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Mishela Temkov[1,2]
Hristina Strinska[2]
Georgi Dobrev[2]
Elena Velickova[1]
Vlad Muresan[3]
Albert Krastanov[2]

[1] Department of Food Technology and Biotechnology, Faculty of Technology and Metallurgy, Ss. Cyril and Methodius University in Skopje, Skopje, Republic of Macedonia

[2] Department of Biotechnology and Molecular Biology, Faculty of Technology, University of Food Technologies, Plovdiv, Bulgaria

[3] Department of Food Engineering, Faculty of Food Technology, University of Agriculture and Veterinary Medicine in Cluj Napoca, Cluj Napoca, Romania

Research Article

Purification of bacterial inulinase in aqueous two-phase systems

In this study, extracellular inulinase from *Bacillus sp.* 11/3 was partially purified and concentrated using aqueous two-phase system (ATPS). Two different phase forming salts and four types of polyethylene glycol (PEG) were used. Binodal curves and tie-length lines (TLLs) for eight ATPS were developed. For inulinase purification, concentrations of PEG and salt according to binodal curves (between 17 and 26%) were chosen. All ATPSs for inulinase purification were characterized. An ATPS consisted of 26% PEG1000 and 26% $MgSO_4$ was found to be the most suitable for inulinase purification. This ATPS has 28.47% TLL, 1.03 of volume ratio, purification factor of 4.65 fold and recovery yield of 66.17%. On the SDS-PAGE electrophoresis two protein bands with molecular weight of around 24 and 56 kDa were observed. The partially purified enzymes had optimal activity at pH 8.0 and 6.5, optimal temperature at 30 and 70°C and kinetic parameters $K_m = 26.32$ mmol and $V_{max} = 526$ mmol/min.

Keywords: Aqueous-two phase systems / Binodal curve / Inulinase / Polyethylene glycol / Purification

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1 Introduction

Inulinase (EC 3.2.1.7, 1-beta-D-fructan fructanohydrolase) is an enzyme that catalyzes the hydrolysis of inulin into fructose and fructooligosaccharides (FOS) [1]. Inulin is a storage carbohydrate often accumulated in the underground organ of several plants such as *Jerusalem artichoke*, chicory root, asparagus root, dahlia tubers or garlic [1, 2]. The natural substrates of inulinase except inulin include levan and sucrose. This enzyme is widely used for biotechnological and industrial applications in converting inulin into high fructose syrup, production of natural prebiotics – fructooligosacharides, or bioethanol fermentation [3, 4]. Microbial inulinases are usually purified by conventional techniques such as: ammonium sulfate precipitation, dialysis, ultrafiltartion, ionic and affinity chromatography or electrophoresis. These techniques for protein purification include several steps and therefore encounter many drawbacks [5]. Low yields, limited reproducibility, separation of the solids, loss of activity, high consumption of time and energy are among the experienced problems [6]. Therefore, efficient methods for recuperation and purification of fermentation products such as enzymes and biologically active proteins are in demand. An alternative method for separation of biomolecules based on aqueous two – phase systems (ATPSs) was proposed by Albertsson, 1985 [7]. These systems are composed of two matters soluble in water that are immiscible beyond critical concentration [8–12]. Varieties of water soluble maters are used for obtaining ATPS. These maters include: two incompatible polymers; polymer and salt at high ionic strength; polymer and ionic liquids; alcohol and salt [12]. The separation into two phases occurs due to the hydration enthalpy and the entropy net balance [10, 13]. The liquid – liquid interface between the phases has low interfacial tension which makes the system suitable for labile components such as plant cells, enzymes, nucleic acids, organelles, etc. [7]. Partitioning of the desired active compound can be improved by repeating the isolation step [14]. The disadvantage of these systems is the high cost of the polymer and the difficulty encountered during isolation of the biomolecules from the polymer phase which complicates the recycling and reusing the polymer [15, 16].

Ho *et al.*, 2017 [17] used the ATPSs composed of PEG/sodium citrate for carboxymethyl cellulase extractions from *Bacillus subtilis*. Zhang *et al.*, 2015 [18] reported extraction and purification of alkaloids from *Sophora flavescens Ait.* by microwave-assisted ATPS consisting of ethanol and ammonia sulfate. Lipolytic

Correspondence: Mishela Temkov (mishela@tmf.ukim.edu.mk), Department of Food Technology and Biotechnology, Faculty of Technology and Metallurgy, Ss. Cyril and Methodius University in Skopje, Rudjer Boskovic 16, 1000 Skopje, Republic of Macedonia

Abbreviations: ATPS, aqueous two phase systems; **DP**, degree of polymerization; **FOS**, fructooligosaccharides; $\mathbf{M_w}$, molecular weight; **PCA**, principal component analysis.; **PEG**, polyethylene glycol; **TLL**, tie line length; $\mathbf{V_R}$, volume ratio

enzymes were purified by Ooi *et al.*, 2009 [15] and Ventura *et al.*, 2012 [5] employing ATPSs consisted of alcohol/salt and PEG/salt respectively, while proteases have been purified by Li *et al.* 2010 [19] using PEG/salt systems and Barros *et al.* 2014 [20] using system of PEG/sodium polyacrylate. Ventura *et al.* 2012 [5] went one step ahead by improving the PEG–based systems with hydrophilic ionic liquids forming ATPSs for partitioning the enzymes. A recent paper demonstrates successful application of this method for purification of inulinase from *Kluyveromyces Marxianus* NRRL Y-7571 in PEG/K_3PO_4 ATPS [12]. So far there is a lack of literature regarding the purification of inulinase from *Bacillus sp.* using polymer/salt ATPSs.

In this study the use of ATPS as a separation method was tested for purification of inulinase from *Bacillus sp.* 11/3. The impact of the molecular weight of the polymer and the type of salt were evaluated, as well as their concentrations. The characteristics of the partially purified enzyme are also described. To overcome the problem posed for the recuperation of the enzyme from the PEG phase, in situ immobilization on different supports was used for our further investigation, as an alternative method.

2 Materials and methods

2.1 Materials

The extracellular inulinase was obtained by submerged cultivation from *Bacillus sp. 11/3*. PEG with M_w of 1000, 3000, 6000 and 8000 g/mol were purchased from Merck, Germany. Potassium dihydrogen phosphate (purity ≥99.5%) and magnesium sulfate (purity ≥99.5%) were obtained from Sigma-Aldrich (St.Louis, MO, USA). Inulin from Jerusalem artichoke (DP > 25) was given by Beneo, Belgium. The Bradford reagent, Coomassie Brilliant Blue G-250 and standard protein marker were obtained from Bio-Rad (California, USA), while 4-hydroxybenzhydrazide (PAH-BAH) was purchased from Alfa Aesar (Karlsruhe, Germany). All other chemicals used in the study were of analytical grade.

2.2 Inulinase production

Extracellular inulinase was obtained by cultivation of *Bacillus sp 11/3* in a liquid medium composed of g/L: inulin, 4.0; yeast extract, 4.0; peptone, 4.0; $MgSO_4$, 0.16; K_2HPO_4, 0.8. The pH was adjusted to 8 with 20% $NaCO_3$. The fermentation was conducted in flasks on a rotary shaker at 50°C for 24 h [21]. Then the culture medium was centrifuged at 14 000 rpm for 3 min for total removal of the microbial cells. The supernatant was collected and stored at -18°C until further analysis. The free cell medium was used as a crude enzyme solution.

2.3 Inulinase activity assay

Inulinase activity assay was performed by incubating 1 mL partially purified enzyme solution from top or bottom phase (Section 2.5.2.) with 2% (w/v) inulin prepared in phosphate buffer pH 8.0 at 50°C for 20 min. After incubation, the reaction tubes were kept in a boiling water bath for 10 min. to stop the enzyme reaction and then cooled to room temperature. The reaction mixture was assayed for reducing sugar by a method described by Lever, 1972 [22] with some modifications. 250 μL of the reaction mixture and 1750 μL solution of PAH BAH dissolved in 10% 0.5 M HCl and 90% 0.5M NaOH was boiled for 10 min. After cooling the absorbance at 410 nm was measured by using a microplate reader SPECTROSTAR – Nano (BMG LABTECH). One unit of enzymatic activity was defined as the amount of enzyme needed to liberate 1 μmol of fructose per minute. Assays were performed in triplicate and average value was used in calculations.

2.4 Protein content assay

Protein concentration was determined according to Bradford method [23], using bovine serum albumin (BSA) as a standard.

2.5 ATPS

2.5.1 Phase diagrams

Binodal curves were constructed by turbidometric titration method, described by Kaul, 2000 [24]. Several ATPS were prepared by mixing PEG and salt solutions in 15 mL tubes at 25°C. The mixture was initially turbid, indicating that two phases would eventually form. Distilled water was then added drop by drop, and each drop was followed by gentle mixing, until the turbidity disappeared. The concentrations of the phase-forming components found in the final system were calculated by measuring the total weight of the added distilled water, and the phase-transition points were determined. The binodal curves were then plotted at varying PEG and salt concentrations.

TLL describes the compositions of the two phases, which are in equilibrium and it was calculated as:

$$\mathrm{TLL} = \left[\left(C_P{}^T - C_P{}^B\right)^2 + \left(C_S{}^T - C_S{}^B\right)^2\right]^{1/2} \quad (1)$$

where, $C_P{}^T$ and $C_P{}^B$ are the concentrations of PEG in top phase and bottom phase and $C_S{}^T$ and $C_S{}^B$ are the concentrations of salt in the top and bottom phase. The concentrations of PEG was analyzed by dry matter (Moisture Analyzing Balance PCE-MA 100), while the concentration of salt in both phases was calculated from the standard conductivity curve (% w/v), using conductometer (WTW Cond 720). The dry weight contribution was subtracted from the weight of the sample.

2.5.2 Purification of inulinase using ATPS

All ATPSs were prepared in 12 mL graduated centrifuge tubes by weighing an appropriate amount of 50% (w/w) stock solution of PEG, 40% (w/w) stock solution of salt and 20% (w/w) crude feedstock of total mass weight. Distilled water was then added to each system to obtain a final mass of 6 g. Each ATPS contained PEG (1000, 3000, 6000, 8000), salt (potassium pfosphate, magnesium sulfate) and cell-free fermentation broth in different concentrations determined by binodal curves. Eight ATPS were developed, each consited of 25 sub systems made of

PEG/salt in concentrations varying from 17 to 29% with 3% increment. The contents were mixed thoroughly at 25°C. The complete phase separation was achieved by centrifugation of each system at 4000 rpm for 15 min at 25°C. The volumes of the top and bottom phases were measured, after which both phases were separated for the inulinase assay and a total protein content determination [15].

2.5.3 Determination of partition coefficients, purification factor, yield, enzyme activity recovery, volume ratio and selectivity

The partition coefficient of inulinase and protein (K_E, K_P) were calculated as a ratio of activities or concentrations in the top and bottom phases.

$$K_E = \frac{A_T}{A_B} \tag{2}$$

$$K_P = \frac{P_T}{P_B} \tag{3}$$

where A_T and A_B are the activities of inulinase (μmol/mL·min) while P_T and P_B are the protein concentration (in μg/mL) in the polymer rich top and salt rich bottom phases, respectively [25].

Selectivity (S) was defined as the ratio of the inulinase enzyme partition coefficient (K_E) to the protein partition coefficient (K_P):

$$S = \frac{K_E}{K_P} = \frac{A_T}{A_B} \cdot \frac{P_T}{P_B} \tag{4}$$

where K_E and K_P are the ratios of inulinase enzyme and protein concentrations found in each phase, respectively.

The purification factor (PF) was calculated according to the Eq. (5) [15].

$$PF = \frac{\text{Specific activity of the top phase}}{\text{Specific activity of crude feedstock}} \tag{5}$$

where specific activity is the ratio of inulinase activity to the total protein concentration of the sample.

The phase volume ratio (V_R) is defined as the ratio of volumes of the top and bottom phases. Phase volume ratio is represented as follows:

$$V_R = \frac{V_T}{V_B} \tag{6}$$

where V_T and, V_B are the volumes of top and bottom phases, respectively [25].

The enzyme activity recovery in the top phase and contaminant *protein recovery* in the bottom phase were calculated using the Eqs. (7) and (8), respectively:

$$R_T{}^E\,(\%) = \frac{100}{1 + \frac{1}{V_R K_E}} \tag{7}$$

$$R_B{}^P\,(\%) = \frac{100}{1 + \frac{1}{V_R K_P}} \tag{8}$$

where V_R represents the volume ratio between top V_T and bottom V_B phases, while K_E and K_P are the enzyme and protein partition coefficients between two phases [5, 26].

The yield (η) of inulinase recovered in the top phase (η_T) is an important parameter for examining industrial extraction processes was determined using Eq. (9):

$$\eta = \frac{V_T \cdot A_T}{V_o \cdot A_o} \cdot 100 \tag{9}$$

where V_T is the volume of the top phase in which a higher proportion of inulinase was extracted, A_T is inulinase activity concentration in the same phase, V_O is the volume of fermented broth subjected to ATPSs, and A_O is inulinase activity in the crude extract . The same Eq. (9) was used for computing the yield of inulinase recovered in the bottom phase (η_B) [12].

2.5.4 PCA analysis

All parameters influencing the purification process of the enzyme for the experimental set-up of 200 samples (8 systems × 25 percentage combinations), described in Section 2.5.3., were applied to PCA in order to represent the characteristics of the data, without loss of information. PCA is a useful multivariate statistical technique usually applied to data treatment of high dimensionality. All variables were preprocessed before PCA application making them dimensionless and with proportional influence.

2.6 Characterization of partially purified inulinase

2.6.1 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed in a Bio-Rad (USA) electrophoresis unit as described by Laemmli, 1970, [27] with 10% resolving gel and 4% acrylamide stacking gel. The electrophoresis was run at 200 V and 20 mA for 150 min. Protein samples recovered from the top phase were precipitated using 4% trichloroacetic acid (TCA) solution, and the precipitate was re – suspended in sample buffer. The gel was stained with 0.05% (w/v) Coomassie brilliant blue R-250.

Molecular masses of the proteins were determined by a standard curve prepared with „BIO-RAD Precision Plus Protein Standards, molecular weight 10 000–250 000 Da", BioRad.

2.6.2 Influence of pH on the enzyme activity and stability

Inulinase activity was measured in the pH range 3.6–10.7 by the method described above. The buffers used were acetate buffers (pH 3.6–5.2), phosphate buffers (pH 5.8–8.0), and carbonate-bicarbonate buffers (pH 9.5–10.7). Inulinase pH stability was tested by pre – incubating partially purified enzyme for 120 min at 50°C in the buffers with the same ionic concentrations and pH values from 3.6 to 10.7. After the incubation the residual inulinase activity was measured at standard conditions.

2.6.3 Influence of temperature on the enzyme activity and stability

Inulinase activity was measured at different temperatures (20, 30, 40, 50, 60, 70, 80 and 90°C) by the method described above. The temperature stability of the partially purified en-

zyme was tested by pre-incubating it at a temperature range of 20–90°C for 120 min. After the incubation the residual inulinase activity was measured at standard conditions.

2.6.4 Kinetics studies

To obtain K_m and V_{max} of inulinase, 5 mL of 0.5, 1.0, 1.5, 2.0, 2.5 or 3.0% inulin was hydrolized with 0.05 mL of the purified inulinase in 0.1 M sodium phosphate buffer (pH 8.0) at 40°C for 20 min. The reaction was terminated at 100°C for 15 min. A Lineweaver-Burk plot was generated to obtain the K_m and V_{max}, and the values were expressed as the mean of the triplicate experiments.

3 Results and discussion

3.1 Phase diagrams of PEG/salt ATPSs

Binodal curves show the areas of concentration for one – phase forming and two – phase forming systems. The phase diagrams of PEG with various molecular weights (1000, 3000, 6000, 8000 g/mol) with the two types of salts (KH_2PO_4 and $MgSO_4$) are shown in Fig. 1A and B. Figure 1 shows the influence of molecular mass of the polymer in ATPSs composed of PEG/KH_2PO_4 and PEG/$MgSO_4$. As seen, with the increase of the molecular mass (binodal D), the minimal concentrations of both polymer and salt for the formation of ATPSs decrease and the binodal curve moves to the water rich region of the phase diagram. This phase separation at lower concentrations could be explained by the reduction of Gibbs free energy of the mixture process by the addition of CH_2 segments to the polymeric chain [28]. The partition driving force (K) of phase separation is linear dependent to the molecular weight of the polymer [29]. Johansson *et al.,* (2011), [29] reported same results for their ternary phase diagrams consisted by PEG (2000, 4000 g/mol), NaPAA and Na_2SO_4 (3, 6 wt%).

The type of the phase forming salt has also an influence on the phase diagrams. The salt increases the mixing enthalpy so that separation is favored [29]. Results reported by Gupta *et al.* (2002) [30] showed that the binodal curve moves to the water-rich corner in a phase diagram when the salt ion was replaced from nitrate to sulfate and even more to phosphate for a fixed molarity of the salt. These findings are in favor of constructing an aqueous two phase systems containing a salt and a polymer that can move the binodal curve closer to the water rich region in a phase diagram due to the lower cost, lower viscosity and biocompatibility. The charge, hydrophobicity and the concentrations of the ions strongly influence the efficiency of the separation and the shape of the binodal curve. The behavior of phase separation depends on the substitution degree of water-cation hydration associations by PEG-ether oxygen-cation interaction, leading to salt-depleted zones and resulting phase formation [31].

In our research the sulfate ions decreased the amount of one – phase region a little bit more than the phosphate ions for the same concentration of the stock solution of the phase forming salt. This is due to the low solubility of potassium hydrogen phosphate in higher concentrations and the noticed effect of crystallization.

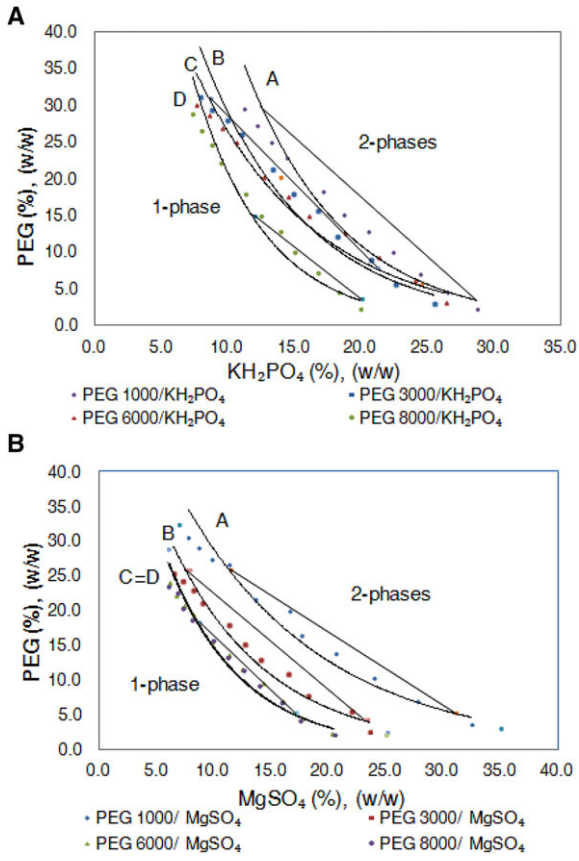


Figure 1. Phase diagrams of PEG/salt ATPSs. Binodal curves of PEG with different molecular weights (A – 1000,◊); (B – 3000,■); (C – 6000,▲); (D – 8000,●) were plotted against (A) KH_2PO_4, (B) $MgSO_4$. The continuous lines are a fitted model for experimentally measured minimal concentrations of the top and bottom phase forming a two phase system. The experiments were measured at 25°C.

TLL calculated for systems with different molecular weights of PEG and the two types of salt are given in Table 1. Increasing the TLL could cause salting out effect which will induce protein precipitation on the interface of two phases if the molecular weight of the polymer is high or the salt concentration is high [32]. This effect was observed in the system composed of PEG 8000 and KH_2PO_4 with the tie line length of 30.83% (Table 1). TLL is also a characteristic for the stability of the ATPSs – the longer TLL is, the system is more stable [24]. As seen from Fig. 1, according to TLL, the most stable ATPSs were with PEG 1000 for both salts.

3.2 Selection of ATPS system

3.2.1 Principal component analysis

PCA calculations were carried out in order to obtain the optimal composition of the APTS in relation with all variables. A PCA plot of 200 samples is presented in Fig. 2. One fictional optimal system was assumed having the highest values of each variable

Table 1. Tie line length (TLL) of various ATPSs at 25°C

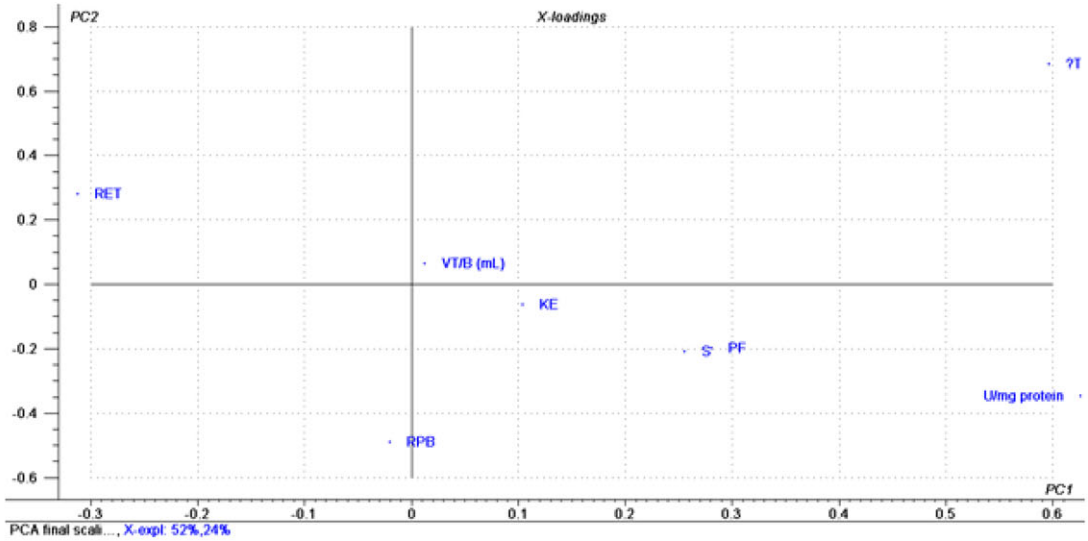
System composition				
PEG (M_w)	Type of salt	TLL (%)	% PEG in the top phase	% of salt in the bottom phase
1000	KH_2PO_4	16.76	28.6	29.8
3000	KH_2PO_4	19.83	21.2	30.9
6000	KH_2PO_4	25.93	24.5	20.3
8000	KH_2PO_4	30.83	14.8	20.0
1000	$MgSO_4$	28.47	31.0	25.9
3000	$MgSO_4$	26.61	23.0	26.6
6000	$MgSO_4$	15.62	17.1	18.3
8000	$MgSO_4$	15.62	17.1	18.3

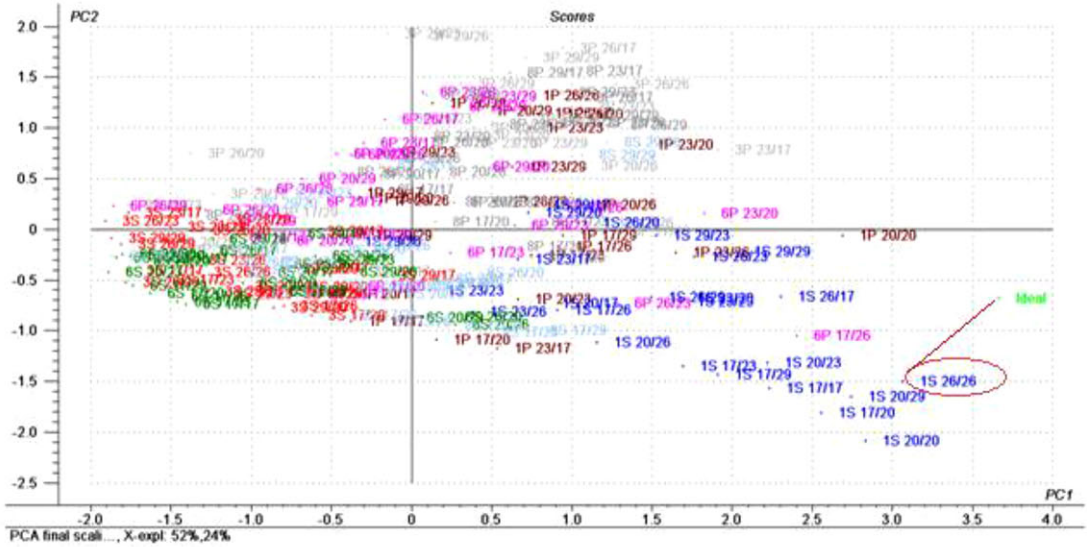
obtained from different systems during the optimization. It can be seen that the samples are grouped according to the PEG molecular weight and the type of salt. In Table 2 the best concentrations out of 25 subsystems of each PEG/salt systems are presented for enzyme purification. However, almost all 25 samples of the system PEG 1000/$MgSO_4$ were suitable for purification, expressing high values for the specific activity, purification factor, partition coefficient and selectivity. They were placed in the closest area to the "ideal" system of the PCA plot. In addition, some samples of the system PEG 1000/KH_2PO_4 exhibit good purification values but they are more scattered on the plot. Systems containing PEG with M_w 3000, 6000 and 8000 have good recovery of the enzyme but low specific activity, purification factor and selectivity.

3.2.2 *Effect of the phase forming polymer*

Polymer characteristics such as molecular weight and concentration play an important role in partitioning behavior of the biomolecules in ATPSs. In order to select a suitable molecular weight of phase forming polymer for inulinase purification, PEG molecular weight was varying in the ATPSs, while the concentration of the phase forming salt and fermentation broth were kept constant. The highest purification factor of 4.65 and selectivity of 18 were obtained with ATPS 26% (w/w) PEG 1000 and 26% (w/w) $MgSO_4$ (Table 2, Figs. 3 and 4). As seen from the Table 2, and Fig. 4 partitioning of inulinase in PEG/KH_2PO_4 or PEG/$MgSO_4$ ATPSs was strongly dependent on M_W of PEG and the highest partition parameters were obtained by PEG 1000. The enzyme was distributed in the top phase ($K_E > 1$) while contaminant proteins were partitioned in the bottom phase ($K_P < 1$) which indicates purification of the enzyme. This partitioning behavior of the enzyme in regard of the molecular size of the polymer was achieved due to the lower interfacial tension. As the molecular mass of PEG increases, the efficiency of the purification decreases. This can be explained with the volume exclusion effect. Namely, the increase of molecular mass will increase the chain length which will cause free volume reduction. Due to this effect, as the M_W of PEG increased towards 8000, inulinase shifted to the bottom phase (Fig. 3). On the other hand, hydrophobic character of PEG increases and proteins with more hydrophobic amino acids will show higher affinity for PEG phase [33]. As the M_W of PEG increases from 1000 to 8000, K_E values decreased from 6.96 to 3.17 (Table 2) and there was precipitation of inulinase on the interface. Partitioning of the biomolecule is better achieved when low molecular weight polymer is used due to its low interfacial tension [33], whereas the reduced free volume in high molecular weight polymer directs protein molecules towards the salt-rich bottom phase and decreases the partitioning coefficient [34]. Low molecular weight polymer has a shorter chain and a hydrophilic end group which reduces hydrophobicity. According to Pico *et al.,* 2007 [35] PEG with low M_W may interact strongly with proteins, while PEG of higher M_W have the ability to form intramolecular bonds. On the contrary, a PEG with a low M_W demands for a higher quantity of the polymer and increases costs. Saravanan *et al.* 2008 [36] investigated the molecular weight of the polymer in partitioning of myoglobin and ovalbumin and found out that the increasing M_W led to decreasing of partition coefficient. In addition, when M_W of PEG increased from 4000 to 10 000, the partition coefficient of myoglobin decreased from 15.77 to 4.3 whereas for ovalbumin the decrease was from 5.51 to 3.09. Hemavathi and Raghavarao, (2011) [37] studied a wide range of PEG molecular mass (400, 1000, 1500, 4000, 6000 and 8000) for partial purification of α-galactosidase and β-glucosidase from barley. They reported that PEG with molecular mass 1500 had better partitioning performances for both enzymes, while using high molecular weight PEG, both the enzymes partitioned to bottom phase. Similar to that, Strinska *et al.*, (2016) [38] purified lipase from *Rhizopus arrhizus* with ATPS composed of PEG (400, 4000, 6000)/$(NH_4)_2SO_4$. The best results in terms of purification factor (5.37 fold) and yield (31.6%) were obtained with 30% (w/w) PEG 4000 and 15% (w/w) $(NH_4)_2SO_4$.

PEG concentrations (17–29%, w/w) had also influence on K_E and K_P. An ATPS 26% (w/w) PEG1000 and 26% (w/w) $MgSO_4$ resulted in high purification factor (4.65 fold) and K_E of 6.96 (Fig. 4). Similarly, high purification factor (4.75 fold) and K_E of 2.22 were obtained when inulinase was partitioned in 20% (w/w) PEG 1000 and 20% (w/w) KH_2PO_4 (Table 2, Fig. 2). If the concentration is higher, there are more polymer units in the system; hence more hydrophobic interactions between PEG and protein are possible. Regarding the PEG concentrations Mehrnoush *et al.,* (2011) [39] showed similar results for serine protease isolated from Kesinai (*Streblus asper*) leaves. Intermediate PEG concentrations (16%, w/w) gave better results





Legend

1 P – PEG 1000 / Potassium Phosphate
3 P – PEG 3000 / Potassium Phosphate
6 P – PEG 6000 / Potassium Phosphate
8 P – PEG 8000 / Potassium Phosphate
1 S – PEG 1000 / Magnesium Sulfate
3 S – PEG 3000 / Magnesium Sulfate
6 S – PEG 6000 / Magnesium Sulfate
8 S – PEG 8000 / Magnesium Sulfate

Figure 2. PCA of the experimental set-up as described in Section 2.5.2.

in partitioning than lowest (8%, w/w) and highest (21%, w/w) concentrations.

3.2.3 *Effect of the phase forming salt*

The other parameter that has an impact on partitioning of target biomolecule is the type of salt and its concentration used to form two phases in ATPSs. The salt has the ability to provide an environment for hydrophobic interactions between molecules. The most common salts used in ATPSs are phosphates, sulfates, citrates. Anions, in order $SO_4^{-2} > HPO_4^{-2} >$ acetate are more effective in partitioning than cations in order $NH_4^+ > K > Na^+ > Mg^{2+} > Ca^{2+}$ [40, 41]. Gupta *et al.*, (2002) [30] explained the effect of salt in ATPSs through their bondage with the water. In fact, they reported that the structure of the water around anions is radially through the O–H bond direction, leaving three other H-bonding vectors for interaction, whereas, around cations, the water orientation is with the dipole axis, colinear with the center

Table 2. Characteristics of the best ATPSs composed of PEG (MW: 1000, 3000, 6000, 8000)/salt (KH_2PO_4, $MgSO_4$) according to PCA

Molecular weight of PEG (M_w)	System composition (%)	U/mg protein	$V_{T/B}$ (mL)	PF	K_E	K_P	R_{PB}	R_{ET}	η_B	η_T	S
PEG/KH_2PO_4											
1000	20/20	2268.52	1.03	4.75	2.22	0.49	66.25	31.69	31.04	97.91	18.95
3000	23/17	2174.68	3.30	2.46	4.92	0.50	37.70	40.15	5.63	91.40	9.82
6000	17/26	3331.00	1.69	0.42	3.00	0.11	84.59	36.09	14.07	71.48	27.89
8000	26/29	2036.53	1.20	0.53	1.07	0.52	61.66	52.83	85.15	96.05	2.06
PEG/$MgSO_4$											
1000	26/26	3522.74	1.03	4.65	6.96	0.37	72.07	12.92	9.20	66.17	18.57
3000	29/17	1176.65	2.10	0.53	0.95	0.45	82.42	33.35	20.51	41.00	2.13
6000	26/20	1460.94	1.32	2.10	2.77	0.33	69.60	32.26	8.68	31.83	8.40
8000	29/26	1208.17	1.24	1.52	3.17	0.52	60.65	28.14	23.85	94.02	6.08

$V_{T/B}$, volume ratio between top and bottom phase; PF, purification factor; K_E – K_P, partition coefficient of enzyme and proteins, respectively; R_{PB} - R_{ET}, recovery of proteins in the bottom phase and enzyme in the top phase respectively, η_B - η_T, yield in the bottom and top phase, respectively; S, selectivity.

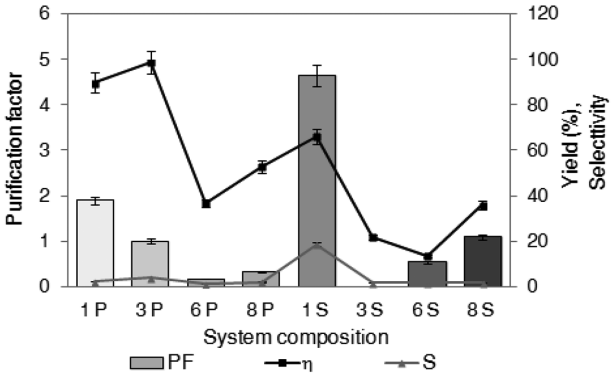


Figure 3. Purification of inulinase by ATPSs 26% PEG/26% salt at 25°C: 1P - PEG 1000/ KH_2PO_4, 3P - PEG 3000/ KH_2PO_4, 6P - PEG 6000/ KH_2PO_4, 8P- PEG 8000/ KH_2PO_4, 1S - PEG 1000/ $MgSO_4$, 3S - PEG 3000/ $MgSO_4$, 6S - PEG 6000/ $MgSO_4$, 8S - PEG 8000/ $MgSO_4$.

of the ion. The partition coefficients of inulinase were higher when using ATPSs PEG/KH_2PO_4 in general, than the ones from ATPSs composed of PEG/$MgSO_4$. This is due to the fact that KH_2PO_4 enhance hydrophobic interactions between molecules. The best partitioning results with 4.75-fold purification factor, inulinase yield 97.91% and selectivity of 18.95 were observed in ATPS composed of 20% (w/w) PEG 1000 and 20% (w/w) KH_2PO_4 (Table 2, Fig. 2). Also, when using $MgSO_4$ in a system with the same M_W of the polymer the purification factor (4.65 fold) and selectivity (18.57) were of great significance, but the yield was considerable lower (66.17%) (Table 2). Despite the fact that KH_2PO_4 gave better results in terms of yield, this salt crystalizes at higher concentrations, causing significant aggregation of proteins. Moreover, the partition coefficient K_E for the system composed of PEG 1000/ KH_2PO_4 is lower (2.22) than the one for the system PEG 1000/ $MgSO_4$ (Table 2). For further investigations an ATPS 26% (w/w) PEG 1000/26% (w/w) $MgSO_4$ was chosen.

The salt concentration has also influence on the partition effect (Fig. 5). If salt concentration is high, the ionic strength increases in the bottom phase which improves biomolecule partition to the top phase or to the interface due to the salting out effect. In general, proteins with positive charge are distributed in the bottom phase, while those with negative charge tend to go in the upper phase [40,42]. When KH_2PO_4 or $MgSO_4$ concentration increased from 17 to 29%, the top phase volume decreased and the bottom phase volume increased. Almost all contaminant proteins retained in the bottom phase. The K_E and PF of inulinase increased with an increase in salt concentration from 17 to 26% (w/w) (Fig. 5). However, there was a decrease in partition parameters when the concentration of salts was increased to 29% (w/w). It can be noticed that the augmentation of salt increased the salting out effect, so that both contaminant protein and enzyme were more partitioned to the top phase which led to decrease in the purification factor and enzyme yield (Fig. 5). Another explanation is that both, the enzyme and the contaminant protein had similar hydrophobic properties on their surfaces [39]. Comparable results were reported by Karkas and Onal, (2012) [42] who purified invertase from yeast with the system PEG/$MgSO_4$, where the salt concentration varied in the range of 15–25% (w/w), and the best partition behavior was shown at 23% (w/w). In other hand, when hydrophobic lipase from *Burkholderia pseudomallei* was purified with an ATPS the highest purification factor of 13.5 and yield of 99% was achieved using 16% (w/w) 2-propanol, 16% (w/w) K_3PO_4 and 4.5% (w/v) NaCl [15].

3.3 Characterization of inulinase purified at optimal concentrations

3.3.1 Determination of molecular weight of partially purified inulinase fractions

In order to estimate the approximate molecular weight of the partially purified inulinase enzyme, SDS-PAGE was performed with standard protein marker. Protein bands were stained with 0.05% (w/v) Coomassie brilliant blue R-250. The molecular weight of inulinase was determined from a standard curve, that was plotted as logarithm of molecular weight of the marker (M_w) versus distance (R_f) moved by each of the protein markers (mm). Inulinase was partially purified by optimal ATPS (Section 3.2.), and the enzyme was distributed in the PEG – rich phase (Lane 3, Fig. 6A,

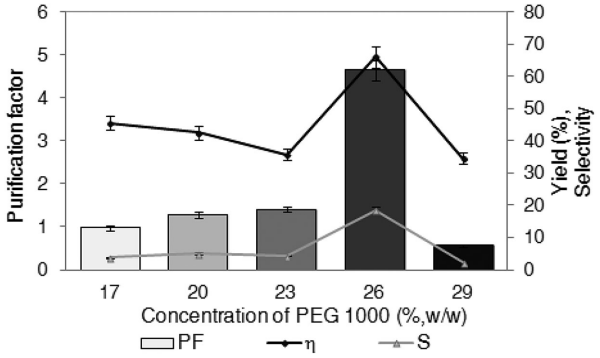


Figure 4. Purification of inulinase in ATPSs at 25°C. Concentrations of PEG 1000 vary from 17 to 29% (w/w), concentration of $MgSO_4$ is constant and equal to 20% (w/w).

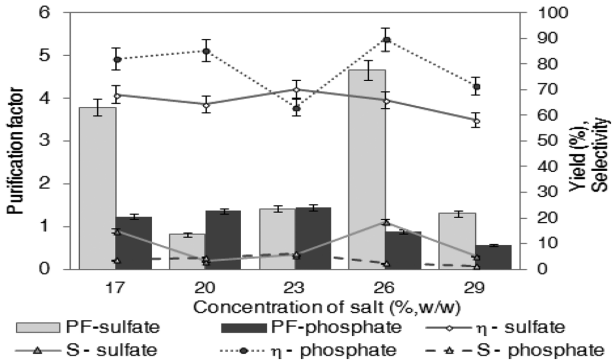


Figure 5. Purification of inulinase in ATPSs at 25°C. Concentration of PEG 1000 is constant and equal to 20% (w/w), concentrations of $MgSO_4$ and KH_2PO_4 vary from 17 to 29% (w/w).

whereas the contaminant proteins were distributed in salt-rich phase (Lane 4, Fig. 6A). In Lane 3 two protein bands are observed which might be multiple forms of the enzyme. The molecular weights were around 37 and 61 kDa, respectively. It was reported that microbial inulinases have molecular weight of around 50 kDa. Hence, Gong *et al.*, (2008) [43] purified inulinase from marine yeast *Pichia guilliermondii* and found molecular weight of 50 kDa, while *Cryptococcus aureus* derived inulinase was estimated to 60 kDa, reported by Sheng *et al.*, (2008) [44]. Inulinases produced by bacterial strains have similar molecular weights to those produced by yeasts. The molecular weight of exo-inulinase from *Geobacillus stearothermophilus* KP1289 was found to be 54 kDa [45].

3.3.2 Effects of pH on enzyme activity

pH profile of partially purified inulinase showed two pH optimums which might indicate two multiple forms of inulinases (Fig. 6B). Two peaks were observed with maximum enzyme activity. The peak with the higher activity was observed at pH 8.0 with 409.2 U, while the peak with the lower activity was noticed at pH 5.8 with 275.8 U (μmol/mL·min). There was complete loss of activity at lower acidic pH, although there was slight activity at pH 5.2.

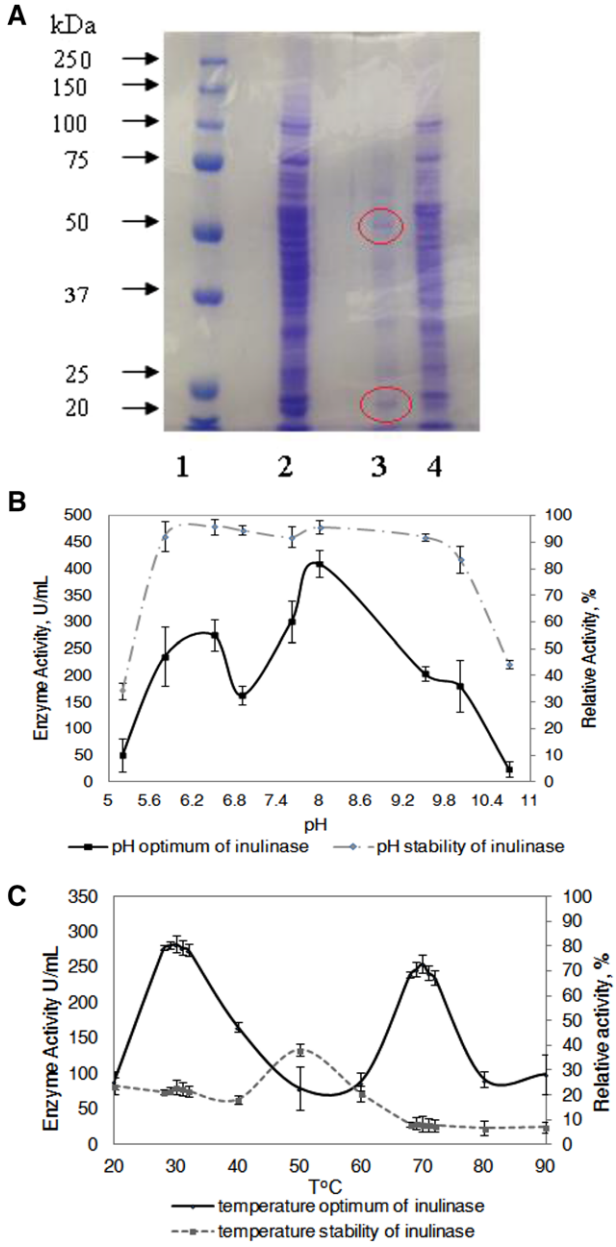


Figure 6. Characteristics of purified inulinase: (A) SDS-PAGE electrophoresis of inulinase from *Bacillus sp.*11/3: 1. Protein markers, 2. Cultural broth. 3. Top phase of ATPS 26% PEG 1000/ 26% $MgSO_4$, 4. Bottom phase of ATPS 26% PEG 1000/ 26% $MgSO_4$, (B) Effects of pH optimum and stability on the enzyme activity, (C) Effects of temperature optimum and stability on the enzyme activity.

Laowklom *et al.*, (2012) [1] reported pH optimum in broad range from 5.5 to 9.0 for inulinase isolated from *Streptomyces* sp. CP01, Sheng *et al.*, (2008) [44] and Gong *et al.*, (2008) [43] observed pH optimum at 6.0 for extracellular inulinases obtained from marine yeasts *Cryptococcus aureus* G7a and *Pichia guilliermondii*, while Meenakshi *et al.*, (2013) [46] showed results for inulinase extracted from bacterium *Bacillus cereus* MU – 31 of

pH 7.0 which was equal either for enzyme production or enzyme activity.

The enzymes have shown stability over pH range of 5.8–10.5 at 50°C retaining their activity up to 95.5% after 2 h of incubation. However, when partially purified inulinases were incubated at values lower than 5.8 or higher than 10.5 for 2 h, the residual activity decreased substantially, demonstrating that the enzymes were not stable outside pH range 5.8–10.5.

3.3.3 *Effects of temperature on enzyme activity and stability*

The temperature profile of partially purified inulinase with ATPSs is shown on the Fig. 6C. As expected, two peaks with maximum enzyme activity were observed at temperature of 30 and 70°C with 283.2 U (μmol/mL·min) and 255.3 U (μmol/mL·min), respectively. Microbial inulinases has temperature optimums of wide range: inulinases isolated from *Streptomyces sp.* CP01 (55°C), *Cryptococcus aureus* G7a (55°C), *Pichia guilliermondii* (60°C), *Bacillus cereus* MU – 31 (30°C), *Kluyveromyces marxianus* var. *bulgaricus* (55°C). [1, 43, 44, 47].

Inulinase thermo stability was measured by incubating the enzyme in phosphate buffer pH 8.0 for 120 min and the residual activity was determined. At 30°C the residual activity remained 23.1% of the maximal after 2 h, while at 70°C after 2 h it decreased to 8.4%. The enzymes were most stable at 50°C retaining its previous activity of 38.2%. In general, the residual activity remained around 20% from 20 to 60°C, after which it dropped substantially to 7%. The loss of activity at 50°C after 1 or 2 h was also reported in the literature [48, 49].

3.3.4 *Kinetics parameters*

The Lineweaver–Burk plot was used for determination the kinetic parameters. K_m was found to be 26.32 mmol and V_{max} – 526 mmol/min. Values for K_m and V_{max} obtained for inulinase isolated from *Streptomyces* sp. CP01 were 2.34 mmol and 440 mmol/min [1]. Our result demonstrated that inulinase from *Bacillus sp. 11/3* does not display affinity toward the inulin as high as inulinases obtained from *Streptomyces* sp. CP01.

4 Concluding remarks

In this study phase diagrams containing binodal curves and TLLs of ATPSs with PEG with different molecular weights and $MgSO_4$ and KH_2PO_4 were developed. Purification of inulinase from *Bacillus sp.* 11/3 in ATPS consisted of 26% PEG1000 and 26% $MgSO_4$ was found to be the most suitable. In this case high purification factor (4.65), selectivity (18.57) and inulinase yield (66.17%) was achieved. On the SDS-PAGE two protein bands (24 and 56 kDa) were separated which indicate multiple forms of inulinase. The partially purified enzyme had pH optimum at 8.0 and 6.5, optimal temperature at 30 and 70°C and kinetic parameters Km = 26.32 mmol and Vmax = 526 mmol/min.

Practical application

ATPS is an alternative economical and high yielding method for purification of biomolecules. The conventional techniques such as hydrophobic interaction chromatography and ultrafiltartion of proteins are costly, low yield and multistep techniques. They are not appropriate methods to apply on large scale due to batch processing and large pressure drops. Proteins are poorly soluble in organic solvents; therefore organic liquid-liquid extraction is also not suitable for protein purification. The research is focused on protein recovery from crude feedstocks by ATPS in downstream processing. ATPS technique has potential in purification and recovery of: enzymes, monoclonal antibodies, viruses, DNA and nucleic acids, cells and organelles, drug residues in food and water, low molecular weight compounds, metal and metal ions, extractive fermentation etc. ATPS have both industrial and analytical applications.

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