FEMS Yeast Research, 2025, **25**, foaf004
DOI: 10.1093/femsyr/foaf004
Advance access publication date: 29 January 2025
Research Article – Food and beverages

Experimental evolution and hybridization enhance the fermentative capacity of wild *Saccharomyces eubayanus* strains

Franco Vega-Macaya[1,2], Pablo Villarreal[1,2,3], Tomas A. Peña[1,2], Valentina Abarca[2,4], Agustín A. Cofré[1,2], Christian I. Oporto[1,4], Wladimir Mardones[2], Roberto F. Nespolo[2,4,5,6], Francisco A. Cubillos[1,2,4,*]

[1]Facultad de Química y Biología, Departamento de Biología, Universidad de Santiago de Chile, Santiago 9170022, Chile
[2]Millennium Institute for Integrative Biology, Santiago 7500574, Chile
[3]Centro Científico y Tecnológico de Excelencia Ciencia & Vida, Fundación Ciencia & Vida, Huechuraba, Santiago 8580702, Chile
[4]Millenium Nucleus of Patagonian Limit of Life (LiLi) Valdivia 5110566, Chile
[5]Instituto de Ciencias Ambientales y Evolutivas, Universidad Austral de Chile, Valdivia 5110566, Chile
[6]Center of Applied Ecology and Sustainability, Facultad de Ciencias Biológicas, Universidad Católica de Chile, Santiago, 6904411, Chile
*Corresponding author. E-mail: francisco.cubillos.r@usach.cl
Editor: [John Morrissey]

Abstract

Lager beer is traditionally fermented using *Saccharomyces pastorianus*. However, the limited availability of lager yeast strains restricts the potential range of beer profiles. Recently, *Saccharomyces eubayanus* strains showed the potential to impart novel aromas to beer, with slower fermentation rates than commercial strains. Here, we applied experimental evolution to nine *S. eubayanus* strains using three different selective conditions to generate improved strains to fermentative environments. We observed environment-dependent fitness changes across strains, with ethanol-enriched media resulting in the greatest fitness improvement. We identified subtelomeric genomic changes in a deficient fermentative strain underlying the greatest fitness improvement. Gene expression analysis and genome sequencing identified genes associated with oxidative stress, amino acid metabolism, sterol biosynthesis, and vacuole morphology underlying differences between evolved and the ancestral strain, revealing the cellular processes underlying fermentation improvement. A hybridization strategy between two evolved strains allowed us to expand the phenotypic space of the F2 segregants, obtaining strains with a 13.7% greater fermentative capacity relative to the best evolved parental strains. Our study highlights the potential of integrating experimental evolution and hybridization to enhance the fermentation capacity of wild yeast strains, offering strengthened solutions for industrial applications and highlighting the potential of Patagonian *S. eubayanus* in brewing.

Keywords: evolution; fermentation; beer; *Saccharomyceseubayanus*; hybridization; Patagonia

Introduction

Beer, the most consumed alcoholic beverage worldwide, is predominantly represented by lager beer, accounting for ∼90% of the beer market (Gibson et al. 2017). Lager beer is traditionally fermented using *Saccharomyces pastorianus*, an interspecies hybrid between *Saccharomyces cerevisiae* and *Saccharomyces eubayanus* (Libkind et al. 2011). Despite the robust fermentative capacity of *S. pastorianus* under diverse lager brewing conditions, the commercial availability of strains remains limited (Gallone et al. 2019). This limited availability of lager strains restricts the range of beer flavours and aromas produced (Mertens et al. 2015, Bonatto 2021). Consequently, there is an industrial concern regarding the limited repertoire of aroma profiles, emphasizing the need to generate new lager strains (Turgeon et al. 2021). A strategy to address this challenge is using alternative cryotolerant yeasts capable of fermenting at low temperatures (below 12°C) and imparting novel organoleptic properties to beer (Turgeon et al. 2021).

Saccharomyces eubayanus is a wild yeast described for the first time in 2011, isolated in the Argentinian Patagonia from *Nothofagus* trees (Libkind et al. 2011), and since then in New Zealand (Goddard and Greig 2015), North America (Peris et al. 2014), Tibeten plateau, East Asia (Bing et al. 2014), Chile (Nespolo et al. 2020), and Ireland (Bergin et al. 2022). Interestingly, *S. eubayanus* was the everlasting missing parent of *S. pastorianus* and is responsible for the hybrid's cryotolerance and ability to efficiently metabolize maltose, the primary sugar source in the wort. Identifying the lager yeast missing parent triggered a series of scientific efforts to study the fermentative potential of *S. eubayanus* to produce beers (Gibson et al. 2013, Mertens et al. 2015, Krogerus et al. 2017). Phylogenetic studies in *S. eubayanus* described five distinct lineages, including Patagonia A 'PA', Holarctic, Patagonia B 'PB-1', 'PB-2', and 'PB-3', together with different admixed strains derived from ancient crosses (Langdon et al. 2020, Nespolo et al. 2020). Interestingly, South America hosts the largest number of lineages and exhibits the greatest genetic diversity, suggesting that the species probably originated in Patagonia and subsequently migrated to the Northern Hemisphere (Langdon et al. 2020, Nespolo et al. 2020). Wild Patagonian *S. eubayanus* strains possess numerous advantages and desirable traits for beer fermentation, making them an attractive choice for brewing. For instance, they can consume

Received 17 December 2024; **revised** 25 January 2025; **accepted** 28 January 2025

maltose and produce a significantly higher number of volatile compounds than lager yeast, resulting in a more complex and aromatic profile (Krogerus et al. 2017, Urbina et al. 2020). However, *S. eubayanus* may exhibit slower fermentation rates at low temperatures than commercial lager-type strains, hampering its industrial use (Krogerus et al. 2015 , Mertens et al. 2015, Mardones et al. 2020). Different strategies have been applied to improve the fermentation capacity of *S. eubayanus* (Steensels et al. 2014, Mertens et al. 2015, Baker and Hittinger 2019, Mardones et al. 2022), including classical techniques such as experimental evolution, which have demonstrated great success in generating food-grade starter cultures (Baker and Hittinger 2019, Brouwers et al. 2019, Mardones et al. 2022).

Experimental evolution approaches are based on the selection of new genetic variants that arise during the clonal growth of a culture (Mardones et al. 2022). In this process, a population of cells is continuously selected for a specific trait of interest. Over multiple generations, novel mutants undergo successive bottlenecks, favouring individuals with improved fitness under the selective environment (Steensels et al. 2014). Although, experimental evolution has successfully yielded various *S. eubayanus* strains with enhanced fermentative capacity under diverse environmental conditions (Baker and Hittinger 2019, Brouwers et al. 2019, Mardones et al. 2022), it remains unclear whether the molecular solutions for adaptation to fermentative environments are shared among different genetic backgrounds depending on the selective media. In this sense, previous studies reported convergent molecular solutions under various environments. For example, evolved lines under carbon-limited chemostat cultures on maltotriose-enriched wort (Brouwers et al. 2019) or low maltose levels (Baker and Hittinger 2019), yielded *S. eubayanus* strains able to grow on maltotriose due to the emergence of a novel mosaic transporter resulting from a translocation event between maltose-encoding transporters. Even though experimental evolution has shown promising results in improving desired traits, the evolving potential of these strains is limited. Experimental evolution strategies generally start from a clonal population and propagated for a limited time (Steensels et al. 2014). The low initial genetic diversity due to the clonal ancestral population and the constant bottlenecks during propagation are the main factors of the limited potential evolvability (Ogbunugafor 2023). An alternative to generating robust strains with fitness levels suitable to reach industrial-quality levels is hybridization. This strategy has proven to be a successful methodology for creating strains with higher fitness than parental strains, known as hybrid vigour, by leveraging a genetic diversity boost (Gerke et al. 2009, Abreu-Cavalheiro and Monteiro 2013, Gabaldón 2020). Unlike mutation accumulation experiments, hybridization is based on combining two distinct genetic backgrounds with desired traits and phenotypes, generating a unique strain that can show higher fitness and phenotypes to their parental strains, known as heterosis (Naseeb et al. 2021). Hybrids not only show better advantages than experimental evolution but can be sporulated to generate transgressive segregants with extreme and superior phenotypes due to the hereditary combination of alleles of the two parental strains (Chimeric effect), providing advantages and further improving the desired phenotypes (Sipiczki 2018), hinting that the combination of these strategies for improving wild yeast could lead to the generation of stable, superior, and novel strains (Mertens et al. 2015, Krogerus et al. 2021).

In this study, we applied experimental evolution to nine distinct *S. eubayanus* strains from diverse lineages across three selective media. We assessed fitness changes across evolutionary lines and environments and identified the genomic changes in strains with notable fitness gains under wort fermentative conditions. Additionally, we evaluated the impact of hybridization and segregant generation alongside experimental evolution, resulting in new *S. eubayanus* strains with enhanced fermentative capacity. These findings offer valuable insights into the genotype-by-environment interactions shaping yeast adaptation for industrial applications.

Materials and methods

Strains and culture media

Nine *S. eubayanus* strains used in this study are isolates collected from different localities in Chilean Patagonia and were previously reported in (Nespolo et al. 2020) (Table S1). Furthermore, we used the *S. pastorianus* Saflager W-34/70 strain (Fermentis, France) strain as a lager fermentation control. All isolates were maintained in YPD agar media (yeast extract 1%, peptone 2%, glucose 2%, and agar 2%) and stored at −80°C in 20% glycerol stocks.

Experimental evolution

The experimental evolution assay was performed as previously described (Mardones et al. 2022). Briefly, a colony of each *S. eubayanus* strain was cultured in 0.67% yeast nitrogen base (YNB) media (Difco, France) supplemented with 2% glucose at 20°C and 150 rpm orbital shaking for 24 h as a preinoculum for the experimental evolution assay. After 24 h, an inoculum was used to perform the experimental evolution assay at a cell density of 1×10^6 cells ml^{-1}. The assay was performed in three different evolution culture mediums for each strain: (a) 0.67% YNB media (Difco, France) supplemented with 2% maltose (hereafter referred to as CLAEM cultures), (b) 0.67% YNB media (Difco, France) supplemented with 2% glucose (w/v) and 9% ethanol (v/v) (hereafter referred to as CLAET cultures), and (c) 12° Brix malt extract medium (hereafter referred to as CLAEX cultures). The CLAEM and CLAET cultures evolved at 20°C with constant orbital shaking at 150 rpm and transferred every three days. For the CLAEX cultures, the assay was carried out at 12°C without agitation, and transfers were performed every 7 days. Subsequently, the cultures were used to inoculate fresh 5 ml media for CLAET and CLAEM cultures, and 50 ml for CLAEX cultures, at an inoculum density of 1×10^6 cell ml^{-1}, and this procedure was sequentially repeated to an estimated number of generations. The number of generations was estimated using the 'generations = log (final cells−log initial cells)/$\log_2$' formula, summing up the number of cells per ml doublings between every culture transfer during the experimental evolution assay.

Phenotyping assay

The micro-cultivation phenotyping assay was performed as previously described (Nespolo et al. 2020). Briefly, isolates were precultivated in 200 μl 0.67% YNB medium supplemented with glucose 2% (w/v) for 48 h at 25°C. Next, strains were inoculated to an optical density (OD) of 0.03–0.1 (wavelength 630 nm) in 200 μl growth media, where the following conditions were considered: glucose 2%, maltose 2%, and ethanol 9% at 12° Plato malt extract. The assays were performed without agitation at 20°C for 24 or 48 h (depending on the condition) using a Tecan Sunrise absorbance microplate reader. The OD was measured every 30 min using a 630 nm filter. Each genotype was grown in triplicate. The maximum growth rate parameter was obtained for each strain using the previously described Gompertz model (Tjørve and Tjørve 2017,

Molinet et al. 2022). Statistical analyses were performed using the R software and the package (Team).

Fermentations on malt extract

Fermentations were carried out as previously described (Mardones et al. 2020, Urbina et al. 2020). Briefly, fermentations were performed in at least three biological replicates, in 12° Plato of malt extract. For this, a colony was transferred to 5 ml 6° Plato of malt extract supplemented with 0.3 ppm $ZnCl_2$ and incubated at 20°C with orbital shaking at 150 rpm for 24 h. Then, the complete preinoculum was transferred to 50 ml 12° Plato of malt extract and incubated in similar conditions for 24 h. Cells were then counted and utilized to inoculate a 50 ml 12° Plato fresh culture of malt extract to a final concentration of 1.8×10^7 cell ml^{-1}. Cultures were maintained at 12°C for 14 days without agitation and CO_2 production was measured. For this, we tracked the weight loss of fermentation bottles over time, which directly correlates with the amount of CO_2 released. Fermentations were conducted in sealed bottles with an airlock to allow gas escape while preventing contamination. Daily weight loss measurements were recorded to calculate the total CO_2 produced during the fermentation process.

A 1 ml aliquot was obtained each day for metabolite quantification, and glucose, fructose, maltose, maltotriose, ethanol, and glycerol were estimated using high-performance liquid chromatography (HPLC) as previously described (Nespolo et al. 2020, Urbina et al. 2020). Segregants fermentative capacity was evaluated on 20 ml microfermentations.

Sequencing of evolved lines and mutation identification

DNA extraction was performed as previously described (Mardones et al. 2020, Nespolo et al. 2020). Sequencing libraries were prepared using the Illumina Nextera protocol and sequencing was performed in a NextSeq 500/550 Mid Output Kit v2.5 (300 Cycles) in the Genomics and Bioinformatics Unit at Universidad de Santiago de Chile. Reads were filtered and trimmed using the Fastp 0.19.4 tool (–3 -l 50 –cut_mean_quality 30) (Chen et al. 2018, Molinet and Cubillos 2020). Reads were aligned against the *S. eubayanus* CBS12357T reference genome (Brickwedde et al. 2018) using BWA-mem (Li). Sequence parameters of evolved lines are available in Table S2. Mapping files were tagged for duplicates using Picard tools 2.18.14 (http://broadinstitute.github.io/picard/). Variant calling and filtering were done using GATK version 4.0.10.1 (DePristo et al. 2011) against the CBS12357T reference genome. For all datasets, we only considered single nucleotide polymorphisms (SNPs) that had no missing data using vcftools option–max-missing 1. Once VCFs for each evolved and parental strains were obtained, we identified variants exclusive to the corresponding evolved strain using bedtools intesect v2.30.0 (Quinlan and Hall 2010). To avoid false positives between evolved and parental strain, we performed an additional filtering step comparing the vcf files against the CL248.1 genetic background (Mardones et al. 2022). The effect of each evolved variant was assessed and annotated using SnpEff version 4.3 (Cingolani et al. 2012). Reads are available in the Biosample Database PRJNA994921.

RNA sequencing and differential expression analysis

Fermentation was carried out as previously described for RNA extraction. A volume of 1 ml of wort supernatant was collected after 24 h of fermentation. The RNA was extracted using the E.Z.N.A. Total RNA Kit I (Omega Bio-Tek, Waltham, MA, USA). RNA was treated with DNase I (Thermo Fisher, USA) and then purified using the RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany). RNA integrity was confirmed using a Fragment Analyzer (Agilent, Saanalynta Clara, CA, USA). The RNA-seq libraries were constructed using the TruSeq RNA Sample Prep Kit v2 (Illumina, San Diego, CA, USA). The sequencing was conducted using paired-end 150 bp reads on an Illumina NextSeq 500 as previously mentioned. Reads were mapped to the *S. eubayanus* $CBS12357^T$ reference genome using STAR ver. 2.7.3 (Dobin et al. 2013) and analysed using featureCounts in R (Liao et al. 2014). Differential expression was analysed using the DESeq2 package in R (Love et al. 2014). Genes exhibiting expression changes > 1.5 and an adjusted $P < 0.05$ were considered differentially expressed genes (DEGs). Reads are available in the Biosample Database Project PRJNA994921.

Gene ontology and biological pathway (KEGG) enrichment analysis was performed with the R package enrichGO using *S. cerevisiae* 288C as a model. The analysis was carried out with a significance threshold of adjusted $P < 0.05$ (Fisher one-tailed test) (https://rdrr.io/github/GuangchuangYu/clusterProfiler/man/enrichGO.html).

Hybrid and segregant strains generation

The intra-specific hybrids were generated by spore-cell mating between CLAET815.1 and a *ura3* auxotrophic haploid derivate of CLEtOH5.1. The haploid derivate was generated as previously described (Molinet et al. 2022), and denominated CLEtOH5.1-α (*Mat* α, *ura3::ho*Δ). Strains were sporulated in 2% of potassium acetate for 10 days at 20°C. A volume of 5 µl of zimolease (1 µg/ml) was added to 100 µl of cells suspension to break the tetrads' cell wall. Spores from CLAET815.1 were dissected and placed next to a CLEtOH5.1-α cell on YPD agar plates using a Singer SporePlay micromanipulator (Singer Instruments, UK). Each YPD plate was incubated at 20°C for 72 h. After that, hybrid status was confirmed by amplifying the *HO* gene, from which hybrids should be heterozygous (Molinet et al. 2022). The selected hybrid was segregated by sporulation and dissection as previously described. Segregant status was confirmed by amplification of *MAT locus*, as we expected that due to the heterozygous *HO* gene nature of the hybrid, each tetrad spores should have a mate type pattern of two heterozygous (a/α) and two homozygous (α/α, a/a) (Catallo et al. 2021).

Results

Characterization of the fermentative profile of *S. eubayanus* strains

To improve the fermentative capacity of *S. eubayanus* strains under brewing conditions, we selected eight strains from Patagonian lineages previously shown to have the highest fermentation capacity in the species (Nespolo et al. 2020). Additionally, we included the CL815.1 strain from Tierra del Fuego, which had previously demonstrated an exceptionally low-fermentative capacity (Table S1), (Nespolo et al. 2020). In this study, all selected strains were initially characterized for their fermentation capacity and metabolite consumption in malt extract under lager temperature fermentation conditions (12°C, Table S3). The assay revealed significantly lower carbon dioxide (CO_2) production levels for six strains compared to the commercial lager strain *S. pastorianus* W34/70 (hereafter referred to as: 'lager yeast') after 14 days of fermentation ($P < 0.05$, ANOVA, Fig. 1A). Notably, the low-

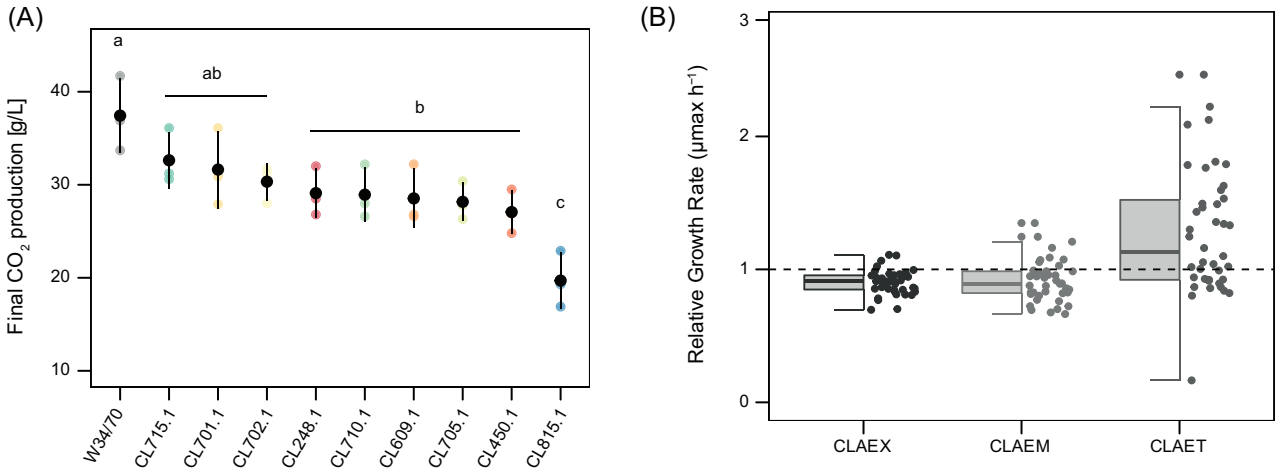

Figure 1. Phenotypic diversity across *S. eubayanus* strains. (A) Final CO_2 production values (g/l) were obtained in 12° Plato malt extract. Each color represents a different parental strain, and the different letters indicate significant differences between the lager yeast W34/70 and the *S. eubayanus* strains ($P < 0.05$, ANOVA). (B) Relative growth rate (μmax) values of the evolved lines compared to the corresponding parental strain in the respective environment. Each dot represents an evolution line.

fermentation strain CL815.1 exhibited 47% lower CO_2 production levels than the lager control strain ($P < 0.05$, Analysis of variance, ANOVA, Fig. 1A). Interestingly, the strains CL702.1, CL701.1, and CL715.1, which belong to the PB-2 lineage, did not show significant differences in CO_2 production compared to the commercial strain ($P > 0.05$, ANOVA, Fig. 1A). Under our microfermentation system, all strains showed a complete glucose, fructose, and maltose sugar consumption after 14 days of fermentation, except CL815.1, which exhibited a 16% of residual maltose (Table S3, $P < 0.0001$, ANOVA). As previously described in *S. eubayanus*, no maltotriose consumption was detected. In this sense, most strains exhibited a lower fermentation capacity than the lager commercial strain *S. pastorianus* W34/70.

To obtain *S. eubayanus* strains with increased fermentative capacity and investigate their adaptation potential to different environments, we performed a parallel evolution assay under three distinct conditions: (i) 12° Plato malt extract (CLAEX), (ii) YNB + maltose 2% (CLAEM), and (iii) YNB + glucose 2% + ethanol 9% (CLAET). The evolution assay was performed over 6 months, resulting in 180 generations for CLAEX, 280 generations for CLAEM, and 250 generations for CLAET. Following the evolution period, we evaluated the microbial growth of the evolved lines under their respective selective media (Table S4). Notably, under both CLAEX and CLAEM conditions, all evolved lines exhibited a significant decrease in μmax compared to their corresponding parental strain (Figure 1B and Figure S1A–D). In contrast, the CLAET-evolved populations showed a significant increase in μmax (Fig. 1B and Fig. S1E–F), indicating greater fitness in an ethanol-enriched environment after the evolution assay.

To estimate the variance explained by genotype and environment, we determine the impact of each variable and their interaction on the evolved lines fitness. We used the μmax data of all evolved strains, measured in the corresponding evolution condition used for genetic improvement. Our data indicated the absence of genotype effect ($F_{1,75} = 0.051$ $P = 0.82$, two-way ANOVA) and the absence of a significant genotype-environment interaction ($F_{1,2} = 0.48$ $P = 0.62$, two-way ANOVA). The only significant effects we found on fitness, was a main statistical significance on medium ($F_{1,2} = 63.8$, $P << 0.001$, two-way ANOVA). These findings suggest that different genotypes reacted similarly to the media (Fig. S2): While some conditions (CLAEX and CLAEM) exerted negative or neutral effects on fitness, others (CLAET) had a positive effect.

Fermentation capacity in evolved lines

Microfermentations were conducted under lager beer conditions for each evolved population to assess the impact of different environmental conditions during evolution on fermentation performance. For this, each evolutionary line's fermentation capacity was evaluated and compared to that of the parental strain (Fig. 2A and Fig. S3). Our result showed a significant increase of 59% and 49% in CO_2 production (g/l) ($P < 0.05$, one-way ANOVA) during fermentation in an evolved population (CL815.1) under CLAEM and CLAET conditions, respectively ($P < 0.05$, Fig. 2A, and Table S5). Interestingly, two populations (CLAEM248 and CLAET715) also exhibited significant results ($P = 0.05$, one-way ANOVA) with an improved fermentative performance compared to the corresponding parental strain. On the other hand, the rest of the evolved lines did not exhibit any significant change compared to parental strains ($P > 0.05$, Fig. S3).

Subsequently, we used HPLC to analyse sugar consumption after fermentation. Our results revealed that the CLAEM815 and CLAET815 evolved lines exhibited an enhanced maltose consumption compared to the parental strain, which likely contributes to their greater fermentative capacity (Table S6). Although the CL450.1 strain showed the lowest fermentative capacity after the evolution assay in all three tested conditions, these strains consumed all available maltose (CLAEX, CLAEM, and CLAET) (Table S6). These findings suggest that CL815.1, the strain with the lowest initial fermentative capacity, experienced the greatest increase in fitness under fermentative conditions compared to all other selected strains in two of the three different environments (CLAEM and CLAET). Overall, these results suggest that the fitness improvement observed during evolution is genotype-dependent, highlighting the role of the genetic background in the adaptive response.

To select individuals with higher fermentative capacity than the parental lines, we isolated colonies from the CLAEM815 and CLAET815 evolved lines. This was done by obtaining colonies from YNB plates supplemented with 3% maltose and 6% ethanol, selecting the largest single colonies from each evolution line. In this

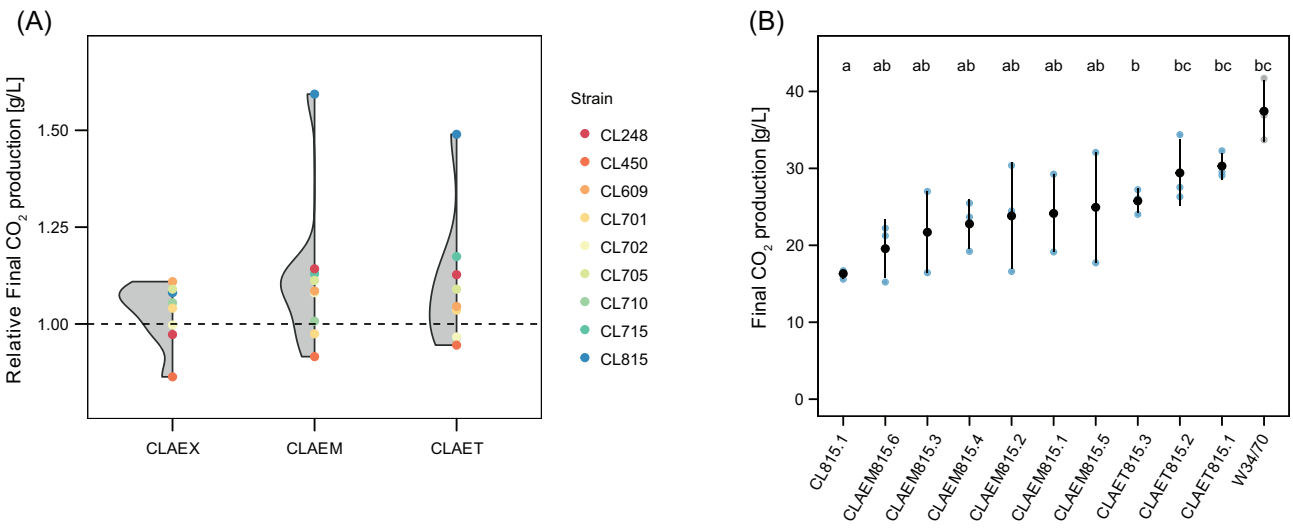

Figure 2. Fermentative capacity of evolved lines. (A) Relative Final CO_2 [g/l] production of all the evolved lines compared to the corresponding parental strain under lager fermentation conditions. Each dot represents the average of three independent replicates. (B) Final CO_2 [g/l] production under lager fermentation conditions in CL815 evolved individuals obtained from CLAEM and CLAET conditions. We used reference values of the lager yeast strain W34/70 for comparisons. Different letters reflect statistically differences between strains with a $P < 0.05$, *t*-test.

way, we isolated nine colonies, six and three, from the CLAEM and CLAET evolution lines, respectively. We then evaluated the CO_2 production capacity of these isolated individuals under lager fermentation conditions. Through this approach, we identified three strains from the CLAET815 population that exhibited significantly enhanced CO_2 (g/l) production compared to the corresponding parental strain: CLAET815.3 (58% higher), CLAET815.2 (80,32% higher), and y CLAET815.1 (85,78% higher), indicating a fitness increase under fermentative conditions (Fig. 2B, Table S7). On the other hand, the individuals obtained from the CLAEM815 line only showed a marginally significantly higher CO_2 (g/l) production compared to the parental strain ($P < 0.1$, *t*-test, Fig. 2B). These findings indicate that the improvement in fermentative capacity was genotype-dependent and influenced by the specific environmental stress, with YNB supplemented with ethanol being the condition that resulted in the greatest increase in fermentation capacity across all evolved lines.

Genomic changes in evolved clones

To investigate whether the genomic changes associated with the fitness increase in the evolved clones from CL815.1 were shared across different environments, we performed whole genome sequencing on two CLAET clones (CLAET815.1 and CLAET815.3) and one CLAEM clone (CLAEM815.4). Sequencing data were carefully filtered to retain only high-quality SNPs, resulting in the identification of 46, 38, and 40 unique SNPs in CLAET815.1, CLAET815.3, and CLAEM815.4, respectively (Fig. 3A, Table S8). We observed that most of these SNPs were located in the subtelomeric regions of most chromosomes in all three individuals (Fig. 3B). Interestingly, 34,8%, 44,7%, and 67,5% of the identified SNPs impacted coding genes in CLAET815.1, CLAET815.3, and CLAEM815.4, respectively (Fig. 3A). Upon further analysis of the mutations, we found that CLAEM815.4 exhibited the highest number of genes (eight genes) carrying mutations, missense, and nonsynonymous mutations. In contrast, CLAET815.1 and CLAET815.3 had fewer mutated genes (3 and 7 genes, respectively) (Table S8). Comparative analysis of the SNP data between the parental lines and evolved clones revealed that none of the mutations were shared across evolved clones, indicating genetic divergence resulting from the experimental evolution process. Furthermore, none of the mutations were found to impact the same gene. Notably, CLAET815.1, which harbored the lowest number of mutations within coding regions, displayed the highest fermentation performance among the evolved clones (Fig. 2B, Table S8).

To identify candidate genes that likely underlie the phenotypic differences between the evolved clones and CL815.1, we looked at those mutated genes in CLAET815.1 exhibiting a predicted functional effect based on a SnpEff analysis (Table S9). In this way, we found two candidate genes: *ECM14*, which encodes for a protein required for normal cell wall assembly (McDonald et al. 2020), and *PEP1*, which encodes for a Type I transmembrane sorting receptor for multiple vacuolar hydrolases (Marcusson et al. 1994). The *ECM14* gene is localized in chromosome XV and contains a missense mutation: 100T > G that changed Tyr34Asp (Table S9). The *PEP1* gene is localized on chromosome XIV and contains multiple missense and synonymous mutations (Table S9). These results suggest that the genomic changes associated with the fitness increase in the evolved clones from CL815.1 may not be fully shared across different environments. Variations in the number and impact of mutations among evolved clones indicate a certain level of genotype-specific adaptation to selective conditions. Since only 34,8% of SNPs mapped to coding regions, we explored the regulatory role of noncoding SNPs using an RNA-seq approach.

Changes in gene expression patterns in evolved clones.

We implemented a transcriptome approach to assess the effect of genetic modifications on the gene expression profile of the evolved individuals during fermentation (Table S10). We used the CLAET815.1 individual, which exerted the greatest fermentation improvement across strains (Fig. 2B). Thus, we identified DEGs between the evolved line CLAET815.1 and the parental strain CL815 after 24 h of fermentation on malt extract, under lager fermentation conditions. Our data showed 547 DEGs in the evolved strain, where 369 were up- and 178 down-regulated during the first 24 h of fermentation (Table S11). Interestingly, within the group of up-regulated DEGs in CLAET815.1 (Fig. 4A, Table S11), we found several genes related to oxidative stress such

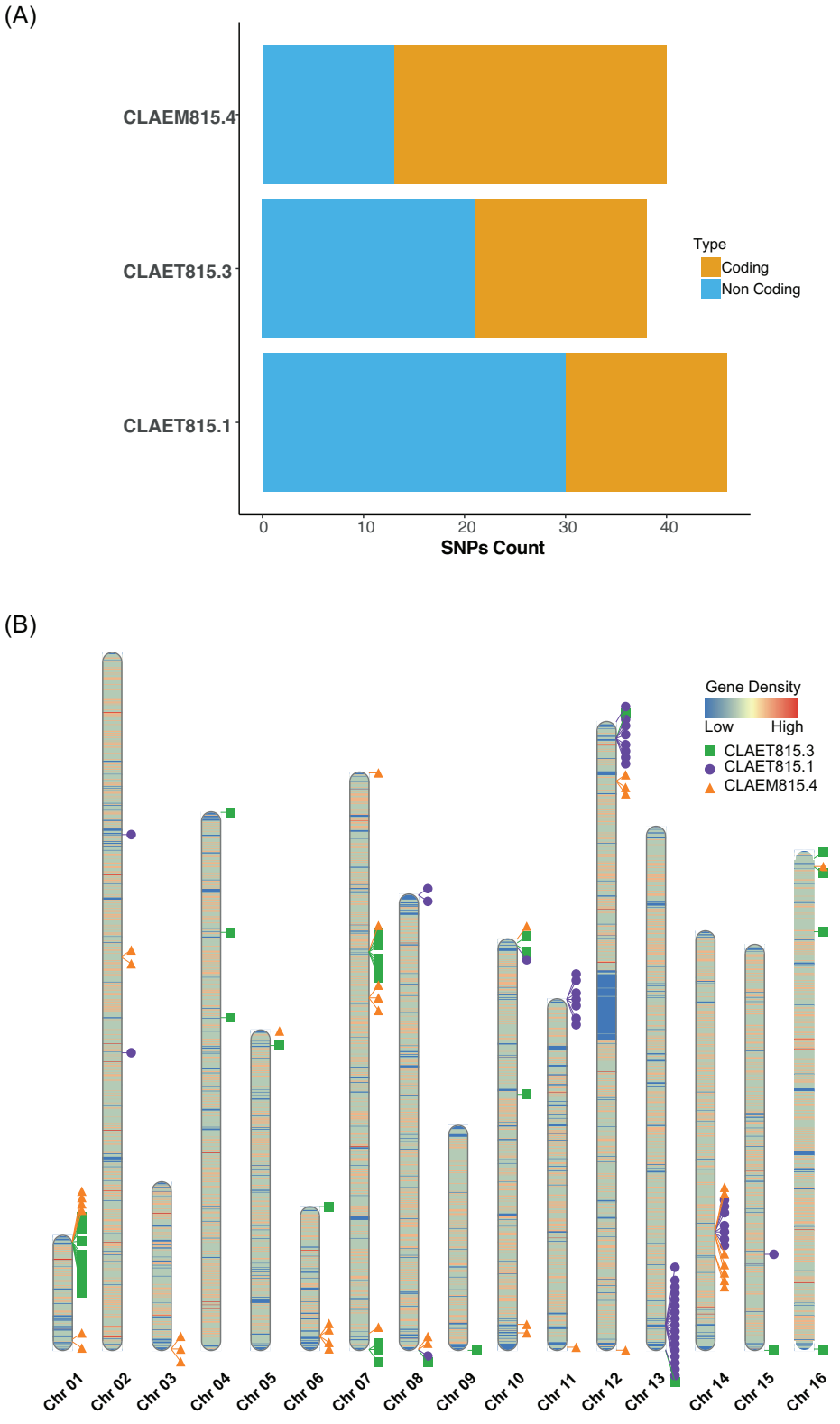

Figure 3. Mutation profile of CL815.1 evolved clones. (A) Total SNPs identified on each evolved strain in coding and noncoding regions. (B) SNPs distribution across the genome. The heatmap represents the gene density on a 1-kb windows analysis. Figures (square, circle, and triangle) and colours (green, purple, and orange) represent SNPs from CLAET815.3, CLAET815.1, and CLAEM815.4, respectively.

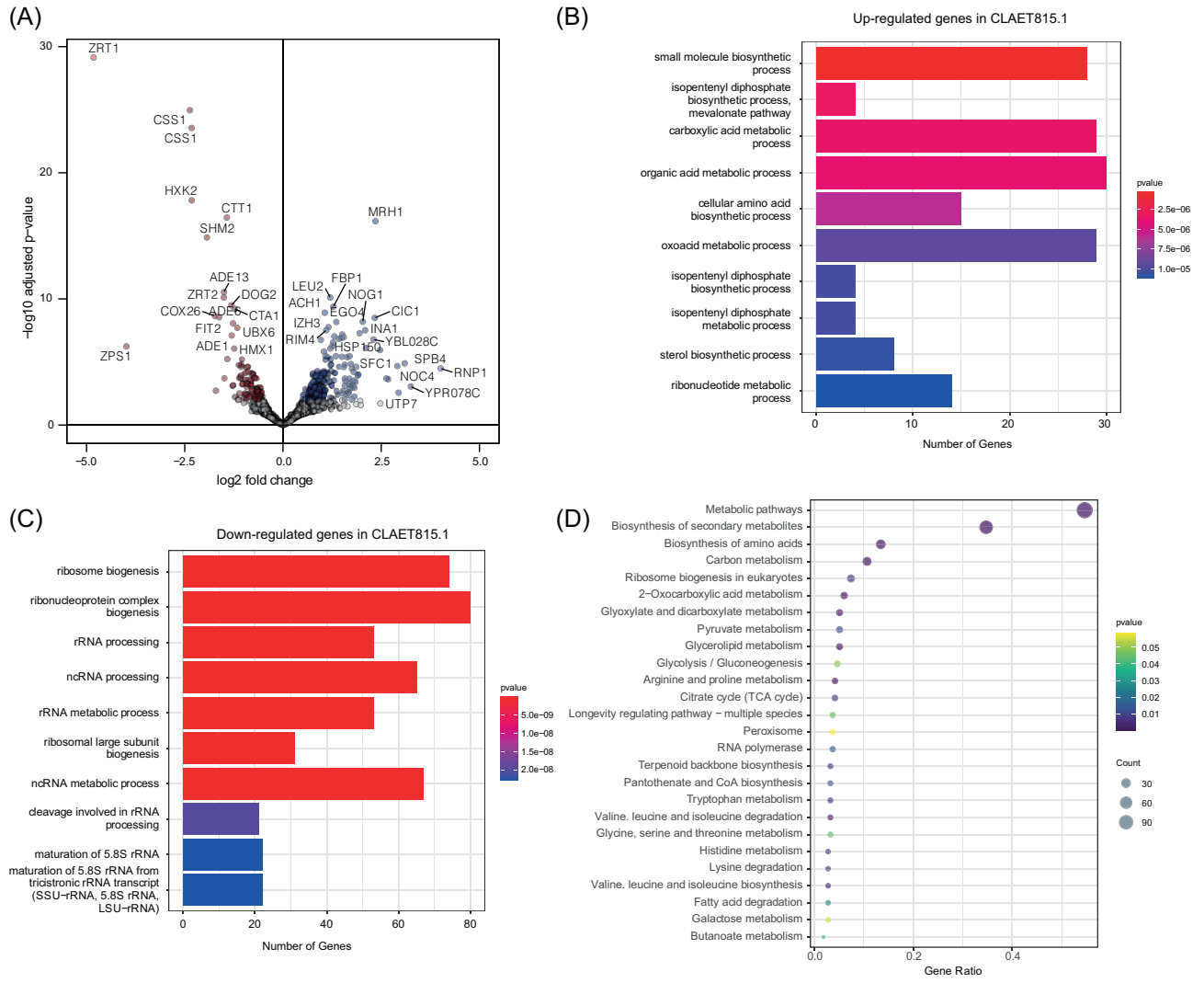

Figure 4. Differential gene expressions between CLAET815.1 and CL815.1 under lager fermentation conditions. (A) Volcano plot depicts differentially expressed genes ($P < 0.01$). Up-regulated and down-regulated genes in CLAET815.1 relative to CL815 are shown in blue and red dots, respectively. (B) Number of genes occurring in gene ontology (GO) terms (biological processes) enriched in CLAET815.1. (C) Number of genes occurring in GO terms (biological processes) enriched in CL815. D. KEGG pathway enrichment analysis of DEGs. The color bar indicates the *P-value* (< 0.05, Fisher's one-tailed test) .

as *HMX1* (encodes for a heme oxygenase), *HXK2* (encodes for a hexokinase involved in glucose repression) and *YBP1* (encodes for a protein involved in cellular response to oxidative stress, Fig. 4A), suggesting a higher tolerance to stress conditions during fermentation.

Enrichment analysis of GO terms associated with DEGs highlighted the upregulation of sterol biosynthetic process genes and the downregulation of ribosomal genes in the evolved strain (Fig. 4B and C). Interestingly, the evolved strain exhibited a series of genes upregulated related to different biological processes, such as diauxic shift (*EGO4*), amino acid metabolism (*LEU2*, *PRO1*, and *PRO3*), and sterol synthesis (*ERG9* and *ERG10*). This upregulation could explain the improvement in fermentation performance relative to its parent and other evolved lines. Next, to identify pathways significantly enriched across DEGs, we performed a KEGG enrichment analysis (Fig. 4D). We found that most of the DEGs were part of the Metabolic pathway (Table S12, Fig. S4, $P < 0.05$, Fisher's one-tailed test) and the Biosynthesis of secondary metabolites pathway KEGG categories (Fig. S5, Table S12, $P < 0.05$, Fisher's one-tailed test). Additionally, the Arginine and Proline metabolism pathway was enriched with DEGs between the CL815.1 parental and the evolved strain (Fig. 4D, Table S12, $P < 0.05$, Fisher's one-tailed test). We found within this pathway DEGs such as *PRO1*, *PRO3*, *PUT1*, *PUT2*, and *PUT3*, all of them encoding for proteins involved in the proline metabolism, likely suggesting intracellular proline accumulation, which has been related to high ethanol concentrations tolerance. Together, these results demonstrate a positive effect of evolution in the CLAET environment on fermentation-related genes (oxidative stress, amino acid metabolism, and sterol biosynthesis), suggesting that they are partly responsible for the increased fermentation performance of the parental strain CLAET815.1.

Generation of interspecies segregants with improved fermentative capacity

To assess whether we could further improve the fermentative capacity of *S. eubayanus* strains, we generated an intraspecific hybrid utilizing CLAET815.1, and CLEtOH5.1, a previously evolved *S. eu-*

bayanus strain with high-fermentation capacity (Mardones et al. 2022). A total of 28 putative hybrids were obtained by spore-to-spore mating, from where three strains were confirmed as hybrids by genotyping the *MAT* locus (ACS15, ACS19, and ACS25). Then, we compared the fermentative capacity between hybrids and parental strains under brewing conditions, where no significant differences were found. These results indicate this trait's lack of hybrid vigor in *S. eubayanus* (Fig. 5A, Table S13).

Considering this, we arbitrarily selected the hybrid ACS25 to generate 210 F2 diploid segregants. To assess many individuals simultaneously, we set up small-volume microfermentations in 20 ml and measured daily CO_2 production. We identified 127 and 6 negative and positive transgressive segregants, respectively (Table S14). Interestingly, the ACS25.37 segregant exhibited the highest values, evidencing a 22.9% increase in CO_2 production relative to the CLEtOH5.1. In comparison, the segregant with the lowest performance showed a 55.7% CO_2 production decline (g/l), relative to CLAET815.1 ($P < 0.05$, one-way ANOVA) (Table S14). To validate our results under larger fermentation volumes, we selected the 10 best and 10 worst segregants based on their mean fermentative capacity and scaled them up to 50 ml fermentations (Fig. 5B, Table S15). Our results indicated that increasing the fermentation volume affected the fermentative capacity of the tested segregants. Interestingly, only ACS25.37 and ACS25.36 showed significant improvement, with CO_2 production levels increasing by 13.7% and 11.6%, respectively, compared to CLEtOH5.1 ($P < 0.05$, one-way ANOVA). On the other hand, 7 out of the 10 worst segregants showed a lower fermentation capacity relative to CLAET815.1. These results demonstrate that an experimental evolution assay coupled with hybridization and a single round of sexual reproduction significantly improves the fermentative capacity of wild *S. eubayanus* strains.

Discussion

In this manuscript, we comprehensively characterize the genomic and phenotypic changes of different *S. eubayanus* strains evolved under different fermentation-related conditions and hybridized generating novel strains with improved phenotypes. Our set of *S. eubayanus* strains exhibited a high fermentative capacity under lager-type fermentation conditions, which is consistent with previous reports (Nespolo et al. 2020). Among these, there are three strains: CL702.1, CL701.1, and CL715.1 strains, showed no significant differences compared to the commercial lager yeast strain W34/70, demonstrating the potential of *S. eubayanus* for brewing. All strains efficiently consumed simple sugars like glucose and fructose and demonstrated efficient consumption of maltose, except for the CL815.1 strain, which exhibited lower maltose consumption. Although our yeast set displayed high levels of CO_2 production (g/l), they reached their maximum CO_2 production levels at later time-points compared to commercial stocks, hampering their broader utilization. In this sense, the fermentative capacity of native strains of *S. eubayanus* can be improved to produce beers with novel organoleptic properties, providing a wider genetic diversity to innovate in lager brewing. Furthermore, our study highlights the potential to generate strains with greater fermentative capacities like commercial strains, often used based on historical grounds (Steensels et al. 2014).

Experimental evolution in yeast represents one of the most successful approaches for acquiring promising new yeasts for brewing. This approach increases the genomic background diversity of native strains, developing novel fermentation profiles and improved properties for lager beers (Hope et al. 2017, Mardones et al. 2022). We employed three evolution media (ethanol, maltose, and malt extract) to evaluate the environmental effects to simulate fermentative environments. Only the ethanol and maltose-based media improved populations among these conditions. In contrast, the malt extract medium, which would better simulate the fermentation environment, failed to produce improved populations without generating genotype-per-strain interaction in fitness. These differences may also be determined by the number of generations and the effective population size per condition, where longer periods between passages and smaller population sizes, such as the one in malt extract, may be inefficient in producing fitter mutants given the lower number of generations (McDonald 2019). Instead, conditions with shorter passages in the period and mutagenic compounds can increase the likelihood of obtaining fitter cells. The variation in the effect of evolution media on our yeast set could be attributed to variations in the mutations accumulated throughout the different passages. Interestingly, our results agree with the prediction by Gao et al. (2016), suggesting that time-dependent mutations should be higher in populations with rapid growth and high division rates. In addition, the spontaneous mutation rate of yeast varies with the environment and stress factors (Liu and Zhang 2019). Stress-induced mutagenesis rates might be higher in media containing ethanol, accelerating the capacity for improvement in these environments and enhancing fermentative abilities in fewer generations. Ethanol has a mutagenic effect, increasing the mutation rate in yeast by recruiting error-prone DNA polymerases and causing DNA replication stress and increased mutation rates (Voordeckers et al. 2020). However, our assay did not detect significant differences in the number of mutations between the ethanol and maltose evolution media. Interestingly, the clone that evolved under maltose exhibited the greatest number of moderate changes in coding regions across the genome. Despite this, none of these mutations showed an evident phenotypic connection to maltose metabolism and highlighted the different mutational landscapes depending on the selective condition. The potential effects of these changes could be further explored in future studies. It is important to note that our experimental design included only three evolved clones, which may need to provide more resolution for a comprehensive comparison but represents an initial assessment. Sequencing a larger number of clones per condition could offer a more detailed and accurate evaluation of the mutational landscape between the different conditions.

Our study contributes to understanding yeast adaptation to stressful biotechnological conditions, such as fermentation environments, which lays the foundations for a more robust characterization of yeast molecular improvement mechanisms in wild strains for subsequent industrial applications. Here, we obtained molecular evidence of the genetic changes in a strain from Tierra del Fuego that evolved under different experimental evolution conditions, proving evidence of alternative mechanisms towards adaptation (Foster et al, 2004 , Brouwers et al. 2019). Our results show that an exceptionally low fermentative strain (CL815.1) recapitulates the fermentative capacity of those strains with high fermentative capacity in *S. eubayanus* (Mardones et al. 2022). This strain was the only genetic background to improve ethanol tolerance and maltose consumption and acquire a greater fermentative capacity. The genomic analysis of mutants exhibiting a greater fitness allowed us to suggest that changes in regulatory regions significantly drove the adaptation. Here, we report the enrichment of the glycolysis and gluconeogenesis KEGG pathway, and the differential expression of a gene related to diauxic shift (*EGO4*). Previous studies have demonstrated that differen-

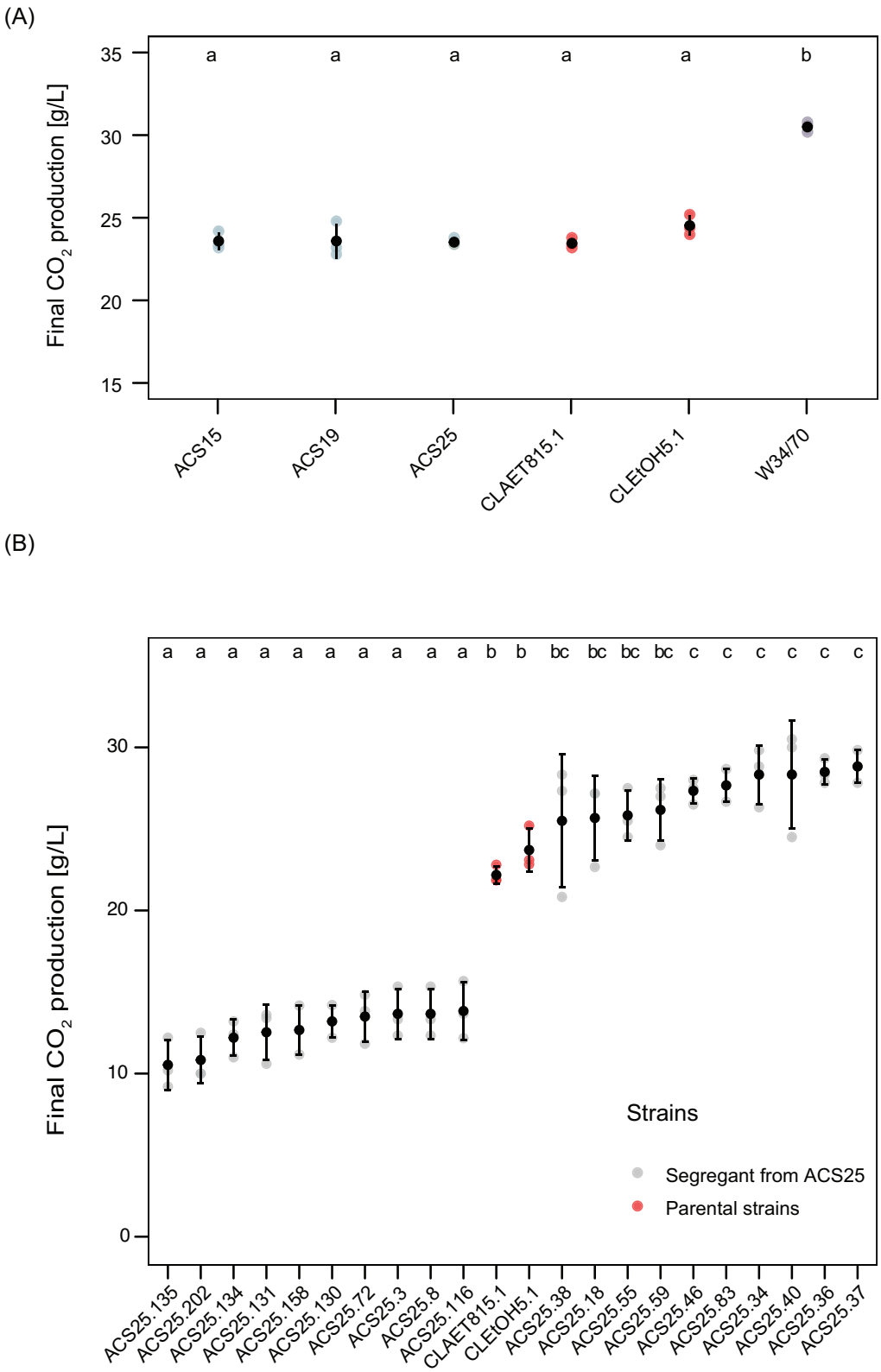

Figure 5. Fermentative capacity of intraspecific hybrid and segregants strains. (A) Final CO_2 [g/l] production under lager fermentation conditions of hybrid strains. Each coloured dot indicates individual replicates ($n = 3$). (B) Top and bottom performance segregants under 50 ml volume fermentations. Letters depict statistical differences between strains with a $P < 0.05$, ANOVA.

tial expression of genes related to glucose uptake and dynamic diauxic shift would positively affect the fermentative capacity of yeast strains (Mardones et al. 2022). Additionally, it has been shown that the *PUT* and *PRO* gene families, which control proline metabolism, are related to ethanol tolerance (Takagi 2008). Consistent with this, increased intracellular proline concentration significantly improves tolerance to high ethanol concentrations (Takagi 2008, Takagi et al. 2016, Villarreal et al. 2024). These findings correlate with genetic changes identified in *ECM14* in CLAET815.1, a Zn-carboxypeptidase required for cell wall assembly, which could be linked to ethanol tolerance. High ethanol concentrations affect yeast cell vitality and viability by impacting cell wall integrity (Fujita et al. 2006). Furthermore, ethanol impacts vacuolar morphology (Meaden et al. 1999), and we identified mutations in *PEP1*, which encodes a type I transmembrane sorting receptor for multiple vacuolar hydrolases and is required for vacuolar biogenesis (Bryant et al. 1998). Our genomics and transcriptomics assays prove these genes may underlie ethanol tolerance and fermentation through proline uptake, cell wall integrity, and vacuole morphology alterations. This suggests that ethanol stress during experimental evolution defines a strain's fermentation potential. Modifications at the genetic level in the CLAET815.1 strain, which impacted the transcriptional profile of genes such as *PRO1*, *PRO3*, *PUT1*, *PUT2*, and *PUT3*, may have played a key role in improving ethanol tolerance, resulting in greater fermentative performance compared to the parental strain.

Experimental evolution techniques are widely employed in the industrial sector since they provide nongenetically modified organisms to the food industry. Genomic and expression analyses of fitter strains have increased the molecular understanding underlying adaptation mechanisms. In this context, the present work provides valuable information on implementing experimental evolution methodologies (CLAET media conditions) that generate observable changes in short generational periods, which depend on the genotype and can be applied to increase genetic variation in strains of industrial interest. Furthermore, our findings suggest that the increased ethanol tolerance in *S. eubayanus* may be attributed to different cellular processes, such as a higher proline, sterol biosynthesis, and oxidative stress-related gene overexpression. Our work demonstrates that how extremely low fermentative capacity strains are more susceptible to genetic improvement than the fittest, demonstrating a greater evolutionary potential. However, the initial genetic diversity (Gamblin et al. 2023) restricts populations' evolvability potential. Clonal populations rely on the original genotype, its interactions with the environment and the evolutionary forces that determine how much a genotype can evolve in a limited period of generations (Izutsu et al. 2024). We overcame the fitness plateau by applying a postexperimental evolution hybridization strategy between the best-evolved individual, CLAET815.1, and a reported high fermentative *S. eubayanus* strain, CLEtOH5.1 (Mardones et al. 2022). The hybrids generated in this study demonstrate that combining these two methodologies can produce novel strains. These strains could benefit from additive effects of orthologous parental genes, epistatic interactions, and newly formed genetic networks, collectively leading to enhanced fermentative performance and greater tolerance to stressful conditions (Shapira et al. 2014). Future studies could explore the fermentative performance of *S. eubayanus* intraspecific hybrids across different temperatures and evaluate heterosis for brewing-related traits, such as aroma production, flavour profile, and post-fermentation viability, to provide deeper insights into their industrial potential.

Acknowledgements

We acknowledge Fundación Ciencia & Vida for providing infrastructure, laboratory space, and experiment equipment. We want to thank V. A. and Antonio Molina for technical help.

Author contributions

Conceptualization: F.V.M., W.M., and F.A.C. Formal analysis: A.C.S., F.V.M., W.M., T.P., P.V., C.I.O., V.A., R.N., and F.A.C. Investigation: F.V.M. and F.A.C. Writing—original draft: F.V.M. and F.A.C. Writing—review & editing: F.V.M., A.C.S., T.P., P.V., R.F.N., F.A.C.

Supplementary data

Supplementary data is available at *FEMSYR Journal* online.

Conflict of interest: None declared.

Funding

This research was funded by Agencia Nacional de Investigación y Desarrollo (ANID) FONDECYT (1220026) and ANID—Programa Iniciativa Científica Milenio ICN17_022 and NCN2024_040. A.C. is supported by grant France Excellence Chili, F.V.M. is supported by ANID grant N° 21190562, T.P. by ANID grant N° 21221095, P.V. by FONDECYT INICIACIÓN (11240649), and Centro Ciencia & Vida, FB210008, Financiamiento Basal para Centros Científicos y Tecnológicos de Excelencia de ANID. W.M. is funded by ANID FONDECYT INICIACIÓN (11240430). All sequences have been deposited in the Sequence Read Archive Database of the National Center for Biotechnology Information (NCBI) under the BioProject accession number PRJNA994921.

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Received 17 December 2024; **revised** 25 January 2025; **accepted** 28 January 2025