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Full Length Research Article

Impact of Halophilic Isolates Producing Keratinase on Reaping of Biofilms Producing Isolates

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Abstract

The emergence of multidrug resistance (MDR) is a major challenge in the fight against infectious diseases. In this context, there is an urgent need to develop advanced enzyme-based formulations that can effectively combat biofilm formation in public health contexts. The present study was focus the isolation of halophilic bacterial isolates from salt pan soil samples in around Thanjavur district, Tamil Nadu, and India, and screening the keratinase enzyme producing isolates. Among the 24 isolates, two isolates were up to tolerate the 20% of salt media, which isolates as strongly halophilic. The S9 isolate was shows potential isolate, which exhibits the 35U/ml of enzyme. Among the 7 genus of clinical isolates, *Enterococcus* was highly inhibited with keratinase which exhibiting the zone of inhibition was ranged from 12 to 18mm and followed by *Klebsiella sp.* The best reduction was achieved at the highest enzyme concentrations 8U/ml, at which the effect on *Enterococcus sp*, with 56% of biofilms inhibition. The results of the antimicrobial activity against biofilm-forming isolates suggest that this enzyme could be a potential agent for the control of biofilms-forming pathogens.

***Key words*:** Halophilic bacteria, biofilms, Keratinase, Antimicrobial activity, Antibiofilm activity

Multidrug resistance (MDR) is a phenomenon in which a microorganism (such as bacteria, fungi, viruses, and parasites) develops resistance to multiple drugs, often being resistant to multiple classes of drugs. This is a major problem in the treatment of infectious diseases, as the same organism can often cause different diseases and respond differently to different drugs. MDR is a major cause of treatment failure and the emergence of drug-resistant strains of organisms, leading to increased morbidity and mortality [1]. Multidrug-resistant bacterial infections are an increasing threat in India. The spread of these infections is driven by the overuse of antibiotics, poor sanitation, and high-density populations [2]. Multidrug-resistant bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant Enterococcus (VRE), and extended-spectrum beta-lactamase-producing Enterobacteriaceae (ESBL-E) are becoming more common in world. These bacteria are resistant to multiple antibiotics and can cause serious, life-threatening infections [3].

The emergence of drug resistance is a major challenge in the fight against infectious diseases. As bacteria and other pathogens evolve, they become resistant to the drugs that are used to treat them, rendering many of the life-saving treatments less effective. This clearly highlights the urgent need for new and improved antibacterial drugs with novel targeting and novel molecular architecture agents to avoid cross-resistance [4]. Antimicrobial enzymes are widely present in nature and are essential in protecting living things from bacterial attack. These enzymes are currently being used against microbial systems more frequently. They are all capable of attacking the microbe directly; preventing the biofilm from forming, destroying the biofilm, and/or catalysing reactions that result in the generation of antimicrobial compounds [5].

Keratinolytic proteases are incrementally gaining traction in industrial processes as an alternative to the classic chemical agents currently used. Keratinase are utilized to various applications including feed production, organic fertilizer production, detergent formulation, leather production, cosmetics, as well as in medicine and nanotechnology [6]. Keratin derived bioactive peptides in which the keratinases have a wide range of activities like antimicrobial [7], antihypertensive [8], anti-inflammatory [9], antioxidants, inhibition of early-stage amyloid aggregation [10], antidiabetic [11] or anti-ageing [12] depending on its required applications. The biocatalytic force accelerates towards the green economy helping in preventing and treating AMR.

Screening new sources of novel enzymes with industrial applications is key research in enzyme biotechnology. For application in industrial processes, enzymes should remain stable in the presence of high temperature, pH, salts, solvents, toxins, etc. Against this background, halophiles have become a huge mine of new enzymes in recent years. Halobacterium-derived enzymes possess unique structural features and

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catalytic capabilities to maintain metabolic and physiological processes under high-salt conditions. Some of these enzymes have been reported to be active and stable under more than one extreme condition. The above point of view, the present of study was focus the isolation of halophilic bacterial isolates from Thanjavur District, Tamil Nadu, and India, and screening the keratinase enzyme producing isolates. Furthermore, current study investigates how to overcome these drug resistance problems by using an eco-friendly, bio-synthesized, affordable and easily available halobacteria producing keratinase enzyme.

MATERIALS AND METHODS

Soil sample collection

Soil samples were collected from various regions of salt pans, located in Thanjavur District, Tamil Nadu, and India. The soil samples were collected from the depth of 10-12 inch in the sterile screw-capped container and stored at 4 °C, to ensure minimal biological activity. The samples were processed within 48 hours of collection. The samples were subjected to identify the salinity.

Isolation and screening of salt tolerant bacterial isolates

The collected samples were serially diluted and spread over the nutrient agar containing various concentration of NaCl (5%, 10%, 15%, 20% and 25%) and the plates were incubated at 37 °C for 48 hours. the potential isolates were selected from highest percentage of salt tolerating isolates, which has called halophilic bacteria. The pure cultures were stowed on agar plates containing 25% NaCl at 4 °C until desired [13].

Screening of bacteria for protease and keratinase activity

Proteolytic screening of bacterial isolates was done on modified nutrient agar plates containing peptone (5g/l), sodium chloride (50g/l) yeast extract (5/l), Skim milk powder (10g/l), agar (15g/l). The plates were spot inoculated with bacterial isolates and incubated at 37 °C for 2 days. A clear zone of skimmed milk around the colony indicated protease production by the isolates [13]. Potential isolates were also screened for keratinase production. All the isolates were spot inoculated in modified Horikoshi agar medium (pH – 7.5) contained soluble starch (5g/l), glucose (5 g/L), peptone (5 g/L), Glucose(5 g/L), K_2HPO_4 (1 g/L), Mg_2SO_4 7H2O (0.2 g/L),Na_2CO_3 (1 g/L), NaCl (50 g/L), Yeast Extract (5 g/L), Feather powder (10 g/L) and agar (15g/L),the plates were incubated at 37°C for 7 days. A clear zone found around the colony indicates the keratinase production. Potential isolates were isolated and subjected to further investigation [14].

Enzyme assays

Keratinolytic activity was measured with soluble keratin (0.5%, w/v) as substrate. Soluble keratin was prepared from chicken feathers by the method of [15]. One unit (U/ml) of keratinolytic activity was defined as an increase of corrected absorbance of 280 nm (A 280) (the modified method by Gradisar *et al*. [16] with the control for 0.01 per minute under the conditions described above and calculated by the following equation:

$$U = 4 \times n \times A280 / (0.01 \times T)$$

Where n is the dilution rate; 4 is the final reaction volume (ml); T is the incubation time (min).

Protein estimation

Lowry's Method was used for the estimation of protein in the sample. A standard quantitative assay for determining the protein content in a solution was used. BSA was used as a reference for protein assay.

Collection of clinical isolates

A total of 7 genera of bacterial isolates were procured from Microteck clinical laboratory, Coimbatore. The source of the isolates collection data and patient location were submitted by clinical laboratory. Primary identification was done on the basis of colony and cell morphology and Gram staining [17]. Representative colonies that appeared on plates were checked for purity through microscopy and pure isolates were streaked on NA slants and stored at 4 °C for further process. All bacterial isolates were maintained on nutrient agar slants at temperature of 4 °C. Antibacterial activities were assayed using the agar diffusion method described by Collins and Lyne [18].

Determination of antibacterial activity of keratinase

The Mueller-Hinton agar plates were inoculated with freshly prepared overnight inoculums, which were swabbed over the entire surface of the medium. Inoculums were of 10^8 CFU/ml of bacteria. The 6mm diameter of the well was made with sterile stainless-steel borer on the agar plates. The different concentrations of keratinase were filled in wells with the help of micropipette and one well filled with PBS only. The 100µl of 10 mg/ml of the standard antibiotic - Ampicillin was used as positive control. The Petri plates were kept for incubation at 37 °C for 24 hours. After incubation, the microbial growth was determined by measurement of zone of inhibition around each well and recorded in millimetres (mm). According to Teh *et al*. [19] procedure, MIC was carryout in titter well plate method.

Antibiofilm activity with keratinase

A 96-well microtiter plate (flat bottom, polystyrene) was used to determine the antibiofilm activity of the keratinases with procedure of Gurunathan *et al*. [20], Barapatre *et al*. [21] with modification. The percentage of inhibition of biofilm formation was calculated using following equation:

$$\% \text{ Biofilm inhibition} = \frac{1 - [\text{OD620 of cells treated with enzyme}]}{\text{OD620 of the non-treated control}} \times 100$$

RESULTS AND DISCUSSION

Antibiotics have long been considered one of the most important inventions of the 20th century. Unfortunately, the age of antibiotics has corresponded with the emergence of the phenomenon of antimicrobial resistance (AMR), the natural process by which microorganisms become resistant to the actions of drugs. In this context, there is a great need to identify new potential antibacterial targets and/or identify new chemical entities as antibacterial drugs. Among the many potential approaches to treat antibiotic resistance to date, the use of antibiotic substance was against non-essential bacterial targets.

Antimicrobial enzymes are abundant in nature and are being used more frequently to fight microbial systems. Together, they have the power to directly combat bacteria, prevent the development of biofilms, dislodge existing biofilms, and/or trigger processes that result in the creation of antimicrobial compounds. There is an urgent need to develop advanced enzyme-based formulations that can effectively combat biofilm formation in manufacturing, environmental protection, and public health contexts. Proteases are proteolytic enzymes, a group of enzymes that differ in structure, target substrate, reaction mechanism, and many physicochemical properties [22]. Among the protease, keratinase is an important

enzyme in industrial sectors, playing an important role in various biological processes. According to the nature of their active site, keratinases belong to serine- and metalloproteases or serine metalloproteases [23]. Indarmawan *et al.* [24] observed the antimicrobial activity producing protease enzyme from fungal isolates of *Xylaria psidii* KT30 against bacteria.

Enzymes that break down proteins possess a wide range of structural, functional, and mechanistic characteristics, all powered by one of the five common chemical processes. Ser proteinases activate the primary hydroxyl of an active site Ser side chain, commonly through Ser-His-Asp triad or Ser-Lys dyad relays, to increase its nucleophilicity and position it for attack on a peptide amide bond. The resulting covalent enzyme complex is then cleaved by hydrolysis. Gongora *et al*. [25] describe the mechanisms of protease enzyme on microbes, including anti-virulence mechanisms include cell wall disruption or membrane pore formation initiated by protease inhibitors to deregulate ion flow and/or membrane disruption to cause leakage of internal cellular components, affecting cell viability. Factors that need to be taken into account while producing the enzyme are the check the source of the isolates, productivity, stability and resistance to unwanted substances. Among those things are in halophilic isolates. Halophytic isolates are increasingly being recognized as a potential source of enzymes for industrial applications. The main advantages of halophytic isolates are their tolerant to extreme environmental conditions, such as high pH, heat, and salinity, and their ability to produce enzymes with high specific activity and stability [26-27]. Additionally, halophytic isolates have been reported to have high resistance to inhibitors and detergents, making them a desirable source of enzymes for applications in detergent and cleaning products [28]. The use of halophilic isolates for enzyme production also has the potential to reduce the risk of introducing non-native species into natural ecosystems [29].

The above point of view, halophilic isolates were isolated from salt pan soil samples in around Thanjavur district. Among the 24 isolates, two isolates were up to tolerate the 20% of salt media, which isolates as strongly halophilic. In India, number of authors have been reported that halophilic micro flora from natural hyper saline habitats (Table 1). Gupta *et al*. [30] observed the various concentrations of salt tolerant isolates were observed. In 2018, Singh and Singh *et al*. [31] found bacteria that could grow up to 35% salt concentration, which were observed in salt pan soil samples. Yadav *et al*. [32] found the halophilic isolates from salt pan and which were tolerate up t0 20% of salt.

Table 1 Screening of salt tolerance isolates from salt pan

Isolates name	Salt concentration									
	2.5%	5%	7.5%	10%	12.5%	15%	17.5%	20%	22.5	25%
1.	+	+	-	-	-	-	-	-	-	-
2.	+	+	+	+	+	+	+	-	-	-
3.	+	+	+	+	-	-	-	-	-	-
4.	+	+	+	+	+	+	-	-	-	-
5.	+	+	+	+	-	-	-	-	-	-
6.	+	+	+	-	-	-	-	-	-	-
7.	+	+	+	+	+	+	-	-	-	-
8.	+	+	+	+	+	+		-	-	-
9.	+	+	-	-	-	-	-	-	-	-
10.	+	+	+	-	-	-	-	-	-	-
11.	+	+	+	+	+	-	-	-	-	-
12.	+	+	+	+	+	+	+	+	-	-
13.	+	+	+	+	+	+	-	-	-	-
14.	+	+	+	-	-	-	-	-	-	-
15.	+	+	+	+	+	-	-	-	-	-
16.	+	+	+	-	-	-	-	-	-	-
17.	+	+	+	+	+	-	-	-	-	-
18.	+	+	+	+	+	-	-	-	-	-
19.	+	+	+	+	+	+	+	+	-	-
20.	+	+	+	+	+	-		-	-	-
21.	+	+	+	-	-	-	-	-	-	-
22.	+	+	+	+	-	-	-	-	-	-
23.	+	+	+	+	-	-	-	-	-	-
24.	+	+	+	+	+	+	+	-	-	-
25.	+	+	-	-	-	-	-	-	-	-
26.	+	+	+	+	+	+	-	-	-	-
27.	+	+	+	+	-	-	-	-	-	-
28.	+	+	+	+	+	+	+	-	-	-

+Growth; -:Non growth

In recent years there has been a great deal of interest in in various extracellular enzymes and other substances produced by halophilic microorganisms Such as DNases, proteases, lipases, pullulanase, potential uses of keratinase in industrial applications. These halophilic enzymes are not only resistant high salt concentration but tolerant and works efficiently over a range of pH and even those resistant to high temperatures. Arokiyaraj *et al*. [33] found that *Bacillus cereus* produces keratinase from a halophilic environment. Numerous authors were reported that keratinase enzyme harbour the antimicrobial activity [34-36], however, there is no evidence of killing of clinical isolates by any of the keratinolytic isolates produced by halophilic isolates. In the present study, high salt tolerant isolates were subjected to screening of keratinase enzyme producers with keratin containing media plant, zone of clearance around the colonies were indicted as keratinase

producers (Plate 1) and furthermore isolates were subjected to enzyme assay method for determination of enzyme producing ability, 12th and 19th isolates were exhibiting the 111.1 U/ml and 85.71 U/ml of keratinase respectively.

In the present study, in order to find new antibacterial drugs and address issues like antibiotic resistance, this study was showing the antibacterial activity of keratinase, a type of serine protease. among the 7 genus, *Enterococcus* was highly inhibited with keratinase which exhibiting the zone of inhibition was ranged from 12 to 18mm and followed by *Klebsiella sp.*, which exhibiting the zone of inhibition was 11 to 16mm. Among the various concentration of enzyme tested, 8 U of enzyme was highest activity against to all isolates (Table 2, Plate 2). In this study it was clearly found that the enzyme has the ability to kill all types of bacteria like gram negative and gram positive without discrimination.

Plate 1 Screening of keratinase producing isolates

Table 2 Antibacterial activity of keratinase enzyme against clinical isolates

S. No.	Isolates name	Con.of enzyme (U/ml) Zone of inhibition in mm				PBS (50µl)	Ampicillin (10µg)
		1	2	4	8		
1.	*E. coli*	-	12	15	17	-	14
2.	*K. pneumoniae*	-	11	13	16	-	13
3.	*E. faecalis*	12	14	16	18	-	15
4.	*S. aureus*	-	-	-	10	-	11
5.	*Salmonella sp*	-	-	-	-	-	-
6.	*Shigella sp*	-	-	12	13	-	12
7.	*P. aeruginosa*	-	-	-	-	-	-

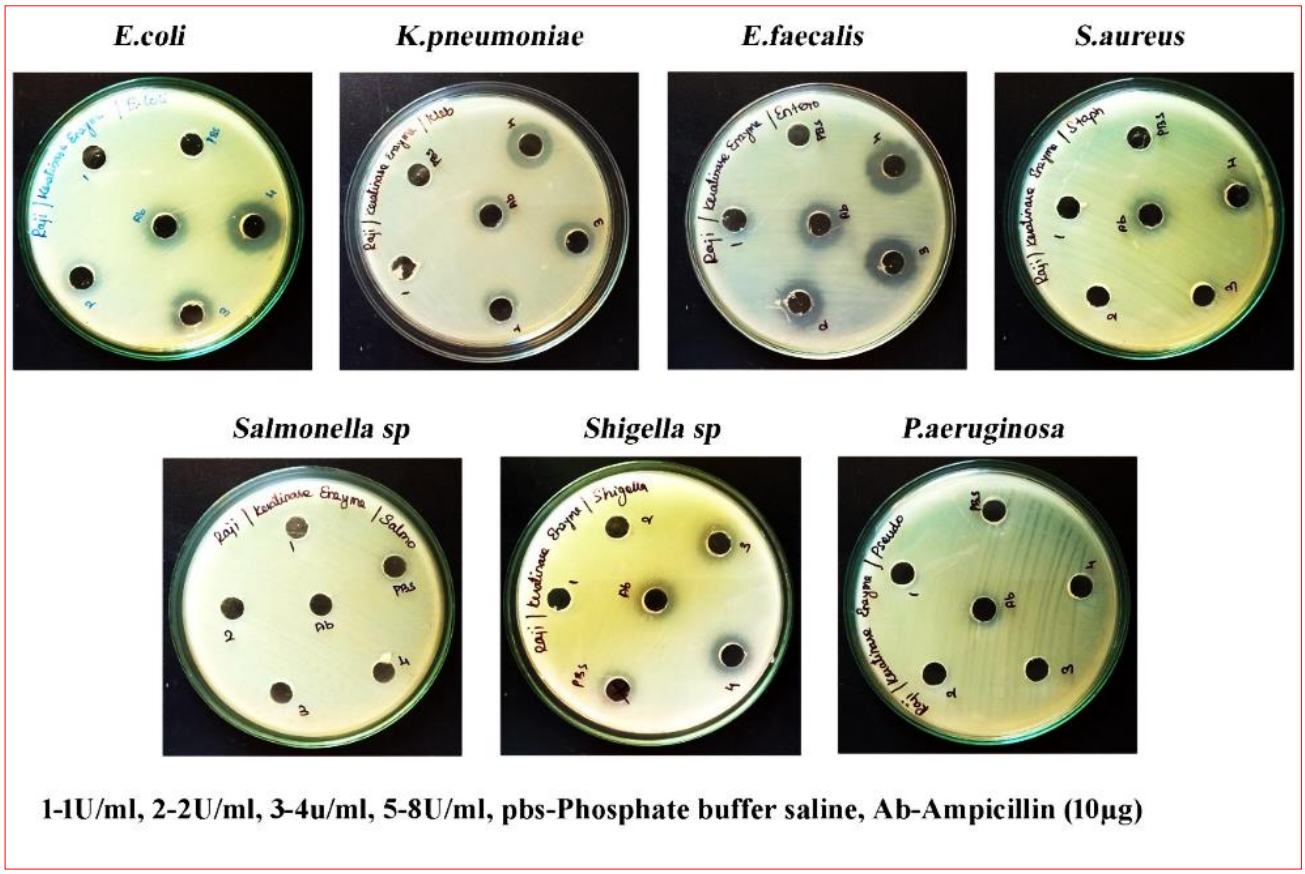

Plate 2 Antibacterial activity of keratinase enzyme against clinical isolates

In this study, all tested bacterial isolates were biofilm producers and this type of bacteria easily escapes from action of antibiotic, although those isolates were also controlled by keratinase enzyme, according to review of literature, no one was inhibited the biofilm producing isolates with keratinase enzyme. In 2015, Bhange *et al*. [37], found the biofilm dispersal and antibiofilm activity of the keratinase produced by *Stenotrophomonas maltophilia* Kb2 was studied against pathogenic bacteria *Staphylococcus aureus* MTCC-96 and *Escherichia coli* MTCC-739. Anti-biofilm enzymes are

considered innovative and environmentally friendly means of biofilm control, as they can degrade the extracellular matrix and facilitate biofilm detachment. Biofilms can be successfully disrupted by amylases and proteases [38].

Bacteria's ability to adhere and form biofilms on biotic and abiotic surfaces results in an increase in virulence, as well as bacterial pathogenicity, where bacteria in biofilms are already thousands of times more resistant to antibiotics than non-biofilms producing isolates [39]. In our study, biofilm reduction percentage was treated with keratinase enzyme (Table 3). The best reduction was achieved at the highest enzyme concentrations 8U/ml, at which the effect of keratinase on *Enterococcus sp*, with 54.9% of biofilms inhibition. Similarly, Maeda *et al*. [40] observed the biofilms inhibition with *Brevibacillus sp*. KH3 producing serine protease enzyme. Biofilm is now considered an important pharmacological development of drugs. Therefore, strategies that can more effectively release biofilm-associated microbes from EPS defenses may help improve the arsenal of enzyme-mediated anti-biofilm therapies [41-42].

Table 3 Antibiofilm activity of keratinase enzyme against clinical isolates

S. No.	Isolates name	Con. of enzyme (U/ml) % of biofilms inhibition			
		1	2	4	8
1.	*E. coli*	9.1	15.6	22.3	44.6
2.	*K. pneumoniae*	12.5	18.9	28.6	49.6
3.	*E. faecalis*	12.7	29.4	42.1	54.9
4.	*S. aureus*	4.6	11.8	31.5	49.6
5.	*Salmonella sp*	2.6	8.9	16.3	34.8
6.	*Shigella sp*	9.7	19.5	27.6	44.8
7.	*P. aeruginosa*	3.6	7.8	16.7	22.4

CONCLUSION

The results of the antimicrobial activity of keratinase enzyme against biofilm producing isolates suggest that this enzyme could be a potential agent for the control of biofilm-forming pathogens. It is important to further investigate its efficacy in controlling biofilm formation and its activity against other bacterial species and research is needed to further elucidate the antimicrobial substance and activity of keratinase enzymes against biofilm producing isolates. Additionally, research should be conducted to determine if the enzyme has any potential toxicity or side effects.

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