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Purification, Activity Assay and Optimization of Antarctic Algae *Chlamydomonas* sp. ICE-L Photolyase Expressed in *E.coli*

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***Chlamydomonas* sp. ICE-L is a type of ice algae capable of withstanding the strong ultraviolet radiation in Antarctic regions. Existing research reveals that DNA photolyase is the key compound responsible for resisting UV-B radiation, repairing DNA damage, and maintaining hereditary stability. We have sequenced and analyzed the transcriptome of *Chlamydomonas* sp. ICE-L and identified the cyclobutane pyrimidine dimmer(CPD) gene fragment. The full-length cDNA sequence has been determined by the RACE method and analyzed using bioinformatics software. As well, a CPD photolyase recombinant gene bacteria have been engineered. To improve the protein concentration and lay foundation for further studies of photolyase function in cells and mouse skin, this article focuses on purifying the protein, sequencing the protein N-terminal, and confirming the optimal conditions for *PHR2* expression. N-terminal sequencing identified the five amino acids at the N-terminal as D/Q/T/A P K R T. Response surface methodology assays confirmed the optimal conditions for recombinant bacteria expressing photolyase to be a pH of 6.98, salinity of 1.40%, 22.01 °C, and IPTG concentration of 0.53 mmol/L.**

Key words: *Chlamydomonas sp*. ICE-L, photolyase, *E.coli*, activity assay, expression optimization.

Numerous studies have confirmed that UV-B radiation will cause various damages to algae (Rail, 1998; Bischof *et al*., 2006). Algae that survive in the high-radiation environment of the Antarctic possess greater resistance to UV-B than common microalgae (Miao *et al*., 2002; Kan *et al*., 2003). Ice algae play an extremely important role in Antarctic ecosystems. Despite exposure to high radiation, Antarctic ice algae are able to grow and reproduce. This indicates that the ability to efficiently self-repair is an important adaptation to this severe environment, in addition to synthesizing compounds to absorb ultraviolet radiation and eliminate free radicals. Relevant studies have also demonstrated that DNA photolyase serves to repair DNA following ultraviolet damage, and maintains reproductive capability (Takahisa *et al*., 2009).

During the past decade, we have conducted thorough and systematic research on the physiological and biochemical aspects of Antarctic ice algae radio-resistance. Utilizing proteomics, we analyzed specific proteins in algae before and after UV-B radiation. Photolyase that acts primarily in life processes also plays a large role in radio-resistance of ice algae (Kan *et al*., 2006). Compared with other organisms, ice algae DNA photolyase must be specific in constructure and functionality, to allow survival in high ultraviolet radiation conditions. We chose *Chlamydomonas* sp. ICE-L as our study subject.

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Photolyase of *Chlamydomonas* sp.ICE-L is a new and peculiar repair enzyme. Preliminary research has been conducted: we have acquired the full-length cDNA of photolyase gene *PHR2* and engineered photolyase-expressing bacteria. Our study focuses on the protein purification, sequencing of photolyase protein, and expression of gene *PHR2* in engineered bacteria, following exposure to various temperatures, pHs, salinities(NaCl%), and IPTG concentrations (mmol/L). We hope to confirm the optimal conditions for expression and lay the foundation for further studies of photolyase structure and function in cells and mouse skin.

MATERIALS AND METHODS

Algae, bacteria and their cultivation

The Antarctic ice algae *Chlamydomonas* sp. ICE-L was isolated from floating ice near the Zhongshan Research Station of Antarctica (69°S, 77°E) (An *et al*., 2013). Cultures were grown in Provasoli seawater medium (Provasoli, 1968) at a light density of 40 μM photons $m^{-2}s^{-1}$, under a 12:12 light/dark cycle, at 5 °C. The bacteria culture medium consisted of 5g/L yeast extract, 10 g/L peptone,10 g/L NaCl, and had an initial pH of 7.0. The selective medium contained 100 μg/mL kanamycin. The bacteria medium was incubated at 37 °C and was agitated overnight at 160 r/min.

Protein expression and purification

In order to express the DNA photolyase in *Escherichia* coli, a full-length photolyase gene was inserted at the EcoRI and XhoI sites of pET-28a (+). The resulting plasmid was transformed into *E.coli* BL21 (DE3) by vector(protocol 25, chapter 1, Molecular cloning, 3rd version). Forward primer *PHR2*-eF and reverse primer *PHR2*-eR (Table 1) were designed according to the sequence of the DNA photolyase genes of *Chlamydomonas* sp. ICE-L. The his-tagged photolyase was then produced and purified by His-Trap column(GE Healthcare). After purification, the enzyme was identified by SDS-PAGE.

Assay of photolyase activity

Oligo $(dT)_{16}$ (5' -TTT TTT TTT TTT TTT T-3') primers were purchased from Invitrogen. DNA oligomers containing thymine dimers were prepared by irradiation of room-temperature deoxygenated(Argon) aqueous solutions (1 ml, 100 μM) for four hours, with a 300 W Hg lamp, through a 280 nm cut-off filter (Zhang *et al*., 2011; Livak *et al*., 2011). The 100 μL assay system contained 1 μM protein, 5 μM UV-oligo $(dT)_{16}$, 1 mM DTT, 0.5 mM FAD, and 3 mM $Na_2S_2O_4$ under anaerobic conditions. The mixtures were incubated for 15 minutes and irradiated with a black light lamp (365 nm) for various time periods. For photo reactivation, the mixtures containing PHR2 were placed above daylight lamps for various time periods. The solutions were then boiled for five minutes and centrifuged at 12 000 *g* for 10 minutes. The detection wavelength was 260 nm. The activity was measured in increments from the peak.

Photolyase N-terminal analyzing

Edman degradation is the most commonly utilized method for protein N-terminal sequencing. The technique has been refined and automated, and is widely used in proteomics research. Purified protein solution was concentrated by ultrafiltration and identified by SDS-PAGE. Bio-rad trans-blot was used to transfer the target protein onto PVDF film. The film was slightly stained with coomassie brilliant blue(CCB) R250 and destained until the film was clean (Wang *et al*., 2012). The film was then dried at 37 °C (Sambrook *et al*., 2002) and sent to Shanghai GeneCore Biotechnologies Co., Ltd for sequencing of N-terminal amino acids.

Different conditions assays

The draw bovine serum albumin (BSA) standard curve (Wang *et al*., 2001; Yang *et al*., 2003; Guo *et al*., 1996) was used to determine the protein content. BSA concentrations were 20, 40, 60, 80, and 100 μg/mL(Fig. 1). Engineered bacteria were activated and transcultivated in new Luria broth containing kanamycin. Various culture conditions (Table 2) were controlled. The bacteria were induced for 24 hours, centrifuged, and resuspended with PBS. The cells were then disrupted and centrifuged at 5 000 r/min. The supernate was collected and diluted to 10^{-1} fold. The detection wavelength was 595 nm (Hartree, 1972). The steepest ascent method was used to help determine the response surface central point (Table 3). Response surface methodology (RSM) and Box-Behnken design were utilized to select N=29 representatives (Table 4 and Table 5) for cross assays (Hu *et al*., 2002; Liu *et al*., 2008). The OD_{595} was detected for all samples. Assays were repeated three times.

RESULTS

PHR2 expression and protein purification

The *PHR2* expressed photolyase was identified by SDS-PAGE. Transformants and *E.coli* BL21 (DE3) were simultaneously induced. The calculated molecular mass of photolyase is approximately 64.3 kDa. Photolyase appeared on gel lane P, while not on lane N(Fig. 2a). Lane P contained transformants and disintegrated supernate, and lane N contained *E.coli*. The photolyase concentration was higher than other proteins in the purified filtrate (Fig. 2b).

Activity Assays *In vitro*

To assess the contribution of *PHR2* to the photorepair of UV-induced DNA damage, *in vitro* photolyase activity assays were performed. The results are shown in Fig. 3. The percentage of repaired DNA was 95% after 60 minutes of photo reactivation.

Protein N-terminal analyzing

N-terminal sequencing identified the five amino acids of the N-terminal as D/Q/T/A P K R T. Target sequence of PHR2 photolyase is MPKRT. The protein can be confirmed to be PHR2 photolyase as the outcome indicated.

Optimization assays

Bacterial growth was influenced by several factors, including media pH and salinity. Expression by engineered bacteria was influenced

Table 1. Forward and reverse primers of *PHR2*

Primers for expression	
PHR2-eF	TAGAATTCATGCCGAAGCGAAC
PHR2-eF	TATCTCGAGTCAAGCTCGTGGC

Table 2. Level control of single factor experiments

Factors	Level					
pH	5	6	7	8	9	
Salinity NaCl%	0	1	3	5	7	
IPTG (mmol/L)	0	0.2	0.4	0.6	0.8	1.0
Temperature (°C)	10	15	20	25	30	37

Table 3. Experimental design and results of steepest ascent

No.	pH	Salinity/NaCl%	T/°C	IPTG/mM	Y
1	5	0	15	0.2	0.419
2	6	1	20	0.4	0.502
3	7	3	25	0.6	0.541
4	8	5	30	0.8	0.364
5	9	7	37	1.0	0.247

Table 4. Factors and level value of Box-Behnken

Level	Factors			
	X_1/pH	X_2/Salinity (NaCl%)	X_3/T(°C)	X_4/IPTG(mM)
-1	6	0	15	0
0	7	1.5	22.5	0.5
1	8	3	30	1

by manipulating the temperature and IPTG concentration. The level of photolyase expression was expressed as OD_{595} value. A higher OD value signified higher photolyase expression. Single factor experiments revealed that *PHR2* expression was highest when the pH was 7, salinity was 1%, inducing temperature was 20°C, and IPTG concentration was 0.6 mmol/L (Fig. 4). The parameters of pH 7, salinity 1.5%, T 22.5°C, and IPTG 0.5 mmol/L were selected as the response surface central point using results of steepest ascent. Minitab15 was used to regressively analyze

Table 5. Experimental design and results of Box-Behnken (N=29)

No.	X_1	X_2	X_3	X_4	Y
1	-1	-1	0	0	0.181
2	-1	0	-1	0	0.219
3	0	1	1	0	0.351
4	-1	0	0	1	0.203
5	0	1	-1	0	0.366
6	0	0	0	0	0.596
7	0	0	0	0	0.585
8	0	0	1	1	0.203
9	0	0	-1	-1	0.315
10	0	1	0	-1	0.259
11	0	-1	0	-1	0.301
12	1	0	0	1	0.194
13	1	-1	0	0	0.205
14	-1	0	0	-1	0.189
15	0	0	0	0	0.592
16	1	0	1	0	0.174
17	0	0	0	0	0.598
18	0	-1	-1	0	0.383
19	0	-1	0	1	0.448
20	-1	0	1	0	0.235
21	1	0	-1	0	0.184
22	0	0	1	-1	0.375
23	0	1	0	1	0.358
24	0	0	-1	1	0.362
25	-1	1	0	0	0.211
26	0	0	0	0	0.593
27	1	1	0	0	0.139
28	1	0	0	-1	0.183
29	0	-1	1	0	0.339

Table 6. Analysis of mean square deviation of regression equation

Source	DOF	Seq SS	Adj SS	Adj MS	F	P
Regression	14	0.594414	0.594414	0.042458	23.41	0.000
Linearity	4	0.008302	0.008302	0.002076	1.14	0.376
Square	4	0.570860	0.570860	0.142175	78.67	0.000
Interaction	6	0.015252	0.015252	0.002542	1.40	0.281
Residual Error	14	0.025396	0.025396	0.001814		
Lack of Fit	10	0.025298	0.025298	0.002530	102.42	0.000
Pure Error	4	0.000099	0.000099	0.000025		
Total	28	0.619811				

Table 7. Model coefficients estimated by multiples linear regression

Item	Coefficient Standard Error	T	P
Constant	0.01905	31.122	0.000
X_1	0.01230	-1.078	0.299
X_2	0.01230	-1.173	0.261
X_3	0.01230	-1.030	0.320
X_4	0.01230	0.990	0.339
X_1^2	0.01672	-16.294	0.000
X_2^2	0.01672	-7.145	0.000
X_3^2	0.01672	-7.406	0.000
X_4^2	0.01672	-8.273	0.000
X_1X_2	0.02130	-1.127	0.279
X_1X_3	0.02130	-0.305	0.765
X_1X_4	0.02130	-0.035	0.972
X_2X_3	0.02130	0.340	0.739
X_2X_4	0.02130	-0.563	0.582
X_3X_4	0.02130	-2.571	0.022
S=0.0543247		PRESS=0.220123	
R^2=95.90%	R^2(anticipated) =76.47%	R^2(modified) =91.81%	

the results of the Box-Behnken design and establish the regression formula:

$$Y = 0.5928 - 0.01325X_1 - 0.0144167X_2 - 0.0126667X_3 + 0.0121667X_4 - 0.272483X_1^2 - 0.119483X_2^2 - 0.123858X_3^2 - 0.138358X_4^2 - 0.024X_1X_2 - 0.0065X_1X_3 - 0.00075X_1X_4 + 0.00725X_2X_3 - 0.012X_2X_4 - 0.05475X_3X_4. \quad ...(1)$$

The mean square deviation of regression equation and estimated model coefficients were determined by multiple linear regression (Table 6 and Table 7). Response surface analysis of the equation helped to build the stereogram and

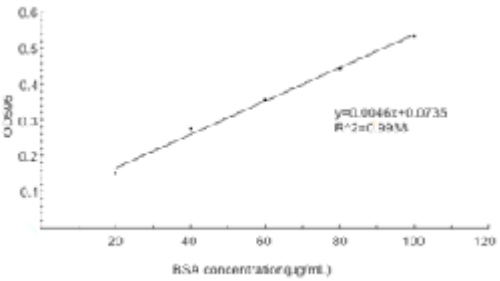

Fig. 1. BSA standard curve(0-100 μg/mL)

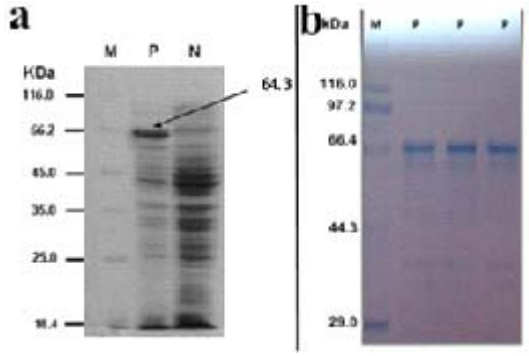

Fig. 2. SDS-PAGE analysis of His-tagged *PHR2* protein(a). The calculated molecular mass is about 64.3 kDa. SDS-PAGE analysis of purified photolyase protein(b).

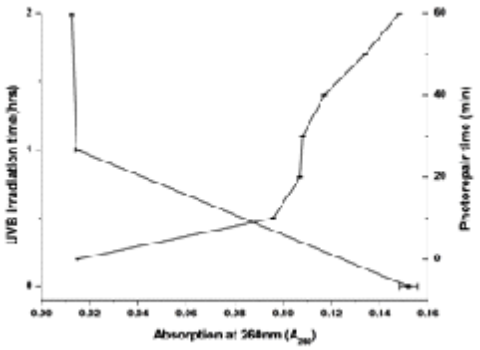

Fig. 3. Changes of absorption at 260 nm when the pyrimidine dimer is repaired by *PHR2* under the white light. Values reported are the mean of three replicates. Error bars indicate the standard deviation.

contour plots (Fig.5, 6, 7, 8, 9, 10). First, the partial derivative of the equation was determined and solved with the simultaneous equations: X_1=6.98, X_2=1.40, X_3=22.01, and X_4=0.53. The results indicated that the optimal conditions for recombinant bacterial expression of photolyase were pH 6.98, salinity 1.40%, 22.01 °C, and IPTG 0.53 mmol/L.

$$-0.01325 - 0.544966X_1 - 0.024X_2 - 0.0065X_3 + 0.00075X_4 = 0$$
$$-0.0144167 - 0.238966X_2 - 0.024X_1 + 0.00725X_3 - 0.012X_4 = 0$$
$$-0.0126667 - 0.247716X_3 - 0.0065X_1 + 0.00725X_2 - 0.05475X_4 = 0$$
$$0.0121667 - 0.276716X_4 - 0.00075X_1 - 0.012X_2 - 0.05475X_3 = 0 \quad ...(2)$$

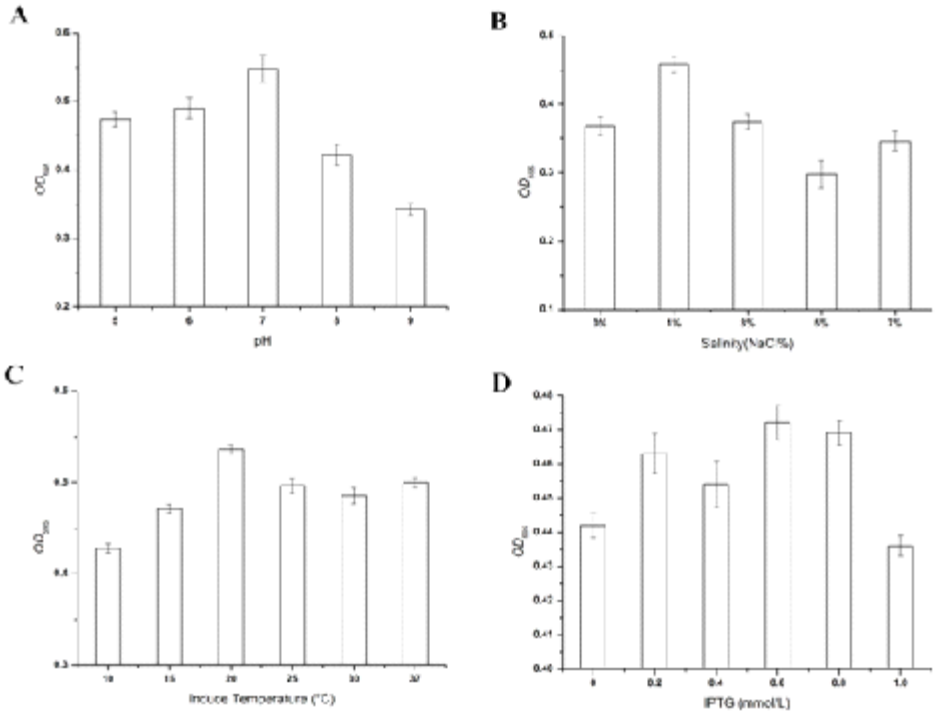

Fig. 4. pH experiment(a). Salinity experiment(b). Inducing temperature experiment(c). IPTG inducing experiment(d).Values reported are the mean of three replicates. Error bars indicate the standard deviation

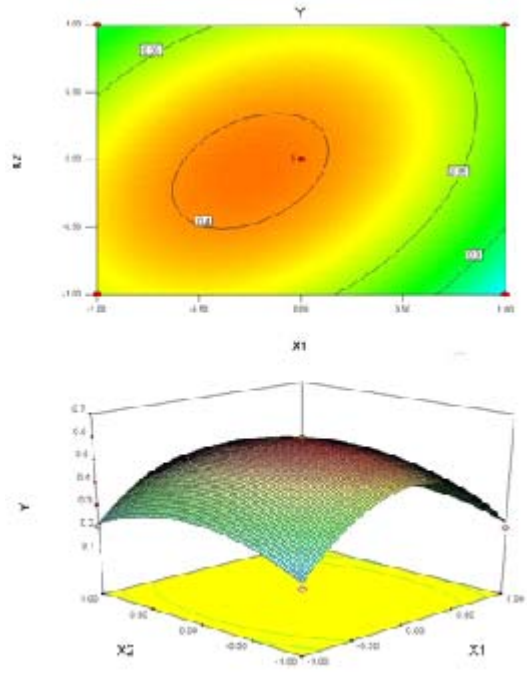

Fig. 5. Y=(X1, X2) contour plot and response surface stereogram

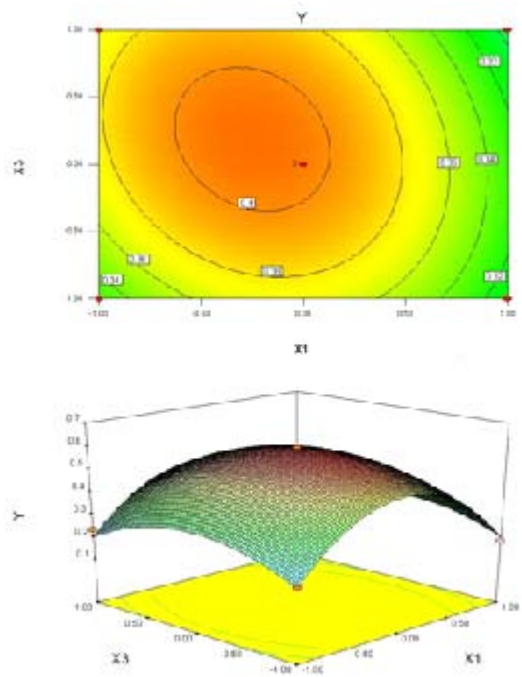

Fig. 6. Y=(X1, X3) contour plot and response surface stereogram.

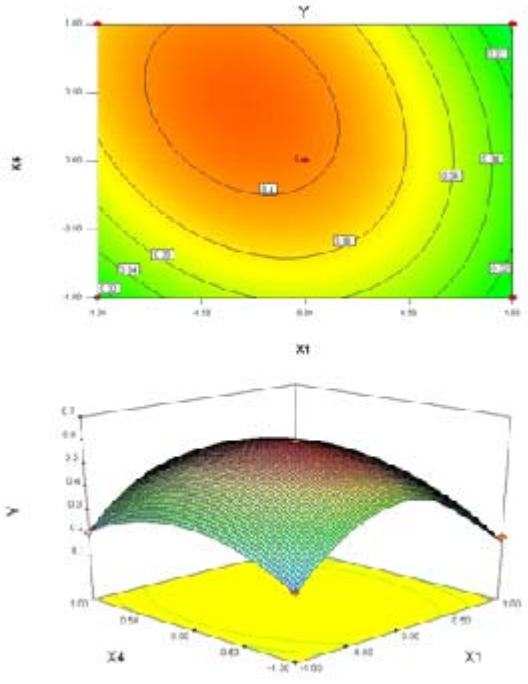

Fig. 7. Y=(X1, X4) contour plot and response surface stereogram

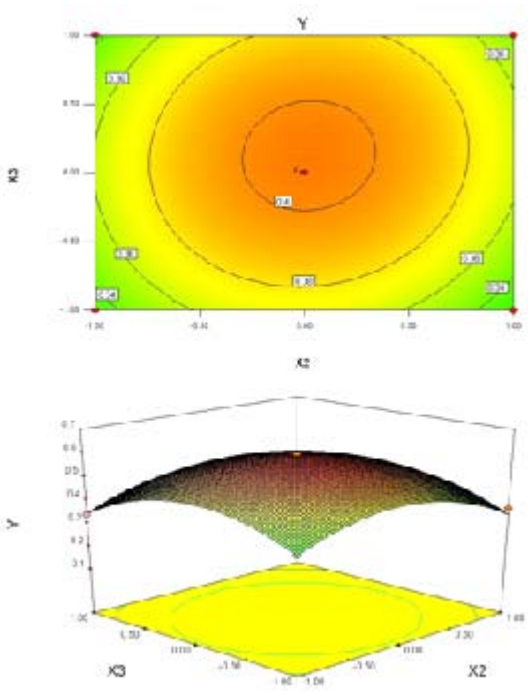

Fig. 8. Y=(X2, X3) contour plot and response surface stereogram

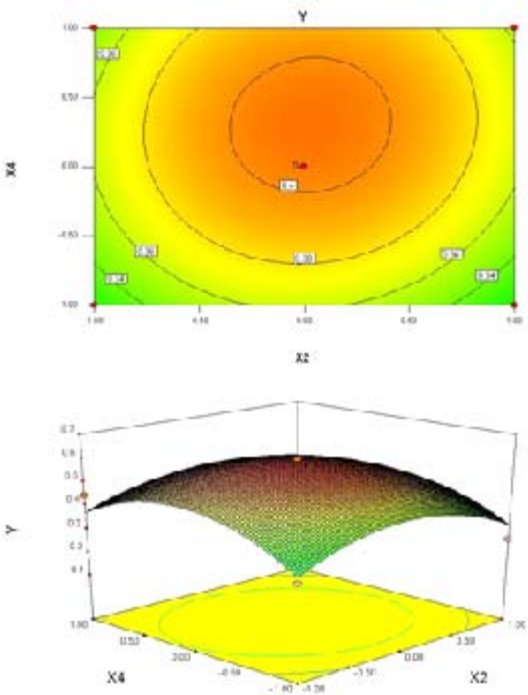

Fig. 9. Y=(X2, X4) contour plot and response surface stereogram.

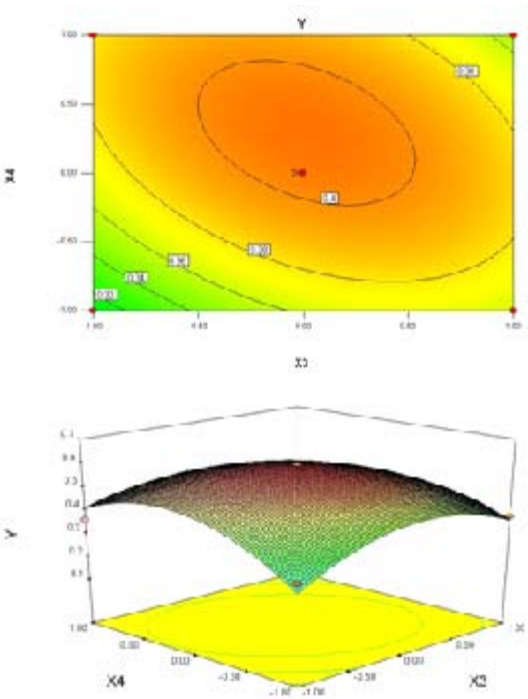

Fig. 10. Y=(X3, X4) contour plot and response surface stereogram

DISCUSSIONS

Ultraviolet (UV) radiation induced damage primarily involves the production of cyclobutane pyrimidine dimmers (CPDs) and pyrimidine (6-4)pyrimidone (6-4PPs) photoproducts (JL Petersen *et al*., 1999). Among them, CPDs are the major induced product, accounting for nearly 75% (Britt, 2004). DNA photolyase is the key compound that reverts pyrimidine dimers to normal bases (Goosen *et al*., 2008). Xu Lei (Xu *et al*., 2005) have carried out numerous, particular research studies involving *Escherichia coli* DNA photolyase. However, studies on UV damage and repair mechanisms of organisms able to survive in the Antarctic high-radiation environment have yet to be reported. Studies on the Antarctic ice algae photolyase gene and its protein expression are also lacking. We have completed *Chlamydomonas* sp. ICE-L photolyase expression in *E.coli* BL21, obtaining protein reaching nearly 95% purity.

Amino acid sequencing is a primary method to analyze protein qualities. N-terminal regions are significant structural and functional sites of proteins and polypeptides, having high sequence specificity (Wilkins *et al*., 1998). Most proteins can be identified using several amino acid residues at the N-terminal region (Zhao *et al*., 2008). All polypeptide synthesis initiates at the N-terminal, and its sequence composition has distinct influences on a protein's biological function. Studies on the N-terminals contribute to understanding the tetrameric structure, and revealing the biological roles of the proteins.

Photolyase usually contains two non-covalent binding cofactors (Gindt *et al*., 1999). One is FADH2, the first chromophore, which possesses catalytic activity. Its active form is FADH ^{ÿ}. The other cofactor is MTHF or 8-HDF (Carell *et al*., 2001), the second chromophore, lacking catalytic activity. MTHF can absorb photons near UV/Vis (300-350 nm) and transfer energy to FADH ^{ÿ}, acting as a light antenna (Sancar, 1994). DNA photolyases are divided into class I and class II, according to amino acid sequence. The similarities between class'!and classa!are less than 20% (Deisenhofer, 2000). Sequence homology exists in all characterized DNA photolyase. Conservatism of the C-terminal 100 amino acid residue sequence is over 52%-75%. N-terminal peptide chain conservatism is relatively lower and depends on photolyase type (Ji *et al*., 2007). The N-terminal sequence confirms that *Chlamydomonas* sp. ICE-L photolyase is a member of class a! photolyases.

Response surface methodology (RSM) is a common statistical tool used to analyze multivariate data (Giovinni, 1982; Yang *et al*., 2005). The methodology relies on a reasonable experimental design and functional relationships between response values and multiple quadric regression equation fitting factors. The equation is analyzed to confirm optimal condition parameters. The softwares Design-Expert 8.0 and Minitab 15 were used to analyze the experimental data, fit curves, and build mathematical models. Optimization was obtained using stereogram and response surfaces (Tao *et al*., 2005).

Single factor experiments indicated that *PHR2* expression was highest when pH was 7, salinity was 1%, inducing temperature was 20 °C, and IPTG concentration was 0.6 mmol/L. Response surface methodology assay confirmed that optimal conditions for recombinant bacteria expressing photolyase were6.98 pH, 1.40% salinity, 22.01°C, and 0.53 mmol/L IPTG. The results demonstrate that the optimal conditions for photolyase expression for transformants are different from the optimal conditions for growth. These single factor experimental results are not completely in agreement with the results of multi-factor response surface assays. The disagreements may be in-part due to interactions between the various experimental factors.

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REFERENCES

1. An M, Mou S, Zhang X, Ye N, Zheng Z, Cao S, Xu D, Fan X, Wang Y, Miao J. Temperature regulates fatty acid desaturases at a transcriptional level and modulates the fatty acid profile in the Antarctic microalga *Chlamydomonas* sp. ICE-L. *Bioresource Technology*, 2013; **134**: 151–157.
2. Britt AB. Repair of DNA damage induced by solar UV. *Photosynth Res*, 2004; **81**: 105-112.
3. Bischof K, Gómez I, Molis M, Hanelt D, Karsten U, Lüder U, Roleda MY, Zacher K, Wiencke C. Ultraviolet radiation shapes seaweed communities. *Reviews in Environmental Science and Biotechnology*, 2006; **5**: 141-166.
4. Carell T. Burgdorf LT., Kundu LM, Cichon M. The mechanism of action of DNA photolyases. *Current Opinion in Chemical Biology*, 2001; **5**: 491-498.
5. Deisenhofer J. DNA photolyases and cryptochromes. Mutation Research, 2000; **460**: 143-149.
6. Giovinni M. Response surface methodology and product optimization. *Food Technology*, 1982; **37(9)**, 41-45.
7. Guo ML, Jiang YM. Effect of ingredients of coomassie brilliant blue color-developing reagent on protein assay. *Progress in Biochemistry and Biophysics*, 1996; **23(6)**, 558-560.
8. Gindt YM, Vollenbroek E, Westphal K, Sackett H, Sancar A, Babcock GT. Origin of transient electron paramagnetic resonance signals in DNA photolyase. *Biochemistry*, 1999; **38**: 3857-3866.
9. Hartree EF. Determination of protein: A modification of the lowry method that gives a linear photometric response. *Analytical Biochemistry*, 1972; **48(2**): 422-427.
10. Hu YH, Shen SB, Ou-yang PK. Application of responses surface method for optimal concentration of culture medium for microorganisms. *Industrial Microbiology*, 2002; **32(1)**: 9-12.
11. Ji XB, Song QH. Recent Progress in Model Studies of Mechanism for DNA Photolyase. *Chinese Journal of Organic Chemistry*, 2007; **27(8)**: 918-924.
12. Kan GF, Miao JL, Li GY. Advance in research on anti-radiation active substances from plankton in the Antarctic. *Chinese Journal of Marine Drug*, 2003; **23(5)**: 35-38.
13. Kan GF, Miao JL, Shi CJ. Changes of soluble protein in Antarctic ice microalga Chlamydomonas sp. ICE-L cultured under UV-B radiation stress. *Acta Oceanologica Sinica*, 2006; **25(6)**: 135-141.
14. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the$2^{-\Delta\Delta CT}$ method. *Methods*, 2011; **25**: 402-408.
15. Liu DX, Ning XB, Zhang JL. Optimization of Growth Condition of Vibrio parahaemolyticus via Response Surface Methodology. *Microbiology China*, 2008; **35(2)**: 306-310.
16. Miao JL *et al*. (2002) Study on Induced Synthesis of Anti-UV Substances in the Antarctic Alga. *High Technology Letters*, **4(11)**, 92-96.
17. Nora Goosen, Geri F Moolenaar. Repair of UV damage in bacteria. *DNA repair*, 2008; **7:** 353-379.
18. Petersen JL, Lang DW, Small GD. Cloning and characterization of a class II DNA photolyase from Chlamydomonas. *Plant Mol Biol*, 1999; **40**: 1 063-1 071.
19. Provasoli L. Media and prospects for the cultivation of marine algae. In: Watanabe A, Hattori A (eds) Culture and collection of algae. Japanese Society of Plant Physiology, Tokyo, Japan, 1968.
20. Rail C. Algal responses to enhanced ultraviolet-B radiation. *Proceedings of the India National Science Academy Part B. Biology Science*, 1998; **64(2)**: 125-127.
21. Sancar A. Structure and function of DNA photolyase. *Biochemistry*, 1994; **33**: 2-9.
22. Sambrook J, Russell DW. Molecular Cloning, 3rd version. Cold Spring Harbor Laboratory Press. 2002.
23. Tao YJ, Daniel T, Zhao YM. Design and Optimization of Falcon Separation Test Using Design-Expert Software. *Journal of China University of Mining & Technology*, 2005; **3**,343-348.
24. Takahisa YamatoÿHirotaka NishiokaÿKei Yura. Identification of Functional Residues of DNA photolyase by Biophysical Computation and Bioinformatics. *Biophysics*, 2009; **49(4)**: 196-197.
25. Wang DN, Zhao YW, Tian FR. Protein quantification with coomassie brilliant blue microplate colormetric. *Journal of Forth Military Medicine University*, 2001; **22(6)**: 528-529.
26. Wilkins MR, Gasteiger E, Tonella L, Ou K, Tyler M, Sanchez JC, Gooley AA, Walsh BJ, Bairoch A, Appel RD, Williams KL, Hochstrasser DF. Protein identification with N and C-terminal sequence tags in proteome projects. *Molecular Biology*, 1998; **278(3)**: 599-608.
27. Wang ZH, Yu HH, Xia JF. Novel GO-blended PVDF ultrafiltration membranes. *Desalination*,

2012; **299**: 50-54.
28. Xu L, Mu WM, Luo SF, Wang YZ. The activity of DNA photolyase *in vitro*. *Chinese Pharmacological Bulletin*, 2005; **21(10)**: 1196-1198.
29. Yang GL, Guo XP. Comparison between Lowry and Bradford method on protein quantification in sodium hyaluronate. *Chinese Journal of Biochemical Pharmaceutics*, 2003; **3**: 131-133.
30. Yang WX, Gao YX., Response surface methodology and its application in food industry. *China Food Additives*, 2005; **2**: 68-71.
31. Zhang PY, Liu SH, Cong BL. A novel omega-3 fatty acid desaturase involved in acclimation processes of polar condition from Antarctic ice algae Chlamydomonas sp. ICE-L. *Marine Biotechnology*, 2011; **13**: 393-401.
32. Zhao LY, Zhang YJ, Qian XH. Research Progress in protein and polypeptide N-terminal sequence technology. *Chinese Medicine Biotechnology*, 2008; **3(3)**: 214-216.