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Full Length Research Paper

To Optimize Beer Production, Potential Yeast Strains that can Hybridize with Saccharomyces Cerevisiae are Screened Metabolically and Fermentatively

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One of the most popular drinks in the world, beer, has been produced with greater efficiency and quality thanks in large part to yeast optimization. In this regard, a promising method for optimizing yeast to produce new and enhanced non-GMO strains is uncommon mating hybridization. The lack of information and comparable data on yeast strains that can hybridize with Saccharomyces cerevisiae, perhaps the most significant yeast species in beer production, is the technique's drawback. It has been reported that yeast belonging to the genera Saccharomyces, Naumovozyma, Nakaseomyces, and Kazachstania can hybridize with S. cerevisiae. 242 yeast strains, including Saccharomyces species (S. cerevisiae, S. kudriavzevii, S. uvarum, S. eubayanus, S. paradoxus, S. mikatae, S. jurei, and S. arboricola) and non-Saccharomyces species (Nau-movozyma, Nakaseomyces, and Kazaschtania), were analyzed under brewing conditions in this study. This represents the entire genetic variability (species and sub-populations) described up until the study began.

In order to ascertain kinetic parameters and CO2 production, the fermentation profile was examined by tracking weight loss during the fermentation process. The concentrations of sugars (maltotriose, maltose, and glucose), alcohols (ethanol, glycerol, and 2,3-butanediol), and organic acids (malic acid, succinic acid, and acetic acid) were measured by metabolic analysis. The main sugars in beer wort are maltose and maltotriose. The qualities of the finished product are determined by the ability to consume these sugars. Then, species, subpopulation, and isolation source comparisons were performed on the dataset. The findings of this study show the wide range of phenotypes found in the genus Saccharomyces and in each of its species, which may help with the creation of brewing hybrids that are optimized. Under brewing conditions, yeasts with varying fermentative capacities and behaviors can be identified. The species that have strains that perform similarly to commercial strains in terms of fermentation are S. cerevisiae, S. uvarum, and S. eubayanus.

Key words: Yeast, Brewing, Hybrids, Maltose, Maltotriose.

INTRODUCTION

One of the most popular alcoholic beverages consumed worldwide is beer. Its manufacturing process involves the hydrolysis and extraction of carbohydrates from cereal grains, followed by yeast fermentation. The genus Saccharomyces is home to the yeasts that are traditionally utilized in the brewing industry (Krogerus et al., 2017). Saccharomyces pastorianus lager yeast, a cross between S. cerevisiae and S. eubayanus, is used to bottom ferment the majority of beers. They can withstand frigid temperatures, and fermentation occurs at 8 to 15 °C. Conversely, top-fermenting S. cerevisiae ale yeasts are employed at temperatures ranging from 16 to 25 °C. According to Querol and Bond (2009), this kind of beer has a high level of aromatic complexity.

Numerous metabolites produced by yeast during wort fermentation add to the scent of the beer. Aldehydes, volatile phenols, esters, and higher alcohols are the primary chemical groups present in beer. Certain fragrance molecules, including vicinal diketones or sulfur compounds, are undesirable, nevertheless (Holt et al., 2019; Krogerus and Gibson, 2013). α-glucosides, maltose (50–60%), a disaccharide, and maltotriose (10–20%), a trisaccharide made of glucose molecules, are the most prevalent fermentable sugars in wort. Glucose, fructose, sucrose, and polymeric α-glucosides (dextrins) comprise the remaining 10% (Zastrow et al., 2001). While maltose and maltotriose enter the cell by proton-symporting active transport, glucose enters the cell by diffusion through a collection of membrane-spanning proteins.

An essential step in controlling the intake of maltose and maltotriose is their passage across the cell membrane. Yeast metabolizes α-glucosides in the cytosol after the enzyme maltase hydrolyzes them to glucose molecules on α1–4 links (Zastrow et al., 2000).

There are presently eight species in the genus Saccharomyces:

S. paradoxus, S. cerevisiae, S. Mikatae, S. Jurei, S. Kudriavzevii, S. Arbor-icola, S. Eubayanus, S. uvarum (Delneri and Alsammar, 2020). hybrids like S. cerevisiae x S. S. pastorianus eubayanus, S. Eubayanus XS. uvarum together with S. cerevisiae x S. Additionally, kudriavzevii are crucial for industrial fermentations. Phylogenetic study of domesticated and wild S. strains of cerevisiae have shown a complicated population structure. Three mosaic groups (M1-M3) and 26 distinct clades were found in one of the most thorough investigations (Peter et al., 2018). Our study will use this information to compare the metabolic patterns of yeasts belonging to various S clades. cerevisiae.

In the other species, many clades have also been described, for example.

S. Paradoxus reveals three distinct subpopulations based on geographic origin: North America, Europe, and the Far East. America A/Europe, America B, and America C are the divisions of the North American population (Alsammar and Delneri, 2020; Warringer et al., 2011). S. Patagonia A (1 and 2), Patagonia B (1, 2, and 3), and Holarctic are the current divisions of Eubayanus strains, which were first isolated in Patagonia (Argentina) (Langdon et al., 2020; Peris et al., 2014, 2016). S. Cryotolerant yeasts called uvarum are typically found in conjunction with fermentation processes. According to Almeida et al. (2014) and McCarthy et al. (2021), they are separated into three clades: Australasia, South America B, and South America A/Holarctic. S. European strains isolated from Portugal, Spain, and France, as well as Asian strains isolated from Japan and Taiwan, represent Kudriavzevii. S's distribution. It seems that Kudriavzevii is only found in Europe and Asia (Alsammar and Delneri, 2020). These subgroups of S. Arboricola are only found in Australasia (New Zealand) and Far East Asia (Yeast separated from China and Taiwan) (Gayevskiy and Goddard, 2016). S. Mikatae has only been isolated in Japan and belongs to a single clade (Naumov et al., 2000). In the same way, S. Jurei only exists in Europe, namely in the French Pre-Alps, and belongs to a single clade (Naseeb et al., 2017).

For brewing purposes, de novo interspecific hybrids produced from other species in the genus Saccharomyces have not been well investigated; however, interspecific hybrids between S. cerevisiae and S. Kudriavzevii, S. Mikatae, S. paradoxus, or S. According to Lopandic et al. (2016), uvarum has demonstrated the ability to enhance aromatic variety in winemaking. By using hybridization techniques, lowering the generation of off flavors, increasing the production of aromas, or merging aroma phenotypes, new aromas can be produced in brewing. It has been demonstrated that producing interspecies lager hybrids can increase the diversity of aromas displayed by natural lager yeast hybrids (Mertens et al., 2015). One of the most effective methods for developing non-GMO yeast strains is interspecific hybridization, which enables the blending and improvement of phenotypic features from many parental strains (Bellon et al., 2015). In addition to species from the formerly known Saccharomyces "sensu lato" group, such as species from the Naumovozyma and Kazachtania, hybrids between species of the Saccharomyces genus have also been found (Marinoni et al., 1999; Morales and Dujon, 2012). This technique is quite effective and has been used, for instance, to increase flavor formation, improve ester formation and fermentation rate (Mukai et al., 2001), introduce dextrin fermentation (Choi et al., 2002), or improve sugar utilisation and fermentation rate (Hebly et al., 2015). (2015) Krogerus et al.

Utilizing this method requires conducting phenotypic research, which enables us to identify the traits of the yeast and choose which ones to hybridize. This kind of work typically entails screening to identify candidate strains with the qualities that make them appropriate for a given usage and that can be employed for that purpose. These studies, such as a screening of native S, have been conducted recently. a screening of several non-Saccharomyces yeasts for their brewing potential (Methner et al., 2019) or cerevisiae strains from Chile in quest of new strains for beer production (Moreira-Ramos et al., 2024). Additionally, it was examined whether non-Saccharomyces yeast might be used to produce low-alcohol beer (Vałstík et al., 2022) or whether yeasts from vineyard and forest ecosystems could be used as brewing yeasts (Iturritxa et al., 2023). In order to identify yeast strains that could be utilized in this business in the future or be potential hybrid production prospects, a large number of strains from all known species and subpopulations were submitted to fermentative and metabolic investigation under brewing conditions. Comparisons at the genus, species, and subpopulation

levels as well as according to the strains' isolation source were used to analyze this data set.

2. Material and methods

2.1. Yeast strains

In this investigation, a total of 242 distinct and very diverse yeast strains were employed (Supplementary Table 1). The 147 yeast strains in this collection are separated into the following categories: 96 S. 11 S. cerevisiae. Kudriavzevii, 10 S. uvarum, 6 S. Eubayanus, 12 S. paradoxus, 6 S. Mikatae, 2 S. 4 S. and jurei. arboricola. Within the genus Saccharomyces, the remaining 32 strains are hybrids: 4 S. x cerevisiae S. S. pastorianus eubayanus, 19 S. cerevisiae x S. Kudriavzevii and nine S. cerevisiae x S. Uvarum. Furthermore, two yeast strains from Naumovozyma castellii and Naumovozyma dairenensis, two yeast strains from Nakaseomyces bacillisporus and Nakaseomyces delphensis, and 51 yeast strains from 34 species of the genus Kazachstania were included. The commercial strain S, lastly. A commercial lager strain control, pastorianus Saflager Weihenstephan 34/70 (WS 34/70), was employed. To compare with other strains that have demonstrated favorable traits under brewing conditions in earlier investigations, eight strains of the species Candida shehatae, Candida tropicalis, Pichia kluyveri, Saccharomycodes ludwigii, Tor-ulaspora delbrueckii, and Zygosaccharomyces rouxii were employed. At —80 °C, the yeasts were cryopreserved. The strains were cultivated for their usage, and the primary kept at 4 °C on solid GPY medium plates with 2% glucose, 2% agar, 0.5% peptone, and 0.5% yeast extract.

Additionally, a classification based on the source of isolation was created in which fall into two primary categories: environments that are fermentative and those that are not. The 56 yeast strains in the fermentative environment group are split up into the following 9 groups: 3 Bakery, 5 Five distilleries, two bioethanols, three sakes, sixteen wines, seventeen beers, three fermentations, and two beverages are included. Twelve groups—two industrial, twenty-six soil, fifteen nature, forty-nine trees, two flowers, sixteen fruits, eight animals, six clinical, five laboratory, five insects, eight food, and two fungi—comprise the 144 yeast strains in the non-fermentative environment group. Since the other 42 strains were either part of the control group or had an unclear source of isolation, they were excluded from this study.

2.2. Wort preparation

Following the procedure outlined in Holt et al. (2018), a representative brewing wort was made. ZnSO4 was added to 200 g/L of Brewferm's 8 EBC blonde malt extract to reach a final concentration of 0.5 mg/L during this process. After bringing the pH down to 4.5, the wort was autoclaved at 110 °C using a software, for fifteen minutes. After letting the wort settle overnight, the sediment was thrown away. After adding hops at a final concentration of 25 mg/L, the wort's sugar content was adjusted to A digital refractomerter (VWR, Leuven, Belgium) with a 15.5◦Bx. For use in microfermentation investigations, the resultant wort was kept in 1-liter bottles at -20°C.

2.3. Microfermentation experiments

The 242 yeast strains underwent triplicate fermentations in wort medium (15.5◦Bx, pH 4.5, 0.5 mg/L ZnSO4, 25 mg/L hops). In microcentrifuge tubes, starter cultures were made with 1.5 mL of wort (15.5◦Bx, pH 4.5, 0.5 mg/L ZnSO4, 25 mg/L hops) and incubated for 24 hours at 28 ◦C. Starter cultures were added to 10 mL bottles that contained 7 mL of wort until the final concentration was 1·106 cells/mL. agitation at 125 rpm and 20 ◦C were the experimental conditions. Every 24 hours, weight measurements were made in order to track the progression of weight loss.

When the daily weight loss was less than 0.01 grams per 24 hours for two days in a row, the fermentation was deemed to have ended. Cells were separated by centrifugation in a 5810 R centrifuge (Eppendorf, Hamburg, Germany) operating at 4000 rpm for 4 minutes at 20 ◦C following fermentation. After discarding the cell pellet, the supernatant was gathered and kept at -20°C until it was needed for analytical analysis.

2.4. Fermentative profile

We were able to estimate the kinetic parameters, the fermentation profile, and the CO2 production of each strain of yeast by measuring the weight loss that occurred during the fermentation process (Daoud and Searle, 1990). Under the assumption that the weight lost at each point is lost as CO2, the concentration of CO2 created was computed from the weight loss. According to the Gompertz equation, "Y = A*(exp(—exp ((μ*2.718282)/A)*(λ-x) + 1))," the growth curve that is obtained In 1990, Zwietering et al. The GraphPad Prism 8.0.1 software was used to fit the weight values to the reparametrized Gompertz equation in order to determine the kinetic parameters A Max and AUC (Area Under the Curve).

2.5. Metabolic analysis

Analysis was done on the extracellular metabolites found in both the starting wort and the finished beer. At the

conclusion of the fermentation process, the primary wort sugars (maltotriose, maltose, and glucose), fermentation by-products (ethanol, glycerol, and 2,3-butanediol), and organic acids (malic, succinic, and acetic) were identified using HPLC (Thermo Fisher Scientific, Waltham, MA), which was outfitted with a HyperREZTM XP Carbohydrate Guard (Thermo Fisher Scientific, Waltham, MA) and a refraction index detector. Eluent, 5 mM H2SO4, 0.6 mL/min flow, and an oven temperature of 50 ◦C were the conditions for the analysis. A comparison was made between the retention periods of the eluted peaks and the commercial analytical standards (Sigma-Aldrich, Madrid, Spain). The calibration graphs (R2 value >0,99) of the standards, which were previously derived using a linear curve fit of the peak regions using 10 standard mixtures, were used to quantify the concentrations, expressed in g/L or % (v/v).

2.6. Modelling, data management and statistical analysis

The mean and standard deviation are used to present the findings from the three biological replicates. GraphPad Prism 8.0.1 was used to create a HeatMap that represented every strain of yeast. GraphPad Prism 8.0.1 software was used to create bar graphs that showed the metabolic study results by subpopulation in each species. The t-Student test ($p < 0.05$) was used for statistical analysis. The Statsoft Statistica 10 tool was used to create box plots that showed the metabolic analyses by species and isolation source. Tukey's HSD test ($p < 0.05$) was used for statistical analysis. The Statsoft Statistica 10 Package was used to conduct principal component analysis (PCA), which gave a summary of the parameter comparisons.

3. Results

3.1. Cell growth and growth parameters in brewing conditions

This project's goal was to examine the metabolic and fermentative variations of a sizable collection of yeast strains that were representative of the majority of species and subpopulations that were phylogenetically similar to S. cerevisiae enough to potentially create hybrids. 242 distinct strains of yeast were microfermented in 10 mL bottles with 7 mL of wort in order to ascertain the differences between these yeasts. Weight loss was measured daily until the fermentation ceased in order to track its development. Following the fermentation process, the growth curve was examined to ascertain the area under the curve and the associated kinetic parameters.

The area under the curve parameter was used to compare the growth profiles of the various species (Fig. 1). Under brewing conditions, strain WS 34/70 (used as a commercial reference) shows good growth. S. uvarum, S. cerevisiae, and

A good growth profile around commercial values is displayed by S. eubayanus. 95 distinct strains of the S. cerevisiae species were examined, and we discovered that they all had varying capacity for development under these particular circumstances. In actuality, the commercial brewing strain of S. cerevisiae exhibits a considerably higher growth behavior than the non-fermentative strains. Conversely, species of S. paradoxus, S. arboricola, S. kudriavzevii, S. mikatae, S. jurei, Naumovozyma, Nakaseomyces, and Kazachstania have reduced area under the curve values, suggesting subpar growth under brewing circumstances.

3.2. An outline of the strain collection that compares the behavior of metabolic analyses during brewing circumstances

We conducted a first global general comparative analysis of the obtained metabolic profile of all the strains (Fig. 2) prior to doing a comprehensive comparison at the species and subpopulation level, which demonstrates that the yeasts under study differ significantly from one another. Generally speaking, the primary variations are in the quantity of residual sugars that remain after fermentation and the quantity of alcohols that are generated. Almost every yeast entirely consumes glucose (10% of the fermentable sugars), with the commercial control WS 34/70 demonstrating a consumption of 100%. Nearly all yeasts exhibit a consumption of >95%. Kazachstania bovina CBS 2760 (63.42 % ± 3.76) and Kazachstania humillis CECT 11871 (85.65 % ± 10.50) are two examples of lesser consumption, nevertheless. Figure 2 demonstrates that nearly all yeast strains have more residual maltotriose at the conclusion of fermentation than WS 34/70 fermentation, which indicates a high consumption of this sugar (86% ± 1.24). The majority of the yeast strains that were examined have minimal maltotriose consumption (less than 30%). Several strains of yeast, mostly from the genus Saccharomyces, were shown to have good maltotriose consumption. RP.10.4, a strain of S. cerevisiae belonging to the Brazilian bioethanol subgroup, exhibits a high maltotriose content of 88.09 percent ± 0.63. This is likewise true for the Ale Beer subgroup's S. cerevisiae CCY_21-4-106 (86.43 % ± 0.14). The S. cerevisiae X S. eubayanus hybrid CECT 11037 (86.21 % ± 0.21) and the S. cerevisiae X S. kudriavzevii hybrid CECT 11002 (85.98 % ± 0.31) are examples of Saccha-romyces hybrids that have good maltotriose consumption.

The yeasts under study also differ greatly in how much maltose they consume. With a maltose consumption of 96.71% ± 0.28, the commercial reference strain WS 34/70 exhibits excellent performance. Maltose and

maltotriose are poorly absorbed by strains of the genus Kazachstania (Fig. 2). Consumption in the genus Saccharomyces varies greatly, ranging from 10% to 100%. S. cerevisiae DJ68 from the African palm wine subpopulation (11.04 % ± 1.24), S. paradoxus CECT 11143 from the Eurasia subpopulation (11.17 % ± 1.82), and S. kudriavzevii CBS 12751 from the Taiwan subpopulation (11.55 ± 2.20) are examples of strains with low consumption. On the other hand, strains like S. cerevisiae Pac6436 from the Wine/European subpopulation (99.71 % ± 0.51), the hybrid S. pastorianus CECT 1463 (100 %), and S. cerevisiae MTF2546 from the West African Cocoa subpopulation (100 %) exhibit extremely high maltose consumption, even surpassing the commercial control strain.

When it comes to producing ethanol, yeast strains belonging to the genera Kazachstania, Nakaseomyces, or Naumovozyma typically exhibit yields of less than 1% (v/v). Depending on sugar consumption, yeast strains in the genus Saccharomyces produce ethanol at varying rates, ranging from 1% to 5.5%. Certain strains, including S. cerevisiae CEY652 with 5.45 ± 0.08 % v/v or S. cerevisiae from the Mosaic Beer subpopulation 6.2_WLP570 with 5.41 ± 0.19 % v/v, were found to produce 5.328 ± 0.37 % v/v, which is slightly more than WS 34/70. There are also several S. cerevisiae x S. kudriavzevii hybrids, like strain PB7, which yields 5.483% v/v ethanol, and CECT 1388, which yields 5.52. Although not as strongly as in the case of ethanol, the creation of glycerol and 2,3-butanediol is linked to the consumption of carbohydrates. In comparison to the other species examined, the strains of S. jurei, S. mikatae, and S. arboricola exhibit a reduced synthesis of 2,3-butanediol. Since there were no discernible changes in the quantities of acetic acid, succinic acid, or malic acid production, these samples were rejected for additional examination by species, subpopulations, and isolated sources.

3.3. Comparing species-level metabolic analyses under brewing conditions

Under brewing laboratory simulation settings, we compared the metabolic behavior of the various yeast species with that of the commercial S. pastorianus control strain WS 34/70 at the conclusion of fermentation (Fig. 3). Because of their comparable behaviors, all species of the genus Kazachstania, Nakaseomyces, and Naumovozyma were placed together. Maltotriose and maltose, the two primary fermentable sugars in the brewing wort, are both excellently consumed by the control yeast WS 34/70. Although the median in the fermentative group is greater than in the non-fermentative group, only the S. cerevisiae species exhibits high maltotriose consumption for the two established groups. The genus Kazachstania and the other Saccharomyces species exhibit limited consumption of this sugar. Maltose exhibits a greater degree of diversity. Like commercial yeast, the species S. uvarum and S. eubayanus have a very high maltose content. Despite more heterogeneity in the non-fermenting group, S. cerevisiae species exhibit good maltose intake. While S. kudriavzevii and S. mikatae exhibit usually low consumption profiles with a few strains that consume maltose, S. arboricola and S. paradoxus exhibit a wide distribution with very varied strains in maltose consumption. The genus Kazachstania and S. jurei do not exhibit any discernible maltose consumption. The consumption of the primary fermentable sugars exhibits a similar tendency to that of CO2 and ethanol production. The process of producing glycerol is comparable to that of producing ethanol. We discover some notable differences in 2,3-butanediol production compared to the industrial control, as it is higher in S. eubayanus than in WS 34/70, while S. jurei and the genus Kazachstania have values that are comparable to those of the majority of the other species. A PCA analysis of the species using the metabolic data from Fig. 3 is shown in Fig. 4. A distribution of the various species in three groups is proposed, and it is evident that the control strain WS 34/70 is distinguished from the other species by its good maltotriose consumption. Although S. uvarum and S. eubayanus are grouped together, most likely because they consume maltose well and produce a lot of alcohol, they differ significantly from WS 34/70 in their production of 2,3-butanediol. Despite being grouped together, the two types of S. cerevisiae strains differ from one another, indicating that yeasts isolated from fermentation environments have a metabolic profile more like to that of an industrial fermentation. Because they consume less sugar and produce less alcohol, the other Saccharomyces species and the genus Kazachstania are farther apart.

3.4. Comparing metabolic analyses during brewing conditions at the subpopulation level

An investigation of the many subpopulations within each species under study was conducted in order to further the metabolic studies of each species (Figs. 5, 6, and 7). Compared to the commercial strain, the various clades of S. cerevisiae (Fig. 5) generally consume less maltotriose. Although some of them exhibit high variances within the clade (e.g., clade 8, mixed origin), there are clades with highly different maltotriose intake, suggesting that there is variety with certain strains exhibiting good maltotriose consumption. The RP.10.4 strain from the "3. Brazilian bioethanol" clade (88.09 ± 0.63 %), the CCY_21–4-106 strain from the "11. Ale beer" clade (86.43 ± 0.14 %), the CECT10120 strain from the "8. Mixed origin" clade (83.32 ± 1.55%), and the 6.2_WLP570 strain from the "7. Mosaic beer" clade (81.37 ± 0.63%) are the S. cerevisiae strains with the highest consumption.

However, there are clades like "M2. Mosaic region 2," "17. Taiwanese," and "19. Malaysian" that consume very

little or no maltotriose. For all clades, maltose consumption is either the same as or greater than maltotriose consumption. Certain clades, such as "1. Wine/European," "2. Mosaic beer," or "11. Ale beer," exhibit nearly complete maltose consumption, similar to the commercial strain WS 34/70. The strain MTF2546 from the "12. West African cocoa" clade (100%) and the strain Pac6436 from the "1. Wine/European (subclade 3)" (99.71 ± 0.51%) and the 2162 strain from the "4. Mediterranean oak" clade (99.20 ± 0.12%) were among the good maltose-consuming yeasts that we observed. Once more, there is a great deal of variation among the groups since some, like "16. CHNI" or "22. Far East Russian," consume far less maltose. When compared to the consumption of carbohydrates, the creation of CO2 and ethanol exhibits a similar pattern.

Saccharomyces non-cerevisiae species were likewise subjected to the same study of identified subpopulations within each species (Fig. 6). With the exception of the "European" clade, which includes two strains that exhibit maltose consumption, CR 85 (95.79 ± 1.03%) and ZP591 = FM1009 (96.79 ± 0.33%), S. kudriavzevii has very low maltotriose and maltose intakes. In every subpopulation examined, S. uvarum and S. eubayanus exhibit low maltose but high maltose intake.

S. paradoxus has a high degree of variability within the groups under investigation; whereas all of them consume little maltotriose, there is a great deal of variation in maltose consumption. While "Eurasia," "America B," "America C," "Hawaii," and "Mix" exhibit extremely low maltose intakes, "America A," "Far East," and "EU" display large intakes that are comparable to the commercial strain's values. Interestingly, the strains yHDPN429 = N44 (97.40 ± 0.07%) and CBS432 = FM472 have significant maltose consumption.

yHDPN23 (78.20 ± 2.89 %) and 97.18 ± 0.12%. With the exception of yHAB336 (88.13 ± 10.57%), a strain of "mikatae Asia B" that consumes maltose, S. jurei and S. mikatae exhibit poor sugar intakes. Two S. arboricola strains with low maltotriose consumption were examined.

Maltose intake is demonstrated by the strain ZP960 = yHDPN432 (96.95 ± 0.10%) in the "Oceania" subgroup. Four types of hybrids—S. cerevisiae x S. eubayanus, S. cerevisiae x S. kudriavzevii, S. cerevisiae x S. uvarum, and S. eubayanus x S. uvarum—were examined in this study. The high maltose consumption of all the hybrids is comparable to that of the commercial WS 34/70. Only in the S. eubayanus x S. uvarum hybrids is there variability; two yeasts, the IV.NK 2.16 (1.55 ± 2.68 %) and the V. BK 2.2 (1.42 ± 1.69 %), exhibit limited consumption of this sugar. With the exception of strain CECT 11135, which exhibits no intake, the S. cerevisiae x S. eubayanus (S. pastorianus) hybrids exhibit the highest maltotriose intakes, approaching 80%. The hybrids of S. cerevisiae and S. uvarum do not consume maltotriose. The several species of the genus Kazachstania and Naumovozyma have extremely low intakes of maltotriose and maltose.

3.5. Comparison of yeast strains' metabolic analyses under brewing conditions, arranged by isolation source

The strains were categorized into nine fermentatively related groups based on the source of isolation: bread, dairy, distillery, bioethanol, sake, wine, beer, fermentation, and drinks. Soil, wild, tree, flower, fruit, animal, clinical, laboratory, industrial, insect, food, and fungal are the 12 non-fermentative linked groups (Fig. 8). Strains in fermentative environments consume more sugar than strains in non-fermentative environments. It is evident that the strains from the non-fermentative environment consume less maltotriose overall.

This is also true for maltose intake, where all subgroups of fermentative isolation sources—including the commercial strain WS 34/70—have consumption rates that are about 100%, but strains from non-fermentative environments exhibit greater variability. The average levels of maltotriose consumption are above 50% for the Bioethanol, Beer, and Bakery categories. While they may not exhibit significant maltotriose consumption, industrial yeasts used to produce other fermented drinks like wine or sake have high maltose intakes. Except for a few high-consuming yeasts, the remaining subgroups from non-fermentative settings exhibit modest maltotriose consumption. Among the various categories, maltose intake varies significantly; the "Clinical," "Fungi," and "Wild" groups exhibit high maltose intakes, whilst the other groups exhibit extremely wide variations. The sugar consumption of yeasts isolated from "fruit," "flower," or "tree" varies greatly, but yeasts isolated from "soil," "crop," or "animal" typically have relatively low intakes. In relation to the overall amount of sugar consumed, the production of CO2 and ethanol has a proportional pattern. There are minor variations in 2,3-butanediol and glycerol synthesis between the subgroups.

A PCA of the metabolic analysis between the various isolation sources is displayed in Fig. 9. The control strain WS 34/70 exhibits optimal consumption of the available sugars by separating from all other groups. Because they comprise yeasts with favorable fermentation profiles tailored to industrial fermentations, the "Bioethanol," "Beer," and "Bakery" groups are the ones that are most similar to the commercial control.

Perhaps because of where these yeasts originated, the "clinical" group is the one that belongs to non-fermentative conditions and is most similar to the commercial strain and the beer group. The close

proximity of the "Fungi" and "Beverages" groups is most likely caused by the strong production of 2,3-butanediol and the low consumption of maltotriose. Because of their low fermentation profile and low sugar intake, the "Animal," "Crop," "Soil," "Tree," and "Flower" categories are the most dissimilar from the others.

4. Discussion

Within a phylogenetically comprehensive collection of S. cerevisiae hybridisable yeast strains, this study examined the metabolic variations among strains, species, subpopulations, and isolation sources. Since we can identify yeasts with varying fermentative powers, the results show the high phenotypic variety that exists within the genus Saccharomyces and within each species of this genus. The brewing business now has access to yeasts with a variety of metabolic behaviors, opening up a world of opportunities.

The strains' varying capacities to ingest sugar were the primary cause of the variations in alcohol production levels that we saw. Because of the wort's low glucose concentration and ease of uptake, nearly every strain tested was able to ingest glucose flawlessly (Hora´k, 2013; Kim et al., 2013). The consumption of maltose and maltotriose, the two main sugars in beer wort, showed a great deal of variation, nevertheless. At least one of the five highly similar and unlinked MAL loci (MAL1–4, MAL6) must be present for the metabolism of maltose and maltotriose (Hora´k, 2013). The existence or lack of these genes, as well as the quantity of copies in which they are present, may be the primary causes of the wide variation in the intake of the major sugars. A major factor in the genetic diversity of these loci is their subtelomeric localization on various chromosomes. Comparative genomic research become more challenging as a result, and in order to comprehend gene-phenotype connections, more comprehensive third-generation sequencing datasets will be needed.

Only strains of S. cerevisiae and S. cerevisiae hybrids that were able to assimilate maltotriose were detected in this investigation; the WS 34/70 strain totally consumes the maltotriose sugar. Fermentation performance is influenced by the ploidy of de novo lager hybrids; hybrids with higher DNA content clearly outperform those with lower ploidy (Krogerus et al., 2016). More maltose/maltotriose transporter genes would be present in hybrid genomes with higher hybrid ploidy, improving the intake of these sugars. During brewing, their uptake restricts fermentation capacity; strains that ferment more quickly also consume maltose and maltotriose more quickly (Krogerus et al., 2016). According to our hypothesis, this explains why the strains that consume the most maltotriose in this investigation are hybrids of S. cerevisiae x S. eubayanus and S. cerevisiae x S. kudriavzevii. Furthermore, constitutive expression of the MALx1, AGT1, MPH2, and MPH3 transporters in S. cerevisiae has been shown in various investigations. Several S. cerevisiae yeasts that are effective at fermenting maltose and maltotriose and contain several MAL loci in their genomes have been examined. It was demonstrated that the consumption of maltotriose is prevented by the presence of MALx1 transporters in their membranes. When the AGT1 permease is removed, the capacity to ferment maltotriose is lost (Alves et al., 2008). The existence of a functional AGT1 transporter may make the maltotriose-positive S. cerevisiae strains identified in our investigation suitable for commercial application.

Despite not consuming maltotriose, all of the S. uvarum and S. eubayanus strains under study exhibit significant maltose consumption. Four maltose transporter genes (SeMALT1–4) were discovered when the genome of the strain of S. eubayanus employed in our investigation, CBS 12357 T, was examined. According to metabolic studies, only SeMALT2/4 transporters are in charge of consuming maltose; they cannot absorb maltotriose (Brickwedde et al., 2018). Thus, it is believed that the parental S. cerevisiae is the source of the hybrid S. pastorianus' capacity to ferment maltotriose. It has been demonstrated that S. cerevisiae ale strains from the beer 1 and beer 2 groups can use this trisaccharide (Gallone et al., 2016). Several strains of S. cerevisiae, including CCY_21–4-106 from the "Ale beer" subpopulation, have been shown to be able to ferment maltotriose in our investigation. This strain is the second best at consuming maltotriose during the entire study, surpassing the commercial control. Our findings support the finding that the AGT1 maltose transporter, which is responsible for the utilization of maltose and maltotriose, is present in Ale strains of S. cerevisiae belonging to the "Beer 1" group (Gallone et al., 2016; Krogerus et al., 2019). However, diastatic strains of S. cerevisiae can produce an extracellular glucoamylase that is encoded by STA1. The "Beer 2" and "Mosaic beer" groups have high levels of the STA1 gene. It has been demonstrated that STA1's extracellular hydrolysis of maltotriose appears to be the primary mechanism permitting the usage of maltotriose during wort fermentation in STA1+ strains by removing STA1 from diastatic strains. According to Gallone et al. (2016) and Krogerus et al. (2019), the formation and maintenance of STA1 seems to represent an evolutionary strategy for the effective use of the sugars found in brewer's wort.

Our findings imply that because of their high consumption of sugars, specifically maltose and maltotriose, S. cerevisiae strains from the Brazilian bioethanol subgroup are appropriate for use in the production of beer. While only RP.10.4 metabolizes 88% of maltotriose, CBS7962, RP.10.4, and RP.10.14 demonstrate a maltose consumption that is nearly 100%. The S. cerevisiae strain LBGA-287, which produces a particularly good beer with excellent efficiency and sensory aspects approved by specialists and the general public, is one of the Brazilian bioethanol strains that have been proposed for beer production in other studies (Lorca Mandujano et al.,

2022).

According to our results, non-conventional yeasts produce less ethanol because they are unable to consume the maltose and maltotriose sugars found in wort. Because of this feature, they can be used to produce beers with lower alcohol concentration. Other investigations have similarly described this (Burini et al., 2021). The use of non-Saccharomyces yeasts in beer production has even gained popularity recently, prompting a hunt for additional natural candidates (Iturritxa et al., 2023).

According to Cordente et al. (2012) and Rodriguez et al. (2017), several species, including S. kudriavzevii, S. mikatae, S. paradoxus, S. jurei, S. eubayanus, and S. uvarum, have already demonstrated promise in alcoholic beverages and have been found to ferment wine, cider, palm wine, tepache, chicha, and other beverages worldwide. According to Peris et al. (2018), S. kudriavzevii is a cryophilic species that is frequently employed in Europe and Australia to ferment wines at lower temperatures (10–15 ◦C). This species could be used in the brewing sector to produce hoppy lager beers. We have verified that S. kudriavzevii cannot eat maltotriose (Pfliegler et al., 2014); yet, as previously mentioned, some S. cerevisiae x S. kudriavzevii hybrids have a high capacity to eat maltotriose. This may be explained by the improved vigor and fermentative activity of some S. cerevisiae x S. kudriavzevii hybrids, as well as the larger contribution of the S. cerevisiae subgenome with maltotriose transporters (Peris et al., 2018). According to Sampaio and Gonçalves (2008), S. kudriavzevii can consume maltose. In this investigation, we discovered two strains of S. kudriavzevii that can metabolize maltose: strain ZP591 = FM1009 and strain CR 85. The brewing sector will benefit from these strains.

Only lately has the brewing potential of S. paradoxus (at 15 ◦C) been studied. At higher beer temperatures, this wild-type yeast species may create intriguing volatile aroma molecules (Majdak et al., 2002). We have examined several strains of S. paradoxus in our study, each of which has unique opportunities for the brewing industry. S. mikatae ferments white wine slowly and gives it fruity, banana, flowery, and sweet scents. Although they cannot metabolize maltotriose, S. mikatae and S. paradoxus can provide unique traits for the manufacture of ale beer; yet, we discovered strains of each species that are maltose-positive.

We found that S. eubayanus can ferment the wort's glucose and maltose but not maltotriose. Consumption tests using labeled maltotriose ([14C]-maltotriose) demonstrated that S. eubayanus cannot ferment this sugar because to its inability to be transported (Brouwers et al., 2019). However, S. eubayanus is an intriguing choice for beer production due to its low temperature growth, usage of maltose, and production of aroma compounds that are preferred for certain beer styles. In 2016, Heineken introduced Lager H41, the first commercial beverage developed with a strain of S. eubayanus from the Patagonia subgroup (Magalhaes et al., 2016; Mardones et al., 2020). De novo hybrids with Mtt + Saccharomyces non-cerevisiae strains were created using the CBS 12357T S. eubayanus strain, which in this work validated its Mtt-phenotype (Gyurchev et al., 2022).

Several strains of S. kudriavzevii, S. paradoxus, S. mikatae, and S. uvarum may be suitable for use in the brewing sector, according to an intriguing study (Bruner et al., 2021). One potential strain for the development of low-alcohol beers is S. mikatae NCYC 288 T.

because it produces very little ethanol. The six strains of S. mikatae that we analyzed for this study all had low maltotriose and maltose consumption, but we also discovered one exception: strain yHAB336. This strain of S. mikatae has a good maltose consumption and can be utilized to make regular beer.

The goal of searching for non-Saccharomyces yeasts for beer production is to provide novel fermentative and organoleptic properties. The primary constraint is the challenge of ingesting maltose and malto-trise. According to this study, strains of the species Kazachstania, Nakaseomyces, or Naumovozyma essentially consume none of these sugars. Nonetheless, we suggest that they might make intriguing hybrids with S. cerevisiae to create novel strains with distinct organoleptic characteristics.

In terms of the source of isolation, this study shows that yeasts from fermentative environments consume somewhat more maltotriose and maltose than those from non-fermentative environments. Compared to yeasts isolated in conditions producing other fermented beverages, such wine or sake, those isolated in beer production environments are better suited to consuming maltotriose, which is mostly found in barley wort (Peris et al., 2018). While yeasts found in non-fermentative settings exhibit a wider range of results, with many strains exhibiting poor maltose consumption, yeasts isolated in fermentative conditions exhibit maltose consumption that is quite similar to WS 34/70. Similarly, yeasts isolated in fermentative settings do not differ from WS 34/70 in terms of CO2 and ethanol generation. On the other hand, those isolated from non-fermentative environments produce less ethanol and exhibit poorer fermentation profiles.

Brewer's and bioethanol yeasts' proximity to WS 34/70 is demonstrated by the PCA. With the exception of clinical or wild yeasts, those from non-fermentative settings are farther removed. The significant production of alcohols and CO2 is indicative of the employment of yeasts for ethanol production, which are included in the "Bioethanol" group (Lorca Mandujano et al., 2022). Since the yeasts of the "Beer" category are used to make beer, they are well suited to consuming maltotriose and maltose (Gibson et al., 2020). One noteworthy finding is that the "Bakery" group is farther away and more in line with the "Bioethanol" group, while the "Clinical" group is closest to the "Beer" group. In contrast, current research indicates that the genesis of clinical strains may be more closely linked to probiotic yeasts such as S. cerevisiae var.

boulardii or bakery yeasts (Morard et al., 2023). One reason would be that the strains designated as clinical origin are only strains that were inadvertently isolated from this anthropic setting through cross-contamination, rather than ones that are actually virulent. However, to verify this theory, particular virulence tests had to be carried out.

In summary, our work has demonstrated the wide variety and potential of numerous strains for application in hybridization-based yeast production for contemporary brewing. That's what we discovered. The strains S. kudriavzevii, S. paradoxus, S. eubayanus, and S. uvarum can be utilized to produce strains with a variety of fragrances but a lesser fermentative ability. Lastly, strains from the species Kazachstania, Nakaseomyces or Naumovozyma, or even S. mikatae or S. jurei, can be employed to produce low-ethanol beer. Additional criteria for yeast selection for yeast optimization through hybridization will be provided by future studies into the variety of flavors produced by the strains.

Declaration of competing interest

The authors state that none of the work described in this study could have been influenced by any known competing financial interests or personal relationships.

Data availability

No data was used for the research described in the article.

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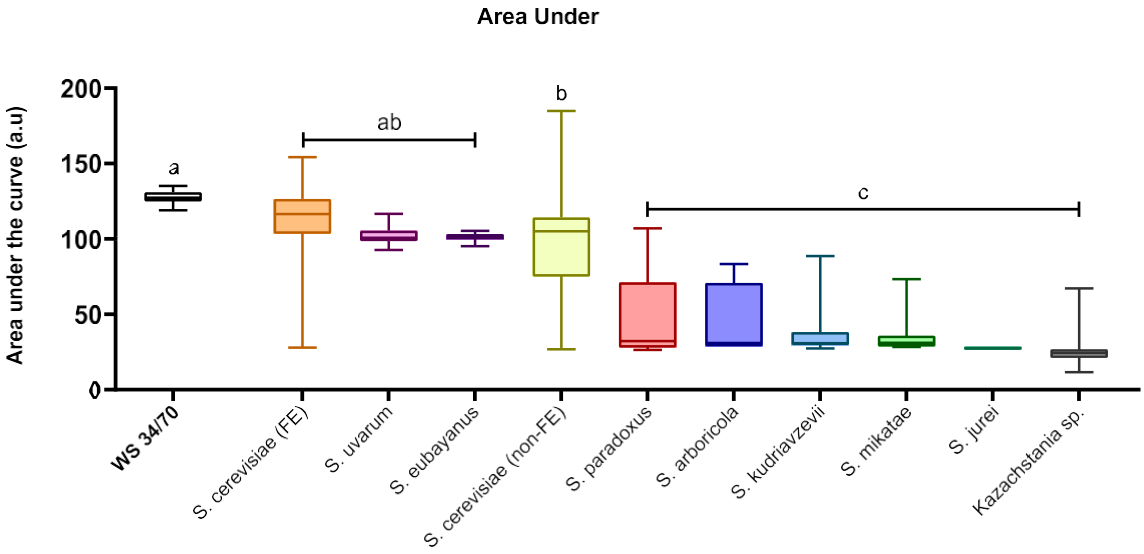

Fig. 1. Area under the curve analysis (arbitrary units) of yeast strains classified by species under brewing conditions in malt extract medium (15.5◦Bx) at 20 ◦C. The commercial reference is *S. pastorianus* strain WS 34/70. Letters indicate significant differences according to Tukey's HSD test ($p < 0.05$). Data for *Naumovozyma* and *Nakaseomyces* strains have been included in the *Kazachstania* sp. data set due to similar behaviour.