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Optimization of Aqueous Two-Phase System (ATPS) of Chicken Liver 3-Mercaptopyruvate Sulfurtransferase (3-MST) through Response Surface Methodology

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ABSTRACT

Background and Objective: The 3-mercaptopyruvate sulfurtransferase (3-MST) is an enzyme known to catalyse the detoxification of cyanide by transferring sulfur from appropriate substrates to cyanide to form a less toxic compound. Aqueous two-phase partitioning system (ATPS) has several advantages as compared with conventional methods of enzyme purification. However, to improve enzyme purification through ATPS, a combination of process variables need to be optimized. **Materials and Methods:** In this study, response surface methodology (RSM) was adopted to determine the optimum condition for the aqueous two-phase partitioning of chicken liver 3-MST. The variables optimized are polyethylene glycol (PEG), ammonium sulphate and NaCl. **Results:** The results obtained validate the predictability of the model. The optimal concentration of independent variables that ensued highest purification yield of 80.989% was 25% (PEG), 6.9% (ammonium sulphate) and 2.8% of NaCl. The R^2 value for the model was 0.9708 ($p<0.05$). **Conclusion:** The experimental values obtained in this study are following those predicted, indicating the suitability of the employed model and the success of the response surface methodology (RSM) in optimizing the purification conditions.

KEYWORDS

Aqueous two-phase system (ATPS), 3-MST, random surface methodology, purification, enzyme yield, chicken liver, polyethylene glycol

INTRODUCTION

Cyanide (CN^-) is extremely toxic to humans and many other living things. It is highly toxic and ineffective detoxification can lead to respiratory inhibition via iron complexation in cytochrome oxidas[1,2]. When cyanide is produced in large quantities by anthropogenic activities such as mining and the electroplating industry, it causes severe environmental problems[3]. Cyanide discharge by accident has occurred in many parts of the world. The pollution of the Danube and Tisza rivers, which collapsed in early 2000, left the greatest impression on the public and was the most serious episode[4]. Cyanide enters the body easily

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through the stomach, lungs, mucosal surfaces and unbroken skin. Depending on the dose, effects begin within seconds of inhalation and within 30 min of ingestion of solution or solid cyanide. Common cyanide poisoning symptoms include slurred speech, vomiting, respiratory distress, convulsions and coma[5].

The 3-mercaptopyruvate sulfurtransferase (EC 2.8.1.2) is an enzyme that catalyzes 3-mercaptopyruvate reactions[6]. The enzyme is of interest because it is part of a cyanide detoxification mechanism. The enzyme is found in the cytosol of both eukaryotes and prokaryotes cells[7]. Enzymes have been purified using time-consuming and expensive methods such as ammonium sulphate precipitation and chromatographic techniques which may result in low enzyme activity and yield[8]. High enzyme yield and purification degree are characteristics of liquid-liquid extraction methods such as aqueous two-phase systems. Protein purification using aqueous two-phase systems is a promising method with a high purification degree[9]. The system is made up of two liquid phases that do not mix above a certain critical concentration. Aqueous two-phase systems are made up of either two different immiscible mixing polymers (e.g., polyethylene glycol/dextran) or one polymer with salt (e.g., polyethylene glycol and ammonium sulphate), both of which are water-soluble at a certain concentration. It has long been recognized as a valuable technique for the separation and purification of biomolecules such as proteins[10]. There are numerous advantages to consider, including the technique's simplicity, rapid separation with minimal enzyme denaturation and selective separation[11]. Some theoretical tools have been used to optimize the purification conditions to reduce the cost, workload and purification time. Among these, is response surface methodology (RSM) which is defined as a set of mathematical and statistical analyses that can be used for modelling and analysis in applications where a response of output (or interest) is affected by a variety of factors[12]. It was previously used to improve the recovery and purification of a variety of enzymes[13]. The primary goal of this study is to purify 3-MST from chicken liver using an aqueous two-phase partitioning system and to optimize the purification conditions using response surface methodology.

MATERIALS AND METHODS

Study area: The study was carried out at the Enzymology Laboratory, Department of Chemical Sciences, Oduduwa University Ipetumodu, Nigeria from December, 2020 to July, 2021).

Materials: Potassium cyanide, sodium thiosulphate, boric acid, sodium borate, formaldehyde, ferric nitrite and nitric acid were obtained from BDH Chemical Limited, Poole, England. Glycerol, Coomassie Brilliant-Blue and bovine serum albumin (BSA) were obtained from Sigma Chemical Company, St. Loius, Mo., USA. Other chemicals used were of analytical grade and were procured from reputed chemical firms.

Sample collection: A chicken was purchased from a local market in Ipetumodu, Ile Ife in Osun state. It was taken to the laboratory and sacrificed. The liver was harvested and washed with normal saline to remove blood stains. Properly washed liver was kept in the refrigerator for further analysis.

Crude enzyme preparation: Twenty grams (20 g) of the liver was homogenized in three volumes of 50 mM Phosphate buffer pH 6.5 using a mortar and pestle. The homogenate was centrifuged at 5000 rpm for 10 min and the supernatant was pipetted into a new tube and assayed for protein and 3-MST activity.

Protein concentration determination: Protein concentration was determined by the modified method of Bradford[14] using bovine serum albumin (BSA) as the standard, where the protein absorbance was extrapolated from the standard curve. The reaction mixture consists of 100 µL of the enzyme solution and 1.0 mL of Bradford reagent. The absorbance was read at 595 nm. For the standard calibration curve, BSA concentration was varied between 100 and 1000 µg mL^{-1}, with the addition of 1 mL of Bradford reagent. The concentration (µg) of protein was plotted against absorbance from which the unknown protein concentration was interpolated.

Enzyme assay: The 3-MST activity was determined according to the modified method of Taniguchi and Kimura[15]. Briefly, the reaction mixture contained 0.38 M Tris-HCl buffer pH 7.8, 0.38 M mercaptoethanol, 0.5 M KCN and 100 µL of enzyme preparation in a final volume of 1.55 mL was incubated for 10 min at 37°C. Reaction termination was done by adding 15% Formaldehyde followed by adding 0.75 mL of Sorbo reagent (which contains 20.20 g of Ferric nitrate in 200 mL of concentrated nitric acid and made up to 400 mL with distilled water). The absorbance was taken at 460 nm after adding the Sorbo reagent[16].

Preparation of aqueous two-phase system: Aqueous Two-phase Partitioning System (ATPS) was carried out by mixing polyethylene glycol (PEG) (15%, w/v) and ammonium sulphate (4%, w/v) and NaCl (2%, w/v) with 20 mL of crude enzyme. The mixture was continuously stirred at 4°C for 2 hrs until the salts were completely dissolved in the enzyme preparation. The mixture was left for 12 hrs at 4°C to achieve phase separation. After phase separation, the enzyme activity and protein concentration of both phases were measured. The bottom phase was dialysed against 50 mM sodium phosphate buffer, pH 6.5 at 4°C for 3 hrs to remove salts. Following that, the specific activity and the yield of the enzyme were calculated.

Response surface methodology (experimental design): Response surface methodology (RSM) with Design-Expert .0.8 software (State-Ease Inc., Minneapolis, MN, USA) was used to establish the optimum conditions for the purification of 3-MST. The independent variables used in this study were polyethylene glycol (PEG) (A (%) w/v), ammonium sulphate (AS) (B (%) w/v) and NaCl (C (%) w/v) while the response was enzyme yield (EY (%)). A second-order polynomial quadratic equation was employed to fit the results (Eq. 1):

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum\sum_{i<j} \beta_{ij} x_i x_j + \sum_{i=1} \beta_{ii} x_i^2 + \in \quad (1)$$

where, Y is the predicted response, β_0, β_i, β_{ii}, β_{ij}, are the correlation coefficients for intercept, linear, quadratic and interaction terms, respectively and x_i and x_j are the levels of the independent variables.

Statistical analysis: The experimental data were analyzed using the response surface regression algorithm. p-values under 0.05 ($p<0.05$) were considered significant. The predictive equation of RSM was used to find the optimal conditions for purification. The validity of the model was determined by comparing the experimental and predicted response values.

RESULTS AND DISCUSSION

Optimization of ATPS experiment: The response surface methodology technique was employed to determine the optimum level of the variables that will result in a high purification yield of 3-MST. The design matrix and results of ATPS purification were summarized in Table 1. Analysis of the variance was employed for the determination of significant variables and their interactions. In this model, two linear (A and C) and five quadratic models (AC, BC, A^2, B^2 and C^2) were found to be significant at the level of $p<0.05$. The result for the fitting quadratic model is listed in Table 2. Analysis of Variance (ANOVA) results indicates that the model was significant ($p<0.05$) for the response of the dependent variables (enzyme yield). The result of the ANOVA also indicates a good model performance with correlation coefficient (R^2) values of 0.9708. This explains 97.08 of the calculated model. The p-value of 0.0001 indicated that the statistical analysis is of a highly significant level, attesting to the goodness of fit for the optimized antioxidants. This result indicates that the statistical model could work well for the 3-MST purification. The fitted quadratic model for rhodanese production is shown in Eq. 2. Several researchers have observed similar outcomes when response surface methodology was used for process optimization[17-21].

$$\text{Enzyme yield} = +79.33+6.82A-1.42B-1.66C-0.75AB+4.50AC+2.00BC-5.75A^2-3.98B^2-4.69C^2 \quad (2)$$

Table 1: Experimental and predicted results for purification yield

Run	PEG	Ammonium sulphate (AS)	NaCl	Enzyme yield (%)	Actual value (%)	Predicted value (%)
1	-1(4.8)	0(7.5)	0(2.5)	50	50.00	51.60
2	+1(30.0)	0(7.5)	0(2.5)	78	78.00	74.53
3	0(17.5)	0(7.5)	0(2.5)	79	79.00	79.33
4	+1(25.0)	+1(10.0)	+1(4.0)	73	73.00	74.39
5	0(17.5)	0(7.5)	+1(5.0)	65	65.00	63.27
6	-1(10.0)	-1(5.0)	+1(4.0)	50	50.00	50.60
7	0(17.5)	-1(3.2)	0(2.5)	73	73.00	70.46
8	0(17.5)	0(7.5)	0(2.5)	79	79.00	79.33
9	+1(25.0)	-1(5.0)	-1(1.0)	71	71.00	73.03
10	-1(10.0)	+1(10.0)	+1(4.0)	54	54.00	53.25
11	-1(10.0)	-1(5.0)	-1(1.0)	67	67.00	66.93
12	+1(25.0)	-1(5.0)	+1(4.0)	72	72.00	74.74
13	-1(10.0)	+1(10.0)	-1(1.0)	63	63.00	61.58
14	0(17.5)	+1(11.7)	0(2.5)	65	65.00	65.67
15	0(17.5)	0(7.5)	0(2.5)	81	81.00	79.33
16	+1(25.0)	+1(10.0)	-1(1.0)	64	64.00	64.72
17	0(17.5)	0(7.5)	0(2.5)	78	78.00	79.33
18	0(17.5)	0(7.5)	-1(0.02)	69	69.00	68.87

Table 2: Analysis of Variance (ANOVA) for response surface quadratic model for the 3-MST purification

Source	SS	DF	MS	F-value	p-value
Model	1541.88	9	171.32	29.54	<0.0001*
A-PEG	634.54	1	634.54	109.40	<0.0001*
B-AS	27.71	1	27.71	4.78	0.0603
C-NaCl	37.82	1	37.82	6.52	0.0340*
AB	4.50	1	4.50	0.7759	0.4041
AC	162.00	1	162.00	27.93	0.0007*
BC	32.00	1	32.00	5.52	0.0468*
A^2	418.17	1	418.17	72.10	<0.0001*
B^2	200.56	1	200.56	34.58	0.0004*
C^2	278.12	1	278.12	47.95	0.0001*

SS: Sum of squares, DF: Degrees of Freedom, MS: Mean square, R^2: 0.9708; R^2 adj: 0.9379, CV: 3.52% (*values statistically significant at $p<0.05$)

It is evident in Eq. 2 that the interaction parameter (AC) had the highest positive effect on the enzyme purification yield while ammonium sulphate (A^2) had the highest negative effect.

Analysis of the response: The effects of 3-MST purification variables are shown in Fig. 1. Figure 1a shows an increase in polyethylene glycol (PEG) up to 25% and a gradual increase in ammonium sulphate led to an increase in enzyme yield. It has been noted that the presence of PEG 6000 at a higher concentration in an aqueous two-phase medium will increase the hydrophobicity between polymer-rich phase with proteins[22]. The free volume available due to the higher concentration of PEG in the top phase becomes one of the migration factors of the 3-MST in that phase. A similar observation was reported when Bromelain was purified through an unconventional aqueous two-phase system (PEG/ammonium sulphate)[18].

A decrease in yield was observed at a high concentration of AS. Figure 1b shows the interaction between the PEG and NaCl also followed the same trend. Enzyme yield increases as the concentration of PEG increases. A sharp drop in yield was noted at a high concentration of NaCl. Sodium chloride has been reported by several researchers to inhibit cyanide degrading enzymes[23,24]. This reduction could be due to interference of the salt with the three-dimensional structure of the enzyme which in turn affects the overall stability and the activity of the enzyme. Arshad and Amid[25] also observed the same pattern when response surface methodology was used to optimize the aqueous two-phase system (ATPS) of recombinant bromelain. In Fig. 1c, high enzyme yield was observed at a low concentration of AS and NaCl. This figure explains the influence of the salts such as NaCl and AS on the enzyme activity and the purification by an aqueous partitioning system.

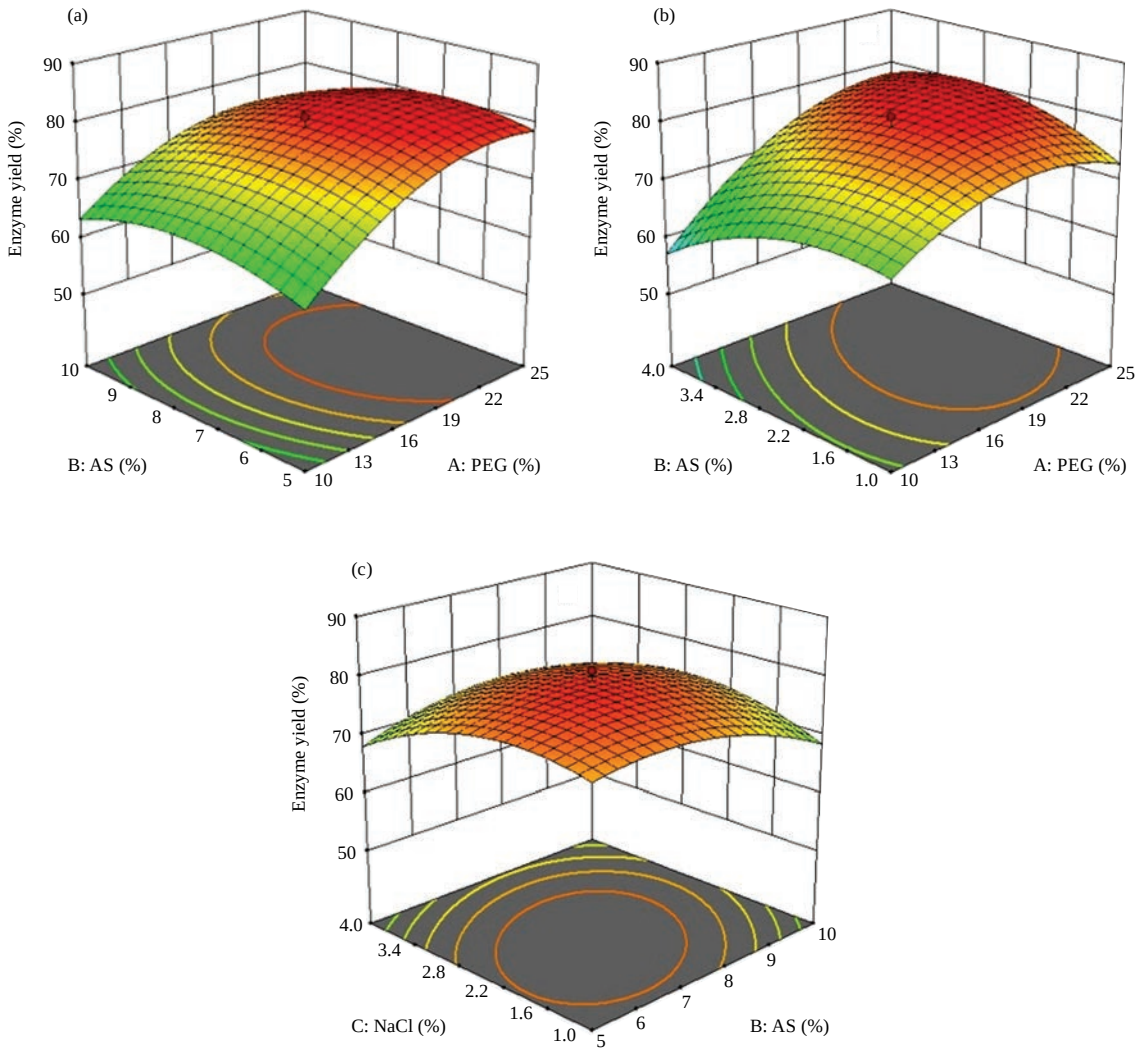

Fig. 1(a-c): Response surface plots showing the effects of AS (% w/v), PEG (% w/v) and NaCl (% w/v) on enzyme yield, (a) Effects of AS (% w/v) and PEG (% w/v) on enzyme yield, (b) Effects of NaCl (% w/v) and PEG (% w/v) on enzyme yield and (c) Effects of NaCl (% w/v) and AS (% w/v) on enzyme yield

Process optimization: The optimal value of the independent variables for the aqueous two-phase partitioning purification of 3-MST was determined using the maximum desirability. The result of optimal conditions to obtain the highest yield of 3-MST were 25% PEG, 2.8% NaCl and AS concentration of 6.9% at which the value for enzyme yield was 81.238%. It is worthy to note that the yield obtained in this study is more than the ones reported by other researchers who used conventional methods such as ammonium sulphate, ion-exchange chromatography and other chromatographic techniques to purify 3-MST. Sanni *et al.*[26] reported a yield of 10.3% after ion exchange chromatography purification of Cane rat 3-MST. The above results showed that the ATPS technique using the RSM approach is suitable for the purification of 3-MST and can be attractive for good the recovery of other enzymes.

CONCLUSION

In this study, the random surface methodology was used to optimise the purification yield of chicken liver 3-MST. The effect of three independent variables (polyethylene glycol, ammonium sulphate and sodium chloride) on purification yield was examined. From the response surface plots, all the three studied independent variables significantly influenced purification yield. Using the response surface methodology, the optimum condition of polyethylene glycol, ammonium sulphate and sodium chloride was obtained.

The optimum conditions were 25% PEG, 2.8% NaCl and ammonium sulphate concentration of 6.9% at which the value for enzyme yield was 81.238%. The results confirm the predictability of the model for the purification of chicken liver 3-MST and this could be applied for the purification of other enzymes.

SIGNIFICANCE STATEMENT

This study discovers the appropriate amount of three independent variables, viz-polyethylene glycol, ammonium sulphate and sodium chloride for high purification yield of chicken liver 3-MST. This study will help the researcher to develop a fast and cost-effective means for the purification of other enzymes. Thus, a new theory on these variable combinations and possibly other combinations, may be arrived at.

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