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Selective Permeation and Organic Extraction of Recombinant Green Fluorescent Protein (gfp_{uv}) from *Escherichia coli*

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Abstract

Background: Transformed cells of *Escherichia coli DH5-α* with pGFPuv, induced by IPTG (isopropyl-β-d-thiogalactopyranoside), express the green fluorescent protein (gfp_{uv}) during growth phases. *E. coli* subjected to the combination of selective permeation by freezing/thawing/sonication cycles followed by the three-phase partitioning extraction (TPP) method were compared to the direct application of TPP to the same culture of *E. coli* on releasing gfp_{uv} from the over-expressing cells.

Material and Methods: Cultures (37°C/100 rpm/ 24 h; $\mu = 0.99\ h^{-1}$ - $1.10\ h^{-1}$) of transformed (*pGFP*) *Escherichia coli DH5-α*, expressing the green fluorescent protein (gfp_{uv}, absorbance at 394 nm and emission at 509 nm) were sonicated in successive intervals of sonication (25 vibrations/ pulse) to determine the maximum amount of gfp_{uv} released from the cells. For selective permeation, the transformed previously frozen (-75°C) cells were subjected to three freeze/thaw (-20°C/ 0.83°C/min) cycles interlaid by sonication (3 pulses/ 6 seconds/ 25 vibrations). The intracellular permeate with gfp_{uv} in extraction buffer (TE) solution (25 mM Tris-HCl, pH 8.0, 1 mM β-mercaptoethanol β-ME, 0.1 mM PMSF) was subjected to the three-phase partitioning (TPP) method with *t*-butanol and 1.6 M ammonium sulfate. Sonication efficiency was verified on the application to the cells previously treated by the TPP method. The intra-cell releases were mixed and eluted through methyl HIC column with a buffer solution (10 mM Tris-HCl, 10 mM EDTA, pH 8.0).

Results: The sonication maximum released amount obtained from the cells was 327.67 μg gfp_{uv}/ mL (20.73 μg gfp_{uv}/mg total proteins – BSA), after 9 min of treatment. Through the selective permeation by three repeated freezing/thawing/sonication cycles applied to the cells, a close content of 241.19 μg gfp_{uv}/mL (29.74 μg gfp_{uv}/mg BSA) was obtained. The specific mass range of gfp_{uv} released from the same cultures, by the three-phase partitioning (TPP) method, in relation to total proteins, was higher, between 107.28 μg/mg and 135.10 μg/mg.

Conclusions: The selective permeation of gfp_{uv} by freezing/thawing/sonication followed by TPP separation method was equivalent to the amount of gfp_{uv} extracted from the cells directly by TPP; although selective permeation extracts showed better elution through the HIC column.

Background

The fluorescent green protein, *gfp* extracted from *Aequorea victoria* shows a maximum excitation under ultraviolet light (UV) in the interval of λ = 395–498 nm and emission in the interval of λ = 490–520 nm, with the maximum peak at λ = 508–509 nm. The recombinant fluorescent green protein, gfp_{uv}, expressed by transformed (*pGFP*, Clontech) cells of *Escherichia coli*, was developed by introducing point mutations in an *in vitro* wild *gfp* DNA, replacing three amino acids (Phe^{99} for Ser; Met^{153} for Thr; and Val^{163} for Ala, based on the amino acid numbering of wild *gfp*). The resulting gfp_{uv} is expressed two to three times faster in *E. coli* strains and shows 18 times brighter fluorescence than the native *gfp;* the maximum peaks for fluorophore excitation being at 395 nm and centered at 508–509 nm for emission [1].

The recombinant gfp_{uv} is a globular protein, compact and acidic, made up of 27 kDa monomers (with 238 amino acids), having a propensity to dimerize. The protein fluorescence is stable between pH 5.5 and 11.5, with the optimum at pH 8.0.

The first step for the gfp_{uv} purification process was the intracellular release and isolation of the protein into an aqueous extraction buffer – TE (25 mM Tris-HCl, pH 8.0, 1 mM β-mercaptoethanol, "β-ME", 0.1 mM phenylmethylsulfonylfluoride, "PMSF"). During extraction procedures, the proteolysis of the C-terminal extremity of gfp_{uv} was inhibited by working at cool temperatures (O°C to 4°C), and in the presence of PMSF. The addition of β-ME and the maintenance of pH at 8.0 enhances protein stability [1].

Several methods are used currently for cellular partial (permeation) or total cellular disruption. For sake of convenience, they may be classified as (i) physical mechanical (ultra sonication, bead mill, blade and high-pressure homogenizers) and non-mechanical treatments (thermal shock, freeze and thaw); and a combination (freezing/thawing cycles interlaid with sonication); (ii) chemical methods for selective protein fractionation by the use of (a) organic solvent (alcohol, toluene, acetone); (b) inorganic acids and bases (pH variation) and salts (salting in and salting out phenomena); (c) combination of salting out (ammonium sulfate) and cosolvent (ethanol, t-butanol); (d) organic water soluble polymers (polyethylene glycol "PEG" molecular weight 4,000 or greater); (iii) affinity methods (chromatography-HIC: hydrophobic interaction chromatography; electrophoresis; affinity phase partitioning); (iv) biological methods with enzyme digestion of microbial membranes (lysozyme); (v) combination of those techniques (physical permeation plus chemical cellular disruption followed by affinity method).

During the isolation procedures from the bacterial cells, the stability of the required protein structure is of fundamental importance. Through the fractionation technique employed as the first step, the maintenance of the protein structure and activity is more important than the purity attained. A protocol selected to release the proteins from the intracellular compartments must provide the best recovery and purity, avoid proteolysis by the addition of protease inhibitors, and minimum binding to either particulates, high molecular weight proteins or non-protein substances that may prevent the recovery of the protein.

For the purpose of gfp_{uv} purification, Yakhnin et al [2] proposed a two-step procedure for the extraction of $gfp_{uv}$with ethanol into the organic phase and subsequent protein re-extraction into the aqueous phase, to reduce the loss of most of the gfp_{uv} during multiple chromatography steps. The ethanol extractions were made from the entire suspension in 70% ammonium sulfate ($(NH_4)_2SO_4$). The re-extraction in the aqueous $(NH_4)_2SO_4$ was performed with n-butanol added to the ethanol extract. The authors observed that one chromatography final step was enough to purify gfp_{uv} from nonprotein substances.

The addition of a polar miscible solvent, such as ethanol or acetone, to an aqueous extract containing proteins has a variety of effects that combined, lead to protein precipitation. The principal effect is the reduction in water activity. The solvating power of water for a charged, hydrophobic protein molecule is decreased as the concentration of organic solvent increases, which causes a bulk displacement of water plus the partial immobilization of water molecules through hydration of the organic solvent molecules. The most hydrophobic molecules or proteins may be soluble in 100% organic solvent [3].

The most advantageous salt for protein isolation and further purification, through fractionation methods, is ammonium sulfate, $(NH_4)_2SO_4$, which forms saline bridges with polar regions, stabilizing the molecule [2–8]. The main advantages of ammonium sulfate fractionation method, through salting out at high salt concentration (2–3 M ammonium sulfate), are (a) the stabilization of protein precipitate over long-term storage, (an easy way of packaging and commercialization); (b) the prevention of proteolysis and bacterial action and (c) the conservation of the protein precipitates, either before centrifuging or as a pellet, a good form in which a sample may be kept for further purification steps [3].

The addition of t-butanol to the ammonium sulfate solution of proteins consolidates the technique known as three-phase-partitioning (TPP), which is characterized by three distinct phases: an upper t-butanol, a lower aqueous

phase containing $(NH_4)_2SO_4$ at high concentration, and a middle layer [4–7]. The mixing of the upper phase with lower phase creates a third intermediate phase of precipitate material. The concentration of the required protein in the third phase depends on the $(NH_4)_2SO_4$ concentration. t-butanol stabilizes protein structure, inhibits proteases and protein/protein interactions [4,5]. These are similar qualities shown by ammonium sulfate during the salting out phenomenon.

$(NH_4)_2SO_4$ at high concentration promotes hydration of polar regions and dehydration of the hydrophobic regions on the protein surface, without direct interaction to molecular structure. Salting out depends on hydrophobic regions on the surface of proteins, where solubility is strongly reduced. At high salt concentration, the salt ions become solvated easier than polar regions on the protein surface. Furthermore, the freely available water molecules become scarce and the bare polar regions that have their water solubility reduced tend to aggregate and form clusters of different sizes [2,3,5].

In contrast, proteins and particulate material with a majority of non-polar surface residues may remain in solution even at the higher salt concentration. With the TPP method, those residues in contact with t-butanol are precipitated and form the first fraction of a floating intermediate phase, characterized by white cluster that is difficult to re-dissolve even in extraction buffer (TE). For the TPP method, the first white middle layer is a collection of high molecular weight contaminants and debris, precipitating at up to 25% ammonium sulfate saturation with conventional salting out fractionation method.

The binding of t-butanol to the hydrophobic regions of the protein molecules in the homogenate depends on the both the concentrations of the $(NH_4)_2SO_4$ and the required protein, which floats into the third phase at the TPP technique. At that time, when t-butanol is added a second time to the homogenate in the presence of a high concentration of ammonium sulfate, a protein is precipitated as an interface between the organic and aqueous phases. Therefore, the t-butanol stabilizes protein aggregates in the middle layer at the second TPP, preventing protein/protein interaction [5]. The thin middle layer is easily dissolved in the extraction buffer (TE, with added EDTA) and may be stored (4°C) for latter purification steps or subjected to further fractionation method by hydrophobic interaction chromatography (HIC – affinity chromatography).

TPP that is an emerging bioseparation technique presents the advantages of conventional salting out, isoionic precipitation, co-solvent precipitation and osmolytic and kosmotropic precipitation of proteins. It is easily scalable and can be used directly with crude suspensions [6,7].

Consequently, TPP was the technique chosen for direct fractionation of gfp_{uv}.

In this study, the bacteria cellular release was blended in 450 µl of TE (25 mM Tris-HCl, β-ME, PMSF). The homogenate was mixed with 300 µL of 4 M ammonium sulfate and 750 µL of t-butanol (ratio 1:1) was added (Ward personal communication) [8]. TPP was performed at room temperature. The sample was vigorously stirred, centrifuged, and the three phases formed were collected separately. The large amounts of gfp_{uv} present in the lower aqueous layer (containing $(NH_4)_2SO_4$), was subjected to TPP several times. The intermediate middle layer containing gfp_{uv}, was precipitated. The precipitated gfp_{uv} was collected and re-suspended in extraction buffer (TE, with added EDTA) and then fractionated on to the HIC column.

Sharma & Gupta [6] employed the TPP method for direct one step purification of protein inhibitor from the crude wheat germ extract. The authors observed best results (25-fold purification with an activity recovery of 85%) with a 1:1 ratio of t-butanol to crude extract and 30% ammonium sulfate (w/v) at 20°C. Sharma & Gupta [7] purified phospholipase from *Daucus carota* by a single step of the TPP procedure and obtained 13-fold purification with an activity of 72%.

The purpose of the research was to compare the association of selective permeation by freezing/ thawing/ sonication (FTS) cycles applied to the transformed cells of *E. coli* followed by the three-phase-partitioning extraction (TPP) method and the direct application of TPP to the same culture of *E. coli* on releasing gfp_{uv} from the over-expressing cells.

Material and methods

Transformation

The *Escherichia coli* DH5-α [2] were transformed with pGFPuv (Clontech, CA, USA) [9], by the standard calcium chloride method [5]. The transformed cells of *Escherichia coli* DH5-α with pGFPuv (Clontech, CA, USA) [2,5] were stored at (-75°C) into LB/amp broth with glycerol added in the proportion of 1:1.

Expression

A 24 h (37°C/100 rpm) culture of *E. coli* was developed in 25 mL Luria Bertani (LB; Difco, USB/Mexico, OH, USA) medium, supplemented with ampicillin (100 µg/mL Boehringer, Mannheim, Germany). When the broth cultures attained a OD_{660nm} = 0.7–0.8 (10^8 CFU/mL) isopropyl-β-D-thiogalactopyranoside (IPTG; dioxane free, USD/Italy,

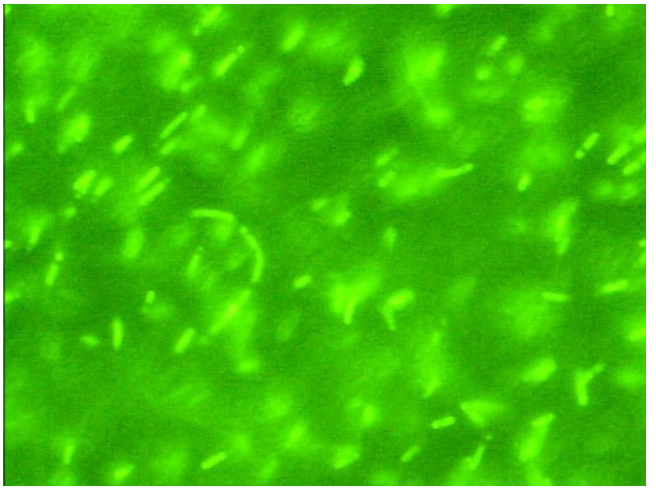

Figure 1
Pelleted cells of *E. coli*, expressing green fluorescent protein (gfp_{uv}).

OH, USA) was added to a final concentration of 0.5 mM [10], as inductor of gfp_{uv} expression by the mutant gfp_{uv}, which is under tight control of the *lacZ* protein β-galactosidase promoter/repressor.

Growth conditions

The growth culture was centrifuged (1000 × g/30 min/ 4°C), the supernatant was decanted, the pelleted cells were observed under UV light (λ = 395 nm) and resuspended in 1.0 mL cold extraction buffer solution (TE: 25 mM Tris-HCl, pH 8.0 – Trizma® Base, Sigma, MO, USA; 1.0 mM β-mercaptoethanol "β-ME", Pharmacia Biotech, Sweden; 0.1 mM phenylmethylsulphonylfluoride "PMSF", USB, Switzerland), dispersed and frozen at -75°C. The length of time the pelleted cells were held frozen was not monitored. (Ultra Freezer-Kelvinator Series 100 – Manitowoc, WI, USA). (Figure 1. ***The pelleted E. coli cells***) The uniform dispersed suspension was thawed (4°C), subjected to blending and pelleted again by centrifugation.

The supernatant was read for total protein (OD_{280nm}) and isolated gfp_{uv} (absorbency = 395 nm; emission = 509 nm) released. The fluorescent pellet was resuspended and dispersed into 1.0 mL TE. Both, supernatant and pelleted cells, were stored at 4°C and processed further:

(i) Sonication to the total permeation of gfp_{uv}: A pelleted frozen (-75 °C) 24 h culture of *E. coli* was dispersed into 20 mL of TE and sonicated (High Intensity Ultrasonic Processor, Vibram cells, model VC 100, Sonic & Materials, CT, USA). A 3 mm microtip ultrasonic processor was inserted in the tube of dispersed suspension (kept immersed in an ice-salt bath) and sonicated with 3, 6, 12, 24, 48, 96, 192, 384-fold pulses, over total intervals of 18s, 54s, 126s, 270s, 558s, 1134s, 1728s, 2880s. Each pulse was at 25 vibration amplitude at alternating cycles of 6 seconds on and 1.0 second off. Between sonication performed intervals, the suspension was centrifuged, a 1.0 mL aliquot of the supernatant was read for total protein and released gfp_{uv} (see Table 1).

Table 1: Sonication of over expressing cells of *E. coli*.

Time (seconds)	Total proteins (BSA) (mg/mL)	gfp_{uv} (μg/mL)	gfp_{uv}/BSA (μg/mg)
0	0.87	25.79	29.53
18	1.62	39.30	24.30
54	3.04	65.89	21.68
126	6.80	167.56	24.63
270	6.19	184.48	29.82
558	15.81	327.67	20.73
1134	9.83	222.68	22.64
1728	20.29	395.14	19.47
2880	14.05	268.28	19.10

(ii) Three-Phase-Partitioning (TPP) extraction method[4–8]. A pelleted frozen (-75°C) 24 h culture of *E. coli* was dispersed into 1.0 mL TE (4°C) and subjected to direct isolation by TPP. To each aliquot of 450 μL, 300 μL of 4 M $(NH_4)_2SO_4$ and 750 μL of t-butanol were added [6–8]. The mixture was vortexed for 1.0 min, allowed to settle for complete phase separation and centrifuged (6,000 × g/ 3 min). The t-butanol upper layer and the white interfacial precipitate were removed and discarded. 750 μL of t-butanol was mixed into the lower aqueous layer. The mixture was allowed to settle and centrifuged. The upper layer was discarded. The interfacial green layer was collected and dissolved in 450 μL TE buffer solution and kept at 4°C for spectrophotometer and spectrofluorometer readings and partial purification through hydrophobic interaction chromatography (HIC). While the lower layer was still fluorescent, it was subjected to repeated TPP by mixing in 750 μL t-butanol, centrifugation and intermediate phase separation. (Figure 2)

(iii) Freezing/ Thawing/ Sonication (FTS) selective permeation of gfp_{uv}[10]: A pelleted frozen (-75°C) 24 h culture of *E. coli* was dispersed into 1.0 mL TE. The re-suspended pellets were subjected to three repeated cycles of slow freezing (0.83°C/min until samples reached -20°C) and thawing (0.83°C/min, at room temperature), in a freezer-dryer (FTS System™, Secfroid, Lyolab G, NY, USA) chamber (Dura Stop™ MP). With PT-100 probes, inserted into the pelleted suspension, the freezing/thawing tempera-

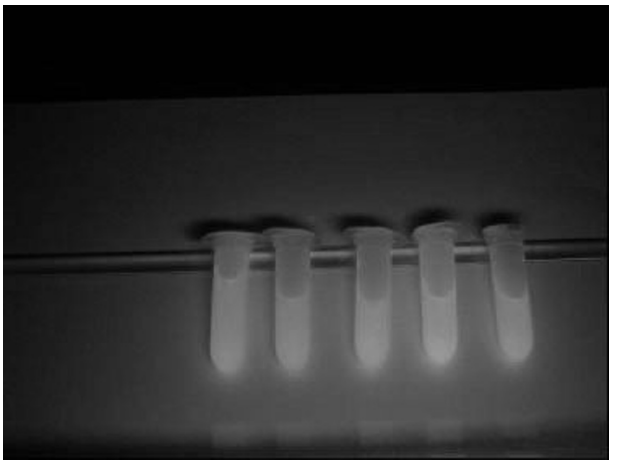

Figure 2
The gfp_{uv} extracted by three phase portioning ("TPP") method and dispersed in extraction buffer.

tures were registered every minute through a software "lyphoware" for Windows (Figure 3). The phenomenon of superfusion on the freezing of the samples was observed to happen between (-11°C) and (-14°C), after which the samples reached (-20°C) at 0.83°C/ min [10]. Between the freezing/thawing cycles, the sample was subjected to three-fold pulse sonication in an ice-water bath over a total interval of 23s. Between the freezing/thawing/ sonication cycle, cells were pelleted by centrifugation, and the supernatant was read for total protein and released gfp_{uv} (see Table 03).

(iv) Three-Phase-Partitioning (TPP) extraction method associated to the FTS procedure. The pellets from the 3rd FTS cycle were re-suspended into 450 µL of TE and subjected to the TPP extraction method.

(v) Direct extraction by the TPP method. Each pelleted frozen (-75°C) 24 h culture (I & J) of E. coli was dispersed into 1.0 mL of TE and vortexed for total uniform dispersion. The suspension was divided into two groups, as follows:

(a) A 450 µL sample of each culture was subjected to: (i) direct 2-step extraction by the TPP method; (ii) followed by sonication procedure (3-fold pulse/ 25 vibration amplitude/ alternating cycles of 6 seconds on and 1.0 second off) in an ice-water bath for 23s and (iii) a final 1-step TPP extraction.

(b) The other 450 µL sample of each culture was previously subjected to: (i) the sonication procedure (three-fold 6s pulse at 25 vibration amplitude) in an ice-water bath for 23s; (ii) followed by one-step TPP extraction method.

(vi) Fast Flow methyl hydrophobic interaction chromatography (HIC) for ion exchange separation and concentration of gfp_{uv} from the extraction mixtures. Equal volumes (1:1) of the TE containing gfp_{uv} and 4 M $(NH_4)_2SO_4$ were mixed, blended and centrifuged. At room temperature, 1.0 mL of the clarified supernatant was loaded onto a methyl HIC column fast flow, pre-equilibrated with 2 M $(NH_4)_2SO_4$. gfp_{uv} was adsorbed at the top of the methyl hydrophobic matrix. The column was loaded with 250 µL of 1.3 M $(NH_4)_2SO_4$ which carried the gfp_{uv}throughout the column bed. The protein was eluted with 1.0 mL buffer solution (10 mM Tris-HCl, 10 mM EDTA, pH 8.0).

Concentrations

The supernatant was read for total protein (based on BSA) and isolated gfp_{uv} released. The fluorescent bright green intact cells with easily defined outlines were observed under an Olympus (BX-60-IV FLA System Attachment, NY, USA) microscope, through filters for the detection of fluorescent cells [U-MWG (510–550 nm); U-MWU (330–385 nm); U-MNV (400–410 nm)]. Recombinant green fluorescent protein (gfp_{uv})absorbs at UV (330–385 nm) and blue (400–410 nm) emission, with a maximum absorbance peak at 395 nm and a minor at 470 nm, emitting maximum green light at 509 nm. The spectrum was confirmed through Fluorescence Spectrophotometer F-2000 – Hitachi (OH, USA) spectra.

Standard Curves

The fluorescence intensity of gfp_{uv} detected in a cleared bacterial cell lysate was measured using the Fluorescence Spectrophotometer, with an excitation filter of 394 nm and an emission filter of 505 nm. The fluorescence intensity of the experimental samples was compared to the standard curve (gfp_{uv} µg/mL = 0.001*(fluorescence intensity) - 0.1133; R^2= 0.995) to determine the amount of gfp_{uv} released from the cells. The standard curve was prepared using known amounts (between 6.66 µg/mL and 0.59 µg/mL) of purified recombinant gfp_{uv} (Clontech) diluted in the same buffer solution (10 mM Tris-HCl, pH 8.0, 1.0 mM β ME, 0.1 mM PMSF).

The total protein released in the medium from *E. coli* cells was expressed in mg/mL and compared with total protein concentrations expressed as purified bovine serum albumin (BSA, mol wt. 66 kDa, Sigma, MO, USA) at λ = 280 nm in a spectrophotometer. The total protein concentrations in the buffer solution ranged from 100 to 1000 µg/mL, the maximum OD_{280nm} being 0.615; and the comparative relationship between total proteins and BSA was made through the standard curve (total protein µg/mL = 1727.2*(OD_{280nm}) - 26.863; R^2 = 0.9943).

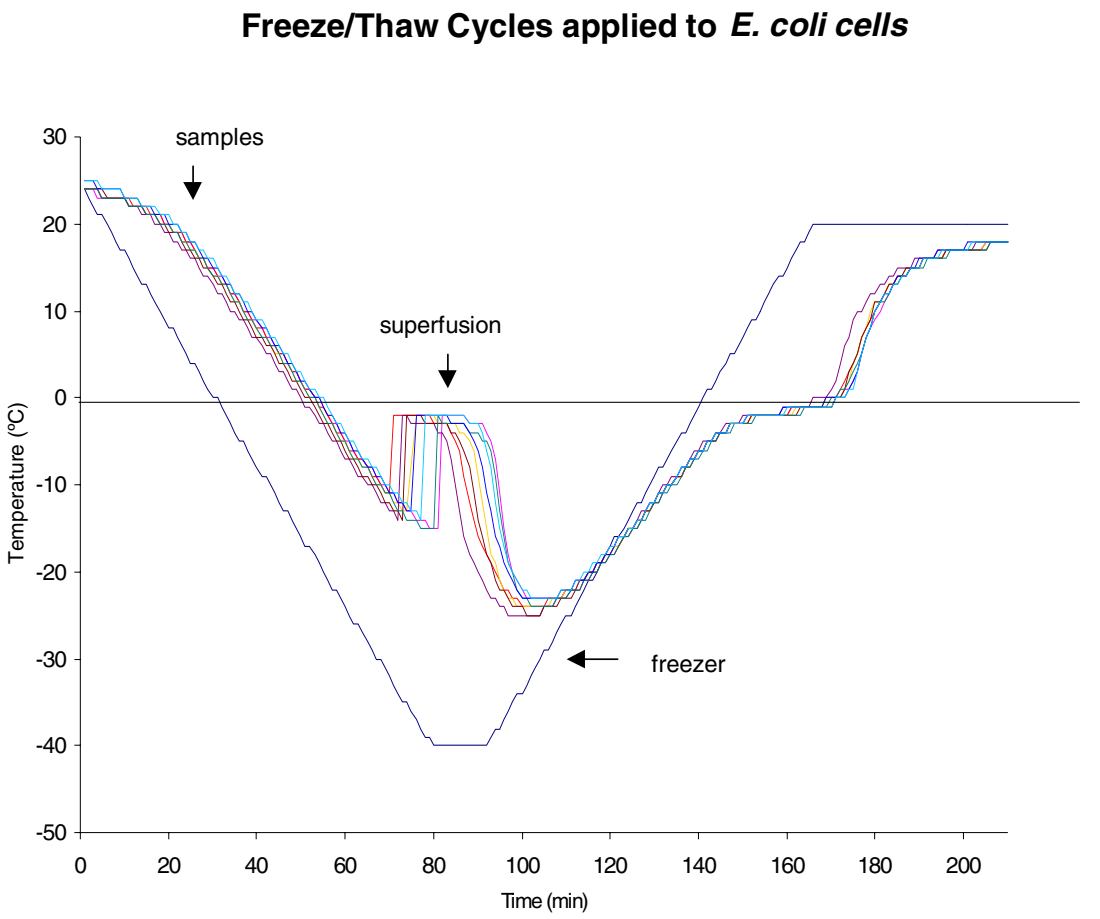

Figure 3
Freeze – Thaw cycles applied to *E. coli* cells.

Results

In Tables 1, 2 and 3, the results were expressed by total protein (BSA, mg/mL), released gfp_{uv} (µg/mL) content, gfp_{uv} yield (%, µg/µg) in relation to the total released gfp_{uv} content obtained; the specific gfp_{uv} mass being expressed by µg of gfp_{uv} in relation to mg of total protein (µg gfp_{uv}/mg BSA).

(i) Sonication of the cells to complete permeation and isolation of gfp_{uv} contents (Table 1)

Sonication was performed on cells of E. coli DH5-α expressing a high concentration of gfp_{uv} to verify the maximum protein content that can be released from the cells. After 558s sonication interval treatment, a maximum concentration of 327.67 µg gfp_{uv}/mL was obtained, equivalent to the specific mass of 20.73 µg gfp_{uv}/mg BSA.

It was interesting to observe that the values of specific mass had varied from 21.68 µg/mg to 29.82 µg/mg, indicating that during the sonication procedure the amount of released gfp_{uv} was proportional to the other proteins and molecules extracted from the sonicated cells.

(ii) Isolation by three-phase partitioning (TPP) method, using ammonium sulfate (final concentration 1.6 M) and t-butanol (Table 2)

In this work, the pelleted sample was diluted in 450 µl of extraction buffer (TE: 25 mM Tris-HCl, pH 8.0, β-ME, PMSF). It was followed by addition of 300 µL of 4 M $(NH_4)_2SO_4$ and, after that, 750 µL t-butanol (ratio 1:1). The sample was stirred for 1.0 min, centrifuged at 10,0000 × g / 3 min and the three phases formed were collected separately. The first white middle layer (an white disc in-

Table 2: Three-phase-partitioning (TPP) method applied directly to cells of E. coli with specific growth rate constant (μ, h-1): of 0.99 h-1 for culture A, 1.05 h-1 for cultures B, C, D, E and of 1.10 h-1 for cultures F, G, H.

	TPP (1)		
Sample	gfp_{uv} (μg/Ml)	BSA (mg/mL)	gfp_{uv}/BSA (μg/mg)
A1	354.08	3.30	107.28
A2	444.05	3.29	135.10
A (Pool)	378.53	3.98	95.11
B1	46.68	2.60	18.05
B2	38.59	2.24	17.26
B (Pool)	45.66	2.42	18.87
C	39.60	1.11	35.65
D	15.92	0.41	38.86
E	28.12	0.73	38.70
F1	15.78	0.52	30.42
F2	11.56	0.22	52.69
F (Pool)	5.47	0.67	8.12
G	19.42	0.79	24.53
H1	16.69	0.82	20.44
H2	9.16	0.19	47.67
H	4.78	0.67	7.09

terface) was a result of remaining high molecular weight contaminants and debris [4,5,8].

At the first stage, gfp_{uv} did not precipitate completely. Then, the lower aqueous layer was subjected to second addition of t-butanol and, at the separation of the three phases, the middle layer containing the precipitated gfp_{uv} was collected and dissolved into 500 μL TE. The solution was eluted through a HIC (*Methyl Support Hydrophobic interaction Chromatography*) for partial purification.

The extraction through the TPP method applied to the 24 h culture A of high expression in gfp_{uv} fluorescence (Table 2), resulted in concentrations that varied from 354.08 to 444.05 μg gfp_{uv}/mL, for a pool (extract mixture 1:1) of 378.53 μg gfp_{uv}/mL, corresponding to specific masses (SM) of 107.28, 135.10, and 95.11 μg gfp_{uv}/mg BSA. Even though the direct sonication of the cells (Table 1) and the direct application of the TPP method (Table 2) released similar concentration ranges of gfp_{uv}, the sonication caused a higher release of other molecules (BSA) that reduced the SM. Therefore, the specific mass for TPP was approximately five times higher than that obtained by the sonication procedure. Through the TPP method, proteins (other than gfp_{uv}), nucleic acids and other molecules were precipitated and separated from gfp_{uv}.

Cultures B, C, D and E showed (Table 2) expressions in μg gfp_{uv}/mL and SM (μg gfp_{uv}/mg BSA), respectively, 45.66 μg/mL and 18.87 μg/mg for culture B; 39.60 μg/mL and 35.65 μg/mg for culture C; 15.92 μg/mL and 38.86 μg/mg for culture D; 28.12 μg/mL and 38.70 μg/mg for culture E. The TPP method extracted lower amounts of contaminants for concentrations lower than 30 μg gfp_{uv}/mL, when the specific mass gradually increased, as shown in cultures F, G and H. The TPP method not only extracted gfp_{uv} but also carried out a selective isolation of the protein.

For higher fluorescence (> 30 μg gfp_{uv}/mL), the formed intermediate white ring proceeding from the contaminant precipitation was observed to entrap low amount of gfp_{uv}, which was lost. On the other hand, the TPP method cleaned the isolated gfp_{uv} solution, making subsequent elution through the HIC easier.

Cultures F, G and H showed (Table 2) half the expression of gfp_{uv}, as follows: 15.78 (F), 19.42 (G) and 16.69 (H_1) μg gfp_{uv}/mL, versus 46.68(B1), 38.59 (B2), 39.60 (C), 15.92 (D) and 28.12 (E) μg gfp_{uv}/mL. However, the specific masses of these cultures B, C, D and E, F, G, H were equivalent, showing that lower concentrations of gfp_{uv} improved their isolation from the cells and contaminants.

Sharma [6] used the three-phase-partitioning (TPP) method to purify crude extract of protease/amylase inhibitors at 20°C; t-butanol was employed at a ratio of 1:1, and $(NH_4)_2SO_4$ at 30%; and the recovery was about 85% and 25-fold purification.

(iii) Permeation by physical procedures (freezing/thawing/sonication procedures) associated to the TPP method (Table 3)

The freezing (-75°C) permeated 69.85 μg gfp_{uv}/mL (A1), five times less than the TPP method (354.08 μg gfp_{uv}/mL, sample A_1, Table 2); however, the specific contents were similar, respectively 86.25 and 107.28 μg gfp_{uv}/mg BSA. Therefore, slow freezing allowed a cleaner permeate than the extraction by TPP, which released four times more contaminants (3.30 mg BSA/mL) than permeation did (0.81 mg BSA/mL).

It was observed that, for culture A1 (Table 2), with a high concentration of gfp_{uv} expressed, the TPP method extracted between 354.08 and 444.05 μg gfp_{uv}/mL from the cells, 70% more than the slow freezing (-75°C). This unit operation was followed by three repeated cycles of freezing/thawing/sonication (FTS), which caused a total permeation of 241.19 μg gfp_{uv}/mL, distributed as follows (Table 3): 29% (69.85 μg/mL) by freezing (-75°C), 24% (57.83 μg/mL), 38% (91.78 μg/mL), and 9% (21.73 μg/mL) after the 1st, 2nd and 3rd FTS cycles, respectively. Penna et al. [10] verified that for increasing amounts of over-expressed

Table 3: Permeation of E. coli cells through independent repeated cycles (1°, 2°, 3°) of slow freezing/ thawing/ sonication (FTS), followed by two three-phase partitioning (TPP) extractions, and elution throughout hydrophobic HIC chromatography column. The specific growth rate constant (μ) of 0.99 h-1 for culture A, 1.05 h-1 for cultures B, C, D, E and 1.10 h -1 for cultures F, G, H.

				Permeation (FTS)														
Sample	(-75°C)			1° FTS			2° FTS			3° FTS			Pool			TPP		
	gfp_{uv} μg/mL	BSA mg/mL	gfp_{uv}/ BSA μg/mg	gfp_{uv} μg/mL	BSA mg/mL	gfp_{uv}/ BSA μg/mg	gfp_{uv} μg/mL	BSA mg/mL	gfp_{uv}/ BSA μg/mg	gfp_{uv} μg/mL	BSA mg/mL	gfp_{uv}/ BSA μg/mg	gfp_{uv} μg/mL	BSA mg/mL	gfp_{uv}/ BSA μg/mg	gfp_{uv} μg/mL	BSA mg/mL	gfp_{uv}/ BSA mg/mL
A1	69.85	0.81	86.25	57.83	2.80	20.62	91.78	3.89	23.61	21.73	0.61	35.87				44.49	1.10	40.32
A2	15.23	1.95	9.24				12.04	5.06	2.38	5.78	5.14	1.12				17.83	0.48	37.52
A3	2.95	0.20	14.81															
(Pool)	44.79	1.75	25.57	57.85	2.80	20.62	55.18	4.18	13.19	21.58	1.66	13.00	41.14	2.15	19.11	23.97	1.64	14.59
B	6.81	0.35	19.72	7.75	1.77	4.36	4.93	0.79	6.27	3.28	0.48	6.84	13.77	1.54	8.93	18.40	1.34	13.73
C	5.39	0.59	9.09	13.73	2.00	6.86	2.77	0.57	4.88	3.37	0.59	5.75	13.46	1.33	10.13	4.28	0.55	7.78
D	5.39	0.47	11.37	7.02	0.92	7.65	2.82	0.59	4.76	1.38	0.20	6.93	12.44	0.62	20.10	2.16	0.40	5.40
E	7.10	0.38	18.70	16.54	1.75	9.43	3.71	0.57	6.54	1.26	0.14	9.13	13.40	0.96	13.97	2.14	0.24	8.81
F	2.52	0.21	11.93	17.62	2.24	7.87	4.50	1.18	3.82	2.57	0.82	3.12	5.35	1.19	4.50	2.53	0.56	4.50
G	1.54	0.47	3.28	20.39	1.45	14.02	4.47	0.74	6.04	3.11	0.51	6.14	5.96	0.91	6.52	20.32	1.28	15.89
H	4.06	0.23	17.43	23.95	4.02	5.96	4.70	1.04	4.50	4.27	0.45	9.56	7.03	0.57	12.26	16.53	0.98	16.79

gfp_{uv} in cells, selective permeation by FTS was proportional to the release of 10–40% gfp_{uv}.

The 1st and 2nd cycles removed about four times as much BSA in relation to the freezing at -75°C, decreasing fourfold the specific contents (Table 3). The concentration of 44.49 μg gfp_{uv}/mL extracted by TPP from the cells, after the FTS cycles, represented 15.6% of the total gfp_{uv} (241.19 + 44.49 = 285.68 μg/mL) released by the association of FTS followed by TPP, for culture A, resulting in specific mass (40.32 μg gfp_{uv}/mg BSA) two times higher than the equivalents obtained in the 1st and 2nd cycles. The combined procedures released four times less BSA (1.10 mg/mL), similar to that one removed from the cells directly subjected to TPP method. To perform an effective permeation, slow freezing at -75°C previously to the TPP method applied to the pelleted cells was effective for high specific gfp_{uv} mass removal.

The repetitive 1st, 2nd and 3rd FTS cycles promoted removal of gfp_{uv}. However, they created a simultaneous increase of BSA concentration, which affected the specific gfp_{uv} content, in agreement with data already obtained [10].

For cultures (B, C, D, E & F) expressing amounts lower than 100 μg gfp_{uv}/mL, the application of TPP method to the pelleted cells exhibited two distinct behaviors as follows:

(i) For amounts greater than 20 μg gfp_{uv}/mL, the TPP method was the best method, both simple and fast when applied directly to the intact cells (Table 1), resulting in concentrations of gfp_{uv}/mL and specific mass, for the cultures, respectively of (B, pool) 45.66 μg gfp_{uv}/mL and 18.87 μg/mg; (C) 39.60 μg gfp_{uv}/mL and 35.65 μg/mg; (D) 15.92 μg gfp_{uv}/mL and 38.86 μg/mg; (E) 28.12 μg gfp_{uv}/mL and 38.70 μg/mg. Comparatively, the application of the TPP method to concentrations previously permeated by FTS cycles (Table 2) resulted in total (sum of every step) equivalent concentrations for the same cultures (B: 41.17, C: 29.54, D: 18.77, E: 30.75 μg/mL). The pool (mixture) of equal parts of the successive FTS samples resulted in a ratio of about 1 gfp_{uv}: 1 BSA: 1 SM (specific mass) showing good performance. Both procedures were shown equivalent.

(ii) For amounts lower than 20 μg gfp_{uv}/mL (Table 2), the concentrations attained by the direct TPP extraction from the intact cells (F:11.56–15.78 μg gfp_{uv}/mL; G:19.42 μg gfp_{uv}/mL; H:9.16–16.69 μg gfp_{uv}/mL) were similar to those permeated (Table 3) by the 1st FTS cycle applied to cells of the same culture (F: 17.62 μg gfp_{uv}/mL; G: 20.39 μg gfp_{uv}/mL; H: 23.95 μg gfp_{uv}/mL) cells. Therefore, the TPP method applied to the cells subjected previously to the FTS cycles (Table 3) showed greater efficiency upon the gfp_{uv} extraction (F:2.53 μg gfp_{uv}/mL; G: 20.32 μg gfp_{uv}/mL; H: 16.53 μg gfp_{uv}/mL) corresponding to large specific contents (F:4.50; G: 15.89; H: 16.79 μg gfp_{uv}/mg BSA). For cultures F, G, H, the maximum gfp_{uv} concentrations [F: 29.74 (=2.52+17.62+4.50+2.57+2.53); G: 49.83; H: 53.51 μg gfp_{uv}/mL] obtained from the combined procedures (FTS plus TPP) were 2–4 times higher than those obtained from the direct application of the TPP method to the pelleted cultures.

The percentage related to the total (-75°C+1st+2nd+3rd+TPP) concentrations of gfp_{uv} released from respective cultures (A: 203.87; B: 41.17; C: 29.54; D: 18.77; E: 30.75; F: 29.74; G: 49.83; H: 53.51) through combined FTS with TPP procedures was as follows:

(i) freezing (-75°C): A: 22%; B: 16.54%; C: 18.25%; D: 28.72%; E: 23.09%; F: 8.47%; G: 3.10%; H: 7.60%;

(ii) 1st FTS cycle: A: 28.37%; B:18.82%; C: 46.48%; D: 37.40%; E: 53.79%; F: 59.25%; G: 40.92%; H: 44.76%;

(iii) 2nd FTS cycle: 27.07%; B: 11.97%; C: 9.38%; D: 15.02%; E: 12.07%; F: 15.13%; G: 8.97%; H: 8.78%;

(iv) 3rd FTS cycle: A: 10.59%; B: 8.00%; C: 11.41%; D: 7.35%; E: 4.10%; F: 8.64%; G: 6.24%; H: 8.00%;

(v) TPP extraction: A: 11.76%; B: 44.69%; C: 14.49%; D: 11.51%; E: 7.00%; F: 8.51%; G: 40.78%; H: 30.89%.

For cultures F, G and H, preliminary freezing at -75°C permeated the lowest amounts, about 3–8% gfp_{uv} (Table 3); however the 1st FTS cycle caused greatest removal – about 40% of the protein, promoting the most efficient permeation. Final TPP extraction applied to the cultures was important in the residual removal of the required protein up to 40% of totalgfp_{uv} obtained for cultures B, G, H.

(iv) Three-partitioning phase (TPP) technique combined with sonication (Table 4)

To cultures over-expressing high concentrations of gfp_{uv}, the TPP method was applied 2–3 times directly to the pellets, while the white middle layer and the lower aqueous phase still containing gfp_{uv}, were visually detected by fluorescence with a UV lamp (λ = 395 nm).

Sonication of the pelleted cultures before the TPP application was employed to increase the release of gfp_{uv} from the cells. Otherwise, sonication turned the homogenate unclear and hindered the TPP efficacy on fractioning proteins. The interaction of high weight proteins entrapped gfp_{uv} to the white intermediate phase, which was the layer not soluble into extraction buffer, led to the loss of the protein. Therefore, the content of BSA was increased and

Table 4: Permeation of the pellets by freezing at -75°C. Three Phase Partitioning (TPP) method applied to the pelleted culture "I" (samples 1, 2, 3, 4), culture "J" (samples 5, 6, 7, 8, 9, 10), directly (TPP) or following sonication of the pellets (S+TPP).

Pellet	Cycle	Final	BSA	gfp_{uv}	gfp_{uv}	Total	Specific mass
Sample		BSA (mg/mL)	yield (%)	(μg/mL)	yield (%)	gfp_{uv} (μg/mL)	gfp_{uv}/BSA (μg/mg)
1	**(-75°C)**	31.49	62.05	337.91	57.50		10.73
	1st S+TPP	5.34	10.53	137.52	23.40		25.74
	2nd S+TPP	13.92	27.42	112.28	19.11	587.71	8.07
2	**(-75°C)**	45.35	68.04	358.35	58.12		7.90
	1st S+TPP	5.82	87.30	165.33	26.82		28.39
	2nd S+TPP	15.47	23.21	92.854	15.06	616.53	6.00
3	**(-75°C)**	10.87	28.78	91.75	23.41		8.42
	1st TPP	4.01	10.65	194.30	49.67		48.41
	2nd S+TPP	22.89	60.59	105.34	26.93	391.20	4.60
4	**(-75°C)**	8.71	46.03	73.207	26.85		8.40
	1st TPP	2.96	15.65	134.53	49.34		45.39
	2nd S+TPP	7.25	38.30	64.94	23.82	272.68	8.96
5	**(-75°C)**	75.14	66.45	1633.78	46.89		21.74
	1st S+TPP	12.12	10.72	645.28	18.52		53.24
	2nd S+TPP	14.82	13.10	929.12	26.67		62.69
	3rd S+TPP	10.99	9.72	275.78	7.92	3483.96	25.10
6	**(-75°C)**	35.54	49.78	1262.9	37.08		35.53
	1st S+TPP	11.74	16.44	998.66	29.32		85.07
	2nd S+TPP	13.99	19.56	873.14	25.64		62.41
	3rd S+TPP	10.11	14.16	271.34	7.97	3406.04	26.84
7	**(-75°C)**	52.66	60.55	1606.06	37.88		30.50
	1st S+TPP	16.45	18.91	1528.08	36.04		92.90
	2nd S+TPP	13.25	15.23	875.68	20.65		66.09
	3rd S+TPP	4.60	5.29	230.03	5.43	4239.85	49.97
8	**(-75°C)**	14.77	32.50	584.69	25.87		39.60
	1st TPP	7.76	17.08	581.31	25.72		74.90
	2nd TPP	14.94	32.88	811.9	35.92		54.35
	3rd S+TPP	7.96	17.53	282.44	12.50	1977.90	35.46
9	**(-75°C)**	18.41	34.54	721.81	21.39		39.21
	1st TPP	12.62	23.68	1199.2	35.54		95.03
	2nd TPP	9.22	17.29	921.12	27.30		99.95
	3rd S+TPP	13.04	24.47	531.49	15.75	2842.13	40.75
10	**(-75°C)**	15.20	27.80	567.94	17.88		37.36
	1st TPP	13.23	24.18	1461.90	46.03		110.47
	2nd TPP	9.14	16.70	699.6	22.02		76.53
	3rd S+TPP	17.13	31.31	446.35	14.02	2729.42	26.05

carried with the gfp_{uv} extraction. For culture I, sonication plus TPP combined procedures extracted similar amounts of gfp_{uv} other than TPP directly applied to the cells. However, specific contents from TPP extracts (45–48 μg gfp_{uv}/mg) were at least twice higher than those obtained from sonication, in addition to TPP (25–28 μg gfp_{uv}/mg). Sonication, applied to the cells after previous TPP treatment, caused an increase of extractedgfp_{uv} content but affected the specific contents 3–6 fold. For samples 3 and 4 (culture I), BSA at 10 to 15% was present in nearly 50% of all gfp_{uv} extracted by the TPP method directly applied to the pellets, and the specific mass was the highest range attained from 45–48 μg gfp_{uv}/mg. After sonication of those pellets, the specific content decreased between 4–8 μg gfp_{uv}/mg, since up to 60% of BSA was shifted with gfp_{uv}.

For culture J, the application of sonication previously to the TPP method did not interfere with the amount of the gfp_{uv} obtained. Through sample 8, it was observed that the 3rd application of the TPP method directly to the pellets did not carry out the gfp_{uv} fractionation. After the sonication of the pellets, the extraction of 12.50% of the protein through the TPP technique was followed by a reduction of the specific content to 35.46 μg gfp_{uv}/mg.

Slow freezing cycle at -75°C was responsible for the gfp_{uv} permeation up to 58% for samples 1 and 2 (culture I), and the average of 37–47% for samples 5, 6 and 7 (culture J). However, simultaneously, near 62–68% of all proteins (BSA) were carried and mixed to the required protein. These samples corresponded to high concentrations of gfp_{uv}, between 338 µg gfp_{uv}/mL (sample 1) and 1634 µg gfp_{uv}/mL (sample 5). For culture I, independently of the concentrations removed of gfp_{uv} and BSA, the specific mass was equivalent to 8–10 µg gfp_{uv}/mg, also for samples 3 and 4, when the freezing removal of gfp_{uv} varied from 23–26%.

For culture J, even though the concentration of gfp_{uv}, ranged from 568 µg/mL (sample 10) to 1634 µg/mL (sample 5), 39% (samples 8 and 9), corresponded to half gfp_{uv}, extracted – respectively, 584.69 µg/mL and 721.81 µg/mL.

The uncontrolled slow freezing (-75°C) speed of the pellets caused variations in the permeation of the pelleted cells.

(v) Three-partitioning phase (TPP) technique directly applied (Table 4)

For samples 3 and 4 (culture I), TPP directly applied to previously frozen cells, extracted two times more gfp_{uv} (49.34–49.67%) than prior freezing permeation (23.41–26.85%), corresponding to a five-fold increase of the specific contents (from 8.4 µg/mg to 45.39–48.41 µg/mg), which showed simultaneous ability by the TPP method of extraction, purity and concentration of gfp_{uv} in the aqueous phase. The sonication following procedure displaced 6 times more BSA mixed to gfp_{uv} reducing up to 10 times the specific contents (sample 3), affecting the purity of the homogenate.

The TPP method applied directly to samples 8, 9, and 10 (culture J) attained the best specific content ranging from 74.90 µg/mg to 110.47 µg/mg for the first application and from 54.35 µg/mg to 76.53 µg/mg for the second turn. Sonication following TPP application to the extracted pellets caused a three-fold decrease of specific contents (26.05 µg/mg, sample 10), confirming that sonication had a negative effect on the isolation and purification procedures of gfp_{uv}.

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