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Original Research Article

Characterization of putative mannoprotein in *Kluyveromyces lactis* for lactase production

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ABSTRACT

Lactase is a member of the β-galactosidase family of enzymes that can hydrolyze lactose into galactose and glucose. However, extracellular lactase production was still restricted to the process of cell lysis. In this study, lactase-producing *Kluyveromyces lactis* JNXR-2101 was obtained using a rapid and sensitive method based on the fluorescent substrate 4-methylumbelliferyl-β-D-galactopyranoside. The purified enzyme was identified as a neutral lactase with an optimum pH of 9. To facilitate extracellular production of lactase, a putative mannoprotein KLLA0_E01057g of *K. lactis* was knocked out. It could effectively promote cell wall degradation and lactase production after lyticase treatment, which showed potential on other extracellular enzyme preparation. After optimizing the fermentation conditions, the lactase yield from mannoprotein-deficient *K. lactis* JNXR-2101ΔE01057g reached 159.62 U/mL in a 5-L fed-batch bioreactor.

1. Introduction

Lactase, also known as β-D-galactosidase (EC. 3. 2. 1. 23, β-gal), can hydrolyze the β-galactoside bond of lactose to produce galactose and glucose [1–3] and has been widely applied in the food industry to improve the taste and quality of dairy products. Two types of lactases are commonly used, namely neutral and acid lactases. Lactase produced by fungi has high activity at acidic pH values of 2.5–5.5 and is mainly used to hydrolyze acid whey and cheese [4]. Yeast and bacterial lactase exhibit high activity at pH 6.0–8.0, which is suitable for preparing lactose-free dairy products in neutral environments [2]. *Kluyveromyces* species, such as *K. lactis* and *K. fragilis*, are the most extensively studied food-grade strains for lactase production. High-throughput screening and genome editing are powerful techniques for developing strains or enzymes with advantageous properties [5,6]. Mastrobattista et al. [7] developed an *in vitro* directed evolution process for improving lactase activity using a fluorescence-activated cell sorter. In addition, strain engineering and fermentation optimization has been applied to improve the yield of lactase. You et al. [8] optimized the fermentation conditions using response surface methodology for lactase production, and achieved a maximum yield of 111.61 U/mL lactase in *K. lactis* grown in fed-batch culture.

According to previous studies, most yeast cannot secrete lactase, and thus the cells must be lysed to collect the enzyme [4,9]. The cell wall of *K. lactis* has a classical sandwich structure that is mainly composed of D-glucan, D-mannose, and a small amount of chitin [10,11]. D-Mannose and proteins are covalently linked to form mannoprotein, which is distributed in the outer layer of the cell wall. The disturbance of mannoproteins mainly affects the cell morphology. Transcriptome analysis revealed that different mannoproteins have different effects on environmental tolerance, cell size, cell wall thickness. Li et al. found that the transcriptional level of multiple genes related to the cell wall structure and integrity was changed after knocking out the mannoprotein CWP2 in *Saccharomyces cerevisiae* [12]. Modifying the components and structure of yeast cell walls may simplify the process of cell wall disruption and improve the extracellular production of the target protein. For example, *SED1* encodes the major cell wall glycoprotein of *S. cerevisiae* in the stationary phase, and the robustness of the cell wall was decreased

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by deletion of *SED1*, which also reduces resistance of *S. cerevisiae* to lyticases [13,14].

In this study, a lactase-producing *K. lactis* was obtained using a fluorescence screening method. The fermentation conditions of isolated *K. lactis* JNXR-2101 were optimized in flasks. To improve the efficiency of cell fragmentation, the putative mannoprotein KLLA0_E01057g was knocked out, and the effects of the knockout on lactase production were examined. Our results demonstrate its potential for extracellular protein preparation.

2. Materials and methods

2.1. Strains, medium, and culture conditions

All strains were grown in yeast extract-peptone-dextrose medium (10 g yeast extract, 20 g tryptone, 20 g glucose) for seed preparation and cultivated in yeast extract-peptone-lactose medium (YPL: 10 g yeast extract, 20 g tryptone, 20 g lactose) for lactase expression. The initial culture was grown in a 250 mL shake flask containing 50 mL of liquid medium and incubated at 30 °C and 220 rpm.

2.2. Characterization of lactase using fluorescent substrate MUGal

The fluorescent substrate (4-methylumbelliferyl-β-D-galactopyranoside, MUGal) was obtained from Bide Pharm (CAS: 6160-78-7; Shanghai, China). MUGal (0.06 g) was dissolved in 1 mL of dimethyl sulfoxide and diluted in 1 L of YPL medium (MUGal-YPL) for culture. The purified enzyme or seed preparation (20 μL) was added to a 96-well plate containing 180 μL of MUGal-YPL medium. The 96-well plate was incubated at 30 °C, and the fluorescence intensity, OD_{600}, and enzyme activity were measured every 30 min using a microplate reader (BioTek, Winooski, VT, USA). The medium without yeast served as the control.

2.3. Lactase activity assay

Lactase activity was measured by referring to the Food Chemicals Codex [15]. One enzyme unit (U) was defined as the amount of enzyme that releases 1 μmol *o*-nitrophenol per minute at 30.0 ± 0.1 °C and pH 6.5. *Kluyveromyces lactis* cells were washed with phosphate-buffered saline, disrupted using FastPrep-24 (MP Biomedicals, Santa Ana, CA, USA) [16], and centrifuged at 10,000×*g* for 10 min to obtain the crude enzyme for analyses. *Ortho*-nitrophenyl-β-galactoside solution (2.5 g/L in 1.5 mL) was pretreated at 30 °C for 10 min and mixed with 300 μL of enzyme solution for the reaction. After incubation for 10 min, 600 μL of Na_2CO_3 solution (50 g/L) was added to stop the reaction. Lactase activity was determined by measuring the specific absorption at 420 nm.

$$Lactase\ activity\ (U/mL) = ((\Delta A_{420} - C_0) \times V \times n)/(K \times T \times v) \quad (1)$$

where ΔA_{420} is the absorbance of the solution after the enzymatic reaction; C_0 is the intercept value of the standard curve; K is the slope of the standard curve; V is the total volume of the reaction solution; v is the volume of the enzyme; n is the dilution factor of the enzyme; and T is the reaction time.

2.4. Properties of lactase

Kluyveromyces lactis JNXR-2101 seed culture (10 mL) was added to 500 mL of YPL medium in 2 L shaking flasks and incubated at 30 °C and 220 rpm for 60 h. The crude enzyme was prepared by high-pressure homogenization (1000 bar, 4 cycles). Lactase was purified using ammonium sulfate precipitation (from 35% to 50% saturation), DEAE Sepharose Fast Flow (Qianchun Bio, China), and Superdex G200 (Cytiva, Marlborough, MA, USA). Lactase was analyzed using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the potential protein band (MW = 118 kDa) was prepared for protein identification by mass spectrometry analysis. The soluble protein concentration was determined using a BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China).

The optimal reaction temperature was determined as the relative activity at pH 6.5 and different temperatures (30–55 °C). To thermostability analysis, the enzymes were incubated at 45 °C for 1 h, and residual activity was measured every 5 min. For optimal reaction pH within 5.5–10.0 was determined (CH_3COOH-CH_3COONa buffer at pH 4.0–6.0, phosphate buffer at pH 6.0–8.0, and glycine-NaOH buffer at pH 8.0–10.0). To analyze the stability of the enzyme at different pH values, residual enzyme activity was determined after incubation for 1 h at different pH values and 30 °C.

2.5. Construction and characterization of mannoprotein-deficient K. lactis JNXR-2101ΔE01057g

pUDP025-gKLLA0_E01057g with guide RNA was constructed using the primer pair P1/P2 targeting the KLLA0_E01057 gene (Table S1). The homology arms of KLLA0_E01057g were amplified using the primer pairs P3/P4 and P5/P6. Donor DNA was obtained by fusing the two homology arms. The T-vector fragment amplified by the primer pair P7/P8 was ligated with the donor DNA to obtain T-vector-ΔKLLA0_E01057g. Next, T-vector-ΔKLLA0_E01057g was used as a template to amplify the donor DNA fragment with the P7/P8 primer pair. Finally, the vector pUDP025-gKLLA0_E01057g and donor DNA fragments were co-transformed into *K. lactis* JNXR-2101 competent cells via electroporation (2000 V, 400 Ω), and the plates were cultured at 30 °C for 3 days. The KLLA0_E01057g knockout strain was confirmed and named as *K. lactis* JNXR-2101ΔE01057g.

Kluyveromyces lactis JNXR-2101ΔE01057g and JNXR-2101 were inoculated into yeast extract-peptone-dextrose medium and cultured for 24 h as the seed preparation. The initial OD_{600} of the fermentation medium was 0.05, and the intracellular and extracellular enzyme activities and OD_{600} were detected every 8 h. The cells were broken using FastPrep-24 to detect enzyme activity.

To determine the cell lysis rate and activity of *K. lactis* JNXR-2101ΔE01057g, the fermentation broth was centrifuged at 5000×*g* for 5 min, and the cells were washed twice with Tris-HCl (pH 7.5) and resuspended in Tris-HCl. The suspension was diluted to an OD_{600} of approximately 0.7. This solution (1.5 mL) was added to a 5 mL tube, and lyticase was added to final concentrations of 1, 5, 10, and 20 U/mL. The cells were cultured in a plate shaker at 30 °C and 300 rpm for 60 min, during which the OD_{600} and activity were detected and the cell lysis rate was calculated [17,18]. Each treatment was conducted in triplicate, and the test was performed twice.

$$Cell\ lysis\ rate\ (\%) = (OD_{600}(0) - OD_{600}(T))/OD_{600}(0) \quad (2)$$

where $OD_{600}(0)$ is the OD_{600} detected at 0 min and $OD_{600}(T)$ is the OD_{600} detected at specified T min.

Cells of *K. lactis* JNXR-2101 and JNXR-2101ΔE01057g were collected from the surface of the plate using a sterile spreader and resuspended in sterile saline solution. We added 1000 U/mL lyticase to the cell suspension at different time points (0, 10, 60 min). Field emission scanning electron microscopy images were acquired using a field-emission scanning electron microanalyzer (SU8200, Hitachi, Tokyo, Japan).

To determine the protein concentration, 1 U/mL lyticase was added to the cell suspension to an OD_{600} of 7.0, reacted at 30 °C for 4 h, and centrifuged to collect the supernatant. The protein concentration was detected using a NanoPhotometer UV/Vis spectrophotometer mobile system (Implen, Germany).

2.6. Flask fermentation

The fermentation conditions were optimized in a 250 mL flask

containing 50 mL of YPL medium incubated at 30 °C and 220 rpm for 60 h. We first evaluated the medium composition, including the type and concentration of the carbon source, nitrogen source, and inorganic salt. Second, an orthogonal experiment (L16_4_5) in medium was designed to analyze the synergistic effect of various factors on lactase production. The orthogonal optimization model is shown in Table S2, and lactase activity and the OD_{600} of the fermentation broth were determined. The fermentation conditions were optimized based on the medium pH, temperature, and liquid volume.

2.7. Fed-batch fermentation

Fed-batch fermentation was conducted in 5 L fermenters; the feeding rate of lactose was 5 g/(L·h) starting at 20 h. The seed culture (40 mL) was transferred into 2 L optimized medium. The culture temperature, airflow, and agitation rate were set to 30 °C, 4 L/min, and 400 rpm. The pH was automatically controlled using hydrochloric acid (6 M) and sodium hydroxide solution (5 M). The fermentation broth was sampled every 8 h to determine the OD_{600}, lactase activity, and residual lactose concentration using high-pressure liquid chromatography (Bio-Rad Aminex HPX-87H Column, Bio-Rad, Hercules, CA, USA; RID-20A photodetector, Shimadzu, Kyoto, Japan).

3. Results

3.1. Screening and characterization of lactase-producing strains

MUGal is a fluorogenic substrate that can be hydrolyzed by lactase and produce water-soluble fluorescent coumarin 4-methylumbelliferone (4-MU) (Fig. 1A). To develop a rapid and easy screening method, the MUGal method was characterized and optimized for a range of reaction conditions (Fig. S1). Commercial lactase (β-galactosidase from *K. lactis*; Aladdin, Shanghai, China) was used as a standard sample and mixed with MUGal for 10 min at 30 °C. The fluorescence intensity was linearly correlated with enzyme activity over the range of 0–23 U/mL (Fig. 1B). Because most lactases were detected as intracellular enzymes in previous studies [9,19], it is necessary to analyze whether MUGal can enter cells and reflect intracellular activity. The previously reported lactase-producing yeast *K. lactis* GG799 was cultured in YPL medium containing MUGal [20], and the fluorescence intensity and enzyme activity were measured. As shown in Fig. 1C, fluorescence intensity and enzyme activity were positively correlated, indicating that the fluorescent substrate MUGal can be used to screen intracellular lactase activity.

To obtain a high-yield lactase-producing strain, a library of microbial strains from different sources was screened using the developed MUGal method, and potential lactase-producing strains were selected as the preliminary collection (Fig. 2A). JNXR-2101 and JNXR-2102 were further characterized in shake flasks because they showed relatively high fluorescence intensities in preliminary screening. The total enzyme activities were 18.06 and 15.39 U/mL in JNXR-2101 and JNXR-2102, respectively, at 60 h, and the extracellular enzyme activity of JNXR-2101 was 3.77 U/mL (Fig. 2B). Considering the enzyme activity and secretion potential, JNXR-2101 was selected for further optimization and characterization. A phylogenetic tree was constructed using the neighbor-joining method in MEGA software, and JNXR-2101 was identified as *K. lactis* based on phylogenetic analysis of the internal transcribed spacer sequence (Fig. S2).

3.2. Enzymatic properties of lactase produced by K. lactis JNXR-2101

Lactase from JNXR-2101 was purified using a combined strategy involving ammonium sulfate precipitation and application of the DEAE Sepharose Fast Flow and Superdex columns G200 (Fig. S3). Mass spectrometry analysis showed that the 118 kDa target protein exhibited 99.9% homology with the reported lactase (accession no.: QLH93946.1). To further characterize lactase produced by JNXR-2101, the effects of temperature and pH on lactase activity were analyzed. As shown in Fig. S4A, lactase showed the highest activity at 45 °C, and the

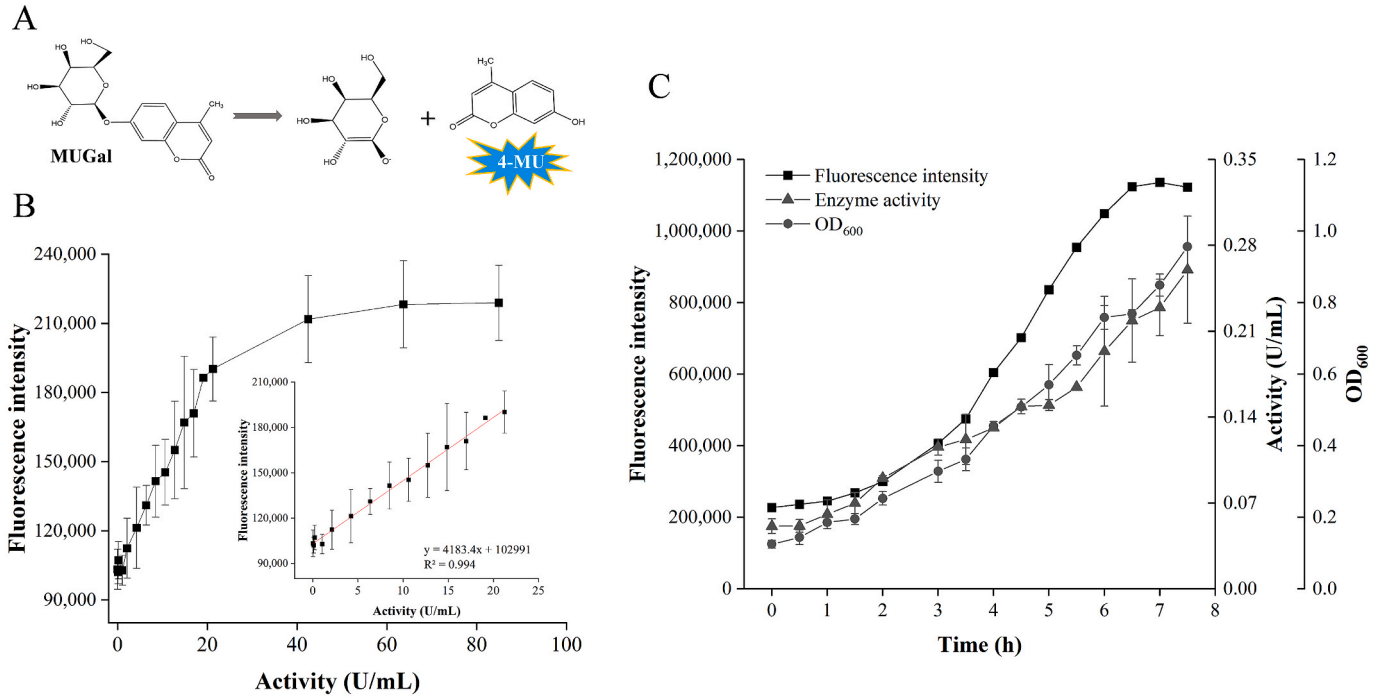

Fig. 1. Lactase activity determined based on the fluorescent substrate MUGal. (A) Fluorescent substrate MUGal can be hydrolyzed by lactase and produce fluorescent coumarin 4-methylumbelliferone (4-MU; $\lambda_{Ex} = 360$ nm/$\lambda_{Em} = 450$ nm). (B) Fluorescence curve of MUGal hydrolyzed by commercial lactase. Briefly, 0.1 mL enzyme with different activity levels was added to 0.7 mL MUGal-YPL medium and incubated for 10 min. (C) Fluorescence curve of MUGal hydrolyzed by lactase-producing *Kluyveromyces lactis* GG799. MUGal was added to YPL medium at the beginning of fermentation, and the enzymatic activity, fluorescence intensity, and OD_{600} were detected.

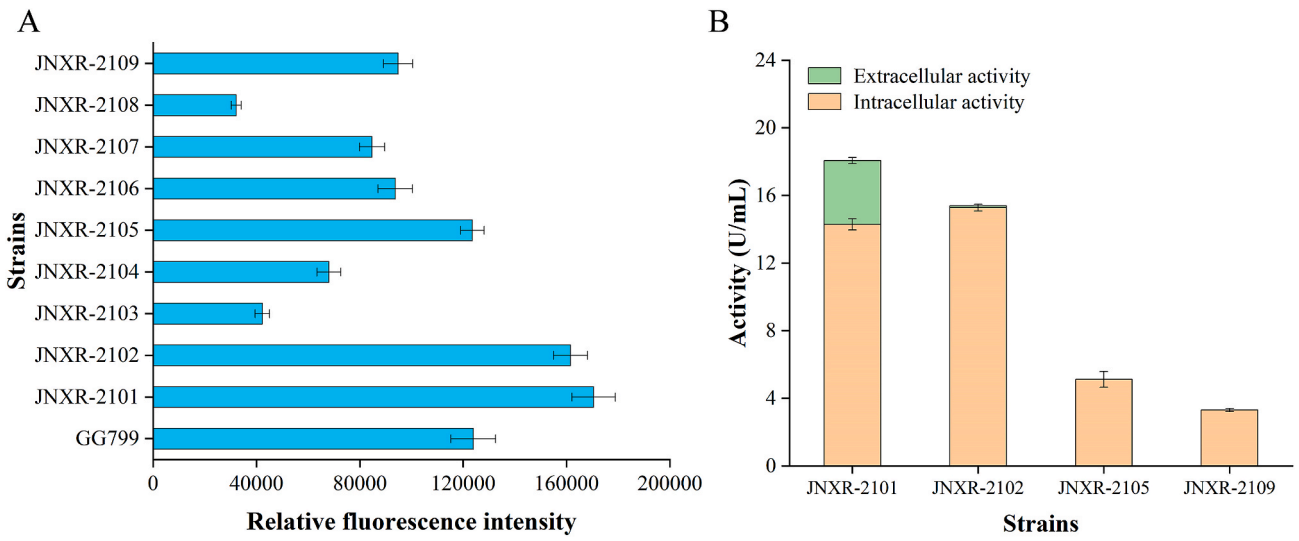

Fig. 2. Characterization of isolated lactase-producing strains. (A) Fluorescence intensity of strains was determined using the MUGal method. The fluorescence intensity of MUGal-YPL medium in a 96-well plate was measured after incubation for 6 h. (B) Lactase activity of selected strains with high fluorescence intensity in shake flasks.

relative enzyme activity remained higher than 60% at 30–50 °C. However, when the temperature was increased to 55 °C or higher, the relative activity decreased to less than 20%. The temperature stability curve also showed that the residual enzyme activity remained at 62.6% after incubation at 45 °C for 1 h (Fig. S4B). The highest lactase activity was detected at pH 9.0, and the relative activity remained above 50% between pH 5.5 and 9.5 (Fig. S4C). The enzyme was almost completely inactivated at pH < 5.0. The relative enzyme activity remained above 60% in the pH range of 6.5–8.0 after treatment at 30 °C for 1 h (Fig. S4D). Thus, lactase from JNXR-2101 was neutral lactase and can be applied in the processing of dairy products.

3.3. Fermentation optimization

The medium composition is a key factor affecting enzyme production during fermentation [21]. To increase lactase production, the medium and fermentation conditions were optimized for *K. lactis* JNXR-2101. First, a single-factor experiment was conducted to determine the concentration ranges for orthogonal experiments [22,23]. Based on the results (Fig. S6 A-G), the medium composition was 30 g/L lactose, 40 g/L complex nitrogen source, 20 g/L corn steep liquor, 1.0 g/L $MgSO_4$, and 0.2 g/L $MnSO_4$. Because the medium components had synergistic effects on enzyme production [24], orthogonal experiments were further performed to obtain the optimal medium. The optimized medium contained 40 g/L lactose, 40 g/L corn steep liquor, 50 g/L nitrogen source (mass ratio of yeast extract and tryptone = 1:2), 0.3 g/L $MnSO_4$, and 0.5 g/L $MgSO_4$, and the enzyme activity reached 50.27 U/mL (Fig. 3). The fermentation conditions, such as the temperature, pH, and dissolved oxygen, were also optimized [25]. Finally, lactase activity increased by 6.5-fold and reached a maximum of 90.2 U/mL at 30 °C and pH 5.0 with

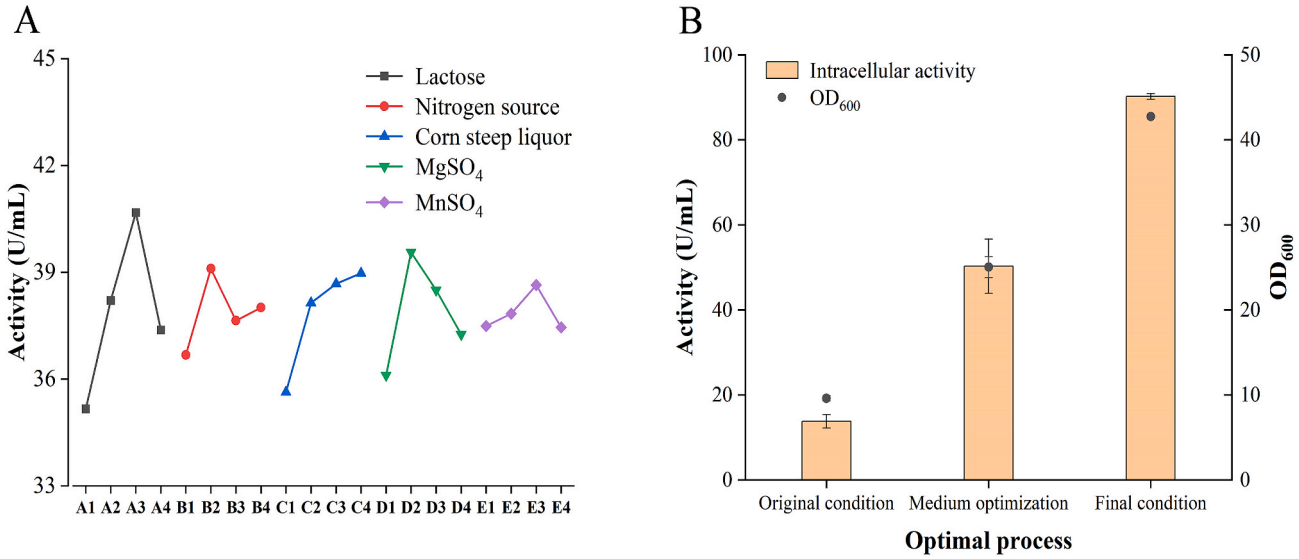

Fig. 3. Optimization of fermentation conditions in shake flasks. (A) Orthogonal experiments of medium composition. A: lactose, B: nitrogen source, C: corn steep liquor, D: $MgSO_4$, E: $MnSO_4$. The orthogonal optimization model is shown in Table S2 (B) Lactase production under each optimized fermentation process. The original condition involved incubation in YPL medium at 30 °C, 50/250 mL. The optimized medium (40 g/L lactose, 40 g/L corn steep liquor, 50 g/L mixed nitrogen source, 0.3 g/L $MnSO_4$, and 0.5 g/L $MgSO_4$) was incubated at 30 °C, 50/250 mL. The final condition involved culture in the optimized medium at 30 °C, 20/250 mL, and pH 5.0.

a loading volume of 20 mL/250 mL (Figs. S6H–J).

3.4. Effect of putative mannoprotein KLLA0_E01057g on lactase production in JNXR-2101

Most *K. lactis* lactases are intracellular enzymes [9,19]; hence, it is important to promote lactase secretion or cell wall disruption to achieve extracellular lactase production. A previous study showed that the cell wall can be modified to increase extracellular protein production [13]. In gene function analysis, the putative mannoprotein-encoding gene KLLA0_E01057g was detected in *K. lactis* and showed 88% homology with *SED1* from *S. cerevisiae* (Table S1). To characterize the function of the putative mannoprotein, KLLA0_E01057g was knocked to obtain mannoprotein-deficient *K. lactis* JNXR-2101ΔE01057g. During fermentation, knockout of KLLA0_E01057g had minimal effects on growth and enzyme production, and *K. lactis* JNXR-2101ΔE01057g showed higher extracellular production (Fig. 4A).

To further analyze the effect of the mannoprotein on cell wall stability, lyticase was used to induce cell fragmentation. The cell lysis rate of *K. lactis* JNXR-2101ΔE01057g was higher than that of the wild-type JNXR-2101, indicating that the mannoprotein-deficient strain was more sensitive to lyticase (Fig. 5). Field emission scanning electron microscopy images revealed no visible differences in the cell morphology between JNXR-2101 and JNXR-2101ΔE01057g. However, cell lysis of *K. lactis* JNXR-2101ΔE01057g significantly increased after adding lyticase, suggesting that the putative mannoprotein E01057p affects the structural stability and sensitivity of the cell wall. When the lyticase concentration was 5 U/mL, the activity of *K. lactis* JNXR-2101ΔE01057g reached a maximum of 12.69 U/mL, which was similar to that obtained by mechanical disruption with FastPrep-24 (Fig. 4B). After treatment with lyticase, the protein concentration of JNXR-2101ΔE01057g was 5.20 mg/mL, which was 1.30-fold higher than that of JNXR-2101 (Fig. S5). These results indicate that knockout of KLLA0_E01057g promotes cell wall degradation. After adding lyticase, JNXR-2101ΔE01057g can be quickly lysed to release intracellular substances, which can reduce the industrial cost of neutral lactase from *K. lactis*.

3.5. Fed-batch fermentation for high-yield lactase production

The optimized medium and culture conditions in shaking flasks were applied in 5 L bioreactors for fed-batch fermentation. The fermentation conditions were set to an airflow rate of 4 L/min, a rotation speed of 400 rpm, and lactose feeding starting at 20 h at 5 g/(L·h) during fermentation referring to previous literature [26,27]. In fed-batch fermentation without pH control, the activity of *K. lactis* JNXR-2101 was 80.11 U/mL at 62 h (Fig. S7). pH is an important factor affecting lactase production, as it influences the electrical properties of the plasmalemma [8]. The lactase activity of *K. lactis* JNXR-2101 increased gradually when the pH was changed from 4.5 to 6.5. Intracellular activity reached 146.70 U/mL at 73 h at pH 6.5 (Fig. 6A). *Kluyveromyces lactis* JNXR-2101ΔE01057g was also fermented under the optimized conditions in a 5 L bioreactor. The intracellular activity reached 159.62 U/mL at 67 h, which was slightly higher than that of JNXR-2101 (Fig. 6B). Nonetheless, minimal extracellular enzyme activity was detected as the yield increased, possibly because of cellular stress. The lactase activities of JNXR-2101 and JNXR-2101ΔE01057g were measured after treatment with lyticases. The enzyme activity of JNXR-2101ΔE01057g treated with lyticase was one-third of that of the enzyme treated using FastPrep-24, whereas JNXR-2101 activity was only one-fifth of the activity of the enzyme obtained using FastPrep-24. Although the lysis effect of JNXR-2101ΔE01057g was much better than that of JNXR-2101, its effect still did not meet application demand.

4. Discussion

High-throughput screening can be used to obtain strains with high enzyme activity and advantageous properties within short periods. High-throughput screening for lactase-producing strains has not been widely performed [28]. Glauche et al. [29] developed an efficient and reproducible method for cell lysis to characterize lactase activity in a 96-well plate by optimizing the chemical reagents. In this study, a rapid and simple method for detecting intracellular lactase activity was used to screen lactase-producing strains. For further screening of a larger mutant library, the method can be optimized by coupling with flow cytometry or microfluidics.

In this study, we obtained lactase activity of 146.04 U/mL in a 5 L

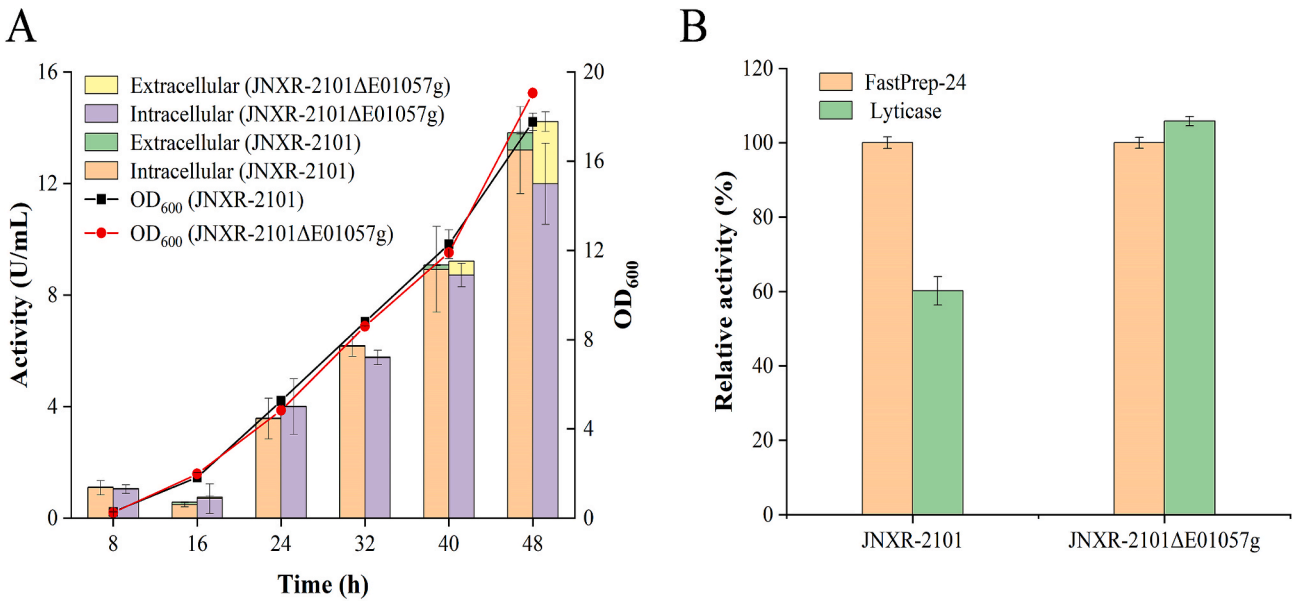

Fig. 4. Effect of putative mannoprotein KLLA0_E01057p on lactase production in *Kluyveromyces lactis*. (A) Growth curve and lactase activity of *K. lactis* JNXR-2101ΔE01057g. (B) Relative enzyme activity of JNXR-2101 and JNXR-2101ΔE01057g after applying different cell wall-disrupting methods. The enzyme activity of *K. lactis* treated with FastPrep-24 was considered as 100%.

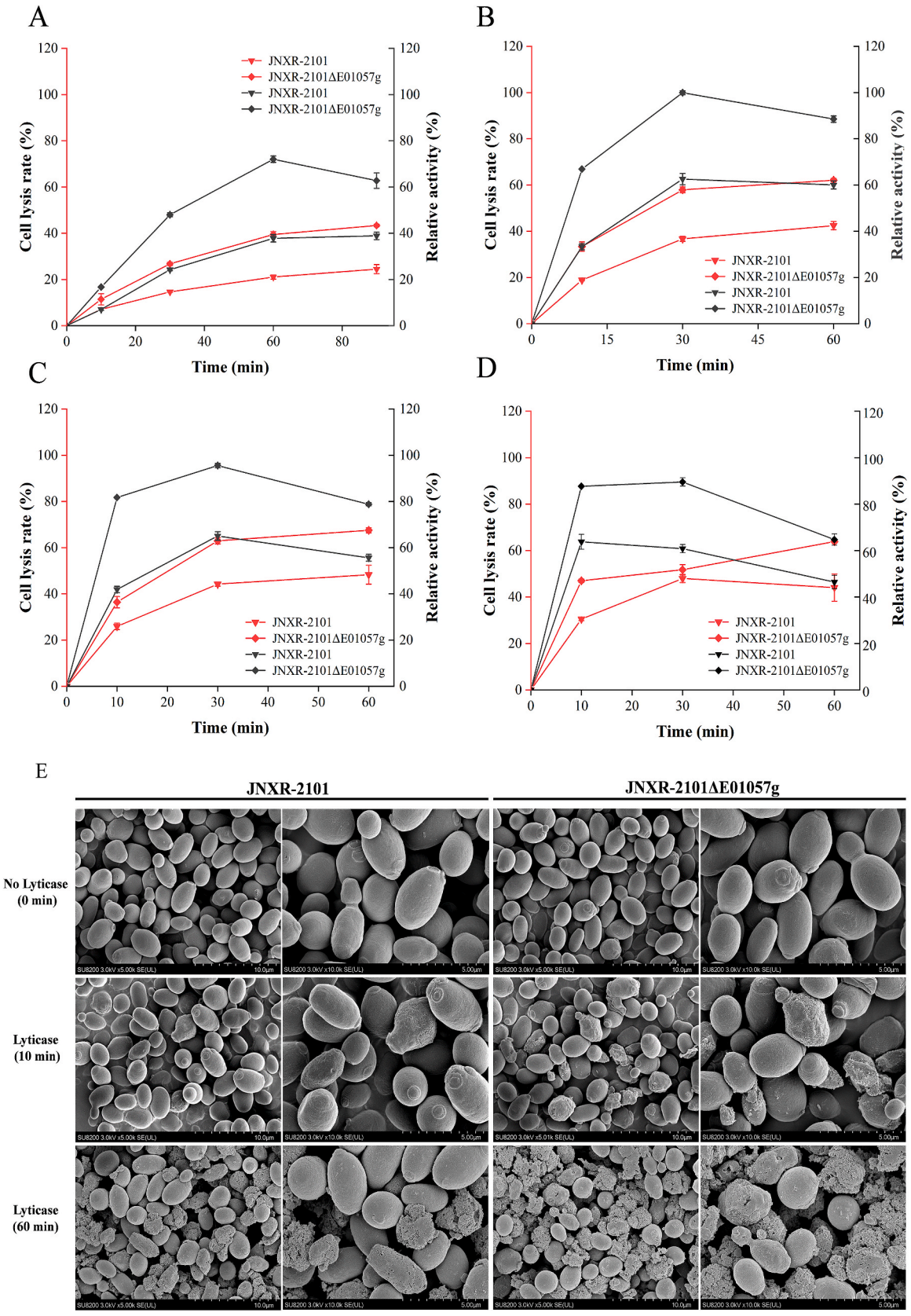

Fig. 5. Characterization of the lysis rate and cell morphology of *Kluyveromyces lactis* JNXR-2101 and JNXR-2101ΔE01057g. (A–D) Cell lysis rate (red line) and relative enzyme activity (black line) after lyticase treatment. Different concentrations of lyticase were added to 1.5 mL yeast suspension (OD_{600} = 0.7). The lyticase concentrations in (A), (B), (C), and (D) were 1, 5, 10, and 20 U/mL, respectively. The cells were incubated at 30 °C and 300 rpm for 60 min. The highest enzyme activity after treatment with 5 U/mL lyticase for 30 min was set to 100%. (E) Field emission scanning electron microscopy images of the yeast surface at different stages. *Kluyveromyces lactis* cells were grown in yeast extract-peptone-dextrose medium solid plate and then scraped off and resuspended in 1 mL normal saline (OD_{600} ≈ 60). Lyticase was added to the cell suspension at different times (0, 10, 60 min).

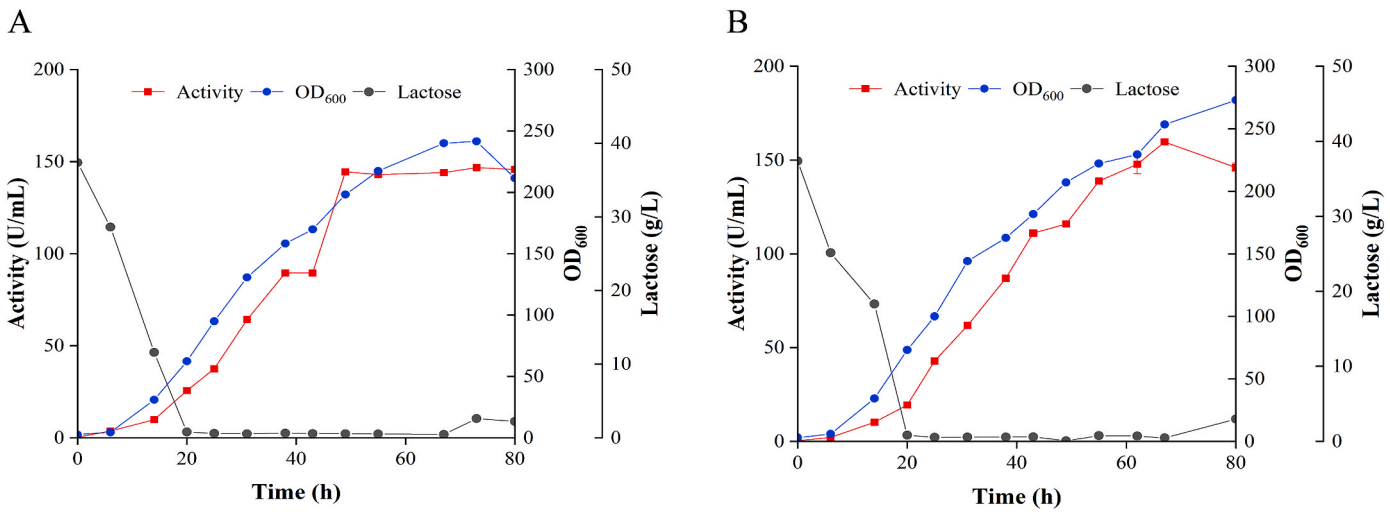

Fig. 6. Lactase production in fed-batch culture in 5 L bioreactors. (A) Fermentation parameters for *Kluyveromyces lactis* JNXR-2101 in a 5 L bioreactor. (B) Fermentation parameters for *K. lactis* JNXR-2101ΔE01057g in a 5 L bioreactor. The fermentation conditions were as follows: feeding rate of lactose 5 g/(L·h) starting at 20 h, culture temperature of 30 °C, airflow of 4 L/min, pH 6.5, and rotation speed of 400 rpm.

fermenter, which is 1.31-fold higher than that of the previously reported non-recombinant *K. lactis* CICC1773 in a 7 L fermenter (111.61 U/mL) [8]. However, lactases from *K. lactis* are intracellular enzymes, leading to high costs in downstream processing, including the cell disruption and separation steps. Mannoprotein-deficient *K. lactis* JNXR-2101ΔE01057g exhibited rapid cell lysis, demonstrating its potential for efficient extracellular lactase production. Although the deficient strain showed slightly improved lactase activity in shake flasks, extracellular lactase activity was not detected in 5 L bioreactors, possibly because cellular stress was triggered when the medium composition was changed, which affected protein secretion [30]. The extracellular protein lactase does not contain signal sequences, suggesting that it is secreted via an unconventional secretory pathway [31]. Indeed, some unconventional secretory events can be triggered by cellular stress [30]. We found that the carbon and nitrogen sources in the medium affected lactase secretion; particularly, 20 g/L lactose and 40 g/L nitrogen increased secretion. Although the lactase yield increased significantly under the optimized fermentation conditions, lactase secretion decreased sharply or even disappeared. Madinger et al. [32] and Becerra et al. [33] also reported that the secretion capacity of *K. lactis* is affected by the culture conditions. To further improve lactase secretion, specific fermentation conditions could be optimized to promote the unconventional secretory pathway in *K. lactis*. Besides, the mechanisms of transmembrane transport should also be explored to achieve efficient lactase secretion.

5. Conclusion

In this work, a rapid and sensitive MUGal-based method was developed for screening lactase-producing strains and a neutral lactase was identified from *Kluyveromyces lactis* JNXR-2101. Based on function prediction and gene knockout, putative mannoprotein KLLA0_E01057g was characterized and demonstrated to promote lyticase-mediated cell lysis for extracellular lactase production. To further improve enzyme activity, the culture conditions were sequentially optimized, and the maximum yield of lactase was 159.62 U/mL in 5 L bioreactors. Taken together, *K. lactis* JNXR-2101ΔE01057g is promising for high-yield lactase production and provides a basis for easier lysis to reduce the cost of lactase production.

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Data availability statement

The data supporting the findings of this study are available within the paper and its supplementary information files. The datasets generated and analyzed during the current study are available from the corresponding author upon request.

CRediT authorship contribution statement

Xiuru Shen: Conceptualization, Investigation, Writing. **Lingtong Liao:** Investigation, Data curation. **Guoqiang Zhang:** Conceptualization, Writing, Funding acquisition. **Jingwen Zhou:** Conceptualization. **Jianghua Li:** Conceptualization, Writing. **Guocheng Du:** Conceptualization.

Declaration of competing interest

All authors declare no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2023.01.001.

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