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Optimization of extraction of luciferase from fireflies (*Photinus pyralis*) using aqueous two-phase extraction

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ABSTRACT

A central composite rotatable design was employed to study the effect aqueous two-phase extraction conditions namely polyethylene glycol-1500 (PEG) concentration (12–16%), $(NH_4)_2SO_4$ concentration (14–20%) and pH (6–9) on the extraction of luciferase from *Photinus pyralis*. Specific activity, activity recovery, purification fold, volume ratio, enzyme and protein partition coefficients were considered as response variables. The significant ($p < 0.05$) response surface models with high coefficients of determination values (R^2) ranging from 0.89 to 0.95 were fitted for the experimental data, which indicated that the polynomial response models fitted well for describing the specific activity, activity recovery, purification fold, volume ratio, enzyme and protein partition coefficients. Based on the design, the optimal conditions were polyethylene glycol concentration 12%, $(NH_4)_2SO_4$ concentration ranging from 16.48% to 17.86% and pH ranging from 8.17 to 8.43. The graphical optimization of superimposed contour plots fulfilled the conditions to obtain specific activity (Y_1) $\geqslant 5.5 \times 10^6$ RLU/mg, activity recovery (Y_2) $\geqslant$ 70%, purification fold (Y_3) $\geqslant$ 6.5, volume ratio (Y_4) $\leqslant$ 0.77, enzyme partition coefficient, K_e (Y_5) $\geqslant$ 95, protein partition coefficient, K_p (Y_6) $\leqslant$ 2.5. The study demonstrated that response surface methodology can be utilized for deriving the optimum conditions for optimizing the conditions for the aqueous two-phase extraction of luciferase.

1. Introduction

Bioluminescence methods employing firefly (*Photinus pyralis*) luciferase enzyme (E.C.1.13.12.7, Molecular weight 62 kDa) finds its applications in numerous areas such as clinical testing, drug screening, development of biosensors for environmental pollutants, detection of microbial contamination [1], genetic reporter assays in molecular biology [2], detection of phosphatase activity [3], DNA sequencing [4] as well as a tool for monitoring *in vivo* protein folding and chaperonin activity [5]. The luciferase catalyzed chemical reaction involves removal of a phosphate group from adenosine triphosphate (ATP) during its oxidation by luciferase resulting in luciferyladenylate, which in turn is oxidized by molecular oxygen to form oxyluciferin and adenosine monophosphate (AMP) leading to the generation of luminescence having a maximum light intensity of 562–570 nm [6,7].

Downstream processing is an integral step to obtain high degree of purity of any biomolecule. Conventional extraction methods for luciferase such as gel filtration [8], precipitation [9] followed by ion exchange chromatography [10] were employed for the enzyme extraction and purification; however, these techniques have a number of drawbacks such as difficulty in scale up, high production cost and lack of suitable biocompatible solvents. The cost effective methods that can separate, concentrate, and purify luciferase and can be easily scaled-up are of great commercial interest. One such alternative method is liquid–liquid extraction using aqueous two-phase extraction (ATPE). The use of aqueous two-phase system (ATPS) in downstream processing is one of the primary methods for purification and concentration of biomolecules. It offers many advantages such as biocompatible environment for the biomolecules, ease of scale-up, lower interfacial tension and scope for continuous operation [11–15]. The partition coefficient of a biomolecule in the system depends on molecular weight, charge of the partitioned particle, the type and concentration of phase forming salt, concentration and molecular weight of phase forming polymer [16–18]. Priyanka et al. [15] studied the conditions for the optimization of downstream processing of luciferase using ATPS by changing one parameter at a time. Recently, many newer applications of ATPS have reported for extraction of vanillin [19], flavonoids from pigeon pea roots [20], citrinin and catalase extraction from fermentation broth [21,22].

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Table 1
Variables and their levels for CCRD.

	Symbols	−1.682	−1	0	1	1.682	Mean	St. deviation
PEG Conc. (%)	X_1	12	12.81	14	15.19	16	14	1.19
$(NH_4)_2SO_4$ Conc. (%)	X_2	14	15.22	17	18.78	20	17	1.78
pH	X_3	6	6.61	7.5	8.39	9	7.5	0.89

Coded values = (Actual value − mean)/standard deviation.

Table 2
Treatment schedule for five-factor CCRD and response in terms.

Exp no.	PEG Conc. (%) X_1	$(NH_4)_2SO_4$ Conc. (%) X_2	pH X_3	Specific activity ($\times 10^6$ RLU/mg)	Activity recovery (%)	Purification fold (−)	Volume ratio (−)	K_e	K_p
1	−1	−1	−1	0.33	40.49	1.40	1.00	10.24	1.06
2	1	−1	−1	3.31	52.03	4.01	1.00	20.59	1.70
3	−1	1	−1	2.67	56.58	3.23	0.50	70.73	1.40
4	1	1	−1	4.83	52.51	5.85	0.50	36.01	1.50
5	−1	−1	1	3.86	62.89	4.70	0.75	79.04	0.88
6	1	−1	1	4.49	102.26	5.43	0.86	101.15	1.06
7	−1	1	1	3.71	76.82	4.49	0.50	62.19	2.75
8	1	1	1	4.51	73.84	5.46	0.50	47.71	1.97
9	−1.682	0	0	4.98	77.45	6.04	0.75	83.57	1.83
10	1.682	0	0	5.78	78.35	6.70	1.00	163.23	2.28
11	0	−1.682	0	2.15	63.20	2.59	1.00	23.15	1.23
12	0	1.682	0	2.42	72.51	2.93	0.50	34.89	2.87
13	0	0	−1.682	0.01	0.14	0.01	0.75	0.31	0.99
14	0	0	1.682	3.08	42.39	3.73	0.50	34.49	1.47
15	0	0	0	3.56	71.99	4.31	0.50	75.00	1.50
16	0	0	0	3.41	72.11	4.13	0.50	70.32	1.77
17	0	0	0	3.50	74.27	4.24	0.50	65.92	1.43
18	0	0	0	3.52	85.27	4.26	0.50	68.36	1.96
19	0	0	0	3.15	73.93	3.82	0.50	64.75	1.58
20	0	0	0	3.13	70.33	4.16	0.50	60.21	1.87

When many factors and interactions affect desired response, response surface methodology (RSM) is an effective tool for optimizing the process [23]. The RSM is a statistical method that uses quantitative data from an appropriate experimental design to determine and simultaneously solve multivariate equation. It usually uses an experimental design such as central composite rotatable design (CCRD) to fit a first- or second order polynomial by a least squares technique. An equation is used to describe how the test variable affects the response, determine the interrelationship among the test variables, and describe combined effect of all the test variables in the response. Many researchers have reported the optimization of conditions employing response surface methodology for the aqueous two-phase extraction of various enzymes such as α and β-amylase [24], xylanase [25], protease [26], xylose reductase [27] and papain [28] and various whey proteins [29] and plasmid DNA [30].

The objectives of the research work were (a) to evaluate the application of response surface methodology to model combined effect of concentration of phase forming polymer (polyethylene glycol) and salt (ammonium sulfate) and system pH on the specific activity, activity recovery, purification fold, volume ratio, partition coefficient of the luciferase as well as the contaminating protein and (b) to study the complex interaction of independent variables on these responses.

2. Materials and methods

2.1. Chemicals

Desiccated whole fireflies (*P. pyralis*) obtained from Sigma Chemicals, St. Louis, MO, USA were stored at −20 °C and used for the extraction of luciferase. Polyethylene glycol (PEG) molecular weight 1500, dithiothreitol (DTT), adenosine triphosphate (ATP), D-luciferin was also procured from Sigma Chemicals, St. Louis, MO, USA. The ammonium sulfate salt was obtained from Ranbaxy Chemicals, SAS Nagar, India.

2.2. Preparation of crude extract

The abdomen portion were separated from the tails and crushed in the pre-cooled mortar and pestle using 0.2 M Tris–HCl buffer pH 7.8 containing 5 mM $MgSO_4$ and 1 mM EDTA. The pH was maintained at 7.5 by addition of 1 M HCl. The mixture was centrifuged (REMI Compufuge CPR-24 Techno instruments, Bangalore) at 27 000*g* for 30 min at 4 °C. The supernatant was referred as crude extract [6].

2.3. Preparation of aqueous two-phase systems

Two phase systems were prepared by the addition of predetermined and weighed quantities of polymer, PEG 1500 and salt namely, $(NH_4)_2SO_4$ (obtained from the phase diagrams reported in the literature) [31,32]. The weight percentage of the crude extract was maintained at 100% w/w basis. The pH of the system was adjusted using 0.1 N NaOH and 0.1 N HCl solutions. The content was thoroughly mixed in a magnetic stirrer for 1 h at 4 °C for equilibration and was subjected to centrifugation at 98*g* for 5 min (REMI Compufuge CPR-24, Techno Instruments, Bangalore, India). After clear separation of two phases, the volume of each phases were measured and analyzed for the enzyme activity and protein concentration. All the experiments were performed three times and average values are reported.

2.4. Determination of enzyme activity and protein content

Luciferase activity was estimated as per the reported procedure [6]. The reaction mixture consisting of 170 μl of 20 mM Tris–HCl buffer pH 7.8 (5 mM $MgSO_4$, 0.5 mM EDTA and 0.5 mM dithiothre-