Localization and Disruption Kinetics of L-Asparaginase from *E. caratovora* Cells by High Power Ultrasound

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High power ultrasound technique was adopted in our study to maximize the release of L-asparaginase from cells of *E. caratovora*. Duty cycle and acoustic power (total power delivered to sample) were found to be the key elements influencing the release of enzyme during this process. Maximum enzyme activity 82 IU mL^{-1} was obtained when cells of *Erwinia* were sonicated at 50 % duty cycle and 50 W acoustic power for 4 minutes. Subcellular enzyme location was estimated by calculating the rate of enzyme release to protein release (location factor) at varying magnitudes of duty cycle (10 %, 30 % and 50 %) and acoustic power (10 W, 30 W and 50 W) during the sonication cycle of 4 minutes. Magnitude of location factor obtained in all the varied conditions was found to be less than unity revealing the cytoplasmic location of enzyme. Furthermore, disruption kinetics was calculated by studying the effect of total power and duty cycle upon percent cell survival. Increased and efficient disruption was achieved at higher values of both duty cycle and acoustic power indicating a direct correlation between degree of disruption and these two independent variables. Magnitude of location factor and disruption constant (β) at 50 % duty cycle and 50 W acoustic power were found to be 0.92 and 0.120 min^{-1} respectively.

Key words:
L-Asparaginase, sonication, location factor, disruption kinetics

Introduction

L-Asparaginase (E.C 3.5.1.1) is a drug of choice in the treatment of childhood acute lymphoblastic leukemia and lymphoma. Leukemic blast cells are devoid of asparagine synthetase activity and thereby depend exclusively upon exogenous L-asparagine supply for their cellular requirements. L-Asparaginase cuts off the supply of this non-essential amino acid, L-asparagine, by hydrolyzing it to ammonia and aspartic acid, leading to cell apoptosis.[1] Apart from its anticancer activity, recently asparaginase is being studied widely in reduction of acrylamide level in fried food products[2] and as an enzyme electrode to detect the asparagine concentration.[3] Microbial as well as plant sources have been screened and reported for their L-asparaginase activity. However high titres of enzyme to date are being produced from *E. coli* and *Erwinia* species.[4] With the exception of a few microbes like *S. cerevisiae,*[5] *P. aeruginosa*[6] and recombinant *E. coli,*[7] this enzyme is produced almost intracellularly. Furthermore, this intracellular enzyme location differs from species to species. *Serratia, Wautersia and Erwinia* produce cytoplasmic L-asparaginase[8] while in *Psuedomonas* the enzyme is found in the periplasmic region in association with glutaminase.[9] *E. coli* is known to produce two distinct asparaginases *viz*, cytoplasmic asparaginase I (devoid of anticancer activity) and periplasmic asparaginase II (exhibiting anticancer activity).[10]

Cellular metabolite location influences the cell disruption method, and knowledge of subcellular metabolite location becomes a prerequisite in setting up of cell disruption protocol to maximize the product release with minimum or no degradation.

Various methods are reported in literature for determining the cellular metabolite location i.e. cell fractionation,[11] disruption kinetics,[12] immuno-electron microscopy,[13] immunocytochemical labelling, cytolabelling coupled with electron or fluorescence microscopy[14] and protein algorithms.[15,16] There are simpler techniques like separating the biomass, dissolving it in buffer and estimating the cell wall bound activity and periplasmic activity[17] or performing cell fractionation with lysozyme and hypotonic solution followed by ultracentrifugation of the cell components.[18] However, these oldest known methods for determination of subcellular metabolite location require the use of sophisticated equipment like ultracentrifuge and are time con-

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suming. Recently developed methods like cytochemical and immunocytolabelling that provide exact cellular and even tissue-based location of the protein or antigen are most commonly employed in clinical studies.[19] Location of β-glucosidase in white rot fungus and glucose oxidase in *Aspergillus* has been reported by these methods.[20,21] Although these techniques are highly specialized and protein-specific, they are very expensive to be applied for routine laboratory work. Deciphering subcellular protein location (SPL) by computational methods based on analyzing either nucleotide or amino acid homology come under the realms of bioinformatics. Only 14 % of existing proteins are submitted to the database making this emerging method difficult to be applied for SPL for all the existing proteins.[16] Studying protein and enzyme release rate kinetics is yet another approach to determine the subcellular metabolite location. Ratio of release rate constants of enzyme to protein is used for calculating location factor.[22] This method of enzyme localization is much faster, inexpensive and requires no expertise or sophisticated equipment as compared to the other above stated methods. Determination of subcellular location by studying release rate kinetics of L-asparaginase by high power ultrasound was thus employed for our current work.

High power ultrasound is the most commonly preferred laboratory method for cell disruption offering few advantages like lower operating cost and easy equipment clean up. However, product denaturation by heat generation narrows down its applicability especially for the heat-sensitive products.[23] Irrespective of these disadvantages, high power sonication yet forms an integral unit operation in purification of many intracellular metabolites especially in recovery of periplasmic inclusion bodies from *E. coli* and yeast.[24–26] Cell disruption by sonication can be explained by the phenomenon of cavitation, wherein continuous growth and collapse of microbubbles impart shear stress upon the microbial cells resulting in cell lysis and protein release. Statistical theory of reliability proposed by Doulah[27] explains the nature of cell disintegration by sonication. Apar and Ozbek[28] studied the protein release kinetics of *S. cerevisiae* by varying power, duty cycle, medium pH and cell concentration. Similarly, Kapucu *et al.*[29] calculated the kinetic constant β for cell disruption of *A. peroxydans* under varying conditions of acoustic power and media pH. James *et al.* [30] found that the rate of protein release of yeast cells is independent of cell concentration but depends linearly on the acoustic power (67–187 W).

In the present study, a high power ultrasound protocol was developed for efficient release of L-asparaginase and total soluble proteins from *E. caratovora* cells. Acoustic power and duty cycle were chosen as key elements to study the release rate kinetics of both enzyme and total protein, and location factor was calculated from the values of these rate constants. Disruption kinetics was also studied simultaneously by applying the Doulah hypothesis of cell disruption, and β value suggesting the pattern of failure during sonication was calculated.

Theory

Concept of location factor

Enzyme and protein release during cell disruption by any cell disruption method essentially follows the first order kinetics.

$$\ln\left(1-\frac{A_t}{A_m}\right) = -k_e t \tag{1}$$

$$\ln\left(1-\frac{P_t}{P_m}\right) = -k_p t \tag{2}$$

where, t = time in minutes

A_m and A_t = maximum amount of enzyme released and enzyme released at time t

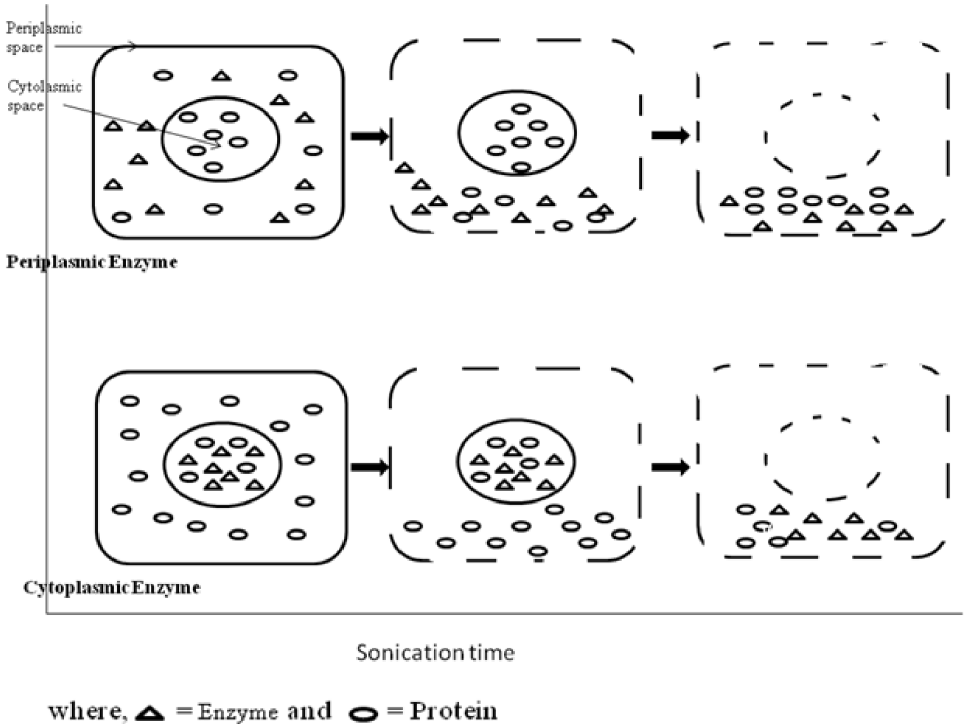
P_m and P_t = maximum amount of protein released and protein released at time t

k_e and k_p = kinetic constants for enzyme and protein release respectively.

The location factor (LF) is ratio of kinetic constant of enzyme to protein release.[22]

$$LF = \frac{k_e}{k_p} \tag{3}$$

Enzymes either have periplasmic or cytoplasmic location and proteins are present in all cell compartments. For example, in the case of a periplasmic location, the enzyme is located between the cell wall and the cell membrane, so when cell lysis occurs the enzyme release rate is much faster than that of the total cellular proteins resulting in location factor greater than one. On the other hand, cytoplasmic enzyme is located in the interior of the cell i.e. beneath the cell membrane, so when lysis takes place, the proteins present in the cell wall and periplasmic region are released first and at a faster rate than the enzyme resulting in location factor value less than one (Fig. 1).



F i g . 1 – *Diagrammatic representation of location factor for periplasmic and cytoplasmic enzymes*

Kinetics of disruption

Cell disruption by sonication is affected by the cavitation phenomenon. Cavitation is of two types, transient and noninertial cavitation. In noninertial cavitation, small bubbles of the liquid simply oscillate under the influence of acoustic field without affecting the bubble collapse and are thus insufficient for affecting microbial cell lysis. On the other hand, cavitation induced under high power ultrasound generates transient cavitation resulting in cell lysis. Continuous breaking and building up of new eddies occurs during the process. During cell disruption, the microbial cells are surrounded by eddies of different sizes and intensities. Eddies larger than the cell size simply dislocate the cell, while eddies smaller than the cell impart pressure on the cells. When this pressure exceeds the strength of cell wall, cell disintegration occurs. Doulah explained this transient cavitation process by Kolmogoroff's theory.[31] Cell suspension when irradiated by sonic waves, the ultrasonic energy is converted to mechanical energy. This energy is dissipated to the surrounding fluid leading to the formation of eddies. Cumulative fraction of cells disrupted at a specified sonication condition is given as follows

$$F_N = 1 - \exp\left[-\left(\frac{t}{\alpha}\right)^{\beta}\right] \qquad (4)$$

Where,

F_N = cumulative fraction of cells disrupted

t = time

α and β are the kinetic constants. By linearising eq. 4, we obtain the values of kinetic constants.

$$\ln\left[\ln\left(\frac{1}{1-F_N}\right)\right] = \beta \ln t - \beta \ln \alpha \qquad (5)$$

β determines the pattern of failure, while α relates the characteristic lifetime of the equipment. $\beta < 1$ indicates early life failure; $\beta > 1$ implies failure results from wear effects and $\beta = 1$ indicates a constant failure.[29]

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, copper sulfate, sodium potassium tartarate, sodium hydroxide, sodium carbonate and tris buffer were purchased from Himedia Pvt. Ltd. Folin's reagent was purchased from SD Fine Ltd. 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.

Cultivation of microorganism

Culture preserved on slant was used to inoculate the seed media (Trypticase soy broth) which was incubated at 30 °C for 24 h on a rotary shaker at 180 rpm. 3 % of the seed adjusted to OD (660 nm) at 1.3 was used to inoculate the 50 mL production medium contained in 250 mL Erlenmeyer flasks and incubated at 25 °C for 16 h at 180 rpm. Production media devised by us had the following composition: 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. Batch was harvested by separating the biomass on remi centrifuge at 8000 rpm for 15 min at 15 °C. Biomass was washed with distilled water and finally suspended in Tris buffer (pH 8.6, 50 mmol L^{-1}).

Cell disruption by sonication

Disruption studies were carried out on Branson Sonifier 450A, USA, with a "1/2" diameter tapped biohorn that delivers ultrasonic sound at a constant frequency of 20 kHz in an ice water bath. Cell disruption was carried out in glass container immersed in a beaker containing ice water mixture. The cell suspension with cell density of 8 % w/v on wet basis and suspension volume of 10 mL was sonicated at duty cycle (10 %, 30 % and 50 %) and acoustic power (10 W, 30 W and 50 W) for 4 minutes. Samples were withdrawn at predetermined time interval, centrifuged on remi centrifuge at 8000 rpm, 15 °C for 15 min, and supernatant thus obtained was used for enzyme and protein analysis. All the experiments were performed in triplicate.

Analytical methods

Enzyme assay

L-Asparaginase catalyzes the hydrolysis of L-asparagine to aspartic acid and ammonia. Ammonia liberated in this reaction was determined by nesslerisation.[32] Reaction mixture containing 1 mL Tris buffer (pH 8.6, 50 mmol L^{-1}), 0.9 mL deionised water and 0.1 mL of (189 mmol L^{-1}) L-asparagine was preheated at 37 °C for 10 min. 0.1 mL of the supernatant was added to initiate the reaction and incubated further for 30 min at 37 °C. 0.1 mL of 15 % TCA was used to stop the reaction. Reaction mixture was centrifuged at 10000 rpm for 10 min at 15 °C. 0.2 mL of the supernatant was added to 4.3 mL of deionised water and color was developed by adding 0.5 mL of nessler's reagent. Yellow color developed was read at 436 nm. Blank was performed replacing enzyme and substrate with deionised water. Standard was performed by using 10 micromole per mL of ammonium sulfate. One international unit of enzyme is defined as the amount of ammonia liberated per minute per mL of enzyme.

Protein assay

Proteins present in the sample were quantified by Folin-Lowry method. [33] Bovine serum albumin was used as a standard.

Total viable count

Fraction of cell survival was performed by usual microbial pour plate technique. The plates were incubated at 37 °C for 24 h. The colonies so formed were counted with the naked eye.

Results and discussion

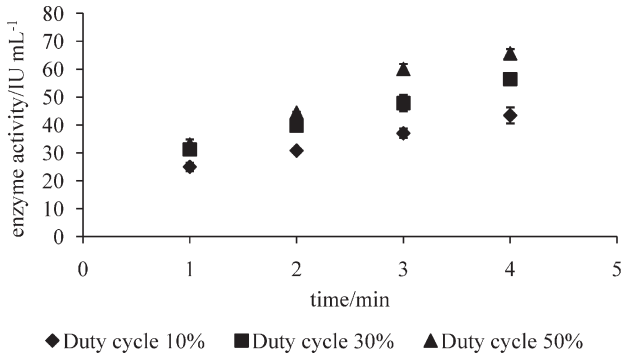
High power ultrasound is a widely used laboratory method for release of intracellular products. Cell lysis by sonication is dependant on a number of variables like cell density, cell volume, pH of sonication buffer, power, duty cycle and peak intensity.[23,34] In our earlier studies, we devised an efficient sonication protocol for maximizing L-asparaginase release from *Erwinia* cells by optimizing cell density, suspension volume, acoustic power, duty cycle and sonication time. Significant variation in enzyme activity from 24 IU mL^{-1} to 65 IU mL^{-1} and 25 IU mL^{-1} to 82 IU mL^{-1} was observed by varying the duty cycle and acoustic power from 10 % to 50 %, and 10 to 50 W respectively. Also, the noticeable increase in enzyme activity i.e. 2.7 fold and 3.28 fold, was attained by optimizing duty cycle and acoustic power respectively. Thus, based on these preliminary experiments, we found that acoustic power and duty cycle exhibited a profound influence on the release of L-asparaginase from *E. caratovora* cells by sonication, and thus decided to study both the protein release kinetics and disruption kinetics by varying the magnitudes of duty cycle and acoustic power. Acoustic power is nothing but the total power transmitted to the sample that governs the size and number of eddies formed during sonication, while duty cycle controls the extent of pulsating period during sonication. During pulsating mode, the ultrasonic waves are transmitted to a solution at a rate of one pulse/sec-

ond. For example, 10 % duty cycle refers to the active interval of transmitting the ultrasonic waves being 0.1 s. Increase in magnitude of duty cycle hence suggests that the active interval of transmitting sonic waves increases thereby reinforcing the cavitation phenomenon.

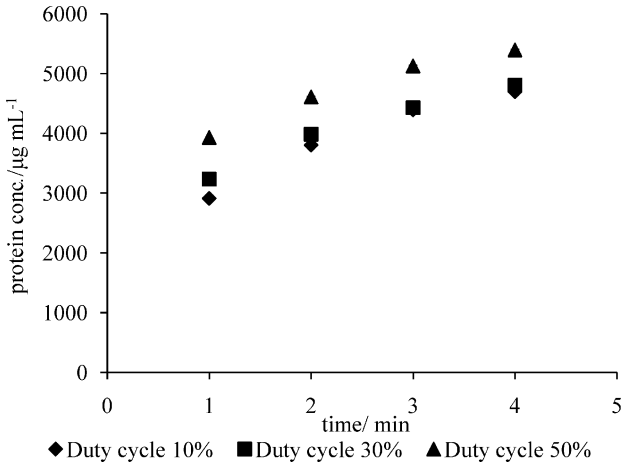
Eddies larger than the bacterial cell are insignificant for cell lysis as they simply move and dislocate the cell rather than disrupt it. A correlation exists between the acoustic power and size, and number of eddies formed. Increase in acoustic power decreases the eddy size and thus a large number of eddies is exposed under the sonic waves with a net increase in disruption of bacterial cells and subsequent release of its intracellular contents. In this paper, we try to analyze the effect of duty cycle and acoustic power on protein release and disruption kinetics of *E. caratovora* cells.

Release rate kinetics and location factor

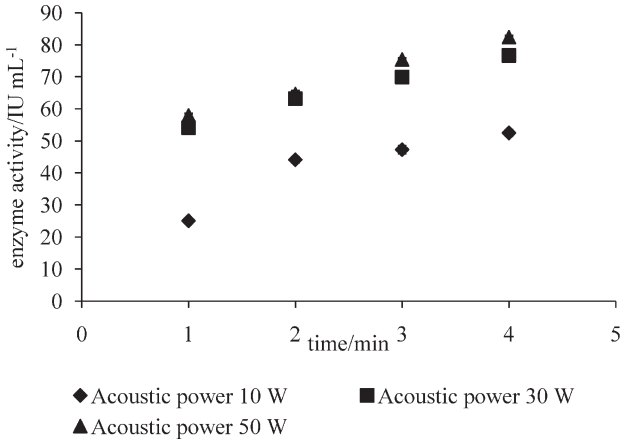
Release of L-asparaginase and total soluble proteins were monitored at varied values of duty cycle (10 %, 30 % and 50 %) and acoustic power (10 W, 30 W and 50 W) by keeping 8 % cell density, 10 mL suspension volume and sonication time not exceeding beyond 4 minutes.[35] Protein release during any cell lysis method obeys the first order kinetics. Increase in release of both enzyme and protein was observed at increasing values of acoustic power and duty cycle implying increased cell lysis and thereby increased release of intracellular contents (Fig. 2(a) and 2(b) and 3(a) and 3(b)). The reason behind this might be due to the formation of a large number of small eddies at higher acoustic power and increased pulsating time at higher values of duty cycle.[1,28] The enzyme and protein activity was found to increase 1.5 fold and 1.6 fold respectively with increase in duty cycle from 10 to 50 % and acoustic power from 10 to 50 W. Maximum release of L-asparaginase 82 IU mL^{-1} was achieved at 50 W acoustic power and 50 % duty cycle with a protein release of 7.08 mg mL^{-1}. Release rate constants for enzyme and protein were calculated in accordance with equations 2 & 3 and by plotting the graph of $\ln[1-(A_t/A_m)]$ and $\ln[1-(P_t/P_m)]$ versus t were enumerated in Table 1. At 50 % duty cycle and 50 W acoustic power k_e and k_p were found to be 0.833 and 0.937 respectively with a location factor of 0.94. Value of location factor less than unity



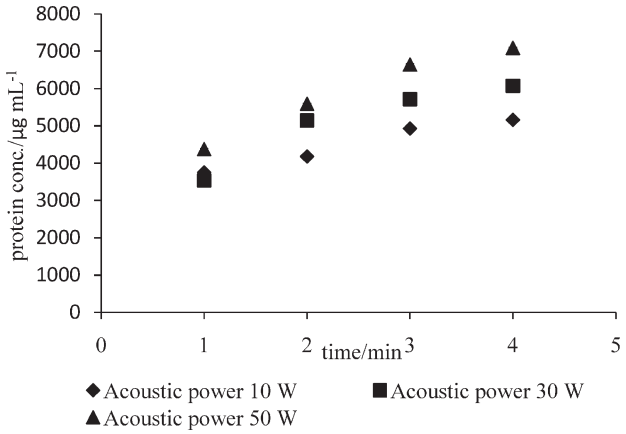
F i g . 2 a – *Release of L-asparaginase from E. caratovora at three different values of duty cycle*



F i g . 2 b – *Release of protein from E. caratovora at three different values of duty cycle*



F i g . 3 a – *Release of L-asparaginase from E. caratovora at three different values of acoustic power*



F i g . 3 b – *Release of protein from E. caratovora at three different values of acoustic power*

Table 1 – *Release rate and location factor of L-asparaginase by varying duty cycle and acoustic power by sonication*

Parameter		Release rate of enzyme, K_e/min^{-1}	Release rate of protein, K_p/min^{-1}	Location factor
duty cycle	10 %	0.648	0.893	0.725
	30 %	0.635	0.878	0.723
	50 %	0.738	1.011	0.729
acoustic power	10 W	0.803	0.994	0.807
	30 W	0.857	0.883	0.914
	50 W	0.835	0.937	0.945

reveals cytoplasmic location of L-asparaginase in *E. caratovora* cells. Table 2 enumerates the values of location factor for different intracellular enzymes explaining the relation between the ratio of k_e/k_p with respect to its cellular location. Location factor of L-asparaginase was found to vary from 0.7 to 0.9 at varied conditions of duty cycle and power. This difference might be attributed to the difference in the release rate pattern of enzyme and protein at these six different experimental conditions due to difference in the shear stress experienced by the microbial cells. Using the similar theory, location factor of galactosidase, penicillin acylase, invertase and glucose oxidase are documented in the literature.[36–38,21] Location factor of enzymes can vary depending upon the release rate of proteins by different cell lysis methods although virtually the original location of enzyme remains unchanged.[22] Farkade and Pandit[39] however have demonstrated the translocation of galactosidase from cytoplasmic to periplasmic space during thermal treatment. Thus maximum release of L-asparaginase was achieved at 50 % duty cycle at 50 W power after 4 minutes of sonication.

Disruption kinetics during high power ultrasound

Duty cycle and acoustic power were varied and total viable count was performed by pour plate technique. The viable count decreased with increasing values of both the variables confirming the increased amount of cell lysis. Based on our initial experimentation, range of duty cycle was varied from 10 %, 30 % and 50 % and that of acoustic power of 10 W, 30 W and 50 W, and percent survival fraction was calculated for 4 minutes of sonication cycle as beyond 4 minutes, a remarkable decrease in enzyme activity was observed.

The survival percent goes on decreasing with increasing duty cycle suggesting the disruption efficiency at increased duty cycle values (Fig. 4). As the duty cycle increases from 10 % to 50 %, the survival fraction decreases from 0.3 to 0.1 % after 4 minutes of sonication. The β value was calculated using eq. 3 and by plotting the graph of $\ln[\ln(1/(1-F_N)]$ vs ln time shown in Fig. 5. β values go on decreasing with increasing values of duty cycle indicating effective disruption. Least β value of

Table 2 – *Comparison among intracellular enzymes and their location factor*

Enzyme and its cellular location	Disruption method	K_e	K_p	K_e/K_p	Ref.
	Sonication (Dakshin horn; 1% cells)				
Alcohol dehydrogenase; cytoplasmic	60 min	0.0412	0.0708	0.584	17
	120 min	0.0235	0.0411	0.572	
	Sonication (Dakshin horn,15 % amplitude and 1% cells)				
invertase; cell wall bound	15 min	0.1194	0.1042	1.146	17
	20 min	0.1160	0.1003	1.156	
	Sonication at				
	40 °C 4 min			0.795	
β-galactosidase cytoplasmic	40 °C 6 min	–	–	0.941	30
	45 °C 3 min			0.854	
	Sonication at				
	40 °C 8min			1.091	
β-galactosidase periplasmic	40 °C 10 min	–	–	1.27	30
	45 °C 5 min			1.5	
	45 °C 8 min			1.224	

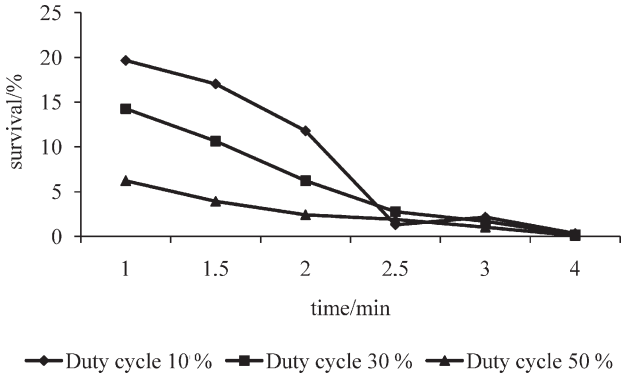


Fig. 4 – *Survival percent by sonication at different values of duty cycle*

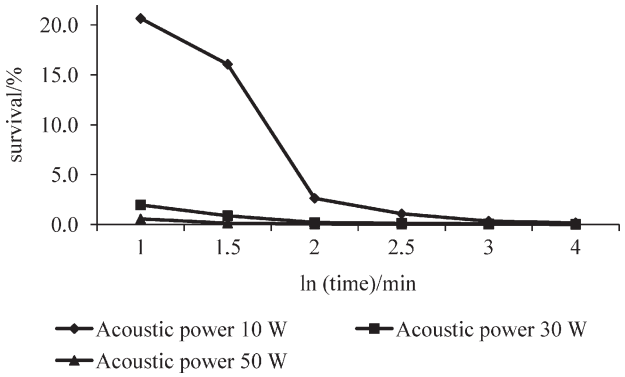


Fig. 6 – *Survival percent by sonication at different values of acoustic power*

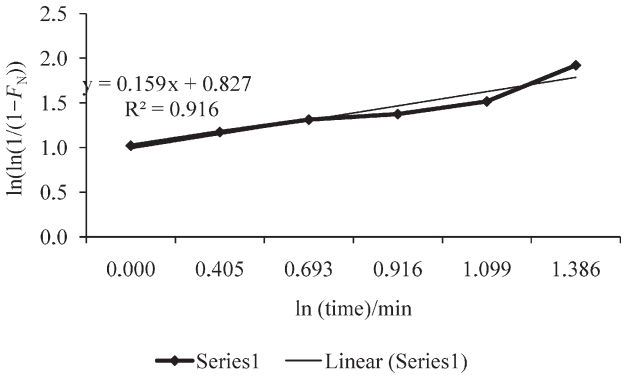


Fig. 5 – *Plot of ln(ln(1/(1−F_N)) vs ln time at 50 % duty cycle and acoustic power of 30 W*

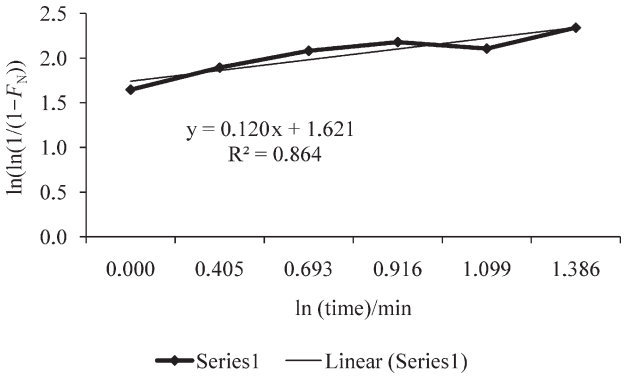


Fig. 7 – *Plot of ln(ln(1/(1−F_N)) vs ln time at 50 W acoustic power and 50 % duty cycle*

0.159 was obtained at 50 % duty cycle, which signifies early life failure. Apar and Ozbek reported duty cycle as crucial parameter during sonication from energy economics point of view while studying the protein release from baker's yeast.[28]

Effect of acoustic power on survival percent is depicted in Fig. 6. With increase in acoustic power from 10 W to 50 W, survival percent was found to decrease from 0.76 to 0.001 indicating almost complete bacterial disruption within 4 minutes of sonication. Values of β were calculated by plotting the graph of ln [ln(1/(1−F_N)] versus ln time (Fig. 7). Similar trend between β value and acoustic power was observed as like with duty cycle. Decreasing values of β with increasing power indicate a gradual decrease in percent survival or effective cell disruption process (Table 3). Doulah had studied the percent survival at 3.3 g of biomass suspended in 20 mL buffer, reported β value of 1.44 indicating wear out damage of cell membrane during sonication[27] while Kapucu *et al.*[29] reported the β value of 0.76 at 100 W power and pH of 3 during 30 minutes of sonication indicating early life failure of bacterial cells. Our results are in agreement with that of Kapucu *et al.*, where β value of 0.120 was attained when power and duty cycle were maintained at 50 W and 50 %, indicating efficient cell disruption and early life failure of *Erwinia* cells during sonication.

Table 3 – *β values during varied conditions of duty cycle and acoustic power*

Parameter		β value
duty cycle	10 %	0.265
	30 %	0.223
	50 %	0.159
acoustic power	10 W	0.304
	30 W	0.149
	50 W	0.120

Correlation between cell disruption and protein release

Increased cell disruption increases the amount of enzyme and protein released. To confirm this hypothesis, both the release rate kinetics (first order kinetic expression) and disruption kinetics (Kolmogoroff's theory) were compared at 10 %, 30 % and 50 % duty cycle and 30 W power for a period of

Table 4 – *Correlation between disrupted cells and enzyme and protein release at different values of duty cycle and 30 W acoustic power*

Sr no	F_N	R_e/IU mL^{-1}	R_p/μg mL^{-1}	β value
Disruption at 10 % duty cycle and acoustic power of 30 W				
1	0.803	24.978	291.071	0.265
2	0.881	30.822	380.357	
3	0.978	37.027	439.286	
4	0.991	43.432	469.643	
Disruption at 30 % duty cycle and acoustic power of 30 W				
1	0.857	31.223	323.214	0.223
2	0.938	39.829	398.214	
3	0.982	47.835	442.857	
4	0.997	56.441	480.357	
Disruption at 50 % duty cycle and acoustic power of 30 W				
1	0.937	33.024	392.857	0.159
2	0.985	44.432	460.714	
3	0.989	60.044	512.500	
4	0.998	65.648	539.286	

4 min and shown in Table 4. It can be clearly stated that with the increase in duty cycle, the amount of cells disrupted increases with a decrease in β value (from 0.265 to 0.159), and an increase in the amount of enzyme and protein released. Hence, we can conclude that in the given range of duty cycle studied, efficient cell lysis was achieved at 50 % duty cycle during 4 minutes of sonication yielding enzyme and protein release of 65 IU mL^{-1} and 5.3 mg mL^{-1} with release rate constant of 0.738 min^{-1} (R^2 0.891) and 1.011 min^{-1} (R^2 0.935) respectively with a β value of 0.159. A similar trend was seen when power was varied by keeping duty cycle at a constant value of 50 % validating our own results. Kapucu *et al.* also reported the similar relationship between cell count and protein release of *A. peroxydans* cells under optimized conditions of acoustic power and pH.[29]

Conclusions

The present study observed that the rate of enzyme and protein release increased as the % duty cycle and acoustic power were increased. Thus, maximum enzyme recovery of 82 IU mL^{-1} and total protein of 7.08 mg mL^{-1} was achieved by high power ultrasound for 4 min at an acoustic power of 50 W with a duty cycle of 50 %. The corresponding k_e (0.835) and k_p (0.937) values were obtained giving a location factor of 0.94 indicating cytoplasmic location of L-asparaginase in *E. caratovora* cells. Disruption kinetics was studied at varying duty cycle and acoustic power. Efficiency of cell disruption increased with decreasing β value, with lowest value of 0.120 exhibiting maximum pressure on the bacterial cell wall thereby causing highest cell disruption.

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List of symbols

t – time, min
A_m – maximum enzyme released, IU mL^{-1}
A_t – amount of enzyme released at time t, IU mL^{-1}
P_m – maximum protein released, μg mL^{-1}
P_t – amount of protein released at time t, μg mL^{-1}
K_e – release rate constant for enzyme, min^{-1}
K_p – release rate constant for protein, min^{-1}
LF – location factor
F_N – cumulative fraction of cells disrupted, %
β – kinetic constant, min^{-1}

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