Recovery of inclusion body protein in *Escherichia coli*: Effects of solubilization methods and process condition

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Abstract. Inclusion bodies (IBs) are produced in *Escherichia coli* cells and solubilization process is required to recover desired protein in bioactive form. Conventional and mild solubilization methods were applied for IBs solubilization and the performances were influenced by respective process condition. Therefore, it is the objective of current work to investigate the effects of solubilization methods on the recovery of soluble enhanced green fluorescent protein (EGFP) from IBs by using urea, alkyl alcohol and freeze thaw method. The present study indicates urea concentration, incubation temperature, type of alcohol and its concentration, freezing duration and freeze thaw cycles influenced the yield and purity of solubilized EGFP. Conventional method using 8 M of urea with incubation temperature of 60°C achieved the highest yield (61%) and purity (10%). Mild IBs solubilization with 6 M of n-butanol and 2 M of urea has solubilized IBs with a yield of 45% and purity of 22%. By freezing and thawing the IBs suspension in 2 M of urea, the yield (66%) and purity (9%) of solubilized EGFP were comparable to that of 8 M of urea in buffer. Hence, mild solubilization using the alkyl alcohol or freeze thaw method is applicable for IBs solubilization.

1. Introduction

Recombinant proteins can be produced in bacteria, yeast, insect cells, mammalian cells, and cell-free system. Among these various expression systems, *Escherichia coli* (*E. coli*) cells have been widely used for the production of recombinant proteins because it is the most well-known species of bacteria that can be rapidly grown on inexpensive and simple media. Previous study reported that the over-expression of recombinant protein in *E. coli* cells often leads to 70% of target protein folded into aggregates called inclusion bodies (IBs) [1]. The expressed protein in IBs have high density than cell fragment and can be easily separated from cell debris by centrifugation after cell disruption. Hence, the IBs exhibits high protein purity that ease the recovery process. However, it cannot be directly used for bioreaction due to their misfolded structure and low solubility.

The general method to recover biologically active protein from IBs, the IBs require to be disaggregated and then refolded back to native conformation. The disaggregation process also called solubilization process that involves the disruption or destruction of both protein secondary and tertiary structures. The interactions in protein aggregates such as the hydrophobic interactions, ionic interactions, disulfide bonds, hydrogen bonds and van der Waals forces are broken during the solubilization process. Traditional method for IBs solubilization used high concentration of chemical
denaturants such as urea and guanidine hydrochloride [2] result in complete disruption of protein structure and lead to protein aggregation during refolding [3,4].

Besides the misfolded proteins in IBs, previous literatures have documented that proteins in IBs have native-like secondary structure that can be used for industrial or medical applications [5-7]. The biologically active protein in IBs can be used as biocatalysts and diagnostic tools in various bioprocesses [8]. In order to recover the native protein in IBs that is embedded in an aggregate, researches have used a mild solubilization process in which consist of a low concentration of chaotropes in combination of detergents, organic solvents, alkaline pH or high pressure conditions to facilitate the IBs solubilization [9]. Urea at lower concentration reduce hydrophobic interaction that cause protein aggregation. Mild solubilization retains the existing native-like secondary structure of protein, reduces protein aggregation during refolding and enhances the recovery of bioactive proteins from IBs [10-12] have successful recovered recombinant human growth hormone from IBs with overall yield of 40% by using mild solubilization method in comparison to 25% achieved with traditional method.

Despite several protocols available for IBs solubilization, the performance is influenced by many factors such as the type and concentration of denaturant [1], solubilizing temperature [13] and buffer pH [14]. By using organic solvents such as alcohols, increase in chain length of alkyl alcohol exhibits variation in solvent hydrophobicity that promotes IBs solubilization [15]. Singe et al. [12] also reported that different amount of solubilized IBs was obtained when concentration of n-propanol based buffer was increased. Besides, freeze thaw process is affected by freeze thaw cycle, sample volume, freezing temperature and incubation duration. The freeze thaw process in fast freezing rate and slow thawing rate caused protein damage [16] and the best freezing temperature of -20°C for IBs was reported by Qi et al. [17].

The influence of various factors resulting different yield and purity of the solubilized protein. Hence, the aim of this study was to investigate the effects of conventional and mild solubilization methods and its process condition on the recovery of the target protein from IBs. Enhanced green fluorescent protein (EGFP) was overexpressed in *E. coli* cells and acted as a model protein in the present study because the protein function can be easily detected based on its fluorescent intensity. These studies demonstrate the benefit of mild solubilization for protein recovery from IBs in which the purity and yield of solubilized protein from the IBs can be maintained and the existing native-like secondary structure of the protein is protected during solubilization.

### 2. Materials and methods

#### 2.1. Preparation of inclusion bodies

*E. coli* strain BL21(DE3) carrying the plasmid pRSETEGFP was used for all experiments [18]. Method of recombinant protein overexpression in the cells as described by Malavasi et al. [19] was slightly modified and used in the present study. Briefly, the cells expressing EGFP gene was grown in Luria-Bertani medium (10 g/L of tryptone, 5 g/L of yeast extract, and 5 g/L of sodium chloride, 100 µg/mL of ampicillin at pH 7.0) with a liquid-to-flask volume ratio of 0.2 and inoculated with 5% (v/v) of inocula. The flask culture was carried out at a shaking frequency of 150 rpm and temperature of 30°C. The expression of EGFP was induced with 1 mM of isopropyl β-D-1-thiogalactopyranoside at the exponential phase (0.8 to 1.0 of optical density at 600 nm). After 16 h of protein induction at 37°C, bacteria were collected by centrifugation at 2,500 g and 4°C for 10 min. The pellet was washed with buffer solution [50 mM of Tris hydrochloride (pH 8.0), 50 mM of NaCl] and centrifuged at the same condition. Cell pellet was then collected and lysed by freeze thaw method [20]. The pellet was frozen at -20°C for 24 h and thawed by resuspending with 10% (w/v) of buffer solution. The suspension was centrifuged at 8,000 g and 4°C for 10 min and the supernatant containing soluble EGFP was discarded. The lysing process was repeated for another cycle and the resulting IBs was stored at -20°C until further process.
2.2. Solubilization of inclusion bodies using urea

For the conventional urea denatured method [17], IBs suspension at 10% (w/v) was prepared in buffer solution [50 mM of Tris hydrochloride (pH 8.0), 50 mM of NaCl] containing different urea concentration (0, 2, 4, 6, and 8 M). All the IBs suspension was then incubated at 50°C for 4 h and centrifuged at 8,000 g and 4°C for 10 min to get clear supernatant. The amounts of solubilized EGFP and total protein in the supernatant were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Bradford assay, respectively. In order to study the effect of incubation temperature, the experiment was repeated by incubating IBs suspension with different molar concentration of urea at temperature of 60, 70, and 80°C.

2.3. Solubilization of inclusion bodies using urea with alkyl alcohol

IBs mild solubilization using 2 M of urea with alkyl alcohol as described in Singh et al. [12]. Six different 6 M alkyl alcohols (methanol, ethanol, ethylene glycol, n-propanol, glycerol, and n-butanol) in presence of 2 M of urea, 50 mM of Tris hydrochloride (pH 8.0), and 50 mM of NaCl were employed to solubilize IBs at 10% (w/v). All the IBs suspension was then incubated at room temperature for 30 min. The IBs suspension was then separated by centrifuging at 8,000 g and 4°C for 10 min and the supernatant was analyzed to determine the amounts of solubilized EGFP and total protein. To investigate the molar concentration of n-butanol (showed the best performance in former experiment) on the solubilization of EGFP from IBs, the experiment was repeated by solubilizing IBs pellet in buffer [50 mM of Tris hydrochloride (pH 8.0), 50 mM of NaCl, and 2 M of urea] with increasing concentration of n-butanol (0 – 6 M).

2.4. Solubilization of inclusion bodies using urea with freeze thaw process

Mild solubilization using 2 M of urea with freeze thaw process reported by Qi et al. [17] was followed. To investigate the effects of freezing incubation period (0 – 4 day) and freeze thaw cycle (0 – 4 cycle) on the solubilization process, IBs suspension at 10% (w/v) was prepared in buffer solution [50 mM of Tris hydrochloride (pH 8.0), 50 mM of NaCl, and 2 M of urea] and was frozen at -20°C for different durations and cycles. The mixture was then thawed at room temperature and centrifuged at 8,000 g and 4°C for 10 min to get clear supernatant for determination of amounts of solubilized EGFP and total protein.

2.5. Polyacrylamide gel electrophoresis analysis

Measurement of denatured EGFP amount in protein samples were based on gel-based imaging using SDS-PAGE [21,22]. Protein samples were mixed with equal volume of 2× SDS sample buffer [125 mM of Tris hydrochloride (pH 6.8), 20% (w/v) of glycerol, 4% (w/v) of SDS, 200 mM of β-mercaptoethanol, and 0.01% (w/v) of bromphenol blue] and boiled for 10 min to denature proteins. Samples were then electrophoresed on a 15% (w/v) of SDS polyacrylamide gel under a constant current of 30 mA for 90 min. After electrophoresis, the gel was stained with a staining solution [0.1% (w/v) of Coomassie Brilliant Blue R-250, 52.5% (v/v) of methanol, and 10.5% (v/v) of acetic acid] and destained with a destaining solution [40% (v/v) of methanol, and 10% (v/v) of acetic acid] until clear protein bands on the gel were obtained. The gel was captured using FluoroChem SP imaging system (Alpha Innotec) and the total amount of denatured EGFP was determined by densitometry analysis of corresponding band using AlphaEase FC software and a standard equation developed by using pure EGFP.

For the determination of functional EGFP amount based on the protein fluorescent intensity, protein samples were remained native and electrophoresed in native polyacrylamide gel as described in Chew et al. [21]. After electrophoresis, the fluorescent intensity of EGFP band on the gel was captured and measured using an imaging system. The intensity value was then compared with a standard equation developed by using pure EGFP to determine the total amount of functional EGFP. The yield of solubilization was calculated as the total amount of EGFP in supernatant sample to the total amount EGFP in IBs suspension.
2.6. Bradford assay
The amount of total protein was determined by following Bradford [23] with bovine serum albumin as the protein standard. Protein samples (20 µL) were mixed with 200 µL of Bradford reagent [0.05% (w/v) Coomasie Brilliant Blue G-250, 23.75% (v/v) ethanol, and 42.5% (v/v) ortho-phosphoric acid] and analyzed with a microplate reader (Infinite 200 PRO, Tecan) under an absorbance value at wavelength of 595 nm. The purity of solubilized EGFP was calculated as the total EGFP amount to the total protein amount in supernatant sample.

3. Results and discussion

3.1. Solubilization of inclusion bodies using urea
Recovery of functional protein from IBs requires solubilization processes. By using conventional IBs solubilization method, different concentrations of urea (0 to 8 M) and incubation temperatures (50 to 80°C) were applied to denature the EGFP IBs and the yield and purity were determined (figure 1). These two factors have a marked influence on the yield and purity of solubilized EGFP. Figure 1a shows that 8 M of urea dissolved most of the IBs. The yield of solubilized EGFP was increased as the concentration of urea increased from 0 to 8 M for every incubation temperature. The present findings are as reported by Zou et al. [24]. The high level intermolecular β-sheet structure in IBs is formed by hydrophobic bonding. Urea as denaturant tends to interact with polar group of protein and this cause the disruption of hydrophobic interaction within the protein structure [25]. After the disruption, hydrogen bonding will be formed between the urea and amino side chain that turns protein structure becomes primary structure [26]. The presence of urea denatures the IBs protein by decreasing the hydrophobic effect, which linearly depends on the urea concentration.

![Figure 1](image_url)

**Figure 1.** Effects of urea concentration and incubation temperature on the solubility of EGFP IBs: (a) Yield of solubilized EGFP. (b) Purity of solubilized EGFP. Error bars represent duplicate independent experiments with duplicate measurements.
For the incubation temperature, the yield and purity of solubilized EGFP increased from 50 to 70°C (figure 1). Under high incubation temperature, IBs are heated and certain amount of energy is absorbed. High incubation temperature increases the enthalpic reaction of non-polar group of protein. The absorbed energy causes the protein molecules start to vibrate rapidly and violently which results in protein structure disruption. When the temperature was increased to 80°C, there were a slight decrease in the yield obtained and the solubilized protein purities are low. This might be due to the huge amount of heat that has changed and broken the covalent bond within the protein structure and causes protein degradation. Hence, IBs solubilization with 8 M of urea concentration and incubated at 60°C were found to be the best condition achieved, wherein high values of yield (61%) and purity (10%) were achieved.

3.2. Solubilization of inclusion bodies using urea with alkyl alcohol
IBs proteins have been reported to have native-like secondary structure [5-7] and the structure can be protected by using mild solubilization. The solubilizing buffer in the mild solubilization containing 2 M of urea which serves in physical separation of the water and protein molecules by disrupting the hydrophobic interactions [27]. In this study, the solubilizing effect of buffer containing 2 M of urea with different 6 M of alkyl alcohols on EGFP IBs was compared. Figure 2a revealed that all types of alkyl alcohols contributed to IBs solubilization with obtained yields higher than the control sample and n-butanol was the best alkyl alcohol for solubilization of EGFP IBs with high value of yield (45%) and purity (22%).

![Figure 2](image-url)

**Figure 2.** Solubilization of EGFP IBs using 2 M of urea with alkyl alcohol. (a) Effect of different alkyl alcohols and (b) Effect of concentration of n-butanol on the yield and purity of solubilized EGFP. Error bars represent duplicate independent experiments with duplicate measurements.
IBs solubilization using alkyl alcohols which have alpha helix stabilizing properties tend to unfold the proteins but remain the existing native-like secondary structure of protein [15]. By adding IBs into Tris buffer containing alcohol, the hydrophilic (polar) amino acid side chains of protein that dislike alcohol tend to be tucked away and moving toward the protein's interior, while the inner hydrophobic (non-polar) amino acid side chains of protein become exposed to solvent [28]. The exchange of protein structures tends to break the hydrogen bonds between amino acids of proteins and unfold the IBs. The hydrogen bond from the protein was then start to produce new link to alcohol molecules during the alcohol denaturation. Higher chain length of carbons increase the hydrophobicity of solvent and able to attracts more inner hydrophobic amino acid side chains of protein for denaturation. Hence, the yield of solubilized EGFP increased when a higher the carbon chain length solvent was applied.

Methanol, ethanol and ethylene glycol only slightly contribute in IBs solubilization (about 10 to 18% higher than control sample). The results are about similar as reported by Singh et al. [12] where Tris buffer containing these alkyl alcohols failed to solubilize recombinant human growth hormone IBs. Effect of ethanol on the stability of bovine serum albumin was investigated by Yoshikawa et al. [29] and discovered that the favourable interaction of ethanol with hydrophobic residues causes protein denaturation, but the unfavourable interaction with charged groups exposed to the solvent causes reduction of protein solubility. When protein in denatured state, the protein consists of 83% of non-polar chains are exposed to solvent [28]. Alkyl alcohols with lower chain length have higher polarity value will not interact with the exposed non-polar chains of protein due to the difference in molecular polarity. Interaction among the non-polar chains of denature proteins increase the likelihood of aggregation and facilitate precipitation. Ethanol has the ability for denaturation, however it does not lead to better solubility of the denatured protein [28,29]. Hence, contributions of the low chain length alcohol based buffers in solubility are lesser compared with n-butanol based buffer.

Besides the effect of increasing hydrocarbon content, effect of increasing hydroxyl group on solubilizing potential of a solvent was investigate by using ethylene glycol and glycerol which consist of two hydroxyl groups with two carbon atoms and three hydroxyl groups with three carbon atoms, respectively. The yield of solubilized EGFP of ethylene glycol was compared with ethanol and the yield of solubilized EGFP of glycerol was compared with propanol as shown in figure 2a. The increments of hydroxyl content of ethylene glycol and glycerol only slightly contribute to the yields of solubilized EGFP. The addition of hydroxyl groups increased the buffer hydrophilicity, however did not much contributed to the IBs solubilization.

The effect of n-butanol concentration (0 to 6 M) on the IBs solubilization was also evaluated (figure 2b). It was found that enhancement of n-butanol concentration in the mixture resulted in the improvement of IBs solubilization. Similar result was reported by Stigter and Dill [30] in which a higher solvent concentration during denaturation enhanced the protein solubility. High solvent concentration may attracts more inner hydrophobic amino acid side chains of protein, make the hydrophilic amino acid side chains on protein surface twist to the internal protein area and lastly break the hydrogen bonds between protein amino acids.

3.3. Solubilization of inclusion bodies using urea with freeze thaw process
Mild solubilization using 2 M of urea with freeze thaw process was conducted by varying the freezing duration and number of freeze thaw cycle. By freezing the IBs suspension for one day and one freeze thaw cycle, higher yield of solubilized EGFP from IBs was observed (figure 3). Further increase the freezing duration and number of freeze thaw cycle did not help much in solubilizing higher yield of solubilized EGFP. The yield of solubilized EGFP was comparable to that of 8 M of urea in buffer. However, addition of increasing freezing period and freeze thaw cycle had no effect on purity of solubilized EGFP.
Figure 3. Solubilization of EGFP IBs using 2 M of urea with freeze thaw process: (a) Effect of freezing period and (b) Effect of freeze thaw cycle on the yield and purity of solubilized EGFP. Error bars represent duplicate independent experiments with duplicate measurements.

Freeze thaw process affects the protein stability through physical and chemical stresses. During the freezing process, water is converted to ice crystal in which a physical stress is applied for denaturing the IBs. Formation of ice reduces the water content and increases the solutes concentration in the IBs mixture. The concentrated urea and protein mixture may affect protein structure through changing in...
pH and ionic strength which cause chemical degradation [16]. The freeze concentration causes severe stresses to protein stability. The freezing duration determines the amount of applied stress on the frozen IBs mixture and the IBs are denatured during the thawing process.

Freeze thaw process is a gentle process for cell disruption where the cell envelope is damaged by repeating freezing and thawing processes to release the desired protein from cells [20]. In this study, freezing condition results physical and chemical stresses on IBs. After thawing, IBs protein is further stressed by recrystallization. Increasing the freeze thaw cycles exert additional interfacial tension or shear on the entrapped proteins and cause protein denaturation. Repeated cycles of freezing and thawing disrupt cells were applied in this study by forming ice on the cell membrane in breaking down the cell membrane. The produced IBs might consist with undisrupted cell membrane which was further broken down in solubilization process. While slight increase in yield of solubilized EGFP, the destroyed cell membrane might increase the level of contaminants in the sample and causing the same purity reading in figure 3.

3.4. Comparative solubilization of inclusion bodies
IBs solubilization is a part of protein production processes for recombinant proteins that are overexpressed in the bacterial host systems. Solubilization using high concentration of urea will cause a complete disruption of protein structure. The conventional urea solubilization method generates random coil structure where the hydrophobic amino acid patches are exposed and this leads aggregation of protein molecule during refolding process [9,17]. Mild solubilization was reported in which the IBs are solubilized under low concentration of urea with addition physical and chemical stresses. Solubilize IBs in mild condition prevents hydrophobic interaction during the initial stage of refolding and enhances the recovery of active protein from IBs [9].

The mild solubilization methods described in this work could be applied for enhancing the recovery of important proteins expressed as IBs in E. coli. The interaction between alcohol and protein molecules have been widely studied because alcohol has potential to modulate the protein structure. Alcohol denatures proteins by disrupting the side chain intramolecular hydrogen bonding. Freeze thaw process affects the protein stability through external stresses. Low concentration of urea reduces hydrophobic interaction and its combination with alcohol or freeze and thaw process may uses as IBs solubilizing agent which destabilise the tertiary structure of protein as well as protect native-like secondary protein structure. These combine effects of this mild denaturant can enhance recovery of desired protein from IBs and decrease protein aggregation during refolding [11].

Solubilization potential of alkyl alcohol based buffers were compared with that of various urea concentration buffers. The 6 M n-butanol based solubilization buffer may solubilized EGFP from the IBs with results comparable to 6 M urea buffer (figures 1 and 2). Besides, the result of mild solubilization using 2 M of urea with freeze thaw process for one day and one freeze thaw cycle was comparable to the traditional 8 M urea denaturation method (figures 1 and 3). It was observed that even though the yield of recombinant protein solubilized in n-butanol based buffer was lower than that achieved with 8 M of urea buffer, the obtained purity was better when solubilized in n-butanol based buffer. Chaotropic agent such as urea is used for cell lysis by breaking hydrophobic and hydrophilic interactions of cells [31]. The IBs undisrupted cell membrane may further destroyed can contribute impurities for low purity. Under the different solubilization temperature and urea concentration, the best obtained purity was around 10% in comparison that of 20% observed for 6 M n-butanol based buffer. Solubilized EGFP with lesser contaminating proteins may reduce aggregation during refolding and enhance the overall renaturation yield.

4. Conclusion
Conventional and mild solubilization methods were conducted by varying its process condition to investigate the effects of the solubilization methods on denaturation of EGFP from IBs. Incubation of IBs suspension at temperature of 60°C with 8 M of urea based buffer achieved a yield of 61% and purity of 10%. However, mild solubilization of IBs protein using the alkyl alcohol and freeze thaw
methods generally applicable. The yield and purity of solubilized EGFP in 6 M of n-butanol and 2 M of urea based buffer were 45% and 22%, respectively. Besides, the yield (66%) and purity (9%) of mild solubilization using 2 M of urea with freeze thaw process was comparable to the conventional 8 M urea denaturation method. These mild solubilization methods can be utilized to prepare large qualities of active soluble proteins from IBs for research and industrial purpose.

Acknowledgments
This work was supported by Universiti Malaysia Pahang under grant RDU1603149.

Reference
[1] Yang Z, Zhang L, Zhang Y, Zhang T, Feng Y, Lu X, Lan W, Wang J, Wu H, Cao C and Wang X 2011 *PloS one* 6 e22981
[2] Marston FAO and Hartley DL 1990 *Methods Enzymol.* 182 264
[3] Dill KA and Shortle D 1991 *Annu. Rev. Biochem.* 60 795
[4] Panda AK 2003 *Adv. Biochem. Eng.Biotechnol.* 85 43
[5] Peternel Š, Jevševar S, Bele M, Gaberc-Porekar V and Menart V 2008 *Biotechnol. Appl. Bioc.* 49 239
[6] García-Fruitós E, González-Montalbán N, Morell M, Vera A, Ferraz R, Aris A, Ventura S and Villaverde A 2005 *Microb. Cell Fact.* 4 27
[7] Peternel S and Komel R 2011 *Int. J. Mol. Sci.* 12 8275
[8] García-Fruitós E 2010 *Microb. Cell Fact.* 9 80
[9] Singh A, Upadhyay V, Upadhyay A, Singh S and Panda A 2015 *Microb. Cell Fact.* 14 41
[10] Khan RH, Rao KB, Eshwari AN, Totey SM and Panda AK 1998 *Biotechnol. Prog.* 14 722
[11] Singh SM and Panda AM 2005 *J. Biosci. Bioeng.* 99 303
[12] Singh S., Sharma A., Upadhyay A., Singh A., Garg L and Panda, A 2012 *Protein Expres. Purif.* 81 75
[13] Day R, Bennion BJ, Ham S and Dagget V 2002 *J. Mol. Biol.* 322 189
[14] Berkelman T, Brubacher MG and Chang H 2004 *Bio-Rad laboratories* 114 30
[15] Kumari NKP and Jagannadham MV 2011 *J. Protein Proteom.* 2 11
[16] Cao E, Chen Y, Cui Z and Foster PR 2003 *Biotechnol. Bioeng.* 82 684
[17] Qi X, Sun Y and Xiong S 2015 *Microb. Cell Fact.* 14 24
[18] Chew FN, Tan WS, Boo HC and Tey BT 2012 *Prep. Biochem. Biotech.* 42 535
[19] Malavasi NV, Cordeiro Y, Rodrigues D and Chura CRM 2014 *Process Biochem.* 49 54
[20] Johnson BH and Hecht MH 1994 *Nat. Biotechnol.* 1970
[21] Chew FN, Tan WS, Ling TC, Tan CS and Tey BT 2009 *Anal. Biochem.* 384 353
[22] Laemmli UK 1970 *Nature* 227 680
[23] Bradford MM 1976 *Anal. Biochem.* 72 248
[24] Zou Q, Habermann-Röttinghaus S and Murphy K 1998 *Proteins: Structure, Function, and Bioinformatics* 31 107
[25] Tanford C 1970 *Adv. Protein Chem.* 23 121
[26] Almarza J, Rincon L, Bahsas A and Brito F 2009 *Biochem.* 48 7608
[27] Patra A, Mukhopadhyay R, Mukhiya R, Krishnan A, Garg L and Panda A 2000 *Protein Expr. Purif.* 18 182
[28] Pace CN, Treviño S, Prabhakaran E and Scholtz JM 2004 *Philos. T. R. Soc. B* 359 1225
[29] Yoshikawa H, Hirano A, Arakawa T and Shiraki K 2012 *Int. J. Biol. Macromol.* 50 1286
[30] Stigter D and Dill KA 1993 *Fluid Phase Equilibr.* 82 237
[31] Islam MS, Aryasomayajula A and Selvaganapathy PR 2017 *Micromachines-Basel.* 8 83