Global Journal of Biotechnology & Biochemistry 5 (2): 97-105, 2010
ISSN 2078-466X

Application of Evolutionary Optimization Technique in Maximizing the Recovery of L-Asparaginase from *E. caratovora* MTCC 1428

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Abstract: L-Asparaginase, an intracellular metabolite of *E. caratovora*, is a potential enzyme-drug used in the treatment of acute lymphoblastic leukaemia (ALL). The current work was carried out to deal with various cell lysis methods employed in maximizing the release of L-asparaginase from the cells of *Erwinia*. Sonication alone gave maximum recovery of enzyme as compared to the other cell lysis methods, was further optimized by a classical factor at a time approach followed by evolutionary optimization (EVOP) technique. The recovery of enzyme increased from 25.618 to 78 IU mL^{-1} in EVOP optimized sonication protocol employing cell volume of 10 mL, sonication time of 3 min at 20 W acoustic power. L-asparaginase from *E. caratovora* is free from associated glutaminase activity. Thus several immunological implications associated with glutaminase activity are absent, which makes this enzyme of great importance in treatment of ALL. The present investigation dealt with maximizing the yield of L-asparaginase from *E. caratovora* cells by focusing upon the release of enzyme using different methods of cell lysis.

Key words: L-asparaginase · *Erwinia caratovora* · Sonication · Evolutionary optimization

INTRODUCTION

L-asparaginase (E.C. no 3.5.1.1) is widely used in the treatment of acute lymphoblastic leukemia and malignant lymphomas [1]. Selectivity of this anticancer enzyme is based on the fact that normal cells possess asparagine synthase activity, while malignant cells are deprived of this enzymatic activity. Thus cancerous cells cannot obtain the nonessential amino acid, L-asparagine from the extracellular sources. This affects their protein, DNA and RNA synthesis leading to cell apoptosis [2].

L-asparaginase is produced by various microbial strains like *Serratia*, *Vibrio*, *Citrobacter*, *Psuedomonas*, *Bacillus*, however commercial production of the enzyme is carried mainly from *E. coli* and *Erwinia* species [3]. L-asparaginse from *E. coli* is associated with glutaminase activity resulting into severe immunological implications. However, such side effects are completely absent with L-asparaginase from *Erwinia* which is virtually devoid of associated glutaminase activity and hence much safer drug in the treatment of ALL [4].

Intracellular location of L-asparaginase from *Erwinia caratovora* demands an additional cell lysis step during its recovery. For any intracellular product depending upon its cellular localization either cell permeablization method using detergent, detergent in combination with tertiary amine and glycerol, pH change, thermolysis (for thermostable compounds) and osmotic shock have been optimized [5-9] or mechanical methods like sonication and bead mill respectively have been attempted [10, 11]. However, among the several methods reported for L-asparaginase, sonication is still the widely used laboratory method.

Sonication is a liquid shear disruption method wherein the cell suspension is exposed to the high frequency ultrasonic wave (15–20 kHz). Cell lysis results due to the cavitation phenomenon which comprises of growth of microbubbles at the rarefaction phase followed by their collapse at the compression cycle of the wave. During collapse there is a conversion of this sonic energy into the mechanical energy that dissipates into the surrounding media imparting a high turbulent motion. When the intensity of this turbulence exceeds the kinetic energy of the cell, cell disruption occurs [12]. Sonication is governed by many factors like acoustic power, sonication time, cell density and suspension volume.

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Success of sonication depends on judicious selection of all these factors. To understand the nature of interactions among the qualitative and quantitative variables, statistical methods are mostly sought.

Evolutionary optimization (EVOP) methodology was adopted in our study to understand the effect of individual parameters as well as the interaction effects during sonication. EVOP is a multivariable sequential technique used to study the system with two or three variables wherein the responses are analyzed statistically to reach the optima. It is also referred to as a "sequential" method because based on the results of previous experiments, the next level of experiments are designed. More number of variables can be studied, but with a large number of process conditions involved makes operation of EVOP a bit more complicated. Experimental design for studying the effect of three factors is given in Table 1. Total of 10 experimental trials are divided into two blocks *viz*, I and II. Run E_{10} and E_{20} are referred to as control or center or mean values and the remaining runs have either higher or lower values than the center values. To eliminate the run to run variations that arises mainly due to measurement or analytical errors or the errors caused due to high impossibility in duplicating precisely the same experimental conditions, center runs should be carried out independently for both the blocks [13]. Based on the average response, the individual and interaction effects and change in mean are calculated while the difference between the responses of two cycles is used in calculating the error limits. Tunga *et al*. [14] have enumerated the formulas for calculating the effects and error limits. Magnitude and sign of the effects are important for analyzing the results. For attaining the optima the change in mean value should be large and negative compared to the error limits and the magnitude of the effects should also be less than the error limits. If magnitude of effects is large and positive then one has to increase the value of the parameter, while the magnitude of effect being large and negative decrease in value of the parameter is suggested. The cycle has to be continued till the final optimum is attained. EVOP is thus a continuing process [15].

The aim of the present investigation was to study the various methods of cell lysis to maximize the release of L-asparaginase and to optimize in detail the sonication protocol. To the best of our knowledge, this is the first report about the application of EVOP methodology in optimizing the sonication protocol for maximizing the L-asparaginase recovery.

MATERIALS AND METHODS

Materials: Tryptone soy broth, agar, sodium acetate, lactose, casein peptone, potassium dihydrogen phosphate, sodium chloride, magnesium sulfate, potassium iodide, mercuric chloride, L-asparagine, trichloroacetic acid, potassium hydroxide, tris buffer and lysozyme were purchased from Himedia Pvt. Ltd. Toluene, butanol, chloroform, hexane, ethanol, tween 20 and 80, triton X-100, cetyl ammonium bromide, SDS were procured from SD Fine, Mumbai.

Microorganism and its Maintenance: *Erwinia caratovora* (MTCC 1428) used in our study was obtained from Microbial Type Culture Collection, Chandigarh. The culture was incubated on trypticase agar slants at 30°C for 24 h. Subculturing was done monthly to preserve the culture and was stored at 4°C.

Inoculum Development and Production of Enzyme: A loop full culture was transferred in tryptone soy broth and incubated at 180 rpm for 24 h at 30°C. Fermentation was carried out in 250 mL Erlenmeyer flasks containing 50 mL of the medium with a composition of sodium acetate 1.25 g L^{-1}, lactose 3.75 g L^{-1}, casein peptone 30 g L^{-1}, dipotassium hydrogen phosphate 1 g L^{-1}, sodium chloride 1 g L^{-1} and magnesium sulfate 0.2 g L^{-1} pH was adjusted to 8.5 before autoclaving. The flasks were sterilized by autoclaving at 121 °C at 15 psi for 15 min. The culture flasks were then inoculated with 3% v/v inoculum and incubated at 25°C for 16 h. Cells were harvested by centrifuging at 10000 rpm for 20 min at 15°C. The pellet was washed twice with distilled water and finally suspended in tris buffer (50 mM, pH 8.6) to attain the desired cell density.

Cell Permeabilization Methods: 3% w/v (on wet basis) cell density and suspension volume of 6 mL was maintained constant throughout the cell lysis experiments. Cell suspension was incubated for one hour at R.T with 0.5% v/v organic solvents (hexane, toluene, ethanol, chloroform and butanol), 0.1% detergent (Tween 20 and 80, triton, SDS and cetyl triammonium bromide) and lysozyme (10000, 30000, 50000, 70000 and 90000 U mL^{-1}) was then centrifuged at 10000 rpm for 10 min at 15°C and the supernatant was analyzed for enzyme activity. Similarly, the cell suspension was sonicated at 40 W power (Branson Sonifier 450, USA) for 4 min at 50% duty cycle and checked for intracellular enzyme activity.

One Factor Methodology for Optimizing Sonication Protocol: Cell pellet was resuspended in tris buffer (50 mM), pH 8.6 to attain a cell density of (4, 6, 8, 10 and 12%). The samples were sonicated on Branson sonifier having a frequency of 20 kHz in an ice-water bath. Sample volume (4, 6, 8, 10 and 12 mL) of respective cell density were sonicated at duty cycle of 50%, acoustic power of (10, 20, 30 40 and 50 W) for a period of (2, 4, 6, 8 and 10 min) on discontinuous mode (1 min of sonication cycle followed by a gap of 30 s). The distance between the sonifier tip and the base of sample holder was maintained at a distance of 2 cm throughout the experiments. All experiments were carried out in triplicate.

Statistical Optimization of Sonication Using EVOP Methodology: 2^3 factorial design was employed to study the effect of three variables *viz*, cell volume, sonication time and acoustic power upon sonication. The design was divided into two blocks i.e. Block I (E_{10}-E_{14}) and Block II (E_{20}-E_{24}) and each variable was varied at two levels i.e. higher and lower with respect to the center values (Run E_{10} and E_{20}). The center values were fixed based upon the results of our initial preliminary experiments. Each run was performed for two cycles and the error limits and effects were calculated.

Enzyme Assay: L-asparaginase converts L-asparagine into asparatic acid and ammonia released in the reaction was quantified by nesslerization [16]. Standard curve was calibrated under identical experimental conditions using 10 mM ammonium sulfate. One international unit (IU) of L-asparaginase is that amount of enzyme which liberates 1 micromole of ammonia per minute at 37°C.

RESULTS

Effect of Various Cell Lysis Methods upon Release of L-asparaginase: Biomass of 3% (w/v) was suspended in 6 mL of 50 mM tris buffer was subjected to various chemical as well as physical methods of cell lysis. Among the various solvents and detergents attempted to permeablise the cell wall, 0.5% toluene and 0.1% triton X-100 gave L-asparaginase activity of 5.564 ± 0.991 IU $mL^{-1}$and 16.372 ± 0.028 IU mL^{-1} respectively. Lysozyme (90000 U mL^{-1}) yielded a very low enzyme activity of 1.441 ± 0.340 IU mL^{-1}. On the other hand, cell suspension when sonicated for 4 minutes, at 40 W power and 50% duty cycle gave maximum enzyme activity of 25.619 ± 0.226 IU mL^{-1} (Fig. 1). Cell lysis was also studied by trying out combination methods for cell lysis i.e. pretreating the bacterial biomass by detergent, lysozyme and their combination followed by sonication. Slight increase in enzyme activity i.e. 28 IU mL^{-1} was observed when cell mass was pretreated for 1 h with combination of 0.1 % v/v detergent and 90000 U mL^{-1} enzyme followed by 4 minutes of sonication cycle made the overall process time consuming and costly.

One Factor at a Time Approach for Optimizing Sonication Protocol

Effect of Cell Density: 2 to 12% (w/v) of cell density was adjusted on wet basis by suspending the pellet in tris buffer (50 mM, pH 8.6). Highest enzyme activity 51.037 ± 0.538 IU mL^{-1} was observed at 8% cell density (Fig. 2). Upto 8% of cell density, the enzyme activity increased linearly but a further increase in the cell density showed negative correlation with enzyme activity.

Effect of Suspension Volume: Effect of suspension volume upon enzyme release was studied in a range of 4–12 mL keeping acoustic power, cell density and time constant. 10 ml suspension volume yielded highest enzyme activity of 56.321 IU mL^{-1} beyond which decrease in enzyme activity was observed (Fig. 3).

Effect of Acoustic Power: Effect of acoustic power on enzyme activity is shown in Fig. 4. Acoustic power more than 40 W was found to be optimum with an enzyme activity 67.674 IU mL^{-1} Acoustic power beyond 40 W showed a drastic decrease with 60 fold reduction at 60 W acoustic power.

Effect of Sonication Time: Sample of 10% (w/v) cell density suspended in 10 mL of tris buffer (50 mM, pH 8.6) sonicated at an acoustic power of 40 W at 50% duty cycle was studied with respect to its sonication time. Enzyme activity of both the supernatant and pellet was measured during each time interval, the results are depicted in Fig. 5. At the beginning of sonication time the enzyme activity in the pellet was found to be 105.471 IU mL^{-1}. At 4 min of sonication almost 66% of enzyme was recovered. Increase in sonication time beyond 4 min resulted into drastic reduction in enzyme activity by 3 folds. Similar trend of protein release with respect to sonication time was observed. Till 4 minutes of sonication release of proteins increased after which their amount declined slowly.

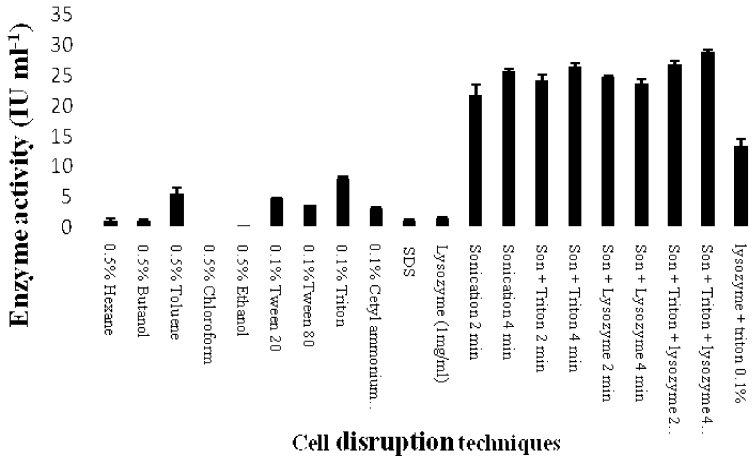

Fig. 1: Comparison of various cell lysis methods for release of L-asparaginase

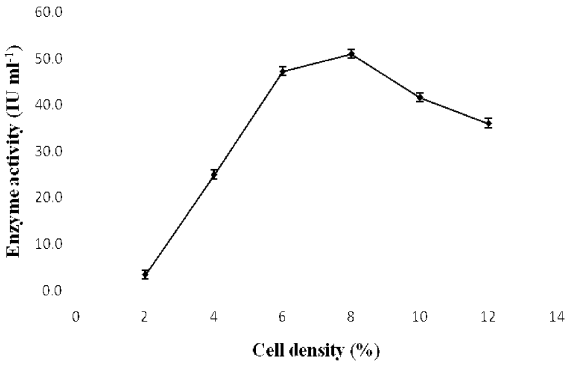

Fig. 2: Effect of cell density upon sonication

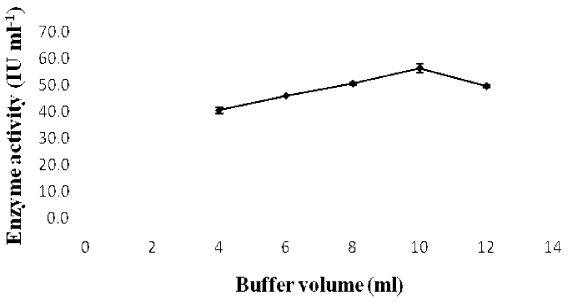

Fig. 3: Effect of buffer volume upon sonication

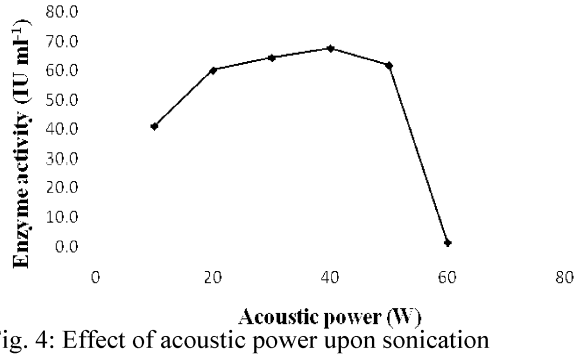

Fig. 4: Effect of acoustic power upon sonication

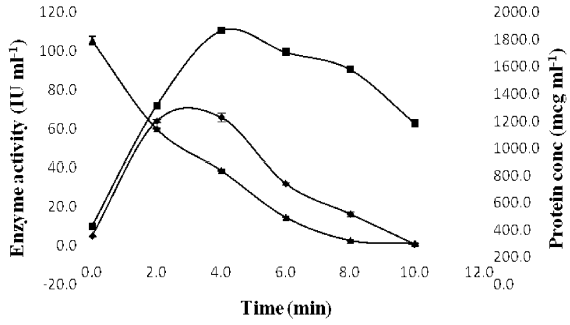

Fig. 5: Effect of sonication time upon enzyme and protein release

Statistical Optimization of Sonication Using EVOP Methodology: In our study, EVOP methodology was applied to optimize the level of three parameters *viz,* cell volume, sonication time and acoustic power for maximizing L- asparaginase release. $2^3 = 8$ experiments were conducted apart from the search levels (E_{10} and E_{20}). The design of experiments is as given in Table 1, whereas both the higher and lower values as compared to the search runs are assigned based on the knowledge of our previous study. The difference between enzyme activity of two cycles and average enzyme activity at respective trials of cycle I is given in Table 2 A and effects and error limits are given in Table 3. From the data analysis, it can be seen that the change in mean is negative and large as compared to the error limits in all the three cycles of EVOP. However, the individual and interaction effects of first two cycles are large as compared to the error limits of the effects that suggests that the true optimum conditions

Table 1: Experimental design of EVOP for three parameters

	Block I					Block II				
Experiment	E_{10}	E_{11}	E_{12}	E_{13}	E_{14}	E_{20}	E_{21}	E_{22}	E_{23}	E_{24}
Parameter										
P1	0	-	-	+	+	0	+	-	+	-
P2	0	-	+	-	+	0	+	-	-	+
P3	0	-	+	+	-	0	+	+	-	-
Response (Cycle I)	A10	A11	A12	A13	A14	A20	A21	A22	A23	A24
Response (Cycle II)	A10	A11	A12	A13	A14	A20	A21	A22	A23	A24
Difference	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10
Average Response	a_{10}	a_{11}	a_{12}	a_{13}	a_{14}	a_{20}	a_{21}	a_{22}	a_{23}	a_{24}

Table 2A: Experimental design for cycle I of EVOP

Exp. Condition	E_{10}	E_{11}	E_{12}	E_{13}	E_{14}	E_{20}	E_{21}	E_{22}	E_{23}	E_{24}
Time (min)	3	2	2	4	4	3	4	2	4	2
Cell Volume (ml)	10	8	12	8	12	10	12	8	8	12
Acoustic power (W)	40	30	50	50	30	40	50	50	30	30
EA IU/ml (I)	61.164	72.572	76.616	2.042	75.134	74.454	13.57	71.852	73.494	76656
EA IU/ml (II)	70.252	70.732	74.534	5.044	76.576	76.416	14.29	71.852	72.732	75936
Difference (I - II)	-9.088	1.84	2.082	-3.002	-1.442	-1.962	-0.72	0	0.762	0.72
Average EA (IU/ml)	65.708	71.652	75.575	3.543	75.855	75.435	13.93	71.852	73.113	76296

Table 2B: Experimental design for cycle II of EVOP

Exp condition	E_{10}	E_{11}	E_{12}	E_{13}	E_{14}	E_{20}	E_{21}	E_{22}	E_{23}	E_{24}
Time (min)	2	1	1	3	3	2	3	1	3	1
Cell volume (ml)	12	10	14	10	14	12	14	10	10	14
Acoustic power (W)	30	20	40	40	20	30	40	40	20	20
EA (I) IU/ml	72.064	74.824	59.276	65.59	59.01	76.308	56.922	62.38	77.726	64964
EA (II) IU/ml	72.504	73.982	59.996	63.148	65.97	75.268	57.242	59.298	77.286	58196
Difference (I - II)	-0.44	0.842	-0.72	2.442	-6.96	1.04	-0.32	3.082	0.44	6.768
Average EA IU/ml	72.284	74.403	59.636	64.369	62.49	75.788	57.082	60.839	77.506	61.58

Table 2C: Experimental design for cycle III of EVOP

Exp. Condition	E_{10}	E_{11}	E_{12}	E_{13}	E_{14}	E_{20}	E_{21}	E_{22}	E_{23}	E_{24}
Time (min)	3	2	2	4	4	3	4	2	4	2
Cell volume (ml)	10	8	12	8	12	10	12	8	8	12
Acoustic power (W)	20	10	30	30	10	20	30	30	10	10
EA (I) IU/ml	76.96	74.89	72.634	73.654	71.98	77.9	74.222	71.616	73.166	74648
EA (II) IU/ml	77.21	74	73.082	73.342	69.82	78.856	73.364	73.2	74.07	73.3
Difference (I - II)	-0.25	0.89	-0.448	0.312	2.16	-0.956	0.858	-1.584	-0.904	1.348
Average EA IU/ml	77.085	74.445	72.858	73.498	70.9	78.378	73.793	72.408	73.618	73974

Table 3: Summary of effects and error limit values of three EVOP cycles

Sr no	Parameters	Cycle I	Cycle II	Cycle III
1	Effect of Time	-32.234	1.247	-0.469
2	Effect of Cell volume	5.374	-9.082	-0.611
3	Effect of Acoustic power	-33.004	-8.513	-0.095
4	Effect of Time*Cell volume	1.191	-2.069	-0.600
5	Effect of Time* Acoustic power	-32.744	-0.759	1.481
6	Effect of Cell volume* Acoustic power	1.681	4.837	-0.983
7	Effect of Time*cell volume* Acoustic power	2.142	-0.973	0.523
8	Change in mean	-10.276	-7.438	-3.635
9	Sigma	2.084	2.474	0.831
10	Error limit of average	2.947	3.498	1.175
11	Error limit of effects	2.092	2.483	0.834
12	Error limit of change in mean	1.857	2.204	0.740

are yet to attain. In the third set of EVOP, optimum parameters of sonication were achieved as both the desired conditions i.e. change in mean (-3.635) is large and negative as compared to the error limit (0.740) and individual as well as interaction effects are small as compared to the error limit of the effects.

DISCUSSION

Success of any cell disruption method lies in maintaining a fine balance between product recovery and its biological activity. Complete lysis of microbial cell doesn't assure complete product recovery, simply because the harsh conditions employed for cell lysis (i.e. pressure or strong acid/alkali) can exert an undesirable effect upon the intracellular product. Thus selection of cell disruption method is a very critical step and is often influenced by the nature of microorganism, subcellular metabolite location and physicochemical properties of the metabolite. As can be seen from Table 4, several methods like homogenizer, ultrasonicator, enzyme and detergent have been attempted either solely or in combination for the release of L-asparaginase. In almost all the cases, the cells were subjected to higher power or pressure for longer period of time (6 min - 24 h) yielded specific activity in range of 0.03 to 6 IU mg^{-1}. The reason for such a low specific activity might be attributed to the process parameters employed during cell disruption. Current research work was thus undertaken with a rationale to increase L-asparaginase release from *Erwinia* cells by optimizing cell disruption conditions with a motive to make it time and energy efficient process.

Cell disruption methods can be broadly divided into permeablization and mechanical disruption methods. In permeablization methods, the cell wall structure is disorganized using detergent, solvents, osmotic shock, enzyme, etc. allowing either the leakage of product or passage of substrate within the cells. Gechkil *et al.*, [26] reported recovery of periplasmic L-asparaginase from *Enterobacter* and *Psuedomonas* by using salt and hexane combination, while Zhao and Yu [27] recovered periplasmic asparaginase by using triton and K_2HPO_4. We failed to get any significant release of enzyme from *E. caratovora* by chemical permeablization techniques mainly because of cytoplasmic location of enzyme [28]. Release of L-asparaginase by lysozyme treatment also failed to enhance its release mainly because of the gram negative nature of the bacterium. Anand *et al.* [29] have reported the enhancement in release of intracellular metabolites by using combination of chemical methods prior to mechanical methods for cell disruption. Pretreatment by triton, or lysozyme or triton (0.1%) + lysozyme (90000 U mL^{-1}) followed by sonication when attempted failed to show any significant positive enhancement in release of enzyme as compared to sonication alone. As can be seen from fig. 1, a slight increase in enzyme activity of 3.252 IU mL^{-1} was observed by combination of triton, lysozyme and sonication against sonication alone with no reduction in sonication time. Thus sonication was chosen for the further optimization of process parameters for enhanced recovery of L-asparaginase.

Table 4: Comparison of various cell disruption methods for L-asparaginase release from different microbial sources

Sr. No	Microorganism	Disruption conditions	Specific activity	Comment	Reference
1	*B. coagulans*	Branson sonifier multiple 3 min runs	0.033	Lower specific activity	[17]
2	*Citrobacter*	APV Homogenizer, 4 passes, 5 atmosphere pressure	0.24	Less operating pressure but lesser specific activity	[18]
3	*E. coli*	High pressure homogenizer, 42%w/w, 4 passes, 100 MPa	23.1	Higher biomass, and shear encorporated	[16]
4	*Proteus vulgaris*	Sonication, 5 kv, 20 min	0.98	Time consuming	[19]
5	*Proteus vulgaris*	1.05 g of lysozyme+50 ml toluene 24 h at 30' with gentle stirring.	1.1	Time consuming	[20]
6	*Serratia marscecens*	Branson sonifier, 12 min	3.4	Higher time requirement	[21]
7	*Serratia marscecens*	Sonication 6 min, 20 KV, max power	0.33	Maximum power with relatively lower sonication time	[22]
8	*Pseudomonas acidivoran*	Gaulin homogenizer, 5500 lb/inch^2 passes	0.039	Higher operating pressure	[23]
9	*Pseudomonas stutzeri*	Sonication, 400 W	1.1	Higher acoustic power	[24]
10	*Erwinia caratovora*	Extracting with anhydrous acetone for 30 min	Enzyme activity 5.8 IU/ml[a]	Time consuming	[25]
11	*Erwinia caratovora*	Sonication 9% cell density, 20 W power and time 3 minutes	18	Rapid and at lower shear	Present work

[a] specific activity not determined.

L-asparaginase being intracellular enzyme, we thought that increase in cell density would increase the enzyme yield. However, our experimental observations showed a different trend. Upto 8% of cell density, the enzyme activity increased linearly but a further increase in the cell density showed negative correlation with enzyme activity. This could perhaps be explained that with increase in cell density (beyond 8% in our case) the cell suspension becomes viscous enough to impede the passage of sonic waves into the suspension and thereby decreases the cavitation zone and hence the cell disruption [30]. Only a part of sonic energy is utilized in this case while the remaining part of the energy is simply wasted. Similar finding was observed by Freil and Kondo [31] in studying ultrasound induced cell lysis at higher cell concentration.

Suspension volume governs the cell disruption process by affecting the size of eddy and actual power dissipated/unit volume into the cell suspension. At lower suspension volume, there is a formation of large number of small eddies. These small eddies are effective in disrupting the cells. But, still a lower enzyme activity is seen at volumes 4, 6 and 8 mL because at these volumes, the power dissipated per unit volume of suspension is more. This results into heating of sample proteins thereby resulting into decreased release of enzyme. At 10 mL of suspension volume, the power dissipated/ unit volume is sufficient enough to bring about cell disruption without enzyme denaturation yielding maxima of 56.321 IU mL^{-1}. At cell volume higher than 10 mL, the power supplied gets diluted resulting into formation of larger eddies. These eddies either move around the cell or dislocate the cell rather than affecting into cell disruption and a decreased enzyme release is observed [32]. Thus at suspension volume of 10 mL we get gradual increase in enzyme release, but beyond that a fall in the release of enzyme is observed (Fig. 3).

Acoustic power governs the wave amplitude. Increase in power supplied increases the amplitude and results into formation of eddies smaller than bacterial cell. Formation of such smaller eddies result into higher disruptive forces with enhanced cell lysis and enzyme activity [32]. Higher heat generation at higher acoustic power denatures the protein and that's why at acoustic power of 50 W almost 3 fold decrease in enzyme activity is observed and the protein gets completely charred at the 60 W power with the residual enzyme activity of 1.241 ± 0.127 IU mL^{-1}.

Cell lysis is a function of time. With increase in sonication time increased number of cells gets disrupted. This was assessed by measuring the release of nucleic acid by taking the OD of cell suspension at 260 nm (data not shown). With more number of cells being disrupted more release of protein and enzyme as expected is observed till 4 min of sonication beyond which magnitudes of both enzyme and protein decline probably because of the sample denaturation due to its overheating.

EVOP is potential statistical tools that have been successfully adapted on laboratory scale like enhancing the production of protease [33], gallic acid [34] and peeling of potatoes [35]. For current investigation experiments were designed by setting the initial values of three parameters at center run (E_{10} and E_{20}) on the basis of our previous preliminary studies. Experimental design is given in Table 2A and its results in table 3. The change in mean effect (-10.275) is negative and large compared to the error limit (1.856) which suggests that the system is approaching towards optima but yet has not attained real optimum conditions [34]. Effect of time and power were found to be large and negative when compared to error limit of the effects implicating that their values need to be reduced in the second cycle while that effect of cell volume was large and positive implicating a higher value in the consecutive cycle. Experimental levels of the highest run of cycle I (E_{24}) were set as center value of cycle II. Design of cycle II of EVOP and its results are enumerated in Table 2B and 3 respectively. As per the decision making rule the change in mean effect (-7.438) is negative and large as compared with the error limit (2.204). Effect of cell volume and acoustic power are negative and large than the error limit of effects implicating a need of another cycle of EVOP to reach the optima however optima of sonication time was attained as its effect was small compared to the error limit of effects.

Design of experiments of cycle III are as given in table 2C wherein run E_{23} of cycle II was set at center value of cycle III. The values of error limits and effects are tabulated in Table 3. Run E_{20} of Cycle III gave maximum enzyme activity of 78.378 IU mL^{-1}. Both the parameters i.e. change in mean effect is large and negative as compared to the error limit and individual and interaction effects of cell volume and acoustic power were small as compared to the error limits indicating that the true optima has been reached. Thus the magnitude of sonication time, cell volume and acoustic power were decreased from 3 to 2 min, 12 mL to 10 mL and 40 W to 20 W respectively leading to effective cell disruption at decreased value of power and time making the process economical in terms of both cost and energy.

It can be concluded that the EVOP based sonication protocol led to almost 80% recovery of enzyme having a specific activity of 18 IU mg^{-1} that is highest than most of the reports of L-asparaginase release at much lesser magnitude of power and time.

ACKNOWLEDGEMENT

The authors would like to thank the University Grant Commission (UGC), New Delhi for financially supporting this research.

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