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Isolation and identification of keratin degrading (keratinolytic) bacteria from poultry feather dumping sites

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Abstract

Poultry industries generate a large quantity of feather waste. These feathers contain high quantity of keratin which can be bio-converted into peptides and amino acids using bacteria to make value added products. Therefore, the aim of this study was to isolate and identify keratinolytic bacteria from poultry feather dumping sites at Jaipur city, Rajasthan, India. The samples such as soil and feathers were collected and screened for keratinolytic bacteria. The samples were inoculated on the feather meal agar plates and incubated at 35° C for 24-48 hrs. The culture obtained from the feather meal agar plate was sub cultured on feather meal broth. The bacterium which showed maximum kertainolytic activity was subjected to morphological and biochemical tests. Molecular characterization of the isolated bacteria was done by 16S rRNA sequencing and identified as *Bacillus subtilis* strain AJ. Culture conditions such as pH, incubation temperature, agitation and duration were optimized for maximum feather degradation. The bacteria *B. subtilis* showed maximum feather degradation at pH 7 at 30° C with an agitation of 140 rpm for 96 hrs. Isolation, purification and characterization of keratinase need to be done to assess the industrial application of the isolate.

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Introduction

The dependence of people on non-vegetarian food is largely fulfilled by poultry farm industries. So the feathers are produced in large amount as a waste by these poultry farms and industries (Lasekan *et al.*, 2013). Feathers are generally disposed by land filling or incineration which causes continuous source of environmental pollution (Gradisar *et al.*, 2000; Wang *et al.*, 2005).Feathers contain approximately 90% protein so the feather meal provides an ultimate source of dietary protein supplement for animal feedstuffs (Gupta and Ramnani, 2006; Kornillowicz-Kowalska and Bohacz, 2011) however, limited by incomplete digestion of keratin protein due to its high stability and rigidity (Gupta *et al.*, 2012). The structural rigidity of keratin is mainly depends on tight packing of α-helix (α-keratin) and β sheet (β-keratin) as well as molecular configuration of its amino acids that makes them recalcitrant (Parry and North, 1998; Brandelli *et al.*, 2010). Therefore, keratin shows resistant to common proteolytic digestion by enzymes such as pepsin, trypsin, and papain (Brandelli *et al.*, 2010; Riffel *et al.*, 2011).

The conventional methods such as thermal/chemical treatment are still being in use to degrade native feathers, which lead to ample loss of important amino acids. Keratinases has the wide application in biotechnology to degrade the keratin wastes by non-polluting methods (Nashy and Ahmady, 2012; Paul *et al.*, 2014).The use of microbial keratinases for feather degradation offers more viable, environment friendly, cheap alternative approach as well as upgrade the nutrition value of feather meal. A number of microorganisms have been used for keratinase production including mesophilic fungi and actinomycetes which are the main producers of keratinases (Cedrola *et al.,* 2012; Laba *et al.,* 2013; Shivkumar and Raveendran, 2015). Some species of *Thermophilic bacillus* are also known to produce these enzymes (Williams *et al.*, 1990; Kim *et al.*, 2001). However, the nutritional enrichment of feather meal depends on the keratinase production by specific microorganism with reference to its optimal growth conditions. The aim of this study was to isolate and identify the keratin degrading bacteria (keratinolytic) from poultry feather dumping sites in and around Jaipur, Rajasthan.

Materials and methods

Collection of samples

The samples such as soil and feather were collected from four poultry feather dumping sites viz. Jaipur Zoo (JZ), Ajmeri gate (AG), Sanganeri Gate (SG) and Mathuradaspura Village (MV) in vicinity of Jaipur, Rajasthan, India. The samples were collected using sterile scalpel and transferred to zipper polythene bags for laboratory experiments.

Preparation of feather meal

The native chicken feathers were used for preparation of feather meal as described by Tork *et al.* (2008). Feathers were cut into 3-4 cm small pieces and washed with tap water thoroughly. Defatting of feathers pieces was done by soaking them in a mixture of chloroform: methanol (1:1) for 2 days followed by chloroform: acetone: methanol (4:3:1) treatment for 2 days, solvent being replaced daily. To remove the solvent residuals, feathers were washed several times and air dried. They were then grinded using an electrical blender.

Screening and isolation of feather degrading isolate

From soil sample

The soil samples were collected from poultry sites and were diluted to 10^{-7} – 10^{-8} times in distilled water. The diluted samples were spread on nutrient agar plate and incubated for 24 h at 35° C for microbial growth. Colonies obtained from these plates were further streaked to feather meal agar plate and incubated for 72 h at 35° C. Colonies from samples (JZ, AJ and SG)were able to degrade the keratin showing the clear zone on feather meal agar plate and further selected for the production of keratinase (a type of protease) enzyme by keratinase assay (Gradisar *et al.* 2005).

From feather sample

Feathers were flooded in peptone broth (5 g L^{-1}) and

incubated for 24 h at 35° C. The suspension was streaked on feather meal agar plates containing10 g feather-meal L^{-1}, 0.5 g NaCl L^{-1}, 0.3 g $K_2HPO_4L^{-1}$, 0.4 g KH_2PO_4 L^{-1}, 15 g agar $L^{-1}$and incubated for 72 h at 35°C. Similar to soil samples single colonies having the ability to hydrolyze the keratin (showing the clear zone on feather meal agar plate) were selected for further screening for efficient keratinase activity.

Keratinase activity

The soluble keratin powder used as substrate in keratinase assay was prepared from white chicken feathers by the method of Wawrzkiewicz *et al .,* (1987). In brief, native chicken feathers (10 g) in 500 ml of dimethyl sulfoxide were heated in a reflux condenser at 100°C for 2 h. Soluble keratin was then precipitated by addition of cold acetone (1 L) at −20°C for 2 h, followed by centrifugation at 10000×g for 10 min. The resulting precipitate was washed twice with distilled water and dried at 40°C in an oven. One gram of quantified precipitate was dissolved in 20 ml of 0.05 mol L^{-1}NaOH. The pH was adjusted to 8.0 with 0.1 mol L^{-1} Tris and 0.1 mol L^{-1} HCl and the solution was diluted to 200 ml with 0.05 mol L^{-1} Tris-HCl buffer (pH 8.0).

Enzyme assay

Keratinolytic activity of isolated strain was measured with soluble keratin (0.5%, w/v) by method of Gradisar *et al.*(2005). In brief, the isolate was cultured in whole feather meal medium from a 10^{-7} CFU ml^{-1} for 96 h and crude enzyme was prepared by centrifugation of culture at 10000×g for 10 min. The 1 ml of crude enzyme was incubated with 1 ml of keratin solution at 50°C in water bath for 10 min. and examined for keratinase activity by measuring absorbance at 280 nm against the control (2.0 ml of crude enzyme with 2.0 ml of TCA solution).

One unit (U/ml) of keratinolytic activity was defined as an increase of corrected absorbance at 280 nm (A280), with the control for 0.01 per minute under the conditions described above and calculated by the following equation:

$$U=4\times n\times A280/ (0.01\times 10)\ 1$$

Where n is the dilution rate; 4 is the final reaction volume (ml); 10 is the incubation time (min).The conditions like temperature, pH, substrate concentration and incubation time were also optimized to achieve maximum enzyme production for the maximum feather degradation.

Identification of isolate

The bacteria were identified based on colony morphology, negative staining, Gram staining, endospore staining and biochemical tests such as catalase, growth on MacConkey agar, Indole test, methyl red test, Voges Proskauer test, citrate utilization, H_2S production, gas from glucose, acid from glucose (TSI test), acid from lactose (TSI test), casein hydrolysis, gelatin hydrolysis, starch hydrolysis, urea hydrolysis, nitrate reduction and Oxidase test.

Light Microscopy

The bacterial morphology analysis was carried out by compound light microscope (Labomed Vision 2000). In brief, the bacterial cells were fixed to clean slides by transferring a loop of the culture and smear was prepared in distilled water for Gram staining, negative staining (Nigrosin dye) and endospore staining (malachite green). Gram staining was performed as per standard method whereas negative and endospore staining were performed using standard method given by Dorner (1926). For further morphology confirmation slides were observed and analyzed by Scanning Electron Microscope.

Scanning electron microscopy (SEM)

The bacterial cells were harvested through centrifugation at 5000 rpm for 10 minutes. The pellet was washed in 0.1M phosphate buffer (pH 7.4) and fixed in a mixture of 2% paraformaldehyde and 2.5% Glutraldehyde in 0.1 M phosphate buffer for overnight at 4° C after centrifugation. The pellet was again resuspended after discarding the fixative and observed using scanning electron microscopy (ZEISS-EVO-18) after gold coating (QUARUM-Q150AS) at

University Science Instrumentation Centre, University of Rajasthan, Jaipur.

Molecular characterization of strain

The molecular characterization of the selected species was carried out by nucleotide sequencing of 16S rRNA genes with the help of Zeal Biologicals, Andhra Pradesh, India. The bacterial DNA samples were prepared by using a pure cultivated bacterium for identification. The colonies are picked up with a sterilized toothpick, suspended in 0.5 ml of sterilizes saline in a 1.5 ml centrifuge tube and centrifuged at 10,000rpm for 10 min. The pellet was suspended in 0.5 ml of InstaGene Matrix (Bio-Rad, USA) and incubated at 56 °C for 30 min followed by heatingat100 °C for 10 min. The polymerase chain reaction (PCR) of the 16s rRNA genes were performed using the forward primer 27F 5'-AGA GTT TGA TCM TGG CTC AG-3' and reverse primer 1492R (TAC GGY TAC CTT GTT ACG ACT T) using a EF-Taq (SolGent, Korea). In brief, PCR reaction was carried out in 20 µl of aliquots containing 25 ng of bacterial DNA. The PCR profile was as follows: Initial denaturation at 95 °C for 2 min followed by 35 amplification cycles at 94 °C for 45 sec, 55 °C for 60 sec, 72 °C for 60 sec and final extension were carried out at 72° C for 10 min. The amplification products about 1,400 bp were purified with a multiscreen filter plate (MilliporeCorp., Bedford, MA,USA). The unincorporated PCR primers and dNTPs were removed from PCR products by using Montage PCR Clean up kit (Millipore).Sequencing reaction was performed using a PRISM Big Dye Terminator v3.1Cycle sequencing Kit. The DNA samples containing the extension products were added to Hi-Diformamide (Applied Biosystems, FosterCity, CA).The mixture was incubated at 95 °C for 5 min., followed by 5 min. on ice and then analyzed by ABI Prism 3730XL DNA analyzer (Applied Biosystems, Foster City, CA).Sequences were further analyzed using BLAST from the National Center of Biotechnology Information (NCBI) database. Phylogenetic analysis of sequences was done with the help of Seaview software (Gouy *et al.*, 2010).

Results

The bacterial colonies obtained from four different sites showed heterogeneous (AJ, SG) as well as homogeneous (JZ, MDV) colonies and were found to be positive for feather degradation.

Table 1. Different samples showing the colony type and their keratinase activity.

Name of Samples	Types of Colonies	Growth on Agar Plate	Keratinase Production	Keratnolytic Activity
AJ	Heterogenous	Positive	++++	++++
JZ	Homogenous	Positive	+++	++
SG	Heterogenous	Positive	+++	+++
MDV	Homogenous	Positive	--	--

++++: maximum enzyme production, +++: Medium enzyme production, ++: Low enzyme production, -- : No enzyme production.

The heterogeneous colonies showed the presence of both coccus and rod shaped bacterium whereas rod shaped bacteria were found to be present only in homogeneous colonies.

The rod shaped bacterium demonstrated more pronounced zone of clearance on feather meal agar plate. The colonies obtained from the AJ sample (both soil & feather) showed the highest feather degradation activity compared to other samples (JZ, SG, MDV). Therefore, the colonies ofAJ samples were further selected, sub cultured in nutrient broth and used for isolate identification and enzyme production (Table 1 and Fig. 1).

Identification of the isolate

The bacterial colony collected from Ajmeri Gate (AJ) gate samples were characterized by morphological as well as biochemical parameters.

Table 2. Morphological and physiological Characteristics of Keratinase Producing Bacterial Strain (**AJ).**

Cultural characteristics	
On Feather meal Agar plate Growth on Feather Meal Browth	White, Abundant mucilage, Abundant, Irregular, Serrate, flat and Translucent Surface Growth-Pellicle, Clouding-light, Sediment-flacky
Morphological characteristics	
Shape	Rods
Gram Stain	Positive
Spore Formation	Positive
Biochemical characteristics	
Growth on MacConkey agar	Negative
Indole test	Negative
Methyl red test	Negative
Voges Proskauer test	Negative
Citrate utilization	Negative
H_2S production	Positive
Acid from glucose (TSI test)	Negative
Acid from lactose (TSI test)	Positive
Gelatin hydrolysis	Positive
Casein hydrolysis	Positive
Urea hydrolysis	Positive
Catalase	Positive
Nitrate reduction	Negative
Oxidase test	Positive
Starch Hydrolysis	Positive

The findings revealed that the colonies were homogenous in nature visualized by colony appearance; culture morphology on agar plate revealed white, abundant, mucilage colonies with clear zone on feather meal agar plates and considered to be of the same organism. The colonies were irregular in shape showed serrate margins with flat elevation.In feather meal broth colonies were translucent, slight cloud forming, pellicle, and shows flaky sediment (Fig. 1B).

Table 3. Conditions optimized for keratin degradation and keratinase production.

SR No.	Parameters	Optimized Conditions	Keratinase Production in u/ml
1	pH	7	45
2	Temperature	30 ˚C	96
3	Incubation Time	4 Days	48
4	Agitation Speed	140 rpm	48

The staining properties revealed that the bacterium is Gram positive, spore forming and rod shaped. The biochemical characteristics such as catalase, starch hydrolysis, citrate utilization, H_2S production, acid from lactose (TSI test), casein hydrolysis, gelatin hydrolysis,oxidase test etc.were found to be positive whereasindole test, growth on MacConkey agar, methyl red test, Voges Proskauer test, acid from glucose (TSI test), nitrate reductionshown a negative results for the isolated strain (Table 2).

Morphological identification by SEM

Scanning electron micrographs clearly depicted the morphology of individual cells, as well as the spatial orientation of cells within the colony; cells are arranged in diplobacillus and streptobacillus forms. Bacterial cells clearly showed the binary fission. (Fig. 2).

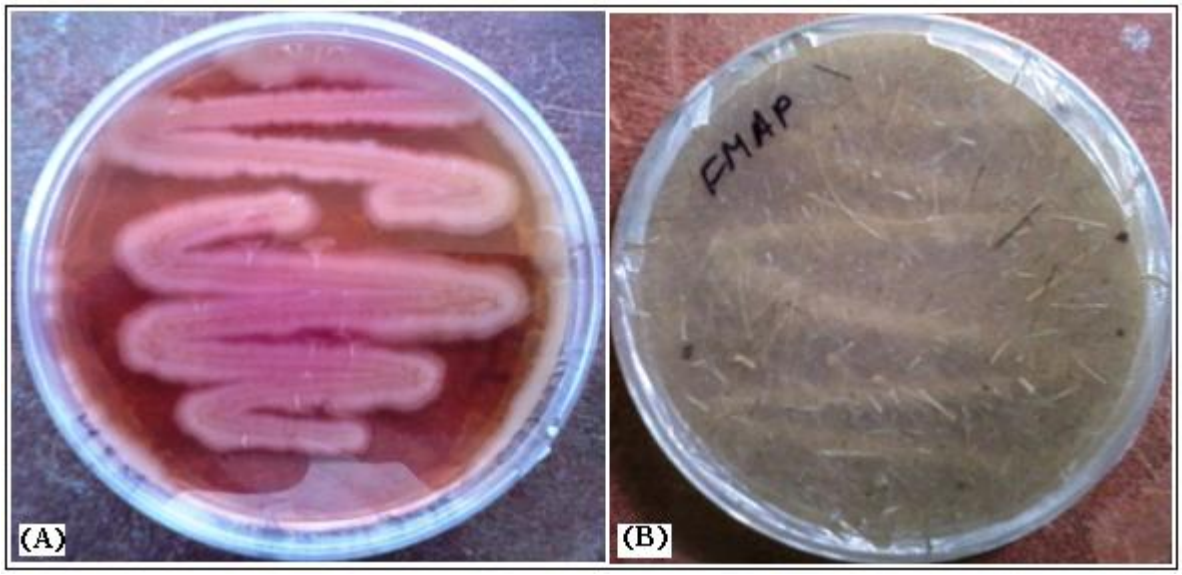

Fig. 1. (A) Growth of isolated strain on MSA plate and (B) Growth of isolated strain on feather meal agar plate.

Optimization of conditions for maximum keratinse production

The different incubation parameters were optimized for maximum degradation of keratin and keratinase production (Table 3). The pH range of 5-9 was scanned for maximum microbial growth and keratin degradation. Maximum growth and keratinase production was observed at pH 7 (Fig.3a). Similarly, a temperature range of 20°-50° C was set for microbial growth in feather meal broth.

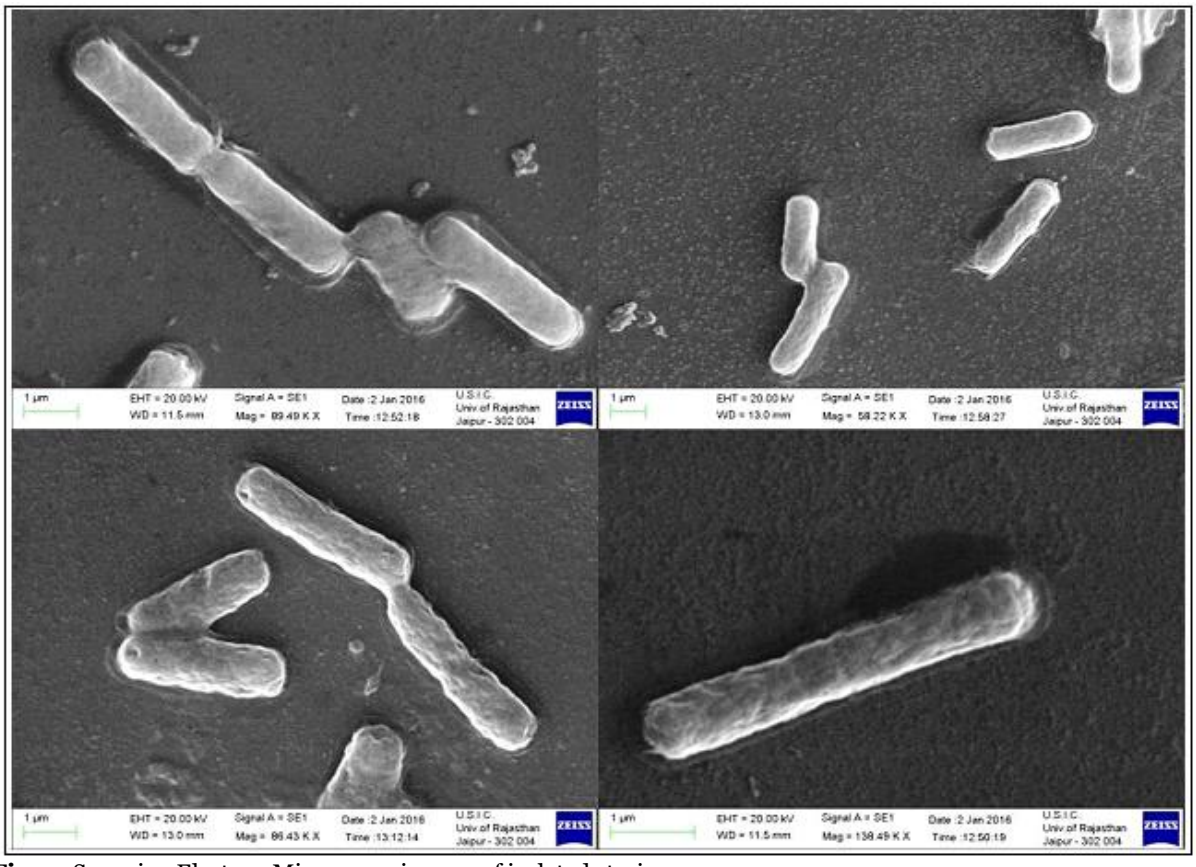

Fig. 2. Scanning Electron Microscope images of isolated strain.

The maximum growth and keratinase production was observed at 30° C (Fig. 3b). While observing the effect of incubation time of isolate on keratin biodegradation, the maximum growth and keratinase production was observed after 96 hours (4 days) of incubation period (Fig. 3c). Various agitation speed (rpm) were used to optimize the enzyme activity of isolated strain. The maximum enzyme production was observed at 140 rpm (Fig. 3d).

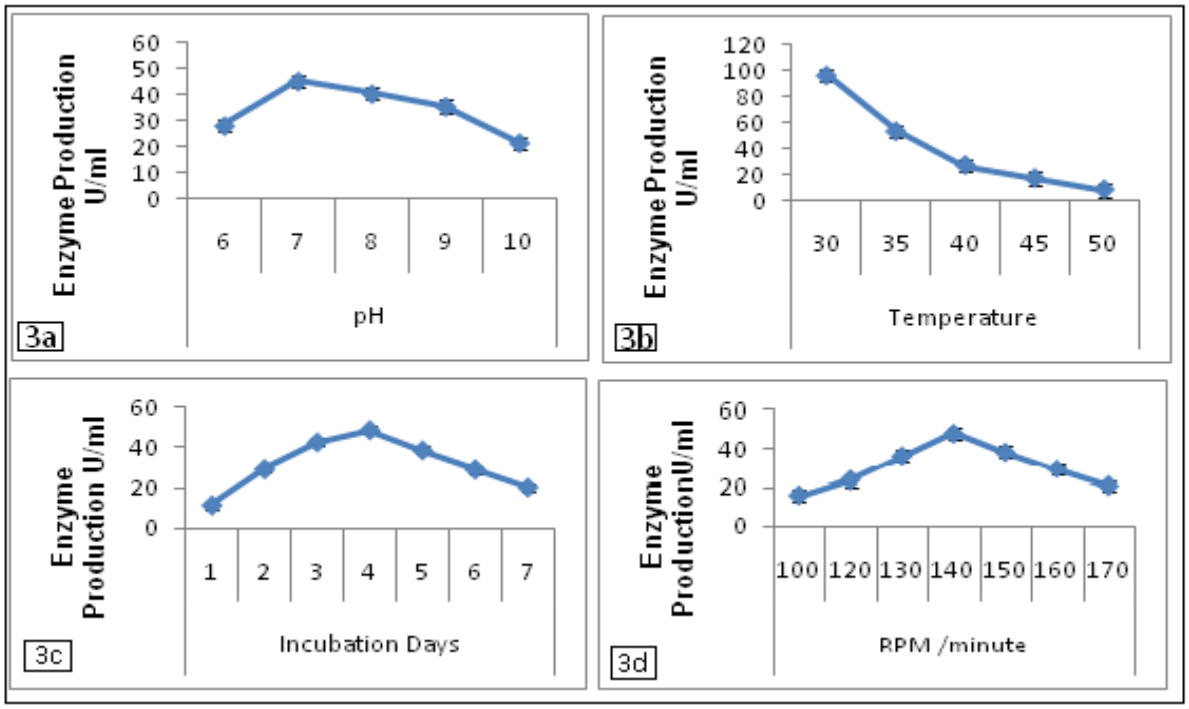

Fig. 3. (3a) Effect of pH, (3b) Temperature, (3c) Incubation Time and (3d) Agitation Speeds on keratinase production of isolated strain. Each point represents the mean value ± SEM.

Identification of bacterial isolates based on 16S rRNA genes

The genus determination of the isolate based on sequences of the 16S rRNA gene showed 96% identity with *Bacillus subtilis* strain YC2 (Accession No. HM770883.1) (Fig.4). Hence, the isolated strain was identified as *Bacillus subtilis*.

Discussion

The keratinolytic activity of microorganisms has been widely documented and out of these most of the isolates being confined to the genera *Bacillus* (Giongo *et al.,* 2007; Cortezi *et al.,* 2008) and *Streptomyces* (Tapia and Simoes,2008). Studies have also reported keratinolytic activity in thermophiles (Riessen and Antranikian,2001; Nam *et al.,*2002) and alkalophiles (Gessesse *et al.,* 2003; Mitsuiki *et al.,*2004). Almost all keratinases are inducible enzymes and various keratin-containing materials such as feathers, hair and wool were used as substrates for keratinase production. Among them, feathers were the mostly utilised substrate (Gupta and Ramnani, 2006). Therefore, by using the appropriate microorganism for feather degradation, the feather keratin could be utilised effectively. Moreover, a lot of microorganism can be inoculated for extended fermentation after peptides and amino acids removal in industrial setup. The products of feather degradation could have a potential to be used as a feed supplement owing to high protein and amino acid content. This could further increase the profit of the poultry industry and utilize feathers instead of disposing them as waste.

In the present study, *B. subtilis* was isolated from both feather and soil samples collected from poultry dumping sites, had shown maximum keratinolytic

activity by utilization of carbon and sulphur as primary source of energy from feathers.The degradation of keratin produced ammonia leads to increase in pH indicates the keratinolysis. In the present study, the *B. subtilis* showed maximum keratin degradation and maximum enzyme production at neutral pH 7. Shivkumar and Raveendran (2015) also reported that the maximum degradation of keratin at pH 7.5. A hike in pH of the medium was observed from pH 7.0 to 9.0 following the 4 days incubation. Several other researchers reported that maximum keratinase production by feather-degrading bacteria was achieved at either neutral or basic conditions (Cheng *et al.*, 1995; Riffel *et al.*, 2003; Werlang and Brandelli, 2005). In contrast, keratinase activity was also reported at extremes, both at alkaline pH or at slightly acidic pH (Mitsuiki *et al.,* 2004; Balaji *et al.,* 2008).

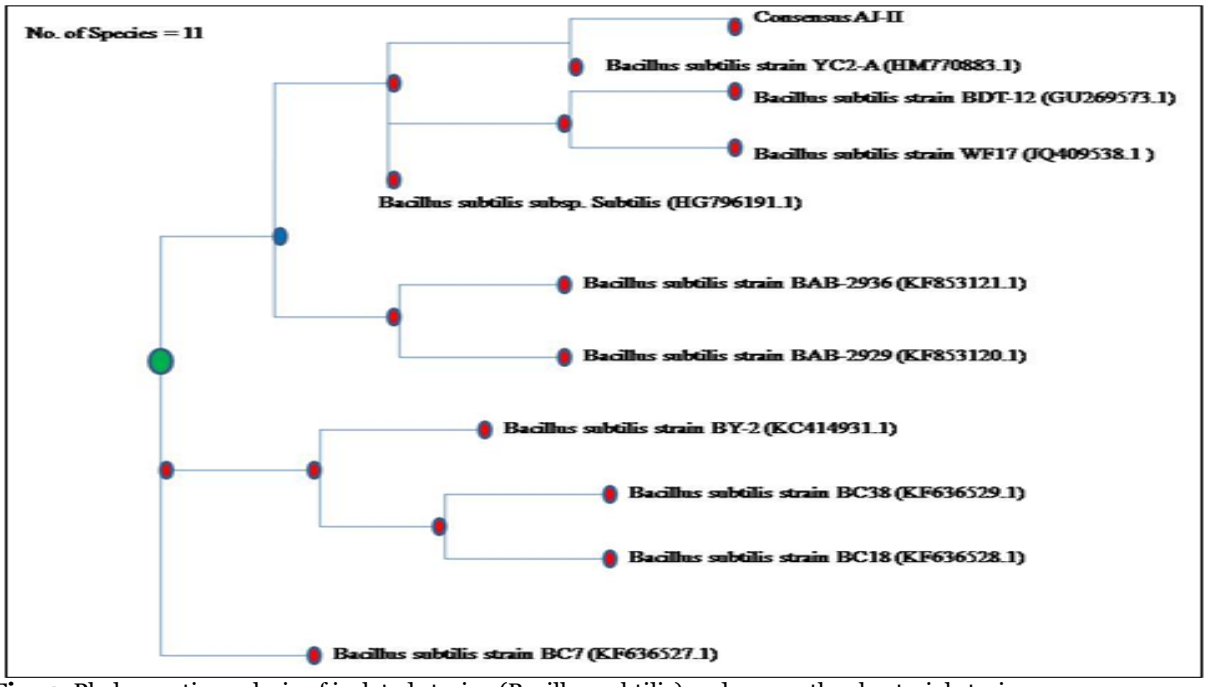

Fig. 4. Phylogenetic analysis of isolated strains (Bacillus subtilis) and some other bacterial strains.

The optimal proteolytic activity of *B. subtilis* was observed at 30°C (mesophillic temperature) for *B. subtilis*. Similar activities were found in *Thermoanaerobacter* and *Fervidobacterium spp.* at 28 to 50°C (Friedrich and Antranikian, 1996; Rissen and Antranikian, 2001; Nam *et al.,* 2002). The keratinase activity has also been reported for psychrotrophic bacteria, *Stenotrophomonas sp.* D1 at 40° C (Yamamura *et al.,* 2002). A variation in incubation period has also been reported and ranging from 24 h to several days for keratin degradation (Kaul and Sambali, 1999; Ramnani and Gupta, 2004). The isolated *B. subtilis* sps showed maximum feather degradation activity at 96 h incubation. Similar results were observed for other *Bacillus spp.* which have shown highest keratin degradation and enzyme production at incubation for 4 days (Pandian *et al.*, 2012).*Bacillus megaterium* also degrade the keratin at condition of 72 h, at 35° C for 3 days (Saibabu *et al.*, 2013), it was also reported that *Streptomyces sp. MS-2* produced maximum keratinase activity after 72 h (3 days) (Mabrouk, 2008). However, *Streptomyces lavendulae*required 7 days (Chitte *et al.*, 1999) and *B. licheniformis* PWD-1 at 50°C in 10 days (Williams *et al.*,1990) for entire feather degradation.

The maximum enzyme production was observed at agitation speed of 140 rpm following the incubation of 96 h at 30° C for *Bacillus subtillis* AJ strain in the present study. Gram negative bacterial spp.*C. indologenes A22, A. hydrophila K12 and S. marcescens P3*have also shown maximum keratinase

activity on feather meal broth at120 rpm during 48 h at 30ºCincubation (Bach *et al.*, 2015).Likewise, Maximum enzyme production were reported at 150 rpm for the spp. of *Streptomyces sp.* MS-2 and *Bacillus amyloliquefaciens* after the incubation 72 h at 35- 40º C respectively (Mabrouk, 2008; Cortezi *et al.*, 2008).

Conclusion

A feather-degrading bacterium was isolated from the chicken feathers dumping sites in Jaipur. Based on morphological, biochemical and phylogenetical characteristics, isolated strain belongs to the family Bacillaceae of the domain Bacteria. The nucleotide base sequencing of 16S rRNA of the isolate were further analyzed using BLAST from the National Center of Biotechnology Information (NCBI) database and phylogenetic tree was prepared. The isolate showed 96% identity with *Bacillus subtilis* strain YC2 Accession No. (HM770883.1) and confirmed as *Bacillus subtilis*

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