

Purification and Crystallographic Analysis of a Novel Cold-Active Esterase (*HaEst1*) from *Halocynthiibacter arcticus*

Sangeun Jeon ^{1,†}, Jisub Hwang ^{2,3,†}, Wanki Yoo ^{1,4}, Joo Won Chang ¹, Hackwon Do ², Han-Woo Kim ^{2,3}, Kyeong Kyu Kim ⁴, Jun Hyuck Lee ^{2,3,*} and T. Doohun Kim ^{1,*}

¹ Department of Chemistry, Graduate School of General Studies, Sookmyung Women's University, Seoul 04310, Korea; sangeun94@sookmyung.ac.kr (S.J.); vlqkshqk61@outlook.kr (W.Y.); cjw1423@sookmyung.ac.kr (J.W.C.)

² Research Unit of Cryogenic Novel Material, Korea Polar Research Institute, Incheon 21990, Korea; hjsub9696@kopri.re.kr (J.H.); hackwondo@kopri.re.kr (H.D.); hwkim@kopri.re.kr (H.-W.K.)

³ Department of Polar Sciences, University of Science and Technology, Incheon 21990, Korea

⁴ Department of Molecular Cell Biology, School of Medicine, Sungkyunkwan University, Suwon 16419, Korea; kyeongkyu@skku.edu

* Correspondence: junhyucklee@kopri.re.kr (J.H.L.); doohunkim@sookmyung.ac.kr (T.D.K.)

† These authors contributed equally to this work.

Abstract: This report deals with the purification, characterization, and a preliminary crystallographic study of a novel cold-active esterase (*HaEst1*) from *Halocynthiibacter arcticus*. Primary sequence analysis reveals that *HaEst1* has a catalytic serine in G-x-S-x-G motif. The recombinant *HaEst1* was cloned, expressed, and purified. SDS-PAGE and zymographic analysis were carried out to characterize the properties of *HaEst1*. A single crystal of *HaEst1* was obtained in a solution containing 10% (w/v) PEG 8000/8% ethylene glycol, 0.1 M Hepes-NaOH, pH 7.5. Diffraction data were collected to 2.10 Å resolution with P2₁ space group. The final R_{merge} and $R_{\text{p.i.m}}$ values were 7.6% and 3.5% for 50–2.10 Å resolution. The unit cell parameters were $a = 35.69$ Å, $b = 91.21$ Å, $c = 79.15$ Å, and $\beta = 96.9^\circ$.

Keywords: esterase; enzyme assay; crystallization; diffraction

Citation: Jeon, S.; Hwang, J.; Yoo, W.; Chang, J.W.; Do, H.; Kim, H.-W.; Kim, K.K.; Lee, J.H.; Kim, D.

Purification and crystallographic analysis of a novel cold-active esterase (*HaEst1*) from

Halocynthiibacter arcticus. *Crystals* **2021**, *11*, 170. <https://doi.org/10.3390/cryst11020170>

Received: 22 January 2021

Accepted: 7 February 2021

Published: 8 February 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Microbial esterase catalyzes the reaction of formation and hydrolysis of chemical bonds between hydroxyl and carboxylic acid groups, which could be used in the preparation of many biological products such as foods, flavors, cosmetics, drugs, and agrochemicals [1–3]. In addition, they are employed in the degradation of carbamates, pesticides, polymer-based plastics, and industrial wastes. These enzymes share the characteristic α/β hydrolase fold, conserved catalytic triad of Ser-His-Asp/Glu, catalytic strategies, substrate specificities, and a lack of cofactors. It has been shown that esterases perform catalytic reactions via a nucleophilic attack on the substrates [1,4–6].

Cold-active enzymes show relatively high activity at low temperatures compared to their mesophilic or thermophilic proteins [7,8]. Due to high demands, there have been a lot of studies to characterize cold-active enzymes [9]. To date, cold-active esterases were identified from *Paenibacillus* sp. [10], *Pseudomonas mandelii* [11], *Lactobacillus plantarum* [12], and *Bacillus halodurans* [13]. However, limited information is still available on the structure and function of these enzymes [7–9].

Halocynthiibacter arcticus is a rod-shaped Gram-negative bacteria from the Arctic region, which could be a valuable resource for biotechnological applications [14,15]. Specifically, structural information of enzymes from this bacterium is largely unknown. Here, we report the identification, purification, and preliminary crystallographic analysis of a novel cold-active esterase (*HaEst1*) from *H. arcticus*. The recombinant enzyme was

purified, characterized, and crystallized for structural studies. Considering that cold-active esterases have great importance for industrial applications, *HaEst1* could be an interesting industrial enzyme with cold-active properties.

2. Materials and Methods

2.1. Chemicals and columns

Nucleic acid modifying enzymes and DNA purification kits were purchased from New England BioLabs (MA, USA) and Intron Korea (Daejeon, Korea). PD-10 column for dialysis and His-tag affinity column for purification were obtained from GE Korea. All other reagents of high purity grade were obtained from Sigma-Aldrich.

2.2. Gene Cloning and Protein Purification

Microbial culture of *Halocynthiaibacter arcticus* (Korea Collection of Type Cultures, KCTC 42129) and purification of its chromosomal DNA were carried out as previously described [16]. The open reading frame of the *HaEst1* gene was amplified and cloned into a pET-21a vector (pET-*HaEst1*) using the following primers with *NheI* and *XhoI* (forward primer: 5'-GTA ACC GCT AGC ATG ACA GAC CCA CAG-3', and reverse primer: 5'-GCT GAC TCG AGT CAG AAT TTC GCC CG-3'). *E. coli* BL21(ΔDE3) cells were transformed and grown in LB medium at 18 °C with 1 mM isopropyl-β-D-1-thiogalactoside (IPTG) induction. Then, bacterial cells were centrifuged and resuspended in a cell disruption buffer (20 mM Tris-HCl pH 8.5, 150 mM NaCl, 20 mM imidazole). Following cell lysis by ultrasonication, supernatants were loaded onto a His-Trap affinity column. *HaEst1* was eluted by a gradient of imidazole method (from 50 mM to 200 mM). The fractions were buffer-changed with PD-10 column. The final proteins were collected and stored at -20 °C.

2.3. Activity Measurement of *HaEst1*

Zymographic assay of *HaEst1* was performed as previously described [17–19]. Protein purities and concentrations of *HaEst1* were determined using SDS-PAGE and Bio-Rad Protein Assay kit. Enzymatic activity of *HaEst1* was studied using 4-methylumbelliferyl (4-MU) acetate and 4-methylumbelliferyl (4-MU) phosphate as substrates. The fluorescence of 4-methylumbelliferone was observed in an Eppendorf tube using UV incubation box. Intrinsic fluorescence was measured using a Jasco FP-6200 spectrofluorometer (MD, U.S.A.).

2.4. Crystallization Method

For effective crystallization trials, purified *HaEst1* (20.0 mg/ml) was used with crystallization kits including MCSG 1T~4T (Anatrace), JCSG-plus (Molecular Dimensions), and PGA Screen (Molecular Dimensions) [20,21]. The screening process was carried out using an automated crystallization robot (SPT Labtech, USA). Initial droplets contained 300 nL of protein solution mixed with 300 nL of reservoir solution by sitting-drop vapor-diffusion method in a 96-well plate. Various crystals of *HaEst1* appeared in several conditions within a week. Diffraction-quality single crystals of *HaEst1* were observed under JCSG-Plus #16 condition of 10% (w/v) PEG 8000/8% ethylene glycol, 0.1 M HEPES-NaOH, pH 7.5.

2.5. Data Collection and Processing

A single crystal of *HaEst1* was removed and transferred to a cryo-protectant solution. After gentle soaking, the crystal was mounted on a synchrotron facility at beamline 5C of the Pohang Light Source (PAL, Pohang, Korea). X-ray diffraction data of *HaEst1* were collected at 100 K using the Eiger X 9M detector (Dectris, Switzerland). For complete X-ray diffraction data collection, the cryo-cooled crystal was rotated throughout 360° rotation with 1° oscillation per frame. Finally, collected x-ray data were processed and

indexed using HKL2000 (see Table 1). Sequences of *HaEst1* and other enzymes were obtained from a public NCBI server, and multiple sequence alignment was prepared using ESPript.

Table 1. Data collection and processing statistics of *HaEst1*.

Data collection	
Synchrotron source	PAL-Korea, beamline 5C
Wavelength (Å)	0.9794
Temperature (K)	100
Detector	Eiger X 9M
Crystal to detector distance (mm)	400
Rotation range per image (°)	1
Exposure time per image (s)	1
Rotation range (°)	360
Data processing	
Crystal parameters	
Space group	$P2_1$
No. of molecules in asymmetric unit	2
Unit-cell parameters (Å, °)	a= 35.69, b= 91.21, c= 79.15 , $\alpha= 90$, $\beta= 96.91$, $\gamma= 90$
Data statistics	
Resolution range (Å)	50.00–2.10 (2.14–2.10)
Total no. of collected reflections	186387 (122)
No. of unique reflections	29269 (1435)
Completeness (%)	99.0 (99.6)
Multiplicity	6.4 (0.1)
$\langle I/\sigma(I) \rangle$	47.8 (7.67)
$R_{\text{merge}} (\%)^\dagger$	8.1 (36.3)
$R_{\text{meas}} (\%)^\ddagger$	8.8 (39.8)
CC(1/2) (%)	99.1 (94.7)
Overall B-factor from Wilson plot (Å ²)	39.7

$$\dagger R_{\text{merge}} = \sum_{\text{hkl}} |I - \langle I \rangle| / \sum_{\text{hkl}} I.$$

$$\ddagger R_{\text{meas}} = \sum_{\text{hkl}} \{N(\text{hkl}) / [N(\text{hkl}) - 1]\}^{1/2} \sum_i |I_i(\text{hkl}) - \langle I(\text{hkl}) \rangle| / \sum_{\text{hkl}} \sum_i I(\text{hkl}).$$

3. Results and discussion

An open reading frame encoding a novel cold-active esterase (*HaEst1*, locus tag: WP_039000957, 756 bp) was detected and obtained from the *H. arcticus* chromosome. *HaEst1* has 252 amino acids with a pI of 5.21. Multiple sequence alignments of *HaEst1* with three related proteins indicated that *HaEst1* showed significant sequence identities with a putative hydrolase from *Agrobacterium vitis* (3LLC, 40.0%), a new family of carboxyl esterase with an OsmC domain from *Rhodothermus marinus* (5CML, 19.8%) [22], and a cinnamoyl esterase from *Lactobacillus johnsonii* LJ0536 (3PF8, 20.6%) [23] (Figure 1). Interestingly, *HaEst1* showed substantial sequence similarity to an alpha/beta hydrolase domain-containing protein 10 (ABHD10) from *Mus musculus* (6NY9, 23.8%). Catalytic triad of Ser-His-Asp as well as G-x-S-x-G motif were also conserved in these two proteins. This ABHD10 was recently shown to be an S-depalmitoylase affecting reduction/oxidation homeostasis [24].

Highly conserved catalytic residues of Ser¹⁰¹, Asp¹⁹⁸, and His²²⁸ were identified, with Ser¹⁰¹ located in a typical GX SXG motif. Sequence analysis revealed that *HaEst1* has a high number of small amino acids such as Gly (11.9 %) and Ala (9.9 %). In addition, high

percentages of Leu (9.1%) and Thr (7.9%) were also observed. The percentage of acidic amino acids (Asp + Glu) was larger than that of basic amino acids (Arg + Lys).

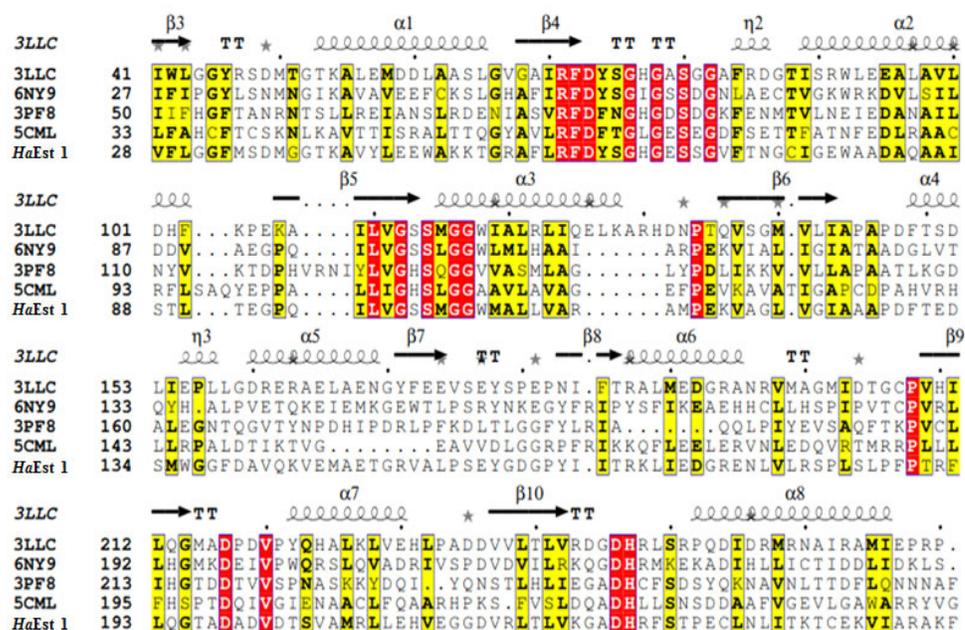


Figure 1. Multiple sequence alignments of *HaEst1*. Identical and highly conserved amino acids among these proteins are displayed in red and yellow.

The recombinant *HaEst1* was purified using an immobilized His-tag metal-binding column (Figure 2A). The enzymatic activity of *HaEst1* was examined using 4-methylumbelliferyl (4-MU) acetate. As shown in Figure 2B, strong fluorescence reaction was observed for 4-MU acetate, although little hydrolysis reaction was carried out for control or 4-MU phosphate.

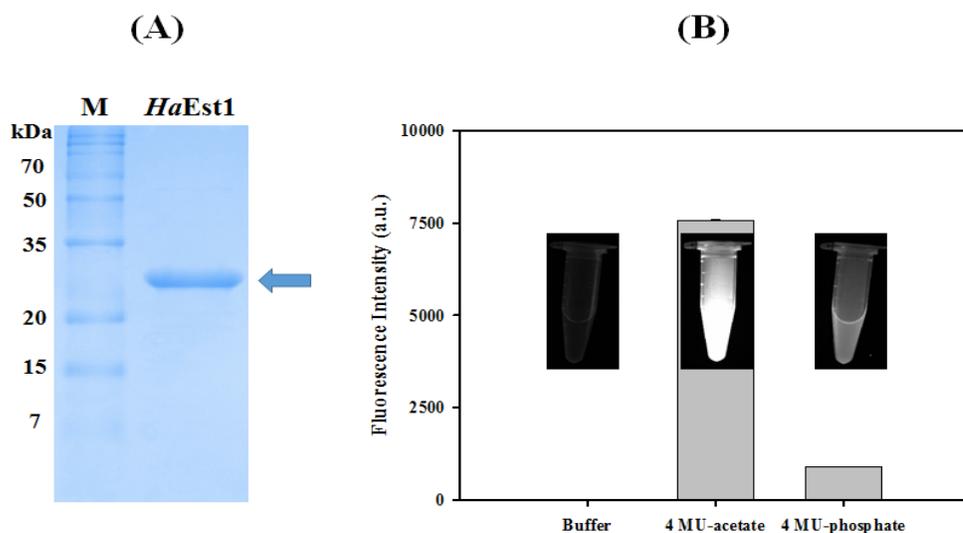


Figure 2. Purification and hydrolytic activity of *HaEst1*. (A) SDS-PAGE analysis of *HaEst1*. Arrow indicates the position of purified *HaEst1*. (B) Hydrolysis of 4-MU acetate was examined with *HaEst1*. Strong fluorescence was observed due to hydrolysis reaction by *HaEst1*.

The diffraction-quality crystals grew to final dimensions of $0.6 \times 0.5 \times 0.2$ mm within three days at 297K (Figure 3), which were transferred to a paratone oil, cryo-protectant solution. The diffraction data set of *HaEst1* was indexed to $P2_1$ space group with unit cell parameter of $a = 35.69$ Å, $b = 91.21$ Å, $c = 79.15$ Å, and $\beta = 96.9^\circ$. The final data were processed using HKL2000 to 2.10 Å resolution with 99.9% completeness. The final R_{merge} and $R_{\text{p.i.m}}$ values were 8.1% and 3.5 % for 50–2.10 Å resolution. The final data collection statistics are summarized in Table 1. Assuming two molecules of *HaEst1* per asymmetric unit, Matthews coefficient (V_M) and solvent content were calculated to be 2.36 Å³/Da and 47.9% [25].

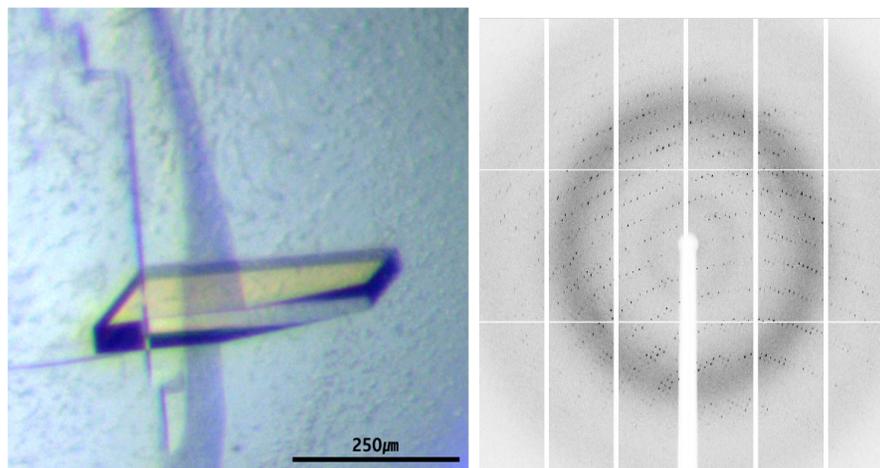


Figure 3. Representative crystal image (left) and diffraction pattern (right) of *HaEst1*. The crystal has final dimensions of $0.6 \times 0.5 \times 0.2$ mm.

We tried to solve the *HaEst1* structure using the molecular replacement method using MOLREP [26]. The crystal structure of a putative hydrolase from *Agrobacterium vitis* (PDB code 3LLC) was used as a search model for the cross-rotation search. For the cross-rotation function calculation, we used data in the resolution range of 39.44–2.41 Å. The results of the cross-rotation showed that the highest peak height was above 7.49σ . The highest peak solution of the rotation function was used for the following translation function search. The solution model also gave a strong correlation coefficient value (above 0.52) in the translation function. Rigid body refinements and individual restrained B-factor refinements were performed using REFMAC5 [27]. After these refinement steps, an interpretable electron density map was calculated, as shown in Figure 4. Model building and further refinement are now underway. Furthermore, substrate or product bound *HaEst1* structure determination with site-directed mutagenesis experiments will be performed. Thus, a detailed structural analysis of *HaEst1* and protein engineering results will be published in an upcoming research paper.

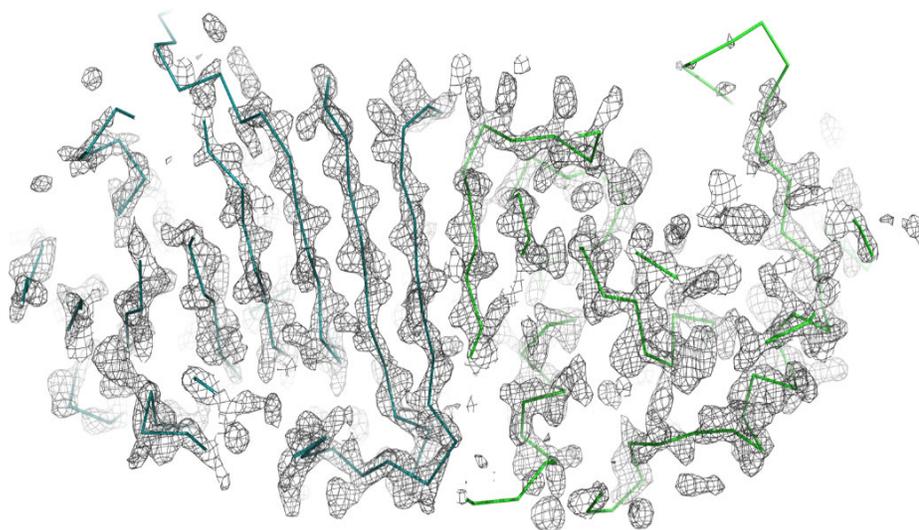


Figure 4. Electron density map of a portion of *HaEst1* structure contoured at 2σ . The initially built Ca trace is shown in green and deep teal color for each chain. The figure was made with Pymol.

Determination of the *HaEst1* structure will allow direct comparison to other mesophilic or thermophilic esterases, which could provide molecular insights on its reaction mechanism as well as its functional properties. In summary, this work deals with the purification and crystallographic analysis of a novel cold-active *HaEst1*, which could be used for biotechnological applications. Furthermore, *HaEst1* could be further improved/mutated through protein or genetic engineering for its useful applications.

Author Contributions: S.J. and W.Y. identified and purified *HaEst1*. J.H., H.D., and H-W. K. got crystals and diffraction data. J.H.L, K.K.K., and T.D.K. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by Medical Research Center Program funded by the Korea government (MSIP) (No. 2011-0030074).

Conflicts of Interest: All authors declare no conflicts of interests.

References

1. Holmquist, M. Alpha/Beta-hydrolase fold enzymes: Structures, functions and mechanisms. *Curr. Protein Pept. Sci.* **2000**, *1*, 209–235.
2. Gupta, R.; Gupta, N.; Rathi, P. Bacterial lipases: An overview of production, purification and biochemical properties. *Appl. Microbiol. Biotechnol.* **2004**, *64*, 763–781.
3. Bhatt, P.; Bhatt, K.; Huang, Y.; Lin, Z.; Chen, S. Esterase is a powerful tool for the biodegradation of pyrethroid insecticides. *Chemosphere* **2020**, *244*, 125507.
4. Sarmah, N.; Revathi, D.; Sheelu, G.; Yamuna-Rani, K.Y.; Sridhar, S.; Mehtab, V.; Sumana, C. Recent advances on sources and industrial applications of lipases. *Biotechnol. Prog.* **2018**, *34*, 5–28.
5. Chen, Y.; Black, D.S.; Reilly, P.J. Carboxylic ester hydrolases: Classification and database derived from their primary, secondary, and tertiary structures. *Protein Sci.* **2016**, *25*, 1942–1953.
6. Oh, C.; Kim, T.D.; Kim, K.K. Carboxylic ester hydrolases in bacteria: Active site, structure, function and application. *Crystals* **2019**, *9*, 597.
7. Santiago, M.; Ramírez-Sarmiento, C.A.; Zamora, R.A.; Parra, L.P. Discovery, molecular mechanisms, and industrial applications of cold-active Enzymes, *Front. Microbiol.* **2016**, *7*, 1408.
8. Siddiqui, K.S. Some like it hot, some like it cold: Temperature dependent biotechnological applications and improvements in extremophilic enzymes. *Biotechnol. Adv.* **2015**, *33*, 1912–1922.

9. Dumorné, K.; Córdova, D.C.; Astorga-Eló, M.; Renganathan, P. Extremozymes: A potential source for industrial applications. *J. Microbiol. Biotechnol.* **2017**, *27*, 649–659.
10. Park, S.-H.; Yoo, W.; Lee, C.W.; Jeong, C.S.; Shin, S.C.; Kim, H.-W.; Park, H.; Kim, K.K.; Kim, T.D.; Lee, J.H. Crystal structure and functional characterization of a cold-active acetyl xylan esterase (PbAcE) from psychrophilic soil microbe *Paenibacillus* sp. *PLoS ONE* **2018**, *13*, e0206260.
11. Truongvan, N.; Jang, S.H.; Lee, C. Flexibility and stability trade-off in active site of cold-adapted *Pseudomonas mandelii* esterase EstK. *Biochemistry* **2016**, *55*, 3542–3549.
12. Esteban-Torres, M.; Mancheño, J.M.; Rivas, B.D.L.; Muñoz, R. Characterization of a cold-active esterase from *Lactobacillus plantarum* suitable for food fermentations. *J. Agric. Food Chem.* **2014**, *62*, 5126–5132.
13. Noby, N.; Hussein, A.; Saeed, H.; Embaby, A.M. Recombinant cold -adapted halotolerant, organic solvent-stable esterase (estHIJ) from *Bacillus halodurans*. *Anal. Biochem.* **2020**, *591*, 113554.
14. Lee, Y.M.; Baek, K.; Lee, J.; Lee, H.K.; Park, H.; Shin, S.C. Complete genome sequence of *Halocynthiaibacter arcticus* PAMC 20958(T) from an Arctic marine sediment sample. *J. Biotechnol.* **2016**, *224*, 12–13.
15. Baek, K.; Lee, Y.M.; Shin, S.C.; Hwang, K.; Hwang, C.Y.; Hong, S.G.; Lee, H.K. *Halocynthiaibacter arcticus* sp. nov., isolated from Arctic marine sediment. *Int. J. Syst. Evol. Microbiol.* **2015**, *65*, 3861–3865.
16. Jeon, S.; Hwang, J.; Yoo, W.; Do, H.; Kim, H.-W.; Kim, K.K.; Lee, J.H.; Kim, T.D. Crystallization and preliminary x-ray diffraction study of a novel bacterial homologue of mammalian hormone-sensitive lipase (*HaLip1*) from *Halocynthiaibacter arcticus*. *Crystals* **2020**, *10*, 963.
17. Le, L.T.H.L.; Yoo, W.; Lee, C.; Wang, Y.; Jeon, S.; Kim, K.K.; Lee, J.H.; Kim, T.D. Molecular characterization of a novel cold-active hormone-sensitive lipase (*HaHSL*) from *Halocynthiaibacter arcticus*. *Biomolecules* **2019**, *9*, 704.
18. Jang, E.; Ryu, B.H.; Shim, H.W.; Ju, H.; Kim, D.W.; Kim, T.D. Adsorption of microbial esterases on *Bacillus subtilis*-templated cobalt oxide nanoparticles. *Int. J. Biol. Macromol.* **2014**, *65*, 188–192.
19. Wang, Y.; Le, L.T.H.L.; Yoo, W.; Lee, C.W.; Kim, K.K.; Lee, J.H.; Kim, T.D. Characterization, immobilization, and mutagenesis of a novel cold-active acetyl esterase (EaAcE) from *Exiguobacterium antarcticum* B7. *Int. J. Biol. Macromol.* **2019**, *136*, 1042–1051.
20. Lee, C.W.; Yoo, W.; Park, S.H.; Le, L.T.H.L.; Jeong, C.S.; Ryu, B.H.; Shin, S.C.; Kim, H.W.; Park, H.; Kim, K.K.; et al. Structural and functional characterization of a novel cold-active S-formylglutathione hydrolase (SfSFGH) homolog from *Shewanella frigidimarina*, a psychrophilic bacterium. *Microb. Cell Factories* **2019**, *18*, 140.
21. Lee, C.W.; Lee, S.; Jeong, C.S.; Hwang, J.; Chang, J.H.; Choi, I.G.; Kim, T.D.; Park, H.; Kim, H.Y.; Lee, J.H. Structural insights into the psychrophilic germinal protease PaGPR and its autoinhibitory loop. *J. Microbiol.* **2020**, *58*, 772–779.
22. Jensen, M.V.; Horsfall, L.E.; Wardrope, C.; Togneri, P.D.; Marles-Wright, J.; Rosser, S.J. Characterisation of a new family of carboxyl esterases with an OsmC domain. *PLoS ONE* **2016**, *11*, e0166128.
23. Lai, K.K.; Stogios, P.J.; Vu, C.; Xu, X.; Cui, H.; Molloy, S.; Savchenko, A.; Yakunin, A.; Gonzalez, C.F. An inserted α/β subdomain shapes the catalytic pocket of *Lactobacillus johnsonii* cinnamoyl esterase. *PLoS ONE* **2011**, *6*, e23269.
24. Cao, Y.; Qiu, T.; Kathayat, R.S.; Azizi, S.A.; Thorne, A.K.; Ahn, D.; Fukata, Y.; Fukata, M.; Rice, P.A.; Dickinson, B.C. ABHD10 is an S-depalmitoylase affecting redox homeostasis through peroxiredoxin-5. *Nat. Chem. Biol.* **2019**, *15*, 1232–1240.
25. Matthews, B.W. Solvent content of protein crystals. *J. Mol. Biol.* **1968**, *33*, 491–497.
26. Vagin, A.; Teplyakov, A. Molecular replacement with MOLREP. *Acta Crystallogr. Sect. D Biol. Crystallogr.* **2010**, *66*, 22–25.
27. Murshudov, G.N.; Skubák, P.; Lebedev, A.A.; Pannu, N.S.; Steiner, R.A.; Nicholls, R.A.; Winn, M.D.; Long, F.; Vagin, A.A. REFMAC5 for the refinement of macromolecular crystal structures. *Acta Crystallogr. Sect. D Biol. Crystallogr.* **2011**, *67*, 355–367.