Biological Forum – An International Journal **15(5): 07-16(2023)**

ISSN No. (Print): 0975-1130
ISSN No. (Online): 2249-3239

Degradation of Keratin by Keratinase Enzyme Producing *Bacillus zhangzhouensis* using Insilico Protocols

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(Received: 17 February 2023; Revised: 16 March 2023; Accepted: 20 April 2023; Published: 20 May 2023)
(Published by Research Trend)

ABSTRACT: The management of keratinous waste generated from different industries is becoming a major challenge across the world, especially in poultry farming. Therefore, there is a need for modern and eco-friendly approaches to disposing of waste products. Due to the environmental challenges of poultry farming, it is necessary to isolate microbial keratinase enzymes with high catalytic functions and identify its mechanisms. Therefore, in the present study, our goal was to identify the potential of keratinase producing isolates that degrade feathers containing keratin through docking studies. The protein structure was predicted using the 16srRNA gene sequence of *Bacillus zhangzhouensis,* and the keratin structure was also predicted with the Insilico method. Then, protein-protein docking studies are performed using an automated protein docking server and their 3D molecular binding interactions are discussed in detail. The overall results clearly show that the amino acids of the enzyme, keratinase efficiently bind at the active sites (motifs) of keratin. Despite these challenges, keratinase enzymes have been successfully used to reduce the levels of keratin in soil, making them a viable option for remediating the environment.

Keywords: 16sr RNA sequence, *Bacillus zhangzhouensis*, Keratin, Protein –Protein Docking.

INTRODUCTION

Keratinases are proteolytic enzymes that are capable of degrading the insoluble and complex protein keratin. Keratinases are complex enzymes consisting of disulfide reductase and polypeptide hydrolase. Keratinase activity involves three steps: denaturation, hydrolysis and transamination. In the first step, disulfide reductase changes the strongly bound structure into its degenerative state by reducing the disulfide link in keratin from cystine (-S-S-) to cysteine (-SH). Next, polypeptide hydrolyase hydrolyzes keratin into polypeptides, oligopeptides, and free amino acids. Finally, there is the transamination process, in which keratin is completely hydrolyzed into free amino acids and sulphides (Fitriyanto *et al.,* 2022). Keratin degradation in microorganisms depends on two protein enzymes cooperation. Shen *et al.* (2022) reported that the keratinase activity of *B. tropicus* Gxun-17 involves the two above enzymes, which will decrease the time of the fermentation process and increase the feather degradation rate. Keratinases are produced by various microorganisms, such as bacteria, fungi, and actinomycetes, which live in environments rich in keratin, such as soil, sediments, and waste materials.

Keratinases are grouped into serine proteases, metallo proteases or serine metallo proteases based on the nature of the active site. Serine proteases are functionally rich with nucleophilic serine residues (Ser) present in the active site of the enzyme; later they attacks the carbonyl part of the peptide bond and form an acyl enzyme intermediate complex. Metalloproteases have a divalent metallic ion, nucleophilic attack on the peptide bond, but they are transmitted through water molecules that coordinate with metal ions. (Zn or Co). A previous study by Gurunathan *et al.* (2021) indicated that keratinolytic proteases from diverse microbial strains are predominantly serine- and metalloproteases. The enzyme has a unique structure and active site that allow it to efficiently hydrolyze keratin. Overall, the evidence suggests that keratinase enzymes have great potential in various fields due to their ability to efficiently degrade keratin and their unique structure and function. In a study by Gupta *et al.* (2017) crystal structure of a keratinase from a feather degrading bacterium was determined. Based on accumulated research, several mechanisms of enzymes have been proposed (Kornilłowicz-Kowalska and Bohacz 2011; Li *et al.,* 2018). The challenge of breaking down keratin is due to the high content of disulfide bonds (Kornillowicz-Kowalska and Bohacz 2011). Since most keratinases are proteases responsible for breaking peptide bonds. There is a previous study that could use bioinformation to understand the mechanism of this breakdown (Song *et al.,* 2018; Pinski *et al.,* 2020). Therefore, enzymes have attracted increasing interest due to their potential applications in various fields such as agriculture, bioremediation, and industrial processes.

Molecular docking methods can be used to model the interactions between molecules and proteins at the

atomic level, which allows us to characterize the behaviour of molecules at the binding sites of target proteins and elucidate fundamental biochemical processes. Several studies have reported on the potential of homology or comparative modelling as well as de novo 3D structure and modelling Insilico for predicting evolutionary relationships using deduced amino acid sequences (Dalton and Jackson 2007). Recently Padmavathi *et al.* (2022) determined the interaction between protein and protein through Insilico analysis. They determined the substrate recognition pattern, and the development of a suitable protease can potentially enhance its applications in keratin degradation. Another study (Kandasamy *et al.,* 2017) also predicted that 3D model of protease from *Bacillus* sp. was generated by using the homology modelling approach. Amal Mahmoud *et al*. (2021) reported that *Bacillus subtilis* AKD9 high alkaline serine protease, has high binding affinities towards substrate casein.

However, molecular docking studies on the role of keratinase enzymes in keratin degradation are not familiar; therefore, the mechanisms of the keratinase enzyme are discussed here through a molecular docking assay. In the present study, an attempt has been made to understand the binding efficiency of the model protease from *Bacillus* sp. and different substrates by molecular docking studies. The proteolytic activity was revealed by using various amino acids, namely, glycine, leucine, valine, arginine, tryptophan and various proteins of keratin, which were utilized, namely, Case in kinase II phosphorylation site, N-myristoylation site, Protein kinase C phosphorylation.

METHODOLOGY

Sequence Selection Method 1. In this study, specific protein sequences were converted from 16srRNA sequence of *Bacillus zhangzhouensis*, which was obtained through the Insilico online tool. (https://biomodel.uah.es/en/lab/cybertory/analysis/trans.htm). The microbial isolation work was done at the Department of Biotechnology, Muthayammal College of Arts and Science, Rasipuram, Tamilnadu, India.16sr RNA sequence of *Bacillus zhangzhouensis* was done at Chromopark life science, Namakkal Tamilnadu, India.

Sequence Selection Method 2. The activities of Swiss-Prot, TrEMBL and PIR protein database have joined together to establish the Universal Protein Knowledgebase (UniProt) consortium in order to deliver a single, centralized, authoritative resource to the scientific community for protein sequences and functional information. The objective was to provide a protein sequence knowledgebase with extensive cross-references and query interfaces that was comprehensive, accurately classified, and richly annotated. (Rolf Apweiler *et al.,* 2004). The sequence of the keratin protein found in chicken feathers (KRFC_CHICK: P02450) was retrieved in FASTA format from the UniProt database (https://www.uniprot.org/).

The computation of various physicochemical properties is done by ProtParam (Gasteiger *et al.,* 2005) which can be deduced from a protein sequence. No extra information is needed about the concerned protein. The protein can either be mentioned as a Swiss-Prot/TrEMBL accession number or ID, or in the form of a raw sequence. ProtParamcomputes parameters such as the molecular weight, amino acid composition, theoretical pI, extinction coefficient, atomic composition, instability index, estimated half-life, aliphatic index and grand average of hydropathicity (GRAVY). The calculation of the molecular weight and theoretical pI is performed as in Compute pI/Mw. The primary sequence was analysed using the ProtParam tool (https://web.expasy.org/protparam/) in order to understand the properties of keratin.

PROSITE (Sigrist *et al.,* 2012) describes protein domains, families, and functional sites along with related patterns and profiles to identify them. It is complemented by ProRule, a collection of rules that augments the discriminatory power of these profiles and patterns by providing supplementary information about functionally and/or structurally critical amino acids. PROSITE signatures are used together with ProRule for the annotation of domains and are a feature of UniProtKB/Swiss-Protentries. The analysis of the keratin protein motif was also carried out using the ScanProSitetool(https://prosite.expasy.org/scanprosite/).

3D Structure Prediction and visualization. The automated comparative modelling of three-dimensional (3D) protein structures is performed using the SWISS-MODEL (Schwede *et al.,* 2003) server. In 1993 a pioneer effort was made in the field of automated modeling and today, it is the most commonly used free web-based automated modelling facility. SWISS-MODEL furnishes multiple levels of user interaction by means of its World Wide Web interface: in the 'first approach mode' only the amino acid sequence of a protein is submitted to construct a 3D model. Complete automation of template selection, alignment and model building is performed by the server. In 'alignment mode', the modelling process pertains to a user-defined target-template alignment. SWISS MODEL (https://swissmodel.expasy.org/), an automated protein modelling server, was used to transform the protein sequence into a three-dimensional structure. In order to investigate the molecular drug docking studies, an advanced molecular visualization tool known as Discovery Studio was used to visualize the modelled 3D structure of *Bacillus zhangzhouensis, p*roducing keratinase and chicken feather keratin protein.

3D protein –protein docking. The HDOCK server (Yan *et al.,* 2020) is an advanced platform to perform template-based modelling, homology search, macromolecular docking, structure prediction, biological information incorporation and job management for effective and fast protein–protein docking. With the arrangement of information data for receptor and ligand particles (either amino corrosive successions or Protein Information Bank structures), the server naturally predicts their cooperation through a mixture calculation of layout based and format free docking. We use the HDOCK server (http://hdock.phys.hust.edu.cn/) to perform protein

docking studies. Here, Keratin is and keratinase ware docked and the 3D results were visualized using Discovery Studio software based on the docking scores obtained. Molecular dynamics studies such as H-bond interactions were also performed.

RESULTS AND DISCUSSION

Keratinase production was significantly high in *Bacillus zhangzhouensis* under invitro conditions. For Insilico analysis, 16s rRNA gene sequence of *Bacillus zhangzhouensis* was converted to protein (keratinase) sequence through a computerized transcription and translation tool, which converts sequences from DNA to RNA and to protein (Fig. 1). The feather keratin1 or keratin gene C protein sequence was retrieved from the UniProt database (Fig. 2).

Various functional domains/motifs (Fig. 3) with corresponding amino acid positions present in the feather keratin protein (*Chicken*). Enzymes bind to substrate and form conformational changes, which expose multiple sites for hydrolytic action (Brandelli *et al.,* 2010). Keratinases are serine and metalloproteases with conserved residues forming their active sites. The crystal structures of various keratinases reveal the structural basis for their activity and can be used to develop more stable and efficient enzymes for industrial use (Betzel *et al.,* 2001). These structures can also be used to generate homology models for other keratinases. Keratin is a valuable source of carbon, nitrogen, and sulphur that can be transformed into a variety of compounds in nature (Wang *et al.,* 2015). Because of their ability to breakdown keratins, keratinases have a wide range of industrial and biotechnological applications (Gegeckas, 2018). Using the single letter code of the protein sequence, it is converted to the amino acid sequence of protein (keratinase) through ProtParam tool computation, Various physical and chemical parameters of the protein (keratinase) sequence ware retrieved from Swiss - Prot or TrEMBL (Fig. 4). It provides the protein molecular weight, theoretical pI, amino acid composition, of the protein (keratinase) molecule. Previous studies reported the best method for three dimensional structure prediction by protein homology based sequences and protein folds using physical chemistry (Piyusha Sharma *et al.,* 2022).

The various functional domains/motifs (Fig. 5) present in the feather keratin protein of poultry using the ScanProSite tool. Scan Prosite tool (De Castro *et al.,* 2006) was used to analyse the protein keratin sequence in order to pinpoint the many functional motifs that were present. The functional motifs found in keratin are displayed based on the overall findings of protein profiling studies: Certain sections of the motifs are crucial in post-docking investigations.

Molecular Mechanism of Protein –Protein Docking. Elucidates that in the 3D structure of the 16sr RNA sequence of *Bacillus zhangzhouensis,* keratinase contains 2 alpha helices (shown in red colour), turns (represented in green colour) and a coiled-coil region (represented in white colour) Fig. 6. The antigenic binding sites are usually present in the coiled-coil regions.

Based on the research, several mechanisms have been postulated (Korniłłowicz-Kowalska and Bohacz 2011). The presence of a large concentration of disulfide bonds makes keratin breakdown difficult. Various research studies also successfully proved protein-protein docking. These researches coincide with the results obtained in various wet lab studies (Remmert *et al.,* 2011; Zashumo *et al.,* 2023). Because most keratinases are proteases that break peptide bonds, other enzymes or chemicals are required to influence disulfide bonds and lower keratin packing forces, allowing proteases to access proteins (Kasperova *et al.,* 2013). HDOCK server used for Protein-Protein docking studies. This result (Table 1, Fig. 7) clearly shows the molecular mechanism explaining how keratin feathers is degraded by the keratinase enzyme by binding to each other. For Insilico studies, this is significant evidence. Keratinase of Bacillus sp serves as the receptor, and our modelled protein target, keratin feather, serves as the ligand. The HDOCK server's docking results (Yan *et al.*, 2020) describe the docking scores based on the -184.68 kcal/mol intermolecular binding affinities.

Four potential binding sites in Keratin protein, namely, Casein kinase II phosphorylation site, N-myristoylation site, Protein kinase C phosphorylation site, and Amidation site (Table 2). It's interesting to note that keratinase binds at amino acid positions (motifs) 66, 87, and 91 at the Phosphosereine and N-myristoylation sites of keratin. The following gives details about the functional part of keratin: 66-71 (GSILSE) and 87-92 (GSRFSG), N-myristoylation site (PS00008), 91-93 (SGR)

Protein kinase C phosphorylation site (PS00005), 91-94 (SGRR) **Amidation** (PS00009). All the results show that keratinase plays a vital role in the breakdown of Keratin. Similar results were reported for the keratinase enzyme from the bacteria *Bacillus flexus* isolated poultry soil sample (Padmavathi *et al.,* 2022). Casein kinase II (CK-2) is a protein serine/threonine kinase that is not affected by cyclic nucleotides or calcium and CK-2 phosphorylates a wide range of proteins (Lim *et al.,* 2015). A significant majority of eukaryotic proteins are acylated via an amide bond by the covalent addition of myristoylated (a C14-saturated fatty acid) to their N-terminal residue (Bonissone *et al.,* 2013). The sequence specificity of the enzyme responsible for this modification, myristoylCoA: protein N-myristoyltransferase (NMT), has been derived from the sequences of known N-myristoylated proteins and experiments utilising synthetic peptides.

In vivo, protein kinase C preferentially phosphorylates serine or threonine residues present near a C-terminal basic residue (Borgo *et al.,* 2021). The presence of extra basic residues at the N- or C-terminus of the target amino acid increases the phosphorylation reaction's Vmax and Km. The precursor of C-terminally amidated hormones and other active peptides is always directly followed. Wang *et al.* (2021) by a glycine residue that provides the amide group and more frequently than not, by at least two consecutive basic residues (Arg or Lys)

that serve as an active peptide precursor cleavage site. Fig. 8-10 shows the docking results of the H-bond interactions between the amino acids of keratin and keratinase using Discovery Studio Software tool. Figs. 11-13 clearly depict that the Keratinase (16s RNA) of *Bacillus sp* binds with keratin protein at various potential binding sites (keratin GLY: 66– LEU: 63 keratinases, keratin GLY: 62 –ARG: 79 keratinases, keratin VAL: 87-TRP: 84 keratinases, keratin ARG: 91-GLN: 88 keratinases).

>16sr RNA –*Bacillus zhangzhouensis* strain
ACGGAGCAACGCCGCTTGAGTGATGAAAGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAG
TGCGAGAGTAACTGCTCGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAG
CCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTC
TTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAAACTTGAGTGCA
GAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCG
AAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATA
CCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAG
CTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGG
GCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACA
TCCTCTG
>16sr RNA –*Bacillus zhangzhouensis* strain - Protein
TEQRRLSDESFRIVKLCCGRTSARVTARTLTVPNQKATANYVPAAAVIRRWQALSGIIGR
KGLAGGFLSLMKPPAQPGRVIGNWETVQKRRVEFHVRNARCGGTPVAKATLWSVTDAEER
KRGERTGLDTLVVHAVNDECVLGGFRPLVLQLTHALRLGSTVARLKLKGIDGGPHKRWSM
WFNSKQREEPYQVLTSS

Fig. 1. 16sr RNA sequence of *Bacillus zhangzhouensis* strain.

The above picture shows the 16sr RNA sequence of *Bacillus zhangzhouensis* and its corresponding amino acid sequence.

>sp|P02450|KRFC_CHICK Feather keratin 1 OS=*chicken* OX=9031 PE=3 SV=2
MSCFDLCRPCGPTPLANSCNEPCVRQCQDSRVVIQPSPVVVTLPGPILSSFPQNTAAGSS
TSAAVGSILSEEGVPISSGGFGISGLGSRFSGRRCLPC

Fig. 2. FASTA sequence of Keratin.

The above is the UniProt FASTA sequence format of the Feather Keratin of *chicken*

KRFC_CHICK (P02450)

Feather keratin 1 (F-ker) (Keratin gene C protein)
Chicken (Chicken)
The computation has been carried out on the complete sequence (**98** amino acids).

Number of amino acids: 98

Molecular weight: 9972.40

Theoretical pI: 7.50

Amino acid composition:

Ala (A)	**5**	**5.1%**
Arg (R)	**6**	**6.1%**
Asn (N)	**3**	**3.1%**
Asp (D)	**2**	**2.0%**
Cys (C)	**8**	**8.2%**
Gln (Q)	**4**	**4.1%**

Glu (E)	3	3.1%
Gly (G)	11	11.2%
His (H)	0	0.0%
Ile (I)	5	5.1%
Leu (L)	7	7.1%
Lys (K)	0	0.0%
Met (M)	1	1.0%
Phe (F)	4	4.1%
Pro (P)	11	11.2%
Ser (S)	16	16.3%
Thr (T)	4	4.1%
Trp (W)	0	0.0%
Tyr (Y)	0	0.0%
Val (V)	8	8.2%
Pyl (O)	0	0.0%
Sec (U)	0	0.0%
(B)	0	0.0%
(Z)	0	0.0%
(X)	0	0.0%

Total number of negatively charged residues (Asp + Glu): 5
Total number of positively charged residues (Arg + Lys): 6

Atomic composition:

Carbon	C	424
Hydrogen	H	687
Nitrogen	N	123
Oxygen	O	136
Sulfur	S	9

Formula: $C_{424}H_{687}N_{123}O_{136}S_9$
Total number of atoms: 1379

Fig. 3. Primary sequence analysis - Keratin (*Chicken).*
The above picture shows the properties of amino acids present in Keratin protein

>16sr RNA –*Bacillus zhangzhouensis* strain - Protein

Number of amino acids: 197
Molecular weight: 21854.26
Theoretical pI: 10.88

Amino acid composition:

Ala (A)	17	8.6%
Arg (R)	22	11.2%
Asn (N)	6	3.0%
Asp (D)	5	2.5%
Cys (C)	4	2.0%
Gln (Q)	8	4.1%
Glu (E)	10	5.1%
Gly (G)	18	9.1%
His (H)	4	2.0%
Ile (I)	6	3.0%
Leu (L)	19	9.6%
Lys (K)	11	5.6%
Met (M)	2	1.0%
Phe (F)	5	2.5%
Pro (P)	9	4.6%
Ser (S)	11	5.6%
Thr (T)	15	7.6%
Trp (W)	5	2.5%
Tyr (Y)	2	1.0%
Val (V)	18	9.1%
Pyl (O)	0	0.0%
Sec (U)	0	0.0%
(B)	0	0.0%
(Z)	0	0.0%
(X)	0	0.0%

Total number of negatively charged residues (Asp + Glu): 15
Total number of positively charged residues (Arg + Lys): 33

Atomic composition:

Carbon	C	961
Hydrogen	H	1571
Nitrogen	N	301
Oxygen	O	270
Sulfur	S	6

Formula: $C_{961}H_{1571}N_{301}O_{270}S_6$
Total number of atoms: 3109

Fig. 4. Primary sequence analysis -16sr RNA –*Bacillus zhangzhouensis* strain – Protein.
The above picture shows the properties of amino acids present in 16sr RNA of *Bacillus zhangzhouensis* strain

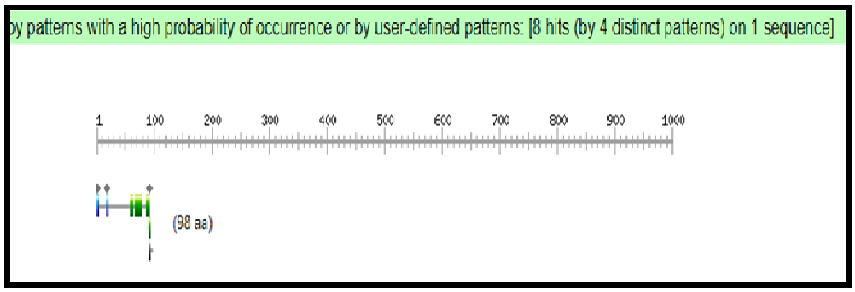

Fig. 5. Protein motif prediction- Keratin (*Chicken*).

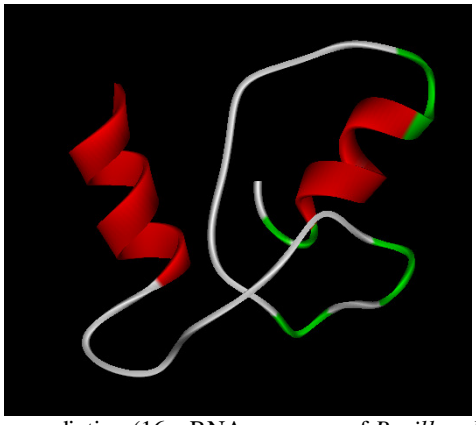

Fig. 6. Protein 3D structure prediction (16sr RNA sequence of *Bacillus zhangzhouensis*_Kertinase.

The 3D structure of the Keratinase enzyme from the *Bacillus zhangzhouensis* strain as seen through Discovery Studio Software is depicted in the image above.

Table 1: Molecular Protein-Protein Docking.

Chicken Feather keratin	**(16sr RNA *Bacillus zhangzhouensis* strain)**
Protein -Keratin (P02450)	**Enzyme - Keratinase**
Keratin	-184.68 kcal/mol

The above table indicates the molecular protein –protein binding affinities score between keratin –Keratinase complex (HDOCK server)

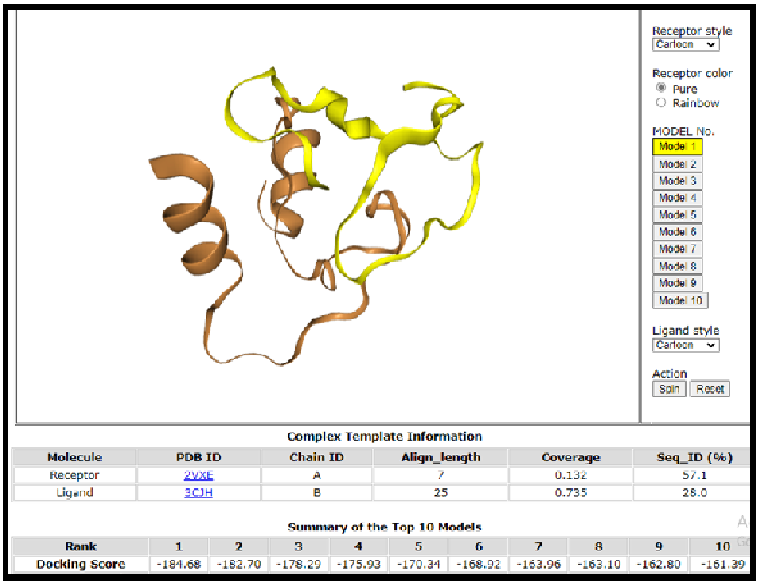

Fig. 7. Protein 3D structure prediction (16sr RNA sequence of *Bacillus zhangzhouensis* Keratinase)

Ribbon model –keratinase

H-bond interaction: Keratin – Keratinase enzyme. Molecular acceptor and donor atoms interaction (Discovery Studio Software)

Table 2: Scan Prosite Tool Results: Keratin.

Casein kinase II phosphorylation site (PS00006) Phosphosereine	N-myristoylation site (PS00008) N-myristoylation site	Protein kinase C phosphorylation site (PS00005) Phosphosereine	Amidation(PS00009) Amidation site
2-5 (SCFD)	58-68 (GSSTSA)	91-93 (SGR)	91-94 (SGRR)
18-21(SCNE)	66-71 (GSILSE)		
	73-78 (GVPISS)		
	87-92 (GSRFSG)		

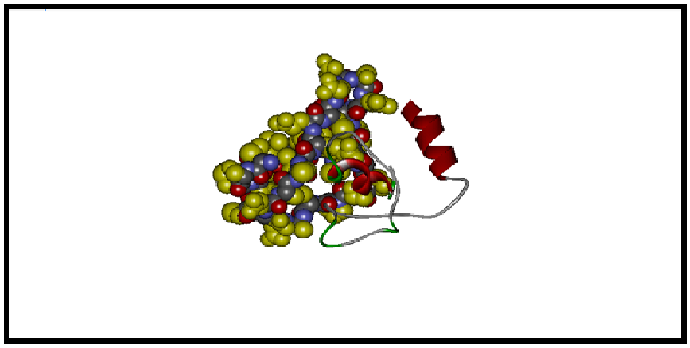

Fig. 8. Protein –Protein Docking (Keratin -Keratinase) Side Chain -Interaction

The above picture shows the complex docking pose of Keratin with Keratinase represented in surface model and ribbon model respectively. It shows the hydrophilic and hydrophobic interaction between Keratin and Keratinase.

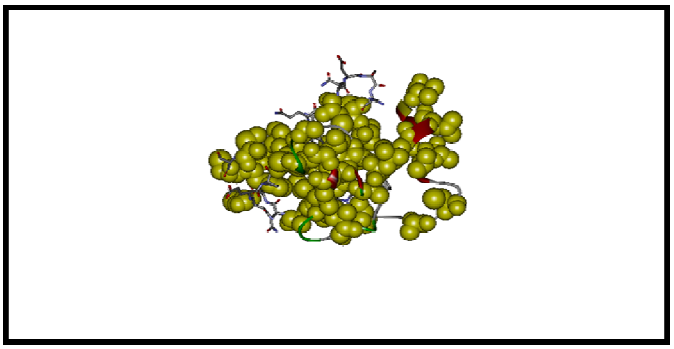

Fig. 9. Protein –Protein Docking (Keratin -Keratinase) Hydrophobic interactions.

The above picture shows the complex docking pose of Keratin with Keratinase represented in surface model and ribbon model respectively. It shows the hydrophobic interaction between Keratin and Keratinase.

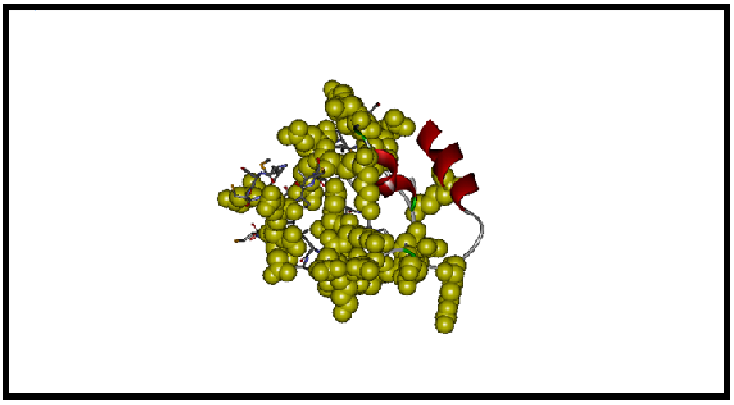

Fig. 10. Protein –Protein Docking (Keratin -Keratinase) Hydrophilic interactions.

The above picture shows the complex docking pose of Keratin with Keratinase represented in surface model and ribbon model respectively. It shows the hydrophilic interaction between Keratin and Keratinase.

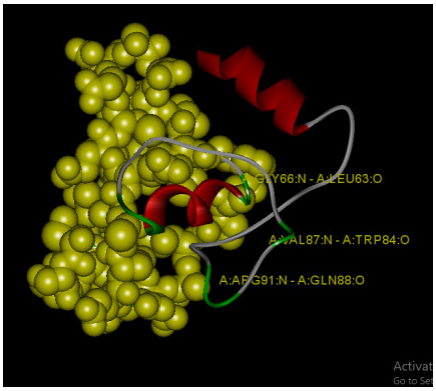

Fig. 11. Protein –Protein Docking (Keratin -Keratinase) H-Bond Interaction 1.

The above image shows Keratinase in ribbon model and Keratin in space fill model which are bound to each other due to the electrostatic interaction between them.

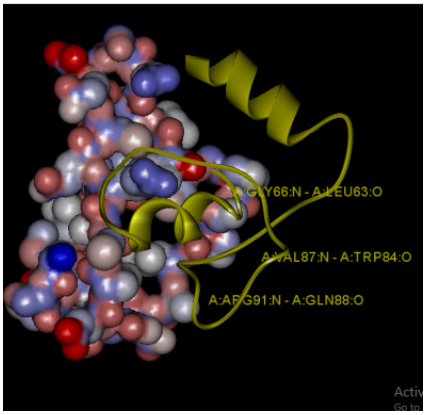

Fig. 12. Protein –Protein Docking (Keratin -Keratinase) H-Bond Interaction 2.

The surface model of keratinase and the spacefill model of keratin are depicted above, along with the corresponding binding amino acids that are bound to one other by electrostatic contact.

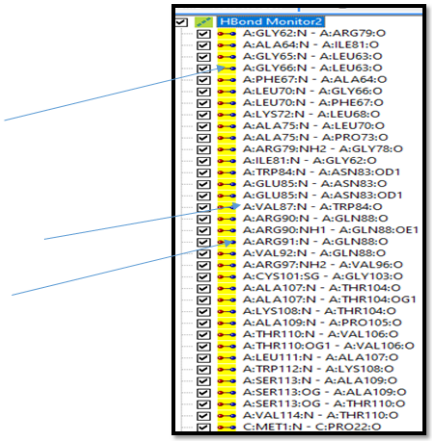

Fig. 13. Protein –Protein Docking (Keratin -Keratinase) Molecular Dynamics: H-Bond interactions.

H-bond interaction between Keratin and Keratinase enzyme. Molecular acceptor and donor atoms interaction shown using Discovery Studio Software(GLY66:N-A;LEU63:O,Val87:N-A:TRP84;Oand A:ARG91:N-A;GLV88:O)

CONCLUSIONS

Keratin is abundantly found in nature, and the volume of keratin-rich waste keeps increasing each year. Keratinase plays an important role in the recycling of keratin and is hence used in different fields. Our protein-protein docking studies revealed that the keratinase enzyme of *Bacillus zhangzhouensis* binds well with the feather keratin protein of *chicken* and aids in the breakdown of disulfide bonds present in keratin. These Insilico results provide evidence that the enzyme of the bacterial sps is eligible for the degradation of keratin.

FUTURE SCOPE

This work can be extended to other species which would be beneficial in learning about environmental science.

Acknowledgement. The authors acknowledge the help extended by Dr. Balaji Munivelan, Ph.D., CEO and Senior Bioinformatician, ABS Geno-informatics, Chennai, for his contribution towards *Insilico drug docking studies.*
Conflict of Interest. None.

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How to cite this article: Rajee T. and Selvamaleeswaran P. (2023). Degradation of Keratin by Keratinase Enzyme Producing *Bacillus zhangzhouensis* using Insilico Protocols. *Biological Forum – An International Journal, 15*(5): 07-16.