enzyme activity became more obvious. Furthermore, the addition of Fe^{2+} showed an apparent promotion that led to an increase (up to 175%) in α-galactosidase activity when compared with α-galactosidase devoid of metal ions. With the increase in Fe^{2+} concentration, the α-galactosidase activity was gradually inhibited. However, compared with other metal ions, Fe^{2+} always exhibited the trend of promoting α-galactosidase activity (Figure 3f). When metal ions were 100 mmol/L, Cu^{2+} and Mn^{2+} had an inhibition effect on α-galactosidase and the relative activity was less than 50%. The results indicate that a low concentration of metal ions had no significant effect on the enzyme activity, while a high concentration of Cu^{2+} had a significant inhibition on the enzyme activity. It has also been shown to inhibit most of the α-galactosidases [18]. It is interesting to note that the same metal ions could have various effects on different α-galactosidase subtypes as well as on α-galactosidase itself. With the existence of Cu^{2+}, the activity of α-galactosidase from *Hericium erinaceus* was increased with the decrease in concentration, whereas Cu^{2+} gradually inhibited the enzyme activity from *Tremella aurantialba* with increasing concentration [31]. These might result from metal ions binding to residues in the active sites of the enzyme, which impacted the structure of the enzyme [11].

3.5. Kinetic Characterization and Substrate Specificity

The kinetic parameters of α-galactosidase including K_m and V_{max} were calculated by Lineweaver-Burk plot, using Origin 2021 software. The K_m and V_{max} of α-galactosidase were found to be 0.983 mmol/L and 1.587 μmol·mL^{-1}·min^{-1}, respectively (Figure 4), exhibiting lower affinity to the substrate, but it was higher than the K_m of other α-galactosidases [29,32]. As shown in Table 2, the α-galactosidase, which was comparable to the majority of other known α-galactosidases [13,33], had the highest sensitivity for *p*-NPG (100%) but exhibited little activity for natural substrates (melibiose and raffinose). The hydrolytic capacity of natural substrates was melibiose > raffinose following treatment with α-galactosidase, which matched their rate of hydrolysis discovered by TLC, as indicated below.

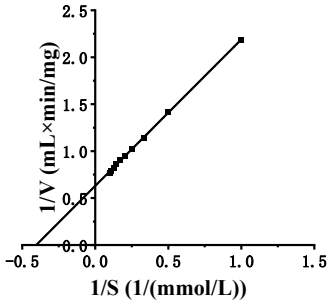

Figure 4. Effect of a substrate (*p*-nitrophenyl α-D-galactopyranoside) on the rate of hydrolysis of α-galactosidase.

Table 2. Hydrolysis of substrates by α-galactosidase.

Substrate	Concentration (mmol/L)	Relative Activity (%)
p-Nitrophenyl α-D-galactopyranoside (*p*-NPG)	8	100.00
Melibiose	50	17.63
Raffinose	50	10.81

3.6. Hydrolysis of Melibiose and Raffinose by α-Galactosidase

3.6.1. TLC Analysis of the Hydrolysate

Three different enzyme dosages (5, 10, and 20 U/mL) were used to hydrolyze melibiose and raffinose at 2, 4, 6, 8, and 10 h. The TLC analysis of the hydrolysate of melibiose and raffinose showed that a majority of them could be rapidly degraded (Figure 5). With the increase in enzyme dosage, the hydrolysis capacity also increased. When the enzyme dosages were 10 U/mL and 20 U/mL, a faint dot of melibiose remained after treatment with α-galactosidase for 2 h (Figure 5b,c). After treatment with 20 U/mL enzyme dosages for 4 h, the spot of raffinose was almost gone (Figure 5f). Furthermore, because the melibiose was a part of raffinose, this may have contributed to the fact that melibiose underwent full hydrolysis more quickly than raffinose. During hydrolysis, the α-1,6 linkage(D-Gal-α-(1,6)-D-Glc) between galactose and glucose may be hydrolyzed first, then the α-1,6-galactoside bonds in raffinose [8]. In agreement with the findings of TLC, melibiose and raffinose were hydrolyzed as evidenced by the progressive increase in the amount of reducing sugar following treatment with α-galactosidase during hydrolysis (Figure 6). Regardless of what happened, it showed that the α-galactosidase had good hydrolysis ability.

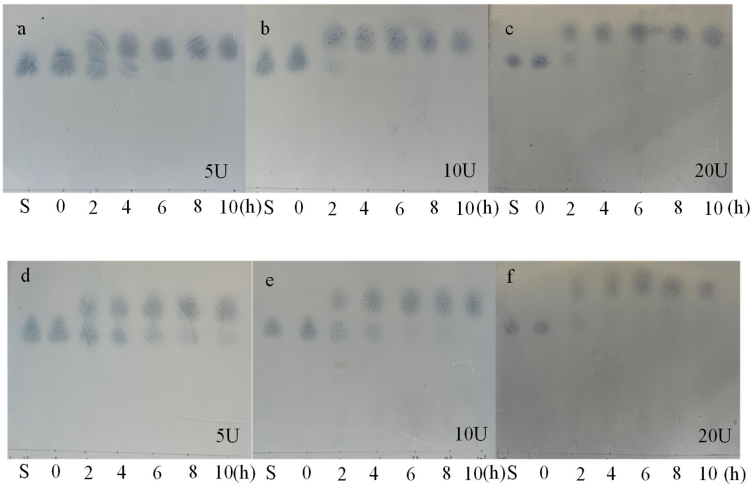

Figure 5. The TLC analysis of hydrolysates of melibiose and raffinose by purified α-galactosidase. (**a**,**d**) Enzyme dosage was 5 U/mL; (**b**,**e**) enzyme dosage was 10 U/mL; (**c**,**f**) enzyme dosage was 20 U/mL.

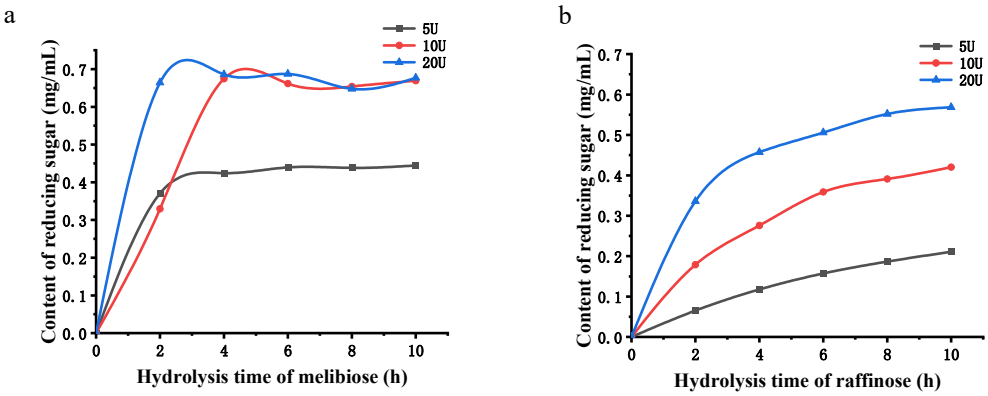

Figure 6. Reducing sugar content after treatment with α-galactosidase. (**a**) Reducing sugar content after the hydrolysis of melibiose; (**b**) Reducing sugar content after the hydrolysis of raffinose. The enzyme dosages are represented by different signals: gray square, 5 U/mL; red circle, 10 U/mL; blue triangle, 20 U/mL.

3.6.2. HPLC Analysis of the Hydrolysis

During the hydrolysis of melibiose and raffinose, high-performance liquid chromatography (HPLC) was also used to detect the hydrolysis products. The external standard method was used to conduct a qualitative study based on the retention durations of each component. By combining the peak area of each component, the content of hydrolyzed products was determined (Figure 7). Generally speaking, under the influence of α-galactosidase, melibiose can be hydrolyzed to galactose and glucose, while raffinose may be hydrolyzed to galactose and sucrose. This capability is consistent with most other α-galactosidases [13,18]. Then, the hydrolytic ability of α-galactosidase was confirmed by HPLC (Figures 8 and 9a–c), which was in accordance with the results of TLC. The degradation rate of melibiose and raffinose was calculated by the decrease in melibiose and raffinose. When the enzyme dosage was 5 U/mL, 10 U/mL, and 20 U/mL, the degradation rate of melibiose at 2 h was 35.17%, 74.02%, and 77.03%, respectively (Figure 8d); the degradation rate of raffinose at 2 h was 30.30%, 61.25%, and 81.69% (Figure 9d). With the increase in the enzyme dosage and treatment time, the melibiose had been completely degraded at the enzyme dosage of 10 and 20 U/mL at 4 h. At this time, the degradation effect of raffinose was also significantly improved. At the enzyme dosage of 20 U/mL, the raffinose was almost degraded and the degradation rate reached 97.30 % at 4 h (Figure 9d). At present, many α-galactosidases have reportedly been used to eliminate RFOs from soybeans. However, the short catalytic efficiency and instability of α-galactosidases may be the reason for their flaws, which included long reaction times and partial hydrolysis [10]. This α-galactosidase hydrolyzed melibiose and raffinose entirely in a substantially shorter amount of time than the α-galactosidase from *Rhizomucor miehei* [34], which required 8 h. In addition, α-galactosidase from *Pleurotus citrinopileatus* [35] needed 9 h to hydrolyze raffinose. The RFOs in soybean milk treated with α-galactosidase from *Aspergillus terreus* [36] could be completely eliminated in 12 h. In contrast, α-galactosidase from *Aspergillus* sp. D-23 can effectively reduce the hydrolysis time and improve the degradation efficiency. These results indicate that this enzyme could effectively hydrolyze RFOs and would be a good candidate for eliminating anti-nutritional factors in soybean in the future.

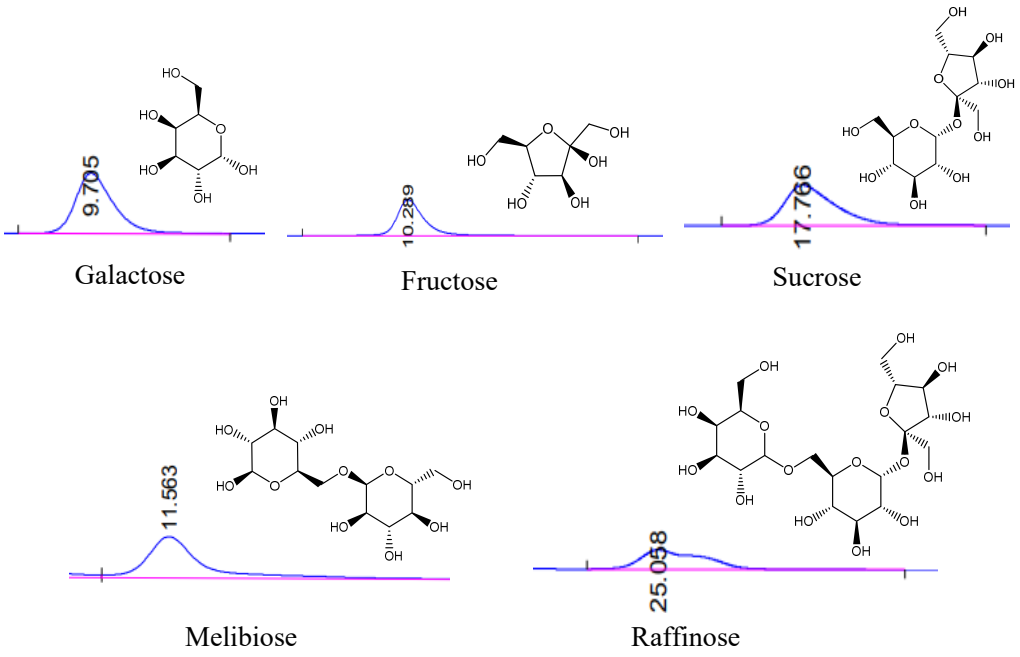

Figure 7. Analysis of the contents of oligosaccharides by HPLC.

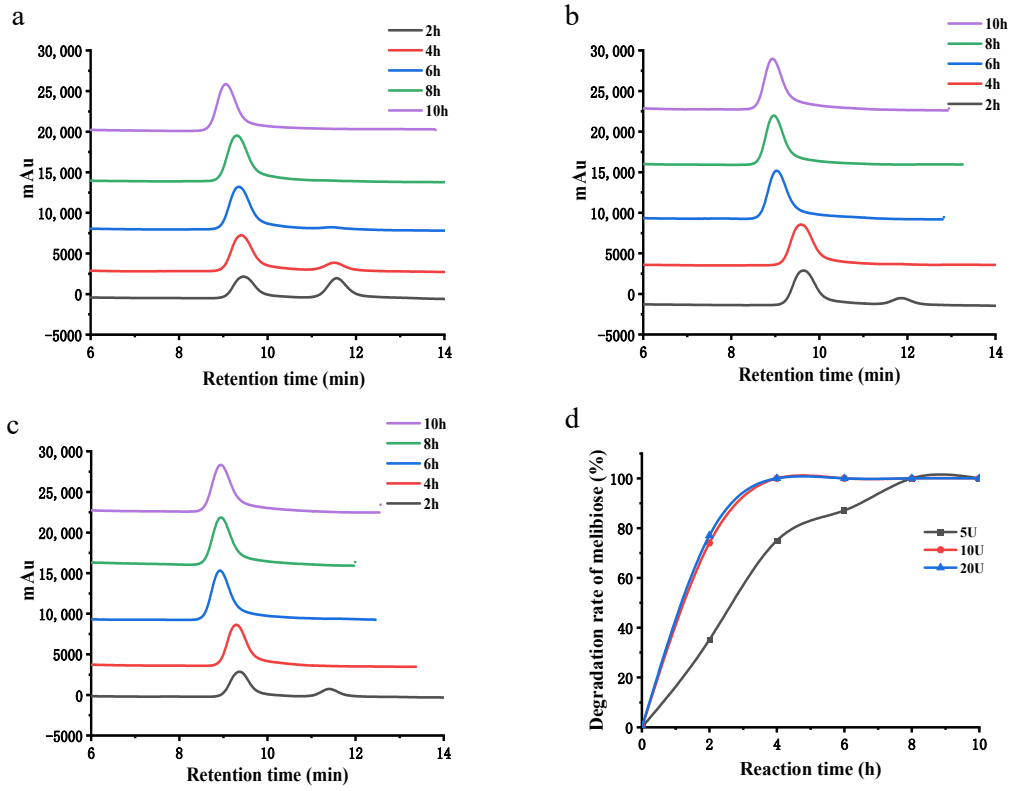

Figure 8. Analysis of the hydrolysis of melibiose by HPLC. (**a**) Enzyme dosage was 5 U/mL; (**b**) Enzyme dosage was 10 U/mL; (**c**) Enzyme dosage was 20 U/mL. (**d**) The degradation rate of melibiose.

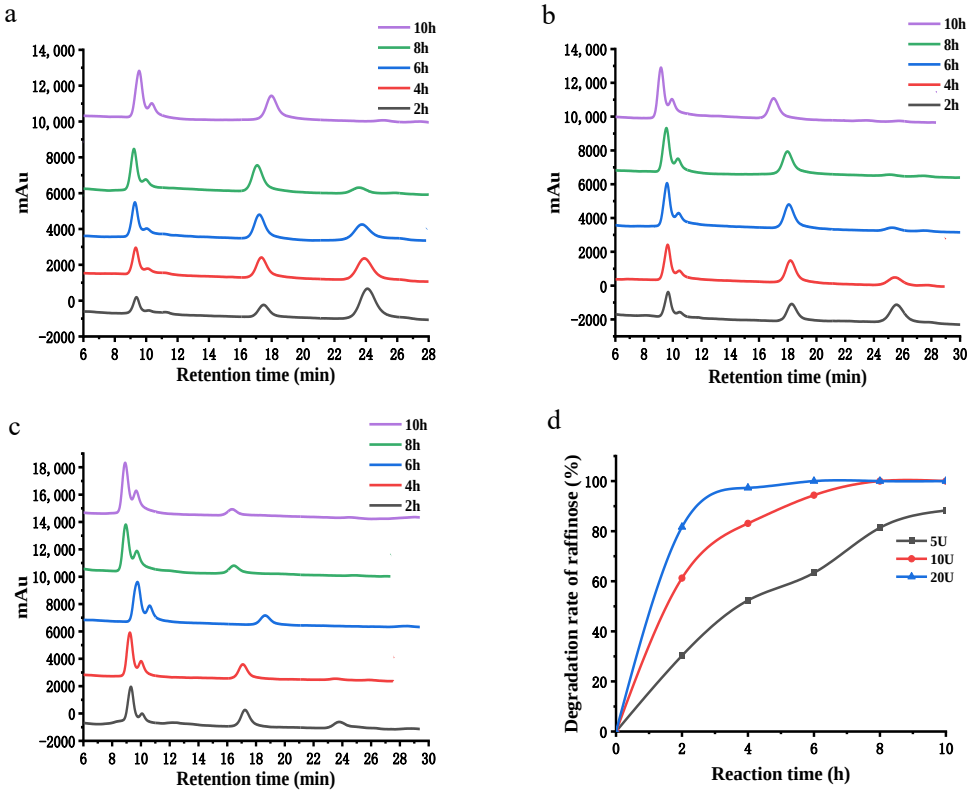

Figure 9. Analysis of the hydrolysis of raffinose by HPLC. (**a**) Enzyme dosage was 5 U/mL; (**b**) enzyme dosage was 10 U/mL; (**c**) enzyme dosage was 20 U/mL. (**d**) The degradation rate of raffinose.

4. Conclusions

Aspergillus sp. D-23 was mutated from *Aspergillus* sp. C18 by ultraviolet-diethyl sulfate (UV-DES) compound mutagenesis, which can purify α-galactosidase from it. In this paper, the purified α-galactosidase displayed a high molecular weight of 125 kDa. It reached its peak activity at 65 °C and pH 5.0 and maintained high stability with a pH between 3.0 and 8.0. This characteristic is favorable for the application of α-galactosidase under acidic conditions. It also displayed good hydrolysis capacity, as this α-galactosidase (20 U/mL) almost completely degraded the melibiose and raffinose (>96.68%) in 4 h. It was possible to thoroughly degrade the anti-nutritive in soymilk. Therefore, this α-galactosidase will be a promising candidate in the food and feed industries such as eliminating the flatulence caused by soybean products and improving the nutritional value of feed as an additive, etc. in the future.

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