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KERATINASE OF *BACILLUS SUBTILIS* N-49: A NOVEL STRAIN OF INDIAN ORIGIN POSSESSING POTENTIAL TO ENHANCE TRANSUNGUAL DRUG DELIVERY

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ABSTRACT

High prevalence of the disease onychomycosis i.e., non-dermatophytic fungal nail infection, has recently been lodged as an underdiagnosed public health issue in India. The causal agent, keratinophilic fungi causes the nails to become thick, brittle and ragged with changes in appearance, slight pain and foul odor. The treatment commonly consists of topical drug employment. However, delivery of these drugs to the site of infection can pose as an obstacle due to poor permeability of antifungal creams across the nail plate. Penetration of such drugs by altering the nail plate barrier via physical and chemical means and usage of penetration enhancers have proven to be minimally invasive. However, this issue can be addressed by the use of keratinases. Keratinases can act as molecular scissors cleaving the firm layers of keratin protein constituting the major part of the nail plate, thereby loosening the plate and effectively enhancing transungual drug permeability. Thus, the present study aimed to isolate an alpha-keratinase producing bacterium. A total of 100 bacterial strains isolated from the soil of a poultry feather dumping site were screened for protease and keratinase production, Of all the shortlisted isolates, isolate N-49 was finally selected as it could completely degrade goat hair in 18h. Further, it was identified as *Bacillus subtilis* based on 16S rRNA sequence homologies. This is the first report of indigenous bacterium *Bacillus subtilis* N-49 possessing alpha-keratinase activity. It has immense potential for enhancing trans-ungual drug delivery.

Keywords: Onychomycosis, Drug permeability, Trans-ungual drug delivery, Keratin, Keratinases

INTRODUCTION

Onychomycosis is a preliminary finger and toe nail fungal infection that causes thickening, changes in appearance like discoloration and separation from nail bed with slight pain, in some cases **[1–3]**. It is generally caused by dermatophytic fungi such as *Trichophyton rubrum*, *Trichophyton mentagrophytes* and *Epidermophyton floccosum* commonly cultivated from public pools, showers, hotel carpets, and soil. The fungus is more prone to grow overtime in the dark, warm and moist environment of shoes creating a micro traumatic pressure on the nail plate which dissociates hyponychial seal and causes the dermatophytes to penetrate into the nail bed **[1]**. There are four types of Onychomycosis on the basis of their route of penetration and clinical presentation-

Distal subungual onychomycosis. The fungus invades through the underside of the nail plate giving rise to a hyponychial infection. Migration of fungus takes place proximally through the underlying nail matrix. This results in thickening of the subungual region and detachment of the nail plate from the nail bed. The subungual region becomes suitable to harbor superinfecting bacteria and mold giving the nail plate a yellowish-brown appearance **[4, 5]**.

Proximal subungual onychomycosis. It occurs when the fungus invades the nail plate proximally through cuticle area and move to the distal area in the newly formed nail plate. It results in the detachment and destruction of the proximal nail plate and production of white color in the distal lunula **[4, 5]**.

White superficial onychomycosis. It is a less common type in which the fungus invades directly through the superficial layers of nail plate. The infection then, spreads to the nail plate and hyponychium. Associated common symptoms might include crumbly, rough and soft nails with appearance of delineating "white chalky islands" increasing progressively as the infection spreads **[4, 5]**.

Total dystrophic onychomycosis. It is considered as an end-stage nail disease and the final result of all types of onychomycosis with the nail completely invaded by the fungus resulting in very thick and dystrophic nails **[4, 5]**.

Traditional treatments for onychomycosis like mechanical methods such as nail avulsion and nail abrasion results in post-operational pain and nail deformity and physical methods such as laser therapy, ultrasound and photodynamic leads to tissue damage, less specificity, dependency on expert help, increased risks and are highly expensive **[6]**.

Therefore, topical drug therapy is a lucrative option for treatment of onychomycosis i.e.,

application of antifungal creams and agents. The method has been found to be advantageous in terms of systemic administration and minimum adverse effects at the site of drug action **[2, 7]**. The application of topical drugs is quite limited due to impermeability and poor penetration of these drugs across the nail plate and easy removal by rubbing or washing owing to the presence of densely keratinized layer **[7–9]**. Keratin is a tough insoluble fibrous structural polypeptide constituting epidermal appendages such as hair, nails, feathers, horns and beaks and forms a rigid barrier between organ and its environment **[10, 11]**. It is highly recalcitrant to proteolytic degradation by enzymes such as trypsin, pepsin and papain due to its molecular structure which is highly stabilized due to the presence of disulfide bonds, hydrogen bonds and ionic bonds **[12, 13]**. Keratin can be classified as α-keratin and β-keratin depending upon the secondary structures of polypeptide chains present in the molecule and composition of amino acids. α-keratin comprises of acidic α-helical coils type I and basic/neutral type II protein chains coiled together as an elongated α-helix filament forming fibrils by interchain bonding and is abundantly found in mammals (human nails). On the other hand, β-keratin comprises of β-sheets and is mainly found in scales of reptiles and avian tissues (feathers) **[14]**. Despite this imperishable structure, keratin can be degraded by the proteolytic activity of Keratinases – an extracellular inducible serine and metalloprotase that breaks the peptide bond between amino acids present in the structure of keratin, are able to recognize hydrophobic substrates and sulfitolyse the disulfide bonds in the structure **[12, 15, 16]**. Certain bacteria such as *Fervidobacterium sp., B. cereus, B. pumilus, Microsporium sp.* and *Thermoanaerobacter sp.* and fungal sources such as *Chrysosporium sp., Aspergillus sp., Cladosprium sp.* and *Microsporum sp.* are already known to be prime producers of keratinase enzyme **[10, 16, 17]**. Keratinases are widely applied in food, chemical and medical industries and are known to be green catalysts for their ecological implications in management of keratinous waste **[10, 17]**. This study contributes to the application of keratinases in medical industry as enzymatic penetration enhancers to be used in the treatment of onychomycosis as a component of topical drug therapy. It is aimed at isolation and identification of an indigenous bacterial strain - *Bacillus subtilis* N-49 on the basis of 16S rRNA homologies by continuous screening of the soil from a poultry feather dumping site possessing alpha keratinase activity to degrade dense keratin layer, in turn, increasing permeability across the nail plate barrier and enhancing penetration of topical drugs efficiently.

MATERIALS AND METHODS

Procurement of soil and isolation of bacteria using enrichment culture method-

1 gram of soil sample from different poultry dumping site was added to a flask containing 50 mL sterile autoclaved enrichment medium (2.5 grams NaCl, 0.5 grams K_2HPO_4, 0.5 grams KH_2PO_4, 0.1 grams $MgSO_4$, 0.5 grams $(NH_4)_2SO_4$, 1-liter Distilled water) and different sources of keratin i.e., Feathers, Nails, Hair, Wool was measured (approximately 0.25-0.5 grams), under sterile conditions. The media was incubated in a shake incubator at 37ºC. The enriched culture was serially diluted up to 10-8 onto nutrient agar plates in 0.95% saline (1.90 grams of NaCl in 200 ml distilled water) for each sample. 1mL of culture from the last four dilutions were plated onto the nutrient agar plates and incubated for 24-48 hours at 37ºC to observe different types of colonies growing on the medium to be subjected to primary screening.

Preliminary screening for proteolytic isolates using skim milk agar plates-

Phosphate buffer agar (160 ml 50 mM phosphate buffer- 10 ml from 61.5 ml of 1M K_2HPO_4 and 38.5 ml of 1M KH_2PO_4 solution, 190 ml distilled water and 4 grams agar) and skim milk (4gms of Skimmed milk powder, 40 mL distilled water) were autoclaved separately at 10 psi for 20 minutes. Both the components were mixed in laminar hood while still warm and poured into petri plates. Colonies from nutrient agar plate were patched on the milk agar plates by making 9 grids on each plate and patching one point-sized colony from nutrient agar plates of nail, feather, and hair sample each. The plates were incubated at 37ºC for 24-48 hours and observed for zone of hydrolysis. Colonies having zone of hydrolysis 0.5 cm or higher were shortlisted for secondary screening and were preserved by storing them in glycerol stock (200 µl of overnight grown culture in 200 µl of glycerol).

Revival of colonies as pure cultures for secondary screening-

Revival of the strains from glycerol stock was done by pouring 1 ml of each stock into culture tubes containing 20 ml nutrient broth. The tubes were incubated at 37ºC for 24 hours. These strains were streaked using nutrient broth agar plates using streak plate method and were incubated at 37ºC for 24 hours to obtain pure cultures of the strains. From 37 isolates as pure cultures, 7 colonies were shortlisted on the basis of their colony and morphology characteristics for secondary screening.

Secondary screening for alpha keratin degraders-

20 ml of feather peptone medium and goat hair peptone medium was poured into conical flask for each isolate and were

autoclaved. 2 ml of each isolate was transferred aseptically into each media. The flasks were incubated in a shaker incubator for 2-3 days and were checked regularly for degradative activity for almost 72 hours. One isolate from each media were observed to show best degradative activity – F-27 (colony number 27 on feather peptone medium) and N-49 (colony number 49 on goat hair peptone media). N-49 colony was finally selected due to the presence of alpha keratin degradation activity. It was further identified by 16S rRNA sequencing.

Sequencing of genome and comparison of sequencing homologies-

The genomic DNA of the selected bacterium N-49 was isolated using genomic DNA isolation kit. 16S rRNA sequence was amplified by PCR using 16S forward and reverse primers and standard protocol. The 1.4kB PCR product obtained was sequenced and the results obtained were analyzed using BLAST alignment tool.

RESULTS & DISCUSSION

Isolation of bacteria using enrichment culture technique-

Bacterial strains were isolated from soil samples by growing on enrichment media containing keratin sources such as hair, feather, nails, wool and were observed for distinct morphologies and colony characteristics.

Preliminary screening of proteolytic isolates using skim milk agar plates-

Preliminary screening was done to identify bacterial isolates which could produce protease enzyme and degrade the milk protein, casein. The proteolytic activity of the bacterial isolates was estimated by measuring the diameter of a clear zone of hydrolysis produced due to casein degradation on skim milk agar plates. 37 out of 100 isolates were observed to produce a clear zone of hydrolysis equal to or greater than a diameter of 0.5 cm. 9 out of these 37 isolates which gave a zone of hydrolysis of more than 1 cm were further shortlisted for secondary screening to test their alpha-keratinase activity.

Figure 1 shows the zone of hydrolysis produced by few isolates. Table 1 represents a detailed list of bacterial isolates and the measurement of their zone of hydrolysis.

The number of colonies highlighted in yellow represents 37 shortlisted colonies producing a zone of hydrolysis equal to or greater than 0.5 cm.

Secondary screening for alpha keratin degraders-

9 bacterial isolates were selected for secondary screening based on their ability to produce a zone of proteolytic hydrolysis of more than 1 cm. To detect alpha keratin degraders, feather and goat hair peptone medium were used where feathers were a source of beta-keratin and goat hair were a source of alpha-keratin which is also present in human nails. After 72 hours of incubation

of the selected colonies on goat hair peptone medium, fastest degradation was observed in colony F-27 i.e., colony number 27 in feather peptone medium and N-49 i.e., colony number 49 in goat hair peptone medium **(Figure 2, Figure 3)**. Therefore, it was found that both colony F-27 and N-49 could produce alpha keratinase for complete degradation of hair. As isolate N-49 was obtained after enrichment in nail supplemented medium, it was finally selected for further identification using 16S r RNA sequencing technique.

Sequencing of genome and comparison of sequencing homologies-

The bacterial isolate N-49 colony was selected as it possessed alpha degradative activity which had the potential to degrade the alpha keratin present in human nails. The bacterium was identified as *Bacillus subtilis*. The homologies of 16s rRNA sequencing were compared using BLAST and the sequence alignment was similar to that of *Bacillus subtilis* by 99.57% identity and 97% query cover **(Figure 4)**.

Table 1: List of samples possessing proteolytic activity and measurement of their zone of hydrolysis

Sample	Hydrolysis zone (cm)	Colony size (cm)	Hydrolysis zone-Colony size (cm)
1. Nail			
N1	**1.8**	**2.0**	**0.2**
N2	**1.5**	**1.9**	**0.4**
N4	**1.7**	**2.2**	**0.5**
N5	**2.1**	**2.5**	**0.4**
N9	**2.3**	**2.7**	**0.4**
N19	**1.4**	**1.7**	**0.3**
N20	**1.7**	**1.8**	**0.1**
N22	**1.7**	**1.8**	**0.1**
N23	**1.6**	**1.8**	**0.2**
N24	**1.25**	**1.7**	**0.55**
N25	**1.0**	**1.5**	**0.5**
N26	**1.2**	**1.7**	**0.5**
N27	**1.3**	**1.7**	**0.4**
N28	**0.8**	**1.4**	**0.6**
N29	**1.4**	**1.7**	**0.3**
N30	**1.0**	**1.4**	**0.4**
N31	**1.4**	**1.8**	**0.4**
N41	**2.0**	**1.0**	**1.0**
N42	**3.2**	**2.4**	**0.8**
N43	**1.4**	**0.5**	**0.9**
N44	**1.9**	**0.8**	**1.1**
N45	**3.0**	**2.7**	**0.3**
N46	**2.6**	**1.8**	**0.8**
N47	**2.4**	**1.5**	**0.9**
N49	**1.6**	**0.5**	**1.1**
N54	**2.8**	**2.3**	**0.5**
N57	**2.2**	**1.4**	**0.8**
2. Hair			
H5	**1.5**	**0.7**	**0.8**
H6	**1.3**	**0.4**	**0.9**
H10	**1.3**	**0.6**	**0.7**
H11	**1.5**	**0.7**	**0.8**
H12	**1.4**	**0.6**	**0.8**
H13	**1.5**	**0.6**	**0.9**
H14	**2.0**	**1.0**	**1.0**

H15	**1.7**	**0.7**	**1.0**
H16	**1.5**	**0.5**	**1.0**
H17	**1.5**	**0.6**	**0.9**
3. Feather			
F4	**2.9**	**2.2**	**0.7**
F5	**1.7**	**0.8**	**0.9**
F18	**1.3**	**0.8**	**0.5**
F19	**1.8**	**1.2**	**0.6**
F20	**1.2**	**0.5**	**0.7**
F21	**1.8**	**0.7**	**1.1**
F22	**2.0**	**0.8**	**1.2**
F23	**1.1**	**0.3**	**0.8**
F24	**1.1**	**0.7**	**0.4**
F25	**1.2**	**0.5**	**0.7**
F27	**1.9**	**1.0**	**0.9**
F28	**1.0**	**0.2**	**0.8**
F33	**0.9**	**0.1**	**0.8**
F36	**1.9**	**0.8**	**1.1**

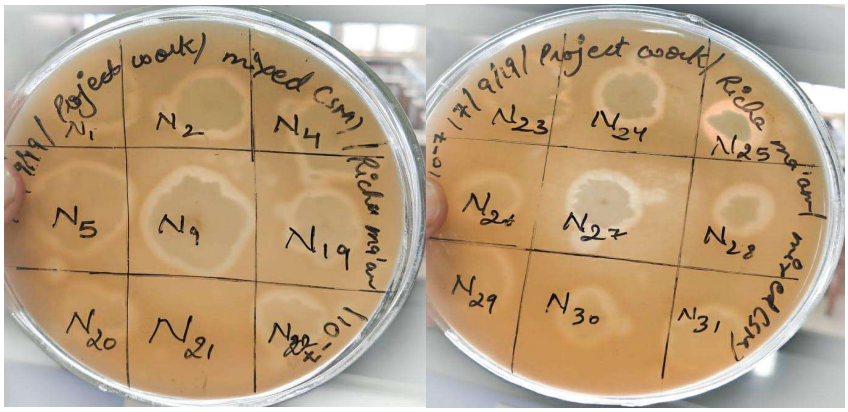

Figure 1: Zone of hydrolysis produced by proteolytic colonies in nail enrichment media on milk agar plates

Figure 2: Goat Hair peptone medium - Colony F-27 showing alpha keratin degradation (on the left) and uninoculated medium (on the right)

Figure 3: Goat hair peptone medium - Colony N-49 showing alpha keratin degradation (on the left) and uninoculated medium (on the right)

Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments Download Select columns Show 100

select all *100 sequences selected* GenBank Graphics Distance tree of results MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bacillus subtilis strain DMN5.8 16S ribosomal RNA gene, partial sequence	Bacillus subtilis	1271	1271	97%	0.0	99.57%	1378	KX809595.1
Bacillus sp. RD_AZPVI_11 16S ribosomal RNA gene, partial sequence	Bacillus sp. RD_AZPVI_11	1271	1271	99%	0.0	99.15%	1481	KU597588.1
Bacillus subtilis strain HO27 16S ribosomal RNA gene, partial sequence	Bacillus subtilis	1271	1271	97%	0.0	99.57%	1502	KF792057.1
Bacillus sp. M94(2010) strain M94 16S ribosomal RNA gene, partial sequence	Bacillus sp. M94(2010)	1271	1271	97%	0.0	99.57%	1451	GQ340484.1
[Brevibacterium] frigoritolerans strain CMG M5 16S ribosomal RNA gene, partial sequence	[Brevibacterium] frigoritolerans	1271	1271	99%	0.0	99.15%	1507	EU081510.1
Bacillus sp. (in: Bacteria) strain CM-CNRG 603 16S ribosomal RNA gene, partial sequence	Bacillus sp. (in: Bacteria)	1269	1269	99%	0.0	99.15%	1521	MK456459.1
Bacterium strain Bacillus subtilis_AF_6 16S ribosomal RNA gene, partial sequence	bacterium	1269	1269	99%	0.0	99.15%	1523	MH027601.1
Bacillus amyloliquefaciens partial 16S rRNA gene, isolate MD8	Bacillus amyloliquefaciens	1269	1269	99%	0.0	99.15%	947	LN899810.1
Bacillus amyloliquefaciens strain SRCF48 16S ribosomal RNA gene, partial sequence	Bacillus amyloliquefaciens	1269	1269	99%	0.0	99.15%	1488	KM658261.1

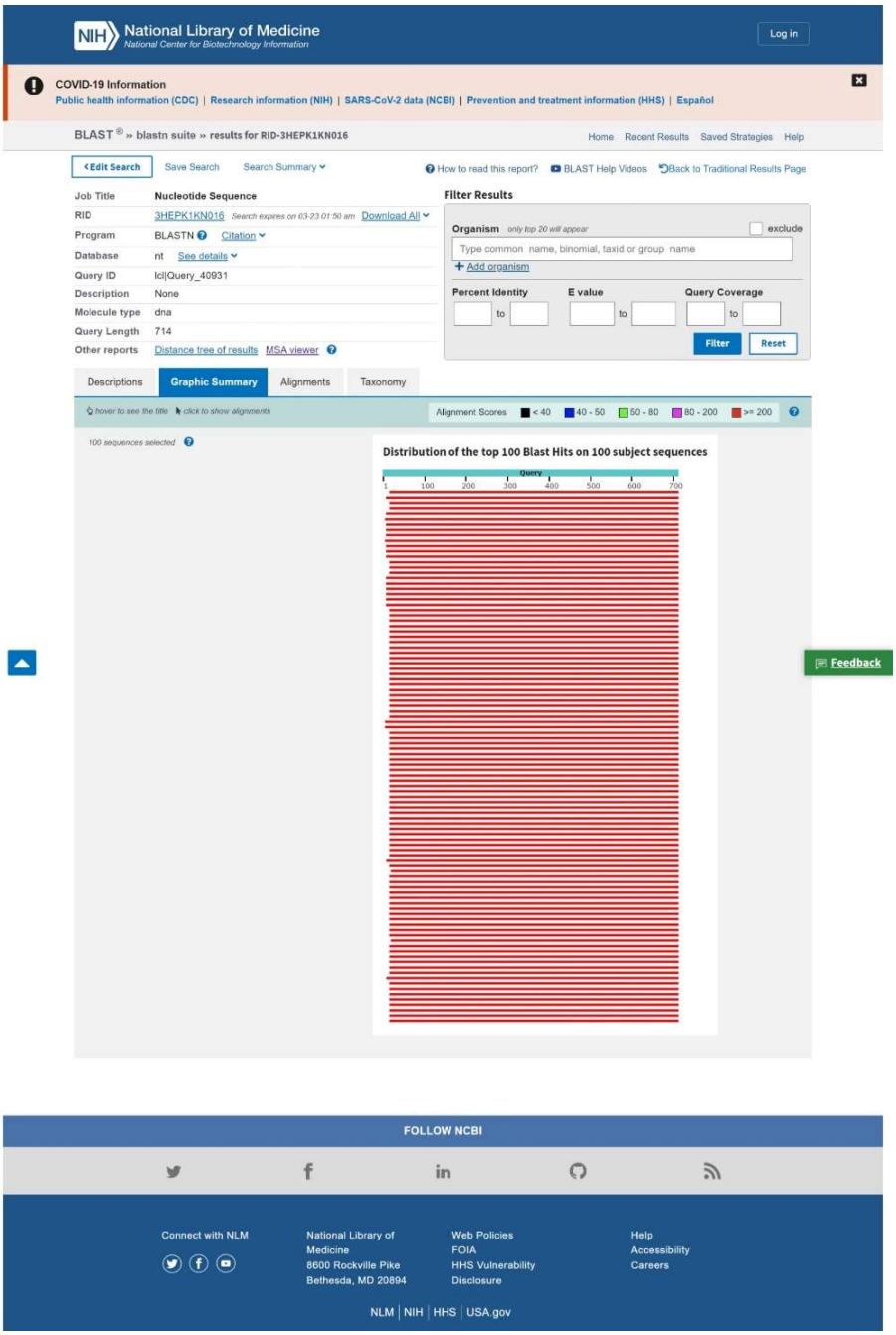

Figure 4: BLAST analysis results for comparing 16s rRNA homologies

CONCLUSION

A goat hair degrading strain of *Bacillus subtilis* N-49 was isolated from the soil of a poultry feather dumping site which efficiently degraded alpha keratin in goat hair present in peptone medium within 72 hours at 37°C. The bacterial isolate was concluded to produce keratinase which is a serine and metalloprotease enzyme known to recognize the hydrophobic substrates of keratin protein, sulfitolyse the disulfide bond present between the its secondary

structures and degrade the peptide bond between amino acid residues **[12, 15, 16]**. A wide variety of bacteria and fungi are already known to produce keratinase enzyme, however, the present strain of *Bacillus subtilis* is a novel report of a bacterium of Indian origin possessing alpha keratinase activity.

The alpha keratin present in the goat hair has been used as an analog to the alpha keratin present in human nail. Thus, keratinase enzyme, when isolated, from *Bacillus subtilis* N-49 strain can be used as a component of topical drugs like antifungal creams employed in the treatment of Onychomycosis to aid in transungual drug delivery system by enhancing the permeability across the nail plate barrier efficiently **[8, 9]**. Sulfitolysis of the disulfide bonds present in keratin can be enhanced in the presence of reducing agents such as mercaptoethanol and thioglycolic acid as present in other topical drugs **[18–20]**. Thus, keratinases can be utilized to separate the nail plate from the nail bed to facilitate penetration of the topical drugs to the fungus-infected live cells. Keratinases can be used as penetration enhancers without the possibility of generation of any hypersensitive response in immunocompromised patients.

Keratinase enzymes isolated from *Bacillus subtilis* N-49 does not account to the chemical waste generated by healthcare industries and instead, possess the potential to replace chemical agents used in transungual drug delivery system. Novel discoveries of indigenous strains such as *Bacillus subtilis* N-49 is an important achievement in the treatment of dermatophytic fungal infections and can be a boon to Indian healthcare systems.

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