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Three-Phase Partitioning OF α-Galactosidase From *Aspergillus Lentulus*: Optimization of System And Characterization of Enzyme

α-Galaktozidazin *Aspergillus lentulus*'dan Üçlü-Faz Ayırımı: Sistemin Optimizasyonu ve Enzimin Karakterizasyonu

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ABSTRACT

Three-phase partitioning (TPP) technique is successfully used for the first time for partial purification of α-galactosidase from *Aspergillus lentulus* in single step. The influence of variations in ammonium sulfate concentration, extract to t-butanol ratio and pH on extraction efficiency of the enzyme are investigated to achieve the highest yield and purity fold. Optimal purification parameters of the system are determined as 55% (w/v) ammonium sulfate concentration with 1:1 (v/v) ratio of crude extract to t-butanol at pH 5.5. This optimized TPP system gave 5.3 purification fold with 178% recovery of α-galactosidase. The maximum activity was registered at pH 6.5 and 50°C. α-Galactosidase showed a good stability within the temperature range of 25-60°C and pH range of 2.6-5.5. The KM and Vmax values were determined as 0.365 mM and 0.093 U, respectively. Among the metals and sugars Na_2CO_3 and galactose showed strong inhibitory effect on the activity of enzyme. The availability of a new α-galactosidase with different biochemical properties from *A. lentulus* by using TPP may be of interest for its various biotechnological applications.

Key Words

α-galactosidase, bioseparation, three-phase partitioning (TPP), enzyme purification, enzyme characterization.

ÖZ

Üçlü-faz ayırma (TPP) tekniği α-galaktozidazın *Aspergillus lentulus*'dan tek adımda kısmi saflaştırılması için ilk kez başarıyla kullanıldı. Yüksek aktivite ve saflaştırma katı elde etmek için enzimin ekstraksiyon etkinliğine amonyum sülfat konsantrasyonu, ekstrakt t-butanol oranı ve pH etkisi araştırıldı. Sistemin optimum saflaştırma parametreleri %55(w/v) amonyum sülfat konsantrasyonu, 1:1 (v/v) ham ekstrakt t-butanol oranı ve pH 5.5 olarak belirlendi. Bu optimize TPP sistemi α-galaktosidaz için 5.3 saflaştırma katı ile %178 aktivite verimi oluşturdu. pH 6.5 ve 50°C'de maksimum aktivite gözlendi. α-Galaktozidaz 25-60°C sıcaklık aralığında ve pH 2.6-5.5 aralığında oldukça iyi bir kararlılık gösterdi. KM ve Vmax değerleri sırasıyla 0.365 mM ve 0.093 U olarak belirlendi. Metal iyonları ve şekerler arasında Na_2CO_3 ve galaktoz enzim aktivitesi üzerinde kuvvetli inhibitor etkisi gösterdi. TPP ile *A. lentulus*'dan farklı biyokimyasal özelliklere sahip yeni bir α-galaktozidazın elde edilmesi onun çeşitli biyoteknolojik uygulamaları açısından ilgi çekici olacaktır.

Anahtar Kelimeler

α-galaktozidaz, biyoayırım, üçlü-faz ayırma (TPP), enzim saflaştırma, enzim karakterizasyonu.

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INTRODUCTION

Three-phase partitioning (TPP) is an elegant, modest and relatively new bioseparation strategy to separate and enrich various biomolecules including proteins, enzymes, enzyme inhibitors, oils and carbohydrates from natural sources such as plants, animals and microorganisms. TPP has several advantages in comparison to traditional extraction and separation techniques like; rapid, efficient, economical, green and scalable. It has generally been used as upstream and downstream separation process. There is no any exact mechanism for TPP, however it is known that a combination of different principles including kosmotropy, electrostatic forces, salting-out, isoionic and co-solvent precipitation, conformation tightening and protein hydration shifts are effective in separation of bioactive molecules [1-3]. According to biochemical properties (hydrophobicity, molecular weight, charge, isoelectric point) of a protein and also temperature and content of the separation medium (salt type and concentration, organic solvent type and concentration, pH and protein amount) proteins show different partitioning behaviour in TPP systems. TPP generally uses ammonium sulfate and t-butanol to precipitate proteins from aqueous solutions. After addition of a known amount of salt to aqueous crude protein extract t-butanol is added to the system to obtain three phases. The upper phase is containing nonpolar compounds like pigments and lipids. The middle phase is precipitated proteins. The lower phase is containing polar compounds like remaining proteins and saccharides. By optimization of TPP, the target proteins selectively partitioned to one phase while contaminating proteins to the other phase [4,5].

TPP was used to purify several biomolecules such as oil [6,7], polysaccharides [8,9], magniferin [10], enzyme inhibitors [11,12], anthocyanin [13]. TPP is also been widely used to separate, concentrate and purify several enzymes; peroxidase [14], serratiopeptidase [15], laccase [16], amylase [17], β-galactosidase [18], naringinase [19] etc.

α-Galactosidases (α-D-galactoside galactohydrolase, EC 3.2.1.22) are important enzymes that broadly distributed in microorganisms, plants and animals. They catalyze the hydrolysis of α-linked terminal non-reducing galactose residues from galactose oligosaccharides, galactomannans and galactolipids. α-Galactosidases have find various industrial applications such as in sugar industry to increase the yield of crystallized sugar, in feed industry as an animal feed additive to improve nutritional value, in pulp and paper industry to improve pulp bleaching and also in clinical applications such as for the treatment of Fabry's disease by enzyme replacement therapy and to convert B blood group to O blood group [20-22]. α-Galactosidases have been purified from different microbial sources with a number of purification protocols including salting-out, ion-exchange, affinity chromatography etc [23-25]. These protocols have generally multiple steps, need large process times and also have high operation costs.

Because of potential and applicability of α-galactosidases in diverse research areas it is necessary to find an alternative source for production. *Aspergillus lentulus* was found as a producer of α-galactosidase and it contain high enzyme activity and could be use as an alternative enzyme source for α-galactosidase production. So, in the present study, *A. lentulus* was used for concentration and purification of α-galactosidase with TPP for the first time. It was aimed to extract, concentrate and purify α-galactosidase from a crude extract of *Aspergillus lentulus* using a TPP protocol and also enzymatic characterization of the enzyme which make them advantageous in applications. It has been reported that physiological conditions influence partitioning of proteins in TPP [4]. The effect of different process parameters such as ammonium sulfate concentration, enzyme extract to t-butanol ratio and pH on partitioning yield of α-galactosidase was optimized to obtain a good TPP system with high performance. The partitioned enzyme was also biochemically characterized. The influence of temperature and pH on activity and stability of partitioned enzyme was investigated. Kinetic constants (K_M and V_{max}) were determined. The selected metal ions and sugars were evaluated for activity of partitioned enzyme.

MATERIALS and METHODS

Chemicals and Microorganism

Ammonium sulfate and t-butanol were acquired from E. Merck (Germany). p-Nitrophenyl-α-D-galactopyranoside (PNPG) and Coomassie Brilliant Blue R-250 were procured from Sigma (USA). Other chemicals were of an analytical grade.

The microorganism was isolated from sand of a hot spring (Eynal, Kütahya/Simav) in Turkey. The fungal strain in which take part in Dr. A. Uzel's collection was coded

and named as 2.2.45 and *Aspergillus lentulus*, respectively, and used as α-galactosidase source.

α-Galactosidase Production

α-Galactosidase was expressed using a thermotolerant fungi (*Aspergillus lentulus*) which was transferred to 1 L sterile production media. The production medium including KH_2PO_4 (7 g), K_2HPO_4 (2 g), $MgSO_4.7H_2O$ (2 g), $(NH_4)_2SO_4$ (1 g), yeast extract (0.6 g) and microcrystalline cellulose (10 g) in 1 L of distilled water was autoclaved to sterilize at 121°C for 20 min. Then the medium was inoculated with small pieces of mycelia of the fungal strain and incubated at 45°C for 7 days with shaking (150 rpm). At the end of incubation period, the culture broth was centrifuged at 9000 rpm for 30 min at 4°C. After separating the cells, the supernatant was taken and analyzed. The activity and protein amounts were found as 0.013 U/mL and 0.005 mg/mL, respectively. The proteins present in supernatant was precipitated with 80% (w/v) ammonium sulfate. The mixture was left overnight at 4°C with stirring. The precipitated proteins was collected by centrifugation at 9000 rpm for 30 min at 4°C. After dissolving the pellet in sodium citrate buffer (0.05 M, pH 6.0) it was dialyzed against the same buffer for overnight. The dialysate was named as "crude α-galactosidase extract" and was analyzed for α-galactosidase activity and protein amount (0.38 U/mL, 3.71 mg/mL, 0.1 U/mg). Afterwards it was stored at -20°C for TPP experiments.

Three-Phase Partitioning of α-Galactosidase

A lentulus α-galactosidase extract (2 ml containing 0.115 U and 0.25 mg protein) was mixed with ammonium sulfate (55%, w/v) at 25°C and thoroughly mixed on a vortex for 2 min for dissolving the salt. The pH of the medium was adjusted to pH 5.5 with HCl followed by addidition of t-butanol 1:1 (v/v). The aqueous/organic system was stirred for 1 min and then left to stand for 1 h at 25°C. After centrifugation at 4000 rpm for 10 min at 4°C the separation of phases was observed. Afterwards the top t-butanol layer was removed and then the bottom aqueous layer and the middle phase were collected separately. The volume of phases were measured. The middle phase was dissolved in 0.5 ml of 0.05 M sodium citrate buffer (pH 6.0). The bottom phase was dialysed for 3 hour against the same buffer. Both bottom and middle phases were then analyzed for enzyme activity and total protein concentration. The purification fold and recovery% were calculated. The initially added enzyme activity was taken as 100%.

The partitioning of enzymes in TPP systems was affected by several key parameters such as salt saturation, ratio of extract to organic solvent, and pH. Therefore, to optimize the TPP system to get best recovery the experimental conditions for α-galactosidase partitioning was described. Ammonium sulfate concentration was changed in the range of 20-70%, w/v. Crude enzyme extract to t-butanol ratio was varied in the following range; 1:0.25-1:2 (v/v). pH was varied between 4.0 to 7.0. The optimized best conditions were used as standard purification protocol for *Aspergillus lentulus* α-galactosidase. After optimization of TPP, the bottom phase which is containing high enzyme activity was collected and dialysed overnight against sodium citrate buffer (0.05 M, pH 6.0) to remove ammonium sulfate. α-Galactosidase was concentrated with ultrafiltration (10 kDa). It was stored at -20°C until use for determination of biochemical characteristic properties. A blank system without crude extract was used as control. All TPP experiments were performed in triplicate and the activity and protein determinations are also mean value of triplicate assay. The difference in the readings was less than ±3%.

Repetition and Scalling-up of TPP

In order to show the repeatability of TPP for α-galactosidase recovery, the system was prepared and repeated for three times under optimum same conditions. For scale-up, TPP system was prepared as x1 to x5 fold in volumes (mL) 15-75 ml under optimum same conditions. After separation of the phases, both middle and bottom phase were analyzed for enzyme activity and protein amount. A blank system without crude extract was used as control. The purification fold and recovery% were calculated. The initially added enzyme activity was taken as 100%.

Analytical Procedures

Enzyme assay

The activity of α-galactosidase was measured by PNPG [26]. The enzyme was appropriately diluted to 0.25 mL using sodium citrate buffer (0.05 M, pH 6.0) (if necessary). 0.25 mL of PNPG solution and 0.5 mL of citrate buffer were added to the enzyme solution. The mixture was incubated at 37°C for 30 min. The reaction was terminated by adding 3.5 mL of 0.2 M sodium borate buffer (pH 9.8). The amount of released p-nitrophenol was determined spectrophotometrically at 400 nm. One unit (1 U) of α-galactosidase activity was defined as the amount of enzyme that liberated one µmol of

p-nitrophenol from PNPG per min at 37°C. The all given data for α-galactosidase activity are mean values of triplicate assay.

Estimation of protein

Amount of protein was determined by Bradford method using Coomassie Blue G-250 dye as a reagent and bovine serum albumin was used as standard [27]. Specific activity is defined as the enzymatic unit per milligram of protein.

Biochemical Characterization of Partitioned α-Galactosidase

Effect of temperature on the enzyme activity and stability

The temperature profile of α-galactosidase was determined by performing the standard enzyme assay procedure using PNPG. The enzyme assays were assessed at different temperatures (25 to 80°C). The substrate solution was first preincubated in the respective temperature and then added to the enzyme solution to start the enzymatic reaction. The relative activities as percentage were expressed as the ratio of the enzyme activity obtained at a certain temperature to the maximum activity obtained at the given temperature range. Thermostability experiments were realized without using any additives. The enzyme was incubated at various temperatures ranging from 25 to 70°C for 30 min. After that, the enzyme aliquots were withdrawn, brought to room temperature and the residual activity was measured at regular intervals of time under optimal assay conditions.

Effect of pH on activity and stability

Optimal pH of α-galactosidase activity was determined in citrate/phosphate buffer (0.05 M) of pH values ranging from pH 2.6 to 7.5. The enzyme in buffer of varying pH was analyzed for its activity and relative activity at each pH was calculated. The pH stability was searched by incubating the enzyme in above buffers with various pHs for 30 min at 4°C and then the remaining activity (%) with respect to control was assayed.

Determination of kinetic constants (KM and Vmax)

Kinetic experiments were performed with PNPG. Its concentration was varied between 0.05-1.25 mM. Michaelis–Menten constant (KM) and the maximum velocity of reactions (Vmax) were calculated from Lineweaver–Burk plot.

Effect of various effectors on activity

The effect of different metal ions (NaCl, $CaCl_2$, $MgCl_2$, $LiCl_2$, $CuSO_4$, $MgSO_4$, Na_2CO_3, $ZnSO_4$, $MnCl_2$, $LiSO_4$, $MnSO_4$) and sugars (galactose, sucrose, glucose, fructose, lactose, maltose, mellibiose and raffinose) in concentrations of 10 mM on α-galactosidase activity was searched. The enzyme was separately preincubated with above effectors for 30 min at room temperature and then the remaining activities were assayed and calculated. The enzymatic activity was assayed in the absence of effectors was considered as 100%. The all given data for relative α-galactosidase activity are mean values of triplicate assay.

Inhibitory effect of galactose on α-galactosidase

D-Galactose is known as an inhibitor of α-galactosidases. Therefore, the inhibition effect of galactose on α-galactosidase activity was studied by using PNPG. For the determination of the effect of galactose on enzyme activity, the enzyme assays were realized at constant substrate concentration and at various galactose concentrations. Enzyme activity without galactose was used as control as 100%. Percent-activity graph was drawn and I50 value was calculated. With three different concentrations of galactose the enzyme activity was measured, the Lineweaver Burk graph was drawn and Ki constant values were also calculated.

RESULTS and DISCUSSION

Partitioning of *Aspergillus lentulus* α-Galactosidase

Three phase partitioning (TPP) is proved to be an excellent procedure for enzyme extraction and purification. The purification fold and activity yield of an enzyme ontained from TPP were influenced both by the source of the enzyme and process parameters. Therefore, in the present work TPP was used as one-step purification protocol for α-galactosidase from a new source; *Aspergillus lentulus*. Fungal strain *A. lentulus* has been proved to be producing highly active extracellular α-galactosidase. The effect of ammonium sulfate saturation (20-70%, w/v), enzyme extract to t-butanol ratio (1:0.25-1:2, v/v) and pH (4-7) on TPP of α-galactosidase was investigated. The optimum partitioning conditions for efficient separation of the enzyme was determined. In all optimization experiments α-galactosidase mainly partitioned and concentrated in aqueous bottom phase and gave high activity recovery and purification fold.

Effect of ammonium sulfate saturation

Salts have key function in the process of TPP as they remarkably affect solubility of proteins. The solubility will vary according to the ionic strength of the solution. Hence, the effect of salt saturation on TPP of α-galactosidase was studied. Ammonium sulfate is chosen as salt that the most widely used sulfate salt because of its low solubility. In comparison to other salts ammonium sulfate also has a high hydration capacity and affinity for water. In the case of sulfate ions which is a member of Hoffmeister series interact well with water, forming H-bonds and dehydrate proteins and has high tendency to crowd proteins [3,14,15]. Critical concentration of salt is needed to be optimized to get the interested enzyme in the desired phase. This also bring out the extraction of the enzyme with minimum enterprice from contaminating substances from complex medium. In order to determine the best ammonium sulfate saturation, salt concentration was varied from 20% to 70% (w/v) The enzyme to t-butanol ratio was fixed to 1:1(v/v). by maintaining the ratio of crude extract to t-butanol ratio constant (1:1). The obtained results are shown in Figure 1. As can be seen from figure, maximum fold purification of 3.84-fold along with highest recovery (113%) of α-galactosidase activity in bottom phase was obtained with 55% (w/v) ammonium sulfate concentration. The enzyme was predominantly recovered at the bottom phase. Sometimes TPP may cause enchancing the enzyme activity due to increased flexibility in the enzyme molecule [3,4]. There are several reports that show the activation of enzymes after TPP such as; invertase [28], α-galactosidase [29], pectinase [30], peroxidase [31] etc.

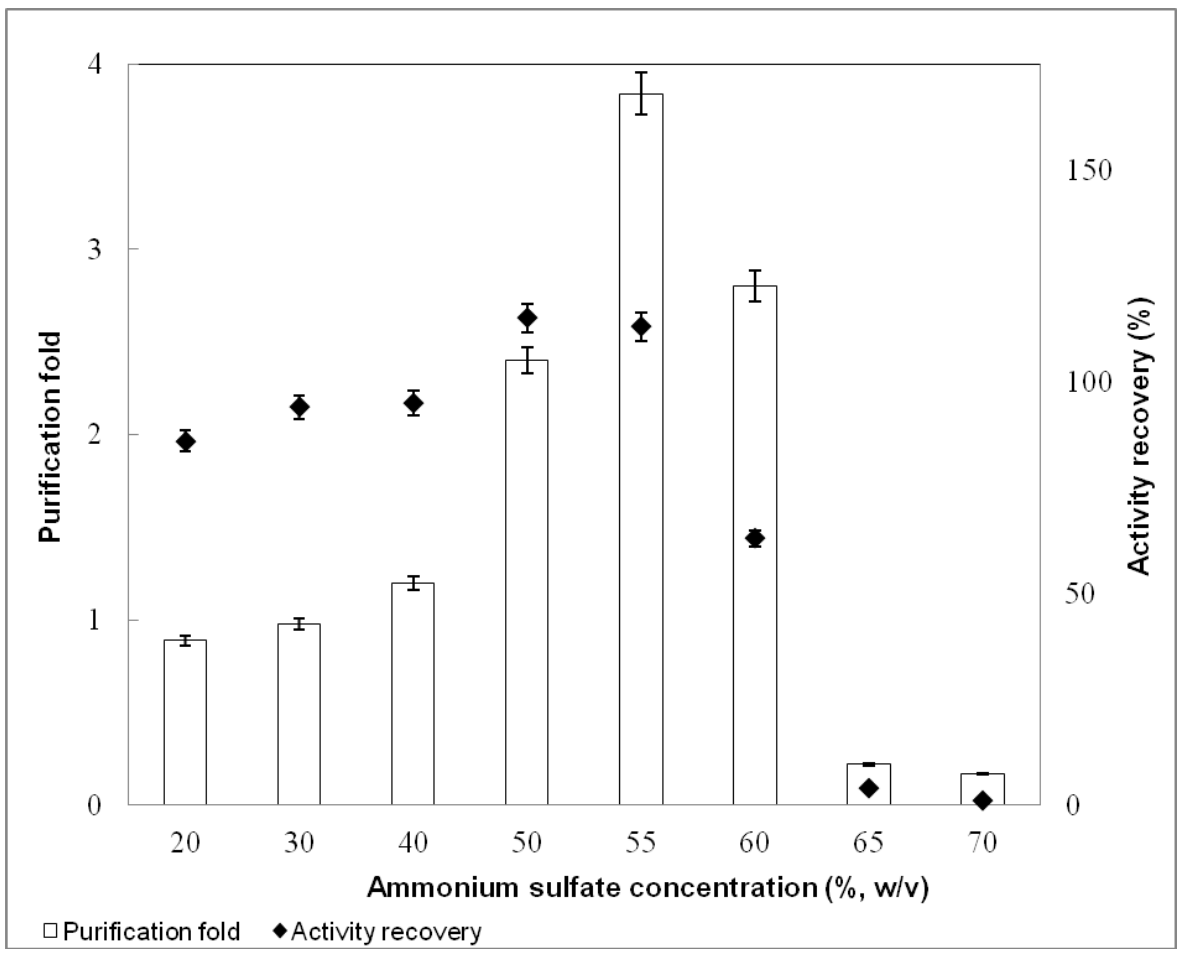

Figure 1. Effect of ammonium sulfate saturation on TPP of *Aspergillus lentulus* α-galactosidase. The enzyme extract (2 ml containing 0,115 U) was brought to different levels of saturation w.r.t. ammonium sulfate (20%, 30%, 40%, 50%, 55%, 60%, 65%, and 70%) and t-butanol was added in the ratio of 1:1 (v/v) with respect to the volumes of the aqueous extract.

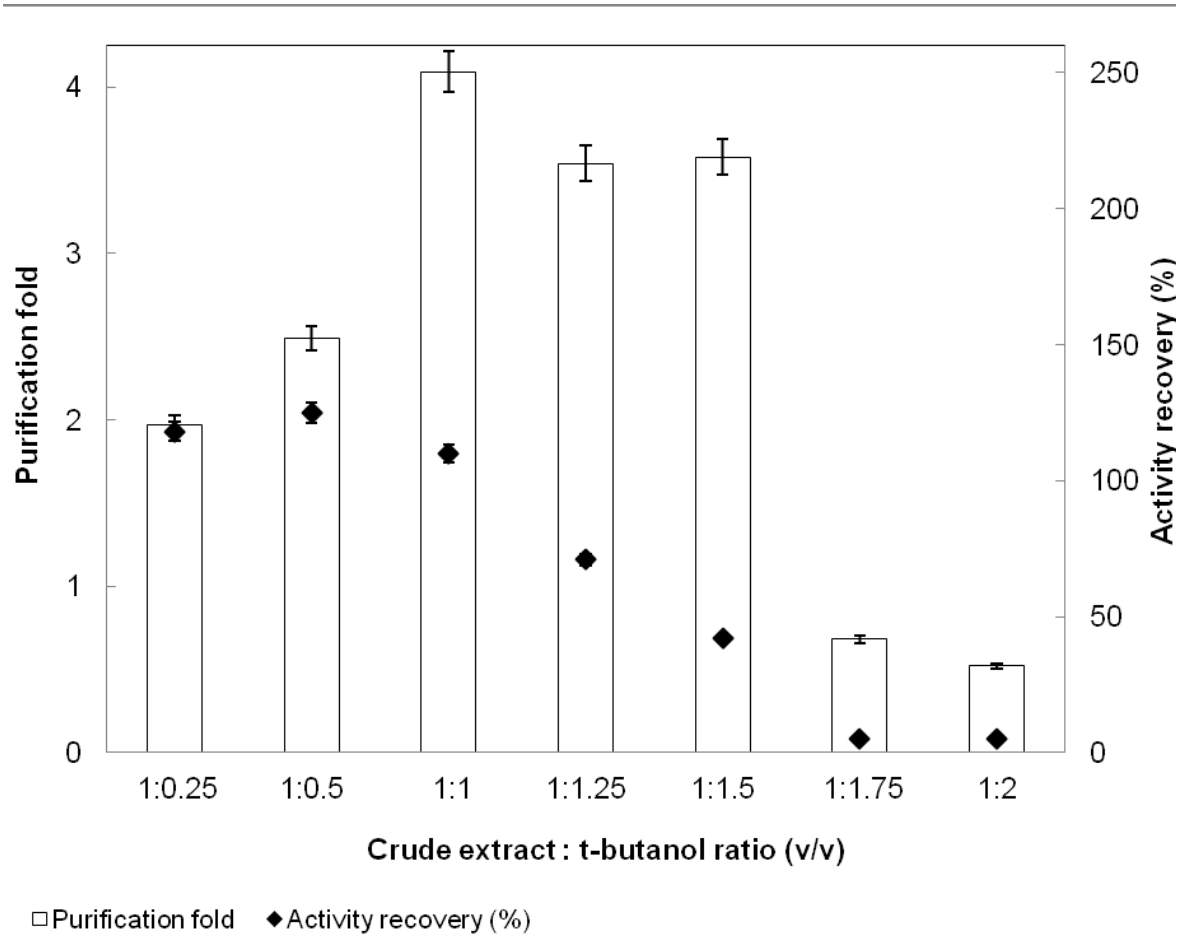

Figure 2. Effect of enzyme extract to t-butanol ratio on TPP of *Aspergillus lentulus* α-galactosidase. Various amount of t-butanol was added to enzyme extract (2 ml containing 0,115 U and saturated with 55% ammonium sulfate) in the following volumetric ratios viz. 1:0,25, 1:0.5, 1:1, 1:1,25, 1:1.5, 1:1,75 and 1:2.

The purification fold and activity recovery values were increased significantly with an increase in concentration of salt from 20% to 50% (w/v). However, beyond 60% (w/v) salt concentration both values were decreased. Especially above 60% (w/v) concentration they have decreased sharply that indicate a decrease in the selectivity of extraction and also degree of purification. At lower concentrations, ammonium sulfate was unable to change the hydrophobic surface of α-galactosidase. By increasing the salt concentration both the surface tension and interfacial tension of the TPP system increases. Therefore, higher tension values point out higher density and polarity differences between phases [31, 32]. Several researchers reported that, in TPP process salting out of a protein by sulfate is linked to kosmotropy, osmotic stressor, ionic-strength effects and the binding of sulfate to cationic sites of protein [3]. Hence 55% (w/v) salt saturation is selected as optimal for further experiments.

Effect of enzyme extract to t-butanol ratio

Selection of organic solvent is very important that affect to the partitioning of an enzyme in TPP. Various C4 alcohols could be used as phase forming solvent in TPP systems. t-butanol is used as the solvent for partitioning of α-galactosidase in TPP. t-butanol could make three-phase and efficiently remove small compounds. It has also been reported that t-butanol generally give the best partitioning results [15, 33]. For the determination of best enzyme to t-butanol ratio, the salt saturation was fixed to 55% (w/v) and enzyme extract to t-butanol ratio was varied from 1:0.25 to 1:2 (v/v). The maximum activity recovery and purification fold were obtained with 1:1 (v/v) ratio (Figure 2).

At lower t-butanol ratios (1:0.25 and 1:0.5, v/v) the recovered activity values were low. When t-butanol amount is lower it could not efficiently synergize with the salt [3]. If the t-butanol amount increases the concentration

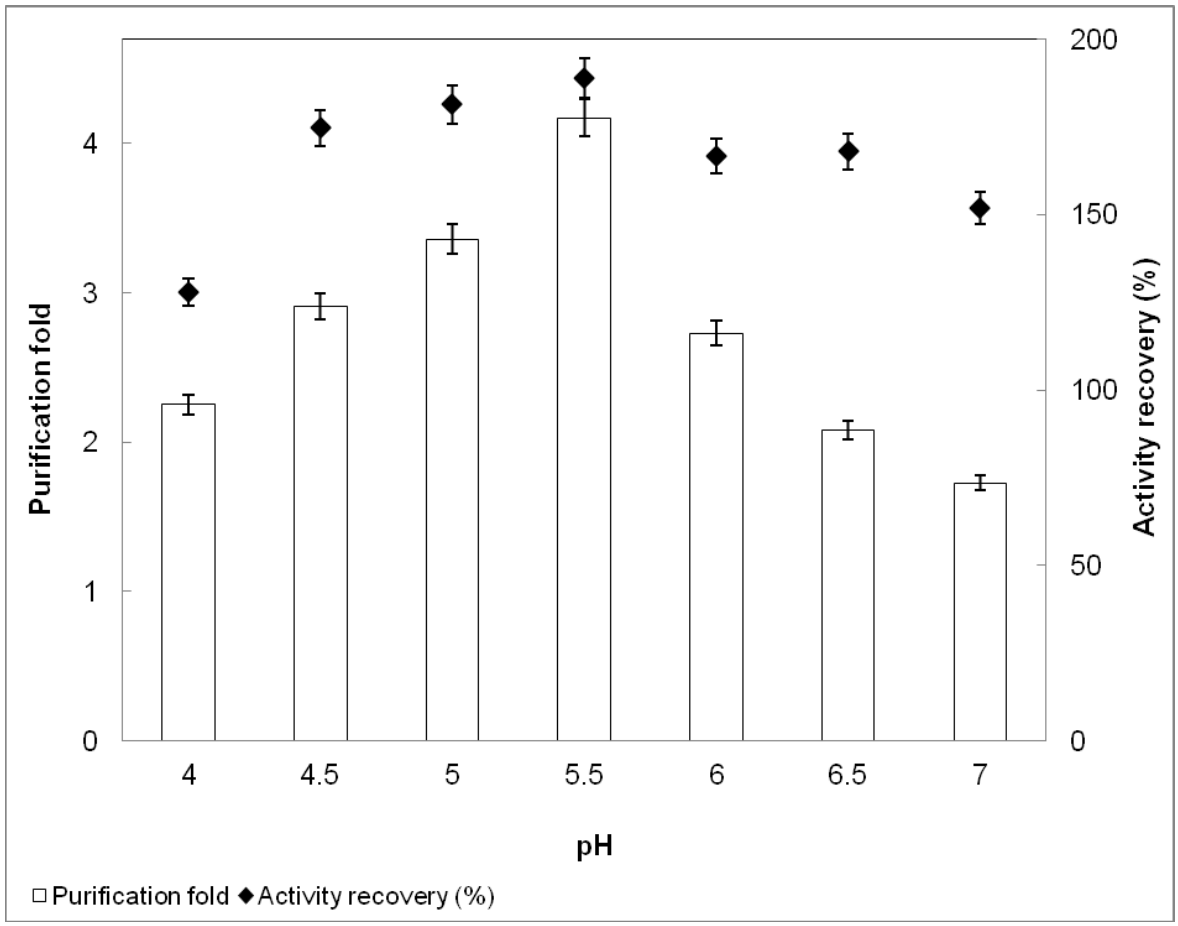

Figure 3. Influence of pH on on TPP of *Aspergillus lentulus* α-galactosidase. Ammonium sulfate (55%, w/v) was added to the enzyme extract of α-galactosidase (2 ml containing 0,115 U). The pH of the medium was adjusted to different pH values. This was followed by addition of t-butanol in a ratio of 1:1 (enzyme extract to t-butanol).

difference between two phases decreases. t-butanol could not easily permeate inside the folded protein molecules due to its size and branched structure. Hence, it does not cause denaturation [3-5, 34]. t-butanol also allows significant crowding effects at room temperature due to its kosmotropic properties which not only intensifies the partitioning process but also makes it convenient [3-5].

Effect of pH

The pH of the medium is another key factor that affect the partitioning of an enzyme in TPP. Protein concentration by salting-out depends on the sulfate concentration and also pH dependent net charge of the proteins. In TPP, sulfate anion binds to the cationic sites in the protein and this is significantly influenced by pH. Proteins tend to precipitate most readily at their isoelectric point. Due to electrostatic interactions between the phases and charged protein partitioning of biomolecules in TPP changes with pH [3,4]. Therefore, the effect of pH on partitioning of α-galactosidase should be searched. The effect of different pH (4-7) values on TPP of α-galactosidase was studied at 55% (w/v) ammonium sulfate concentration with crude extract to t-butanol ratio of 1:1 (v/v). TPP behavior of α-galactosidase is illustrated in Figure 3. It can be seen and stated from figure that, the enzyme was partitioned selectively to the aqueous phase at pH 5.5. 55% (w/v) ammonium sulfate saturation with 1:1 (v/v) ratio of crude extract to t-butanol at pH 5.5 gave a maximum 4.2 fold purification and 189% activity recovery of the enzyme. The raised recovery at this pH could be a result of a better conformational stability of enzyme towards t-butanol at that pH. The extraction efficiency was low between pH 4-5 and pH 6-7 as compared to pH 5.5.

The net charge of a protein can be altered by facilitating change in pH and can directly influence the partitioning

Table 1. Overall purification of α-galactosidase from *Aspergillus lentulus* by three-phase partitioning.[a]

Step	Total activity (Unit)	Total protein (mg)	Specific activity (Unit/mg)	Purification fold	Activity yield (%)
Crude extract (80%, w/v, ammonium sulfate fraction)	0.41	1.25	0.33	1.00	100
TPP-interfacial precipitate	0.10	0.90	0.11	0.33	24
TPP-aqueous phase	0.73	0.42	1.74	5.3	178

a The ammonium sulfate (55%, w/v) was added to the enzyme extract of *Aspergillus lentulus* α-galactosidase (2 ml containing 0.115 U) , after then pH was adjusted to pH 5.5. Afterwards, t-butanol was added to the enzyme extract to the ratio of 1:1 (v/v) (crude extract: t-butanol). Three phases were spied on clearly. The upper phase was decanted and then the lower aqueous phase and interfacial precipitate were tested for enzyme activity and protein amount. Each experiment was carried out in triplicate and the difference in the readings was less than ±5%.

performance of biomolecules in the system [35]. In TPP, it is sighted that with an increase in pH beyond the pI infers net negative charge on protein and partitioned it to aqueous phase. So, α-galactosidase has tendency to participitate to the bottom phase that is releated to its structure. Generally TPP systems suddenly change around the isoelectric point of proteins and it is linked to the pH of the system [3,4].

Evaluation of α-galactosidase partitioning

The partitioning and concentration of *Aspergillus lentulus* α-galactosidase with one step TPP is summarized in Table 1. The enzyme is concentrated in bottom phase of the system depending to its structure. α-Galactosidase was efficiently partitioned and concentrated with high activity recovery (178%) and degree of purification (5.3-fold) under best conditions (55% (w/v) ammonium sulfate concentration, 1:1 enzyme extract to t-butanol ratio, pH 5.5).

There are several reports that have been used for purification of various biomoecules like proteins, enzymes, oils, saccharides etc [6,8,11,15,19]. TPP based separations have several superiorities in comparison to conventional techniques; selective partition and concentration of desired enzyme to one phase, inexpensive, recycle of t-butanol, less steps, at room temperature [5,30]. Beside of this, it is possible to concentrate proteins from crude extracts with good activity yields and fold purification than traditional concentration methods. TPP is also as a concentrating and dewatering protocol that several enzymes could increase their catalytic activities in system. Sometimes this protocol may lead to contemporaneous activation of enzyme. The observed activity yield may be due to increased flexibility in enzyme molecule [36]. The results showed that, TPP is a useful and efficient technique for concentration and purification of α-galactosidase from *A. lentulus* under optimized conditions.

Repetition and scale-up of TPP of α-galactosidase

Under optimized partitioning conditions the TPP system was established separately for three times. The results are shown in Figure 4a. The activity recovery values were 162%, 178% and 165%, respectively. For scalling-up, five TPP system under optimized conditions was prepared in different volumes (x1 to x5) as 15 to 75 ml. The activity recovery values were found as 161%, 171%, 165%, 163% and 178%, respectively (Figure 4b). The repeatability and scalling-up of a TPP system is considerable for industrial and economical purposes. The results showed that TPP system for α-galactosidase could be operated by giving similar findings. The bottom phase of TPP obtained from scalling-up was dialysed and used for further biochemical characterization studies.

Biochemical Properties of Partitioned α-Galactosidase

Concentration and partial purification of α-galactosidase from *A. lentulus* has been carried out using TPP. Biochemical characterization of partitioned enzyme which make it a good alternative for useability in several industries such as food, feed, paper, sugar also studied. The features of α-galactosidase concerning remaining activity, the effect of temperature, pH, substrate concentration and different effectors on the activity of enzyme and the effect of temperature and pH on the stability of enzyme were also investigated. The results

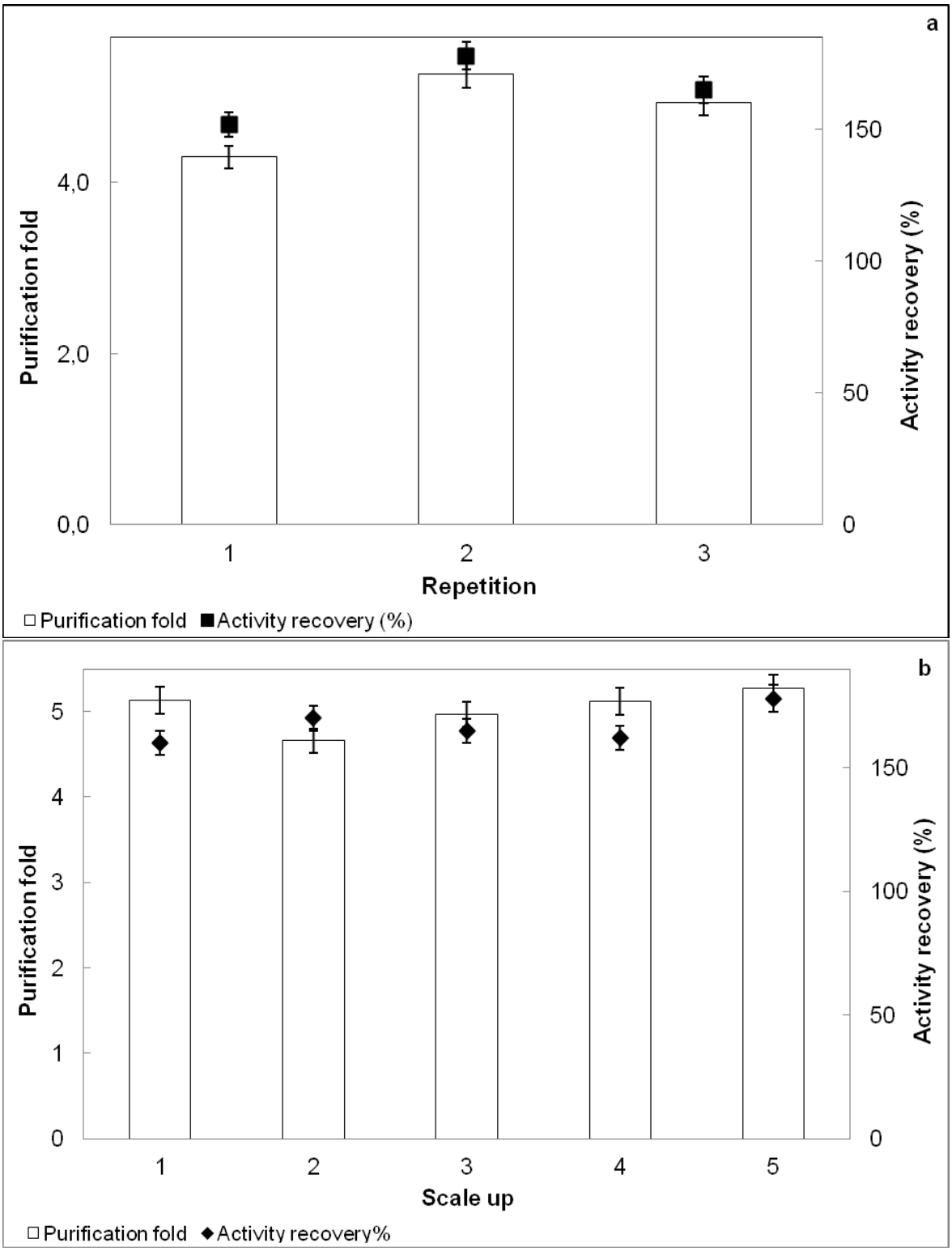

Figure 4. Repetition (a) and scalling-up (b) of TPP for *Aspergillus lentulus* α-galactosidase recovery.

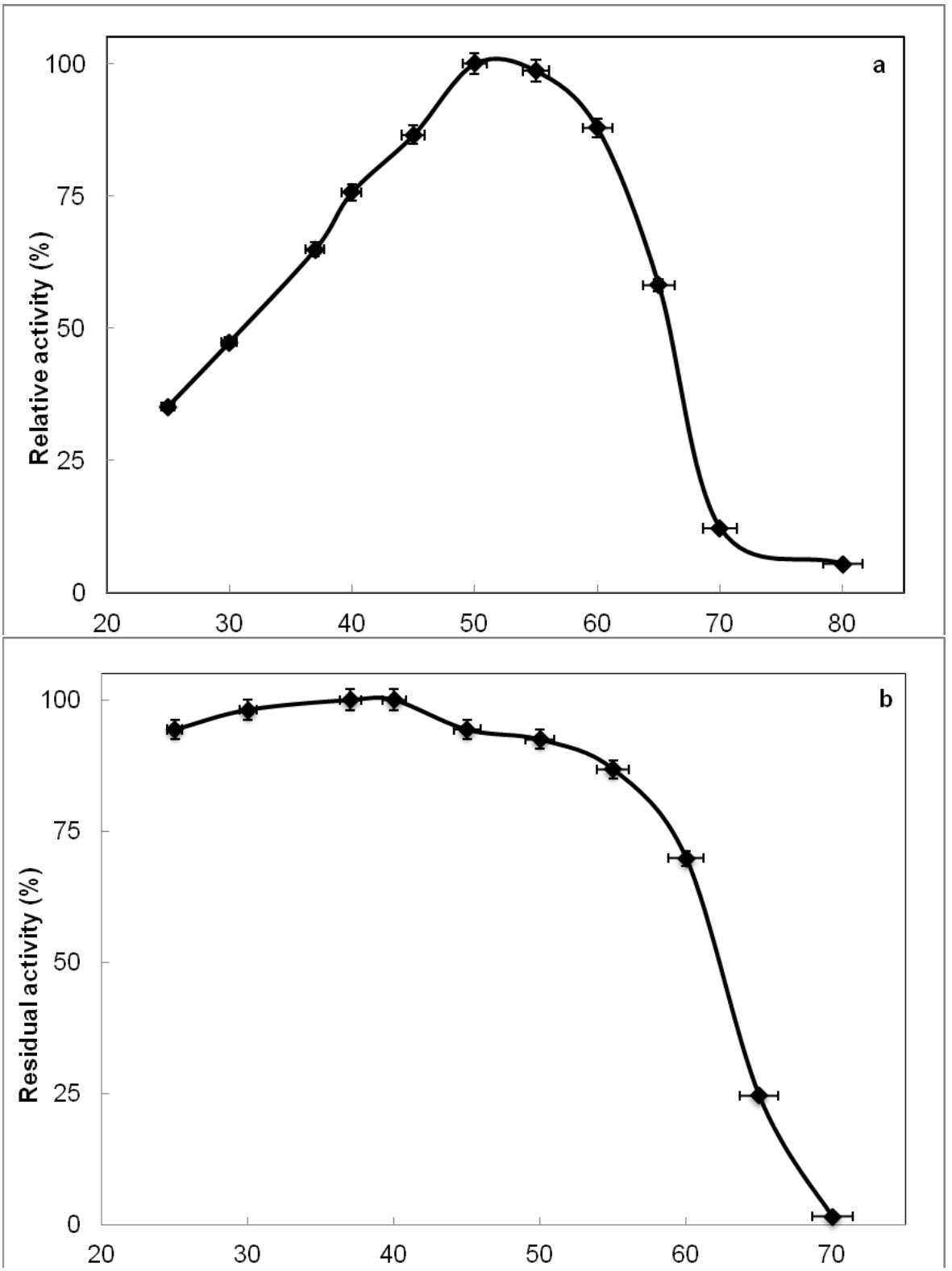

Figure 5. Influence of temperature on the activity (a) and stability (b) of *Aspergillus lentulus* α-galactosidase.

were given in proper sections by regarding figures and tables and evaluated. All characterization experiments were performed in triplicates and the activity determinations are mean value of triplicate assay. The difference in readings was less than ±3%.

Influence of temperature on activity and stability of partitioned α-galactosidase

Three dimensional structure of enzymes must be protected for its activity. Temperature is one important parameter that affect the enzymeatic activity. The effect of temperature on catalytic activity of α-galactosidase was determined in the temperature range of 25-80°C. The maximum activity was observed at 50°C and at temperatures higher than 60°C the enzyme lost its activity quickly(Figure 5a). High temperatures could break the ionic and hydrogen bonds between the aminoacids of the enzyme. This cause denaturation and inactivation of enzyme. Thermostability of α-galactosidase is also studied by measuring the residual activity of the enzyme after incubation at various temperatures (25-70°C) for 30 min. The thermostability of α-galactosidase is very good in the temperature range of 25-60°C and it retains more than 50% of its initial activity at these temperatures (Figure 5b). About 70% of its original activity was maintained at the end of incubation at 60°C for 30 min. This high temperatures are favorable for its industrial application because of lower risk of contamination.

There are several profiles that show the effect of temperature on α-galactosidase activity and stability which is depending to enzyme source. For instance, the optimum activity for F. oxysporum α-galactosidase was found at 35°C. After 1 h incubation at 40°C all the enzyme activity has been retained [23]. Optimum temperature of P. djamor α-galactosidase was reported as 53.5°C and the enzyme was preserved 68% of its initial activity after incubation at 50°C for 1 h [24]. Zhou et al. [23] have informed the optimal activity of recombinant α-galactosidases from Mesorhizobium and Streptomyces were 45°C and 35°C, respectively. The first enzyme was also found as very stable between 35 -50°C. α-Galactosidase from T. leycettamus had a temperature optimum of 70°C and showed good thermostability at 65°C after 1 h incubation and retained more than 60% of its initial activity [37]. P. purpurogenum α-galactosidase have an optimum at 50°C. It is also compared with other fungal α-galactosidases [20]. As most the fungal enzymes have high temperature optimums (50-75°C) and thermostability (40-70°C) for various times [20,37].

Influence of pH on activity and stability of partitioned α-galactosidase

Biochemical reactions taking place in vivo in an aqueous environment, pH affects the activity of the enzyme due to the charge status. Optimum pH of *A. lentulus* α-galactosidase was found as pH 6.5 (Figure 6a). The enzyme was found to retain more than 50% of its activity at a broad pH range of 3.5-7.0. The results revealed that, α-galactosidase is more active in the neutral range as compared to the acidic range. pH stability of an enzyme is affected by many factors such as buffer type, its concentration, ionic strength and also incubation time. As shown in Figure 6b, α-galactosidase is fairly stable between pH range of 2.6-6.5 and remains initial activity more over 50%.

The results compare well with the previous results obtained by otherl authors using different α-galactosidases. Hu et al. [24], indicated that the optimum pH of P. djamor α-galactosidase is pH 5.0 and the enzyme is quite stable over a broad range of pH 3-10. It is known that, several fungal α-galactosidases are active in acidic and/or neutral pH range. Morales-Quintana have showed an optimal pH for P.purpurogenum as pH 5.0. The various α-galactosidases with different pH optimum [24] and pH stability [25] could be available. For instance, recombinant α-galactosidase from Mesorhizobium exhibited more than 70% of its activity within pH range of 7-9 and from Streptomyces exhibited more than 40% of its activity within pHrange of 8 to 10.5 [25].

Kinetic constants of partitioned α-galactosidase

The variation of enzymatic activity with the PNPG concentration in the range from 0.05-1.25 mM was investigated at 37°C to determine the kinetic constants. A Lineweaver-Burk plot of the data indicates that the enzyme follows simple Michaelis-Menten kinetics. KM and Vmax values were estimated and calculated from Lineweaver-Burk graph as 0.365 mM and 0.093 U, respectively (data not shown). The KM value of *A. lentulus* α-galactosidase is in agreement with those presented in literature. KM from other fungal α-galactosidases ranging from 0.11 to 11 mM. This indicate that the enzyme has high affinity towards the PNPG [20,22,23].

Influence of various effectors on partitioned α-galactosidase activity

The effect of various metal ions and sugars on the activity of α-galactosidase was searched. The results are summarized for metal ions and for sugars in Table 2 and

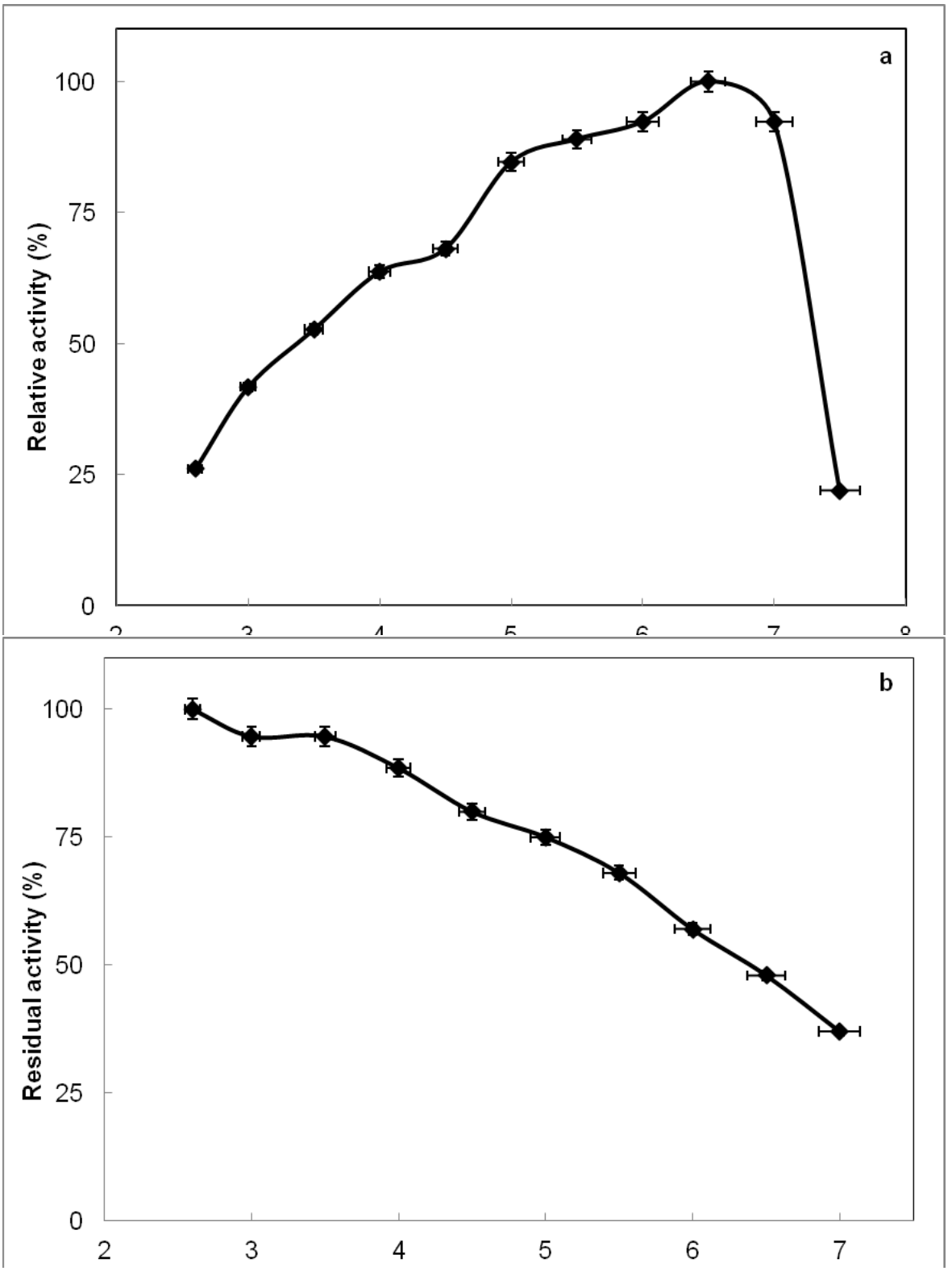

Figure 6. IInfluence of pH on the activity (a) and stability (b) of *Aspergillus lentulus* α-galactosidase.

Table 2. Effects of various ions on the activity of *Aspergillus lentulus* α-galactosidase [a]

Metal ions	Relative activity (%)
Control	100
NaCl	82
$CaCl_2$	105
$MgCl_2$	118
$LiCl^2$	100
$CuSO_4$	145
$MgSO_4$	107
Na_2CO_3	7
$ZnSO_4$	130
$MnCl_2$	118
$LiSO_4$	95
$MnSO_4$	107

a After preincubation of *Aspergillus lentulus* α-galactosidase with different compounds at 10 mM concentration of them at 25°C for 30 min the remaining enzyme activity was measured with standard activity assay procedure. Activity without added compounds taken as 100% activity. Data present the means of three determinations.

Table 3, respectively. As it could be observed from the tables, all these effectors had different effects on the activity of α-galactosidase. As is seen from the Table 2, among metal ions Na_2CO_3 showed a strong inhibitory effect and had nearly 7% of inhibition. $CuSO_4$ and $ZnSO_4$ strongly activated the enzyme and had 145% and 130% activatory effect, respectively. $CaCl_2$, $MgCl_2$, $MgSO_4$, $MnCl_2$ and $MnSO_4$ have slightly activated the enzyme. Beside of this, NaCl and $LiSO_4$ have slightly inhibited the enzyme.

For sugars tested, all sugars have an inhibitory effect with different levels (Table 3). However, galactose strongly inhibited the enzymatic activity. Galactose is known as a common inhibitor of α-galactosidases.

There are various results in literature that α-galactosidases were influenced with different metals at various degrees. Maruta et al. [23] have reported that several metal ions (Ag^+, Hg^{+2} and Zn^{+2}) inhibited the F. oxysporum α-galactosidase completely. The enzyme was moderately inhibited with Co+2 and Cu+2. Recombinant T. leycettanus α-galactosidase was found very resistant to different metal ions and chemicals [22]. The enzymatic activity of α-galactosidase from Mesorhizobium was enhanced by $ZnSO_4$ and $Pb(CH_3COO)_2$ at 0.5-30 mM [25]. Hu et al. [24] have indicated that, α-galactosidase from P djamor was significantly enhanced in the presence of Zn^{+2}, Fe^{+2}, Mn^{+2} and Ca^{+2}. Pb^{+2} and Mg^{+2} showed an inhibitory effect on enzyme

Table 3. Effects of various carbohydrates on the activity of *Aspergillus lentulus* α-galactosidase [a]

Carbohydrates	Relative activity(%)
Control	100
Mellibiose	42
Maltose	89
Lactose	83
Raffinose	76
Glucose	83
Galactose	2
Sucrose	93
Fructose	91

a After preincubation of *Aspergillus lentulus* α-galactosidase with different carbohydrates at 10 mM concentration of them at 25°C for 30 min the remaining enzyme activity was measured with standard activity assay procedure. Activity without added carbohydrates taken as 100% activity. Data present the means of three determinations.

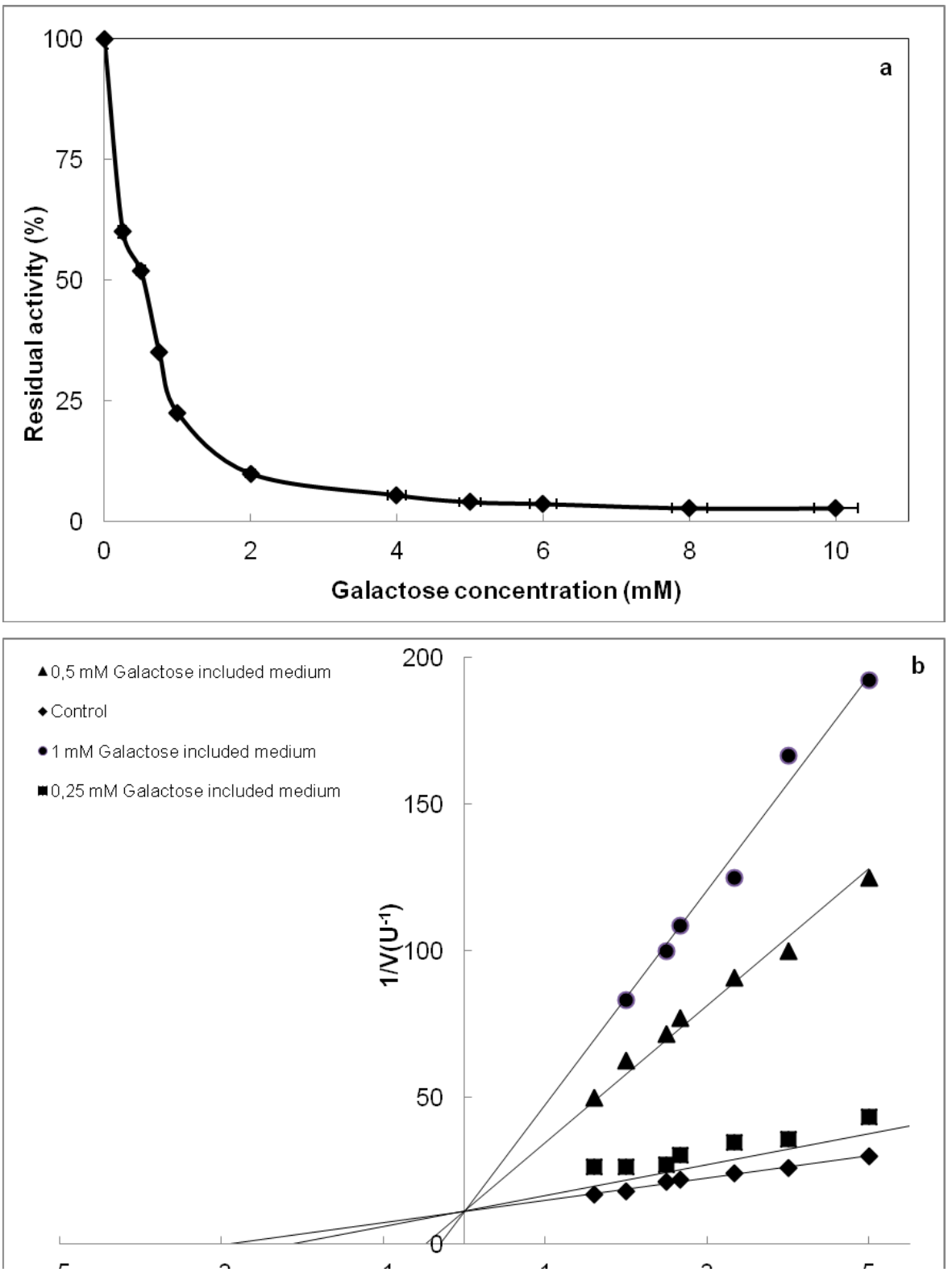

Figure 7. Effect of galactose concentration on the activity (a) of *Aspergillus lentulus* α-galactosidase and Lineweaver –Burk plot (b).

activity. The effects of different sugars on P djamor α-galactosidase activity was also searched. They have found that, the enzyme was severely inhibited with melibiose and galactose [24].

Inhibitory effect and kinetics of galactose on partitioned α-galactosidase

D-Galactose is also an inhibitor of α-galactosidases from microbial sources [20,24,38]. The effect of various concentrations (0.25-10 mM) of galactose on α-galactosidase activity was assayed.

The reciprocals of the activity recovery (%) as a function of the initial galactose concentration is reported in Figure 7a. IC50 value was calculated from graph as 0.42 mM. By using 0.25, 0.5 and 1.0 mM of galactose concentrations the L. Burk plot was drawn (Figure 7b). KI values were calculated from the graph as 0.322, 0.475 and 2.102 mM for 0.25, 0.5 and 1.0 mM galactose concentrations, respectively. Our results showed that, data were fitted to the model of competitive inhibition by galactose. Galactose, one of the products of the catalytic action of α-galactosidases on α-D-galactosides, is reported as being a competitive inhibitor of α-galactosidases from different sources [39-41].

Conclusion

Aspergillus lentulus was found as a good source for α-galactosidase. Fungal α-galactosidases have several advantages such as their extracellular localization, acidic or neutral pH optima and broad stability profiles. Because of this, it is very easy to produce them, have better safety and very suitable for its biotechnological applications. α-Galactosidase was concentrated and simultaneously purified from *A. lentulus* with 5.3 purification fold and 178% activity recovery by TPP under optimal partitioning conditions (55% (w/v) ammonium sulfate concentration with 1:1 (v/v) ratio of crude extract to t-butanol at pH 5.5). TPP as an emerging elegant bioseparation technique has been widely used for the recovery and purification of various enzymes fron newer sources that have many advantages in comparison to traditional separation and purification techniques like fast, simple, scale-applicable and economic. The number of unit operation involved in the classical chromatographic protocols is also reduced. α-Galactosidase from *A. lentulus* was also characterized. Biochemical characterization of the enzyme revealed its superior properties like stability, over a broad temperature and pH range, strong resistance to metal ions and sugars. α-Galactosidase obtained from TPP with these good characteristics could be applied safetly especially in food, feed, sugar, pulp and paper industries.

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