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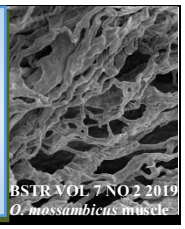

Optimisation of Acetylcholinesterase Extraction from the Brain of *Clarias batrachus* using Response Surface Methodology

Nazri, M.A.F.S.[1], Basirun, A.A.[1], Manogaran, M.[1], Khalidi, S.A.M.[2], Sabullah, M.K.[2] and Shukor, M.Y.*

[1]Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[2]Fakulti Sains dan Sumber Alam, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia.

*Corresponding author:
Prof. Dr. Mohd Yunus Abd Shukor
Department of Biochemistry,
Faculty of Biotechnology and Biomolecular Sciences,
Universiti Putra Malaysia,
UPM 43400 Serdang,
Selangor, Malaysia.
Email: mohdyunus@upm.edu.my

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ABSTRACT

Acetylcholinesterase is an important enzyme in biosensing works. It is utilized to detect insecticides and cheaper sources of the enzyme is constantly being sought. Optimisation of the acetylcholinesterase extraction from the brain of *Clarias batrachus* using Response Surface Methodology is reported for the first time in this study. AChE from the brain of this fish was extracted and partially purified using ammonium sulphate. The optimum range of ammonium sulphate cut was between 40 and 50%. RSM was conducted using Box-Behnken design and the optimisation value for the extraction was determined for each variable at the end of the study. The optimum concentrations of Triton X-100, PMSF, phosphate buffer and the pH for AChE extraction based on RSM were 0.75%, 1.00 mM, 0.075 M and 7.5, respectively.

INTRODUCTION

Acetylcholinesterase (AChE) is the primary enzyme responsible for the hydrolytic metabolism of the neurotransmitter acetylcholine (ACh) into choline and acetate. AChE exists as three different isoforms and is primarily localized to the cytoplasm of the outer cell membrane of cholinergic neurons of the central nervous system (CNS) and neuromuscular junction (NMJ) facilitating rapid hydrolysis of Acetylcholine. The enzyme is very important in insecticides detection in many sensor works [1,2]. Response surface methodology (RSM) is a statistical technique used for the improvement and optimization of complicated processes. The approach has several blessings over conventional experimental or optimization strategies wherein one variable at a time is used. RSM affords a big quantity of facts and is extra economical technique due to the fact a small number of experiments are performed for tracking the interplay of the impartial variables on the response.

By using RSM to optimise the extraction of AChE from fish brain of *Clarias batrachus*, the optimum parameter for every variable may be determined for this reason minimising the time and quantity of experiments needed to extract AChE. Current issues that had been encountered when studying enzyme extraction are that enzyme extraction require optimum conditions for better yield output and high number of experimental trials needed to evaluate multiple parameters. Effectiveness of Response Surface Methodology (RSM) in optimisation also needed to be tested as there is no paper on the optimisation of AChE production using RSM from fish as judged by literature review.

The aim of this study was to determine the optimum variables mainly buffer concentration, Triton X-100 percentage, PMSF concentration, and pH buffer on extraction of Acetylcholinesterase in the brain of *Clarias batrachus* using Response Surface Methodology and to understand the interaction between buffer concentration, Triton X-100 percentage, PMSF concentration, and pH buffer on optimisation of Acetylcholinesterase extraction.

MATERIALS AND METHODS

Preparation of crude homogenate

Clarias batrachus were bought alive from a fish dealer in Selangor, Malaysia. The weight of the fishes was approximately 300 grams. The fishes were freeze-killed by immersing them into the ice cube within 30 min, decapitated and their brains were collected after that. The extraction process was carried out by homogenising samples with 0.1 M Sodium Phosphate buffer, pH 7.0 containing 1 mM phenylmethylsulfonyl fluoride (PMSF) with a buffer ratio of 1:4 (w/v). The crude was homogenised using Ultra-Turrax T25 homogeniser. The homogenate was then centrifuged at 10,000× g with the temperature of 4 °C within 10 min. The supernatant was collected and stored at -20 °C for further purification process [3–6].

Ammonium sulphate precipitation

The supernatant was transferred into a beaker that was placed in iced condition. The sample were stirred while adding the ammonium salt slowly. The amount of ammonium sulphate powder required to give the desired percentage of saturation was determined based on the ammonium sulphate precipitation table [7]. The mixture was centrifuged at 15,000× g for 10 min at 4 °C. The pellet was collected and redissolved by adding a small amount of 0.1 M sodium phosphate buffer followed by dialysis to remove ammonium salt. This technique was repeated at the salt concentration of 0-20, 20-30, 30-40, 40-50, 50-60 and 60-70%.

Ellman assay and protein content determination

The enzyme activity of cholinesterase enzyme was tested using developed Ellman assay method [8]. This method has been slightly modified to be tested using spectrophotometer to assure absorbance through spectrophotometer at the wavelength of 405 nm. The assay has used synthetic substrates of acetylthiocholine iodide (ATC), butyrylthiocholine iodide (BTC) and propionylthiocholine iodide (PTC) to determine the most abundant type of protein contained. 1 mL of assay was prepared which to be tested using spectrophotometer that contained 800 µL 0.1M of Sodium Phosphate buffer pH 7, 80 µL 0.1mM DTNB and 40 µL fish brain supernatant. After 10 minutes of incubation, the substrates were added with amount of 20 µL. The mixtures were incubated for 10 min after adding the substrates and the absorbance was read after that. Protein content quantification of enzyme was done using Bradford protein assay to prepare BSA standard curve. The reaction samples contained 80 µL samples and 800 µL Bradford reagent. The mixtures were then incubated for 10 minutes at room temperature. After incubation, an absorbance at 405 nm was taken.

Experimental design

Response Surface Methodology (RSM) was utilized in this study to work out the optimum conditions for the extraction of acetylcholinesterase from fish brain samples. The experimental design and applied math analysis were performed victimization design knowledgeable computer code. The experiments were supported a Box-Behnken style with a quadratic model so as to check the combined effects of 4 freelance variables (Triton X-100CJ concentration, PMSF molarity, buffer concentration and pH buffer). Each independent variable had 3 levels which were -1, 0 and +1, as shown in **Table 1**. The dependent variable was known as response function.

Table 1. Response function for AChE optimisation using RSM factors levels.

Factors	Levels		
	-1	0	+1
Triton X-100	0.50 %	0.75 %	1.00 %
PMSF	0.50 mM	1.00 mM	1.50 mM
Buffer	0.05 M	0.08 M	1.00 M
PH Buffer	7.0	7.5	8.0

RESULTS AND DISCUSSIONS

Enzyme Assay

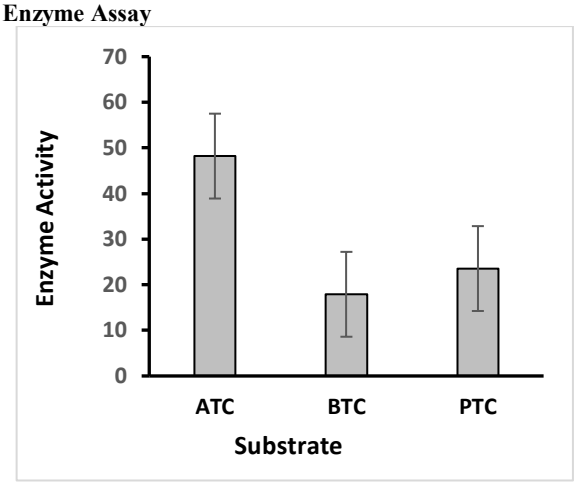

Fig. 1. Enzyme activity bar based on several AchE substrates.

The data showed that the AChE reaction obeyed Michaelis-Menten kinetics in hydrolysing the three different substrates, namely, ATC, BTC, and PTC, at varying concentrations (**Fig. 1**). ATC recorded the highest values than that of BTC and PTC, indicating high concentration of Acetylcholine in the samples.

Protein Purification

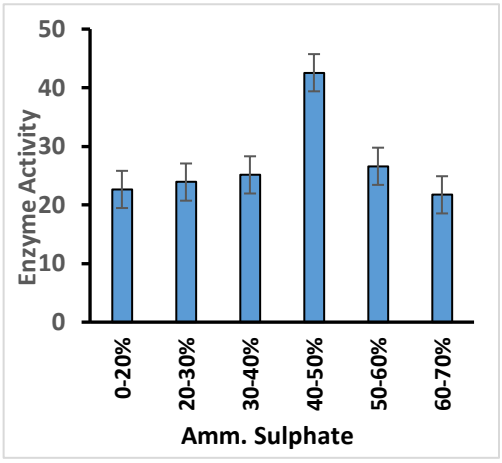

Fig. 2. Ammonium sulphate precipitation bar chart.

The highest activity was recorded at ammonium sulphate concentration of 40-50%. The solubility of globular proteins increases upon the addition of salt, an effect termed salting-in.

At higher salt concentrations, protein solubility usually decreases, leading to precipitation; this effect is termed salting-out [7]. Salts that reduce the solubility of proteins also tend to enhance the stability of the native conformation. In contrast, salting-in ions are usually denaturants. The mechanism of salting-out is based on preferential solvation due to exclusion of the cosolvent (salt) from the layer of water closely associated with the surface of the protein (hydration layer). Ammonium sulphate has been utilized as a method of purification for a variety of AChE sources with similar percentages of ammonium sulphate reported in this study [9–17].

Response Surface Methodology

Response surface methodology by Box–Behnken design employing the multivariate approach enables substantial improvement in the method development using fewer experiments, without wastage of large volumes of organic solvents, which leads to high analysis cost. The response was measured in terms of actual factors of absorbance. The model was validated by analysis of variance (ANOVA). The statistical analysis (**Table 2**) showed that the model represents the phenomenon quite well and the variation of the response was correctly related to the variation of the factors.

Table 2. Statistical analysis of model.

ANOVA	
R-Squared	0.9871
Adj R-Squared	0.9677
Pred R-Squared	0.9528
Adeq Precision	16.124

Diagnostic model plot was utilised to judge the the fitness of data (**Fig. 3**). The plots are important particularly in the assessment of data error that differs from model predictions, which aids in assessing and improving model adequacy [18]. The plot of actual versus predicted CFU obtained from the experiment (**Fig. 3**) revealed a not so close relationship between the actual and predicted value as the data points assembled roughly close to the line. This indicate a weakness in the model that needs further improvement in future studies.

Visualization of the optimum conditions for all of the factors needed for maximum response is presented through 3-dimensional and 2-dimensional responses and contour plots (**Figs. 4 to 6**). The plots are important in analysing the growth at zero or intermediate levels of different combinations of independent factors before a real experiment is performed [19,20]. The 3D response plot shows that there is a strong interaction between Triton X-100 and PMSF concentrations and mild interactions between the rest of the pair of factors.

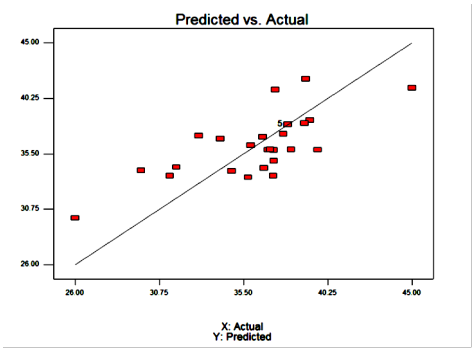

Fig 3. Model diagnostic plots; predicted versus actual.

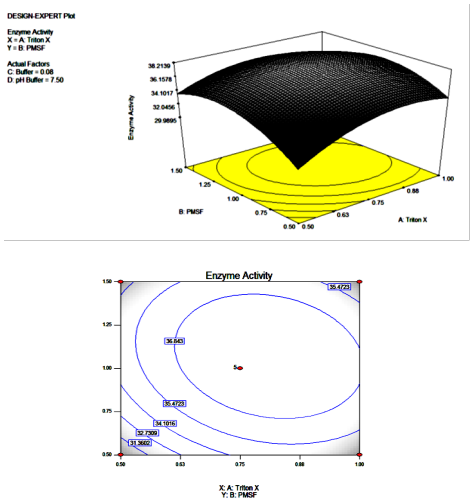

Fig. 4. 3D and 2D surface response view showing the interaction between Triton X-100 and PMSF concentrations.

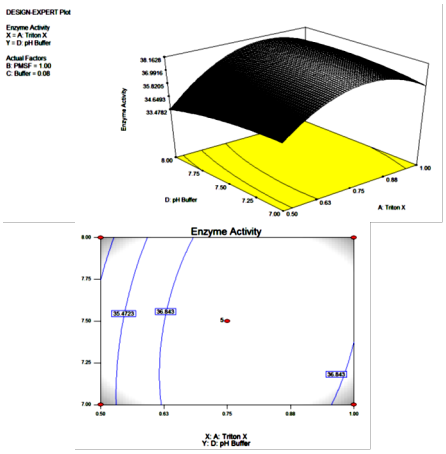

Fig. 5. 3D and 2D surface response view showing the interaction between Triton X-100 concentration and pH.

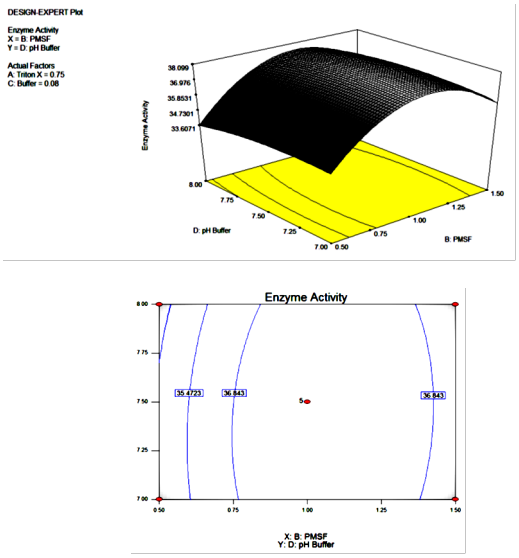

Fig. 6. 3D and 2D surface response view showing the interaction between PMSF concentration and pH.

This contour plots show the relationship between the PMSF and Triton X-100, pH buffer and Triton X-100 and pH buffer and PMSF in AChE optimisation. Using lower concentration of both factors results in lower enzyme activities. However, higher value of factors also results in low enzyme activates due to denaturation of protein of interest. The optimisation results based on Response Surface Methodology (RSM) were as follow:

Table 3. Optimisation based on RSM.

Factors	Optimum level
Triton X-100	0.75%
PMSF	1.00 mM
Buffer concentration	0.075 M
pH buffer	7.5

Phenylmethylsulfonyl fluoride (PMSF) was used for preparation of cell lysates because it binds specifically to the site aminoalkanoic acid residue in serine hydrolases. The effective concentration of PMSF was at 1 mM. Higher concentrations is inhibitory to cholinesterase activity [21]. Triton X-100 has been used to extract AChE. In one study, a major increase in acetylcholinesterase activity from human brain of up to 360% after extraction with 1% Triton X-100 is reported [22].

CONCLUSION

The AChE from *C. batrachus* has been successfully extracted and optimized using response surface methodology. AChE from the brain of this fish was extracted and partially purified using ammonium sulphate. The optimum range of ammonium sulphate cut was between 40 and 50%. RSM was conducted using Box-Behnken design and the optimisation value for the extraction was determined for each variable at the end of the study. The optimum concentrations of Triton X-100, PMSF, phosphate buffer and the pH for AChE extraction based on RSM were 0.75%, 1.00 mM, 0.075 M and 7.5, respectively.

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