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Docking Studies and Molecular Cloning of Alpha/Beta Hydrolase from Novel *Streptomyces werraensis* KN23 for Enhanced Keratinase Activity

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ABSTRACT

Alpha/beta hydrolases constitute an expansive functional group of enzymes present in bacteria, archaea, and eukaryotes. The present study demonstrates the gene encoding alpha/beta hydrolase (consisting of 1596 bp nucleotides) of novel *Streptomyces werraensis* KN23, the sample was subjected to isolation, sequencing, and then uploaded to the NCBI GenBank database with the accession number OQ511280. Validation of the modeled protein's 3D structure was conducted through Ramachandran's plot, revealing that, for the template proteins *S. werraensis* FS97 and KN23, 92.51% and 97.48%, respectively, of the 532 the residues were placed in the area with the highest preference. Docking studies revealed optimal binding affinities of the alpha/beta hydrolase with beta keratin, of values -259.20 kcal/mol and -227.70 kcal/mol for the template strains *S. werraensis* FS97 and *S. werraensis* KN23, respectively. Expression of the *S. werraensis* KN23 alpha/beta hydrolase gene, the sample had a keratinase activity of 51.60 U/ml. The binding affinities between the deduced protein and beta keratin were calculated using computational methods. The alpha/beta hydrolase gene was transcribed and translated in *E. coli DH5α*, leading to a substantial increase in keratinase activity (93.38 U/ml).

Keywords: alpha/beta hydrolase, beta keratin, modeled structure 3D, Docking studies, molecular cloning

1. Introduction

The alpha/beta hydrolase (ABH) superfamily is known for its wide range of metabolic activities found in all three domains of life., has a wide distribution and a protein fold that can adapt structurally (Mindrebo *et al*., 2016).

The ABH fold was first characterized three decades ago (Ollis *et al*., 1992), and the ESTHER database, specifically dedicated to proteins exhibiting this fold, was established in 1995. Over the years, there has been a consistent expansion in the array of enzymatic activities and biological functions linked to members of this protein superfamily. Notably, the most recent publications introducing updates to the database date back a decade (Lenfant *et al*., 2012 and Lenfant *et al*., 2013).

Since then, numerous discoveries have been made. For instance, the number of subfamilies increased from 148 to 235, and the count of experimentally determined 3D structures has doubled, reaching 3000. Various reviews have explored the superfamily, such as those by Nardini and Dijkstra (1999); Carr and Ollis (2009) and Rauwerdink and Kazlauskas (2015). Additionally, sister databases focusing on specific structural folds or activities have been introduced, including the Lipase Engineering Database (LED), the alpha/beta hydrolase fold 3DM database (ABHDB**)** (Kourist *et al*., 2010), and the peptidase database MEROPS (Rawlings and Bateman 2021).

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Microbial keratinolytic proteases have been identified as a valuable group of enzymes with promising applications in various industries (Gupta *et al*., 2013). Keratinases, a subset of these proteases, are of particular interest as they can break down the tough, insoluble structure found in keratin substrates. These enzymes have garnered increased attention due to their potential uses in hydrolyzing keratinous materials and other applications (Bouacem *et al*., 2016).

Keratin, an indissoluble protein, is the primary constituent of robust defensive tissues in animals, including hair, feathers, wool, nails, and claws. Annually, a substantial amount of keratin, amounting to millions of tonnes, is generated worldwide as a notable by-product in diverse industrial procedures. The structure of keratin is densely interwoven as a result of its dense arrangement of hydrogen bonds, hydrophobic interactions, and strong disulfide bonds, making it stable and resistant to typical enzymes. Keratinase, however, has the unique ability to break down keratin. This capability offers an environmentally friendly method for managing keratin waste, contributing to sustainable development (Zhang *et al*., 2020).

The chicken business at a worldwide scale produces millions of tonnes of waste each year, mostly in the form of feathers. The surge in poultry meat consumption has resulted in the widespread accumulation of feathers, constituting a significant environmental challenge with potential detrimental effects on human health (Alahyaribeik *et al*., 2020). Consequently, researchers are actively exploring methods to harness the keratin content within feathers, both in its original high-molecular-weight polymer state (Shanmugasundaram *et al*., 2018) and its derived products (Tamreihao *et al*., 2019; Yusuf *et al*., 2015). These investigations aim to identify applications for keratin in the realms of medicine and agriculture.

Presently, scientists possess the ability to readily separate or fabricate genes and introduce them into a compatible expression system. The feasibility of using this prokaryotic expression system is attributed not only to its cost-effectiveness but also to the availability of diverse instruments that streamline the execution of this procedure (Saccardo *et al*., 2016; Ariyaei *et al*., 2019).

Scientists have employed protein engineering to manage and control genes, leading to the creation of proteases with distinct specificity and improved stability. This approach also aids in comprehending the connections between the composition and operation of enzymes. However, exploring the vast microbial diversity to identify producers of novel proteases with desired industrial traits remains a significant and promising area of research (Singh and Bajaj, 2015). Several microbial proteases have been identified and examined in bacteria (Joshi and Satyanarayana, 2013) and fungi (Choudhary, 2013). Proteases from *Bacillus* species are known for their ability to tolerate extreme conditions and function effectively in challenging environmental settings (Suberu *et al*., 2019).

Our main goals in this research included identifying, sequencing, and uploading the alpha/beta hydrolase gene of S. *werraensis* KN23 to the NCBI Gen Bank. Employing the molecular modelling techniques, aimed to generate and validate a 3D model of the alpha/beta hydrolase protein through Ramachandran's plot, employing template proteins from *S. werraensis* strains FS97 and KN23. Additionally, aimed by implementing molecular docking studies to identify the respective alpha/beta hydrolase's binding with beta keratin and assessing in-silico interaction potencies of the deduced protein. Lastly, attempted to develop an expression system for the alpha/beta hydrolase gene within *E. coli* DH5α strain with a view to comparably assessing and optimizing the keratinase activity over that of the original strain.

2. Material and Methods

2.1. Plasmids, reagents, media and strains

The strain *S. werraensis* KN23 with the NCBI Accession No. OK086273, known for its effective feather degradation capabilities, has been preserved in our laboratory according to the method given by Abd El-Aziz *et al*. (2023a). *E. coli* DH5a was cultured at a temperature of 37 °C for a duration of 12–18 hours in either Luria–Bertani (LB) broth or low-salt LB medium. The solution has the following concentrations (g/L): NaCl, 10.0; yeast extract, 5.0; and tryptone, 10.0. Actinomycetes were cultured on Plate Count (P.C) agar medium (Himedia, West Chester, Pennsylvania, USA). The main composition for isolating and fermenting microorganisms that degrade feathers was NaCl (0.5 g/L), KH2PO4 (0.7 g/L), K2HPO4 (1.4 g/L), MgSO4 (0.1 g/L), and feathers (10 g/L), with a pH of 7.2 (Cai *et al*., 2008). The pGEM-T Easy-cloning vector was acquired from Promega Co. in Madison, WI, USA.

It has a length of 3015 base pairs and contains the ampicillin resistance gene. The entire genomic DNA was isolated from the *S. werraensis* strain KN23 using a genomic DNA isolation kit provided by GeneDireX, Inc. in Taoyuan, Taiwan. The extraction of plasmid DNA from E. coli DH5a was performed using the QIA prep spin miniprep kit. The T4 DNA ligase was acquired from NEB (USA) and TaKaRa (China).

2.2. Amplification of α/β-hydrolase encoding gene

Designed primers for the alpha/beta hydrolase gene based on its DNA sequence available of *S. werraensis* FS97 at NCBI GenBank. Genomic DNA was then extracted from the *S. werraensis* KN23 (NCBI Accession No. OK086273 by Abd El-Aziz *et al*. (2023a). The strain was subjected to genomic DNA isolation using a Genomic DNA Isolation kit from GeneDireX, Inc. in Taoyuan, Taiwan. The isolated DNA was then used as a template for amplifying the alpha/beta hydrolase gene. The primers used were specifically chosen to have a high sequence similarity with alpha/beta hydrolase genes found in *S. werraensis* strains. The primers used were specifically: Primer used to initiate DNA synthesis in the forward direction alpha/beta-F (5′-ATGCCGACACGACGTACGA-3′) and reverse primer alpha/beta-R (5′-TCACTCCTTGCCCTGGAGC-3′), The deductions were made via the Primer3 programme. The PCR experiment was performed using 2 µL of DNA (50 ng) as the template, 2.5 µM of each primer, 10× PCR buffer, 2 U of Taq polymerase (Invitrogen), and 0.5 mM dNTPs in a 25 µL reaction volume. The alpha/beta hydrolase gene's DNA band, obtained from the agarose gel, was isolated using the FavorPrep GEL Purification kit (FAVORGEN, Biotech Corp., Ping Tung, Taiwan). This DNA was used as the template for polymerase chain reaction (PCR) amplification of the alpha/beta hydrolase gene. The PCR reaction included of 35 cycles, commencing with an initial denaturation at 95°C for 5 minutes, followed by denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute per kilobase, culminating in a final extension at 72°C for 3 minutes. The PCR results were evaluated on a 1% agarose gel, and the amplified gene was extracted using a Gel purification kit. The gel-eluted product was subjected to sequencing, and the gene sequence was verified using BLAST at NCBI. The PCR result was then used for cloning.

2.3. Bioinformatics analysis

The alpha/beta hydrolase protein sequence was derived from the experimentally determined alpha/beta hydrolase gene of *S. werraensis* KN23 using the ExPASY online translate tool. To assess similarity analysis of the alpha/beta hydrolase sequence, the Blastp program at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was utilized, with sequences chosen based on similarity percentage identity. For aligning multiple sequences, the Clustal Omega program was employed (https://www.ebi.ac.uk/Tools/msa/clustalo/). The construction of a phylogenetic tree was conducted using MEGA-X software (Mei *et al*., 2013; Eswar *et al*., 2006). Additionally, the secondary structure prediction of the alpha/beta hydrolase protein was carried out through the online PDBsum server, accessible at the PDBsum home page (ebi.ac.uk)/ thornton-srv/databases/pdbsum/ (Yang and Yang, 2015).

2.4. Structural homology modeling, validation and binding pockets prediction of alpha/beta hydrolase

The Robetta program (robetta.bakerlab.org) was used for an iterative homology modeling process. To create 3D models of novel proteins related to the alpha/beta hydrolase reference protein family, a modeling approach was employed (Yang and Yang, 2015). An optimised alpha/beta hydrolase homology model was constructed utilising the "DOPE profile" settings from the produced models (Banerjee *et al*., 2014). The best model was chosen based on its confidence level (C-score) and potential energy for further investigation (Sahi *et al*., 2012; Singh *et al*., 2013). The model underwent validation using the "Molprobity" online tool and evaluation through the "Ramachandran Plot Analysis" online tool (Kulkarni *et al*., 2014). Furthermore, a stereo picture depicting the surface groove structure of the alpha/beta hydrolase model was generated using the "PyMol" programme. (Gupta *et al*., 2017). The Site-Map module of the deepsite/playmolecule online programme was used to forecast binding

locations in the alpha/beta hydrolase protein and the protein-ligand beta keratin (Kesharwani and Misra, 2011). The binding site predictions took into account the physical attributes of amino acids, including their size, degree of enclosure or exposure, hydrophobic or hydrophilic nature, tightness, and ability to form hydrogen bonds (Banerjee *et al*., 2014).

2.5. Protein–protein docking studies

The 3D structure of alpha/beta hydrolase underwent processing using the MOE software, it included the extraction of water molecules, ions, and preexisting ligands. The receptor molecule was hydrogenated using PyMOL. The substrate, beta keratin, was created in pdb format using MOE and PyMOL. Beta keratin is also known as beta keratin and has the PubChem CID: 395651 and PF02422. The HDock online programme was used to conduct docking experiments, aiming to examine the binding mechanism between beta keratin and the alpha/beta hydrolase protein. The macromolecule file was stored in pdb format for the docking procedure (Gupta *et al*., 2017; Banerjee *et al*., 2014; Sahi *et al*., 2012).

2.6. Molecular cloning and expression of alpha/beta hydrolase encoding gene in *E. coli* host cells.

The amplified product alpha/beta hydrolase of *S. werraensis* KN23 Abd El-Aziz *et al*. (2023a) The insertion was performed by ligating the DNA into the appropriate locations of the pGEM® -T Easy cloning vector using a ligation cloning kit from Promega Co., located in Madison, WI, USA. To generate the recombinant plasmid, a ligation procedure using the T4 ligase enzyme is performed. The DNA that had been ligated (20 µl) was combined with 0.1 ml of recently generated competent cells, which had a transformation efficiency of 2.98 x 105 colony-forming units per microgram of DNA. The recombinant plasmid was introduced into *E. coli* DH5α cells by the heat shock technique, and the samples were then subjected to a 1-hour incubation at 37°C.Following incubation, the samples were spread onto LB agar plates supplemented with 0.5 mM IPTG, 50 µg/mL ampicillin, and 40 µg X-gal. The plates were then incubated at 37 °C overnight (Barman *et al*., 2014). Colonies of alpha/beta hydrolase were streaked once again on a suitable plate containing antibiotics and then placed in an incubator at a temperature of 37 °C for a duration of 16-20 hours. Extracellular expression of alpha/beta hydrolase activity was assessed in positive colonies at 37°C. A 50 mL culture of Luria-Bertani (LB) broth medium supplemented with ampicillin (50 µg/mL) was prepared by inoculating it with a single colony of *E. coli* DH5α that had been transformed with plasmid pGEM-T-alpha/beta hydrolase. The culture was then incubated at 37°C with shaking for 16 hours. The mature culture was subjected to centrifugation at a speed of 4000 revolutions per minute for a duration of 10 minutes at a temperature of 4 degrees Celsius. Next, the liquid portion above the sedimented cells was removed, and the solid cell material was used for extracting the modified DNA molecule using the QIA prep spin miniprep kit from Germany, following the instructions provided by the manufacturer (Mei *et al*., 2013; Maha, 2017). The colonies that underwent transformation and included the plasmid carrying the alpha/beta hydrolase gene (pGEM-T-alpha/beta hydrolase) were examined using colony PCR. This screening process involved the use of primers specific to the alpha/beta hydrolase gene and followed the same PCR conditions used for amplifying the alpha/beta hydrolase gene (Kesharwani and Misra, 2011; Froger and Hall, 2007; Gupta *et al*., 2017; Singh *et al*., 2012; Mei *et al*., 2013).

2.7. Preparation of keratin solution

The assessment of keratinolytic activity included the use of soluble keratin (0.5%, w/v) as the substrate, which was derived from white chicken feathers. The procedure described in the reference by Wawrzkiewicz *et al*. (1987) was followed to create the substrate. Feathers from indigenous chickens weighing 10 grammes were collected from several local poultry shops in Cairo, Egypt. These feathers were treated with dimethyl sulfoxide (DMSO), subjected to heat, and then precipitated using cold acetone. The resultant precipitate was rinsed, dehydrated, and dissolved in a solution of sodium hydroxide (NaOH). The pH was modified, and the solution was thinned with Tris-HCl buffer (pH 8.0) according to the technique specified by Cai *et al*. (2008).

2.8. Preparation of crude enzyme extract and enzyme assay

In order to initiate the production of keratinase, a selected strain was introduced to a fermentation medium containing 1% feather. The liquid obtained, which included the enzyme, was collected for a quantitative keratinase test. The efficacy of the enzyme was assessed by using a keratin solution as the substrate, employing the methodology described in the reference provided by Cai *et al*. (2008). More precisely, the diluted crude enzyme (1.0 ml) was combined with the keratin solution (1 ml) and subjected to incubation at a temperature of 50°C for a duration of 10 minutes. The reaction was terminated using trichloroacetic acid (TCA). After centrifugation, the absorbance of the liquid was quantified at 280 nm relative to a control sample. A single unit of keratinolytic activity was defined as a rise in corrected absorbance of 280 nm (A_{280}) at a rate of 0.01 per minute. The activity (U/ml) was determined using the formula: $U = 4\times n\times A_{280} / (0.01\times 10)$, where n is the dilution rate (Gradisar *et al*., 2005).

3. Results

3.1. Alpha/beta hydrolase encoding gene amplification from *S. werraensis* KN23

The primers specifically designed to target the alpha/beta hydrolase gene were able to effectively amplify the gene that encodes it, which was then subjected to sequencing. The obtained sequence was analysed using a BLAST search against the NCBI database, which identified an open reading frame consisting of 1596 base pairs. This reading frame corresponds to the expected length of the alpha/beta hydrolase gene in *S. werraensis* KN23. The genetic sequence encodes a protein composed of 532 amino acids. A comparative investigation revealed a 99% match between the alpha/beta hydrolase gene of *S. werraensis* KN23 and that of *S. werraensis* strain FS97. The sequence for the alpha/beta hydrolase gene in *S. werraensis* KN23 was submitted to the NCBI database and assigned the accession number GenBank OQ511280. Afterwards, the nucleotide sequence was converted into its matching amino acid sequence, which represents the protein under investigation. By comparing it to three other alpha/beta hydrolase sequences from the UniProt protein database and analysing it using the InterProScan service (EMBL), it was determined that amino acid residues 1 to 532 belong to the alpha/beta hydrolase family.

3.2. Multiple sequence alignment (MSA)

The examination of various sequence alignment revealed the presence of consensus areas in the alpha/beta hydrolase sequences derived from three distinct sources. Out of all the sequences, *S. werraensis* KN23 had the most resemblance to the alpha/beta hydrolase sequence of *S. werraensis* FS97, suggesting a close connection between them within the Streptomyces genus. Significantly, the research were centred on the active site of the alpha/beta hydrolase enzymes found in *S. werraensis* KN23 and *S. werraensis* FS97. These enzymes were shown to have ASP220, ASP90, ARG401, and LYS440 at certain locations, as seen in Figure 1.

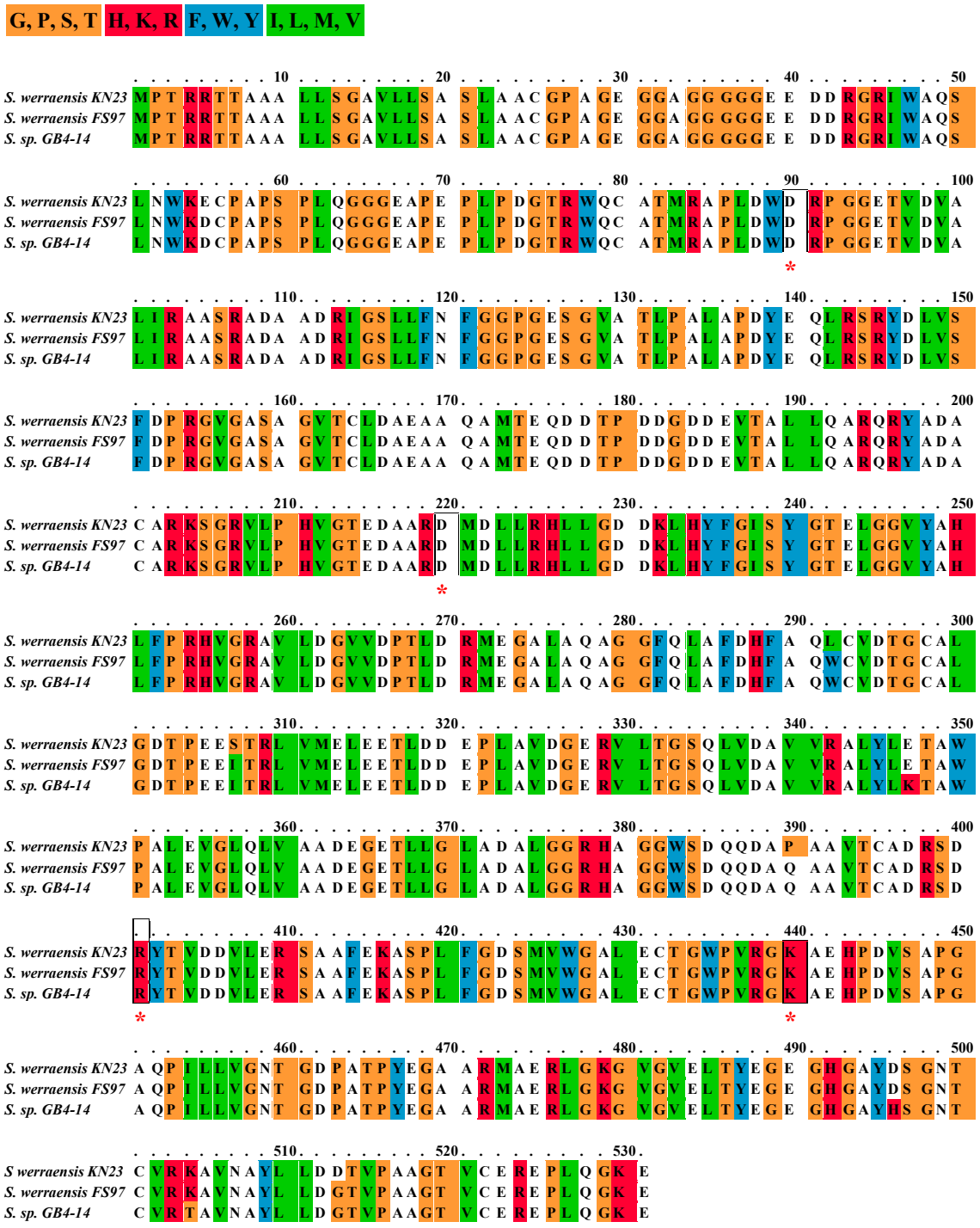


Fig 1: Multiple sequence alignment of amino acids in experimentally determined alpha/beta hydrolase sequences from the *streptomyces* family, with identification of active site residues marked by a red '*' symbol.

3.3. Cluster analysis (phylogenetic tree) of *S. werraensis* alpha/beta hydrolase sequence

A phylogenetic analysis was performed to ascertain the placement of the alpha/beta hydrolase protein within the larger framework of established alpha/beta hydrolase family members. For this investigation, a meticulously curated dataset consisting of 11 alpha/beta hydrolase proteins from different species was picked from the UniProt protein database. One of the proteins included in this group is the alpha/beta hydrolase from *S. werraensis* KN23. The comparative investigation showed a notable 99% sequence similarity between the alpha/beta hydrolase of *S. werraensis* KN23 and the homologous alpha/beta hydrolase proteins of *S. werraensis* FS97, both belonging to the Streptomyces genus. To create the phylogenetic tree, the Molecular Evolutionary Genetics Analysis (MEGAX) software was utilized, employing the alpha/beta hydrolase sequences from the 11 Streptomyces strains. The results unveiled the classification of Streptomyces genotypes into seven primary clusters. The results indicated that the template *S. werraensis* FS97 and S. *werraensis* KN23, closely related to alpha/beta hydrolase and in the same group. The details of The taxonomic connections between these strains are depicted in Figure 2.

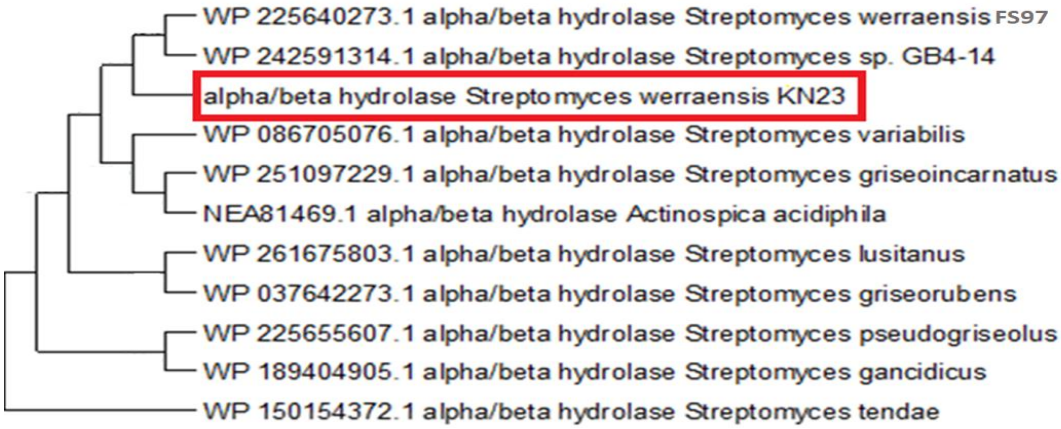
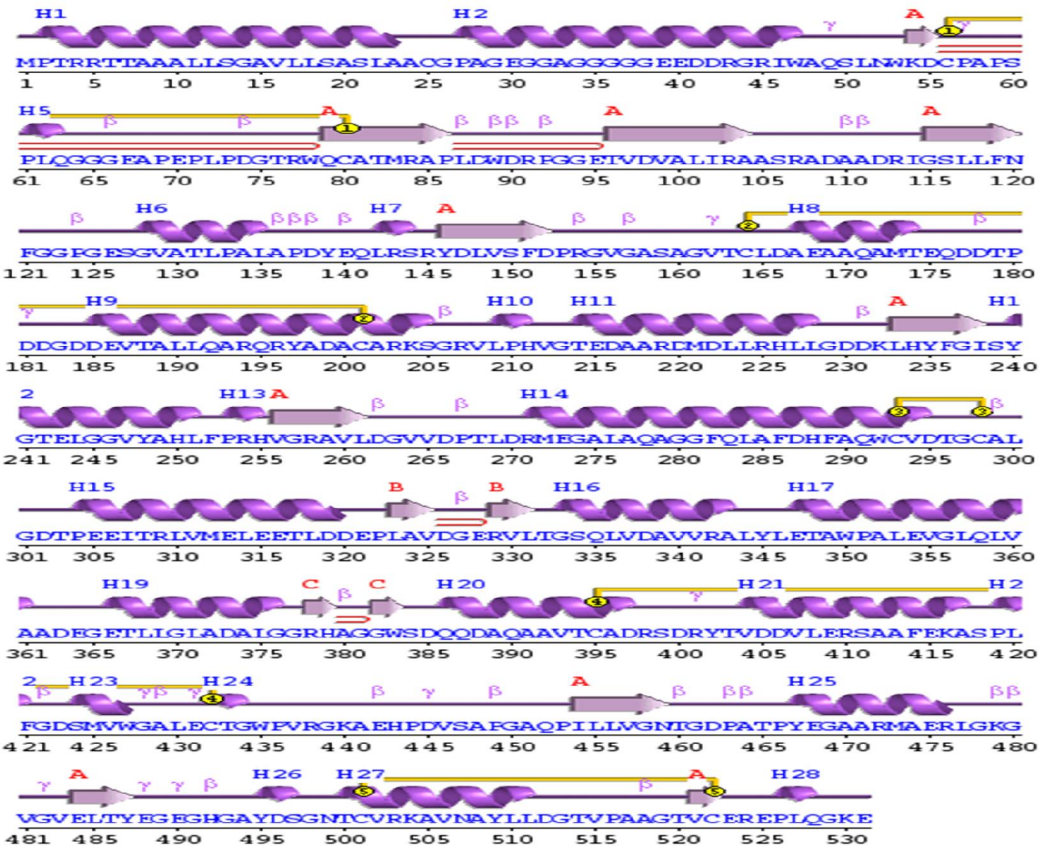


Fig 2: Phylogenetic Tree Construction for 11 *Streptomyces* Alpha/Beta Hydrolase Protein Sequences Utilizing the Neighbor-Joining Method in MEGAX Software.

3.4. Secondary structure prediction of alpha/beta hydrolase encoding gene.

The alignment of sequences and the prediction of the secondary structure for the alpha/beta hydrolase protein derived from the reference template *S. werraensis* FS97 and *S. werraensis* KN23 Experiments were performed with the PDBsum server. According to the characterization given by PDBsum, the predicted arrangement and secondary structure of the alpha/beta enzyme in S. werraensis FS97 is composed of 28 α-helices (H) and 34 β sheets (E). Similarly, it consists of 29 α-helices (H) and 34 β sheets (E) for *S. werraensis* KN23, as illustrated in Fig 3.

A



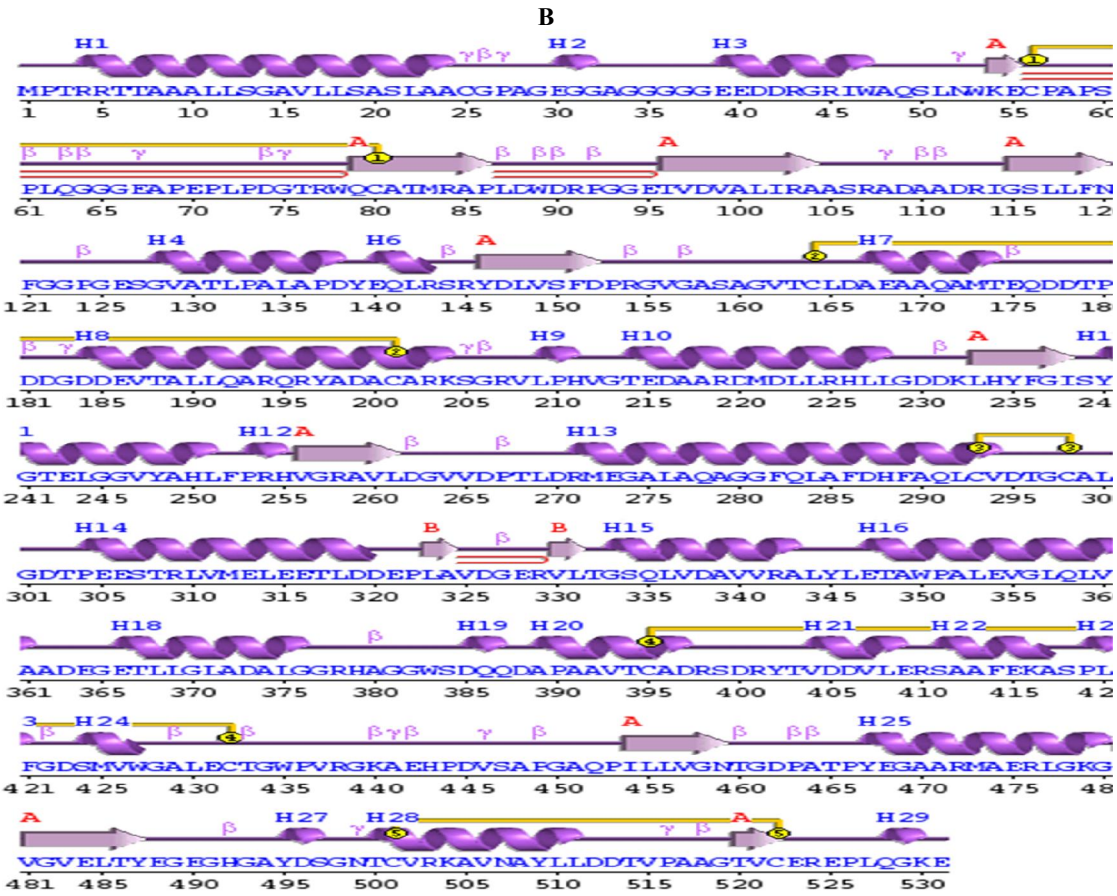


Fig. 3: Predicted secondary structure of alpha/beta hydrolase protein of; **A;** template *S. werraensis* strain FS79; **B;** strain *S. werraensis* strain KN23.

3.5. Homology modeling and validation of alpha/beta hydrolase encoding gene.

Utilizing the Robetta tool, five models were generated for the alpha/beta hydrolase protein of *S. werraensis* KN23, based on cluster density and using the template of the *S. werraensis* FS97 alpha/beta hydrolase with 532 residues. Model 1 of the *S. werraensis* FS97 template, exhibiting the highest Confidence score (C-score), Was chosen for further examination. A high C-score indicates a model with heightened confidence, and the estimated Template Modeling score (TM-score) for this model was 0.78, with an estimated Root Mean Square Deviation (RMSD) of 18.1±3.7Å. Similarly, Model 1 of the *S. werraensis* KN23 strain, Selected for further examination due to its highest C-score. The TM-score estimation for this model was 0.81, and the estimated RMSD was 13.4±5.4Å. These details are depicted in Figures (4a and 4b)**.**

The selected alpha/beta hydrolase structure model underwent energy minimization through the Swiss-Pdb Viewer's force fields via the YASARA Server. Subsequently, the model was evaluated using various stereochemical parameters provided by PROCHECK, VERIFY3D, and ERRAT tools on the SAVES server. Additionally, a Ramachandran plot was generated to assess the conformational quality of the energy-minimized model.

The Ramachandran plot, categorized into quadrants representing low energy, allowed, generously allowed, and disallowed regions, illustrated that 92.51% and 97.48% of residues in the 3D-modeled templates of *S. werraensis* FS97 and *S. werraensis* KN23 alpha/beta hydrolase proteins, respectively, resided in the most favored regions. This discovery confirms the stability and high quality of the Ramachandran plot for computational research. In the PROCHECK investigation of the template strain *S. werraensis* FS97 alpha/beta hydrolase protein, 91.7% of residues were found in the most

preferred areas of the Ramachandran plot, with 6.7% in other acceptable regions, 0.5% in moderately acceptable regions, and 1.2% in unacceptable regions. For the template strain *S. werraensis* KN23 alpha/beta hydrolase protein, the PROCHECK analysis revealed that 88.7% of residues were located in the most favourable regions of the Ramachandran plot. Additionally, 9.3% were found in other permissible regions, 1.4% in generously permissible regions, and 0.7% in regions that are not permissible. Further information and numerical data may be seen in Figure 5a and 5b.

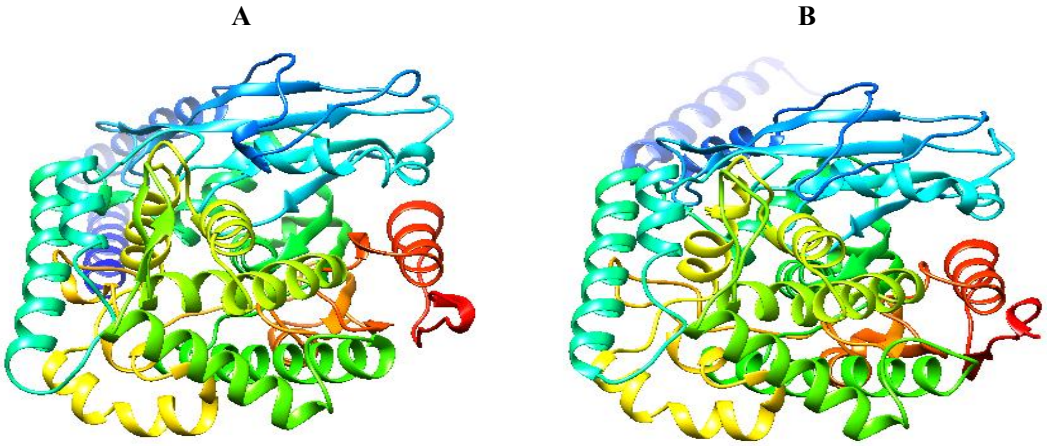


Fig. 4: Modeled 3D structure of; **A,** template *S. werraensis* FS97, **B,** strain *S. werraensis*.KN23 alpha/beta hydrolase protein.

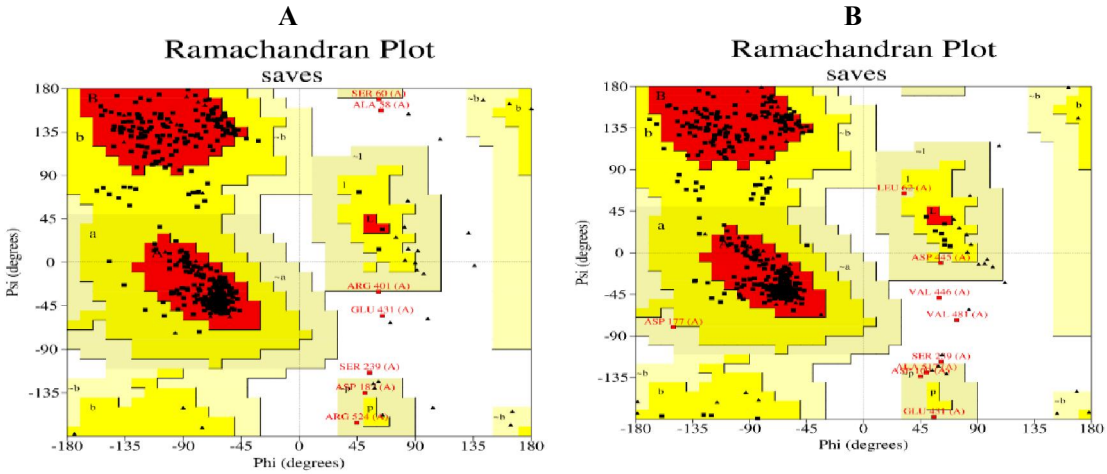


Fig 5: Ramachandran plot of; **A,** template *S. werraensis* FS97, **B,** strain *S. werraensis*.KN23 alpha/beta hydrolase protein.

3.6. Alpha/beta hydrolase binding pocket prediction

The playmolecule/deepsite server (http://playmolecule.com/deepsite/) was employed to detect the amino acids in the active site. The forecasted active sites of the alpha/beta hydrolase in the template *S. werraensis* FS97 showed that the active site, scoring 1.00, comprised one amino acid, ASP220 and ASP90, positioned at the receptor center. Similarly, the active site forecast for the beta keratin ligand identified one amino acid, PRO30 and GLN28, at the center, with score of 0.951 for receptor and ligand. For the alpha/beta hydrolase in the strain *S. werraensis* KN23, the active site prediction resulted in a score of 1.090, including one amino acid, ARG401 and LYS440, at the center. Furthermore, the active site prediction for the beta keratin ligand revealed one amino acid, PHE67 and PRO39, at the active site center, with score of 0.993 for receptor and ligand.

3.7. Docking and molecular interaction studies

Docking tests were undertaken using the HDock online programme to explore the interaction between the substrate keratin and the 3D model of the alpha/beta hydrolase protein. The receptor and ligand files were converted to pdb format for the docking operation. Polar hydrogen atoms were allocated Gasteiger charges, whereas nonpolar hydrogen atoms were combined with carbons. Modifications were made to internal degrees of freedom and torsions as required.

The results of the docking research between beta keratin and the 3D model of alpha/beta hydrolase demonstrated unique patterns of interaction. The template *S. werraensis* FS97 alpha/beta hydrolase exhibited an affinity score of -259.20 kcal/mol, a Confidence score of 0.8988, Ligand RSMD (Å) of 46.41, and active site amino acids including receptor ASP220 and ligand PRO30 with an interface residue(s) score of 0.951. In addition, the active site amino acids of the receptor, namely ASP90, and the ligand, specifically GLN28, were determined. The interface residue(s) score was found to be 2.123, as shown in Figure 6a.

The alpha/beta hydrolase of *S. werraensis* KN23 exhibited an interaction with an affinity score of -227.70 kcal/mol, a Confidence score of 0.8255, Ligand RSMD (Å) of 81.33, and active site amino acids involving receptor ARG401 and ligand PHE67 with an interface residue(s) of 1.644. Furthermore, the active site amino acids of receptor LYS440 and ligand PRO39, as well as an interface residue(s) with a distance of 1.809, were discovered and shown in Figure 6b.

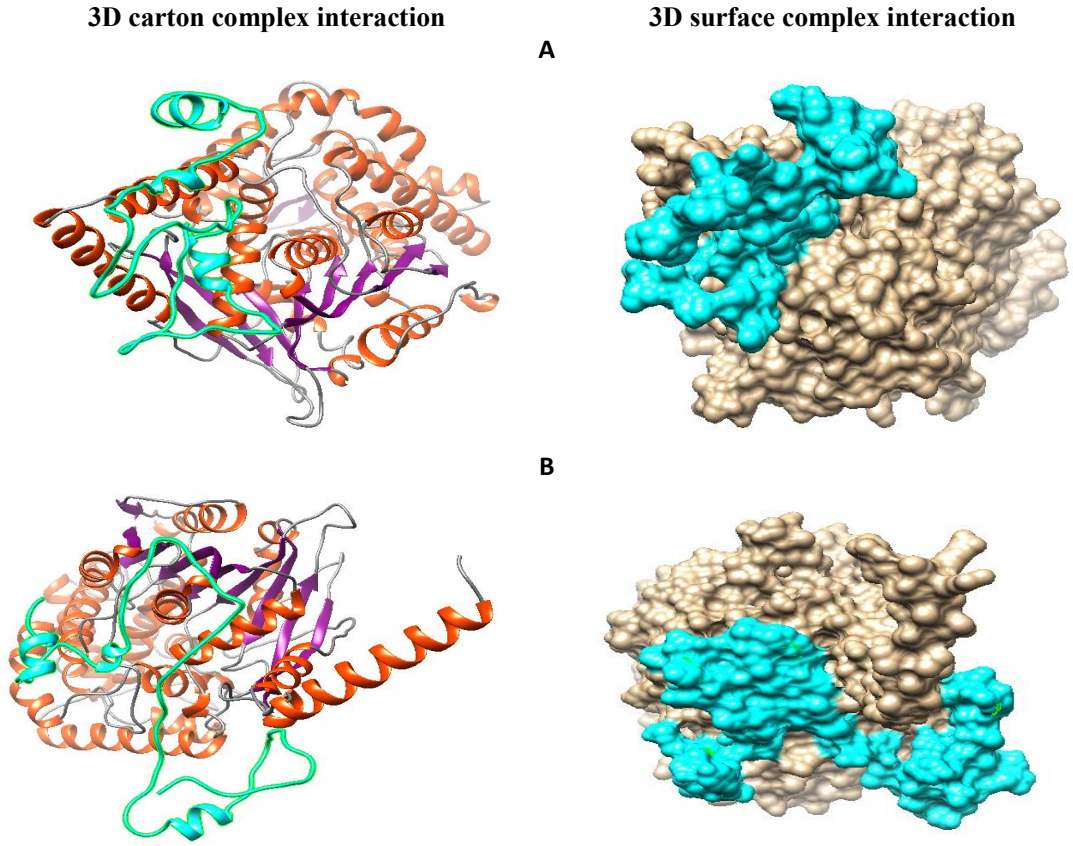


Fig 6: HDock is used to visually represent the protein-protein docking chimaera. It shows the interactions between the 3D cartoon and 3D surface complexes of two strains: Template *S. werraensis* Strain FS97 and *S. werraensis* Strain KN23. The illustration depicts the molecular complex interaction between beta keratin (in cyan) and the amino acids within the 3D model of the alpha/beta protein receptor.

3.8. Cloning and expression of alpha/beta hydrolase encoding gene.

The gene responsible for encoding alpha/beta hydrolase, spanning 1596 bp in length from *S. werraensis* KN23 (GenBank: OQ511280), The amplification of the gene was efficiently enhanced utilising primers specific to the gene. A gel electrophoresis analysis was performed to evaluate the PCR results, resulting in the detection of a 1.596 kilobase product derived from genomic DNA. The findings demonstrated that an annealing temperature of 55°C provided the most favourable conditions for amplifying the desired 1596 bp DNA fragment. Afterwards, the DNA band (1596 bp) that had been purified from the agarose gel using the Qiagen Gel Purification kit, was joined to the pGEM®-T Easy Vector using a ligation cloning kit. The resulting recombinant plasmid was named pGEM-T-alpha/beta. The process of heat-shock treatment was used to introduce the recombinant plasmid into *E. coli* DH5α, which served as the host for gene expression. The transformed cells were then chosen based on their ability to resist ampicillin and using the white/blue screening approach, which involves the use of IPTG and X-gal. The *E. coli* strain underwent a successful transformation, resulting in the production of a wide range of transformants. Plasmids were extracted from *E. coli* transformants selected at random and then analysed using agarose gel electrophoresis. This examination confirmed the presence of plasmid DNA bands in all of the tested *E. coli* transformants. The presence of distinct DNA bands in each transformant indicated the presence of diverse types of plasmid DNA, such as supercoiled, open circular, or linear plasmids. Following that, plasmids from four *E. coli* DH5α (pGEM-T-alpha/beta) transformants were examined using PCR with alpha/beta hydrolase gene-specific primers, using the same PCR conditions as those used for amplifying the alpha/beta hydrolase gene. Additional analysis was conducted using agarose gel electrophoresis.

3.9. Alpha/beta hydrolase expression

The evaluation of alpha/beta hydrolase activity was performed using a fermentation medium that included 1% feather as the only carbon source. The experimental setup consisted of *E. coli* recipient strains, the donor strain *S. werraensis* KN23, and four *E. coli* strains with the alpha/beta hydrolase plasmid (*E. coli* (pGEM-T-alpha/beta)). The cultures were subjected to incubation at a temperature of 37°C while being shaken at a speed of 120 rpm for a period of up to 4 days. Each culture was sampled, and the resulting liquid after centrifugation, known as the crude enzyme, was used for the alpha/beta hydrolase activity test, following a previously published process. The findings indicated that the donor strain *S. werraensis* KN23 had alpha/beta hydrolase activity, but the recipient strains of *E. coli* did not demonstrate this activity. On the other hand, the *E. coli* cultures that obtained the alpha/beta hydrolase plasmid (*E. coli* (pGEM-T-alpha/beta)) exhibited alpha/beta hydrolase functionality. The results validated the biological functionality of the cloned alpha/beta hydrolase gene. The alpha/beta hydrolase activity was consistently present for the whole 4-day incubation period with feather in both strains of *E. coli* recombinant. The peak activity of around 93.38±23.12 U/mL was seen on day 3 for E. coli DH5α (pGEM-T-alpha/beta), exceeding the activity of the wild-type strain *S. werraensis* KN23, which had an activity of 51.60 U/mL, as reported by Abd El-Aziz *et al*. (2023a).

4. Discussion

In a preceding investigation by Abd El-Aziz *et al*. (2023a), The strain KN23 of *S. werraensis*, a kind of actinomycetes, was obtained from several farms in Egypt. The researchers effectively amplified and sequenced the alpha/beta hydrolase gene from *S. werraensis* KN23 using primers they specifically developed. The BLAST analysis identified an open reading frame (ORF) consisting of 1596 base pairs (bp), which codes for a protein with 532 amino acids. The findings showcased that the alpha/beta hydrolase from *S. werraensis* KN23 shared a 99% similarity with that of *S. werraensis* FS97.

Multiple sequence alignment analysis has disclosed variations in the active site location of the alpha/beta hydrolase in *S. werraensis* strain KN23. To delve into the evolutionary connections, a phylogenetic tree was crafted utilizing MEGAX software, encompassing alpha/beta hydrolase sequences from eleven *Streptomyces* strains. The findings from the analysis revealed that *Streptomyces* genotypes could be categorized into seven primary clusters. It was indicated that *S. werraensis* strain

KN23 is closely related to the template *S. werraensis* FS97, both tightly associated with alpha/beta hydrolase. The use of protein 3D structure prediction tools is crucial for modelling protein sequences that lack structural knowledge. It facilitates the investigation of interactions between proteins and ligands, offering significant understanding of the connections between protein sequence, dynamics, structure, and function (Xu and Zhang, 2013).

In their study, Gupta *et al*. (2017) conducted a multiple sequence alignment using the experimentally identified *ker* enzyme and other subtilisins from the Bacillus family. The PDB codes for these enzymes are given. In a similar manner, Nahar *et al*., (2016) presented a comparative analysis of the amino acid sequence alignment of the mature protein (274 aa) in *B. licheniformis* strains MZK-05, with a focus on the extremely comparable KerA and Subtilisin Carlsberg proteins present in other strains. SC is an abbreviation for Subtilisin Carlsberg. The KerA protein from *B. licheniformis* MZK-05 exhibited significant variations, including the substitution of Phe26 with Tyr26, and the replacement of Ser86 with Asn86 and Asn211 with Ser211, in comparison to other sequences.

Peng *et al*. (2021) Demonstrated a correlation between the pro-peptide sequences of keratinases obtained from various sources. For the recombinant keratinase KerZ1, eight sequences linked to keratinases that are both highly expressed and active were compared. This comparison was done by a procedure called multiple sequence alignment of pro-peptides, which resulted in a range of 16% to 66%.

In their research, Rahimnahal *et al*. (2023) performed a protein sequence alignment of the keratinase *Bacillus licheniformis* KRLr1 with many closely similar proteins. In a similar manner, Almahasheer *et al*. (2022) conducted an extensive examination of several amino acid sequences of the KerS gene. The investigation was conducted using peptidase sequences from the Bacillus cereus group's S8 family, which were retrieved from the GenBank database. Moreover, Abdel-Naby *et al*. (2020) Performed a comprehensive investigation of the amino acid sequences of *B. cereus*. The results showed that there were 19 amino acid changes that increased the activity of the protease by roughly 31.17% compared to the original form. Significantly, nine of these amino acid changes were shown to greatly enhance the enzyme's catalytic performance.

In the investigation carried out by Abd El-Aziz *et al*. (2023b) In order to examine the connections between these strains, a phylogenetic tree was constructed using MEGAX software. This investigation used sequences from eight strains of *Pichia kudriavzevii* metalloprotease proteins. The findings demonstrated that *P. kudriavzevii* YK46 showed the most similarity to the reference *P. kudriavzevi*i 129, indicating a strong correlation with metalloprotease. Furthermore, Rahimnahal *et al*. (2023) performed a circular phylogenetic tree analysis on amino acid sequences and found that KRLr1 and comparable sequences had the most similarity with *Bacillus licheniformis* keratinase, which belongs to the serine peptidase/subtilisin-like S8 family. Subsequently, a neighbor-joining (NJ) tree without a root was created using the alignment of protein sequences that showed significant similarities.

In the process of designing the structure of an enzyme, a crucial step involves understanding its three-dimensional (3D) configuration. To accomplish this, homology modeling was undertaken by utilizing X-ray structural coordinate files, aiming to construct the 3D structure of the alpha/beta hydrolase enzyme produced by *S. werraensis* KN23. It is worth noting that, currently, there are no reported crystal structures of the enzyme from *S. werraensis* KN23. Nevertheless, the structure of the alpha/beta hydrolase from *S. werraensis* KN23 demonstrated homology with the alpha/beta hydrolase from *S. werraensis* FS97. In situations where protein sequences lack structural details, protein 3D structure prediction software, such as Robetta, proves value for reliably modeling their structures. This programme enables the research of the relationship between protein sequence, structure, dynamics, and function. It also allows for the study of protein interactions with other molecules or ligands. Therefore, Robetta, serving as a homology modeling tool, was employed to generate five models for the alpha/beta hydrolase proteins of both template *S. werraensis* FS97 and strain *S. werraensis* KN23. The model displaying the highest C-score, indicative of heightened confidence, was chosen for subsequent analysis (Zheng *et al*., 2021; Yang and Yang, 2015).

Tertiary structure modeling for the protein was conducted by Patni *et al*. (2021) using I-TASSER. The predictions resulted in a tertiary structure characterised by a C score of -0.68, a TM-score of 0.63 ± 0.14, and an RMSD of 8.4 ± 4.5 Å. A TM-score more than 0.5 implies a viable topological model, but a TM-score lower than 0.17 shows random similarity. In addition, the researchers conducted structural validation by carefully examining the refined I-TASSER structure, which was obtained using the Galaxy refine tool and the SAVES v5.0 server. Upon inspection of the PROCHECK Ramachandran

plot, it was found that 82.7% of protein residues were situated in the most preferred region, while 13.0% and 1.7% of residues were positioned in the permissible and very permissible regions, respectively. The forbidden area had just 2.7% of residues, indicating that the modelled protein maintains its structural integrity.

In the investigation carried out by Rahimnahal *et al*. (2023), The Bacillus subtilis keratinase KRLr1 PDB structure was modelled using MODELLER V.9, with the 3WHI PDB structure used as the template. Subsequently, the KRLr1 PDB structure was refined using the ModRefine server, and its validation was performed using the PROSA and PROCHECK servers. The Verify-3D scores and ERRAT quality factors were 58 and 93.06%, respectively. The Ramachandran graph findings showed that, after refinement, 90% of the amino acids were located in the favoured zone, 8% in the permitted region, and 2% in the outlier region. This discovery indicates that the arrangement of amino acids in KRLr1 is well aligned at the phi (φ) and psi (ψ) angles. The validation results confirm the precise representation of KRLr1.

Furthermore, the work by Almahasheer *et al*. (2022) presented findings on the validation of the keratinase's 3D structure model through the utilization of a Ramachandran plot. The results indicated that 91.98% of the structure fell within the favored region, while 1.43% resided in the disallowed region. Notably, the amino acids located outside the favored region encompassed A324 SER, A46 GLN, A160 VAL, A137 ASP, and A114 PRO.

The quality of the generated models was evaluated using various assessments, including the Ramachandran plot. The analysis from the plot indicated that a majority of the 532 residues in both the models of template S. *werraensis* FS97 and strain S. *werraensis* KN23 were situated in the most favored region. Specifically, these residues accounted for 92.51% and 97.48% of the total residues in their respective models. These findings underscore the reliability and stability of the Ramachandran plot, along with the overall effectiveness of ERRAT, VERIFY3D, and PROSA models in facilitating in silico studies (Sahi *et al*., 2012).

The identification of active sites within both *S. werraensis* FS97 and *S. werraensis* KN23 templates was predicted to reveal a singular active site in each, centered around the amino acids ASP220, ASP90, ARG401 and LYS440 (Kesharwani and Misra, 2011). Molecular docking is a commonly used technique to study the interactions between drugs and receptors, as well as to assess the strength of the bond between small molecules and target proteins (Karumuri *et al*., 2015), uncovered favorable binding affinities in alpha/beta hydrolase with beta keratin. Specifically, the affinities were -259.20 kcal/mol for the *S. werraensis* FS97 template and -227.70 kcal/mol for the *S. werraensis* KN23 strain (Gupta *et al*., 2017; Banerjee *et al*., 2014). Docking studies have also played a crucial role in enhancing the catalytic effectiveness of keratinases derived from *Bacillus licheniformis* and *Stenotrophomonas sp.* (Banerjee *et al*., 2014; Fang *et al*., 2016). In a distinct investigation by Gupta *et al*. (2017), The process of expressing the cloned keratinase gene from Bacillus subtilis RSE163 was carried out, and the subsequent protein's binding affinities were measured using in-silico methods. The keratinase gene that was expressed showed a significant increase in activity, and the accuracy of the modelled structure was confirmed using the Ramachandran plot. In addition, by using the extra precision (XP) technique of Glide, docking studies revealed the most favourable binding affinities between psoriasis medications (such as Acitretin, Clobetasol propionate, Fluticasone, Desonide, Anthralin, Calcipotreine, and Mometasone) and the target proteins. Yet another investigation made by Banerjee *et al*. (2014). The study used molecular docking techniques using phenylmethylsulfonyl fluoride (PMSF) to predict the active site of Bacillus licheniformis alkaline serine protease. This enzyme had a sequence that was 100% identical to the chosen *Bacillus* genus sequence structure. Out of the 10 docking sites that were found, two were chosen as the active sites for the keratinase enzyme from the *Bacillus* species based on predictions.

Abd EL-Aziz *et al*. (2023c) study employed molecular docking methods revealed high interactions affinities for three active sites for *Rhodotorula mucilaginosa* PY18 endo-PG1 and mutant *Rhodotorula mucilaginosa* E54 endo-PG1.

Peng *et al*. (2020), successfully amplified the keratinase gene from the strain *Bacillus* licheniformis BBE11-1. Moreover, the keratinase gene was amplified from *Bacillus licheniformis* KRLr1 using a process called amplification. (Rahimnahal *et al*., 2023), the generation of a 1050 base pair (bp) fragment was followed by its assignment in the NCBI database under the accession number MT482301.1. As shown by Almahasheer *et al*. (2022) the researchers amplified the Keratinase (KerS)

gene from different wild-type isolates (S1, S13, S15, S26, and S39) and their corresponding mutants (S1ems, S13uv, S13uv + ems, S15ems, S26uv, and S39ems). The comparison of these genes with the keratinase, thermitase alkaline serine protease, and thermophilic serine protease from the *B. cereus* group showed a high level of similarity, ranging from 95.5% to 100%. Furthermore, Wang *et al*. (2019) recorded the effective amplification of a 1170 base pair kerT1 gene derived from the feather-degrading *Thermoactinomyces sp*. strain YT06.

4. Conclusion

This study highlights the significance of alpha/beta hydrolases as a diverse and widespread group of enzymes present in various life forms. The investigation focused on the alpha/beta hydrolase gene from *S. werraensis* KN23, revealing its detailed genetic composition and encoding a protein with 3D structural characteristics validated through Ramachandran's plot. The docking studies underscored the enzyme's optimal binding affinities with beta keratin, providing insights into its potential for keratin degradation. Notably, the expression of the *S. werraensis* KN23 alpha/beta hydrolase gene in *E. coli* DH5α resulted in a substantial increase in keratinase activity, emphasizing its applicability in industrial processes involving the degradation of keratinous substrates. This comprehensive analysis contributes valuable knowledge about the alpha/beta hydrolase from *S. werraensis* KN23, offering perspectives for potential biotechnological applications.

Supplementary Materials

All the supplementary materials of this study are available within the article.

Author Contributions

The conception and design of the study, execution of experimental procedures, and engagement in data analysis, statistical analysis, and data visualization were collaborative efforts among all authors, with equal contributions. Additionally, active participation from each author was observed in the writing, revision, and editing of various sections of the article. It is important to note that all authors have meticulously reviewed and endorsed the final version of the manuscript.

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Data Availability Statement:

The identified *S. werraensis* KN23 sequences have been submitted and can be found in the NCBI database with the accession numbers OK086273. Additionally, the alpha/beta hydrolase gene that was sequenced are also available in the NCBI database with the accession number OQ511280. All other relevant data that support the results and conclusions of this study are provided within the article itself.

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Declarations

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