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Original Article

Simultaneous improvement of fructophilicity and ethanol tolerance of *Saccharomyces cerevisiae* strains through a single Adaptive Laboratory Evolution Strategy

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ABSTRACT

Saccharomyces cerevisiae is the main yeast used in the winemaking industry. Its innate glucophilicity provokes a discrepancy in glucose and fructose consumption during alcoholic fermentation of grape must, which, combined with the inhibitory effect of ethanol accumulated in the fermentation broth, might lead to stuck or sluggish fermentations. In the present study, we realized an Adaptive Laboratory Evolution strategy, where an alcoholic fermentation of a 20 g/L fructose broth was followed by cell selection in a high ethanol concentration environment, employed in two different *S. cerevisiae* strains, named CFB and BLR. The evolved populations originated from each strain after 100 generations of evolution exhibited diverse fermentative abilities. One evolved population, originated from CFB strain, fermented a synthetic broth of 100 g/L glucose and 100 g/L fructose to dryness in 170 h, whereas the parental strain did not complete the fermentation even after 1000 h of incubation. The parameters of growth of the parental and evolved populations of the present study, as well as of the ethanol tolerant populations acquired in a previous study, when grown in a synthetic broth of 100 g/L glucose and 100 g/L fructose, were calculated through a kinetic model, and were compared to each other in order to identify the effect of evolution on the biochemical behavior of the strains. Finally, in a 200 g/L fructose synthetic broth fermentation, only the evolved population derived from CFB strain showed improved fermentative behavior than its parental strain.

1. Introduction

Saccharomyces cerevisiae is the main microorganism used worldwide for the production of alcoholic beverages, bioethanol, as well as bread and pastries. *S. cerevisiae* cells can ferment sugars in the form of hexoses to produce pyruvic acid, through the metabolic path of glycolysis. Subsequently, under aerobic conditions, pyruvic acid is oxidized to carbon dioxide and water through the tricarboxylic acid cycle, whereas, when oxygen is absent, pyruvate is transformed into ethanol and carbon dioxide, through a process known as alcoholic fermentation. *S. cerevisiae* is also capable of performing alcoholic fermentation aerobically, a phenomenon known as "Crabtree effect"[1–4]. The ethanol produced during alcoholic fermentation is particularly stressful for the yeast cells, affecting the permeability and elasticity of their cell membrane and altering the concentration of its fatty acids [5–10]. Ethanol toxicity also affects the sugar transport systems of *S. cerevisiae* [11–13]. The resulting reduced cell viability leads to impaired fermentation rate and decreased ethanol production [14–18].

Wine is one of the most popular alcoholic beverages, traditionally produced by grape must, fermented by various yeasts. During the grape ripening, the sucrose from the leaves is transferred in the berries in the form of glucose and fructose [19]. Thus, the crashed berries typically produce a grape must composed of an equimolar amount of glucose and fructose [20,21]. In the winemaking process, both sugars should be consumed by the yeast cells so that residual fermentable sugar in the wine is less than 2–4 g/L [22,23]. *S. cerevisiae* is characterized as a

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"glucophilic" yeast, which means that glucose is the preferred carbon source of its cells when other carbon sources, including fructose, are available [24,25]. Owing to this partiality, in a typical wine fermentation, glucose is assimilated with a higher rate than fructose, leading to a discrepancy between the two sugars in kinetics of wine production [26]. During the final stages of fermentation, when nutrients are exhausted and ethanol concentration has reached the highest level, often *S. cerevisiae* cells are incapable of fermenting the remaining undesirable fructose in the fermentation broth, leading to sluggish or stuck fermentations [17,27–29]. Consequently, the produced wine might be undesirably sweet, since fructose is twice as sweet as glucose [30] and it also faces the risk of contamination by lactic acid bacteria and excessive production of acetic acid [31,32].

Although the reason for the preference of *S. cerevisiae* for glucose over fructose is yet to be thoroughly investigated, the specialization of the plasma membrane sugar transport system is considered to be involved. Hexoses are transported in the plasma membrane of the yeast cell via hexose transporters, by passive facilitated diffusion. There are 20 genes in *S. cerevisiae* which encode hexose transporters, i.e. *HXT1* to *HXT17*, *GAL2*, *SNF3*, and *RGT2* [33,34]. It has been proven that there are no specific transporters for the hexoses glucose, fructose and mannose, meaning that they are all transported by the same transporters [35,36]. Hxt2, Hxt6 and Hxt7 are high-affinity transporters for all three sugars, Hxt1 and Hxt3 present low-affinity, whereas other Hxt transporters have intermediate affinity [34,37–39]. Both the high and low affinity transporters have greater affinity for glucose than fructose [39]. The Hxt3 transporter is supposed to play the most important role in a wine fermentation, since it is capable of carrying out a normal fermentation on its own [37]. Also, this transporter interferes with energy production, possibly affecting the ability for better adaptation to ethanol stress in the early stages of fermentation [40]. Additionally, it has been shown that the expression of a mutated *HXT3* allele of a commercial fructophilic wine yeast (Fermichamp), in a *S. cerevisiae* strain unable to ferment hexoses, resulted in a higher fructose utilization capacity than when the standard *HXT3* allele was expressed [41], indicating the importance of hexose transport in the rates of fructose and glucose consumption during alcoholic fermentation. It was also observed that in high ethanol concentrations fructose consumption was inhibited in a greater degree than glucose consumption [26,42] whereas elsewhere [43] it was shown that the presence of fructose reduces ethanol tolerance in yeasts. Evidently, the increased preference of *S. cerevisiae* cells for fructose, combined with improved ethanol tolerance, would be of great biotechnological interest for the winemaking industry.

The amelioration of fructose consumption by *S. cerevisiae*, combined with enhanced ethanol tolerance, could be also of great interest for such fermentations in the bioethanol industry, as the use of bioethanol as a renewable biofuel is gradually increasing worldwide [44]. Renewable biofuels are utilized as substitutes of fossil fuels to reduce carbon dioxide emission to the environment [45]. Lately, there is an effort to produce bioethanol and other fermentation products from renewable raw materials, which are inexpensive and do not interfere with food [46–48]. Jerusalem artichoke, an easy and cheap to cultivate plant, is an excellent source of biomass for biofuels [49]. Its tubers contain more than 600 g/kg inulin [50], which is a water-soluble D-fructose polymer linked by β-(2,1) glycosidic bonds, with glucose linked through α-(1,2) at a terminus, used as a storage material for the plant [51]. Inulin is also produced in other plants, such as agave, asparagus, coffee, chicory, dahlia, dandelion, garlic, onion and yacon [51,52]. The extract from Jerusalem artichoke tuber which contains inulin can be hydrolyzed and then used as substrate for ethanol production by *S. cerevisiae*. In particular, *exo*-inulinases are used to catalyze removal of the terminal fructose residues from the nonreducing end of the inulin molecule, producing fructose as a main product, which can subsequently be fermented to ethanol from *S. cerevisiae* cells [52–54]. Adaptive laboratory evolution (ALE) is a laboratory procedure in which the principles of natural evolution are applied to a population, in a selected environment, with the desired conditions, towards the acquisition of a desired phenotype [55]. The core of such an experiment is the combination of the suitable selective pressure and the optimum duration [56–58]. An ALE experiment on microbial populations is either realized by serial transferring to shake flasks containing a liquid medium with the desired selective pressure (constant or gradually increasing), or in chemostats with the addition of inhibitors as selective pressure [59,60]. ALE is one of the techniques used for the optimization of yeast strains for winemaking [61]. In the present study, we performed a two-step ALE experiment, in which an alcoholic fermentation of a 20 g/L fructose broth by *S. cerevisiae* cells is followed by cell selection in a high ethanol concentration, i.e., 18 % v/v, environment. The ultimate aim was to obtain new, ethanol tolerant, *S. cerevisiae* populations, able to assimilate fructose more effectively in a fermentation broth with equimolar amounts of glucose and fructose, in order to achieve improved fermentation rates. The kinetic parameters of the evolved populations and the parental strains were calculated using a detailed mathematical model in order to estimate the effect of the evolution on the biochemical behavior of the strains. Additionally, the newly acquired evolved populations were evaluated for their fermentation capacity in synthetic broth containing 100 g/L glucose and 100 g/L fructose and compared to their parental strains and to the ethanol tolerant cell populations generated in previous work [62].

2. Material and methods

2.1. Microorganisms and culture conditions

Saccharomyces cerevisiae ho_SB and *Saccharomyces cerevisiae* DBVPG1973 were used. In this work, as well as previously [62] we use the names *Saccharomyces cerevisiae* CFB and *Saccharomyces cerevisiae* BLR, respectively, as they appear in the original paper [63]. CFB is a diploid homozygous strain with aneuploidy in chromosomes 2 and 9 and 45,420 SNPs, isolated from wine in Bordeaux, France. BLR is diploid, heterozygous, with 45,338 SNPs, isolated from grape must in Greece. The strains were kept at − 80 °C in 30 % v/v glycerol. From the frozen stock the strains were rejuvenated in submerged cultures at 28 °C on potato dextrose broth (PDB, Himedia, Mumbai, India), or on yeast extract, peptone, dextrose (YPD) medium containing 2 % w/v glucose (AppliChem, Darmstadt, Germany), 1 % w/v peptone (HiMedia) and 1 % w/v yeast extract (Condalab, Madrid, Spain). Media were sterilized at 121 ∘C for 20 min.

Evolution experiments were performed in a medium containing Yeast Nitrogen Base (YNB, Condalab) 1.7 g/L, 1.32 g/L $(NH_4)_2SO_4$ (Merck, Darmstadt, Germany) and fructose 20 g/L. Fermentation experiments were performed in a 100 mL semi-synthetic (wine simulating) medium containing Yeast Nitrogen Base (YNB, Condalab) 1.7 g/L, 1.32 g/L $(NH_4)_2SO_4$ (Merck, Darmstadt, Germany), 100 g/L fructose and 100 g/L glucose, or 200 g/L fructose. Medium pH in all cases was adjusted to 3.5 using a KH_2PO_4 (Fluka, Steinheim, Germany) solution 1 M. Media were sterilized by filtration through membrane of pore diameter 0.2 μm.

2.2. Evolution experiment

A schematic representation of the evolution experiment used in this study is shown in Fig. 1. The applied strategy was performed independently in each strain (*S. cerevisiae* CFB and *S. cerevisiae* BLR) and it comprised three steps:

Step 1 (Evolution): 10^6 yeast cells from fresh PDB culture were inoculated in 250 mL Duran bottle containing 100 mL medium consisted of 20 g/L fructose as described above, 1.32 g/L $(NH_4)_2SO_4$ and 1.7 g/L YNB, pH 3.5. The Duran bottle was incubated in a rotary shaker at 180 rpm and 28 °C until complete fructose consumption.

Step 2 (selection): 10^6 cells from the Duran bottle were transferred to McCartney glass bottle containing 10 mL of an aqueous ethanol solution 18 % v/v supplemented with 1.7 g/L YNB. The McCartney glass bottle was incubated at 28 °C for 3 h. The ethanol concentration used as

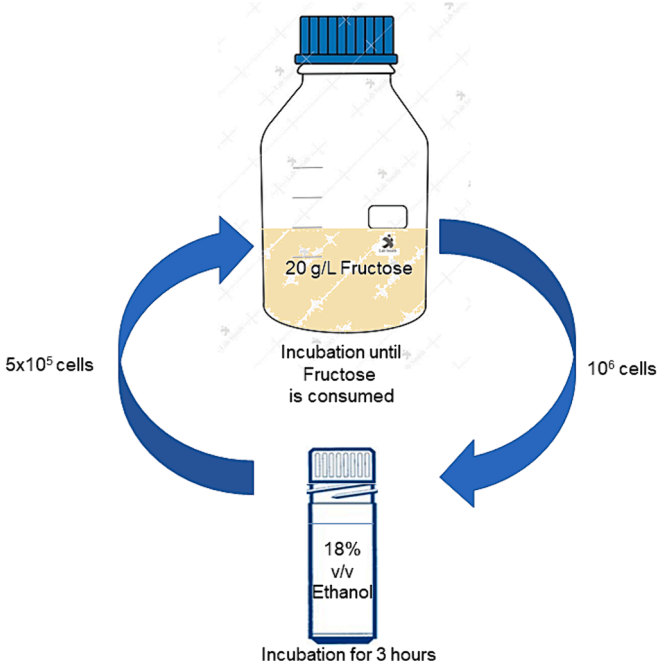

Fig. 1. Schematic representation of the Adaptive Laboratory Evolution (ALE) strategy used in this study for the generation of ethanol tolerant *S. cerevisiae* populations able to assimilate fructose more effectively than their parental strains. Details are discussed in the text.

selective pressure and the duration of incubation were estimated previously [51].

Step 3: The survived cells from the McCartney glass bottle (around $5x10^5$ cells) were transferred to a new Duran bottle containing fresh medium consisted of 20 g/L fructose until complete fructose consumption and the procedure was repeated in a loop.

The number of yeast cells in each ALE cycle was determined using a hemocytometer (HBG Henneberg-Sander, Gießen, Germany). Cell density during growth was estimated by measuring the Optical Density (OD) values of the culture broth at $\lambda = 660$ nm. The number of generations (n) during each evolutionary cycle was estimated according to the formula:

$$N_t = N_0 \times 2^n$$

where N_0 is the number of cells per mL of culture broth at the beginning of the cycle and N_t is the number of cells per mL of culture broth at the end of the cycle.

Each cycle corresponded to 6–7 generations. The ALE experiment was repeated for 15 cycles for each yeast strain, which corresponded to 100 generations of evolution.

2.3. Evaluation of evolved populations and chemical analyses in the fermentation broth

The fermentation capabilities of the evolved populations were tested in 250 mL Duran bottles with 100 mL medium containing 100 g/L glucose, 100 g/L fructose or 200 g/L fructose, as described above. In both cases, the Duran bottles were aseptically inoculated with 10^6 cells of a 48-hours PDB preculture and incubated in a rotary shaker at 180 rpm and 25 °C. The Duran bottles were kept with a loosely closed lid for the first 2 h of incubation, for oxygen supplementation, and the lid was well closed for the rest of the fermentation, for anaerobic conditions, and the agitation was reduced to 100 rpm. At regular intervals during the fermentation, 2 mL of the fermentation broth were collected aseptically for further analysis.

Glucose, fructose and ethanol were determined in the culture medium using a Waters 600E High Performance Liquid Chromatography (Waters Corporation, Milford, Massachusetts) equipped with an Aminex HPX-87H (300 mm × 7.8 mm, Bio Rad CA) column, coupled to a differential refractometer (RI Waters 410) with the following operating conditions: Sample volume 20 μL; mobile phase 10 mM H_2SO_4; flow rate 0.5 mL/min; column temperature 65 °C. Samples were centrifuged at 9000 rpm for 10 min at 4 °C (Hettich Universal 320-R, Tuttlingen, Germany) and the supernatant was filtered through a 0.2 μm membrane filter before the injection to the chromatograph.

2.4. Cell density and dry cell mass determination

From a fermentation in 250 mL Duran bottles with 100 mL semi-synthetic medium containing 100 g/L glucose and 100 g/L fructose (described above) incubated in a rotary shaker at 180 rpm and 25 °C, 5 mL of the broth were collected at regular intervals. Cell density was estimated by measuring the Optical Density (OD) value of the culture broth at $\lambda = 660$ nm. Subsequently, the broth was filtered through a Whatman 0.8 μm paper. The remaining pellet was dried at 80 °C until constant weight. Dry cell mass was determined gravimetrically. A standard curve with correlation between OD at $\lambda = 660$ nm and dry cell mass (g/L) was created for each parental strain and evolved cell population. Dry cell mass during the fermentation experiments was estimated indirectly through measurement of the Optical Density (OD) value of the culture broth at $\lambda = 660$ nm.

2.5. Modeling

The Aiba kinetic model [64], which is widely applied to describe the alcoholic fermentation process, including ethanol inhibition effect on the cell growth and ethanol production rate, was used to describe the process. The balance equations for biomass synthesis, ethanol production, glucose and fructose consumption are

$$\frac{dX}{dt} = (\mu_1 + \mu_2)e^{(-K_{px}P)}X \quad (1)$$

$$\frac{dP}{dt} = (q_1 + q_2)e^{(-K_{pp}P)}X \quad (2)$$

$$-\frac{dS_1}{dt} = \left(\frac{\mu_1 e^{(-K_{px}P)}}{Y_{xs_1}} + \frac{q_1 e^{(-K_{pp}P)}}{Y_{ps_1}}\right)X \quad (3)$$

$$-\frac{dS_2}{dt} = \left(\frac{\mu_2 e^{(-K_{px}P)}}{Y_{xs_2}} + \frac{q_2 e^{(-K_{pp}P)}}{Y_{ps_2}}\right)X \quad (4)$$

where X, P, S_1, S_2 are biomass, ethanol, glucose and fructose concentrations (g/L), respectively, t is the fermentation time (h), μ_1 is the specific growth rate on glucose (1/h), μ_2 is the specific growth rate on fructose (1/h), q_1 is the specific ethanol production rate on glucose (1/h), q_2 is the specific ethanol production rate on fructose (1/h), K_{px} and K_{pp} are the inhibition constants (g/L), Y_{xs_1} is the yield coefficient for biomass production with respect to glucose (g biomass/g glucose), Y_{xs_2} is the yield coefficient for biomass production with respect to fructose (g biomass/g fructose), Y_{ps_1} is the yield coefficient for ethanol production with respect to glucose (g ethanol/g glucose) and Y_{ps_2} is the yield coefficient for ethanol production with respect to fructose (g ethanol/g fructose).

The specific growth rates on glucose (μ_1) and fructose (μ_2) as well as the specific ethanol production rates on glucose (q_1) and fructose (q_2) are:

$$\mu_1 = \mu_{1_{max}} \cdot \frac{S_1}{K_{sx_1} + S_1} \quad (5)$$

$$\mu_2 = \mu_{2_{max}} \cdot \frac{S_2}{K_{sx_2} + S_2} \quad (6)$$

$$q_1 = q_{1_{max}} \cdot \frac{S_1}{K_{sp_1} + S_1} \quad (7)$$

$$q_2 = q_{2_{max}} \cdot \frac{S_2}{K_{sp_2} + S_2} \quad (8)$$

where $\mu_{1_{max}}$ is the maximum specific growth rate on glucose (1/h), $\mu_{2_{max}}$ is the maximum specific growth rate on fructose (1/h), $q_{1_{max}}$ is the maximum specific ethanol production rate on glucose (1/h), $q_{2_{max}}$ is the maximum specific ethanol production rate on fructose (1/h) and K_{sx_1}, K_{sx_2}, K_{sp_1} and K_{sp_2} are the saturation constants (g/L).

The fitting of the model on the experimental data sets was carried out using the commercial numerical code Aquasim (Version 2.1d), which uses a fully implicit finite-difference spatial discretization Gear scheme in conjunction with the algorithm DASSL. Aquasim uses a weighted least-squares method to estimate the values of parameters from experimental data [65].

2.6. Statistical analysis

Standard error from two experimental replications in Fig. 2 was calculated using Microsoft Excel.

The results in Figs. 3–6 derive from 2 independent cultures and are presented without statistical analysis.

3. Results

3.1. Cell growth during ALE experiment

The aim of this experiment was to generate ethanol tolerant yeast populations able to assimilate fructose more effectively than the strain of origin. During cell growth in the first step of the ALE experiment, on Duran bottles with 20 g/L fructose (pH=3.5) under agitation at 28 °C, there was a lag phase of 60 h in both *S. cerevisiae* CFB and *S. cerevisiae* BLR strains. In CFB the lag phase was reduced in 23 h in the second cycle of ALE (Fig. 2a), whereas in BLR, the lag phase did not reduce before the fourth cycle of ALE, where it reached the value 16 h (Fig. 2b). In both cases the cells reached the stationary phase in 44–48 h.

3.2. Fermentation ability of the parental strains and the evolved populations

The fermentation capabilities of the parental strains and the evolved populations of the present and the previous study in which the populations were evolved on glucose [62], were tested in semi-synthetic glucose-fructose broth. The experimental data and model fitting are illustrated in Figs. 3 and 4, while the estimated values of the kinetic parameters along with the coefficient of determination (R^2) are shown in Table 1. Experimental fermentation parameters of *S. cerevisiae* parental strains and evolved populations are shown in Table 2. The CFB parental strain did not complete the fermentation even after 1000 h of incubation (Fig. 3a), leaving 8.76 g/L residual glucose and 36.31 g/L residual fructose (Table 2). In contrast, both sugars were consumed in 495 h by the CFB population evolved on glucose for 150 generations (Fig. 3b) and in 170 h by the CFB population evolved on fructose for 100 generations (Fig. 3c). Mathematical model predictions were in accordance with the above experimental observations. For example, the values of $q_{1_{max}}$ and $q_{2_{max}}$ in the parental strain were lower compared to the values obtained from the evolved populations (Table 1). Similarly, the highest $\mu_{2_{max}}$ value was estimated by the model in the population evolved on fructose for 100 generations, while the highest $\mu_{1_{max}}$ value was predicted in the population evolved on glucose for 150 generations. However, $\mu_{2_{max}}$ presented a lower value (0.331 1/h) in the CFB population evolved on glucose for 150 generations than in the CFB parental strain (0.431 1/h) (Table 1).

The BLR strain exhibited a different physiological behavior. The parental strain consumed all sugars in 354 h (Fig. 4a), whereas the population evolved on glucose for 200 generations completed the fermentation in 312 h (Fig. 4b) and the population evolved on fructose for 100 generations in 436 h (Fig. 4c). The mathematical model estimated the lowest $\mu_{1_{max}}$ and $\mu_{2_{max}}$ values in the BLR parental strain, while the highest representative values were predicted in the population evolved on glucose for 200 generations. The model also predicted higher $q_{1_{max}}$ and $q_{2_{max}}$ values in the evolved populations in comparison with the BLR parental strain and the highest values were estimated in the population evolved on glucose for 200 generations (Table 1). Comparisons between the parameters of the present study and the parameters mentioned in literature [66–70], estimated by other *S. cerevisiae* strains that produce ethanol during growth on renewable materials are shown in Table 3.

Alcoholic fermentations were also realized in synthetic fructose broth, with the parental strains and the populations derived after 100 generations of evolution on fructose (present study). The fermentation parameters of *S. cerevisiae* parental strains and evolved populations are shown in Table 4. Both the CFB parental strain and the evolved population did not contrive to complete the fermentation even after 2100 h of incubation, leaving 54.27 g/L and 17.54 g/L residual fructose, respectively (Fig. 5a; 5b). In the case of BLR, the parental strain completed the fermentation relatively early, consuming the whole fructose quantity in 400 h (Fig. 6a), whereas the evolved population had a remaining of 11.99 g/L fructose in 2000 h of fermentation (Fig. 6b). In all cases, the ethanol yields (g of ethanol/ g of fructose consumed) ranged between 0.41 and 0.43 (Table 4).

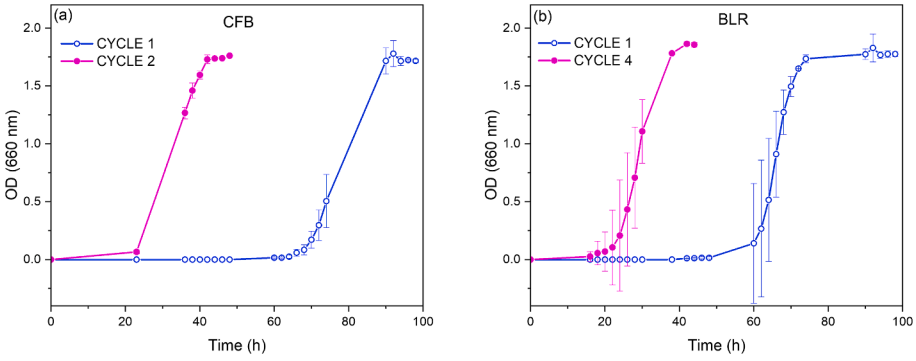

Fig. 2. Growth kinetics of the first two cycles of *S. cerevisiae* CFB (a) and the first and fourth cycle of *S. cerevisiae* BLR (b) during the first step of the ALE experiment realized on Duran bottles with 20 g/L fructose (pH=3.5) under agitation at 28 °C. Growth was estimated using the optical density (OD) values of the culture broth at λ = 660 nm.

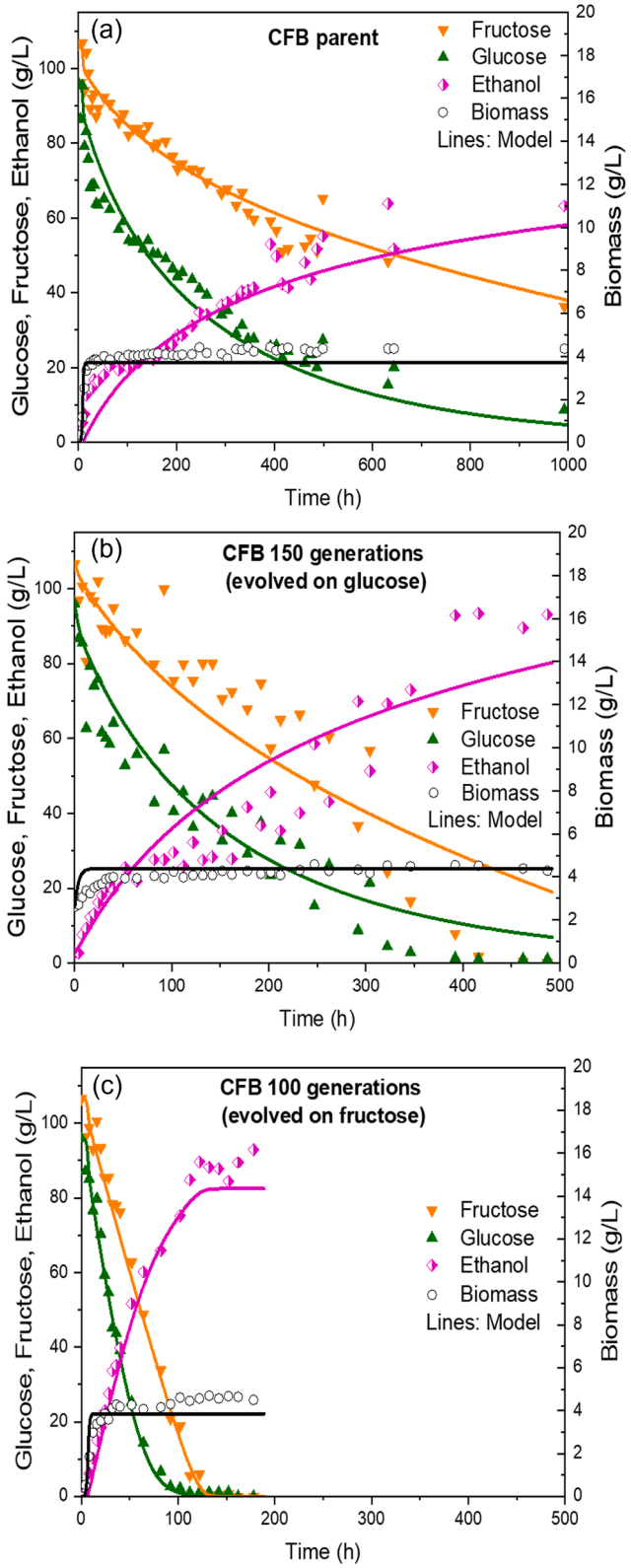

Fig. 3. Glucose and fructose consumption (g/L) and ethanol production (g/L) of *S. cerevisiae* CFB parental strain (a) and the evolved populations after 150 generations of evolution on glucose (b) and 100 generations of evolution on fructose (c) in synthetic broth containing 100 g/L glucose and 100 g/L fructose, pH=3.5. Points and lines represent experimental data and model prediction, respectively.

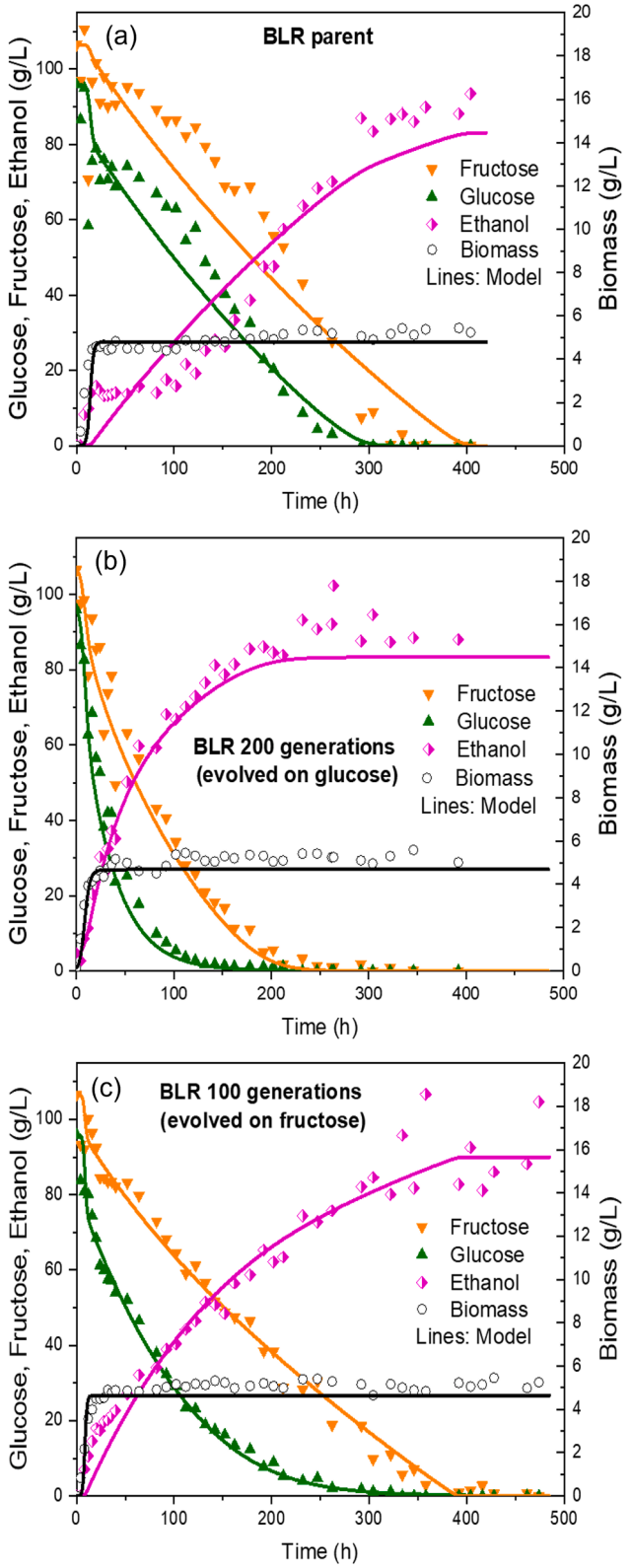

Fig. 4. Glucose and fructose consumption (g/L) and ethanol production (g/L) of *S. cerevisiae* BLR parental strain (a) and the evolved populations after 200 generations of evolution on glucose (b) and 100 generations of evolution on fructose (c) in synthetic broth containing 100 g/L glucose and 100 g/L fructose, pH=3.5. Points and lines represent experimental data and model prediction, respectively.

Table 1
The values of the kinetic parameters obtained by fitting the model to the experimental data derived from fermentations performed by the parental strains and the evolved populations. For details see text. *Experimental values.

Parameter	CFB parental	CFB 150 generations (evolved on glucose)	CFB 100 generations (evolved on fructose)	BLR parental	BLR 200 generations (evolved on glucose)	BLR 100 generations (evolved on fructose)
K_{pp} (g/L)	0.034	0.019	0.001	0.007	0.013	0.009
K_{px} (g/L)	3.441	0.712	1.040	1.713	0.267	1.658
K_{sp_1} (g/L)	29.944	45.807	25.476	1.844	41.233	22.571
K_{sp_2} (g/L)	0.668	0.162	3.785	0.873	6.900	0.166
K_{sx_1} (g/L)	1.587	10.466	2.856	0.56	0.899	2.540
K_{sx_2} (g/L)	0.701	18.21	1.679	26.99	0.257	45.98
$q_{1_{max}}$ (1/h)	0.068	0.107	0.254	0.038	0.389	0.101
$q_{2_{max}}$ (1/h)	0.025	0.0506	0.109	0.036	0.132	0.048
$\mu_{1_{max}}$ (1/h)	0.549	0.813	0.779	0.507	0.706	0.686
$\mu_{2_{max}}$ (1/h)	0.431	0.331	0.471	0.125	0.688	0.464
Y_{ps_1} (g/g)	0.398	0.437	0.454	0.455	0.533	0.519
	*0.266	*0.362	*0.429	*0.434	*0.440	*0.442
Y_{ps_2} (g/g)	0.405	0.484	0.428	0.447	0.499	0.532
	*0.324	*0.397	*0.436	*0.440	*0.446	0.450
Y_{xs_1} (g/g)	0.253	0.209	0.261	0.263	0.073	0.153
	*0.024	*0.023	*0.022	*0.026	*0.025	*0.025
Y_{xs_2} (g/g)	0.247	0.203	0.238	0.270	0.123	0.133
	*0.030	*0.026	*0.023	*0.026	*0.026	*0.025
R^2	0.872	0.874	0.933	0.849	0.919	0.936

Table 2
Actual fermentation parameters of *S. cerevisiae* parental strains and evolved populations when grown in synthetic broth with 100 g/L glucose and 100 g/L fructose.

S. cerevisiae population	Fermentation time (h)	Residual glucose (g/L)	Residual fructose (g/L)	$Y_{P/S}$ (g of ethanol/ g of total sugars consumed)
CFB parental	1000	8.76	36.31	0.40
CFB 150 generations (evolved on glucose)	495	1.09	0.18	0.44
CFB 100 generations (evolved on fructose)	170	0	0	0.46
BLR parental	354	0	0	0.44
BLR 200 generations (evolved on glucose)	312	0	0	0.45
BLR 100 generations (evolved on fructose)	436	0	0.63	0.47

4. Discussion

During alcoholic fermentation for wine production, *S. cerevisiae* cells are summoned to ferment glucose and fructose simultaneously. *S. cerevisiae* is a glucophilic yeast, a fact that, in combination with nutrient deprivation and ethanol stress during the final stages of the fermentation, renders total fructose consumption by its cells a remarkably challenging task. Fructose fermentation by *S. cerevisiae* is also of great importance in the bioethanol industry, since fructose is produced from hydrolysis of inulin-rich plants which produce biomass for bioethanol production. In our previous study [62] we performed an ALE experiment realized in two steps, alternating between weak selective pressure (20 g/L glucose) and strong selective pressure (18—25 % v/v ethanol). The evolved *S. cerevisiae* populations exhibited improved fermentation capabilities and improved ethanol yields in synthetic fermentation broth with 200 g/L glucose. Such populations could be particularly useful in alcoholic fermentation for bioethanol production, where high ethanol yield is the main priority. In the present study, we attempted to induce evolved *S. cerevisiae* populations capable of consuming fructose more effectively than the parental strains, while at the same time being more tolerant to ethanol toxicity. Similarly, we realized an ALE experiment in two steps; in the first step the yeast cells conducted alcoholic fermentation in the less preferred (due to transporter specificity) environment of 20 g/L fructose and in the second step they were exposed to the toxic environment of high ethanol concentration. We assume that such separation of the ALE experiment in two steps could result highly beneficial for alcoholic fermentations in winemaking.

4.1. Cell growth during the ALE experiment

In the first step of the evolution experiment there was an extended lag phase during growth on fructose broth, as long as 60 h. This fact was somehow anticipated, owing to the low preference of *S. cerevisiae* cells for fructose. There has already been observed extended lag phase in an OD/time curve of *S. cerevisiae* cells grown in YNB medium supplemented with high fructose concentrations [71]. The cells of the CFB strain managed to reduce the lag phase in 23 h already in the second cycle of the ALE experiment, i.e., after 7 generations, whereas the BLR strain achieved a decrease of the lag phase to 16 h in the fourth cycle, corresponding to 20 generations. Albeit this difference between the two strains might seem odd, it is not a cause for concern, given that the lag phase is not only strain specific, but it might also differ between cells of the same population [72] and is triggered by different other factors apart from stress [73]. It should also be taken into consideration that ALE experiments affect each strain differently, since the mutations occurred are unpredictable and strain specific [58,74]. The fact that the lag phase did not reduce below 16 h in the whole ALE experiment is also unsurprising, since the same pattern was presented in our previous ALE experiment using the same parental strains [62].

4.2. Alcoholic fermentations in wine simulating broth

The evolved populations acquired in the present study were evaluated by their performance in alcoholic fermentation in a synthetic broth with equimolar concentrations of glucose and fructose, which mimics the sugar composition of the grape must. Successfully evolved

Table 3
Comparisons between the parameters of the present study and the parameters mentioned in literature estimated by other *S. cerevisiae* strains that produce ethanol during growth on renewable materials.

K_{pp}(g/L)	K_{px}(g/L)	K_{sp_1}(g/L)	K_{sp_2}(g/L)	K_{sx_1}(g/L)	K_{sx_2}(g/L)	$q_{1_{max}}$(1/h)	$q_{2_{max}}$(1/h)	$\mu_{1_{max}}$(1/h)	$\mu_{2_{max}}$(1/h)	Y_{ps_1}(g/g)	Y_{ps_2}(g/g)	Y_{xs_1}(g/g)	Y_{xs_2}(g/g)	Reference
0.034	3.441	29.944	0.668	1.587	0.701	0.068	0.025	0.549	0.431	0.398	0.405	0.253	0.247	CFB parental (present study)
0.0191	0.712	45.807	0.162	10.466	18.21	0.107	0.0506	0.813	0.331	0.437	0.484	0.209	0.203	CFB 150 generations (evolved on glucose) (present study)
0.000354	1.04	25.476	3.785	2.856	1.679	0.254	0.109	0.779	0.471	0.454	0.428	0.261	0.238	CFB 100 generations (evolved on fructose) (present study)
0.00665	1.713	1.844	0.873	0.56	26.99	0.038	0.036	0.507	0.125	0.455	0.447	0.263	0.27	BLR parental (present study)
0.0134	0.267	41.233	6.9	0.899	0.257	0.389	0.132	0.706	0.688	0.533	0.499	0.073	0.123	BLR 200 generations (evolved on glucose) (present study)
0.00907	1.658	22.571	0.166	2.54	45.98	0.101	0.048	0.686	0.464	0.519	0.532	0.153	0.133	BLR 100 generations (evolved on fructose) (present study)
N/A	N/A	213.6	N/A	N/A	N/A	N/A	N/A	0.084	N/A	0.668	N/A	0.136	N/A	[66] (Monod model)
0	0.115	2.206	N/A	18.31	N/A	0.4	N/A	0.18	N/A	2.795	N/A	0.031	N/A	[67] (Aiba model)
N/A	181.1	N/A	N/A	N/A	N/A	N/A	N/A	0.509	N/A	N/A	N/A	N/A	N/A	[68] (Andrews model)
N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.54	N/A	0.11	N/A	[69] (Proposed model)
N/A	0	N/A	N/A	N/A	N/A	4.8	N/A	0.30	N/A	0.53	N/A	0.44	N/A	[70] (Monod-based model)

Table 4
Actual fermentation parameters of *S. cerevisiae* parental strains and evolved populations when grown in synthetic broth with 200 g/L fructose.

S. cerevisiae population	Fermentation time (h)	Residual fructose (g/L)	$Y_{P/S}$ (g of ethanol/ g of fructose consumed)
CFB parental	2100	54.27	0.42
CFB 100 generations (evolved on fructose)	2100	17.54	0.41
BLR parental	400	0	0.43
BLR 100 generations (evolved on fructose)	2000	11.99	0.43

S. cerevisiae cells should be able to finalize the fermentation faster than the parental strains, due to their adaptation to high ethanol concentrations and by consuming fructose more effectively, by reducing the "area" between glucose and fructose in the kinetic diagram of alcoholic fermentation [39]. For the CFB strain, the evolved populations seemed to have attained the desired fitness; the parental strain left residual sugars of 8.76 g/L glucose and 36.31 g/L fructose after 1000 h of fermentation, whereas the population evolved on fructose for 100 generations consumed both sugars in a brief period (i.e., 170 h). This performance possibly indicates that the hexose transporters in this evolved population acquired greater affinity for fructose during the evolution, a fact that could also have been subserved by their expedited ethanol tolerance, since hexose transporters are known to be inhibited by ethanol [11–13]. Hxt6 and Hxt7 transporters were probably the ones

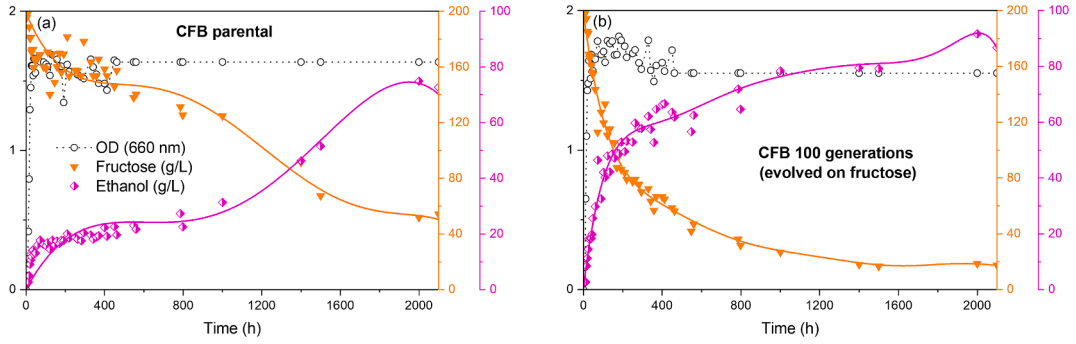

Fig. 5. Fructose consumption (g/L) and ethanol production (g/L) of *S. cerevisiae* CFB parental strain (a) and the evolved population after 100 generations of evolution on fructose (b) in synthetic broth containing 200 g/L fructose, pH=3.5. Growth was estimated using the optical density (OD) values of the culture broth at λ = 660 nm.

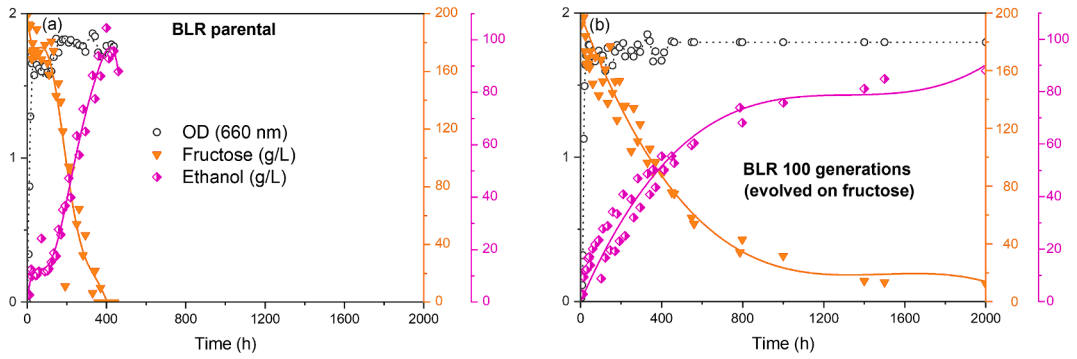

Fig. 6. Fructose consumption (g/L) and ethanol production (g/L) of *S. cerevisiae* BLR parental strain (a) and the evolved population after 100 generations of evolution on fructose (b) in synthetic broth containing 200 g/L fructose, pH=3.5. Growth was estimated using the optical density (OD) values of the culture broth at λ = 660 nm.

affected, since they are essential at the final stages of the alcoholic fermentation, when the ethanol concentration in the broth reaches its peak [37]. The evolved CFB population acquired in our previous study [62] was also tested in the same alcoholic fermentation conditions. We observed better fermentation kinetics than the parental strain, i.e., the fermentation was completed, although as late as in 495 h. This performance could possibly have been unexpected, considering that this population was evolved for 150 generations with glucose as the sole carbon source, which could have resulted to an even lower preference for fructose than the parental strain. However, considering that one key factor that inhibits fructose consumption during the final stages of fermentation, is ethanol toxicity [17], we can attribute the fructose consumption ability of the CFB population evolved on glucose to its resistance to ethanol, which was proved particularly high. Indeed, the difference in fructose consumption between the parental strain and the population evolved on glucose, occurred after 170 h of fermentation, when the fermentation broth began accumulating ethanol. This was also confirmed by the mathematical model which estimated K_{pp} value close to zero for the population evolved on fructose for 100 generations indicating a negligible inhibition effect of the produced ethanol concentration [67]. Also, the value of $\mu_{2_{max}}$ was lower than that of the parental stain. According to our previous study [62], in a fermentation with 200 g/L glucose the parameter μ_{max} exhibited the same value between the CFB parental strain and the population evolved on glucose for 150 generations, whereas Y_{ps} was higher in the evolved population than in the parental strain. These indications led us to the conclusion that the improved ethanol tolerance in this strain had a positive impact solely on ethanol production and not on cell growth. It is known that ethanol inhibition can affect both ethanol productivity and cell growth during alcoholic fermentation [64,75–77]. The fact that the population evolved on glucose completed the fermentation 325 h later than the population evolved on fructose, indicates the effectiveness of the ALE strategy employed in the present study.

In the case of BLR strain all three cell populations, the parental strain, the population evolved for 200 generations on glucose [62] and the population evolved for 100 generations on fructose, managed to complete the fermentation. It seems that the BLR strain is more fructophilic than the CFB strain by its nature, since the fermentation was completed in 354 h. The mathematical model was also consistent with the experimental data as the predicted values of Y_{xs_1}, Y_{xs_2}, Y_{ps_1} and Y_{ps_2} were higher in the BLR parental strain than in the CFB parental strain (Table 1). The value of $q_{2_{max}}$ in the BLR parental strain was also higher compared to the respective value in the CFB parental strain. However, the model failed to predict a higher value of $\mu_{2_{max}}$ in the BLR parental strain compared to the CFB parental strain, which is possibly in accordance with our previous study, where the μ_{max} of the BLR parental strain in a fermentation with 200 g/L glucose was 0.12 1/h, in comparison with 0.23 1/h of the CFB parental strain [62]. Strain specificity in the ability for fructose consumption during alcoholic fermentation has already been reported [26,78]. Surprisingly, the evolved population of the present study completed the fermentation 82 h later than the parental strain, presenting an improved ethanol yield. This could indicate that the ALE procedure did improve this cell population in the aspect of fructose consumption, yet, it should possibly have been prolonged for more generations, in order that more ethanol tolerance would also have been achieved. The fact that the population evolved on glucose for 200 generations exhibited the best fermentation performance, completing the fermentation 42 h earlier than the parental strain and 124 h earlier than the population evolved on fructose, proves the point that ethanol tolerance is of greater importance for improved fructose consumption in the BLR strain. Mathematical model was also able to predict this behavior since the values of $\mu_{1_{max}}$ and $\mu_{2_{max}}$ were higher in the population evolved on glucose for 200 generations in comparison with the values obtained from the parental strain and the population evolved on fructose (Table 1). Besides, it has been observed that high ethanol concentration hampers fructose consumption in a greater degree than glucose consumption [26,42]. Moreover, the mathematical model predicted that the initial concentrations of glucose and fructose did not have a limitation effect neither on biomass nor on ethanol production, as kinetic parameters of K_{sx_1}, K_{sx_2}, K_{sp_1} and K_{sp_2} were significantly lower than glucose and fructose concentrations. The estimated values of the above parameters were in the range of those obtained using the Aiba mathematical model to simulate the growth of the yeast *S. cerevisiae*, where glucose was used as the sole carbon source [67]. In Table 3 are also listed the parameters estimated in other studies by *S. cerevisiae* strains which produced ethanol during growth on glucose [66,68,69] or sweet sorghum juice [70] utilizing different mathematical models. Interestingly, the values of $\boldsymbol{\mu}_{1_{max}}$, $\boldsymbol{Y}_{ps_1}$ and $\boldsymbol{Y}_{xs_1}$ were similar to the ones estimated in the present study in all cases. It is noted that the theoretically predicted values of Y_{ps_1} and Y_{ps_2} were close to the experimental values, although these parameter values were optimized, in comparison with the maximum theoretical ethanol yield of alcoholic fermentation (0.51 g/g) [3] (Table 1). The differences between experimental and theoretically predicted values of Y_{xs_1} and Y_{xs_2} may be due to the formation of inhibitory byproducts (e.g. glycerol) during the fermentation process [79] that were not included in the Aiba model.

The fermentative abilities of commercial *S. cerevisiae* wine yeasts mentioned previously vary, depending on the medium used, the fermentation temperature, as well as the yeast strain. The strain *S. cerevisiae* N96, in a fermentation trial in Colombard must with 110.86 g/L glucose and 107.23 g/L fructose at 20 °C, consumed glucose in 14 days and fructose in 24 days [26]. In contrast, the strain *S. cerevisiae* T73 assimilated both sugars of a 'Tempranillo' red must with 112 ± 21 g/L glucose and 116 ± 17 g/L fructose in 7 days at 28 °C [80]. Liccioli et al. [81] selected 20 commercial *S. cerevisiae* wine yeasts that had been proven capable of consuming fructose effectively and realized alcoholic

fermentations in a chemically defined grape juice medium (CDGJM) with 115 g/L glucose and 115 g/L fructose at 28 °C. All strains consumed both sugars in less than 150 h. Nevertheless, in another study [82], the commercial strain *S. cerevisiae* UVAFERMPM completed a fermentation with CDGJM of 110 g/L glucose and 110 g/L fructose at 20 °C in more than 20 days, whereas the strain *S. cerevisiae* UVAFERM228 left almost 20 g/L residual sugars after almost 27 days of fermentation. Based on these results, the fermentative ability of the evolved population *S. cerevisiae* CFB of the present study (evolved on fructose), which consumed both sugars of a synthetic medium with 100 g/L glucose and 100 g/L fructose at 25 °C in 170 h (7 days) can be considered competent. However, it should be noted that the comparison is not necessarily fair, since the model medium used in this study renders a simulation solely of the sugar composition of grape must.

4.3. Alcoholic fermentations in synthetic fructose broth

The parental and evolved populations of the present study also underwent alcoholic fermentation in synthetic broth with 200 g/L fructose. The CFB parental strain was unable to complete the fermentation after 2100 h, leaving residual fructose of 54.27 g/L. The evolved population, although also unable to bring the broth to dryness, exhibited better fermentation ability than its parental strain, which was obvious in 400 h of fermentation, when it had residual fructose 66.43 g/L, in contrast to the parental strain which still had 158.42 g/L fructose remaining in the broth. The final residual fructose after 2100 h of fermentation was 17.54 g/L. This is an indication of better fitness of the evolved population, although the fermentation was sluggish in both cases. Previous studies have shown faster fructose consumption of a *S. cerevisiae* strain when fermenting both glucose and fructose than when fermenting solely fructose [81,83]. Although this phenomenon should be furtherly interpreted, it could explain why the CFB evolved population of the present study presented remarkable fermentation ability in a broth with both glucose and fructose, whereas it did not consume all fructose when used alone in the fermentation broth. Also, it is worth mentioning that an ALE experiment which targets the improvement of a specific phenotypic characteristic, can potentially provoke trade-offs, i.e., some characteristics will improve while other characteristics might actually deteriorate [61,84]. In the case of BLR, the cell population of the parental strain consumed all fructose in 400 h, confirming the natural ability of this strain to assimilate fructose. The fermentation realized by the evolved population was sluggish, leaving residual fructose of 11.99 g/L in 2000 h of fermentation, indicating that the ALE procedure realized in this study was not particularly impactful in this strain, possibly requiring more generations of evolution and/or a stronger selective pressure. This comes as no surprise, since ALE experiments are highly unpredictable and strain specific [58,74], meaning that the outcome of such cannot always be beneficial. Evolution is a non-linear process depending on the combination of a set of beneficial, neutral and deleterious mutations. Although ALE experiments are designed in order to purge deleterious mutations, the presence of clonal interference (2 or more beneficial mutations competing with each other) and genetic hitchhiking (increase of neutral and deleterious mutations' frequency) in asexual populations can occasionally impede the survival of the fittest phenotype [60].

4.4. Utilizing ALE to improve biochemical abilities of S. cerevisiae

To our knowledge, optimizing the ability of *S. cerevisiae* to consume fructose has not been extensively targeted. Walker et al. [85] recently realized random mutagenesis combined with directed evolution to generate *S. cerevisiae* strains with improved fructophilicity. Using the commercial strain *S. cerevisiae* AWRI 796 as parental strain, 4 different evolved isolates exhibited diverse fermentative abilities in a fermentation of CDGJM with 115 g/L glucose and 115 g/L fructose at 30 °C. In particular, the parental strain consumed all sugars in 153 h, two evolved isolates needed 117 h and 135 h, respectively, for total sugar consumption, whereas two other isolates did not consume all sugars in 153 h. Elsewhere [86] mild pre-treatment of *S. cerevisiae* cells with 14 % v/v ethanol resulted to improvement solely of glucose consumption, although the initial plan was the improvement of fructose consumption. Concerning the improvement of ethanol tolerance in wine yeast strains, an ALE experiment utilizing high ethanol concentrations as selective pressure was realized to the industrial wine yeast strains *S. cerevisiae* L76 and L78 [87]. In a synthetic must with 140 g/L glucose and 140 g/L fructose at 25 °C, both parental strains and the evolved populations did not complete the fermentation in 30 days, with the evolved populations leaving less concentration of residual sugars in all cases. Furthermore, in Chardonnay and Grenache natural musts with 150 g/L sugars at 16–17 °C, both strains and the evolved populations completed the fermentations in 30 days, with the evolved populations presenting faster fermentation kinetics [87]. In the present study, we conducted an ALE strategy which aimed to ameliorate both fructophilicity and ethanol tolerance of *S. cerevisiae* cells. The result was an evolved population, generated by the strain CFB, that combined the two desired traits, when tested in a synthetic broth with 100 g/L glucose and 100 g/L fructose.

5. Conclusion

ALE experiments have the potential to result particularly useful in the winemaking industry, where both the low preference of *S. cerevisiae* for fructose, as well as the toxicity of accumulated ethanol in the fermentation broth are causes for concern. The Aiba mathematical model used to estimate the biochemical performance of parental strains and evolved populations confirms that ALE is an effective method to improve *S. cerevisiae* in terms of fructophilicity and ethanol tolerance. Two different *S. cerevisiae* strains were used, producing two entirely different cell populations, proving that strain specificity plays a major role in wine yeast strains. One population derived from CFB parental strain exhibited exceptionally improved fermentation ability when fermenting a synthetic broth with 100 g/L glucose and 100 g/L fructose. Such population could be highly beneficial for the winemaking industry. Inferentially, such evolved populations with improved fermentation characteristics should subsequently conduct alcoholic fermentations under real winemaking conditions, to further be evaluated.

CRediT authorship contribution statement

Maria Mavrommati: Writing – original draft, Visualization, Software, Methodology, Investigation, Data curation, Conceptualization. **Christina N. Economou:** Software, Methodology, Formal analysis, Data curation. **Stamatina Kallithraka:** Writing – review & editing, Validation, Supervision. **Seraphim Papanikolaou:** Writing – review & editing, Validation, Supervision, Resources. **George Aggelis:** Writing – review & editing, Validation, Supervision, Resources, Conceptualization.

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.

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