ISSN 0100-2945 DOI: http://dx.doi.org /10.1590/0100-29452022849

Revista Brasileira de Fruticultura

Processing

Extraction, purification and characterization of invertase from *Candida guilliermondii* isolated from peach solid wastes

Tariani Lemos Avila[1], Ricardo Peraça Toralles [2], Estefani Tavares Jansen[3], Marcela Vega Ferreira[4], Claudio Rafael Kuhn[5], Walter Augusto Ruiz[6]

Abstract - The best conditions for *in vitro* sucrose hydrolysis based on invertase from *Candida guilliermondii* (ICg) were studied and the kinetic parameters K_M, V_{max}, and thermal stability of ICg were determined. *Candida guilliermondii* (Cg) yeast isolated and lyophilized from peach solid wastes was identified using the API 20C AUX method. Subsequently, the Cg was submitted to an autolysis process using $NaHCO_3$ at 200 mM under 200 rpm stirring and 40 °C for 24 h. The enzyme extracts obtained were recovered through precipitation with acetone followed by dialysis and ion-exchange chromatography. The extract purified through precipitation with acetone had activity of 27.7 $U.mg^{-1}$ and 56% recovery whereas the chromatography process yielded 46.5 $U.mg^{-1}$ and 44.8%. The optimal sucrose hydrolysis conditions were pH 5.0 and 50 °C, resulting in K_M of 30.5 mM and 28.7 mM sucrose, respectively, at 25 °C and 50 °C, both with Michaelian behavior. Thermal inactivation of ICg exhibited first-order apparent kinetics and its residual activity was typically linear between 40 °C and 70 °C. Three isoenzymes were detected through electrophoresis.
Index terms: Yeast; autolysis; isoenzymes; inverted sugar.

Extração, purificação e caracterização da invertase de *Candida guilliermondii* isolada de resíduos sólidos de pêssego

Corresponding author:
toralles@pelotas.ifsul.edu.br

Received: September 11, 2021
Accepted: February 07, 2022

Resumo - Estudaram-se as melhores condições de hidrólise da sacarose *in vitro* a partir da invertase de *Candida guilliermondii* (ICg), também se determinou os parâmetros cinéticos K_M, V_{max} e a estabilidade térmica da ICg. A levedura *Candida guilliermondii* (Cg) extraída, isolada e liofilizada de resíduo de pêssego foi identificada usando o método API 20C AUX. Posteriormente, a levedura Cg foi submetida a um processo de autólise, usando $NaHCO_3$ a 200 mM sob agitação a 200 rpm e 40°C, por período de 24 horas. Os extratos enzimáticos obtidos foram recuperados por precipitação com acetona seguido de diálise e cromatografia de troca iônica. O extrato purificado por precipitação com acetona teve a atividade de 27,7 $U.mg^{-1}$ e 56% de recuperação, sendo que, por cromatografia, obtiveram-se 46,5 $U.mg^{-1}$ e 44,8%. As condições ótimas na hidrólise da sacarose foram pH de 5,0 e 50°C, apresentando K_M de 30,5/mM e 28,7 mM de sacarose, respectivamente, a 25°C e a 50°C, ambas com comportamento Michaeliano. A inativação térmica da ICg mostrou uma cinética aparente de primeira ordem, e sua atividade residual foi tipicamente linear entre 40°C - 70°C. Por meio de eletroforese, foram detectadas três isoenzimas.
Termos para indexação: Levedura; autólise; isoenzimas; açúcar invertido.

[1]Graduate in Chemical Engineering, Instituto Federal Sul Riograndense, Pelotas-RS, Brazil. E-mail: tarianilemos@hotmail.com (ORCID 0000-0002-9036-0688)

[2]Dr. in Food Science, Instituto Federal Sul Riograndense, Pelotas-RS, Brazil. E-mail: toralles@pelotas.ifsul.edu.br (ORCID 0000-0002-9836-1693)

[3]Graduate in Chemical Engineering, Instituto Federal Sul Riograndense, Pelotas-RS, Brazil. E-mail: estefani_tj@hotmail.com (ORCID 0000-0001-8760-1666)

[4]Ms. in Food Science, Universidade Federal de Pelotas, Pelotas-RS, Brazil. E-mail: marchii20@yahoo.es (ORCID 0000-0002-5096-3142)

[5]Dr. in Food Science, Instituto Federal Sul Riograndense, Pelotas-RS, Brazil. E-mail:crkuhn@pelotas.ifsul.edu.br (ORCID 0000-0003-0898-1803)

[6]Dr. in Food Science, Universidade Federal do Rio Grande, Rio Grande-RS, Brazil. E-mail:dqmwar@furg.br (ORCID 0000-0001-8242-0938)

Introduction

Invertase (EC 3.2.1.26 β–D fructofuranosidase) is an enzyme that catalyzes the hydrolysis of the sucrose molecule at the non-reducing terminal of the β–D fructofuranosidase residue (CANTARELLA et al., 2003), resulting in an equimolar blend of glucose and fructose known as inverted sugar (OYEDEJI et al., 2017).

Inverted sugar is a major product in the food industry, where it is used as syrup, an ingredient in jams, candy drops, and sweets in general due to its slow crystallization, high sweetening power (approximately 40% higher than that of sucrose), and longer shelf life (BATISTA et al., 2021).

The process most often employed to produce inverted sugar is acid hydrolysis. However, the product easily undergoes changes in color and flavor that hinder its shelf life (RADOVANOVIC et al., 2017). Such alterations are a consequence of the formation of hydroxymethylfurfural (HMF), a product of high carcinogenic potential for humans, formed during its processing at low pH and high temperatures. The process requires the use of bases to neutralize the formation of toxic residues (GARGEL et. al., 2014; ORTU;CABONI, 2017).

The enzymatic process is an important alternative as it uses milder conditions such a pH between 4.5 and 5.0 and temperature in the 30-50 °C range (VITOLO, 2004), which lead to lower impact to the environment. However, it has the downside of high cost when compared with the acid process. Nonetheless, the enzymatic process may provide a high degree of hydrolysis when the kinetic criteria of an enzyme are well balanced with the operation, e.g., the use of enzymes immobilized in insoluble solid supports (CABRAL et al., 2017; DI ADDEZIO, 2014). The product yielded is of superior quality, with low contents of ash, color, and HMF. It also does not require neutralization (GARGEL et. al., 2014).

Invertases are found in organisms such as invertebrates, vertebrates, green algae, fungi, and yeasts (AMJAD et al., 2020; WANG et al., 2020). *Saccharomyces cerevisiae* yeast is the main source of invertase and is classified as generally recognized as safe (GRAS). *Saccharomyces. cerevisiae* synthesizes two invertases: One glycosylated and the other non-glycosylated. The non-glycosylated enzyme is found in the cytoplasm, while the glycosylated one is located in the periplasm bound to the cell wall of the yeast, which is the predominant form (GUIMARÃES et al., 2007; CANTARELLA et al., 2003). *Candida utilis* yeast also produces both isoforms of the enzyme (BELCARZ et al., 2002).

Over 160 types of microorganisms have been identified in fruits such as peach and its processing waste, among which *Saccharomyces cerevisiae* yeast stands out for its fermentative potential and significant thermal resilience (LOPES et al., 2014). Ferreira et al. (2016) identified *Saccharomyces cerevisiae*, *Rhodotorula mucilaginosa* and *Trichosporum mucoidesi* yeasts from spoiled peach puree. All those yeasts are sucrose and raffinose positive, thus bearing potential for invertase production.

Here, invertase was extracted from *Candida guilliermondii* (Cg) from solid peach waste through autolysis, purified through precipitation followed by dialysis, and identified through horizontal electrophoresis. The best conditions for in *vitro* sucrose hydrolysis as well as the kinetic parameters K_M, V_{max}, and thermal stability of ICg were also studied. The identification of yeasts isolated from solid peach waste using API 20C AUX and a Commercial *Saccharomyces cerevisiae* yeast as witness were also studied.

Material and Methods

Microorganism

The microorganism used in this investigation was isolated from local industrial peach solid wastes. The stock cultures were maintained in potato dextrose agar (PDA) at pH 5 and counted as described by Siqueira (1995) after five days of incubation at 25 °C. The initial count was approximately 10^6 CFU.mL^{-1}. The yeast's growth was prepared as described by Ferreira et al. (2016). Next, the colonies were then successively cultured in PDA and stored 4 °C. Afterwars, 400 g of PDA culture was lyophilized and stored at 4 °C for enzyme extraction. The lyophilized yeast samples yielded about 100 g. The experiment was carried out in the applied biochemistry laboratory of IFSUL-Pelotas-RS, Brazil.

The yeast colonies were then successively cultured in PDA, lyophilized, and stored at 4 °C for enzyme extraction. The experiment was carried out in the applied biochemistry laboratory of IFSUL-Pelotas-RS, Brazil.

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 (Biomérieux, Ref. 20210) 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. A commercial *Saccharomyces cerevisiae* (Fleischmann ®) yeast was used as witness. 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 analytique API 20C AUX from 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).

Invertase Extraction

Invertase was extracted using the autolysis method as described by Ferreira et al. (2018). To 100 g samples of lyophilized yeast, 300 mL 200 mM $NaHCO_3$ were added and the mixture was placed in stirring in an orbital shaker at 200 rpm and 40 °C for 24 h. The sample was then centrifuged at 2,025 *g* for 10 min. The supernatant liquid was called the raw extract (RE) as a source of invertase released by autolysis. Next, the protein content was determined by the method by Lowry et al. (1951) in an AJX-1000 UV/VIS spectrophotometer (Acros, New Jersey, USA) with 750 nm transmittance using bovine serum albumin (BSA, Inlab) as standard. The RE was stored at -20 °C.

Invertase Activity

Invertase enzyme activity was determined by the reaction of reducing sugars (RS) with 3,5-dinitrosalicylic acid (3,5-DNS, Inlab) with 490 nm transmittance. The reaction medium consisted of 1.0 mL extract, 1.0 mL of Mcllvaine pH 5.0 buffer, and 1.0 mL of 120 mM sucrose solution as substrate, resulting in a final reactive mixture with 40 mM sucrose. The blank assay employed 1.0 mL water in place of the substrate. The reaction was carried out at pH 5.0 and 25 °C. After 10 min, the content of RS was determined by 3,5-DNS using glucose as standard (Ferreira et al., 2018). One enzyme activity unit was defined as the amount of enzyme that leads to the increase by 1 µg RS as glucose.min^{-1}.

Raw Extract Purification

The RE was purified through precipitation with acetone. The cold solvent was slowly added to the RE at a 1:1 ratio under stirring and cooling. The mixture was kept under refrigeration for 15 min, centrifuged for 10 min at 2,025 *g*, and then left to sit overnight at 4 °C. The precipitate obtained was resuspended in distilled water and disodium EDTA at 0.10% m/v and pH 7.2 at a 4:1 water:disodium EDTA ratio. The suspension underwent dialysis using cellulose membranes (12 - 16 kDa 25 Å diameter) for 12 h with water replaced periodically. After this period, the content of soluble proteins and enzyme activity were determined.

The purification factor (PF) and recovery (R) were calculated using Equations 1 and 2, where the PF takes into account the increase in specific activity of the enzyme after the purification step and recovery is the percentage of enzyme recovered in relation to the enzyme fed.

$$PF = \frac{PEA}{REA} \quad (1)$$

$$R\,(\%) = \frac{PPE}{PRE}.PF.100 \quad (2)$$

where PEA is purified extract activity ($U.mg^{-1}$), [PPE] is the protein concentration in purified extract ($mg.mL^{-1}$), REA is the raw extract activity ($mg.mg^{-1}$), and PRE is the protein concentration in the raw extract.

The dialyzed extract was purified through ion-exchange chromatography in a 1.0x10 cm DEAE-Sephadex A-25 chloride column balanced and eluted with 50 mM acetate buffer at pH 5.0. The content of soluble proteins and invertase activity were determined in 2 mL eluates, which were then stored at -20 °C for later use in electrophoresis. The α-glucosidase residual activity of raw extracts and fractions of Cg was measured using p-nitrophenyl- α-D-glucopyranoside (Sigma) as substrate for α-glucosidase.

Effect of pH and Temperature on Enzyme Activity

The effect of pH on invertase activity was determined in the 3-8 pH range using Mcllvaine buffer at 25 °C. Once the optimal pH was defined, it was used in all further trials. The effect of temperature was determined between 10 °C and 90 °C using a temperature-controlled water bath (model Nova Ética).

Effect of Substrate Concentration and kinetic Parameters

The effect of substrate concentration on invertase activity was determined using sucrose at final concentrations between 0 mM and 100 mM in the reaction medium under the best pH conditions and constant temperature at 25 °C and 50 °C, respectively. The Michaelis-Menten constant (K_M) and maximum rate (V_{max}) were determined by non-linear regression.

Thermal Stability of ICg

1 mL aliquots of purified enzyme extract were placed in capped tubes with 9 mm internal diameter and 1 mm wall thickness, which were heated to 40 °C and 70 °C, respectively, over different periods of time. The reaction medium was stored soon after and enzyme activity was determined as described in item 2.4.

Thermal denaturation of invertase was monitored for each heating time. The first-order denaturation rate constants (k) were determined from the slopes of the denaturation curves according to Equations 3 or 4.

$$\log \frac{A_t}{A_0} = -\left(\frac{k}{2.303}\right) . t \quad (3)$$

or

$$\ln \frac{A_t}{A_0} = -k.t \quad (4)$$

where A_0 is the initial enzyme activity, A_t is the activity after the heating time, and t is the heating time.

The curve slopes were determined through linear regression and the rate constants were used to create the Arrhenius plot. The activation energy (E_a) for either temperature in study were also calculated using linear regression from the slopes of the Arrhenius plots of ln (k) *versus* 1/T according to Equation 5.

$$\ln (k) = -\frac{E_a}{RT} + \ln A \quad (5)$$

where R is the universal gas constant (8.314 $J.mol^{-1}.K^{-1}$), T is temperature in K, and A is the pre-exponential factor.

The half-life time ($t_{½}$), i.e., the time for enzyme activity to decrease from A_0 to ½ A_0, was obtained through Equation 7, which derives from algebraic modifications of Equation 6:

$$k.t_{\frac{1}{2}} = -\ln\left(\frac{\frac{1}{2}.A_0}{A_0}\right) = -\ln\left(\frac{1}{2}\right) = \ln(2) \quad (6)$$

$$t_{\frac{1}{2}} = \frac{\ln 2}{k} \quad (7)$$

where $t_{½}$ is the half-life time of invertase and A_0 is the initial enzyme activity.

Electrophoresis

The purified extract from ICg was submitted to 6% polyacrylamide gel horizontal electrophoresis using a pH 8.3 buffer prepared according to Scandalios (1969). 40 µL samples of enzyme extract (1,000 $µg.mL^{-1}$) were distributed in each gel gap and a 90 V tension was applied for 4 h at 4 °C. The bovine serum albumin (BSA), standard for protein quantification, was stained with Coomassie blue for 24 h followed by bleaching with a 40:10:50 methanol:acetic acid:water solution. The isoenzymes were revealed by a system containing 0.4 $mg.mL^{-1}$ tetrazolium blue chloride (Sigma-Aldrich), 0.2 $mg.mL^{-1}$ phenazine methosulfate (Sigma-Aldrich), and 2% sucrose at pH 5.

Statistical Analysis

The TIBCO Software Inc. (2018) was used to calculate the linear regression coefficients, analysis of variance, and coefficient of determination, as well as to generate the two-dimensional plots. The Quasi-Newton method was used for non-linear regression using the same computation resource. The confidence intervals of the coefficients were calculated by multiplying the standard error by Student's t (t_{n-2}) adjusted to the degrees of freedom (p = 0.05). The computer simulation of chemical and kinetic equilibrium of sucrose hydrolysis by ICg was carried out in the software Scilab (2017).

Results and Discussion

Yeast identification

Table 1 shows the identification of the yeasts by the API 20C AUX system. *Candida guilliermondii* (6776277) was identified with 90.5% accuracy, beige color and round shape, while the witness was identified as *Saccharomyces cerevisiae* (2044073), 90.5%, beige color and convex shape. Both are sucrose and raffinose positive (Table 1, Figure 1).

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

	Peach waste	Witness
Shape	round	convex
Color	beige	beige
Urea	-	-
BK	-	-
GLU	+	+
GLY	+	-
2KG	+	-
ARA	+	-
XYL	+	v
ADO	+	-
XLT	+	-
GAL	+	+
INO	-	-
SOR	+	-
MDG	+	+
NAG	-	-
CEL	+	-
LAC	-	-
MAL	+	+
SAC	+	+
TRE	+	+
MLZ	+	v
RAF	+	+
Hypha	+	-
Numeric profile	6776277	2044073
Identification	*C. guilliermondii*	*S. cerevisiae*
% accuracy	90,5	90.5

Figure 1. Yeast colonies identified in PDA agar: (a) *Candida guilliermondii* (6776277); in witness: (b) *Saccharomyces cerevisiae* (2044073).

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 (CANTARELLA et al., 2003). *C. guilliermondii* was melezitose positive (Table 1). In melezitose, the glucosyl residue attached to fructose is not modified and, thus, α-glucosidase can hydrolyze melezitose *in vitro*, but Cg-6776277 extracts did not show α-glucosidase residual activity using p-nitrophenyl- α-D-glucopyranoside as substrate. Invertase and α-glucosidase from *S. cerevisiae* were also unable to hydrolyze melezitose (YOON et al., 2003). Bramono et al. (2011), when working with comparison of α-glucosidase activities from clinical isolates of *Candida* species, demonstrated that *C. albicans*, *C. parapsilosis* and *C. tropicalis*, showed higher α-glucosidase activities, and that *C. krusei*, *C. glabrata*, and *C. guilliermondii* didn't show activity. On the other hand, Songpim et al. (2011) reported the potential of *C. guilliermondii* for invertase production.

Extract Characterization

The results obtained from purification with acetone for invertase recovery by precipitation using a 1:1 solvent:raw extract ratio are shown in Table 2.

Table 2. Activity of ICg extracts raw, purified with acetone, and in a Sephadex A-25 column.

	Specific activity ($U.mg^{-1}$)	Protein content ($\mu g.mL^{-1}$)	Recovery	
			RF	R%
RE*	18.0 ± 2.2	2196	1.0	100
Acetone**	27.7 ± 0.7	796	1.54	56.0
F1***	46.5 ± 2.2	984	2.58	44.8

*RE is the raw extract 50% in volume (initial volume= 100 mL). . **Acetone extract 20% in volume. ***F1 is the first fraction collected from the chromatographic column. PF = recovery factor and R(%) = recovery. 1 U = 1 µg $RS.min^{-1}$.

Comparing the activities of the raw ICg extract (18.0 $U.mg^{-1}$) with the extract purified through precipitation with acetone followed by dialysis shows the purification factor was 1.5-fold (56.0%) and specific activity was 27.7 $U.mg^{-1}$. The fraction collected from the chromatographic column (F1) achieved purification factor of 2.6-fold (44.8%) in relation to the raw extract, with specific activity of 46.5 $U.mg^{-1}$. Ferreira et al. (2018), when studying purification of invertase from *S. cerevisiae* isolated from peach purée, reported a 2.1-fold purification factor with 89.9% recovery using the acetone:water system. Andjelković et al. (2010) achieved recovery of 79.2% for yeast invertase using the same system. As expected, precipitation, dialysis, and chromatography separation allow separating enzymatic proteins from other analytes present in the initial extract.

Effect of pH and Temperature on Enzyme Activity

As seen in Figure 2, the maximum ICg activity was observed at pH 5.0, at which the relative activity (RA) of 100% corresponds to specific activity of 27.7 ± 0.7 $U.mg^{-1}$. This result is interesting for application of this invertase in extraction of sugars for technology of fruit juices since most of the fruits rich in sucrose exhibit acidic pHs (ASHURST, 2005). Plascencia-Espinosa et al. (2014), when working with invertase from *Candida guilliermondii*, reported optimal activity at pH 5 and 65 °C. Belcarz et al. 2002, when working with invertase from *Candida utilis*, observed high activity between pH 3.6 and 5 and optimal conditions for invertase activity at pH 4.4. Barbosa et al. (2018), using invertases produced from *Saccharomyces cerevisiae* CAT-1 and *Rhodotorula mucilaginosa*, reported optimal pH and temperature were 4.0 and 70 °C for *Rhodotorula mucilaginosa* invertase and 4.5 and 50 °C for *Saccharomyces cerevisiae* invertase. Most of the invertases have an acidic optimum pH and display some activity with sucrose and raffinose as substrates.

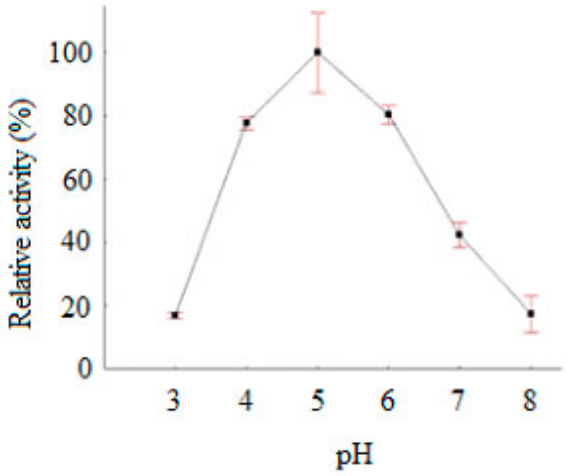

Figure 2. Effect of pH on the activity of ICg from peach waste. Reaction conditions: McIlvaine buffer pH 5 (100 mM citric acid – 200 mM sodium phosphate). Temperature: 25 ± 0.1 °C. Enzyme: 1 mL in 3 mL of reaction medium with 40 mM sucrose. The points are the means of three repetitions ± 0.95*standard deviation. 100% of relative activity is 27.7 ± 0.7 U.mg^{-1}.

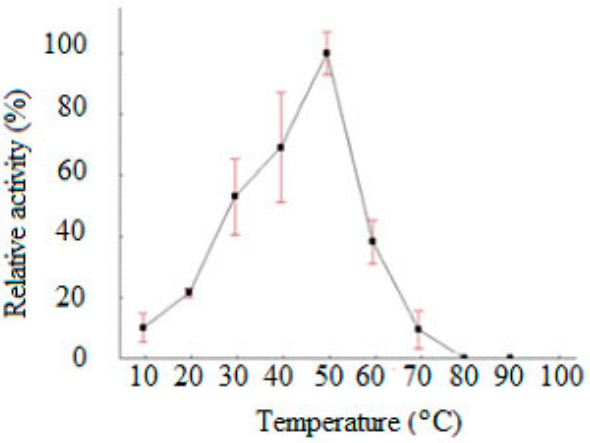

Figure 3. Effect of temperature on the activity of ICg from peach waste. Reaction conditions: McIlvaine buffer pH 5 (100 mM citric acid – 200 mM sodium phosphate). Enzyme: 1 mL in 3 mL of reaction medium with 40 mM sucrose. The points are the means of three repetitions ± 0.95*standard deviation. 100 % of relative activity is 74.9 ± 2.1 U.mg^{-1}.

The optimal temperature observed was 50 °C, as shown in Figure 3. Stability decreased after the optimal point and no residual activity was detected at 80 °C. This result corroborates previous studies that described yeast invertases with optimal activity around 50-55 °C (VALÉRIO et al., 2013; GARGEL et al., 2014; TORALLES et al., 2014). The dependence on temperature is an important characteristic for the production of inverted sugar with control of non-enzymatic darkening (RADOVANOVIC et al., 2017).

Kinetic Parameters of ICg

The kinetic parameters for hydrolysis rate of sucrose by peach waste ICg were determined through non-linear regression at 25 °C and 50 °C, as shown in Table 3. A hyperbolic behavior was found at either temperature between hydrolysis rate (U.mg^{-1}) and sucrose concentration (mM) throughout the reaction, which suggests Michaelis-Menten kinetics according to Figure 4.

Table 3. Michaelis-Menten constant (K_M) and maximum rate (V_{max}) of ICg at 25 °C and 50 °C.

Parameters	ICg	
	25 °C	50 °C
K_M[a] (mM)	30.5 ± 20.3**	28.7 ± 11.4**
V_{max}[a] (U.mg^{-1})	47.7 ± 10.7**	120.6 ± 15.8**
V_{max}/K_M	1.56	4.20
R^2	0.98	0.99

[a] value ± confidence interval at p = 0.05. **Significant (p ≤ 0.01).

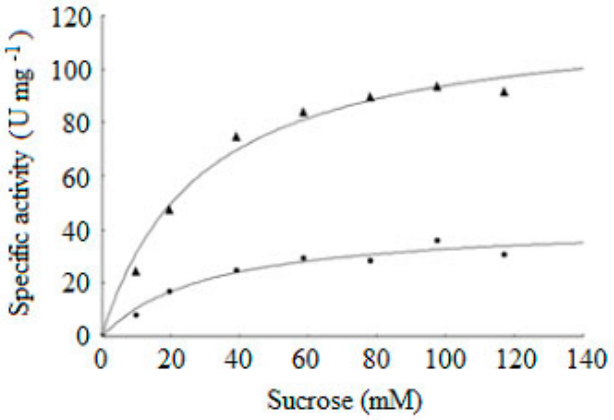

Figure 4. Effect of sucrose concentration on ICg hydrolysis rate. Reaction conditions: McIlvaine buffer pH 5 (100 mM citric acid – 200 mM sodium phosphate). Enzyme: 1 mL in 3 mL of reaction medium with sucrose. Temperatures: (●) 25 ± 0.1 °C and (▲) 50 ± 0.1 °C.

The values of the Michaelis apparent constant (K_M) for 25 °C and 50 °C were 30.5 mM and 28.7 mM sucrose, respectively. At 50 °C, the maximum rate (V_{max}) was about three times higher than at 25 °C and the specificity coefficient (V_{max}/K_M) was about 2.7 times higher.

Under the same hydrolysis conditions, Toralles et al. (2014) found K_M of around 24 mM and V_{max} of 47 $U.mg^{-1}$ for raw extract from commercial invertase from *S. cerevisiae* and, later, K_M of around 6.61 mM sucrose and 300 $U.mg^{-1}$ for purified invertase. That shows purification enhances affinity to the substrate (TORALLES et al., 2021).

Thermal Stability of ICg

Enzyme thermal stability is a major factor in its use. Figure 5 presents the thermal stability of ICg. The initial invertase activity was 27.7 $U.mg^{-1}$ at pH 5, equivalent to 100% of the relative activity (RA). In Figure 5, this value is the same in residual activity (log(RA) = 2) for 0 min. Afterwards, the thermal inactivation of invertase exhibited first-order apparent kinetics and its residual activity was typically linear at all temperatures employed in this study.

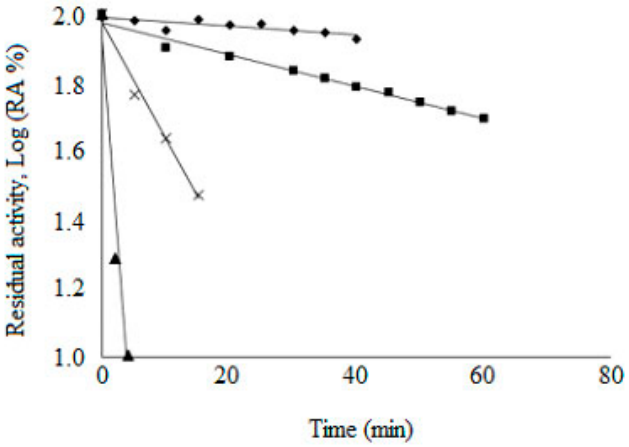

Figure 5. Thermal stability of ICg as a function of time at different temperatures. Reaction conditions: Mcllvaine buffer pH 5 (100 mM citric acid – 200 mM sodium phosphate). Enzyme: 1 mL in 3 mL of reaction medium with sucrose. Temperatures: (¨) 40 °C, (¡) 50 °C, (x) 60 °C, and (▲) 70 °C.

At 50 °C, ICg remained stable with about 85% of its activity after 60 min. Above that temperature, activity rapidly decreased and, after 4 min at 70 °C, 90% of the initial activity had been lost.

That suggests the catalytic structure of invertase is significantly altered as temperature increases. That likely occurs due to the importance of the non-covalent bond in preserving enzyme structure. Such bonds involve Van der Waals forces, electrostatic interactions, hydrogen bonds, and hydrophobic interactions and, when high temperatures interrupt those interactions, the proteins denature (WHITAKER, 2003; OLIVEIRA et al., 2019).

Figure 6 features the graphical representation of Arrhenius. The magnitudes of the Arrhenius parameters, determined by linear regression analysis from Figure 6 and are shown in Table 4. The activation energy (E_a) found for ICg denaturation was $1{,}56.10^2$ $KJ.mol^{-1}$ ($R^2 = 0.98$). The inactivation rates (k) were $3.20x10^{-3}$ and $1.08x10^{-2}$ min^{-1} at 40 °C and 50 °C, respectively.

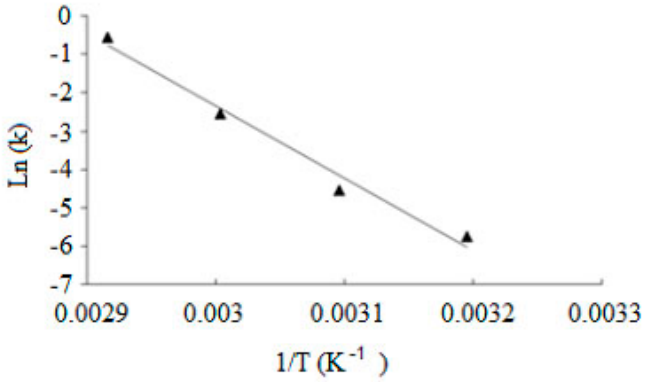

Figure 6. The graphical reprentation of Arrhenius for ICg.

Table 4. ICg thermal inactivation parameters.

Temperature (°C)	$K_{observed}$ (min^{-1})	$K_{calculated}$ (min^{-1})	E_a ($KJ.mol^{-1}$)	t ½ (min)
40 °C	$3.29x10^{-3}$	$2.43x10^{-3}$	155.0	210.4
50 °C	$1.08x10^{-2}$	$1.56x10^{-2}$		64.0

The thermal stability of the activity of an enzyme comprises thermodynamic and kinetics stability. The latter is often expressed as its half-life at a given temperature (OLIVEIRA et al., 2019). The half-life allows proving invertase at 40 °C is kinetically more stable than at 50 °C as the half-life at the former was three-fold higher than at the latter (Table 4).

Identifying Isoenzymes by Electrophoresis

Three isoenzymes were identified (Figure not shown), two of which with 0.1 and 0.3 relative mobility (RM) with molecular mass (MM) below 66 KDa of the BSA standard. A third band at RM = 0.55 and MM above 66 KDa of the standard was observed. Plascenia-Espinosa et al. (2014) reported one isoform of *Candida guilliermondii* MpIIIa invertase with the highest percentage of glycosylation and its deglycosylated form with an estimated MM of 63 kDa.

The MM of active yeast invertases is variable, as well as the number of isoforms (CANTARELLA et al., 2003). For example, the precursor work of Gascón et al. (1967) suggests that *Saccharomyces cerevisiae* synthesizes two invertases: one is non-glycosylated (internal, 135 kDa) and the other is glycosylated (external, 270 kDa), about half of which is mannan. Andjelković et al. (2010), when studying invertase extracted from *Saccharomyces cerevisiae* cells using a similar extraction methodology as the present research, reported the existence of four external invertase isoforms.

Candida utilis yeast also produces both forms. The non-glycosylated monomeric form has an estimated MM of 60 kDa and the glycosylated form has MM of 300 kDa. The MM values of both invertase forms were established using a commercial molecular mass markers kit (BELCARZ et al., 2002).

In contrast to most of the reported yeast invertases, Álvaro-Benito et al. (2007) described one glycosylated monomeric invertase form (85 kDa) from *Schwanniomyces occidentalis*, as well as one form of 165 kDa using gel filtration on Sephadex G-200.

It has been reported that the glycosylated nature of native invertase can affect the MM estimate (CANTARELLA et al., 2003). In addition to variation from one yeast species or strain to another, the methods chosen for extraction, purification, and characterization can also affect the presence or absence of internal and external invertases. For example, Klein et al. (1989) described one invertase form of *Schwanniomyces occidentalis* with MM in the 60-65 kDa range. Such mass is slightly lower than that reported by Álvaro-Benito et al. (2007). In another example, the external invertase is very stable when in contact with autolysis products between 40-45°C (ANDJELKOVIĆ et al., 2010; FERREIRA et al., 2018), but much of the mannan component content of external invertases can be removed. On the other hand, $T < 40^{o}C$ favors mannan component, but decreases invertase content in extract. The mannan is covalently linked to the protein moiety of the enzyme (VITOLO, 2019; SCHIAVONE et al., 2015)

So far, ICg extracted by autolysis has three isoforms, two with MM below 66 kDa and one, above. In addition, the three isoforms were sucrose positive using the system containing tetrazolium blue chloride, phenazine methosulfate, and sucrose at pH 5, which is specific for identifying invertase. The results in Table 1 are consistent with the action of yeast sucrose invertase. The extraction technique used favors external isoforms, but protein structural analysis will be required to fully understand the biological function of the enzyme and to improve its potential.

Conclusions

Purification of invertase from *C. guilliermondii* (6776277) through precipitation with acetone followed by dialysis achieved 56% recovery from the initial extract. Maximum ICg activity was found at pH 5.0 and 50 °C. ICg activity follows a Michaelian behavior and its activity is more specific at 50 °C than at 25 °C. At 50 °C, ICg remained stable with about 85% of its activity after 60 min. Above that temperature, activity rapidly decreased. Thermal inactivation of ICg exhibited first-order apparent kinetics and its T-dependence was well described by Arrhenius' law. Separation by electrophoresis yields three fractions with 0.1, 0.3, and 0.55 relative mobility. All isoforms with action sucrose positive. Finally, temperature at 50 °C enhances ICg affinity and specificity, but reduces the half-life, an important kinetic characteristic when designing bioreactors. At 40 °C, the half-life is four-fold longer.

Acknowledgment

The MEC/SETEC/CNPq (Government Agency linked to the Ministry of Education, Brazil) and PROPESP (Pro-Rectory of post-Graduate Studies and Research at the IFSUL, Brazil) supported this work financially.

References

AMJAD, A.; JAVED, M.S.; HAMEED, A.; HUSSAIN, M.; ISMAIL, A. Changes in sugar contents and invertase activity during low temperature storage of various chipping potato cultivars. **Food Science and Technology**, New York, v.40, n.2, p.340-345, 2020.

ÁLVARO-BENITO, M.; ABREU, M.; FERNÁNDEZ-ARROJO, L.; PLOU, F. J.; JIMÉNEZ-BARBERO, J.; BALLESTEROS, A.; POLAINA, J.; FERNÁNDEZ-LOBATO, M. Characterization of a β-fructofuranosidase from *Schwanniomyces occidentalis* with transfructosylating activity yielding the prebiotic 6-kestose. **Journal Biotechnology**, Amsterdam, v.132, n.1, p.75-81, 2007.

ASHURST, P. R. **Chemistry and technology of soft drinks and fruit juices**. 2nd ed. Hereford: Blackwell Publishing, 2005. 393 p.

ANDJELKOVIĆ, U.; PIĆURIĆ, S.; VUJČIĆ, Z. Purification and characterisation of *Saccharomyces cerevisiae* external invertase isoforms. **Food Chemistry**, London, v.120, n.1, p.799-804, 2010.

BARBOSA, P.M.G.; MORAIS, T.P.; SILVA, C.A.A.; SANTOS, F.R.S.; GARCIA, N.F.L.; FONSECA, G.G.; LEITE, R.S. R.; PAZ, M.F. Biochemical characterisation and evaluation of invertases produced from *Saccharomyces cerevisiae* CAT-1 and *Rhodotorula mucilaginosa* for the production of fructooligosaccharides. **Preparative Biochememistry & Biotechnology**, New York, v.48, n.6, p.506–513, 2018.

BATISTA, R.D.; MELO, F.G.; AMARAL SANTOS, C. DO; DE PAULA-ELIAS, F.C.; PERNA, R.F.; XAVIER, M.; VILLALBA MORALES, S.A.; DE ALMEIDA, A.F. Optimization of β-fructofuranosidase production from agrowaste by *Aspergillus carbonarius* and its application in the production of inverted sugar. **Food Technology and Biotechnology**, Zagreb, v.59, n.3, p.306–313.

BELCARZ, A.; GINALSKA, G.; LOBARZEWSKI, J.; PENEL, C. The novel non-glycosylated invertase from *Candida utilis* (the properties and the conditions of production and purification). **Biochimica et Biophysica Acta**, Amsterdam, v.1594, n.1, p.40–53, 2002.

BRAMONO, K.; YAMAZAKI, M.; TSUBOI, R.; OGAWA, H. Comparison of proteinase, lipase and alfa-glucosidase activities from the clinical isolates of *Candida* Species. **Japanese Journal of Infectious Diseases**, Tokyo, v.59, n.2, p.73-76, 2006.

CABRAL, B.V.; SANTOS, L.D.; FALLEIROS, L.N.S.S.; CARMO, T.S.; FREITAS, F.F.; CARDOSO, S.L.; RESENDE, M.M.; RIBEIRO, E.J. Sucrose hydrolysis by invertase immobilized on Duolite A-568 employing a packed-bed reactor. **Chemical Engineering Communications**, London, v.204, n.9, p.1007–1019, 2017.

CANTARELLA, L.; ALFANI, F.; CANTARELLA, M. β-D-fructofuranoside fructohydrolase. *In*: WHITAKER, J.R., VORAGEN, A.G.J.; WONG. D.W.S. (ed.). **Handbook of food enzymology**. New York: Marcel Dekker, 2003. p.787-804.

CATALOGUE ANALYTIQUE API 20C. Logiciel d'identification apiweb TM. ATB TM instrument or mini API (consult bio Mérieux). 2021. Disponível em: http://biotech.spip.ac-rouen.fr/IMG/pdf/API_20C_Aux.pdf. Acesso em: 21 nov. 2021.

DI ADDEZIO, F.; YORIYAZ, E.J.; CANTARELLA, M.; VITOLO, M. Sucrose hydrolysis by invertase using a membrane reactor: effect of membrane cut-off on enzyme performance. **Brazilian Journal of Pharmaceutical Sciences**, São Paulo, v.50, n.2, p.257-260, 2014.

FERREIRA, M.V; AVILA, T.L.; TORALLES, R.P.; KUHN, C. R.; ROMBALDI, C.V. Identifying yeast isolated from spoiled peach puree and assessment of its batch culture for invertase production. **Food Science and Technology**, New York, v.36, n.4, p.701–708, 2016.

FERREIRA, M.V.; ROSSLER, A.F.; TORALLES, R.P.; RUIZ, W.A; ROMBALDI, C.V. Extracción optimizada y purificación parcial de invertasa aislada de S. Cerevisiae en puré de durazno. **Revista Brasileira de Fruticultura**, Jaboticabal, v.40, n.2, p.1-9, 2018.

GARGEL, C.A.; BAFFI, M.A.; GOMES, E.; SILVA, R. Invertase from a *Candida stellata* strain isolated from grape: production and physico-chemical characterization. **Journal Microbiology Biotechnology and Food Sciences**, Nitra, v.4, n.1, p.24-28, 2014.

GASCÓN, S.; NEWMAN, N.; LAMPEN, J.O. Comparative study of the properties of the purified internal and external invertases from yeast. **Journal of Biological Chemistry**, Baltimore, v.243, n.7, p.1573-1577, 1968.

GUIMARÃES, L.H.S.; TERENZI, H.F.; POLIZELI, M.L.T.M.; JORGE, J.A. Production and characterization of thermostable extracellular β-D-fructofuranosidase produced by Aspergillus ochraceus with agroindustrial residues as carbon sources. **Enzyme and Microbial Technology**, London, v.1, p.52–57, 2007.

KLEIN, R.D.; DEIBEL JR, M.R.; SARICH, J.L.; ZURCHER-NEELY, H.A.; REARDON, I.L.; HEINRIKSON R.L. Purification and characterization of invertase from a novel industrial yeast, *Schwanniomyces occidentalis*. **Preparative Biochemistry**, New York, v.19, n.4, p.293–319, 1989.

LACAZ, C.S; PORTO, E.; MARTINS, J.E.C; HEINS-VACCARI, E.M; MELO, N.T. **Tratado de micologia médica.** 9th ed. São Paulo: Sarvier, 2002.

LOPES, A.M; TORALLES, R.P; ROMBALDI, C.V. Thermal inactivation of polyphenoloxidase and peroxidase in Jubileu clingstone peach and yeast isolated from its spoiled puree. **Food Science and Technology**, Hoboken, v.34, n.1, p.150-156, 2014.

LOWRY, O.H.; ROSEBROUGH, N.J.; FARR, A.L.; RANDALL, R.J. Protein measurement with the Folin phenol reagent. **Journal Biological Chemistry**, Irvine, v.193, n.1, p.265-275, 1951.

OLIVEIRA, R.L.; SILVA, M.F.; CONVERTI, A.; PORTO, T.S. Biochemical characterization and kinetic/thermodynamic study of Aspergillus tamari URM4634 β-fructofuranosidase with transfructosylating activity. **Biotechnology Progress**, Washington, v.35, n.6,p.e2879-n/a, 2019.

ORTU, E.; CABONI, P. Levels of 5-hydroxymethylfurfural, furfural, 2-furoic acid in sapa syrup, Marsala wine and bakery products. **International Journal of Food Properties**, Philadelphia, v.20, n.S3, p.S2543–S2551, 2017.

OYEDEJI, O.; BAKARE, M.; ADEWALE, I.; OLUTIOLA, P.; OMOBOYE, O. Optimized production and characterization of thermostable invertase from *Aspergillus niger* IBK1, using pineapple peel as alternative substrate. **Biocatalysis and Agricultural Biotechnology**, Amsterdam, v.9, p.218–223, 2017.

PLASCENIA-ESPINOSA, M.Á.; SANTIAGO-HERNÁNDEZ, A.; PAVÓN-OROZCO, P.; VALLEJO-BECERRA, V.; TREJO-ESTRADA, S.; SOSA-PEINADO, A.; BENITEZ-CARDOZA, C.G.; HIDALGO-LARA, M.E. Effect of deglycosylation on the properties of thermophilicinvertase purified from the yeast *Candida guilliermondiiMpIIIa*. **Process Biochemistry**, Barking, v.49, n.9, p.1480-1487, 2014.

RADOVANOVIC, M.; RACIC, B.; TANASKOVIC, S.; MARKOVIC, G.; TOMIC, D.; PANTOVIC, J. The catalytic effect of honey on formation sugars during sucrose hydrolysis. **Hemijska Industrija**, Beograd, v.71, n.2, p.105-110, 2017.

SCANDALIOS, J.G. Genetic control of multiple molecular forms of enzymes in plants: a review. **Biochemical Genetics**, New York, v.3, n.1, p.37-39, 1969.

SCHIAVONE, M.; SIECZKOWSKI, N.; CASTEX , M.; DAGUE, E.; FRANÇOIS, J.M. Effects of the strain background and autolysis process on the composition and biophysical properties of the cell wall from two different industrial yeasts. **FEMS Yeast Research**, Oxford, v.15, n.2, p.1-11, 2015.

SIQUEIRA, R.S. **Manual de microbiologia de alimentos**. Brasília: EMBRAPA, 1995.

SONGPIM, M.; VAITHANOMSAT, P.; VANICHSRIRATANA, W.; SIRISANSANEEYAKUL S. Enhancement of Inulinase and Invertase production from a newly isolated *Candida guilliermondii* TISTR 5844. **Kasetsart Journal - Natural Science**, Amsterdam, v.45, n.4, p.675-685, 2011.

TIBCO Software. **Statistica** (data analysis software system). Version 13. Palo Alto, 2018. Disponível em: http://tibco.com.

TORALLES, R.P.; KUHN, C.R.; SILVA, P.; RUIZ, W.A. Extração e caracterização parcial de invertase de levedura de purê e resíduo de pêssego. **Revista Brasileira de Tecnologia Agroindustrial**, Ponta Grossa, v.8, n.2, p.1399–1415, 2014.

TORALLES, R.P.; FERREIRA, M.V.; RUIZ, W.A. Imobilização e cinética da invertase de *Sacharomyces cerevisiae* em agarose. *In*: VIEIRA, V.B.; PIOVESAN, N. (ed.). **Investigação científica no campo de engenharia e da tecnologia de alimentos**. Ponta Grossa: Atena, 2021. p.153-159.

VALÉRIO, S.G.; ALVES, J.S.; KLEIN, M.P.; RODRIGUES, R.C.; HERTZ, P.F. High operational stability of invertase from *Saccharomyces cerevisiae* immobilized on chitosan nanoparticles. **Carbohydrate Polymers**, Orlando, v.92, n.1, p.462-468, 2013.

VITOLO, M. Invertase. *In*: SAID, S.; PIETRO, R.C.L.R. (Ed.). **Enzymes as biotechnological agents**. Ribeirão Preto: Leggis Summa, 2004. p.207-221.

VITOLO, M. Autolysis of baker´s yeast and partial purification of invertase in the presence of surfactants. **European Journal of Pharmaceutical and Medical Research**, Bhandara, v.6, n.5, p.84-90, 2019.

WANG, X.; CHEN, Y.; JIANG, S.; XU, F.; WANG, H.; WEI, Y.; SHAO, X. PpINH1, an invertase inhibitor, interacts with vacuolar invertase PpVIN2 in regulating the chilling tolerance of peach fruit. **Horticulture Resarch**, Tokyo, v.7, n.1, p.1-14, 2020.

WHITAKER JR. Protein structure and kinetics of enzyme reaction: a historical perspective. *In*: WHITAKER JR.; VORAGEN A.G.J.; WONG, D.W.S. (ed.). **Handbook of food enzymology**. New York: Marcel Dekker, 2003. p.16-25.

YOON S.H.; MUKERJEA R.; ROBYT J.F. Specificity of yeast *Saccharomyces cerevisiae* in removing carbohydrates by fermentation. **Carbohydrate Research**, Amsterdam, v.338, p.1127-1132, 2003.