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Investigation of the cell disruption methods for maximizing the extraction of arginase from mutant *Bacillus licheniformis* (M09) using statistical approach

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Abstract−Arginase, an intracellular enzyme produced by *Bacillus licheniformis* (NRS-1264) is effectively used as a drug in the treatment of arginine-dependent cancers, and it is essential for controlling acute neurological disorders. We investigated the effect of various cell disruption methods for maximizing the extraction of intracellular arginase from mutant *Bacillus licheniformis* (M09), followed by comparing optimization methods, one factor at a time (OFAT), evolutionary operation (EVOP) and response surface method (RSM). Ultrasonication for 2-5 min having a suspension volume in the range of 12-20 mL at a radio frequency power between 30-70 W appeared to be the most effective extraction technique for arginase. The arginase yield decreased in the range of 50-70 W of RF power/16-20 mL suspension volume and 4-5 min sonication time. EVOP predicted a maximum arginase extraction of 3,910 IU·L^{-1} at 2 min sonication having 16 mL suspension volume at 30 W RF power. However, response surface optimization suggested an optimized condition of 3 min sonication having 14.5 mL suspension volume at 35 W RF power in which the maximum arginase activity in the medium was 3,600 IU·L^{-1}.

Keywords: Mutant *Bacillus licheniformis* (M09), Arginase, Ultra-sonication, One Factor at a Time (OFAT) Optimization, Evolutionary Operation Technique (EVOP), Response Surface Method

INTRODUCTION

Arginine is one of the key amino acids involved in the synthesis of agmatine, glutamate, nitrous oxide, proline and polyamines through different metabolic pathways. In mammals, the metalloenzyme arginase (L-arginine amidinohydrolases, E.C. 3.5.3.1) catalyzes the depletion of arginine level by hydrolyzing it into ornithine and urea [1]. There are many evidences stating that arginase also inhibits the growth of various cancer cells, especially against hepatocellular carcinoma [2] and malignant melanoma [3]. Being an arginine-degrading enzyme, the potential of arginase as a target entity has been explored in order to control the metabolic pathway of all the above-mentioned compounds [4]. There is a wide range of microbial sources for arginase which include (many *Bacilli*, *Agrobacterium-Rhizobium* group) [5], cyanobacteria, *Proteus* spp. [6], yeast (*Saccharomyces cerevisiae*) [7] and fungi (*Neurospora crassa*, *Aspergillus nidulans*, *Agaricus bisporus*) [8]. In the case of microbial sources, arginase is generally present as an intracellular enzyme. Hence, the energy efficient recovery of intracellular metabolites such as arginase is very crucial for the industry.

Microbial cell disruption is an important unit operation for the production of many intracellular products [9,10], and the process industry demands this operation to be energy and time efficient [11,12]. There are a number of simple and direct methods available for the isolation of extracellular enzymes, whereas recovery of intracellular enzymes often requires a complicated downstream process [13]. The recovery of particular intracellular metabolites from Gram-positive bacteria are hindered by a rigid cell wall, which requires an added cell disruption step to enable the extraction of intracellular metabolites. Cell disruption appears to be a vital downstream processing step as it affects final yield and thereby the cost of production of bioactive compound [14]. Numerous chemical, mechanical and physical approaches are used for disrupting microbial cells. The commonly used cell disruption methods are bead mill, sonication, french press, high-pressure homogenizers (HPHs), osmotic and chemical lysis. Other potential methods are enzyme-assisted extraction that facilitates the possibility of completing in a short time, with lower temperature, less energy utilization and high extraction yield [15]. On the other hand, the added enzyme may imply additional associated cost and complicate purification processing of desired metabolites. However, mechanical methods have received major attention towards large-scale processing [13]. Before selecting any cell disruption technique, various factors have to be taken into account: characteristics of the product of interest, the composition of the cell wall, and the location of the product inside the cell [16-18].

Evolutionary operation (EVOP) is a multivariable progressive method used to give the training to the system with at least two or more variables to obtain the optima for the responses analyzed. It is a relatively simple and practical technique, which is moreover referred to as a "sequential" method. Basically, it works based on the results obtained in the earlier experiments, and thereby the subsequent experimental points are designed, and the loop continues until it reaches the optima. EVOP technique was introduced as an optimization tool long back by Banerjee and Bhattacharyya [19] in which the simultaneous effect of two or more parameters was cor-

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roborated to enhance the enzyme productivity in a laboratory scale fermentation process. Furthermore, this approach is equally useful to understand the relative impact of individual parameters as well as the interaction effects during any extraction technique like ultra-sonication.

On the other hand, response surface methodology (RSM) has been widely used for optimizing the ultrasound-assisted extraction processes like intracellular β-galactosidase from *Kluyveromyces lactis* [20], polysaccharides from *Tremella mesenterica* and *Lycium barbarum* [21]. Response surface method (RSM) is an optimization technique which employs an experimental design followed by regression and numerical optimization, which finally provides the statistically optimized condition at a minimum number of experimental data. Concomitantly, it develops a quadratic polynomial relationship between the independent factors and the dependent variables or responses through the number of experiments set by the design. Simultaneously it quantifies the interaction between the variables affecting the response [22].

The present investigation aimed to select the best technique from different methods of cell disruption targeting a maximum extraction of arginase from mutant *Bacillus licheniformis* (M09). Furthermore, three optimization methods, one factor at a time (OFAT), evolutionary operation (EVOP), and response surface modelling techniques, were applied to optimize the condition of the selected technique targeting a maximum arginase extraction.

MATERIALS AND METHODS

1. Materials

All the chemicals used in the study were of analytical grade. Agar, beef extract, peptone, yeast extract, sucrose, glucose, sodium chloride, arginine, urea, and other media components were procured from HIMEDIA (Hi-Media Limited, Mumbai, India). Buffer salts like Tris, dipotassium hydrogen phosphate, sodium chloride and HCl were purchased from Merck (Merck India Limited, Mumbai, India). Butanol, hexane, chloroform, ethanol, toluene, tween 80 and 20, SDS, cetyl ammonium bromide, triton X-100 were supplied by SDFCL (SDFCL, Mumbai, India).

2. Bacterial Strain and its Maintenance

The microbial strain of *Bacillus licheniformis* (NRS-1264) was procured from Agricultural Research Service (USDA Agricultural Research Service, Salinas, California, USA). The growth medium used for its maintenance contained the concentration ($g \cdot L^{-1}$) of peptone : beef extract : yeast extract : sodium chloride : agar as 5 : 1.5 : 1.5 : 5 : 20 at a pH value of 7.0±0.2. The parent strain *Bacillus licheniformis* (NRS-1264) was mutagenized for the improved production of arginase by treating it under UV and with ethyl methane sulfonate. After extensive screening, a total of 21 different arginase producing mutants were selected and the best yielding strain was labelled as M09 and used for further experiments. The mutant bacterial cells were incubated in agar slants at 37 °C for 48 h and stored at 4 °C. To maintain the potency of strain, subculturing was performed periodically after every month.

3. Inoculum and Fermentation

A loopful of cells picked from a slant was inoculated into 50 mL pre-sterile seed medium and incubated at 37 °C/18 h/180 rpm. Further 1% (v/v) of 18-h old *B. licheniformis* (M09) seed culture having a cell density of approximately 3×10^7 cells·mL^{-1} was allowed to grow in production medium for 36 h in an incubator shaker (Remi Instruments Ltd., Mumbai, India) at 37±2 °C and 180 rpm.

4. Preparation of Cell Suspension

The cell broth was centrifuged at 12,100×g at 15 °C for 15 min and the obtained cell pellets were washed twice with distilled water. The washed cell pellets were dispersed in 50 mM tris buffer (pH 8.5) to attain the desired cell density of 3.4×10^9 cells·mL^{-1} corresponding to 1% w/v for all the experiments. Hence 2, 4, 6, 8, 10, 12 and 14% (w/v) of cell density correspond to 6.10×10^9, 1.20×10^{10}, 1.94×10^{10}, 2.65×10^{10}, 3.29×10^{10}, 3.91×10^{10} and 4.80×10^{10} cells·mL^{-1}, respectively.

5. Arginase Assay

Arginase activity was quantified by measuring the rate of urea released from the hydrolysis of L-arginine following the method reported by Andersen and Strange [23] with a slight modification. In brief, the reaction mixture having 0.2 mL of glycine buffer (pH 9.5), 0.5 mL broth (enzyme source) and 0.1 mL of $MnCl_2$ was incubated at 37 °C for 10 min. To this 0.1 mL of arginine was added and the mixture was further incubated at 37 °C for 30 min. The reaction was stopped by adding 1 mL of perchloric acid to it, followed by the addition of 1.0 mL of H_3PO_4-H_2SO_4 mixture (3 : 1 v/v) and 0.1 mL of 4% α-isonitrosopropiophenone. The mixture was treated at 100 °C for 1 hour followed by cooling to room temperature. The absorbance of the released urea was measured at 540 nm.

6. Effect of Different Parameters on Arginase Extraction During Cell Disruption

6-1. Effect of Different Organic Solvents and Detergents

The effect of different organic solvents and detergents was analyzed targeting a maximum extraction of arginase in the medium. In one set of experiments, five different organic solvents--hexane, toluene, ethanol, butanol and chloroform at three varying concentrations (3, 6 and 9% v/v)--were used as an extraction medium. In the second set, the effect of anionic (sodium dodecyl sulfate), cationic (cetyl ammonium bromide) and non-ionic (tween 20, 80 and triton X-100) detergents on the disruption of cell wall facilitating the arginase extraction was studied at 0.5% detergent concentration. In both the cases, 0.1 M tris buffer (pH 8.5) served as the suspension medium and the reaction volume was kept fixed at 10 mL in which the biomass concentration was 3% w/v. The mixture at the desired concentration of solvent or detergents was further incubated at 28±2 °C for 1 h on a rocker shaker. The suspension was centrifuged at 8,000 rpm for 20 min at 4 °C, and arginase activity within the supernatant was measured following the protocol as mentioned earlier.

6-2. Effect of Ultra-sonication on Arginase Extraction

A probe sonicator (Branson Sonifier W450A, Germany), having a maximum radio frequency power of 400 W, was used for the ultra-sonication experiment. The experimental setup for the ultra-sonication treatment is represented in Fig. 1(a). The instrument had the flexibility to adjust the duty cycle (length of the pulses) ranging from 10-90%. For instance, 30% duty cycle represented that the ultra-sonic output was activated for 30% of each second of operation, and for the remaining 70% duration, ultra-sonic output was deactivated. The radio frequency power supply was adjusted in such

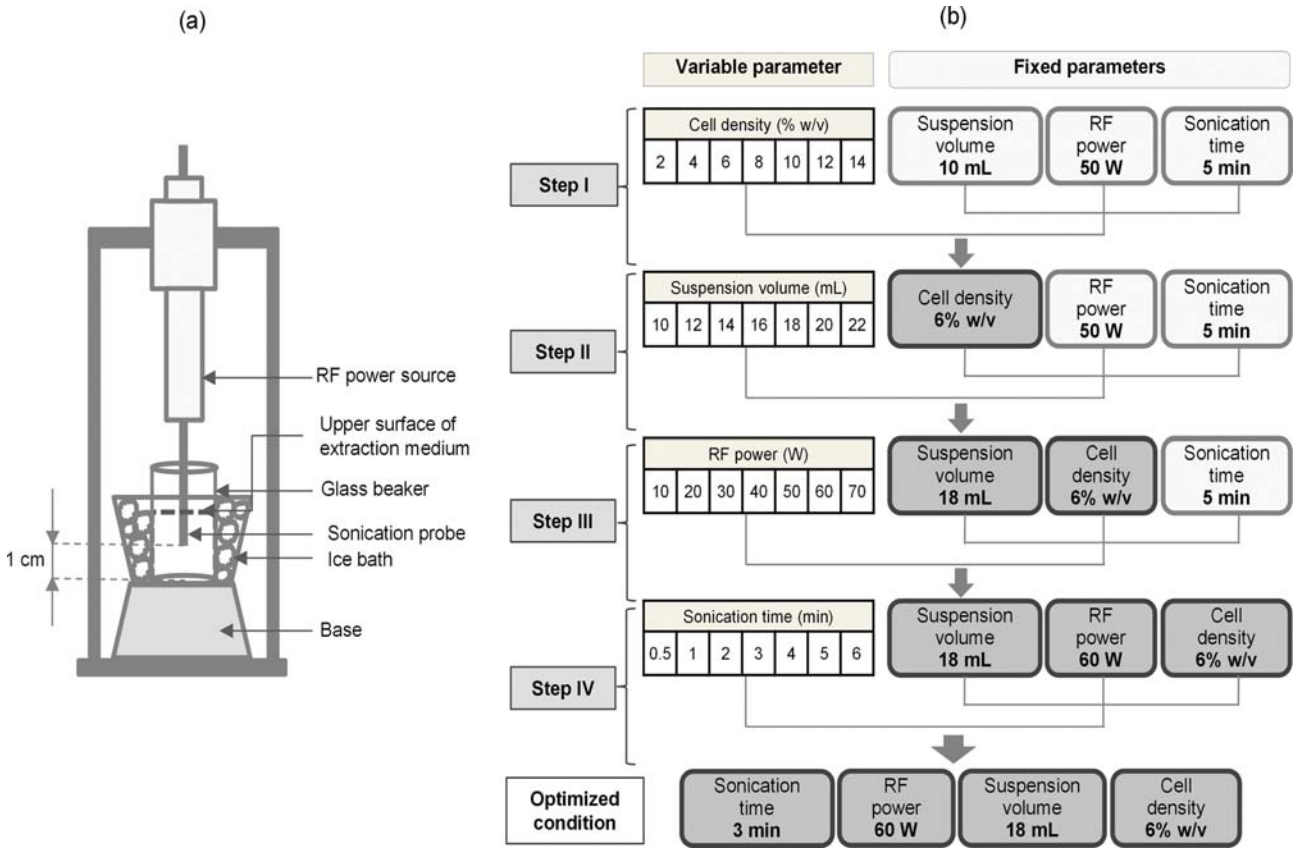

Fig. 1. (a) Schematic of the experimental setup used for ultra-sonication treatment, (b) experimental design matrix used for optimizing the ultrasonication conditions through one factor at a time (OFAT) approach.

a way that converted electrical energy output ranged around 20 kHz frequency with a typical amplitude of ~40 μm. The horn was attached to the converter, which amplified the vibration within the solution through the sonifier tip (Φ1.2 cm). The sample was taken in a 50-mL glass beaker (Φ4 cm), and a certain distance of 1 cm between the sonifier tip and the base of sample holder was maintained throughout the experiment. The glass beaker containing the suspension was kept in an ice bath during the experiment. The temperature of the medium was recorded by a J-type thermocouple at the point 0.5 cm apart from the wall of the beaker. The temperature gradient was reported after one minute from the sonication start.

7. Optimization of Sonication Protocol

7-1. One Factor at a Time Approach (OFAT)

The preliminary trials confirmed that cell density, suspension volume, radio frequency (RF) power and sonication time were significantly affecting the efficiency of arginase extraction in the medium. Therefore, optimized conditions for these four variables were found through one factor at a time (OFAT) approach. The experimental design for OFAT approach is summarized in Fig. 1(b). The effect of variation in a single factor on the arginase extraction was studied, keeping rest of the three variables fixed. The OFAT approach was completed in four steps by optimizing cell density, suspension volume, RF power and sonication time sequentially in each of the four steps. Each of the four variables was varied in seven equidistant levels within their corresponding domain leading to a total 28 (7×4) experiments for OFAT approach. The corresponding domain of cell density, suspension volume, radio frequency (RF) power and sonication time was 2-14% w/v, 10-22 mL, 10-70 W and 0.5-6 min, respectively (Fig. 1(b)). In all the experiments, the duty cycle was kept constant at 30% and tris buffer (100 mM, pH 8.5) served as the suspending medium. The arginase activity in the extraction medium was quantified spectrophotometrically, following the method discussed in the earlier section.

7-2. Optimization Using Evolutionary Operation (EVOP) Technique

The sonication protocol was further optimized using evolutionary operation (EVOP) technique following the methods discussed by Banerjee and Bhattacharyya [19]. EVOP was performed by inducing a slight variation from the initial optima in specific operating conditions and determining the process responses. Two levels-three factors full factorial design (2^3) was employed to study the effect of three variables--sonication time (P1), suspension volume (P2) and radio frequency power (P3)--targeting a maximum extraction of arginase. EVOP approach was conducted through a series of experiments in sequential phases. The phase-wise experimental conditions and the corresponding response are summarized in Table 1. Each phase was divided into two blocks (Block I and II) resulting in ten experimental runs (E1 to E10), while three factors P1, P2 and P3 were varied at two extreme levels (lower labeled as '–' and higher labeled as '+') and one experiment at the initial

Table 1. Experimental conditions for EVOP approach and results of phase I, phase II and phase III for the extraction of arginase from *B. licheniformis* (M09)

Parameters		Block I					Block II				
		E1	E2	E3	E4	E5	E6	E7	E8	E9	E10
Design	Time (min) (P1)	0	(−)	(−)	(+)	(+)	0	(+)	(−)	(+)	(−)
	Suspension volume (mL) (P2)	0	(−)	(+)	(−)	(+)	0	(+)	(−)	(−)	(+)
	RF power (W) (P3)	0	(−)	(+)	(+)	(−)	0	(+)	(+)	(−)	(−)
	Response (Cycle I)	M1	M2	M3	M4	M4	M5	M6	M7	M8	M9
	Response (Cycle II)	M1	M2	M3	M13	M4	M5	M6	M7	M8	M9
	Difference	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10
	Average response	ar1	ar2	ar3	ar4	ar5	ar6	ar7	ar8	ar9	ar10
Phase I	Time (min)	3	2	2	4	4	3	4	2	4	2
	Suspension volume (mL)	18	16	20	16	20	18	20	16	16	20
	RF power (W)	60	50	70	70	50	60	70	70	50	50
	EA IU/mL (I)	1.27	2.36	0.35	0.51	0.37	1.29	0.12	0.72	2.11	1.06
	EA IU/mL (II)	1.37	2.31	0.29	0.65	0.32	1.34	0.19	0.65	2.01	0.94
	Difference (I-II)	−0.10	0.05	0.06	−0.14	0.05	−0.05	−0.07	0.06	0.10	0.12
	Average EA (IU/mL)	1.32	2.34	0.32	0.58	0.34	1.32	0.16	0.69	2.06	1.00
Phase II	Time (min)	2	1	1	3	3	2	3	1	3	1
	Suspension volume (mL)	16	14	18	14	18	16	18	14	14	18
	RF power (W)	50	40	60	60	40	50	60	60	40	40
	EA IU/mL (I)	2.31	1.37	1.09	0.83	0.87	2.42	1.36	0.84	3.02	1.44
	EA IU/mL (II)	2.45	1.24	1.25	0.89	0.81	2.37	1.28	0.72	2.93	1.59
	Difference (I-II)	−0.14	0.13	−0.16	−0.06	0.06	0.05	0.08	0.12	0.09	−0.15
	Average EA (IU/mL)	2.38	1.31	1.17	0.86	0.84	2.40	1.32	0.78	2.98	1.52
Phase III	Time (min)	3	2	2	4	4	3	4	2	4	2
	Suspension volume (mL)	14	12	16	12	16	14	16	12	12	16
	RF power(W)	40	30	50	50	30	40	50	50	30	30
	EA IU/mL (I)	3.04	2.59	2.24	2.93	2.90	2.95	2.01	2.94	2.34	3.96
	EA IU/mL (II)	2.92	2.21	2.72	2.99	2.74	3.04	2.51	2.47	2.40	3.86
	Difference (I-II)	0.12	0.38	−0.48	−0.06	0.16	−0.09	−0.50	0.47	−0.06	0.10
	Average EA (IU/mL)	2.98	2.40	2.48	2.96	2.82	3.00	2.26	2.71	2.37	3.91

Table 2. Calculation worksheet of effects of the three-variable system, standard deviation and error limits for arginase extraction using EVOP approach

Effect of Parameters	Calculation of effects
Time (min)	¼(ar4+ar5+ar7+ar9−ar2−ar3−ar8−ar10)
Suspension volume (mL)	¼(ar3+ar5+ar7+ar10−ar2−ar4−ar8−ar9)
RF power (W)	¼(ar3+ar4+ar7+ar8−ar2−ar5−ar9−ar10)
Time*Suspension volume	¼(ar2+ar5+ar7+ar8−ar3−ar4−ar9−ar10)
Time* RF power	¼(ar2+ar4+ar7+ar10−ar3−ar5−ar8−ar9)
Suspension volume* RF power	¼(ar2+ar3+ar7+ar9−ar4−ar5−ar8−ar10)
Time*Suspension volume* RF power	¼(ar7+ar8+ar9+ar10−ar2−ar3−ar4−ar5)
Change in mean effect	1/10 (ar2+ar3+ar4+ar5+ar7+ar8+ar9+ar10−4ar1−4ar6)
Standard deviation (σ)	½(σ_1+σ_2)
σ_1	R_1 * f
σ_2	R_2 * f
R_1	(largest difference-smallest difference) in block I
R_2	(largest difference-smallest difference) in block II
F	Statistical constant, 0.3 for 2^3 factorial
Error limits:	
For average	±1.414 σ
For effects	±1.004 σ
For change in mean	±0.891 σ

center values (0, 0, 0) was repeated in each of the two blocks. The center values were fixed and selected to be the optimum concentration of individual factors as measured by the OFAT approach. Each experimental run was conducted for two cycles, and their differences and average values of all phases are presented in Table 1. The deviation in each of the three parameters was uniform during EVOP approach. For instance, the interval set for sonication time, suspension volume and RF power was ±1 min, ±2 mL and ±10 W, respectively. The shifting from Phase I to Phase II was monitored through the decision-making loops generated from the standard deviation and error limits obtained in the former phase. The worksheet for calculating the error limits and evaluating the corresponding effects are summarized in Table 2. The sequence of continuing the experiments in following phases was terminated until the magnitude of the error limit of summative effects surpassed its individual counterparts. On the contrary, the optimum condition within the searched region in a particular phase was achieved until the magnitude of '*change in mean*' became less with respect to the '*error limit of change in mean*' (Table 2).

7-3. Response Surface Optimization

A mixed level full factorial design with 3-factors was applied for optimizing the sonication time (min), suspension volume (mL) and input radio frequency (RF) power (W). The enzyme activity after the treatment was analyzed corresponding to each of the 36 runs (4×3×3) of the design matrix and the optimization was targeted to obtain a maximum extraction of arginase in the medium. The lower and upper limits of the independent parameters were selected based on the one-factor optimization data. All the independent variables, the sonication time (min), suspension volume (mL) and input radio frequency power (% of maximum wattage), were varied at three equidistant levels. For instance, in the case of sonication time, the samples were treated for 1, 2, 3 and 4 min keeping the suspension volume at 12, 16 and 20 mL, respectively. The RF power was varied within the range of 30-70% keeping the duty cycle time fixed at 30% for all the treatments. The cell density in the suspension was maintained at 6% w/v in all the cases. The initial medium temperature was maintained at 20 °C. The experiment was repeated six times at the center of the domain (50 W RF power, 16 mL suspension volume, 2.5 min sonication time) to analyze the lack of fit for the developed model.

A quadratic polynomial (Eq. (1)) was fitted for the enzyme extraction (EA, $IU \cdot L^{-1}$) as a function of coded counterparts of three independent variables: the sonication time, suspension volume and RF power.

$$EA=\beta_0+\beta_1x_1+\beta_2x_2+\beta_3x_3+\beta_4x_1x_2+\beta_5x_2x_3+\beta_6x_1x_3+\beta_7x_1^2+\beta_8x_2^2+\beta_9x_3^2 \quad (1)$$

where, β_0 to β_9 are the regression coefficients; x_1, x_2 and x_3 represent the dimensionless coded values of the sonication time (t, min), suspension volume (V, mL) and RF power (P, W), respectively. The conversion from real to coded forms of all three variables is expressed in Eqs. (2), (3) and (4).

$$x_1=(t-2.5)/1.5 \quad (2)$$

$$x_2=(V-16)/4 \quad (3)$$

$$x_3=(P-50)/20 \quad (4)$$

7-4. Numerical Optimization

Numerical optimization was employed to find the best sonication condition for a maximum enzyme extraction. It was targeted towards minimizing the sonication time and RF power with a maximum acoustic intensity inculcated through the medium. Iteration through numerical optimization was performed in Excel Solver (MS Office 2016, USA). The optimized condition was validated further by performing the experiment at the nearest possible condition as of the optimized counterpart.

8. Statistical Analysis

All experiments were conducted in duplicate and analyzed in triplicate (N=6). Model significance and analysis of variance (ANOVA) were evaluated using Microsoft Excel (MS Office 2016, USA) and SPSS software (IBM® SPSS® Statistics v23, USA), respectively. Duncan's multiple range test was employed to determine the statistical significance of the differences between the means at $p \leq 0.05$ keeping the confidence interval at 95% of the mean.

RESULTS

1. Effect of Different Organic Solvents and Detergents on Arginase Extraction during Cell Disruption

Different solvents had a significant effect on the extraction of arginase during the cell disruption method. Among all the five different organic solvents used, toluene seemed to be the better medium, facilitating the enzyme extraction during sonication followed by hexane, butanol and ethanol, respectively (Fig. 2). All the solvents used for extraction of arginase (except chloroform) at increasing concentration (from 3 to 9% v/v) exhibited a lesser enzyme activity. Solvents (toluene/hexane/butanol/ethanol) used at 3% (v/v) concentration showed varying arginase extraction from 0.15 to 0.57 $IU \cdot mL^{-1}$ at 3% biomass concentration. However, the arginase extraction at 3 and 9% v/v chloroform was statistically similar ($p > 0.05$), revealing a counteracting effect towards its extraction. The maximum extraction of arginase (0.572 $IU \cdot mL^{-1}$) was attained with 3% (v/v) concentration of toluene (Fig. 2) and an increase in toluene concentration led to a reduction in enzyme activity, which

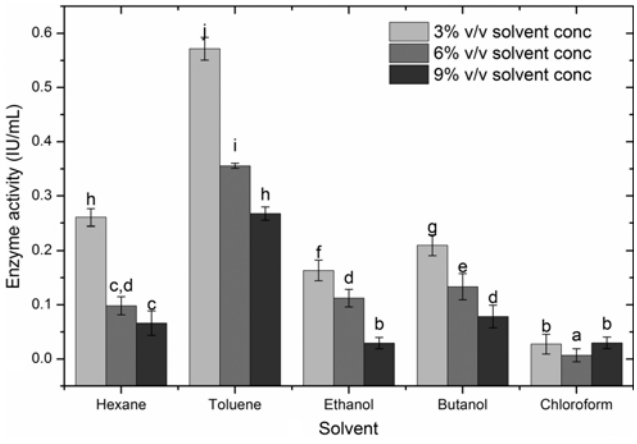

Fig. 2. Effect of different types of organic solvent and their concentrations on the extraction of arginase from *Bacillus licheniformis* (M09). Different alphabets denote that the corresponding mean values belong to varying subsets at $p<0.05$.

perhaps was due to denaturation of the protein of interest. A similar trend was reported by Toshio et al. [24] while working on various solvents to find its effect on protein denaturation. The extent of protein denaturation was increased up to 4-fold when the solution was exposed to a higher concentration of toluene. In the same line, Dange et al. [25] reported the same trend in case of succinate dehydrogenase and acetylcholinesterase, the key enzymes involved in the Kreb's cycle and chemical synapses, respectively.

Amongst the different anionic (sodium dodecyl sulfate), cationic (cetyl ammonium bromide) and non-ionic (tween 20, 80 and triton X-100) detergents, a maximum enzyme activity of 0.524 IU·mL^{-1} was obtained in 0.5% triton X-100 (Fig. 3(a)). While optimizing the concentration of triton-X100 itself, a maximum extraction of arginase 0.746 IU·mL^{-1} was obtained at 1% detergent (Fig. 3(b)).

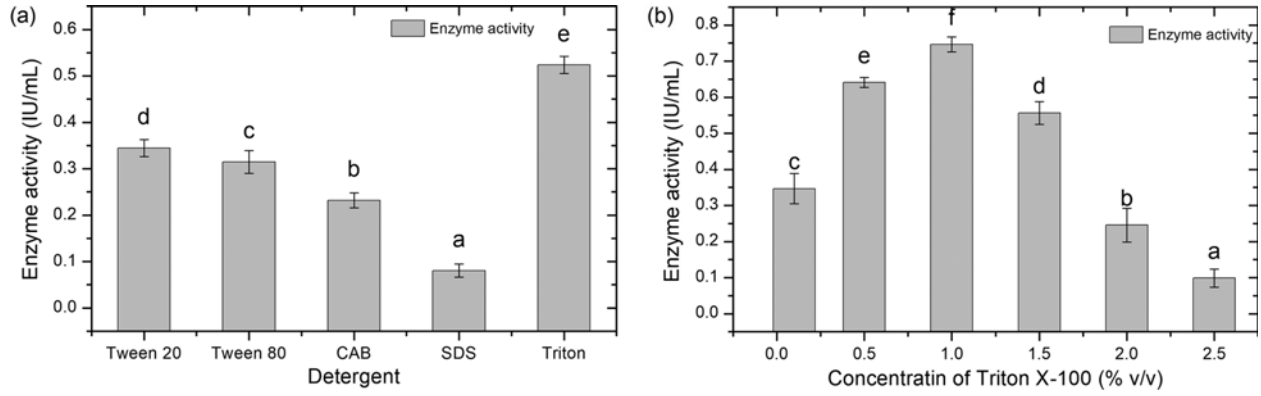

Fig. 3. Effect of (a) different detergents at 0.5% concentration and (b) varying concentration of triton X-100 on the extraction of arginase from *Bacillus licheniformis* (M09). Different alphabets denote that the corresponding mean values belong to varying subsets at $p<0.05$.

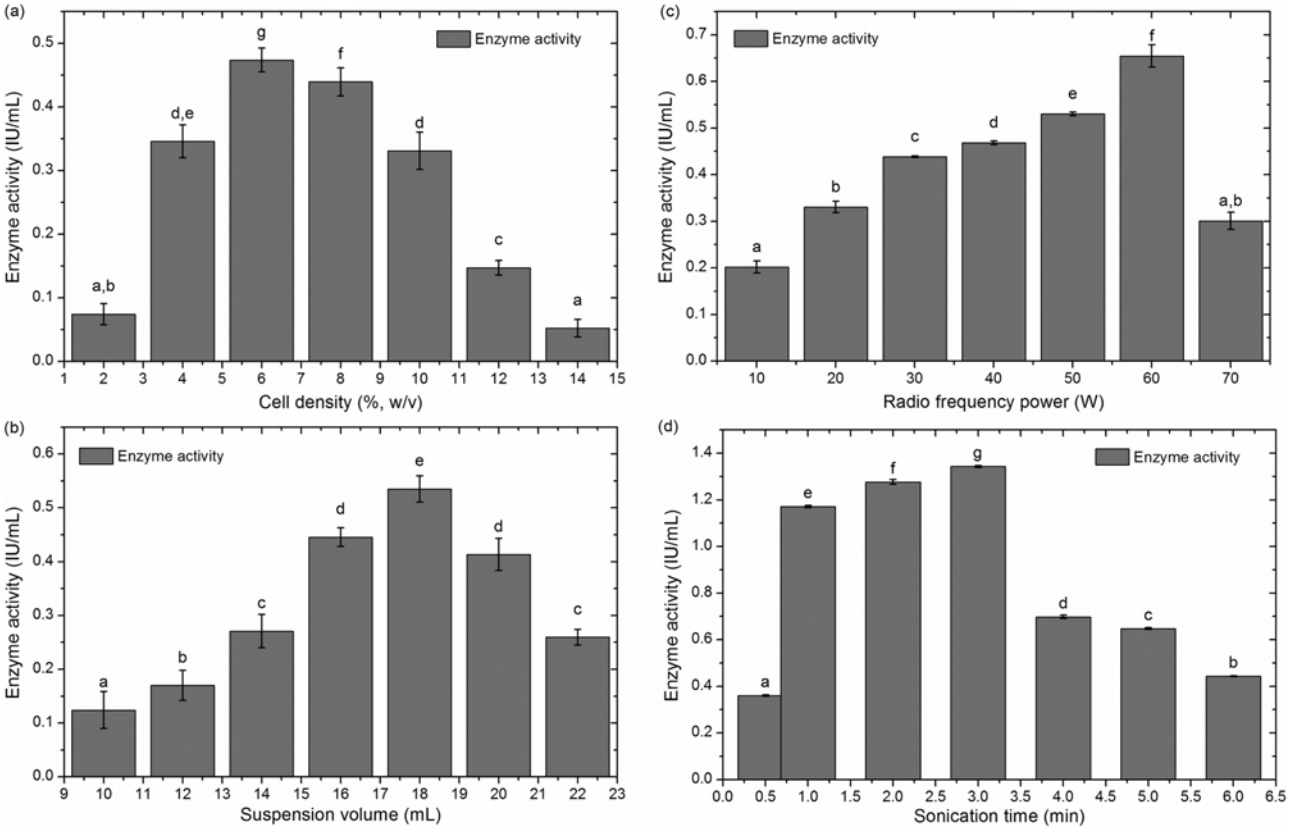

Fig. 4. Effect of different ultrasonic parameters on the extraction of arginase from *Bacillus licheniformis* (M09): (a) Effect of different cell densities sonicated for 5 min at 50 W radio frequency power, (b) effect of varying suspension volume sonicated for 5 min at 50 W radio frequency power, (c) effect of varying radio frequency power keeping 6% cell density and sonication time of 5 min, (d) effect of different sonication time at 60 W radio frequency power keeping 6% cell density within 18 mL suspension volume. Different alphabets denote that the corresponding mean values belong to varying subsets at $p<0.05$.

2. One Factor at a Time Approach for Optimizing Sonication Protocol

2-1. Effect of Cell Density

The effect of different cell densities (2-14% w/v) at 50 W RF power keeping the suspension volume and sonication time fixed at 10 mL and 5 min, respectively, is represented in Fig. 4(a). As expected, being an intracellular enzyme, an increase in cell density resulted in a higher extent of arginase extraction up to 6% w/v; however, an opposite trend was profound thereafter. The highest enzyme activity (0.474 IU·mL^{-1}) was obtained at 6% cell density (Fig. 4(a)).

2-2. Effect of Suspension Volume

The extraction of arginase as affected by different suspension volume during ultra-sonic extraction at 6% w/v cell density, 50 W RF power and 5 min of sonication time is summarized in Fig. 4(b). The enzyme activity obtained in the mixture ranged between 0.12 and 0.535 IU·mL^{-1} having the maximum enzyme extraction corresponding to 18 mL. An increased enzyme activity was obtained in the extraction medium when the suspension volume was raised to 18 mL, beyond which the enzyme concentration was found to be less.

2-3. Effect of Radio Frequency Power

The effect of different levels of radio frequency (RF) power on enzyme activity at 18 mL suspension volume, 6% w/v cell density and 5 min sonication time is represented in Fig. 4(c). At a constant cell density, suspension volume and sonication time, an increase in RF power up to 60 W resulted in a higher amount of arginase extraction in the medium. The maximum arginase activity of 0.654 IU·mL^{-1} was obtained at 60 W RF power; however, a further increase in RF power by 10 W led to a 54% reduction in the enzyme activity in the extraction medium.

2-4. Effect of Sonication Time

The effect of different sonication time (0.5-6 min) on the arginase extraction at 60 W RF power, 18 mL suspension volume and 6% cell density can be observed in Fig. 4(d). Higher enzyme activity was observed in the extraction medium with an increase in sonication time. A maximum enzyme extraction of 1.35 IU·mL^{-1} was obtained till 3 min of sonication, and there was a reduction of 67% enzyme activity when the sonication was extended up to 6 min. The highest enzyme activity of 1.35 IU·mL^{-1} was observed at 3 min (Fig. 4(d)).

Based on the above results obtained, it is clear that ultra-sonication appears to be a suitable method of cell disruption for arginase extraction from *Bacillus licheniformis* (M09). In continuation, OFAT approach revealed that the sonication for 3 min at 60 W RF power, taking 6% w/v biomass dispersed in 18 mL tris buffer (pH 8.5), is capable of releasing a maximum arginase activity of 1.35 IU·mL^{-1} in the medium. The EVOP and response surface methodologies were further applied to fine-tune the optimized zone of ultra-sonication suggested by the OFAT approach.

3. Optimization of Sonication Protocol by EVOP

The optimized ultra-sonication conditions obtained from the OFAT approach were considered as the starting point for evolutionary operation (EVOP). Keeping the cell density fixed at 6% w/v, the other three variables, sonication time (P1, min), suspension volume (P2, mL) and RF Power (P3, W), were optimized through EVOP protocol. As per the decision-making rule described by Banerjee and Bhattacharyya [19], the phase sequence was designed.

In Phase I the initial or control condition (0, 0, 0) was 3 min sonication/18 mL suspension volume/60 W RF power, which resulted in 1.32 IU·mL^{-1} arginase activity in the medium. After completing all the 10 runs in Phase I, the conditions for E2 (2 min sonication/16 mL suspension volume/50 W RF power) were set as the starting point for next phase (i.e., Phase II) as it yielded a maximum enzyme activity of 2.34 IU·mL^{-1}. The corresponding change in mean effect (−0.306) was negative and large compared to the error limit (0.051) as shown in Table 3. It suggests that the system is approaching an optimum but has not reached the real optimum condition [26]. It is clear from Table 3 that the effects of P1, P2 and P3 are negative in Phase I and the magnitudes are very high compared to the 'error limit of change in mean' which triggers to shift in Phase II targeting to find a newer and narrow domain. It also indicates that the corresponding values of P1, P2 and P3 need to be reduced in Phase II. The change in mean effect (−0.834) is negative and large compared to the error limit (0.074) in Phase II. Effects of P2 and P3 are also negative and larger than the error limit of effects,

Table 3. Effects of different parameters investigated and their error limits of three phases of EVOP

Parameters	Error limits in different phases		
	Phase I	Phase II	Phase III
Effect of time	−0.299	0.306	−0.270
Effect of suspension volume	−0.961	−0.270	0.258
Effect of RF power	−0.998	−0.626	−0.273
Effect of time*Suspension volume	−0.111	−0.568	−0.383
Effect of time* RF power	0.166	0.189	0.288
Effect of suspension volume* RF power	0.565	0.693	−0.720
Effect of time*Suspension volume* RF power	0.080	0.605	0.145
Change in mean	−0.306	−0.834	−0.199
Standard deviation	0.057	0.084	0.274
Error limit of average	0.081	0.118	0.388
Error limit of effects	0.057	0.084	0.275
Error limit of change in mean	0.051	0.074	0.244

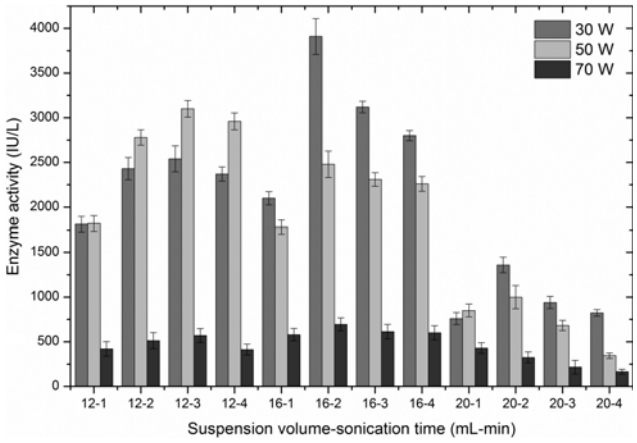

Fig. 5. The effect of different combinations of sonication time, suspension volume and radio frequency power on the extraction of intracellular arginase from *Bacillus licheniformis* (M09) during ultra-sonication according to the mixed level full factorial design.

suggesting that their values need to be reduced in Phase III, while it was the reverse in case of sonication time. In a similar manner, in phase III, the change in mean effect was smaller as compared to the error limit. In addition, the individual effects of P1, P2 and P3 were also small as compared to the error limits, indicating that the true optima had been reached. Thus, the corresponding conditions for E10 in Phase III (2 min of sonication/16 mL suspension volume/30 W RF power) were set as the optimized condition for a maximum arginase extraction of 3. 91 IU·mL^{-1}.

4. Response Surface Optimization of Sonication Parameters

The changes in the magnitude of arginase extraction as influenced by three independent parameters, the sonication time, suspension volume and RF power, are represented in Fig. 5. In most of the cases, a higher amount of enzyme extraction was obtained having the suspension volume at 16 mL. However, 30 W RF power facilitated the process to a maximum in all other combinations. When the quadratic regression model (Eq. (1)) was developed for the response as a function of independent variables, it was clear that experimental data were well explained by the developed polynomial model. The coefficients in the developed polynomial model are summarized in Table 4. The analysis of variance (ANOVA) for the regression model shows a high coefficient of determination (adjusted R^2>0.85), reflecting that the quadratic polynomial model explains the variability well enough. The lack of fit tests for the model reveals a high p-value (p>0.07), suggesting that the data within the system do not have a default variation arising from the system or any type of noise; instead, the variation in the magnitude of enzyme extraction is due to change in independent variables. A higher F-value (18.9) and lower p-value (<0.0001) also support the model adequacy.

In view of the relative effect of the individual variable on the enzyme extraction, it is clear that RF power is most significantly contributing towards response followed by suspension volume and sonication time (Table 4). In the quadratic model developed, all the terms except the interaction term between sonication time and RF power (x_1 x_3) were significantly affecting the enzyme extraction.

Table 4. ANOVA data and coefficients for the polynomial model developed for arginase extraction as a function of different sonication parameters

Terms	Coefficients for the model with	
	Coded values	Real value
Constant	2550.21	−8316.551
x_1 (Sonication time)	86.25	1656.471
x_2 (Suspension volume)	−576.29	1175.463
x_3 (RF power)	−809.42	59.452
x_1 x_2	−213.32	−35.537
x_1 x_3	−94.23**	−3.126
x_3 x_2	280.62	3.507
x_1 x_1	−392.46	−174.861
x_2 x_2	−703.04	−43.941
x_3 x_3	−592.92	−1.482
F-value	18.9	-
p-value	<0.0001	-
p_{lof}	Not significant	-
Adj R^2	0.85	-

All the terms are significant at p<0.05 otherwise mentioned as * and **..
* Represents 0.05<*p*<0.1 and ** stands for *p*>0.1

The negative sign of the linear terms of suspension volume and RF power depict that the extent of enzyme extraction was compromised at a higher value of any of the parameters. However, sonication time positively influenced the enzyme extraction phenomenon.

The interacting effect of suspension volume with sonication time and RF power contributing towards arginase extraction separately has been shown in Fig. 6. From the contours in Fig. 6(a) and 6(c), it is evident that the extraction of the enzyme is synergistically influenced by suspension volume and RF power (Fig. 6(a)) whereas suspension volume acted antagonistically towards sonication time (Fig. 6(c)). The square terms also contributed significantly leading to an elliptical surface in Fig. 6(b) and 6(d). From Fig. 6(b), it is clear that there is a steep reduction in enzyme extraction with RF power at a constant suspension volume (10-20 mL).

The response surface model and contours (Fig. 6(c) and Fig. 6(d)) reveal that at the fixed value of 50 W RF power, the sonication time and suspension volume act antagonistically towards enzyme extraction. Eventually, at any particular dilution (12 or 20 mL suspension volume), the extent of released arginase reached a maxima within 2.5-3.5 min sonication time. After that, an opposite trend was prominent. In the same line, for a fixed sonication time (1 or 4 min), the optimum enzyme extraction was found at 14-16 mL suspension volume followed by a reduction in enzyme extraction with dilution.

Numerical optimization focused on a maximum desirability value when targeting a maximum enzyme extraction in the solution (Table 5). From industrial facet, it is always beneficial to have a minimum sonication time along with a reduced power consumption. The numerical optimization suggested that a maximum enzyme extraction of 3.5 IU·mL^{-1} could be obtained at 3.2 min sonication having 14.6 mL suspension volume at 36 W of RF power (Table 5).

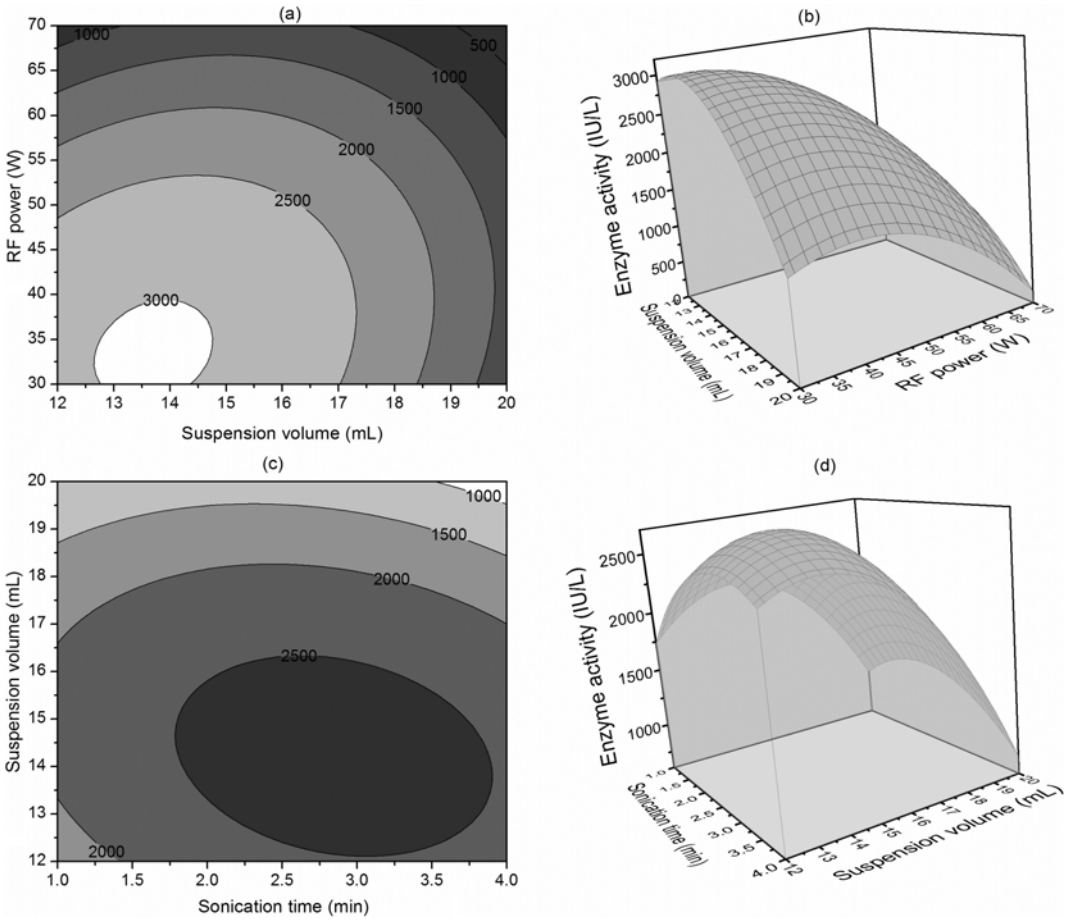

Fig. 6. Response surface model and contours describing the interaction between two parameters on the extraction of intracellular arginase from *Bacillus licheniformis* (M09) during ultra-sonication; (a) contour plot, (b) response surface plot between radio frequency power and suspension volume at 2.5 min sonication time, (c) contour plot, (d) response surface plot between suspension volume and sonication time at radio frequency power of 50 W.

Table 5. The set of constraints for different parameters for optimizing the ultra-sonicated extraction of arginase

Parameter	Goal	Lower limit (L_i)	Upper limit (U_i)	Relative importance (r_i)	Optimized value at D=0.77	Experimental data*
Enzyme activity ($IU \cdot mL^{-1}$)	Maximize	165	3950	5	3511	3620±215
Sonication time (t, min)	Minimize	1	4	4	3.2	3.0
Suspension volume (V, mL)	In range	12	20	3	14.6	14.5
RF power (P, W)	Minimize	30	70	4	36.0	35.0

*Values are reported as mean±standard error (N=6)

The desirability (D_i) value obtained in this case was 0.77, which is well accepted for any type of numerical optimization method. When the experiment was conducted at the nearest possible condition as of the suggested optimized condition, it was evident that the variability in the response was within 8% of the predicted counterpart. Hence, the condition of 3 min sonication time at 14.5 mL suspension volume at 35 RF input power was taken as the optimized condition.

DISCUSSION

The superiority of toluene as a solvent facilitating the diffusion of metabolites from the microbial cell disruption, as obtained in the present study, was reported by many researchers earlier. Middelberg [27] obtained an efficient recovery of β-galactosidase from *Kluveromyces lactis* using toluene as solvent. In a similar way, Jayakar and Singhal [28] reported 8% recovery of lipoic acid from *Saccharomyces cerevisiae* with 1% v/v toluene concentration. The authors also stated that lowering the concentration of solvent resulted in an increase in the percentage recovery of the desired metabolite in the medium. In general, when added to biomass a non-polar organic solvent like toluene tries to invade within the lipo-protein structure of the microbial cell wall, resulting in swelling followed by rupture

within it. Thus, the leaching of the proteins from the perturbed counterpart is facilitated [29-31]. Being Gram-positive bacteria with a thin hydrophobic lipid layer, permeabilization of intracellular material through the cell wall was facilitated in case of *Bacillus licheniformis* (M09). On the other hand, 3% v/v ethanol and butanol had comparatively lower values of hydrophobicity (log P of 0.88 and 0.19, respectively) and thus being ineffective towards releasing the intracellular enzyme. However, due to its highest lipophilicity hexane exhibited an insignificant positive effect on the enzyme release from cellular fragments.

In the case of detergent selection based on the maximum enzyme extraction, non-ionic detergents had a better effectivity than cationic and anionic counterparts, respectively. It might be attributed to their charges, which made the cell disruption process difficult in comparison with the non-ionic surfactants [31]. Jayakar and Singhal [28] reported that using 1% v/v triton-X100 with solvent yielded an optimum recovery of 14% lipoic acid from *Saccharomyces cerevisiae*. Galabova et al. [32] also supported the ability of triton-X100 towards reducing the surface energy between solvent and cell wall interface. The authors obtained a maximum phosphatase activity while disrupting yeasts cells within the range of 0.1-0.2% triton-X100. Being a non-ionic detergent, triton X-100 possesses a very high binding affinity for hydrophobic species and thus is capable of solubilizing phospholipids effectively from both inner and outer membrane fragments [33]. On the other hand, tween 20 and 80 are less water soluble than triton X-100 and thus able to release a lesser quantity of intracellular protein [34].

The present study shows the pathway to reach the optimized condition sequentially through one factor at a time (OFAT), evolutionary operation (EVOP) and response surface method (RSM) targeting towards an effective ultra-sonication condition for maximizing the extraction of intracellular arginase.

To begin with OFAT technique, the arginase extraction was first estimated at different levels of cell densities and it suggested optima at 6% w/v of the biomass while fixing the RF power, dispersion volume and sonication time at 50 W, 10 mL and 5 min, respectively. Initially, enzyme extraction was increasing with an increase in cell density up to 6% w/v following a reverse trend afterwards. Lovitt and Jones [35] and Feril et al. [36] observed a linear relationship between the protein release and operating pressure during a hydraulically operated cell disrupter for baker's yeast. The authors obtained an increase of 15 g/L in protein concentration when the yeast concentration was increased from 40 to 90 g/L (cell dry weight) at 170 MPa disrupter pressure. On the other hand, Lee and Row [37] and Feril et al. [36] reported a decrease in metabolite concentration beyond the optimum cell density during ultrasound-assisted cell disruption. It might be attributed to the viscosity of the suspension containing more than 6% biomass which was impeding the passage of sonic waves disseminating into the medium and thereby narrowed the cavitation zone. Thus, only a part of sonic energy was utilized within the medium, which eventually compromised the efficacy of cell disruption [38].

The arginase extraction in the medium during ultra-sonication was influenced by different suspension volume in a manner similar to the cell density. In this case, an optimum enzyme extraction was obtained at 6% biomass dispersed in 18 mL buffer which was sonicated at 50 W RF power for 5 min. The results are in agreement with the trend reported by Kamarudin et al. [39]. The authors obtained a maximum recovery of HBcAg antigen from *E. coli* at a suspension volume of 15 mL within the range of 10-20 mL. Feliu et al. [40], however, reported that the rate of intracellular β-galactosidase extraction from *E. coli* was found to be reduced with an increase in medium volume from 10 to 50 mL. It can be hypothesized that at a lower suspension volume like 10 mL, the specific density of small eddies effective in disrupting the cells were higher, which led to an increased acoustic power dissipated per unit volume. This acoustic power finally converted to heat energy and increased the temperature of suspension, which was more likely to be capable of enzyme denaturation. Concomitantly, at the suspension volume higher than 18 mL, the disseminated acoustic power became diluted, resulting in the formation of larger eddies which focused on moving around instead facilitating the arginase extraction [39].

Within the domain of 10-70 W RF power, 60 W led to the maximum arginase extraction and following the same trend obtained for cell density and suspension volume. A similar trend was registered in case of β-galactosidase extraction from *L. acidophilus* [41] and protein release from *Acetobacter peroxydans* within 30-100 W RF power [42]. It is clear that radio frequency power supply governs a maximum power in order to maintain the amplitude. Thus, an increase in RF power leads to more amount of acoustic power concentrated within a small amount of suspension volume like 18 mL which triggers the formation of small eddies having shape even smaller than bacterial cells. Thus, the penetration of these eddies through disruptive stresses enhances the cell lysis and arginase concentration in the medium [39,42]. On the other hand, the heat generated at higher radio frequency power of 70 W denatures the protein, thus leading to a reduced enzyme activity in the medium.

In the case of visualizing the effect of sonication time on enzyme extraction, the optima was at 3 min at 60 W RF power and 6% biomass dispersed in 18 mL buffer. The overall trend of initial rise, reaching the optima and certain drop thereafter also held true in case of sonication time as of the other three parameters. A similar trend has been reported by Chisti and Moo-Young [13] and Ho et al. [39]. Lateef et al. [43] conveyed that fructosyltransferase extraction from *Aureobasidium pullulans* was increased up to 9 min of ultrasonication and thereafter decreased with sonication duration. As expected, the extraction of arginase increased linearly with an increase in sonication time till 3 min; however, beyond which extraction of enzyme declined probably because of the protein denaturation due to its overheating. As discussed earlier, the more time the solution is sonicated, higher will be the cell lysis, leading to an enhanced extraction of arginase. However, it is also true that the most amount of dissipated acoustic energy is converted into heat energy within the medium [13]. Thus, sonication beyond 3 min might surpass the threshold temperature of arginase denaturation, which eventually leads to a reduced enzyme activity within the medium [39].

EVOP factorial technique has been successfully used in case of statistical optimization of several biological processes like the production of poly-lysine from *Streptomyces noursei* [44], serratiopeptidase from *Serratia marcescens* [45], lipase from *Penicillium chrysogenum* [46] and *Rhizopus oligosporus* [47]. The obtained narrower domain was further optimized through response surface optimi-

zation method to quantify the interaction effect between P1, P2 and P3 affecting the arginase extraction.

While applying the response surface methodology to optimize the sonication protocol, the trend followed by the three individual parameters, suspension volume, RF power and sonication time, was the same as obtained from OFAT and EVOP approaches. For instance, at a certain value of RF power within 30-40 W, the enzyme extraction was almost constant with extraction volume, followed by a rapid decrease in the same for a more diluted solution. A similar type of trend was reported in case of the extraction of intracellular invertase from *Aspergillus niger* which was obtained at 40 W [48]. Apar and Özbek [49] also registered the same trend in case of protein extraction from baker's yeast, following the same intended for the hydrolysis of lactose recovered from whey [50] and polysaccharides from some marine algae [51]. Lateef et al. [43] suggested that a maximum amount of fructosyl-transferase could be extracted from *A. pullulans* at 50 W of radio frequency power for a sonication up to 9 min followed by a steep reduction in the same.

It is already established that cell disruption using ultrasound energy is the effect of the implosion of the cavitation bubbles, which leads to the formation of shock waves eventually generating the eddies [52]. In addition, it is also clear that the smaller the shape of eddies, more will be the lethal effect from the shear stress evolved and likewise essential for cell disruption. In the present study, increasing trend of arginase extraction with radio frequency power up to 50 W might be due to the tiny dissipative eddies produced at a high radio frequency power facilitating the cell disruption process [38,50]. However, a steep decline in arginase extraction beyond 50 W/3 min radio frequency power for an extended period of time up to 70 W/5 min might be attributed to the extraction of other loosely bound biomass other than arginase in the solution [43]. It was also supported by the turbid solution obtained at those sonicated conditions (50-70 W/3-5 min).

Eventually, RSM revealed that the interaction obtained between the sonication time and suspension volume at a fixed RF power influencing the arginase extraction was antagonistic in nature. A similar trend of obtaining a higher extent of enzyme release with an increase in reaction volume was reported in the case of lactose hydrolysis from whey and alpha-amylase from barley [50,53]. The authors also stated an opposite trend after that; Pchelintsev et al. [54] registered a maximum sonication efficiency for protein extraction while the suspension volume was in between 100-700 μL. Ho et al. [39] reported the same trend in case of ultrasound extraction of intracellular Hepatitis-B core antigen from *Escherichia coli*. Authors obtained the maximum efficiency of the ultra-sonication at 15 mL suspension volume, while the range was 10-20 mL. The compromised efficiency of the ultra-sonication at a higher suspension volume was also obtained in case of *β*-galactosidase extraction from *Escherichia coli* [40].

For efficient extraction of intracellular arginase, homogeneous dissipation of acoustic intensity is very much essential [51]. The suspension volume range of 12-16 mL might be the narrow range for obtaining a maximum extraction of the same because of the probable distribution of the shear stress developed across the cell wall. On the other hand, beyond 16 mL suspension volume the lower acoustic intensity or reduced specific sonication energy per unit volume of the mixture was restricting the formation of small eddies obligatory for cell disruption [40]. Thus, it led to a compromised yield of arginase in the diluted medium (16-20 mL suspension volume).

CONCLUSIONS

The present study shows the comparison between evolutionary and response surface optimization techniques leading to an effective ultra-sonication method for maximizing the extraction of intracellular arginase. The evolutionary operation technique confronted a combination of 16 mL suspension volume, 2 min sonication time at 30 W of RF power for a maximum arginase extraction. This result was very much supplemented by the condition predicted from response surface method. Combining both the results, it can be concluded that 14.5 mL suspension volume, 3 min sonication time at a 35 W of RF power appeared to be the desirable and optimized condition for a maximum extraction of intracellular arginase from mutant *Bacillus licheniformis* (M09). The study indicated that RSM is slightly more significant for optimizing the cell disruption protocol in comparison with the same from EVOP, as the former technique considers the interaction between two independent parameters. Being less sophisticated and relatively economical, the result of the study will help in commercializing the ultra-sonic extraction of high valued products like arginase.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest

The authors declare that they have no conflict of interest towards this research.

Ethical Approval

This article does not include any studies with human participants or animals performed by any authors.

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