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Development of freezing-resistant hybrid yeast from *Saccharomyces cerevisiae* for French bread dough

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ABSTRACT

This study aimed to develop non-GMO freezing-resistant hybrid yeast for frozen French bread dough. The hybrids were generated by direct mating, using *Saccharomyces cerevisiae* strains from the ethanol industry (PE-2) and a baker's yeast (FLE). Four viable hybrids were generated and named F5P1, F5P33, F5P40, and F5P42. According to the 96-well microplate analyses, the growth curves of the hybrids after freezing and subsequent thawing presented similar behavior to those without freezing. In a performance test after the freezing step, F5P1 and F5P40 achieved a higher development index, with values of (147.1 ± 2.5) % and (102.1 ± 5.6) %, respectively. The peak reached by F5P1, after 50 min, was similar ($p > 0.05$) to that of FLE, which was at 110 min. The difference between the time required for F5P1 to achieve the growth peak with and without freezing was 10 min, whereas for FLE, this difference was three times higher, which highlights the improving freezing resistance of the hybrid. Therefore, the hybrid F5P1 showed a leavening capacity similar to FLE ($p > 0.05$) and improved freezing resistance, which indicates its potential for application to frozen French bread dough.

1. Introduction

Bread is a staple food widely consumed by humanity; it has many forms and is usually made using wheat flour (Cauvain & Young, 2007). The worldwide revenue in the bread market amounted to US$ 450 billion in 2022, while in Brazil, this revenue was US$ 14.53 billion (Statista, 2023). French bread is the main product of the Brazilian bread market, accounting for 45 % of the sales volume. A typical French bread is (12.5–14.0) cm long, (5.5–7.0) cm in diameter, 50 g of final weight, has a brown and glossy crust and has a white and soft crumb (Carr & Tadini, 2003).

Freezing is a well-known technique to extend the shelf-life of food and beverages. For French bread, freezing can occur after each step of production, and depending on the step it is applied to, the products have different advantages. For example, freezing bread before fermentation, which is called frozen French bread dough (FFBD), results in a lower specific volume of bread due to a loss in the leavening capacity of the yeast. However, since dough has a lower volume than baked bread, FFBD allows for a greater amount of storage and transportation simultaneously. Another example is frozen part-baked French bread (FPBFB), which is the product obtained when freezing is applied after a short baking process. As a consequence, rigorous temperature control is required to prevent the collapse of the crust. In addition, a loss in bread weight is observed after 2 days of storage, and a reduction in specific volume occurs after 4 days (Carr, Rodas, Della Torre, & Tadini, 2006; Carr & Tadini, 2003).

The enhancement of yeast can be an innovative possibility in breadmaking, allowing it to overcome the loss of the leavening capacity of yeast after the freezing process. To accomplish that, hybridization methods can be applied by selecting previously known yeasts with target characteristics (Krogerus, Magalhães, Vidgren, & Gibson, 2017; Lu et al., 2021). The use of hybrid yeasts is increasing since it has a high potential to overcome industrial problems due to being more resistant to stresses (Bendixsen, Frazão, & Stelkens, 2022; Lu et al., 2021). Furthermore, as molecular biology advanced, it was discovered that most industrial yeasts are natural hybrids from different species. Thus, genome complexity was increased and the yeast performance for industrial processes has been improved (Lopandic, 2018).

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The development and application of hybrid yeasts are widely common in the beverage industry, due to the search for new flavors for beer and wine (Bellon et al., 2011; Bellon, Schmid, Capone, Dunn, & Chambers, 2013; Bellon, Yang, Day, Inglis, & Chambers, 2015; Hart et al., 2019; Kanter et al., 2019; Krogerus, Magalhães, Vidgren, & Gibson, 2015; Lu et al., 2021). In breadmaking, there are few papers about hybrid yeasts in the literature. Oda, Tanizaki, Yokoyama-Ohtsuka, and Sakurai (2020) developed a hybrid between two different species, *S. mikatae* and *S. cerevisiae*, for fresh dough. However, the bread prepared with the hybrid presented a different flavor compared to the control one. Magalhães, Calton, Heiniö, and Gibson (2021) developed hybrids from *S. cerevisiae* and the species *S. eubayanus*, *S. jurei* and *S. arborícola* for frozen dough. Although one of the hybrids showed potential for bread application, these hybrids showed poor growth under industrial conditions, since they were developed by mating non-industrial species.

Based on what was previously presented, this study aimed to develop a non-GMO freezing-resistant hybrid from two industrial strains of *S. cerevisiae*, one highly stress-tolerant from the ethanol industry and a traditional baker's yeast, for applications to frozen French bread dough. The hybridization was made by the direct mating protocol and we have used growth rates, final optical density, freezing resistance, and leavening capacity to select both haploids derived from the parental strains, as well as the hybrids generated by the selected haploids.

2. Material and methods

The methodology presented in this work to generate and select the hybrids is summarized in Fig. 1.

2.1. Parental strains

In this work, two *Saccharomyces cerevisiae* strains were mated to generate hybrid yeasts. PE-2, from the ethanol industry (Basso, de Amorim, de Oliveira, & Lopes, 2008), and FLE, which is a baker's yeast.

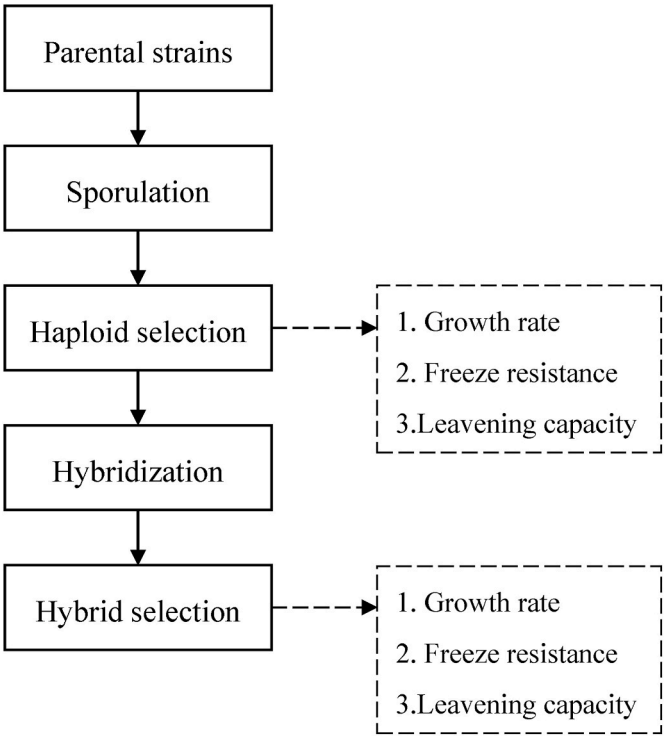

Fig. 1. Main steps for the development and selection of hybrids. Continuous lines indicate the main process and dashed lines indicate the performance tests.

Both strains were donated by Prof. Luiz Carlos Basso from ESALQ-USP (Escola Superior de Agricultura "Luiz de Queiroz" - Universidade de São Paulo).

2.2. Growth media and stock culture preservation

This methodology was adapted from Green and Moehle (1999), as follows: A YP medium (10 g/L yeast extract and 20 g/L bacto peptone) was used as growth media, supplemented with 20 g/L of dextrose (YPD20) or 50 g/L of dextrose (YPD50). The strains were cultivated overnight at 30 °C and 180 rpm using an orbital shaker (New Brunswick Scientific, C24 Incubator Shaker, USA). As for growth in solid media, YPD20 was solidified by adding 20 g/L of agar (YPD20-A), and the strains were streaked and incubated (Nova Ética, N480, Brazil) at 30 °C for 48 h. Yeast stock cultures were maintained at – 80 °C (Eppendorf, CryoCube F570, Germany) in YPD20 supplemented with 10 mL of a glycerol solution (20 g/100 g).

2.3. Strains characterization

The methodologies presented in this section were applied to all strains, starting with the parental strains, secondly to haploids, and finally to hybrids. For haploids and hybrids, each method was a selection step to determine which strains would be discarded or kept. The order presented is that in which the experiments were conducted.

2.3.1. Growth rate and maximum optical density

This methodology was adapted from Teunissen et al. (2002), as follows: The strains were evaluated by optical density measurements (Quimis, Q898U2M5, Brazil) at 600 nm (OD_{600nm}), specific growth rate (μ), and lag phase (λ). μ and λ were calculated using Python programming language. Two media were used for haploids: YPD20 for the first screening and YP medium supplemented with 10 g/L maltose (YPM10) for the selected haploids. As for hybrids, only YPD20 was used. For all strains, growth experiments were performed in a 96-well microplate using 200 μL of the medium and washed biomass pellet, pre-grown in YPD20 overnight culture, was added to reach 0.1 of the starting OD_{600nm}. The experiment was carried out on a plate reader (TECAN, Infinite® M200PRO, Switzerland) at 30 °C and OD_{600nm} was measured every 20 min for 20 h.

2.3.2. Freezing resistance

This methodology was adapted from Teunissen et al. (2002), as follows: The freezing resistance was evaluated using the same 96-well-microplates methodology described in item 2.3.1 using YPD20 and with the addition of freezing and thawing steps. The microplates were frozen at – 20 °C (Consul, CVU26, Brazil) for 1 h and thawed for 10 min at room temperature. Subsequently, microplates were inserted into the plate reader.

2.3.3. Leavening capacity test

The leavening capacity of the strains was evaluated using a methodology adapted from Codón et al. (2003). The experiment was conducted using 20 mL tubes containing 3.3 mL of Milli Q water, 2 g of wheat flour, and supplemented with 0.2 g of yeast cream. The aqueous suspension of wheat flour was mixed using a mechanical stirrer (Fisatom, Modelo 715, Brazil) until a homogeneous suspension was obtained and fermented (Nova Ética, N480, Brazil) at 30 °C for fresh dough. For the frozen dough, the tubes were frozen at – 20 °C for 1 h (Electrolux, H500, Sweden) and thawed for 30 min at room temperature before the fermentation process.

The height of the suspension was measured every 10 min for 3 h using a Nikkon D60 camera and two graduated rulers, fixed on both sides of the incubator. The Development Index (*DI*) was calculated according to Equation (1).

$$DI = \frac{H_{max} - H_0}{H_0} \times 100 \quad (1)$$

wherein: H_{max} is the maximum height (cm) achieved and H_0 is the initial height (cm).

2.4. Sporulation and tetrad dissection for obtaining haploids

This methodology was adapted from Basso (2015), as follows: The *S. cerevisiae* strains (PE-2 and FLE) were grown in a raffinose-acetate sporulation medium (0.02 g/100 g raffinose and 0.3 g/100 g potassium acetate) at 30 °C and 220 rpm (New Brunswick Scientific, C24 Incubator Shaker, USA) for 5 days until sporulation occurred. The tetrad suspension (2 mL) was centrifugated (Beckman Coulter, GS-15 Centrifuge, USA) for 10 min at 4000 rpm and resuspended in 450 μL of Milli Q water and 40 μL of zymolyase enzyme solution (ZymoReasearch® 10 units/uL, 10 mM mercaptoethanol, 50 mM potassium phosphate buffer, pH 7.5). These resuspended suspensions were incubated for 15 min at 37 °C in a thermostatic bath (FANEM, model 102, Brazil) and 10 μL of treated cells were streaked on a solid medium for micromanipulation (5 g/L yeast extract, 5 g/L peptone, 20 g/L dextrose, and 20 g/L agar). Tetrad dissections were performed using a micromanipulator (Carl Zeiss, Scope A1 AXIO, Germany) and the mating type of isolated spores was determined (Mortimer & Hawthorne, 1800) using the testers strains KFY138 for MATα and KFY139 for MATa.

2.5. Hybridization by direct mating

Hybrids were generated by the spore-to-spore method, crossing the selected haploids of PE-2 and FLE according to their mating type. Biomass from the selected haploids, pre-grown in YPD20-A for 48 h at 30 °C, was mixed in another YPD20-A and kept at room temperature for 3 h until zygote formation. The resulting biomass containing the zygotes was streaked in a solid medium and their separation was made using a micromanipulator (Carl Zeiss, Scope A1 AXIO, Germany). The plates were incubated at 30 °C for 72 h (FANEM, B.O.D model 347 CD, Brazil). The hybrid formation was confirmed by the mating type, the sporulation test and cell size evaluation under microscope.

2.6. Statistical analyses

All experiments were performed in triplicate. All data from growth rate, maximum optical density, freezing resistance, and leavening capacity were submitted to statistical analysis using Statgraphics Centurion XVI v.16.1.15. Analysis of variance (ANOVA) and Tukey tests for multiple ranges with a significance level of $p < 0.05$ were conducted.

3. Results and discussion

3.1. Haploids

After the sporulation, tetrad dissection, incubation, and mating type test, 43 viable haploids from PE-2 (29 MATα and 14 MATa) and 10 from FLE (9 MATα and 1 MATa) were obtained. The PE-2 haploids were named from Ha-PE-1 to Ha-PE-43 and the FLE haploids from Ha-FLE-1 to Ha-FLE-10.

3.1.1. Growth rate and maximum optical density of the haploids

Since FLE is a common bakery yeast, the first screening was based on its values of μ and OD_{max} on YPD20. The reason for using growth rate (μ) as the selecting physiological parameter is because yeast cell biomass formation is directly linked to CO_2 evolution during carbon source dissimilation, which is responsible for dough leavening. Therefore, 16 PE-2 haploids and 1 FLE haploid (Ha-FLE-5) that did not show significant differences ($p > 0.05$) in these parameters, were selected. However, since direct mating requires haploids with different mating types and Ha-FLE-5 was MAT**a**, the MAT**a** PE-2 haploids were discarded. The values of μ and OD_{max} in YPD20 from the 11 selected PE-2 haploids and Ha-FLE-5 are presented in Table 1.

These parameters from selected haploids were evaluated on YPM10. Although all haploids showed significant differences ($p \leq 0.05$) in the μ values, they did not show significant differences ($p > 0.05$) in the OD_{max} values. Therefore, they were maintained for the freezing resistance test.

3.1.2. Freezing resistance of the haploids

The lag phase (λ) was evaluated to select freezing-resistant haploids. Dough proving varies between 45 and 90 min depending on relative humidity (Carr et al., 2006; Rouillé, Chiron, Colonna, Della Valle, & Lourdin, 2010; Sommier, Chiron, Colonna, Della Valle, & Rouillé, 2005); consequently, haploids showing a lag phase higher than 1 h were disposed of. Therefore, in this first screening, six PE-2 haploids and the Ha-FLE-5 were selected.

In the second screening, a comparison of the experiment values was made with and without the freezing step (Table 2). After the freezing and thawing processes, an increase in λ is expected due to the high stress that cells undergo. An increase of 20 min was observed for Ha-PE-18, Ha-PE-33, and Ha-PE-42, and 40 min for Ha-PE-20 and Ha-FLE-5. Since Ha-PE-1 and Ha-PE-40 did not show differences, it indicates the higher freezing resistance of these haploids. Although these alterations in λ values were observed, the 7 haploids were maintained for the performance test.

3.1.3. Leavening capacity test of the haploids

As dough proving varies between 45 and 90 min, the haploid selection was based on their Development Index (*DI*) and the time required to achieve it (Table 2). Ha-FLE-5 presented the highest *DI* of all haploids (96.8 ± 21.5) % after 60 min. From PE-2 haploids, only Ha-PE-33 and Ha-PE-42 presented higher *DI* values achieved up to 90 min of the experiment. Ha-PE-1, Ha-PE-18, and Ha-PE-20 reached their *DI* after 90 min and did not show significant differences ($p > 0.05$) in comparison to those of Ha-PE-33 and Ha-PE-42.

The haploid from the baker's yeast showed better results on leavening capacity, whereas the PE-2 haploids performed better on the microplate freezing resistance test. These results were expected based on the application of each strain. Thus, mating one haploid from PE-2 with another from FLE could result in a hybrid with acceptable leavening capacity and higher freezing resistance. Therefore, the 7 haploids were kept for the hybridization step.

Table 1
Values of specific growth rate (μ) and maximum optical density (OD_{max}) on YPD20 of 11 selected PE-2 and Ha-FLE-5 haploids, obtained from the haploid selection step, in comparison to the parental strain FLE. Three replications were made, and values are presented as means ± standard error.

Strain	μ (1/h)	OD_{max}
FLE	0.5507 ± 0.0163^{a}	$0.8761 \pm 0.0038^{a.b.c.d.e}$
Ha-PE-1	0.5764 ± 0.0861^{a}	$0.8376 \pm 0.0324^{a.b.c.d.e}$
Ha-PE-15	0.5667 ± 0.0198^{a}	$0.9140 \pm 0.0092^{c.d.e.f}$
Ha-PE-17	0.5342 ± 0.0653^{a}	$0.9211 \pm 0.0225^{d.e.f}$
Ha-PE-18	0.5884 ± 0.0341^{a}	0.9501 ± 0.0762^{f}
Ha-PE-20	0.5697 ± 0.0655^{a}	$0.8895 \pm 0.0573^{b.c.d.e}$
Ha-PE-21	0.5490 ± 0.0534^{a}	$0.8958 \pm 0.0310^{b.c.d.e}$
Ha-PE-23	0.5478 ± 0.0239^{a}	0.9316 ± 0.1109^{f}
Ha-PE-28	0.5740 ± 0.0217^{a}	$0.9193 \pm 0.0364^{d.e.f}$
Ha-PE-33	0.5545 ± 0.0286^{a}	$0.9224 \pm 0.0117^{e.f}$
Ha-PE-40	0.5546 ± 0.0594^{a}	0.9324 ± 0.0244^{f}
Ha-PE-42	0.5361 ± 0.0040^{a}	0.9313 ± 0.0115^{f}
Ha-FLE-5	0.5482 ± 0.0395^{a}	$0.7333 \pm 0.0197^{a.b}$

Means with the same letter in the column are not significantly different ($p > 0.05$).

Table 2
Values of lag phase (λ) of the six haploids from PE-2 and one from FLE, obtained from freezing resistant experiments, and the values of Development Index (*DI*) with their respective achievement time from the same haploids obtained from leaving capacity tests. Three replications were made, and values are presented as means ± standard error.

Haploid	λ (min)		*DI* (%)	Time (min)
	Fresh	Frozen		
Ha-PE-1	20	20	55.2 ± 12.3[b]	150
Ha-PE-18	20	40	58.4 ± 18.6[b]	180
Ha-PE-20	20	60	52.2 ± 9.8[b]	150
Ha-PE-33	20	40	52.1 ± 14.7[b]	90
Ha-PE-40	20	20	29.4 ± 8.9[c]	50
Ha-PE-42	20	40	56.4 ± 13.9[b]	70
Ha-FLE-5	20	60	96.8 ± 21.5[a]	60

Means with the same letter in the column are not significantly different ($p > 0.05$).

3.2. Hybrids

Of the 53 haploids obtained, 6 from PE-2 haploids, and 1 from FLE haploids were selected, making the development of 6 hybrids possible. After hybridization, the hybrids developed with Ha-PE-18 and Ha-PE-20 were not viable. As a consequence, four hybrids were obtained and named F5P1, F5P33, F5P40, and F5P42. They were submitted to the mating type and sporulation tests and were checked on the microscope to compare cell size. For all hybrids, no halo was observed in the mating type test, indicating that they are diploids. F5P33 and F5P42 could sporulate and generate tetrads, while F5P1 and F5P40 did not sporulate. This indicates that these two hybrids lost their ability for sporulation or they need another sporulation media. Furthermore, all hybrid cells had larger sizes than both haploid parents.

3.2.1. Growth rate and maximum optical density of hybrids

Firstly, the hybrids were compared to their parents to evaluate how the characteristics of both parents were inherited by the hybrid, as shown in Table 3. For OD_{max}, all hybrids presented higher values than their parents and there were no significant differences among them ($p > 0.05$). Moreover, the μ of F5P1 was not significantly different ($p > 0.05$) compared to all haploids; F5P33 and F5P42 showed lower values of μ than those of all haploids; and F5P40 showed the highest value of μ from all strains.

When hybrid results were compared with that of FLE (Table 3), its OD_{max} was higher than all hybrids. As for μ, FLE and F5P1 did not show significant differences ($p > 0.05$), whereas F5P33 and F5P42 had lower values, whereas F5P40 had the highest of all.

Table 3
Values of specific growth rate (μ) and maximum optical density (OD_{max}) on YPD20, and the lag phase (λ) of the 5 haploids used in hybridization and their 4 hybrids, in comparison to the parental strain FLE. Three replications were made, and values are presented as means ± standard error.

Strain	μ (1/h)	OD_{max}	λ (min)
FLE	0.5507 ± 0.0163[a]	1.4078 ± 0.1247[d]	40[a]
Ha-FLE-5	0.5482 ± 0.0395[a]	0.7333 ± 0.0197[a]	60
Ha-PE-1	0.5764 ± 0.0861[a]	0.8376 ± 0.0324[a,b]	20
Ha-PE-33	0.5545 ± 0.0286[a]	0.9224 ± 0.0117[b]	40
Ha-PE-40	0.5546 ± 0.0594[a]	0.9324 ± 0.0244[b]	20
Ha-PE-42	0.5361 ± 0.0040[a]	0.9313 ± 0.0115[b]	40
F5P1	0.5561 ± 0.0186[a]	1.2392 ± 0.0630[c]	40
F5P33	0.5066 ± 0.0148[b]	1.2535 ± 0.0123[c]	40
F5P40	0.6290 ± 0.0049[c]	1.1861 ± 0.0067[c]	20
F5P42	0.4959 ± 0.0080[b]	1.2690 ± 0.0323[c]	40

Means with the same letter in the column are not significantly different ($p > 0.05$).
[a] without the freezing step.

3.2.2. Freezing resistance of the hybrids

The comparison of the λ values after freezing and thawing of the hybrids and their parental strains is shown in Table 3. The hybrids F5P33, F5P40, and F5P42 showed the same λ values as their PE-2 haploid parents, while F5P1 presented a λ value higher than its PE-2 haploid parent and lower than its FLE haploid parent. F5P40 had the lowest lag phase time, while all the other hybrids had the same time as the FLE without the freezing step (40 min).

Moreover, the lag phase of all hybrids was the same with or without the freezing and thawing steps. These results can indicate that the hybrids have higher freezing resistance, especially in the case of F5P40, which presented the lowest lag phase time.

3.2.3. Leavening capacity test of the hybrids

The *DI* values of the hybrids in comparison to that of FLE and the respective time to reach it are shown in Fig. 2. The *DI* values of FLE, with (165.8 ± 5.50) % and without the freezing step (162.4 ± 0.4) %, and of F5P1 (147.1 ± 2.5) % after freezing, did not show significant differences ($p > 0.05$), and represent the highest growth. F5P42 had the lowest *DI*, with similar values for frozen (55.9 ± 8.4) % and fresh (54.4 ± 1.8) % suspensions. Of all hybrids, only F5P1 showed significant differences ($p \leq 0.05$) between frozen and fresh suspensions, and the frozen suspension had a higher *DI* than the fresh one (97.4 ± 16.3) %. For better visualization, the suspensions of the hybrids and the parental strains are shown in Fig. 3.

Another parameter that is crucial for the baking process is the time required to reach the *DI,* which is indicated in Fig. 2. Two hybrids did not show differences in time between frozen and fresh suspensions, 80 min for F5P33 and 120 min for F5P42. The lowest time was observed for F5P1, which achieved its *DI* after 50 min for frozen and 40 min for fresh suspensions. This result is interesting for the baking process since this time is shorter than the maximum time usually considered for fermentation (90 min). Almost the same was observed for F5P40, which has 20 min of difference between frozen and fresh suspensions, values higher than those of F5P1.

The results for F5P1 and F5P40 indicate an increase in freezing resistance compared to FLE. FLE *DI* was achieved after 80 min for fresh suspension and 110 min for frozen suspension, which represents an increase of 30 min in time. In addition, the time required to achieve the *DI* in frozen suspension is higher than the upper limit of fermentation time. The increase of 30 min observed in FLE is three times higher than the difference observed in F5P1 (10 min). Comparing those two, the hybrid F5P1 was capable of acquiring the same growth as FLE ($p > 0.05$) after being frozen and it was observed 30 min before the FLE fresh suspension and 60 min before the FLE frozen suspension. This result can indicate a satisfactory leavening capacity and freezing resistance of F5P1. Three hybrids showed lower times to reach the *DI* than FLE, indicating that they present better freezing resistance, which was inherited from the PE-2 parent.

Since the aqueous suspension of wheat flour is a model bread system, it is important to mention that it does not contain added salt. Salt is usually applied to doughs as a way to control fermentation until the full development of gluten structure via osmotic stress (Cauvain & Young, 2007), which induces a stress response on yeast cells, increasing glycerol formation and decreasing growth rate and CO_2 evolution (Feldmann, 2012). PE-2 is an industrial strain with generally better resistance to osmotic stress as compared to other yeast strains (Basso et al., 2008), and as the hybrid might inherited its resistance, this could indicate that it can be more tolerant to salt addition.

4. Conclusions

Hybridization is a consolidated method for the development of new strains and the direct mating method is one of the options to accomplish non-GMO new strains. Of the four hybrids developed in this work, one of them, F5P1, showed an improvement in freezing resistance compared to

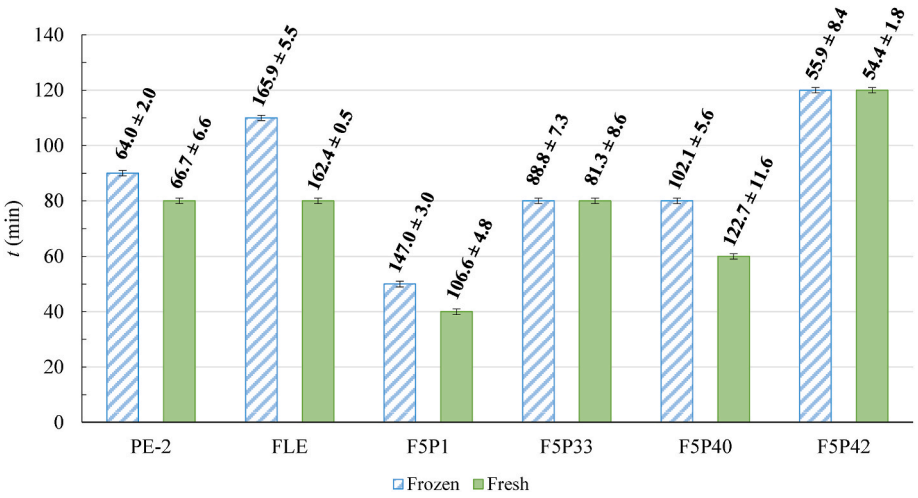

Fig. 2. Time (min) required to reach the Development Index (*DI*), with and without the freezing and thawing steps. Bold numbers on each bar indicate the *DI* of the hybrids in comparison to that of FLE. Three replications were made and the *DI* values are presented as means ± standard error.

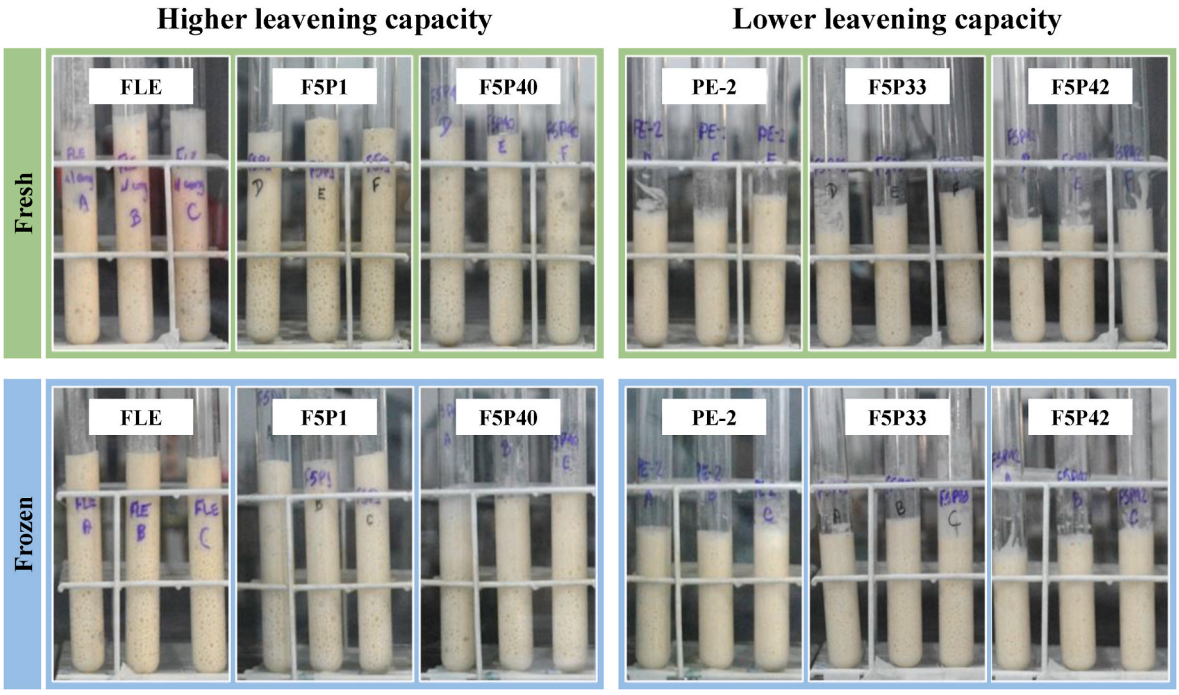

Fig. 3. Pictures of fresh and frozen suspension of hybrids and the parental strains at their maximum development index.

the FLE parent and a better leavening capacity than the PE-2 parent. While FLE required 110 min to reach its *DI* (165.8 ± 5.5 %) after the freezing and thawing processes, F5P1 required less than half of this time, 50 min to reach its *DI* (147.1 ± 2.5 %), with similar growth peaks ($p > 0.05$). This indicates that the hybrid inhales the target parameters from its parents (FLE and PE-2) and can be an option for applying to frozen French bread dough. Although this work focused on French bread dough for its economic importance as the main product in the Brazilian bread market, the hybrid F5P1 can also be an option for applying to other frozen doughs.

CRediT authorship contribution statement

Giulliana Petean Torrano: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Gabriel Aparecido Atanazio-Silva:** Methodology, Investigation. **Thalita Peixoto Basso:** Writing – review & editing, Resources, Methodology, Formal analysis, Conceptualization. **Thiago Olitta Basso:** Writing – review & editing, Resources, Methodology, Formal analysis, Conceptualization. **Carmen Cecilia Tadini:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Acknowledgments

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References

Basso, T. P. (2015). Improvement of Saccharomyces cerevisiae by hybridization for increased tolerance towards inhibitors from second-generation ethanol substrate. In *Thesis (Doctor in Science. Area: Agricultural Microbiology), Escola Superior de Agricultura Luiz de Queiroz*. Universidade de São Paulo. https://doi.org/10.11606/T.11.2015.tde-29042015-132341. Piracicaba.

Basso, L. C., de Amorim, H. V., de Oliveira, A. J., & Lopes, M. L. (2008). Yeast selection for fuel ethanol production in Brazil. *FEMS Yeast Research, 8*(7), 1155–1163. https://doi.org/10.1111/j.1567-1364.2008.00428.x

Bellon, J. R., Eglinton, J. M., Siebert, T. E., Pollnitz, A. P., Rose, L., de Barros Lopes, M., et al. (2011). Newly generated interspecific wine yeast hybrids introduce flavour and aroma diversity to wines. *Applied Microbiology and Biotechnology, 91*(3), 603–612. https://doi.org/10.1007/s00253-011-3294-3

Bellon, J. R., Schmid, F., Capone, D. L., Dunn, B. L., & Chambers, P. J. (2013). Introducing a new breed of wine yeast: Interspecific hybridisation between a commercial Saccharomyces cerevisiae wine yeast and Saccharomyces mikatae. *PLoS One, 8*(4), Article e62053. https://doi.org/10.1371/journal.pone.0062053

Bellon, J. R., Yang, F., Day, M. P., Inglis, D. L., & Chambers, P. J. (2015). Designing and creating Saccharomyces interspecific hybrids for improved, industry relevant, phenotypes. *Applied Microbiology and Biotechnology, 99*(20), 8597–8609. https://doi.org/10.1007/s00253-015-6737-4

Bendixsen, D. P., Frazão, J. G., & Stelkens, R. (2022). Saccharomyces yeast hybrids on the rise. *Yeast, 39*(1–2), 40–54. https://doi.org/10.1002/yea.3684

Carr, L. G., Rodas, M. A. B., Della Torre, J. C. M., & Tadini, C. C. (2006). Physical, textural and sensory characteristics of 7-day frozen part-baked French bread. *LWT - Food Science and Technology, 39*(5), 540–547. https://doi.org/10.1016/j.lwt.2005.03.012

Carr, L. G., & Tadini, C. C. (2003). Influence of yeast and vegetable shortening on physical and textural parameters of frozen part baked French bread. *LWT - Food Science and Technology, 36*(6), 609–614. https://doi.org/10.1016/S0023-6438(03)00079-3

Cauvain, S. P., & Young, L. S. (2007). *Technology of breadmaking* (2nd ed.). New York: Springer New York. https://doi.org/10.1007/0-387-38565-7

Codón, A. C., Rincón, A. M., Moreno-Mateos, M. A., Delgado-Jarana, J., Rey, M., Limón, C., et al. (2003). New Saccharomyces cerevisiae baker's yeast displaying enhanced resistance to freezing. *Journal of Agricultural and Food Chemistry, 51*(2), 483–491. https://doi.org/10.1021/jf020701a

Feldmann, H. (2012). *Yeast: Molecular and cell biology* (2nd ed.). Weinheim: Wiley-VCH Verlag GmbH & Co. KGaA. https://doi.org/10.1002/9783527659180

Green, S. R., & Moehle, C. M. (1999). Media and culture of yeast. *Current Protocols in Cell Biology, 4*(1.6), 1–1.6.12. https://doi.org/10.1002/0471143030.cb0106s04

Hart, R. S., Jolly, N. P., & Ndimba, B. K. (2019). Characterisation of hybrid yeasts for the production of varietal Sauvignon blanc wine - a review. *Journal of Microbiological Methods, 165*, Article 105699. https://doi.org/10.1016/j.mimet.2019.105699

Kanter, J. P., Benito, S., Brezina, S., Beisert, B., Fritsch, S., Patz, C. D., et al. (2019). The impact of hybrid yeasts on the aroma profile of cool climate Riesling wines. *Food Chemistry X, 5*, Article 100072. https://doi.org/10.1016/j.fochx.2019.100072

Krogerus, K., Magalhães, F., Vidgren, V., & Gibson, B. (2015). New lager yeast strains generated by interspecific hybridization. *Journal of Industrial Microbiology & Biotechnology, 42*(5), 769–778. https://doi.org/10.1007/s10295-015-1597-6

Krogerus, K., Magalhães, F., Vidgren, V., & Gibson, B. (2017). Novel brewing yeast hybrids: Creation and application. *Applied Microbiology and Biotechnology, 101*(1), 65–78. https://doi.org/10.1007/s00253-016-8007-5

Lopandic, K. (2018). Saccharomyces interspecies hybrids as model organisms for studying yeast adaptation to stressful environments. *Yeast, 35*(1), 21–38. https://doi.org/10.1002/yea.3294

Lu, S., Zheng, F., Wen, L., He, Y., Wang, D., Wu, M., et al. (2021). Yeast engineering technologies and their applications to the food industry. *Food Biotechnology, 35*(3), 252–271. https://doi.org/10.1080/08905436.2021.1942037

Magalhães, F., Calton, A., Heiniö, R. L., & Gibson, B. (2021). Frozen-dough baking potential of psychrotolerant Saccharomyces species and derived hybrids. *Food Microbiology, 94*, Article 103640. https://doi.org/10.1016/j.fm.2020.103640

Mortimer, R. K., & Hawthorne, D. C. (1800). Yeast genetics. In A. H. ROSE, & J. S. HARRISON (Eds.), *The yeast* (pp. 386–460). New York: Academic Press.

Oda, Y., Tanizaki, S., Yokoyama-Ohtsuka, M., & Sakurai, H. (2020). Interspecific hybrid between Saccharomyces mikatae and Saccharomyces cerevisiae as an alternative strain for bread making. *Journal of Microbiology, Biotechnology and Food Sciences, 10*(1), 127–129. https://doi.org/10.15414/jmbfs.2020.10.1.127-129

Rouillé, J., Chiron, H., Colonna, P., Della Valle, G., & Lourdin, D. (2010). Dough/crumb transition during French bread baking. *Journal of Cereal Science, 52*(2), 161–169. https://doi.org/10.1016/j.jcs.2010.04.008

Sommier, A., Chiron, H., Colonna, P., Della Valle, G., & Rouillé, J. (2005). An instrumented pilot scale oven for the study of French bread baking. *Journal of Food Engineering, 69*(1), 97–106. https://doi.org/10.1016/j.jfoodeng.2004.07.015

Statista. (2023). *Consumer market insights – bread*. Retrieved from https://www.statista.com/outlook/cmo/food/bread-cereal-products/bread. (Accessed 24 October 2023).

Teunissen, A., Dumortier, F., Gorwa, M. F., Bauer, J., Tanghe, A., Loïez, A., et al. (2002). Isolation and characterization of a freeze-tolerant diploid derivative of an industrial baker's yeast strain and its use in frozen doughs. *Applied and Environmental Microbiology, 68*(10), 4780–4787. https://doi.org/10.1128/AEM.68.10.4780-4787.2002