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Identifying yeast isolated from spoiled peach puree and assessment of its batch culture for invertase production

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Abstract

The identification of yeasts isolated from spoiled Jubileu peach puree using the API 20C AUX method and a commercial yeast as witness were studied. Subsequently, the yeast's growth potential using two batch culture treatments were performed to evaluate number of colonies (N), reducing sugar concentration (RS), free-invertase (FI), and culture-invertase activity (CI). Stock cultures were maintained on potato dextrose agar (PDA) slants at 4 °C and pH 5 for later use for batch-culture (150 rpm) at 30°C for 24 h, then they were stored at 4 °C for subsequent invertase extraction. The FI extract was obtained using $NaHCO_3$ as autolysis agent, and CI activity was determined on the supernatant after batch-cultured centrifugation. The activity was followed by an increase in absorbance at 490 nm using the acid 3,5-DNS method with glucose standard. Of the four yeasts identified, *Saccharomyces cerevisiae* was chosen for legal reasons. It showed logarithmic growth up to 18 h of fermentation with positive correlation CI activity and inverse with RS. FI showed greater activity by the end of the log phase and an inverse correlation with CI activity. Finally, it was concluded that treatment "A" is more effective than "B" to produce invertase (EC 3.2.1.26).

Keywords: spoiled peach puree; invertase; *Saccharomyces cerevisiae*; autolysis.

Pratical Application: Potential production of glucose and fructose syrup by yeast invertases from spoiled peach pure.

1 Introduction

Yeasts are fungi usually and predominantly found in unicellular form. Their vegetative reproduction takes place mainly through gemmulation. They have cell walls and are easily differentiated from bacteria due to their larger dimensions and morphological properties. Each species has a characteristic shape, but, even in pure cultures, there is considerable variation in individual cell size and shape depending on the age and environment (Choudhary & Johri, 2009). Flowers and fruit are important micro-habitats for a range of yeast species due to their favorable sugar concentration and pH. Yeast are mainly found in ripe and spoiled fruits due to the visits of vector insects and the wind. Moreover, they are ubiquitous eukaryote microorganisms with an active role in fermented foods, pulps, and drinks (Fazio, 2006; Silva et al., 2011). In fruit pulps, the most commonly isolated yeasts belong to the genera *Saccharomyces, Pichia, Cryptococcus, Kluyveromyces*, and *Candida* (Sancho et al., 2000; Melo et al., 2007; Aleklett et al., 2014).

The genus *Saccharomyces* is the most widely investigated and attractive to work with for being an organism classified as GRAS (generally recognized as safe) and for being a reference of eukaryote cells in biology, besides its wide application in biotechnology (Ostergaard et al., 2000). Its colonies' color range between white (predominantly) and beige (occasionally), with convex and smooth shape. The yeasts may form pseudohyphae consisting of simple chains of spherical, elliptic, or cylindrical cells. They most times reproduce by multilateral gemmulation, with the new gemmule being formed on the side of the mother cell (Pretorius, 2000; Choudhary & Johri, 2009).

Properly identifying new isolated yeasts is crucial and relevant for applications in both biotechnology and clinical practice. Traditionally, yeasts have been identified through phenotypic methods to assess morphological, physiological, and biochemical characteristics, but the results are not very safe. Several genotypic techniques, including new ones such as polymerase chain reaction (PCR), molecular markers and DNA sequencing have become the way to properly identify yeasts (Araújo et al., 2005; Guamán-Burneo & Carvajal-Barriga, 2009). The downside of these techniques is the effort to extract and purify the DNA. On the other hand, a simple method based on the assimilative ability of substrates known as API 20C AUX has been widely used to identify yeasts (Silva & Candido, 2005; Chen et al., 2010).

In fermentative yeasts, respiration in aerobic conditions prevails – Pasteur effect. On the other hand, in anaerobic conditions, the dominant mode is fermentation. *Saccharomyces cerevisiae*, through the Crabtree effect, is one of the few yeasts able to grow well in either aerobic or anaerobic conditions, with fermentation in both cases as long as the sugar concentration is not

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low (Díaz-Montaño et al., 2010; Nevoigt, 2008). When glucose is below a critical concentration, between 0.09 and 0.9% in mass, it favors diauxic growth onset and the yeast may have both growth profiles, the second of which is followed by the consumption of another non-fermentable carbon source in the medium, usually ethanol aerobically. In the absence of a carbon source, the cell enters decline (Galdieri et al., 2010; Rettori & Volpe, 2000).

Another important characteristic of this yeast is its thermal resistance and several studies have assessed the *Saccharomyces cerevisiae* inactivation in foods, such as those by Guerrero-Beltrán & Barbosa-Cánovas (2006) from mango nectar, Reveron et al. (2003) for pilsen beer, Guerrero-Beltrán & Barbosa-Cánovas (2005) from apple juice, and Lopes et al. (2014) from spoiled peach puree yeast. Such properties are particularly important for the yeast's broad industrial use, comprehending the production of bread, enzymes, and alcoholic and non-alcoholic beverages (Beuchat, 2005; Chaves-López et al., 2014; Souza et al., 2014).

The enzymes obtained from these yeasts have been widely studied, including β-D-fructofuranoside fructohydrolase (EC 3.2.1.26), known as invertase, which catalyzes sucrose hydrolysis to produce a mix of glucose and fructose called inverted sugar, which is used in the food and pharmaceutical industries. Besides in yeasts, particularly in the Species *S. cerevisiae*, invertases are also found in invertebrates, vertebrates, green algae, bacteria, plants, and fungi (Deuner et al., 2005; Oliveira et al., 2006; Goulart et al., 2013). In *S. cerevisiae*, two different invertase forms have been identified: One non-glycosylated found in the cytoplasm and one glycosylated found in the periplasm and bound to the yeast's cell wall, mainly in the extracellular side (Bofo et al., 2005; Cantarella et al., 2003). *Candida utilis* also produces these two isoforms (Guimarães et al., 2007).

Concentrated spoiled peach puree is a commodity usually marketed internationally and has broad application in the food industry. Several microorganisms have been identified and quantified in different processing steps, in particular the yeast *S. cerevisiae* with fermentative potential and significant thermal resistance (Garza et al., 1994; Bian et al., 1994; Toralles & Vendruscolo, 2007; Lopes et al., 2014). However, no studies have been found that identify yeast isolated from Brazilian cultivars with culture invertase production and its potential with free invertase activity and the growth curve and carbon source. To that end, the first objective of this work was to isolated and identify yeast species from spoiled Jubileu peach puree using the API 20C AUX method and a commercial yeast as witness. In the second objective step we selected one of the yeasts identified to study the yeast's growth potential using two batch culture treatments ("A" with 20 g cultures in PDA and "B" with 10 g) with fermentation times of 0, 18, and 24 h and refrigerated storage times of 1, 10, 17, and 24 days to assess the number of colonies (N), reducing sugar concentration (RS), free-invertase activity (FI), and culture-invertase activity (CI).

2 Materials and methods

2.1 Microorganism and culture

The microorganisms used in this work were isolated from spoiled peach puree at 22 °Brix, prepared as was described by Lopes et al. (2014). The cultures were maintained in potato dextrose agar (PDA) at pH 5 and counted as described by Siqueira (1995) after 5 days of incubation at 25 °C. The initial count was 22×10^2 CFU.mL^{-1} for spoiled peach puree. The experiment was carried out in the food technology laboratory of IFSUL-Pelotas-RS, Brazil.

2.2 Yeast identification

The colony isolated from PDA was aseptically transferred to test tubes containing 2 mL sterilized saline solution and adjusted to turbidity equivalent to 2 in the McFarland scale. One drop of this suspension was added to the API 20C AUX basal medium and homogenized. Next, each well of the identification strip was filled. The composition of the API 20 C AUX strip is given below in the list of tests: D-glucose (GLU), glycerol (GLY), calcium 2-keto-gluconate (2KG), L-Arabinose (ARA), D-Xylose (XYL), Adonitol (ADO), Xylitol (XLT), D-Galactose (GAL), Inositol (INO), D-Sorbitol (SOR), Methyl-αD-Glucopyranoside (MDG), N-acetylglucosamine (NAG), D-Cellobiose (CEL), D-Lactose (LAC), D-Maltose (MAL), D-Saccharose (SAC), D-Trehalose (TRE), D-Melezitose (MLZ), and D-Raffinose (RAF). The strip was incubated in a previously prepared wet chamber and placed in an oven at 30 °C for up to 72 h, with readings at 48 and 72 h. The results were considered positive or negative, respectively, if the wells of each carbohydrate were or were not opaque. A seven-digit code was obtained, which was interpreted with the catalogue from Apilab Plus, Bio-Merieux. Each yeast isolated underwent the urea hydrolysis test, which considers urease-positive those that release the ammonia that makes the medium alkaline. The medium contains phenol red, thus its color changes to bright pink. The hypha identification was confirmed in rice agar with tween 80 (Lacaz et al., 2002).

2.3 Yeast growth potential

The yeast's growth potential was assessed using two batch-growth treatments: "A" with 20 g PDA culture and "B" with 10 g. For each treatment, the culture was carried out with 20 g.L^{-1} glucose, 1 g.L^{-1} $(NH_4)_2SO_4$, 2 g.L^{-1} KH_2PO_4, 0.5 g/L^{-1} $MgSO_4$.7, 0.5 g.L^{-1} $FeSO_4$, and 5 g.L^{-1} peptone under orbital shaker (Quimis-Q816M22) Erlenmeyer flasks (150 rpm) at 30 °C for 24 h. Next, the colonies were stored at 4 °C for analysis and enzyme extraction. The variables selected were: number of colonies (N), reducing sugar concentration (RS), free-invertase activity (FI), and culture-invertase activity (CI), measured at 0, 18, and 24 h of fermentation and at 10, 17, and 24 days of storage.

2.4 Reducing sugars determination

The reducing sugar concentration (RS) were determined by two methods due to the quantification limits. At time zero of fermentation, the method by Eynon-Lane was used as described by the Association of Official Analytical Chemists (2000). At the other times, the RS was determined by an increase in absorbance at 490 nm using the colorimetric method resulting from the glucose-DNS reaction as described by Toralles et al. (2014). The transmittance was measured using an AJX-1000 UV/VIS spectrophotometer.

2.5 Invertase extration

The enzyme extracts were obtained by autolysis using the extraction method with $NaHCO_3$, known as autolysis. For every 100 g biomass, 300 mL of 200 mM $NaHCO_3$ solution were used in shaker Erlenmeyer flask (200 rpm) at 40 °C for 24 h. Next, the sample was centrifuged at 2,025 *g* for 10 min so that the clear supernatant liquid was obtained, which corresponds to the enzyme extract as a source of invertase released by autolysis, i.e., free invertase (FI). Afterwards, the protein content was determined by the method of Lowry et al. (1951) using BSA as standard (Acros, New Jersey, USA) and stored at –20 °C. Culture-invertase (CI) activity was determined on the batch-culture supernatant fluid after centrifugation at 2025g for 10 min.

2.6 Invertase activity

The Invertase enzyme activity was determined by the acid 3.5-DNS method with glucose standard. The transmittance resulting from this reaction was measured at 490 nm by spectrophotometry. For both FI and CI invertase, the reaction medium contained 1.0 mL Mcllvaine buffer, 1.0 mL sucrose solution as substrate, and 1.0 mL enzyme extract. The final reactive mix contained 40 mM substrate. The reaction was carried out at pH 5.0 and 25 °C and the glucose content was then dosed. The blank consisted of 1.0 mL deionized water replacing the substrate in the reaction mix. The enzyme activity was calculated in the linear part of the slope. One activity unit was defined as the amount of enzyme that leads to the increase of absorbance equivalent to 1 μg glucose.min^{-1}.

2.7 Osmotic pressure

Osmotic pressure was calculated according to the Equation 1:

$$p = CRTi \quad (1)$$

where p is the osmotic pressure (atm), C is the salt's molar concentration, R is the universal gas constant (0.082 atm.L.K^{-1}.mol^{-1}), T is the temperature (K), and (i) is the van't Hoff factor.

2.8 Statical analysis

The software Statistica was used to generate the two-dimensional plots. The differences between means were determined using Tukey's multiple comparison tests. To assess the relationship N, RS, FI, and CI for each treatment, Pearson's correlation coefficients were calculated with 95% confidence.

3 Results and discussion

3.1 Yeast identification

Table 1 shows the identification of the yeasts found in spoiled Jubileu peach puree by the API 20C AUX system. Both for the puree and for the witness, the results indicated accuracy superior to 90% after 72h for *S. cerevisiae*, being from different species (Table 1).

Rhodotorula mucilaginosa (2642073) was identified with 85.7% accuracy and salmon color, while *Tricosporon mucoides* (6745776) was identified with higher accuracy (90.5%) and beige-yellow color. Both are urease positive (Table 1, Figure 1), which matches Chen et al. (2010) for the urea hydrolysis test. Beckenkamp (1997), when working with pasteurized fruit pulp samples, also identified the genera Rhodotorula & Trichosporon, while Sancho et al. (2000) identified *R. mucilaginosa* in pear pulp. Guamán-Burneo & Carvajal-Barriga (2009), while working with different yeasts species of the genus Rhodotorula, found different salmon and pink hues for *R. mucilaginosa* cultured in CPM agar for 72 h at 20, 30, and 37 °C. Silva & Candido (2005), while working with the same yeast identification system, showed that the API 20C AUX system correctly identified 92% of the 50 yeast species tested.

Table 1. Yeast identification profile by the API 20C AUX system.

Tests	Puree			Witness
Shape	convex	round	round	convex
Color	beige	beige yellow	pink to red	beige
Urea	-	-	+	-
BK	-	-	-	-
GLU	+	+	+	+
GLY	-	+	-	-
2KG	-	+	-	-
ARA	-	+	v	-
XYL	-	+	v	v
ADO	-	-	-	-
XLT	-	-	-	-
GAL	+	+	v	+
INO	-	+	-	-
SOR	-	v	+	-
MDG	v	+	-	+
NAG	-	+	-	-
CEL	-	+	-	-
LAC	-	+	-	-
MAL	+	+	+	+
SAC	+	+	+	+
TRE	-	+	+	+
MLZ	-	-	+	v
RAF	+	v	+	+
Hypha	-	+	-	-
Numeric profile	2040032	6745776	2642073	2044073
Identification	*S. cerevisiae*	*Trichosporon mucoides*	*Rhodotorula mucilaginosa*	*S. cerevisiae*
% accuracy	95.2	90.5	87.5	90.5

All yeasts identified, were sucrose and raffinose positive (Table 1). These results are consistent with the action of yeast sucrose invertase or β-D-fructofuranosidase (EC 3.2.1.26). This enzyme hydrolyzes the glucosidic bond between C(2) and O of sucrose. α-glucosidase can also hydrolyze sucrose between C(1) and O, but not raffinose. *R. mucilaginosa* was melezitose positive (Table 1). In melezitose, the glucosyl residue attached to fructose is not modified and, thus, α-glucosidase can hydrolyze melezitose (Cantarella et al., 2003).

However, *S. cerevisiae*, whose numeric profile is 2040032, was chosen to be investigated for its invertase-production

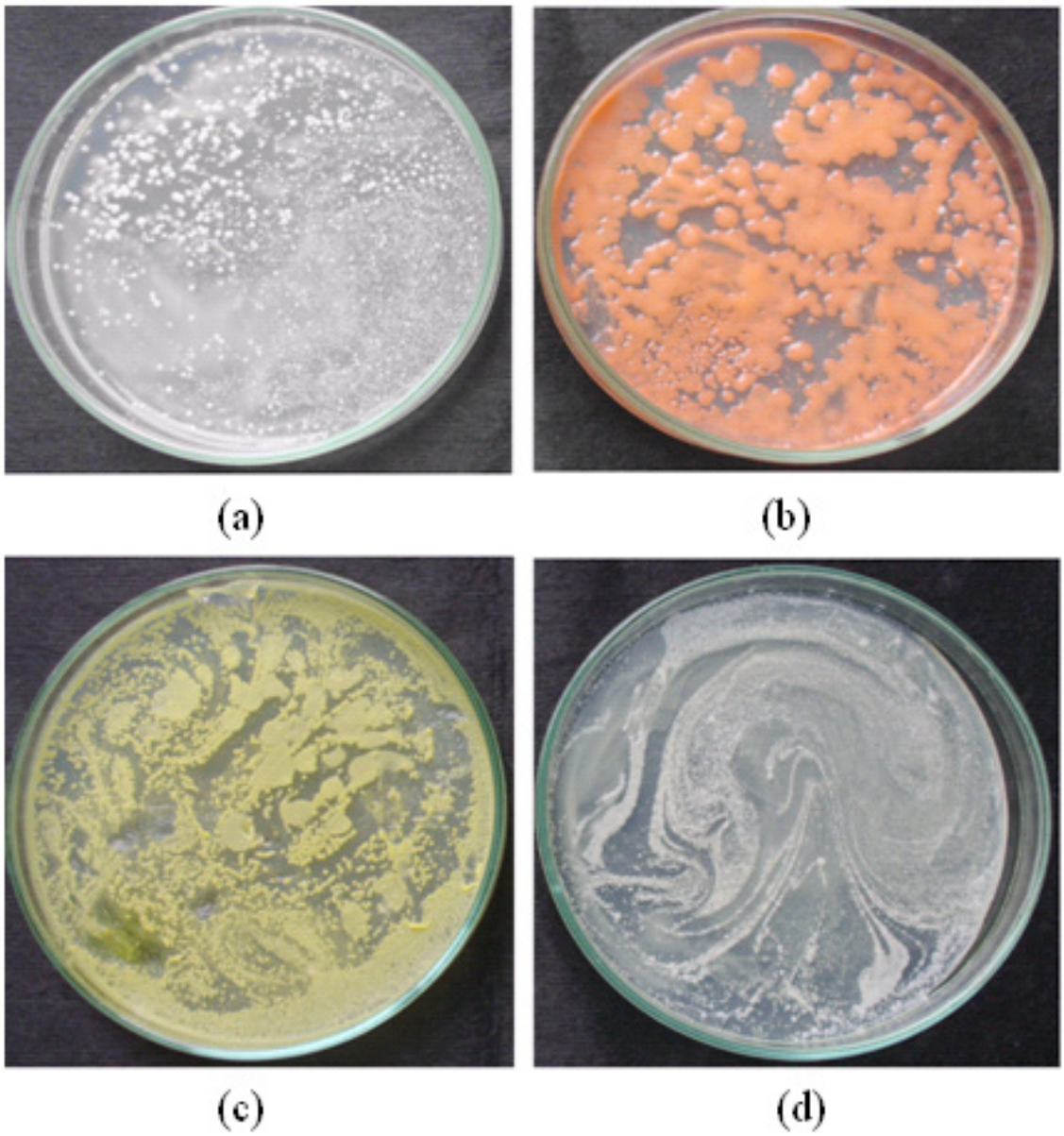

Figure 1. Yeast colonies identified in PDA agar from spoiled Jubileu peach puree: (a) *Saccharomyces cerevisiae* (2040032); (b) *Rhodotorula mucilaginosa* (2642073) and (c) *Trichosporon mucoides* (6745776); in witness: (d) *Saccharomyces cerevisiae (*2044073).

potential due to legal reasons, based on RDC resolution no. 26 of 2009, which establishes the list of enzymes allowed to be used in foods for human consumption according to their microbial origin (Brasil, 2006).

3.2 Assessing the invertase production by S. Cerevisiae in batch culture

The effect of the fermentation and storage periods and of the batch culture treatments on *S. cerevisiae* (2040032) varied widely for colony count, reducing-sugar content, FI and CI activity (Table 2). As for the colony count, a significant ($p < 0.05$) and growing gain was observed for both treatments until 17 days of storage, with a higher colony count for culture treatment A (5.0×10^7) than B (3.8×10^7) by the 17th day of culture. After that, a decrease in the count was observed for both treatments, more so for B, which was initially inoculated with 70% of the initial count of A ($N = 4.3 \times 10^6$). On average, it was observed that, during the fermentation and storage periods, treatment A had 42% higher viable cell count than B. For both treatments (Figure 2) a very quick initial growth until 18 h of culture – log phase – followed by a stationary phase until the 17th day for culture system B.

On the other hand, batch culture treatment A kept growing for up to ten days, although at a lower growth rate than in the log phase. After 17 days, the yeast entered a decline phase for both treatments. According to Lima et al. (2001), the decline is

followed by cell lysis, i.e., microorganism autolysis or rupture, caused by the action of intracellular enzymes.

As for the reducing sugar, given the initial concentration of about 20 g.L^{-1}, a conversion of over 97% was observed in the fermentation period for both treatments. Here, glucose is the main carbon source and its high consumption corresponded to an exponential growth in the log phase (Figure 2). Souza (2013), when working with optimized fermentation kinetics of sweet cassava, also reported that cell growth in the log phase is followed by the high consumption of reducing sugar concentration, besides CO_2 and ethanol production. Here, during the storage period, sugar concentration remained low, which suggests zero-order kinetics throughout storage for both treatments (Table 2). Moreover, between 18 h and 24 days of storage, glucose concentration was below the critical concentration that favors the onset of diauxic shift and the yeast may show two growth profiles. Guidi et al. (2010), when working with the effect of different glucose concentrations on diauxic shift of a wild-type *S. cerevisiae*, reported a diauxic-shift-free curve for the initial concentrations of 20% m/v glucose, but not for 2% m/v. Haurie et al. (2003) also reported diauxic shift for initial 2% m/v glucose concentration. At this initial glucose concentration, a second growth profile significant for treatment A was observed between the first and tenth days of storage ($p < 0.05$). Mielniczki-Pereira et al. (2008) observed this phenomenon between 16 and 20 h of fermentation using a wild-type *S. cerevisiae* species. On the other hand, Gray et al. (2004) reports that this might occur after one day.

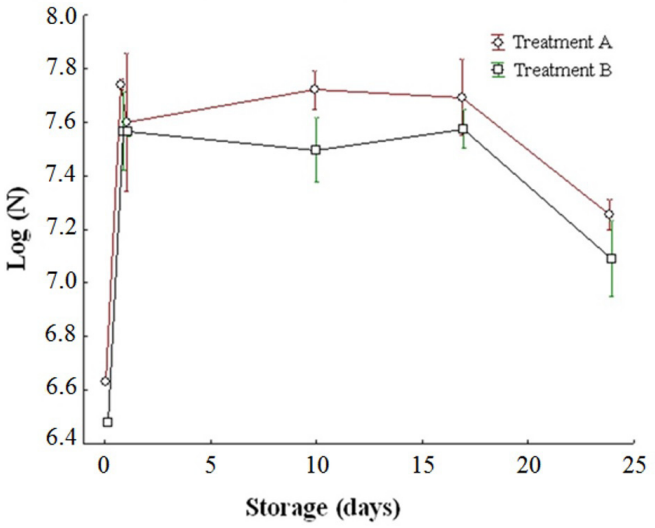

Figure 2. Growth curve of *S. cerevisiae* (2040032) isolated from spoiled peach puree for both batch culture treatments: (A) with 20 g cultures in PDA and (B) with 10 g. Yeast growth took place at 30 °C and 150 rpm stirring for 24 h of orbital fermentation followed by refrigerated storage for 24 days. The mean count values are from triplicate plates.

Regarding invertase activity in the batch culture supernatant, the highest values were observed between the tenth and 17th days. That value for treatment A (27.1 U.mL^{-1}) on the tenth day of storage was higher ($p < 0.05$) than the other values (Table 2). On the other hand, by the end of the log phase and in the decline phase, the lowest CI activity values were observed for the culture. This enzyme has been long known and many consider the activity in the batch culture supernatant, here called CI, an extracellular invertase, including invertase from *A. parasiticus* (Lucca et al., 2013), from *A. ochraceus* (Guimarães et al., 2007), and from *Penicillium chrysogenum* (Poonawalla et al., 1965). According to Myers et al. (1997), the expression of the gene that codes invertase is repressed at high free glucose concentrations. In this research, high free glucose concentration was only observed in the beginning of the log phase, which may be related to the low CI activity observed after 24 h of fermentation (Table 2). Table 3 shows a significant inverse correlation between CI activity and RS for both treatments.

For free invertase extracted from the biomass of *S. cerevisiae* culture isolated from spoiled peach puree, no viable cell count was detected after 24 h of autolysis. The maximum activity was observed for treatment A after 24 h of fermentation, with specific activity of 15.7 U.mL^{-1} (20.0 U.mg^{-1}) at pH 5 and 25 °C. Under those same reaction conditions, Toralles et al. (2014) found 8.86 U.mg^{-1}. At that occasion, the yeasts present in spoiled peach puree could not be isolated or identified, nor could the best culture period for invertase production be determined. Furthermore,

Table 2. Effect of the fermentation and storage periods on viable cell count (N), reducing sugar concentration (RS), free-invertase activity (FI), and culture-invertase activity (CI) for two batch culture treatments: (A) with 20 g cultures in PDA and (B) with 10 g. The yeast chosen for the culture was *Saccharomyces cerevisiae* (2040032) isolated from spoiled peach puree.

Variable	Treatment[b]	Period of[a]												Mean CV (%)
		Fermentation/culture (hours)				Storage (days)								
		0		18		1		10		17		24		
N (UFC. mL^{-1})	A	4.3×10^6	aD	5.5×10^7	aA	4.1×10^7	aB	5.3×10^7	aAB	5.0×10^7	aAB	1.8×10^7	aC	8.39
	B	3.0×10^6	aC	3.7×10^7	bA	3.7×10^7	bA	3.2×10^7	bA	3.8×10^7	bA	1.2×10^7	bB	7.59
RS (mg.L^{-1})	A	19732	aA	521.0	aB	492.6	aB	371.0	aB	465.5	aB	483.2	bB	4.93
	B	20235	aA	532.8	aB	470.1	bB	474.9	aB	373.3	aB	505.6	aB	4.96
CI (U.mL^{-1})	A	-	-	-	-	5.91	bB	27.1	aA	10.51	bB	13.94	aB	14.2
	B	-	-	-	-	7.97	aB	14.35	bA	14.53	aA	0.00	bC	2.71
FI (U.mL^{-1})	A	-	-	-	-	15.73	aA	0.00	aC	0.00	aC	7.21	bB	14.1
	B	-	-	-	-	4.86	bB	0.00	aC	0.00	aC	11.1	aA	7.38

[a] Mean values followed by the same small letter in the column or by the same capital letter in the row do not statistically differ according to Tukey's test ($p < 0.05$); [b] Treatments A and B were carried out at 30 °C and 150 rpm for 24 h orbital batch fermentation differing only in the number of plates inoculated in the culture medium. Storage took place at 6 °C for 24 days for both treatments.

an extraction technique optimized for commercial-yeast invertase was employed, whith recommended osmotic pressure of 5.1 atm. Here, osmotic pressure of 10.3 atm was used, which corresponds to a 200 mM $NaHCO_3$ concentration. On the other hand, no free-invertase activity was observed between ten and 17 days of storage. This is shown by the lack of cell autolysis and, therefore, enzyme extract with no invertase activity. According to Galdieri et al. (2010), in this period the stationary cells are resistant to heat and osmotic change the accumulation reservation as trealoses molecules that protect cells against different strains. Further studies are suggested that report on the optimal invertase autolysis conditions for *S. cerevisiae* from peach regarding osmotic pressure, stirring velocity, and extraction time.

The correlation coefficients calculated for the fermentation and storage periods, N, RS, glucose conversion (%X), CI, and FI for the two batch culture treatments are found in Table 3 and Figure 3. A significant positive correlation was found between RS contents and FI in treatments "A" ($r = 0.5$, $p < 0.005$) and "B" ($r = 0.62$, $p<0.005$), however, the correlation was inverse for N, log (N), CI and %X for both treatments. Over the period, a positive correlation was observed with %X for both treatments. The correlation was also invertase between FI and CI activities, as shown in Figure 3. On the other hand, a positive correlation was found between cell growth in terms of log (N) and CI (Table 3).

Table 3. Correlation coefficients (r) between treatment period (P), colony count (N), reducing sugar concentration (RS), culture-invertase activity (CI), and free-invertase activity (FI) for both batch culture treatments: (A) with 20 g cultures in PDA and (B) with 10 g. The yeast chosen for the culture was *S. cerevisiae* (2040032) isolated from spoiled peach puree.

	Treatment A			Treatment B		
	P	RS	Log (N)	P	RS	Log (N)
N	0.16^{ns}	−0.75*	0.96*	0.15^{ns}	−0.76 *	0.96*
CI	0.10 ^{ns}	−0.72*	0.53*	−0.45^{ns}	−0.64*	0.53*
FI	0.42 ^{ns}	0.50*	−0.63*	0.46 ^{ns}	0.62*	−0.63*
%X	0.66*	−1.00*	−0.90*	0.66*	−1.00*	−0.90*

*significant at $p \leq 0.05$ and $n = 18$; ns = non-significant.

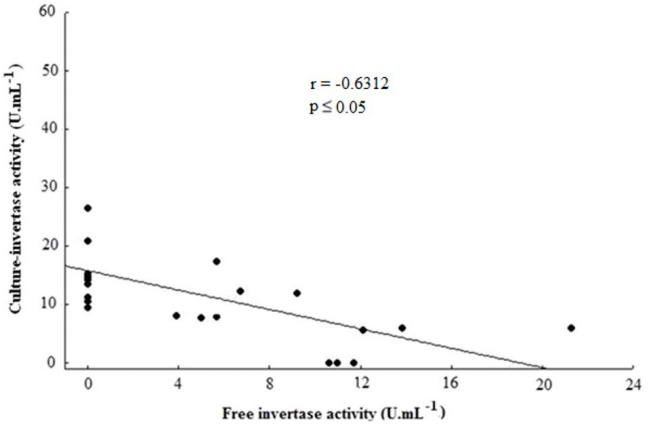

Figure 3. Correlation between culture-invertase activity (CI) and free-invertase activity (FI) extracted by autolysis from *S. cerevisiae* (2040032) isolated from spoiled peach puree. The figure represents the correlation of the enzyme activities for both treatments.

4 Conclusion

In the spoiled peach puree sample at 22 °Brix, with an initial count in accordance with the legislation, three different types of yeasts were identified, while only one type of yeast was identified in the witness. *S. cerevisiae* was chosen due to legal reasons to have its culture potential assessed regarding invertase (EC 3.2.1.26) production, despite the other yeasts also with having potential since all were sucrose and raffinose positive. *R. mucilaginosa* showed potential for α-glucosidase (EC 3.2.1.20) production.

S. cerevisiae had logarithmic growth for the first 18 h of fermentation. After that, the yeast entered stationary phase for 17 days of refrigerated storage and then a decline phase for both treatments, while treatment "A" apparently developed diauxic growth after one day of fermentation. This fact is because the diauxic phenomenon and stationary phase the cells accumulate invertase are more resistant to autolysis. The reducing sugar concentration decreased with a conversion rate above 97% at the end of the log phase. FI had greater activity in the log phase and in the decline phase than CI. During the decline, spontaneous cellular lysis takes place, which might have favored FI production. Overall, the strong inverse correlation between RS and CI allows concluding that the low reducing-sugar content after one day of fermentation favored the production of invertase in the culture, with CI inversely proportional to FI.

Finally, it is concluded that invertase production in the culture is correlated with cell growth and the number of viable cells, with treatment "A" being superior throughout the fermentation and storage periods. Free-invertase activity doubles when osmotic pressure doubles compared to the value reported in the literature and its production through biomass autolysis is recommended after one day of fermentation or in the decline phase under the following batch autolysis conditions: 200 mM $NaHCO_3$, 200 rpm, 24 h at 40 °C.

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