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Partial purification of invertase from *Momordica charantia* (bitter melon) by three phase partitioning (TPP) method

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The single step three-phase partitioning (TPP) method was evaluated for the purification of invertase from *Momordica charantia.* Optimum conditions for TPP method, that is, 70% ammonium sulfate saturation, 1:0.5 t-butanol ratio and 30 min incubation time lead to 20.28% efficiency and 10.48 fold partial purification. Total protein decreased from 34.86 mg to 0.68 mg in the homogenate, and the specific activity of the enzyme increased from 21.67 to 227.02 U/mg protein. The purified enzyme showed maximum activity in 0.3 M pH 5.0 buffer at 50°C. In conclusion, the optimized single step TPP method revealed better purification.

Key words: Three-phase partitioning (TPP), invertase, *Momordica charantia,* enzyme purification.

INTRODUCTİON

Invertase (β-fructofuranosidase, EC 3.2.1.26) catalyses the hydrolysis of sucrose into glucose and fructose. In general, *Saccharomyces cerevisiae* invertase is used in the food and beverage industries. Invertase is commonly used for the production of non-crystallizable (invert) sugar syrup from sucrose. Invert sugars have a wide range of uses from fermentation to the manufacture of artificial honey and plasticizing agents. (Guimaraes et al., 2009; Hussain et al., 2009; Kotwal and Shankar 2009; Akardere et al., 2010). Invertase has been purified using various conventional purification processes, such as salting out, gel filtration, ultrafiltration, ion-exchange and affinity chromatographies (Wiseman, 1995). However, most of these processes involve multi-steps that decrease enzyme yields. These multi-step processes also affects product efficiency in terms of application difficulties and increased costs (Wiseman, 1995).

A simple three-phase partitioning (TPP) method was developed by Tan and Lovrien (1972) for the separation and purification of target proteins, which is more economically efficient This technique comprises mixing the crude protein extract with certain proportions of ammonium sulfate and t-butanol to generate three phases. The upper t-butanol phase contains non-polar compounds; whereas, the lower aqueous phase contains polar compounds with the two phases seperated by an interfacial protein precipitate containing the target protein. It is generally believed that the separation process involves a combination of different operating principles including kosmotropy, salting out, co-solvent precipitation,

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isoionic precipitation, osmolytic electrostatic forces, conformation tightening, and protein hydration shifts, which all contribute to protein precipitation at the medium phase (Dennison and Lovrien, 1997; Lovrein et al., 1987). Proteins display various behaviors under these conditions, depending upon their source, molecular weight, hydrophobicity, charge, temperature and isoelectric point (*pI*) (Dhananjay and Mulimani, 2009; Pike and Dennison, 1989; Dennison and Lovrein, 1997). Since the method was first reported in 1972, more than 100 research studies have been published on the use of TPP separation technology for the extraction and purification of a variety of biomolecules including proteins, enzymes, enzyme inhibitors, edible oils or lipids, carbohydrates and small-molecule organic compounds from diverse natural sources (Yan et al., 2017). Presently, invertase has been purified from five plant sources by the TPP methodology (Lovrein et al., 1987; Dhananjay and Mulimani, 2008; Kat and Yılmazer, 2013).

The objective of this study was to optimize the TTP purification of invertase from *Momordica charantia* fruits. *M. charantia* (bitter melon or bitter gourd) is a flowering vine in the Cucurbitaceae family. It is a tropical plant that is widely cultivated in Asia, India, East Africa, and South America. Its immensely bitter fruits are commonly used as an edible and natural remedy for treating diabetes (Abascal and Yarnell, 2005) and a wide range of illnesses (Bakare et al., 2010). Bitter melon is known to play an important role in glucose metabolism (Shibib et al., 1993; Platel and Srinivasan, 1997). Invertase is an enzyme which plays a role in glucose metabolism also, so, we choosed the bitter melon as an invertase source.

The methods used to purify different enzymes from other sources may not be suitable for a novel enzyme or source, because enzymes can be affected by various factors (ammonium sulfate concentration, ratio of t-butanol, pH and incubation time) in the method. Therefore, the method must be optimized, hence in the case of enzyme purification with TPP, the method has been optimized in the literature (Akardere et al., 2010; Chaiwut et al., 2010; Dhananjay and Mulimani, 2008; Duman and Kaya, 2013; Gagaoua et al., 2014; Hussain et al., 2009; Liu et al., 2006, Sagu et al., 2015).

This study is new in terms of the source from which the invertase is purified and the optimization of the TPP method for invertase.

MATERIALS AND METHODS

Ammonium sulfate ($(NH_4)_2SO_4$), copper (II) sulphate pentahydrate, Folin and Ciocalteu's phenol reagent, phosphoric acid, trisodium citrate, sodium carbonate, dialysis tubing cellulose membrane, sodium bisulfate, potassium sodium tartrate, t-butanol, sodium hydroxide, sucrose, 3,5-dinitro salicylic acid, ethylene diamine tetra acetic acid (EDTA), sodium dodecyl sulfate (SDS), bovine serum albumin (BSA) and trichloroacetic acid (TCA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other analytical grade chemicals were obtained from Merck (Darmstadt, Germany). *M. charantia* fruits were harvested from the southern region of Adana in Turkey.

Preparation of crude extract from *M. charantia*

M. charantia was obtained from a farm in August 2016 and then frozen at -20ºC. Frozen fruits were added to acetate buffer (0.2 M pH:5.0) at a 1:9 (w/v) ratio and ground in a blender. The milled sample was filtered through Whatman paper No.1. Preparation of this crude extract was carried out at 4°C. This sample was referred to as “crude extract” and used for further three-phase partitioning experiments.

Three-phase partitioning (TPP) of invertase

The TPP method described by Roy and Gupta (2002) was modified for enzyme purification. Solid crystalline ammonium sulfate was added to the crude extracts until desired saturation percentage was achieved, t-butanol was added at different ratios after complete dissolution of the ammonium sulfate. The mixture was allowed to stand for different times at room temperature and then centrifuged at 4500 rpm for 10 min to make easy the separation of phases. The upper phase was removed carefully and interfacial precipitate was dissolved in acetate buffer (0.2 M, pH 5.0). The samples were dialyzed against acetate buffer (0.2 M, pH 5.0), then total protein content of the samples and total and specific activity of invertase were determined. The optimum conditions (ratio of ammonium sulfate, ratio of t-butanol and incubation time) which resulted into maximum recovery of invertase were used in the purification procedure. The purified enzyme was characterized in order to determine some biochemical properties of the enzyme.

Optimization of TPP method

Optimization of ammonium sulfate ratio for invertase partitioning

The ammonium sulphate precipitation of crude extract was carried out at ratios of 40, 50, 60 and 70% (w/v) saturation at 4°C. Optimum concentration of $(NH_4)_2SO_4$ were investigated by using the 1:1 (v/v) ratio of t-butanol. The interfacial precipitate was collected and dissolved in 0.2 M acetate buffer (pH 5.0) for determination of enzyme activity and protein content. The ammonium sulphate concentration providing the highest invertase acticity was chosen for further study.

Optimization of t-butanol ratio for invertase partitioning

The crude extract was saturated with the optimal $(NH_4)_2SO_4$ concentration. Then, t-butanol was added to the samples at ratios of 1:0.5, 1:1, 1:1.5, and 1:2 (v/v). The t-butanol ratio that gave the highest enzyme activity was chosen for further study.

Optimization of ıncubation time for invertase partitioning

Incubation times evaluated were 15, 30 and 60 min. The incubation time with the highest invertase activity was chosen for further study.

Determination of invertase activity

The enzymatic activity of invertase was determined through the initial rates of sucrose hydrolysis reaction by the 3,5-dinitrosalicilic acid (DNS) method (Miller 1959; Sumner 1921). The assay mixture consisted of 0.2 ml of 0.5 M sucrose, 0.6 mL of acetate buffer (0.2 M, pH 5.0) and 0.2 ml of a sample containing invertase, which was then incubated at 37ºC for 30 min. Next, 1 ml of DNS reagent was

added and samples were heated in a boiling water bath for 10 min. Then samples were cooled to room temperature and the amount of reducing sugar was analysed with UV-visible spectrophotometer at 546 nm wavelength. One unit of invertase activity was defined as the amount of enzyme that resulted in the released 1 µmol of glucose from sucrose per minute.

Determination of protein content

Protein amounts of samples were determined by Lowry method using bovine serum albumin (BSA) as a standard (Lowry et al., 1951).

Characterization of invertase from M. charantia

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SDS-PAGE was performed following the procedure of Laemmli (1970) using 15% separating gel on a Peqlab gel system unit with Biorad power suppy (45 mA, 200 V). Gels were then silver stained (Oakley et al., 1980) to visualize proteins.

Optimum temperature for M. charantia invertase

Standard assay procedure was performed at 25, 37, 50 and 60ºC. The specific activities were expressed as the ratio of enzyme activity obtained at certain temperature to the maximum activity obtained at the given temperature range.

Optimum pH for M. charantia invertase

Using the standard assay procedure, 0.2 M of the buffer systems (sodium acetate; pHs 4.0, 5.0, 6.0 and sodium phosphate; pHs 7.0, 9.0) were tested at optimum temperature. The remaining activity was determined and described as the specific activity.

Optimum buffer concentration for M. charantia invertase

The standard assay was applied using buffer concentrations at 0.2, 0.3, 0.5 and 0.7 M at optimum temperature and pH. Specific activity of the enzyme was calculated.

RESULTS AND DİSCUSSİON

Three-phase partitioning of invertase from *M. charantia*

For the single-step purification of invertase from *M. charantia*, TPP method was optimized in this study. There are several factors that affected the recovery of the desired protein in this process. So, the method needs some optimization to obtain suitable phase system for efficient TPP. The starting protein concentration (containing 7.56 U/ml of invertase activity) was 0.35 mg/ml. Invertase showed the highest activity at the interfacial precipitate predominantly at this study. If pI of protein are below the pH values of the process , the protein will remain in the aqueous phase because the protein is loaded negatively, thanks to negatively charged amino acid residues on the surface. Conversely, when pI of the protein is above the pH of the TPP medium, protein is precipitated and enriched in middle phase (Dennison and Lovrein, 1997 and Wang et al., 2011) There are some studies in the literature that the enzyme is in the middle phase. For example alpha-galactosidase enzyme was purified from pepino using TPP method, in which enzyme concentrated in the interfacial phase (Sen et al., 2011). In another study for the purification of Invertase from *Vitis labrusca*, the enzyme enriched middle phase after the TPP procedure (Kat and Yilmazer, 2013). Specific activity, ativity fold and yield were presented in Table 1.

As seen from the results, this single step invertase partitioning method depends on the amounts of ammonium sulfate and t-butanol, and also incubation time. pH is also the most important factor for this procedure. In the literature catalytic activities of invertases were measured at pH 5.0 (Miller, 1959; Sumner,1921) and also most of the invertase purification studies especially which are used plants as source at around pH 5.0 (Fotopoulos, 2005), therefore, pH wasn't optimized in this study.

The results of ammonium sulfate optimization are given in Figure 1. The highest invertase activty (1.23 U/mL) was observed at 70% (w/v) ammonium sulfate saturation. Optimum ammonium sulphate saturation for invertase purification is variable based on the source of the enzyme such as *Aspergillus oryzae* 30% (w/v) (Dhananjay and Mulimani, 2008), tomato (*Lycopersicon esculentum*) 50% (w/v) (Özer et al., 2010), Baker's yeast (*S. cerevisiae*) 50% (w/v) (Akardere et al., 2010), *Aspergillus sojae* 80% (w/v) (Lincoln and More, 2018), *Saccharum officinarum L.* 100% (w/v) (Hussain et al., 2009).

t-Butanol is the most convenient co-solvent, owing to its size and furcate structure, and also does not cause denaturation (Dennison and Lovrein, 1997; Dhananjay and Mulimani, 2008). The best results with 2.70 U/mL activity were obtained with 1:0.5 (v/v) crude extract: t-butanol ratio as illustrated in Figure 2. As the amount of t-butanol increase, the activity of the enzyme decreased in this study. Higher amounts of t-butanol can cause protein denaturation (Yan et al., 2017; Chaiwut et al., 2010). Similar ratios were reported for other species with 1:1 (v/v) for *A. oryzae* (Dhananjay and Mulimani, (2008), *V. labrusca* (Kat and Yılmazer, 2013) and tomato (*L. esculentum*) (Özer et al., 2010), the ratio is 1:0.5 (v/v) for *Baker's yeast (S. cerevisiae)* (Akardere et al., 2010).

Maximum activity of invertase was seen in 30 min incubation time as presented Figure 3. Invertase activity decreased sharply after 60 min. It means that enzyme may be denatured at this time period at that conditions.

M. charantia invertase was partialy purified 10.48-fold with a recovery of 20.28% by the TPP procedure as presented in Table 1. These results are similar to other studies that used multi-stage purification of invertase. For instance, invertase from *Aspergillus sojae JU12*. was purified by size exclusion chromatography with 5.41 fold and 10.87% recovery (Lincoln and More, 2018).

Table 1. Activity of invertase from *Momordica charantia* purified by three-phase partitioning.

Step	Activity (U/mL)	Protein (mg/mL)	Total protein (mg)	Specific activity (U/mg protein)	Total activity (U)	Yield (%)	Purification fold
Crude extract	7.56	0.35	34.86	21.67	755.50	100	1
TPP-Interfacial precipiate	5.11	0.02	0.68	227.02	153.24	20.28	10.48

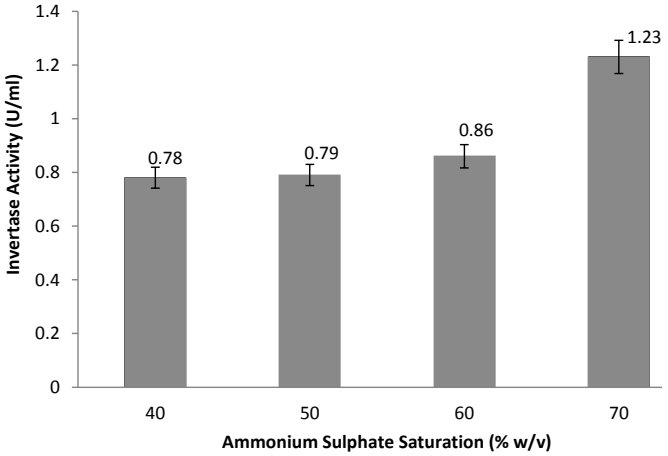

Figure 1. Effect of varying saturations of ammonium sulfate on purification of invertase by TPP. Ammonium sulfate was added to crude extract at ratios of 40, 50, 60 and 70% (w/v).

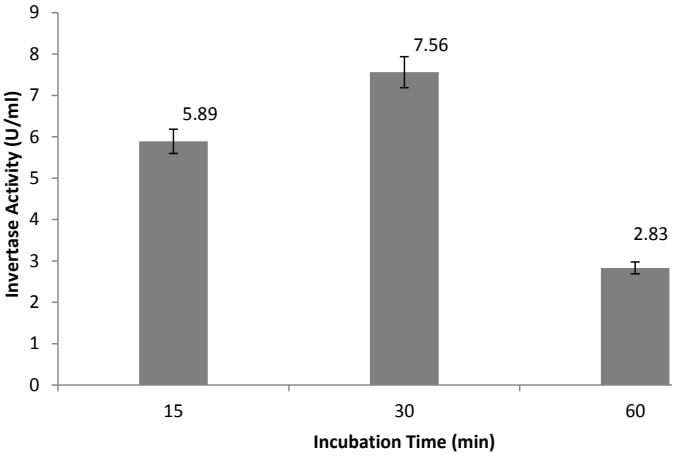

Figure 3. Optimization of incubation time for invertase partitioning. Ammonium sulphate and t-butanol were incubated in crude extract at 15, 30 and 60 min for the TPP.

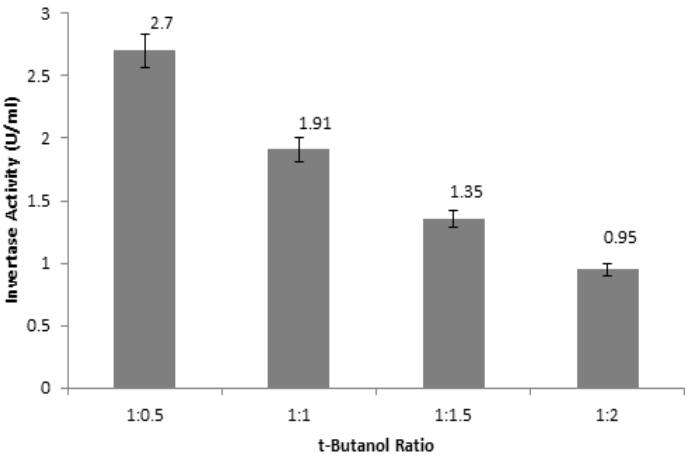

Figure 2. Optimization of crude extract to t-butanol ratio for invertase partitioning. t-butanol was added at ratios of 1:0.5, 1:1, 1:1.5, and 1:2 (v/v) to crude extract saturated with 70% ammonium sulfate.

Purification of invertase from bamboo cells achieved 64-fold purification with a recovery of 26% using interaction of ammonium sulfate precipitation, Dimethylaminoethyl (DEAE)-sephacel and gel filtration chromatographies (Liu et al., 2006). Invertase from sugarcane was purified by muli-stage; ammonium sulfate precipitation, anion exchange, hydrophobic interaction chromatography and gel filtration resulting in 13-fold purification with 35% recovery (Hussain et al., 2009). Guimaraes et al. (2009) used DEAE-cellulose and Sephacryl S-200 chromatographic methods resulting in 7.1-fold with a recovery of 24% for the purification of invertase from *Aspergillus ochraceus*.

The purification findings of current study (10.48-fold with a recovery of 20.28%) as seen in Table 1 are also better or close to some other TPP studies. For example AAO (aryl alcohol oxidase) from *Pleurotus ostreatus* was purified using TPP 10.19-fold with 10.95% activity recovery (Kumar et al., 2011). Dogan and Tari (2008) were also purified using exo-polygalacturonase from *Aspergillus sojae* to 6.7 purification fold with 25% activity recovery. PPO (polyphenol oxidase) from *Trachystemon orientalis L.* purified 3.59-fold with 68.75% activity recovery (Alici and Arabaci, 2016). Purification of inulinase from *Aspergillus niger* was achieved with 10.2 purification fold and 88% activity recovery (Kumar et al., 2011) which is enough to make TPP preferable. Moreover TPP also enhances catalitic activities of some enzymes by concentrating or dewatering proteins. This may be the result of increased flexibility of the enzyme molecule at TPP conditions. A noteworthy increase in the catalytic activity and yield of some enzymes obtained from TPP was reported (Akardere et al., 2010; Chaiwut et al., 2010; Duman and Kaya, 2013; Dogan and Tari, 2008;

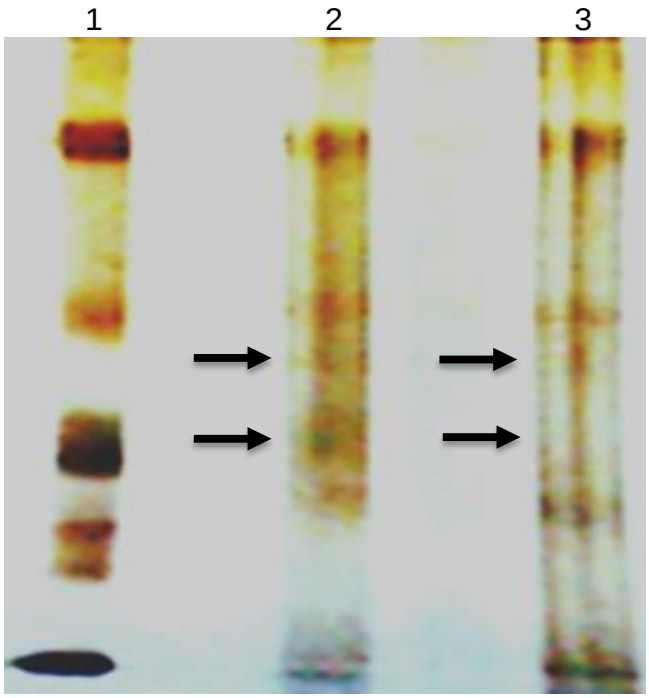

Figure 4. SDS-PAGE analysis of invertase from *M. charantia* [Lane 1; molecular weight markers (14-66 kDa), lane 2; crude extract, and lane 3; after TPP (medium phase)].

Sharma and Gupta, 2001a; Gagaoua et al., 2014, 2016).

In some studies two-step TPP was applied because of the low activity recoveries of one-step. Dhananjay and Mulimani have used the TPP as a two-step process to purify invertase from A. oryzae. In the first step, 61-fold purification with 1.4% recovery was achieved. The second TPP was resulted from 12-fold purification and 54% activity recovery (Dhananjay and Mulimani, 2008). Purification of laccase from Submerged cultures of *Ganoderma sp.* WR-1 (Rajeeva and Lele, 2011), purification of phospholipase D from *Dacus carota* (Sharma and Gupta, 2001b), and purification of protease from *Calotropis procera latex* (Rawdkuen et al., 2010) were also achieved by two-step TPP. In this study we planned to reveal the results of only one-step TPP for purification of invertase from bitter melon. The results show that while the purification fold (10.48) is better than some previous studies (Akardere et al., 2010; Chaiwut et al., 2010; Dhananjay and Mulimani, 2008; Duman and Kaya, 2013; Hussain et al., 2009; Liu et al., 2006), activity recovery (20.28%) is inadequate when only one-step TPP is used, and it may be increased by applying TPP again or other steps.

Characterization of invertase

SDS-PAGE analysis

A reduction in the number of protein bands in SDS-PAGE is evidence that some proteins have been eliminated. As shown in Figure 4, density of protein bands of the sample obtained from TPP application (lane 3) decreased according to crude extract (lane 2). So, simplier image in lane 3 is noteworthy which showes that *M. charantia* invertase had been partially purified by TPP.

Optimum temperature, pH and buffer concentration of M.charantia invertase

The effect of temperature on enzymatic activity of invertase presented in Figure 5 shows that the temperature at which the invertase activity is maximum is 50°C. Generally, invertases exhibit high catalytic activity in the temperature range of 35-75°C depending on the enzyme sources (Akardere et al., 2010; Tang et al., 1996; Kern et al., 1992; Persike et al., 2002).

The highest activity of invertase was found to be pH 5. As presented in Figure 6. Thus, we can conclude that *M.charantia* invertase is an acidic enzyme. The optimum pH values of invertases purified from different sources varies between 3.5 to 8.0 in the literature (Akardere et al., 2010; Lincoln and More, 2018; Tang et al., 1996; Persike et al., 2002 and Belcarz et al., 2002).

As shown in Figure 7, significant differences were observed in invertase activities at different buffer concentrations. Maximum activity of the enzyme was observed for 0.3 M pH 5.0 sodium acetate buffer solution.

Conclusion

The present study focuses on the purification and characterization of invertase from *M.charantia* using single step TPP and also optimization of the purification metod. Under different ammonium sulfate and t-butanol amount, different enzyme recoveries were obtained. And also incubation time was important parameter at this optimization procedure. Interfacial phase was containing the target protein. This work shows that, under optimized conditions, TPP is a useful method for partial purifiction or concentration of the invertase from bitter melon. We purified the enzyme using this technique partially. This technique has been fast and it is a very important feature for maintaining the enzyme activity; maybe more pure invertase can be obtained by applying the second or third TPP step under optimised condition in further sudies.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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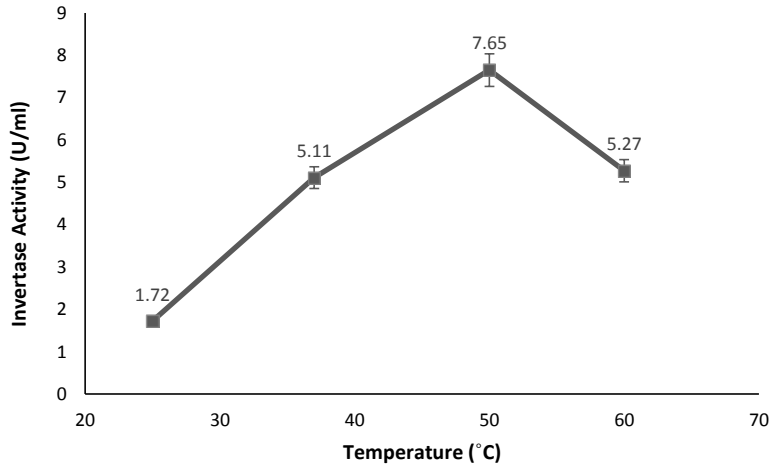

Figure 5. Determination of optimum temperature on invertase activity purified from *M. charantia*.

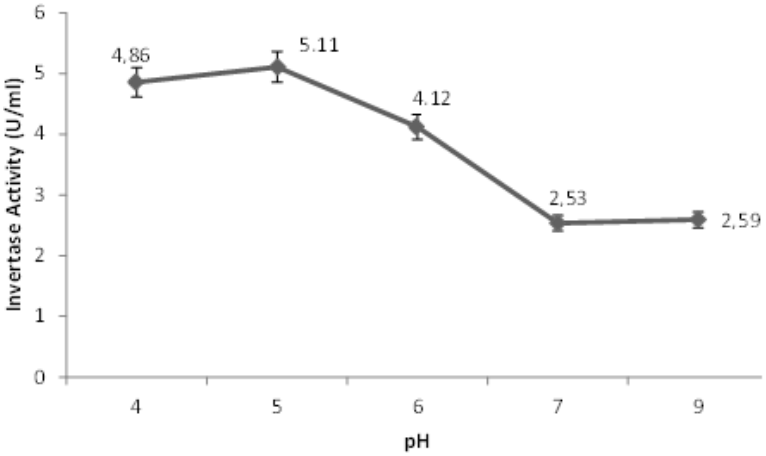

Figure 6. Determination of optimum pH on invertase activity purified from *Momordica charantia.*

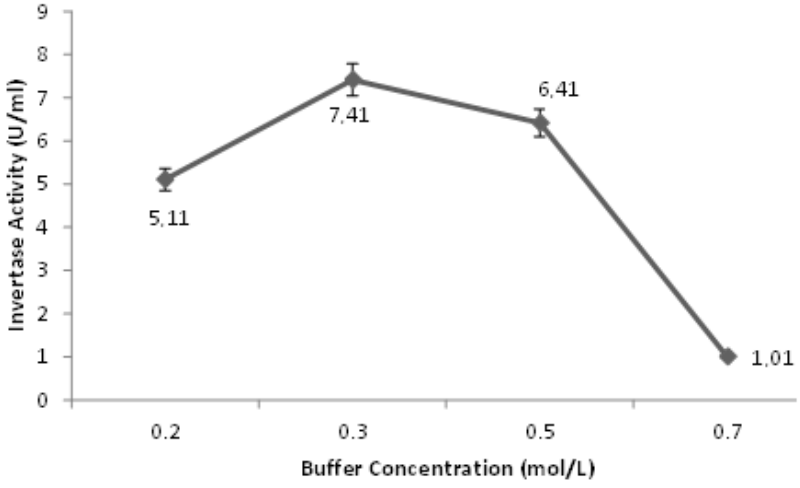

Figure 7. Determination of optimum buffer concentration on invertase activity purified from *M.charantia.*

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