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Evolved *Saccharomyces cerevisiae* strains to reduce ethyl carbamate in Sherry wines

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ABSTRACT

Urea is the main precursor of ethyl carbamate in fortified wines, which is in turn mostly produced by *Saccharomyces cerevisiae* due to the arginine catabolism during alcoholic fermentation. Due to its potential safety risks, efforts have been taken to reduce ethyl carbamate content by reducing the urea produced. However, most of them have been made through genetic manipulation, and their use in the food industry is therefore limited by legal constraints. In the present study, the adaptive laboratory evolution technique had been used to improve this trait in a diploid wine yeast already used at industrial level to obtain Sherry base wine. For this purpose, the genetic variability of the yeast population was increased by sexual reproduction and subsequently canavanine, a toxic arginine analogue, was applied as selective pressure to select yeast variants with lower urea production. Finally, an evolved variant that showed 62% lower urea content than the parental strain, also displaying an enhanced fermentative performance, was selected. The base Sherry wine obtained at industrial level not only showed a lower urea and ethyl carbamate content, but also an improvement in the aromatic profile, being fruitier and fresher than that obtained with the parental strain mainly due to an increase in ester content.

1. Introduction

Ethyl Carbamate (EC), also known as urethane, is considered a probably carcinogenic to humans, being classified into Group 2 A by The International Agency for Research on Cancer (Schehl et al., 2007). EC is mainly formed during alcoholic fermentation, distillation, ageing and storage processes to obtain fermented foods and alcoholic beverages (Abt et al., 2021), through a spontaneously chemical reaction of ethanol with cyanate or compounds containing a carbamoyl functional group (Guo et al., 2016). Among these precursors, urea is the most important one (Zhao et al., 2013), being produced by yeasts due to L-arginine catabolism during alcoholic fermentation.

In *Saccharomyces cerevisiae*, arginine is transported into cells yeast by three different amino acid permeases encoded by *CAN1*, *GAP1,* and *ALP1* (Zhang et al., 2016). Once inside the cell, arginine can either be transported to the vacuole by Vb2ap or be degraded by arginase (CAR1p) into urea and proline (Zhang et al., 2018). Urea can in turn be metabolised into carbon dioxide and ammonium by urea amidolyase (encoded by *DUR1,2*); or be transported outside the cell. Urea, as well as some polyamines, can in turn be reabsorbed by the cell via a transporter encoded by the *DUR3* gene.

Sherry wines constitute a wide range of products from the Jerez-Xérès-Sherry Appellation of Origin, in southern Spain, being considered as fortified wines (Cordero-Bueso et al., 2018). Their elaboration is sophisticated and includes typical techniques of white wine vinification, a subsequent fortification with ethanol to raise their alcoholic content, to allow their ageing through the traditional and dynamic "criaderas and soleras" system. Therefore, this implies that such wines undergo a long ageing period, which, together with their high alcoholic content, promotes the generation of EC. Palomino Fino is the grape variety most

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commonly used to obtain Sherry wines from this Apellation of Origin (Pozo-Bayón & Moreno-Arribas, 2011). In fact, it is considered a key element in the production of both sweet and dry Sherry wines via ageing, either biological or oxidative one (Roldán et al., 2021). This is due to that this grape variety contains a low amount of aromatic precursors, with no remarkable aromatic attributes.

In wine, ethyl carbamate is often found in significant concentrations (0.01–0.025 mg/l), especially in wines that have undergone biological ageing or long storage. Due to its potential health risk to consumers, some countries, including Canada, the USA and Brazil, have set legal limits for EC concentrations in fermented beverages (Jiao et al., 2014), whereby restrictions are expected to become increasingly stringent.

There are a number of studies aimed towards diminishing ethyl carbamate through the reduction of urea excreted by the yeast strains during the alcoholic fermentation. Several studies have been performed to date based on the inactivation of the *CAR1* gene in yeast by genetic engineering to reduce the amount of urea produced and, consequently, the generation of ethyl carbamate (Chin et al., 2016). The *CAN1* gene has also been deleted in commercial yeast strains using CRISPR-Cas9 (Vigentini et al., 2017). The resulting recombinant strains reduced urea production by 18–36% compared to the parental strains under laboratory winemaking conditions, although they displayed a reduced growth and fermentation rate. Furthermore, using metabolic engineering, Wu et al. (2015) deleted the *CAR1* gene and induced constitutive expression of *DUR1,2* in an industrial strain. They carried out pilot-scale (500 L) rice wine fermentations, obtaining a reduction of urea and EC by 89.1% and 55.3%, respectively, while overexpression of *DUR1,2* led to a reduction of urea and EC by 75.6% and 40%, respectively. The directed evolution strategy has also been employed to reduce urea production (Zhang et al., 2018). The *S. cerevisiae* parental strain was grown for 360 rounds with urea as the sole source of nitrogen, applying it as a selective pressure towards its utilization. A variant capable of decreasing the residual urea concentration by 47.9% in synthetic must was obtained. As for the EC concentration, the levels decreased by 40.5% compared to the parental strain. However, also in this case, the yeast variants obtained showed a lower growth capacity. Although commercially available yeast strains with lower urea production are already available, they have been genetically engineered (Coulon et al., 2006). Therefore, although accepted by the FDA, these approaches are not yet legally accepted in Europe.

Laboratory adaptive evolution (ALE) has emerged in recent years as a strategy that does not imply genetic modification to evolved industrial strains, considered as a relative low-consuming technique which allows to obtain desirable traits in different industrial domesticated strains (De Vero et al., 2017). During ALE, cells are cultured under some specific conditions, thus promoting the accumulation of beneficial mutations that allow cells to be adapted to the specific trait. If the genetic variability of the population itself is increased beforehand by means of sexual reproduction, the number of screening rounds until the yeast population adapts can be considerably reduced (Steensels et al., 2014).

L-canavanine is a L-arginine toxic analogue naturally found in several leguminous plants, playing a key role in plant defence against different depredators, including fungi (Suizu et al., 1989). It is considered a very valuable drug for both basic and clinical research. Due to its cytotoxic activity, it offers valuable therapeutic potential for different types of cancer (Karatsai et al., 2020). The primary mode of action of canavanine has been extensively described in the literature and seems to be related to the alteration of the structure of arginine-rich proteins (Akaogi et al., 2006; Staszek et al., 2017). Arginyl-tRNA synthetase activates and aminoacylates canavanine, which can then be incorporated into proteins in place of arginine. However, some of the canavanine-producing plants have evolved adaptive mechanisms that protect against self-poisoning. It has been shown that the arginyl-tRNA synthetase of such plants could distinguish between arginine and canavanine (Igloi & Schiefermayr, 2009). Data based on analysis of in vitro synthesised proteins showed that up to 30% of arginine residues can potentially be substituted by canavanine (Rosenthal, 2001). It was accompanied by an increased level of ubiquitin-conjugated proteins associated with degradation by cytosolic proteasomes (Akaogi et al., 2006). Furthermore, the impact of canavanine on protein structure has been linked to a marked reduction in the stability of mitochondrial proteins (Konovalova et al., 2015).

In the present work, an industrial wine strain *S. cerevisiae* was evolved to produce a lower urea content during alcoholic fermentation, thus promoting a reduced EC formation. For that purpose, genetic variability of the yeast population was exploited and then canavanine was applied as a selective pressure to select putative yeast variants with lower urea production than the parental yeast strain. Once the variants were screened, fermentations of Palomino Fino white variety were carried out at pilot-scale, and finally on an industrial scale, obtaining a base Sherry wine with reduced urea, and ethyl carbamate, accompanied by an overall improvement of the wine's aromatic profile.

2. Materials and methods

2.1. Yeast strain and culture media

The strain *S. cerevisiae* var. bayanus UCA-Y-0012 was used as parental yeast strain to obtain the evolved variants in this work. It is both a genetic and phenotypic isolate of the commercial wine yeast strain Lalvin EC1118™ (Lallemand, Germany). Yeast cultures were routinely maintained on solid YPD agar plates (2% glucose, 2% peptone, 1% yeast extract, and 2% agar). For long-term preservation, yeast strain cultures were stored at −40 °C in cryotubes supplemented with glycerol (final concentration 40%).

2.2. Laboratory adaptive evolution and variant screening

The procedure followed in this work was that previously described (De Vero et al., 2011; Ruiz-Muñoz et al., 2022) with slight modifications. The parental strain was induced to sporulation on acetate medium (0.5% sodium acetate and 2% agar) at 25 °C during 10 days. After tetrad formation, asci and cells were digested by using a standard Zymolyase protocol. Spores, recovered by centrifugation, were suspended in 2 ml YPD medium and periodically checked for 2 h under a confocal microscope for restoration of the diploid state by conjugation of gametes. Subsequently, 25 μl of suspension was inoculated into tubes with SDC medium, consisting of 0.67% YNB medium without amino acids and 5 g/l of ammonium sulphate (Difco, Thermo Fisher Scientific®), with 2% glucose, and supplemented with 200 μg/ml L-canavanine (Sigma-Aldrich, St. Louis, MO, USA). Such toxic concentration was chosen as it previously led to a significant growth inhibition, both to cells and spores, within 10 days of incubation at 28 °C in SD medium (0.67% YNB medium without amino acids, 5 g/lammonium sulphate and 2% glucose) supplemented with different canavanine concentrations. From each tube in which cell growth was observed, 100 μl of culture was plated on SDC agar medium. Colonies that grew were isolated from each plate, and seeded again on SDC agar plates. Thereafter, the isolates that preserved canavanine resistance were inoculated for several rounds in YPD medium to stabilise the phenotype as well as to avoid aberrant phenotypes.

The first screening of putative yeast variants was based on their ability to produce urea and/or consume arginine. For this purpose, the selected variants were grown SD medium supplemented with 2% L-arginine (SDA medium). After 4 days incubated at 25 °C, supernatants were collected to determine the arginine and urea content using a standard enzymatic kit (Megazyme, Ireland) following the manufacturer's instructions, employing 96-well polystyrene plates and a plate reader Nunc™ 96-well (Thermo Fisher Scientific, MA, USA), for photometric measurements. In all cases, the parental strain was used as a control.

2.3. Real time quantitative PCR (qRT-PCR) assay

Total RNA was extracted as described previously (Fernández-Morales et al., 2021) with some modifications. Each yeast cell pellet was first homogenised in 2 ml tubes containing 450 mg of 450–600 μm glass beads (Sigma-Aldrich), two 1/4″ CeramicSpheres (Q-Biogene, Carlsbad, CA, USA) and 1 ml of Trizol reagent (Invitrogen) using a Fast-Prep instrument (3 cycles: 6 m/s for 30 s) (Fast-Prep®-24, MP Biomedicals, Santa Ana, CA, USA). Total RNA was treated with RQ1 RNase-Free DNase (Promega, Madison, WI, USA), and both concentration and integrity was determined using a Qubit™ 4 Fluorometer (Thermo Scientific, Waltham, MA, USA).

For quantitative RT-qPCR expression analyses, 5 μg of total RNA from each sample was reverse transcribed into cDNA using the PrimeScript™ RT (Perfect Real Time) reagent kit (Takara, San Jose, CA, USA) according to the manufacturer's protocol. Control reactions were performed without reverse transcriptase to verify the absence of gDNA contamination.

Sequences of the three genes encoding arginine permeases (*CAN1*, *GAP1*, *ALP1*), the urea amydolase (*DUR1,2*) as well as the actin (*ACT1*) genes in the genome of *S. cerevisiae* EC1118 strain were retrieved from the Saccharomyces Genome Database (SGD). The *ACT1* gene was used as a reference gene (Nadai et al., 2015). Primer3 and Beacon Designer 8.0 softwares were used to design the primers employed (Table 1).

All real-time PCR amplifications were carried out using the CFX96 Touch™ Real-Time PCR Detection System (BioRad, Hercules, CA, USA) and Maxima SYBRGreen/ROX qPCR Master Mix kit (Thermo Scientific) following the manufacturer's instructions. Primer concentrations were optimised, using a range of 100–300 nM for each one. Annealing temperatures were also optimised, testing a temperature range from 50 to 62 °C. For each experiment, efficiency curves were drawn for each primer set; a negative control was used, and each reaction was repeated in five technical replicates. Amplification conditions was an initial step for enzyme activation at 50 °C for 2 min, a denaturation step at 95 °C for 10 min, and 45 cycles at 95 °C for 15 s and 1 min at 60 °C. Melting curve analysis was set up from 65 to 95 °C, with an increment of 0.5 °C for 5 s and plate read. The $2^{-\Delta\Delta Ct}$ method was used to quantify the relative transcription level of the selected genes thought the fold-change normalized to *ACT1* gene.

2.4. Fermentation trials

Laboratory-scale fermentations for the selected evolved variants were run in 1-L conical flasks measuring 500 ml of natural pasteurised must of Palomino Fino grape variety. Flasks were inoculated in triplicate with 1×10^6 viable cells/ml of each variant yeast grown in YPD medium at 28 °C for 24 h, washed twice with sterile water and re-suspended in the natural must prior to inoculation. Total and viable cells were counted under an optic microscope (Motic) using a Neubauer chamber. The inoculated flasks were incubated in static at 25 °C. Each flask was daily weighed in order to monitor the fermentation process of each yeast strain (Rodríguez et al., 2010).

On the other hand, Sherry base wines were performed under industrial scale in a winery placed in the Sherry winemaking area (Chipiona, Cádiz, Spain). They were carried out in stainless steel vessels of 20,000 l, performing the inoculum of each yeast strain as previously described (Ruiz-Muñoz et al., 2022). A control of the implantation of each inoculated yeast variant in each fermentation trial was carried out to ensure that they were responsible for carrying out the process.

Table 1
Oligonucleotides designed for RT–PCR.

Gene	Sequence (5′-3′)
ACT1 Fw	CGTCTGGATTGGTGGTTCTA
ACT1 Rv	GTGGTGAACGATAGATGGAC
ALP1 Fw	TTTGTCTCTTCATCGCCCTTTATGA
ALP1 Rv	ACACATTAGAGTTACCTGCGGAAAG
CAN1 Fw	GTTTATCCACACCTCTGACCAACGC
CAN1 Rv	ATGTAGCCATTTCACCCAAGGACTG
GAP1 Fw	ATCACTGTTGTTGGGTTCATCATCT
GAP1 Rv	CTTTGAATTTAGCACCTGGAGTGTC

2.5. Analysis of the wines obtained

The alcoholic titer was analysed by density measurement of the distillate in a DMA-5000 densimeter (Anton Paar, Ashland, OR, USA). A pH-Meter Basic 20 (Crison Instruments, Barcelona, Spain) was used to measure the pH. Titratable and volatile acidity, as well as free and total SO_2, were determined by volumetry according to the official methods established by the International Organization of Vine and Wine (OIV, 2019).

Extraction of the volatile compounds was performed following the methodology previously described (Ibarz et al., 2006) (LiChrolut EN, Merck, 0.2 g of phase, Darmstadt, Germany) using as internal standard 4-nonanol (0.1 g/l). Volatile compound analysis was carried out as stated previously (Izquierdo-Cañas et al., 2021). GC-MS conditions were the same that those described by (Pérez-Navarro et al., 2019). For the identification of volatile compounds, chromatographic retention times and mass spectra were used, using commercial standards. The characteristic m/z fragment for each compound was analysed using the internal standard method for quantification (Table S1).

The odour activity value (OAV) of each identified volatile compound was determined. It was calculated as the ratio between the concentration of a compound in wine and its detection threshold according to literature. It was considered that compounds with OAV > 1 values could have a direct sensory impact on wine. In addition, volatile compounds whose OAV > 0.1 were also selected in order to consider possible interactions between different aromas.

The wines obtained at the laboratory scale were tested by a trained panel of six expert judges for the laboratory-wines, while the industrial-scale wines were tested by a second group of 16 people (6 women and 10 men) regular white wine consumers with no formal wine training, as proposed previously (Pagliarini et al., 2013). Furthermore, wines elaborated with the parental strain, as well as with its evolved variants, at an industrial-scale, were compared by triangle tests (ISO 4120:2007) to assess whether aroma differences existed between the wines. Sensory descriptive analysis was used to describe and quantify attributes of the wines based on a scale from one (low intensity) to ten (high intensity).

2.6. Statistical analysis

All experiments were performed in biological replicates with three independent measurement of each sample. Mean values, as well as standard deviation, were used for further calculations.

The difference between population means in the experiments to test the urea and ammonium content of the evolved strains compared to the parental one, to test the main differences in fold-change values of the transcription of the genes analysed, as well as for the OAV of each identified volatile compound, Student's t-test was employed. In all cases, a p-value ≤ 0.05 was considered as statistically significant.

3. Results

3.1. Canavanine-resistant yeast variants selection

Out of 65 tubes with SDC medium (containing 200 μg/ml canavanine) that were inoculated with the newly conjugated spores, there was growth in nineteen of them, yielding resistance to this arginine analogue in 29% of the total inoculated. They were then spread on Petri dishes with SDC agar medium and, after being incubated for 4 days at 28 °C, only nine of them showed grown. Different colonies were isolated from each plate, isolating a total of 160 yeast colonies. They were inoculated

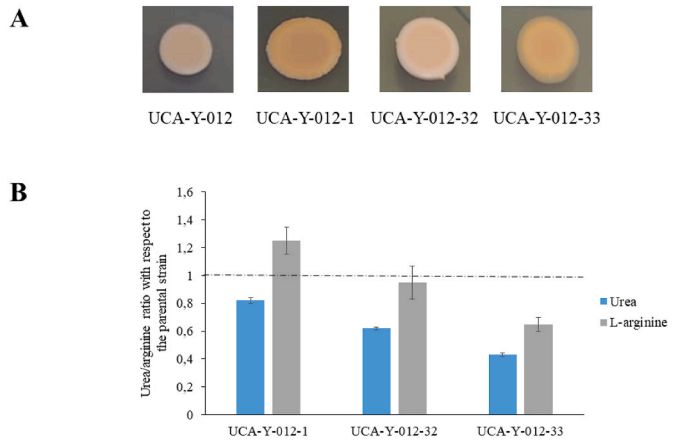

Fig. 1. Screening of putative yeast evolved variants after applying the ALE technique. A) Colony phenotype on plate; B) Ratio of L-arginine and urea present in the medium compared to the parental strain. These ratios were obtained by spectrophotometric quantification of the concentration of urea and arginine present in the medium with respect to the parental strain (UCA-Y-012).

for several rounds in YPD medium to ensure normal growth and to avoid aberrant phenotypes. Finally, 56 colonies were selected, also displaying different colony phenotypes on plate due to a pinkish colouration (Fig. 1A).

The selected yeast colonies were further analysed for the urea excreted, as well as the arginine consumption, on a medium supplemented with L-arginine (SDA medium). Data were normalized according to the urea and arginine initially present in the medium, as well as the values obtained from the parental strain assays. Out of the 56 yeast colonies isolated, three of them showed a significantly reduction in residual urea, although the arginine content present in the medium was similar or even lower than in the parental strain (Fig. 1B).

Finally, based on the overall results of this initial screening, three evolved variants were selected to carry out fermentation trials, namely UCA-Y-012-1, UCA-Y-012-32 and UCA-Y-012-33.

During laboratory fermentations, the growth traits of the three evolved variants were similar to the parental strain, and no stuck fermentation was recorded (Fig. 2). The fermentative traits of the evolved variants were similar to the parental one, with the UCA-Y-012-B33 variant standing out for its relatively higher fermentative capacity.

Once the wines were obtained at laboratory scale, they were tasted by an expert panel to determine the sensory impact on the wine due to the evolution of the parental strain. The hedonic sensory analysis carried out showed that there were differences among the wines obtained (Fig. 3). In general, the aroma of the wine made with the parental strain

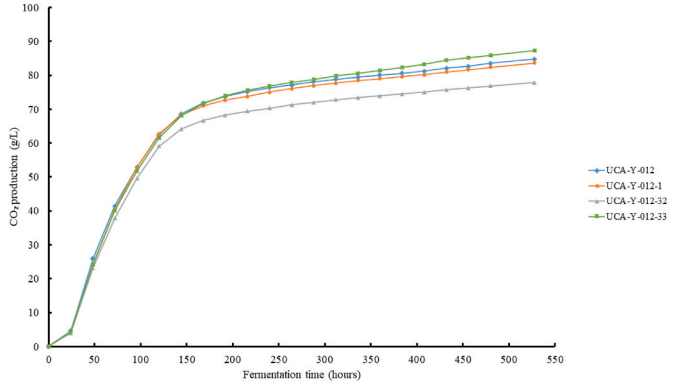

Fig. 2. Fermentation performance of selected evolved yeast variants, expressed as production of CO_2 (g/L/h) over fermentation time. UCA-Y-012 is the parental strain.

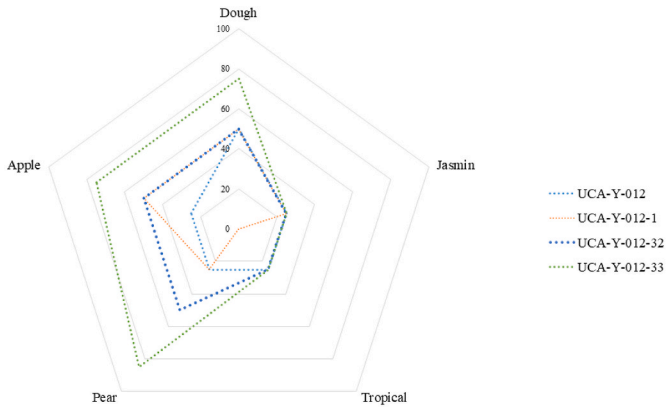

Fig. 3. Spider graph of the principal attributes in the obtained wines at laboratory scale according to sensory analysis. UCA-Y-012 is the parental yeast strain.

was enhanced by the evolved variants, while maintaining a similar sensory profile, as expected and desired. Mainly, the pear, apple and dough notes were intensified, especially in the wine made with the variant UCA-Y-012-33.

3.2. Expression analysis of evolved variants

The transcriptional level of arginine metabolism-related genes was analysed in the selected evolved variants compared with the parental yeast strain (Fig. 4), in order to understand the improvement achieved at the molecular level. The transcriptional expression of *DUR1,2* was upregulated in the three evolved variants, being especially significant in UCA-Y-012-B33 by 8.4 fold-change (p-value <0.001). As for the three arginine-permeases, different behaviours were found among the evolved variants. UCA-Y-012-1 showed transcriptional level of both *GAP1* and *ALP1* significantly upregulated (5.1 and 4.6 fold-change, respectively), while in UCA-Y-012-33, *CAN1* and *ALP1* transcription were upregulated (3.5 and 4.8 fold-change, respectively) compared to the parental strain.

3.3. Aromatic profile of industrial Sherry base wines

Considering its suitable fermentation capacity as well as its enhanced sensory profile, the evolved yeast variant UCA-Y-012-33 was employed to elaborate wine on an industrial scale under winery conditions. Once the wines were obtained at industrial level, both from the parental strain and from the finally selected evolved variant (UCA-Y-012 and UCA-Y-012-33, respectively), they were analysed both from an analytical and sensory standpoint.

Considering the main oenological parameters (Table 2), the alcoholic strength was slightly lower in the evolved yeast variant, also recording a

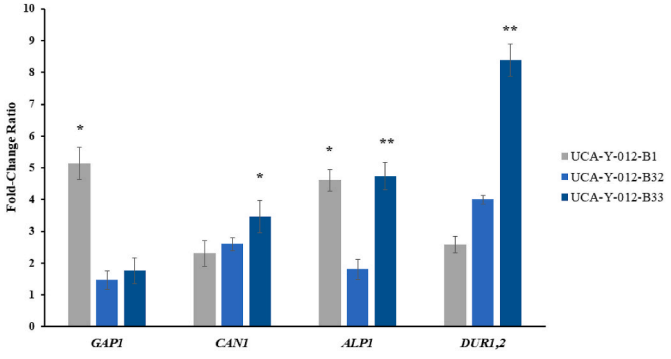

Fig. 4. Fold changes in gene regulation measured by qPCR of the selected evolved yeast variants, compared to the parental yeast strain.

Table 2
Main oenological parameters (mean value ± standard deviation, n = 3) of white wines elaborated with the evolved variant, UCA-Y-012-33 and the parental strain UCA-Y-012. P-value was obtained through Student's t-test.

	UCA-Y-012	UCA-Y-012-33	p-value
Alcoholic strength (% v/v)	12.13 ± 0.02	11.81 ± 0.01	0.024
Total acidity (g/l)	3.17 ± 0.06	3.04 ± 0.01	0.072
pH	3.51 ± 0.03	3.50 ± 0.02	0.102
Total sulphur (mg/l)	33 ± 1.41	38.50 ± 0.70	0.029
Free sulphur (mg/l)	2.00 ± 0.00	3.50 ± 0.70	0.102
Residual sugars (g/l)	0.34 ± 0.03	0.29 ± 0.08	0.210
Acetic acid (g/l)	0.44 ± 0.03	0.39 ± 0.01	0.062
Malic acid (g/l)	0.43 ± 0.04	0.51 ± 0.06	0.229
Lactic acid (g/l)	0.00 ± 0.00	0.00 ± 0.00	–
Tartaric acid (g/l)	1.79 ± 0.06	1.94 ± 0.10	0.201
Citric acid (g/l)	0.07 ± 0.02	0.03 ± 0.01	0.045
Succinic acid (g/l)	0.51 ± 0.04	0.42 ± 0.03	0.161
Glycerine (g/l)	4.94 ± 0.11	4.54 ± 0.08	0.102
Meso-butanediol (mg/l)	0.07 ± 0.01	0.18 ± 0.01	4.762 E−10
Levo-butanediol (mg/l)	0.24 ± 0.04	0.19 ± 0.01	0.215
Ethyl carbamate (μg/l)	6.73 ± 0.40	4.80 ± 0.60	0.0225
Urea (g/l)	5.61 ± 0.09	3.48 ± 0.02	1.967 E−05
Acetaldehyde (mg/l)	52.93 ± 0.25	49.96 ± 0.41	0.012
Ethyl acetate (mg/l)	71.51 ± 2.11	62.36 ± 0.91	0.029

lower acidity and residual sugars. It also noted a higher concentration of both total and free sulphur. Especially interesting was the reduction of recorded ethyl carbamate (4.80 ± 0.60 μg/l compared with 6.73 ± 0.40 μg/l, achieved by the parental strain). The remaining parameters were in the usual range for this type of wine.

Regarding volatile compounds, of the 57 metabolites initially detected by gas chromatography in both wines, 35 of them were considered to contribute to the wine aroma with an OAV > 0.1 (Table 3), whereas only 18 of them showed an OAV >1. Among them, ethyl octanoate, ethyl hexanoate, isoamyl acetate and damascenone showed the highest values.

In order to predict the overall impact of the improvement strategy on the aroma of the dry white wines at industrial level, these compounds were grouped into families according to the aromatic series to which they belong (Sánchez-Palomo et al., 2017). The total intensity of each aromatic family was the result of the sum of the OAV of each compound grouped (Fig. 5). The evolved variant recorded a significant increase in fruity, fresh, sweet and fatty families, compared to the wine elaborated with the parental one.

The triangular test showed that 61.5% of the tasters could distinguish the wines; hence, there were significant differences between the wines performed with the parental and the evolved yeast strains at a significance level of 5% (according to the tables of Roessler et al., 1948).

Regarding the description of the wines obtained, the hedonic tasting showed a similar trend to that obtained from the laboratory-scale wines, with fruitier aroma notes in the wine elaborated with the evolved yeast variant (Fig. 6). In this case, it was also noticed that the wine made with the evolved yeast variant recorded more tropical notes and less bread dough than the obtained with parental one, and even compared with the obtained at the laboratory-scale. In addition, at the industrial level, an aromatic defect was found in the control wine related to reductions, such as that caused by hydrogen sulphide.

Table 3
OAV values of volatile compounds (mean value ± standard deviation, n = 3) of the white wines obtained with the evolved variant and the parental yeast strain (UCA-Y-012-33 and UCA-Y-012, respectively). P-value was obtained through Student's t-test.

	Odour descriptors	Odorant series	Odour Threshold (μg/l)	UCA-Y-012	UCA-Y-012-33	p-value
Ehyl octanoate	Sweet, fruity	1,2,4	5 (a)	351.88 ± 4.98	353.98 ± 1.86	0.478
Ethyl hexanoate	Green apple	1	14 (a)	162.14 ± 28.00	157.01 ± 0.27	0.418
Isoamyl acetate	Banana	1	30 (b)	148.42 ± 11.81	133.80 ± 3.35	0.123
Damascenone	Sweet, fruit	1,4	0.05 (c)	134.94 ± 0.04	250.26 ± 11.85	0.023
Acetaldehyde	Pungent, ripe apple	1,6	500 (c)	117.14 ± 21.36	92.18 ± 2.76	0.190
Octanoic acid	Sweat, cheese	5	500 (b)	39.76 ± 0.39	53.04 ± 0.73	0.005
Hexanoic acid	Sweat	5	420 (a)	39.55 ± 1.20	50.73 ± 0.62	0.011
Isovaleric acid	Sweet, acid, rancid	4,5	33 (b)	37.53 ± 6.57	68.79 ± 3.23	0.023
2,3-butanodiol	Butter, creamy	5	100 (d)	26.28 ± 0.09	27.05 ± 5.88	0.440
Ethyl butirate	Fruity	1	20 (c)	15.30 ± 6.01	9.38 ± 0.04	0.197
Butiric acid	Rancid, cheese, sweat	5	173 (a)	8.62 ± 0.35	14.90 ± 0.73	0.014
Ethyl acetate	Fruit, solvent	1.6	7500 (c)	8.24 ± 0.53	6.33 ± 0.35	0.100
Decanoid acid	Rancid fat	5	1000 (a)	7.20 ± 1.48	10.72 ± 0.07	0.088
Isoamyl alcohols	Fusel	6	30000 (c)	4.71 ± 0.79	5.18 ± 0.42	0.339
Ethyl decanoate	Sweet, fruity	1,4	200 (b)	2.31 ± 0.51	2.91 ± 0.06	0.154
2-phenylethyl alcohol	Floral, roses	2	10000 (c)	1.40 ± 0.00	1.35 ± 0.03	0.149
3-etoxi-1-propanol	Fruity	1	100 (e)	1.26 ± 0.11	1.16 ± 0.02	0.248
2-phenylethyl acetate	Floral	2	250 (c)	0.97 ± 0.22	1.39 ± 0.03	0.100
g-nonalactona	Coconut	4	30 (b)	0.75 ± 0.06	0.78 ± 0.03	0.214
Vanillin	Vanillin	5,6	60 (a)	0.82 ± 0.10	1.22 ± 0.06	0.026
c-3-hexen-1-ol	Green, cut grass	3	400 (c)	0.69 ± 0.00	1.14 ± 0.04	0.018
Linalol	Floral	2	15 (c)	0.45 ± 0.05	0.48 ± 0.01	0.300
Isobutanol	Oily, bitter, green	3.6	40000 (a)	0.35 ± 0.03	0.42 ± 0.02	0.016
3-metil-thio-propanol	Cooked, vegetable	6	500 (c)	0.32 ± 0.00	0.71 ± 0.02	0.014
4-vinylphenol	Almond shell	6	180 (f)	0.27 ± 0.05	0.22 ± 0.00	0.169
4-vinylguaiacol	Spices/curry	5	40 (c)	0.24 ± 0.01	0.39 ± 0.02	0.023
Eugenol	Spices, clove, honey	6	6 (b)	0.21 ± 0.02	0.27 ± 0.01	0.039
Hexyl acetate	Green, floral	2,3	1500 (b)	0.20 ± 0.01	0.25 ± 0.01	0.072
Diethyl succinate	Vinous	6	200000 (a)	0.19 ± 0.04	0.27 ± 0.00	0.096
Geraniol	Roses, geranium	2	30 (c)	0.16 ± 0.01	0.10 ± 0.00	0.066
Benzaldehyde	Sweet, cherry, almond	1,4	350 (b)	0.14 ± 0.01	0.33 ± 0.02	0.040
Methanol	Chemical, medicinal	6	668000 (a)	0.13 ± 0.00	0.14 ± 0.01	0.293
Isobutyric acid	Rancid, butter, cheese	5	2300 (a)	0.13 ± 0.01	0.28 ± 0.01	0.002
1-hexanol	Flower, green, cut grass	2,3	8000 (c)	0.11 ± 0.00	0.17 ± 0.00	0.018
Acetovanillone	Vanilla, clove	6	1000 (a)	0.11 ± 0.01	0.19 ± 0.00	0.027

1 = Fruity; 2 = Floral; 3 = Green, fresh; 4 = Sweet; 5 = Fatty; 6 = Others; (a) Etievant, 1991; (b) Ferreira et al., 2000; (c) Guth, 1997; (d) Rocha et al., 2004; (e) Peinado et al., 2004; (f) Boido et al., 2003

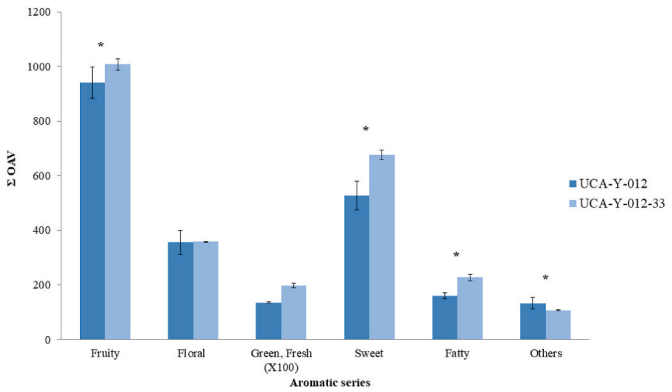

Fig. 5. Aromatic series (expressed as the total sum of OAV) in the white wines obtained with the parental strain UCA-Y-012 and the evolved variant, UCA-Y-012-33. Data are expressed as mean value ± standard deviation, n = 3.

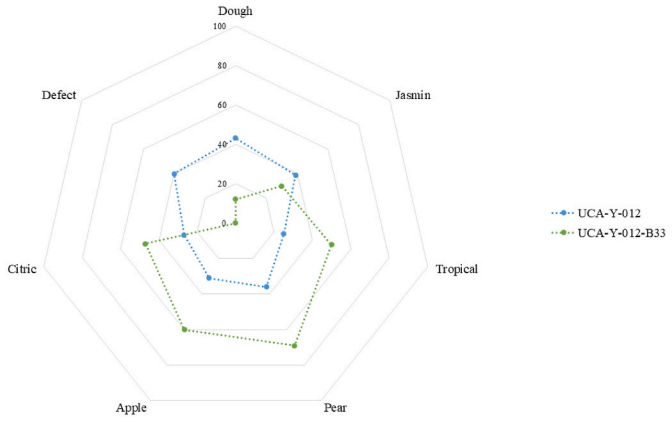

Fig. 6. Sensory characterization of Palomino Fino white wines at industrial scale with the parental strain (UCA-Y-012) and the evolved yeast variant (UCA-Y-012-33).

4. Discussion

In the present study, the ALE technique has been applied on an industrial *S. cerevisiae* strain already used to produce a base Sherry wine. The aim was to reduce the urea accumulated during alcoholic fermentation, thus reducing the concentration of ethyl carbamate generated, not only during alcoholic fermentation but also during subsequent ageing of such wines. Prior to applying selective pressure on the yeast population, their genetic variability was increased by sporulation and later conjugation of the spores, as already proposed and successfully carried out in other works carried out with ALE strategy (De Vero et al., 2011; Mezzetti et al., 2014). In this way, selecting variants which phenotype of interest could be obtained without the need for numerous rounds of screening to adapt the population to the new conditions. It should be noted that the viability of the spores of the parental strain was low (data not shown). This has a direct impact on the efficiency of spore mating, as one of the haploid gametes may be non-existent in the population, while the rest will undergo autodiploidization, i.e. conjugation of gametes of the same type (Bonciani et al., 2016). However, the resulting phenotypic variability after applying the different screens to the variants obtained shows that the applied ALE-based breeding programme was successful.

In yeasts, canavanine resistance has widely been used as a positive marker for genetic programmes to select transformants (Suizu et al., 1989). In these cases, canavanine resistance was supposed to be due to a defect in arginine permease (encoded by *CAN1*). On the other hand, a culture medium containing canavanine, arginine and ornithine (CAO medium) was developed to select arginase-deficient yeast mutants (i.e., *CAR1* mutants) (Kitamoto et al., 1993). Despite this medium seemed to accomplish its purpose, the mechanism by which this mutation and variant selection occurs has not been clarified yet (Kuribayashi et al., 2017).

In the present study, it was initially thought that canavanine-resistant variants would be due to alterations in their permeases, showing a reduced arginine/canavanine uptake. However, expression analysis of the three arginine permeases known up to now in *S. cerevisiae* (CAN1p, GAP1p, and ALP1p) was found to be upregulated, in addition to urea amidolyase, encoded by *DUR1,2*. These three arginine permeases have been reported to show both synergistic and antagonistic effects on arginine metabolism and regulation (Zhang et al., 2016), which could explain the difference in expression observed in the different evolved variants analysed in this work. Interestingly, overexpression of *ALP1* and *GAP1* showed a strong capacity for extracellular urea reduction, although it significantly inhibits cell growth (Sopko et al., 2006). The same effect was also achieved when *DUR1,2* was overexpressed in *S. cerevisiae* (Wu et al., 2015). However, in the present work, an improved growth and fermentative capacity compared to the parental strain has been observed. Furthermore, despite the lower urea production, the arginine intake was similar or even higher (in the case of the UCA-Y-012-33 variant) than in the parental strain. Given these results, studies at the genomic and/or transcriptomic level are needed to elucidate which regions have been altered in order to better understand the effect of canavanine-resistance on eukaryotic cells.

During the first screening rounds, some of the evolved variants showed a pink colouring, having found three different colour phenotypes. Such colour change could be due to a modification in the *ADE2-1* gene, which causes red pigmentation by accumulation of an intermediate compound in the vacuoles of yeast cells (Bharathi et al., 2016; Nevzglyadova et al., 2011). This phenotype has been related to an adenine auxotrophy, but evolved yeast variants did not show any auxotrophy in this case (data not shown). A possible reason for this may be that arginine, besides being metabolised to urea, is being more actively transported into the vacuole (via an ATP-dependent H+/Arg anti-transport pump). This would also explain the high arginine intake observed in the evolved variants. Thus, during ALE strategy, those yeast variants which sequestered more canavanine in the vacuole may have had resistance to this toxic analogue, as a defence mechanism. Once inside the vacuole, detoxification may have generated this difference in colouring by combining with other compounds (Cools et al., 2020).

Although the molecular basis has not been fully elucidated yet, the phenotype of the selected variant, besides reducing urea produced during alcoholic fermentation as well as ethyl carbamate, showed a remarkably fermentative performance. The same number of volatile compounds were detected in the two wines obtained on an industrial scale, although differences in concentration were found. These phenotypic differences, which had a direct repercussion on the wines obtained, may have been due to the breeding programme itself. When selecting freshly conjugated spores from the same parental strain, the resulting phenotypic variability in the population may produce other advantages to the yeasts, resulting in slight differences in oenological traits other than the specific one to be improved. The compounds that contributed most according to their OAV were esters, especially ethyl octanoate, ethyl hexanoate, and isoamyl acetate. Esters are formed during the alcoholic fermentation of wines due to yeast metabolism and play a key role in wine aroma, contributing to the fruity aroma series (Amores-Arrocha et al., 2021). The differences found in the concentration of these compounds, as well as norisorprenoids (mainly β-damascenone), are due to metabolic and enzymatic changes in the evolved yeast variant during the applied breeding programme. Fatty acid ethyl esters and higher alcohol acetates are used by flor yeast during biological ageing, playing a key role in the production of tertiary aromas (Pozo-Bayón & Moreno-Arribas, 2011) and being employed to the esterification equilibrium during ageing (Denat et al., 2022). The wine obtained at

industrial level with the evolved variant was fruitier, fresher and sweeter than that obtained with the parental strain, despite having a higher alcohol content and a similar concentration of residual sugars. It was also found to be fattier, due to an increase in metabolites such as octanoic, hexanoic, or isovaleric acid. These carboxylic acids contribute to the quality of young white wine by increasing its aroma complexity (Sánchez-Palomo et al., 2017). Moreover, these carboxylic acids are very important during biological ageing, as they are used as nutrients by flor yeasts through their oxidative metabolism.

Results obtained in this work have been highly successful, since it has been achieved a 62% urea reduction compared to the parental strain at industrial level. Furthermore, the selected variant showed a greater fermentation performance than the parental one, as well as a sensorial improvement in the base wine obtained for Sherry production, may even be considered to be directly marketed as a white, young wine.

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CRediT authorship contribution statement

Marina Ruiz-Muñoz: Data curation, Writing – original draft, Visualization, Investigation, Experiment Performance. **Gustavo Cordero-Bueso:** Conceptualization, Methodology, Funding, Funding acquisition, Supervision, Writing – review & editingWriting- Reviewing and Editing. **Lorena González-García:** Data curation, Writing – original draft. **Pedro Miguel Izquierdo-Cañas:** Visualization, Investigation, Experiment Performance. **Alejandro Centeno-Cuadros:** Software, qPCR, Validation. **Adela Mena-Morales:** Visualization, Investigation, Experiment Performance. **Sergio Martínez-Verdugo:** Visualization, Investigation, Experiment Performance. **Jesús Manuel Cantoral:** Conceptualization, Methodology, Funding, Funding acquisition, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2023.109958.

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