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Article

Effect of environment factors on the expression of soluble PDE8A1 in *E. coli*

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SUMMARY

PDE8, a type of phosphodiesterase (PDE), is proven to be crucial in various cellular activities and physiological activities by influencing second messenger systems. It is involved in a wide range of diseases, including Alzheimer's disease and various heart diseases. However, there is limited information about PDE8 selective inhibitors. The lack of studies is mainly due to the difficulty of obtaining large amounts of active, soluble enzymes, as only a limited amount of PDE8 is expressed by cells. Hence, this work aimed to improve the solubility and yield of PDE8 in the supernatant by exploring suitable culture conditions, including temperatures and different additives. We hypothesized that the presence of additives including glycerol, betaine, L-arginine, and L-glutamate, as well as different culture temperatures can positively affect the concentration of soluble PDE8A1 expressed by cultures of *Escherichia coli*. We conclude that PDE8A1 catalytic domain, the only splice variant of PDE8A with a complete functional domain, expression is favored at higher temperatures, at about 16°C. A glycerol concentration of 8% promotes the solubility of PDE8A1 expressed greatly. Betaine did not increase the concentration of soluble PDE8A1, but could potentially increase the its stability. L-glutamate and L-arginine had similar effects on the expression of soluble protein and were able to evidently increase the solubility of PDE8A1.

INTRODUCTION

Phosphodiesterase (PDE) is a family of enzymes that can hydrolyze second messengers cAMP and cGMP (1). The balance between PDEs and these two second messengers regulates physical activities of the human body (2). Phosphodiesterase 8 (PDE8) is cAMP-specific and carries various important roles in the human immune system, nervous system, steroidogenesis, and insulin secretion by islet cells (3–8). PDE8 also has a possible role in various diseases including Alzheimer's disease, heart disease, and diabetes (5, 8, 9).

Although PDE8 is considered to be involved in a range of diseases, there has been little research into it compared with other members in the PDE family, especially research focusing on PDE8 selective inhibitors and their therapeutic uses. One reason for this could be that it is difficult to obtain active, soluble PDE8 for researchers to study its structural and kinetic characteristics (10). This difficulty is partly because of unexpected expression of PDE8 in the inclusion body, and to reduce this, either refolding or expression regulation is needed.

The PDE8 family contains two isoforms, PDE8A and PDE8B (11), and the expression of PDE8A is higher than that of PDE8B in human body (12). The growth of *E. coli* cells depends greatly on the culture conditions in which cells are grown (11). The components, additives, and temperature of the culture may regulate the expression of PDE8A1catalytic domain, which is the only splice variant with a complete functional domain (1). By modifying the expression condition, the solubility of expressed PDE8A1 can be improved. Additives commonly used by researchers to promote the expression of proteins are glycerol, L-arginine, L-glutamic acid, and betaine (12–14). Research has been able to explain their effect in terms of molecular interactions (12–14).

Glycerol, an osmolyte, is widely used as a solubility enhancer in aqueous solution. It acts by influencing the dielectric constant and refractive index of the solution (12). The expression and yield of recombinant protein produced by *Escherichia coli* is greatly increased when glycerol is present in the culture medium. The proteins expressed in environments containing glycerol are more stable than proteins expressed in the absence of glycerol when exposed to denaturing conditions (15). Furthermore, glycerol is able to promote crystal nucleation of proteins by decreasing the energy barrier to nucleation (16). The beneficial effect of glycerol and other solubility enhancers could help produce PDE8 crystals for various structural studies.

L-arginine and L-glutamate are able to effectively prevent protein aggregation and precipitation and could greatly increase the long-term stability of proteins up to 4–8 times (13). The addition of 50 mM L-arginine and L-glutamate to a protein solution is proven to significantly decrease protein aggregation and increase their solubility. The stability and lifetime of proteins are also increased (17). This effect could be due to the extra hydrogen bonds between the excipients and the protein surface when both excipients are present (17). L-arginine alone plays an important role in protein refolding, solubilization, and purification (18). It enhances the surface tension of solvents and interacts with amino acid side chains and peptide bonds (19). The positive effects of L-arginine and L-glutamate make them ideal additives to prepare stable, long-lasting crystals for structural studies.

Betaine is an osmolyte that was proven to stabilize proteins (14). Research has shown that different betaine concentrations

could affect protein aggregation differently. At a concentration of 5–7.5 mM betaine, proteins tend to aggregate, but at 10–20 mM, betaine effectively decreases aggregation and can disrupt preformed protein aggregates (14). Studies have been carried out to determine the structural characteristics that underlie its stabilizing ability. This effect could be due to the exclusion of betaine from aliphatic carbons and amide oxygens on protein surfaces (20). Due to its stabilizing effect, betaine plays an important role in cells. It is able to increase heat tolerance and stabilize various proteins when a hyperosmotic shock occurs (21,22). The stabilizing effect of betaine could effectively help the successful expression of protein, and could potentially increase the concentration of soluble protein samples for future study.

Temperature has also been shown to be critical for solubility of expressed protein (23). Modification of expression temperature can greatly and successfully increase the solubility of various proteins (23). It is shown that low temperature can promote protein solubility and decrease the formation of inclusion bodies when the proteins are expressed from *E. coli* cells. Temperature reduction can partially decrease the expression of heat shock protease and inhibit hydrophobic interactions which favor aggregation reactions (24).

We hypothesized that a culture environment with suitable temperature and additives could help *E. coli* cells express high quality PDE8A1 in an active and soluble form. Therefore, our primary aim was to find the optimal temperature and additives to facilitate high quality PDE8A1. The production of enough PDE8A1 can rener it as a target for further studies aimed at determining selective inhibitors in the context of multiple diseases. We postulated that all additives tested under optimized temperature could positively affect the expression of soluble PDE8A1, as they are commonly used as protein solubility promoters and stabilizers. The results of our study supported this hypothesis, as we identified positive effects on solubility of PDE8A1 with addition of glycerol, L-arginine, and L-glutamate, while betaine promoted the stability of PDE8A1.

RESULTS

Flocculent precipitation observation

This test aimed to determine the stability of the expressed PDE8A1. Generally, the more unstable expressed PDE8A1 is, the more flocculent precipitation is presented. The control sample contained obvious, large amounts of visible flocculent precipitation at both 12°C and 16°C (**Table 1**).

In cell samples grown in culture with glycerol as additive at both 12°C and 16°C, large amount of flocculent precipitation existed at 1%, while moderate amount of precipitation was observed in 4% and 8% (**Table 1**). This suggests that glycerol, as an osmolyte, is able to moderately increase the stability of PDE8A1 at a higher concentration level.

Most samples collected from cells grown in culture with betaine added showed limited or no presence of flocculent precipitation at both temperatures (**Table 1**). This suggests that betaine is either an effective protein stabilizer and can successfully stabilize the expressed PDE8A1 to maintain its solubility or that it is an inhibitor of PDE8A1 expression.

Glycerol			Betaine		
	12 ℃	16 ℃		12 ℃	16 ℃
0	++	++	0	++	++
1%	++	++	1 mM	-	+
4%	+	+	5 mM	-	-
8%	+	+	10 mM	-	-
L-Arginine			L-Glutamate		
	12 ℃	16 ℃		12℃	16 ℃
10 mM	++	++	10 mM	++	++
30 mM	++	++	30 mM	++	+
50 mM	++	+	50 mM	+	++

Table 1: Effect of glycerol, betaine, L-arginine and L-glutamate concentration and temperature on PDE8A1 flocculent precipitation in supernatant. The table shows the amount of flocculent precipitation observed in each supernatant sample (n=3). Cell samples were grown in LB culture with glycerol (0, 1%, 4%, 8%), betaine (1 mM, 5 mM, 10 mM), L-arginine or L-glutamate(10 mM, 30 mM, 50 mM) added at 12°C or 16°C, and overexpression was induced by 0.1 mM IPTG for 24 hours. Large amount of precipitant is noted as "++", moderate amount of precipitant is noted as "+", limited amount of precipitation is noted as "-".

Supernatant samples from cells grown in culture with L-arginine or L-glutamate added showed large amounts of precipitation (**Table 1**). Although there were some samples with moderate amounts of precipitation, there was no obvious trend and relationship between the additive concentration and precipitation amount. This suggests that L-arginine and L-glutamate wouldn't be able to promote the stability of PDE8A1 at concentration levels we tested.

Biuret test

This test aimed to determine whether PDE8A1 is presented in the supernatant samples. The control group did not undergo apparent color change in the biuret test at both 12°C and 16°C (**Table 2**). The color is light, purplish-blue. This indicates that the concentration of soluble PDE8A1 is low.

Cell samples that were grown and induced in cultures containing glycerol at all concentrations (1%, 4%, and 8%), at both 12°C and 16°C, showed moderate color change in the biuret test (**Table 2**). The color changed from blue to purplish blue. This showed that low amount of PDE8A1 is expressed under these conditions.

In cell samples with betaine as the additive, at 12°C the color change in the biuret test slightly differed with betaine concentration (**Table 2**). The sample with the lowest concentration of betaine, 1 mM, showed little color change. Cell samples at both 5 mM and 10 mM indicated moderate color change, suggesting a higher concentration of expressed protein. Cell samples with betaine of all concentrations and cultured at 16°C all showed moderate color change, showing the presence of soluble PDE8A1 in the supernatant (**Table 2**).

Cells grown in culture with L-arginine added at 12°C all showed moderate color changes in the biuret test, suggesting

		12 ℃	16 ℃
Control		-	-
Glycerol	1%	+	+
	4%	+	+
	8%	+	+
Betaine	1 mM	-	+
	5 mM	+	+
	10 mM	+	+
L-arginine	10 mM	+	+
	30 mM	+	+
	50 mM	+	++
L-glutamate	10 mM	-	-
	30 mM	-	-
	50 mM	-	-

Table 2: Effect of glycerol, betaine, L-arginine and L-glutamate concentration and temperature on results of PDE8A1 supernatant samples in biuret tests. The table shows the extent of color change in the biuret test (n=1). Cell samples were grown in LB culture with glycerol (0, 1%, 4%, 8%), betaine (1 mM, 5 mM, 10 mM), L-arginine or L-glutamate(10 mM, 30 mM, 50 mM) added at 12°C or 16°C, and overexpression was induced by 0.1 mM IPTG for 24 hours. Apparent change in color is noted as "++", moderate change in color is noted as "+", limited change in color is noted as "-".

the presence of soluble PDE8A1 (**Table 2**). However, when expressed at 16°C, the cells grown in 50 mM L-arginine showed a more apparent color change than the other tested groups while both the cells grown in 10 mM and 30 mM showed moderate color change (**Table 2**).

Cells grown in L-glutamate at a concentration of 10 mM, 30 mM, or 50 mM at both 12°C and 16°C, showed no difference in color compared to the control group (**Table 2**). This suggests the presence of L-glutamate may not affect the expression of PDE8A1.

Ultraviolet spectrophotometry

We tested the A280 of supernatant samples that were collected to determine the concentration of soluble PDE8A1 in them. By comparing the results obtained in the control group at different temperatures, it was clear that 16°C resulted in a higher yield of soluble PDE8A1, with an average A280 value of 0.232, higher than 0.128 of the 12°C control group (**Figure 1**). The significant difference (p=0.0006) indicated that the expression of PDE8A1 might favor a higher temperature around 16°C, instead of a lower temperature of 12°C.

In cells grown in culture with glycerol added, the A280 values of cells grown at both temperatures with glycerol added at all concentration levels are significantly different from the corresponding control groups (p<0.0001) according to the Dunnett's multiple comparison test. The one-way ANOVA test of samples grown in both 12°C (f(3)=214.2, p<0.0001) and 16°C (f(3)=102.3, p<0.0001) showed that variation in results was more likely a consequence of glycerol added and not due to chance. The A280 increased as the concentration of glycerol increased from 1% to 8% (**Figure 2A**). This suggests that the presence of glycerol has a positive effect on the

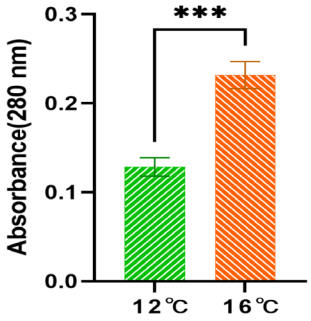

Figure 1: Effects of temperature on PDE8A1 expression. Bar graph showing the 280 nm absorbance of supernatant samples (n=3). Cell samples were grown in LB culture with no additives and at 12°C or 16°C, and overexpression was induced by 0.1 mM IPTG for 24 hours. Independent samples t-test was conducted to analyze the data. *** p<0.001.

expression of PDE8A1 at concentrations of 1% to 8%, and might continue to promote the solubility when the additive concentration increases (19).

For the cells that were grown in culture with betaine added, the A280 values with different concentrations of betaine grown at 12°C were similar with no significance differences (p>0.1). However, cells grown in culture with added betaine at 16°C showed a significant difference in A280 values between 0 and 5 mM treatments(p<0.01) (**Figure 2B**). One-way ANOVA results showed that there was no significant difference among the means of groups at 12°C (f(3)=0.75, p=0.5523) but was a significant difference observed at 16°C (f(3)=6.015, p=0.0190). The increase of betaine concentration from 1 mM to 10 mM did not significantly affect the A280 values in treatment groups maintained at 12°C. In fact, in comparison to the control group, it seems like betaine did not necessarily affect the concentration of expressed PDE8A1 (**Figure 2B**).

For the samples from cells grown in culture with L-arginine, we found a statistically-significant difference in A280 values

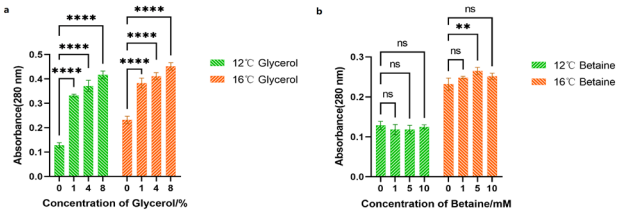

Figure 2: Effects of different concentrations of additives on PDE8A1 expression at selected temperatures. (a) The effect of glycerol concentration on PDE8A1 expression at 12°C and 16°C. Bar graph showing the 280 nm absorbance of supernatant samples (n=3). Cell samples were grown in LB culture with glycerol (1%, 4%, 8%) added at 12°C or 16°C, and overexpression was induced by 0.1 mM IPTG for 24 hours. **(b)** The effect of temperature and betaine concentration on PDE8A1 expression. Bar graph showing the 280 nm absorbance of supernatant samples. (n=3) Cell samples were grown in LB culture with betaine (1 mM, 5 mM, 10 mM) added at 12°C or 16°C, and overexpression was induced by 0.1 mM IPTG for 24 hours. One-way ANOVA test and Dunnett's multiple comparison test was conducted to analyze the data.**p<0.01, ****p<0.0001.

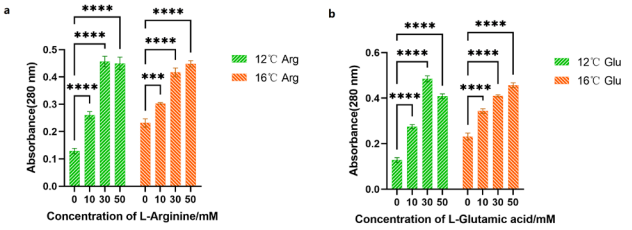

Figure 3: Effects of different concentrations of amino acids on the PDE8A1 supernatant expression. (a) Effect of temperature and L-glutamate concentration on PDE8A1 expression. Bar graph showing the 200 nm absorbance of supernatant samples. (n=3) Cell samples were grown in LB culture with L-arginine (10 mM, 30 mM, 50 mM) added at 12°C or 16°C, and overexpression was induced by 0.1 mM IPTG for 24 hours. One-way ANOVA test was conducted to analyze the data. **(b)** Effect of temperature and L-arginine concentration on PDE8A1 expression. Bar graph showing the 280 nm absorbance of supernatant samples. (n=3) Cell samples were grown in LB culture with L-glutamic acid (10 mM, 30 mM, 50 mM) added at 12°C or 16°C, and overexpression was induced by 0.1 mM IPTG for 24 hours. One-way ANOVA test and Dunnett's multiple comparison test was conducted to analyze the data. ***p<0.001, ****p<0.0001.

between the treatments of concentration at 0, 10, 30 and 50 mM at 12°C (f(3)= 260.1, p<0.0001) and similar differences was observed at 16°C (f(3)=191.3, p<0.0001) (**Figure 3A**). The A280 values of samples with different concentrations and at both temperatures were all significantly different from the control group according to the Dunnett's multiple comparison test (p<0.001), suggesting a positive effect of L-arginine on expression of soluble enzyme (**Figure 3A**). The maximum concentration achieved by adding 50 mM of L-arginine was similar to that of samples with 8% glycerol added at 16°C, with A280 reaching about 4.6.

The results of L-glutamate as an additive are similar to the results from L-arginine (**Figure 3B**). Variations in results between 0,10, 30, and 50 mM treatment groups at both 12°C (f(3)=631.9, p<0.0001) and 16°C (f(3)=240.8, p<0.0001) were significantly different among group means. The results of all groups with L-glutamate added at both temperatures are all significantly different from the control groups (p<0.0001), suggesting a noticeable positive effect of it on PDE8A1 expression. Moreover, the A280 values of samples grown at 16°C were generally higher than that of samples grown in 12°C, except for the concentration of 30 mM. This again proves that temperature has a role in the expression of soluble PDE8A1.

DISCUSSION

This work determined the effects of four different additives and two different temperature levels on *E. coli* cells, which were tested for the concentration of soluble PDE8A1 following cell lysis. We conclude that the results partially support the hypothesis that a culture environment with suitable temperature and additives could help *E. coli* cells express PDE8A1 in a high quality, active and soluble form. For the presence of glycerol, L-arginine, and L-glutamate at different concentrations, and for an expression temperature of 16°C, the concentration of PDE8A1 expressed is positively affected, according to biuret test and ultraviolet spectrophotometry. On the other hand, the presence of betaine has the greatest positive effect on protein stability, according to the flocculent precipitation presented.

From the results, it is clear that glycerol has a positive effect on the expression of soluble PDE8A1 at both 12°C and 16°C, supporting the fact that glycerol is a common solubility promoter. In ultraviolet spectrophotometry, the experimental groups with glycerol added presented A280 results greatly higher than that of the control groups. The effect of glycerol is due to the modification of the dielectric constant and refractive index of the solvent (12). Using the intrinsic fluorescence spectroscopy, we determined that glycerol does not modify the structure of the protein (15). This ensures the protein is functional, promoting solubility without changing its basic characteristics. Some studies have also shown that protein expressed in the presence of glycerol is also more stable in denaturing conditions (16). However, in our experiment, glycerol did not show stabilizing ability, still allowing the PDE8A1 to form flocculent precipitates. This contrast could be due to the experimental environment, since the samples are centrifuged at room temperature, which may negatively affect the folding of protein. As PDE8A1 originally has low stability, the high temperature could have covered the effects of glycerol and caused the precipitation. One advantage of using glycerol as an additive is that it can promote both solubility and crystallization (12). Research has shown that glycerol can effectively decrease the energy barrier to nucleation, interfering with the attraction force between protein molecules (12). The addition of glycerol could effectively produce large amount of PDE8A1 crystal structures, and can be used in structural studies.

The effect of betaine on the expression of soluble PDE8A1 differed from that of glycerol. Our results in ultraviolet spectrophotometry showed that betaine did not have a significant effect on PDE8A1 solubility, according to the comparison with control groups, but can promote the stability of protein expressed. This can be concluded by the fact that protein samples with betaine as an additive showed little flocculent precipitation compared to the samples with glycerol added. The stabilizing effect of betaine has been well studied (20). It is shown to be dependent on its concentration (14). At a concentration of 5 mM to 7.5 mM, proteins tend to form macroscopic prefibrillar structures (14). At a concentration higher than 10 mM, proteins tend to disaggregate and form soluble assemblies (14). Different concentrations of betaine could be a useful stabilizer for PDE8A1. Although there were no positive effects on the concentration of protein expressed, betaine probably could be used with other additives that are effective to increase the solubility of PDE8A1 to produce protein that is more soluble and more stable.

The effects of L-glutamate and L-arginine are very similar, as the A280 value from samples with these additives were

very similar, suggesting a similar mechanism of interacting with protein expression. All groups with L-arginine and L-glutamate as additives showed significantly different results from the control groups, suggesting significant positive effects. The effect of L-arginine can be explained by its suppression of protein interactions, as it interacts with most amino acid chains and peptide bonds favorably (19). Further tests can be carried out to see the differences and similarities of the two additives in a wider concentration range to find out the most effective one. Both additives enhance the solubility of PDE8A1 better than glycerol. The use of them in preparation of a large amount of PDE8A1 could effectively increase the efficiency. Additionally, some research has shown that the mixture of L-arginine and L-glutamate can produce even better protein solubility, which can successfully increase the solubility and long-term stability, providing a much simpler method to produce a large amount of soluble proteins (13). This method should be tested on the expression of PDE8A1 to see if it is more effective than using the additives alone.

The research can still be improved. While we tested several additives, additional additives remain to be tested that could affect the expression and stability of PDE8A1. More additives should be tested as combination of different additives could also produce good or even better results than using them alone. Other additives like β-ME, trehalose, $CuCl_2$, and proline should be tested (25). Beside the kinds of additives, the additive concentrations tested should be wider and intervals should be smaller. This allows the results to show a clear tendency of solubility change under a wide range of environments, and makes the results more practical. More temperature levels can be used for expression, as temperature greatly influences the expression of proteins (26). An optimum temperature can greatly increase the yield of soluble, active PDE8A1. Possible temperatures that can be tested are 20°C, 24°C, 28°C, 32°C, 37°C, and 40°C, which are shown to have different effects on the expression of proteins (27).

Other than variables, our experimental method could be modified. More cells should be grown in different environments, so the amount of protein expressed can be easily determined and compared. Each environment should contain more than one group of cells, have an increased number of cells, and measure their mean to be scientific. Several detailed steps of the experiment are tricky and challenging. For instance, PDE8A1 could be unstable under room temperature, and centrifugation at room temperature could cause negative effects on activity and stability of soluble protein. There are also other possible factors that can affect active PDE8A1 expression. For instance, the presence of several chaperones is shown to be essential for the correct folding of various proteins, preventing aberrant folding and aggregation (28). The sequence optimization of the *E. coli* strain can also affect protein expression.

By increasing the scale of experiments, the concluded pattern could be likely generalized for all laboratory conditions. Therefore, the additives tested can be applied to the preparation of PDE8A1 in different studies, especially for research of its characteristics and screening of its selective inhibitors. This method can greatly increase the yield of PDE8A1 expressed, decreasing the need to refold the protein in the inclusion body. It can be included in a more efficient, effective procedure of preparing PDE8A1. This can be time- and cost-saving, so more research centered on PDE8A1 and PDE8A1 inhibitors can be carried out, leading to better understanding of the physiological roles of PDE8A in the human body, its characteristics, and how it can be successfully inhibited to treat related diseases. The experimental method could also be applied to the preparation of other proteins of the PDE family, especially ones that are shown to be important for physiological events. For example, this method could benefit the preparation of PDE6 and PDE10. PDE6 is shown to play a crucial role in various retinal diseases, while PDE10 is accounted in treatment of schizophrenia and cancer (29-31). A more efficient protocol of active enzyme preparation could empower the study of the PDE family in multiple dimensions, from characteristic studies to inhibitor design and testing.

While our study supports the proposed hypothesis, it would be worthy to further investigate a wider range of manipulated variables to increase the validity and applicability of the study outcomes.

MATERIALS AND METHODS

Plasmid Transformation

A plasmid encoding PDE8A1 was transferred into *E. coli* BL21 cells. BL21 frozen competent cells (100μL) were thawed on ice, then 2 μL of plasmid is added. The cells were placed in an ice bath for 25 min, then put in a thermostat water bath at 42°C for 90 s, followed by an ice bath for 5 min. 800 μL of LB culture, containing 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, were added to the cells. Cells were grown in the shaker at 37°C, 200 rpm for 45 min. The cell sample was then centrifuged at 600 rpm for 5 min, followed by the removal of 650 μL supernatant. The strain was dripped on a petri dish, containing 100 mL LB culture, 1.5g agar, 50 μg/mL ampicillin (AMP) that is filtered by 0.22 μm syringe filter. Strain was grown in 37°C for 12 h.

Cell growth

A colony of transformed strain was obtained and placed in 3 mL of modified LB culture, containing 10 g/L tryptone, 5 g/L yeast extract, 10g/L NaCl ,50 μg/mL ampicillin that was filtered, and 30 μg/mL chloramphenicol (CAP). The cells were grown in a shaker at 37°C, 200 rpm overnight to produce monoclonal strains. The *E. coli* strain was then mixed with 50% glycerol with volume ratio 1:1, and stored in -80°C.

The strain was diluted 1:200 in 25 mL modified LB culture, containing 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 50 μg/mL AMP, 30 μg/mL CAP, and 0.2% glucose. Strains were then grown in a shaker at 37°C, 200 rpm until OD600 reached 0.6–1.0.

Protein expression

Isopropyl-1-thio-β-D-galactopyranoside (IPTG) that had been filtered by 0.22 μm syringe filter was added to the LB medium reach 0.1 mM to induce overexpression for 24 hours. At the same time, different groups of additives were added (1, 4, and 8% glycerol; 10, 30, and 50 mM L-arginine at pH 7.04; 10, 30, and 50 mM L-glutamate at pH 7.40; 1, 5, and 10 mM betaine; or a control group with no additive) while expression occurred at different temperature (12°C and 16°C). The cells were then centrifuged at 4000 rpm for 30 minutes. Pellet was collected and stored at -80°C.

Every cell sample was added with 200 μL of lysis buffer containing 20 mM Tris-HCl pH 8.0, 300 mM NaCl, 15 mM imidazole, and 1 mM β-Mercaptoethanol (β-ME). A hand grinder (Jinxin Technology, LC-MY-10) was used to break the cells, grinding 3 to 5 minutes. Samples were then centrifuged at 12000 rpm for 15 minutes, and the supernatant which contain the soluble enzyme was then obtained.

Tests and data analysis

The supernatant sample was first observed to determine if visible flocculent precipitation exists. Samples that did possess such precipitation was recorded as having low protein stability, and the additives were considered to have no stabilizing effect.

The biuret test was first carried out on half of the supernatant samples. Three drops of solution A (0.1 g/mL NaOH) were added to the sample, then three drops of solution B (0.01 g/mL $CuSO_4$). The color change indicates whether protein is presented in the supernatant. A change from blue to purple suggests the presence of proteins.

The ultraviolet spectrophotometer (Shanghai JingHua Technology, UV spectrophotometer 745) was then used to test the other half of the supernatant samples. One hundred μL of the supernatant was obtained, then mixed with 400 μL of lysis buffer. The A280 value of the samples were tested to determine the concentration of PDE8A1 expressed. Each sample was tested three times, and the average value was obtained.

The results were plotted and analyzed using GraphPad Prism (Version 9.4.0). Samples were catergorised under two treatments conditions i.e concentration of additives and temperature. The A280 value of each sample group at different additive concentrations were compared within each temperature condition i.e. 12°C or 16°C using a One-way ANOVA. The One-way ANOVA compared the means of independent groups under each concentration to determine whether there was statistical evidence to indicate significant difference across the means. To identify the pairs of means (control and experimental groups) showing significant differences, the Dunnet's multiple comparison post-hoc test was run if One-way ANOVA tests showed significant findings (Figure 2 and 3). The A280 values of the control groups i.e those without additives but under different temperature conditions were compared using an independent samples t-test (Figure 1), The values were then plotted as a bar graph where significances were denoted with an asterisk.

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