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Reexamining the Effects of Media and Selective Pressures in *Saccharomyces cerevisiae* Isolation from Floral Samples

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Abstract

Background: The yeast *Saccharomyces cerevisiae* plays a pivotal role in fermentation industries, yet its ecological behavior and methods for effective isolation from natural environments, particularly floral sources, remain insufficiently understood. Conventional enrichment media are often assumed to selectively favor *S. cerevisiae*, facilitating its dominance during cultivation. **Methodology:** This study evaluated the effectiveness of several enrichment media based on osmotic pressure tolerance, ethanol resistance, and raffinose utilization, using both batch and chemostat cultivation systems. *Camellia* flower samples served as the primary isolation source. ITS region sequencing was used for taxonomic identification. We also compared colony recovery through direct streaking versus dilution plating, and attempted *S. cerevisiae* isolation via pAUR plasmid-based transformation. **Results:** Contrary to expectations, *S. cerevisiae* was not dominant in any culture condition. Instead, genera such as *Lachancea thermotolerans* and *Metschnikowia* spp. predominated across conditions. Only a single *S. cerevisiae* isolate was recovered from batch cultures, exclusively through dilution plating. Transformation with pAUR plasmids failed to yield positive isolates, possibly reflecting limited plasmid receptivity in wild strains. **Conclusion:** These results challenge the effectiveness of traditional media for specifically enriching *S. cerevisiae* and underscore the importance of dilution plating in recovering rare, non-dominant strains. The findings invite further exploration of ecological and genetic factors affecting wild *S. cerevisiae*, including their transformation competence.

Keywords

Saccharomyces cerevisiae, Wild Yeast Isolation, Selective Media, Enrichment Culture

1. Introduction

Saccharomyces cerevisiae, also known as budding yeast, has been integral in various industries, notably brewing and bread making, for its sugar fermentation capability. Its use dates back to before the Common Era, and extensive breeding has led to strains for specific applications [1]-[3]. In Japan, Sake Yeast *S. cerevisiae* Kyokai no. 7 exemplifies this, with numerous derivatives developed for unique sake flavors and aromas, reflecting the meticulous breeding in sake brewing [4] [5]. Yeasts with superior brewing traits like high alcohol fermentation are often isolated from residues of alcoholic beverages such as sake, wine, and beer [6]. This reflects a long tradition of brewers selectively breeding yeast strains with desired characteristics. In contrast, wild yeasts from natural environments generally show lower brewing performance than industrially cultivated strains. As a result, these wild yeasts have been less focused on for commercial production, and there is limited knowledge about their ecological roles [7].

Recent mycobiome studies show *Saccharomyces cerevisiae*'s widespread presence in nature with vast genetic diversity [8], indicating many unexplored, valuable strains. The concept of "flower yeast", introduced in Japan in 1999 [9], refers to wild yeasts isolated from flowers. Contrary to previous beliefs, these yeasts demonstrate alluring brewing characteristics, not just effective alcohol fermentation. Since the 2000s, sake brewed with flower yeast has gained prominence, with its origin in aesthetically pleasing flowers enhancing consumer appeal. Beyond mere aesthetics, flower yeast is increasingly valued for its unique brewing properties.

It is believed that the unique metabolic pathways acquired by flower yeasts in adapting to their specific growth environments contribute to their distinct characteristics. Namely, flower yeasts produce a variety of fermentation by-products not generated by existing brewing yeasts, thereby imparting unique aromas and flavors [10]. Against this backdrop, product development using flower yeasts, isolated from region-specific flora, is becoming increasingly popular across Japan. However, a significant barrier in developing products with flower yeast is the challenge of isolation. In natural environments, other less nutritionally demanding yeasts (such as those from the genus *Candida*) are predominant, leading often to the isolation of these non-target yeasts from environmental samples like flowers. This potential barrier in brewing using flower yeast boils down to a simple issue: the difficulty in isolating *S. cerevisiae*. One reason for this challenge is the scarcity of this particular yeast species in the environment. Enrichment cultivation becomes essential for isolation of this yeast. Although media reported to be effective for isolating the desired species already exist [9] [11], the yeasts isolated and used in brewing as "flower yeasts" are not limited to *S. cerevisiae*; rather, they often include numerous species from genera like *Lachancea* and *Hanseniaspora* [12] [13]. Recent studies have suggested that floral environments, particularly nectar-rich flowers, may serve as microhabitats for wild *S. cerevisiae* populations. For example, Hisatomi and Toyomura [12] reported the successful isolation of diverse

budding yeasts, including potential *S. cerevisiae* strains, from rose petals. Such environments are hypothesized to offer transient yet sugar-rich niches conducive to yeast colonization and diversification. Therefore, the exploration of flowers as sources of novel *S. cerevisiae* strains is ecologically meaningful, potentially uncovering genotypes with unique fermentation properties and broadening the genetic resource pool for brewing applications.

The resolution of the "difficulties" associated with isolating *S. cerevisiae* from flower yeast and proposing viable solutions could provide significant insights into the brewing industry. This study aims to evaluate the effectiveness of various media reported to enrich and isolate *S. cerevisiae* from floral samples, examining the types of yeasts enriched and whether they include *S. cerevisiae*. The overall experimental workflow, including sample processing, enrichment, and isolation strategies, is outlined in Figure 1. We investigated the suitability of these media based on cultivation methods. Both batch and chemostat enrichment methods were employed, and the microbial communities within each system were analyzed through these cultivation methods.

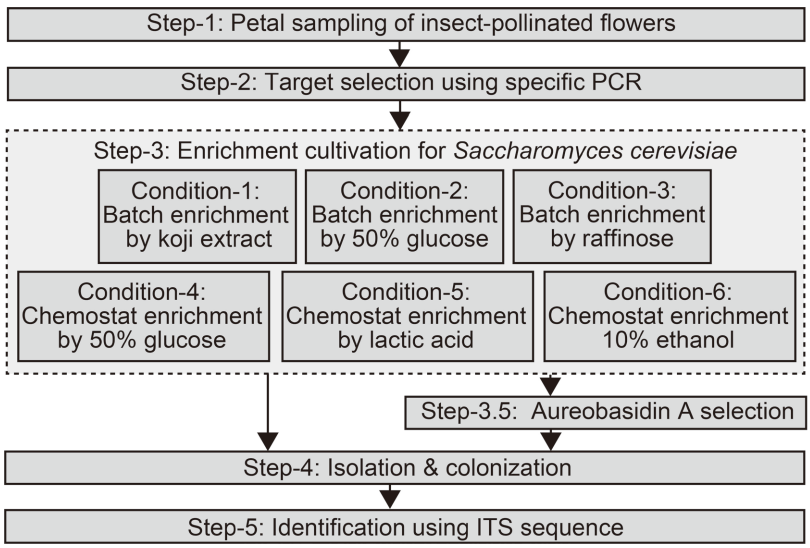

Figure 1. Sequential enrichment and isolation protocol for *Saccharomyces cerevisiae* from floral petals. This schematic depicts the comprehensive methodology employed for the enrichment and isolation of *Saccharomyces cerevisiae* strains from the petals of insect-pollinated flowers. The initial step involves the collection of petal samples (Step 1), succeeded by the amplification of target yeast DNA using specific PCR primers (Step 2). Step 3 encompasses a series of enrichment cultures under varied conditions: Condition-1 employs koji extract; Condition-2 uses a high-glucose medium with a 50% concentration; Condition-3 involves raffinose; Condition-4 continues enrichment in a chemostat with 50% glucose; Condition-5 utilizes a chemostat with lactic acid; and Condition-6 employs a chemostat with 10% ethanol to select for ethanol-tolerant strains. Step 3.5 introduces a selection phase with Aureobasidin A to further refine the yeast population. Isolation and colonization of the yeast are executed in Step-4. Finally, Step-5 involves the identification of the isolated strains through ITS sequencing, characterizing the genetic signature of the yeast.

These results indicate that the microbial community in the chemostat cultiva-

tion system tends to simplify, and that the various media components traditionally thought to be "key factors" for the enrichment of *S. cerevisiae*, such as lactic acid and ethanol, do not always have a direct effect. The observed diversity of microbial communities in the batch system suggests that the breakdown products of media components, or secondary metabolites from other fungal species, may contribute to the enrichment and dominance of *S. cerevisiae*. Additionally, we attempted a novel approach for isolation using the host specificity of plasmids, but it did not lead to successful isolation. This raises new discussions about the plasmid receptivity of wild *S. cerevisiae* strains.

2. Materials and Methods

2.1. Selection of Isolation Source (Step 1)

In this study, we extracted DNA from insect-pollinated floral samples (*Lilium japonicum*, *Gypsophila elegans*, *Camellia japonica*) and grape berry surfaces (both fresh and dried) to detect *S. cerevisiae* using specific primers. The *S. cerevisiae* strain IFO10217 and grape berry surfaces, known to harbor *Saccharomyces*, served as positive controls to validate this methods. *Lilium* and *Gypsophila* were collected from the KOSEN, Kure campus (E132˚36'21" N34˚13'44"). *Camellia samples were* collected from the Irifuneyama Memorial Museum, Kure city (E132˚33'50" N34˚14'25").

2.2. Enrichment Cultivation Conditions (Step 3)

Enrichment was performed using both batch (conditions 1 to 3) and chemostat (conditions 4 to 6) cultivation. Condition-1: involved a medium primarily composed of koji saccharification broth. Rice koji (Masuyamiso Co., Ltd., Hiroshima, Japan) was saccharified overnight at 56˚C with four times its volume in distilled water. The solution was then decanted to obtain the broth, which contains Yeastcidin produced by *Aspergilus oryzae*, known for its antifungal and antiyeastal action against non-*S. cerevisiae* species [14]. The saccharification liquid was supplemented with 2 g/L sodium propionate and 100 mg/L chloramphenicol, and the pH was adjusted to 4.0 with lactic acid. Condition-2: utilized *S. cerevisiae*'s high tolerance for sugar and osmotic pressure [15], comprising a medium of 500 g/L glucose, 10 g/L yeast extract, 20 g/L hypolypepton (Shiotani MS, Japan), 2 g/L sodium propionate, and 100 mg/L chloramphenicol. Condition-3: leveraged *S. cerevisiae*'s unique ability to assimilate raffinose [16], consisting of a medium with 10 g/L raffinose, 6.7 g/L yeast nitrogen base (BD, USA), 80 mL/L ethanol, 2 g/L sodium propionate, and 100 mg/L chloramphenicol. For batch cultivation, the culture media under these conditions were left undisturbed for two weeks before being used for isolation cultivation.

Condition-4 had a similar medium composition to Condition-2 but was used in chemostat cultivation to minimize changes in medium components. Condition-5 was based on the knowledge that lactic acid produced by *Lactobacillus* during sugar decomposition is a factor in *S. cerevisiae*'s dominance [17]. The medium

was adjusted with lactic acid to a pH of 5.0, containing 400 g/L glucose, 10 g/L yeast extract, 20 g/L hypolypepton (Shiotani MS, Japan), 2 g/L sodium propionate, and 100 mg/L chloramphenicol. Condition-6 was designed based on *S. cerevisiae*'s high ethanol tolerance as a dominance factor [18], creating a medium with an elevated ethanol concentration: 400 g/L glucose, 100 mL/L ethanol, 10 g/L yeast extract (Difco, USA), 20 g/L hy-polypepton (Shiotani MS, Japan), 2 g/L sodium propionate, and 100 mg/L chloramphenicol. The chemostat cultivation vessels were modified medium bottles with a working volume of 200 mL. The culture medium was stirred at a low speed to maintain homogeneity, and the system was continuously supplied with fresh medium to maintain a dilution rate (D) of 2.0 day^{-1}, corresponding to a hydraulic retention time of 0.5 days. This rate was intentionally set lower than the specific growth rate of *S. cerevisiae* to impose steady selective pressure. The enriched material, similar to that in the batch system, was harvested after two weeks of cultivation for isolation.

2.3. Introducing pAUR Plasmid into the Enriched Material for Selective Isolation (Step 3.5)

Given the expectation that the enriched material contains multiple yeast species, employing selective pressures beyond substrate assimilation capabilities could potentially enable the isolation of *S. cerevisiae* strains with a broader range of characteristics. In Step-3.5, we adopted a microbial isolation technique based on this patent [19], using the species-specific plasmids pAUR101 and pAUR112, both purchased from Takara Bio Inc. (Shiga, Japan) [20]. These plasmids confer resistance to Aureobasidin A specifically in *S. cerevisiae* and have been previously validated in laboratory strains. No further modifications or re-cloning were performed prior to use. The enriched culture material was washed with sorbitol buffer, then diluted to an O.D. 600 of 0.1 for electroporation using the Gene Pulser Xcell™ Electroporation System (Bio-Rad Laboratories) and 0.2 cm gap cuvettes, under conditions of 1.5 kV, 500 Ω, and 50 μF. Two micrograms of each plasmid were added per transformation. Following electroporation, the cells were recovered in YPD medium for 2 hours and then plated on PDA medium containing 0.1 mg/L Aureobasidin A for selection.

2.4. Isolation Cultivation (Step 4)

For general yeast culture, Potato Dextrose Agar (Fuji film wako pure chemical Co., Osaka, Japan) medium was prepared. The enriched culture material was colonized on this medium. In particular, for the samples that underwent Step 3.5, PDA medium supplemented with 0.5 mg/L Aureobasidin A (PDA + AUR) was used. Two methods were employed to achieve colonization: direct streaking of the enriched samples onto the medium and dilution plating [21]. All colonies obtained, irrespective of the method used, were preserved as individual strains.

2.5. DNA Extraction, PCR, and Identification (Steps 2 & 5)

In this study, DNA was extracted from all petal samples (Maple, Lilium, Gypsoph-

ila), as well as from dried and fresh grape samples, and the IFO10217 strain culture, using the CTAB method [22]. Additionally, DNA from clones isolated from the enriched material was crudely extracted using the cellysate method [23]. To verify the presence of *Saccharomyces cerevisiae* in the environmental samples, PCR was performed with specific primers SC1 (5'-AACGGTGAGAGATTTCTGTGC-3') and SC2 (5'-AGCTGGCAGTATTCCCACAG-3') [24]. The PCR reactions were conducted using KOD one (TOYOBO, Japan), and the resulting amplicons were purified using AMPure (Beckman Coulter, Germany). For the identification of the isolated strains at the genus and species levels, a similar PCR process was employed using different primers for the ITS region of 16S-23SrRNA. The primers ITS1F (GTAACAAGGTYTCCGT) and ITS1R (CGTTCTTCATCGATG) were used [25]. The sequencing was performed on the ABI 3100avant Sanger sequencer to determine the microbial communities of the colonies originating from the enrichment systems. The obtained sequences were then analyzed for nucleotide sequence homology using BLASTn [26].

3. Results and Discussions

3.1. Reevaluating the Presence of *S. cerevisiae* in Natural Environments (Steps 1 to 2)

In this study, *Saccharomyces cerevisiae* was not detected on grape surfaces, which

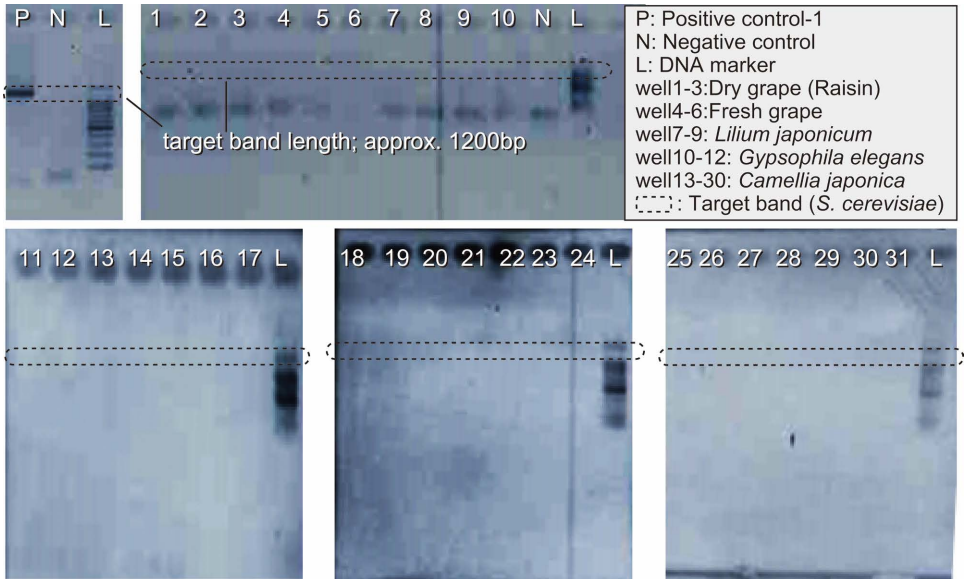

Figure 2. PCR amplification results for detection of *Saccharomyces cerevisiae* in various floral samples. This agarose gel electrophoresis image represents the outcomes of Step-2, targeting the identification of *Saccharomyces cerevisiae* from different floral samples using specific PCR. Lanes 1 to 30 correspond to PCR products from various samples, with "P" representing the positive control, "N" the negative control, and "L" the DNA ladder used for size comparison. Samples include dry grapes (lanes 1 - 3), fresh grapes (lanes 4 - 6), *Lilium japonicum* (lanes 7 - 9), *Gypsophila elegans* (lanes 10 - 12), and *Camellia japonica* (lanes 13 - 30). The dashed line indicates the expected size of the target DNA band for *S. cerevisiae*, which is absent in all samples, suggesting that *S. cerevisiae* was not present at detectable levels in the samples tested.

contradicts our initial expectations of grapes as a reliable positive control (**Figure 2**). This finding diverges from the results reported in [27], where *S. cerevisiae* was successfully identified in similar samples. Employing a potent KOD one polymerase and executing 35 PCR cycles, this study suggests that the presence of *S. cerevisiae* in natural environments might be more sporadic and less abundant than previously assumed. Successful detection was achieved only with the control strain IFO10217, showing the necessity of enriching sufficient yeast densities for detection and isolation. Given these challenges in detecting *S. cerevisiae* even on grape surfaces, this study indicates that flower petals may not be the ideal source for isolating wild *S. cerevisiae*. Investigating more specific microhabitats within floral environments, such as nectar, could be crucial, as these may harbor higher concentrations of the yeast. However, the absence of *S. cerevisiae* on grape surfaces also implies that even these localized microbial communities might not demonstrate a predominance of *S. cerevisiae*. This study, therefore, underscores the importance of targeting specific microhabitats and the need for more nuanced approaches to effectively isolate *S. cerevisiae* from natural environments.

Considering these observations, while it's unlikely that a significant amount of *Saccharomyces cerevisiae* adheres to the bulk of *Lilium*, *Gypsophila*, and *Camellia* petals, past studies suggest that isolation becomes possible through enrichment cultivation. Therefore, in light of these insights, *Camellia* petals were selected as the enrichment source for the experiments detailed in the following sections.

3.2. Identification of Isolated Strains (Steps 3 to 5 Excluding 3.5)

Currently, there is no reported medium that selectively cultivates *Saccharomyces cerevisiae* directly from environmental samples. Most media capable of supporting the growth of *S. cerevisiae* and other yeasts also permit the proliferation of other fungi, such as molds. Therefore, antifungal components like propionic acid are commonly added to yeast isolation media. However, these additives do not completely inhibit the growth of competing mold species. Consequently, when environmental samples are directly applied to such isolation media, molds tend to dominate due to their inherent microbial community structure, preventing effective yeast isolation. For bacteria, strong inhibitors like chloramphenicol can be used to significantly reduce their presence [28].

As revealed in the previous section, *S. erevisiae* is extraordinarily rare in environmental microbial communities, even in places like grape surfaces, traditionally thought to be rich in brewing seed yeasts. This rarity necessitates enrichment culture as a key strategy for isolation. Indeed, several enrichment media have been proposed previously. In this study, we used a koji extract-based enrichment medium (condition-1), leveraging the fact that Yeasticidin, produced by koji mold during sake brewing's saccharification process, inhibits non-*S. cerevisiae* yeasts [14]. We also utilized a medium tailored for *S. cerevisiae*'s resilience to the osmotic pressure of high glucose concentrations, containing over 50% glucose (condition-2) [15]. Additionally, a medium was prepared based on *S. cerevisiae*'s unique abil-

ity to metabolize raffinose, a sugar most other yeast species cannot utilize (condition-3) [16].

The identification of isolated strains, as depicted in **Table 1**, showed similar fungal communities across all three conditions, predominantly consisting of *Lachancea*, *Metschnikowia*, *Hanseniaspora*, and *Candida* genera. This indicates that the specific medium used, whether influenced by Yeasticidin, high glucose concentration, or raffinose utilization, did not significantly alter the diversity of yeast and fungal species isolated from the *Camellia* petals. A particularly noteworthy outcome from condition-3 was the isolation of BR-38-1, which is closely related to, and perhaps a strain of, *S. cerevisiae*. This marked a unique instance in this study where *S. cerevisiae* was successfully isolated, an observation not replicated in this chemostat cultivations, which will be discussed in a later section. The batch enrichment system (denoted by "B" in strain names in **Table 1**) yielded a relatively diverse group of colonies, including one *Saccharomyces cerevisiae* strain. While species-level resolution based on ITS sequencing can sometimes be limited, especially among closely related taxa, the fact that BR-38-1 was the only isolate assigned to the genus Saccharomyces—whereas all others belonged to distinct genera—supports the robustness of this identification at least at the genus level. Further genomic analysis would still be necessary to confirm its precise species classification within the Saccharomyces genus.

While comprehensive reports detailing yeast colony profiles from environmental samples are scarce, Iturritxa's study on wild yeast strains isolated from tree bark, soil, and acorns in northern Spanish forests provides a valuable comparison [29]. They utilized a Sniegowski enrichment medium containing moderate sugar and ethanol concentrations, along with chloramphenicol to inhibit bacterial growth. Notably, the sugar concentration in their medium was much lower than in our high-glucose condition-2, suggesting that a lower concentration of around 1% sugar is effective for yeast enrichment. Parallel to Iturritxa's findings, *S. cerevisiae* did not emerge as the dominant species in our enriched material across conditions 1 - 3. This observation underlines that while certain media compositions are known to support *S. cerevisiae* growth, they may not exclusively promote its enrichment, showing the need for more targeted approaches to isolate *S. cerevisiae* specifically.

The batch enrichment system (denoted by "B" in strain names in **Table 1**) yielded a relatively diverse group of colonies, including one *Saccharomyces cerevisiae* strain. Over time, batch enrichment involves the fermentation of sugars into ethanol, lactic acid, and acetic acid, leading to dynamic substrate composition changes and consequent shifts in the fungal community. For instance, raffinose, a trisaccharide composed of glucose, fructose, and galactose, may not exclusively favor *S. cerevisiae* during selective enrichment as its hydrolyzed components can serve as substrates for other yeast species. This raises a question about the effectiveness of raffinose as a selective factor for *S. cerevisiae* enrichment. If the failure to achieve *S. cerevisiae* dominance in batch enrichment is attributed to dynamic

Table 1. Isolation and characterization of yeast strains from various enrichment conditions. This table summarizes the isolation and characterization of various yeast strains from batch and chemostat enrichment conditions, indicated by the initial letters "B" and "C" respectively, followed by a letter denoting the specific enrichment substrate (Koji, Glucose, Raffinose, Lactic acid, and Ethanol). Strain codes (e.g., BK-C-1, CG-1), closest relative sequences, and similarity percentages are listed alongside GenBank Accession Numbers. Seed origins are provided when applicable, indicating the sample source. Notably, strain BR-38-1 was identified as *Saccharomyces cerevisiae* with a 99.8% similarity, isolated under Condition-3.

Strain	Closest relative sequence	Similarity (%)	Accession No.	Seed origins	Enrichment conditions
BK-C-1	*Lachancea thermotolerans* strain AUMC 10763	99.3	KY495731	*Camellia japonica*	Condition-1
BK-C-2	*Metschnikowia vanudenii* strain CBS: 9134	99.1	KY104212		
BK-C-3	*Candida* sp. WLT-20 12 strain DBT 157	95.7	JX420688		
BK-C-4	*Hanseniaspora valbyensis* strain CBS 311	98.8	KU674848		
BK-C-5	*Hanseniaspora valbyensis* strain CBS 311	99.1	KU674848		
BK-C-6	*Pichia kudriavzevii* strain NCL 45	94.3	FJ231424		
BK-L-1	*Hanseniaspora valbyensis* strain CBS 311	99.1	KU674848	*Lilium japonicum*	
BK-L-2	*Candida* sp. WLT-20 12 strain DBT 157	95.7	JX420688		
BK-G-1	*Lachancea thermotolerans* strain AUMC 10763	99.3	KY495731	*Gypsophila elegans*	
BK-G-2	*Hanseniaspora valbyensis* strain G101	98.8	KP205834		
BG-03-1	*Hanseniaspora valbyensis* strain G101	98.8	KP205834	*Camellia japonica*	Condition-2
BG-05-1	*Lachancea thermotolerans strain* AUMC 10763	99.3	KY495731		
BG-09-1	*Hanseniaspora valbyensis* strain GE2 03	99.7	KP205835		
BG-15-1	*Candida* sp. WLT-20 12 strain DBT 157	95.7	JX420688		
BG15-3	*Metschnikowia vanudenii* strain CBS: 9134	99.1	KY104212		
BG-21-1	*Hanseniaspora valbyensis* strain CBS 311	98.0	KU674848		
BG-24-1	*Hanseniaspora valbyensis* strain CBS 311	98.8	KU674848		
BG-24-2	*Meyerozyma caribbica* strain F222	97.7	KY497943		
BG-24-3	*Meyerozyma caribbica* strain F222	99.5	KY497943		
BG-24-4	*Meyerozyma caribbica* strain F222	99.5	KY497943		
BG-24-5	*Hanseniaspora valbyensis* strain CBS 311	97.9	KU674848		
BG-24-6	*Meyerozyma caribbica* strain F222	97.0	KY497943		
BG-24-7	*Meyerozyma caribbica* strain F222	98.7	KY497943		
BG-24-8	*Meyerozyma caribbica* strain F222	97.7	KY497943		
BG-35-1	*Hanseniaspora valbyensis* strain CBS 311	99.1	KU674848		
BG-50-1	*Zygotorulaspora florentina* culture CBS: 6078	99.5	KY106085		
BG-50-2	*Zygotorulaspora florentina* culture CBS: 6078	98.7	KY106085		
BR-06-1	*Pichia kudriavzevii* isolate NCL 45	94.3	FJ231424		Condition-3
BR-07-1	*Hanseniaspora vinae* culture CBS: 2568	97.5	KY102212		
BR-11-1	*Metschnikowia vanudenii* strain CBS: 9134	98.2	KY104212		
BR-11-1	Uncultured soil fungus clone ITS11/(S2)	95.7	AM229065		
BR-11-2	*Metschnikowia vanudenii* strain CBS: 9134	99.1	KY104212		

Continued

BR-12-2	*Metschnikowia vanudenii* strain CBS: 9134	99.1	KY104212	
BR-13-10	*Metschnikowia vanudenii* strain CBS: 9134	99.6	KY102256	
BR-13-11	*Candida oleophila* culture CBS: 4371	99.6	KY102256	
BR-13-12	*Metschnikowia vanudenii* strain CBS: 9134	94.8	KY104212	
BR-14-16	Uncultured soil fungus clone ITS11/(S2)	98.4	AM229065	
BR-14-5	*Metschnikowia vanudenii* strain CBS: 9134	99.1	KY104213	
BR-22-1	*Metschnikowia vanudenii* strain CBS: 9134	99.1	KY104212	
BR-27-1	Uncultured fungus clone CMH228	98.4	KF800319	
BR-27-2	Uncultured fungus clone CMH228	99.0	KF800319	
BR-38-1	*Saccharomyces cerevisiae* strain CBS: 6282	99.8	KY105061	
BR-52-1	*Zygotorulaspora florentina* strain CBS: 6078	99.5	KY106085	
CG-1	*Lachancea thermotolerans strain* AUMC 10763	100.0	KY495731	
CG-2	*Lachancea thermotolerans strain* AUMC 10763	100.0	KY495731	
CG-3	*Lachancea thermotolerans strain* AUMC 10763	100.0	KY495731	
CG-4	*Lachancea thermotolerans strain* AUMC 10763	100.0	KY495731	Condition-4
CG-5	*Lachancea thermotolerans strain* AUMC 10763	100.0	KY495731	
CG-7	*Lachancea thermotolerans strain* AUMC 10763	100.0	KY495731	
CG-8	*Lachancea thermotolerans strain* AUMC 10763	100.0	KY495731	
CL-1	*Lachancea thermotolerans strain* AUMC 10763	100.0	KY495731	
CL-2	*Lachancea thermotolerans strain* AUMC 10763	100.0	KY495731	
CL-3	*Lachancea thermotolerans strain* AUMC 10763	100.0	KY495731	
CL-4	*Lachancea thermotolerans strain* AUMC 10763	100.0	KY495731	Condition-5
CL-5	*Lachancea thermotolerans strain* AUMC 10763	100.0	KY495731	
CL-6	*Lachancea thermotolerans strain* AUMC 10763	100.0	KY495731	
CL-7	*Lachancea thermotolerans strain* AUMC 10763	100.0	KY495731	
CE-1	*Lachancea thermotolerans strain* AUMC 10763	100.0	KY495731	
CE-2	*Lachancea thermotolerans strain* AUMC 10763	100.0	KY495731	
CE-3	*Lachancea thermotolerans strain* AUMC 10763	100.0	KY495731	
CE-4	*Torulaspora* sp. strain E21254	100.0	MK267654	Condition-6
CE-5	*Lachancea thermotolerans strain* AUMC 10763	100.0	KY495731	
CE-6	*Lachancea thermotolerans strain* AUMC 10763	100.0	KY495731	
CE-7	*Lachancea thermotolerans strain* AUMC 10763	100.0	KY495731	
CE-8	*Lachancea thermotolerans strain* AUMC 10763	100.0	KY495731	

changes in medium composition, then stabilizing these changes through chemostat cultivation might enable easier and more pronounced dominance of a species well-adapted to the medium, ideally *S. cerevisiae*. Based on this hypothesis, conditions 4 - 6 utilized chemostat cultivation for enrichment, resulting in 23 isolates.

Intriguingly, all isolates from these systems, except for one (Torulaspora sp. strain CE-4), were identified as *Lachancea thermotolerans* with 100% sequence match. This unprecedented result challenges the traditional understanding of *S. cerevisiae*'s "final" dominance in brewing, attributed to high sugar osmotic pressure [15], pH reduction by lactic acid [17], and the bactericidal effect of ethanol [18]. It suggests that these factors may not necessarily lead to the dominance or exclusion of *S. cerevisiae* and other yeast species under constant selective pressure. Instead, *Lachancea* species seem more adaptable to these specific media conditions.

These findings clearly indicate that *S. cerevisiae* dominance is not solely achieved through selective pressure by medium composition. This raises a crucial question: how is *S. cerevisiae* dominance achieved in the brewing process? While this study does not provide a conclusive answer, the occasional isolation of *S. cerevisiae* in the "B" series suggests a complex interplay of dynamic substrate changes, secondary metabolites produced by other yeast species, and the source of inoculation. These results imply that a combination of multiple factors, rather than just medium composition, influences the ecological dynamics and dominance of *S. cerevisiae* in natural and industrial settings.

3.3. The Critical Role of Dilution Plating in Yeast Isolation

In the batch enrichment systems, as identified by the "B" series in Table 1, we obtained a diverse array of colonies, including a strain of *Saccharomyces cerevisiae*. This diversity prompts a crucial question: which is more effective for isolating non-dominant yeast species such as *S. cerevisiae*—streaking or dilution plating? Our literature review revealed a lack of documentation on the use of dilution plating for wild yeast isolation, a method commonly employed in bacterial isolation. This suggests that dilution plating, despite its potential effectiveness, has been underutilized in yeast isolation, particularly for species without mycelial growth. Given that streaking is traditionally used for purifying dominant species within a culture, its effectiveness for isolating non-dominant species like *S. cerevisiae* is questionable. In contrast, dilution plating allows for the isolation of less dominant species, provided their presence in the culture is around 0.5% or more. However, it's important to note that the probability of isolating less prevalent species proportionally decreases with their occupancy rate in the culture. These findings indicate that, considering *S. cerevisiae*'s status as a non-dominant species in enrichment cultures, dilution plating is the preferable method.

Remarkably, no prior reports on wild yeast isolation have discussed the limitations of current batch cultivation methods or the significance of dilution plating. Most literature vaguely describes the process as "spreading the enrichment culture on agar media to form colonies," without specifying the method used (e.g. [9] [10] and [27]). We propose that the lack of emphasis on *S. cerevisiae*'s non-dominance and the underuse of dilution plating have been significant barriers to successfully isolating wild strains of *S. cerevisiae* in subsequent research. Supporting this hypothesis, we obtained some strains directly through streaking

from the enrichment cultures (e.g., BG-09-1, BG15-3, BR-11-1), all of which were identified as belonging to dominant genera *Metschnikowia* and *Hanseniaspora*. In contrast, the closely *S. cerevisiae*-related strain BR-38-1 was isolated using dilution plating, reinforcing the effectiveness of this method for isolating non-dominant species.

3.4. Incorporating Plasmid-Based Isolation: Insights from Step 3.5

In our final exploration, we attempted to isolate *Saccharomyces cerevisiae* using a selection pressure different from substrate utilization, albeit unsuccessfully (**Figure 3**). As Liti *et al.* have demonstrated [8], the environmental presence of *S. cerevisiae* is broader than previously assumed, and humanity has likely accessed only a fraction of its genetic diversity. This raises the question: does substrate utilization-based selection merely isolate a limited subset of *S. cerevisiae* strains? To advance this discussion, future genomic sequencing of our isolated strain BR-38-1 and further analysis of the genetic diversity among flower-derived *S. cerevisiae* strains are warranted. To access a broader range of *S. cerevisiae* bioresources, we explored a novel selection method based on genetic transformability—another hallmark trait of yeast biology alongside fermentative metabolism.

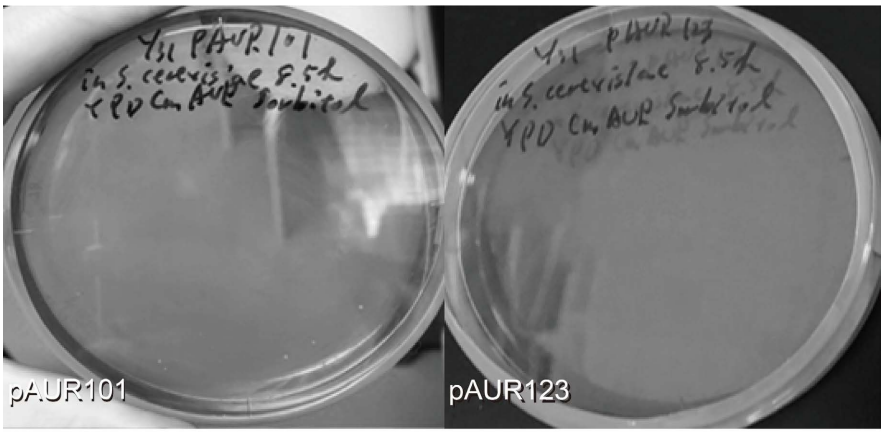

Figure 3. Electroporation efficacy of Aureobasidin A-Resistant Plasmids into enriched Samples for *Saccharomyces cerevisiae* Isolation. This figure presents the results of an electroporation attempt using aureobasidin A-resistant plasmids (pAUR112 and pAUR101) directly into enriched samples to isolate *Saccharomyces cerevisiae*. The absence of colonies on both agar plates indicates a lack of successful transformation. This outcome suggests potential issues related to the low concentration of *S. cerevisiae* cells in the samples, the receptivity of the yeast to the plasmids, or a combination of both factors affecting plasmid uptake and expression.

Specifically, following the method reported by Kimura [19], we introduced plasmids pAUR112 and pAUR101—both conferring aureobasidin A resistance—into the enrichment culture under condition-3. These plasmids require either autonomous replication or genomic integration, and their function hinges on the successful intracellular expression of resistance genes. Consequently, this system

imposes dual selection pressures: plasmid compatibility and drug resistance.

4. Conclusions

This study embarked on an exploration to isolate and understand the dominance mechanisms of *Saccharomyces cerevisiae* from environmental samples, focusing on floral sources. Various enrichment cultivation conditions were tested, revealing insights into the rarity of *S. cerevisiae* in the environment and the complexity of its dominance process. These findings show that both batch and chemostat enrichment often led to the dominance of yeast species other than *S. cerevisiae*, suggesting that traditional enrichment conditions might not be specifically effective for its selective dominance. The effectiveness of dilution plating emerged as a crucial technique for isolating non-dominant species like *S. cerevisiae*, representing a superior approach compared to traditional streaking methods in yeast isolation strategies. Attempts to isolate *S. cerevisiae* using pAUR plasmids did not yield success in this study. However, they provided new insights into the transformation capabilities of yeast, hinting that wild *S. cerevisiae* might possess different properties compared to domesticated strains.

Overall, this study offers new perspectives on isolating and understanding the dominance of *S. cerevisiae* from environmental samples, suggesting new pathways for future yeast research and the utilization of bioresources.

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Authors' Contributions

Z.K. designed the research and supervised the overall project. M.E., K.K., R.K., S.I., Yuya Itoiri (Y.I.1), K.S., H.K., T.F., and Yuki Iwasaki (Y.I.2) conducted the experiments and data collection. Data analysis was performed by all authors. Z.K., Y.I.2 and M.E. led the drafting of the manuscript, with substantial input from K.K., Y.I.1 and R.K. in the results and discussion sections. All authors reviewed and approved the final manuscript.

Declaration of Generative AI and AI-Assisted Technologies in the Writing Process

During the preparation of this article, the corresponding author, Kimura, used SciSpaceGPT for English proofreading. After using this tool, the corresponding author reviewed and edited the content as needed and takes full responsibility for the content of the publication.

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Availability of Data and Materials

Data is contained within the article.

Conflicts of Interest

No potential conflict of interest was reported by the authors.

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