# Production of Cross-Linked Enzyme Aggregate-Lipase from Channel Catfish (*Ictularus Punctatus*) Viscera

Safa Senan Mahmod[1], Faridah Yusof [2] and Mohammed Saedi Jami [3]

***Abstract*—** Cross linked enzyme aggregates technology is one of the latest methods in immobilization of enzymes. This technique provides biocatalysts with higher activity, stability and reusability in industrial applications. In this research, lipase was extracted from the viscera of river channel catfish (*Ictularus punctatus*) and was immobilized based on cross-liked enzyme aggregate methodology. Glutaraldehyde was used as a cross-linker, acetone as precipitant and bovine serum albumin as an additive to improve the formation of CLEA-lipase. Experimental design was carried out by Response Surface Methodology using central composite design to find the optimum conditions for immobilization which was 50 mM of glutaraldehyde, 60% (v/v) Acetone and 0.11 mM of BSA. Finally, CLEA-lipase's specific activity was 1.713 U/mg that is 20.43% higher than the free lipase activity.

***Keywords*—** BSA, CLEA, cross-linker, fish viscera, *Ictularus punctatus*, lipase, precipitant.

## I. Introduction

CROSS-LINKED enzyme aggregates (CLEAs) have been reported to have various interesting benefits compared to free enzymes such as a higher concentrated activity, better stability, lower production cost, simple synthesis procedure, recyclability feature due to their heterogeneous character and the fact that enzyme purification step is not required [1]. Combination of three steps; purification, precipitation and immobilization in one step attracted the researchers and scientists for the simplicity, time-saving and low cost process. Moreover, the cross-linked enzyme aggregates method led to the development of new family of immobilized enzymes that is high in stability and activity [2]. The main aim of immobilizing enzymes is to be able to isolate the enzyme from the reaction mixture and reuse it in order to increase its reusability [3].

Glutaraldehyde has been considered as the most common cross-linker in the CLEA technology, it is a bifunctional reactive agent that is capable of reacting with the surface amino groups of enzymes and carriers [5].

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For precipitation, many salts and organic solvents have been reported to be used in the CLEA immobilization technology; such as ammonium sulfate [6-7-8], acetone [8-9], polyethylene glycol (PEG 6000) [8-10], etc.

Bovine serum albumin (BSA) as a proteic co-feeder is used in many studies as an additive to the process of CLEA preparation [6], it is mainly used to facilitate CLEAs preparation in cases in which the protein concentration is low and/or the enzyme activity is susceptible to a high concentration of the cross-linker required to obtain the aggregates [6-11].

Usually, fish waste including (guts, bones, skin, head, fins) is collected and dumped into the waste sites or in the seas leading to many health and environmental problems including contamination of the soil and water sources and sickening odors. The significance of this study is to make use of this wasteful by-product to produce a commonly used enzyme in industry nowadays. However, several hydrolases have been screened from the extract of fish viscera; lipase was positively identified in the extract of channel catfish (*Ictularus punctatus*) viscera [17].

Lipase is known to have many industrial applications for its role in catalyzing the hydrolysis of various forms of fatty acyl esters and in contrast to other esterases needs an oil-water interface for optimum activity. Lipases are found in nature and have been found in animals, higher plants and microorganisms [4]. CLEA-lipase has many potential applications in industry such as detergent, cosmetics, pharmaceutical, biodiesel, etc.

In this study, lipase extracted form channel catfish's viscera was immobilized using gulteraldehyde as cross-linker, acetone as precipitant and bovine serum albumin as an inert additive. The specific activity and the enzyme recovery of produced lipase-CLEA was calculated and compared with the free enzyme.

## II. Method

### *A. Materials*

All chemicals used for analysis are obtained from Essen Haus Sdn. Bhd and Merek Sdn. Bhd. Tecan microplate reader (Switzerland) was used to measure the absorbance, Sartorius Shaker (Germany) for preparation of CLEA-lipase.

### *B. Sample preparation*

Visceral parts of channel catfish was washed and weighed (749 g) for blending with 1M phosphate buffer (pH7.3) to viscera 2:1 then filtered by muslin cloth.

The extract was then centrifuged at 12,000 rpm at 4°C for 1 hour; the supernatant collected was used to prepare the crude enzyme by precipitating with 4M ammonium sulfate for 24 h at 4°C with continuous stirring. The sample was dissolved in PBS and centrifuged at 3000 rpm for 15min at 4°C. Followed by dialysis against minimal phosphate buffer saline pH 7 using 10000 MWCO and left 4 hours with continuous mixing at 4°C. Sample is stored at -20 °C for further steps.

*C. Protein Concentration*

The protein content of the prepared sample was measured by using the famous Bradford assay using BSA as standard [12].

*D. Lipase Activity Assay*

Modified method of reference [13] was used to measure free-lipase and CLEA-lipase activity of channel catfish viscera using *p*-nitrophenyl palmitate as substrate and the optical density was measured at the wavelength of 410 nm after incubation at 37°C for 15 min. The substrate solution was prepared by mixing 10 mL isopropanol, 30 mg *p*NPP, 0.5M Tris-HCL buffer, 0.4% Triton X-100 and 100 mg of gum Arabic, the substrate solution was incubated at 40°C for 10 min then the enzyme was added to the substrate solution, 2 ml of 96% ethanol was added after the 10 min. Lipase activity is determined by using *p*-Nitrophenyl palmitate standard curve.

One unit of lipase activity is defined as the required enzyme to liberate 1 μM of *p*-nitophenol/min under experimental conditions.

*E. Preparation of CLEA-Lipase*

Cross linked enzyme aggregate-lipase was prepared by adding 1 mL of the prepared sample to different concentration of the cross-linker (Glutaraldehyde 50, 65, 80 mM) and organic solvent precipitant of different concentrations (Acetone 30, 45, 60% (v/v)) and Bovine Serum Albumin BSA as an inner additive to facilitate the formation of CLEA-lipase in different concentrations (0.04, 0.11, 0.19 mM) that is equivalent to (10, 30, 50 mg) in 1 mL of enzyme. Samples were kept in the shaker at room temperature for 17 h at 200 rpm as shown in Fig 1.

Samples are taken out and centrifuged at 4000 rpm at 4°C for 30 min and washed 3 times with 100% (v/v) acetone. Finally lipase activity assay was conducted on the immobilized enzymes and stored at -20°C for further tests.

*F. Experimental Design and Statistical Analysis of Optimization of CLEA-Lipase*

Design Expert (version 6.0.8) computer software was used to determine the optimum parameters in preparation of CLEA - lipase from fish viscera. Response Surface Methodology (RSM) was used for the experimental design using central composite design (CCD). Three factors were selected for this design; glutaraldehyde, acetone and BSA. The design has 17 runs the interaction between these factors is based on 3-level factorial. While the temperature, time and speed were fixed as room temperature, 17 hours and 200 rpm, respectively. The response in this design was the specific enzyme activity of CLEA-Lipase. Regression analysis was carried out and regression equation was developed by statistical analysis (ANOVA).

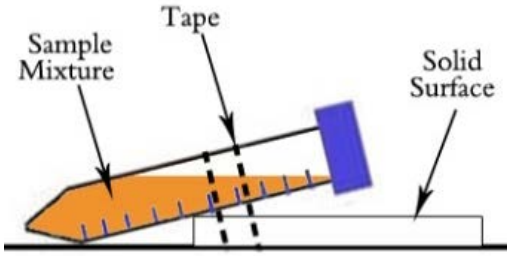


Fig. 1 The position of the sample mixture (free enzyme, glutaraldehyde, acetone, BSA) to be immobilized in the shaker

## III. Results and Discussion

*A. Preparation of free enzyme stock solution*

4M ammonium sulfate was used to precipitate all proteins and to discard the other molecules present in the mixture, it was left to precipitate overnight under slow stirring at 4°C. the precipitate is collected in the next day and centrifuged at 5000 x g for 15min at 4°C, the supernatant was discarded and the precipitate were dissolved in minimal phosphate buffer saline PBS (pH7). Then, dialysis of the solution with 10,000 MWCO against PBS was carried out for 4 hours at 4°C. Dialysis let the smaller unwanted molecules to leave the mixture to the buffer leaving the lipase molecule inside membrane because of its large size. Also, the removal of any low MW contaminants present in the sample mixture occurs during the dialysis process, because Snake Skin Dialysis Tubing is made from the same type of regenerated cellulose as flat tubing, its dialysis performance matches that of conventional tubing [14]. Sample was stored at -20 °C for further experiments.

*B. Preparation of CLEA-Lipase*

The assayed CLEAs did not go through previous drying; based on reference [6] where recovered activities of fresh CLEAs found to be similar to that of CLEAs vacuum dried for 16 h.

Different CLEA-Lipase were prepared by varying the amounts of Glutaraldehyde, Acetone and BSA as shown in Table I, the highest lipase specific activity determined was at 50 mM, 60 % (w/v) and 0.11 mM of glutaraldehyde, acetone and BSA, respectively.

The analysis of variables (ANOVA) facilitated the validation of the model (1) for CLEA-lipase specific activity, where the correlation coefficient of 0.89, F-value is 6.35 implies that the model is significant, there is only a 1.18% chance that a "Model F-Value" could occur due to noise and p=0.0118. In general, the model was significant and the response surface is presented in Fig 3 and Fig 4 where the optimum glutaraldehyde concentration was shown to be at 50 mM. The specific activity increased as acetone concentration increased and the activity increased as BSA

increased till some point around 0.13 mM and a drop in the graph was shown after that Fig 4.

Specific activity = 1.05 - 0.13 * A +0.17 * B + 0.068 * C - 0.30 * $A^2$+ 0.27 * $B^2$ - 0.15 * $C^2$ -0.22 * A * B - 0.072 * A * C + 0.15 * B * C (1)

Where A is glutaraldehyde, B is acetone and C is BSA concentrations.

TABLE I
RESULT OF ENZYME SPECIFIC ACTIVITY OF CLEA-LIPASE

| Run | Gluteraldehyde (mM) | Acetone %(v/v) | BSA (mM) | Lipse specific activity (U/mg) |
|---|---|---|---|---|
| 1 | 65.00 | 60.00 | 0.11 | 1.58 |
| 2 | 80.00 | 60.00 | 0.19 | 0.74 |
| 3 | 50.00 | 60.00 | 0.19 | 1.71 |
| 4 | 65.00 | 30.00 | 0.11 | 0.95 |
| 5 | 65.00 | 45.00 | 0.11 | 1.28 |
| 6 | 50.00 | 45.00 | 0.11 | 0.87 |
| 7 | 80.00 | 30.00 | 0.19 | 0.79 |
| 8 | 65.00 | 45.00 | 0.11 | 1.00 |
| 9 | 80.00 | 30.00 | 0.04 | 0.92 |
| 10 | 65.00 | 45.00 | 0.11 | 1.07 |
| 11 | 80.00 | 45.00 | 0.11 | 0.51 |
| 12 | 50.00 | 60.00 | 0.04 | 0.96 |
| 13 | 50.00 | 30.00 | 0.19 | 0.56 |
| 14 | 50.00 | 30.00 | 0.04 | 0.71 |
| 15 | 80.00 | 60.00 | 0.04 | 0.59 |
| 16 | 65.00 | 45.00 | 0.19 | 0.87 |
| 17 | 65.00 | 45.00 | 0.04 | 0.82 |

The specific activity of the immobilized enzyme has increased 20.43% compared to free lipase activity; this is the main aim of the immobilization of the enzyme, but the increase is not too high for many reasons: (1) Enzymes tend to form aggregates in organic solvents (in this case acetone) and hence tend to be poorly accessible for the substrate (2) the enzyme molecule will be cross-linked or attached to the additive providing less active sites for the substrate (Singh et al, 2012).

### *C. Effect of Additive*

In a separate experiment, the effect of BSA as an additive in the formation of CLEA-lipase was evaluated. 2 runs were carried –with BSA the specific activity was 1.68 U/mg and without BSA it was 0.4998 U/mg - by fixing the values of the cross-linker and precipitant at 60 mM and 60 % (v/v), respectively.

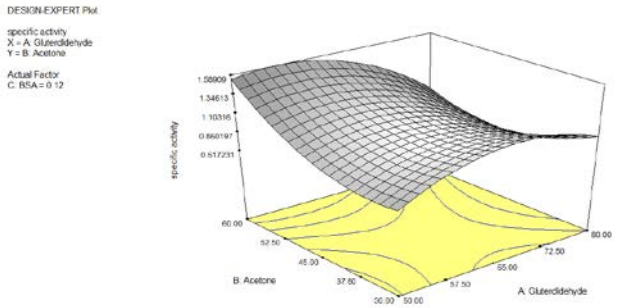


Fig. 3 Response surface for CLEA-lipase specific activity, showing the interaction between glutaraldehyde and acetone

The result shows 70.25% increase in the CLEA-lipase specific activity with BSA, this can be explained as this protein provides lysine residues which glutaraldehyde can bind and avoid the denaturing of the target catalytically active protein; this is shown clearly in Fig 5. Addition of BSA as a co-feeder allowed the cross-linking step to act effectively, and lead to better stabilization of CLEA-lipase produced [6].

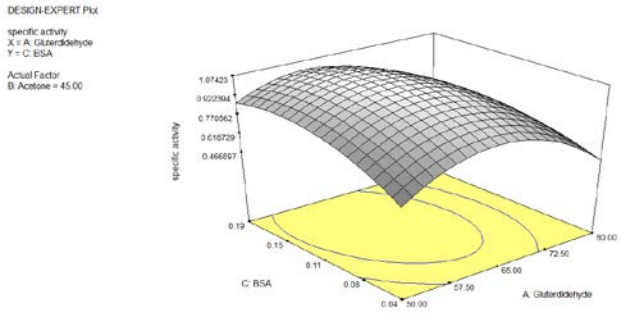


Fig. 4 Response surface for CLEA-lipase specific activity, showing the interaction between glutaraldehyde and BSA

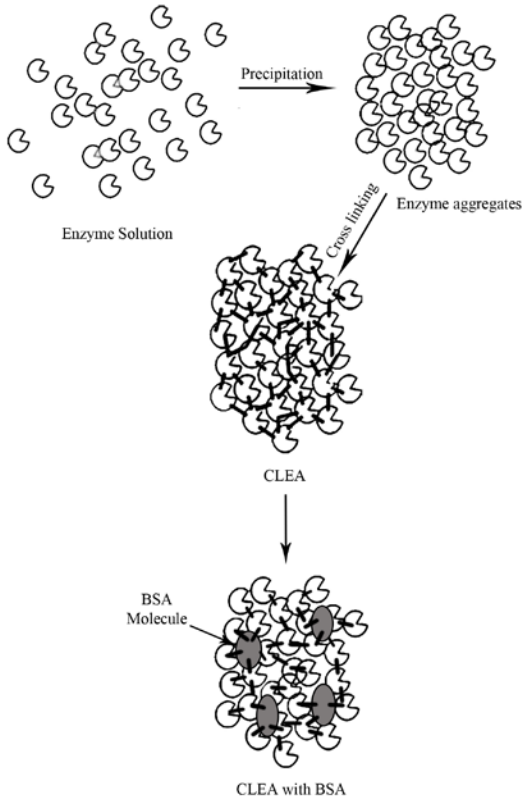


Fig. 5 Schematic diagram of preparation of CLEA-lipase

### *D. Effect of Precipitant*

The use of organic solvents as reaction media for biocatalytic reactions has been proven to be an extremely useful approach to expand the range and efficiency of the practical applications of biocatalysts. Precipitation helps in forming the aggregates that is initial step for cross linking;

from Fig. 3 it is shown that the higher acetone concentration has higher specific activity for the formed CLEA-lipase.

Based on reference [15] it is stated that the unfortunate fact that the majority of naturally available biocatalysts are usually not optimally suited for catalysis in non-aqueous solvents (e.g. acetone) in industrial processes.

### *E. Effect of Glutaraldehyde*

Glutaraldehyde is the most common cross-linking agent to prepare CLEAs. According to reference [16], Glutaraldehyde is described to be crucial for enzyme catalytic activity, knowing that glutaraldehyde is a small molecule which could penetrate the internal structure of the protein and react with the amino residues. The results in Table I showed that 50 mM glutaraldehyde was a suitable concentration for cross-linking. For higher concentrations of glutaraldehyde the activity observed was too low, this can be explained as an excessive cross-linking took place resulting in a loss of the enzyme flexibility that is necessary for its activity [8], or more cross-linking occurred leading to too strong CLEA with a strong diffusion resistance [10].

## IV. Conclusion

CLEA-lipase from the viscera of channel catfish (*Ictularus punctatus*) was synthesized using glutaraldehyde as cross-linking agent, acetone as precipitant and bovine serum albumin as an additive. The effect of each of the factors involved in the CLEA-lipase production was discussed based on the comparison made between the specific activity of free lipase and CLEA-lipase. Immobilized lipase showed higher activity than the free lipase. Also, the effect of the co-feeder additive on the specific activity of CLEA-lipase was discussed with and without BSA. Optimum parameters for the CLEA-lipase production procedure were determined using experimental design and statistical analysis to verify the model.

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