A versatile and economical method for the release of recombinant proteins from Escherichia coli by 1- propanol cell disruption

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A versatile and economical method for the release of recombinant proteins from Enterobacter cloacae by 1-propanol cell disruption†

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Introduction

The release of intracellular target proteins from microbial cells necessitates an additional step of cell disruption prior to the purification step. The cell disruption process plays a critical role in bioprocessing because it affects the quantity, biological activity and subsequent purification of the target protein.1 Mechanical methods of cell disruption normally cause a complete release of the cellular content, thus complicating the downstream processing of target proteins. These methods also demand a high power consumption in stages such as the mechanical process and homogenate cooling.2 Much of the energy input needed by mechanical methods of cell disruption is dissipated as heat, which may degrade the heat-sensitive proteins. On the other hand, chemical cell disruption does not require energy-intensive instruments for the operation and therefore the costs of setup and operation are relatively lower.

Alcohol can serve as a cell-permeabilizing agent. The application of alcohol in permeabilizing a broad range of microorganisms [e.g., Enterobacter cloacae (E. coli), Saccharomyces cerevisiae, Lactococcus lactis and Kluyveromyces sp.3–6] has been reported. For example, 2-propanol and butanol were used to permeabilize microbial cells acting as the whole cell biocatalysts in bioconversion, and a remarkable increase in the biocatalysis reaction was recorded.3–8 Butanol was also used in the controlled permeabilization of cheese-starter bacteria involved in the cheese-ripening processes.2 Besides, permeabilization of yeast cells used in lactose-hydrolysed milk production was achieved by ethanol.9 Moreover, methanol was successfully used to extract astaxanthin, i.e., a high-value antioxidant, from Haematococcus algae.10

In view of the potential of alcohol in cell permeabilization, we aimed to investigate the release of intracellular proteins from microbial cell using alcohol-based cell disruption. *E. coli* expressing an enhanced green fluorescent protein (EGFP) was used in this study as a model system owing to the following reasons: (i) *E. coli* is the most widely used bacterial expression system for the production of recombinant proteins; (ii) EGFP has an extreme resistance to thermal/chemical degradation and is stable over a wide range of temperatures.5–7 Our preliminary screening result showed that the permeabilization of *E. coli* by 1-propanol (1-PrOH) gave a higher selective in EGFP release as compared to that by ethanol and 2-propanol. This alcohol-based cell disruption method was optimized using response surface methodology (RSM). The process parameters such as alcohol concentration, temperature and pH, were chosen as the factors in the RSM optimization. The alcohol cell disruption method was also compared to the mechanical methods of cell disruption (i.e., glass beads vortexing and ultrasonication) in terms of efficiency and power consumption. With the establishment of this robust alcohol-based cell disruption on the widely used *E. coli* host cell, this strategy aims to provide a straightforward and universal technique for unleashing the expressed recombinant protein without the reliance on energy-intensive equipment.

### Experimental section

#### Materials

Luria–Bertani (LB) broth, isopropyl β-d-thiogalactopyranoside (IPTG), 1-PrOH (purity ≥ 99.5%) and sodium chloride (NaCl) were purchased from Merck (Darmstadt, Germany). Sodium phosphate monobasic, sodium phosphate dibasic, citric acid, trisodium citrate dihydrate, sodium carbonate, sodium bicarbonate, sodium bicarbonate, sodium hydroxide, bicinechonic acid assay (BCA) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, USA). Ampicillin was purchased from Amresco (Ohio, USA).

#### Apparatus and procedure

**Fermentation of culture.** *E. coli* strain Rosetta-gami 2 (DE3) carrying plasmid pRSETB-EGFP was prepared using the method mentioned in ESI.† The cells were cultured in a shake flask containing 50 mL LB broth and 100 μg mL⁻¹ ampicillin. The culture was incubated with shaking (200 rpm) at 30 °C. When the biomass concentration reached a constant optical density (OD₆₀₀) of ~0.8, the protein expression was induced by adding 1 mM IPTG into the culture. The culture was further incubated overnight at 200 rpm and 30 °C. Then, the cells were harvested by centrifugation at 2739 × g and 4 °C for 10 min.

**Cell disruption.** Three types of cell disruption techniques, namely alcohol-based cell disruption, glass bead vortexing and ultrasonication, were assessed in this study. The concentration of feedstock (*E. coli* cells) treated by all three types of cell disruption methods was set at 0.1 g mL⁻¹ wet cell weight. The feedstock was prepared by resuspending the harvested *E. coli* cell pellet in phosphate buffer (20 mM monosodium phosphate; 20 mM disodium phosphate; 0.5 mM NaCl; pH 7.4). After the cell disruption process, the treated cell suspension was centrifuged at 2739 × g and 4 °C for 30 min. The supernatant was then sampled for the measurements of total protein and active EGFP. The selective EGFP release was calculated using eqn (1) as shown below:

\[
\text{Selective EGFP release} = \frac{\text{amount of active EGFP release (mg mL}^{-1})}{\text{amount of total protein release (mg mL}^{-1})} 
\]

**Alcohol treatment.** From our preliminary screening studies (data not shown), 1-PrOH resulted in the highest EGFP release as compared to ethanol and 2-propanol. Thus, 1-PrOH was used in this study as the cell-disrupting agent. The pH and concentration of 1-PrOH for each experimental run were in accordance to the experimental design shown in Table 1. The preparation of 1-PrOH solution at a desired pH is described in ESI.† The buffered 1-PrOH solution was added to the pre-loaded wet cells in a 2 mL micro-centrifuge tube. Then, the micro-centrifuge tube was transferred to a rotating mixer kept in a static incubator. The mixture was subjected to rotational mixing at 35 rpm and at a desired temperature for 30 min.

**Glass bead vortexing.** *E. coli* containing the recombinant EGFP were subjected to glass bead vortexing.14 In brief, the acid-washed glass beads were loaded to a 2 mL micro-centrifuge tube. The *E. coli* cells suspended in phosphate buffer (20 mM monosodium phosphate; 20 mM disodium phosphate; 0.5 mM NaCl; pH 7.4) were then transferred to the micro-centrifuge tube. The mass ratio of cell suspension to glass bead was set at 1 : 1. The suspension was vortexed at 3000 rpm for 5 min followed by a cooling interval of 5 min on ice. The vortexing-cooling cycle of the suspension was repeated two times.

**Ultrasonication.** The ultrasonic disruption of *E. coli* cells was performed using an ultrasonic homogenizer (Cole Palmer, USA) equipped with a microtip stepped probe of 3 mm diameter. The ultrasonic homogenizer was operated at 20 kHz and 35% amplitude. The *E. coli* cells were incubated in an ice bath, and the total disruption period was 40 min, with intervals of 10 s off period for every 10 s operation time.15

**Calculation of power consumption for cell disruption process.** The power consumption for each of the cell disruption methods was calculated using eqn (2) as shown below:

\[
\text{Power consumption (kW)} = \frac{\text{watt} \times \text{no. of hour}}{1000} 
\]

**Time-course release of protein during 1-PrOH cell disruption.** A 20 mL of wet cell suspension (0.1 g mL⁻¹ wet cell weight) was subjected to the optimized 1-PrOH cell disruption condition [32.2% (v/v) 1-PrOH; pH 8.8; 25 °C] under rotational mixing at 35 rpm. At every 5 min over a total of 60 min, 500 μL sample was withdrawn from the suspended mixture of wet cells and 1-PrOH. The total protein content and the EGFP concentration in the collected samples were then analysed using the methods described below.

**Measurements of active EGFP and total protein.** The concentration of active EGFP was determined based on the
fluorescent intensity. The samples (100 µL) loaded in a black 96-well microplate were analyzed by a microplate fluorescence spectrometer (Infinite® 200 PRO, Tecan) at an excitation wavelength of 478 nm and an emission wavelength of 512 nm. The concentration of EGFP was calculated according to a calibration curve obtained via the EGFP standard solution. The protocol of preparation of EGFP standard solution was described in ESI.† The total protein concentration was measured with the BCA assay using BSA as a standard. To a 96-well microplate, 1 part of sample solution was mixed with 8 parts of BCA working reagent. The microplate was then incubated at 37 °C for 30 min. Subsequently, the reading of absorbance at 562 nm was taken by using a micro-plate reader (Sunrise model, Tecan). All samples were analysed in triplicate.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE was performed as described by Laemmli.14 The protein samples were separated in a SDS-polyacrylamide gel made of 5% stacking gel and 12% resolving gel. The electrophoresis was run at 110 V and 36 mA for 75 min using the Mini Protean 3 apparatus (Bio-Rad, USA). Then, the gel was incubated in a staining solution composed of 0.05% (v/v) of Coomassie Brilliant Blue R-250, 30% (v/v) methanol and 10% (v/v) acetic acid. The de-staining of gel was achieved by using a solution made of 30% (v/v) methanol and 10% (v/v) acetic acid.

Microscopy. The E. coli cells treated with different methods of cell disruption were viewed under a fluorescence microscope (Olympus IX81) at 40× magnification.

Statistical design of 1-PrOH cell disruption. The statistical design and analysis were performed using the Design Expert software (version 10, Stat-Ease, USA). Central composite design (CCD) was used to optimize the cell disruption process for the maximum recovery of EGFP. Three factors, namely percentage of alcohol (5–50%, v/v), pH (6–10) and temperature (5–45 °C), were varied over three levels and two additional alpha levels (1.682) to evaluate their effects on the release of EGFP and selective EGFP release from the permeabilized cells. The ranges of values for the investigated parameters were selected based on the EGFP stability test (see ESI: Fig. S1–S3†). A set of 20 experiments was carried out according to Table 1.

Results and discussion

Optimization of 1-PrOH cell disruption

For 1-PrOH cell disruption, operating parameters such as alcohol concentration, temperature and pH affect the active EGFP release and their influences may be either independent or interactive. RSM was adopted to identify the significant parameters and the mutual interactions among them. The CCD matrix along with the experimental runs is shown in Table 1. The results of RSM indicated that all three parameters (1-PrOH concentration, temperature and pH) were very significant (p < 0.001) for the active EGFP release, whereas the influences of temperature and pH on the total protein release were far greater than the parameter of 1-PrOH concentration (see Table S1: ESI†). It was observed that a considerably high level of total protein release was achieved regardless of the level of 1-PrOH concentration used (Table 1). The interactions between the factors on the releases of active EGFP can be observed from Fig. 1. A dome-shaped pattern of surface plot (Fig. 1A) indicates that the optimal active EGFP release can be achieved at the middle ranges of 1-PrOH concentration and temperature.

<table>
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<tr>
<th>Standard order</th>
<th>A: 1-PrOH concentration (% (v/v))</th>
<th>B: temperature (°C)</th>
<th>C: pH</th>
<th>Experimental value</th>
<th>Predicted value</th>
<th>Total protein release Experimental value</th>
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Table 1 CCD matrix of 1-PrOH cell disruption and its responses
temperature condition (55 °C) was used in the 1-PrOH cell disruption of E. coli, the EGFP release and the total protein release were low. It is well known that the tertiary or quaternary structure of a protein would be disrupted beyond a critical temperature, causing the denaturation of protein.\textsuperscript{3,24} A past study\textsuperscript{25} reported that the far-UV circular dichroism (CD) signal from GFP decayed as the temperature was raised to 80 °C. The losses in CD signal and the fluorescent intensity of GFP were attributed to the conformational instability of GFP, \textit{i.e.}, loss of secondary/tertiary structure of GFP.\textsuperscript{26} Moreover, the concentration of soluble proteins might be reduced by the aggregation of the released proteins due to the high temperature condition applied in the 1-PrOH cell disruption.

The RSM results showed that the alkaline pH (greater than 8) condition favoured the releases of active EGFP and total protein. In general, an extremely alkaline condition was responsible for the increase in permeability of bacterial cell, which has resulted in a partial protein release without a complete cell breakage.\textsuperscript{27} An alkaline treatment of bacteria cells at pH 11.5–12.5 for 20–30 min may cause the cell lysis.\textsuperscript{28} The alkaline hydrolysis of peptide bonds in proteins could be induced by the high pH and the elevated temperature. An extreme pH condition denatures the membrane channels proteins, which in turn increases the membrane permeability. The present study demonstrated that the acidic pH condition yielded a low protein release. At acidic pH, the metastable and fluorescent intensity of GFP variants reduced over time, especially at the elevated temperature.\textsuperscript{29}

The optimum condition for the maximum active EGFP release was achieved at 32.2% (v/v) 1-PrOH, 25 °C and pH 8.8. The predicted value of active EGFP release (\textit{i.e.}, 1.39 mg mL\textsuperscript{−1}) was close to the validated experimental results (\textit{i.e.}, 1.27 mg mL\textsuperscript{−1}) (see ESI: Table S2†), thereby confirming the effectiveness of the statistical design of experiments in optimizing the process parameters of 1-PrOH cell disruption. Based on the
high F-value (428.53) and the very low p-value (<0.0001) for the response of active EGFP release, the response surface quadratic model was suggested to be significant (i.e., with a high confidence level). The low standard deviation (0.035) and pure error \((5.33 \times 10^{-3})\) obtained for the model of active EGFP release indicates a good reproducibility of the experimental data. The equation of the fitted model in terms of coded factors for EGFP release is shown in eqn (3):

\[
Y = +1.26 - (0.13 \times A) - (0.18 \times B) + (0.29 \times C)
- (0.33 \times A^2) - (0.30 \times B^2) - (0.024 \times C^2)
- (0.051 \times A \times B) - (0.086 \times A \times C) - (0.14 \times B \times C)
\]

(3)

Versatility of 1-PrOH cell disruption

The 1-PrOH cell disruption can be optimized to suit the tolerance level of a target protein. For example, 1-PrOH cell disruption (see RSM standard order no. 5 from Table 1) is capable of releasing the active EGFP at low 1-PrOH concentration \((15\%, \text{ v/v})\) and low temperature \((15 \, ^\circ \text{C})\) but at a high pH \((9.8)\), whereby the active EGFP release was approximately 90\% of that achieved under mid-level operating conditions (i.e., average of the RSM standard order no. 15–20 from Table 1). Moreover, the 1-PrOH cell disruption conducted at pH 11 and at moderate levels of 1-PrOH concentration \((32.5\%, \text{ v/v})\) and temperature \((30 \, ^\circ \text{C})\) (see RSM standard order no. 14 from Table 1) achieved a relative active EGFP release of about 87\%. The versatility of 1-PrOH cell disruption method can further be noted from the high level of total protein release over the wide range of working parameters, i.e., 1-PrOH concentration \((3–62\%, \text{ v/v})\), pH \((8–11)\) and temperature \((15–30 \, ^\circ \text{C})\). Typical proteins have only a minimum tolerable range to organic solvent, and the versatility of this 1-PrOH cell disruption is of great importance because the target protein can be extracted according to its tolerance levels to the operating parameters of 1-PrOH cell disruption. For example, this method can be applied to the extraction of solvent-tolerable proteins such as interferon-\(\alpha\)-2b,\textsuperscript{29} lipase,\textsuperscript{30} and serine protease.\textsuperscript{31}

The release profiles of total protein and EGFP obtained from the RSM-optimized 1-PrOH cell disruption are shown in Fig. 2. A rapid release of active EGFP (67\%) and total protein (80\%) was observed after 5 min, while the maximal releases of active EGFP and total protein were achieved in less than 25 min.

In an attempt to assess the scalability of the optimized 1-PrOH cell disruption, a scale-up experiment was conducted by increasing the wet cell weight of \(E. \text{ coli}\) to 10 fold (i.e., from 0.1 g to 1 g). The active EGFP release and selective EGFP release for the scaled-up 1-PrOH cell disruption were found to be comparable to that for the small-scale 1-PrOH cell disruption (see Table 2), thereby showing the reliability of this 1-PrOH cell disruption for a larger scale of operation. As a whole, the developed 1-PrOH cell disruption system is anticipated to be a simple technique to disrupt a high volume of cells in a short period of time.

Performance and energy consumption for different cell disruption methods

The performances of 1-PrOH cell disruption and other mechanical methods, i.e., glass bead vortexing and ultrasonication were compared, and the result is shown in Table 2. The glass bead vortexing yielded the poor results of active EGFP release \((0.71 \, \text{mg mL}^{-1})\) and selective EGFP release \((0.17 \, \text{mg mL}^{-1})\). In comparison, the optimum 1-PrOH cell disruption showed a 2-fold increase in both active EGFP release and selective EGFP release than the glass bead vortexing method. For ultrasonication, the active and selective EGFP releases were 1.35 mg mL\(^{-1}\) and 0.19 mg mL\(^{-1}\), respectively. The active EGFP release obtained from 1-PrOH cell disruption was close to that from the ultrasonication treatment. Nonetheless, 1-PrOH cell disruption excels the ultrasonication in term of selective EGFP release. The samples extracted by the investigated cell disruption methods were also analysed by SDS-PAGE (see Fig. 3). All the protein samples (lanes 1–3) shared the similar protein band profiles. The EGFP band was clearly observed at about 27 kDa in all the protein samples. The band of EGFP in lane 2 (i.e., from glass bead vortexing) was noticeably less intense than that of lane 1 (i.e., 1-PrOH cell disruption) and lane 3 (i.e., ultrasonication); this confirmed the result of low active EGFP release \((0.71 \, \text{mg mL}^{-1})\) acquired by glass bead vortexing. In addition, the bands of contaminants in lane 1 (i.e., 1-PrOH cell disruption) were fainter than those in lane 2 (i.e., glass bead vortexing) and lane 3 (i.e., ultrasonication). The EGFP release achieved by the 1-PrOH cell disruption was proved to be highly selective because the total intracellular proteins from the cells are not completely released.

These quantitative results also corroborated the fluorescent intensity of the treated \(E. \text{ coli}\) cells (see the insets of Fig. 4) obtained from 1-PrOH cell disruption (i.e., faint fluorescence), glass bead vortexing (i.e., bright fluorescence) and ultrasonication (i.e., minimal fluorescence). In comparison to the non-treated \(E. \text{ coli}\) sample (Fig. 4A), a small reduction in the count of fluorescent rod-shape \(E. \text{ coli}\) cells was observed in the sample undergone glass bead vortexing (Fig. 4C). Contrastively, the fluorescent integral \(E. \text{ coli}\) cells were hardly found in the samples treated by either ultrasonication (see Fig. 4B) or 1-PrOH (see Fig. 4D), thus proving that the active EGFPs were released as soluble protein from the cells to the surrounding.
The power consumption for 1-PrOH cell disruption, glass bead vortexing and ultrasonication is presented in Table 2. The 1-PrOH cell disruption had the lowest power consumption demand. The power consumption for ultrasonication was approximately 4 times greater than the 1-PrOH cell disruption, while the glass bead vortexing method consumed about twice the amount of power than the 1-PrOH cell disruption. However, the selective EGFP release achieved by both ultrasonication and glass bead vortexing methods was only half of that from the 1-PrOH cell disruption.

Apparently, 1-PrOH cell disruption consumed less power as compared to glass bead vortexing and ultrasonication. The application of ultrasonication in cell disruption is mainly limited to the laboratory scale because of the high operation cost and the limitation in handling a large processing volume. In general, ultrasonication treatment is not energy efficient because most of the kinetic energy imparted to the cell medium is transformed into heat energy. The excess heat generated around the proximity of the sonication probe often leads to the degradation of protein. During ultrasonication, cells that are impacted directly by the ultrasonic waves will be disrupted, while turbulence caused by the ultrasonic wave will push other non-disrupted cells away. As the number of intact cells reduced exponentially, the ultrasonic wave energy will act on the breakage of the cell fragments instead on the remaining whole cells. In addition, the increasing fragments of cells present in the solution can inhibit the propagation of ultrasonic waves, thus reducing the effectiveness of ultrasonication process.

For glass bead vortexing, one of the typical concerns over the lysis process is the increase in solution’s viscosity, leading to a less pronounced abrasive force and a drop in disruption efficiency. Previous studies also reported that cell disruption based on glass bead vortexing generally yielded a low level of protein release (∼50%). On the other hand, alcohol-based cell disruption demonstrated in this study does not rely on special or high-end equipment, making the technique within reach of the most budget-conscious laboratories. In general, alcohol and rotator mixer are commonly available in most of the well-established fermentation laboratories. Best of all, the alcohol-based cell disruption is possible to be conducted at room temperature, hence eliminating the need of cooling system during the operation.

### Conclusions

The efficiency of 1-PrOH cell disruption in releasing the intracellular EGFP was demonstrated. A high relative EGFP release can be achieved over a wide range of disruption parameters [3–62% (v/v) 1-PrOH, pH 8–11 and 15–30 °C], showing the

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<th>Cell disruption methods</th>
<th>Wet cell weight (g)</th>
<th>Working volume (mL)</th>
<th>Active EGFP release (mg mL⁻¹)</th>
<th>Selective EGFP release (mg mg⁻¹)</th>
<th>Power consumption (kW)</th>
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<td>1-PrOH treatment</td>
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<td>Glass bead vortexing</td>
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![Fig. 3](image3.png)

**Fig. 3** SDS-PAGE of protein samples collected from 1-PrOH cell disruption, glass bead vortexing, and ultrasonication. Lane M: protein markers (11–190 kDa); lane 1: sample from 1-PrOH cell disruption (based on 0.1 g mL⁻¹ wet cell weight); lane 2: sample from glass bead vortexing; lane 3: sample from ultrasonication; lane 4: EGFP standard (~27 kDa).

![Fig. 4](image4.png)

**Fig. 4** Fluorescence micrographs (40×) of *E. coli* cells disrupted by different methods: (A) non-treated; (B) ultrasonication; (C) glass bead vortexing; and (D) 1-PrOH treatment. Right inset of each micrograph shows the colour of the cell pellet obtained after the cell disruption process.
versatility of this alcohol-based cell disruption. The active EGFP release and the selective EGFP release obtained from 1-PrOH cell disruption were comparable to that from ultrasonication treatment. The 1-PrOH cell disruption was also less energy intensive as compared to ultrasonication and glass bead vortexing methods, hinting that a lower cost of operation can be achieved even at large-scale cell disruption process. The flexibility of process parameters, combined with its economical and rapid operation, make the 1-PrOH cell disruption expandable to the disruption of E. coli expressing other types of intracellular recombinant proteins. Ultimately, the potential of this 1-PrOH cell disruption strategy in replacing the conventional methods of cell disruption can be further pursued.

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