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Characterization of Invertase from Saccharomyces cerevisiae MK obtained from toddy sample

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ABSTRACT

Activity and stability of invertase obtained from Saccharomyces cerevisiae MK were characterized with the following parameters like pH, temperature, metal ions, surfactants and chemical inhibitors. The pH stability of this enzyme was observed between pH 2 to pH 9 with an average of 80 percent retaining activity for 24 hours. The crude enzyme showed optimum activity at pH 6 and 30ºC. Enzyme activity was increased in the presence of 5mM CaCl2 (92.74%). Maximum invertase activity of 35.88% was recorded at polyethylene glycol (1%). Maximum invertase activity of 29.16% was recorded at EDTA for Saccharomyces cerevisiae MK. The kinetic parameters (Km and Vmax) were determined at 30°C and pH 6 for Saccharomyces cerevisiae MK invertase for concentrations ranging between 0.5 to 5 mg/ml of sucrose as substrate the Km and Vmax of Saccharomyces cerevisiae MK invertase are 0.3410 mg/ml and 0.5953 µm/min/mg.

Keyword: Saccharomyces cerevisiae MK, invertase, specific activity, relative activity, Enzyme characterization.

INTRODUCTION

Invertase is special kind of enzyme. Invertase catalyses α-1,4glycosidic linkage between α-D-glucose and β-D-fructose molecules of sucrose by hydrolysis releasing monosaccharides such as glucose and fructose. This mixture is called invert syrup. It also hydrolyses β-fructans such as raffinose into simple sugars [1]. *Saccharomyces cerevisiae* is particularly interesting microorganism, it synthesizes two Invertase is biosynthesized by yeast strains, *Aureobasidium* sp. *Rhodotorulaglutinis, Saccharomyces cerevisiae* [2], *Saccharomyces carlsbergenesis* [3]. Invertase: a glycosilated periplasmic protein and a cytosolic non glycosilated protein [4]. The same culture of yeast invertase exists in several forms. For example, intracellular invertase has a weight of 135 000 Daltons, while extracellular invertase has a weight of 270 000 Daltons [5].

Sucrose is considered to be the sole carbon source for invertase production as the availability of glucose for yeast is dependent on sucrose hydrolysis by invertase. *Saccharomyces cerevisiae* is the organism of choice for invertase production because of its characteristic high sucrose fermentability [6]. It is one of the most widely used enzymes in the food industry where fructose is preferred than especially in the preparation of jams and candies, because it is sweeter and does not crystallize easily D-glucose and D-fructose at concentrations lower than 10% sucrose, thus making these enzymes suitable for biotechnological applications. It has wide range of commercial applications including the production of confectionery with liquid or soft canters, chocolates, candy products, fondants, after dinner mints, fermentation of cane molasses into ethanol and production of lactic acid [7].

Most of food and pharmaceutical industries utilize this enzyme. It is also used in pharmaceutical industry as digestive aid tablets, powder milk for infant's foods, as calf feed preparation, assimilation of alcohol in fortified wines and in manufactured inverted sugars as food for honeybees [8]. On industrial scale citric acid fermentation uses molasses as a feedstock, which contains principally sucrose as a carbon source [9].

MATERIALS AND METHODS

Invertase assay

The culture medium was centrifuged at 10000 rpm for 10 minutes at 4°C. The supernatant was used as crude enzyme source for invertase assay. Invertase activity was assayed as per the method of Sumner and Howells (1935) [10] using 0.5ml of sucrose as the substrate in 0.03M acetate buffer (pH- 5.0) and incubated at 45°C for 30 minutes. The reaction was terminated by addition of 1ml of DNS reagent and tubes were kept at

boiling water bath for 5 minutes. After cooling the tubes at room temperature, 3ml of distilled water was added in each tube. The intensity of the colour was read at 540nm in UV-Vis spectrophotometer (Systronics, 119). Standard curve was performed with glucose solution. One unit of enzyme activity was defined as the amount of enzyme required for release 1µ mol of glucose/ml/minute under assay condition. Enzyme activity was expressed in International units.

Invertase activity was calculated using this formula:

IU/ml = concentration of glucose / 0.5×30×0.180

Acetone precipitation

For protein precipitation, double the amount of acetone was added to the culture supernatant solution and the solution was left overnight at 4ºC, the supernatant was removed and pellet of precipitated protein was kept and dried at laboratory temperature. The pellet which contained invertase was dissolved in 5ml of double distilled H_2O and it was dialyzed against double distilled H_2O for 48 hours at 4ºC. This was further dialyzed against 50% (W/V) PEG in order to concentrate the protein sample (Mawadza *et al*., 2000).

Effect of pH on activity and stability

The optimum pH of the crude invertase was determined by incubating the mixture of the crude invertase in the presence of the buffer (0.1M citrate buffer) at pH 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12. The pH effects on invertase activity were assayed at pH values ranging from 2 to 12 for 30 minutes. To determine pH stability, the enzyme was pre-incubated at 30ºC for 24 hours at pH 2 to 12 [11].

Effect of temperature on invertase activity and stability

The effect of temperature on the activity of invertase was determined in the temperature range of 10-90ºC in 0.1M citrate buffer at pH 6 for 30 minutes. The invertase activity was determined under standard assay condition. To determine temperature stability, the invertase was pre-incubated at 30ºC for 24 hours at pH 2 to 12 [13].

Effect of different metal ions on invertase activity

The crude invertase was mixed with 5mM concentration of various salts such as $CaCl_2$, $CoCl_2$, $MgCl_2$, $ZnSo_4$, $NiSo_4$, $CuSo_4$ and KCl for 30 minutes at 30°C pH 6 before adding the substrate and subsequently invertase activity was determined. To determine metal ions stability, the enzyme was pre-incubated at 30ºC for 24 hours at pH 6 [3].

Effect of various surfactants on activity

The effect of surfactants on the activity of crude invertase was determined by pre-incubating the enzyme in the presence of Triton X-100 (1%), Triton X-100 (2%), Triton X-100 (3%), Tween-20 (1%), Tween-20 (2%), Tween-20 (3%), SDS (0.1%), SDS (0.3%), SDS (0.5%), Poly ethylene glycol (0.1%), Poly ethylene glycol (0.3%), Poly ethylene glycol (0.5%) for 30, 60, and 90 min at 30ºC before adding the substrate. Subsequently relative invertase activities were measured at optimum temperature [14].

Effect of different chemical inhibitors on invertase activity and stability

The effect of different chemical inhibitors on invertase activity and stability were determined individually for crude invertase of *Saccharomyces cerevisiae* MK. The crude invertase was mixed with 0.1mM concentration of different chemical inhibitors such as DMSO, EDTA, β-mercaptoethanol, H_2So_4 and H_2O_2 for 30 minutes at 30°C pH 6 before adding the substrate and subsequently invertase activity was determined. The relative activities were based on the ratio of the activity obtained at specific chemical inhibitors to the maximum activity obtained and expressed as percentage. To determine chemical inhibitors stability, the enzyme was pre-incubated at 30ºC for 24 hours at pH 6 [15].

DETERMINATION OF KINETIC PARAMETERS FOR CRUDE INVERTASE

The kinetic parameters (Michaelis-Menton constant) Km and maximal velocity Vmax of invertase activity *Saccharomyces cerevisiae* MK were determined individually from Lineweaver Burk plot optimal assay conditions 45°C, pH 6 at 30 minutes for sucrose concentrations ranging from 0.5mg to 5mg/ml. The evaluation of these graph yielded the kinetic parameters for the invertase activity (Graph pad Prism 5.04 software) [16].

RESULT

Effect of pH on activity and stability

The effect of pH on the activity of crude invertase was determined in the pH range of 2-12. Maximum invertase activity of 93.83% was recorded at pH 6. The invertase activity was decreased to 16.41% at pH 12. The pH stability of invertase was measured by the standard assay method. An average 80% of retaining activity was observed between pH 4 and 8 (Fig. 1).

Effect of temperature on invertase activity and stability

The effect of temperature on the activity of crude invertase was determined in the temperature range of 10-90ºC. Maximum invertase activity of 83.33% was recorded at 30ºC. Minimum invertase activity of 22.91% was recorded at 90ºC. The original invertase activity was retained from 10 to 90ºC approximately above 84% from 10-40ºC (Fig. 2).

Effect of various metal ions on invertase activity

The crude invertase was pre-incubated at 30ºC for 30 minutes at different concentration of the metal ions prior to standard invertase activity assay with sucrose. Maximum invertase activity of 92.74% was recorded at calcium chloride. Minimum invertase activity of 23.25% was recorded at potassium chloride. Partial inhibition of the crude invertase was in the order of KCl > $MnSo_4$ > $ZnSo_4$ > $Niso_4$ > $MgSo_4$ > $CoCl_2$ (Fig. 3).

Effect of different surfactants on invertase activity

The relative activity of invertase was decreased with increased in concentration of surfactants and also by time of exposure. At 1% surfactants concentration the relative activity was high at the same time at 5% surfactants concentration it gradually reduced but not completely inhibited. Maximum invertase activity of 35.88% was recorded at polyethylene glycol (1%). Minimum invertase activity of 10.46% was recorded at triton X-100 (1%). The residual enzyme activity for surfactants was given in Fig. 4a and 4b.

Effect of various chemical inhibitors on invertase activity and stability

The crude invertase was pre-incubated at 30ºC for 30 minutes at different concentration of the chemical inhibitors prior to standard invertase activity assay with sucrose. Maximum invertase activity of 29.16% was recorded at EDTA. Minimum invertase activity of 10.41% was recorded at DMSO. The

residual invertase activity for chemical inhibitors was given in Fig. 5

DETERMINATION OF KINETIC PARAMETERS FOR *SACCHAROMYCES CEREVISIAE* MK

The kinetic parameters (Km and Vmax) were determined at 30°C and pH 6 for *Saccharomyces cerevisiae* MK for concentrations ranging between 0.5 to 5 mg/ml of sucrose as substrate. The Km and Vmax of *Saccharomyces cerevisiae* MK are 0.3410 mg/ml and 0.5953 µm/min/mg (Table. 1). The kinetic parameters of enzymatic reaction were calculated by the Lineweaver-Burk linearization using the Michaelis-Menton kinetic model the -1/Km value is -1.6792 and the –rVmax is 4.18 (Fig. 6).

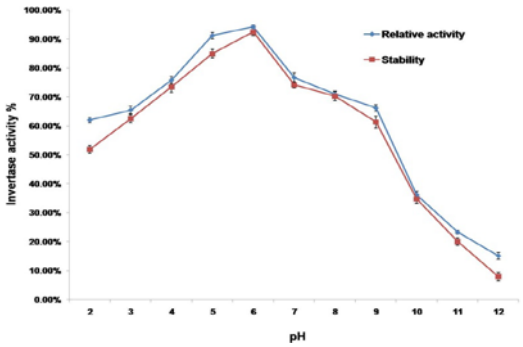

Fig. 1. Effect of pH on invertase activity and stability by Saccharomyces cerevisiae MK

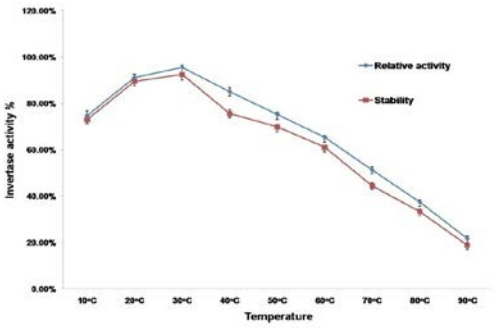

Fig. 2. Effect of temperature on invertase activity and stability by Saccharomyces cerevisiae MK

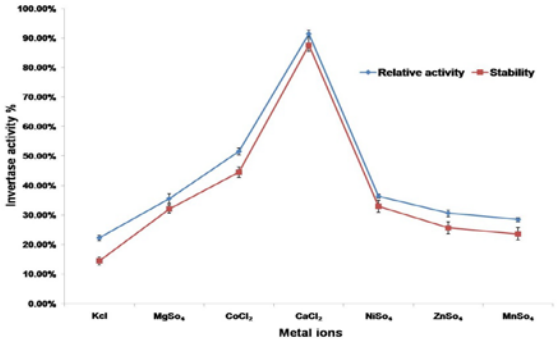

Fig. 3. Effect of various metal ions on invertase activity and stability by Saccharomyces cerevisiae MK

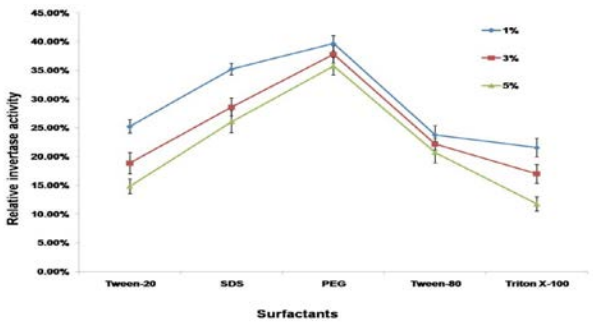

Fig. 4a. Effect of different surfactants on invertase activity by Saccharomyces cerevisiae MK

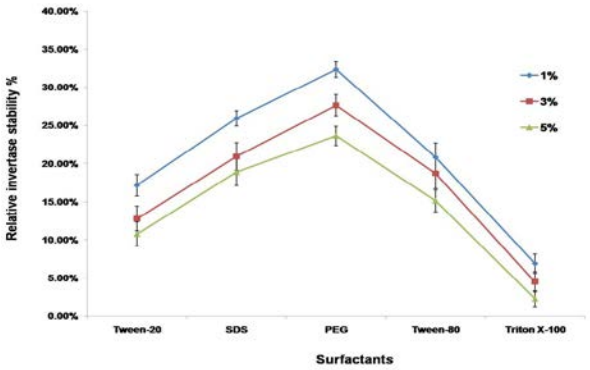

Fig. 4b. Effect of different surfactants on stability by Saccharomyces cerevisiae MK

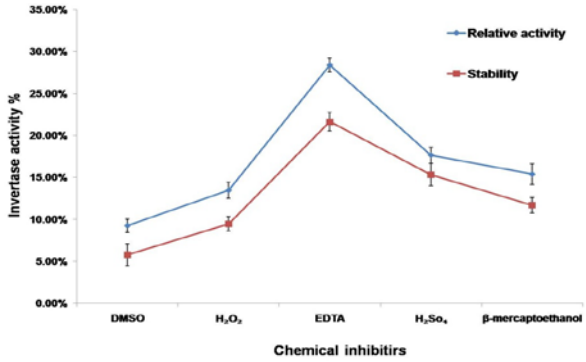

Fig. 5. Effect of various chemical inhibitors on invertase activity and stability by Saccharomyces cerevisiae MK

Table 1. Michaelis-Menton constant for Saccharomyces cerevisiae MK

Michaelis-Menton	***Saccharomyces cerevisiae* MK**
Best-fit values	
Vmax	0.3410
Km	0.5953
Std. Error	
Vmax	0.04650
Km	0.3791
95% Confidence Intervals	
Vmax	0.2338 to 0.4482
Km	0.0 to 1.467

Goodness of Fit	
Degrees of Freedom	8
R square	0.4857
Absolute Sum of Squares	0.02243
Sy.x	0.05295
Constraints	
Km	Km > 0.0
Number of points	
Analyzed	10

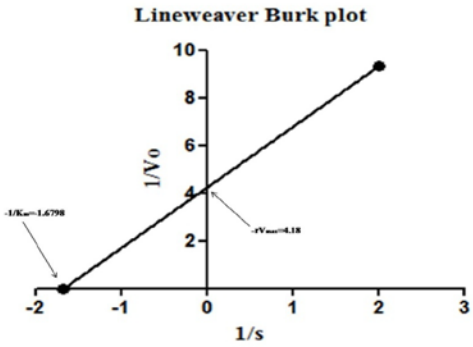

Fig. 6. Lineweaver Burk plot for Saccharomyces cerevisiae MK

DISCUSSION

In the present work the effect of pH for invertase activity by *Saccharomyces cerevisiae* MK were assessed. The effect of pH on the activity of crude invertase was determined in the pH range of 2-12. Maximum invertase activity of 94.28% was recorded at pH 6. The invertase activity was decreased to 15.14% at pH 12 in *Saccharomyces cerevisiae* MK. Uma *et al*. (2012) also reported that maximum invertase activity was recorded at pH 6.0 for invertase by *Cladosporium cladosporioides*. Kaur and Sharma, (2005) reported that the initial pH 5.0 gave the best invertase activity for invertase by an actinomycetes strain. Qureshi *et al*. (2012) stated that the maximum invertase activity was recorded at pH 5.0 for invertase from *Mucor geophillus* EFRL 03. Whereas, Talekar *et al*. (2010) stated that maximum invertase activity was recorded at pH 4.2 for invertase by *Saccharomyces cerevisiae*.

In the present study the effect temperature on invertase activity by *Saccharomyces cerevisiae* MK were investigated. The effect of temperature on the activity of crude invertase was determined in the temperature range of 10°C-90°C. Maximum invertase activity of 91.60% was recorded at 30°C. Maximum invertase activity of 95.54% was recorded at 30°C, the invertase activity was decreased to 21.70% at 90°C in *Saccharomyces cerevisiae* MK. Oda and Tonomura, (2010) reported that maximum invertase activity was recorded at 45°C for invertase by *Torulaspora pretoriensis*. However, Patil *et al*. (2012) evaluated the invertase by *Aspergillus* sp., it gave the good invertase activity at 60°C. At temperature other than optimal, a decline in enzyme activity was recorded. The higher temperature than optimal caused decrease in rate of reaction due to thermal denaturation of enzyme as reported by Resa *et al*. (2009).

The effect of metal ions on invertase activity by *Saccharomyces cerevisiae* MTCC 170 and MK were investigated in the present work. The various metal ions such as $CaCl_2$, $CoCl_2$, $MgCl_2$, $ZnSo_4$, $NiSo_4$, $CuSo_4$ and KCl were tested. Maximum invertase activity of 91.45% was recorded in calcium chloride. The minimum invertase activity was recorded to 22.28% at potassium chloride for *Saccharomyces cerevisiae* MK invertase. Whereas, Kaur and Sharma, (2005) reported that the $CoCl_2$ gave the maximum invertase activity for invertase by an actinomycete strain. All metal ions expect Na^+, Hg^{2+} and Ca^{2+} were well tolerated and did not adversely affect invertase activity.

Similarly, Talekar *et al*. (2010) stated that maximum invertase activity was recorded at calcium chloride for invertase by *Saccharomyces cerevisiae*. Patil *et al*. (2012) investigated that invertase by *Aspergillus* sp., gave the best invertase activity for calcium chloride. The enzyme activity was enhanced by Ca^{2+} and Cd^{2+}, while Hg^{2+} inactivates the enzyme. The cations Hg^{2+}, Ag^+, Cu^{2+} and Cd^{2+} exhibited a noticeable inhibition of the enzyme (Workman and Day 1983).

In the present work the effect of surfactants on invertase activity by *Saccharomyces cerevisiae* MK were investigated. Various metal ions such as Triton X-100, Tween-20, SDS, polyethyleneglycol and Tween-80 were determined. Maximum invertase activity of 35.88% was recorded at poly ethylene glycol (1%), minimum invertase activity of 10.46% was recorded at Triton X-100 in *Saccharomyces cerevisiae* MK invertase.

The effect of chemical inhibitors on invertase activity by *Saccharomyces cerevisiae* MK was investigated in the present study. The different chemical inhibitors such as DMSO, H_2O_2, EDTA, H_2SO_4 and β-mercaptoethanol were tested. Maximum invertase activity of 29.16% was recorded in EDTA, minimum invertase activity was recorded at 10.41% at DMSO in *Saccharomyces cerevisiae* MK. The invertase activity was significantly inhibited by bivalent metal ions (Ca^{+2}, Cu^{+2}, Cd^{+2} and Hg^{+2}), dithiothreitol and beta-mercaptoethanol, and partially enhanced by ethylene diamine tetra acetic acid (EDTA) (Gine *et al.*, 2000).

In the present investigation the kinetic parameters (Km and Vmax) were determined at 30°C and pH 6 for *Saccharomyces cerevisiae* MK for concentrations ranging between 0.5 to 5 mg/ml of sucrose as substrate. The Km and Vmax of *Saccharomyces cerevisiae* MK are 0.3410 mg/ml and 0.5953 µm/min/mg. Uma *et al*. (2010) reported the similar result for invertase by *Aspergillus flavus* in Lineweaver-Burk plot of the enzyme affinity for sucrose gave a straight line plot from which the Km as 0.23 mg/ml and Vmax was 15.8 U/mg. Ribeiro and Vitolo, (2005) stated the similar result for conventional Lineweaver-Burk plot (reciprocal initial reaction rate versus reciprocal substrate concentration), the kinetic constants for soluble (Km = 18.3mM and Vmax = 0.084 U/mgE) and insoluble (Km = 29.1mM and Vmax =0.075 U/mgE) invertase were calculated.

Similarly Almeida *et al*. (2005) investigated the kinetic parameters of enzymatic reaction were calculated by the Lineweaver-Burk linearization using the Michaelis-Menton kinetic model. For the auto-immobilized enzyme it was obtained a Km of 447 mM and Vmax of 2,805mmol/min.

Similarly Uma *et al*. (2012) also stated the Michaelis-Menton kinetics for and constants were determined from a Lineweaver-Burk plot. The kinetic constant of invertase was Vmax 28.57U/mg and Km of 0.26mg/ml.

Whereas, Mona *et al*. (2009) evaluated the invertase from *Saccharomyces cerevisiae* NRRL Y-12632 in Michaelis constant (Km) value of the pure enzyme was found to be 60 mM while its Vmax was 35.5 min mg protein as calculated by Hanes-Woolf plot. The rate of sucrose hydrolysis; decreased by increasing substrate concentration, which may be due to substrate inhibition. Whereas, Talekar *et al*. (2010) reported the kinetic constants for soluble invertase (Km = 10.80mM and Vmax = 21.59 µmol/minutes) were calculated from nonlinear regression. Whereas, Guimaraes *et al*. (2007) stated that β-fructofuranosidases from *Aspergillus niveus* had Km value of 5.78 mM and Vmax of 28.46 U per mg of protein per min and Chavez *et al*. (1997) investigated that invertase from *Candida utilis* and *Saccharomyces cerevisiae* exhibited Km values of 11 and 25 mM, respectively.

CONCLUSION

The yeast (*Saccharomyces cerevisiae* MK) capable of producing invertase was isolated from toddy sample. The invertase activity and stability for the *Saccharomyces cerevisiae* MTCC 170 was found to be at pH 6, 30°C, calcium chloride (metal ions), poly ethylene glycol (surfactants), EDTA (chemical inhibitors). The Km and Vmax of *Saccharomyces cerevisiae* MK invertase are 0.3410 mg/ml and 0.5953 µm/min/mg.

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