

A PRIMARY APPROACH FOR SEPARATION AND CHARACTERIZATION OF α -AMYLASE FROM WHITE PITAYA (*HYLOCEREUS UNDATUS*) PEELS BY POLYMER/SALT TWO PHASE SYSTEM

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ABSTRACT

α -Amylase was isolated from white pitaya peel with two phase system in aqueous media and then characterized. The effects of polyethylene glycol (PEG), PEG concentration, molecular weight, sodium citrate, and sodium chloride (NaCl) on yield and purification factor were studied. The highest purification factor (4.5) and yield (83%) were obtained in system PEG 6000 (14% w/w)-sodium citrate (18% w/w) with 6% (w/w) NaCl. The purified α -amylase molecular weight exhibited a band above 40 KDa by SDS-PAGE. α -Amylase showed optimum activity at pH of 6.0 and temperature of 55 °C. The activity of this enzyme was stable in the pH scope of 6-8 and temperature scope of 10-55 °C. Various metal ions were evaluated for amylase activation/inhibition effect. Na⁺ and Ca²⁺ were exhibited to have appropriate activating effect, where as Mn²⁺, Zn²⁺, Fe³⁺, Ni⁺, Ba⁺ and Cu²⁺ had inhibition effect. In addition, this enzyme showed remarkable stability in the existence of TritonX-100, SDS, sodium perborate, Tween 20 and Tween-80. In conclusion, this enzyme has high stability and activity at suitable temperature and pH, and in the existence of different surfactants and metal ions. This approach has a potential in progress of various industrial applications by introducing a method for applying new, low-cost, and biocompatible amylases source.

Keywords: Aqueous two-phase system; Amylase; Characterization; *Hylocereusundatus*; Yield

INTRODUCTION

α -Amylase is one of the major components for converting starch through catalyzing the hydrolysis of 1,4-a-D-glucosidic linkages, directly in polysaccharides (Hashemi *et al.*, 2013). They comprise 30% of the enzyme wholesale in the global trade (Van Der Maarel *et al.*, 2002). Produced enzymes through this process, have countless applications in many industries including foods, detergents, pharmaceuticals, and animal feeds (Wang *et al.*, 2011). Amylase enzymes can be found in microorganisms, plants and even animal tissues. Nowadays, α -amylases which were derived from bacterial sources mainly from genus *Bacillus* are the most abundant in market and considered among enzymes that are more available commercially and used mainly in industry (Kelly *et al.*, 1995). However, this amount of production is not enough to meet the needs of the industry. This is mainly due to the growing demand for this compound. Furthermore, about 15% of the world's population is affected by the allergic effects of these compounds (Gómez *et al.*, 2018). Therefore, the discovery of new sources is essential for producing this valuable compound.

Pitaya fruit (dominant agricultural product in *Cactaceae* family), also commonly known as dragon fruit, has received significant attention in the recent years due to their economic value, striking color, and health benefits (Hani *et al.*, 2015). Pitaya peels are mainly discarded during processing, especially in the production of beverages (Bakar *et al.*, 2011). This results in environmental problems and the treatment of which waste puts a great burden on the industry. The peel comprises nearly 33% of a complete fruit (Wu *et al.*, 2006; Tenore *et al.*, 2012), which can be used as a substantial, economical and achievable source for producing enzyme, naturally.

Aqueous Two-Phase Systems (ATPS) is a fast, high throughput, low-priced and simple technique which was used for purification of biological products i.e. proteins, enzymes, and antibiotics through mixing two immiscible compounds in a non-organic solution such as polyethylene glycol (PEG) and dextran or PEG and salt more above a critical concentration to attain a distinct liquid-liquid phase (Zhang *et al.*, 2016). Furthermore, ATPS as a first purification step can remove contaminants such as nucleic acids and undesirable proteins (Fernandes *et al.*,

2020). Therefore, ATPS has become an appealing method with industrial application as it is more quick and results in improved efficiency with better purity for separated enzyme (Andersson *et al.*, 1985).

Each industrial process needs precise properties of the bio-catalyst, hence the characterization of enzyme is necessary and vital for defining their correct biotechnological application. Amylase characteristics such as specificity, stability, temperature, and pH dependence of amylase are important properties for its applications in industry (Souza, 2010). Amylases having the optimum activity in acidic pH are applied primarily in baking industries and glucose syrup, while those that are active at alkaline pH have been used in the formulation of laundry detergents (Ghorbel *et al.*, 2009). Extraction and purification of thermo labile compounds was studied in various sample matrices with different techniques. Recently, members of our research group applied polymer based materials for extraction and purification of penicillin G from fermentation broth (Javanbakht *et al.*, 2012), and modified cellulose acetate membrane for extraction of melamine from dry milk to identify its possible adulteration (Akbari-adergani *et al.*, 2017). With the aim of using the benefits of aqueous two-phase purification as an efficient extraction technique, in other study we reported a response surface methodology modeling for purification of amylase from white pitaya peel (Shad *et al.*, 2018). In this study we focused on the purification of amylase separated from white pitaya peel using PEG/sodium citrate ATPS as well as its characterization. Effect of molecular weight of the polymer, concentration of PEG, concentration of sodium citrate, and NaCl on the purification factor and yield of the enzyme were investigated. Beside this, effects of pH and temperature, metal ion and detergents on amylase activity and stability were also examined.

MATERIAL AND METHODS

Plant Materials and Chemicals

White pitayafruits (*Hylocereusundatus*) with the weight of 400-500 g were purchased from the retail marketplaces in Selangor, Malaysia. They were collected based on no apparent defects, being in the same size and shape and when it was

reach under enough ripened economical maturity stage. The fruits were preserved freshly in a refrigerator at 4 °C, before their application in the extraction phase. All chemicals and reagent were selected with enough purity. Bovine serum albumin (BSA), Triton-100, Tris-HCl, 3, 5-dinitrosalicylic acid (DNS) and Tween 80 were supplied from Sigma Chemical Co. (St. Louis, MO, USA). Bradford reagent was supplied from Amresco (AMRESCO LLC, Solon, OH, USA). Polyethylene glycol (g/mol) was obtained from Fluka. Co. (USA). Citric acid monohydrate (C₆H₈O₇·H₂O), Monobasic sodium phosphate (NaH₂PO₄·H₂O), sodium hydrogen phosphate dihydrate (Na₂HPO₄·2H₂O), maltose, trisodium citrate dihydrate (C₆H₅Na₃O₇·2H₂O), sodiumdodecyl sulfate (SDS) and N, N, N, N-Tetramethylethylenediamine (TEMED) were bought from Merck (Darmstadt, Germany) while soluble starch and potassium tartrate sodium salt (NaKC₄H₄O₆·4H₂O) were supplied from Fisher Scientific. Acrylamide/Bis mixed solution was obtained from Nacalai Tesque (Kyoto Japan).

Feedstock Preparation

Preparation of white pitaya peel was performed according to Shad et al. procedure with slightly modification (Shad et al., 2018). Briefly, it was washed and disinfected completely in the first step and after enough cutting and homogenizing in phosphate buffer, it was filtered thoroughly. The filtrate phase preserved at refrigerator at 4 °C for the subsequent cleanup step (Kumar et al., 2011).

Preparing Aqueous Two-Phase System

Preparation of two-phase system was performed according to Ng et al. procedure with some modifications. Briefly, a mixture of sodium citrate and PEG was prepared in various PEG molecular weight and different PEG concentration as well as in different citrate and NaCl concentrations. Then appropriate amount of feedstock was added to the system and mixed thoroughly. Homogenization of the prepared solution was performed by an IKA® vortex mixer (Genius 3, VG 3 S2) and for phase separation it was centrifuged at 4000 rpm for 10 min. Obviously, a small amount between bottom and upper phases were remained that facilitate removing the separated phases with Mohr pipet. Finally, an aliquot of each phase were analyzed for determination of protein amount and enzyme activity (Ng et al., 2011).

Analytical Test Methods

Enzyme Assay

Enzyme activity test was performed based on the Bernfeld method, with modifications that reported in our previous study (Bernfeld, 1955; Shad et al., 2018). Briefly, A solution containing 0.5 ml of 1.0% w/v starch in phosphate buffer (0.01 M, pH=6.0) and 0.5 ml of the extracted enzyme was prepared. This solution was incubated in a water bath at 55 °C for 10 min and the reaction was stopped by 1.0 ml addition of 3,5-Dinitrosalicylic acid. After an upward and downward heating of the solution, the final volume was adjusted to 12 ml and its absorbance was recorded at 540 nm. Finally, the enzyme activity was recorded as the amount of enzyme under the assay condition that releases 1.0 μmol of maltose per min.

Protein Assay

A colorimetric protein assay according to Bradford reference method by using bovine serum albumin as the standard protein, was applied for accurate determination of protein in the samples (Bradford, 1976).

Determination of partition coefficient, purification factor and yield for purified samples by ATPS

The partition coefficient for purified enzyme was calculated by dividing amylase activity in the top phase (a.t) to its corresponding value in the bottom phase (a.b) according the following equation (Kammoun et al., 2009) :

$$Ke = \frac{a.t}{a.b} \quad [1]$$

Specific activity of the enzyme was recorded by calculation this ratio : the enzyme unit per mL (U/mL) divided by the protein concentration (mg/mL). This was performed according to the following equation (Lawal et al., 2014) :

$$SA[U/mg] = \frac{Totalactivity[U]}{Totalprotein[mg]} \quad [2]$$

For calculating purification factor (PF), the ratio of specific activity in the top phase (a_t) and its corresponding protein concentration (C_t) as well as the ratio of special activity of initial amylase (a_i) and protein concentration in the initial extract (C_i) were recorded and then PF was reported by dividing these two ratios by the following equation: (Mayerhoff et al., 2004; Kammoun et al., 2009)

$$PF = \frac{(a_t/C_t)}{(a_i/C_i)} \quad [3]$$

Amylase production yield (Y_{top} %) in the upper phase was calculated using the ratio of top to bottom phase volumes ($\frac{V_t}{V_b}$) as VR in the following equation (Porfiri et al., 2011):

$$Y_{top}, \% = \frac{100}{1+(\frac{1}{VR.Ke})} \quad [4]$$

SDS-PAGE Electrophoresis

SDS-PAGE as a common electrophoresis technique was used to evaluate samples' proteins following the method of Laemmli (1970). The molecular weight and purity of amylase were investigated using 12% SDS-PAGE electrophoresis. A specific mini protean II cell electrophoresis unit (Bio-Rad Laboratories, Richmond, CA, USA) with a discontinuous buffer system was used to fractionate protein. Coomassie Brilliant Blue R-250 was used to stain the gels for detection of protein after electrophoresis, and a mixture of 7% acetic acid and 40% methanol in a destaining solution was used to destain until a clear background appeared.

Characterization of Aqueous Two Phase Extracted a-amylase

Some variables such as pH, temperature, presence of metal ions and detergents were investigated for their effects on amylase activity and stability.

pH effect

To determine the optimum pH, 0.5 ml of amylase extracted enzyme were incubated with 0.5 mL of soluble starch (1.0 %) prepared in 0.01 M buffers at different pH ranges (3-10). Phosphate buffer, Acetate buffer, glycine-NaOH buffer and Tris-HCl buffer were utilized at several pH intervals i.e. 3.0-4.0, 5.0-6.0, 7.0-8.0, and 9.0-10. The residual activity of amylase enzyme was determined under the assay condition. The stability of the amylase enzyme at different pH values was established by determination of residual activity for the enzyme incubated at pH 3.0-11.0 for 1 h at 37 °C (Wang et al., 2011).

Temperature effect

The optimum temperature for the enzyme activity and stability was studied by measuring the enzyme activity of approximately 0.5 mL of extracted enzyme mixed with 0.5 mL of sodium phosphate buffer (0.01 M, pH= 6.0) containing soluble starch (1.0 %). The residual activity was determined by incubating the reaction mixtures at different temperatures 20-100 °C for 10 min. For determining temperature effect on enzyme stability, the amylase extracted enzyme was pre-incubated at various temperatures for 30 min (20 °C to 100 °C). The residual activity of enzyme was evaluated under enzyme assay conditions mentioned earlier (Aygan et al., 2008).

Metal ions effects

To evaluate the effects of different metal ions on the activity of amylase with the following chemicals in concentration - 10mM: KCl, LiCl, BaCl₂, CaCl₂, MgCl₂, CuCl₂, MnCl₂, ZnSO₄, and FeCl₃ - the amylase extracted enzyme with different metal ions were incubated at 55°C for 10 min and then residual activity was followed by enzyme activity assay (Liu et al., 2006).

Detergent Effects

The effect of four surfactants such as SDS, Tween-20, Tween-80 and Triton X-100 as well as oxidizing agent namely sodium perborate (NaBO₃) on the enzyme stability were analyzed and then tested for the residual activity. The enzyme activity without any additive was taken as 100% (Hmidet et al., 2009).

Statistical Analysis

All experiments were performed using a completely randomized design (CRD) having three replicates, each repeated twice for reproducibility. The one-way analysis of variance (ANOVA) followed by the Turkey's test (when significant differences were found at $P \leq 0.05$) was used for testing the hypothesis about equal means between each data set. The experimental results were recorded as the "means ± standard deviation" of independent trials. The statistical analysis was performed by Minitab V.16 (Minitab Inc., State College, PA, USA).

RESULTS AND DISCUSSION

The results for investigating the effect of several variables such as PEG molecular weight, PEG 6000, sodium citrate and sodium chloride concentrations on

purification factor and amylase production yield were presented in the tables 1 to 4 with the following descriptions:

Effect of PEG Molecular Weight

The PEG effect with different molecular weights (2000-10,000 g/mol) was studied to determine the PF and amylase production yield from initial substrate. As the molecular weight of the PEG increases from 2000 to 6000 g/mol, its surface hydrophobicity increases, resulting in higher PF and yield (Table 1). In other words, PEG corresponding to lesser molecular weight of 6000 may not be an appropriate candidate for the system since this low weight may draw all desired proteins and contaminants to the upper phase that caused low purification factors and poor separation of the protein (Ng et al., 2011).

Higher molecular weights of PEG (>6000), affected negatively the partitioning of the enzyme. This can be explained by the fact that by increasing the molecular mass of PEG, it reduces the volume of the top phase by increasing the length of the PEG polymerchain. It should be noted that for the same concentration of the polymer, there were lesser hydroxyl groups and hence causing increase in the polymer-richer top phase in hydrophobicity (Priyanka, Rastogi et al., 2012; Ibarra-Herrera et al., 2011; Yücekan and Önal, 2011). According to the data obtained, the maximum yield of the enzyme and purification factor were achieved at 6,000 g/mol PEG. This is mainly because of exclusion volume effect that causes the transfer of contaminated protein to the bottom phase and reduces total protein in the upper phase. Accordingly, the final amylase purification factor causes an obvious increase in the top phase.

Table 1 Effect of PEG molecular weight on PF and production yield of amylase

PEG molecular weight (% w/w)	PF	Production Yield (%)
2000	2.30± 0.14 ^d	34.5± 1.71 ^f
3000	3.36± 0.17 ^b	49.7± 0.77 ^d
4000	3.11± 0.11 ^{bc}	63.9± 0.60 ^b
6000	4.17± 0.10 ^a	75.6± 0.53 ^a
8000	2.93± 0.07 ^c	56.7± 0.96 ^c
10000	2.52± 0.12 ^d	39.2± 0.24 ^e

^{a-f} Different lower-case letters show a significant difference among mean values ($p < 0.05$).

Effect of PEG 6000 concentration

Based on the screening results, the effect of PEG 6000 at different concentrations (12-20%) on PF and enzyme production yield was evaluated. As it was illustrated in Table 2, the PEG 6000 concentration shows a significant effect on PF and production yield of amylase ($p \leq 0.05$). The maximum value for PF (4.42) and yield of enzyme production (76.6%) were obtained at 14% (w/w) for concentration PEG. This can be due to the more hydrophobic interaction between PEG and enzyme. Therefore, more amylase can be transferred to the top phase. Similarly, Navapara et al.(2011), Kammoun et al. (2009) and Alhelli et al. (2016) found that the PF and production yield of enzyme were significantly raised by increasing the PEG concentration in the system.

Table 2 Effect of PEG 6000 concentration on PF and production yield of amylase

PEG 6000 concentration (% w/w)	PF	Production Yield (%)
12	2.61± 0.03 ^d	63.7± 2.36 ^c
14	4.42± 0.09 ^a	76.6± 1.49 ^a
16	3.69± 0.05 ^b	70.9± 0.90 ^b
18	3.30± 0.06 ^c	64.4± 0.90 ^c
20	2.04± 0.05 ^e	45.1± 2.14 ^d

^{a-e} Different lower-case letters show a significant difference among mean values ($p < 0.05$).

Effect of Sodium Citrate concentration

The effect of different sodium citrate concentrations (8-20% (w/w)) on the PF and production yield are illustrated in Table 3. A significant increase ($p \leq 0.05$) can be seen in the PF and yield percent of amylase production with only including 18% (w/w) concentration for sodium citrate. Adding salts to the aqueous PEG solution led to the arrangement of ordered water molecules around the PEG molecules because of the molecular bond breaking effects of the water structure (Nalinanon et al., 2009). Moreover the formation and development of the water layer around the cation led into a more compact structure with a little volume of PEG molecule. It was observed that at higher concentrations of sodium citrate, there was an increase in the salting-out effect of salt. As a result, this caused the biomolecule solubility to decrease in the bottom phase, leading to partitioning of biomolecule in the upper phase, hence decreasing the enzyme purity (Amid et al., 2011; Nalinanon et al., 2009; Niphadkaret et al., 2015).

Babu et al. (2008) and Navapara et al. (2011) reported that excess upraising in the salt concentration can affect on the protein precipitation and even on its accumulation at the ATPS interface and also a reduction in the partition coefficient of enzyme may be observed. However, at the lower levels of sodium citrate

concentration where proteins exists in a more solubilized state, the partition coefficient is less, obviously. Our results agree with those of Chavan et al. (2015) and Lu et al. (2013). The maximum yield (83.4%) with a PF value of 4.32 was observed at sodium citrate concentration of 18%. Hence, a system comprising of PEG 6000 (14%) and sodium citrate (18%) was chosen for more investigations.

Table 3 Effect of sodium citrate concentration on PF and production yield of amylase

Sodium citrate concentration (% w/w)	PF	Production Yield (%)
8	3.29± 0.15 ^b	50.4± 2.36 ^{cd}
10	3.53± 0.10 ^b	52.7± 1.49 ^{cd}
12	3.56± 0.06 ^b	53.7± 0.90 ^c
14	4.09± 0.06 ^a	59.7± 0.90 ^b
16	4.10± 0.16 ^a	62.9± 2.14 ^b
18	4.32± 0.09 ^a	83.4± 1.90 ^a
20	2.78± 0.08 ^c	48.7± 1.18 ^d

^{a-d} Different lower-case letters show a significant difference among mean values ($p < 0.05$).

Effect of NaCl concentration

The effect of sodium chloride concentration (0-15% (w/w)) on the PF and production yield was evaluated for 14% PEG 6000/ 18% sodium citrate system. It can be seen in Table 4 that with increase of the salt concentration from 0 to 6% (w/w) caused an increase in the purification factor and production yield of amylase. Thus, the maximum purification factor (4.57) and yield (83) were achieved in the system with a 6% (w/w) NaCl concentration, because more amylase was partitioned to upper phase due to the chemical potential of solutes replacement that was applied by the salt. This implies the addition of 6% (w/w) NaCl which has caused positive effect towards the ATPS of amylase. It can be stated that the addition of neutral salts (i.e. NaCl) in general, will affect partitioning in ATPS by (i) accelerating the separation phase, (ii) reducing protein hydrophobicity, or (iii) affecting the phase potential (Guo-qing et al., 2005). However, the more concentrations of neutral salts (NaCl) over 6% may thoroughly denature proteins that exist in the system. This is mainly due to the presense tremendous amount of water molecules that intensely bound to the salts. Therefore, the interactions between protein molecules become stronger than that among water and protein molecules (Kianmehr et al., 2014). Amid et al. (2012) reported the same observation in the purification of serine protease extracted from mango peel using similar aqueous two phase system.

Table 4 Effect of NaCl concentration on PF and production yield of amylase

NaCl concentration (% w/w)	PF	Production Yield (%)
0	3.38± 0.10 ^c	50.7± 1.88 ^c
2	2.49± 0.05 ^e	52.1± 0.71 ^c
4	4.05± 0.06 ^b	75.4± 1.08 ^b
6	4.57± 0.03 ^a	83.0± 0.82 ^a
8	3.20± 0.11 ^c	46.7± 1.08 ^d
10	2.88± 0.14 ^d	41.7± 0.82 ^e
15	1.93± 0.03 ^f	28.6± 0.71 ^f

^{a-f} Different lower-case letters show a significant difference among mean values ($p < 0.05$).

SDS-PAGE analysis of purified amylase

The SDS-PAGE method was used to evaluate the purification degree of the resulting enzyme preparation after ATPS extraction (Laemmli, 1970). The standard protein marker is shown in Fig. 1 (Lane 1). In Lane 2, crude enzyme showed a dense intensity of bands representing large amounts of contaminant proteins and impurities in the crude extract. However, fainter and lesser bands, comparing to crude feedstock, were shown in Lane 3 that contains the bottom phase of ATPS. In Lane 4, which contains the top phase of the ATPS, it can be seen a thicker and distinct band above 40 kDa. It represents the purified amylase from the crude extract of white pitaya peel.

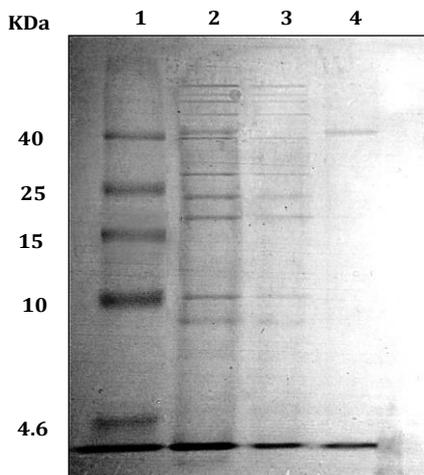


Figure 1 SDS-PAGE analysis. Lane 1: Molecular weight marker; Lane 2: crude enzyme amylase; Lane 3: ATPS bottom phase; Lane 4: ATPS top phase

Characterization of Aqueous Two Phase Extracted α -amylase

The results for the effect of several variables such as pH, temperature, metal ions, surfactants and oxidizing agents on amylase activity and stability were presented in the figures 2 and 3 with the following descriptions:

Effect of pH

In general, α -Amylases have good stability when the pH is within the range of 5.5 to 8.0 but there can be exceptions on both sides, especially in microbial-originated enzymes (Mohamed et al., 2010). In this study, the optimum pH for hydrolysis of starch by amylase was found to be 6.0. The protein retains above 50% of the activity in the pH range of 5-8 (Fig. 2a). The effect of pH on amylase stability was evaluated by measuring the residual activity of the enzyme after 1h incubating in different pH buffer solutions at 37 °C. It was found that the extracted enzyme was very stable when kept at pH between 6 and 8, maintaining more than 70% of the initial activity (Fig. 2b).

Effect of temperature

The enzyme activity was determined at different temperatures from 10 to 100°C and the results are presented in Fig. 2(c). The enzyme maintained its activity up to 50% in a range of temperatures from 45–75 °C. It was found that the enzyme exhibited the highest activity at 55°C, and this activity continued to increase with gradual rise in temperature up to 55 °C. Any increase in the temperature beyond 55 °C resulted in a steady drop in the activity, demonstrating the enzyme’s loss of active conformation. In addition, at the evaluated temperature, enzyme begins to denature. These findings are in agreement with other reports on purified amylase enzyme from plants (Biazus et al., 2009; Khemakhem et al., 2013; Mohamed et al., 2014). According to the current findings, heat stability was assessed in a wide temperature range (Fig. 2d). After 30 min pre-incubation at different temperatures, amylase retained more than 70% of its activity at a wide range of temperature from 10 to 55 °C; but, incubating at higher temperatures quickly inactivated the enzyme.

Effect of metal ions

Metal ions are able to catalyze an enzyme reaction through many ways such as modification of the flow of electron in the reaction of enzyme substrate or with the alteration in the substrate orientation with respect to the functional group that is located at active site. Metal ions can provide or receive electrons and therefore act as electrophiles. They can mask nucleophiles to stop undesirable side reactions that bind enzyme and substrate by coordinating bonds. They can also hold the reacting groups in the required 3D molecular orientation, and hence stabilizing the enzyme’s catalytically active conformation (Palmer and Bonner, 2007). The significant effects of several metal ions were tested at 10 mM concentration for amylase activity at pH= 6.0 and temperature 55 °C ($p \leq 0.05$) by adding the respective cations to the reaction mixture and the outcomes are shown in Fig. 3(a). Ca^{2+} and Na^+ have an active effect in increasing the activity by 10% and 6%, respectively.

It is a known fact that α -amylases in plants and animals are metalloenzymes containing a Ca^{2+} -binding domain which is crucial in stabilizing the tertiary structure (Berbezy et al., 1996; Greenwood and MacGregor, 1965; Vallee et al., 1959). The role of Mg^{2+} and Ca^{2+} in the maintenance of α -amylase stability and structure has been reported (Parkin, 1993). This outcome suggests that calcium is required for the optimal enzyme stability and activity (Noman et al., 2006).

Enhancing the amylase activity of Ca^{2+} cations is on the basis of its ability to interact with amino acid residues with negative charge including aspartic and glutamic acids, which helps to stabilize and maintain the enzyme conformation. Additionally, calcium has been observed to play a part in binding the substrate (Mohamed et al., 2009). It is also reported that Ca^{2+} binding to amylase is popularly favoured over other cations like Mg^{2+} (Bush et al., 1989). Li^+ , K^+ and Mg^{2+} did not affect the amylase activity, while Mn^{2+} , Zn^{2+} , Fe^{3+} , Ni^+ , Ba^+ and Cu^{2+} led to the reduction of amylase activity. Other divalent cations can also be responsible for the inhibitory effects probably because of the competition for binding sites of calcium, while Mg^{2+} and monovalent cations might compete weakly to bind calcium (Shaw and Oulee, 1984). The inhibitory impact of tested metals might be because of their binding to either catalytic residues or by substituting the Ca^{2+} from the binding site of the enzyme substrate (Elarbi et al., 2009; Muralikrishna and Nirmala, 2005).

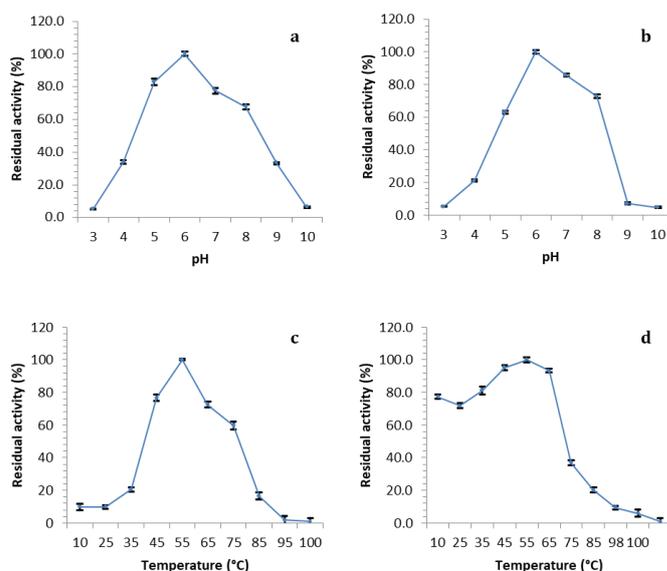


Figure 2 Effect of pH on amylase activity (a) and stability (b), and effect of temperature on amylase activity (c) and stability (d)

Effect of surfactants and oxidizing agents

In order for a detergent to have an effective washing behavior, the forming enzyme must exhibit stability and compatibility with other involving detergent compounds (i.e., oxidizing agents, surfactants, and other similar additives) which are found in many detergent formulations (Gupta et al., 2002). The stability was assessed by incubating the enzyme extract with surfactants and oxidizing agents (SDS, Tween-20, Tween-80, Triton X-100 and sodium perborate) at 1% w/w for 1 h at 40 °C. The amylase is very stable when non-ionic surfactants such as Triton X-100, Tween-20 and Tween-80 are present (Fig. 3b).

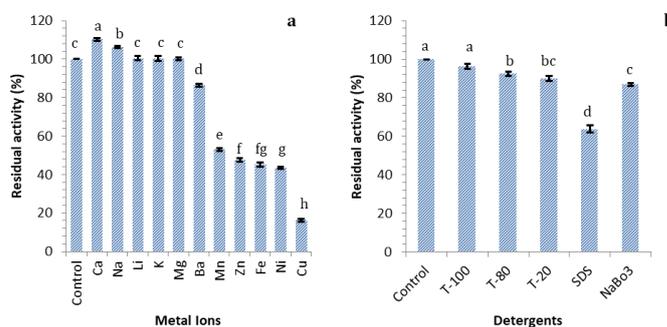


Figure 3 Effect of metal ions on amylase activity extracted from white pitaya peel a-h significant ($p \leq 0.05$) different (left), and Effect of surfactants and oxidizing agent on amylase activity from white pitaya peel a-d significant ($p \leq 0.05$) different (right)

Moreover, the enzyme has relative stability in the presence of a strong anionic surfactant (i.e. SDS); as it can be seen the enzyme was able to retain about 64% of its initial activity after incubation in the presence of 1% SDS. The α -amylase also shows relative stability toward the oxidizing agent, and is able to retain 87% of its initial activity after being incubated for 1 h in the presence of sodium perborate (1% w/v). According to the current results, amylase activity showed a reasonably

good stability in the presence of different detergents, which makes it suitable to be used for liquefaction of starch and particularly in the detergent industry.

CONCLUSION

In this study, we addressed a primary approach for separation, partial purification and characterization of amylase from white pitaya peel. The methodology and results explored the effects of some process parameters on amylase extraction. It was shown that using a low-cost yet an efficient technique; enzyme can be purified with 83% yield and 4.5 purification factor. It was concluded that PEG concentrations, the PEG molecular weight, NaCl as well as citrate concentrations, are major parameters which play a significant performance in amylase partitioning into the upper PEG phase. The optimal system consisted of 14% (w/w) PEG 6000, 18% (w/w) sodium citrate and 6% (w/w) NaCl for amylase purification. These results also showed that ATPS can be employed as one of the potential preliminary step in the amylase purification due to its low-cost and environmental friendly nature. The molecular weight of amylase was illustrated to be 42 KDa. In addition, enzyme characterization studies revealed that purified amylase by this polymer/salt extraction system has been found to be thermostable, active under wide pH ranges and stable in the presence of an oxidizing agent and surfactant, making this enzyme an helpful biocatalyst with potential application in various industrial uses. This amylase which is obtained from a rich and cheap source could be used as a valuable enzyme in food, detergents and other industries.

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