# Significance of Location of Enzymes on Their Release During Microbial Cell Disruption

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**Abstract**: The release kinetics of the enzyme invertase and alcohol dehydrogenase from yeast and penicillin acylase from *E. coli* during disruption using various techniques has been investigated. The disruption techniques used were sonication, high-pressure homogenization, and hydrodynamic cavitation. The first-order-release kinetics was applied for the determination of release rate of these enzymes and total soluble proteins. Location factor (LF) values were calculated using these release rates. The location of the enzymes as given by the values of location factor coincided well with those reported in the literature. Varying values of location factor for the same enzyme by different disruption techniques gave some indications about the selectivity of release of a target enzyme by different disruption techniques. Varying values of location factor for the same enzyme with the use of a particular equipment or disruption technique at different conditions reveals the degree to which the cell is disrupted. Few plausible applications of this location factor concept have been predicted and these speculations have been examined. This location factor concept has been used for monitoring the heat-induced translocation of ADH and location of penicillin acylase during the growth period of *E. coli* cells. © 2001 John Wiley & Sons, Inc. *Biotechnol Bioeng* **75**: 607–614, 2001.

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## INTRODUCTION

A large proportion of the potentially useful microbial products are retained within the cells. The microbial cell can be genetically manipulated to make the product extracellular or to make the cell leaky with respect to the target microbial product. The genetic manipulation of microbial cells to make them leaky is limited in scope (Chisti and Moo-Young, 1986). Most recombinant gene products have been produced as intracellular products (Sauer et al., 1988). The secretion of recombinant protein to the periplasmic space has numerous advantages over its expression in the cytoplasm (French et al., 1996). Hence, disruption of the cell becomes inevitable (Feliu et al., 1988). Of the methods available, physical methods are often favored due to the economic and operational limitations of the chemical and enzymatic methods. The cell-disruption process is dependent on cell properties such as the physical strength of the cell wall of the microorganism (Sauer et al., 1988) and on the intracellular location of enzyme in the microbial cell (Follows et al., 1971: Hettwer and Wang, 1989: Kuboi et al., 1995: Kula and Schutte, 1987: Melendres et al., 1993; Schutte et al., 1983). Thus, location of the enzyme can influence its release rate during the process of disruption. The release kinetics of a particular enzyme and total soluble proteins has been used by Kuboi et al., (1995) for identifying the location of an enzyme. This concept of identification of location of enzyme based on the release kinetics was given the term as release selectivity. Later, it was renamed as location factor by Umakoshi et al. (l998). This location factor value depends on the release rate of the enzyme and total soluble proteins. The location factor values for the same enzyme could probably vary with different equipment or even within the use of the same equipment at different disruption (operating) conditions because the cells get disrupted to a different degree and might result in the variation of the release rate of enzyme and protein.

The objective of the present work was to disrupt the microbial cells using different disruption equipment and determine the location factor values. The enzymes whose location are well-known were selected for the study. In the present study, yeast was disrupted for the release of Alcohol dehydrogenase (ADH) and invertase and *E. coli* cells were disrupted for the release of penicillin acylase by various disruption equipments. The disruption equipments included a sonicator, high-pressure homogenizer, and hydrodynamic cavitation set-up. The release kinetics of these enzymes and total soluble proteins have been studied and the location factor values have been calculated.

## MATERIALS AND METHODS

### Microorganism

Bakers yeast (Tower brand) was obtained from a local supplier as a source of protein and enzymes (invertase and alcohol dehydrogenase).

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*Escherichia coli* ATCC 10799 obtained from National Chemical Laboratories (NCL) Pune was used as a source of penicillin acylase enzyme. Agar slants in test tubes (nutrient agar) were inoculated and incubated for 24 h at a temperature of 28–30°C. Culture was stored on nutrient agar slants at 5°C for not more than 1 week. A 24-h-old culture on nutrient agar slant was washed with 10 mL of saline and used as inoculum. 0.5 mL of inoculum (i.e., 1%) with an OD of 0.3 to 0.4 at 660 nm was added into the medium. Medium contained nutrient broth (dehydrated medium reconstituted) 1.3%, phenyl acetic acid 0.02%, and yeast extract 2.5%. Fifty milliliters of medium in 250- mL conical flasks were incubated in a rotary shaker (175 rpm) at room temperature (28–32°C) for 18 h. Eighteen-hour incubation was used in all experiments except for the study of location of enzyme during various growth stages for which cells were grown up to 10, 12, 14, and 16 h. Except for the incubation period, other conditions were similar for all the experiments. The pH of the medium was adjusted to 7.0 using IN sodium hydroxide as maximum biomass is formed at this pH. Cells cultivated were centrifuged for separation from medium using Remi centrifuge at 8000 rpm for 17 min at room temperature. Then, they were resuspended in phosphate buffer of pH 7.4 to study its disruption in various equipments.

## Cell Disruption

### *Sonication*

Cell disruption for the release of invertase and penicillin acylase was performed with Ultrasonic Ace horn (Ace horn, New York) at an operating frequency of 20 kHz with a power rating of 600 W. One percent yeast suspension in acetate buffer of pH 5 (100 m*M*) was subjected to sonication for a time period up to 15 min and 20 min separately at amplitude of 15% using Ace horn. One percent yeast suspension in phosphate buffer of pH 8 (50 m*M*) was disrupted for 60 min and 120 min each using Dakshin horn (Pune, India) at an operating frequency of 22 kHz and power consumption of 240 W. Then, 5%, 10%, and 20% of *E. coli* cells (wet weight) suspended in phosphate buffer of pH 7.4 (100 m*M*) were disrupted using Ace horn at an amplitude of 15% for 25 min. The temperature of the cell suspension was maintained throughout the period of operation at 30°C by surrounding the suspension in ice-bath.

### *High Pressure Homogenization*

For invertase a suspension of 1% yeast cells in acetate buffer of pH 5 (100 m*M*) and for ADH 1% of yeast cells suspended in phosphate buffer of pH 8 (50 m*M*) were disrupted using Manton-Gaulin APV Homogenizer (GmbH, model lab 160-10 TBS) at discharge pressures ranging from 3000 to 5000 *psig* for 15 passes separately. *Escherinichia coli* (5%, wet weight) cells suspended in phosphate buffer of pH 7.4 (100 m*M*) were disrupted for 10 passes using Manton-Gaulin APV Homogenizer (GmbH, model lab 160-10 TBS) at 2000, 3000, 4000, and 5000 *psig* each. During homogenization the cell suspension was cooled by the passage of chilled water (8°C) through a heat exchanger.

### *Hydrodynamic Cavitation*

The set-up essentially consisted of a closed loop circuit comprised of a holding tank of 100-L volume, a centrifugal pump (2900 rpm, 5.5 kW, Calama Industries Ltd., Noida, India) control valves and flanges to accommodate the orifice plates as shown in the Figure 1. The suction side of the centrifugal pump is connected to the bottom of the tank. The discharge branches into two lines. The main line consists of a flange which houses the orifice plates and a hard glass tube is available next to the flange for visual observation. A bypass line is provided to control the liquid flow through the main line. Both the bypass and the main line terminate well inside the tank, below the liquid level, to avoid any induction of air into the liquid due to the plunging liquid jet. The inside diameter of delivery line of centrifugal pump is 38 mm. Control valves (V1, V2, V3) are provided at appropriate places to control the flow rate through the main line. The tank, valves, and the entire flow line is constructed of stainless steel (SS316). The tank is provided with cooling jacket to maintain the temperature (30°C) of the circulating fluid during the operation. Pressure gauges are provided to measure the inlet pressure (P1) and fully recovered downstream pressure (P2). The orifice plate in the main line consisted of 33 holes of

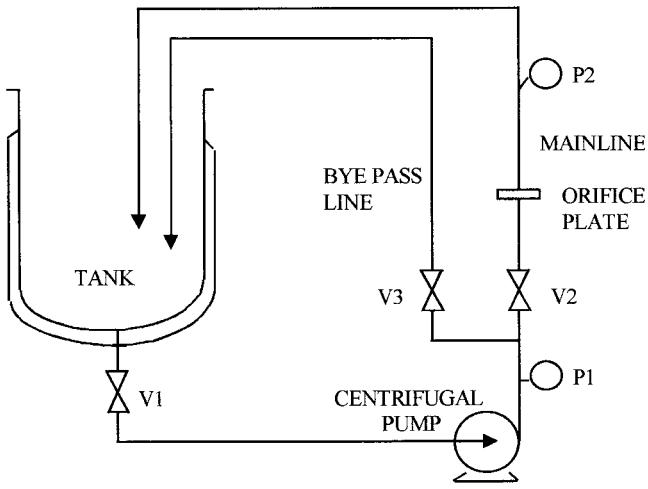


**Figure 1.** Hydrodynamic cavitation set-up.

1 mm diameter. Fifty liters of 1% yeast suspension in acetate buffer of pH 5 (100 m$M$) were disrupted at 75 psig pump-discharge pressure for 50 min.

### Centrifugation

Samples of disrupted cell suspension were centrifuged at 8000 rpm in Remi cooling centrifuge at 15°C for 17 min. The clear supernatant was collected for protein and enzyme analysis, i.e. invertase and alcohol dehydrogenase.

Samples of disrupted cell suspension were centrifuged using Remi centrifuge at 17°C for 40 min at 14,000 rpm and the clear supernatant was decanted for analysis of penicillin acylase and protein as described below.

### Analytical Methods

Invertase acts on sucrose, a nonreducing disaccharide and converts it to glucose and fructose, which are reducing monosaccharides (Gascon and Lampen, 1968). The amount of invertase can be estimated from the amount of glucose formed. The reducing property of glucose is used for its colorimetric estimation. Glucose can reduce 3,5-dinitrosalicylic acid (DNSA) to give an orange color, the intensity of which can be measured at 540 nm using UV-visible spectrophotometer. A calibration curve with known concentration of standard invertase (Sigma, St. Louis, mo; 45 units/mg) was obtained and was used to estimate the concentration of invertase in the clear disrupted sample.

Yeast alcohol dehydrogenase uses ethanol as substrate and oxidizes it to acetaldehyde with stoichiometric consumption of $NAD^+$. In this equilibrium redox reaction there is simultaneous production of reduced form of the cofactor, i.e., NADH from $NAD^+$, which gives a characteristic absorbance band at 340 nm ($\varepsilon_{340}$ = 6.2 m$M$ cm$^{-1}$). The rate of formation of NADH is used for the calculation of enzyme activity (Rocker, 1955). One enzyme unit of ADH is defined as that amount of enzyme, which brings about the formation of 1 μ$M$ of NADH per minute under the reaction condition.

For the analysis of penicillin acylase the substrate penicillin G was hydrolyzed by the action of the enzyme and the amount of 6-APA formed was determined by the PDAB method. A Schiff's base is formed by the reaction of 6-APA with paradimethylamino benzaldehyde, which is measured by its absorbance at 415 nm (Balasingham et al., 1972). One unit of penicillin acylase enzyme activity is defined as the activity producing 1 μ$M$ of 6-APA per minute at pH 7.5 and 37°C. The calibration curve was obtained by using dilutions of standard 6-APA. The total soluble proteins were estimated by using bovine serum albumin (BSA) as the standard (Lowry et al., 1951).

### The Concept of Location Factor

First-order kinetic relationship is reported to be applicable to the cell-disruption process. (Carlson et al., 1995; Follows et al., 1971; Hetherington et al., 1971; Kuboi. et al., 1995; Melendres et al., 1993; Sauer et al., 1988). Rate constant for enzyme release ($k_{\mathrm{I}}$) and protein release ($k_{\mathrm{T}}$) is given by an expression of the form

$$ln\left[\frac{R_{I,m}}{R_{I,m}-R_I}\right] = lnD^{-1} = k_I \times t \qquad (1)$$

where, $R_I$ is the released amount of total protein ($I = T$) and enzymes ($I = 1, 2, 3$) in time "$t$" s and $R_{I,m}$ is the maximum amount of total protein ($I = T$) and enzymes ($I = 1, 2, 3$) obtainable from the cells, with the respective disruption technique used. The In $D^{-1}$ values are plotted against time to get the release rate of enzyme and protein. The location factor value is obtained by taking a ratio between the release rate of enzyme to protein (Umakoshi et al., 1998).

$$LF_I = \frac{k_I}{k_T} \qquad (2)$$

The proteins are located throughout the cell, but enzyme is confined in most cases to either periplasm or cytoplasm. Hence, when the release rate of enzyme ($k_I$) is greater than that of protein ($k_{\mathrm{T}}$) the ratio becomes greater than unity. This happens when the enzyme is periplasmic. When the enzyme is cytoplasmic, the release rate of enzyme would be less than or equal to that of protein and hence, the location factor value will be less than one. Thus, the above concept is based on the relative rates of release of the enzyme to protein.

## RESULTS AND DISCUSSION

### Location Factor of Invertase (Cell Wall-Bound)

A well-defined periplasmic space cannot be differentiated in a yeast cell, unlike gram-negative bacteria, yet the extracytoplasmic region of yeast is often referred as periplasmic space. (Follows et al., 1971). There are two forms of invertase present in a yeast cell. The outer cell wall-bound form is the predominant form and is referred as external invertase and the cytoplasmic form is referred as internal invertase. This internal form represents only 2–6% of the total invertase present in the cell (Gascon and Lampen, 1968). The pH stability profile of these two forms varies considerably. The external form is said to be stable between pH 3–7.5, while the internal invertase is said to stable between pH 6–9. Also, the internal invertase undergoes a reversible inactivation at acid pH (Gascon et al., 1968). Analysis of the invertase in the present study was performed at a pH of 5, thus it can be safely concluded that invertase determined in the present study refers only to the external invertase (Cell wall- bound).

**Table I.** Release rate and location factor of invertase by different disruption techniques.

| Disruption technique | Rate of release of enzyme ($k_I$) $min^{-1}$ | Rate of release of protein ($k_T$) $min^{-1}$ | Location factor ($LF_I = k_I/k_T$) |
|---|---|---|---|
| Sonication (Ace horn, 15% amplitude, 1% cells) | | | |
| 15 min | 0.1194 | 0.1042 | 1.146 |
| 20 min | 0.1160 | 0.1003 | 1.156 |
| High-pressure homogenization (1% cells, 15 passes) | | | |
| 3000 psig | 0.1491 | 0.1374 | 1.085 |
| 4000 psig | 0.1420 | 0.1316 | 1.079 |
| 5000 psig | 0.1938 | 0.1798 | 1.103 |
| Hydrodynamic cavitation (75 psig, 1% cells) | | | |
| 50 min | 0.0612 | 0.0398 | 1.538 |

Using different techniques of cell disruption, under the specified conditions, the release rate of enzyme and proteins were estimated. These rates were used to calculate the location factor values by the method described above. The results have been summarized in Table I.

In Table I, it can be seen that by using all three methods of disruption, which were used for the release of invertase the value of the location factor of the enzyme was found to be greater than one. As per the concept of location factor the value of greater than one suggests periplasmic location of the enzyme confirming the report by Follows et al. (1971). The values obtained for the same enzyme by different techniques were found to vary although physically the location of the enzyme remains constant. The reason could be due to the fact that the estimated value of location factor depends on the release rate of the enzyme and protein. The release rate of the enzyme was found to vary with each technique and also at different operating conditions for the same technique. There was a significant difference between the location factor values obtained by hydrodynamic cavitation and the other two (i.e., high-pressure homogenization and sonication). This was because the difference between the release rate of the enzyme and protein ($k_I - k_T$) is very high with hydrodynamic cavitation ($0.0612 - 0.0398 = 0.0214\ min^{-1}$) while the same difference was less for high-pressure homogenization ($0.1491 - 0.1374 = 0.0117\ min^{-1}$ at 3000 psig) and sonication ($0.1194 - 0.1042 = 0.0152\ min^{-1}$ using 15 min disruption). Thus, the value of the location factor which is nothing but the ratio of the release rate of the enzyme and protein is also high for hydrodynamic cavitation while it is less by the other two methods of disruption. This implies that the enzyme is released relatively faster than other proteins by disruption in hydrodynamic cavitation set-up compared to other methods. This faster release of invertase could possibly indicate selective release of the enzyme and hence, the amount of contaminating proteins released is low. The total enzyme released in hydrodynamic cavitation set-up is also low. This suggests that an optimization, i.e., lower total activity and higher purity or higher total activity but lower purity can be managed by manipulating the disruption condition in the hydrodynamic cavitation set-up.

## Location Factor of Alcohol Dehydrogenase (ADH) (Cytoplasmic)

The release rate of ADH and total soluble protein were estimated by disruption using sonicator and high-pressure homogenizer. The location factor values obtained are presented in Table II.

As shown in Table II, it is found that though the release rate of ADH and protein varied with sonication and high-pressure homogenization (for varying discharge pressures), the differential in the release rate of ADH and protein is maintained in all these cases. Hence, there was no appreciable difference in the values of location factor obtained with different techniques indicating that these two techniques used for the disruption are similar in terms of their disruption intensity as far as the release rate of cytoplasmic enzyme is con-

**TABLE II.** Release rate and location factor of alcohol dehydrogenase by different disruption techniques.

| Disruption technique | Rate of release of enzyme ($k_I$) $min^{-1}$ | Rate of release of protein ($k_T$) $min^{-1}$ | Location factor ($LF_I = k_I/k_T$) |
|---|---|---|---|
| Sonication (Dakshin horn, 1% cells) | | | |
| 60 min | 0.0412 | 0.0708 | 0.584 |
| 120 min | 0.0235 | 0.0411 | 0.572 |
| High-pressure homogenization (1% cells, 15 passes) | | | |
| 3000 psig | 0.0546 | 0.1149 | 0.475 |
| 4000 psig | 0.0605 | 0.1085 | 0.558 |
| 5000 psig | 0.0693 | 0.1541 | 0.450 |

cerned. The value of location factor as approximately 0.5 (<1.0) obtained with both the techniques suggests cytoplasmic location for ADH confirming the reports by Follows et al. (1971) and Keshavarz et al. (1990).

### Location Factor of Penicillin Acylase (Periplasmic)

To study the effect of concentration of cells on the location factor values, different concentrations *of E.coli* cells were taken for disruption by sonication. The release rates of penicillin acylase and total soluble proteins and location factor values estimated by the disruption *of E.coli* cells using sonicator and high-pressure homogenizer are presented in Table III. For all three different cell concentrations used in the disruption studies, the location factor values were greater than one indicating the periplasmic location of penicillin acylase (Novella et al., 1994). The results of high-pressure homogenization at different discharge pressures (LF > 1) confirmed the periplasmic location of the enzyme. There was a relatively larger difference in the location factor values obtained at 2000 psig and 5000 psig discharge pressure of high-pressure homogenization. The possible reason for such behavior will be discussed later.

### Variation in Location Factor Values Due to Variation in Operating Conditions

Table III shows the variation in location factors for penicillin acylase. In high-pressure homogenization the contribution of the each mechanism (shear, impingement, and cavitation) for disruption differs at different discharge pressure. At higher discharge pressure cavitation plays an important role in cell disruption. Thus, with an increase in the discharge pressure, the cells are likely to get disrupted completely and the cytoplasmic protein(s) gets released at a rate similar to that of periplasmic enzyme. This reduces the difference between the release rate of the enzyme and protein leading to the decrease in the value of location factor (1.8 to 1.4). The $LF_I$ value approaches one at high-discharge pressure indicating increased rate of protein release. Thus, the location factor value indicates the degree of disruption and cell mutilation achieved or rather the relative difference in the degree of disruption (small nick or complete mutilation) obtained at various discharge pressures of high pressure homogenization. The location factor value also indicates the predominant mechanism of disruption.

### Variation in Location Factor Values of the Same Enzyme by Different Disruption Techniques

Different values of location factor were obtained for the same enzyme with the use of different disruption techniques (Table I). The location factor value of invertase by hydrodynamic cavitation was very high when compared to others (1.53 against 1.1 and 1.15). ADH (cytoplasmic) could not be released at any rate using hydrodynamic cavitation at the conditions used for the disruption in the present study. Total soluble proteins, most of which are located in cytoplasm (French et al., 1996, Huang et al., 1991) were also released at a very low rate (0.039 $min^{-1}$) compared to invertase (0.061 $min^{-1}$) using hydrodynamic cavitation. This-implies that cytoplasmic products are not released at a significant level by hydrodynamic cavitation. Significant level of the release of invertase shows that the outer wall of yeast is broken resulting in the release of periplasmic contents, while only a nick or scraping of the cytoplasmic membrane does not allow the release of cytoplasmic ADH to a significant degree. Thus, the higher value of location factor by hydrodynamic cavitation indicates a selective release of invertase, a periplasmic enzyme as compared to the cytoplasmic enzyme ADH. Hence, location factor could be used for identifying whether a particular equipment or particular technique used for the cell disruption gives a selective release of enzyme or not.

### Comparison of Location Factor Values of Different Periplasmic Enzymes

Comparison of Tables I, II, and III reveals some interesting facts. It was found that for a similar extracyto-

**Table III.** Release rate and location factor of penicillin acylase by different disruption techniques.

| Disruption technique | Rate of release of enzyme ($k_I$) $min^{-1}$ | Rate of release of protein ($k_T$) $min^{-1}$ | Location factor ($LF_I = K_I/k_T$) |
|---|---|---|---|
| Sonication (Ace horn, 15% amplitude, 25 min, using cells grown up to 18 h) | | | |
| 5% cells | 0.1592 | 0.0917 | 1.736 |
| 10% cells | 0.1907 | 0.1210 | 1.576 |
| 20% cells | 0.1834 | 0.1160 | 1.581 |
| High-pressure homogenization (5% cells, 10 passes, using cells grown up to 18 h) | | | |
| 2000 psig | 0.3047 | 0.1688 | 1.805 |
| 3000 psig | 0.3425 | 0.2055 | 1.666 |
| 4000 psig | 0.7252 | 0.4990 | 1.453 |
| 5000 psig | 0.7344 | 0.4957 | 1.481 |

plasmic enzyme in a different microbe the location factor value shows a significant variation. There was a large difference between the location factor values obtained by sonication for the enzyme invertase and penicillin acylase, though both of them are extracytoplasmic in nature. On comparing the location factor value of invertase and penicillin acylase it was found that the value for invertase is very closer to one (1.1) while that for penicillin acylase the value is much higher than one (1.7). This could be due to the entanglement of invertase in the cell-wall matrix (Gascon and Lampen, 1968; Melendres et al., 1993) of yeast cell. Hence, a larger disruption force, which is possibly equivalent to that required for the release of cytoplasmic contents, is necessary for the release of the invertase, a wall-bound enzyme. Using sonication, the release rate of enzyme invertase (0.1194 $min^{-1}$) is very close to the release rate of cytoplasmic protein (0.1042 $min^{-1}$) and the location factor value is very close to unity, while it is not in the case of penicillin acylase.

## Selectivity

Selectivity values for the release of invertase has been calculated and presented in Table IV. The selectivity of an enzyme is calculated by taking the ratio of the maximum concentration of invertase (mg/mL) obtained to that of maximum concentration of the proteins obtained (mg/mL). Higher value of selectivity for an enzyme in the particular equipment indicates that the amount of contaminating proteins released at the given operating conditions is less.

It was found that the value of selectivity for invertase by hydrodynamic cavitation (for 10 min operation) is high (0.5) as compared to high-pressure homogenization (0.207) at 3000 psig discharge pressure. Thus, the selective release of invertase by hydrodynamic cavitation is once again confirmed. Based on the value of selectivity, sonication (0.0177) was found to release more contaminating proteins when compared to the other two techniques of disruption, when the selective release of extracytoplasmic enzymes is the aim of the disruption.

## Speculative Uses of Location Factor

As explained earlier, variation in the location factor values for the same enzyme using different techniques indicates whether the particular equipment gives selective release of enzymes or not. Variation of location factor values of the same enzyme at different conditions using the same equipment indicates the extent and the quality of disruption achievable at different conditions for that particular technique.

Apart from these inferences a few more applications of the location factor concept have been proposed. They are:

When a microbial cell is subjected to mechanical or thermal stresses enzyme/protein migration is known to occur. The concept of location factor could be used to monitor this process of translocation of the enzyme (Umakoshi et al., 1998).

Enzymes are synthesized in the cell and translocated to various regions within the cell. The process of secretion (translocation) of an enzyme from cytoplasm to periplasm and hence, the secretion rate could be determined using the concept of location factor.

Cells that are genetically manipulated for secretion could be differentiated from the parent cell (which does not secrete the enzyme).

Thus, an attempt has been made in the present study to translocate the cytoplasmic ADH and to determine the location of the periplasmic enzyme penicillin acylase at various growth stages of *E. coli*.

## Heat-induced Translocation

To study the enzyme/protein migration or translocation due to thermal and/or mechanical stresses the concept of location factor can be used to get semiquantitative information about this process.

Yeast cells were heated to 55°C for 20 min and then disrupted by sonication using Dakshin horn for 120 min. The release rates of the enzyme and protein were determined and the location factor value is calculated. There was no change in the release rate of total soluble

**Table IV.** Selectivity of release of invertase by different disruption techniques.

| Disruption technique | Conc. of invertase (mg/mL) (A) | Conc. of total soluble proteins (mg/mL) (B) | Selectivity (A/B) |
|---|---|---|---|
| Sonication (Ace horn, 15% amplitude, 1% cells) | | | |
| | 0.0053 | 0.580 | 0.0177 |
| High-pressure homogenization (1% cells, 15 passes) | | | |
| 3000 psig | 0.0510 | 0.252 | 0.207 |
| 4000 psig | 0.0630 | 0.337 | 0.186 |
| 5000 psig | 0.0757 | 0.541 | 0.139 |
| Hydrodynamic cavitation (75 psig, 1% cells) | | | |
| 10 min | 0.00462 | 0.009 | 0.500 |
| 20 min | 0.00504 | 0.022 | 0.225 |
| 35 min | 0.00570 | 0.091 | 0.063 |
| 50 min | 0.00669 | 0.155 | 0.043 |

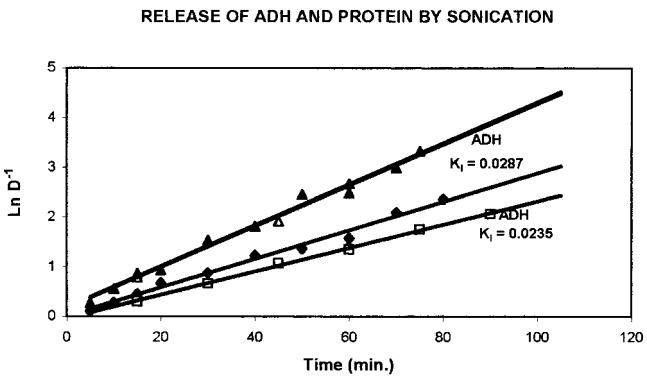


**Figure 2.** Release rate of ADH and total soluble proteins by sonication after heating yeast cells at 55°C for 20 min. (■) Release of ADH from heated cells; (▲) release of total soluble proteins, from heated cells; (□) release of ADH from control cells; (△) release of total soluble proteins from control cells.

protein but the release rate of ADH was found to be increased marginally on heat treatment (0.0235 to 0.0287). Hence, there was a slight increases in the value of location factor of ADH from 0.57 to 0.69 (Fig. 2). It is reported (Umakoshi et al., 1998) that on heating a microbial cell there is a change in hydrophobicity of the enzyme and the cytoplasmic membrane which results in an increased interaction between the two resulting in the translocation of the cytoplasmic enzyme to periplasm.

The reason for the marginal increase in the release rate of enzyme ADH after heating of the cells is not yet known. Further studies regarding the change in hydrophobicity of the cell wall and cytoplasmic membrane and surface property of ADH have to be undertaken to determine if complete translocation of ADH to periplasm would be possible and also to know the precise mechanism of translocation (Umakoshi et al., 1998).

## Location of Penicillin Acylase During Various Time Periods of Cell Growth

The process of secretion of enzyme from cytoplasm to periplasm as discussed earlier can be monitored by the concept of location factor. Penicillin acylase is known as a periplasmic enzyme. The release rate of penicillin acylase from l0-, 12-,14-, and 16-h culture of *E. coli* cells was determined by disrupting the cells using sonication with Ace horn. The results are tabulated in Table V.

The location of penicillin acylase as given by location factor values at 10 h of fermentation appears to be cytoplasmic while it was found to be periplasmic at 12 h and further (LF changed from 0.59 for 10- h culture to 1.04 for 12- h culture and further). In the present study the inducer for enzyme synthesis was added before inoculation itself, so the enzyme gets synthesised along with the growth of the cells.

But there are certain periplasmic enzymes that are produced by the addition of inducer to cells in stationary phase. The synthesis and the translocation of an enzyme from cytoplasm to periplasm in such a case could be monitored by location factor concept. The time it takes for the secretion of enzyme from cytoplasm to periplasm, i.e., the secretion rate could be determined by the location factor concept. This secretion rate could probably be useful to predict the amount of time period that would be required to keep the cells in the stationary phase for the production of the enzymes, which gets synthesized and secreted to the periplasmic space after the addition of the inducer to the cells in the stationary phase.

**TABLE V.** Location factor of penicillin acylase during growth period of *E. coli* cells.

| Time of culture (h) | Location factor values (Sonication, Ace horn, 15% amplitude, 25 min, 5% cells) |
|---|---|
| 10 | 0.59 |
| 12 | 1.04 |
| 14 | 1.35 |
| 16 | 1.68 |

The translocation of the polypeptide precursor of an enzyme in a microbial cell occurs either by cotranslational translocation or posttranslational translocation. Though cotranslational translocation is the common mode of secretion of an enzyme in a prokaryotic cell, penicillin acylase is one of the exceptions. Penicillin acylase is reported to undergo posttranslation mechanism of translocation. The polypeptide precursor of penicillin acylase is translocated to the periplasmic space where the signal peptide is removed to give the active penicillin acylase enzyme (De Leon et al, 1996). The reason for location factor value of 0.59 for penicillin acylase, from *E.coli* cells of 10-h culture, indicating it as active cytoplasmic enzyme is not yet known. It is reported that incubation under aerobic conditions with high dissolved oxygen concentration have resulted in higher penicillin acylase activity (De Leon et al., 1996). Sonication is found to be useful for oxidation of potassium iodide (Gogate et al., 2001; Senthil Kumar et. al., 2000) and hence, the oxidative environment of the sonication could have resulted in maturation of the cytoplasmic polypeptide precursor of penicillin acylase. Further experiments with hydrodynamic cavitation, as it is proved to selectively release extracytoplasmic enzyme, are necessary to confirm the above hypothesis and this application of location factor can only be used in the cases, where the polypeptide precursor of an enzyme can be activated in vitro to quantify them.

## CONCLUSIONS

The location of the enzyme invertase, alcohol dehydrogenase, and penicillin acylase as determined by the location factor concept was found to coincide well with

the literature. Much information is revealed by determining the value of the location factor by different equipment. When the location factor value is very close to unity for the disruption equipments under consideration then the equipment that gives a higher value will give a selective release for that particular enzyme. In the present study invertase was released selectively by hydrodynamic cavitation set up (LF–1.5) as compared to sonication (LF–1.14) and high-pressure homogenization (LF–1.03). Location factor concept reveals the relative difference in the quality of the disruption obtained by different techniques (equipment) and also at various disruption conditions for the same technique (equipment) as shown by the various location factor values of penicillin acylase using high-pressure homogenization operating at various discharge pressures.

Location factor concept can be used to identify the usefulness of an equipment for DPR (differential product release). Hydrodynamic cavitation set-up could be used for this purpose, as only the periplasmic wall is broken at the said operating conditions. After the release of periplasmic enzyme it could then be operated at more severe conditions to release cytoplasmic enzymes. The location factor concept could be used for monitoring this DPR technique.

Location factor concept could be used to monitor the translocation of the enzyme during conditions of mechanical or thermal stress. This concept could also be used to identify the location of an enzyme during various growth stages of cells. Hence, it could probably be used in the optimization of secretion rate (from cytoplasm to periplasm) of a periplasmic enzyme, which gets synthesized after the addition of an inducer to growing cells in stationary phase. But this application of location factor cannot be concluded before further studies are conducted. Location factor study could also be used to differentiate between a genetically engineered cell, which secretes enzyme from one compartment to another or to extracellular medium and the parent cell where enzyme is intracellular.

A more detailed study of the location factor of enzymes located in other parts of the microbial cell like cytoplasmic organelles (fumerase in mitochondria), and microscopic observations of the disrupted cells and cell debris characterization will lead to a better understanding of this concept of location factor and its useful applications.

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