

Extraction of recombinant proteins from *Escherichia coli* by cell disruption with aqueous solutions of surface-active compounds

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Abstract

BACKGROUND: Green fluorescent protein (GFP) is used extensively as a biomarker due to its unique spectral and fluorescence characteristics. GFP is usually obtained from recombinant strains of *Escherichia coli* (*E. coli*) producing the protein intracellularly. However, the common methods of extraction are cumbersome leading to an increase in the downstream process costs and complexity, sometimes leading to a higher risk of biomolecule degradation.

RESULTS: This work proposes a new method to extract recombinant intracellular GFP from *E. coli* BL21 by using aqueous solutions of surface-active compounds.

CONCLUSION: By comparing the fluorescence intensity of the extracted GFP, it was concluded that some of these compounds, namely ionic liquids with an alkyl chain of 10 or more carbons (best solvent being tributyl-1-tetradecylphosphonium, [P_{4,4,4,14}]Cl) are more effective than an ultrasonic-assisted extraction, even at low concentrations, being able to extract the whole GFP content from the cells.

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Keywords: green fluorescent protein; recombinant proteins; extraction; intracellular protein; surface-active compounds; cell disruption

INTRODUCTION

The green fluorescent protein (GFP) is a protein that has been widely used for many biotechnical applications in which it serves as marker for gene expression and tracking of analyses within living cells or organelles.^{1,2} GFP was first isolated from the bioluminescent jellyfish *Aequorea victoria*, being successfully expressed in many organisms such as bacteria, fungi, plant, and animal cells.³ However, like most of the other recombinant proteins, GFP is usually expressed in the *Escherichia coli* (*E. coli*) strain, the most common heterologous system for protein expression.⁴ The GFP has been recognized for its high stability towards denaturing conditions/compounds, such as temperature, pH, proteases, organic solvents, detergents, or chaotropic agents like urea up to 8 mol L⁻¹.^{3,5} In addition, GFP has a unique spontaneous and natural emission of green light fluorescence when excited by UV light,⁶ which is useful in real-time imaging applications. However, since GFP is produced intracellularly in *E. coli*, an additional cell disrupting stage is required. The available methodologies for the recovery of intracellular products from cells can be either mechanical (e.g. multiple cycles of freezing/thawing⁷ and/or ultrasonic homogenization⁸) or non-mechanical (enzymatic treatments⁹ or with organic solvents¹⁰). However, these methods have major drawbacks, such as long processing time, high consumption of energy, and/or risk of biomolecules degradation. Moreover, when using the enzymatic treatments, there is an increase of protein

contaminants in the final extract that will hinder downstream additional steps.⁹

Above a lower critical concentration, some amphiphilic compounds were proven to disrupt cell membranes due to their interaction with lipid bilayers. Depending on the type of compounds and their concentration, the changes in cell membrane can lead to permeability alteration, or even the entire disintegration of the membrane.¹¹ The amphiphilic compounds previously mentioned include tensioactive ionic liquids (ILs).^{11–14} This class of compounds has been proposed for a wide range of different applications, from detergent formulation to biomedicine applications, due to their main characteristics. The low-charge density and low symmetry of their ions lead to their melting points close to room

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temperature.¹⁵ ILs are usually known as 'designer solvents' due to the possibility to design an IL with a set of properties for a given application.¹⁶ This versatility allows control of their structure and hydrophilic–lipophilic balance, leading to the design of a new class of surfactants.¹⁶ Besides, these aqueous raw extracts composed of surfactants and extracted molecules can be easily integrated in a downstream process using an aqueous micellar two-phase system (AMTPS). This platform allows the formation of two distinct phases when a certain concentration of a tensioactive compound is combined with a temperature increase, and thus, allowing the fractionation of the molecules in between the two phases, and consequently, the concentration and purification of the target compound.¹⁷

In this work, a new method of GFP extraction from *E. coli* BL21 cells by using tensioactive compounds is developed. GFP is used in this work as a model protein being produced intracellularly. Aqueous solutions of common surfactants and tensioactive ILs were evaluated for their ability to release the intracellular GFP by their action on the cell membranes. To obtain further insights about the cell disrupting agents' mechanisms over the *E. coli* membrane, and the morphologic changes of the cell walls were evaluated by scanning electron microscopy (SEM). The results are compared with those obtained by an optimized conventional ultrasonic-assisted extraction.

MATERIALS AND METHODS

Materials

The microorganism *E. coli* BL21(DE3) pLysS carrying the plasmid pET-28(a) encoding the gene for expression of GFP recombinant was kindly provided by the Molecular and Cellular Biology Laboratory of the School of Pharmaceutical Sciences from Universidade Estadual Paulista 'Júlio de Mesquita Filho'.

The components of Luria-Bertani (LB) culture media, namely tryptone and yeast extract, were purchased from Oxoid while NaCl was acquired from Panreac. The antibiotics used in cell culture, kanamycin disulfate salt from *Streptomyces kanamyceticus* and chloramphenicol (≥ 98 wt%), were supplied by Sigma as well as the isopropyl β -D-1-thiogalactopyranoside (IPTG, ≥ 99 wt%). The components of the Tris–HCl buffer, namely tris(hydroxymethyl)aminomethane and chloridric acid (37%), were acquired from Pronalab and Sigma-Aldrich, respectively.

The series of 1-alkyl-3-methylimidazolium chloride ILs [C_nmim]Cl, including the 1-butyl-3-methylimidazolium chloride, [C₄mim]Cl (99 wt%), 1-hexyl-3-methylimidazolium chloride, [C₆mim]Cl (98 wt%), 1-methyl-3-octylimidazolium chloride, [C₈mim]Cl (99 wt%), 1-decyl-3-methylimidazolium chloride, [C₁₀mim]Cl (98 wt%), 1-dodecyl-3-methylimidazolium chloride, [C₁₂mim]Cl (98 wt%), 1-methyl-3-tetradecylimidazolium chloride, [C₁₄mim]Cl (98 wt%), 1-hexadecyl-3-methylimidazolium chloride, [C₁₆mim]Cl (98 wt%), as well as 1-methyl-1-propylpiperidinium chloride, [C₃mpip]Cl (99 wt%), 1-butylpyridinium chloride [C₄py]Cl (98 wt%), and 1-butyl-1-methylpyrrolidinium chloride, [C₄mpyr]Cl (99 wt%) were supplied by IoLiTec. The phosphonium-based ILs such as tetrabutylphosphonium chloride, [P_{4,4,4,4}]Cl (> 95 wt%), tributyl-1-tetradecylphosphonium, [P_{4,4,4,14}]Cl (97 wt%), and tetrabutylphosphonium bromide, [P_{4,4,4,4}]Br (> 95 wt%), were provided by Cytec. The series of ammonium ILs such as tetrabutylammonium chloride, [N_{4,4,4,4}]Cl (97 wt%), trimethyldecylammonium chloride, [N_{1,1,1,10}]Cl (98 wt%), and hexyltrimethylammonium bromide, [N_{1,1,1,6}]Br (98 wt%), were purchased from Sigma-Aldrich, TCI, and Alfa Aesar, respectively. Other

surfactants such as dodecyltrimethylammonium bromide, [N_{1,1,1,12}]Br (98 wt%), myristyltrimethylammonium bromide, [N_{1,1,1,4}]Br (99 wt%), hexadecylpyridinium chloride, [C₁₆py]Cl (99 wt%) dioctyl sulfosuccinate sodium salt, AOT (98 wt%), sodium dodecyl-benzenesulfonate, SDBS, polyethylene glycol hexadecyl ether, Brij C10, polyethylene glycol oleyl ether, Brij 93, polyethylene glycol dodecyl ether, Brij L4, polyethylene glycol sorbitan monooleate, Tween 20, and polyethylene glycol sorbitan monooleate, Tween 80, and Merpol A were supplied by Sigma-Aldrich. Sodium dodecylsulfate, SDS (pure), and polyethylene glycol *tert*-octylphenyl ether, Triton X-114 were purchased from Acros Organics. The chemical structures are depicted in Supporting information (Figs A1 to A4).

Cell cultivation for production of GFP

Initially, the *E. coli* BL21(DE3) pLysS was transferred to a Petri dish containing LB agar and IPTG (inductor of fluorescent protein in cells production) to a final concentration of 0.5 mmol L⁻¹ and 50 μ g mL⁻¹ of the kanamycin and chloramphenicol antibiotics. The plate was maintained at 37 °C for 24 h. Then, a colony was transferred to 25 mL of LB medium containing both antibiotics (kanamycin and chloramphenicol at 50 μ g mL⁻¹) and incubated overnight at 150 rpm and 37 °C in an orbital shaker (New Brunswick™ Excella® E24 Incubator), in which Erlenmeyer flasks with no baffles were used. 50 mL of fresh media was then inoculated with 2.5 mL of overnight fermented inoculum [optical density at 600 nm (OD₆₀₀) of 0.1] and grown at 37 °C and 150 rpm in the same orbital shaker. After incubation for 6 h, the IPTG (0.25 mmol L⁻¹) was added to induce the GFP production. After 17 h of protein induction, the fermented broth was centrifuged in a Thermo Scientific Heraeus Megafuge 16 R Centrifuge at 2655 *g* for 30 min at 4 °C. Then, the supernatant was discharged and the weight of wet cells determined. The cell pellets containing GFP were resuspended in 50 mmol L⁻¹ of Tris–HCl buffer (pH 8) at approximately 0.025 wet cells (wt%).

GFP release: cell disruption

First, a conventional method of cell disruption for *E. coli* BL21 was adapted and optimized from literature.¹⁸ The wet cells at 0.025 wt% were homogenized by a digital ultrasonic cell disrupter (Branson 450 Digital Sonifier) at an amplitude of 40% (corresponding to 112 W). During the ultrasonication, to avoid temperatures higher than 40 °C preventing protein denaturation, the cell suspension was maintained in an ice-water bath. The number of cycles of the ultrasonication process was optimized using pulses of 5 s ON/5 s OFF. Once the number of cycles was optimized and fixed, the effect of the ON/OFF interval time was also studied.

An alternative method of GFP release was designed using aqueous solutions of tensioactive surface compounds. A screening of common surfactants (cationic, anionic, and non-ionic) and ILs with different properties (cations, anions, and alkyl chain lengths for different cations) was performed to assess their ability to permeabilize the cell membrane, releasing the GFP content to the extracellular medium (chemical structures of all studied compounds are presented in Supporting information, Figs A1 to A4). The cell suspension (at 0.025 wt% of wet cells) was mixed with surfactants and ILs to a final concentration of 250 mmol L⁻¹, except for SDBS in which 100 mmol L⁻¹ was used due to solubility limits. The samples were subjected to constant stirring (25 rpm) for 30 min in an orbital mixer and at room temperature. The tensioactive compounds with the best cell disrupting performance were selected to

evaluate the effect of the surfactant/IL concentration (50, 100, 250 and 500 mmol L⁻¹) on cell lysis and GFP release.

The cell suspensions were centrifuged at 12 000 rpm for 10 min in a VVR microstar 17 centrifuge, and the pellet containing the cellular debris was used for SEM and cells viability analyses.

Analytical methods

Protein content analysis

The lysate supernatant was analyzed in term of GFP content, measured by the fluorescence in the maximum of the variant used (GFPuv) (excitation at 485 nm and emission at 530 nm) in a microplate reader (Synergy HT microplate reader – BioTek). The sonicated samples were also analyzed in terms of total protein content present by measuring their absorbance at 280 nm in the same microplate reader.

Scanning electron microscopy (SEM)

Cell debris were fixed with glutaraldehyde at room temperature. 50 μL of glutaraldehyde (2% (v/v) in distilled water) was added to a 0.5 mL of each sample. After 2 h, the samples were washed three times with distilled water and were then freeze dried. The specimens were deposited on carbon conductive tape and coated with a carbon layer before observation by SEM using a Hitachi SU-70 microscope operating at 15 kV.

RESULTS AND DISCUSSION

Conventional cell disruption: ultrasonication

Figure 1 represents the GFP and total proteins released to the extracellular medium as a function of the number of ultrasonication cycles applied.

As shown in Fig. 1 the amount of GFP released increased (fluorescence intensity parameter) with the number of cycles up to 30 ultrasonication cycles (corresponding to 5 min), being almost constant after that. Since the trends of GFP and total proteins (including the contaminants) releases were identical, 30 cycles of ultrasonication were adopted for further studies and as a benchmark.

Thus, to determine if ON/OFF ultrasonication interval in each cycle influenced the GFP and protein release, a new set of 30 cycles of ultrasonication was performed by maintaining the 5-s ON time as a constant condition and varying the OFF time (5, 10, 15, and 20 s). It is shown (Fig. 2) that for longer OFF times (5 s ON, 10, 15 and 20 s OFF), there was a slight increase of the GFP content in the extracts.

Since the effective ultrasonication time (ON) applied to cells was the same (5 s), this increase of GFP release was not expected. Thus, it is evident that the high GFP content at longer OFF times in the supernatant was not promoted by better cell disruption, but with shorter OFF intervals (5 s) some GFP denaturation occurred, decreasing the overall GFP concentration. Therefore, the standard method of cell disruption using ultrasonication was optimized to a total of 30 cycles, each cycle with 5 s ON/10 s OFF (corresponding to total of 7.5 min).

Alternative cell disruption: aqueous solutions of surface-active compounds

The study of an alternative GFP extraction process using aqueous solutions of surface-active compounds to recover the GFP from the intercellular environment was carried out. The screening of the various surface-active compounds over a cell suspension

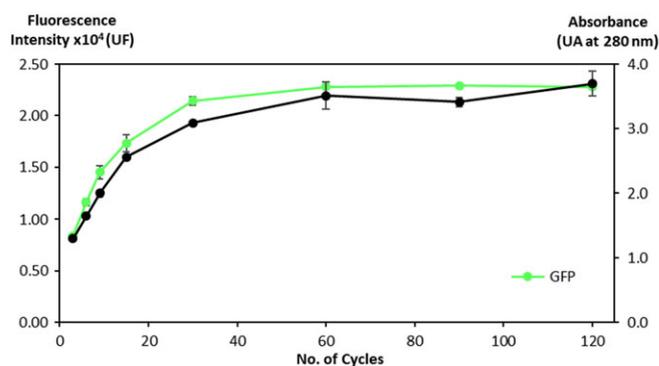


Figure 1. GFP fluorescence intensity in units of fluorescence (UF) and total proteins in units of absorbance (UA) release as a function of the number of ultrasonication cycles (5 s ON/5 s OFF) applied. The fluorescence was measured using excitation at 485 nm and emission at 530 nm.

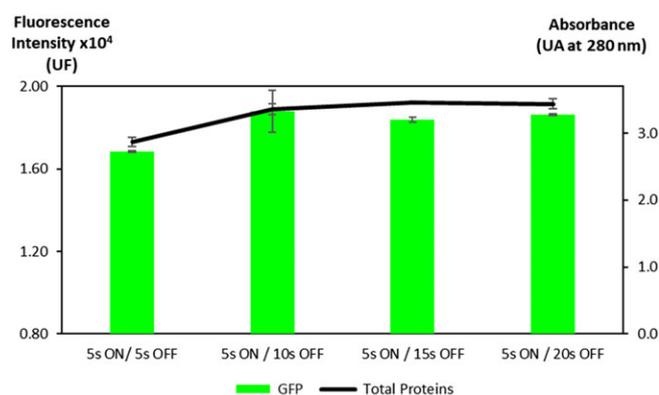


Figure 2. GFP and total protein release in assays with pulses of 5 s (time ON), different OFF times and a fixed total of 30 cycles. The fluorescence was measured using excitation at 485 nm and emission at 530 nm.

(≈ 0.025 wt% of wet cells) was performed. The results of the alternative disruption methodology were compared with the conventional method previously optimized (ultrasonication), as can be observed in Fig. 3.

A comparative assay using only the Tris–HCl buffer was also performed to ensure that the medium in which the cells were suspended was not able to promote cell disruption. The results reported in Fig. 3 show that the Tris–HCl buffer has no effect on GFP extraction from the *E. coli* BL21 cells. They also show that the non-tensioactive ILs tested were not effective in the GFP release. Similarly, the non-ionic surfactants were unable to permeabilize the cell membrane, and lead to the GFP release.

On the other hand, the cationic surfactants, in particular the tensioactive ILs, with alkyl chain lengths larger than 8 carbon atoms, allow the disruption of cell membranes. Several studies have suggested that the interactions between these amphiphilic compounds and cell membranes and their proteins, promote cell changes, namely expansion and/or permeabilization, leading to the release of intracellular material and cell disruption.^{11,20}

As can be observed in Fig. 3 not all the tensioactive compounds have the ability to induce these changes in cell permeability, which is expressed by low GFP fluorescence intensity in the lysate supernatant. Comparatively, the anionic tensioactive compounds have poor efficiency of GFP release. This may be explained by the repulsive electrostatic interactions between the anionic surfactants and the negatively charged phospholipids.¹² Moreover,

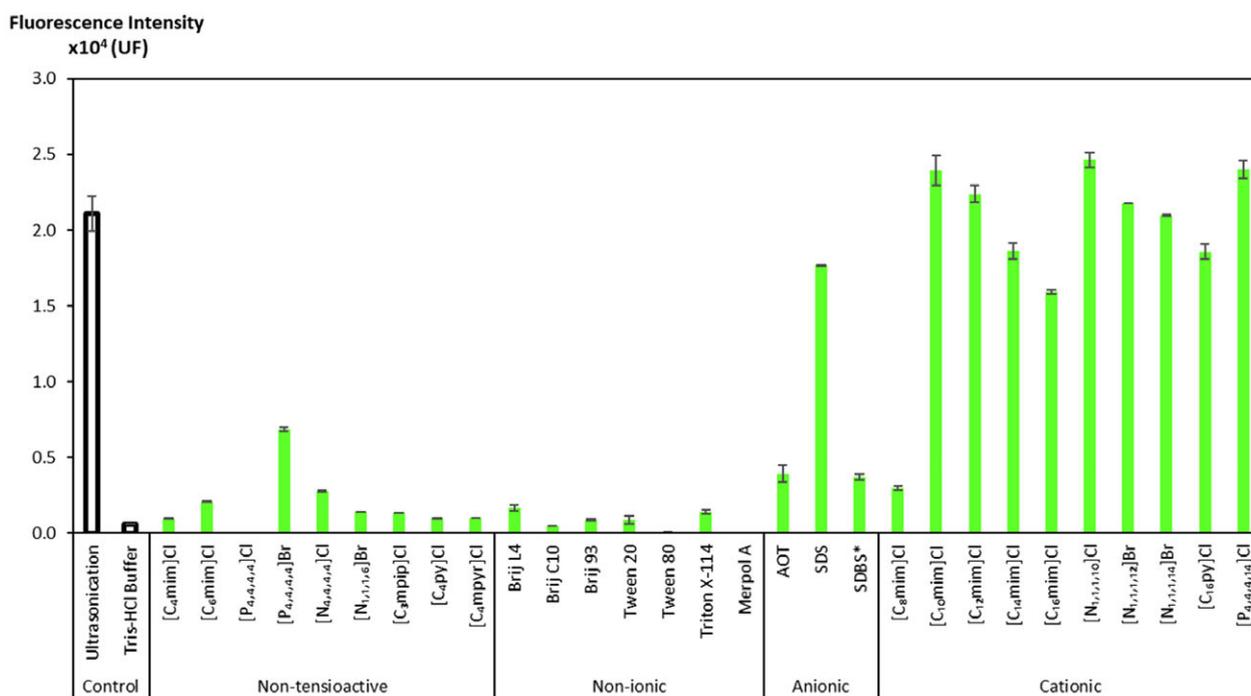


Figure 3. Release of GFP using common disruptor techniques (ultrasonication and Tris-HCl buffer – black empty bars) and aqueous solutions of surfactants and ILs (green full bars), with a concentration of 250 mmol L⁻¹ (except for SDBS, with 100 mmol L⁻¹) after 30 min stirring (25 rpm) at 25 °C. The fluorescence was measured using excitation at 485 nm and emission at 530 nm. The results represent the average of two independent experiments with the corresponding standard deviation errors.

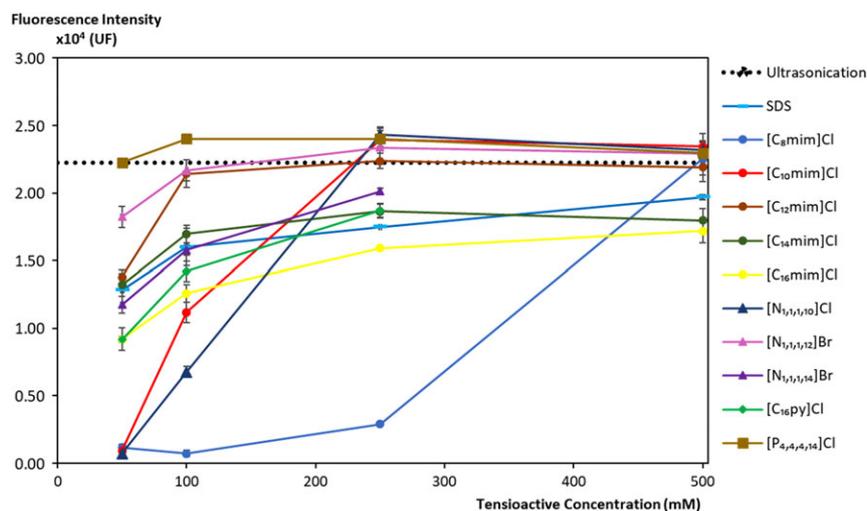


Figure 4. Influence of concentration (mmol L⁻¹) of tensioactive aqueous solution on GFP release. Results at 500 mmol L⁻¹ of [N_{1,1,1,12}]Br and [N_{1,1,1,14}]Br were not obtained due to solubility limitations. The fluorescence was measured using excitation at 485 nm and emission at 530 nm.

according to Yoo *et al.*,¹⁴ the more hydrophilic anionic surfactants will more freely disperse in the aqueous environment, reducing the contact with the cells and thus exhibiting less disruptive effect. A further evaluation of the effect of tensioactive ILs on the *E. coli* BL21 cells show, for the imidazolium and ammonium families studied, that the dominant effect was the interaction of the alkyl chain with the cell membrane, which plays a key role in the permeation of GFP. Actually, the compounds better performing GFP extraction have in their constitution a single alkyl chain composed of 10 carbons. With the increase of the length of the alkyl chains, namely chains composed of 12, 14, and 16 carbons, a progressive decrease in GFP release to the extracellular environment occurred.

This may be explained by the decrease of the solubility of compounds with higher alkyl chains. Moreover, the performance of these extractions is more efficient, in term of GFP release, than that from ultrasonic-assisted extraction previously optimized.

Depending on the concentration of surfactant used, it may lead to either swelling of the lipid bilayers or even, at higher concentrations, cell disruption and the formation of surfactant-lipid micelles in aqueous suspensions.¹¹ Thus, having selected the compounds with better GFP release performances, the effect of tensioactive compounds concentration in aqueous solution was studied at concentrations ranging from 50 to 500 mmol L⁻¹, as reported in Fig. 4.

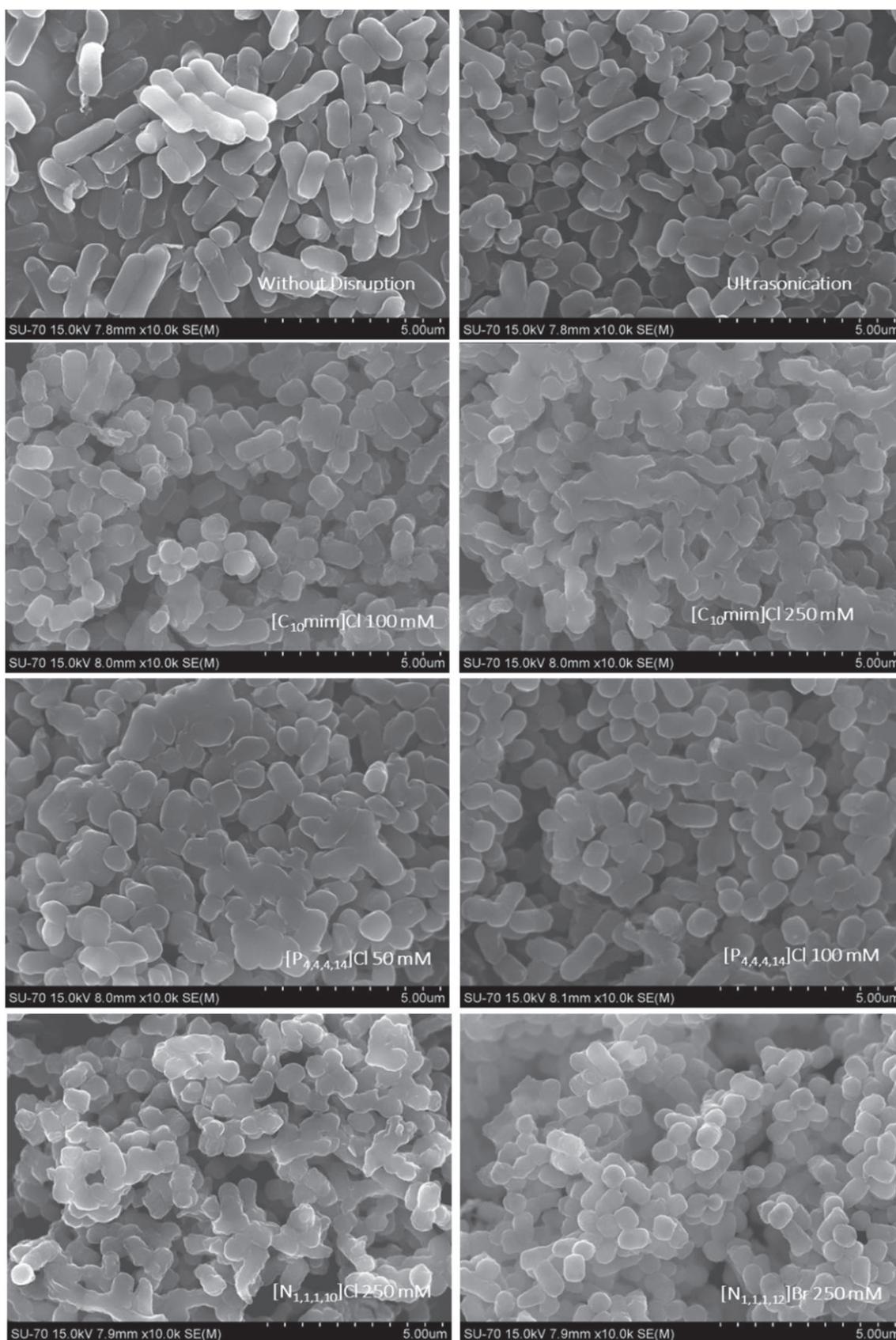


Figure 5. Scanning electron microscopy (SEM) images of *E. coli* cells (or cell debris) under different conditions: without disruption (control); after ultrasonic treatment; after treatment with $[C_{10}mim]Cl$ at 100 mmol^{-1} , $[C_{10}mim]Cl$ at 250 mmol^{-1} , $[P_{4,4,4,14}]Cl$ at 50 mmol^{-1} , $[P_{4,4,4,14}]Cl$ at 100 mmol^{-1} , $[N_{1,1,1,10}]Cl$ and $[N_{1,1,1,12}]Br$ at 250 mmol^{-1} , respectively.

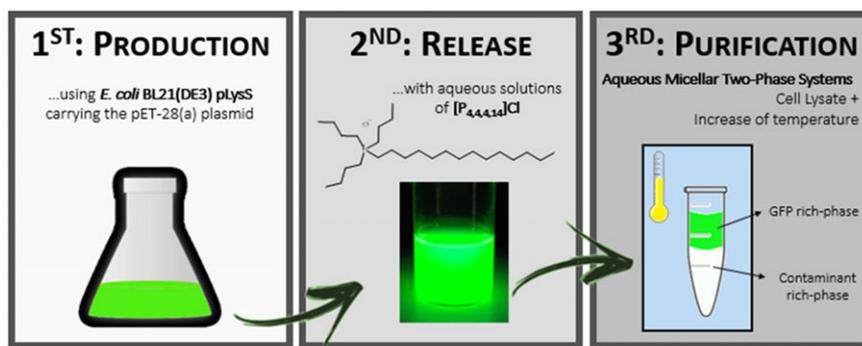


Figure 6. Example of complete downstream process from GFP production to purification.

In general, for most of the compounds tested up to a concentration of 250 mmol L^{-1} , the fluorescence of the extract then remaining almost constant for higher concentrations. Two exceptions occurred with $[\text{C}_8\text{mim}]\text{Cl}$ and $[\text{P}_{4,4,4,14}]\text{Cl}$ aqueous solutions. It is clear that when using concentrations of tensioactives below or even close to their critical micelle concentration (CMC) values, the extraction is not successful, explaining the poor results obtained at $50, 100, \text{ and } 250 \text{ mmol L}^{-1}$ of $[\text{C}_8\text{mim}]\text{Cl}$, 50 mmol L^{-1} of $[\text{C}_{10}\text{mim}]\text{Cl}$, and 50 mmol L^{-1} of $[\text{N}_{1,1,1,10}]\text{Cl}$ (further information about CMCs is detailed in Supporting information, Table A1).

However, it is important to note that there is not necessarily an increase in GFP extraction with the decrease of the CMC. On the other hand, using $[\text{P}_{4,4,4,14}]\text{Cl}$ as cell disrupting agent, 50 mmol L^{-1} was enough to release the same amount of GFP as that from the ultrasonic-assisted extraction, the maximum GFP release being attained at the concentration 100 mmol L^{-1} . Considering the overall results, the best system to promote the GFP release is the use of $[\text{P}_{4,4,4,14}]\text{Cl}$ at 100 mmol L^{-1} .

To obtain further information about the mechanisms behind the GFP extraction, some samples of *E. coli* BL21 cells and cell debris undergoing different treatments were analyzed using SEM, and compared with whole bacterial cells (without any disruption treatment). The morphology of the cells and debris without any disruption treatment, after ultrasonication, and after exposure to different tensioactive agents at different concentrations are compared in Fig. 5.

As expected, the images of the control presented the rod-shaped characteristic structure of *E. coli* cells. For the cells exposed to the ultrasonic treatment it is possible to distinguish some plasmolized cells and the presence of smaller cells with spherical morphology. In addition, the cells after exposure to the different tensioactive compounds seemed to lose the rod-shaped structure, giving rise to smaller spherical cells, perhaps due to disruption and the release of intracellular material including the GFP.

The viability of the samples studied previously by SEM was evaluated by their ability to re-grow in a LB solid-agar medium containing the same antibiotic content as the cultivation medium. Only the cells that did not suffer any disruption and the cells that were subject to ultrasonic treatment were capable of growing, which proves that the use of these surface-active compounds leads to severe changes in the cells including its irreversible disintegration and death.

CONCLUSION

In this work a simple and efficient methodology to extract GFP from a strain of recombinant *E. coli* BL21 was developed. A

conventional method using an ultrasonic-assisted extraction was first optimized and used as a benchmark for the new methodology proposed. Aqueous solutions of ILs and surfactants were used to induce the GFP release from the bacteria cells. From the tensioactive compounds studied, the cationic structures, namely the compounds with alkyl chains of more than 10 carbons induced the full release of GFP from the cells for concentrations above their CMC values. The best disrupting agent found was the $[\text{P}_{4,4,4,14}]\text{Cl}$, which promoted the total release of GFP using aqueous solutions with a concentration of only 100 mmol L^{-1} . Some of these tensioactive compounds were shown to be even more effective for GFP extraction than the ultrasonic-assisted extraction. The cells were evaluated in terms of: (i) their morphology by SEM; and (ii) viability, being demonstrated that the tensioactive compounds promoted irreversible changes in the cells originally rod-shaped, inducing the formation of smaller spherical structures with no cellular viability.

As final remarks, in this work, the treatment of *E. coli* cells using tensioactive agents was shown to be advantageous not only because of the high capacity to disrupt the cells and release the cytoplasmic content, but also the potential of this chemical-based disruption method in overcoming the limitations of upscaling the ultrasonication technologies. Thus, the proposed approach appears to be a promising, simple and effective method of cells (*E. coli*) disruption for industrial application. Apart from that, the use of tensioactive aqueous solutions as solvents can also be easily integrated with further stages of downstream processing by using aqueous micellar two-phase systems,^{17,21} as schematized in Fig. 6.

Associated Content

Supporting information. General information of the surface-active compounds structures used, as well as their CMCs, and experimental results on the cell viability after GFP release using the best surface-active compounds.

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Supporting Information

Supporting information may be found in the online version of this article.

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