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Screening of Bacterial Isolates and their Combinatorial Effect on Poultry Feather Degradation

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ABSTRACT

Poultry feather waste contribute a major challenge in the urban solid waste management across the globe. Feather is composed of 90 % keratin which is recalcitrant in nature for biodegradation. However, keratinolytic bacteria are found to have feather degradation potential. As feather is rich in protein, a suitable biodegradation process will be useful in the fields of agriculture and allied industries. The current practice of feather meal production is of low industrial viability due to the use of alkali and thermal processes. On the other hand, fermentation based feather degradation has more industrial viability as they are cheap and involve ecologically guarded process. Hence the present study focused on exploring potential bacterial isolates for degradation of poultry feather. Bacteria were isolated from feather dumping pit of different poultry farms around Bengaluru and screened for feather degradation potential. The isolates were identified using 16S rRNA gene sequencing and BLAST analysis. Best three isolates viz. *Pseudomonas* KDB2, *Klebsiella* KDB6 and *Klebsiella* KDB16 were used individually and in combination for feather degradation. The experimental results showed that, on 10th day of the incubation, maximum keratinolytic activity (68.39 U/ml) and degree of degradation (70%) was observed in the treatment received with all three best isolates in combination compared to other treatments. As there are diverse group of bacterial species involved in the feather degradation process, the isolates that we report here could also be of potential use in specific industrial and agricultural applications.

Keywords Screening, Bacterial consortia, Poultry feather, Degradation.

INTRODUCTION

Poultry feather waste is one of the critical problems as part of urban solid waste management across the globe. Annually 24 billion chickens were killed across the world which is discarding 8 to 8.5 billion tons of poultry feather, of which India's contribution alone is 350 million tonnes (Gupta *et al*. 2011). Feather waste generated from poultry farms, poultry process-

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ing industries, leather industry and slaughter houses, are generally considered as waste. These waste acts as habitats for sustaining many pathogenic microorganisms and emits various pollutants like ammonia, nitrous oxide, hydrogen sulphide (Tamreihao *et al.* 2019). Feather contains a significant quantity of protein which is less utilized and can be utilized in various agriculture and industrial process, it approximately contains 15% N, so that it can be used in agricultural field as nitrogenous fertilizer and a potential Biofertilizer (Joardar and Rahman 2018). Over the last 30 years, research has been conducted to improve the agronomic utilization of animal wastes, including poultry wastes, *via* composting. The nutrients are also biologically and chemically stabilized to more stable organic forms before application to agricultural soils (Tiquia and Tam 2002). These changes could help to degrade and convert waste feathers into a useful resource (compost), which improves soil fertility, in industries it is mainly used to produce feather meal, hence attention should be given for recycling or utilizing the poultry waste in a scientific way. Poultry feather is composed of about 90 % keratin, (Lange *et al.* 2016) which is an insoluble structural protein, with tightly packed β-sheet polypeptide chain and extensively cross-linked with di-sulphide bonds, hydrogen bonds and hydrophobic interactions (Riffel *et al.* 2003). Due to the recalcitrant nature of keratin (Godheja *et al*. 2015), which is resistant to common proteolytic enzymes such as trypsin, pepsin and papain, specific keratinases produced by keratinolytic bacteria are found to be effective (Ningthoujam *et al*. 2018). As the current practice of feather meal production using alkali hydrolysis and thermal treatment is of low-nutritive-value and high energy demanding, fermentation based keratin production has more industrial viability as they are cheap and involve ecologically guarded process (Sahoo *et al.* 2017). Therefore, in fermentation technology, keratinase producing microorganisms have prominent applications in tanneries, sewage treatment, food, agriculture, textiles, medicine and cosmetics industries (Jeevana Lakshmi *et al.* 2013, Joardar and Rahman 2018, Tiquia and Tam 2002, Verma *et al*. 2017). Hence, as there should be a continuous exploration of novel microorganisms and study their potentials under different environmental situations, this work was aimed to explore kerationolytic bacterial isolates to degrade poultry feather.

MATERIALS AND METHODS

The experiment was conducted in the Department of Agricultural Microbiology and Plant Biotechnology, University of Agricultural Sciences, GKVK, Bangalore 560065, India during the year 2019 to 2020.

Isolation of bacteria

For the isolation of feather degrading bacteria, soil samples at 8-10 cm depth were collected from feather dumping pit of different poultry farms around Bengaluru. Ten gram of soil samples was homogenized in 90 ml of sterile saline and serially diluted up to 10-6. The diluted suspension (0.1ml) of dilutions 10-4 onwards were inoculated (spread plate method) on tryptic soya agar (TSA) and incubated at 37± 2°C for 24 hr (de Oliveira *et al.* 2016). Pure culture technique was followed for colony selection and preservation.

Primary screening for protease activity

The isolated bacteria were screened for protease activity in skim milk agar (SMA) at 30°C for 24hr. Plates were observed for clear zones around the colonies, which indicates the protease activity. Then clear zone diameter was measured to understand the relative potential of the bacterial isolates (Sekar *et al*. 2016).

Screening for keratinase activity

To test the keratinase activity, bacterial isolates with protease activity on SMA were inoculated into feather meal agar (FMA) plate with the following composition; NH_4Cl - 0.5 g, NaCl- 0.05 g, $MgCl_2$. $6H_2O$–0.1 g, K_2HPO_4 – 0.03 g, KH_2PO_4 – 0.04 g, yeast extract – 0.1 g, feather meal – 1g. The plates were incubated for 72 hr at 40°C. The colonies producing clear zones on this medium were selected for further studies (Sekar *et al*. 2016).

Identification of the bacterial isolates

Molecular identification of the bacterial isolates was done by using 16s rRNA gene sequencing method. Bacterial genomic DNA was isolated (Birnboim

Fig. 1. Zone of clearance by feather degrading bacterial isolates on SMA.

and Doly 1979) and Polymerase Chain Reaction of purified DNA was performed (Bartlett and Stirling 2003) with two primers (22 bp forward primer 5' GGAGAGTTAGATCTTGGCTCAG 3' and 20 bp reverse primer 5'AAGGAGGGGATCCAGCCGCA 3') already reported for 16S rRNA gene sequence from the NCBI (http://www.ncbi.nlm.nih.gov). The primers were custom synthesized by Sigma-Aldrich (Sigma, Bangalore) and amplicon sequenced by Biokart India Pvt Ltd Bangalore. Sequence obtained thus was subjected to BLAST analysis in 16S rRNA sequence database (www.ncbi.nlm.nih.gov/blast).

Evaluation of the bacterial isolates for poultry feather degradation

Inoculum preparation

One hundred ml sterile TS broth was inoculated with a loop full of each of the three bacterial isolates and incubated for 24 hrs on a rotary shaker (150 rpm) at 30°C.

Poultry feather preparation

Clean and defatted poultry feathers were initially cut to an average size of 1-3 cm and dried for 3 days at 50 °C. Dry feather was ground, which looked like cotton wool, using a laboratory grinder and stored in aseptic condition at 4 °C.

Experimental setup

Two parallel experiments were setup each one for continuous progressive sampling and for destructive sampling for 14 days under identical conditions, with eight treatments (Individual isolate and in combinations with one control), in three replications to evaluate the potentiality of the selected bacterial isolates for feather degradation. For continuous progressive sampling, the experiment was set up in the laboratory using 250 ml conical flask with 100 ml mineral medium (K_2HPO_4 - 0.3g, NH_4Cl - 0.2g, $MgSO_4.7H_2O$ - 0.2 g, NaCl - 0.5 g, $FeSO_4.7H_2O$ - 0.01g, KH_2PO_4 – 0.4g), 10% chicken feathers (as sole source of carbon and nitrogen) and a total of 1 ml inoculum (109 cfu/ml) individually and in combination with pH adjusted to 7.0. The experiment was performed at 30 in °C an orbital shaker (125 rpm) for 14 days and samples were withdrawn at every 48 h to determine the, pH, soluble protein, proteolytic and keratinolytic activity (Arima *et al*. 1970, de Oliveira *et al.* 2016). To study the degree of degradation, destructive sampling experiment was setup using 50 ml test tube with 25 ml mineral medium, 10 % chicken feathers and a total of 0.25 ml inoculum (10^9 cfu/ml) individually and

Table 1. Screening of bacterial isolates based on the production of keratinase and protease.

Sl. No.	Isolates	Keratinase clearance index	Protease clearance index
1.	KDB 1	1.25 ±0.02^{g}	1.25 ± 0.02^{hi}
2.	KDB 4	1.25 ±0.02^{g}	1.23 ± 0.03^{i}
3.	KDB 2	1.88 ±0.03^{b}	1.85 ± 0.04^{b}
4.	KDB 6	2.10 ±0.04^{a}	2.00 ± 0.04^{a}
5.	KDB 8	1.50 ±0.03^{e}	1.66 ± 0.03^{de}
6.	KDB 15	1.66 ±0.03^{d}	1.33 ± 0.03^{gh}
7.	KDB 13	1.42 ±0.02^{ef}	1.45 ± 0.03^{f}
8.	KDB 14	1.36 ±0.02^{f}	1.36 ± 0.03^{fg}
9.	KDB 16	1.77 ±0.03^{c}	1.83 ± 0.04^{bc}
10.	KDB 18	1.44 ±0.02^{ef}	1.33 ± 0.03^{gh}

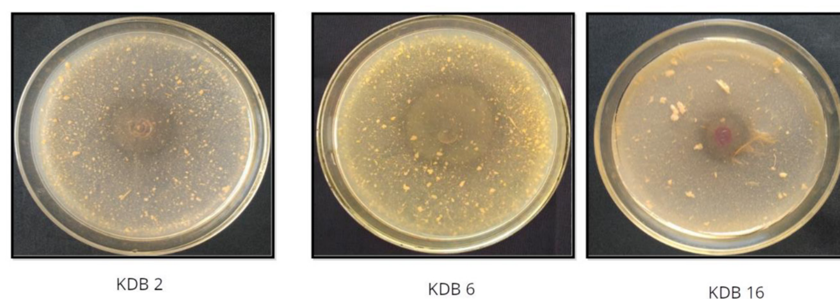

Fig. 2. Zone of clearance by feather degrading bacterial isolates on FMA

in combination with pH adjusted to 7.0. The experiment was performed at 30 for 14 days, and °C samples were withdrawn at every 48 and the residual feather was washed, dried and scaled to calculate degree of degradation (Basheer and Umesh, 2018).

RESULTS AND DISCUSSION

Isolation, screening and identification of keratinolytic bacterial isolates

Forty bacterial colonies were isolated (named as KDB 1 to 40) from different locations of poultry farming units in Bangalore as the soil from feather dumping pits are found to be good source of feather degrading bacteria (Kim and Patterson 2000). Out of 40 isolates, 15 isolates showed positive by forming clear zone around the colony on SMA medium, which indicates the proteolytic activity of the bacterial isolates. Among the 15 positive isolates, KDB2, KDB6 and KDB16 showed maximum clear index compared to others (Table 1, Fig. 1). Further, all 15 isolates were also subjected to secondary screening on FMA medium and observed the similar results, where KDB2, KDB 6 and KDB16 showed maximum clear index, indicating keratinolytic activity of the bacterial iso-

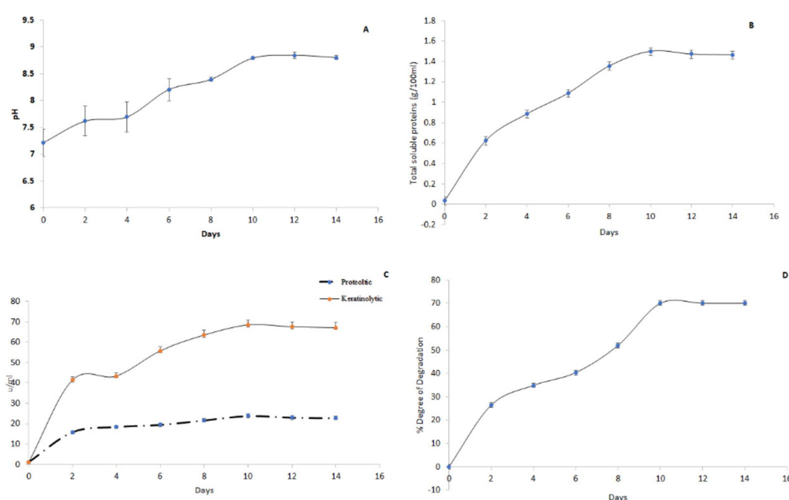

Fig. 3. Changes in the pH (A), total soluble proteins (B), proteolytic and keratinolytic activity (C) and degree of degradation (D) during the poultry feather degradation process.

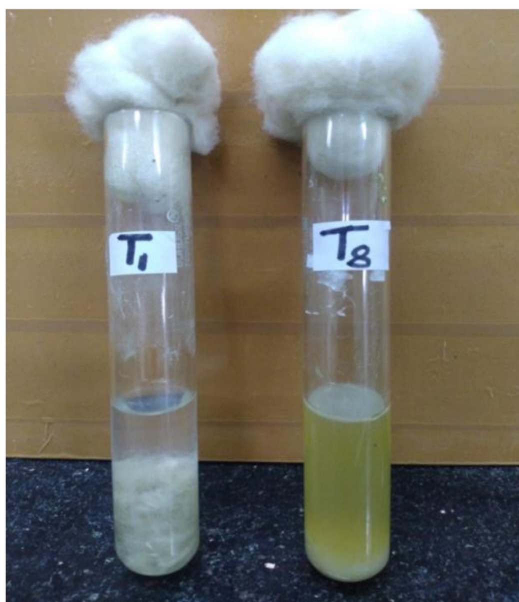

Fig. 4. Treatment comparison on 10th day, T_1 - Control and T_8 - 10th Day (with three bacterial isolates).

lates (Table 1, Fig. 2). Three best isolates viz., KDB2, KDB6 and KDB16 were subjected to 16s rRNA gene sequencing for identification. The 16 s rRNA gene sequences of all three best isolates were subjected to BLAST search in NCBI's nucleotide database. The sequence BLAST search with each of the isolates resulted similarity with more than 100 accessions from the database. Out of more than 100 similarity hits, KDB2 had 100 % sequence similarity with six *Pseudomonas* sp., KDB6 had 100 % similarity with one *Klebsiella* sp. and KDB16 had 100% similarity with 99 *Klebsiella* sp. The sequence was submitted to the NCBI Gene Bank with the accession number MT734026 (KDB2), MT734025 (KDB6) and MT734024 (KDB16). Feather degradation has been studied extensively in the recent past due to its recalcitrant nature and threats regarding environmental and health issues. In the last decade, many reports showed the potential use of *Bacillus* sp. besides other genera including *Pseudomonas*, *Brevibacillus*, *Micrococcus, Xantnthomonas* (Li 2019). The current study as well as previous reports show that the diverse bacterial populations are involved in the degradation of poultry feathers. Therefore, there are ample opportunity for selection of microbial source when poultry feather degradation is to be integrated with other industrial process as well as agricultural application.

Feather degradation potential of bacterial isolates

Based on the results obtained from previous analysis, the isolates viz. *Pseudomonas* KDB2 *Klebsiella* KDB6 and *Klebsiella* KDB16 were tested individually as well as in combination for their ability to degrade the poultry feather. Inoculation of feather degrading bacterial isolates either individually or in combination increased the growth media pH value upto 8.84 compared to the control treatment which is an indirect evidence of buffer action due to the degraded peptides and amino acids by keratinolytic bacterial isolates. Fig 3-A shows the changes in pH value in the feather degradation system with a combination of all three isolates which was found to be the best treatment in which maximum degradation was observed. The present results are in compliance with some of the previous studies wherein higher keratenolytic activity was observed in the pH range from 7 to 9.5 by various isolates (Friedrich and Antranikian 1996, Jain *et al*. 2012, Kim *et al.* 2001, Sahoo *et al*. 2017, Suntornsuk and Suntornsuk 2003). The total soluble protein content during feather degradation was significantly highest in treatment T8 wherein combination of all three isolates (*Pseudomonas* KDB2 , *Klebsiella* KDB6 and *Klebsiella* KDB16) are used. A highest total soluble protein (1.5g/100ml) was observed on 10th day of incubation compared to all other treatments (Fig 3-B). The increase in total soluble protein content indicates the degradation of complex keratein structure. It has been previously reported that, the feather degradation is corroborated by the increase in soluble protein which is an indirect measure of degradation of feather keratin (de Oliveira *et al.* 2016, Bishmi *et al*. 2015). The same trend was observed in all treatments with different rate. In all the treatments the total soluble protein content was found increased upto 10th day of incubation and subsequently stable. Besides analysis of total soluble protein as a measure of feather degradation, proteolytic and keratinolytic activities were also measured and found in accordance with the formation of total soluble protein. On 10th day of the incubation, maximum Proteolytic (23.81

U/ml) and keratinolytic (68.39 U/ml) activity was observed in the treatment wherein combination of all three bacterial isolates (*Pseudomonas* KDB2, *Klebsiella* KDB6 and *Klebsiella* KDB16) were used (Fig 3-C). Based on the total soluble protein, proteolytic and keratinolytic activities, gravimetric estimation of degree of feather degradation was also performed to understand the degradation potential of isolates. The gravimetric estimation of residual feathers showed that, the degradation was started from 2nd day of incubation onward. A linear response in degradation was observed in all the treatments at different rate. The maximum degradation (70%) (Fig 3-D and 4) was observed on 10th day in the treatment with all three inoculants in combination. Incubation period plays a major role in keratinolytic activity of microorganisms and it differ from genera to genera. Many studies reported the optimum incubation period for keratinolytic activity in the ranges of 3 – 11 days (Kanchana 2012, Me *et al*. 2017).

In the present study, isolation and screening of feather degrading bacteria from poultry feather dumping pits has lead to the identification of three potent bacterial genera viz. *Pseudomonas* KDB2, *Klebsiella* KDB6 and *Klebsiella* KDB16 based on 16 s rRNA gene sequencing and BLAST analysis. However, further studies are required for species level identification. The degradation potential evaluation showed that, these isolates are performing better in combination than that of their individual performance. The degradation potential of bacterial isolates of the present study is on par with the previously reported bacterial isolates. Previous reports as well as the results of the present study show that, there are diverse group of bacterial isolates involved in the feather degradation process. The isolates that we report here could also be of potential use and hence a better combination of bacterial consortia that suit specific industrial and agricultural applications may be developed for better utilization of poultry feather waste.

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