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Protease purification from *Bacillus amyloliquefaciens* B7 using Aqueous Two-Phase System (ATPS)

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Abstract

Bacillus amyloliquefaciens B7 was isolated from the fermented fish sauce and identified as protease producer. Generally, in downstream processing, purification of enzymes consumes higher cost regarding reagents and equipment used. Moreover, harsh purification methods used might cause denaturation of the enzymes. Therefore, there is a high demand for efficient and low-cost extraction and purification methods. Aqueous two-phase system (ATPS) is an alternative method that should be considered as it is simple, rapid separation yet cause little denaturation. Protease produced by *B. amyloliquefaciens* B7 was partitioned using two different ATPS, which were PEG/potassium phosphate and PEG/sodium citrate. Results showed the highest enzyme activity was found in interface phase with the ATPS system of 27% (w/w) PEG1500/ 34% (w/w) sodium citrate. Later, the ATPS conditions (pH, temperature, the concentration of selected salt and PEG) were optimized by using response surface methodology. The optimum conditions for ATPS purification were observed in ATPS conditions at pH 7 and 35°C with the enzyme activity of 0.20± 0.01 U/ml.

Introduction

Traditional fermented food such as fish sauce, fish, and fermented soybean are rich in protein content and suitable to become sources in the isolation of protease-producing microorganisms (Choorit and Prasertsan, 1992; Singh *et al*., 2014; Oke and Onilude, 2014). Fish fermented sauce (Budu) traditionally made by mixing small fish such as anchovies with salt (NaCl) at the ratio of 3:1 (w/w) and the mixture is allowed to ferment for at least six months naturally and above in the closed tank (Sim *et al*., 2009). According to Lopetcharat *et al.* (2001), the bacteria present in budu was producing proteolytic enzymes which associated with protein degradation and flavor development from the type of *Bacillus* sp. Previous studies discovered that among the *Bacillus* species, gram- positive and spore-forming *Bacillus amyloliquefaciens* was known as one of the protease producers (Sudhakar *et al.*, 2014). Protease produced by this strain usually used in the detergent industries due to the high thermal stability and stable in alkaline environments. Also, Anjali *et al*., (2014) found that proteases from *Bacillus amyloliquefaciens* have an ability to degrade native feather in a short time which identified as a keratinolytic protease. Moreover, Sai-Ut *et al.* (2015) discovered an extracellular gelatinolytic protease that worth to produce because it had various bioactivity including angiotensin-I-converting enzyme inhibition and antioxidant from degradation of collagen and gelatin-derived peptides. There are several *B. amyloliquefaciens* strain isolated from fermented soybean products such as *B. amyloliquefaciens* DC-4 from Douchi (Yong and Yizheng, 2002), *B. amyloliquefaciens* MJ5-41 from Meju (Jo *et al*., 2011) and *B. amyloliquefaciens* LSSE-62 from Chinese soybean paste (Wei *et al*., 2011). Our previous study had successfully isolated and identified *B. amyloliquefaciens* B7 from the fermented fish sauce (Abd Samad *et al.*, 2017) which also proved by Lopetcharat *et al.* (2001) that bacteria involved in most fish sauce mostly from *Bacillus* sp. and *Staphylococcus* sp.

However, in the large scale of enzyme productions, recovering of the enzymes from the fermentation broth is an important part and usually, requires many steps which result in a high tendency of enzymes denaturation. Numerous purification methods have been described for enzyme purification including gel filtration, affinity chromatography and ion-exchange chromatography (Gupta *et al*., 2002) which involve expensive multistep methods, having difficulties to scale-up and time-consuming. There is a high demand for efficient and low-cost extraction methods

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for commercial enzyme production. Aqueous two-phase system (ATPS) is an alternative that should be considered. ATPS is a liquid-liquid extraction method formed by mixing polymer and polymer such as PEG/Dextran system or polymer and salt, which is PEG/Potassium phosphate system. This method is simple, rapid separation, easy to scale up, and low risk of enzyme's denaturation during the separation of enzymes (Ratanapongleka, 2010). Therefore, this study aims to separate the protease from the culture broth of *Bacillus amyloliquefaciens* B7 in different PEG/salt systems and optimize the parameters that might affect ATPS conditions, which are pH and temperature.

Materials and methods

Materials

Polyethylene glycol with molecular weights of 1500, 4000, 6000 and 8000 (g/mol), Tri-potassium citrate monohydrate ($K_3C_6H_5O_7$) was purchased from Merck-Schuchardt (Munich, Germany). Nutrient Broth, casein, sodium carbonate, trichloroacetic acid were obtained from Sigma (St. Louis, MO, USA) while Bradford reagents were from Bio-Rad (Germany). All chemicals were of analytical grade.

Media and culture conditions

The method of bacteria culture was adopted from Sudhakar *et al*. (2014) with slight modification. About 1 ml of starter culture was inoculated into 250 ml of Nutrient Broth in the Erlenmeyer flask and incubated for 24 hours at 37°C. After incubation, to collect the enzyme, the culture was centrifuged at 12000 rpm for 15 mins at 4°C. The supernatant was utilized as a source of crude enzyme.

Protease activity and total protein determination

Protease activity (U/ml) was measured by using casein as a substrate. The method was followed Sigma's non-specific protease activity (Cupp-Enyard, 2008). One milliliter of casein (0.65% w/v in 50 mM potassium phosphate buffer, pH 7.5) was mixed to 0.2 ml of the enzyme, and the reaction mixture was incubated for 10 mins. Then, 1 ml of trichloroacetic acid reagent (110mM) was added to stop the reaction, and the mixture was incubated for 30 mins. Next, the supernatant was collected by centrifuging the mixture at 5000 rpm for 15 mins. About 0.5 ml of filtrate was mixed with 1.25 ml sodium carbonate solution and 0.25 ml Folin and Ciocalteu's Phenol Reagent. Absorbance was measured at 660nm. One unit of the protease was defined as the amount of the enzyme required to liberate 1 μmol of tyrosine per minute under the defined assay conditions (Shivanand and Jayaraman, 2009). Protein concentration was determined by Bradford method using bovine serum albumin (BSA) as standard. About 1 ml of Bradford reagent and 0.2 ml of sample were added to a dry and clean microcentrifuge tube. After 5 mins of incubation, the solution was transferred into a cuvette, and an absorbance at 595nm was measured.

Purification of protease by ATPS

Purification of the protease was carried out in PEG/Sodium citrate and PEG/Potassium phosphate system. Phase systems were prepared by weighing out the desired concentration of selected PEG, sodium citrate stock solutions, water and 2% of the crude enzyme to make the total weight of the system became 100% (w/w) in the 15 ml of the graduated centrifuge tube. The tube was centrifuged at 3000 rpm for 20 mins to speed up the phase separation, and the tube was placed at 20°C for 24 hrs for complete equilibration according to the method applied by Ramyadevi *et al*., (2013). The steps above were repeated for different pH of sodium citrate (pH 6, 7, 8) and different temperature of tube placement (20°C, 35°C, 50°C). Protease activity (Cupp-Enyard, 2008) and total protein determination (Bradford, 1976) were conducted for each phase of separation (top, interface and bottom phase) to determine the enzyme separation.

Optimization of ATPS conditions

Response surface methodology (RSM) using Design-Expert 8.0.5 software (State-Ease Inc., Minneapolis MN, USA) was used to determine the optimum conditions for enzyme separation in aqueous two-phase system. The complete design consisted of 11 experiments were coded using the face-centered central composite design of response surface methodology (RSM) based on the factors of pH and temperature with the response of specific activity.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was conducted to analyze the enzyme present in the samples of ATPS, as described by Laemmli (1970). 12% resolving gel and 4% stacking gel was used to separate the protein bands, and the ATPS samples were prepared by diluting it in distilled water in 1:4 ratios. The electrophoresis was performed at 200 V for 30 mins, and the gels were stained with silver staining.

Table 1. Specific activity for different PEG/Salt system

Molecular weight of PEG	Specific Activity (U/mg)	
	Sodium Citrate	Potassium Phosphate
1500	6.11± 0.07	0.19± 0.13
4000	4.13± 0.15	2.98± 0.07
6000	4.00± 0.13	2.41± 0.05
8000	2.48± 0.05	3.52± 0.08

Table 2. Analysis of variance of quadratic model for specific activity of protease

Source	**Enzyme Activity (U/ml)**	
	Sum of squares	p-Value Prob > F
Model	0.033	0.0023(significant)
A-pH	4.183E-004	0.2994
B- Temperature	3.128E-003	0.0250
AB	1.000E-006	0.9571
A^2	5.137E-003	0.0098
B^2	0.017	0.0007
Lack of Fit (p-value)	0.7429 (not significant)	
R^2	0.9546	
Adjusted R^2	0.9092	
Predicted R^2	0.8066	

Results and discussion

Purification of protease by ATPS

According to Ratanapongleka (2010), the partitioning of the protein depends on many parameters such as the concentration and molecular weight of the polymer, type, and concentration of salts, temperature, system pH and biomolecule surface properties. In the recent study, the molecular weight of PEG and phase forming salt were selected based on the highest protease specific activity. The protease activity was conducted when the volume ratio (Vr) of phase separation equal to one and from previous research, Arshad (2016) reported that the highest enzyme activity was obtained at Vr = 1.

The PEG/Sodium citrate system showed the higher specific activity compared with PEG/ Potassium phosphate based on the results observed in Table 1. The 27% (w/w) of PEG 1500 and 34% (w/w) of Sodium citrate had the highest specific activity with the value of 6.11±0.07 (U/mg) and the lowest value of specific activity was observed at 23% (w/w) of PEG 1500 and 25% (w/w) of potassium phosphate system (0.19±0.13 U/mg). As stated by Nandini and Rastogi (2011), potassium phosphate system was discovered to be more suitable for lipase (Bandmann *et al.*, 2000), alcohol dehydrogenase (Madhusudhan *et al.*, 2008) and c-phycocyanin (Patil and Raghavarao, 2007), while sodium citrate was found to be more suitable for protease (Porto *et al*., 2008) and bromelain (Babu *et al*., 2008). The use of citrate salts in ATPS is favored due to their high selectivity, biodegradable and non-toxic, thus reducing the environmental pollution (Goja *et al.*, 2013).

When separated into two phases, the position of the enzyme was found at the interphase instead of at the top or the bottom phase. The high concentration of PEG 1500 used in this study affects the physicochemical equilibria between phases, and the enzyme becomes precipitated in the interphase. Precipitation at the interphase is related to the volume effect of PEG which encourages the ability of PEG to precipitate protein as described by Kuznetsova *et al.* (2015). Moreover, high PEG concentration increases the viscosity and surface tension; hence obstruct the partitioning of protease to the other phase and causing the protease at the interphase (Zhao *et al.*, 2013).

Optimization of ATPS conditions

The separation of protein in PEG/Sodium citrate was optimized using a statistical experimental design

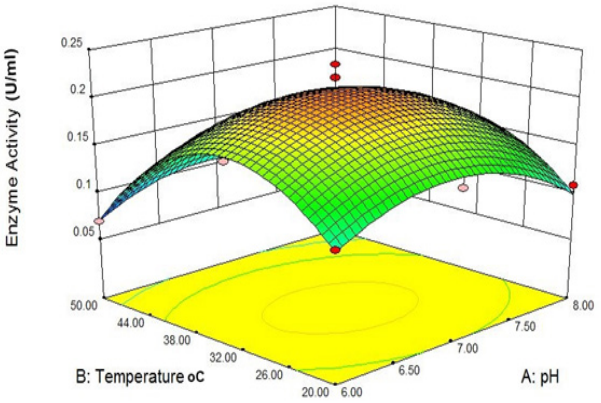

Figure 1. The three-dimensional surface plot of response surface analysis showing the effect of the interaction of pH and temperature on the specific activity of the protease.

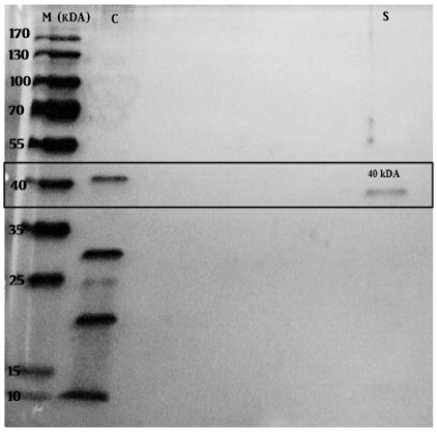

Figure 2. SDS-PAGE analysis of protease after ATPS purification. M: PageRuler® marker, C: crude enzyme before ATPS and S, purified enzyme after ATPS.

involving two variables, pH (A) and temperature (B) at a concentration of 27% (w/w) of PEG and 34% (w/w) of sodium citrate. The concentration of PEG 1500 and sodium citrate was screened by using the OFAT (one factor at a time) technique from a previous study (Samad, 2017). Table 2 presents the ANOVA of a quadratic model where the chosen response is enzyme activity. According to the analysis of variance, the F-value for the overall model (17.69) with a value of p-value (0.0023) are significant at 0.05 level, and the p-value of lack of fit is not significant (0.7429) indicates that the first-order model with interaction is fit in approximating the response surface of the experimental design. In this model, the linear model in terms of pH was insignificant (0.2994) while for linear and quadratic model of temperature variables were significant. Besides, the interaction of the pH and temperature variables is not significant, hence shows that pH parameter does not play the significant role in protein separation in this PEG/Sodium citrate system. The pH can be related to the isoelectric point of the proteins (Raja and Murty, 2013). In general, negatively charged protein should move to the PEG-rich phase while positively charged protein prefers in salt phase. In this study, the protein tends to precipitate at the interphase indicates the protein has reached its isoelectric point and cause the insignificant effect of pH on the system (Ketnawa *et al*., 2014).

The coefficient of determination (R^2) and adjusted R^2 values were 0.9546 and 0.9092, respectively. These values demonstrate a correlation between actual and predicted data (Ramyadevi *et al*., 2012). The closeness between the actual and predicted coefficient of determination (R^2) confirms good correlation for protease partitioning (Chavan *et al*., 2015). Besides, the value of R^2 implies that the predicted model could explain 95.46% of the variability in the response. From the three-dimensional surface plot in Figure 1, the central lump showed the maximum enzyme activity was found when the ATPS condition at pH 7 and 35°C with purification fold at 1.05. The result was supported by the equation from the previous study (Abd Samad, 2017) as mentioned below:

Enzyme Activity = -2.33880 + 0.62322* pH + 0.024247 *Temperature - 3.33333E-005* pH * Temperature - 0.045029* pH^2 - 3.64795E-004* $Temperature^2$

The result showed that the most favorable condition for partitioning of biomolecule products such as enzyme was found in neutral pH rather than the acidic or alkaline pH (Mohamed Ali *et al*., 2014). A similar finding was conducted by Loc *et al*. (2013) showed that the best temperature to carry out ATPS for a recombinant neutral protease was 30°C with 1.2 purification fold. For SDS-PAGE analysis, the presence of a single band with a molecular weight around 39-40 kDa after ATPS purification of the crude enzyme as showed in Figure 2 confirm the separation was occurred.

Conclusion

This study had demonstrated the potential application of ATPS processes for the recovery of protease enzyme from the fermentation broth. The maximum enzyme activity of 0.20± 0.01 U/ml was observed in ATPS conditions at pH 7 and 35°C. The system of PEG/Sodium citrate could be explored in details for future works as it needs the improvement of types of PEG and salt used in the system.

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