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Isolation from grain based starter and fermentation performance of high aroma strains of *Saccharomyces cerevisiae* in Korean rice wine

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Abstract

Why was the work done: The quality and sensory differentiation of Cheongju rice wine require the selection of yeast strains that combine stable fermentation performance with desirable aroma profiles. However, indigenous yeasts from traditional fermentation starters have received little attention or comparative evaluation under standardised Cheongju fermentation conditions.

How was the work done: Strains of *Saccharomyces cerevisiae* were isolated from traditional Nuruk used in Cheongju production and screened using glucose and ethanol tolerance. Two selected strains were evaluated through seed (500 mL) and 5 L fermentations, with analysis of alcohol production, volatile aroma compounds, taste attributes, and enzymatic activity profiles. These were compared with a commercial reference strain of *S. cerevisiae*.

What are the main findings: The yeasts exhibited stable fermentation performance, producing alcohol concentrations of 17-19% (w/v). Cheongju fermented with these strains showed an enrichment of fruity ethyl ester compounds, with no differences in acetate ester formation. Taste analysis using an 'electronic tongue' showed reduced bitterness and a modest increase in umami-related attributes. Further, the strains had higher esterase and esterase-lipase activities than the reference strain.

Why is the work important: These findings highlight the potential of indigenous yeast strains to enhance aroma quality and sensory balance in Cheongju fermentation. The results provide a practical basis for yeast selection in rice wine brewing and support the development of optimised fermentation strategies for Cheongju production.

Keywords

Cheongju; Korean rice wine; isolation; *Saccharomyces cerevisiae*; fermentation; alcohol production; aroma profile.

Introduction

Korean rice wine or 'Cheongju' is a traditional fermented beverage produced by saccharification of steamed rice with Nuruk (grain based fermentation starter containing *Aspergillus* species, lactic acid bacteria and yeasts), followed by alcohol fermentation and filtration (Kim et al. 2017a; Lee 2022). The wine is characterised by its clear appearance and balanced flavour profile. In recent years, Cheongju has attracted attention as a premium traditional alcoholic beverage in both domestic and international markets (Kim et al. 2010; Yang et al. 2013; Kim et al. 2017b).

The quality of Cheongju is largely determined by the alcohol concentration, volatile compounds, and sensory attributes. Esters such as isoamyl acetate, ethyl caproate, and ethyl octanoate contribute fruity notes, whereas phenethyl acetate and phenethyl alcohol are major contributors to floral aromas (Takahashi et al. 2017; Gong et al. 2025). These volatile compounds primarily originate from yeast metabolism and enhance aroma complexity contributing to consumer preference. Their composition and abundance can vary depending on yeast strain used during fermentation (Lee et al. 2007; Saerens et al. 2010; Yoshimoto and Bogaki 2023). *Saccharomyces cerevisiae* is commonly used in the fermentation of Cheongju and other traditional alcoholic beverages (Kim et al. 2010; Lee et al. 2011; Kim et al. 2017b). This yeast produces volatile compounds, including esters and higher alcohols, via amino acid and lipid metabolism and its genetic stability facilitates strain improvement and industrial application (Saerens et al. 2010). However, strain dependent differences in ester production, ethanol tolerance, and fermentation efficiency have been reported, even under identical fermentation conditions (Sahana et al. 2024; Ji et al. 2025). These observations highlight the importance of selecting yeast strains with desirable fermentation and aroma forming characteristics to enhance and differentiate Cheongju quality.

Commercial yeast strains used in the production of alcoholic beverages are selected for fermentation robustness and process consistency. However, reliance on a limited number of commercial strains has been associated with practical constraints. For traditional rice wine production systems these include supply stability, production cost, and in maintaining consistent product quality (Jung et al. 2014; Park et al. 2021). In addition, commercially available strains may exhibit limited adaptability to fermentation environments, resulting in atypical aroma profiles in traditional rice wines. Accordingly, studies have suggested the identification and application of yeast strains with fermentation and sensory characteristics tailored to specific rice wine contexts is necessary (Kim et al. 2017a; Park and Sathiyaraj 2024). Such strain selection may facilitate the development of differentiated premium products through enhanced volatile compound diversity.

Previous studies have focused on isolating and characterising yeast strains from traditional fermentation starters for potential application in rice wine fermentation. Kim et al (2017b) isolated yeasts and moulds from Nuruk and evaluated their fermentation properties, providing insights into the microbial contributions to the quality of rice wine. Additionally, Park et al (2021) reported that the isolation of yeasts exhibiting favourable fermentation traits and enhanced volatile compound profiles during rice wine production (Park et al. 2021). Nonetheless, systematic evaluation and direct comparative analyses under standard fermentation conditions remain limited, hindering a clear assessment of the fermentation potential of such strains in Cheongju production.

In this study, yeast strains with high alcohol and aroma producing potential were isolated from grain based fermentation starters and assessed for their suitability in Cheongju fermentation. The evaluation included glucose and ethanol tolerance, fermentation capacity, and volatile compound production which were compared to a commercial reference strain. This study sought to identify indigenous yeast strains from Nurak with fermentation performance and ethyl ester profile comparable to - or exceeding that - of a commercial reference strain.

Methods and materials

Isolation and identification of yeast strains

Yeasts were isolated from 17 Nuruk samples from traditional markets in the Gunsan region (Jeollabuk-do, Korea). Samples were suspended in sterile

physiological saline, serially diluted, and plated on YPD agar (BD Difco™, Franklin Lakes, NJ, USA), followed by incubation at 30°C for 48 h. Colonies were purified by repeated subculturing. Genomic DNA was extracted using the DNeasy® Blood & Tissue Kit with the manufacturer's yeast protocol (Qiagen, Hilden, Germany). Molecular identification was performed by sequencing the internal transcribed spacer (ITS) region and the D1/D2 domain of the 26S rDNA following PCR amplification using previously described primers (Park and Sathiyaraj 2024). Sequencing was conducted by Macrogen (Seoul, Korea), and species identification was achieved by comparison with reference sequences from public databases. A total of 27 yeast strains approved for food use by the Ministry of Food and Drug Safety of Korea were isolated, and all isolates were identified as *S. cerevisiae*.

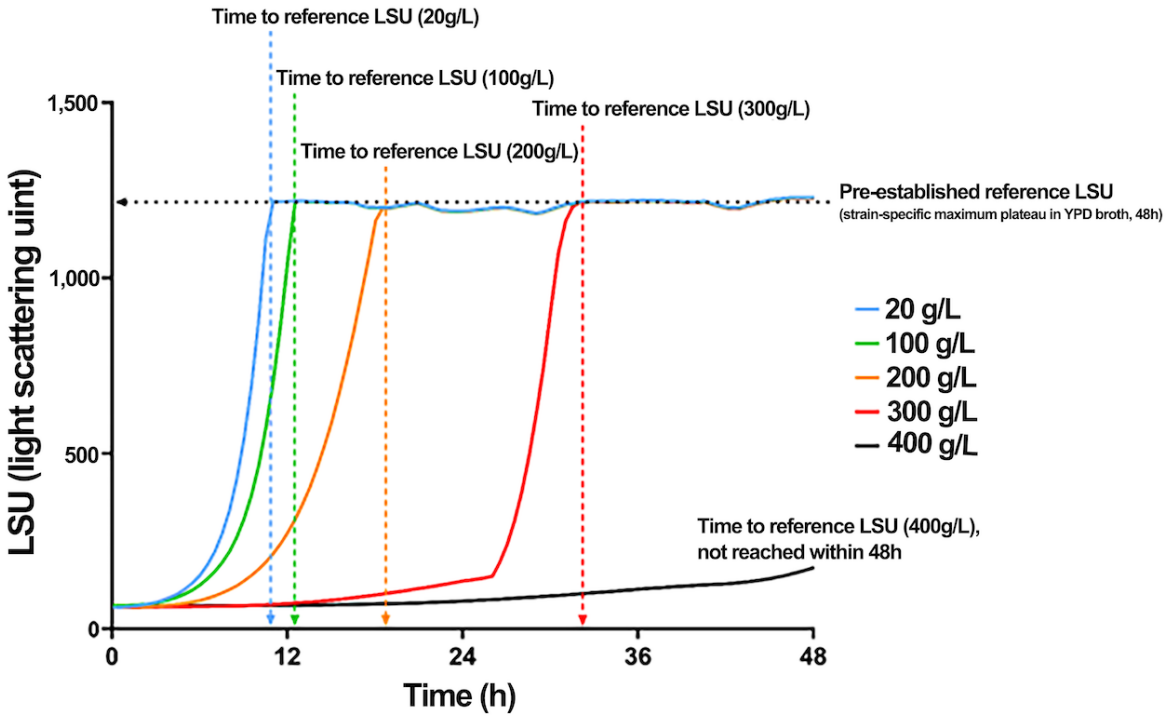
Glucose and ethanol tolerance

Glucose tolerance of the isolated yeasts was evaluated using a BioLector® microbioreactor system (G-BL100-1, M2P-Labs, Aachen, Germany). Yeast cells were cultivated in YPD medium and inoculated into 48 well FlowerPlates®, followed by incubation at 30°C with continuous agitation, and growth was monitored at 620 nm for 48 h. The maximal light scattering unit ('LSU') was defined as a strain specific reference LSU. To assess glucose tolerance, yeast isolates were cultivated in glucose free YPD basal medium supplemented with glucose at final concentrations of 10–40% (w/v), and tolerance was evaluated based on the time required to reach the reference LSU. The workflow used to define the strain-specific reference LSU and determine the time required to reach this value for each glucose concentration is illustrated in Figure 1.

Ethanol tolerance was evaluated as previously described by Kim et al (2010) with minor modifications. Yeast strains were cultivated in YPD broth supplemented with 0–10% (v/v) ethanol at 30°C for 24 h, and cell density was measured at 600 nm. Ethanol tolerance was assessed based on relative changes in OD_{600} compared with the ethanol free control.

Based on performance in both glucose and ethanol tolerance assays, ten strains exhibiting more rapid attainment of the strain specific reference LSU under high glucose conditions and higher relative growth under different ethanol concentrations

Figure 1. Strain specific reference 'light scattering unit '(LSU) and impact of glucose concentration.



LSU from the BioLector system and a proxy for microbial growth; reference LSU = is strain specific and pre-established as the maximal value after 48 h cultivation in YPD broth; time to reference LSU = time point at which the LSU achieves he reference LSU for each glucose concentration.

were selected as candidates for subsequent screening.

Rice extract medium for Cheongju fermentation

The rice extract medium was prepared by mixing rice Nuruk with distilled water, according to the methods of Kim et al (2017b) and Lee et al (2007). Rice Nuruk was purchased from a local market (Gyeongdong Market, Seoul, Korea), mixed with distilled water at a ratio of 1:10 (w/v), and heated at 55°C for 2 h under constant stirring. The extract was filtered through Whatman No. 1 filter paper (Cytiva, Amersham, UK) to remove solids, sterilised at 121°C for 15 min, and cooled to 25-30°C. The resulting filtrate was used as the rice extract medium, and the pH was adjusted to 5.0 prior to fermentation where specified.

Fermentation – 500 mL and 5 L

The Cheongju procedure, including staged mashing and low-temperature fermentation, was conducted as described by Yang et al (2013) and Kim et al (2017a), with modifications to fermentation scale and temperature profile.

For the seed stage fermentation, the selected yeast strains were inoculated at 1×10^8 CFU/mL into 500 mL Erlenmeyer flasks containing rice extract medium and incubated statically at 30°C for 2 days. The cultures were then transferred to a starter fermentation medium prepared from rice extract medium supplemented with sterile lactic acid (0.8%, v/v) and fermented statically at 13°C for 14 days. Yeast strains selected from the glucose and ethanol tolerance screening were evaluated during seed stage fermentation. The resulting seed cultures were used as the starter (jumo) for 5 L scale Cheongju fermentation. Here, the seed culture was transferred to a BIOSTAT B-DCU benchtop fermenter system (Sartorius, Germany) equipped with a cylindrical glass vessel and operated at a 5 L working volume, with approximately 15% headspace to accommodate foaming and CO_2 release. Fermentation was performed under static conditions, with no air or oxygen supplied during fermentation - dissolved oxygen was not actively controlled. Following inoculation, the process proceeded through a three-step mashing process followed by fermentation. During the three-step mashing process, steamed rice, Nuruk, and water were incorporated at a ratio of approximately 1:0.25:2.5 (w/w/v). In the first mashing step, the starter and added ingredients together constituted approximately 25% of the final working volume. The second and third mashing steps subsequently increased the fermentation volume to approximately 50% and 100% of the final working volume.

Following the staged additions, fermentation was continued for an additional 20 days under low temperature conditions, gradually reduced from 13 to 11°C. Accordingly, the total duration of the main scale mashing and fermentation process was 24 days. Samples were collected during and at the end of fermentation for analysis of alcohol concentration, pH, titratable acidity, and reducing sugars. For each yeast strain, fermentations were performed in triplicate. Based on fermentation performance, the strains *S. cerevisiae* SC8247 and SC8326 were selected for further evaluation. A commercial sake yeast, *S. cerevisiae* WLP705/SC705; White Labs, San Diego, CA, USA), was included as a reference strain.

Physicochemical parameters

Physicochemical parameters of Cheongju fermentation were analysed at 500 mL and 5 L scale, including total soluble solids, titratable acidity, pH, and alcohol concentration. Total soluble solids were measured using a digital hand held refractometer (0–32 °Bx; Atago Co., Saitama, Japan). Titratable acidity was determined by titration of culture supernatant with 0.1 N NaOH to pH 8.2 and expressed as acetic acid equivalents (g/L) (Kim and Seo 2021). pH was measured using a calibrated pH meter (SevenCompact, Mettler Toledo, Columbus, OH, USA). For alcohol determination, fermentation samples were clarified by centrifugation (8000xg, 10 min) and filtration, followed by distillation using a VAPODEST automated steam distillation system (Gerhardt GmbH & Co. KG, Königswinter, Germany). The distillate was analysed using a density meter (DMA 5000M, Anton Paar, Graz, Austria) to determine the alcohol concentration (Bruner et al. 2021; Lee et al. 2025).

Analysis of volatile compounds and taste attributes

Samples of Cheongju for volatile and taste analysis were obtained from the main scale (5 L) fermentations. Volatile compounds were analysed by solid-phase microextraction–gas chromatography/mass spectrometry (SPME–GC/MS) (Kang et al. 2016; Wong et al. 2023). Samples (3 g) was placed in a 20 mL vial and equilibrated at 40°C for 30 min. Headspace volatiles were extracted using an 85 µm CAR/PDMS fibre (Supelco, Bellefonte, PA, USA) for 30 min and analysed using an Agilent GC–MS system (7890B/5977A; Agilent Technologies, Santa Clara, CA, USA). Separation was performed on a DB-WAX capillary column with helium at 1 mL/min. Mass spectra were acquired in electron ionisation mode (70 eV, m/z 35–450), and compounds identified using the Wiley12/NIST20 library. For comparative profiling, volatile compound data were expressed as relative proportions (% of total peak area), (Jung et al. 2014; Kim et al. 2017b).

Taste attributes were evaluated using an electronic tongue system (SA402B, Insent, Japan) equipped with six sensors (GL1, CT0, C00, AAE, CA0, and AE1) based on previously reported procedures (Kang et al. 2014; Liu et al. 2020). Prior to analysis, the sensors were conditioned and calibrated using a reference solution (30 mM KCl and 0.3 mM tartaric acid), followed by sequential cleaning and stabilisation steps (Ren et al. 2023; Wang et al. 2026). Sensors were cleaned with the standard cleaning solution and equilibrated in the reference solution before each measurement cycle to ensure signal stability and reproducibility. Samples were analysed under controlled conditions, and each sample was measured four times, with data from the final three measurements used for analysis.

Enzymatic activity analysis using API-ZYM

Extracellular enzymatic activities, including esterase and proteases, were evaluated using the API-ZYM system (bioMérieux, Marcy l'Etoile, France). The two selected yeast strains and the reference strain SC705 were precultured in YPD broth and adjusted to an OD_{600} of approximately 1. Cell suspensions were inoculated to API-ZYM strips (No. 25200) with reagents ZYM A and ZYM B and incubated at 37°C for 4 h. Enzymatic activities were assessed based on colour development using the API-ZYM reference chart and scored on a six-point scale from 0 (no activity) to 5 (maximum activity) (Pennacchia et al. 2008; Liszkowska et al. 2024).

Statistical analysis

All experiments were performed in triplicate. Statistical analyses were conducted using SPSS Statistics 24 (IBM, USA) and GraphPad Prism 10.3.1 (GraphPad Software, San Diego, CA, USA). Data were analysed by one way analysis of variance (ANOVA), followed by Tukey's honest significant difference (HSD) test for multiple comparisons. Differences were considered statistically significant at $P < 0.05$.

Results

Selection of yeast strains based on glucose and ethanol tolerance

The growth of yeast strains were compared under different glucose and ethanol concentrations. For glucose tolerance (Table 1), all strains exhibited comparable growth at 20 g/L (2%, w/v), whereas differences became evident at higher concentrations. Some strains, including SC8255 and SC8326, reached the reference LSU with 100 g/L (10%) glucose, whereas SC8289 exhibited delayed growth. No significant differences were observed at 200 g/L, while at 300 g/L (30%), some isolates failed to reach the reference LSU, and no strains achieved this within 48 h at 400 g/L (40%). Ethanol tolerance decreased with increasing ethanol concentration, with more pronounced differences at higher levels. Several strains, including SC8247 and SC8326, exhibited higher OD_{600} values at 10% ethanol, whereas SC8255 and SC8262 showed reduced tolerance under these conditions (Table 2). To facilitate comparison, glucose and ethanol tolerance profiles are summarised in Table 3, from which 10 strains with the highest tolerance were selected for further analyses.

Assessment – 500 mL fermentation

The fermentation characteristics of 10 yeast strains were evaluated in 500 mL seed scale fermentations. Alcohol production varied among strains, with final concentrations ranging from 12.86 ± 0.51 to 17.54 ± 0.41% (w/v). Strains SC8247 and SC8326 exhibited higher alcohol production, with SC8255 the lowest (Table 4). In contrast, titratable acidity and pH remained stable across strains, with no significant difference. Final °Brix values showed no significant

Table 1. Time to the reference LSU for the *Saccharomyces cerevisiae* isolates at different glucose concentrations

Strain	Glucose 20 g/L		Glucose 100 g/L		Glucose 200 g/L		Glucose 300 g/L		Glucose 400 g/L		Total score
	Time to rfLSU(h)	Score	Time to rfLSU(h)	Score	Time to rfLSU(h)	Score	Time to rfLSU(h)	Score	Time to rfLSU(h)	Score	
SC8245	11.33±0.33	75	11.56±0.48	83	23.13±1.96	74	31.28±1.82	71	N/R	-	**303**
SC8246	11.71±0.70	73	11.75±0.11	81	18.02±1.80	76	30.41±0.62	66	N/R	-	**295**
SC8247	9.56±0.49	89	11.30±0.30	97	19.44±1.25	79	34.76±2.10	76	N/R	-	**340**
SC8250	11.42±0.46	74	11.46±0.25	78	18.06±.140	78	32.51±2.93	69	N/R	-	**299**
SC8253	12.12±1.45	70	13.44±0.30	76	19.01±0.17	74	32.93±0.22	66	N/R	-	**286**
SC8254	9.54±0.08	89	11.52±0.51	95	17.88±0.72	94	27.64±0.20	78	N/R	-	**356**
SC8255	9.47±0.51	90	11.83±0.41	99	20.54±2.43	87	31.33±1.34	68	N/R	-	**344**
SC8256	9.58±0.26	89	13.46±0.64	98	17.01±0.54	94	30.04±0.94	73	N/R	-	**353**
SC8257	11.76±0.82	72	13.58±0.37	79	20.33±1.44	73	34.63±1.73	73	N/R	-	**297**
SC8258	11.39±1.02	75	11.21±0.53	77	17.91±0.59	76	23.62±0.45	71	N/R	-	**298**
SC8261	12.60±0.38	67	13.45±0.68	82	21.13±3.46	74	33.27±3.99	69	N/R	-	**292**
SC8262	9.99±0.47	85	13.88±0.64	83	22.38±2.21	89	36.06±2.88	72	N/R	-	**330**
SC8263	11.56±0.58	73	14.46±0.71	85	21.86±2.01	83	34.08±2.58	68	N/R	-	**309**
SC8264	10.01±0.29	85	14.76±0.73	97	23.09±1.17	95	35.54±2.28	85	N/R	-	**363**
SC8268	12.74±1.78	67	14.22±0.58	80	23.37±2.30	72	32.47±1.60	67	N/R	-	**285**
SC8269	11.03±0.43	77	14.56±0.31	95	22.49±1.80	83	33.32±1.32	75	N/R	-	**330**
SC8272	11.05±0.38	77	13.60±0.43	83	22.95±0.41	100	34.44±0.47	79	N/R	-	**339**
SC8289	10.74±0.21	79	13.24±0.50	82	20.44±2.62	84	34.93±0.88	68	N/R	-	**313**
SC8295	11.88±0.71	71	14.04±0.82	80	23.55±0.37	74	35.36±0.80	66	N/R	-	**292**
SC8299	11.81±0.94	72	13.96±0.46	82	23.09±0.41	80	35.55±0.98	69	N/R	-	**302**
SC8300	12.21±0.24	70	13.72±0.57	77	21.28±1.34	71	34.30±0.63	66	N/R	-	**283**
SC8315	12.37±1.36	69	14.57±0.31	79	23.98±4.06	73	36.05±3.25	67	N/R	-	**288**
SC8316	11.47±0.57	74	14.14±0.83	79	23.25±2.13	75	35.18±2.38	65	N/R	-	**292**
SC8321	11.25±1.01	75	14.25±0.57	81	22.72±0.25	72	36.57±0.62	71	N/R	-	**300**
SC8324	11.74±0.18	72	13.79±0.54	77	23.71±1.95	74	33.08±0.30	65	N/R	-	**288**
SC8326	8.49±0.32	100	14.50±0.44	100	23.11±1.66	95	36.21±0.79	100	N/R	-	**395**
SC8327	12.36±0.87	69	14.62±0.78	77	24.45±1.85	70	33.88±2.55	70	N/R	-	**285**

Glucose tolerance = the time (h) to reach the strain specific reference growth level (rfLSU). Measurements were made in YPD medium with different glucose concentrations at 30°C, with growth monitored at 30 min intervals (BioLector® system). LSU (Figure 1) from the BioLector system; rfLSU = strain specific maximum growth plateau as LSU; Score = relative performance as (mean time to rfLSU of the fastest strain/mean time to rfLSU of each strain) × 100; total score = sum of scores across glucose concentrations (excluding 400 g/L); SC = *S. cerevisiae*. Data are as mean ± SEM; NR = LSU not reached within 48 h. Strains highlighted in grey were selected for further evaluation.

variation, with SC8255 (highest) and SC8326 (lowest). Correlation analysis between alcohol concentration and ΔBrix (before-after fermentation) showed a marginal positive relationship (R^2 = 0.1817).

Assessment – 5 L fermentation

Five strains from the 500 mL evaluation were compared in 5 L fermenters. Alcohol production differed, ranging from 17.34 ± 0.24 (SC8269) to 19.04 ± 0.12% (w/v) (SC8326) (Table 5) reflecting fermentation performance (Figure 2). The control sake yeast (*S. cerevisiae* WLP705/SC705) produced 18.83 ± 0.39 (%, w/v). In contrast, titratable acidity and pH remained relatively consistent across the strains. Final °Brix values also showed no significant variation, with minor differences between strains. A positive correlation was observed between alcohol concentration and ΔBrix (R^2 = 0.7608). Accordingly, SC8326 and SC8247 (18.60 ± 0.22%, w/v) were selected for further analyses given their superior alcohol production.

Volatile and sensory characteristics of fermented Cheongju

The volatile profiles of fermented Cheongju from the commercial reference yeast SC705 together with SC8247 and SC8326, are presented in Table 6. Total volatile compound levels, expressed as GC–MS peak area (×10^3), were significantly higher in SC8247 and SC8326 than the reference strain (Supplementary Infomation Table 1). Individual volatile compounds were normalised to relative proportions (%) of the total volatile content to enable comparison of strain dependent aroma composition. Consistent with these results, significant differences were observed across sensory categories (Figure 3). 'Fruity' and 'waxy' compounds were most abundant in SC8326 and higher in SC8247 than in the reference strain, which exhibited higher proportions of 'ethereal' and 'sweet' compounds. 'Floral', 'honey' and 'fermented' attributes were higher in the reference strain, and lowest in SC8326.

Table 2. Ethanol tolerance of the *Saccharomyces cerevisiae* isolates

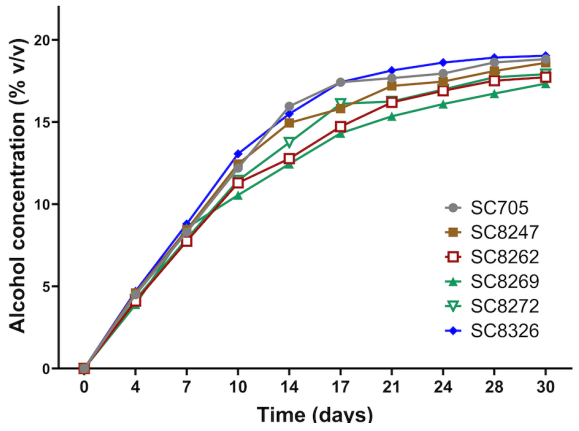
Strain	Ethanol 0% v/v		Ethanol 2% v/v		Ethanol 4% v/v		Ethanol 6% v/v		Ethanol 8% v/v		Ethanol 10% v/v		Total score
	OD_{600}	Score	OD_{600}	Score	OD_{600}	Score	OD_{600}	Score	OD_{600}	Score	OD_{600}	Score	
SC8245	1.77±0.07	87	1.45±0.09	74	1.37±0.07	75	0.95±0.04	68	0.54±0.01	45	0.07±0.00	16	**366**
SC8246	1.73±0.07	85	1.57±0.02	80	1.23±0.01	68	1.00±0.01	71	0.38±0.00	31	0.08±0.01	18	**353**
SC8247	1.78±0.09	87	1.66±0.13	85	1.53±0.12	84	1.37±0.05	97	1.00±0.09	83	0.45±0.04	100	**536**
SC8250	1.69±0.07	83	1.43±0.08	73	1.37±0.08	76	1.05±0.07	75	0.39±0.01	32	0.06±0.00	14	**353**
SC8253	1.82±0.07	90	1.64±0.22	84	1.44±0.11	80	0.85±0.06	61	0.40±0.00	33	0.03±0.01	7	**354**
SC8254	1.96±0.04	96	1.95±0.11	100	1.81±0.11	100	1.27±0.10	90	0.77±0.08	64	0.43±0.06	94	**544**
SC8255	1.83±0.07	90	1.66±0.06	85	1.38±0.06	76	0.93±0.05	66	0.45±0.09	37	0.09±0.02	19	**373**
SC8256	2.03±0.04	100	1.96±0.10	100	1.52±0.02	84	1.24±0.04	88	0.77±0.01	63	0.37±0.05	82	**518**
SC8257	1.76±0.07	86	1.67±0.09	85	1.43±0.07	79	0.89±0.05	63	0.44±0.02	36	0.06±0.00	13	**362**
SC8258	1.73±0.04	85	1.58±0.07	81	1.34±0.05	74	0.89±0.05	63	0.54±0.02	45	0.08±0.00	18	**366**
SC8261	1.84±0.04	90	1.60±0.08	82	1.39±0.07	76	0.93±0.06	66	0.44±0.02	36	0.05±0.00	12	**363**
SC8262	1.99±0.12	98	1.52±0.04	78	1.45±0.00	80	1.21±0.01	86	0.67±0.08	56	0.12±0.01	26	**423**
SC8263	1.64±0.08	81	1.57±0.18	80	1.32±0.08	73	0.71±0.10	51	0.33±0.02	27	0.23±0.02	50	**362**
SC8264	1.71±0.03	84	1.51±0.05	79	1.31±0.00	72	1.04±0.07	74	0.81±0.07	67	0.19±0.01	41	**417**
SC8268	1.76±0.04	87	1.54±0.07	77	1.58±0.07	87	0.82±0.11	58	0.55±0.03	45	0.05±0.03	11	**366**
SC8269	1.89±0.08	93	1.76±0.05	90	1.35±0.01	75	0.90±0.02	64	0.32±0.00	27	0.10±0.01	21	**369**
SC8272	1.79±0.05	88	1.65±0.08	84	1.53±0.07	85	1.41±0.08	100	1.21±0.05	100	0.43±0.05	95	**552**
SC8289	1.72 ±0.01	85	1.85±0.17	94	1.38±0.04	76	1.11±0.09	79	0.94±0.14	78	0.27±0.08	60	**472**
SC8295	1.82±0.03	90	1.72±0.08	88	1.27±0.06	70	0.77±0.07	55	0.43±0.02	35	0.13±0.03	28	**366**
SC8299	1.75±0.06	86	1.77±0.17	91	1.12±0.04	62	0.81±0.07	58	0.44±0.02	36	0.14±0.02	31	**364**
SC8300	1.70±0.08	84	1.50±0.07	77	1.38±0.05	76	0.81±0.01	58	0.37±0.02	30	0.17±0.02	37	**361**
SC8315	1.76±0.06	86	1.48±0.06	75	1.34±0.08	74	0.88±0.07	62	0.23±0.01	19	0.23±0.01	50	**367**
SC8316	1.75±0.02	86	1.55±0.02	79	1.21±0.01	67	0.75±0.01	54	0.39±0.03	32	0.19±0.00	42	**360**
SC8321	1.80±0.02	89	1.47±0.02	75	1.12±0.08	73	0.91±0.13	65	0.30±0.03	25	0.10±0.01	22	**349**
SC8324	1.73±0.04	85	1.44±0.07	74	1.48±0.07	82	0.91±0.07	64	0.34±0.03	28	0.14±0.02	30	**363**
SC8326	1.93±0.05	95	1.91±0.07	98	1.71±0.06	94	1.28±0.9	91	0.97±0.05	80	0.44±0.06	97	**555**
SC8327	1.71±0.07	84	1.49±0.09	76	1.30±0.11	72	0.81±0.08	57	0.30±0.03	25	0.20±0.02	43	**357**

OD_{600} = optical density measured at 600 nm after 24 h cultivation; Score = relative growth score (mean OD600 of each strain/highest mean OD600 at each ethanol concentration) × 100; total score = sum of scores calculated across ethanol concentrations (0–10%); data are presented as mean ± SEM. Strains highlighted in grey were selected for further evaluation.

Relative compositional patterns (% of total volatiles) are presented in Figure 3A. The reference strain was characterised by higher proportions of phenethyl alcohol, isoamyl alcohol, and benzaldehyde, whereas the selected isolates showed increased proportions of ethyl caproate and ethyl octanoate, with the highest level observed in SC8326. Comparative analysis of representative aroma compounds (Figure 3B) showed that ethyl caproate and ethyl alcohol were higher in the isolates, whereas isoamyl alcohol, and benzaldehyde were higher in the reference strain.

Electronic tongue analysis (Figure 3C) showed that Cheongju from the isolates exhibited lower sourness and bitterness than with the reference strain, while other taste attributes showed no significant differences.

Figure 2. Ethanol concentration during 5 L Cheongju fermentation by isolates of *Saccharomyces cerevisiae*



The control - SC705 - is a commercial sake yeast.

Enzymatic activity assessment and profile

Extracellular enzymatic activities of the reference strain SC705 and the two selected isolates SC8247 and SC8326 were analysed using API-ZYM (Table 7). Overall, the three strains exhibited similar enzymatic profiles, with strong activity observed for acid phosphatase and naphthol-AS-BI-phosphohydrolase. However, the selected isolates showed higher activities of esterase (C4) and esterase lipase (C8) compared with the reference strain. Several hydrolase activities, including α-glucosidase, β-glucosidase, and valine arylamidase were detected in all strains, whereas alkaline phosphatase activity remained low.

Table 3. Glucose and ethanol tolerance of *Saccharomyces cerevisiae* isolates

Glucose tolerance based on time to strain-specific reference LSU at different glucose concentrations

Glucose	g/L	SC8247	SC8254	SC8255	SC8256	SC8262	SC8264	SC8269	SC8272	SC8289	SC8326
Time to rfLSU (h)	20	9.56 ± 0.49^{ab}	9.54 ± 0.08^{ab}	9.47 ± 0.51^{ab}	9.58 ± 0.26^{ab}	9.99 ± 0.47^{ab}	10.01 ± 0.29^{ab}	11.03 ± 0.43^{b}	11.05 ± 0.38^{b}	10.74 ± 0.21^{b}	8.49 ± 0.32^{a}
	100	11.30 ± 0.30^{ab}	11.52 ± 0.51^{ab}	11.83 ± 0.41^{a}	13.46 ± 0.64^{ab}	13.88 ± 0.64^{ab}	14.76 ± 0.73^{ab}	14.56 ± 0.31^{ab}	13.60 ± 0.43^{ab}	13.24 ± 0.50^{b}	14.50 ± 0.44^{a}
	200	19.44 ± 1.25^{a}	17.88 ± 0.72^{a}	20.54 ± 2.43^{a}	17.01 ± 0.54^{a}	22.38 ± 2.21^{a}	23.09 ± 1.17^{a}	22.49 ± 1.80^{a}	22.95 ± 0.41^{a}	20.44 ± 2.62^{a}	23.11 ± 1.66^{a}
	300	34.76 ± 2.10^{b}	27.64 ± 0.20^{ab}	31.33 ± 1.34^{b}	30.04 ± 0.94^{b}	36.06 ± 2.88^{b}	35.54 ± 2.28^{ab}	33.32 ± 1.32^{b}	34.44 ± 0.47^{ab}	34.93 ± 0.88^{b}	36.21 ± 0.79^{a}
	400	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R

Ethanol tolerance based on growth (cell density) under different ethanol concentrations (OD_{600} at 24 h)

Ethanol	(%, w/v)	SC8247	SC8254	SC8255	SC8256	SC8262	SC8264	SC8269	SC8272	SC8289	SC8326
Cell density (OD600)	0	1.78 ± 0.09^{a}	1.96 ± 0.04^{a}	1.83 ± 0.07^{a}	2.03 ± 0.04^{a}	1.99 ± 0.12^{a}	1.71 ± 0.03^{a}	1.89 ± 0.08^{a}	1.79 ± 0.05^{a}	1.72 ± 0.01^{a}	1.93 ± 0.05^{a}
	2	1.66 ± 0.13^{a}	1.95 ± 0.11^{a}	1.66 ± 0.06^{a}	1.96 ± 0.10^{a}	1.52 ± 0.04^{a}	1.54 ± 0.05^{a}	1.76 ± 0.05^{a}	1.65 ± 0.08^{a}	1.85 ± 0.17^{a}	1.91 ± 0.07^{a}
	4	1.53 ± 0.12^{ab}	1.81 ± 0.11^{b}	1.38 ± 0.06^{ab}	1.52 ± 0.02^{ab}	1.45 ± 0.00^{ab}	1.31 ± 0.00^{a}	1.35 ± 0.01^{a}	1.53 ± 0.07^{ab}	1.38 ± 0.04^{a}	1.71 ± 0.06^{ab}
	6	1.37 ± 0.05^{bc}	1.27 ± 0.10^{bc}	0.93 ± 0.03^{ab}	1.24 ± 0.04^{ab}	1.21 ± 0.01^{ab}	1.04 ± 0.07^{ab}	0.90 ± 0.02^{a}	1.41 ± 0.08^{c}	1.11 ± 0.09^{ab}	1.28 ± 0.09^{bc}
	8	1.00 ± 0.09^{c}	0.77 ± 0.08^{bc}	0.45 ± 0.09^{ab}	0.77 ± 0.01^{bc}	0.67 ± 0.08^{ab}	0.81 ± 0.07^{bc}	0.32 ± 0.01^{a}	1.21 ± 0.05^{c}	0.94 ± 0.14^{bc}	0.97 ± 0.05^{bc}
	10	0.45 ± 0.04^{b}	0.43 ± 0.06^{b}	0.11 ± 0.02^{a}	0.37 ± 0.05^{b}	0.12 ± 0.01^{a}	0.19 ± 0.01^{ab}	0.10 ± 0.01^{a}	0.43 ± 0.05^{b}	0.27 ± 0.08^{ab}	0.44 ± 0.06^{b}

Table 4. Fermentation characteristics of *Saccharomyces cerevisiae* isolates in 500 mL fermentations

Reference yeast SC705. Data as mean ± standard error; abcd indicates significant differences among groups in the same row (P < 0.05).

		SC705	SC8247	SC8254	SC8255	SC8256	SC8262	SC8264	SC8269	SC8272	SC8289	SC8326
Alcohol (%, w/v)		17.11 ± 0.42^{c}	17.26 ± 0.39^{c}	14.78 ± 0.50^{ab}	12.86 ± 0.51^{a}	14.28 ± 0.77^{ab}	16.74 ± 0.33^{bc}	13.79 ± 0.58^{ab}	16.66 ± 0.96^{bc}	16.68 ± 1.08^{bc}	14.39 ± 0.28^{ab}	17.54 ± 0.41^{c}
Titratable acidity (g/L, acetic acid)		0.51 ± 0.02^{a}	0.49 ± 0.01^{a}	0.54 ± 0.03^{a}	0.54 ± 0.02^{a}	0.52 ± 0.03^{a}	0.51 ± 0.02^{a}	0.51 ± 0.04^{a}	0.44 ± 0.05^{a}	0.49 ± 0.03^{a}	0.54 ± 0.04^{a}	0.49 ± 0.01^{a}
pH		3.81 ± 0.02^{a}	3.78 ± 0.03^{a}	3.68 ± 0.06^{a}	3.69 ± 0.02^{a}	3.73 ± 0.04^{a}	3.79 ± 0.03^{a}	3.73 ± 0.05^{a}	3.82 ± 0.02^{a}	3.78 ± 0.02^{a}	3.59 ± 0.07^{a}	3.80 ± 0.03^{a}
Brix	Day 0	44.8 ± 0.27										
	Day 14	17.27 ± 0.39	17.36 ± 0.51^{a}	19.76 ± 0.41^{a}	21.10 ± 0.08^{a}	19.31 ± 1.29^{a}	17.90 ± 0.96^{a}	20.46 ± 1.57^{a}	17.46 ± 0.09^{a}	16.99 ± 0.53^{a}	19.07 ± 0.83^{a}	16.82 ± 0.45^{a}
	ΔBrix	27.53	27.44	25.04	23.7	25.49	26.9	24.34	27.34	27.81	25.73	27.98

Table 5. Fermentation characteristics of *Saccharomyces cerevisiae* isolates in 5L fermentations

Reference yeast SC705. Data as mean ± standard error; abcd indicates significant differences among groups in the same row (P < 0.05).

		SC705	SC8247	SC8262	SC8269	SC8272	SC8326
Alcohol (%, w/v)		18.83±0.39^{b}	18.60±0.22^{b}	17.73±0.37^{ab}	17.34±0.24^{a}	17.91±0.17^{ab}	19.04±0.12^{b}
Titratable acidity (g/L, acetic acid)		0.23±0.02^{ba}	0.22±0.01^{a}	0.24±0.02^{a}	0.19±0.01^{a}	0.20±0.01^{a}	0.22±0.02^{a}
pH		4.34±0.13^{a}	4.24±0.04^{a}	4.15±0.04^{a}	4.39±0.13^{a}	4.23±0.10^{a}	4.27±0.16^{a}
Brix	Day 0	44.8±0.27					
	Day 14	13.12±0.27^{a}	13.02±0.13^{a}	14.42±0.30^{a}	14.84±0.24^{ab}	14.60±0.67^{a}	13.53±0.45^{a}
	ΔBrix	31.68	31.78	30.38	29.96	30.2	31.27

Discussion

The performance of yeast in fermentation systems is determined by strain-specific physiological responses to dynamically changing environmental conditions. These responses shape fermentation efficiency and metabolic profiles that define product quality. Accordingly, a systematic evaluation of yeast strains integrating stress tolerance with fermentation performance and quality was required for strain selection and process design focussed on improving the quality of Cheongju.

Cheongju fermentation involves a sequential shift in physicochemical conditions, with high sugar availability during early saccharification followed by progressive ethanol accumulation. These stage-specific stresses impose distinct physiological constraints on yeast growth and metabolism, enabling clearer differentiation of strain dependent fermentation performance. Although increasing glucose concentrations impose osmotic stress, most yeast strains reached comparable

maximum growth levels given sufficient cultivation time. Therefore, glucose tolerance was evaluated using a time-based metric, as monitoring how rapidly strains grow at different glucose concentrations provides better discrimination than measuring biomass at the end of cultivation. In contrast, ethanol acts as a direct inhibitory stressor that limits yeast growth in a concentration-dependent manner, and ethanol tolerance was therefore assessed based on endpoint cell density under defined ethanol concentrations. In alcohol fermentation, high initial sugar concentrations can enhance ethanol yield. Excessive sugar levels impose osmotic stress, which can delay fermentation, increase by-product accumulation, and leave residual sugars that compromise product quality (Douradinho et al. 2023). As fermentation progresses, increasing ethanol concentration compromises yeast viability by altering membrane fluidity and destabilising protein structures, resulting in premature death in strains with poor ethanol tolerance (Ji et al. 2025). Conversely, strains capable of maintaining metabolic activity under high ethanol stress continue to metabolise residual sugars during late fermentation, ensuring consistent fermentation kinetics and the accumulation of aroma compounds that enhance product complexity and sensory quality (Sahana et al. 2024). Therefore, tolerance to both elevated sugar and ethanol concentrations are a key determinant of fermentation and quality in Cheongju production. Kim et al (2010) evaluated yeast strains for Yakju (a rice wine like Cheongju) fermentation and reported that only a few isolates that exhibited high viability with 20% (w/v) ethanol or 40% (w/v) glucose. Here, superior strains (Y183-2, YA-6) maintained metabolic activity at ethanol concentrations of 16–18% (w/v), but viability declined above 20% (w/v). Lee et al (2011) reported that several *Wickerhamomyces anomalus* and *Pichia anomala* strains from grapes exhibited superior growth compared with a commercial *S. cerevisiae* strain in media containing 30% (w/v) glucose. However, the commercial strain performed better at glucose concentrations of >40% (w/v). Consistent with these findings, the results reported here revealed pronounced strain-dependent differences at 300 g/L (~30%, w/v) glucose, while growth was not demonstrated by any isolate at 40% (w/v).

The present study employed a combined assessment of glucose and ethanol tolerance to identify candidate strains. Such an approach aligns with the physiological constraints inherent to Cheongju fermentation and provides a framework for selecting yeasts capable of stable fermentation and consistent aroma formation. Previous studies reported strain and condition dependent variability in Cheongju and Yakju fermentations, particularly with alcohol production and sugar utilisation. Yang et al (2013) reported that for fermentations (500 mL) using *S. cerevisiae* K1001, the alcohol levels ranged from 10.2 to 14.3% (w/v) depending on the *Aspergillus oryzae* strain used. Similarly, Kim and Seo (2021) observed alcohol concentrations of 9.9–12.6% (w/v) in Cheongju fermentations (500 mL) using *S. cerevisiae* INRA7013, with °Brix values varying according to the type of Nuruk, while pH and total acidity remained within narrow ranges. Consistent with these reports, this work demonstrated strain dependent differences in alcohol production and sugar consumption in small (500 mL) and large scale (5 L) fermentations, whereas acid related parameters remained stable across strains. Alcohol concentrations in this study were generally higher than previously reported, which is likely to reflect differences in fermentation capacity of the strains. At the larger scale, alcohol levels of 17.34-19.04% (w/v) exceeded those (14.90-16.07%, w/v) of Chae et al (2021) in 3.8 L Yakju fermentations while comparable ranges of pH and titratable acidity were found between studies. Notably, the consistency of alcohol production and acidity profiles between the fermentations (500 mL and 5 L) underscores the importance of strain stability for scale-up. Indeed, these results are consistent with previous work demonstrating stable fermentation performance at different scales using *S. cerevisiae* (Kuribayashi et al. 2022), However, as the present study was at a volume of 5 L, validation at pilot and industrial scales is required. At larger production scales, strain stability alone may not be sufficient to ensure fermentation performance, with optimisation required of process parameters, including oxygen transfer, mixing efficiency, heat dissipation, foaming control, and CO_2 management.

Aroma compounds that influence the quality and consumer acceptance of Cheongju include ethyl caproate, ethyl octanoate, isoamyl acetate, and phenethyl alcohol (Kang et al. 2016; Wong et al.

2023). These volatiles are produced by yeast during fermentation from fatty acid and amino acid metabolism. Among them, ethyl caproate and ethyl octanoate are recognised as contributors to fruity and floral notes and are associated with the characteristic ginjo aroma in sake style beverages (Kang et al. 2016; Takahashi et al. 2017; Kuribayashi et al. 2022). Isoamyl acetate, formed via esterification of isoamyl alcohol derived from leucine catabolism, also contributes to ginjo aroma, whereas accumulation of the precursor, isoamyl alcohol, negatively affects sensory quality through harsh alcoholic notes (Hu et al. 2018; Yoshimoto and Bogaki 2023). Accordingly, the balance between ester and higher alcohol levels is a critical determinant of aroma quality. Phenethyl acetate, with floral and honey-like aromas, has similarly been identified as an important volatile in traditional rice wines, including Cheongju and sake (Kang et al. 2016; Wong et al. 2023).

In this study, strain SC8326 and, to a lesser extent SC8247, exhibited an enrichment of fruity ethyl esters, particularly ethyl caproate and ethyl octanoate, whereas the commercial control yeast SC705 had higher proportions of phenethyl alcohol and higher alcohols. Previous studies have highlighted ethyl esters, together with isoamyl acetate, as major contributors to desirable fruity aroma notes. Aroma perception is determined not only by the relative composition of volatiles but also by their overall abundance, with compositional balance influencing qualitative attributes and total volatile levels affecting sensory intensity (Czerny et

Table 6. Volatile compounds in fermented Cheongju produced with three *Saccharomyces cerevisiae* strains
Reference yeast SC705. Data as mean ± standard error; abcd means significant differences among groups in the same row ($P < 0.05$).

Sensory characteristic	CAS no.	Compounds	Relative proportion (%, of total GC-MS peak area)		
			SC705	SC8247	SC8326
Fruity & Waxy	109-60-4	propyl acetate	0.24±0.01^{a}	-	-
	110-19-0	isobutyl acetate	0.19±0.01^{a}	-	-
	105-54-4	ethyl butyrate	0.14±0.00^{b}	0.08±0.00^{a}	0.16±0.01^{b}
	123-92-2	isoamyl acetate	3.82±0.21^{a}	4.66±0.17^{b}	3.09±0.24^{a}
	123-66-0	ethyl caproate	1.08±0.03^{a}	7.19±0.21^{b}	13.03±0.72^{c}
	626-77-7	propyl hexanoate	-	-	0.03±0.00^{a}
	106-30-9	ethyl heptanoate	-	-	0.04±0.00^{a}
	106-32-1	ethyl octanoate	1.69±0.14^{a}	4.21±0.25^{b}	7.25±0.23^{c}
	2198-61-0	isoamyl hexanoate	-	0.02±0.00^{a}	-
	110-38-3	ethyl decanoate	0.19±0.02^{a}	0.30±0.01^{b}	0.23±0.01^{a}
	2035-99-6	isoamyl octanoate	-	-	-
	123-25-1	diethyl succinate	0.06±0.01^{a}	-	-
	142-62-1	hexanoic acid	0.07±0.00^{a}	0.37±0.02^{c}	0.20±0.01^{b}
	124-06-1	ethyl myristate	0.05±0.01^{a}	-	-
	124-07-2	octanoic acid	0.07±0.00^{a}	0.07±0.00^{a}	0.06±0.01^{a}
	628-97-7	ethyl palmitate	0.12±0.01^{a}	-	-
	Subtotal		7.70±0.10^{a}	16.90±0.09^{b}	24.10±0.73^{c}
Floral & Honey	103-45-7	phenethyl acetate	2.09±0.17^{b}	1.26±0.01^{a}	1.14±0.02^{a}
	60-12-8	phenethyl alcohol	7.64±0.58^{c}	5.89±0.16^{b}	3.83±0.14^{a}
	Subtotal		9.72±0.73^{c}	7.15±0.16^{b}	4.97±0.14^{a}
Fermented	71-36-3	butyl alcohol	-	0.05±0.01^{b}	0.04±0.00^{a}
	123-51-3	isoamyl alcohol	5.91±0.07^{c}	4.35±0.07^{b}	2.47±0.12^{a}
	Subtotal		5.91±0.07^{c}	4.40±0.07^{b}	2.50±0.12^{a}
Ethereal	141-78-6	ethyl acetate	2.83±0.13^{b}	1.61±0.08^{a}	1.71±0.05^{a}
	78-83-1	isobutyl alcohol	1.42±0.13^{b}	0.10±0.00^{a}	0.26±0.02^{a}
	137-32-6	2-methyl-1-butanol	1.05±0.07^{b}	0.36±0.01^{a}	0.36±0.01^{a}
	Subtotal		5.30±0.14^{b}	2.07±0.09^{a}	2.34±0.07^{a}
Sweet	100-52-7	benzaldehyde	4.10±0.36^{b}	0.47±0.00^{a}	0.54±0.02^{a}
Others	64-19-7	acetic acid	-	-	-
	107-92-6	butyric acid	-	-	-
	64-17-5	ethanol	66.35±0.02^{a}	68.56±0.37^{a}	65.08±0.37^{a}
	71-23-8	propyl alcohol	0.74±0.02^{c}	0.31±0.01^{a}	0.37±0.01^{b}
	100-42-5	styrene	0.08±0.01^{a}	0.05±0.00^{a}	0.06±318.08^{a}
	111-70-6	1-heptanol	-	-	-
	104-76-7	2-ethyl-1-hexanol	0.09±0.00^{b}	0.08±0.01^{a}	0.09±0.00^{b}
	96-48-0	gamma-butyrolactone	-	-	-
	Subtotal		67.26±1.04^{a}	69.01±0.37^{a}	65.54±0.38^{a}
Total		Relative proportion (%)	100	100	100
		GC–MS peak area (×10^{3})	4,921,561±121,324^{a}	5,971,334±267,235^{b}	6,541,587±211,352^{b}

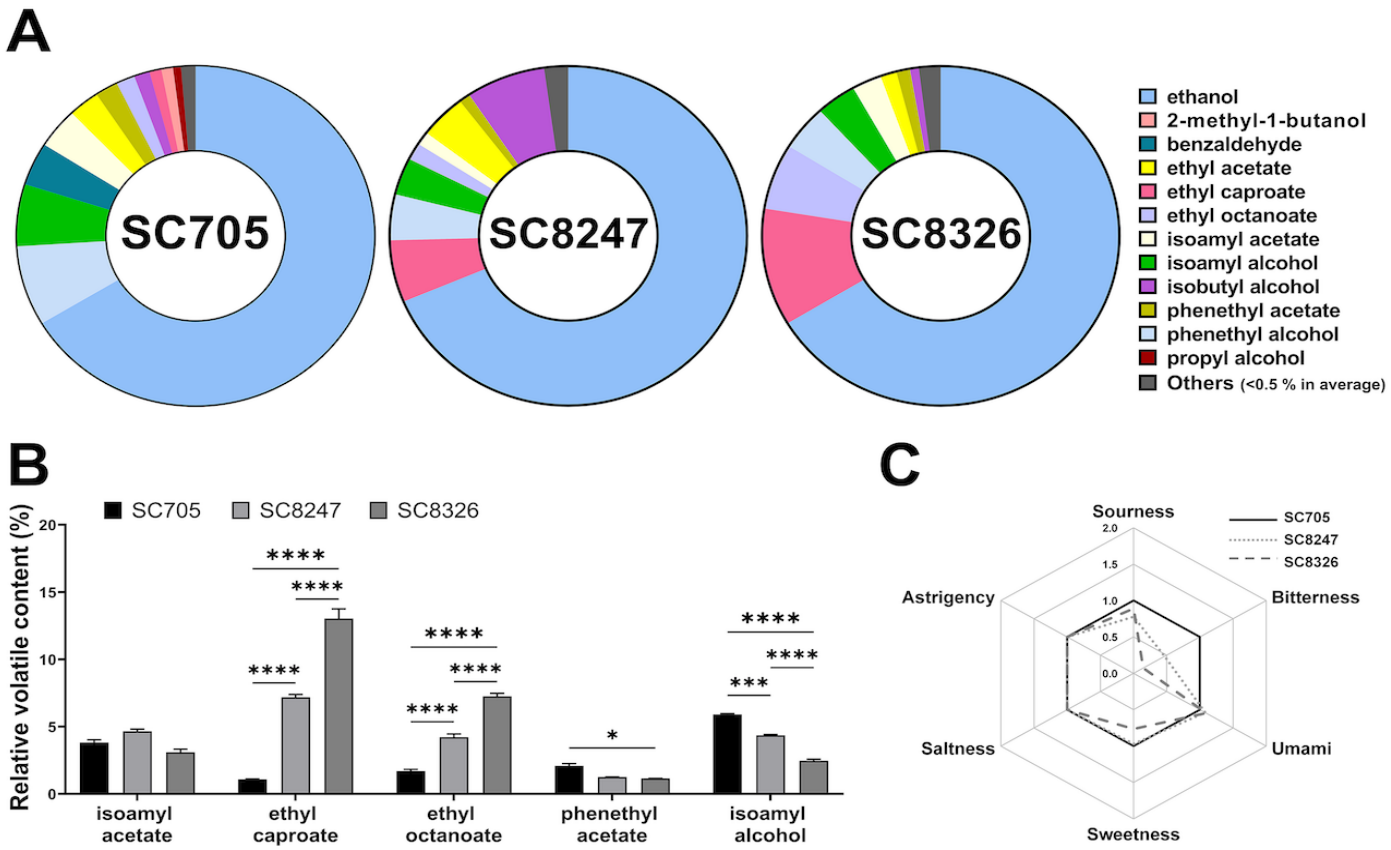
al. 2008; Rocha et al 2022). In this context, the ester enriched composition combined with higher total volatile production observed in SC8326 are likely to contribute to a greater perception of fruity attributes. E-tongue analysis revealed a reduction in bitterness, which may be associated with reduced accumulation of higher alcohols or hydrophobic peptides during fermentation. Suzuki et al (2012) reported a strong correlation between bitterness intensity and fluorescence signals indicative of hydrophobic peptides in sake, supporting the view that reduced bitterness is favourable to consumer acceptance. The intensity of umami was also increased, possibly reflecting the enhanced release of free amino acids and peptides, which are known contributors to the perception of umami and kokumi (Vinther Schmidt et al. 2021; Yang et al. 2022). Although a slight decrease in sweetness was observed, previous studies have identified fruity and floral attributes as the primary drivers of preference in sake-like beverages. Accordingly, the elevated production of fruity esters with strains SC8247 and SC8326 may compensate for minor changes in sweetness.

Esterases are known to influence yeast aroma metabolism by regulating the hydrolysis and turnover of fatty acid derived intermediates, thereby affecting the intracellular availability of medium chain acyl substrates. Although the direct biosynthesis of medium chain fatty acid ethyl esters is catalysed by acyl-CoA:ethanol O-acyltransferases - particularly *Eeb1* with a secondary contribution from *Eht1* - substrate availability remains a key determinant of ester production efficiency in *S. cerevisiae* (Verstrepen et al. 2003; Saerens et al. 2010; Kruis et al. 2018). In support of this, previous studies have reported that strains exhibiting higher esterase (C4) and esterase lipase (C8) activities in API-ZYM assays tend to produce higher levels of fruity ethyl esters, including ethyl caproate and ethyl octanoate (Liszkowska et al. 2024). In the present study, the selected yeasts exhibited elevated esterase activities compared with the reference strain, and this was associated with significantly higher proportions of ethyl caproate and ethyl octanoate in the volatile profiles of Cheongju. Although API-ZYM does not directly measure ester-synthesising enzymes, the observation suggests that enhanced extracellular esterase activity may reflect strain specific conditions that promote the turnover and availability of medium chain fatty acid intermediates, facilitating ethyl ester formation during fermentation. In contrast, SC705 exhibited higher valine arylamidase activity, and its volatile profile was characterised by increased levels of branched chain and higher alcohols, such as isoamyl alcohol and 2-methyl-1-butanol. As valine arylamidase is linked to branched chain amino acid catabolism, which provides

Table 7. Enzymic profiles of strains of *Saccharomyces cerevisiae* using API-ZYM assay
Reference yeast SC705. Level of enzyme activity, from none (0) to maximum (5).

Enzyme	Substrate	SC705	SC8247	SC8326
Alkaline phosphatase	2-naphthyl phosphate	1	1	1
Esterase (C4)	2-naphthyl butyrate	2	3	3
Esterase lipase (C8)	2-naphthyl caprylate	2	3	4
Lipase (C14)	2-naphthyl myristate	0	0	0
Leucine arylamidase	L-leucyl-2-naphthylamide	3	3	3
Valine arylamidase	l-valyl-2-naphthylamide	3	2	2
Cystine arylamidase	l-cystyl-2-naphthylamide	3	3	3
Trypsin	N-benzoyl-dl-arginine-2-naphthylamide	0	0	0
α-chymotrypsin	N-glutaryl-phenylalanine-2-naphthylamide	0	0	0
Acid phosphatase	2-naphthyl phosphate	5	5	5
Naphthol-AS-BI-phosphohydrolase	Naphthol-AS-BI-phosphate	5	5	5
α-galactosidase	6-Br-2-naphthyl-αd-galactopyranoside	0	0	0
β-galactosidase	2-naphthyl-βD-galactopyranoside	0	0	0
β-glucuronidase	Naphthol-AS-BI-βd-glucuronide	0	0	0
α-glucosidase	2-naphthyl-αD-glucopyranoside	3	3	3
β-glucosidase	6-Br-2-naphthyl-βd-glucopyranoside	2	2	2
N-acetyl-β-glucosidaminidase	1-naphthyl-N-acetyl-βd-glucosamide	0	0	0
α-mannosidase	6-Br-2-naphthyl-αd-mannopyranoside	0	0	0
α-fucosidase	2-naphthyl-αl-fucopyranoside	0	0	0

Figure 3. Volatile profiles and electronic tongue analysis of Cheongju fermented with *Saccharomyces cerevisiae*.



(A) Distribution of volatile compounds in Cheongju from 5 L fermentations analysed by SPME–GC/MS, as relative proportions (%) calculated from individual GC–MS peak areas normalised to the total volatile peak area. **(B)** Comparative analysis of representative aroma compounds among strains based on relative GC–MS peak area (%). Data as mean ± SEM (n = 3). Statistical significance using one-way ANOVA followed by Tukey's post hoc test - not significant (ns), *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. **(C)** Electronic tongue analysis of taste attributes.Reference yeast SC705.

precursors for higher alcohol synthesis through the Ehrlich pathway (Isogai et al. 2023), these results likely reflect strain-dependent differences in amino acid metabolism rather than direct effects captured by the API-ZYM assay. Despite this, higher esterase activities observed in SC8247 and SC8326 did not translate into significant differences in isoamyl acetate levels, highlighting limitations of API-ZYM interpretation. As the method provides semi-quantitative measurements under non-fermentative conditions, the results are indicators of potential enzymatic capacity rather than direct predictors of aroma compound formation during fermentation. Ester biosynthesis in *S. cerevisiae* is controlled by a tightly regulated network involving acyltransferase activity, substrate availability, and intracellular redox balance (Verstrepen et al. 2003). Accordingly, the data does not allow linkage between API-ZYM enzyme profiles and volatile ester production. Indeed, clarification of strain dependent aroma formation will require quantitative characterisation of key acyltransferases, transcriptional analysis of aroma related metabolic pathways, together with targeted metabolomic profiling during Cheongju fermentation. In addition, as this work was conducted at laboratory scale with API-ZYM and electronic tongue analyses performed under non-fermentative or instrumental conditions, there should be caution in extrapolating these findings to human sensory perception and industrial scale Cheongju production.

Conclusions

The *S. cerevisiae* isolates - SC8247 and SC8326 - showed stable fermentation performance at 500 mL to 5 L scale and produced Cheongju characterised by a relative enrichment of fruity ethyl esters, particularly ethyl caproate and ethyl octanoate. Sensory evaluation showed reduced bitterness and moderately increased umami intensity compared with the commercial reference strain. This suggests that strain selection may influence both fermentation efficiency and flavour attributes associated with Cheongju quality. Although the

present study focused on comparative fermentation performance and functional characterisation, the underlying metabolic and genetic mechanisms governing aroma formation were outside the scope of this work. The current findings provide a basis for future investigation aimed at quantitatively assessing aroma related enzymes and metabolic pathways, and for the development of strain specific fermentation strategies to improve Cheongju sensory quality. The present study demonstrates that a stepwise screening framework based on evaluation of stress tolerance facilitates the identification of Nuruk derived indigenous yeasts with fermentation performance comparable to that of a commercial reference strain of *S. cerevisiae*. Further, the selected strains exhibited an enriched fruity ethyl ester profile, providing a basis for the utilisation of indigenous yeasts as practical alternatives in Cheongju production.

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Miri Park: conceptualisation, investigation, data curation, writing (original draft).
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EunJu Lee: methodology, validation.
Jeongwon Kim: investigation.
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Conflict of interest

The authors declare no conflicts of interest. The research was conducted with support from Lotte Chilsung Beverage Co. Ltd. This organisation had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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