Physicochemical Properties of Invertase Partitioned in an Aqueous Two-Phase System of Poly(Ethylene Glycol)/Sodium Sulfate

Poli(Etilen Glikol)/Sodyum Sülfat Sulu İkili-Faz Sisteminde Ayrılan İnvertazın Fizikokimyasal Özellikleri

Research Article

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ABSTRACT

Biochemical properties of an enzyme are very important for its applications in various fields. In the present work, we have investigated some parameters affecting to the invertase activity and stability which was partitioned in poly(ethylene glycol)/sodium sulfate aqueous two-phase system (ATPS). The optimum temperature and pH of the invertase were found as 50°C and pH 5.0, respectively. The enzyme was very stable at the temperature ranged from 25 to 50°C and also in a pH range of 4.0-7.5. The enzyme was stored at 4°C and lost about only 47% of its initial activity at the end of two months. Kinetic parameters; K_m and V_{max} were determined as 4.65 mM and 30 U, respectively. The effect of various effectors on the activity of the invertase was also searched. Especially, Mn^{+2} ions showed an activator effect for invertase. Invertases are still efficient tools in various biotechnological applications especially in the food and beverage industry. The results obtained in this study indicate that, characteristic properties of invertase partitioned by PEG/Na_2SO_4 aqueous two-phase system is very suitable for its industrial applications. The relatively wide range of pH and temperature for activity and stability exhibited by invertase could be very interesting for the food processes.

Key Words
Aqueous two-phase system, PEG/salt systems, Partitioning, Invertase.

ÖZET

Enzimlerin biyokimyasal özellikleri çeşitli alanlardaki uygulamaları için oldukça önemlidir. Bu çalşmada, poli(etilen glikol)/sodyum sülfat sulu ikili-faz sistemi (ATPS) ile ayrılan invertaz enziminin aktivitesi ve kararlılığına etki eden bazı parametreler incelendi. İnvertaz enziminin optimum pH'sı 5.0 ve optimum sıcaklığı 50°C olarak belirlendi. Enzim 25-50°C sıcaklık ve 4.0-7.5 pH aralığında oldukça kararlıdır. Enzim 4°C'de depolandı ve iki ay sonunda başlangıç aktivitesinin sadece % 47'sini kaybetti. Kinetik parametrelerden K_m ve V_{max} sırasıyla 4.65 mM ve 30 U olarak belirlendi. İnvertaz aktivitesine çeşitli efektörlerin etkisi de incelendi. Özellikle, Mn^{+2} iyonları invertaz için aktivatör etkisi gösterdi. İnvertazlar özellikle gıda ve içeçek sanayindeki çeşitli biyoteknolojik uygulamalarda hala etkin araçlardır. Bu çalışmadan elde edilen sonuçlar, PEG/Na_2SO_4 sulu ikili-faz sisteminde ayrılan invertazın karakteristik özelliklerinin endüstriyel uygulamaları için oldukça uygun olduğunu gösterdi. İnvertaz aktivitesi ve kararlığı için sıcaklık ve pH'nın geniş aralıkta değişmesi özellikle gıda prosesleri için ilgi çekici olacaktır.

Anahtar Kelimeler
Sulu ikilifaz sistemi, PEG/tuz sistemi, Ayrılma, invertaz.

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INTRODUCTION

Invertase (β-fructofuranoside fructohydrolase, EC 3.2.1.26) is a hydrolytic enzyme that catalyzes breakdown of disaccharide sucrose into the correspondent monosaccharides. Hydrolysis of sucrose by invertases produces an equimolar mixture of D-glucose and D-fructose, known as invert sugar, at concentrations lower than 10% sucrose. This make the enzyme suitable for many biotechnological applications especially in the food and beverage industry. Invert sugar is sweeter and easier to incorporate in industrial preparation than sucrose. Moreover, it has the advantage of being colourless in contrast to the coloured products obtained by acid hydrolysis and also has a lower crystallinity than sucrose at the high concentrations employed. Invert sugar is widely used in the production of non-crystallising creams, jams, artificial honey and liquid sugar [1-3].

As the high commercial potential of the invertase several attempts have been made to obtain a highly active enzyme suitable for industrial applications. Various traditional purification processes have been used to purify a number of invertases from different sources. However, most of these purification protocols involve a number of steps, scalling-up is difficult and also very expensive [4-8]. From this point of view, an effective and economically viable method for the separation and purification of invertase is partitioning in an aqueous two-phase system (ATPS). ATPS is an emerging technique which has applications in partitioning and purification of various biomolecules. An ATPS forms readily upon mixing aqueous solutions of two hydrophilic incompatible polymers, or a polymer and an inorganic salt, above a certain critical concentration [9-12]. Mixtures of biomolecules added to this two phase system tend to partition unequally between the phases thus allowing for the extraction of a particular biomolecule. Some operating parameters like; type, concentration and molecular mass of phase forming polymer, type and concentration of phase forming salt, type and concentration of cosolute and pH of the system have to be optimized [9,10]. The molecular mass, shape, specific binding sites and the surface hydrophobicity of the biomolecules also affect the partition behavior [10,12,13]. Compared to other separation techniques, this method offers many advantages such as; a low processing time, low energy consumption, biocompatible environment and the relative ease of its scalling-up [14]. The effectiveness of ATPS in downstream processing has been confirmed on the extraction, separation, concentration and primary purification of several enzymes including protease [15] , lysozyme [16], lipase [17] , α-galactosidase [18], xylose reductase [19], polyphenol oxidase [20], trypsin and α-chymotrypsin [21], β-glucosidase [22] and invertase [23]. In spite of various ATPSs have been developed for the separation and purification of different enzymes their use for invertase extraction is quite limited.

In previous work [23] we have been developed an ATPS (polyethylene glycol/sodium sulfate) for the partitioning of tomato invertase. The parameters that effect the partition behavior of invertase have also been studied and optimized for the efficient separation of the enzyme. As the biochemical properties of the enzyme are very important for evaluation of its use in various industrial applications characterization and determination of biochemical properties of the partitioned invertase aimed in the present study.

MATERIALS AND METHODS

Materials

Tomatoes were purchased from a local market (Turkey). Polyethylene glycol (PEG) with molecular masses of 3000 and sodium sulfate were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and used without further purification. All other chemicals used were of analytical grade.

Extraction of invertase from tomato

Freshly harvested tomato fruits (full ripen-red stage) were selected as an enzyme source. Tomatoes (500 g) were first washed throughly with distilled water and then after the removal of the peels and seeds tomato fruit was cut into small pieces. The tomato flesh was homogenized (Silverson STL 2, UK) at 4°C for 4-5 minutes with 0.2 M sodium acetate buffer (pH 5.0) containing 1 M NaCl. The homogenate was filtered from two layers of cheese-cloth and then the pH of the filtrate was adjusted to pH 5.7-6.0 with 1 M NaOH. The suspension was magnetically stirred for 1 h and then centrifuged at 11 200 x g for 15 min at 4°C

(Hettich Universal 30 RF). The clear supernatant (22 U/mg) was subjected 85% (w/v) ammonium sulfate precipitation and then the mixture was allowed to stand overnight at 4oC with continuous stirring. The precipitate was collected by centrifugation at 11 200 x g for 30 min at 4°C. The pellet was dissolved in sodium acetate buffer (0.2 M, pH 5.0) and then dialyzed against same buffer for overnight. The dialysate represented as "tomato invertase extract" and stored at -20°C until aqueous two-phase partitioning. The protein concentration and specific activity of the enzyme were determined as 2.1 mg/mL and 153 U/mg, respectively.

Preparation of the aqueous two-phase system and partitioning of invertase

Stock solutions of PEG-3000 (50%, w/w) and sodium sulfate (30%, w/w) were prepared in deionized water. The system parameters for PEG-salt ATPS were selected based upon previous report [24]. Polymer, buffer, salt and deionized water were mixed before adding the invertase extract to avoid protein precipitation. The total weight of the phase system was 10 g. The pH of the phase system was adjusted with concentrated NaOH or HCl. After mixing of stock solutions the phases were dispersed by gentle mixing for 15 min at 25°C. Complete phase separation was achieved by low-speed batch centrifugation at 4000x g for 10 min. Measurement of the volumes of top and bottom phases were visually made in gradual tubes. The volumes of the phases were then used to estimate the experimental volume ratio (Vr). The top and bottom phases were withdrawn separately with Pasteur pipette. After the separation of PEG from invertase (bottom phase) by ultrafiltration both the phases were analysed for invertase activity and protein amount. In order to correct for PEG and Na_2SO_4 interference, a blank system (without enzyme extract) was prepared for each set of conditions and the samples were read against blank. The reported results are the average of two independent experiments and standard errors were judged to be ±5% of the mean value. The protein content and specific activity of the partitioned invertase were determined as 0.291 mg/mL and 677 U/mg, respectively. This partitioned enzyme was used for characterisation studies.

Assay of invertase activity

The enzymatic activity was determined through the initial rates of sucrose hydrolysis reaction method, thus being determined the formed reduced sugar by the 3,5-dinitrosalicilic acid (DNS) method [25]. The assay mixture consisted of 0.2 mL of 0.5 M sucrose, 0.6 mL of sodium acetate buffer (0.2 M, pH 5.0) and 0.2 mL of enzyme solution was incubated at 37°C for 30 min. After that, 1 mL of DNS reagent was added and heated in a boiling water bath for 10 min. It was then cooled to room temperature (23°C) and the amount of reducing sugars was measured spectrophotometrically at 546 nm. The data presented for all invertase activity determinations are mean values of triplicate assay. One unit of invertase activity was defined as the amount of enzyme which released one μmol of glucose from sucrose per minute at pH 5.0 and 37°C.

Protein determination

Protein concentration was determined by Bradford method using Coomassie Blue G-250 dye as a reagent and bovine serum albumin (BSA) as a standard [26]. Assays were performed in triplicate and the averages were used in calculations. Specific activity was expressed as units per milligram of protein with sucrose as a substrate.

Characterisation of tomato invertase partitioned in PEG/Na_2SO_4 ATPS

Influence of temperature on the activity and stability of invertase

Temperature profile of the invertase was conducted from 25 to 70°C using the standard invertase assay at given temperature. The relative activities (%) were expressed as the ratio of the partitioned invertase activity obtained at certain temperature to the maximum activity obtained at the given temperature range. In order to determine the thermal stability of invertase, the enzyme was incubated at different temperatures (4-70°C) for 30 min with continuous shaking. After desired incubation periods enzyme aliquots were withdrawn, cooled immediately and then assayed at standard conditions to determine the residual invertase activity.

Effect of pH on the activity and stability of invertase

The effect of pH on the activity of invertase was studied by incubating the enzyme with sucrose in 0.2 M of buffers (acetate; 4.0-5.5 and phosphate; 6.0-8.0) at 37°C. The relative activity (%) was expressed as the ratio of the invertase activity obtained at certain pH to the maximum activity obtained at the given pH. For pH stability, the enzyme was incubated in above buffers for 3 h at 4°C and then the remaining activity was estimated using the standard activity assay procedure.

Effect of substrate concentration on the activity of invertase

The influence of substrate concentration on the enzyme activity was carried out with the initial concentration of sucrose 2.5-125 mM at pH 5.0 and 37°C. Kinetic parameters include the maximum reaction rate of the enzymatic reaction (V_{max}) and the Michaelis-Menten constant (K_m) were determined. The values were calculated from Lineweaver-Burk plot which is a plot of 1/V against 1/[S] for systems obeying the Michaelis-Menten equation.

Storage stability of invertase

The partitioned invertase was stored at 4°C in 0.2 M acetate buffer (pH 5.0). The storage stability of the enzyme was investigated by measuring its activity after being stored at 4°C for a two month period and the remaining activity measurements were performed once a week.

Effect of various effectors on the activity of invertase

The influence of various effectors (NaCl, KCl, $MgCl_2.6H_2O$, $MgSO_4.7H_2O$, $BaCl_2.2H_2O$, $CaCl_2$, $MnSO_4.H_2O$, $ZnSO_4.7H_2O$, Na_2CO_3, $CuSO_4.5H_2O$, $FeCl_3.6H_2O$, $MnCl_2.4H_2O$) (in concentrations of 0.1-2.5 mM) on the invertase activity was investigated by preincubating the enzyme with different effectors in acetate buffer (0.2 M, pH 5.0) for 30 min at room temperature. Residual activity was calculated against control. Control invertase activity without added effectors was taken as 100% activity.

RESULTS AND DISCUSSION

Partitioning of tomato invertase in PEG/Na_2SO_4 ATPS

Aqueous two-phase systems (ATPSs) have been employed as an efficient tool in several biotechnological processes for the partition of proteins and enzymes. This system seems to be an ideal technology where clarification, concentration and partial purification of the target product can be integrated in one step. The simplicity of the process and low cost of the phase forming materials make it feasible for large-scale protein purification using appropriate scale-up technique. Moreover, compared to other separation techniques ATPSs are less energy-consuming, less time processing and ensure a high yield. [9,10,15,18]. In previous work [23] we have been found that poly(ethylene glycol)/sodium sulfate (PEG/Na_2SO_4) ATPS could be employed as a viable and potentially useful process for the partitioning of the invertase. All the partitioning experiments have been carried out using crude invertase extract from tomato. The selection of the suitable phase system is very important for the efficiacy of the process. Since, multiple factors affect the recovery of the desired protein the influence of process parameters like; molecular mass and concentration of PEG, type and concentration of salt and cosolute (added salt), amount of protein, weight and pH of the system on invertase partitioning was studied. The best optimal ATPS for the partitioning of invertase was 15% (w/w) PEG-3000, 12% (w/w) Na_2SO_4 and 5% KCl (w/v) at pH 4.5. Partitioning of invertase with optimized ATPS resulted in a high activity yield of 88% and a 5.5-fold increase of enzyme purity with only one operation step. SDS-PAGE analysis of the purified invertase showed that, the enzyme preparation was nearly homogenous with a molecular weight corresponding to 20 kDa [23]. As the phase forming species PEG and salt are non-toxic and safe to use in food applications, the partitioned invertase could be used in the food processing industry.

Biochemical properties of invertase partitioned in PEG/Na_2SO_4 ATPS

In the present study, in order to characterize and determine the biochemical properties of the purified invertase some parameters affecting to the enzyme activity and stability were searched. The results were compared with invertases which were purified by using different purification processes. The good properties of an enzyme offer potential for use in production processes. The biochemical properties of the invertase are very important for evaluation of its potential use especially in food industry. Therefore, we have determined the properties of the enzyme regarding remaining activity, pH and thermal stability as well as the influence of the temperature, pH and substrate concentration on the activity of the invertase. The results were given below and discussed by comparing the similar studies.

Effect of temperature on the activity of invertase

As is known, temperature enhance the enzyme-catalyzed reactions. The temperature dependence of the sucrose hydrolysis reaction catalyzed by invertase was studied in the temperature range of 25-70°C and the results are shown in Figure 1. The activity of the enzyme was found to be dependent upon the temperature. The optimum temperature was found to be 50°C. As the temperature was further increased, the activity reduced significantly by 70°C. The enzyme retained more than 40% of its activity at a broad temperature range of 40-60°C. This result is compared well with the previous reports.

Generally, plant invertases show high activity in the temperature range of 35-70°C depending to their sources and also incubation time [27-29]. Alam et al. have been reported that the tomato invertase showed maximum activity at 35°C and the activity was decreased sharply with the rise of temperature above 50°C [27].

Thermal stability of invertase

Thermal stability of an enzyme is one of the most important criteria with respect to applications. It is the ability of enzyme to resist against thermal unfolding in the absence of substrates. Thermal stability of the invertase was determined by measuring the residual activity of the enzyme exposed to various temperatures (4-70°C) for 30 min with continuous shaking and the results are shown in Figure 2. As is seen from the figure, the invertase is very stable between the temperature range of 4-50°C. The enzyme was retained more than 55% of its initial activity at 50°C. However, relative activity (%) was sharply decreased to 17% at 60°C and nearly complete inactivation was observed at 70°C. Similar results have been reported previously by other researchers. Takehana et al. have been reported that, invertase was stable at 30°C for 5 min, however completely inactivated by the incubation at 60°C [30]. Alam et al. have also shown a drastic drop in the invertase activity above 50°C and complete inactivation have occured nearly at 80°C [27]. Thermal stability of invertase is very important property that food processing and preparation commonly involve exposure to elevated temperature.

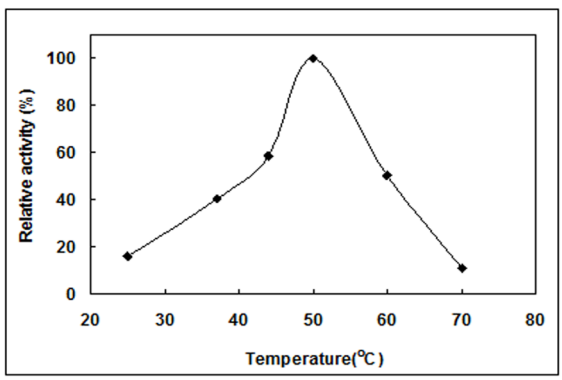

Figure 1. The effect of temperature on the activity of invertase (activities were assayed at indicated temperatures by using 0.5 M sucrose prepared in 0.2 M acetate buffer at pH 5.0).

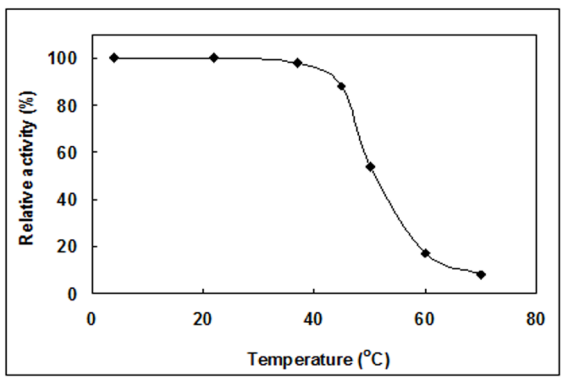

Figure 2. Thermal stability of invertase (after incubation at indicated temperatures activities were assayed at 37°C by using 0.5 M sucrose prepared in 0.2 M acetate buffer at pH 5.0).

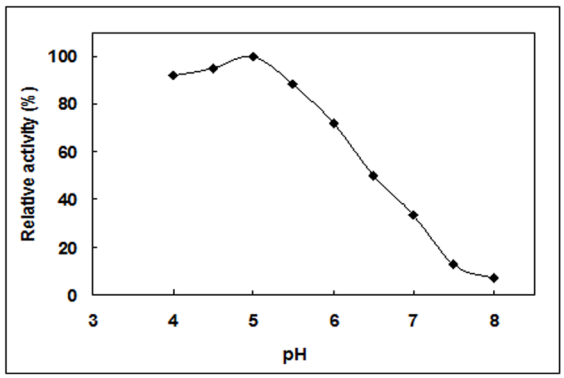

Figure 3. The effect of pH on the activity of invertase (activities were assayed at 37°C by using 0.5 M sucrose prepared in appropriate buffer solution).

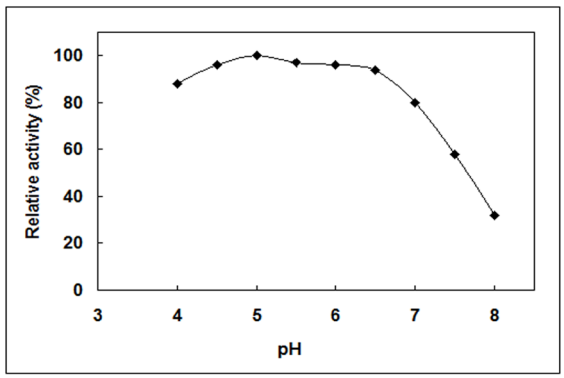

Figure 4. pH stability of invertase (after incubation at indicated pH's activities were assayed at 37°C by using 0.5 M sucrose prepared in 0.2 M acetate buffer at pH 5.0).

Influence of pH on the activity of invertase

The activity of an enzyme may change dependent upon the pH conditions of its immediated environment. Hence, the effect of pH on invertase activity was studied by changing the pH of the medium from 4.0 to 8.0 at 37°C. The variation of the relative activity of the invertase at different pH values is shown in Figure 3. As can be seen from the figure, the pH optima was found to be at pH 5.0. pH profile of the enzyme was also very broader in a pH range of 4.0-6.5 and more than 50% of its initial activity was recovered. The enzyme is also virtually inactive above pH 7.0. Very similar pattern of pH profiles has been reported for different plant invertases. The pH optimum of plant invertases changes in the range of pH 4.0-8.0 [8,31]. Takehana and Nakagawa have reported that, the activity of tomato invertase was optimum at pH 4.7 and pH 3.5 for sucrose and pH 5.7 for raffinose, respectively [30]. Similarly, optimum pH of the tomato invertases have also been determined as pH 5.5 [27] and pH 5.0 [32] by other researchers.

pH stability of invertase

The pH stability of the enzyme was also studied by incubating the enzyme in buffers of varying pH for 3 h at 4°C and then determining the remaining catalytic activity was determined under standard activity assay conditions. As is shown in Figure 4, the enzyme was very stable at a pH range of 4.0-7.0 retaining more than 80% of its initial activity whereas activity decrease was observed after pH 6.5. This broad pH stability can be a potential candidate for different applications of invertase in industry. The findings are in agreement with the previous works. For tomato invertases purified with different protocols it has been reported that, the enzyme was very stable from pH 3.5 to 7.0 [32] and from pH 2.6 to 6.0 [30].

Effect of substrate concentration on the activity of invertase

The enzyme kinetics obeyed simple Michaelis-Menten kinetics. The kinetic constants (K_m and V_{max}) of the enzyme were determined from Lineweaver-Burk graph. The K_m and V_{max} values were calculated as 4.65 mM and 30 U, respectively (Figure 5). The K_m value of invertase is in agreement with those presented in literatures [8,27,29]. Takehana and Nakagawa have reported that, the K_m value of tomato invertase was 4 mM at pH 5.7 and 6.9 mM at pH 4.7 for sucrose as substrate [30] Higher K_m values were also obtained for raffinose in the same work. The K_m values of tomato invertases against sucrose as substrate were also found to be 4.5 mM at pH 5.0 by Alam et al. [27] and 4.35 mM by Konno et al. [33].

Storage stability of invertase

One of the most important parameter to be considered for practical purposes is the stability of the enzyme. Storage stability is of considerable importance for various applications of biocatalysts in a commercial point of view. Enzymes can be easily lose their catalytic activity and denaturated, thus careful storage and handling conditions are essential. The tomato invertase was stored at 4°C in 0.2 M acetate buffer (pH 5.0) and the activity measurements were carried out for a period of 2 months. By this period, the enzyme was lost about 47% of its initial activity (Figure 6). The decrease

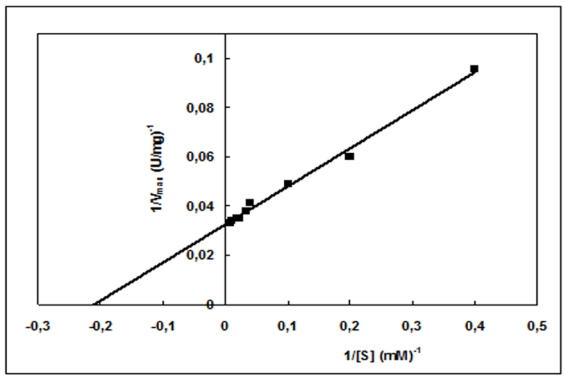

Figure 5. Lineweaver-Burk plot of tomato invertase (activities were assayed at 37°C by using sucrose prepared in 0.2 M acetate buffer at pH 5.0).

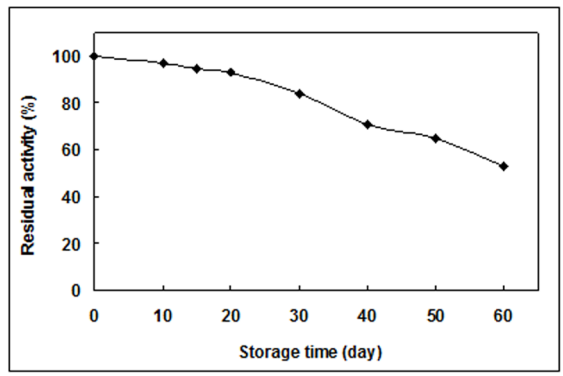

Figure 6. Storage stability of invertase (activities were assayed at 37°C by using 0.5 M sucrose prepared in 0.2 M acetate buffer at pH 5.0).

in the activity was explained as a time-dependent natural loss in enzyme activity. The storage stability of an enzyme varies depending on the source of the enzyme and storage conditions.

Influence of various effectors on the activity of invertase

The influence of various effectors (at different concentrations; 0.1-2.5 mM) on the activity of invertase was also determined by using sucrose as the substrate. The results are shown in Table 1. As is seen from the table, Mn^{+2} ions showed an activator effect for tomato invertase. The enzyme activity was also effectively enhanced in the presence of 1 mM $MnSO_4$ and $MnCl_2$. In previous work, it has been reported that tomato invertase required Mn^{+2}, Mg^{+2}, K^+, Na^+ and Ba^{+2} for the stability of enzyme and invertase activity was increased slightly at 5 mM [27] The activity of tomato invertase decreased drastically with the increased concentration of Cu^{+2}. Relative activities (%) were found as 84.6, 79.6 and 48.6% at concentrations of 0.1, 1.0 and 2.5 mM, respectively. On the other hand, moderate inhibition effects on invertase activities have been found by other compounds. Nakagawa et al. [34] have observed that, tomato invertase is strongly inhibited by Ag^+ and Cu^{+2} ions and also by p-mercurybenzoate. Plant invertases are characteristically inhibited by various metal ions like; Ag^+, Cu^{+2}, Hg^{+2}, Mg^{+2}, Ni^{+2}, Ba^{+2} and also with different compounds [8,35-37].

Table 1. Effect of various effectors on the activity of invertase*

Effector	Relative Activity(%)*		
	0.1 mM	1.0 mM	2.5 mM
Control	100	100	100
NaCl	98.1	97.8	97.5
KCl	101.3	96.2	96.1
$MgCl_2.6H_2O$	96.1	95.6	92.7
$MgSO_4.7H_2O$	97.4	95.7	90.4
$BaCl_2.2H_2O$	103.5	95.3	95.8
$CaCl_2$	97.7	96.2	84.1
$MnSO_4.H_2O$	112.2	161.3	120.9
$MnCl_2.4H_2O$	123.8	150.3	138.4
$ZnSO_4.7H_2O$	92.5	93.7	72.3
Na_2CO_3	90.6	89.8	82.7
$CuSO_4.5H_2O$	84.6	79.6	48.6
$FeCl_3.6H_2O$	99.4	97.6	93.2

*Invertase activity without added compounds(control) was taken as 100% activity.

CONCLUSION

Invertases are still efficient tools in various biotechnological applications especially in the food and beverage industry. The results obtained in this study indicate that, characteristic properties of invertase partitioned by PEG/Na_2SO_4 aqueous two-phase system (ATPS) is very suitable for its industrial applications. From

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the obtained catalytic properties of tomato invertase we can say that, the temperature, pH and effectors had different effects on its respect activity. The relatively wide range of pH and temperature for activity and stability exhibited by the tomato invertase could be very interesting for the food processes.

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