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ARTICLE *in* JOURNAL OF CHROMATOGRAPHY. B, BIOMEDICAL APPLICATIONS · MAY 2015

DOI: 10.1016/j.jchromb.2015.04.034

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Journal of Chromatography B, 993–994 (2015) 1–8

Contents lists available at ScienceDirect

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A novel liquid/liquid extraction process composed of surfactant and acetonitrile for purification of polygalacturonase enzyme from *Durio zibethinus*

Mehrnoush Amid[a,*], Yazid Manap[a,b], Farhana Azmira[a], Muhaini Hussin[a], Zaidul Islam Sarker[c]

[a] Department of Food Technology, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
[b] Halal Products Research Institute, Universiti Putra Malaysia (UPM), 43400 Serdang, Selangor, Malaysia
[c] Department of Pharmaceutical Technology, Faculty of Pharmacy, International Islamic University Malaysia, 25200 Kuantan, Pahang, Malaysia

ARTICLE INFO

Article history:
Received 7 September 2014
Accepted 23 April 2015
Available online 1 May 2015

Keywords:
Novel liquid/liquid extraction process
Surfactant
Acetonitrile
Recycling of phase components
Polygalacturonase
Durio zibethinus

ABSTRACT

Polygalacturonase is one of the important enzymes used in various industries such as food, detergent, pharmaceutical, textile, pulp and paper. A novel liquid/liquid extraction process composed of surfactant and acetonitrile was employed for the first time to purify polygalacturonase from *Durio zibethinus*. The influences of different parameters such as type and concentration of surfactants, concentrations of acetonitrile and composition of surfactant/acetonitrile on partitioning behavior and recovery of polygalacturonase was investigated. Moreover, the effect of pH of system and crude load on purification fold and yield of purified polygalacturonase were studied. The results of the experiment indicated the polygalacturonase was partitioned into surfactant top rich phase with impurities being partitioned into acetonitrile bottom rich phase in the novel method of liquid/liquid process composed of 23% (w/w) Triton X-100 and 19% (w/w) acetonitrile, at 55.6% of TLL (tie line length) crude load of 25% (w/w) at pH 6.0. Recovery and recycling of components also was measured in each successive step of liquid/liquid extraction process. The enzyme was successfully recovered by the method with a high purification factor of 14.3 and yield of 97.3% while phase components were also recovered and recycled above 95%. This study demonstrated that the novel method of liquid/liquid extraction process can be used as an efficient and economical extraction method rather than the traditional methods of extraction for the purification and recovery of the valuable enzyme.

1. Introduction

Extraction process is traditionally based on a polymer/salt system, such as PEG/potassium phosphate, or a polymer/polymer system, including polyethylene glycol (PEG)/dextran [1]. The slow segregation [2], the high cost of the polymers [3] and complications in isolating the purified bio-molecules from the polymer [4] phase are the main disadvantages of traditional methods of aqueous two phase systems that employs a polymer/polymer or polymer/salt mixture. In addition, the main drawbacks of the traditional methods of aqueous two phase system are the fact that the phase-forming chemicals cannot be recycled effectively, which results in large consumption of chemical/polymers and high production costs [5]. It has also been widely reported that additional, tedious operations, such as ultra-filtration, diafiltration and crystallization, are needed to remove the phase-forming chemicals/polymers from the desired proteins recovered from these traditional ATPS [6]. To improve the traditional aqueous two phase system, a more economical and environmental friendly aqueous two phase system with the ability to retain the biological activity of enzymes is preferable compared to other conventional aqueous two phase system.

Abbreviations: BSA, bovine serum albumin; DNS, dinitrosalicylic acid; DTAB, dodecyltrimethylammonium bromide; DMRT, Duncan's multiple range test; ANOVA, one-way analysis of variance; PEG, polyethylene glycol; P_F, purification fold; SA, specific activity; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TLL, tile line length.

* Corresponding author at: Department of Food Technology, Faculty of Food Science and Technology, University Putra Malaysia (UPM), UPM, 43400 Serdang, Selangor, Malaysia. Tel.: +60 03 8946 8413; fax: +60 03 8948 5970.
E-mail addresses: Mehrnoush@upm.edu.my, mehrnoush_amid@yahoo.com (M. Amid).

http://dx.doi.org/10.1016/j.jchromb.2015.04.034
1570-0232/

A novel liquid/liquid extraction process composed of a surfactant and acetonitrile overcomes this drawback of the traditional ATPS method. This system makes it possible to create two phases and the surfactant rich top phase and acetonitrile bottom phase can be recycled with high recovery of purified enzyme [7]. The acetonitrile, CH_3CN is a polar organic solvent has an ability to dissolve with water in any concentrations [8]. Acetonitrile has been widely used in many industries such as solvent in manufacture of pharmaceutical [9], used in molding the plastic materials [10], used to extract fatty acids from animal and vegetable oils [11], as a chemical intermediate in perfume production [12], and also as a mobile phase in high-performance liquid chromatography [13]. Thus, the improved (liquid/liquid extraction process) can minimize the overall cost and the separation process of target proteins from phase solution will be simplified. Furthermore, recycling the solution components can minimize environmental pollution. Polygalacturonase is pectin-degrading enzyme complex that is the most abundant among all of the pectinolytic enzymes. It acts as a hydrolytic depolymerizing group that hydrolyzes polygalacturonic acid chains by water addition [14]. It has been reported that the enzyme accounts for 25% of the global food enzyme sales [15]. Polygalacturonase shave been used in many industrial and biotechnological processes, such as fruit and beverage juice clarification, coffee and tea fermentation, oil extraction, wastewater treatment, textile and plant fiber processing and paper making [16]. Durian (*Durio zibethinus*) is one of the important tropical commercial fruits in the world and production of the fruit is increasing due to high demand for durian as a healthy and nutritive table fruit [17]. Durian is typically eaten fresh; however, only one-third of durian is consumable, whereas the seeds (20–25%) and shell are commonly considered waste [18,19]. While, durian seed can be used as a valuable, economical and abundant media source to produce natural enzymes such as polygalacturonase. There are some researches to report of employing of acetonitrile for sample separation [20,21,22]. However, to the best knowledge of the researchers, there is no information regarding the recovery of polygalacturonase from durian seed using liquid/liquid extraction process composed surfactant and acetonitrile up to date. In this study, the feasibility of recovering polygalacturonase by recycling the phase components in a novel liquid/liquid extraction process for the first time was investigated. The partitioning efficiency of polygalacturonase in the liquid/liquid extraction process and the effects of the type and concentration of surfactants, concentration of acetonitrile, composition of surfactants/acetonitrile and crude load and pH were investigated to achieve a high purification factor and yield of the polygalacturonase enzyme. Moreover, the recycling recovery of the surfactant and acetonitrile at each recycling step was also investigated.

2. Material and methods

2.1. Materials

All chemicals and reagent were analytical grade. Bradford reagent, 3,5-dinitrosalicylic acid (DNS), bovine serum albumin (BSA) and polygalacturonic acid were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Triton X-100, Tween 80, Dodecyltrimethylammonium bromide (DTAB), sodium dodecyle sulphate (SDS), acetic acid, sodium citrate, citric acid, D-galacturonic acid, sodium potassium tartrate ($NaKC_4H_4O_6{\cdot}4H_2O$) were obtained from Merck (Darmstadt, Germany). Durian (*Durio zibethinus*) fruits were purchased from local market (Selangor, Malaysia). Ripened durian fruits free of visual defects were selected based on the size uniformity at the same stage of ripening. The fruits were stored in a cold room at 4 °C until used for the extraction procedure.

2.2. Extraction of polygalacturonase

Durian fruits (*Durio zibethinus*, 2 kg) were cleaned and rinsed thoroughly with sterile distilled water and dried with tissue paper. The fruit was de-husked, by cutting along the suture on the back of the lobules. Durian seeds were washed and chopped, then quickly blended for 5 min at 1:6 g/ml sample to solvent ratio (Model 32BL80, Dynamic Corporation of America, New Hartford, CT, USA) with Tris–HCl buffer (pH 6.0) at room temperature. The blended sample was filtered through chessecloth (0.6 mm) and then centrifuged for 10 min at 4 °C [23]. The feedstock was kept in a refrigerator at 4 °C until use for liquid/liquid extraction process experiment.

2.3. Liquid/liquid extraction process composed of non-ionic surfactants and acetonitrile

The systems for purification of polygalacturonase from durian seed were prepared in graduated glass centrifuge tubes, weighing the appropriate amounts of each surfactant, acetonitrile and crude feedstock with concentration of 25% (w/w) in the system. Deionised water was added to the mixture to achieve the final mass of 10 g. After the complete mixing of all components for a given mixture composition, each system was centrifuged at 4000 × *g* for 10 min. After the two phases had become clear and transparent, and the interface was well defined, the bottom phase was carefully removed using a long needle syringe and a pipette for removing the top phase. The volumes of both top and bottom phases were recorded. Subsequently, the samples were then analyzed by polygalacturonase activity assay and the quantification of protein was carried out based on the Bradford analysis. Another tube with the same phase-forming components but without feedstock was prepared as a blank to avoid interference. Acetonitrile was appropriately measured with its appropriate dosage and concentration and all the experiments were carried out at temperature below than 0 °C.

2.4. Phase diagram

The binodal curve, as determined by the cloud-point method described by Albertsson [24], separates the one-phase and two-phase areas. Several liquid/liquid extraction process was prepared by mixing surfactant and acetonitrile solution in each tube. 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 phase-transition points were approximated by measuring the total weight of the added distilled water. The concentrations of the phase-forming components found in the final system were calculated. The binodal curves were then plotted at varying surfactant and acetonitrile concentrations. Tie line length (TLL) describes the compositions of the two phases, which are in equilibrium and it was calculated as follows:

$$TLL = \sqrt{\Delta P^2 + \Delta C^2} \quad (1)$$

where ΔP and ΔC are the difference between surfactant and acetonitrile concentration, respectively, in the two phases. The concentrations of surfactant and acetonitrile were analyzed by refractive index and conductivity measurement, respectively.

2.5. Polygalacturonase activity assay and protein concentration determination

Polygalacturonase activity was measured by determining reduction groups released from polygalacturonic acid as substrate.

The reaction mixture contained the enzyme (0.5 mL) and polygalacturonic acid (0.5 mL) which is dissolved in 100 mM solvent. The mixture was incubated at 70 °C for 1 h in water bath. After incubation, DNS (1 mL) was added to the mixture to stop the reaction and then the sample was boiled for 5 min. The released reducing sugar was determined by spectrophotometry (BioMate™-3, Thermo Scientific, Alpha Numerix, Webster, NY, USA) at 575 nm using galacturonic acid as standard reducing sugar. One unit (U) of enzyme activity is defined as the amount of enzyme that catalyses the release of 1 μmol polygalacturonic acid per minute [25]. The protein contents of samples were determined using dye binding method as described by Bradford [26] and BSA was used as standard.

2.6. Determination of the enzyme partitioning

The partition coefficient (K) of the polygalacturonase was calculated as the ratio of the polygalacturonase activity in the two phases (Eq. (2)):

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

where A_T and A_B are the polygalacturonase activities in units/ml in the top phase and bottom phases, respectively.

The specific activity (SA) was defined as the ratio between the enzyme activity (U) in the phase sample and the total protein concentration (mg) (Eq. (3)):

$$\text{SA (U/mg)} = \frac{\text{Enzyme activity (U)}}{\text{[Protein] (mg)}} \tag{3}$$

The selectivity (S) was defined as the ratio of the lipase enzyme partition coefficient (K_e) to the protein partition coefficient (K_p) (Eq. (4)):

$$S = \frac{K_e}{K_p} \tag{4}$$

The volume ratio (V_R) was defined as the ratio of volume in the top phase (V_T) to that in the bottom phase (V_B) (Eq. (5)):

$$V_R = \frac{V_T}{V_B} \tag{5}$$

The purification fold (P_{FT}) was calculated as the ratio of the lipase specific activity in the top phase to the initial lipase specific activity in the crude extract (Eq. (6)):

$$P_{FT} = \frac{\text{SA of phase sample}}{\text{SA of crude stock}} \tag{6}$$

Yield of lipase in top phase was determined using (Eq. (7)):

$$Y_T\,(\%) = \frac{100}{1 + (1/V_R * K)} \tag{7}$$

where K is partition coefficient and V_R is the volume ratio [27].

2.7. Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 6% stacking gel and 12% resolving gel were used to analyze the samples from crude extract, top and bottom phase in liquid/liquid extraction process. Samples were diluted in a sample buffer and heated at 100 °C for 5 min. Electrophoresis was run at 50 V and 12 mA for 1 h. The use of coomassie brilliant blue R-250 staining method followed by destaining in a solution containing 40% (v/v) methanol and 10% (v/v) acetic acid allowed the detection and observation of desired protein bands [28].

Table 1
Effects of various phase compositions on the polygalacturonase activity of *Durio zibethinus*.

Phase composition	Concentration (%,w/w)	Polygalacturonase activity
Triton X-100	20	100.1 ± 1.33 [a]
	40	103.2 ± 0.01[a]
	60	130.4 ± 0.21[b]
	80	110.2 ± 0.00[c]
Tween 80	20	99.3 ± 0.11[a]
	40	97.1 ± 0.21[b]
	60	106.4 ± 0.22[c]
	80	98.6 ± 0.57[ab]
DTAB	20	86.6 ± 0.10[a]
	40	88.1 ± 0.07[b]
	60	97.2 ± 0.12[c]
	80	83.2 ± 0.52[d]
SDS	20	66.3 ± 0.32[a]
	40	78.2 ± 1.42[b]
	60	63.2 ± 0.09[a]
	80	67.1 ± 0.32[a]
Acetonitrile	20	126.1 ± 0.07[a]
	40	118.6 ± 1.10[b]
	60	111 ± 0.22[ab]
	80	98.2 ± 0.52[c]

The sample sizes for all experiments were three. Mean values followed by different letters (a–d) differ significantly ($p < 0.05$).

2.8. Statistical design and analysis

All the experiments were organized using a completely randomized design with three replicates, repeated twice for reproducibility. Mean values of triplicate data for all the parameters were obtained and subjected to one-way analysis of variance (ANOVA). The statistical significance was accepted at $p < 0.05$ using Duncan's multiple range test (DMRT).

3. Result and discussion

3.1. The effect of surfactants and acetonitrile on polygalacturonase activity

To determine the effect of each phase composition on the polygalacturonase activity, crude feedstock polygalacturonase was mixed with various compounds. Based on our preliminary studies, the enzyme is stable in the presence of Triton X-100, Tween 800, DTAB, SDS, and acetonitrile, which make the enzyme suitable for partitioning in this novel method of liquid/liquid extraction process. It is believed that surfactant promotes the availability of reaction sites, which would increase the hydrolysis rate [29]. In addition, the surfactant could also increase the activity of the enzyme and thus, reduce enzyme denaturation during the hydrolysis [29,30]. It is also mentioned that the surfactant could increase enzyme activity by affecting the enzyme-substrate interaction e.g. adsorbed enzymes are prevented from inactivation by addition of surfactant, which facilitates desorption of enzymes from substrate [31–33]. The polygalacturonase was slightly activated by high concentration [60% (v/v)] of Triton X-100, Tween 80, DTAB, though SDS partially inactivated the polygalacturonase activity (Table 1). The phenomenon could be due to binding of ionic surfactant (SDS) molecules to proteins, which can disrupt the native structure of most globular proteins. Polygalacturonase activity was also determined for polygalacturonase mixed into different concentrations of acetonitrile. It should be considered that the polygalacturonase showed the high enzyme stability in the presence of acetonitrile. It could be probably due to help of acetonitrile to maintain the enzyme's open conformation by exposing the active site crevice and thereby stimulating polygalacturonase activity [34]. The result

Table 2A
Partition behavior of polygalacturonase in different surfactant/acetonitrile systems.

System	Concentration of surfactant/acetonitrile (% w/w)	TLL (%, w/w)	Selectivity	Purification factor
TritonX-100/acetonitrile	16/19	52.2	65.81 ± 0.2^{a}	4.21 ± 1.1^{a}
	21/23	55.6	82.14 ± 1.3^{b}	9.11 ± 0.5^{b}
	25/26	56.0	51.04 ± 0.1^{c}	7.55 ± 0.3^{c}
	30/28	63.8	26.01 ± 2.1^{d}	5.84 ± 0.2^{d}
Tween-80/acetonitrile	18/16	33.9	32.20 ± 2.1^{e}	6.33 ± 1.3^{ab}
	21/20	37.0	33.82 ± 0.5^{e}	4.32 ± 2.1^{e}
	23/23	44.1	28.15 ± 0.2^{ed}	3.81 ± 0.1^{d}
	28/24	46.1	23.41 ± 0.3^{e}	4.62 ± 0.3^{e}
DTAB/acetonitrile	17/15	38.4	18.02 ± 0.3^{f}	3.41 ± 0.3^{c}
	23/21	43.2	19.62 ± 1.1^{g}	2.14 ± 0.2^{c}
	24/20	41.6	11.11 ± 0.4^{h}	1.03 ± 1.1^{a}
	32/30	47.5	12.04 ± 2.1^{i}	1.95 ± 0.3^{cd}
SDS/acetonitrile	14/12	33.6	5.23 ± 1.2^{j}	0.12 ± 0.4^{g}
	19/17	35.4	3.15 ± 1.3^{k}	0.09 ± 0.2^{h}
	23/20	46.5	3.05 ± 0.2^{k}	0.16 ± 0.3^{i}
	28/24	56.3	2.42 ± 0.4^{jk}	0.08 ± 1.1^{j}

(Table 1) showed that the higher concentration of the acetonitrile (60%, w/v) reduces the enzyme activity compared to the polygalacturonase activity observed in 20% (w/v) acetonitrile solutions. It is because of denaturation of the enzyme in a higher concentration of the acetonitrile.

3.2. Phase diagram of surfactants and acetonitrile

The phase diagrams of the different surfactants (Triton X-100, Tween 80, DTAB and SDS) with the acetonitrile are shown in Fig. 1. The experimental phase diagrams for each surfactant and acetonitrile were determined at 298 K and atmospheric pressure. The binodal curves associated with the Triton X-100 and Tween 80 were further from the origin than DTAB/acetonitrile and SDS/acetonitrile curves, indicating that non-ionic surfactants improved the overall solubility and miscibility in the system. Amongst the nonionic/acetonitrile systems, the binodal curves of the Triton X-100/acetonitrile systems were closer to the origin than the binodal curves of Tween80/acetonitrile systems. This phenomenon was attributed to Tween's higher hydrophobicity [35]. Higher hydrophobicity causes poor acetonitrile solubility and miscibility; thus, a lower concentration of phase components (surfactant and acetonitrile) is needed to form the two-phase system. It should be noted that the aggregation number of micelles in Triton X-100-rich phase is twice that in Tween 80-rich phase and the micelle size of Triton X-100 is slightly bigger [36] than that of Tween 80 based on their micelle molecular weight.

3.3. Selection of the optimal surfactants/aceronytryl liquid/liquid extraction process for the enzyme partitioning

Selectivity and purification factors of polygalacturonase in various surfactants and acetonitrile systems are presented in Table 2A. It should be noted that the effect of surfactants on the enzyme partitioning is dependent on the chemically selective interactions between the molecules, which may be influenced by the enzyme structure and chemical property of the surfactant. Based on the results, the selectivity and purification factor of the polygalacturonase in non-ionic surfactant/acetonitril system was significantly ($p < 0.05$) higher compared to ionic and cationic surfactants liquid/liquid extraction process (Table 2A). It could be because the active site of enzyme in 'closed' form is shielded from the surface by protective amino acids surface loops called the 'lid' [37,38]. In the presence of non-ionic surfactants, the lid undergoes a conformational rearrangement exposing the active site and creating the active, open form of the enzyme [39]. Such a conformational change may also be responsible for the observed higher activity and partitioning of the enzyme in the presence of non-ionic surfactants. It should be considered that in this case, Triton X-100 increased the enzyme partitioning more than in Tween 80. The partitioning of the enzyme was decreased in the presence of cationic surfactant (DTAB). In fact, cationic surfactants are able to electrostatically bind to negatively charged amino acid residues (acidic amino acids), those involved in the active site interacting within the protein, and can also disrupt the enzyme native conformation through hydrophobic interaction by their non-polar tail [40]. SDS, an anionic surfactant had the lowest efficient effect on partitioning of the enzyme. As mentioned earlier, it seems that the binding of ionic surfactant molecules to proteins could be interrupting the tertiary structure of proteins. It could be explained that ionic surfactants interact with proteins through a combination of electrostatic and hydrophobic forces [41], thus, the surfactant head group will play a determining role in protein–surfactant interactions, which preferentially begins with the formation of strong ionic bonds between the surfactant polar groups, especially the charged sites on the protein surface [42]. Therefore, it could decrease the proper partitioning of the enzyme in the system, thus, reducing

Table 2B
Partition of polygalacturonase in different concentrations of Tritonx-100/acetonitrile systems.

Triton X-100 (%, w/w)	Acetonitrile (%, w/w)	Purification factor	Yield (%)
21	19	3.11 ± 0.5^{a}	65.3 ± 0.5^{a}
21	21	4.21 ± 1.1^{b}	68.6 ± 0.2^{b}
21	23	2.55 ± 0.3^{c}	70.4 ± 1.1^{c}
21	25	5.64 ± 0.2^{d}	66.3 ± 0.3^{ab}
21	28	5.58 ± 1.1^{e}	78.3 ± 0.5^{d}
23	19	10.71 ± 0.3^{f}	87.6 ± 0.2^{e}
23	21	8.83 ± 1.3^{g}	84.4 ± 1.1^{f}
23	23	8.42 ± 2.1^{g}	78.3 ± 0.3^{g}
23	25	7.61 ± 0.1^{h}	79.3 ± 0.5^{g}
23	28	6.72 ± 0.3^{b}	77.6 ± 0.2^{e}
25	19	3.83 ± 1.1^{ab}	63.4 ± 1.1^{f}
25	21	2.68 ± 1.3^{i}	61.3 ± 0.3^{g}
25	23	2.31 ± 0.3^{i}	67.3 ± 0.5^{h}
25	25	2.12 ± 0.2^{i}	60.6 ± 0.2^{i}
25	28	1.03 ± 1.1^{i}	57.4 ± 1.1^{j}
30	19	1.95 ± 0.3^{j}	52.3 ± 0.3^{k}
30	21	2.31 ± 0.2^{i}	51.3 ± 0.5^{jk}
30	23	1.82 ± 1.1^{k}	53.6 ± 0.2^{j}
30	25	0.11 ± 0.4^{l}	48.4 ± 1.1^{l}
30	28	0.08 ± 0.2^{m}	32.3 ± 0.3^{m}

The sample sizes for all experiments were three. Mean values followed by different letters (a–m) differ significantly ($p < 0.05$).

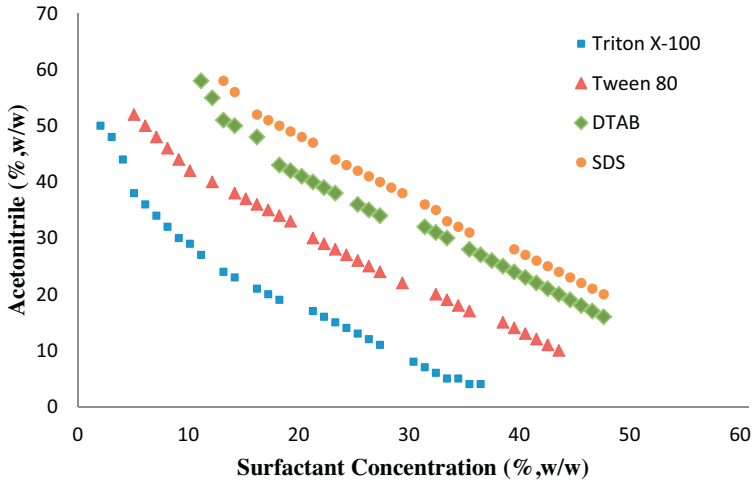

Fig. 1. The binodal curves for Triton X-100 (■), Tween-80 (▲), DTAB (♦), SDS (●) were plotted against acetonitrile.

the enzyme purification factor. Based on the result the maximum selectivity (82.14) and purification factor (9.11) was achieved in the Triton X-100/acetonytril system, hence, this system was selected for future optimization of the surfactant/acetonitrile liquid/liquid extraction process.

To optimize the polygalacturonase partition efficiency in a Triton X-100/acetonytril system, 20 systems were evaluated. These systems systematically varied the Triton-100/acetonytril concentrations. According to Table 2B the optimal condition for partitioning polygalacturonase was observed in the Triton X-100 23% (w/w) and acetonitrile 19% (w/w) system, which had a purification factor of 10.71 and a yield of 87.6%. The results indicated that the polygalacturonase partitioning is better at low concentration of surfactants and acetonitrile. It should be noted that the high concentration of surfactants could negatively affect the amount of solubilized enzyme and its catalytic actions. A similar trend was observed when the acetonitrile concentration was increased. This was due to the gradual dehydration of the bottom phase as the no-ionic surfactant concentration in the top phase increased, thus causing an imbalance that did not favor the polygalacturonase retention in the top phase [43].

3.4. The effect of crude feedstock concentration and total volume of aqueous phase

An increment in crude load would be advantageous in the recovery process of larger feed volumes by the liquid/liquid extraction process. The impact of the loaded mass on the partition of enzymes is important, as the loaded feed stock can alter the phase volume ratio [44] and the partition behavior of target protein [45]. The increasing amounts of both polygalacturonase and contaminants in the systems could result in a decrease of the liquid/liquid extraction process performance. Liquid/liquid extraction process experiments were carried out by varying the crude load up to 45% (w/w). Fig. 2 illustrates the effect of crude load on top polygalacturonase recovery. Based on the results, crude load of 25% (w/w) is the maximum capacity on the basis of 10 g liquid/liquid extraction process. The selectivity and yield for the 25% (w/w) crude load liquid/liquid extraction process was 86.4 and 93.6%, respectively. A higher amount of the sample loading into the liquid/liquid extraction process will decrease the volume ratio and affect the composition of the process. It appeared that the components in the crude stock had changed the properties of the liquid/liquid extraction process; hence the process was not optimum for purification of polygalacturonase. Such behavior can be explained by the increasing accumulation of precipitate at the interface, showing the loss of polygalacturonases together with other contaminants in the purification. Therefore, it is clear that a 25% of sample loading would be feasible for the maximum top phase recovery of polygalacturonase from the crude extract.

3.5. The effect of system pH on polygalacturonase partitioning

The partitioning of polygalacturonase in liquid/liquid extraction process at different pH value was shown in Fig. 3. It is well known that biomolecules partition in liquid/liquid extraction process is obviously influenced by pH of the system and generally system pH affects the partitioning behavior of protein by changing the charge of the target protein itself. In fact, the manipulation of pH in liquid/liquid extraction process correlated with electrochemical interactions between protein and solvent in the system. These electrochemical interactions play an important role in partition behavior of enzymes [46]. Polygalacturonase has a (isoelectric point) p*I* about 5.9, so at pH 6.0 the polygalacturonase is slightly negatively charged and the partitioning will mostly depend on the surface properties rather than the net charge. Polygalacturonase, being a negatively charged molecule prefers to partition to the hydrophobic phase. However, partitioning direction changed for the target enzyme, and it partitioned to the more hydrophobic surfactant enriched top phase. This change of the partition behavior of polygalacturonase was caused by the influence of the protein charge. Notably, the purification factor and yield of the enzyme were decreased at pHs above 6.0. This phenomenon could be due to the decreased enzyme activity at pHs above 6.0 because the enzyme is active and stable at acidic pH, and the activity may have decreased in the presence of natural/alkaline pH. Thus, the maximum purification factor (14.3) and yield (97.3%) were obtained at pH 6.0. This phenomenon was reported by Shahbaz and Omidinia [47], who purified recombinant phenylalanine dehydrogenase by an liquid/liquid extraction process. Thus, pH 6.0 was selected as the optimal pH in this study.

3.6. Recycling of phase components

The very important advantage for this novel liquid/liquid extraction process was that both phase-forming components could be recycled with high recovery and more than 95% recoveries still could be achieved at the fifth recycle relating to initial run (Table 3). As shown in Table 3, there is only minor loss of the surfactant

Table 3
The recycles recovery of surfactant and acetonitrile systems.

System	Initial	Recycle systems				
		First	Second	Third	Forth	Fifth
Recovery of surfactant (%)	99.6	99.2 ± 0.02	98.1 ± 0.2	97.7 ± 0.11	97.5 ± 0.03	97.3 ± 0.02
Recovery of acetonitrile (%)	98.9	98.2 ± 0.01	97.8 ± 0.3	97.3 ± 0.02	95.2 ± 0.13	95.0 ± 0.01

and acetonitrile in the recycling steps whereas the recovery of the surfactant could still be maintained at over 97% after five cycles in relation to the initial amount. This could be explained by the good ability of the surfactant to take desirable protein into the top phase after being reused several times. The surfactant reached its maximum capacity to accommodate the negatively-charged protein; therefore the protein newly dispensed into the liquid/liquid extraction process could be partitioned into the surfactant-rich top phase. The result also exhibited that almost 95% of acetonitrile was recovered in the recycling procedure, which makes the new system economical and suitable for application in industry as well as protecting the environment.

3.7. Polygalacturonase recovery

The optimum condition of polygalacturonase recovery was obtained in liquid/liquid extraction process comprising Triton X-100/acetonitrile with TLL of 55.6% (w/w), 25% (w/w) crude load at pH 6.0. The purity of the polygalacturonase from durian seed was investigated employing 12% SDS-PAGE (Fig. 4). As shown in Fig. 4, Lane 1 was identified as crude feedstock with lots of impurity bands. Line 2 contained the sample of the aqueous phase, which indicates lesser, and fainter bands than crude feedstock. The sample recovered from the top phase indicated just one dark band at 34.4 kDa (Lane 3). As such, this SDS-PAGE result indicated the efficacy of the

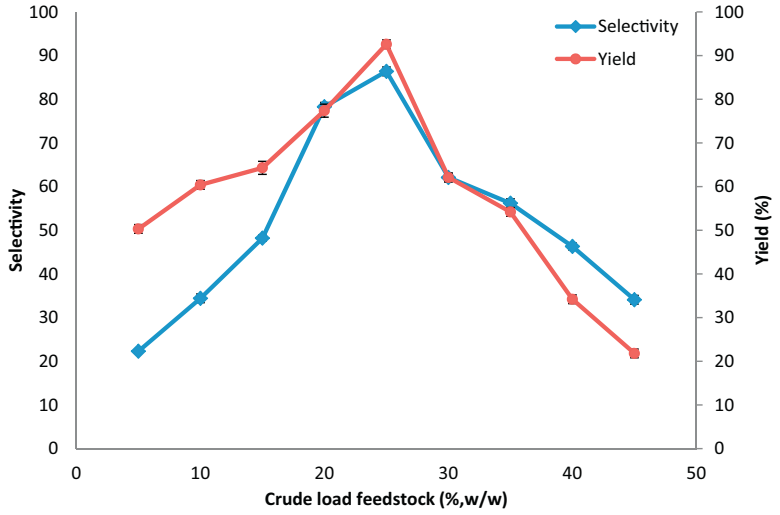

Fig. 2. The optimization of Triton X-100/acetonitrile system was performed by varying the crud load of liquid/liquid extraction process. The partition efficiency of polygalacturonase was measured in terms of selectivity and yield.

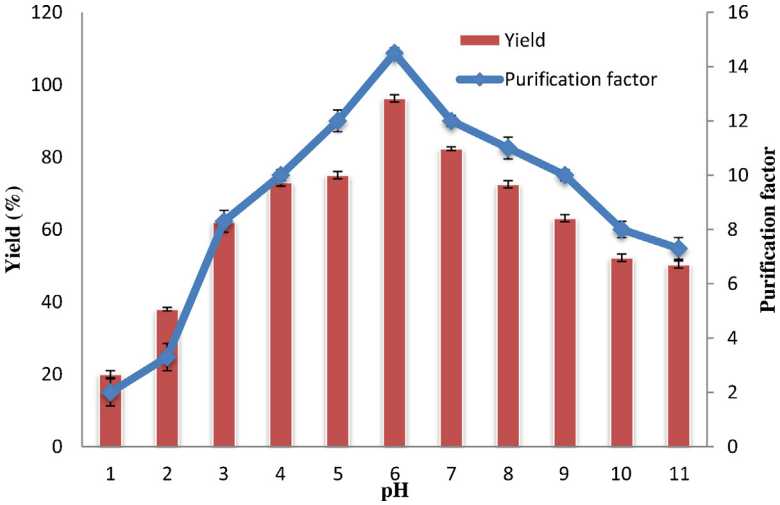

Fig. 3. In all experiments, the pH of liquid/liquid extraction process was varied between 1.0 and 11.0. The yield and purification factor of polygalacturonase from *Durio zibethinus* were calculated according equations 5 and 6, respectively.

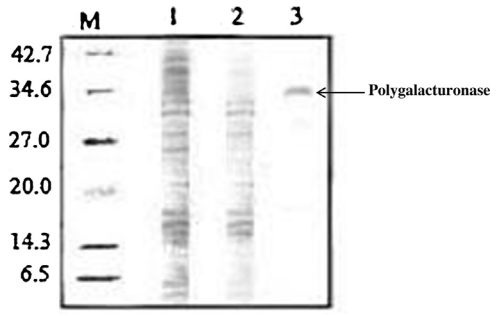

Fig. 4. The purity of the partitioned polygalacturonase was assessed by 12% SDS-PAGE analysis. Molecular weight of standard protein marker ranged 6.5–42.7 kDa. M: protein molecular marker; Lane 1: crude feedstock, Lane 2: liquid/liquid extraction process bottom phase; Lane 3: liquid/liquid extraction process top phase.

purification technique in this study which gives maximum recovery of polygalacturonase from *Durio zibethinus*.

4. Conclusion

In this study, the effect of type and concentration of surfactants, concentration of acetonitrile, TLL, crude load feedstock, and pH was evaluated due to optimization of novel liquid/liquid extraction process employed for future recycling experiments. The optimum condition was obtained at 23% (w/w) Triton X-100 and 19% (w/w) acetonitrile with 55.6%TLL, 25% crude load at pH 6.0. In the optimized condition, a high yield of 97.3% and a purification fold of 14.3 of purified polygalacturonase were obtained. More than 95% of phase components were recycled from the liquid/liquid extraction process based on surfactant/acetonitrile. The direct recovery of polygalacturonase from durian waste employing the liquid/liquid extraction process based on surfactant/acetonitrile was shown to be a successful method for purification of the enzyme from fruit source. This study also indicated that the phase components can be recycled and reused, resulting in this procedure being more efficient, economical and providing a mild environment especially for large scale production of the enzyme from fruit waste.

Acknowledgements

We gratefully appreciate the financial support of this work by the Ministry of Science, Technology and Innovation of Malaysia through Science Fund (02-01-04-SF1800) and grant of Research Management Center (RMC) of University Putra Malaysia (9400500). We would like to thank the staff of the Enzyme Laboratory in Food Science and Technology Faculty of University Putra Malaysia for their help, support and the use of all facilities which were needed in conducting the study.

References

[1] M. Rito-Palomares, Practical application of aqueous two-phase partition to process development for the recovery of biological products, J. Chromatogr. B 807 (2004) 3–11.
[2] J.S. Becker, O.R.T. Thomas, M. Franzre, Protein separation with magnetic adsorbents in micellar aqueous two-phase systems, J. Sep. Purif. Technol. 65 (2009) 46–53.
[3] A. Chavez-Santoscoy, J. Benavides, W. Vermaas, M. Rito-Palomares, Application of aqueous two-phase systems for the potential extractive fermentation of cyanobacterial products, J. Chem. Eng. Technol. 33 (2010) 177–182.
[4] O.K. Ashipala, Q. He, Optimization of fibrinolytic enzyme production by *Bacillus subtilis* DC-2 in aqueous two-phase system (poly-ethylene glycol 4000 and sodium sulfate, J. Bioresour. Technol. 99 (2008) 4112–4119.
[5] J. Persson, A. Kaul, F. Tjerneld, Polymer recycling in aqueous two-phase extractions using thermoseparating ethylene oxide-propylene oxide copolymers, J. Chromatogr. B 743 (2000) 115–126.
[6] F. Luechau, T.C. Ling, A. Lyddiatt, Partition of plasmid DNA in polymer-salt aqueous two-phase systems, J. Sep. Purif. Technol. 66 (2009) 397–404.
[7] M. Li, J.W. Kim, T.L. Peeples, Amylase partitioning and extractive bioconversion of starch using thermo-separating aqueous two-phase systems, J. Biotechnol. 93 (2002) 15–26.
[8] C. Gustavo de Brito, T. Mourão, F.M. Pereira, M.G. Freire, A.T. Fricks, C.M.F. Soares, A.S. Lima, The pharmaceutical is aqueous two-phase systems based on acetonitrile and carbohydrates and their application to the extraction of vanillin, J. Sep. Purif. Technol. 104 (2013) 106–113.
[9] P. Chakravarty, K.S. Alexander, A.T. Riga, K. Chatterjee, Crystal forms of tolbutamide from acetonitrile and 1-octanol: effect of solvent, humidity and compression pressure, Int. J. Pharm. 288 (2005) 335–348.
[10] A. Jhoany, A. Alberto, R. Eva, S. Ana, A thermodynamic study on binary and ternary mixtures of acetonitrile, water and butyl acetate, J. Fluid Phase Equib 203 (2002) 83–98.
[11] B. Wang, T. Ezejias, H. Feng, H. Blaschek, Sugaring out for separation of acetonitrile and extraction of proteins and antibiotics, J. Chem. Eng. Sci. 63 (2008) 2595–2600.
[12] R.B. Tôrres, M.I. Ortolan, P.L.O. Volpe, Volumetric properties of binary mixtures of ethers and acetonitrile: experimental results and application of the Prigogine–Flory–Patterson theory, J. Chem. Thermdyn. 40 (2008) 442–459.
[13] M. Taha, H.L. Teng, M.J. Lee, Phase diagrams of acetonitrile or (acetone + water + EPPS) buffer phase separation systems at 298.15 K and quantum chemical modelling, J. Chem. Thermdyn. 54 (2012) 134–141.
[14] W. Schnitzhofera, H.J. Weber, M. Vrsanska, P. Biely, A. Cavaco-Paulo, G.M. Guebitz, Purification and mechanistic characterisation of two polygalacturonases from *Sclerotium rolfsii*, J. Enzyme Microb. Technol. 40 (2007) 1739–1747.
[15] R.S. Jayani, S. Saxena, R. Gupta, Microbial pectinolytic enzymes: a review, J. Process Biochem. 40 (2005) 2931–2944.
[16] P. Palanivelu, Polygalacturonases: Active site analyses and mechanism of action, Indian J. Biotechnol. 5 (2006) 148–162.
[17] L.H. Ho, R. Bhat, Exploring the potential nutraceutical values of durian (*Durio zibethinus* L.) – an exotic tropical fruit, J. Food Chem. 168 (2015) 80–89.
[18] K.Y. Foo, B.H. Hameed, Transformation of durian biomass into highly valuable end commodity: trends and opportunities, J. Biomass Bioenerg. 35 (2011) 2470–2478.
[19] M.H.C. Maciel, C.A. Ottoni, P.N. Herculano, T.S. Porto, A.L.F. Porto, C. Santos, N. Lima, K.A. Moreira, C. Souza-Motta, Purification of polygalacturonases produced by *Aspergillus niger* using an aqueous two-phase system, J. Fluid Phase Equilbr. 371 (2014) 125–130.
[20] G. de Brito Cardoso, I.N. Souza, M.M. Pereira, L.P. Costa, M.G. Freire, C.M.F. Soares, Á. Silva Lima, Poly(vinyl alcohol) as a novel constituent to form aqueous two-phase systems with acetonitrile: Phase diagrams and partitioning experiments, J. Chem. Eng. Res. Des. 94 (2015) 317–323.
[21] G.S. Cardoso, I.N. Souza, M.M. Pereira, M.G. Freire, C.M. Faria Soares, Á.S. Lima, Aqueous two-phase systems formed by biocompatible and biodegradable polysaccharides and acetonitrile, J. Sep. Purif. Technol. 136 (2014) 74–80.
[22] D.C. Gustavo, T. Mourão, F.M. Pereira, M.G. Freire, A.T. Fricks, S. Lima, Aqueous two-phase systems based on acetonitrile and carbohydrates and their application to the extraction of vanillin, J. Sep. Purif. Technol. 104 (2013) 106–113.
[23] M. Amid, M.Y.A. Manap, M. Shuhaimi, Purification of pectinase from mango (*Mangifera indica* L. cv. Chokanan) waste using an aqueous organic phase system: a potential low cost source of the enzyme, J. Chromatogr. B. 931 (2013) 17–22.
[24] E. Albertsson, G. Johansson, F. Tjerneld, J.A. Asenjo, Separation Processes in Biotechnology, New York and Basel, 1990.
[25] M.G. Antov, D.M. Peričin, G.R. Dimić, Cultivation of *Polyporus squamosus* for pectinase production in aqueous two-phase system containing sugar beet extraction waste, J. Biotech. 91 (2001) 83–87.
[26] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, J. Anal. Biochem. 72 (1976) 248–254.
[27] G.A. Mirjana, Partitioning of pectinase produced by *Polyporus squamosus* in aqueous two phase system polyethylene glycol 4000 crude dextran at different initial pH values, J. Carbohydr. Polym. 56 (2004) 295–300.
[28] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, J. Nature 2 (1999) 680–685.
[29] H.U. Hebbar, B. Sumana, K.S.M.S. Raghavarao, Use of reverse micellar systems for the extraction and purification of bromelain from pineapple wastes, J. Bioresour. Technol. 99 (2008) 896–4902.
[30] J.Y. Imm, S.C. Kim, Convenient partial purification of polyphenol oxidase from apple skin by cationic reversed micellar extraction, J. Food Chem. 113 (2009) 302–306.
[31] V.I. Padma, A. Laxmi, Enzyme stability and stabilization - aqueous and non-aqueous environment, J. Process Biochem. 43 (2008) 1019–1032.
[32] D. Das, P.K. Das, Superior activity of structurally deprived enzyme carbon nanotube hybrids in cationic reverse micelles, J. Langmuir 25 (2009) 421–4428.
[33] C.M.L. Carvalho, J.M.S. Cabral, Reverse micelles as reaction media for lipases, J. Biochim. 82 (2000) 1063–1085.
[34] J.G. Liu, J.M. Xing, R. Shen, C.L. Yang, H.Z. Liu, Reverse micelles extraction of nattokinase from fermentation broth, J. Biochem. Eng. 21 (2004) 273–278.
[35] N. Streitner, V.C. Flaschel, Reverse micellar extraction systems for the purification of pharmaceutical grade plasmid DNA, J. Biotechnol. 131 (2007) 188–196.
[36] E.A. Ermakova, N.L. Zakhartchenko, Y.F. Zuev, Effect of surface potential of reverse micelle on enzyme–substrate complex formation, J. Colloids Surf. A 317 (2008) 297–302.

[37] H. Zhong, G.M. Zeng, X.Z. Yuan, H.Y. Fu, G.H. Huang, F.Y. Ren, Adsorption of dirhamnolipid on four microorganisms and the effect on cell surface hydrophobicity, J. Appl. Microbiol. Biotechnol. 77 (2007) 447–455.
[38] H. Zhong, G.M. Zeng, J.X. Liu, X.M. Xu, X.Z. Yuan, H.Y. Fu, G.H. Huang, Z.F. Liu, Y. Ding, Adsorption of monorhamnolipid and dirhamnolipid on two *Pseudomonas aeruginosa* strains and the effect on cell surface hydrophobicity, J. Appl. Microbiol. Biotechnol. 79 (2008) 671–677.
[39] U. Sivars, F. Tjerneld, Mechanisms of phase behaviour and protein partitioning in detergent/polymer aqueous two-phase systems for purification of integral membrane proteins, J. Biochim. Biophys. Acta 1474 (2000) 133–146.
[40] W.W. Wang, X.Z. Yuan, G.M. Zeng, Y.S. Liang, Y. Chao, Enzymatic hydrolysis of cellulose in reverse micelles (in Chinese), J. Environ. Sci. 31 (2010) 2202–2207.
[41] G.H. Ma, P. Wang, Z.G. Su, Nanoscience and enzyme (in Chinese), J. Chin. Basic. Sci. 5 (2009) 49–54.
[42] C.M.L. Carvalho, J.M.S. Cabral, Reverse micelles as reaction media for lipases, J. Biochim. 82 (2000) 1063–1085.
[43] R.C.F. Bonomo, L.A. Minim, J.S.R. Coimbra, R.C.I. Fontan, L.H. Mendes da Silva, V.P.R. Minim, Hydrophobic interaction adsorption of whey proteins: effect of temperature and salt concentration and thermodynamic analysis, J. Chromatogr. B 844 (2006) 6–14.
[44] W.O. Chien, L.H. Siew, M.K. Mazlina, A. Arbakariya, C.L. Tau, Extractive fermentation using aqueous two-phase systems for integrated production and purification of extracellular lipase derived from *Burkholderia pseudomallei*, J. Process. Biochem. 46 (2011) 68–73.
[45] P.L. Show, C.P. Tan, M.S. Anuar, A. Ariff, Y.A. Yusoff, S.K. Chen, T.C. Ling, Primary recovery of lipase and recycling phase components in aqueous two phase system, J. Biochem. Eng. 60 (2012) 74–80.
[46] N.K. Zohdi, M. Amid, Optimization of extraction of novel pectinase enzyme discovered in Red Pitaya (*Hylocereus polyrhizus*) peel, J. Mol. 18 (2013) 14366–14380.
[47] H. Shahbaz, M. Eskandar Omidinia, Process integration for the recovery and purification of recombinant *Pseudomonas fluorescens* proline dehydrogenase using aqueous two-phase systems, J. Chromatogr. 15 (2013) 11–17.