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Precipitation of Cellulase and Xylanase for Cross-Linked Enzyme Aggregates

Nurul Jannah Sulaiman, Roshanida A. Rahman*, Norzita Ngadi

Faculty of Chemical Engineering, UniversitiTeknologi Malaysia, 81310 UTM Johor Bahru, Johor, Malaysia

*Corresponding author: roshanida@cheme.utm.my

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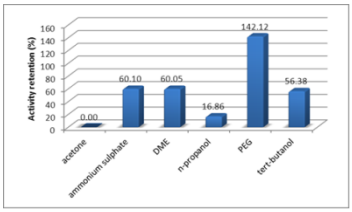
Graphical abstract



Abstract

Cross-linked enzyme aggregate is a promising strategy among other enzyme immobilization technologies such as solid matrix linking and gel entrapping. Despite of having the advantage of being reused, cross-linked enzyme aggregate (CLEA) also offers greater stability during operation and storage. Preparation of CLEA involves two steps which are precipitation and cross-linking of the enzymes. The purpose of this study is to find the best precipitant for cross-linked enzyme aggregate of cellulase and xylanase. The tested precipitants were acetone, ammonium sulphate, dimethoxyethane (DME), n-propanol, polyethyleneglycol (PEG), and tert-butanol. The enzymes were precipitated and cross-linked using glutaraldehyde. The enzyme activities were determined through DNS method and the relative activities for resulted CLEA were compared. It was found that PEG was the best precipitant for CLEA-cellulase while DME, ammonium sulphate and tert-butanol contributed the highest activity retention for CLEA-cellulase-xylanase under cellulase and xylanase assay, and CLEA-xylanase, respectively.

Keywords: Precipitation; cross-linked enzyme aggregates; glutaraldehyde; cellulase; xylanase

1.0 INTRODUCTION

Owing to the rise in green awareness, reactions that being catalyzed by enzymes are of growing importance for the industrial production of chemicals and pharmaceutical. The ability to produce a stable and reusable enzyme through immobilization has established a crucial step to make the process of enzymatic economically feasible [1]. Currently, the immobilization of enzyme that is properly designed has been discovered to be a very great tool to improve the enzyme stability, selectivity as well as specificity and also the enzymatic activity [2]. A carrier-free immobilization among other technologies that has been revealed as a promising strategy is cross-linked enzyme aggregates (CLEAs) [3]. It produce immobilized enzyme through the reaction of aggregation and cross-linking of the molecules of enzyme [4-5]. The low cost, efficiency and simplicity of this immobilization technology makes it attractive compared to other conventional methods.

Protein precipitation and cross-linking using glutaraldehyde as cross-linking agent is the common method for CLEAs preparation [6-8]. Separation of enzymes from its solution was achieved by transformation of soluble proteins to an aggregated state. Precipitating reagent can be categorized into three types; salt, organic solvent and non-ionic polymer. Salts are said to be very soluble, thus can stabilize the protein structure, and has low density, inexpensive and also readily available in pure form [9]. Most of organic solvents consist of alcohol component that contributes in preserving the hydrophobic analyte in solution that consequently results in protein precipitation and conjugation as well [10]. Non-ionic polymers promoted the proteins exclusion from the solvent regions sterically and precipitation happens after proteins concentration that subsequently make the solubility exceed [11]. Since different precipitants have different effect towards protein structure and characteristics, therefore, in this study, a few precipitants had been put into test to decide which precipitants act better.

In this study, multipurpose enzymes which are cellulase, cellulase-xylanase, and xylanase were used as the target for finding the best precipitants for their CLEA preparation. Acetone, ammonium sulphate, DME, n-propanol, PEG and tert-butanol were used as precipitants and glutaraldehyde was used as cross-linker to form enhanced activity CLEAs which is the same as the previous work [12]. This new method was being estimated to find the best precipitants that could yield more stable CLEAs because the previous literature conclude that different enzyme has different structure, yet produce different changes towards enzymatic activity.

Cellulase, cellulase-xylanase and xylanase CLEAs were prepared by aggregating using several precipitants and cross-linking enzyme molecules using glutaraldehyde as cross-linker. Precipitants were used as aggregating agent that can stabilize the enzyme structure based on previous work [13]. As is known, the addition of salts, or water-miscible organic solvents or non-ionic polymers, to aqueous solutions of proteins leads to their precipitation as physical aggregates, held together by non-covalent

bonding without perturbation of their tertiary structure [14]. Cellulase and xylanase were chosen because of its versatility as biocatalysts in biorefineries, textile, food, paper and pulp, feed and technical industry [15-16]. Glutaraldehyde is usually the chosen cross-linking agent, as it is inexpensive and readily available in commercial quantities [8].

Table 1 Types of precipitants used

Salt	Water-miscible organic solvents	Non-ionic polymer
Ammonium sulphate	Acetone DME n-propanol tert-butanol	Polyethylene glycol

Table 1 shows the precipitants that were chosen for this study. Proteins are precipitated from solutions with high salt concentrations as the salt ions become hydrated and the available water molecules decrease, drawing the water away from the protein hydrophobic surface regions which in turn results in aggregation of protein molecules via protein–protein hydrophobic interactions. Ammonium sulphate was reported to be the most effective salt precipitant [17]. Organic solvent precipitants lower the dielectric constant of the plasma protein solution, which increases the attraction between charged molecules and facilitates electrostatic protein interactions, and then the organic solvent displaces the ordered water molecules around the hydrophobic regions on the protein surface. Hydrophobic interactions between proteins are minimized as a result of the surrounding organic solvent, while electrostatic interactions become predominant and lead to protein aggregation [9]. PEG is a common polymer used in CLEA preparation [8, 12, 18-20]. This is because PEG has low tendency to denature when present at elevated temperature and high concentration [21].

■2.0 MATERIALS AND METHODS

2.1 Materials

Cellulysin Cellulase from Trichodermaviride, DME and n-propanol were purchased from Merck Sdn. Bhd. (Shah Alam, Selangor). Cellulase-xylanase and xylanase were supplied by Novozymes South Asia Ptd. Ltd. (Bangalore, India). Substrate; filter paper Whatman No. 1 was obtained from Ichem Solution Sdn Bhd. (Skudai, Johor). Other precipitants that were used in this study beside DME and n-propanol were acetone, ammonium sulphate, PEG and tert-butanol. Cross-linker that was used was glutaraldehyde. All of the chemicals were purchased from Sigma-Aldrich (Subang Jaya, Selangor) unless otherwise noted.

2.2 Preparation of CLEAs

Aggregates of cellulase (1ml from 1g/L), cellulase-xylanase (1ml) andxylanase (1ml) were prepared by adding varying precipitants (5ml) at 4˚C to precipitate the enzyme. Table 2 summarizes the concentration of precipitants used. After 15 minutes, droplets of glutaraldehyde were added under shaking by hand for cross-linking the enzyme aggregate, and the mixture was shaken at 4˚C for 4 hours. The supernatants produced were continually washed for three times with 0.05 M citrate buffer at pH 4.8 and separated by centrifugation at 10,000 rpm for 5 minutes at 4˚C for each wash. The final enzyme preparation was kept in the same buffer (1 ml) at 4°C.

Table 2 Precipitants concentration

Precipitants	Concentration
Acetone	99.5%
Ammonium sulphate	1 g/ml
DME	99.5%
n-propanol	99.5%
Polyethylene glycol	1 g/ml
tert-butanol	99.0%

2.3 Determination of Cellulase Activity

Free cellulase and CLEAs activity was determined by an assay method that was previously stated with the substrate of filter paper [22]. Citrate buffer (0.05 M) of pH 4.8 was used as blank, while other mixtures consist of 0.5 ml buffer and the remaining were of appropriate dilution of enzyme solution. All of the mixtures were made up until 0.75 ml. The mixtures were kept in the water bath at 50˚C for 60 minutes. Dinitrosalicyclic acid (1.5 ml) was added into the mixture to stop the reaction after the incubation. The absorbance of the solution was measured at 540 nm. The amount of enzyme that exhibit 1 µmol of glucose every minute was considered as one unit activity of cellulase. Immobilized enzyme residual activity was calculated according to the previous work [12]. All of the experiments were done in triplicate.

2.4 Xylanase Activity Assay

Free xylanase and CLEAs activity was determined by an assay method that was previously stated with the substrate of xylan [23]. Citrate buffer (0.05 M) of pH 4.8 was used as blank, while other mixtures consist of 0.5 ml buffer and the remaining were of appropriate dilution of enzyme solution. All of the mixtures were made up until 0.75 ml. The mixtures were kept in the water bath at 50˚C for 5 minutes. Dinitrosalicyclic acid (0.75 ml) was added into the mixture to stop the reaction after the incubation. The absorbance of the solution was measured at 575 nm. The amount of enzyme that exhibit 1 µmol of xylose every minute was considered as one unit activity of xylanase. Immobilized enzyme residual activity was calculated according to the previous work [12]. All of the experiments were done in triplicate.

■3.0 RESULTS AND DISCUSSION

Figure 1 shows the activity retention for cross-linked cellulase aggregates using various precipitants. As can be seen, immobilization of cellulase by using PEG as a precipitant gives the highest activity retention, about 142%. It seems that PEG constructs a more stable structure for the enzyme. Similar finding was observed when PEG was used as a precipitant on lipase, penicillin acylase, peroxidase and tyrosinase [24-28]. The enzymes were still in its origin conformational even after the changes induced by aggregated state [12]. Unlike for acetone, the retained activity of cellulase CLEA decreased, and the activity decreased to a complete inactivation of the enzymatic activity. This may be due to excessive cross-linking that happened when acetone, cellulase and glutaraldehyde reacted together [27].

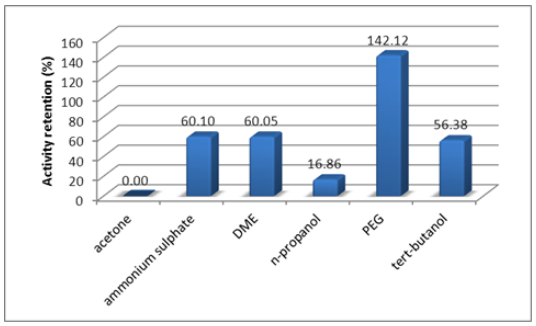


Figure 1 CLEA-cellulase activity retentionusing different precipitants

The influence of varying precipitants towards cellulase-xylanase CLEAs activity retention under cellulase assay is shown in Figure 2. DME was found suitable for the cellulase-xylanase aggregate to form stable structure of CLEAs. DME and tert-butanol retained 37.65% and 1.4% of the original activity, respectively. However, the CLEAs residual activity that were aggregated using acetone, ammonium sulphate, n-propanol and PEG were completely inactivated. This observation was contradicted with the findings reported from other studies [12, 15, 22, 25, 29-30]. This indicates that DME is more suitable for the cellulase structure in this enzyme than other precipitants for preparing stable cellulase-xylanase. Figure 3 shows the activity retention of cellulase-xylanase CLEAs under xylanase assay upon varying precipitants. It was found that the effect of retention activity diversed from the effect given under cellulase assay. This observation was believed due to different in structure between cellulase and xylanase. Ammonium sulphate was discovered to be more suitable for the xylanase conformational structure in cellulase-xylanase than other precipitants. Surprisingly, DME that gives the highest activity retention under cellulase assay gives the lowest activity under xylanase assay.

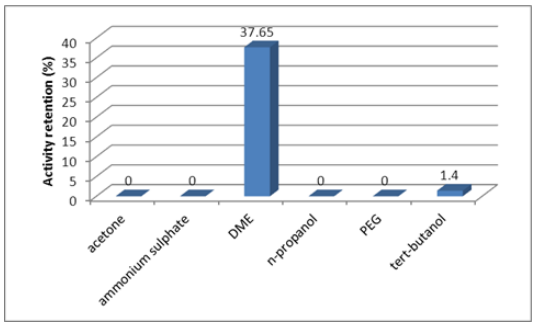


Figure 2 CLEA-cellulase-xylanase activity retention under cellulase assay using different precipitants

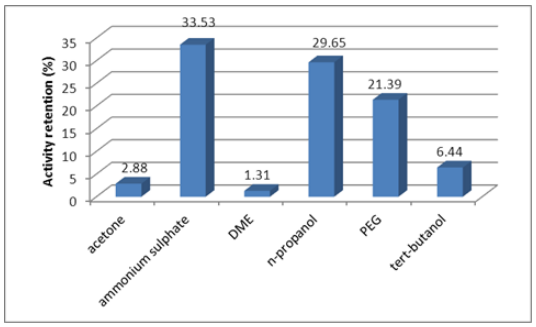


Figure 3 CLEA-cellulase-xylanase activity retention under xylanase assay using different precipitants

Similar to the previous studies, activity retention of CLEAs preparation showed distinguished variances from each other [12, 15, 20, 21, 27, 31]. The retained activity of xylanase CLEAs prepared using tert-butanol was 12.31% of its initial activity (Figure 4), which is three times higher than that of xylanase CLEAs prepared with ammonium sulphate. It has been reported that precipitation process will purify and stabilize proteins as it decreases the surface area of the enzyme that in contact with the solvent [30].

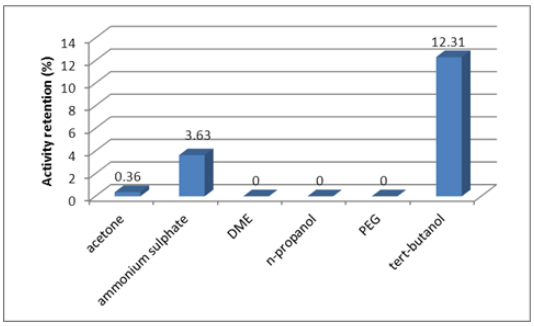


Figure 4 CLEA-xylanase activity retentionusing different precipitants

4.0 CONCLUSION

This study established a modest and effective method to attain stable cellulase, cellulase-xylanase, and xylanase CLEAs using varying precipitants and glutaraldehyde as a cross-linker.The cellulase CLEAs prepared using PEG had shown to exhibit even better activity retention compared to other precipitants. DME was found to be the best precipitant for cellulase structure in cellulase-xylanase enzyme while ammonium sulphate was the best for xylanase structure. For xylanase, tert-butanol was the most suitable precipitant for CLEA preparation of xylanase. Most of all, these preparations can be further optimized by manipulating the parameters involved during the enzyme preparation. This strategy can be utilized in improving preparation of enzyme that being used in some chemicals and biocatalysis production. This strategy is also tremendously simple and may be of general use to produce rigid and stable CLEAs.

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