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OPTIMIZING THE PREPARATION OF CROSS-LINKED ENZYME AGGREGATES (CLEA) - AMYLASE FROM SUPERMEAL WORM *(ZOPHOBAS MORIO)*

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ABSTRACT

Cross-linked enzyme aggregates (CLEA) technology has been gaining more attention recently due to their advantages in industrial application. Various CLEA- enzymes has been successfully prepared with diverse advantages. Among the benefits of CLEA are; more stability, easy to prepare, cheap and reusable as compared to the free enzymes. This particular study has attempted to optimize the production of CLEA-amylase, which sourced from supermeal worm protein extract. One Factor at a Time (OFAT) and Response Surface Methodology (RSM) had been used to achieve the goal. Screening of several additives had been conducted to enhance the recovery activity of CLEA- amylase. The final preparation of CLEA-amylase was done by applying acetone as precipitant, glutaraldehyde as cross-linking agent and BSA as proteic feeder. Under Face Centered Central Composite Design (FCCCD), the interactions between those three influential parameters were observed. From the 20 runs, the optimum activity was recorded at Run 11 (26.65%), under the condition of using 60% acetone, 90mM glutaraldehyde and 2.5 mg/ml bovine serume albumin (BSA) as additive. The model equation was considered valid after been tested through validation test. Acetone and glutaraldehyde concentration had shown the most dominant factor from this study, while BSA concentration had insignificant effects.

Keywords: cross-linked enzyme aggregate, supermeal worm, response surface methodology.

INTRODUCTION

Supermeal worm (Zophobas morio) is the larvae stage for darkling beetles insects. They exist naturally all over the world and also available in Malaysia due to its capability to adapt with tropical climate [1]. Previous studies have found high protein concentration encompasses in various insects species. Generally, palatable insects will have around 9-25% of protein content [2]. According to [3], supermeal worm larvae contains 45% of crude protein from the dry matter composition of processed insects. Since enzymes are made up from protein, high protein composition would suggest eminent enzyme hold by this insect. Due to its eating habit, which consume mostly on starchy foods like wheat, supermeal worm larvae possess high level of amylase activity, which is important for their digestive system.

Hydrolases enzymes like amylase are extremely important industrially. Amylases have been used widely in food and detergent industries [4] and currently marked 25% of the overall enzyme market [5].

Unfortunately, enzymes can be denatured easily and become unstable during industrial applications. This is because; most of the industrial processing condition would expose enzymes to harsh environments like extreme temperature and pH [6]. Therefore, immobilization of enzymes has been utilized so that the stability, selectivity, activity and productivity of the enzymes could be enhanced. Furthermore, the technology also aims to produce reusable, practicable, safe-to-use as well as cost-efficient enzymes.

There are few immobilization techniques available such as entrapment, encapsulation, support-based immobilization as well as self-immobilization. Carriers used in support-based immobilization are undesirable since it can reduce more than half of the catalytic activity due to large amounts of non-catalytic mass. Besides that, they are laborious, time consuming and expensive.

In contrast, carrier-free immobilization is considered as the best method to date since immobilization is achieved by cross-linking the enzymes molecules with cross-linking agents, usually glutaraldehyde; hence remove the necessity for any carriers. In addition, non-purified enzymes could be used in the CLEA preparation since it combines two unit process; namely purification and immobilization in one single operation [7].

To prepare the CLEA enzymes, salts (eg; ammonium sulphate), water-miscible organic solvents (eg; acetone) and non-ionic polymers can be used to form non-covalent bonding, which helps to aggregate the protein molecules. As a consequence, the protein would turn permanently insoluble while still maintaining their superstructure and catalytic activity. Acetone has been studied as a precipitant in several previous researches [8]–[10]. Most of the works resulted in good CLEA activity, however there are also studies which showed negative effects of using acetone as a precipitant [9]. This is because; different enzymes have dissimilar structure upon reacting with the acetone, thus lead to various consequences.

Next, the aggregates protein molecules will be cross-linked with cross-linker reagents, usually glutaraldehyde. This is a vital component in preparing CLEA since it helps to lock the aggregated enzyme in less favourable condition, hence maintaining the enzyme activity. It is noteworthy to pay attention on the glutaraldehyde concentration used in preparing CLEA.

Insufficient cross-linking might occur when the glutaraldehyde concentration is too low, thus reducing the stability of CLEA. Contrastingly, too high level of glutaraldehyde might lead to total loss of CLEA's activity and flexibility [7].

Another important point to consider is that glutaraldehyde might decrease the active site available on CLEA surface. This is because; it might negatively select functional amino groups like lysine to react during the process. The problems will turn bigger if the enzymes encompasses only little amount of protein and lysine residue, hence reducing the efficacy of the cross-linking procedure [11]. To solve the issue, additives like bovine serum albumin (BSA), heptane and sodium dodecyl sulphate (SDS) can be added during the preparation process. In fact, there are two main reasons of adding additives; (1) to stabilize the enzyme activity, (2) to provide glutaraldehyde with additional lysine groups to react with [12]

In this particular study, acetone, glutaraldehyde and BSA concentration were optimized. One Factor at a Time (OFAT) method was used initially to get the optimum range for all three factors. Next, Response Surface Methodology (RSM) under Face Centered Central Composite Design was employed to optimize the CLEA-amylase recovery activity.

MATERIALS AND METHODS

Materials

Supermeal worm larvae were grown in several plastic containers, with holes on the lid to promote aeration. Ground wheat and chicken bran in the ratio of 2:1 was provided as their food bedding, whereas carrot was supplied once in every two days as their water source. They were grown for at least 4 weeks and only those which achieved more than 600mg were used in this study. Most of the chemicals used were analytical grades, purchased from different companies (Friendmann Schmidt, Sigma Aldrich, Merck, and Systerm). Thermo Scientific Multiskan Go spectrophotometer was used for measuring absorbance. All experiments were done in triplicates.

Extraction of crude sample from supermeal worm

Worms with weight more than 600mg were used in this study. They were washed under continuous tap water to remove any dirt before placed in -200 ^{o}C freezer for 15 minutes to make it senseless.

For each extraction, 20 g of worms were used and blend for 1 minute with phosphate buffer (pH 6.11) in ratio 1:1.4 (w/v). These parameters were the optimum condition for amylase extraction based on earlier study. Next, the solution was filtered by muslin cloth to separate solid residue before subjected to 12,000 rpm centrifugation for 1 hour at 4 ^{o}C. The resulting supernatant was then precipitated by 4M ammonium sulphate under gentle stirring for 60 minutes at 4 ^{o}C for salting out the protein. Another centrifugation step was applied at 10,000 rpm for 1 hour at 4 ^{o}C. Here, the supernatant was discarded, whereas the solid residue was dissolved in minimal phosphate buffer and stored in -20 ^{o}C.

Amylase activity assay

Bernfeld method was followed with slight modification to determine amylase activity assay [13]. In 0.02M sodium phosphate buffer (pH 6.9), 1% (w/v) starch was dissolved together with 0.006M NaCl by heating and continuous stirring until clear solution was obtained. 0.5ml enzymes was added into a glass before added with 0.5ml pre-incubated starch solution. The mixture was then incubated for 15 minutes at 37 ^{o}C. To stop the reaction, 1ml of dinitrosalicyclic acid (DNS) reagent was added and immediately boiled for 5 minutes. The tube was then left cool at room temperature before added with 10ml distilled water. Tube was then inverted for several times to ensure even mixing. The absorbance was then measured at 540nm and compared with a standard curve drawn using various concentration of maltose stock solution.

1 unit of amylase is defined as the amount of enzyme which hydrolyse 1 µmoles of maltose per minute under specified conditions.

To calculate recovery activity of CLEA- amylase, the following equation 1 was applied:

$$Recovery\ activity\ (\%) = \frac{Total\ CLEA\ activity}{Total\ activity\ of\ free\ enzyme\ for\ CLEA\ preparation} \quad (1)$$

Protein concentration

Bradford assay, which use BSA as a standard was employed to determine protein content available in the supermeal worm extraction. The absorbance was then read at 595nm [14].

CLEA preparation

Lopez and co-workers' method was referred with slight modification to prepare CLEA-amylase [15]. 0.5 ml of enzyme solution from supermeal extract was poured into a 15ml Falcon tube. Next, acetone, glutaraldehyde and additives were added in varying concentrations until the final volume reached 4ml. 200rpm agitation was used at room temperature and left to agitate for 16 hours. After the process was completed, the insoluble CLEA was washed by adding 3ml of acetone into the tube before centrifuged at 4000 rpm at 4 ^{o}C for 30 minutes. The resulting supernatant was decanted, while the pellet was washed with acetone for two more times. The amylase assay was done directly. If the CLEA need to be stored, 3ml acetone was added and kept at 4 ^{o}C before the assay was done.

One factor at a time (OFAT) studies

OFAT studies were done to investigate the optimum range that will be used in the optimization part later. 4 influencing parameters were studied in this conventional method; namely (1) acetone concentration, (2) glutaraldehyde concentration, (3) BSA concentration and (4) cross-linking time.

Screening of several additives

To screen the additives, acetone and glutaraldehyde concentration were fixed at 60% and 60mm respectively. These are the optimum parameters as obtained from OFAT studies.10mg of Sodium dodecyl sulphate (SDS), 2.5 mg/ml of BSA and 50% of heptane was screened to observe the effect on the CLEA-amylase recovery activity.

Optimizing the preparation of CLEA-amylase

In this particular study, the optimization process was done with the help of Design of Experiment (DOE) ver 7.0.0 software. Response Surface Methodology (RSM) under Face Centered Central Composite Design (FCCCD) was chose to analyse the result.

Various range of acetone (40, 60, 80%), glutaraldehyde (30, 60, 90 mM) and BSA (0.5, 2.5, 4.5 mg/ml) concentrations were used to cross-link the crude enzyme. The optimum range from the OFAT studies were set as centre points in RSM FCCCD with another two minimum and maximum points. Total of 20 runs were obtained, with 6 replications at the centre point.The entire process was done similarly as aforementioned steps.

RESULTS AND DISCUSSIONS.

Extraction of crude sample from supermeal worm

Optimum condition for buffer pH and concentration were vital to get maximum activity of amylase before immobilization was done. From earlier study, 0.05M phosphate buffer (pH 6.11) with the ratio of 72% (w/v) supermeal worm was found to yield the highest amylase activity (around 75.12 U/ml).

OFAT studies for acetone concentration

In the absence of any additives, glutaraldehdye concentration was kept at 60mM, while acetone was studied at varying level (20-80%). As shown in Figure-1, 60% acetone concentration had displayed the best CLEA-amylase recovery activity, thus were kept constant in the next study. There is probably not enough precipitation below 60% acetone concentration, whereas, beyond that level, the protein might experience too much precipitation. This is undesirable since it can lead to mass transfer limitation of substrate to react with the enzyme [11].

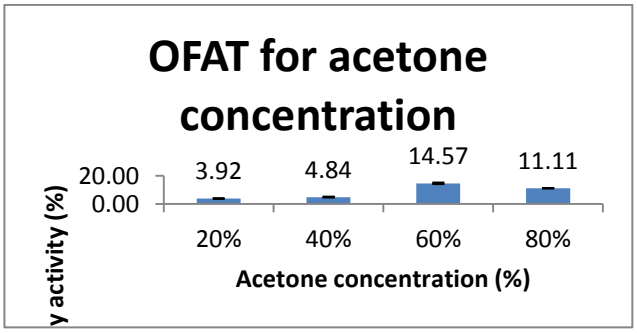

Figure-1. OFAT studies on effect of acetone concentration on CLEA-amylase recovery activity.

OFAT studies for glutaraldehyde concentration

As 60% acetone concentration had shown the best recovery activity from the previous test, thus it was kept constant. In contrast, glutaraldehyde concentration was varied from 30-150mM to study their effect on the recovery activity. Similarly, no additives were added in the CLEA preparation. As shown in Figure-2, 60mM glutaraldehyde concentration was found to exhibit the maximum CLEA-amylase recovery activity. Hence, in the subsequent CLEA preparation, 60mM glutaraldehyde concentration was applied.

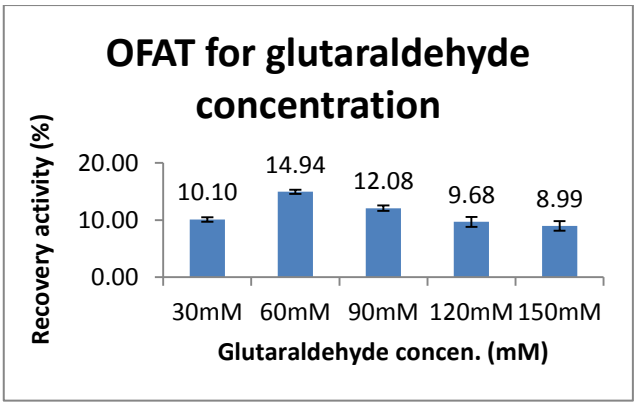

Figure-2. OFAT studies on effect of glutaraldehyde concentration on CLEA-amylase recovery activity.

OFAT studies for agitation time

Both acetone and glutaraldehyde concentration was kept fixed at 60% and 60mM respectively in this study without the presence of any additives. Agitation time plays significant role on the stability of CLEA since it effect the cross-linking efficiency of glutaraldehyde during the preparation. As shown in Figure-3 below, there is only slight difference between all tested agitation times, with 16 hours demonstrated the best recovery activity. The results obtained here was acceptable to those reported by [16], [17] where they found 17 hours to be the best cross-linking time.

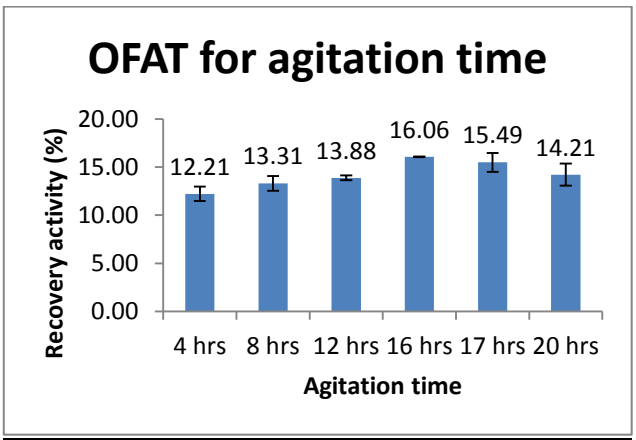

Figure-3. Effect of agitation time on CLEA-amylase recovery activity.

Screening of additives

Three common additives were screened in this study which has different influence on CLEA recovery activity. BSA was expected to behave as a proteic feeder [18], SDS as surfactants [11] while heptane as an interfacial activation [11]. From Figure 4 below, presence of BSA in CLEA preparation had improved the CLEA-amylase recovery activity in organic solvent medium to 174.19% relative activity. In this study, CLEA which was prepared in the absence of additives was considered as 100% relative activity. One of the compelling reasons is because; BSA might facilitate the enzyme activity to be more stable. This is important since high glutaraldehyde concentration used to achieve aggregates during CLEA preparation might impair the enzyme activity [12]. Besides that, additional amino groups of lysine might be provided by the BSA, thus avoiding glutaraldehyde from cross-linking with amino groups related to the active site of enzymes [11]. For instance, studied by [19], they found 100% recovery activity of CLEA-lipase in addition of BSA. Contrastingly, only 0.4% recovered activity was achieved in the preparation without BSA. Likewise, the presence of 10 mg BSA per 100 mg aminoacylase had increased recovery activity of CLEA-aminoacylase from 24 – 82% [18].

SDS had exhibit less relative activity as compared to the non-additive CLEA in this study. The idea of using SDS come from the fact that it can affect the enzyme activity since protein structure might be altered by the interaction of this surfactants with enzyme's binding sites [17]. Moreover, SDS might inactivate the globular proteins in the enzyme thus, reducing the enzyme activity.

On the other hand, heptane was applied as an interfacial activation for CLEA preparation as suggested by Guauque Torres *et al*. [11]. When added in solution, heptane shows hydrophobic properties. In this study, the presence of heptane led to higher recovery activity as compared to CLEA prepared without additives. The feasible reason is because, heptane might provide the enzyme with extra interfacial surface as reflected by its function.

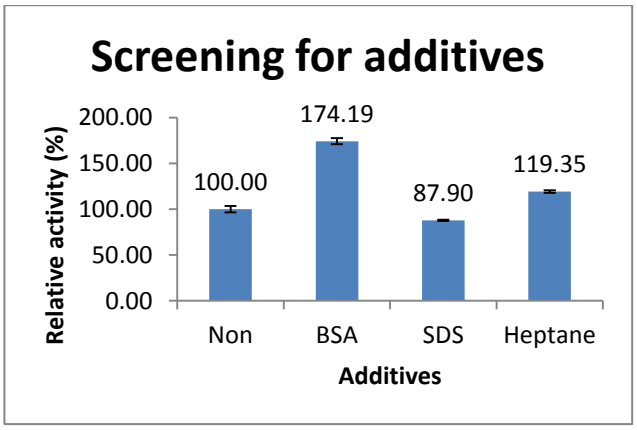

Figure-4. Effect of several additives on CLEA-amylase relative activity.

OFAT studies on BSA concentration

Since BSA had the best effect on CLEA-amylase recovery activity from the previous screening study, it was added for the next preparation. However, it is important to investigate the optimum BSA concentration to give the maximum activity. Too little BSA might give insignificant result on the recovery activity, whereas excessive addition might lead to competition between free amino group of amylase with free amino groups supplied by the BSA. This is undesirable since it could reduce the CLEA recovery activity by preventing necessary cross-linking of amylase molecules. As shown in Figure-5 below, 2.5 mg/ml BSA concentration had demonstrated the highest recovery activity of CLEA-amylase by 22.33%. In contrast, CLEA without BSA presence only marked 13.29%.

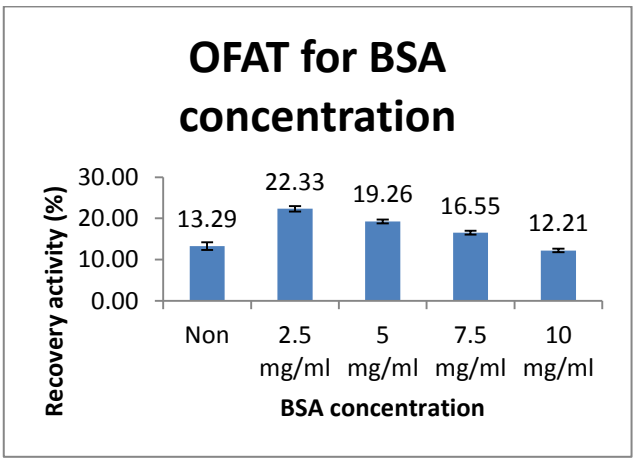

Figure-5. Effect of BSA concentration on CLEA-amylase recovery activity.

Experimental design of CLEA-amylase preparation

Statistical analysis was done by using Design of Expert (DOE) ver. 7.0.0 software to get better understanding on the parameters used in CLEA-amylase preparation. As a consequence from the OFAT studies, optimization was done on the 3 most significant factors that would influence the CLEA-amylase recovery activity which are; acetone, glutaraldehyde and BSA concentration. These are the 3 factors studied previously by [16], [17] on CLEA-lipase.

Response Surface Methodology (RSM) under Face Centered Central Composite Design (FCCCD) was chosen to analyze the data. Total of 20 runs were conducted with six replications of center points. The varying parameters used and the corresponding response were tabulated in Table-1 below.

Table-1. Experimental design using FCCCD showing the parameters applied and response of CLEA-amylase recovery activity.

Run	A	B	C	Response
	Acetone concen. (%)	Glutaral .concen. (mM)	BSA concen. (mg/ml)	Recovery activity (%)
1	40 (-1)	90 (+1)	0.5 (-1)	5.77
2	40 (-1)	30 (-1)	4.5 (+1)	5.50
3	80 (+1)	60 (0)	2.5 (0)	13.72
4	40 (-1)	90 (+1)	4.5 (+1)	12.89
5	60 (0)	60 (0)	0.5 (-1)	21.70
6	40 (-1)	60 (0)	2.5 (0)	11.38
7	80 (+1)	30 (-1)	4.5 (+1)	7.89
8	60 (0)	60 (0)	4.5 (+1)	20.55
9	60 (0)	60 (0)	2.5 (0)	21.79
10	80 (+1)	90 (+1)	4.5 (+1)	16.74
11	60 (0)	90 (+1)	2.5 (0)	26.65
12	80 (+1)	90 (+1)	0.5 (-1)	21.06
13	60 (0)	60 (0)	2.5 (0)	23.30
14	60 (0)	60 (0)	2.5 (0)	23.58
15	80 (+1)	30 (-1)	0.5 (-1)	8.53
16	60 (0)	60 (0)	2.5 (0)	21.88
17	60 (0)	60 (0)	2.5 (0)	23.21
18	60 (0)	30 (-1)	2.5 (0)	13.99
19	60 (0)	60 (0)	2.5 (0)	22.89
20	40 (-1)	30 (-1)	0.5 (-1)	6.06

Run 11 had displayed the highest CLEA-amylase recovery activity (26.65%) when 60% acetone, 90 mm glutaraldehyde and 2.5 mg/ml of BSA concentration were used. The activity of CLEA-amylase was found to be smaller than in free enzyme, particularly due to the immobilization process. This is because; immobilization might blocked the active site of the enzyme; thus hindering the substrate from having efficient catalytic reaction [20–22]. It is also important to take note that the substrate used (starch) for amylase assay is macromolecule, thus create internal mass-transfer limitations on enzyme [23], [24].

In order to get the best-fitted model, non-transformed model was chosen with manual selection. Reduced cubic model was obtained with significant model, insignificant lack-of-fit, and high value of determination coefficient R2 (0.973), adjusted R2 (0.966) and predicted R2 (0.959). Table 2 below shows the analysis of variances (ANOVA) obtained from the reduced-cubic model for CLEA-amylase recovery activity.

Based on the ANOVA results, the overall model and glutaraldehyde concentration is highly significant as reflected by very low p-value (<0.0001). Acetone is also significant as the p-value is less than 0.05, whereas BSA is not significant (p-value> 0.05). Insignificant lack of fit is desirable since the model should be fit.

Table-2. Analysis of variance of reduced cubic model for CLEA-amylase recovery activity.

Source	Sum of squares	F-value	p-value (prob >F)
Model	2810.62	129.55	< 0.0001
Acetone, A	8.24	4.94	0.0313
Glutaraldehyde, B	240.42	144.06	< 0.0001
BSA, C	1.98	1.19	0.2813
AB	75.86	45.46	< 0.0001
AC	49.39	29.60	< 0.0001
BC	5.89	3.53	0.0666
A^2	737.93	442.18	< 0.0001
B^2	23.36	14.00	0.0005
C^2	6.40	3.84	0.0562
ABC	47.80	28.64	< 0.0001
A^2B	36.62	21.94	< 0.0001
A^2C	2.87	1.72	0.1960
AB^2	16.01	9.59	0.0033
Lack of fit	5.85	3.71	0.0604

Most of the parameter interaction is significant except for BC, C^2 and A^2C, most likely due to insignificant effect of BSA concentration. Next, a mathematical prediction model between the parameters and variables was derived as shown in Equation 2:

(Recovery activity) = +22.42 + 1.17*A + 6.33*B – 0.57*C + 1.78 *A*B -1.43 *A*C + 0.50 *B*C – 9.46 *A2 – 1.68 *B2 -0.88*C2 -1.41 *A*B*C -2.76 *A2*B + 0.77 *A2*C + 1.83 *A*B2 (2)

As expected, acetone concentration would affect the recovery activity of the prepared CLEA. Acetone acts as a precipitant in this study to form protein molecules aggregates prior to cross-link with glutaraldehdye. As shown by 3D plot in Figure 6, optimum acetone concentration was observed approximately around 60-70%. Too little acetone concentration might not enough to form enzyme aggregation, whereas too high concentration might lead to protein denaturation [24]. This is because; presence of organic solvents like acetone might impair the structure as well as the function of enzyme since it can remove water from the protein. It is noteworthy to mention that, some water is crucial in maintaining the activity of enzyme [25].

In terms of the glutaraldehyde concentration, increasing trend of the recovery activity could be observed when the concentration was increased from 30 to 90 mM. The optimum level of glutaraldehyde was still remaining unknown since no decreasing pattern was displayed from the range tested. Therefore, the range of glutaraldehyde concentration studied should be increased in the future researches. Insufficient cross-linking might happen when the concentration applied was too low, thus lead to unstable enzyme which can leach easily in water. In contrast, excessive cross-linking might result in loss of flexibility in enzymes, which is vital for their activity [26].

Besides that, strong diffusion resistance to the substrate also might occur as suggested by Dong *et al*. [18].

BSA concentration was not significant in this study, suggesting that it just gave little effects on the CLEA-amylase recovery activity. The results obtained here were contradicted with several studies. For example, as reported by [17], they found significant effect of BSA on the CLEA-lipase preparation as shown by the p-value= 0.0007 from the ANOVA test. [16], [19] also has found substantial improvement on their CLEA-lipase preparation which source from fish viscera and Pseudomonas cepacia, respectively. One of the plausible reasons why it was not significant in this study is might due to the narrow range of BSA concentration tested (0, 2.5 and 4.5 mg/ml), thus producing little effect on the response.

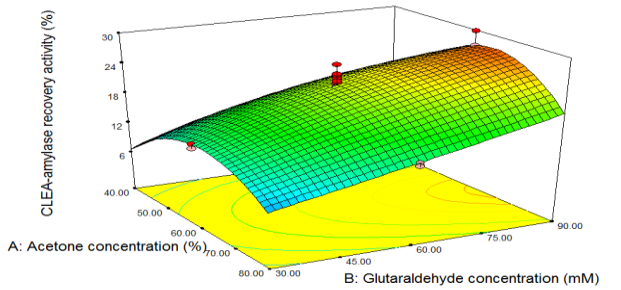

Figure-6. 3D response surface showing the interaction between acetone and glutaraldehyde concentration on CLEA-amylase recovery activity.

Lastly, validation test was conducted to observe the reliability of the model obtained from the RSM FCCCD (Equation. 2). Maximum goal was set for the response recovery activity, while the other 3 parameters (acetone, glutaraldehyde and BSA concentration) were set as in range. The results from the validation run were depicted as in Table-3 below. The difference between predicted and experimental values for the CLEA-amylase recovery activity suggested that the model was repeatable and reliable.

Table-3. Validation test on optimization of CLEA-amylase recovery activity.

No	Parameters			CLEA-amylase recovery activity (%)	
	Acetone conc. (%)	Glut. conc. (mM)	BSA conc. (mg/ml)	Pred.	Exp.
1	65.03	90	1.60	27.65	27.84
2	65.09	90	1.42	27.64	28.49
3	65.06	90	1.33	27.63	28.53

CONCLUSIONS

Supermeal worm has high amylase activity which can be manipulated industrially. OFAT studies were conducted primarily to find the optimum range for several factors; which are acetone concentration (precipitant), glutaraldehyde concentration (cross-linker) and agitation time. Next, screening of several additives (SDS, heptane and BSA) were done to observe the effect of each chemical on the recovery activity of CLEA-amylase. BSA had displayed the best activity, thus had been chosen as additives in this study. To optimize the CLEA-amylase preparation, RSM under FCCCD had been used. Acetone and glutaraldehyde concentration had the significant effect on the recovery activity, whereas BSA displayed insignificant effects based on the range tested. Finally, to verify the model, validation experiment was conducted. In future, the stability of the prepared CLEA-amylase will be tested in wide range of pH and temperature in addition to the reusability test. This is necessary to investigate the robustness of this CLEA-amylase as compared to the free enzymes.

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