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Identification and Phylogenetic Analysis of Keratinase Producing Bacteria SNP1 from Poultry Field

Divya Balakrishnan and Nithadas Sathyadas Padmanabhan

Department of Biotechnology, Sree Narayana College, Kollam-691001, Kerala, India.

*(*Corresponding author)*

Abstract

The study was conduct to select the best promising keratinolytic bacterial strain. A keratinase positive bacterial strain was isolated from the soil samples of poultry field, Attingal, Thiruvananthapuram. Each sample was plated on skim milk agar and feather meal agar plates containing 5 g feather. The well grown isolates which produced the largest clear zone on skimmed milk plate were selected for keratinase assays. Out of 26 bacterial isolates, 7 isolates were selected. Among the selected strain, the best keratinase producing bacterium SNP1was selected for further analysis. The SNP1 potential strain was later confirmed as *Bacillus haynessi* based on molecular and phylogenetic analysis. The medium components and culture conditions were optimized to enhance keratinase production through optimization. Keratin and feather powder (10g/l) were identified as good substrates for the highest keratinase production. The strain SNP1 resulted maximum enzyme production at 96h of incubation at 37°C and pH 8 under 120 rpm. Therefore, *Bacillus hayneisi* might be used for large scale production of keratinase for industrial purposes.

Keywords: Keratinase, *Bacillus sp*., Optimization, Enzyme activity, 16SrRNA

Keratin is a hard-degrading fibrous and recalcitrant structural protein, which forms the third most abundant polymer in nature after cellulose and chitin (Lene *et al* ., 2016). In the native state, the feather keratin resists the degradation by proteolytic enzymes such as trypsin and pepsin due to tightpeptide-chains held together by disulfide bridges by means of cysteine residues (Weeranut, 2017; Korniłłowicz-Kowalska and Bohacz, 2011). Keratin provides animals more tough against both abiotic stress and

biotic attacks. Since microbial degradation of keratin is not widespread in nature, keratin can serve as an efficient defense even against microbial attack (McKittrick *et al*., 2012)

The main interest in feather-degrading bacteria recently originated from engineers involved in waste disposal. The Worldwide consumption of chicken leaves behind tons of feathers as a waste, around 18,500 lakhs tons of poultry feather is generated annually, of which India's contribution alone is 3500 tons (Sharad *et al*., 2017). The poultry industry is one of the major branch of the food industry where the amount of waste generated is enormous, whose keratin composition is quiet difficult to process and degrade. Hence, the isolation of feather-degrading bacteria from poultry waste opened new potential for generation of contamination-free waste disposal in poultry industry (Paweł Kowalczyk, *et al*., 2018).

Keratinase are extracellular in nature and are usually produced by microorganisms growing in a basal medium containing keratinous substrates (Jaouadi, *et al*., 2011). It is believed that in the future, microbial keratinase will occupy a special niche among proteases as valuable enzymes for the bioprocessing of the keratinous wastes, which are released as huge amounts into the environment due to human activities (Gopinath, *et al*., 2015 and Verma, *et al*., 2016). Because of their higher specificity, keratinases will emerge as new potential for generation of contamination-free waste and also replace proteases in the leather industry and detergents (Daroit and Brandelli, 2014).

Diverse groups of microorganisms are reported to produce keratinase, among bacteria, particularly *Bacillus subtilis* (Gröhs Ferrareze, *et al*., 2016) and *Chryseo bacterium* (Hong, 2015). Moreover, actinomycetes from the *Streptomyces* genus are known to produce keratinases (Allure, 2014). The most common active keratinolytic fungi belong to *Aspergillus* (Mazotto 2013 and Lopes, 2011, *Microsporum* (Sowjanya and Chary, 2012) *Trichoderma* (Cao, *et al*., 2008) and *Chrysosporium* genera (Bohacz, 2017 and Maruthi, 2011). Enzymatic degradation of keratin either using the bacterial colonies or using the enzymes isolated from bacterial cells has been proposed as a cheap and clean method of transforming feather keratin into polypeptides and single amino acids. In the present work, we isolate keratinolytic bacteria from poultry field that can reach commercial exploitation as keratinase producers. We also present the molecular methods enabling deeper insight into the genetic background of feather-degrading strain.

MATERIALS AND METHOD

Microbiological analysis

Sample collection and Primary Screening : The soil samples were collected in a sterile polythene bag from the various poultry locations in Thiruvananthapuram, Kerala. Isolation of the bacterial strains were done by serial dilutions up to $10^{-6.}$ All the dilutions were plated on Nutrient agar medium (Himedia M-001) and incubated at room temperature for 3days. Skim Milk Agar (Himedia M-763) were used for the primary screening of keratinolytic bacteria (Sharad, *et al*., 2017). Suspected bacterial

isolates were inoculated on skim milk agar plates, incubated for 5 days and further examined for zone formation.

Secondary screening of keratinolytic bacteria: Raw fresh chicken feathers collected from poultry farm, repeatedly washed with tap water and distilled water to remove the blood strains and later dried in room temperature.The bacterial isolates were inoculated in 50 ml of sterile medium modified basal medium ($MgSO_4.7H_2O$ 0.2 g/l; K_2HPO_4 0.3 g/l; KH_2PO_4 0.4 g/l; $CaCl_2$ 0.22 g/l) supplemented with chicken feather (5g) andthe pH was adjusted to 8.0. The medium was incubated in a rotary shaker at a speed of 120 rpm for 37°C for 24 hours. After incubation, the cells were removed by centrifugation at 10,000 rpm for 10 minutes and the supernatant was collected and examined for further enzyme activity (Saber et al., 2010; Selvam et al., 2013)

Bacterial inoculums preparation: 50 ml of nutrient broth (13 g/l at pH 7.4 ± 0.2) was prepared and sterilized in an autoclave (NatSteel (Big), India) at 15 lbs/in 2 pressure, 121°C for 20 min. Freshly grown 1ml of the inoculum were aseptically transferred to media and incubated at 37°C overnight at 120 rpm in a incubator shaker (Lab Tech, India).

Enzyme production: The keratinase enzyme production was carried out using the basal medium composition of (g/l): feather meal powder (10 g), NH_4Cl (1 g), NaCl (1 g), K_2HPO_4 (0.6 g), KH_2PO_4 (0.8 g), $MgCl_2 \cdot 6H_2O$ (0.48 g) and yeast extract (0.2 g) with pH 7.5 (Rajesh, *et al*., 2014). 1ml of the bacterial inoculum were aseptically transferred to sterile medium and incubated for 96 h of incubation.The cell free supernatant were collected after centrifugation at 5000 rpm for 20 min at 4 °C and further used for keratinase assay.

Enzyme assay: Keratinase assay was determined by taking 2 ml of reaction mixture, contained 1 ml of keratin solution and 1 ml of supernatant as crude enzyme. The reaction mixture was incubated for 10 minutes at 40° C in water bath. To the cooled mixture, 2ml of 10% chilled TCA was added and kept for 20 minutes incubation.The mixture was later centrifuged at 5000 rpm for 10 minutes. 0.1 ml of the supernatant was added to 0.9ml of distilled water and 0.5 ml 500 mM sodium carbonate was incubated at 35°C for 10 minutes. 2ml folinciocalteu reagent (1:3 v/v) was added and incubated for 20 minutes. A blue colour developed and the absorbance was measured at 660nm.A standard graph was generated using standard tyrosine solution of 10-100ug/ul. One unit of keratinolytic activity is defined as the amount of enzyme that liberates 1ug of tyrosine equivalent per minute under the described assay condition (Krishna Rayudu, 2014).

Optimization of cultural conditions for keratinase production: The production of keratinase by bacterial inoculums was studied by considering the media components and culture conditions. All the experiments were carried out in triplicate and the standard value were presented.

Effect of Incubation Time: The optimum incubation period for maximum keratinase production was studied with 45 ml of sterile feather meal inoculated with 5% bacterial culture in a shaker for 144 hrs at 120 rpm at 37°C. Enzyme activity was then quantified after every 24 h using standard assay protocol.

Effect of pH: Effect of pH on keratinase production was observed at different pH values i.e. from 4 to 11 with the 45ml of sterile feather meal with 5% bacterial culture. The pH was adjusted to prior inoculation using 1M NaOH and 1M HCl. After inoculation, the flasks were incubated at 37 °C for 144hrs with shaking (120rpm).

Effects of substrates on keratinase production

Various substrates such as keratin, casein, peptone, skim milk powder and feather meal powder were used (10 g/l) as main nutrient sources separately for the production of keratinase. Fermentation was carried out with 45 ml of sterile feather meal inoculated with 5% bacterial culture in a shaker at 37 °C for 144hrs at 120 rpm. All the experiments were performed in triplicates and results were expressed as mean±S.D.

Statistical Analysis : Each experiment had three replicates. Means of variable and standard deviation were recorded were carried out to detect any significant differences between the results of control and the treated sample

Molecular identification of keratinase species: Strains grown onbasal medium enriched with chicken featherswere identified with a molecular procedure, which includes extraction of total DNA, partial amplification of 16S rRNA genes and finally sequence analysis.

DNA extraction and Amplification of 16S rRNA genes: The total genomic DNA was extracted using NucleoSpin® Tissue Kit (Macherey-Nagel). 16S rDNA sequence were amplified from genomic DNA using universal primer (Integrated DNA Technology, India) 16S-RS-F-5-CAGGCCTAACACATGCAAGTC-3 and 16S-RS-R 5-GGGCGGWGTGTACAAGGC-3in a PCR thermal cycler (Nubel, *et al*., 1997). PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1X PCR buffer, 0.2mM each dNTPs, 2.5mM $MgCl_2$, 1 unit of Ampli Taq Gold DNA polymerase enzyme, 0.1 mg/ml BSA, 4% DMSO, 5pM of forward and reverse primers and 2µl of template DNA. PCR amplifications were carried out for 35 cycles with a following procedure of initial denaturation at 95°C for 5 min, final denaturation of 95°C for 30 sec, annealing at 60ºC for 40 sec and final extension at 72ºC for 60 sec using Thermal cycler (Gene Amp PCR System 9700, Applied Biosystems, india). Almost 5 µl of the PCR products were analyzed by 1.2% agarose gel in 0.5X TBE buffer at 75 V for 1-2 hours (Alegría, *et al*., 2009).

Sequencing and sequence comparison

The 5 µl of PCR product were incubated with 2 µl of ExoSAP-IT at 37ºC for 15 minutes, for the removal of unwanted primers and dNTPs followed by enzyme inactivation at 80ºC for 15 minutes. Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) https://www.thermofisher.com/in/en/home/brands/applied-biosystems.html.
Averagely, 1200 bp was obtained per sequence, which was then compared with similar sequences in GenBank using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

Phylogenetic analysis: The sequences of 16S rRNA gene were aligned using the Bioedit program after deleting the regions containing ambiguous nucleotides. The phylogenetic trees were constructed by neighbor-joining statistical method using MEGA X (*https://www.megasoftware.net/dload_win_gui)*. In order to determine the stability of phylogenetic tree, the sequence data were sampled 1000 times for bootstrap analysis using MEGA X with 50% cut-off (Kumar *et al*., 2018).

Result and Discussion: A total of 26 well-formed single colonies were selected finally from ten different soil samples based on their morphological difference in nutrient agar plates. The strains were SNP1, SNP2, SNP3, SNP7, SNP16, SNP19, SNP22. Among them SNP1, SNP2 and SNP3 were selected for the further study. (Fig1.). The isolates those showed the distinct clear zone on both media were considered as keratinase producing bacterial strains.

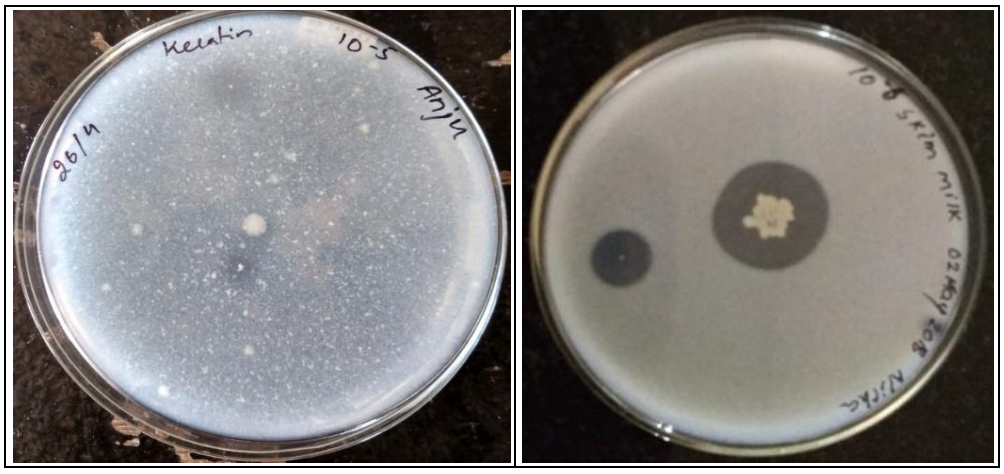

Fig 1. A: Isolation of protease producing bacteria from poultry field.; B : Zone formation of the bacteria in Skim milk agar plate.

The total bacterial population (CFU/ml) was observed at 10^{-5} dilution for all the tested soil samples. The population was high of 2.93×10^{7}CFU/ml in the soil sample (SNP1) collected from Attingal, followed by the soil sample (SNP2) of Pettah with 1.98×10^{7}CFU/ml. The least bacterial population was found in Alamcode (SNP3) soil sample, where only 1.2×10^{7}CFU/ml was observed.Among them, SNP1 resulted more zone formation and selected forfurther experiment. It has creamy white colonies with, moderate round, lobated margin on feather meal agar. The bacterium was gram positive and cocci shape in nature (Fig 2.) The strain SNP1 produced higher level of zone of 16.3mm by its protease production. Selected strain SNP1, SNP2 and SNP3 were inoculated with feather meal medium for feather degradation at different time intervals.

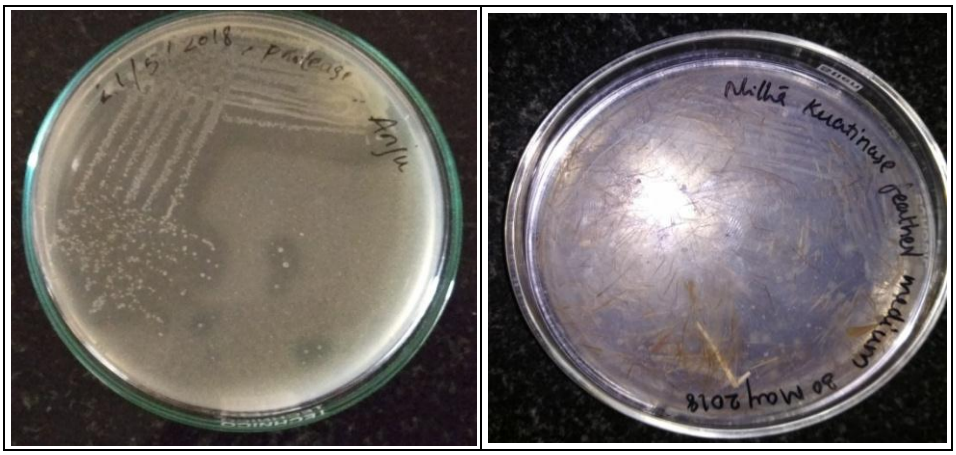

Fig 2. A: Growth of bacteria in Skim milk agar; B: Growth of bacteria in feather meal agar plate.

Keratinase activity of the bacterial strain

The assay was carried out by inoculating the seven identified isolates individually in feather minimal medium with keratin as the sole carbon source. Among three of them resulted zone formation in feather meal media. Based on the keratinase assay, SNP1 strain showed higher keratinase activity.

Optimization of growth conditions for maximum keratinase production

A number of factors play a very crucial role in enzyme production such as incubation time, temperature, pH, aeration, and composition of the fermentation medium (Ashraf et al.,2018). The enzyme production was assayed by bacterium before optimizing the parameter of media components and culture condition.

Effect of incubation period

The effect of incubation period for keratinase production from *Bacillus haynessi* was studied for the incubation period from 0 to 144 h as shown in (Fig 3.). It was observed

that the maximum enzyme production (16.2 U/ml) was attained at 96 h of incubation period. Keratinase production beyond the optimum time result rapid decline in the enzyme yield. The optimum incubation period in this study was found similar to the results *of Bacillus subtilis* (Singh *et al*., 2017) Maximum extracellular alkaline keratinase production were resulted after 72 h incubation when *B. megaterium* was grown in the feather meal medium (Saibabu, *et al*., 2013).

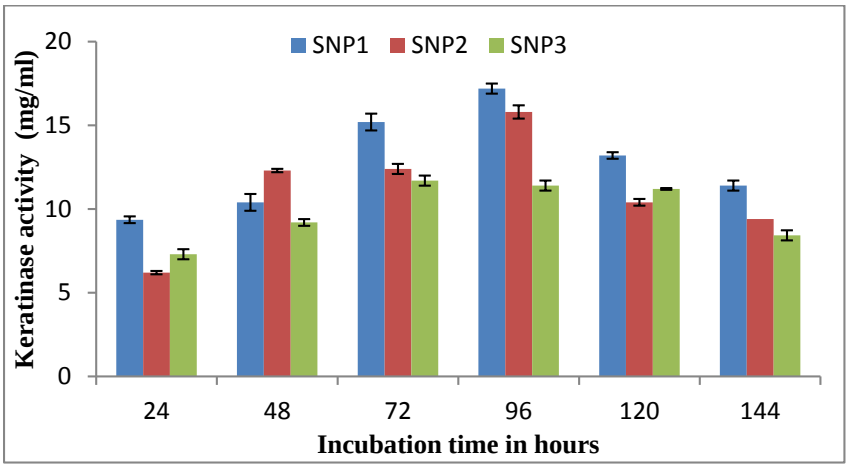

Fig 3. Effect of incubation time on the production of Keratinase enzyme from selected strains.

Effect of pH on the enzyme production: pH of the media affect the reaction of environment and transport of nutrients across the cell membrane of bacteria. Maximum production takes place when a suitable pH in culture media is maintained (Fig 4.). It was observed that the maximum enzyme production (18.3 U/ml) was attained at pH 8.The maximum enzyme production was also found at moderate pH. The optimum pH for keratinase production by *Arthrobactersp.* NFH5 was observed at pH 7.0.

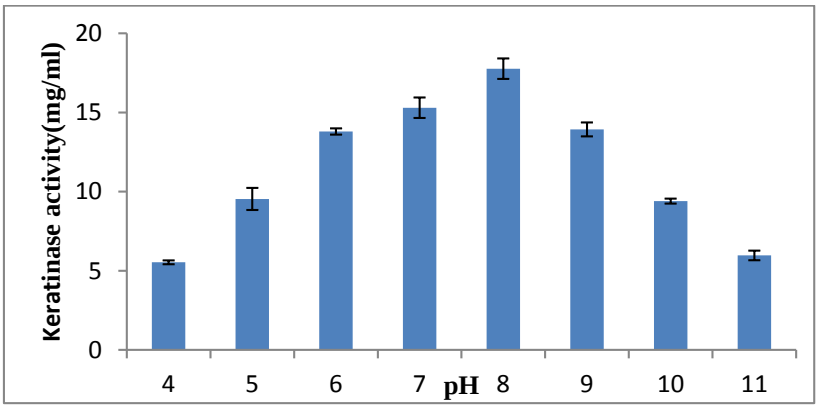

Fig 4. Effect of pH on the production of Keratinase enzyme from SNP1strain

Effect of different substrate on enzyme production: The effect of different nitrogen sources such as Skim milk, keratin, peptone, and feather meal (10g/l) on keratinolytic enzyme production from *Bacillus sps* was studied as shown in (Fig. 5). Maximum enzyme (25.21 U/ml) production was obtained in presence of Keratin as additional nitrogen source and minimum of (7.22 U/ml) obtained in presence of peptone. *Bacillus subtilis* also produced significant level of keratinolytic enzyme when cultivated in a medium containing feather meal. The isolated *Bacillus sps* produced maximum keratinase when keratinous protein elements were present in the media. Similar result was found for *Bacillus cereus SKH1* when grown in medium containing keratin as the keen nitrogen sources.

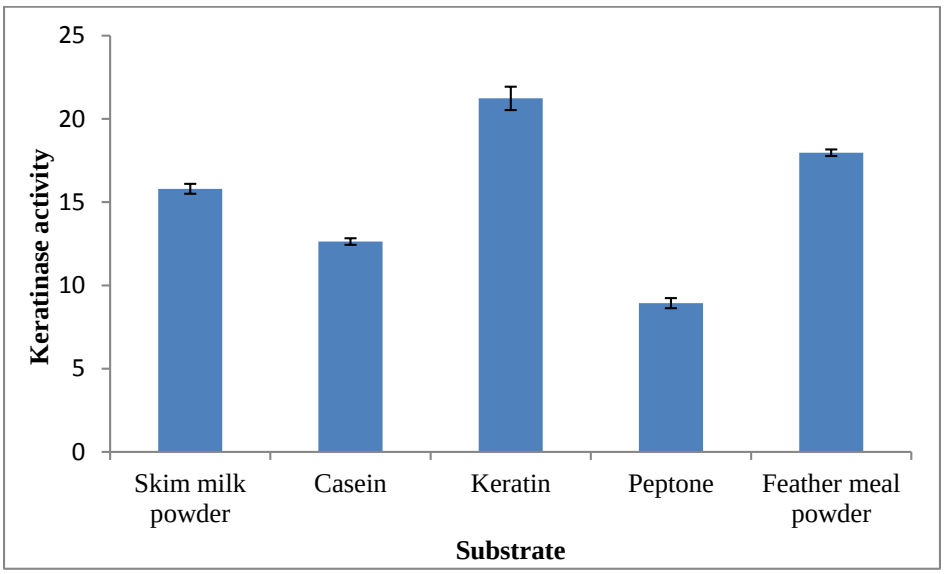

Fig 5: Effect of different substrate on the production of Keratinase enzyme from SNP1 strain

Molecular identification of keratinase producing bacteria

The keratinase producing bacteria were recovered from feather meal agar media. The bacterial stain was further subjected to molecular identification procedure which includes extraction of total DNA, amplification by 16S rDNA primer, finally the comparison of phylogenetic sequence.

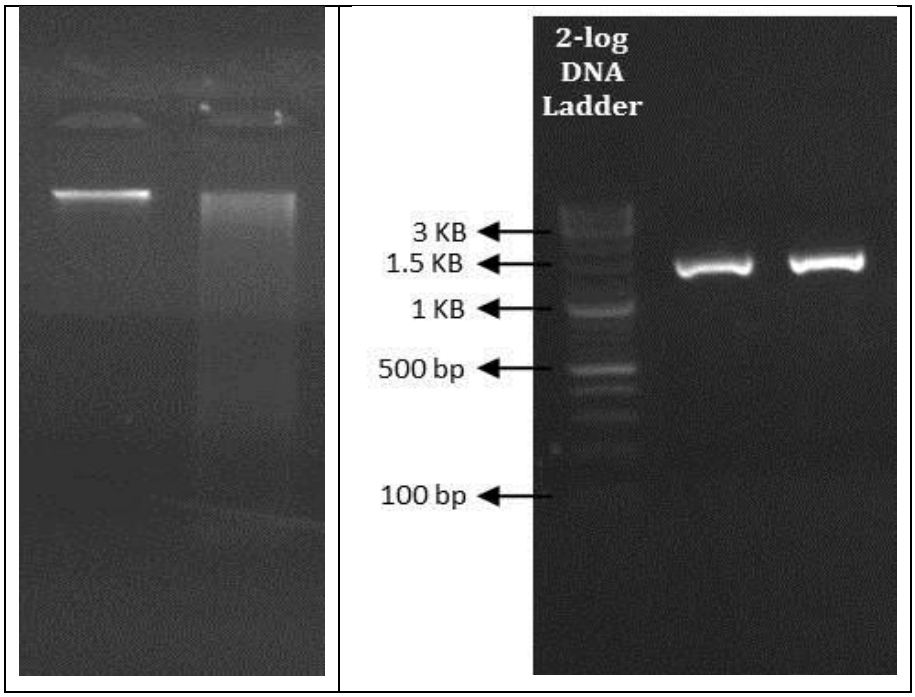

Fig. 6. A: Genomic DNA from bacterial strain using DNA isolation kit loaded in 0.8% agarose gel

B: PCR amplification of 16s rDNA fragment from bacterial sample.

DNA extraction: Total genomic DNA was extracted from keratinase producing bacteria Fig 6A. For further identification the amplification of the genomic DNA was performed using 16S rDNA primer (Fig.6. B). Later, gene sequencingand construction of phylogenetic tree was done for the species identification.

Sequence Analysis: In this work, we have provided the molecular identification of bacteria on the basis of 16S rDNA analysis. The results of the forward and reverse primers were aligned together in Bioedit tool to retrieve the complete aligned sequences. The NCBI BLAST was performed to confirm identity of specimens (Altschul, *et al*.1990). The 16S rRNA gene sequence was compared to the Gen-bank of database using the BLAST. The 16S rRNA gene sequence of the isolate SNP1 showed high levels of sequence similarity with members of the genus *Bacillus.* The 'BLASTn' tool was used for sequence assignment against NCBI database and highest-scoring hit from each query is taken for the bacterial identification. The Blast result predicted based on percent match given bacterial sequence belongs to *Bacillus* genus and alignment of gene sequence with 10 closely related gene sequence was performed and phylogenetic tree was constructed using MEGA X (Fig 7.) The bacterial strain was identified as *Bacillus haynesii*.

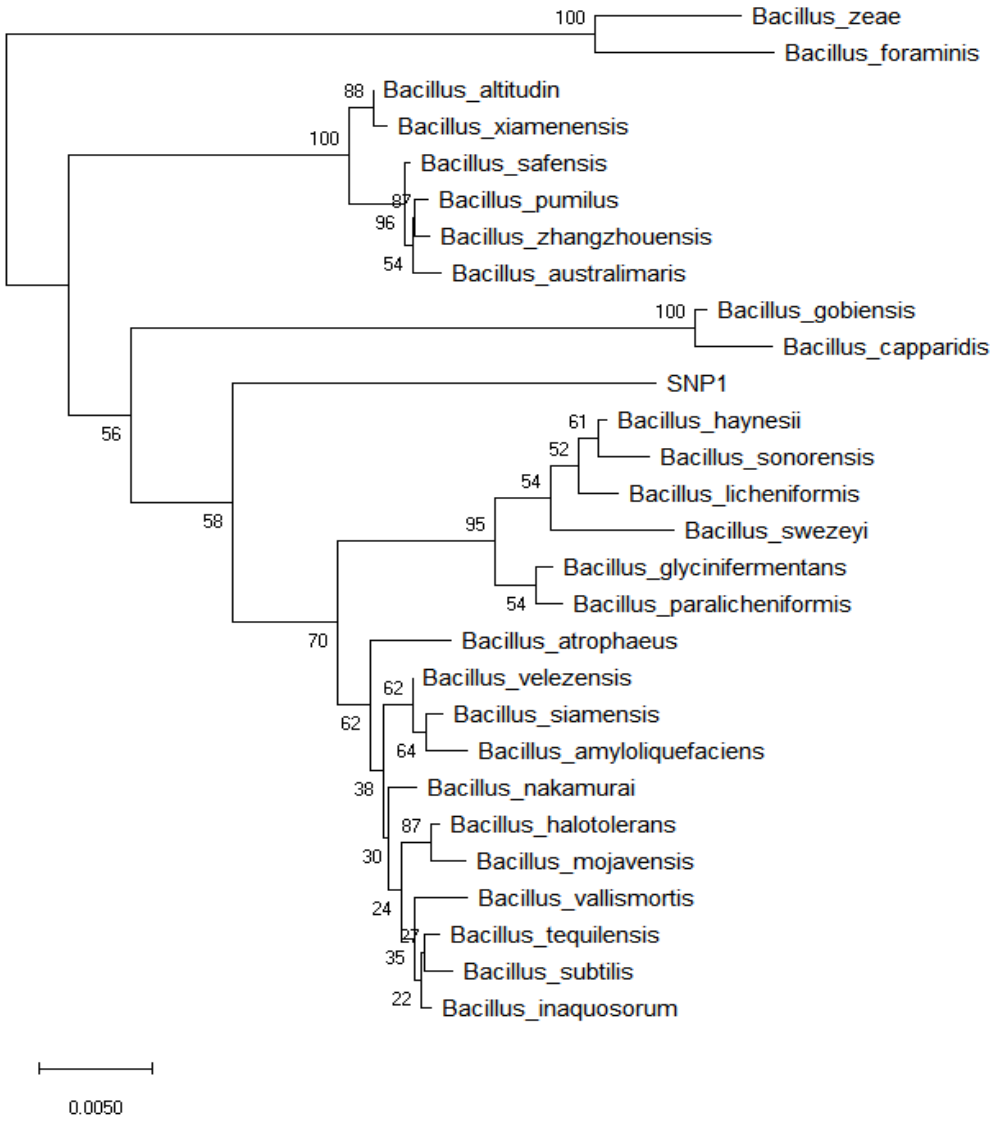

Fig 7. Phylogenetic Tree of Strain SNP1. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.17128572 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 28 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 1501 positions in the final dataset. Evolutionary analyses were conducted in MEGA X

CONCLUSION

The hydrolysis of a feather by *Bacillus haynesii* confirm that the bacterium has a substantial keratinase activity and can break keratin disulphide bonds. Measuring enzymatic activity in seven isolated strains showed that *Bacillus* strain may be the best candidate in various industries.

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