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Extracción y caracterización de β-fructofuranosidasas producidas por *Kluyveromyces marxianus* CCMB 322

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Resumo

A invertase (β-frutofuranosidase, EC 3.2.1.26) catalisa a hidrólise da sacarose em glicose e frutose, e é uma das mais simples carboidrases. Estas enzimas ocorrem amplamente na natureza e a sua presença tem sido relatada em microrganismos e plantas. As leveduras são as principais fontes industriais, a maioria das pesquisas sobre esta enzima se concentrou em invertase extraída de tal fonte. Este estudo extraiu e caracterizou invertase intracelular (Inv-I) e extracelular (Inv-E) de *Kluyveromyces marxianus* CCMB 322 isolada na região do semiárido baiano. *Kluyveromyces marxianus* CCMB 322 produz invertase intracelular e extracelular com características diferentes. A atividade ótima foi alcançada a um pH de aproximadamente 3,9 e 45ºC, para Inv-I e Inv-E. As invertases produzidas por *K. marxianus* CCMB 322 mostrou estabilidade térmica semelhante ao encontrado em outros estudos. Os valores de K_m e V_{max} da enzima Inv-I foram de 61.12mM e 5,56 µmol/mL.min^{-1}, mas os valores de K_m e V_{max} da enzima Inv-E foram de 76,5mM e 0,364 µmol/mL.min^{-1}. Invertases de *K. marxianus* têm uma afinidade elevada para a sacarose em comparação a enzimas obtidas de outras fontes.

Palavras-chave: Enzima; β-fructofuranosidase; *Kluyveromyces marxianus*.

Abstract

Invertase (β-fructofuranosidase, EC 3.2.1.26) catalyzes sucrose hydrolysis into glucose and fructose and it is one of the simplest carbohydrases. These enzymes occur widely in nature and their presence has been reported in microorganisms and plants. Since yeasts are the main industrial source, most researches concerning this enzyme have focused on invertase extracted from such source. This study extracted and characterized inverted intracellular (Inv-I) and extracellular (Inv-E) of *Kluyveromyces marxianus* CCMB 322 isolated in the baiano semi-arid region. *Kluyveromyces marxianus* CCMB 322 produces intracellular and extracellular

invertase with different characteristics. The optimum activity was achieved at approximately pH 3.9 and 45ºC, in Inv-I and Inv-E. The invertases produced by *K. marxianus* CCMB 322 showed thermal stability similar to that found in other studies. The Km and Vmax values of the Inv-I enzyme were 61.12mM and 5.56 µmol/mL.min-1, but the Km and Vmax values of the Inv-E enzyme were 76.5mM and 0.364 µmol/mL.min-1. Inverted from K. marxianus is a higher affinity for sucrose compared to enzymes from other sources.

Keywords: Enzymes; β-fructofuranosidase; *Kluyveromyces marxianus*.

Resumen

La invertasa (β-fructofuranosidasa, EC 3.2.1.26) cataliza la hidrólisis de sacarosa en glucosa y fructosa, y es uno de los carbohidratos más simples. Estas enzimas se encuentran ampliamente en la naturaleza y su presencia ha sido reportada en microorganismos y plantas. Las levaduras son las principales fuentes industriales, la mayor parte de la investigación sobre esta enzima se ha centrado en la invertasa extraída de dicha fuente. Este estudio extrajo y caracterizó la invertasa intracelular (Inv-I) y extracelular (Inv-E) de *Kluyveromyces marxianus* CCMB 322 aislado en la región semiárida de Bahía. *Kluyveromyces marxianus* CCMB 322 produce invertasa intracelular y extracelular con diferentes características. La actividad óptima se logró a un pH de aproximadamente 3.9 y 45ºC, para Inv-I e Inv-E. Las invertasas producidas por *K. marxianus* CCMB 322 mostraron una estabilidad térmica similar a la encontrada en otros estudios. Los valores de K_m y V_{max} de la enzima Inv-I fueron 61.12mM y 5.56 µmol/mL.min^{-1}, pero los valores de K_m y V_{max} de la enzima Inv-E fueron 76.5mM y 0.364 µmol/mL.min^{-1}. Las investigaciones de *K. marxianus* tienen una gran afinidad por la sacarosa en comparación con las enzimas obtenidas de otras fuentes.

Palabras clave: Enzimas; β-fructofuranosidasa; *Kluyveromyces marxianus*.

1. Introduction

Invertase (β-fructofuranosidase, EC 3.2.1.26) catalyzes sucrose hydrolysis into glucose and fructose and it is one of the simplest carbohydrases. They are widely found in nature and their presence has been reported in microorganisms and plants. Since yeasts are their main industrial source, most researches concerning this enzyme have focused on invertase extracted from such source (Lincoln & More, 2018). The *Kluyveromyces marxianus* species has been used by industry for presenting characteristics such as the capacity to assimilate key sugars, namely lactose, sucrose and inulin and for being thermotolerant (Marcišauskas, Ji, & Nielsen,

2019).

Invertases are widely used in food and beverage industries. Invertases are widely used in food and beverage industries. An important application of invertases is the production of non-crystallizable sugar syrup (inverted sugar syrup) from sucrose. Inverted sugar is used as humectant in soft candies and fondants manufacture. Invertase is also used whenever substrates containing sucroses are subjected to fermentation such as in alcoholic beverages, lactic acid and glycerol production and used in the manufacture of artificial honey and plasticizing agents (Do Nascimento et al., 2019).

The Brazilian semi-arid region represents a large bioprospection area since it naturally fosters microorganisms adapted to tropical semi-arid environments which present high temperature, low humidity throughout the year and may show some features of great industrial interest.

The search for enzyme-producing microorganisms with industrial applications is essential to supply human needs (Santos et al., 2020). The current study analyzed the extraction and characterization of intracellular (Inv-I) and extracellular (Inv-E) invertases from *K. marxianus* CCMB 322 isolated in the Brazilian semi-arid region.

2. Methodology

The work consists of an experimental quantitative research, in which the experiments were carried out in the Pharmacotechnics laboratory allocated to the Department of Sciences and Technologies - DCT of the State University of Southwest Bahia - UESB.

2.1 Microorganism

The *K. marxianus* CCMB 322 yeast strain used in the current study was isolated from necrotic plant tissues (Agave sp.) collected in natural environments of the Brazilian semi-arid region, Bahia, Brazil and obtained at the Microorganisms Culture Collection of Bahia (CCMB) - UEFS, Brazil. Molecular identification was performed according to Oliveira et al., (2009).

2.2 Production and extraction of invertase

The yeast strain of *K. marxianus* CCMB 322 isolate was previously grown in YM agar

(Difco, USA) at 28ºC for 18 hours, diluted in sterile distilled water to approximately 10^8 cells.mL^{-1} and 10% (v/v), and inoculated in 25 mL flasks containing mineral medium for fermentation (Patching & Rose, 1970). The medium contained the following (g.L^{-1}): 3 $(NH4)_2SO4$, 4.5 KH_2PO_4, 0.25 MgSO4, and 0.25 CaCl2, pH 5.0 and it was supplemented with 10 g.L^{-1} sucrose (Sigma). After incubation at 28 °C for 48 hours in an orbital shaker at 150 rpm/min, the microorganisms were separated by centrifugation at 10.000×g for 10 min at 4ºC. The culture media supernatants were used as extracellular fractions (Inv-E). The cell pellet was lysed in 50mM phosphate buffer (pH 8.0) by sonication, followed by centrifugation at 12.000 rpm for 30 min. The clarified crude lysate was used as intracellular enzymatic crude extract (Inv-I).

2.3 Enzyme assay

The invertase activity was spectrophotometrically (A_{540}) assayed using the dinitrosalicylic reagent method described by (Miller, 1956). The reaction mixture consisted of 100 µL of 0.02M sucrose in 50 mM acetate buffer, pH 5.5, and 100 µL of enzymatic crude extract. The reaction mixture was incubated for 15 min at 50ºC. After incubation, the mixture was boiled at 100ºC for 15 min with 200 µL of dinitrosalicylic. After cooling, 2 mL of distilled water were added to the reaction medium. The reducing sugars were measured by reaction with dinitrosalicylic reagent at 540 nm (UV-Visible spectrophotometer - Varian-Cary 50). One unit of enzyme activity (UA) of invertase was defined as the amount of the enzyme that catalyzed the formation of 1 µmol of glucose min^{-1}.

2.4 Experimental design for determination of optimum pH and temperature

Doehlert design (DD) - for two factors three and five levels leading to 9 sets of experiments and performed in triplicate - was used to determine optimum pH and temperature. The response surface analysis was based on multiple linear regressions by taking into account the main quadratic and interaction effects, according to the following Equation 1:

$$Y = \beta_0 + \beta_1 X_1 + \beta_{11} X_1^2 + \beta_2 X_2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 \quad (1)$$

in which Y is the predicted response; β_o, the constant; β_1 and β_2, the linear effects; β_{11} and β_{22}, the second order effects; and β_{12}, the interaction effect between variables 1 and 2.

The optimal pH of invertase activity was assayed at pH 3.0, 4.0, 5.0, 6.0, and 7.0. Blank spaces missing invertases were used in each determination. The invertase activity was tested under standard assay conditions under the temperature range of 30 - 70ºC to determine the optimum temperature. The temperatures were controlled using circulating water bath.

2.5 Thermostability analysis

Invertase samples were incubated in water at different temperatures (50 and 60ºC) and during different periods of time (0, 10, 20, 30, 40 and 50 min) within uniform sized test tubes in order to assess the enzyme's stability at high temperatures. The tubes were cooled in ice bath after heating, and the residual activity measurement was carried out at pH 5.5 and temperature of 50ºC.

2.6 Cations effect

The Na^+ and K^+ effect on invertase activity was investigated. The following concentrations of both NaCl and KCl were used: 0.2, 0.4, 0.6, 0.8 and 1.0 mol L^{-1}. The invertase activity was determined by a previously described assay, which used sucrose as substrate.

2.7 Determination of kinetic parameters

A reaction containing 100 µL of sucrose (0.02, 0.04, 0.06, 0.08 and 1.0 mol L^{-1}) and 100 µL of invertase in 0.05 M acetate buffer (pH 5.5) was incubated at 50ºC for 15 min to obtain the Michaelis-Menten kinetic parameters for sucrose hydrolysis by invertase. The invertase values K_m and V_{max} were determined by the Lineweaver-Burk plotting method.

2.8 Statistical analysis

The data were analyzed using STATISTIC 7.0 software (StatSoft, Inc.) to generate a design matrix and a dimensional response surface plot, and for the analysis of variance (ANOVA) and Tukey test (5%).

3. Results and Discussion

Invertase enzymes have promising applications in several sectors. The use of yeasts as a source for obtaining enzymes has attracted attention, due to the ease of large-scale production and the possibility of optimization to leverage production (Yuivar, Barahona, Alcaíno, Cifuentes, & Baeza, 2017). Table 1 shows the Doehlert design applied to temperature and pH optimization in invertase from *K. marxianus* CCMB 322. It is possible to observe that the interaction between variables is associated with enzymatic activities.

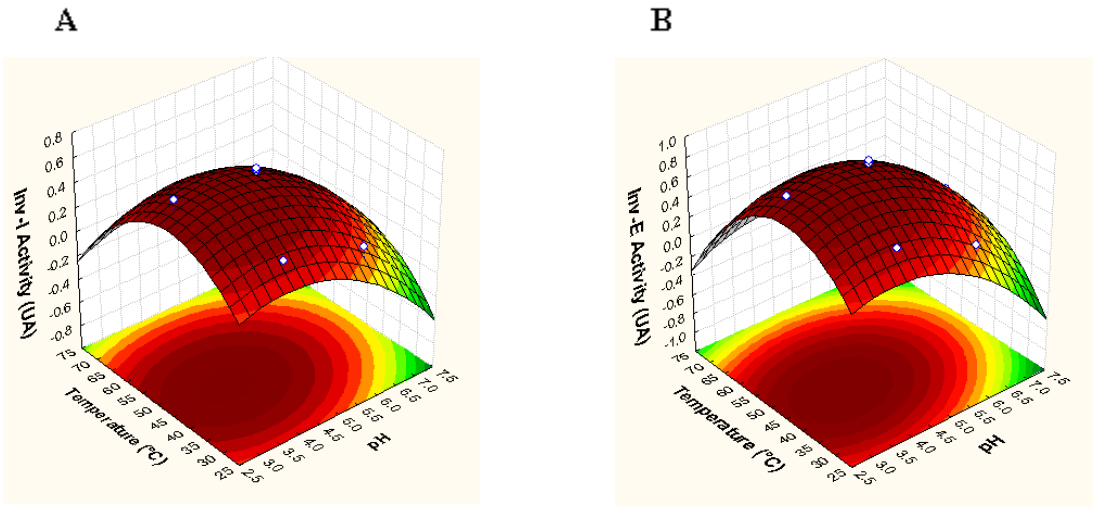
Table 1 - Doehlert design results of temperature and pH on invertase from *K. marxianus* CCMB 322.

Experiment	pH	Temperature (°C)	Inv-I Activity (UA)	Inv-E Activity (UA)
1	4 (-0.5)	70 (+0.866)	0.098	0.188
2	6 (+0.5)	70 (+0.866)	0.027	0.069
3	3 (-1.0)	50 (0)	0.609	0.837
4C	5 (0)	50 (0)	0.565	0.770
5C	5 (0)	50 (0)	0.516	0.818
6C	5 (0)	50 (0)	0.584	0.845
7	7 (+1.0)	50 (0)	0.055	0.232
8	4 (-0.5)	30 (-0.866)	0.334	0.584
9	6 (+0.5)	30 (-0.866)	0.150	0.258

Fonte: Direct search.

Temperature and pH influence on the enzymes' activity was investigated in *K. marxianus* CCMB 322 using the surface response methodology. The results are shown in Figure 1. In the regions with the highest color intensity, the best conditions for enzymatic activity are found.

Figure 1 - Response surface plot showing the effects of pH and temperature (°C) on *K. marxianus* CCMB 322 invertase activity. (A) Inv-I enzyme. (B) Inv-E enzyme.



Fonte: Direct search.

The regression model developed for enzymes activity linked to pH and temperature within the experimental design is expressed by Equation 2 and 3:

Inv-I Activity (UA) = –1.875(±0.344) + 0.372(P) (±0.091) – 0.055$(P)^2$(±0.008) – 0.075 (±0.007)(T) + 0,001(P)x(T). (II)

Inv-E Activity (UA) = –2.210(±0.372) + 0.422(P) (±0.099) – 0.069$(P)^2$(±0.008) – 0.096 (±0.008)(T) – 0.001$(T)^2$ + 0,003(P)x(T). (III)

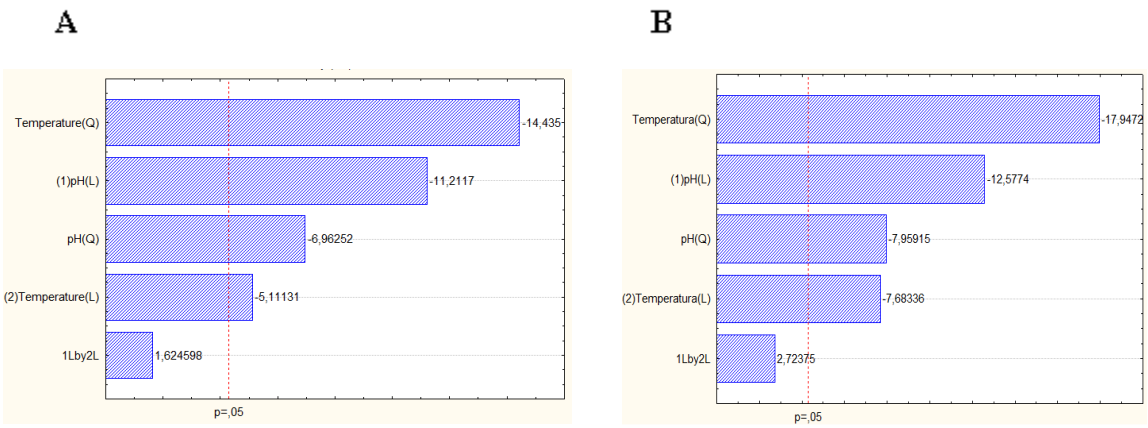
This equation illustrates the relation between these two variables (pH and temperature) and the enzyme activity (UA), which P is the pH and T is the temperature (ºC). The maximum enzymatic activity points could be obtained through the equation's derivation.

Invertases are more active at temperatures between 40 and 60 ° C and in the pH range of 3 to 5 (Singh, Singh, & Sachan, 2019). According to such methodology, the optimal pH is 3.9 and temperature of 46.5ºC in Inv-I, and pH 3.9 and 45.6ºC in Inv-E. Such values are close to those found for invertases obtained from *Saccharomyces cerevisiae*, with optimum pH and temperature of 4.5 and 50ºC, respectively, as reported in the study conducted by Barbosa et al. (2018). *K. marxianus* invertase obtained using by-products of food processing as a substrate

showed better activity under conditions of pH 4.5 and pH of 55ºC (Ragauskaite & Cizeikiene, 2019).

The Pareto Chart (Figure 2) shows that the pH and temperature variables are linearly and quadratically significant in Inv-I and Inv-E invertase activities, because both variables have P value greater than 0.05.

Figure 2 - Pareto Chart showing invertase activity of *K. marxianus* CCMB 322. (A) Inv-I enzyme. (B) Inv-E enzyme.



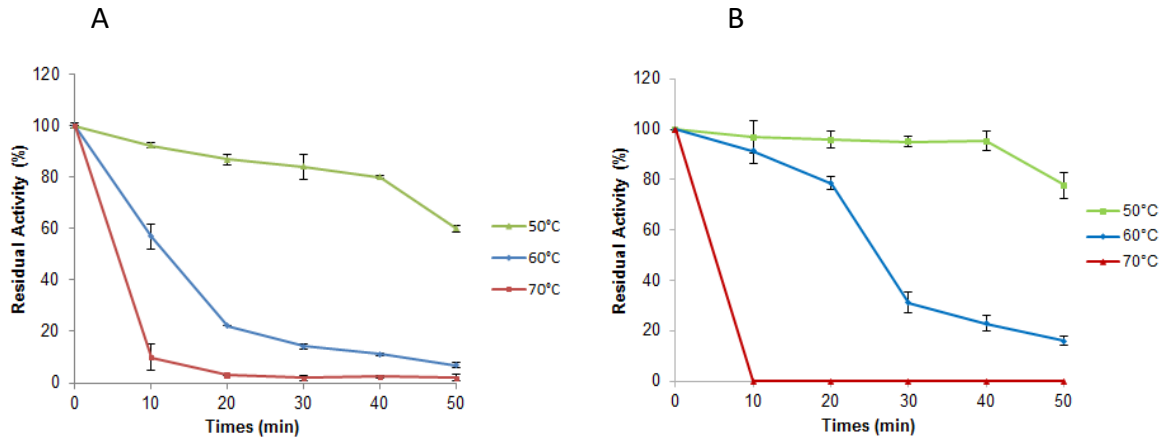
Fonte: Direct search.

The ANOVA test used the experimental data to evaluate the fitted quadratic model. The test based on Fisher distribution (F-test) has indicated that the fitted equation is statistically significant (F= 16.14 > 9.01) and (F= 68.11 > 9.01) in Inv-I and Inv-E, respectively. A lack-of-fit sum of squares (F= 12.13 < 18.51) and (F= 2.99 < 18.51) in Inv-I and Inv-E, respectively, indicates good matching between the model's predicted response and the experimental values studied in each variable. The Lagrange criterion applied to this equation (1) and (2) indicates that the critical point is characterized as maximum. The coefficient of determination R^2, calculated as 0.99, implies that 99% of the total variation in residual activities is explained by the fitted model.

According to Dinarvand, Rezaee, & Foroughi (2017) temperature is an aspect that can influence the rate of production of invertase from fungi. The Inv-I kept approximately 70% of its original activity after heating for 50 min at 50ºC in 50mM acetate buffer (pH 4.5). However, when subjected to temperatures higher than 50ºC, the enzyme probably loses its native structure, thus leading to its complete inactivation. The Inv-E enzyme kept approximately 78% of its original activity after heating for 50 min at 50ºC in 50 mM acetate

buffer (pH 5.5). However, it kept approximately 16% of its original activity after heating for 50 min at 60ºC, and it completely lost its activity when subjected to the temperature of 70ºC for 10 min (Figure 3).

Figure 3 - Thermal stability profiles of *K. marxianus* CCMB 322 invertase at 50, 60 and 70°C. (A) Inv-I enzyme. (B) Inv-E enzyme.

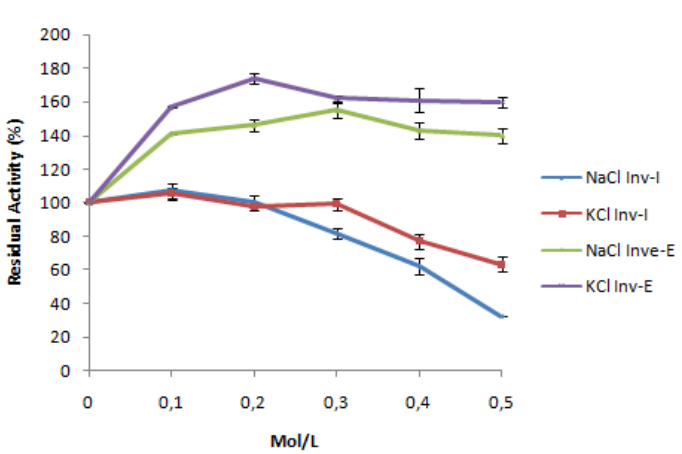


Fonte: Direct search.

The thermostability reported in the study of *K. marxian* CCMB 322 invertase is similar to that of recombinant invertase obtained from *S. cerevisiae* as described by Mohandesi, Siadat, Haghbeen, & Hesampour (2016) in which, after being submitted to a temperature above 65ºC, there was a decrease in its activity.

Ions can affect enzyme activity and influence binding to the substrate (Czyrko et al., 2018). The effect of NaCl and KCl salts on Inv-I and Inv-E invertase activity is presented in Figure 4. Regarding Inv-I, the concentration 0.10 mol.L^{-1} of NaCl and KCl increased the activity in approximately 6% and 7%, respectively. KCl concentration higher than 0.50 mol.L^{-1} decreases enzyme activity in 30%. The obtained Inv-E increased the activity in all NaCl and KCl concentrations (Figure 4).

Figure 4 - Effect of salts on the invertase activity of *K. marxian* CCMB 322.

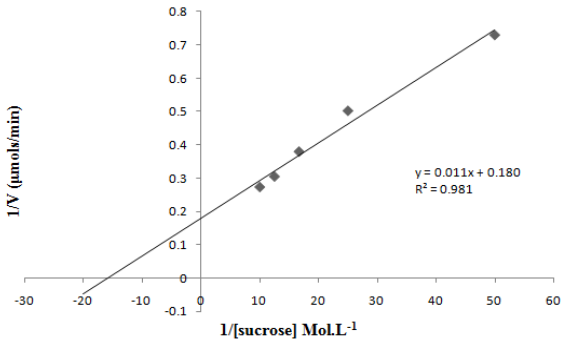


Fonte: Direct search.

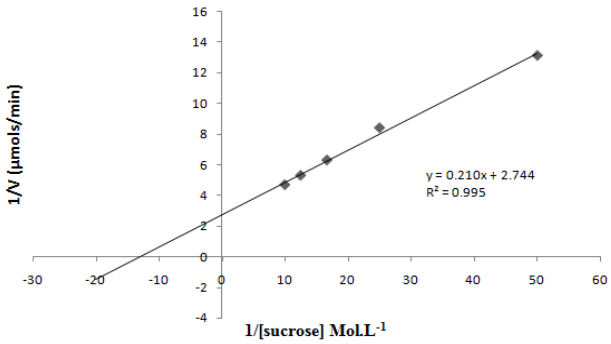
The Michaelis-Menten constant (K_m) determines the affinity of an enzyme for its substrate, while the maximum rate (V_{max}) of the reaction refers to the maximum reaction rate when the system is saturated with the concentration of the substrate. The Lineweaver-Burk plot showed that K_m and V_{max} values of the Inv-I enzyme were 61.12mM and 5.56 µmol/mL.min-1, but the K_m and V_{max} values of the Inv-E enzyme were 76.5mM and 0.364 µmol/mL.min-1, respectively (Figure 5). Thus, the *K. marxian* CCMB 322 Inv-I has a higher affinity for sucrose compared to Inv-E. The kinetic properties of invertases vary considerably depending on the microbial species for which it is obtained (Nadeem et al., 2015).

Figure 5 - Substrate concentration influence on the invertase activity. (A) Inv-I enzyme. (B) Inv-E enzyme.

A



B



Source: Direct search.

For the use of microbial enzymes in industrial processes, it is necessary to stimulate research involving the characterization and optimization of production in order to favor the performance of biocatalysis (Santos et al., 2020). The results found infer that the enzymes obtained from *K. marxianus* CCMB 322 have the potential to be explored under the conditions proposed in this study, in industrial processes.

4. Final Considerations

This research demonstrated the optimal conditions for enzyme activity obtained from *K. marxianus* CCMB 322. It was possible to observe that *K. marxianus* CCMB 322 produces intracellular and extracellular invertase with different characteristics. By using the response surface methodology, it was possible determining optimum pH and Temperature for the enzyme activity.

The optimum activity was achieved at approximately pH 3.9 and 45 °C, in Inv-I and Inv-E. Such enzymes showed thermal stability similar to that found in other studies. Inv-E seems to be more thermostable than Inv-I, therefore, it is better to industrial use. NaCl and KCl 0.1 concentrations increase the activity of the invertase produced by *K. marxianus*.

Invertases from *K. marxianus* has a higher affinity for this substrate than the enzymes from other sources. The founded results in this study indicate that invertases from *K. marxianus* CCMB 322 can be applied to industrial processes, since the studied conditions are followed. However, research that seeks its purification and boost its application is necessary.

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