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INVERTASE FROM A *CANDIDA STELLATA* STRAIN ISOLATED FROM GRAPE: PRODUCTION AND PHYSICO-CHEMICAL CHARACTERIZATION

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ABSTRACT

Invertases are enzymes which hydrolyze the sucrose and are widely employed in food and pharmaceutical industries. In this work, the screening of autochthonous grape yeasts from Brazil was carried out in order to investigate their invertase production potential. Yeasts belonging to *Saccharomyces*, *Hanseniaspora*, *Sporidiobolus*, *Issatchenkia*, *Candida*, *Cryptococcus* and *Pichia* genera were analyzed by submerged fermentation (SbmF) using sucrose as substrate. Among them, *Candida stellata* strain (N5 strain) was selected as the best producer (10.6 U/ml after 48 hours of SbmF). This invertase showed optimal activity at pH 3.0 and 55°C, demonstrating appropriate characters for application in several industrial processes, which includes high temperatures and acid pHs. In addition, this invertase extract presented tolerance to low concentrations of ethanol, suggesting that it could also be suitable for application at the beginning of alcoholic fermentation. These data provide promising prospects of the use of this new invertase in food and ethanol industry.

Keywords: Grape yeasts, non-*Saccharomyces*, invertase, food industry

INTRODUCTION

The invertases, also named β-D-fructofuranosidases (EC 3.2.1.26), are one of the most widely employed enzymes in industries with a wide range of commercial applications including lactic acid production (**Acosta *et al.*, 2000**); fermentation of sugarcane into ethanol (**Lee and Huang, 2000**) and fructose syrup production (**de Almeida *et al.*, 2005**). It is also used in pharmaceutical industry as digestive aid tablets, powder milk for child nutrition as calf feed preparation and assimilation for alcohol in fortified wines (**Uma *et al.*, 2012**).

Invertases catalyze the hidrolysis of the glicosidic bond from sucrose in its respective monomers glucose and fructose. The product of this hydrolysis, the glucose and fructose syrup, is known as "inverted sugar" and has important features: it is sweeter than sucrose (about 40%), it is stable at high concentrations, it is more soluble than the original disaccharide and it has higher boiling and lower freezing points (**Uma *et al.*, 2012**). As a consequence, the crystallization phenomenon is avoided, improving the texture of candies, jams, ice creams, and other food products (**Valerio *et al.*, 2013**).

These enzymes can be synthesized by plants (**Hussain *et al.*, 2009**), some filamentous fungi (**Chelliappan and Madhanasundareswari, 2013**) and yeasts such as *Saccharomyces cerevisae* (**Pataro *et al.*, 2002**), *Candida utilis* (**Belcarz *et al.*, 2002**), *Rhodotorula glutinis* (**Rubio *et al.*, 2002**) and *Pichia fermentans* (**Caputo *et al.*, 2012**), being *S. cerevisiae* the chief strain used for the production of invertases (**Kulshrestha et al., 2013**).

In yeasts, they are present either as extracellular as well as intracellular forms, being 80% from extracellular location (**Nakano *et al.*, 2000**). The secreted invertase is a glycoprotein with around 50% of carbohydrates, while the intracellular form is composed only by aminoacids. For the production of invertases useful in food industry, extracellular invertases are preferable and more appropriate since they are released directly in the culture broth avoiding additional steps of cell rupture to extract the enzyme (**Kulshrestha et al., 2013**).

In spite of the the majority of the studies concerning yeast invertases has focused on *Saccharomyces cerevisiae* (**Pataro *et al.* 2002**), there are few works describing invertase production by other yeast genera (**Turkel *et al.* 2006**). Besides, up to this moment there is not any study reporting authoctonous grape yeasts as invertase producers. Thus, the investigation of new yeast species able to produce such enzymes could be of great importance for different industrial processes.

The objective of our study was the screening of yeast strains isolated from grape surfaces invertase biosynthesis and secretion. The physico-chemical properties from the selected best producer strain were evaluated in order to characterize this new crude enzyme and suggest possible applications in food industries which operate at similar pH/temperature conditions.

MATERIAL AND METHODS

Yeasts

Saccharomyces and other yeast strains were obtained from the Grape Yeast Collection (Sao Paulo State University, Brazil) and were previously assigned to species level by analysis of the 5.8S-ITS ribosomal DNA (rDNA) region (**Baffi *et al.*, 2011**). The stock culture was preserved in YPD agar (1% yeast extract, 1% peptone and 2% glucose) supplemented with choramphenicol (100 mg l^{-1}), ampicillin (25 µg ml^{-1}) and sodium propionate (0.25 g l^{-1}) at room temperature. A total of 34 yeast strains was screened for invertase production and compared to a *Saccharomyces cerevisae* commercial strain. Yeast strains are listed in Table 1.

Invertase production by SbmF

An isolated colony from each yeast strain was pre-grown in YPD medium overnight at 28-30°C under shaking. The submerged fermentation (SbmF) was carried out under aerobic conditions, using a 18 h-old culture with an initial inoculum concentration of 1.0 x 10^6 CFU ml^{-1}, in erlenmeyer flasks containing 50 ml of liquid YP media, with 2% sucrose (**Shafic *et al.*, 2003**, with modifications). Cultures were incubated for 120 h at 28◦C and 200 rpm. To ascertain the time of the peak enzyme synthesis, aliquots were withdrawn and the invertase activity was determined at time intervals of 24 h. Samples were centrifuged at 12,000 *g* for 15 min at 5°C. Afterwards, the supernatant was discarded and the precipitate was washed with NaCl 0.8% solution for 15 min and submitted to centrifugation at the same conditions described above. Afterwards, the cells were suspended in 5 ml of 0.2 mol l^{-1} sodium acetate buffer and macerated with washed sand. Finally, it was centrifuged again for 10 min and the enzymatic extract was filtrated in a Millipore membrane.

Table 1 Yeast species and respective strains screened for invertase production

Yeast species	Strains
Hanseniaspora uvarum	U6, U17, N1, N7, N36, 10A, 3A
Saccharomyces cerevisae	N24, N37, N38, 3
Candida quercitrusa	N17, N19
Pichia orientalis	N40, 52, 18, 28
Pichia kluyveri	N31
Pichia terricola	22A, 5A
Pichia occidentalis	39, 41, 43
Sporidiobolus pararoseus	8A
Candida stellata	N5, N9
Aureobasidium pullulans	1A, 4A, 12A
Cryptococcus laurenti	U2
Cryptococcus flavescens	U8, U10
Meyerozyma quilliermondii	U5, U9

Cellular growth and pH analysis

The cell viability was monitored at fermentation medium, at intervals of 24 h, by counting of viable cells in a Neubauer chamber after differential staining using Erythrosine B (ERB) (**Karwoski *et al.*, 1995**). The acidity of the fermentation media was also monitored by measuring the pH value at intervals of 24 h. These data were compared with the ones obtained with a commercial *S. cerevisiae* strain.

Invertase activity assay

The assay mixture consisted of 0.2 ml of 0.5 mol l^{-1} sucrose, 0.6 ml of sodium acetate buffer (0.2 mol l^{-1}, pH 5.0) and 0.2 ml of the enzymatic extract. The mixture was incubated at 37°C for 30 min. The amount of reducing sugars released was determined spectrophotometrically at 546 nm using the 3.5 dinitrosalicylic acid (DNS) method (**Miller, 1959**). Enzyme activity was expressed in U ml^{-1}. One unit (U) of enzymatic activity was defined as the amount of enzyme that releases 1 µmole of glucose/fructose from sucrose per minute under the conditions of the assay. Assays were conducted in duplicate.

Physico-chemical characterization

Optimum pH was determined by measuring the invertase activity over a pH range of 2.0–8.0 at 37°C, in citrate phosphate buffer (100 mM–pH range 2.0–4.0), sodium acetate buffer (100 mM pH range 3.0–5.0), Tris-maleate buffer (100 mM, pH 5.0–7.0) and Tris-HCl (100 mM pH range 7.0--8.0). The optimum temperature was determined at optimum pH, in the temperature range of 20–70°C. The pH stability was investigated storing the enzyme diluted 5-fold for 24 h at room temperature in buffers with increasing pHs (2.0-8.0). The remaining activity was measured under standard conditions at optimum pH and temperature. The thermostability was studied by incubating the crude enzyme, without substrate, for 1 h, over a temperature range of 20–70°C. The aliquots were assayed for residual activities at optimum conditions. Assays were done in triplicate. Enzymatic activity was calculated from the amount of reducing sugars released, using the DNS method.

Effect of ethanol on invertase activity

Increasing concentrations of ethanol from 0 to 20%, v/v) were added to the reaction mixture to check their effects on invertase activity. Assays were carried out under optimal conditions of pH/temperature and in triplicate. A maximum value of activity was considered in the reaction mixture without ethanol.

RESULTS AND DISCUSSION

Invertase production

Among all the yeast strains tested, *Candida stellata* (N5 strain) was the only strain able to present significant invertase activity, with peak of production after 48 hours of SmF of 7.8 U/ml of fermented broth (Fig1a) (Table 2). It was compared with the invertase production by a commercial *Saccharomyces cerevisiae* strain at the same conditions. However, it presented a lower invertase activity (2.5 U/ml) (Fig1a). Other authors showed higher invertase activity for *Saccharomyces cerevisiae*. A value of 8.35 U/ml of invertase was obtained by **Shafic et al. (2003).** This result suggests *C. stellata* N5 strain is a good producer of this enzyme in short time (48 hours). At optimal conditions, 10.6 U/ml of activity were produced. Lower invertase activity values were found for other non-*Saccharomyces* yeast strains **(Rubio *et al.* 2002; Caputo *et al.* 2012**).

Table 2 Invertase production by SmF by yeast strains investigated in this study.

Yeast species	Strain	Peak of activity (h)	Maximum invertase activity (U/ml)
Hanseniaspora uvarum	U6	-	ND*
Hanseniaspora uvarum	U17	-	ND
Hanseniaspora uvarum	N1	-	ND
Hanseniaspora uvarum	N7	-	ND
Hanseniaspora uvarum	N36	-	ND
Hanseniaspora uvarum	10A	-	ND
Hanseniaspora uvarum	3A	-	ND
Saccharomyces cerevisae	N24	48	2.5
Saccharomyces cerevisae	N37	48	2.8
Saccharomyces cerevisae	N38	48	2.6
Saccharomyces cerevisae	3	48	2.3
Candida quercitrusa	N17	48	0.4
Candida quercitrusa	N19	48	0.7
Pichia orientalis	N40	48	1.2
Pichia orientalis	52	48	1.0
Pichia orientalis	18	48	1.0
Pichia orientalis	28	48	0.9
Pichia kluyveri	N31	-	ND
Pichia terricola	22A	-	ND
Pichia terricola	5A	-	ND
Pichia occidentalis	39	48	1.3
Pichia occidentalis	41	48	1.5
Pichia occidentalis	43	48	1.2
Sporidiobolus pararoseus	8A	-	ND
Candida stellate	N5	48	7.8
Candida stellate	N9	48	7.3
Aureobasidium pullulans	1A	-	ND
Aureobasidium pullulans	4A	-	ND
Aureobasidium pullulans	12A	-	ND
Cryptococcus laurenti	U2	-	ND
Cryptococcus flavescens	U8	-	ND
Cryptococcus flavescens	U10	-	ND
Meyerozyma quilliermondii	U5	-	ND
Meyerozyma quilliermondii	U9	-	ND

*ND: not detected at fermentation broth.

After counting the viable cell number, it was observed that the peak of cellular multiplication for *C. stellata* N5 strain was up to 48 h (*log* stage), followed by a *stationary* stage (between 48 and 96 hours of fermentation) and a *decline* stage (96 to 120 h) (Figure 1b). After 96 hours of fermentation, a small increase of the cell viability was once more observed. This oscillating behavior can probably be due to some experimental error or also by enzyme competition or other reasons. Nevertheless, this small variation is irrelevant since the peak of maximum growth and maximum enzyme production was at 48 h, and, afterwards, both growth and enzyme production have decreased, with a low oscilation at 96 hours.

In this study, the number of viable cells between N5 strain and the commercial strain was also compared. Thus, it was observed that the cellular viability in *C. stellata* was six fold higher than in *S. cerevisae* (Table 3) at the analyzed conditions.

Table 3 Cell viability among *C. stellata* and *S. cerevisiae* during SbmF.

Time (hours)	Viability *C. stellata* (*10^7 cells/ml)	Viability *S. cerevisiae* (*10^7 cells/ml)	Viability *C. stellata/ S. cerevisiae* (*10^7 cells/ml)
0	1	1	1
24	8	3	2.67
48	22	2	14
72	14	3.5	4
96	17.5	3.2	5.468
120	12	2.8	4.285
Average			6.08

As far as it concerns the pH of the medium during the fermentation, the pH of *C. stellata* medium remained acidic during the whole process, being advantageous to yeast growth (Fig 1c). In contrast, the pH of the medium inoculated with the commercial strain increased, reaching a pH range of 6.0 to 8.0 during the fermentation. These pH values are not appropriate for yeast growth which can justify the lower invertase biosynthesis by this strain. In addition, basic pHs are favorable for most of the bacteria development which can spoil the fermentation broth. These results indicated that *C. stellata* N5 strain showed a better capacity to grow in a fermentation broth containing sucrose as the sole carbon source, suggesting it as promising yeast for application in food industries.

Effects of pH and temperature

The highest invertase activity was observed at pH 3.0 (Fig 2a). **Andjelkovic *et al.* (2010)** observed an optimum pH around 3.5 for an extracelular invertase produced by *S. cerevisiae* and **Alegre *et al.* (2009)** described extra and intracellular invertases of *Aspergillus caespitosus* with maximum activities at the pH range of 4.0-6.0. This result is interesting for application of this invertase in food industries, for example extraction of sugars for juice fabrication since the most of the fruits rich in sucrose exhibit acid pHs, such as pineapple, peaches, apricots, organges, grapefruits and many others (**Anon, 1962**). Optimal temperature was achieved at 55°C (10.6 U/ml at optimal conditions) (Fig 2b). This result is in accordance with previous studies which described yeast invertases with optimal activity around 50-55°C (**Kulshrestha *et al.*, 2013**; **Valério *et al*., 2013**).

The enzyme was stable in a broad range of pH (2.0 to 8.0) (Fig 2c) and temperatures (20-55 °C) (Fig 2d). It was stable under low temperatures, retaining around 95% of residual activity after incubation for 1 h at 20°C and up to 55°C (with a residual activity of 83% at this temperature. These results agreed with previous reports, which showed invertases with similar stabilities up to 55°C (**Kulshrestha *et al.*, 2013**).

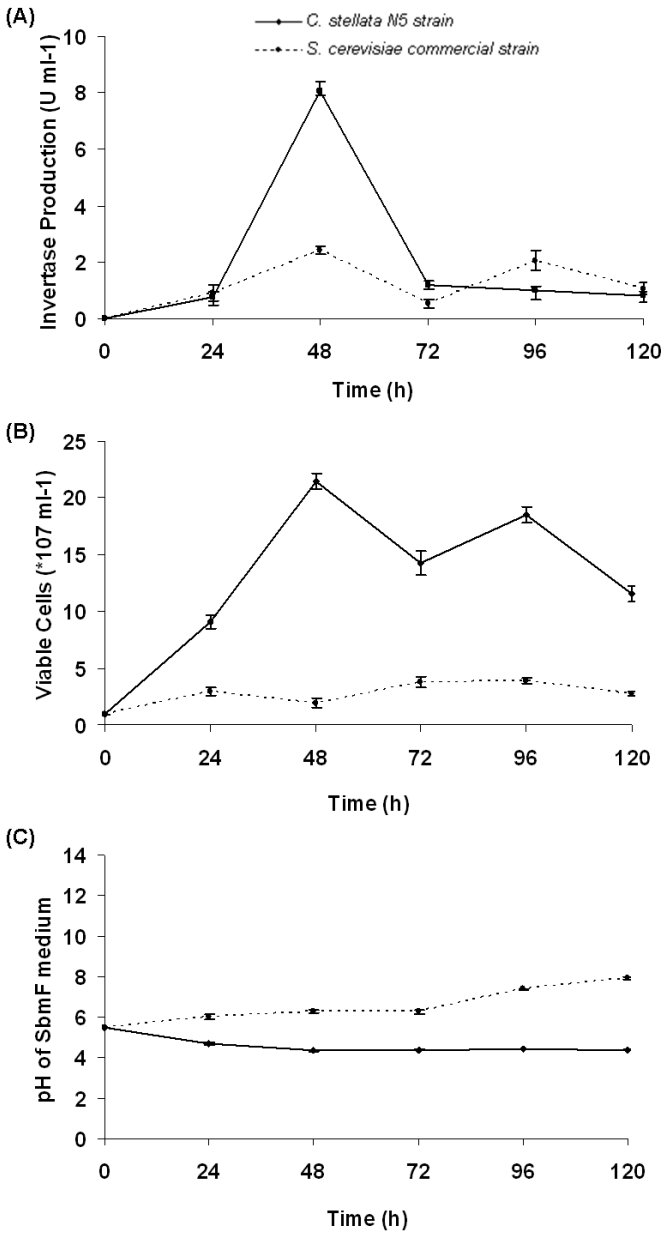

Figure 1 Comparison of the invertase biosynthesis (a), cell viability (b) and pH of fermentation media (c) among *C. stellata* and *S. cerevisiae*, during SbmF. Invertase activity expressed as U ml^{-1}; cell viability expressed in number of viable cells x 10^7 ml^{-1}. Results are mean values from duplicate experiments (error bars indicate standard deviation ($P < 0.05$)

Ethanol effect

The *C. stellata´*s invertase was tolerant at low concentrations of ethanol, remaining 84.0, 63.0 and 58.0% of its original activity at 2.5, 5.0 and 7.5% of ethanol (w/v), respectively (Figure 3). However, at high concentrations of this alcohol, the activity was meaningfully reduced. This moderate tolerance to ethanol suggests that this invertase could also be suitable for application in the initial steps of alcoholic fermentation in ethanol production processes. These data indicate that the enzyme could be added at the beginning of alcoholic fermentation because at this stage low concentrations of ethanol are found, being useful to the increase of the amount of reducing sugars (**Baffi et al. 2011**). At industrial conditions, the maximum ethanol production achieves usually among 10-14% (v/v) at the end of fermentation (**Bai *et al.* 2008**).

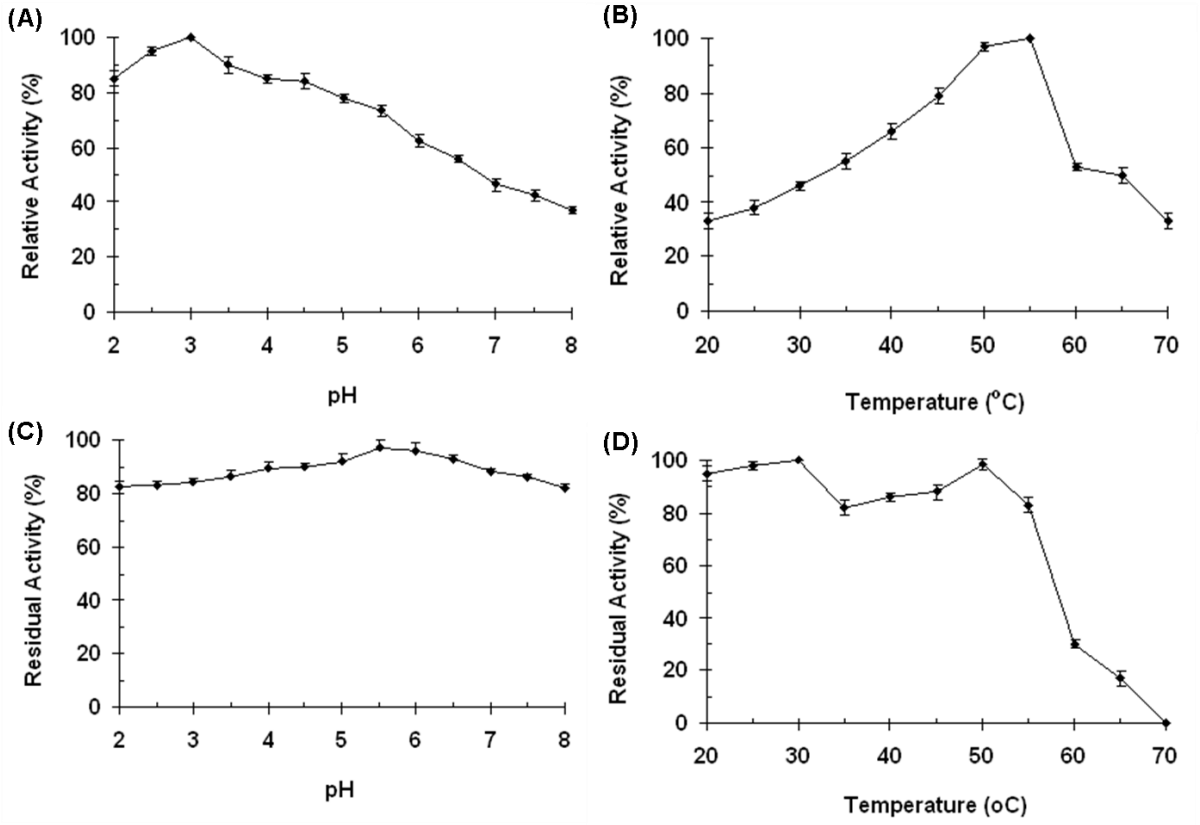

Figure 2 Effects of pH and temperature on *C. stellata* invertase. (a) Optimum pH; (b) Optimum temperature; (c) pH stability and (d) thermostability. Results are mean values from duplicate experiments and error bars indicate standard deviation ($P < 0.05$)

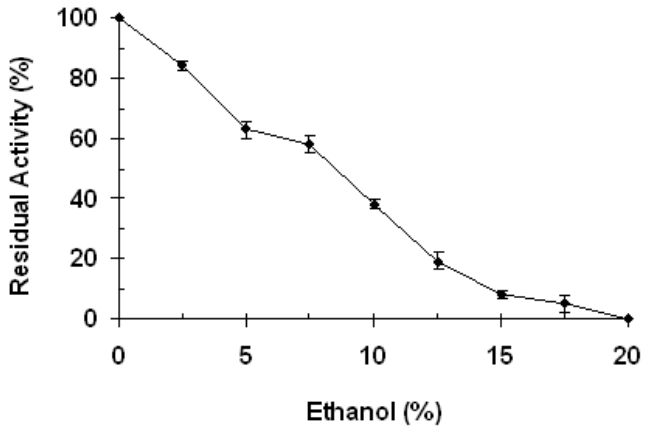

Figure 3 Effect of ethanol on the activity of *C. stellata* invertase. Results are mean values from duplicates and error bars indicate standard deviation (P<0.05)

CONCLUSION

This work showed evidence that *C. stellata* N5 strain is a potential producer of invertase. The results demonstrated that the enzyme was active and stable in a broad range of pH and temperatures and at low concentrations of ethanol. These data support this new invertase as a promising catalytic agent for use in several biotechnological processes in the food industry and alcoholic fermentations.

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