A comparative study on cell disruption methods for release of aspartase from E. coli K-12

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Applicability of different mechanical cell disruption techniques namely sonication, bead milling and French press for the release of aspartase from E. coli K-12 was compared. Various operating parameters of each technique were optimized to obtain maximum aspartase release. The efficiency of aspartase release and cell disruption by all the methods was also compared under optimal conditions. The maximum release of aspartase (98.22%) and maximum cell breakage (84.25%) was observed using French press, while 92% of aspartase release was obtained by both sonication and bead milling. The order of cell disruption constant ($k$) for aspartase release by these methods was French press > bead milling > sonication. Disruption of cells using French press also demonstrated maximum protein release (14.12 mg/mL). The crude enzyme preparations can be further used for purification and its applications.

Keywords: Aspartase, Cell disruption, E. coli, Liquid-shear, Solid-shear, Sonication

Aspartase (L-aspartase ammonia-lyase; EC 4.3.1.1) catalyzes the reversible conversion of L-aspartate to fumarate and an ammonium ion. The pH of the medium controls the direction of the reaction and at alkaline pH, fumaric acid and ammonia are converted to L-aspartic acid. The equilibrium constant of aspartase-mediated reaction favours the formation of aspartate. It is among the most specific enzymes which catalyze the reaction with high specificity and catalytic efficiency. Aspartase contributes significantly to Krebs cycle and amino acid metabolism, and is an important enzyme in microbial metabolic pathway. It is an industrially important enzyme for its ability to produce L-aspartic acid, an important ingredient of artificial sweetener aspartame. There are a few reports on aspartase activity of higher plants and bony fishes. However, aspartase from these sources is not well characterized because of poor yield and extensive time required as compared to microbial sources. Several microorganisms from fungi, yeast and bacteria are reported as sources of aspartase. Typically, aspartase is a bacterial enzyme which is inducible and intracellular. It has been reported from E. coli, E. alcalescens, Bacillus subtilis, B. cereus, B. stearothermophilus, Pseudomonas putida, P. fluorescens, Pasteurella pestis, Hafnia alvei, Cytophyga sp., Erwinia sp., Brevibacterium sp., Rhizobium sp. etc. It has also been reported from Aeromonas media.

Whole cells as well as partially purified aspartase preparations have been used for L-aspartate synthesis. Most studies report the use of immobilized whole cells as aspartase source for the production of L-aspartic acid. As aspartase is intracellular, immobilization of whole cells can pose mass transfer limitations in addition to the limitations imposed by cell membrane. Immobilization of crude enzyme can eliminate or decrease such diffusional limitations and the developed biocatalyst can also be used repeatedly in the bioprocess. Cell disruption is the first step of downstream processing of an intracellular product used for food, pharmaceutical and agrochemical industries. To make the downstream processing more economically viable, a feasible cell disruption method should be developed to ensure a low operating cost and high product recovery. The present investigation was undertaken to compare and evaluate the effect of sonication, French press and bead milling on aspartase release from E. coli K-12. The crude enzyme released can be used further for the development of an efficient immobilized biocatalyst for the production of aspartic acid.

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Materials and Methods

**Bacterial culture—*Escherichia coli* K-12 (MTCC 1302) was procured from CSIR-Institute of Microbial Technology, (CSIR-IMTECH) Chandigarh, India. It was maintained periodically on agar slants made from medium containing tryptone 1%, yeast extract 0.5%, sodium chloride 1%, agar 2.5% and adjusted to pH 7.5.**

**Aspartase production and cultivation conditions—** The inoculum was prepared in Erlenmeyer’s flasks containing 100 mL of maintenance medium except agar and incubated at 37 °C for 24 h under agitation (150 rpm) on a rotary shaker. Aspartase production was carried out in a laboratory scale bioreactor (Biolab, B. Braun, Germany) equipped with a ruston turbine impeller with six blades and automatic control of aeration, agitation and temperature. Production medium (1L) containing 1% sodium-L-aspartate, 0.5% peptone, 0.2% glycerol, 0.3% dipotassium hydrogen phosphate, 0.2% potassium dihydrogen phosphate, 0.2% sodium chloride, 0.01% magnesium sulphate and adjusted to pH 7.2 was sterilized in the bioreactor and equilibrated at 37 °C before inoculation. Bioreactor inoculated with 10% (v/v) of inoculum was operated at 37 °C for 24 h under agitation (200 rpm) and aeration (1 vvm). To control foam formation during fermentation, sterilized silicon antifoaming agent (0.002%, w/v) was added in the beginning of fermentation. Foam formation was regularly monitored during the fermentation run.

**Cell disruption methods for release of aspartase—** After cultivation for 24 h, cells were harvested by centrifugation (4000 g, 10 min, 4 °C) in a refrigerated centrifuge (5804R, Eppendorf AG, Germany), washed twice in potassium phosphate buffer (0.5M, pH 6.8) and resuspended in the same buffer to make the appropriate cell concentration (100 mg/mL). Different disruption methods were optimized for the release of aspartase from cells of *E. coli* K-12 as mentioned below.

**Sonication**—Cell suspension in aliquots of 2 mL was subjected to sonication with 30 sec pulse-on and pulse-off time each, using an Ultrasonicator (VCX 500, Sonics & Materials Inc., USA). The effect of various parameters like sonication time (1-8 min), acoustic power (50-200 W) and cell concentration (50-250 mg/mL) was monitored. During sonication, the sampling tubes were kept in ice-bath to avoid the heat-mediated denaturation of crude enzyme. After centrifugation (8000 g, 10 min, 4 °C) of sonicated suspensions, supernatant and debris were analyzed for enzyme activity and protein content.

**French press (Liquid-shear)—** Aliquots of 2 mL cell suspension (100 mg/mL, unless otherwise mentioned) were passed through Thermo Spectronic French Press (Rochester, USA) in a 40K cell to optimize aspartase release from *E. coli* K-12 cells. The effect of various parameters like pressure (4,000-12,000 psi), number of passages (1-4), flow rate of the cell suspension (0.5-3.0 mL/min) and cell concentration (50-250 mg/mL) was investigated. Aspartase activity and protein content in supernatant and debris was determined after centrifugation (8000 g, 10 min, 4 °C) of homogenized slurry. The 40K cell was stored at 4 °C for 12 h prior to high pressure homogenization and the cell suspensions were also cooled in an ice-bath after each passage.

**Bead milling (Solid-shear)**—The cell suspension was poured into the bead-beater chamber (300 mL, Willy A. Bachofen Maschinenfabrik, Basel, Switzerland) loaded with lead-free glass beads and operated at a rotational speed of 2,000 rpm. The effect of parameters like milling time (5-30 min), bead size (0.25-1.5 mm), bead volume (50-80%) and cell concentration (50-200 mg/mL) on the aspartase release were assessed. During milling, a mixture of glycerol and water at 0 °C was passed through the jacket of the chamber for its cooling. The ground slurry was centrifuged (8000 g, 10 min, 4 °C), and both supernatant and debris were analyzed for aspartase activity and protein content.

**Differential interface contrast microscopy (DIC)—** The cell debris obtained from each method after centrifugation was appropriately diluted and the images were captured using DIC Microscope (Model E 600) equipped with DIC module with a mounted Nikon (Japan) camera. The images were visualized by Image Pro Express (Media Cybernetics, Madison, USA) at X 100 magnification.

**Aspartase assay**—Aspartase activity was determined as described earlier. Briefly, reaction mixture (2 mL) containing sodium-L-aspartate (0.5 M) and MgCl₂ (0.1 M) in Tris-HCl buffer (0.5 M, pH 8.8) and an appropriate quantity of crude enzyme was incubated at 30 °C in a UV-Visible Spectrophotometer (Pharamspec 1700, Shimadzu, Japan) equipped with a Peltier-type cell temperature control unit. The increase in absorbance at 240 nm due to the formation of fumarate was measured and molecular coefficient of 2.53×10³/M/cm at 240 nm for...
fumarate was used for determination of aspartase activity. One unit of aspartase is defined as the amount producing one µmol of fumarate/min under standard assay conditions. Aspartase activity was expressed in units/g of fresh biomass (u/g fresh biomass).

**Protein determination**—Protein content was determined as described by Lowry et al.

### Results

Majority of the enzymes produced by microorganisms are intracellular and some of these have been successfully produced on an industrial scale. To release intracellular microbial products, it is important to use an efficient method of cell disruption, but at the same time does not cause denaturation of biological substances. Sonication, homogenization, bead milling and nobulization are some of the efficient methods of disintegration for the release of proteins from microbial cells.

**Sonication**—Ultrasonication is an active method of cell disruption at laboratory scale. It has low operating cost and does not require sophisticated equipment or extensive technical training. However, inactivation of released products by ultrasonication can be caused by shear stress developed by eddies arising from shock waves. Enzyme released by sonication can be calculated as:

\[
R = R_m (1 - e^{-kt}) \tag{1}
\]

where \( R \) and \( R_m \) are the released enzyme activity and maximum enzyme activity that can be released, respectively (U/mg/min), \( k \) is disruption constant for sonication and \( t \) is time of sonication (min). The enzyme released (%) and enzyme retained (%) in the debris is the enzyme activity expressed relative to the activity of whole cells. The release of aspartase from the whole cells was investigated at an acoustic power of 150 W for 1 to 7 min. Maximum aspartase was released within 3 min of sonication, beyond which a decline in aspartase release was observed (Fig. 1a). However, total cellular protein release increased up to 7 min. The influence of acoustic power on aspartase release was monitored by sonicating the cell suspensions at 50-200 W for 3 min. An increase in aspartase release was observed up to 150 W (Fig. 1b) and this acoustic power was used in further investigations. The cell concentration (50-200 mg/mL) in suspension did not show any impact on release of aspartase under optimized conditions (Fig. 1c).

**French press (Liquid-shear)**—High-pressure homogenization was initially used in the dairy industry and emulsion dairy, but eventually adopted for application in cell disruption. High-pressure homogenization forces the cell suspension through a small orifice and the cells are disrupted because of the extremely high-shear forces generated by the pressure drop, \( \Delta P (P_{\text{appl}} - P_{\text{atm}}) \). Enzyme release by high-pressure homogenizer can be described by the following equation:

\[
\ln \left( \frac{R_m}{R - R_m} \right) = kt \tag{2}
\]

where \( R_m \) is the maximum releasable protein, \( R \) is the protein released from the cells, \( k \) is the rate constant for disruption and is a function of homogenizer pressure and valve geometry.

![Fig. 1—Effect of various parameters of sonication on the release of aspartase from E. coli K-12 (a) Sonication time, (b) Acoustic power, (c) Cell concentration (Sample volume 2.0 mL; Microtip probe diameter 1/8”).](image-url)
To determine how the applied pressure affects the disruption of whole cells and release of aspartase, cell suspensions (100 mg/mL) were subjected to a pressure range of 4,000-12,000 psi. The increase in pressure up to 10,000 psi enhanced the aspartase release and thereafter, no increase in aspartase release was observed (Fig. 2a). A 93.33% aspartase release was observed after single passage of cell suspension through the orifice of the homogenizer and subsequent cycles demonstrated only a marginal increase (Fig. 2b). The cell suspension flow rate of 1 mL/min has been found optimal for the release of aspartase (Fig. 2c). The cell concentrations (50-250 mg/mL) used for disintegration did not show any influence on the release of aspartase (Fig. 2d).

Bead Mill (Solid-shear)—A bead mill used for disruption of cells consists of a chamber with specially designed agitator discs mounted on the shaft which transfers kinetic energy to the spherical beads. When the cell suspension inside the chamber and the impeller are rotated, the beads generate high shear forces, resulting in cell wall disintegration. The enzyme released by bead milling can be described by the following equation:

\[
\ln \left( \frac{R_m}{R - R} \right) = \frac{k(1 - \Phi)V}{F} \times N 
\]

where \( R \) and \( R_m \) are the released enzyme activity and maximum enzyme activity that can be released, respectively (U/mg/min), \( t \) is the milling time, \( k \) is the disruption rate constant, \( V \) is the volume of the grinding chamber, \( \Phi \) refers to the chamber volume occupied by the beads and \( F \) is the flow rate\(^{17} \).

Maximum release of aspartase from \( E. coli \) K-12 cells was observed at 20 min milling time, beyond which saturation was observed (Fig. 3a). The bead size and bead volume also showed strong impact on the release of aspartase from \( E. coli \) K-12 cells. Bead size of 0.75 mm and bead loading of 70% resulted in maximum release of aspartase (Fig. 3b and c). Similar to the results of sonication and liquid-shear, no effect of cell concentration on aspartase release was observed during bead milling (Fig. 3d).

Comparison of cell disruption methods employed for aspartase release—Comparison of methods for cell disruption was made at their optimal conditions of aspartase release from \( E. coli \) K-12 cells and the results are shown in Table 1. Maximum aspartase release (98.22%) was observed by liquid-shear technique. Ultrasonication and solid-shear resulted in more than 90% aspartase release under their respective optimized conditions.

Discussion

The method of choice for the release of enzyme of interest depends upon the enzyme yield and its overall cost. In most of studies on L-aspartic acid production, either whole cells or partially purified aspartase preparation has been used.
Fig. 3—Effect of various parameters of bead mill on the release of aspartase from *E. coli* K-12 (a) Milling time, (b) Bead size, (c) Bead volume, (d) Cell concentration (Sample volume 150 mL).

Table 1—Comparison of different disruption methods for the release of aspartase from *E. coli* K-12

<table>
<thead>
<tr>
<th>Method employed</th>
<th>Aspartase released (%)</th>
<th>Total protein in CFE (mg/mL)</th>
<th>Cell breakage (%)</th>
<th>Disruption constant (k)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sonication</td>
<td>92.66</td>
<td>10.65</td>
<td>78.95</td>
<td>0.55</td>
</tr>
<tr>
<td>French press</td>
<td>98.82</td>
<td>14.12</td>
<td>84.25</td>
<td>0.72</td>
</tr>
<tr>
<td>Bead mill</td>
<td>92.05</td>
<td>12.95</td>
<td>80.04</td>
<td>0.68</td>
</tr>
</tbody>
</table>

CFE: Cell free extract

The characteristics of crude enzymes can never depict the actual catalytic activities because the other competitive enzymes present in the crude extract may pose rearrangement and other chemical changes in the substrate. Therefore, purification of an enzyme is mandatory to clearly identify its molecular mechanism responsible for a discrete function. During ultrasonication, sonication time and acoustic power are two important parameters affecting the release of enzyme of interest. The decline in release of aspartase from *E. coli* cells after 3 min of sonication may be due to the release of active proteases, which might have degraded the desired enzyme or denaturation of the enzyme may have occurred because of the heat generated by ultrasonication. The decrease in aspartase release when acoustic power was increased beyond 150 W may be due to increased heat generation associated with higher acoustic power. It has been reported that both sonication time and acoustic power have a strong impact on the stability of enzymes during sonication. During ultrasonication, sonication temperature and acoustic power are two important parameters affecting the release of enzyme of interest. The decline in release of aspartase from *E. coli* cells after 3 min of sonication may be due to the release of active proteases, which might have degraded the desired enzyme or denaturation of the enzyme may have occurred because of the heat generated by ultrasonication. The decrease in aspartase release when acoustic power was increased beyond 150 W may be due to increased heat generation associated with higher acoustic power. It has been reported that both sonication time and acoustic power have a strong impact on the stability of enzymes during sonication.

The maximum release of aspartase at 10,000 psi as compared to ultrasonication indicates that the enzyme is more stable under the conditions of release by liquid-shear method. This may be attributed to the less heat generated during liquid-shear as compared to sonication. The lesser release of aspartase at pressure below 10,000 psi may be attributed to the fact that at low pressure, the cells experience the point-break, losing the soluble content partly but without the total disintegration of cell wall. A single passage through
French press has been found sufficient for the release of aspartase and subsequent cycles resulted in only a marginal increase in aspartase yield. The use of French press for more than two cycles resulted in generation of very fine debris which may pose severe problems in downstream processing. It has also been reported that the increase in number of passes leads to maximum protein release and micronization of cell debris. The increase in flow rate above 1 mL/min of the cell slurry through the orifice of the French press significantly decreases the aspartase release. This may be due to the fact that at higher flow rates (>1 mL/min), cells retained the pressure drop for a lesser time resulting in incomplete cell disruption. An electrically-assisted extraction of bioproducts using high pressure disruption of yeast cells has been reported as an efficient method and may have a good potential in biotechnological and food applications.

Amongst the optimized parameters of bead milling, milling time, bead size and bead loading showed a very significant impact on the release of aspartase from E. coli K-12 cells. The less release of aspartase from the cells by smaller beads may be attributed to the fluidization effect of the slurry in the chamber. In comparison, larger beads were too heavy to be significantly influenced by the flow of slurry and therefore, less cell disruption was achieved. The change in cell concentration during milling showed no influence on aspartase release. In contrary, it has been reported that cell concentration in case of yeasts has a strong impact on disintegration during bead milling. As compared to ultrasonication, bead mill and enzymatic lysis, grinding in liquid nitrogen has been reported most effective in terms of disruption efficiency and time for the release of lipids from Chlorella vulgaris. All the methods employed were found suitable and efficient for the release of aspartase from E. coli K-12. However, the choice for method to be opted for disruption of cells should be made according to objectives of the experiments.

Conclusions

Sonication, liquid-shear and solid-shear have been found very efficient for the release of aspartase from E. coli cells. The cell concentration in the suspension has not shown any impact on the aspartase release. The present results corroborate the findings of nitrilase release from Alcaligenes faecalis. Sonication can be applied at laboratory scale for the release of aspartase, and liquid-shear and solid-shear techniques can be used on large scale for the release of aspartase.

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