**Jilin Daxue Xuebao (Gongxueban)/Journal of Jilin University (Engineering and Technology Edition)**
ISSN: 1671-5497
E-Publication: Online Open Access
Vol: 42 Issue: 07-2023
DOI 10.17605/OSF.IO/5VAKC

# SCREENING OF HYDROLYTIC AND NOVEL ISOMERASE ENZYMES FROM EXTREMOPHILES: *GEOBACILLUS STEAROTHERMOPHILUS* MB600 AND *HALOMONAS ELONGATA* MB591

**MONAZA BIBI**
Microbiology and Biotechnology Research Lab, Department of Biotechnology, Fatima Jinnah Women University Rawalpindi, Pakistan. Corresponding Author E-mail: Khanmonaza31@gmail.com

**AZRA YASMIN**
Microbiology and Biotechnology Research Lab, Department of Biotechnology, Fatima Jinnah Women University Rawalpindi, Pakistan.

**RIHAF ALFARAJ**
Department of Pharmaceutics, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia.

**Abstract**

Extremophile bacteria originally isolated from the hot springs of Gilgit Baltistan and Khewara salt mine, i.e., *Geobacillus stearothermophilus* strain MB600 and *Halomonas elongata* strain MB591, were tested for their production of extremozymes. The targeted enzyme class was hydrolytic enzymes (Lipase, Gelatinase, Protease, & Cellulase), and isomerase enzymes (aconitase, hydratase, and phosphoglycerate mutase). The thermophilic bacteria MB600 produced all hydrolytic enzymes after 24 hours of incubation. Matrix-assisted laser desorption/ionization-time of flight (MALDI TOF) analysis confirmed the presence of Aconitase hydratase isolated from *Geobacillus stearothermophilus strain* MB600 with a molecular weight of 98 KD. Similarly, all the hydrolytic enzymes were produced by *Halomonas elongata* strain MB591. The production of Phosphoglycerate mutase by *Halomonas elongata* strain MB591 was also confirmed, and its molecular characterization was performed using Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and MALDI-TOF with a molecular weight of 56 KD. Later, insilico analysis was also performed for predicting the 3D structure of enzymes, and their bonding interactions with guaiacol were explained by docking using CB Dock

**Index Terms**— Docking, Extremophiles, Extremozymes screening, *Geobacillus, Halomonas,* Insilco analysis, MALDI-TOF

## 1. INTRODUCTION

Extremophiles are a group of organisms that can survive and thrive in harsh environments. Due to the complexity of examining ecological niches and isolating extremophilic microorganisms, it is one of the more challenging fields to investigate, and as a result, the majority of extremophiles are still unknown and are still considered to be components of the microbial dark matter [1]. Extremozymes are enzymes made by extremophiles that are resistant to extreme environments. Because of their amazing strength, they perform better than their mesophilic counterparts under extreme settings on a functional level [2]. Enzymes produced by thermophilic organisms are known as Thermozymes. Due to their resistance to chemical denaturants, great thermostability, broad hydrolysis ability, high catalytic activity, pH adaption, and least contamination at high temperatures, thermozymes can withstand harsh environments [3], [4]. They are a possible contender for businesses including food, textile biofuel manufacturing, and paper

**Jilin Daxue Xuebao (Gongxueban)/Journal of Jilin University (Engineering and Technology Edition)**
ISSN: 1671-5497
E-Publication: Online Open Access
Vol: 42 Issue: 07-2023
DOI 10.17605/OSF.IO/5VAKC

because of these outstanding qualities. Due to their extraordinary qualities, they are a possible contender for the food, paper, and textile biofuel industries [5]. Halozymes are the name given to the enzymes made by halophilic microorganisms. These enzymes had some modifications, mostly to their structural organization, but otherwise shared the same characteristics as ordinary enzymes. One of these characteristics is the high need for Sodium Chloride (NaCl) for appropriate biological functioning [5]. The focus of this study was the screening of halozymes from halophilic bacteria isolated from the Mayo Salt Mine and thermozymes from thermophilic bacteria isolated from the hot springs of Gilgit-Baltistan.

## 2. MATERIALS AND METHODS

### 2.1 Chemicals

The following chemicals were used: syringaldazine (SGZ), guaiacol, gelatin, skimmed milk, polysorbate, and carboxymethyl cellulose. All of the compounds were of analytical grade and purchased from Merck (Germany). Mini-PROTEAN TGX Precast Gels were purchased from BIORAD (USA).

### 2.2 Source of Microorganism

The *Geobacillus stearothermophilus* strain MB600 (GenBank: MG720004) and *Halomonas elongata strain* MB591 (GenBank: MG685893) were obtained from Microbiology and Biotechnology Research Lab at Fatima Jinnah Women University Rawalpindi. Bacterial culture was maintained on Nutrient agar (NA) medium and stored at -20C.

### 2.3 Extremozymes (Hydrolases) Screening Using Plate Assay

#### 2.3.1 Screening of Protease

Using skimmed milk as the substrate, an initial screening of protease enzymes from halophiles and thermophiles was carried out (Tables 2.16 and 2.22). Nutrient agar (NA) was supplemented with skimmed milk (1%), and bacterial colonies were streaked and spotted on the NA plate as described by [7]. For thermophiles, while halophiles were studied using LB agar with 2.5 M NaCl and 1% skimmed milk [8]. After autoclaving, each medium was poured into petri plates and allowed to solidify. The test organisms were added to these plates, and they were then incubated for 24-48 hours at 37°C for halophiles and 80°C for thermophiles. Clear zones that formed surrounding bacterial colonies served as indicators of proteolytic activity.

#### 2.3.2 Screening of Lipase

On nutritional agar and LB agar petri plates supplemented with 0.5% polysorbate 80, screening of lipase was conducted [8]. Halophilic bacteria on LB agar received 2M NaCl as an addition. Separately autoclaved Polysorbate 80 was then added to the media as a supplement. After becoming solid, these plates were streaked with the test organism and incubated at specific temperatures (37°C for halophiles and 80°C for thermophiles). Lipase synthesis by extremophilic bacteria was suggested by the presence of distinct halos.

**Jilin Daxue Xuebao (Gongxueban)/Journal of Jilin University (Engineering and Technology Edition)**
ISSN: 1671-5497
E-Publication: Online Open Access
Vol: 42 Issue: 07-2023
DOI 10.17605/OSF.IO/5VAKC

### 2.3.3 Screening of Gelatinase

The substrate employed for the screening of the gelatinase enzyme was 1% gelatin. On nutritional agar and LB (2M NaCl) agar petri plates with 1% gelatin (w/v), extremophilic bacteria were streaked. These plates were exposed to acidified mercuric chloride for 10 minutes after 24 hours of incubation. According to Balan the development of a clear zone around bacterial colonies suggested the production of gelatinase

[9].

### 2.3.4 Screening of Cellulase

A minimum medium containing 2M NaCl and 1% carboxymethyl cellulose (CMC) was autoclaved. After that halophilic bacterial colonies were streaked on the hardened petri plates and incubated at 37°C for halophiles and 80°C for thermophiles. By forming a large zone of hydrolysis around bacterial colonies after 24 and 48 hours, cellulase production was demonstrated [10].

## 2.4 Extracellular production of Aconitase hydratase and Phosphoglycerate mutase (PGAM) and culture conditions

Luria-Bertani (LB) broth media was used for the extracellular production of Aconitase hydratase and Phosphoglycerate mutase (PGAM).LB broth media (pH 7) containing 0.5 mM guaiacol was inoculated with 24hr fresh culture of thermophile bacteria i.e. MB600 and incubated at 80°C for 3 days, for the production of Aconitase hydratase. For Phosphoglycerate mutase (PGAM) production LB media (pH 7) containing 2 M NaCl was inoculated with 24hr fresh culture of halophile bacteria i.e. MB591, and incubated at 37°C for 6 days. After 3 days for MB600 and 6 days for MB591, the culture was harvested by centrifugation at 8,000 rpm for 15 min at 4°C. The supernatant was used as a crude extracellular enzyme [11]

## 2.5 Partial purification of thermophilic Aconitase hydratase and halophilic Phosphoglycerate mutase

The six-day-old MB591 and three-day-old MB600 bacterial cultures were centrifuged at 10,000 rpm for 20 min at 4°C. The pellet was discarded and the cell-free supernatant was precipitated with 60 % ammonium sulphate for 24 h with constant stirring at 4°C. After 24 hr the ammonium sulphate precipitated protein samples were then centrifuged for 20 min at 10,000 rpm and 4°C. The brown color pellet was then dissolved in a small amount of buffer (20 mM sodium phosphate buffer, pH 6.4), and dialyzed for 24 hours at 4°C using a dialysis tube with an average width of 25 mm and a pour size of 12 KDa. The dark brown dialysates were then concentrated by centrifuging at 4,000 rpm at 4°C for 8 minutes after being washed three times with 0.1M sodium acetate buffer (pH 4.5) using 30 KD vivaspin and served as partially purified proteins [11].

## 2.6 Molecular weight identification using SDS-PAGE

A standard molecular mass marker of 10-180 kDa was used in the SDS-PAGE of partially purified protein samples [12].

**Jilin Daxue Xuebao (Gongxueban)/Journal of Jilin University (Engineering and Technology Edition)**
ISSN: 1671-5497
E-Publication: Online Open Access
Vol: 42 Issue: 07-2023
DOI 10.17605/OSF.IO/5VAKC

### 2.7 Molecular identification using MALDI- TOF

Two protein bands of interest were cut from SDS gel and picked in a 0.2 ml microcentrifuge tube. Trypsin digestion of excised protein was done by the method [13]. The peptide mass fingerprinting was performed on a MALDI-TOF-TOF MS analyzer (ABSCIEX TOF/TOF 5800, Applied Biosystems, USA) and the protein identification (ID) was obtained using result-dependent analysis (RDA) by ProteinPilot™ software (Version 3.2, USA). The resulting peptide mass finger-printings (PMFs) were used to identify both proteins by searching the SWISS-PROT and NCBI-nr databases using the Mascot 2.0 search engine with fragment mass tolerance of ±0.3 Da.

### 2.8 3D proteins structure using Alpha fold

3D structures of proteins identified from MALDI-TOF were retrieved from the alpha fold. The protein sequence obtained after the mascot database similarity was used as a template for structure prediction [14].

### 2.9 Docking of enzyme and substrate

Docking was performed between ligand (guaiacol) and proteins using CB Dock a web server to perform the blind docking by the method of Liu et al. [15].

## 3. RESULTS AND DISCUSSION

### 3.1 Screening of hydrolytic from thermophile and halophile

Extremophiles are thought to have special genes that allow them to produce extremozymes, which are metabolites (proteins and enzymes) that can endure extreme environmental conditions [16] (Dumorne *et al*., 2017). A similar approach opted in the present study to explore the enzyme production potential of one halophilic and one thermophilic bacteria. Initial screening of enzymes from the thermophilic bacteria *Geobacillus stearothermophilus* strain MB600 and halophilic bacteria *Halomonas elongata strain* MB591 was performed. As makers of thermostable hydrolytic enzymes, thermophiles are drawing attention in the biotechnology industry. [17] Reported thermophilic *Bacillus sp.* protease activity. Additionally, the economically significant hydrolytic enzymes lipase, cellulase, gelatinase, and protease were examined in the hyperthermophilic bacteria *Geobacillus stearothermophilus* strain MB600 investigated here. *Halomonas elongata* strain MB591 hydrolyze gelatin (1%) by producing gelatinase after 24 hr of incubation at 37°C. The formation of clear zones around the bacterial colony indicated protease activity. MB591 was also given positive results for the screening of lipase, gelatinase, and cellulase enzymes production by the hydrolysis of (0.5% polysorbate80), 1% gelatinase, and 1% Carboxy methyl cellulose giving the significant zone of hydrolysis after 24 and 48hr of incubation at 37°C (Table 1). Similar results were investigated by [18].

Jilin Daxue Xuebao (Gongxueban)/Journal of Jilin University (Engineering and Technology Edition)
ISSN: 1671-5497
E-Publication: Online Open Access
Vol: 42 Issue: 07-2023
DOI 10.17605/OSF.IO/5VAKC

**Table 1: Screening of Hydrolytic enzymes from Halophilic and thermophilic bacteria**

| Strain | Lipase Polysorbate 80 (0.5 % ) | Cellulase CMC (1%) | Gelatinase Gelatin (1% ) | Protease Skimmed milk (1%) |
|---|---|---|---|---|
| **MB591** | +ive | +ive | +ive | +ive |
| **MB600** | +ive | +ive | +ive | +ive |

### 3.2 Isomerase enzymes from thermophiles and halophiles

Production of two different isomerase enzymes, aconitate hydratase, and phosphoglycerate mutase, was monitored using LB broth as a growth medium from the thermophilic strain *Geobacillus stearothermophilus* strain MB600 and the halophilic bacteria *Halomonas elongata* strain MB591. For extracellular aconitate hydratase production, a three-day-old thermophilic bacterial culture incubated at 80 °C was centrifuged, the pellet was discarded, and the supernatant was used as crude aconitate hydratase. Similarly, for phosphoglycerate mutase production, a six-day-old halophilic bacterial culture incubated at 37 °C was centrifuged, the supernatant was separated and served as crude laccase, while the pellet was discarded (Figure 2).

### 3.3 Molecular characterization through SDS-PAGE

Molecular weight characterization of aconitate hydratase and phosphoglycerate mutase using gel electrophoresis revealed that aconitate hydratase had a molecular weight of 98 KD and phosphoglycerate mutase had a molecular weight of 56 KD (Fig. 1).

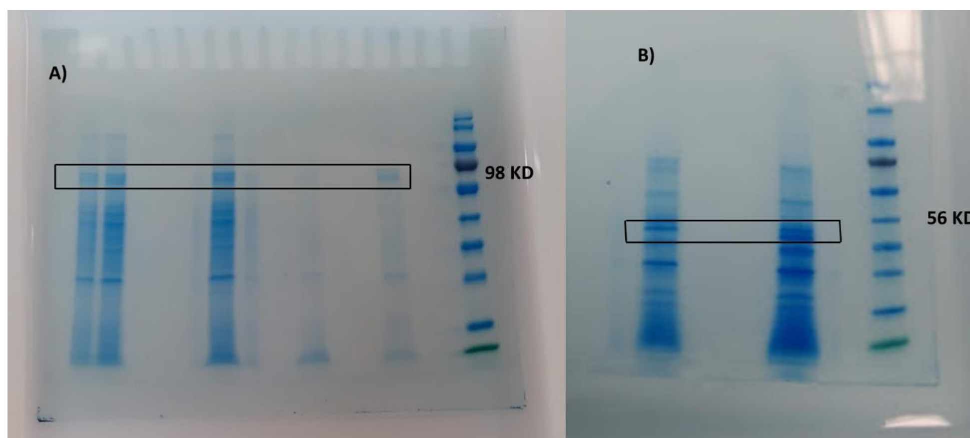



**Fig 1: SDS-PAGE of A) Aconitate hydratase, B) Phosphoglycerate mutase**

### 3.3 Molecular characterization through MALDI-TOF

The enzymatically digested peptide fragments were identified using mass spectrometry and specialized software such as "mascot" based on their distinctive masses and patterns. The identified protein from *Geobacillus stearothermophilus* strain MB600 was aconitate hydratase similar to the one present in *Sporosarcina globispora* (GN=AF332_18615 PE=3 SV=1) (Table 2). Aconitase hydratase from thermophilic bacteria can also shed light on the composition and mechanism of this enzyme. This

**Jilin Daxue Xuebao (Gongxueban)/Journal of Jilin University (Engineering and Technology Edition)**
ISSN: 1671-5497
E-Publication: Online Open Access
Vol: 42 Issue: 07-2023
DOI 10.17605/OSF.IO/5VAKC

knowledge can be put to use to increase the enzyme's activity and stability and to create new uses for it. While halophilic protein identified from halophilic bacteria *Halomonas elongata strain* MB591 was phosphoglycerate mutase similar to the one present in *Halomonas sp*. (GN=CME74_01610 PE=4 SV=1)(Table 3). Due to its potential usage in numerous industries, phosphoglycerate mutase produced by *Halomonas* is of interest to both scientists and business. Phosphoglycerate mutase can be used, for instance, to create 2PG, a precursor to a number of significant chemicals, including glycerol and glyceraldehyde-3phosphate.

**Table 2: MALDI-TOF sequence identification of Thermophilic Aconitate hydratase**

| 3::A0A0M0GFS4_SPOGL Mass: 98837 Score: 100 Matches: 3(0) Sequences: 3(0) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Aconitate hydratase OS=*Sporosarcina globispora* GN=AF332_18615 PE=3 SV=1 | | | |
| **Query** | **Observed** | **Mr(expt)** | **Mr(calc)** | **Delta** | **Miss** | **Score** | **Expect** | **Rank** | **Unique** | **Peptide** |
| 883 | 473.7081 | 945.4016 | 944.3988 | 1.0028 | 0 | 26 | 2.3e+02 | 5 | | R.DFNSYGSR.R |
| 3609 | 788.4542 | 1574.8939 | 1573.8902 | 1.0037 | 0 | 45 | 1.7 | 1 | | R.SNLVLMGVLPLQFK.A |
| 4085 | 872.9756 | 1743.9367 | 1743.9367 | 0.0001 | 0 | 30 | 57 | 6 | U | K.AVAESDLLITSVLSGNR.N |

**Table 3: MALDI-TOF sequence identification of Halophilic Phosphoglycerate mutase**

| 2::A0A2D5MFZ8_HALSX Mass: 56261 Score: 82 Matches: 3(0) Sequences: 3(0) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Phosphoglycerate mutase (2,3-diphosphoglycerate-independent) OS=*Halomonas sp*. GN=CME74_01610 PE=4 SV=1 | | | | | | |
| **Query** | **Observed** | **Mr(expt)** | **Mr(calc)** | **Delta** | **Mis** | **Score** | **Expect** | **Rank** | **Unique** | **Peptide** |
| 301 | 408.2348 | 814.4551 | 814.4548 | 0.0002 | 0 | 32 | 1.1e+02 | 3 | | R.VVEAIER.A |
| 1142 | 521.7735 | 1041.5324 | 1040.5291 | 1.0033 | 0 | 30 | 1.6e+02 | 2 | | R.IVYQDFTR.I |
| 4532 | 650.9624 | 1949.8654 | 1948.8611 | 1.0042 | 0 | 23 | 5.6e+02 | 1 | U | K.TYDEKPEMSAYEITEK.L |

### 3.4 Insilco analysis of aconitate hydratase and phosphoglycerate mutase

The 3D structure of aconitate hydratase and phosphoglycerate mutase was predicted from Alpha Fold (Fig. 2) and the 3D structure of ABTS and guaiacol were retrieved from the Zinc database. The molecular docking was performed by CB- Dock online docking server. When doing the docking, CB Dock used the CurPocket curvature-based cavity detection method to more accurately estimate the target protein's binding site while AutoDock Vina was used to determine the binding poses of the query ligands. The first

**Jilin Daxue Xuebao (Gongxueban)/Journal of Jilin University (Engineering and Technology Edition)**
ISSN: 1671-5497
E-Publication: Online Open Access
Vol: 42 Issue: 07-2023
DOI 10.17605/OSF.IO/5VAKC

binding mode with a Vina score of (-5.5) and cavity size of 1521 was chosen for Guaiacol (Fig.3). Docking of Guaiacol with Aconitate hydratase (Fig.4). The Docking for halophilic enzyme phosphoglycerate mutase with guaiacol was also performed by using CB Dock. First binding mode with a Vina score of (-5.2) and cavity size of 3048 was chosen for Guaiacol (Fig. 5). Docking of Guaiacol with phosphoglycerate mutase (Fig. 6). It has been determined through docking experiments how guaiacol, aconitase hydratase, and phosphoglycerate mutase interact when they are bound to one another. According to these investigations, guaiacol can bind to both enzymes in a variety of ways. The hydroxyl groups in guaiacol and the amino acid residues in the enzymes act as mediators for the binding interactions.

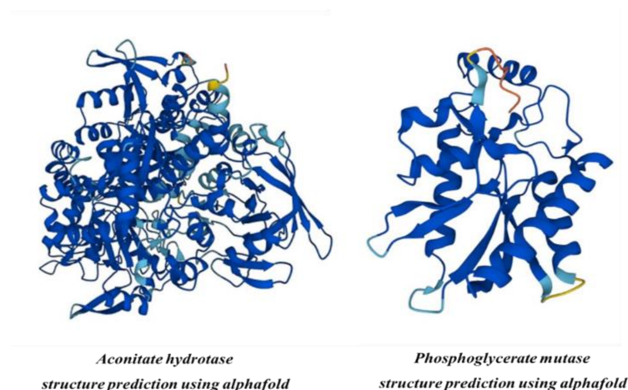
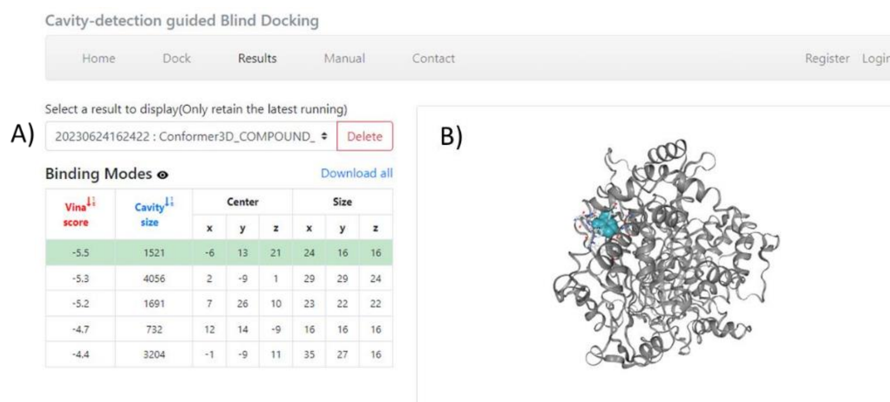



**Fig 2: 3D structure of proteins**




| Vina score | Cavity size | Center x | Center y | Center z | Size x | Size y | Size z |
|---|---|---|---|---|---|---|---|
| -5.5 | 1521 | -6 | 13 | 21 | 24 | 16 | 16 |
| -5.3 | 4056 | 2 | -9 | 1 | 29 | 29 | 24 |
| -5.2 | 1691 | 7 | 26 | 10 | 23 | 22 | 22 |
| -4.7 | 732 | 12 | 14 | -9 | 16 | 16 | 16 |
| -4.4 | 3204 | -1 | -9 | 11 | 35 | 27 | 16 |

**Fig 3: The web interface of CB-Dock. A) Vina scores and cavity information of results for guaiacol with aconitate hydratase, B) the interactive 3D viewer illustrating selected binding modes**

ISSN: 1671-5497
E-Publication: Online Open Access
Vol: 42 Issue: 07-2023
DOI 10.17605/OSF.IO/5VAKC

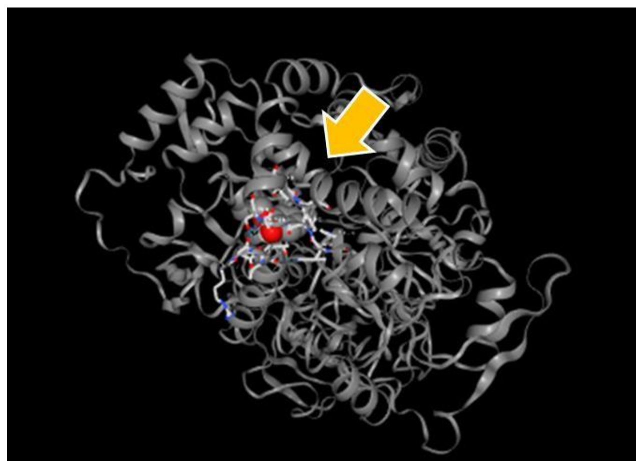
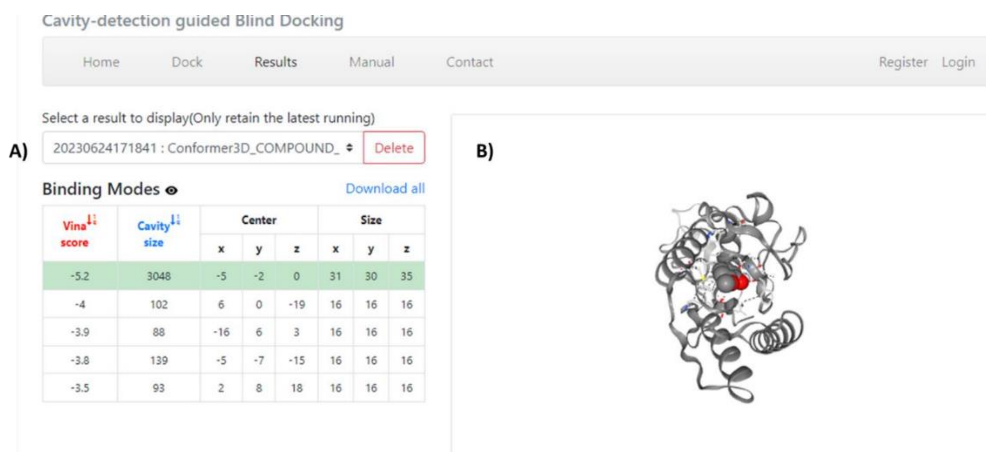


**Fig 4: Docking of Guaiacol with Thermophilic Aconitate hydratase**




| Vina score | Cavity size | Center | | | Size | | |
|---|---|---|---|---|---|---|---|
| | | x | y | z | x | y | z |
| -5.2 | 3048 | -5 | -2 | 0 | 31 | 30 | 35 |
| -4 | 102 | 6 | 0 | -19 | 16 | 16 | 16 |
| -3.9 | 88 | -16 | 6 | 3 | 16 | 16 | 16 |
| -3.8 | 139 | -5 | -7 | -15 | 16 | 16 | 16 |
| -3.5 | 93 | 2 | 8 | 18 | 16 | 16 | 16 |

**Fig 5: The web interface of CB-Dock. A) Vina scores and cavity information of results for guaiacol with phosphoglycerate mutase, B) the interactive 3D viewer illustrating selected binding modes**

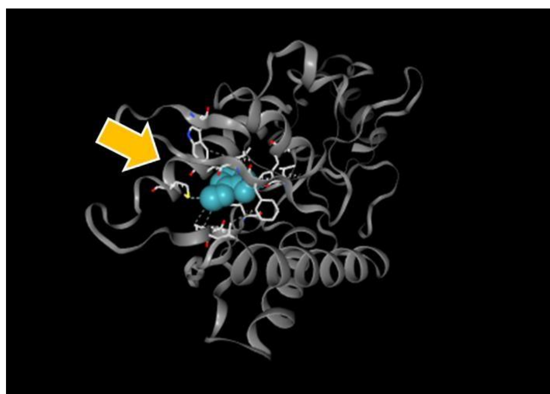


**Fig 6: Docking of Guaiacol with Halophilic *phosphoglycerate mutase***

ISSN: 1671-5497
E-Publication: Online Open Access
Vol: 42 Issue: 07-2023
DOI 10.17605/OSF.IO/5VAKC

## CONCLUSION

In the current investigation, thermophilic bacteria from the genus *Geobacillus* and halophilic bacteria from the species *Halomonas* that possess an outstanding combination of multiple industrially significant enzymes were shown to be effective. This research serves as a foundation for continued investigation of these extreme-temperature and extreme salt tolerant enzymes for improvement in industrial applications, particularly in the medical and industrial domains.

**Disclaimer**: None to Declare

**Conflict of interest**: None to Declare

### Reference


1) Bernard, G., Pathmanathan, J. S., Lannes, R., Lopez, P., and Bapteste, E. (2018). Microbial dark matter investigations: how microbial studies transform biological knowledge and empirically sketch a logic of scientific discovery. *Genome Biology and Evolution,* 10(3): 707715.

2) Kuddus, M., & Bano, N. (2022). Microbial screening for extremozymes. In Microbial Extremozymes (pp. 1-7). Academic Press.

3) Arora, R., Behera, S., and Kumar, S. (2015). Bioprospecting thermophilic/thermotolerant microbes for production of lignocellulosic ethanol: a future perspective. *Renewable and Sustainable Energy Reviews*, 51: 699-717.

4) Akram, F., ul Haq, I., Imran, W., and Mukhtar, H. (2018). Insight perspectives of thermostable endoglucanases for bioethanol production: a review. *Renewable Energy*, 122: 225-238.

5) Atalah, J., Caceres-Moreno, P., Espina, G., and Blamey, J. M. (2019). Thermophiles and the applications of their enzymes as new biocatalysts. *Bioresource Technology*, 280: 478-488.

6) Edbeib, M. F., Wahab, R. A., and Huyop, F. (2016). Halophiles: biology, adaptation, and their role in decontamination of hypersaline environments. *World Journal of Microbiology and Biotechnology,* 32(8): 1-23

7) Baltaci, M. O., Genc, B., Arslan, S., Adiguzel, G. and Adiguzel, A. (2017). Isolation and characterization of thermophilic bacteria from geothermal areas in Turkey and preliminary research on biotechnologically important enzyme production. Geomicrobiol. J., 34(1), 5362.

8) Kumar, S., Karan, R., Kapoor, S., Singh, S. P. and Khare, S. K. (2012). Screening and isolation of halophilic bacteria producing industrially important enzymes. Braz. J. Microbiol., 43(4): 1595-1603.

9) Balan, S. S., Nethaji, R., Sankar, S., and Jayalakshmi, S. (2012). Production of gelatinase enzyme from *Bacillus spp* isolated from the sediment sample of Porto Novo Coastal sites. *Asian Pacific Journal of Tropical Biomedicine*, 2(3): S1811-S1816

10) Shanmugapriya, K., Saravana, P. S., Krishnapriya, M. M., Mythili, A., and Joseph, S. (2012). Isolation, screening and partial purification of cellulase from cellulase producing bacteria. *International Journal of Advanced Biotechnology and Research*, *3*(1): 509-514.

11) Shah AI, Jobanputra JB. Production and partial purification of laccase produced by *Bacillus species* isolated from contaminated soil. Int j innov. 2017; 6(9): 17862-17869.

12) Laemmli UK. Cleavage of structural proteins during the assembly of the head of *bacteriophage* T4. Nature. 1970; 227(5259): 680-685.

13) Shevchenko A, Tomas H, Havli J, et al. In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc. 2016; 1(6): 2856-2860.

**Jilin Daxue Xuebao (Gongxueban)/Journal of Jilin University (Engineering and Technology Edition)**
ISSN: 1671-5497
E-Publication: Online Open Access
Vol: 42 Issue: 07-2023
DOI 10.17605/OSF.IO/5VAKC

14) Jumper, J et al. Highly accurate protein structure prediction with AlphaFold. Nature (2021).

15) Liu Y, Grimm M, Dai WT, et al. CB-Dock: a web server for cavity detection-guided protein–ligand blind docking. Acta Pharmacologica Sinica. 2020; 41(1): 138-144.

16) Dumorné, K., Córdova, D. C., Astorga-Eló, M., & Renganathan, P. (2017). Extremozymes: a potential source for industrial applications.

17) Panda, M. K., Sahu, M. K. and Tayung, K. (2013). Isolation and characterization of a thermophilic *Bacillus sp*. with protease activity isolated from hot spring of Tarabalo, Odisha, India. Iran. J. Microbiol., 5(2): 159–165.

18) Rohban, R., Amoozegar, M. A., & Ventosa, A. (2009). Screening and isolation of halophilic bacteria producing extracellular hydrolyses from Howz Soltan Lake, Iran. *Journal of Industrial Microbiology and Biotechnology*, *36*(3), 333-340.