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Thermoanaerobacter tengcongensis esterase resists denaturation by urea and sodium dodecyl sulfate

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Summary

Introduction: The broad applications of lipolytic enzymes in various industrial processes have led to increased interest in esterases with distinctive features. Thermophiles are promising source of esterases with inherent thermal and chemical stability. *Thermoanaerobacter tengcongensis* esterase (TTE) is one of such esterases with thermostable potential, however, its resistance to protein denaturants, detergents and molecular docking studies are yet to be fully characterised. **Aim:** Therefore, this study investigated the *in vitro* and *in silico* effects of urea and sodium dodecyl sulfate on TTE activity. **Experimental:** TTE activity was determined spectrophotometrically at 405 nm. TTE was active over a pH range of 3.0 to 12.0 and its activity was optimal at alkaline range of 9.0 and 12.0. **Results:** TTE was found to be most active at 60 °C with the highest thermal stability at the same temperature. Urea at 0.1 to 4.0 mM had a concentration dependent activating effect on TTE; SDS (0.5 to 4.0 mM) had similar effect on the enzyme. Urea at 0.5, 1.0 and 2.0 mM increased maximum reaction rate (V_{max}), catalytic constant (K_{cat}) and Michaelis constant (K_m) of TTE. All concentrations of SDS (0.5 to 2.0 mM) investigated increased V_{max} and K_{cat}, while the K_m value of TTE reduced in the presence of 1.0 and 2.0 mM SDS. Structural characterization of TTE substantiates the *in vitro* thermostability claim. The

molecular docking analysis revealed that donepezil demonstrated optimal binding with TTE. **Conclusion:** the findings from this study showed that TTE strongly resists denaturation by optimal concentrations of urea and SDS.

Keywords: Thermoanaerobacter tengcongensis, esterase, thermophiles, protein denaturants, para-nitrophenyl dodecanoate

Resumen

La esterasa de *Thermoanaerobacter tengcongensis* resiste la desnaturalización por la urea y el dodecilsulfato de sodio

Introducción: Las amplias aplicaciones de las enzimas lipolíticas en diversos procesos industriales han llevado a un mayor interés en esterasas con características distintivas. Los termófilos son una fuente prometedora de esterasas con estabilidad térmica y química inherente. La esterasa de *Thermoanaerobacter tengcongensis* (TTE) es una de esas esterasas con potencial termoestable; sin embargo, su resistencia a los desnaturalizantes de proteínas, los detergentes y los estudios de acoplamiento molecular aún no se han caracterizado por completo. **Objetivo:** Por lo tanto, este estudio investigó los efectos *in vitro* e *in silico* de la urea y el dodecilsulfato de sodio sobre la actividad del TTE. **Parte experimental:** la actividad TTE se determinó espectrofotométricamente a 405 nm. El TTE estuvo activo en un rango de pH de 3,0 a 12,0 y su actividad fue óptima en un rango alcalino de 9,0 y 12,0. **Resultados:** Se encontró que el TTE era más activo a 60 °C y tenía la mayor estabilidad térmica a la misma temperatura. La urea de 0,1 a 4,0 mM tuvo un efecto activador dependiente de la concentración sobre el ETT; SDS (0,5 a 4,0 mM) tuvo un efecto similar sobre la enzima. La urea en concentraciones de 0,5, 1,0 y 2,0 mM aumentó la velocidad de reacción máxima (V_{max}), la constante catalítica (K_{cat}) y la constante de Michaelis (K_m) de TTE. Todas las concentraciones de SDS (0,5 a 2,0 mM) investigadas aumentaron V_{max} y K_{cat}, mientras que el valor de K_m de TTE se redujo en presencia de SDS 1,0 y 2,0 mM. La caracterización estructural de TTE fundamenta la afirmación de termoestabilidad *in vitro*. El análisis de acoplamiento molecular reveló que donepezilo demostró una unión óptima con TTE. **Conclusión:** los hallazgos de este estudio mostraron que el TTE resiste fuertemente la desnaturalización por concentraciones óptimas de urea y SDS.

Palabras clave: Thermoanaerobacter tengcongensis, esterasa, termófilos, desnaturalizantes de proteínas, dodecanoato de paranitrofenilo

Resumo

A esterase de *Thermoanaerobacter tengcongensis* resiste à desnaturação pela uréia e dodecil sulfato de sódio

Introdução: As amplas aplicações de enzimas lipolíticas em diversos processos industriais têm levado ao aumento do interesse em esterases com características distintivas. Os termófilos são fontes promissoras de esterases com estabilidade térmica e química inerente. A esterase de *Thermoanaerobacter tengcongensis* (TTE) é uma dessas esterases com potencial termoestável, no entanto, sua resistência a desnaturantes de proteínas, detergentes e estudos de acoplamento molecular ainda não foram totalmente caracterizadas. **Objetivo:** Portanto, este estudo investigou os efeitos *in vitro* e *in silico* da uréia e dodecilsulfato de sódio na atividade do TTE. **Parte experimental:** A atividade do TTE foi determinada espectrofotometricamente a 405 nm. O TTE foi ativo em uma faixa de pH de 3,0 a 12,0 e sua atividade foi ótima na faixa alcalina de 9,0 e 12,0. **Resultados:** Descobriu-se que o TTE é mais ativo a 60 °C, com maior estabilidade térmica à mesma temperatura. A uréia em concentrações de 0,1 a 4,0 mM teve um efeito ativador dependente da concentração no ETT; SDS (0,5 a 4,0 mM) teve efeito semelhante na enzima. A uréia a 0,5, 1,0 e 2,0 mM aumentou a taxa máxima de reação (V_{max}), a constante catalítica (K_{cat}) e a constante de Michaelis (K_m) do TTE. Todas as concentrações de SDS (0,5 a 2,0 mM) investigadas aumentaram V_{max} e K_{cat}, enquanto o valor de Km de TTE reduziu na presença de 1,0 e 2,0 mM de SDS. A caracterização estrutural do TTE fundamenta a alegação de termoestabilidade *in vitro*. A análise de acoplamento molecular revelou que o donepezil demonstrou ligação ideal ao TTE. **Conclusão:** os resultados deste estudo mostraram que o TTE resiste fortemente à desnaturação por concentrações ótimas de uréia e SDS.

Palavras-chave: Thermoanaerobacter tengcongensis, esterase, termófilos, desnaturantes de proteínas, para-nitrofenil dodecanoato

Introduction

Esterases (E.C 3.1.1.x) are lipolytic enzymes of the α/β hydrolases superfamily that catalyse the hydrolysis of a variety of substrates containing ester linkages, such as aryl esters, carboxylic esters and acylglycerols, resulting in the formation of an alcohol and carboxylic acid [1, 2]. Esterases are widely distributed in nature and have been isolated from various species, however, a significant amount of the enzymes originates from bacteria [3, 4]. The structural analyses of microbial esterases revealed that they consist

of a "cap" domain that participate in substrate binding and a catalytic domain which contains a functional serine in a conserved pentapeptide Gly-Xaa-Ser-Xaa-Gly [3, 5]. The serine of the catalytic domain together with aspartate and histidine make up the highly conserved catalytic triad (Figure 1) [4]. Esterases can catalyze three basic types of reactions: esterification, interesterification and transesterification reactions [6]. Furthermore, esterases are independent of cofactors and have been observed to be active in aqueous and non-aqueous solvents [7]. Although having preference for short to medium chain monoesters, esterases are able to hydrolyse long chain water insoluble triglycerides up to twelve carbon atoms [4, 6].

Typically, esterases display a wide pH, temperature and substrate spectra, and they also exhibit tolerance to metal ion, salt and solvent to varying extents [4]. These features in addition to their regio- and enantio-selectivity make esterases attractive as a biotransformer for the synthesis of optical prodrugs, additives and polyesters [3]. Esterases are widely used in food, pharmaceutical, detergent, pulp and paper industries, biodiesel production, fats and oils production, as well as environmental applications for the degradation of lipid wastes [4, 8, 9].

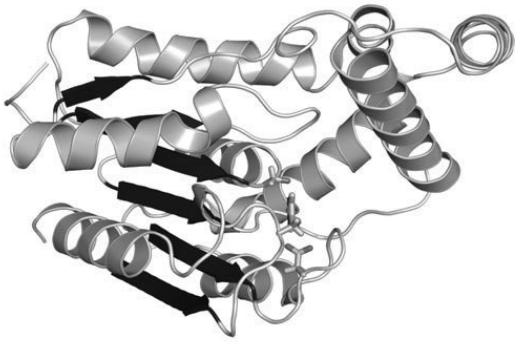

Figure 1. The overall structure of the C-terminal domain of esterase D. The central β-sheet and surrounding α-helixes are shown in black and grey, respectively. Catalytic triad residues are indicated [10].

Most industrial lipolytic hydrolase and esterification reactions occur under harsh conditions (i.e. at high temperature and presence of organic solvent) which constrain enzyme stability, thus the demand for esterases with intrinsic thermal and chemical stability has increased [3]. Extremophiles, particularly thermophiles have overtime been observed to be a promising source of esterases with high inherent stability, and over the years, some esterases with the aforementioned features have been characterised from thermophiles and hyperthermophiles such as *Archaeoglobus fulgidus*, *Pyrococcus*

furiosus, *Pyrobaculum calidifontis, Thermoanaerobacter tengcongensis* and *Thermotoga maritima* [3, 10].

Urea and Sodium dodecyl sulfate (SDS) are well established protein denaturants with denaturing effects similar to thermal denaturation above protein's melting temperature (T_m) of 75 °C [11-13]. For *Thermoanaerobacter tengcongensis* esterase (TTE) to maintain industrial relevance and competitiveness, it is necessary for it to be stable and effective in complex mixtures of compounds such as organic compounds and anionic detergent [14]. The thermostability of TTE has been established but its resistance to protein denaturants, urea and SDS, is yet to be elucidated. In this study, we investigated both *in vitro* and *in silico* the effects of protein denaturants, urea and sodium dodecyl sulfate (SDS) on *Thermoanaerobacter tengcongensis* esterase (TTE) activity.

Materials and methods

Materials

Homogenous *Thermoanaerobacter tengcongensis* esterase (TTE) was synthesized and purified at Institute of Molecular Cell and Systems Biology, University of Glasgow, Bower Building, Glasgow G12 8QQ, Scotland, UK. Para-nitrophenyl dodecanoate (pNPD) also known as 4-Nitrophenyl ester ($C_{18}H_{27}NO_4$) and sodium dodecyl sulfate (Lauryl Sulfate) sodium salt ($C_{12}H_{25}O_4SNa$) were products of Sigma-Aldrich Co, St. Louis, USA. Urea (NH_2CONH_2) is a product of NAAFCO, Scientific Supplies Ltd. New York. All other reagents used in this study were purchased from Fisher Scientific, UK, and were of analytical grade.

Methods

Overexpression and purification of Thermoanaerobacter tengcongensis esterase

The gene coding for putative esterase from *T. tengcongensis* was expressed using *E. coli* BL21 (DE3) pLysS competent cells, this produced His-tagged fusion protein. *E. coli* BL21 (DE3) pLysS culture induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG) showed the over-production of His-tagged TTE. His-tagged TTE was extracted from BL21 (DE3) pLysS cells through cell lysis with the aid of a Sonicator according to the procedure earlier described by Olorunniji *et al.* [15]. His-tag purification was carried out using immobilized metal affinity chromatography (IMAC) technique. His-tagged TTE was eluted with a buffer containing imidazole since it competes with his-tag for binding to the nickel charged resin leaving the protein of interest [15].

Determination of Thermoanaerobacter tengcongensis esterase activity

Thermoanaerobacter tengcongensis esterase (TTE) activity was determined by monitoring the rate of hydrolysis of para-nitrophenyl dodecanoate (pNPD) as previously described by Gopinath *et al*. [16] with some modifications. TTE hydrolyses the colourless synthetic substrate, pNPD, to produce a yellow-coloured, para-nitrophenol (pNP). Reaction mixture containing 0.1 M Glycine-NaOH buffer (pH 9.4) and 0.001 μM TTE was pre-incubated in water bath at 37 °C for 10 minutes. The reaction was initiated by the addition of 0.08 mM pNPD and the mixture was incubated at 37 °C for 10 minutes. The reaction was then terminated by the addition of 0.5 M TCA and 0.5 M NaOH. The amount of the para-nitrophenol (pNP) released from the hydrolysis of pNPD catalysed by TTE according to Beer-Lambert's law were measured spectrophotometrically at 405 nm. All assays were carried out in triplicates.

Effect of pH on Thermoanaerobacter tengcongensis esterase activity

Reaction mixture containing 0.1 M Glycine-NaOH buffer at varying pH (3 - 12) and 0.001 μM TTE was firstly pre-incubated in a water bath at 37 °C for 10 minutes. The reaction was initiated by the addition of 0.08 mM pNPD and then incubated for 10 minutes at 37 °C. The reaction was terminated by the addition of 0.5 M TCA and 0.5 M NaOH.

Effect of temperature and thermal stability of Thermoanaerobacter tengcongensis esterase activity

To the reaction mixture containing 0.1 M Glycine-NaOH buffer (pH 9.4) and 0.001 μM TTE, 0.08 mM pNPD was added to initiate the reaction. The reaction mixture was incubated for 10 minutes at varying temperatures (20 - 100 °C) and then stopped by the addition of 0.5 M TCA and 0.5 M NaOH.

For the effect of high temperatures on stability of the enzyme, reaction mixture containing 0.1 M Glycine-NaOH buffer (pH 9.4) and 0.001 μM TTE was pre-incubated in water bath at varying temperatures (30, 35, 40, 45, 50, 60, 70, 80 °C) for 10 minutes and then placed on ice to cool. Reaction was initiated by the addition of 0.08 mM pNPD and then incubated for 10 minutes at 37 °C. Reaction was terminated by the addition of 0.5 M TCA and 0.5 M NaOH.

Effect of urea and sodium dodecyl sulfate on Thermoanaerobacter tengcongensis esterase activity

Reaction mixture containing 0.1 M Glycine-NaOH buffer (pH 9.4), varying concentrations of urea or SDS (0.1 – 4.0 mM) and 0.001 μM TTE was pre-incubated in a

water bath at 37 °C for 10 minutes. Reaction was initiated by the addition of 0.08 mM pNPD and then incubated at 37 °C for another 10 minutes. Reaction was terminated by the addition of 0.2 ml of 0.5 M TCA and 0.25 ml of 0.5 M NaOH.

Kinetics of Thermoanaerobacter tengcongensis esterase catalysed hydrolysis of para-nitrophenyl dodecanoate

Reaction mixture containing 0.1 M Glycine-NaOH buffer (pH 9.4) and 0.001 μM TTE was pre-incubated in water bath at 37 °C for 10 minutes. Reaction was initiated by the addition of 0.01 – 0.50 mM pNPD separately and incubated at 37 °C for another 10 minutes. Reaction was terminated by the addition of 0.2 ml of 0.5 M TCA and 0.25 ml of 0.5 M NaOH.

Kinetics of Thermoanaerobacter tengcongensis esterase catalysed hydrolysis of para-nitrophenyl dodecanoate in the presence of urea/sodium dodecyl sulfate

Reaction mixture containing 0.1 M Glycine-NaOH buffer (pH 9.4), with 0.5, 1.0 and 2.0 mM urea or SDS and 0.001μM TTE was pre-incubated in a water bath at 37°C for 10 minutes. Reaction was initiated by the addition of 0.01 – 0.50 mM pNPD separately and incubated at 37 °C for another 10 minutes. Reaction was terminated by the addition of 0.2 ml of 0.5 M TCA and 0.25 ml of 0.5 M NaOH.

In silico study

In silico study was done to gain better insight on the binding affinity of TTE active site to urea, SDS and pNPD compared to donepezil. The selected ligands (urea and SDS) are known molecules with high enzyme denaturation potentials, while donepezil is a standard inhibitor. The protein and ligands were converted into dockable pdbqt format using Autodock tools. Pdbqt format of the protein, as well as those of the ligands, was dragged into their respective columns and the software was run. Blind docking of the ligands to the protein target was done and binding scores determination was carried out using PyRx-Python Prescription 0.8 (The Scripps Research Institute) [17]. The dimensions for TTE were set as grid center: x = -21.9200, y = 10.7710, z = -41.6009 and size: x = 66.7970, y = 61.4321, z = 39.3460. The binding scores of evaluated ligands and pNPD were compared to the binding score of donepezil.

Ligands and protein preparation

The three-dimensional (3D) SDF structures of urea, SDS, pNPD and donepezil with CIDs: 1176, 8778, 74778 and 3152 respectively were retrieved from PubChem database (www.pubchem.ncbi.nlm.nih.gov) [18]. The direct investigation of TTE through

in silico approach was hindered due to non-availability of its 3D crystal structure in the protein data bank. Thus the theoretical characterization of TTE was carried out. The TTE primary sequence with UniProtKD ID: Q8RC83 was retrieved from UniProt database (https://www.uniprot.org/) [19]. The obtained primary sequence was used for the modeling of the TTE tertiary structure using the swiss model webserver (https://swissmodel.expasy.org/interactive/) [20]. The quality of the foreseen TTE model for structural characterization was checked using the online PROCHECK webserver (https://saves.mbi.ucla.edu/) [21, 22] via the Ramachandran plot.

Results

Effect of pH on Thermoanaerobacter tengcongensis esterase activity

The pH dependency of *Thermoanaerobacter tengcongensis* esterase (TTE) was investigated at different pH ranging from 3.0 – 12.0 in this study. TTE was observed to be active over a pH range of 3.0 – 12.0 and the activity of the enzyme progressively increased with increase in pH from 3.0 to 9.0. Between pH 9.0 and 12.0, there was no noticeable increase in TTE activity (Figure 2). The TTE activity at pH 9.0 was observed to be 12% higher when compared with the activity at pH 3.0.

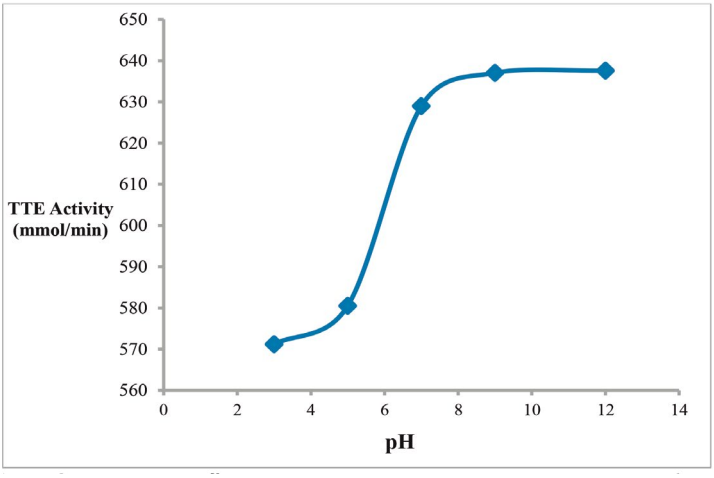

Figure 2. Effect of varying pH on *Thermoanaerobacter tengcongensis* esterase catalysed hydrolysis of para-nitrophenyl dodecanoate. Data are the means of three independent assays.

Effect of temperature on Thermoanaerobacter tengcongensis esterase activity

The effects of varying assay temperatures (20 – 100 °C) and varying pre-incubation temperatures (30 – 80 °C) at assay temperature of 37 °C on TTE activity were investigated in this study. TTE activity increased steadily with increase in assay temperature from 20 °C to 60 °C, at 60 °C activity spiked up by 124% when compared with activity at 20 °C (Figure 3). As assay temperature approached 100 °C, TTE activity declined by approximately 17% when compared with the peak observed at 60 °C. Increasing the pre-incubation temperature from 30 °C to 60 °C resulted in a corresponding increase in activity, which peaked at 60 °C with a 44% increase when compared with the activity recorded at 30 °C (Figure 4). At 80 °C pre-incubation temperature, TTE activity was observed to have dropped by approximately 30% when compared with the peak at 60 °C.

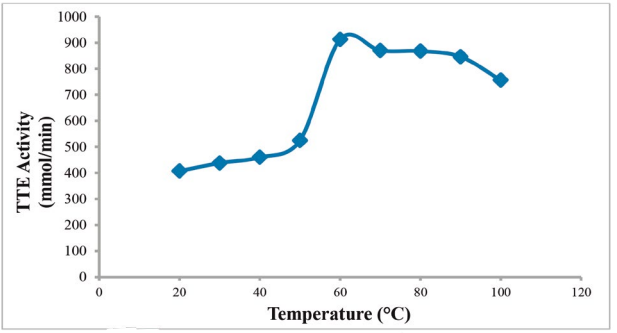

Figure 3. Effects of varying assay temperatures (20 – 100 °C) on *Thermoanaerobacter tengcongensis* esterase catalysed hydrolysis of para-nitrophenyl dodecanoate. Data are the means of three independent assays.

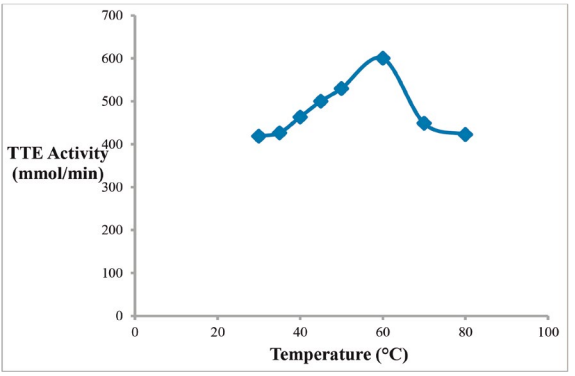

Figure 4. Effects of varying pre-incubation temperatures (30 – 80 °C) at assay temperature of 37 °C on *Thermoanaerobacter tengcongensis* esterase catalysed hydrolysis of para-nitrophenyl dodecanoate. Data are the means of three independent assays.

Effect of urea and sodium dodecyl sulfate on Thermoanaerobacter tengcongensis esterase activity

In this study, the modulatory effect of varying concentrations of urea and sodium dodecyl sulfate (SDS) on TTE activity was examined in the presence of 0.08 mM pNPD. In the presence of 0 to 4.0 mM urea, TTE activity increased in a concentration dependent manner (Figure 5). TTE activity peaked in the presence of 4.0 mM urea with an 82% increase compared with the activity observed in the absence of urea. Similarly, SDS also had a concentration dependent activating effect on TTE; activity peaked in the presence of 4.0 mM SDS with 119% increase when compared to activity in the absence of SDS (Figure 6).

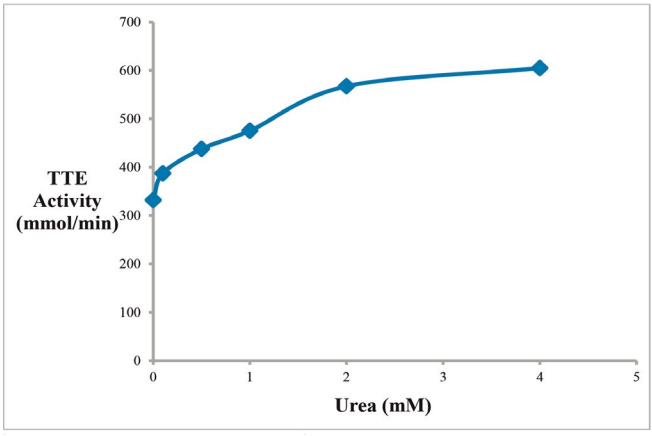

Figure 5. Effects of varying concentrations of urea on *Thermoanaerobacter tengcongensis* esterase catalysed hydrolysis of para-nitrophenyl dodecanoate. Data are the means of three independent assays.

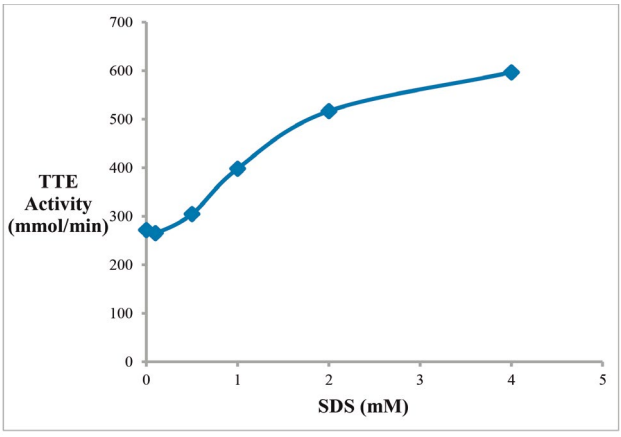

Figure 6. Effects of varying concentrations of sodium dodecyl sulfate (SDS) on *Thermoanaerobacter tengcongensis* esterase catalysed hydrolysis of para-nitrophenyl dodecanoate. Data are the means of three independent assays.

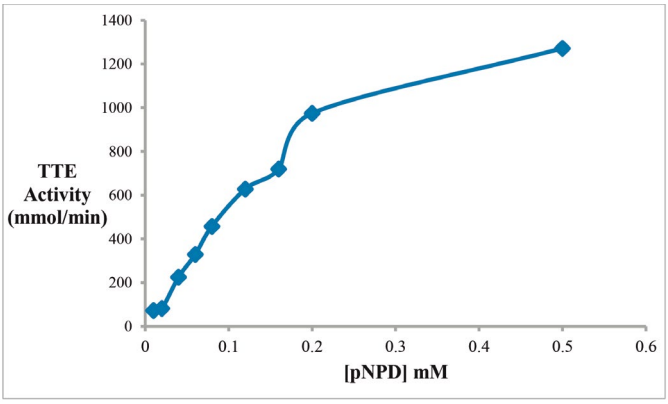

Figure 7. Michealis-Menten curve of *Thermoanaerobacter tengcongensis* esterase catalysed hydrolysis of para-nitrophenyl dodecanoate. Data are the means of three independent assays.

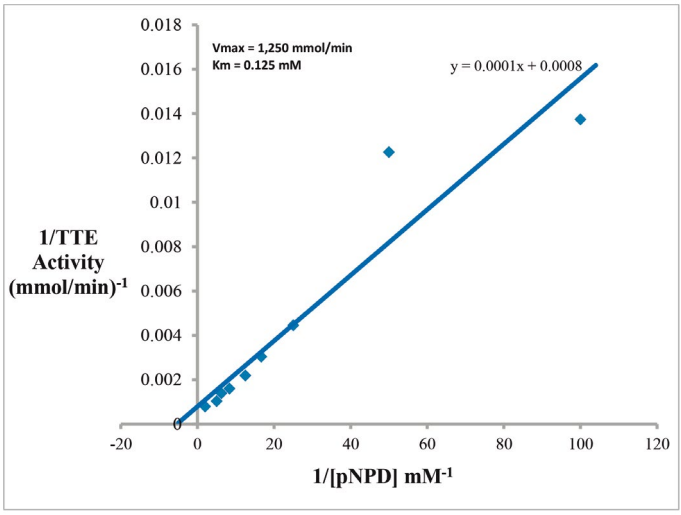

Figure 8. Lineweaver-Burk plot of *Thermoanaerobacter tengcongensis* esterase catalysed hydrolysis of para-nitrophenyl dodecanoate. Data are the means of three independent assays.

Kinetics of Thermoanaerobacter tengcongensis esterase catalysed hydrolysis of para-nitrophenyl dodecanoate in the presence of urea

The substrate kinetics of TTE catalysed hydrolysis of pNPD in the presence of urea follows the classical Michealis-Menten hyperbola curve (Figure 9). From the Lineweaver-Burk plot in Figure 10, V_{max}, K_m and K_{cat} values were calculated and are shown in Table 1. The result showed that 0.5 and 1.0 mM urea concentrations, had similar effect on TTE with both showing approximately 14.3% increase in V_{max} and K_{cat} when compared with

what was observed in the absence of urea. The K_m value of TTE was also observed to increase slightly with 0.5 and 1.0 mM urea. At 2.0 mM concentration, urea had peak effect on TTE, yielding 33.3 and 33.5% increase in V_{max} and K_{cat}, respectively.

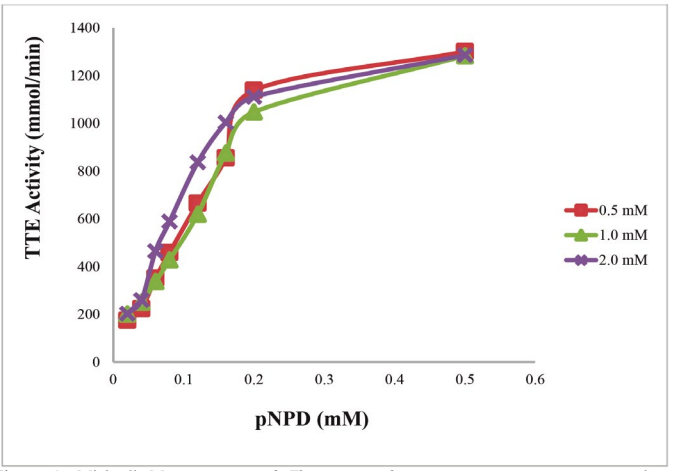

Figure 9. Michealis-Menten curve of *Thermoanaerobacter tengcongensis* esterase catalysed hydrolysis of para-nitrophenyl dodecanoate in the presence of urea. Data are the means of three independent assays.

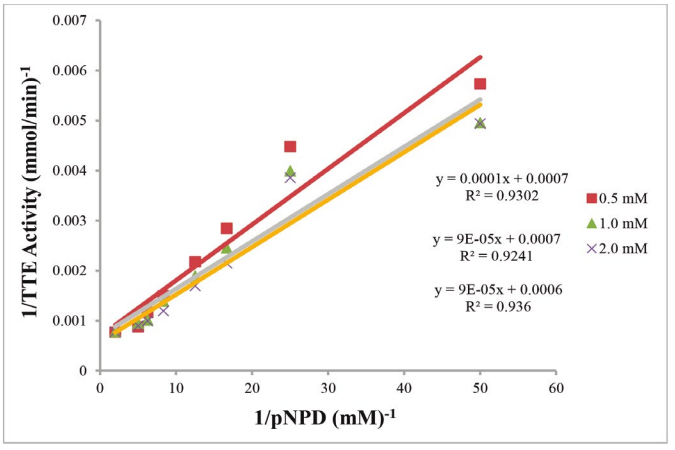

Figure 10. Lineweaver-Burk plot of *Thermoanaerobacter tengcongensis* esterase catalysed hydrolysis of para-nitrophenyl dodecanoate in the presence of urea. Data are the means of three independent assays. Correlation coefficient (R^2) values > 0.90.

Kinetics of Thermoanaerobacter tengcongensis esterase catalysed hydrolysis of para-nitrophenyl dodecanoate in the presence of sodium dodecyl sulfate (SDS)

The substrate kinetics of TTE catalysed hydrolysis of pNPD in the presence of sodium dodecyl sulfate (SDS) follows the classical Michealis-Menten hyperbola curve (Figure 11). V_{max}, K_m and K_{cat} values were calculated from the Lineweaver-Burk plot in Figure 12 and are shown in Table 1. SDS at 0.5, 1.0 and 2.0 mM increased the V_{max} and K_{cat} values of TTE catalyzed hydrolysis of pNPD. Also, SDS at 0.5, 1.0 and 2.0 mM increased the V_{max} of TTE, with the peak in the presence of 0.5 mM (approximately 300% increase) when compared to the V_{max} in the absence of SDS. Similarly, the K_m value of TTE was also observed to be the highest (with about 300% increase) in the presence of 0.5 mM SDS; concentrations beyond this resulted in decrease in K_m up to 20%. The K_{cat} of TTE also increased in the presence of SDS when compared to its absence, highest at 0.5 mM with 300% increase.

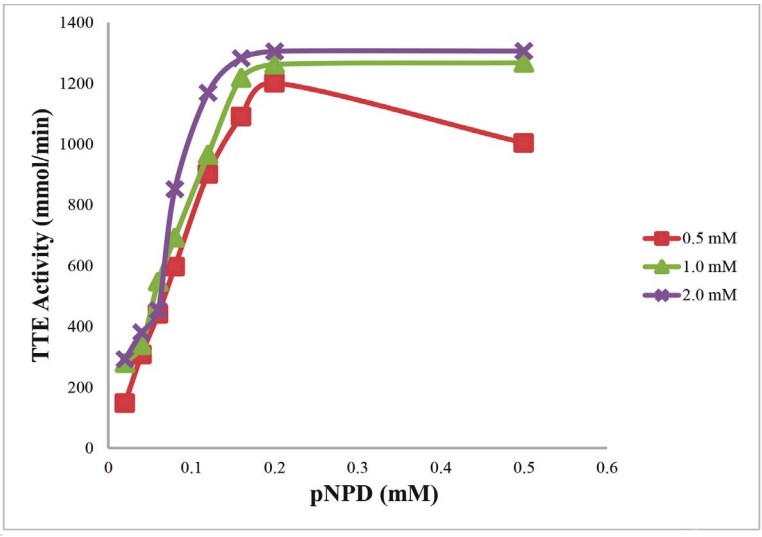

Figure 11. Michealis-Menten curve of *Thermoanaerobacter tengcongensis* esterase catalysed hydrolysis of para-nitrophenyl dodecanoate in the presence of sodium dodecyl sulfate (SDS). Data are the means of three independent assays.

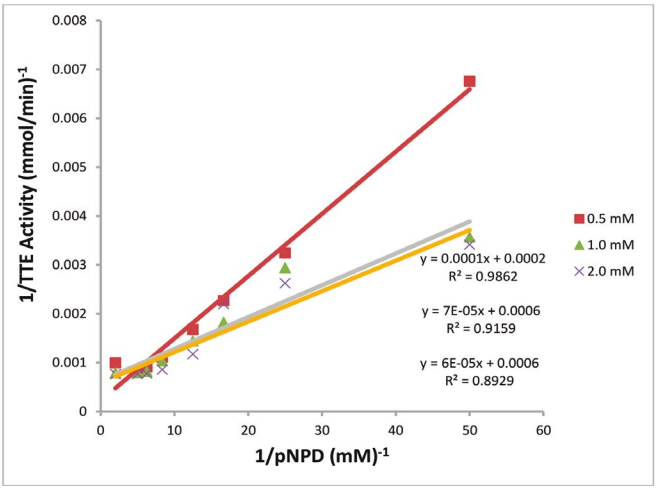

Figure 12. Lineweaver-Burk plot of *Thermoanaerobacter tengcongensis* esterase catalysed hydrolysis of para-nitrophenyl dodecanoate in the presence of sodium dodecyl sulfate (SDS). Data are the means of three independent assays. Correlation coefficient (R^2) values > 0.88.

Table 1. Kinetic parameters of *Thermoanaerobacter tengcongensis* esterase catalysed hydrolysis of pNPD in the presence of urea and sodium dodecyl sulfate

Reaction	V_{max} (mmol/min)$^{-1}$	K_m (mM)	K_{cat} (sec^{-1}) × 10^4
TTE	1,250.000	0.125	3.940
TTE + 0.5 mM Urea	1,428.570	0.143	4.510
TTE + 1.0 mM Urea	1428.570	0.128	4.510
TTE + 2.0 mM Urea	1666.67	0.15	5.26
TTE + 0.5 mM SDS	5000.000	0.500	15.780
TTE + 1.0 mM SDS	1666.670	0.117	5.260
TTE + 2.0 mM SDS	1666.670	0.100	5.260

Theoretical characterization of Thermoanaerobacter tengcongensis esterase

The swiss model for TTE with GMQE and QMEAN Z-Scores 0.15 and -3.58 using *Burkholderia stabilis* cholesterol esterase (PDB ID: 7COG; resolution 3.0 Å) as template was used for the modeling (Figure 13). Also, the PROCHECK summary result for TTE without refinement via Ramachandran plot is 91.1% with 123 amino acid residues (91.1%) found in favored region (A, B, and L; Red color), 10 amino acid residues (7.4%) found in the additional allowed region (a, b, l and p; yellow color), 1 amino acid residue (0.7%) found in the generously allowed region (~a, ~b, ~l, and ~p; light green and cream colors) and 1 amino acid residue (0.7%) found in the disallowed region (white color) (Figure 14).

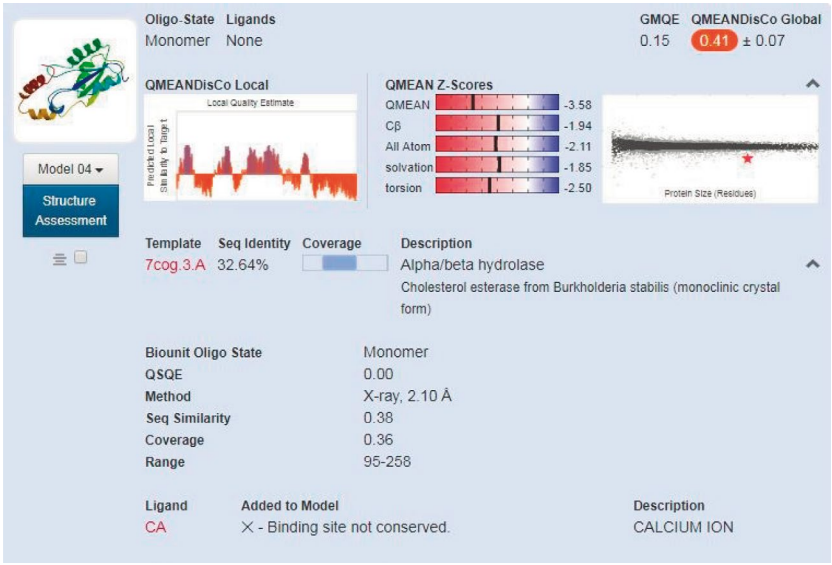

Figure 13. The Swiss model 3D crystal structure of TTE

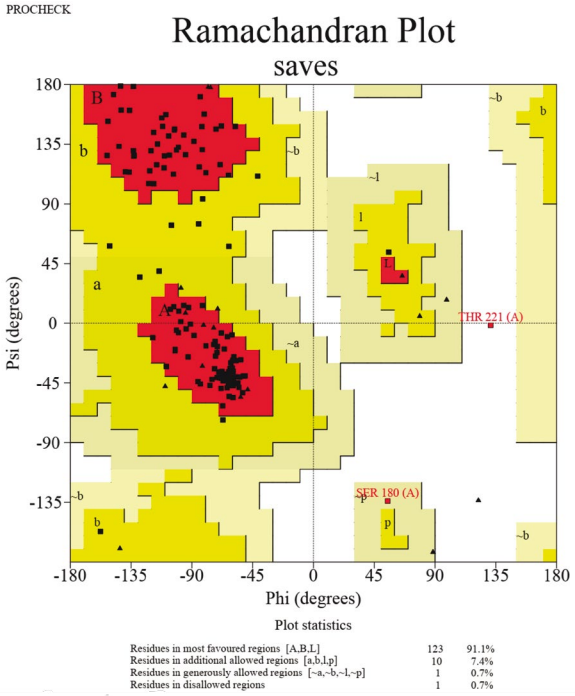

Figure 14. The PROCHECK Ramachandran plot of TTE

Molecular docking

The molecular docking study was carried on the predicted TTE model where the binding scores and interacting residues were obtained as well as the type of non-covalent interactions between the respective ligand towards the TTE model as shown in Table 2 and Figures 15a and 15b.

Table 2. Molecular docking binding scores of pNDP, urea, SDS, and donepezil towards TTE and the non-covalent interacting residues

Parameters / Ligands CIDs	Binding Scores (Kcal/mol)	Conventional Hydrogen Bond	Other non-covalent bonds
74778 1176	-5.37±0.21[d] -3.20±0.20[a]	Thr126, His179 Asp122, Gly249, Tyr252, Asn253	Leu105, His107, Phe115, Phe116
8778 3152	-4.70±0.00[c] -7.37±0.15[b]	Ser124, Thr126 Thr126	Leu105, Met129, Ala141, His179 Leu105, His107, Phe116, Asp122, Leu128, Val177

ANOVA followed by Duncan multiple range test for correlation. Different superscript indicates significant different at $P < 0.05$

Discussion

The ongoing global demand for novel biocatalyst with impeccable biochemical characteristics was expected to have reached approximately $6.2 billion as of 2020 [4]. The bid to meet the continuous high demand for sustainable and environmental-friendly biocatalyst has driven the need to explore new environments for these enzymes [23]. Microbial communities which are adapted to extreme environment such as temperature, salinity, pressure, and low levels of light have overtime been proven to be promising source of enzymes that may be uniquely suited for various industrial processes [24]. One of such extreme environment is the Tengcong hot spring in China where extreme thermophile, *Thermoanaerobacter tengcongensis*, was isolated [6].

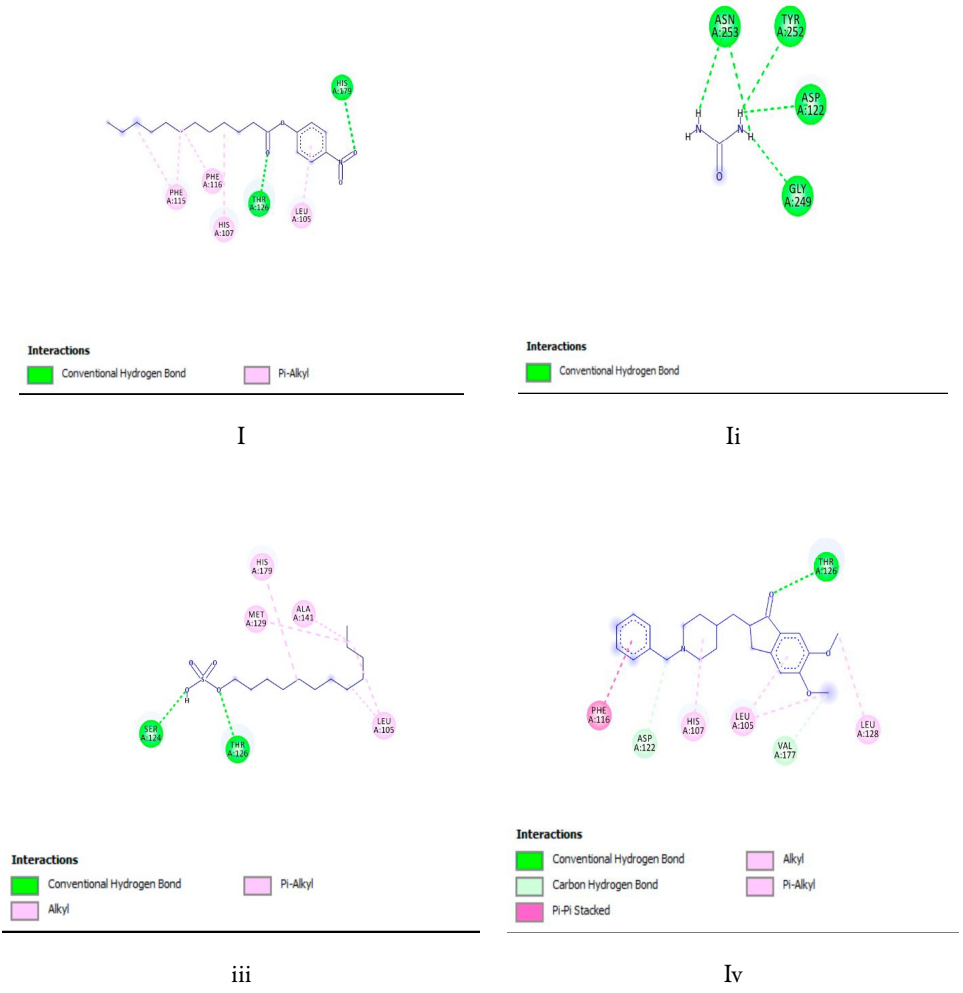

Figure 15a. 2D molecular binding structures of TTE with pNPD (i), urea (ii), SDS (iii) and Donepezil (iv)

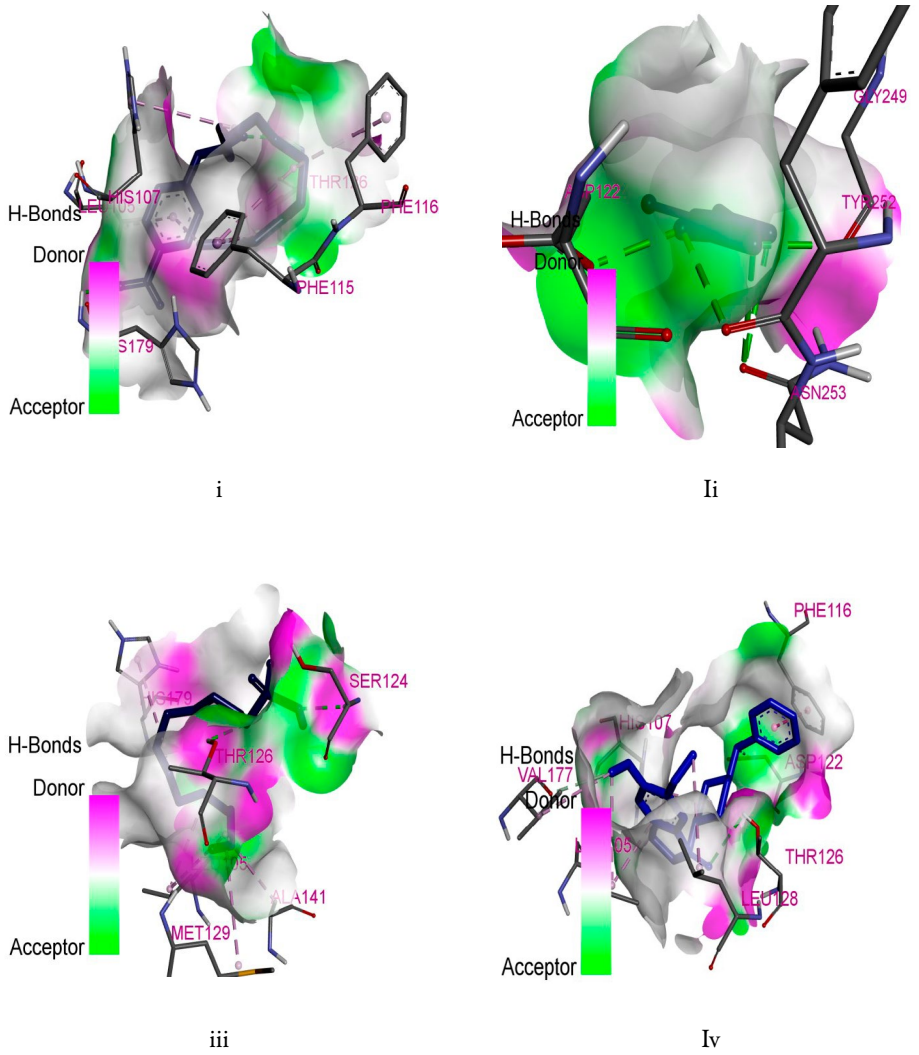

Figure 15b. 3D molecular binding structures of TTE with pNPD (i), urea (ii), SDS (iii) and Donepezil (iv)

Esterases from thermophilic sources present higher industrial potentials than those from other sources and are usually stable in a broad pH range [25]. pH stability in addition to reasonable thermostability reinforces esterases suitability for various industrial applications [6]. In this study, *Thermoanaerobacter tengcongensis* esterase (TTE) was active over a range of acidic and neutral pH and also showed excellent stability in pH range of 9.0 – 12.0. This result is in agreement with the findings of Rao *et al*. [6],

where the activity of TTE was reported to peak at pH 9.5 and stable within the pH range of 8.0 – 10.0. Similarly, Zhang *et al*. (2005) reported optimal activity for TTE at pH 9.0; however unlike the findings here, no activity was recorded at pH below 6.0. Similar to the findings herein, Borchert *et al*. [4] reported *Stelletta normani* esterase activity at all pH values investigated, however, 80 % activity was observed at alkaline pH of 9.0 and 10.0.

Esterases typically exhibit a broad temperature spectrum and several research groups have reported successful identification and development of thermostable esterases from extremophiles particularly thermophiles [4, 6]. This coupled with other factors such as tolerance to metal ion, salts and solvent, regio-, chemo-, and stero-selectivity make esterases desirable industrial biocatalyst [26]. *Thermoanaerobacter tengcongensis* esterase (TTE) displayed optimal activity at 60 °C and like other esterases from thermophiles, TTE maintained high and stable activity between 70 to 90 °C, however at 100 °C, TTE maintained 83 % of its maximal activity. The optimal temperature obtained in this study falls within the survival temperature range for *Thermoanaerobacter tengcongensis* which is between 50 to 80 °C [27]. In this study, the optimal temperature reported for TTE was 10 °C lower than what was reported by Rao *et al*. [3], where optimal activity for *T. tengcongensis* esterase was reported at 70 °C, and this might be due to the difference in other assay conditions. Similar to the findings in this study, *Geobacillus* specie esterase, a thermostable enzyme, has been reported to display optimal activity at 60 °C [25]. Thermostability assays in this study showed that TTE was most stable at 60 °C after 10 minutes pre-incubation and displayed moderate stability at higher temperatures, 70 and 80 °C, where it maintained 74 and 70 % of its maximal activity, respectively. The findings of this study are in line with earlier reports on the thermostability of *T. tengcongensis* esterase by Rao *et al*. [3] where the enzyme was reported to have retained its original activity at 60 °C for longer duration.

Besides being thermostable, the structural characteristics of esterases from thermophilic organisms enables them to have high resistance against organic solvents as well as different denaturing conditions [25]. This, in addition to other factors, reinforces the suitability of esterases for application in detergent industry, waste treatment, oil biodegradation, biodiesel production and pharmaceutical industry [26]. Small organic molecules in aqueous solutions have been observed to have significant effect on the structure, stability and function of proteins and one of such is urea [11]. Overtime, urea has been used to access protein stability and its characteristic denaturing effect has been attributed to its ability to cause unfolding of polypeptides [11]. Many manufacturing industries especially those involved in the production of fertilizers, detergent, glue and feed supplements make use of urea, as such potential biocatalysts are required to have certain degree of stability to be effective in these industries [28]. Findings of

this study showed that concentrations of urea from 0.1 to 4.0 mM progressively activated TTE activity (Figure 5). This finding showed that TTE resisted denaturation by urea up to 4.0 mM and as opposed to denaturation, urea in this test condition activated the enzyme. Urea is known to cause polypeptide unfolding and denaturation at high concentrations [11]; as such the low concentration dependent activating effect on TTE observed in this study might be as a result of mild urea-induced unfolding of TTE thereby exposing the active site to substrate. Similarly, Igunnu and co-researchers reported the activating effect of urea within the concentration range of 0.1 to 4.0 mM on *Thermomyces lanuginosus* lipase [29]. Zhu *et al*. [25] also reported the stability of *Geobacillus* sp. esterase in the presence of urea, with the enzyme maintaining 79.4 % of its activity in 2.5 M urea. High concentrations of urea (1 to 5 M) were reported to induce conformational changes at the active site of *Exiguobacterium antarcticum* esterase resulting in progressive activity decline [1].

The kinetic analysis of *Thermoanaerobacter tengcongensis* esterase in the absence of denaturant/detergent showed that the enzyme had a high turnover of product per unit time and appreciable affinity for the alternative substrate (pNPD). The K_{cat} and K_m value of TTE reported here, 0.125 mM and 3.9×10^4 s^{-1}, respectively, were observed to be higher than those reported by Rao *et al*. [6] and this might be as a result of the difference in experimental conditions. In similar manner, Levisson *et al*. [10] reported a low K_{cat} and K_m value of 1.3 s^{-1} and 0.072 mM, respectively, for *Thermotoga maritima* esterase.

Furthermore, the kinetic analysis of TTE revealed that urea at 0.5 and 1.0 mM had same impact on maximum reaction rate (V_{max}) and catalytic constant (K_{cat}) with a noticeable 14.2% increase when compared with those recorded in the absence of urea (Table 1). However, in the presence of 1.0 mM urea, TTE had higher affinity (lower K_m) for pNPD as a result of increased unfolding. Urea at 2.0 mM had the highest increasing effect on V_{max} and K_{cat}, this implies that at this concentration of urea, TTE had the highest turnover of product per seconds.

With the emergence of biotechnology, esterases have been widely used in various industrial processes; however, their use is restricted by industrial conditions that require the use of complex mixtures of compounds including anionic and nonionic detergents [14, 30]. Therefore the ability to remain stable in the presence of detergents confers an advantage and might be crucial for certain industrial applications [14]. Anionic surfactant such as sodium dodecyl sulfate (SDS) is able to destroy non-covalent bonds within enzyme molecules, thereby resulting in conformational changes that lead to loss of activity [30]. For TTE to be efficient in detergent industry as a facilitator of fat stain removal in washing powders, its ability to be stable in detergents is imperative. From this study, it was observed that TTE was stable at all tested concentrations of

SDS with 0.5 to 4.0 mM showing a concentration dependent activating effect on the enzyme. The findings in this study showed that TTE strongly resisted denaturation by SDS up to 4.0 mM and may be applicable in industries where moderate concentrations of anionic detergent are necessary for manufacturing. The activating effects at low concentrations of detergents have earlier been reported for *Thermomyces lanuginosus* lipase, where concentrations between 0.1 to 5.0 mM were observed to have led to increase in activity [29, 31]. The increased TTE activity observed in the presence of SDS in this study might be due to the increased exposure of the active site as the surfactant unfolds the protein. In a like manner, a recombinant esterase from *Geobacillus sp*. was reported to show good tolerance for SDS [25]. Zhang *et al*. [5] reported that TTE was unstable in 1% SDS which shows that TTE was unable to resist the denaturing effect of SDS at a concentration as high as 34 mM. Similarly, the presence of 1% SDS was reported to completely inhibit *Thermotoga maritima* esterase [10]; likewise, Gao *et al*. [30] reported the strong inhibitory effect of SDS on alkaline-stable esterase from *Stenotrophomonas maltophilia*.

Furthermore, the findings in this study showed that in the presence of abundant supply of pNPD, all concentrations of SDS (0.5 to 2.0 mM) increased the maximum reaction velocity and turnover rate of TTE. This finding is in line with the previous observation here in that SDS within concentration 0.5 to 4.0 mM had activating effect on TTE. It was also deduced from the kinetic analysis that 0.5 mM had the highest impact on the turnover of product. Similar to the findings here in, Igunnu *et al*. [29] had earlier reported that 2.0 mM SDS increased the V_{max} and K_{cat} of *Thermomyces lanuginosus* lipase. On the other hand, the K_m value of TTE increased in the presence of 0.5 mM SDS and decreased as concentration approached 2.0 mM. This finding shows that in the presence of 0.5 mM SDS, the affinity of TTE for pNPD was slightly reduced indicating that at this concentration, the surfactant reduced enzyme binding affinity but enhanced its catalytic power.

To substantiate the thermostability study, the structural characterization result revealed closest GMQE, QMEANDisco (between 0 and 1) and QMEAN Z-score (between -4.0 and 0) values for TTE model suggesting a good quality, reliability and the degree of nativeness of the built model to the experimental structure of similar size [32-34]. In addition, the TTE model quality check result using Ramachandran plot depicted a good quality check with 91.1% amino acid residues in the favored region and less than 1% in the disallowed region. Interestingly, the amino acid residue (THR221) in the disallowed region did not interact with any of the docked molecule towards TTE. This result suggested that the phi and psi backbone dihedral angles in the predicted structure of TTE model is reasonably accurate [35, 36]. Furthermore, the molecular docking study showing the molecular interactions between the investigated ligands

and TTE complement its homology assessment. It is evidence from the docking result that none of the investigated compounds (urea and SDS) showed better binding score than pNPD except donepezil (-7.37 ± 0.15 Kcal/mol), a known inhibitor for esterase.

Conclusion

In conclusion, findings from this study showed that TTE strongly resists denaturation by optimal concentrations of urea and SDS. In fact, moderate concentrations of the denaturants (urea and SDS) increased the activity of *Thermoanaerobacter tengcongensis* esterase. More so, TTE was stable within a broad range of temperature and pH values; these impeccable biochemical characteristics make it an ideal enzyme for use in detergent industry, waste treatment, oil biodegradation, biodiesel production, glue and feed supplements industry where harsh operating conditions might be required.

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Conflicts of interest

The authors declare no conflict of interest.

References

1. C. W. Lee, S. Kwon, S. Park, B. Y. Kim, W. Yoo, B. H. Ryu, H. W. Kim, S. C. Shin, S. Kim, H. Park, T. D. Kim, J. H. Lee, Crystal structure and functional characterization of an esterase (EaEST) from *Exiguobacterium antarcticum*, *PLoS One*, **12**(1), e0169540 (2017).

2. C. Oh, T. D. Kim, K. K. Kim, Carboxylic ester hydrolases in bacteria: Active site, structure, function and application, *Crystals*, **9**, 597 (2019).

3. L. Rao, Y. Xue, C. Zhou, J. Tao, G. Li, J. R. Lu, Y. Ma, The thermostable esterase from *Thermoanaerobacter tengcongensis* opening up a new family of bacterial lipolytic enzymes, *Biochimica et Biophysica Acta*, **1814**, 1695–1702 (2011).

4. E. Borchert, J. Selvin, S.G. Kiran, S.A. Jackson, F. O'Gara, A.D.W. Dobson, A novel cold active esterase from a deep sea sponge *Stelletta normani* metagenomic library, *Frontiers in Marine Science*, **4**, 287 (2017).

5. J. Zhang, J. Liu, J. Zhou, Y. Ren, X. Dai, H. Xiang, Thermostable esterase from *Thermoanaerobacter tengcongensis*: High-level expression, purification and characterization, *Biotechnology Letters*, **25**, 1463–1467 (2003).

6. T.E. Rao, M. Imchen, R. Kumavath, Marine enzymes: Production and applications for human health, *Advances in Food Nutrition Research*, **80**, 149–163 (2017).

7. L.F. Godinh, C.R. Reis, P.G. Tepper, G.J. Poelarends, W.J. Quax, Discovery of an *Escherichia coli* esterase with high activity and enantioselectivity towards 1,2-O-isopropylideneglycerol esters, *Application Environment Microbiology*, **77**(17), 6094–6099 (2011).

8. L. Ramnath, B. Sithole, R. Govinden, Classification of lipolytic enzymes and their biotechnological applications in the pulping industry, *Canadian Journal of Microbiology*, **63**, 179–192 (2017).

9. F. Sasso, A. Natalello, S. Castoldi, M. Lotti, C. Santambrogio, R. Grandori, *Burkholderia cepacia* lipase is a promising biocatalyst for biofuel production, *Biotechnology Journal*, **11**, 954–960 (2016).

10. M. Levisson, J.V. Oost, S.W.M. Kengen, Characterization and structural modeling of a new type of thermostable esterase from *Thermotoga maritime*, *FEBS Journal*, **274**, 2832–2842 (2007).

11. D.R. Canchi, A.E. Garcia, Cosolvent effects on protein stability, *The Annual Review of Physical Chemistry*, **64**, 273–293 (2013).

12. H. Wang, K.K. Andersen, P. Sehgal, J. Hagedorn, P. Westh, K. Borch, D.E. Otzen, pH regulation of the kinetic stability of the lipase from *Thermomyces lanuginosus*, *Biochemistry*, **52**(1), 264–276 (2013).

13. H. Monhemi, R.M. Housaindokht, A.A. Moosavi-Movahedi, M.R. Bozorgmehr, How a protein can remain stable in a solvent with high content of urea: insights from molecular dynamics simulation of *Candida antartica* lipase B in urea: Choline chloride deep eutectic solvent, *Physical Chemistry Chemical Physics*, **16**(28), 14882–14893 (2014).

14. R. Fernandez-Lafuente, Lipase from *Thermomyces lanuginosus*: Uses and prospects as an industrial biocatalyst, *Journal of Molecular Catalalysis B: Enzymatic*, **62**, 197–212 (2010).

15. F.J. Olorunniji, A.L. McPherson, S.J. Rosser, M.C.M. Smith, S.D. Colloms, W.M. Stark, Control of serine integrase recombination directionality by fusion with the directionality factor, *Nucleic Acids Research*, **45**(14), 8635–8645 (2017).

16. S.C.B. Gopinath, Extracellular enzymatic activity profiles in fungi isolated from oil-rich environments, *Mycoscience*, **46**(2), 119–126 (2005).

17. O. Trott, A.J. Olson, AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization and multithreading, *Journal of Computational Chemistry*, **31**(2), 455–461 (2010).

18. S. Kim, J. Chen, T. Cheng, A. Gindulyte, J. He, S. He, Q. Li, B.A. Shoemaker, P.A. Thiessen, B. Yu, L. Zaslavsky, J. Zhang, E.E. Bolton, PubChem 2019 update: Improved access to chemical data, *Nucleic Acids Research*, **47**(1), 1102–1109 (2019).

19. The UniProt Consortium, UniProt: The universal protein knowledgebase in 2021, *Nucleic Acids Research*, **49**(D1), D480–D489 (2021).

20. A. Waterhouse, M. Bertoni, S. Bienert, G. Studer, G. Tauriello, R. Gumienny F.T. Heer, T.A.P. de Beer, C. Rempfer, L. Bordoli, R. Lepore, T. Schwede, SWISS-MODEL: Homology modelling of protein structures and complexes, *Nucleic Acids Research*, **46**(W1), W296–W303 (2018).

21. R.A. Laskowski, M.W. MacArthur, D.S. Moss, J.M. Thornton, PROCHECK: A program to check the stereochemical quality of protein structures, *Journal of Applied Crystallography*, **26**(2), 283–291 (1993).

22. R.A. Laskowski, J.A. Rullmannn, M.W. MacArthur, R. Kaptein, J.M. Thornton, AQUA and PROCHECK-NMR: Programs for checking the quality of protein structures solved by NMR, *Journal of Biomolecule NMR*, **8**(4), 477–486 (1996).

23. M.A. Salameh, J. Wiegel, Effects of detergents on activity, thermostability and aggregation of two alkalithermophilic lipases from *Thermosyntropha lipolytica*, *The Open Biochemistry Journal*, **4**, 22–28 (2010).

24. R. Singh, M. Kumar, A. Mittal, P.K. Mehta, Microbial enzymes: Industrial process in 21st century, *3 Biotech*, **6**, 174 (2016).

25. Y. Zhu, W. Zheng, H. Ni, H. Liu, A. Xiao, H. Cai, Molecular cloning and scharacterization of a new and highly thermostable esterase from *Geobacillus* sp. JM6, *Journal of Basic Microbiology*, **55**, 1219–1231 (2015).

26. A.K. Singh, M. Mukhopadhyay, Overview of fungal lipase: A review, *Applied Biochemistry and Biotechnology*, **166**, 486–520 (2012).

27. Z. Qian, J. Zhao, X. Bai, W. Tong, Z. Chen, H. Wei, Q. Wang, S. Liu, Thermastability of glucokinases in *Thermoanaerobacter tengcongensis*, *BioMed Research International*, **2013**, 646539 (2013).

28. J.H. Meessen, H. Petersen, Urea, *Ullmann's Encyclopedia of Industrial Chemistry*, Wiley-VCH, Weinheim, 2010.

29. A. Igunnu, A. Aberuagba, S.O. Oyeyipo, B.T. Bakare, E.E. Onwurah, S.O. Malomo, Effects of urea and sodium dodecyl sulphate on stability profile of *Thermomyces lanuginosus* lipase, *Bioscience Research Journal*, **33**(2), 55–67 (2020).

30. X. Gao, X. Mao, P. Lu, F. Secundo, C. Xue, J. Sun, Cloning, Expression, and characterization of a novel thermostable and alkaline-stable esterase from *Stenotrophomonas maltophilia* OUC_Est10 catalytically active in organic solvents, *Catalysts*, **9**(5), 401 (2019).

31. J.E. Mogensen, P. Sehgal, D.E. Otzen, Activation, inhibition, and destabilization of *Thermomyces lanuginosus* lipase by detergents, *Biochemistry*, **44**, 1719–1730 (2005).

32. M. Alcaide, P.J. Stogios, A. Lafraya, Á. Tchigvintsev, R. Flick, R. Bargiela, Pressure adaptation is linked to thermal adaptation in salt-saturated marine habitats, *Environmental Microbiology*, **17**, 332–345 (2015).

33. J.M. Cardoso, L. Fonseca, C. Egas, I. Abrantes, Cysteine proteases secreted by the pinewood nematode, *Bursaphelenchus xylophilus*: *In silico* analysis, *Computational Biology and Chemistry*, **77**, 291–296 (2018).

34. B. Patel, V. Singh, D. Patel, *Essentials of Bioinformatics*, vol. I., Springer, Cham, Switzerland, 2019, pp. 169–199.

35. H. Yamaguchi, T. Akitaya, Y. Kidachi, K. Kamiie, H. Umetsu, Homology modeling and structural analysis of human γ-glutamylcysteine ligase catalytic subunit for antitumor drug development, *Journal of Biophysical Chemistry,* **3**(3), 238–248 (2012).

36. I. Olaoye, B. Oso, A. Aberuagba, Molecular insights into the binding mechanisms of human and mouse Glutamate-cysteine ligases, *Revista Colombiana de Ciencias Químico Farmacéuticas*, **52**(1), 311–336 (2023).

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