Yogurt usually contains 5–7% sugar and 3–5% lactose. As β-galactosidases can hydrolyze lactose and improve sweetness, they have the potential to produce lactose-free (LF) and no-sugar-added (NSA) yogurt. In this study, β-galactosidase AoBgal35A from Aspergillus oryzae was engineered by site-saturation mutagenesis. Results of 19 variants of T955 residue showed that lactose hydrolysis rate of T955R-AoBgal35A was up to 90.7%, much higher than 78.5% of the wild type. Moreover, the optimal pH of T955R-AoBgal35A was shifted from pH 4.5 to pH 5.5 and the optimal temperature decreased from 60°C to 50°C. The mutant T955R-AoBgal35A was successfully expressed in Komagatella pastoris, which produced extracellularly 4528 U/mL of β-galactosidase activity. The mutant T955R-AoBgal35A was used to produce LF yogurt. Streptococcus thermophilus counts of LF yogurt increased from 7.9 to 9.5 log cfu/g, significantly higher than that of the control group (8.9 log cfu/g). Residual lactose content of LF yogurt was 0.13%, meeting the requirement of “lactose-free” label (<0.5%, GB 28050–2011, China). Furthermore, sugar in yogurt was replaced by whey powder to produce LF-NSA yogurt. The optimal addition content of whey powder was 7.5%. The texture, WHC and titratable acidity of LF and LF-NSA yogurt achieved good stability during the shelf life. Therefore, this study provides an insight for technological implications of β-galactosidases in the dairy industry.

Keywords: β-Galactosidase, Saturation mutagenesis, Lactose-free yogurt, No-sugar-added yogurt

INTRODUCTION

Lactose is the predominant carbohydrate in milk and it is composed of glucose and galactose (Misselwitz et al., 2019). However, lactose intolerance affects around 70% population over the world, 90% of them is Asian (Oak et al., 2019; Singh et al., 2022). As lactose prevents several people consuming dairy product, lactose-free (LF) dairy products get great attention. However, threshold levels around world for the use of the terms “lactose-free”; e.g., a final product should contain less than 10 mg/100 g lactose to be “lactose-free” in Denmark, whereas this limit is 100 mg/100 g in Germany. The threshold for ‘low-lactose’ products is 1 g/100 g in the EU. In China, a final product should contain less than 0.5 g/100 g lactose to be “lactose-free” (EFSA, 2010). Western Europe is the biggest and fastest growing LF market, followed by Latin America (Dekker et al., 2019). Globally, the LF claim is no longer only used for niche products; it is, in fact, among the top health claims in new milk product launches (Mintel Global New Product Database). LF dairy products market will reach $18.2 billion by 2026, especially yogurt will be the fastest-growing product (Sutay et al., 2022). Thus, a high percent of population with lactose intolerance has raised the demand for LF yogurt.

β-Galactosidases (EC 3.2.1.23) are applied to hydrolyze lactose for LF dairy production. So far, numerous β-galactosidases from bacteria, fungi, plants and animals have been discovered and characterized (Damin et al., 2021). The commercially available β-galactosidases used in the dairy industry have been mainly obtained from fungi, such as Kluyveromyces sp. and Aspergillus sp. (Córdoa et al., 2023). Aspergillus oryzae is one of the most important fungal sources producing β-galactosidases. Its β-galactosidase (AoBgal35A) has several advantages in hydrolyzing whey and producing galacto-oligosaccharides (GOS), with high acidic stability and thermal stability, which has valid generally recognized as safe (GRAS).
status (Shi et al., 2021). However, its acidic pH optimum and severe product inhibition limit its application for lactose hydrolysis in dairy (Shi et al., 2021). Therefore, it is very essential to modify the pH optimum of AoBgal35A to the neutral range and increase hydrolysis efficiency for decreasing the cost of downstream in dairy production (Zhang et al., 2018). To improve the applicability in milk, several strategies have been successfully attempted to modify AoBgal35A. Shi et al. (2021) determined the mutants of AoBgal35A to shift the pH optimum from pH 4.5 to pH 5.5–6.0 by conducting structure-based sequence alignment. The mutant Y364F from Aspergillus oryzae increased lactose hydrolysis efficiency in milk. Li et al. (2022) found that the T955 residue of AoBgal35A is related to lactose hydrolysis rate by directed evolution. The mutant T955A-AoBgal35A was efficiently expressed in Komagatella pastoris to prepare lactose-free milk, and the hydrolysis efficiency was increased by 10%. In the hydrolysis reaction, galactose will linger in the active sites of β-galactosidases because of its interactions with the binding sites, thus leading to product inhibition (Godoy et al., 2016). Thus, complete hydrolysis of lactose with high concentrations is difficult to achieve (Liu et al., 2021). Milk powder and whey powder are usually added to dairy as important ingredients, which lead to a higher concentration of lactose (>7.5%, wt/wt) than normal milk (about 4.6%, wt/wt) (Misselwitz et al., 2019). Thus, it is necessary to improve hydrolysis efficiency of β-galactosidases at high lactose concentration. Zhang et al. (2018) found that the mutant Y364F of Aspergillus candidus increased the hydrolysis rate from 78% to 90% at the high lactose content (24%, wt/wt) after 48 h. Liu et al. (2021) reported that the replaced specific N148 residue of the β-galactosidase from Bacillus coagulans is correlated with the reduction of galactase inhibition. The N148D mutant was selected for lactose hydrolysis and increased hydrolysis rate from 78% to 90% by saturation mutations. Although many β-galactosidases have been mined and engineered for hydrolyzing whey or milk, only few attention has been paid on yogurt (Walaa, et al., 2021).

Lactose hydrolysis has merits not only for lactose-intolerant individuals but also for consumers who want to reduce sugar intake because glucose and galactose are sweeter than lactose (McCain et al., 2018). Application of β-galactosidase and glucose isomerase in yogurt and pudding samples allowed for 10–20% (wt/wt) reduction in the total sugar content, while retaining equal sweetness (Luzzi et al., 2020). Therefore, β-galactosidases have potential to produce no-sugar-added (NSA) yogurt. So far, NSA yogurts on market have several shortcomings, such as the sour taste, poor texture or addition of artificial sweeteners (Wan et al., 2021). However, there is growing epidemiological evidence linking the consumption of artificial sweeteners to adverse cardiometabolic phenotypes, such as type 2 diabetes and cardiovascular disease (Witkowski et al., 2023). Consumers desire new types of health and delicious NSA yogurt with “clean label.” Whey is a high nutritional value by-product of the dairy industry (Ozel et al., 2022). Addition of whey powder cannot only improve yogurt texture, also increase the lactose content in milk, and increase the content of hydrolyzed monosaccharides, which enhance the sweetness of dairy to produce LF and NAS (LF-NSA) yogurt.

β-Galactosidases are widely used in milk and cheese, while several studies focus on other dairy products such as yogurt (Walaa, et al., 2021; Zhao et al., 2022). To prepare LF yogurt, milk is generally treated with β-galactosidases before or concurrently with fermentation, hydrolyzing lactose into glucose and galactose (Dekker et al., 2019). β-Galactosidases have been reported to exert influence on fermentation properties of yogurt. Nagaraj et al. (2009) produced the lactose hydrolyzed yogurt by pre-hydrolysis of lactose, and found that the lactose hydrolyzed yogurt setting time reduced 30–45 min over control. Skryplonek et al. (2019) prepared LF frozen yogurt by commercial Ha-lactase®, and the reaction occurred simultaneously with fermentation by L. bulgaricus and S. thermophilus (YF-L903, Chr. Hansen, Denmark). They found the acidity of LF product was significantly higher than the control, suggesting that the β-galactosidase enhanced the fermentation process. Karnyaczki et al. (2017) found that the yogurt fermentation from LF milk was extended about 1 h. The starter culture included Lactobacillus acidophilus, Bifidobacterium, S. thermophilus and L. bulgaricus (YoFast88, Chr. Hansen, Denmark). Additionally, the texture and flavor of yogurt are influenced by metabolites from lactose hydrolysis and fermented monosaccharides. Khabibullaev et al. (2019) found that viscosity of LF yogurt was lower than the control. A higher level of exopolysaccharides was synthesized in LF yogurt than the regular yogurt fermented by S. thermophilus and L. bulgaricus (Schmidt et al., 2016). Therefore, it is important to improve the properties of β-galactosidases for the production of LF yogurt with high quality and stability during shelf life.

In this study, AoBgal35A was engineered by site-saturation mutagenesis to improve the applicability in yogurt processing. The mutant T955R-AoBgal35A was successfully expressed in K. pastoris to produce LF yogurt. Whey powder is added to milk and resulted in a high lactose concentration. Lactose is hydrolyzed by β-galactosidases into glucose and galactose, which are much sweeter than lactose. Thus, sugar has the potential to be replaced by whey powder to produce LF-NSA yogurt. This study provided a candidate β-galactosidase for LF dairy products, and afforded an efficient method to produce LF-NSA yogurt.
Miao et al.: Engineering the β-galactosidase...

MATERIALS AND METHODS

Materials

The pPIC9K-AoBgal35A was constructed as described by Li et al. (2021). Escherichia coli DH5α and K. pastoris GS115 were used as hosts for gene cloning and expression, respectively. The heterologous protein expression system of K. pastoris GS115 and pPIC9K was purchased from Invitrogen (Carlsbad, CA). o-Nitrophenyl(oNP)-β-galactopyranoside and lactose were purchased from Sigma (St. Louis, MO, USA). Milk, whey powder (Friesland Campina, Netherlands) and yogurt starter (Deep-frozen powder, Chr Hansen, Denmark) were provided from Mengniu (Beijing, China). Unless otherwise stated, all other chemicals were of analytical grade.

Site-saturation Mutagenesis

Site-saturation mutagenesis on the residue Thr955 of AoBgal35A (Genebank: KF857462) was carried out as described in the previous study (Li et al., 2022). The plasmid pPIC9K-AoBgal35A was used as template and the primers were designed using NNK degeneracy (Table S1). The PCR products were transformed into E. coli DH5α competent cells by electroporation transformation. The transformants were subsequently cultured on Luria-Bertani (LB) plate at 37°C overnight. The DNA sequences of single colonies were performed to collect all 20 variants. The 20 recombinant plasmids linearized by PmeI was transformed into K. pastoris GS115 competent cells according to the manufacturer’s instructions (Invitrogen). The colonies on minimal dextrose plates were used to express the mutant β-galactosidases in BMGY/BMMY medium. The activity of crude enzyme was examined after methanol induction for 3 d at 30°C. The β-galactosidase (10 U) was mixed with 1 mL of 5% (wt/vol) lactose. After incubation at 42°C, the samples were withdrawn at 6 h. Hydrolysis products were analyzed using thin layer chromatography (TLC) to select mutants preliminarily. These reaction mixtures were deionized and spotted on to a silica gel plate, and developed twice in a solvent system containing butan-1-ol: acetic acid: water (5:3:2, vol/vol). Saccharides were detected by heating in an oven after spraying the plates with a mixture of methanol: sulfuric acid (95:5, vol/vol).

Biochemical Properties of Mutants

Three mutants, viz. T955A-AoBgal35A, T955R-AoBgal35A, T955W-AoBgal35A, and wild type were purified by fast protein liquid chromatography (FPLC, AKTA purifier, GE Healthcare) using Q-Sepharose column (10 × 100 mm, GE Healthcare). The optimal pH of the enzymes was determined at 65°C in 50 mmol/L different buffers within pH 3.0 to 7.5, including McIlvaine (pH 3.0–4.0), acetate (pH 4.0–6.0), phosphate (pH 6.0–7.0), Tris-HCl (pH 7.0–8.0). The optimal temperature was assessed at temperature range of 30–70°C in 50 mmol/L acetate buffer (pH 4.5).

High Cell Density Fermentation

The mutant T955R-AoBgal35A was selected to ferment at high cell density. The selected transformant with the highest β-galactosidase activity was cultivated in a 5-L fermentor for high cell density fermentation according to the guidelines of Pichia fermentation (Version B, 053002, Invitrogen). Briefly, the transformant was cultured in 150 mL BMGY medium and further grown at 30°C until the optical density at 600 nm reached 10.0. The medium was then inoculated into the fermentor, which contained 1.5 L of the fermentation medium. The cells were grown at 30°C and pH 5.5 with mechanical agitation of 600 rpm. After the exhaustion of initial glycerol (about 6 h), fed-batch fermentation was performed for 4 h. The dissolved oxygen level was kept at ≥20% air saturation throughout the fermentation process. At this stage, the fermentation medium pH was constant at pH 6.0 with agitation at 800 rpm. During the whole induction phase, wet cell mass, and β-galactosidase activity were monitored. The mutant T955R-AoBgal35A expression was analyzed by sodium dodecyl sulfate-PAGE (SDS-PAGE). Fermentation supernatant samples were withdrawn at 12, 24, 48, 72, 96, 120, 144, and 168 h, respectively, after methanol induction.

LF Yogurt Preparation and Analysis

Two types of yogurts were prepared: regular yogurt (Control) and LF yogurt with the mutant T955R-AoBgal35A. The technological process of yogurt is shown in Figure S1. Sugar (7%, wt/wt) was suspended in milk by stirring (600 rpm) at room temperature for 10 min. The milk was homogenized at 65°C (150–200 bar), then heated at 95°C for 5 min to sterilize. Milk was cooled to 42°C and inoculated with the yogurt starter containing Lactobacillus bulgaricus and Streptococcus thermophilus (100 μL/kg). Meanwhile, the β-galactosidase (T955R-AoBgal35A) was added into milk for LF yogurt. After incubation at 42°C for 6 h, milk was fermented up to the final pH of 4.5. Yogurt was broken before cooling in water bath at 4°C. The fermented yogurt was stored at 4°C for 16 h. Samples were taken at different time points of the fermentation stage. The pH, viable count and carbohydrate contents were measured per 1 h.

The pH value of each yogurt sample was measured in triplicate by digital pH meter at 25°C. S. thermophilus
was counted using MC agar (pH 7.2) incubated at 37°C for 48 h under aerobic conditions. MRS agar (pH 5.2) was incubated at 37°C for 72 h under anaerobic conditions for counting *L. bulgaricus*. Titratable acidity expressed as Thorner degree (°T) was determined by titration of a sample and distilled water with 0.1 mol L⁻¹ NaOH in the presence of phenolphthalein as an indicator, according to the description of Comunian et al. (2017). The contents of lactose, glucose and galactose were quantified by high-performance liquid chromatography (HPLC) equipped with Agilent-1260 Infinity system equipped with a refractive index detector (RID) using a BP-800 Pb ++ column (Benson Polymeric, Sparks, NE, USA). The column was controlled at 80°C and eluted with ultra-pure water at a flow rate of 0.6 mL/min.

**LF-NSA Yogurt Preparation and Quality During Shelf Life**

The processing technique for the LF-NSA yogurt was similar to LF yogurt in the section 2.5. Differently, sugar was replaced by whey powder, which was suspended in milk by stirring (600 rpm) at 45°C for 10 min, and left to set for 20 min. Yogurts were supplemented with 0%, 2.5%, 5.0%, 7.5% and 10.0% (wt/wt) of whey powder, denoted as the Blank, WP-2.5%, WP-5.0%, WP-7.5% and WP-10.0%, respectively. The enzyme dosages were 13 U/mL, 18 U/mL, 24 U/mL, 28 U/mL and 32 U/mL. The contents of lactose, glucose and galactose were quantified as the section 2.5. Preference sensory evaluation of yogurts was analyzed using the method described by Kárnyáczki et al. (2017) with some modifications. Fifteen volunteers were recruited from students in the College of Food Science & Nutritional Engineering, China Agricultural University (7 males and 8 females, aged between 20 and 35). The products were evaluated at d 1 of storage at 4°C. Volunteers were required to rate yogurt samples for sweetness, acidity, color, firmness, creaminess and flavor ranging from dislike (1) and extremely like (10). They were given water to clean their palate between samples. This study was approved by the Human Body Research Ethics Committee of China Agricultural University, China (CAUHR-20231201).

Yogurts were stored at 4°C, samples were collected in 1, 7, 14, 28 d. Texture, titratable acidity and water-holding capacity (WHC) were tested during the shelf life. The texture analysis was done by measuring the force of penetration of a probe in the sample at a well-defined speed. A cylindrical probe was used in the analysis, with the crosshead speed of 1.0 mm/s and the penetration depth of 10 mm. Each force-time curve was obtained by 2 successive compressions of each sample, and the texture profile parameters were determined. WHC of yogurts was measured according to Kárnyáczki et al. (2017) with slight modification. Approximately 30 g yogurt samples were centrifuged at 3000 rpm for 10 min at room temperature, and the precipitate was weighed as gram. The WHC was calculated as the following equation:

$$WHC(\%) = \frac{\text{Weight of precipitate (g)}}{\text{Weight of yogurt sample (g)}} \times 100.$$  

**Statistical Analyses**

Statistical analysis and comparison among means were carried out using the statistical package SPSS 24.0 (IBM SPSS Statist cs, Chicago, IL, USA). One-way ANOVA test using first “formulation.” Tukey test was used for means comparison (95% confidence level).

**RESULTS AND DISCUSSION**

**Biochemical Properties of the β-Galactosidase Mutants**

To investigate the effect of Thr955 on AoBgal35A, the site-saturation at Thr955 was performed. Among the 20 mutants of Thr955, a few substitutions obviously improved the hydrolysis ability of AoBgal35A, including T955A-AoBgal35A, T955W-AoBgal35A and T955R-AoBgal35A (Figure S2). To further select the most suitable mutant for yogurt processing (fermented at 42°C and pH decreased from pH 6.5 to pH 4.5), the optimal pH and temperature of T955A-AoBgal35A, T955W-AoBgal35A, T955R-AoBgal35A and AoBgal35A were evaluated (Figure 1). Compared with AoBgal35A, the optimal pH values of T955A-AoBgal35A, T955W-AoBgal35A, T955R-AoBgal35A and AoBgal35A were evaluated (Figure 1). Compared with AoBgal35A, the optimal pH values of T955A-AoBgal35A, T955W-AoBgal35A and T955R-AoBgal35A were pH 5.0, pH 5.0 and pH 5.5 respectively (Figure 1A). Meanwhile, the optimal temperatures of 3 mutants decreased from 60°C to 55°C, 50°C and 50°C respectively (Figure 1B). The mutant T955R-AoBgal35A displayed the highest optimal pH (pH 5.5) and the lowest optimal temperature (50°C) (Figure 1). Thus, the T955R-AoBgal35A might be the most suitable mutant β-galactosidase for LF yogurt fermentation among 3 mutants.

The pH of yogurt is dynamical in production and storage stage. During yogurt fermentation, the pH of milk decreases from pH 6.5 to pH 4.5 (Sutay et al., 2022), thus an ideal β-galactosidase for yogurt production should possess high hydrolysis activity within pH 4.5–6.5. The optimal pH of mutant T955R-AoBgal35A was improved from pH 4.5 to pH 5.5, and maintained 75% activity within pH 4.5–6.5 (Figure 1A). Some neutral β-galactosidases
are unsuitable for yogurt fermentation, because most of them are seriously inactivated under acidic conditions, such as β-galactosidases from Bacillus coagulans T242 (pH 6.8, relative activity <50% below pH 6.0) (Xu et al., 2021), Bifidobacterium animalis (pH 6.0, relative activity <50% below pH 5.5) (Xu et al., 2019) and Alteromonas sp. ML117 (pH 8.0, relative activity <40% below pH 6.5) (Yao et al., 2019). The decrease of enzyme activity might result in extending hydrolysis time and high level of the residual lactose. A few neutral β-galactosidases can hydrolyze most of lactose within a short time (1–2 h). The β-galactosidase from Bacillus coagulans degraded lactose totally (5.0%, wt/wt) in 90 min (Liu et al., 2021). However, rapid hydrolysis of lactose led to a great change of carbohydrate composition in yogurt, and then influenced the growth and metabolism of lactic acid bacteria (Yamamoto et al., 2020). Likewise, some acidophilic β-galactosidases are unsuitable in yogurt, such as β-galactosidases from Aspergillus niger CICIM F0215 (pH 3.5) (Niu et al., 2017), Teratosphaeria acidotherma AIU BGA-1 (pH 1.5) (Chiba et al., 2015) and Aspergillus lacticoffeatus (pH 3.5) (Cardoso et al., 2017). The optimal temperatures of most β-galactosidases are mesothermal (40–60°C), which are lower than that of AoBgal35A (65°C), such as β-galactosidases from Kluyveromyces lactis CECT1931 (40°C) (Rodriguezclosinas et al., 2011), Aspergillus niger (50–60°C) (Niu et al., 2017) and Aspergillus lacticoffeatus (50°C) (Cardoso et al., 2017). In general, yogurt fermentation temperature is between 35–45°C (Kárnýaczkzi et al., 2017). The optimal temperature of the mutant T955R-AoBgal35A decreased from 65°C to 55°C (Figure 1B), which is beneficial for yogurt fermentation. Thus, enzymatic properties of T955R-AoBgal35A may be suitable for yogurt processing conditions.

### Hydrolysis Properties of the Mutant T955R-AoBgal35A

After hydrolysis at 42°C for 6 h, the mutant T955R-AoBgal35A showed the least residual lactose content (0.11%), and contents of glucose and galactose were 2.38% and 2.16% respectively (Figure 2A). The hydrolysis rates of T955R-AoBgal35A and AoBgal35A were 90.7% and 78.5%, respectively (Figure 2B).

Hydrolysis efficiency of β-galactosidases play an important role in the LF dairy production. Long hydrolysis time may raise production cost and limit the β-galactosidase’s application in dairy production (Shi et al., 2021). The β-galactosidase from Arthrobacter sp. 32cB (2 U/mL milk) hydrolyzed 90% lactose after 24 h (Pawlak-Szukalska et al., 2014). Erwinia sp. E602-orgin β-galactosidase with the enzyme addition of 3 U/mL hydrolyzed 60% lactose after 12 h of reaction (Xia et al., 2018). After the addition of 10 U/mL, the wild type AoBgal35A took 48 h for degrading 90% lactose at 37°C in milk (Zhao et al., 2014). To overcome this obstacle, some strategies have been used to improve the hydrolysis efficiency. In this study, AoBgal35A was engineered by site-saturation mutagenesis. Lactose (5%, wt/wt) was hydrolyzed with 10 U/mL enzyme addition at 42°C for 6 h. Hydrolysis efficiency of the T955R-AoBgal35A mutant increased from 78.5% (wild type) to 90.7% (Figure 2B). Similarly, Li et al. (2022) reported that the mutant T955A-AoBgal35A modified by directed evolution hydrolyzed 90.0% lactose in milk after 72 h at room conditions.
temperature with enzyme dosage of 2 U/mL. Shi et al. (2021) found that hydrolysis rate of mutant Y364F of AoBgal35A improved from 88.5% to 96.9% at 37°C for 20 h. Compared with them, the mutant T955R-AoBgal35A have the potential application to hydrolyze lactose completely during yogurt fermentation.

**High-level expression of T955R-AoBgal35A in *K. pastoris***

The mutant T955R-AoBgal35A was successfully expressed in *K. pastoris* GS115. Among all transformant on YPD geneticin (G418, 1–4 mg/mL) plates, one transformant with the highest β-galactosidase activity (45 U/mL) was selected for high cell density fermentation. The protein content and β-galactosidase activity of the fermentation supernatant were up to 20 mg/mL and 4528 U/mL, respectively, after 168 h incubation (Figure 3A). The proteins in the fermentation supernatant during the induction process was analyzed by SDS-PAGE (Figure 3B). A protein band at 110 kDa, which was corresponding to T955R-AoBgal35A in the fermentation supernatant, was detected and increased with fermentation time.

Many β-galactosidases with efficient hydrolysis ability have been reported, but low level of expression limits their use in dairy industry. The β-galactosidase from *Bacillus coagulans* degraded lactose totally (10.0%, wt/wt) in 3 h (Liu et al., 2021), and the β-galactosidase from *Alteromonas* sp. ML52-origin β-galactosidase hydrolyzed 94% lactose after 5 h at 25°C (Sun et al., 2018). However, those have been expressed in *Escherichia coli* BL21 (DE3), which is unsuitable for dairy industry. In this study, the mutant T955R-AoBgal35A was high-level expressed in *K. pastoris*. The enzyme achieved 4528 U/mL (Figure 3A), which is similar to the mutant T955A expressed in *K. pastoris* GS115 (4760 U/mL) (Li et al., 2022) and wild type AoBgal35A expressed in *K. pastoris* KM71 (4239 U/mL) (Zhao et al., 2014). The expression level of T955R-AoBgal35A in *K. pastoris* is much higher than those of other β-galactosidases from *Sulfolobus solfataricus* (204.9 U/mL) (Duan et al., 2014), *Aspergillus candidus* (3600 U/mL) (Zhang et al., 2005), *Arthrobacter* sp. (1926 U/mL) (Hildebrandt et al., 2009) and *Aspergillus awamori* (347.8 U/mL) (Vidya et al., 2020).

**Properties of LF Yogurt Fermented with T955R-AoBgal35A**

Microbiological counts of *L. bulgaricus* and *S. thermophilus* in the milk fermented with T955A-AoBgal35A are shown in Table 1. During the initial 2 h of fermentation, *S. thermophilus* counts in LF yogurt ranged from 7.9 to 9.5 log cfu/g, significantly higher than that of the control group (8.9 log cfu/mL). At the end of fermentation, LF yogurt exhibited a higher amount of *S. thermophilus* (10.2 log cfu/mL) than the control group (9.8 log cfu/mL). *L. bulgaricus* counts in LF yogurt was significantly higher than the control yogurt in the later fermentation (4–6 h) (Table 1). At the end of fermentation, *L. bulgaricus* counts in LF yogurts increased from 4.8 to 7.2 log cfu/mL, compared with the control yogurt (6.5 log cfu/mL). In addition, milk fermentation was characterized by a drop of the pH value from pH 6.5 to approximately pH 4.5 within 6 h (Figure 4D). LF yogurt showed similar starting and ending pH compared with the control, while the drop of pH in T955R-AoBgal35A yogurt was

**Figure 2.** Hydrolysis property of the mutant T955R-AoBgal35A. (A) Contents of lactose (●), glucose (▲) and galactose (■) were analyzed in 5% lactose (pH 5.5) at 42°C. (B) Hydrolysis rate of T955R-AoBgal35A (●) and AoBgal35A (▲) were compared. Samples at various time-intervals were analyzed by HPLC for the content of residual lactose.
considerably accelerated within the first 2 h of fermentation. Contents of lactose, glucose and galactose during yogurt fermentation are shown in Figure 4A, B and C, respectively. At the end of fermentation, residual lactose concentration of T955R-AoBgal35A group was 0.24%, which is much lower than that of control group (3.01%). Final contents of glucose and galactose in LF yogurt were 2.2% and 2.1%, respectively (Figure 4B and 4C).

Lactose hydrolysis has been reported on influence the fermentation properties of yogurt, due to the changes in carbohydrate composition (Kárnyáczki et al., 2017). *S. thermophilus* counts in the LF yogurt were significantly higher than the control group at the initial stage of fermentation (0 ~2h) (Table 1), perhaps because of the high lactose content (from 10.5% to 4.6%) in LF yogurt (Figure 4A). The majority of *S. thermophilus* preferentially consumes lactose over glucose and galactose, and displays low growth rate in the absence of lactose (Gas Ser et al., 2022). Furthermore, the growth and metabolic changes of *S. thermophilus* are mainly dependent on the concentration of lactose but not on the ratio of lactose to glucose concentration. At the end of fermentation (4~6 h), the rapid growth of *L. bulgaricus* was observed in the LF yogurt (Table 1). This might be down to the massive accumulation of glucose. The number of *L. bulgaricus* 2038 significantly increased in the monoculture with the glucose medium compared with lactose medium (Yamamoto et al., 2021). In addition, *S. thermophilus* produced a higher concentration of formic acid in the glucose medium than in the lactose medium, which boosted the growth of *Lactobacillus bulgaricus* (Yamamoto et al., 2021). At the late stage of fermentation, total viable count of LF yogurt was higher than the control yogurt (Table 1), might due to the high content of total sugar in yogurt.

**LF-NSA Yogurt Sensory Evaluation and Quality During Shelf Life**

LF-NSA yogurts were prepared with the mutant T955R-AoBgal35A and whey powder according to Figure S1. After addition of the optimized enzyme dosage and fermentation, lactose contents of 5 yogurts decreased from 4.7%, 6.5%, 8.6%, 10.6% and 12.5% to 0.13%, 0.33%, 0.34%, 0.46% and 0.57%, respectively (Figure 5B). To explore the impact of whey powder on LF-NSA yogurt taste, yogurts with different amounts of whey powder from 0% to 10.0% were tested for sensory evaluation (Figure 5A). When whey powder was added up to 5.0%, subjects could taste sweetness obviously. WP-7.5% group gained highest preference degree, especially in sweetness and acidity. Moreover, whey powder imparted more salient milk flavor than blank group, and creaminess of yogurt was increased. With addition of whey powder increasing, yogurt color became light yel-

![Figure 3. Time-course of the T955R-AoBgal35A expressed in Komagatella pastoris in a 5-L fermentor (A) and the extracellular protein analysis (B) during high cell density fermentation. Enzyme activity (●), and wet cell mass (▲) of fermentation media was measured every 24 h. Lane M, low molecular weight protein standards; lanes 1–8, fermentation supernatant samples withdrawn at 12, 24, 48, 72, 96, 120, 144, and 168 h, respectively, after methanol induction.](image) Low gradually within the range of consumer acceptance. The optimal addition of whey powder in LF-NSA yogurt was found to be 7.5% on the basis of sensory evaluation. With the addition of whey powder, the LF-NSA yogurt protein was 3.27%, significantly higher than that of control yogurt (2.97%, Table 2). Similarly, the LF-NSA yogurt fat was 3.04%, significantly higher than that of control yogurt (2.88%, Table 2). The texture parameters, WHC and titratable acidity of 2 kinds of yogurt during 28 d shelf life are presented in Table 3. Firmness of LF-NSA yogurt was significantly higher than the control yogurt in shelf life. In accordance with firmness, the WHC of LF-NSA yogurt (97.1%) was little higher than the control yogurt (94.2%). There was not significant difference in cohesiveness between 2 kinds of yogurt samples. Overall, LF-NSA yogurt made with T955R-AoBgal35A exhibited good stability during shelf life.
Table 1. *Streptococcus thermophilus* and *Lactobacillus bulgaricus* counts of control yogurt and T95SR-AoBgal35A LF yogurt during fermentation

<table>
<thead>
<tr>
<th>Fermentation Time (h)</th>
<th><em>Streptococcus thermophilus</em> (lg cfu/mL)</th>
<th><em>Lactobacillus bulgaricus</em> (lg cfu/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>LF yogurt</td>
</tr>
<tr>
<td>0</td>
<td>6.85 ± 0.28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.90 ± 0.13&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1</td>
<td>7.26 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.28 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>8.38 ± 0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.78 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>9.03 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.93 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>8.86 ± 0.09&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.20 ± 0.09&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>8.78 ± 0.16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.20 ± 0.09&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Control yogurt contained sugar. Lactose-free (LF) yogurt contain sugar and was treated with the β-galactosidase T95SR-AoBgal35A. Results are presented as the mean ± standard deviation (n = 3). Values in a row with different letters differ significantly (P < 0.05).

Figure 4. Fermentation changes of the control yogurt and lactose-free (LF) yogurt. Lactose (A), glucose (B) and galactose (C) were analyzed by HPLC. The medium acidity was measured by pH meter (D). Samples at various time-intervals were analyzed during fermentation. Two groups of yogurts were prepared: regular yogurt with sugar (Control); lactose-free yogurt with the T95SR-AoBgal35A.
Lactose hydrolysis is an important method for sugar reduction in dairy, such as milk (Li et al., 2015), milk powder (Dantas et al., 2021) and ice cream (Skryplonek et al., 2019). As common ingredients, milk powder and whey powder are usually added to milk, resulting in a high lactose concentration. With the use of β-galactosidases, whey powder has the potential to produce LF dairy. Few studies applied whey powder for dairy production, because of the low sweetness and salty taste. The addition of whey permeate powder created an overwhelming salty taste because of the minerals in permeate, which made this an unsuitable approach to sweeten chocolate milk (Li et al., 2015). In this study, desalted whey powder was used to avoid this problem, and improved the balance between sweetness and acidity owing to the addition of mutant T955R-AoBgal35A. The optimized addition amount of whey powder was 7.5% (Figure 5A). Complete hydrolysis of high concentration lactose is difficult (Liu et al., 2021), because transglycosidation reaction was found for many β-galactosidase from Kluyveromyces lactis (Córdoval et al., 2023), S. thermophilus (Zhao et al., 2022) and Bacillus circulans (Han et al., 2022). In this study, with the optimized enzyme dosage, lactose contents of 5 yogurts decreased from 4.68%, 6.53%, 8.57%, 10.59% and 12.52% to 0.13%, 0.33%, 0.34%, 0.46% and 0.57%, respectively (Figure 5B). As shown in Table 2, lactose in LF and LF-NSA yogurts met the requirement of “lactose-free” label in China. The increase of yogurt firmness might be due to the addition of whey powder (Table 3). Textural differences of yogurt depend on the protein interaction during acidifying with fermentation, which dominates the formation of yogurt gels (Wang et al., 2023). This result is in agreement with the increase of protein content in LF-NSA yogurt previously observed (Table 2). Thus, T955R-AoBgal35A has great potential application in LF and LF-NSA yogurt production.

**CONCLUSIONS**

This study was aimed to produce lactose-free and no-sugar-added (LF-NSA) yogurt with the modified β-galactosidase and whey powder. The β-galactosidase (AoBgal35A) from Aspergillus oryzae was engineered by site-saturation mutagenesis to improve the enzyme properties. The increase of optimal pH and the decrease of optimal temperature made the mutant T955R-AoBgal35A being suitable for yogurt processing. The mutant T955R-AoBgal35A was high level expressed in K. pastoris, making it highly cost-effective for dairy industrial applications. The mutant T955R-AoBgal35A could pro-

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**Table 2.** Fundamental composition of control, LF and LF-NSA yogurts stored at 4°C for 3 d

<table>
<thead>
<tr>
<th>Samples</th>
<th>Total solids (%)</th>
<th>Protein (%)</th>
<th>Fat (%)</th>
<th>Sugar (%)</th>
<th>Lactose (%)</th>
<th>Glucose (%)</th>
<th>Galactose (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control yogurt</td>
<td>18.1 ± 0.27a</td>
<td>2.97 ± 0.06a</td>
<td>2.88 ± 0.05a</td>
<td>6.87 ± 0.11a</td>
<td>3.71 ± 0.26a</td>
<td>0.56 ± 0.09a</td>
<td>0.22 ± 0.07a</td>
</tr>
<tr>
<td>LF yogurt</td>
<td>17.9 ± 0.42b</td>
<td>2.84 ± 0.12b</td>
<td>2.94 ± 0.09b</td>
<td>6.91 ± 0.19b</td>
<td>—</td>
<td>1.85 ± 0.02b</td>
<td>1.73 ± 0.16b</td>
</tr>
<tr>
<td>LF-NSA yogurt</td>
<td>18.4 ± 0.87b</td>
<td>3.27 ± 0.11b</td>
<td>3.04 ± 0.01b</td>
<td>—</td>
<td>—</td>
<td>4.89 ± 0.08b</td>
<td>4.56 ± 0.19b</td>
</tr>
</tbody>
</table>

Control yogurt contained sugar. Lactose-free (LF) yogurt contain sugar and was treated with T955R-AoBgal35A. Sugar in lactose-free and no-sugar-added (LF-NSA) yogurt was replaced by whey powder and was treated with T955R-AoBgal35A. Values in a row with different letters differ significantly (P < 0.05). “—” meant the content of carbohydrate was below 0.1%.
promote growth of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. Sugar in yogurt was further replaced by whey powder to produce LF-NSA yogurt. This study modified the β-galactosidase to produce LF dairy, and provide an efficient method to reduce sugar in dairy.

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**REFERENCES**


Zhao, Q., F. Liu, Z. W. Hou, C. Yuan, and X. Q. Zhu. 2014. High level production of β-galactosidase exhibiting excellent milk-lactose deg-
Miao et al.: Engineering the β-galactosidase...


**ORCIDs**

Miao Miao  [https://orcid.org/0009-0001-2850-6009](https://orcid.org/0009-0001-2850-6009)
Shaoqing Yang  [https://orcid.org/0000-0002-8708-677X](https://orcid.org/0000-0002-8708-677X)
Qiaojuan Yan  [https://orcid.org/0000-0003-4568-5544](https://orcid.org/0000-0003-4568-5544)
Zhengqiang Jiang  [https://orcid.org/0000-0002-9573-2890](https://orcid.org/0000-0002-9573-2890)