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Micrococcus luteus: A Potential Keratinolytic Bacteria Isolated from Poultry Waste Dumping Site at Namakkal District, Tamil Nadu, India

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Abstract: In this study, the experimental soil samples were collected from poultry waste dumping site at Namakkal district, Tamil Nadu, India. From the five soil samples, 15 bacterial colonies were isolated based on Keratinolytic activity with source of agar nutrient medium, nitrogen, carbon and feather meal powder. After the incubation period only 6 isolates showed clear zone of hydrolysis around colony in feather meal agar medium incubated at 37 C for 72 h. The Bacterial species were identified by the morphological and biochemical tests compared with Bergey's manual of systematic bacteriology IV edition. The obtained bacterial genera from poultry waste dumping sites were *Escherichia coli*, *Enterococcus faecalis*, *Micrococcus luteus*, *Klebsiella pneumoniae*, *Proteus* sp., *Staphylococcus aureus*, *Salmonella* sp. and *Pseudomonas aeruginosa.* During the Keratinolytic activity the maximum zone of inhibition was noticed in the isolate No.3, and it was identified as *Micrococcus luteus.* The identified potential keratinolytic bacterial species culture was optimized with different temperature, pH, nitrogen and carbon sources for bulk production of enzyme. The bulk production of enzyme was precipitated with 20% of ammonium sulphate and it was purified with dialysis bag and subjected to assay procedure for determination of enzyme activity. The antibiotic sensitivity was tested on the isolated bacterial isolates and the highest antibiotic resistance was observed in *Staphylococcus aureus* (75%) and second most *Salmonella* (70%) and followed by *Klebsiella pneumoniae* (60%), *Enterococcus faecalis* (50%) and *Escherichia coli* (45%) .

Keywords: Keratinolytic activity, Poultry waste, Bacterial isolates, Antibacterial resistance, *Escherichia*, *Enterococcus*, *Micrococcus*, *Klebsiella*, *Proteus*, *Staphylococcus*, *Salmonella*, *Pseudomonas*

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Introduction

The poultry farm is one of the largest growing agro-based industries in India. Chickens are the most acceptable animal source for meat and eggs to fulfill a huge demand of non-vegetarian food around the world. Among them, feathers are the most mind boggling integumentary members found in vertebrates and are framed in little follicles in the epidermis or external skin layer,

that create keratin proteins (Esawy, 2007). Due to poor management of poultry wastes, especially feathers become one of the major pollutants due to their recalcitrant nature (Khardenavis *et al.*, 2009) and gradually increasing day by day. Each bird contains up to 125 g of feather and about 2 million tons of feathers wastes are generated annually as a by-product of poultry industry globally (Verma *et al*., 2017). Untreated and improper storage of feather wastes produced many pathogenic microorganisms and emit various pollutants such as nitrous oxide, ammonia and hydrogen sulfide, which are a threat to the environment and people's health (Tesfaye *et al*., 2017; Tamreihao *et al*., 2019). Therefore, converting feathers into economically important products using microbial methods were of great interest to many researchers (Kang *et al*., 2018). Microbial conversion of feathers into value-added products such as bio-fertilizer and animal feeds were used in poultry industries (Tamreihao*et al*. 2019). Keratin of feather is a soluble protein having low molecular weight. Keratin contains more cystine or proline residues than silk or collagen, high in glycine, serine, proline, leucine and glutamic acid. Feather colour usually correlates with the types of carotenoids deposited, particularly the number of conjugated double bonds that they bear. Carotenoids had highly unsaturated hydrocarbons and soluble in fat and organic solvents.

An important use of feather is to convert it into feather meal using physical and substance treatments. However, these strategies can wreck certain warmth heat-sensitive amino acids, such as, methionine, lysine and tryptophan and other nonnutritive amino acids, like lanthionine and lysinoalanine (Ana *et al*., 2011). Biodegradation of chicken feather by keratinases is an environment friendly biotechnological process, which converts this abundant waste into low-cost, nutrient-rich animal feed. Keratinolytic enzymes have applications in the detergent, medical, cosmetic and leather industries and as pesticides (Muller *et al*., 2006; Kumar *et al*., 2008). Keratinase producing microorganisms are being more and more applied for degradation and recycling of poultry feather waste. Hydrolysis of poultry waste via microbial keratinase and enhancing the nutritional cost constitute the progressive and eco-friendly method for treatment of keratinous waste. Various authors have reported that some species of *Bacillus*, actinomycetes and fungi are able to produce these keratinases and peptidases (Ana *et al*., 2011). The Keratinolytic micro-organisms gives considerable decrease in environmental contamination and increase in feather waste nutritional value (Kumawat *et al*., 2017; Holkar *et al*., 2018; Peng *et al*., 2019). Hence, in the present study we assessed the Keratinolytic bacteria from poultry waste dumping site. The potential isolate was characterized and optimized with different culture methods.

Materials and Methods

Sample Collection and Serial Dilution:

The soil samples were aseptically collected from waste soil disposal site and feather samples were collected from the poultry farm in Namakkal district, Tamil Nadu, India. The collected samples were immediately transferred to laboratory and stored in refrigerator for further experiments. Serial dilution for each soil sample was prepared by adding 1 g m of the soil sample to 9 ml of sterile saline. Then serial dilution up to 10 was done using sterile saline and from appropriate dilutions (10^{-7} and 10^{-8}) and 1 ml of sample was poured into the Nutrient Agar plates, respectively. Sample and control plates were incubated at 37 C for 24 h. After the incubation, different bacterial colonies were picked up by inoculation loop and were streaked on respective plates containing nutrient agar. Then the colonies were continuously streaked on nutrient agar plates by different streaking plate methods until we get purified colonies of isolates. Obtained nine different purified colonies were sub-cultured on nutrient agar slants.

Isolation and Screening for Keratinolytic Bacteria:

One loop full of bacterial isolate was taken from nutrient agar slants and inoculated into the conical

flasks containing sterilized nutrient broth. Inoculated broths containing conical flasks were incubated at 37 C for 24 h and used as inoculums for screening of keratinase -activity of isolates. Chicken feathers were washed, boiled and dried in hot air oven at 50 C for 4 h. The dried feathers were crumbled and the powder was used as feather meal. The plates containing feather meal agar plates were prepared and bacterial isolates were inoculated on media and the plates were incubated at 37 C for 24 h. The positive results were indicated by zone of clearance around the colonies. Keratinolytic organisms were cultured in nutrient broth for 7-8 h. Then it was transferred to flask containing liquid basal medium (Riffel *et al*., 2003). Whole feather was cut into pieces and mixed with sole source of carbon and nitrogen. The flask was incubates 47 C for 7 days with 120 rpm shaking. The per cent of keratinous waste degradation was determined by the method of Kim *et al*. (2011):

$$\text{DD (\%)} = \frac{\text{TF} - \text{RF}}{\text{TF}} \text{X100}$$

Where, TF is the total feather and RF is the residual feather.

Morphological and Biochemical Study of Isolated Bacteria:

The bacterial isolates were physically and biochemically analyzed by morphological characters, Gram's nature, motility, Indole test (I), Catalase test, Oxidase test, Urease production test (U), Methyl red test (MR), Voges-Proskauer test (VP), Citrate utilization test (C), TSI agar test and Carbohydrate fermentation test.

Optimization of Keratinase Production:

Maximum enzyme production was done with different carbon sources (Glucose, Maltose, Sucrose, Fructose and Lactose), organic nitrogen sources (Tryptone, Malt extract, Beef extract, Yeast extract and Ammonium sulphate), temperature (17 to 47 C) and pH (6-10).

Purification of the Enzyme (Ammonium Salt Precipitation):

The crude enzyme extract was prepared as described above. 10 ml of this crude extract was taken in a pre-washed glass beaker and solid ammonium sulfate was added with stirring to give different saturations of ammonium sulfate. The solution was centrifuged at 1000 rpm at 4 C for 10 min. The supernatant was collected without disturbing the pellet. Pellet was dissolved in sterile distilled water depending on the quantity of pellet. The pellet was dissolved with appropriate volume and desalted the enzyme with dialysis bag method.

Determination of Antibacterial Activity:

This test was carried out according to the method of Jahir and Kumar (2011). 20 ml of sterilized Mueller Hinton agar (MHA) was poured into each Petri plate (90 mm diameter) and allowed to solidify. One wheel of 6 mm diameter was bored with the medium of each plate with the help of sterile cork-borer. Different concentration of sample (1 to 4 µl/ml) was filled each well with the help of micropipette and Ampicillin (5 µg/ml) was used as positive control. After 24 h incubation period, the inhibition zone was measured around the wells.

Results and Discussion

In the present investigation from the five soil samples, 15 bacterial colonies were selected based on the morphological non-similarity and each soil sample had at least one colony. Among them, 6 isolates showed clear zone of hydrolysis around colony on feather meal agar medium on incubation at 37 C for 72 h (Fig. 1). Among 6 isolates, third bacterial strains (IS3) showed high degree of feather degradation rate than the other 5 species (Table1). Keratinolytic bacteria was identified based on cell morphology, colony morphology and several biochemical tests (Table 2). Among the microbes, keratinase enzyme producing Gram-positive bacterium was isolated and identified as *Micrococcus luteus* (IS3). Although members of the Micrococcaceae family were scarcely used in keratin degradation, increasing reports like this study and other recent studies descried the potential kertinolytic activity of *Micrococcus* species (Laba *et al.*, 2015). The

excellent characteristics of the crude keratinase may lead to the potential application in waste treatment and recovery in poultry, leather industry, medicine and cosmetic development as studied by Chao *et al*. (2007) and Saha *et al*. (2013). This is in accordance with previous report of Vijay Kumar *et al.* (2011) as *Bacillus altitudinis* GVC11, a chicken feather degrading bacterium, was identified and isolated from waste-dump-yard in Hyderabad. Pintubala (2017) isolated 26 keratinolytic bacteria from feather dumping sites in North East India.

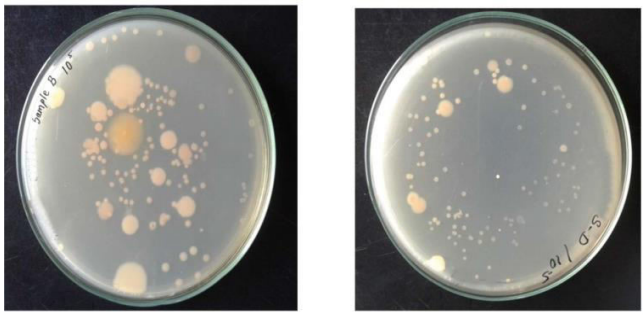

Fig. 1: Isolation of bacterial species from poultry waste soil samples collected from Namakkal, Tamil Nadu, India.

Optimization of Keratinase Synthesis by pH:

Several cultivation factors like temperature, pH and other nutritional sources play vital roles in the production of enzymes. The Keratinolytic bacterial isolate growth was optimized with culture medium pH of 6, 7, 8 and 10. The maximum enzyme synthesis was observed at pH 7 and it was calculated as 0.62 U/ml and followed by pH 8 (0.4 1U/ml) (Table 3). During the enzyme production assessment the moderate production was found in pH 6, 9 and 10. Changes in pH affect the enzyme production and also change the properties of substrate. Similar kind of result was also observed by Bo Xu *et al*. (2009) and they reported thatmaximum keratinase production was obtained when medium pH was 7 and microbes growth rate also maximum. Samuel *et al.* (2012) stated that high pH leads to low metabolic action of bacterium thus suppressing the colony growth and inhibits the enzyme production. This is in accordance with previous reports which indicated that the keratinase enzyme produced by *Bacillus* species could be classified as an alkaline protease and that it is most active under neutral or alkaline conditions (Mohamedin, 1999; Suntornsuk and Suntornsuk, 2003; Jayalakshmi *et al*., 2011). Physical factors likes pH, temperature, aeration and agitation are important in any fermentation for optimization of biochemical production.

Optimization of Keratinase Synthesis by Temperature:

Temperature has great impact on enzyme production. The production of keratinase was greatly induced by the temperature and the maximum Keratinase synthesis was reached by the *Micrococcus* sp. at 37 C where its production reached up to 0.52 U/ml. The other temperatures such as 27 C and 47 C were also seen to induce the less amount of keratinase production (Table 4). Thus 37 C was the optimum temperature and it leads to support to optimize the bacterial population as well as enzyme production. During this study keratinase production was not found in 57 C because of absence of bacterial cell growth at such high temperature. Studies also revealed that the enzymes from fungi, bacteria and other microbes needs optimal temperature which gives to high efficiency in enzyme production (Samuel *et al.,* 2012; Kanoksilapatham and Intagun, 2017; Abdel-Fattah *et al*., 2018).

Optimization of Keratinase Synthesis by Different Carbon Sources:

In this experiment five different carbon sources namely Glucose, Maltose, Sucrose, Fructose and Lactose were utilized to produce maximum quantity of keratinase enzyme. In this study, maltose (0.51 U/ml/min) showed maximum support on keratinase enzyme production followed by fructose, lactose, sucrose and glucose, respectively (Table 5). A similar study was done by Ire and Onyenama (2017).

Optimization of Keratinase Synthesis by Different Nitrogen Sources:

In this study 5 different organic nitrogen sources were subjected for the optimization process

Table 1: Screening of Keratinolytic potential bacterial isolates from poultry waste dumping site from Namakkal district, Tamil Nadu

S. No.	Isolates	Keratinase activity (U/ml/min)	Percentage of Degradation
1.	Isolate 3	**0.19**	**37**
2.	Isolate 6	0.14	29
3.	Isolate 8	0.12	22
4.	Isolate 9	0.14	24
5.	Isolate 12	0.13	22
6.	Isolate 15	0.12	19

Table 2: Biochemical characterization of poultry soil bacteria collected from Namakkal, Tamil Nadu

S. No.	Biochemical tests	IS3	IS6	IS8	IS9	IS12	IS15
1.	Gram staining	+	-	-	+	-	-
2.	Indole test	-	-	-	-	+	-
3.	Catalase test	+	-	+	+	-	+
4.	Oxidase test	+	-	-	+	-	+
5.	Urease production test	+	-	+	-	+	-
6.	Methyl Red test	-	+	+	+	+	+
7.	Voges-Proskauer test	-	+	+	-	-	-
8.	Citrate utilization test	-	-	+	-	+	-
9.	TSI	K/K	A/A	A/A	A/A	K/A	A/A
10.	Glucose	+	+	+	+	+	+
11.	Lactose	-	+	+	-	-	-
12.	Maltose	+	-	+	+	-	+
13.	Sucrose	-	V	+	-	-	-
		M. luteus	***E. coli***	***K. pneumoniae***	***Enterococcus* sp.**	***Proteus* sp.**	***Salmonella* sp.**

+ Positive, - Negative, V- Variable, K- Alkaline, A- Acidic

Table 3: Optimization of Keratinase enzyme production with different pH

S. No.	pH	Keratinase activity (U/ml/min)
1.	6	0.32
2.	**7**	**0.62**
3.	8	0.41
4.	9	0.22
5.	10	0.18

(Table 6). Among the nitrogen sources tested, Beef extract was found to be the most suitable organic nitrogen source for *Micrococcus* and the enzyme activity observed was 0.50 U/ml. The lowest Keratinase production was observed in Ammonium sulphate supplied medium. The beef extract consists of high content of minerals, vitamins and coenzymes were present in the nitrogen components which enhance the enzyme production. Similar results have been reported by

Table 4: Effect of different Temperature on Keratinase enzyme production

S. No.	Temperature	Keratinase activity (U/ml/min)
1.	17 C	0.31
2.	27 C	0.42
3.	**37 C**	**0.52**
4.	47 C	0.47
5.	57 C	0.20

Table 5: Effect of carbon source on Keratinase production

S. No.	Carbon sources	Keratinase activity (U/ml/min)
1.	Glucose	0.30
2.	**Maltose**	**0.51**
3.	Sucrose	0.32
4.	Fructose	0.47
5.	Lactose	0.32

Table 6: Effect of nitrogen sources on keratinase production

S. No.	Nitrogen sources	Keratinase activity (U/ml/min)
1.	Tryptone	0.37
2.	Malt extract	0.42
3.	**Beef extract**	**0.50**
4.	Yeast extract	0.32
5.	Ammonium sulphate	0.30

Ramachandran *et al*. (2004) and Chaturvedi and Verma (2014) who have also reported the increased enzyme activity by using the beef extract.

Purification of the Enzyme (Ammonium Salt Precipitation):

In the present study, after optimization process, bulk production was carried out with optimized parameters (Table 7). The production (0.79 U/ml/min) of keratinase enzyme was done by using the beef extract, maltose, pH 7 and temperature 37 C. The enzyme was precipitated with 20% of ammonium sulphate and subjected to assay procedure for determination of enzyme activity (Table 8). The result was compared with crude enzyme, the activity was improved when precipitated the enzyme with ammonium sulphate (1.18 U/ml). Furthermore, partially purified enzyme was desalted with dialysis bag and 1.48 U/ml of purified enzyme was obtained.

Antibiotic Resistance of Isolated Bacterial Genera from Soil:

Antibiotic resistance is a serious problem worldwide which emerged as one of the foremost problem in public health concerns of the 21st century. Antibiotic resistance leads to higher medical costs, prolonged hospital stays

Table 7: Bulk production of Keratinase with optimized nitrogen source, pH and Temperature

Carbon source	Nitrogen source	pH	Temperature	Enzyme activity (U/ml/min)
Maltose	Beef extract	7	37 C	0.79

Table 8: Purification of Keratinase enzymes

S. No.	Purification steps	Enzymes activity (U/ml/min)
1.	Crude	0.84
2.	Ammonium sulphate	1.18
3	Dialysis	1.48

Table 9: Antibiotic resistance patterns of microbial isolates from soil collected from poultry waste dumping site

S.No	Name of the Poultry isolates	Antibiotics										% of Resistance
		P	AMP	AK	AMX	CFM	K	CTR	CPD	E	N	
1.	*M. leutues*	S	S	S	S	I	I	R	R	R	R	40
2.	*E. coli*	R	R	S	S	R	R	I	R	R	S	60
3.	*K. pneumoniae*	S	S	R	S	R	R	S	S	I	S	30
4.	*Enterococcus* sp.	S	R	S	R	S	R	S	R	R	R	60
5.	*Proteus* sp.	R	S	S	S	R	R	R	S	S	S	40
6.	*Salmonella* sp.	R	R	R	I	R	R	R	S	R	I	70

R-Resistance, I-Intermediate, S-Sensitive, P-Penicillin, AMP- Ampicillin, E-Erythromycin, AK- Amikacin, AMX-Amoxicillin, CFM- Cefixime, K- Kanamycin, CTR- Ceftrizone, CPD- cefphotoxime, N- Novobiocin

and increased mortality. Recently, the global consumption of antimicrobials in livestock has indicated the hotspots of antibiotics use across the continents that will have economic and public health impacts in upcoming years. The antibiotics are commonly used in cattle, chicken and pigs and the usage would gradually increase in 2030 up to 67% in the most populated countries of the world (Van Boeckel *et al*., 2015). A total of seven types of bacterial genera were obtained from poultry waste dumping side, as *M. luteus, E. coli, K. pneumoniae, Enterococcus* sp., *Proteus* sp. and *Salmonella* sp. Their antibiotic resistance has been illustrated in Table 9.

The worrying aspect of the current study is that 6 isolates were resistant to 6 or more antibiotics. Among the 6 types of bacterial isolates, the maximum percentage of resistance was observed in *Salmonella* sp. (70%) and second most *E. coli* (60%) and *Enterococcus* sp. (60%), followed by *Proteus* sp. (40%), *M. leutues* (40%) and *K. pneumoniae* (30%). The antibiotic resistance pattern demonstrated by the bacterial isolates in this study revealed that resistance was highest in most tested antibiotics such as Penicillin and Ampicillin and followed Erythromycin. The antibiotic resistance pattern obtained in this study is a serious challenge to public health because most of the antibiotics were converting to resistance in the isolates.

The current result provided indication that poultry waste can serve as an environmental

reservoir for multiple antibiotic resistance bacteria and hence can serve as possible routes for the entrance of multidrug resistance zoonotic pathogen in the human being. This has very significance inference for human health, as multidrug resistance infections were problematic to treat and frequently required costly antibiotic and long term therapy. This can considerably raise the cost of treatment and even death. Antibiotic resistance is a serious problem worldwide and emerged as one of the foremost problem in public health concerns of the 21st century. This phenomenon mainly occurred by the indiscriminate and inappropriate use of antibiotics for the treatment of the common bacterial infection. The bacterial species are able to survive after exposure to one or more antibiotics. Pathogenic isolates are resistance to multiple antibiotics and is considered as MDR (multiple drug resistances). This is very dangerous to human and animals because resistance has been shown to increase with duration of treatment (Chakrapany *et al*., 2014)

Enzymes are proteinaceous in nature. All enzymes are programmed to carry out a special task. Like a key in a lock, enzyme fits together with a specific substrate modifying it in a proper way. Microbial enzymes and co-enzymes are widely used in many industries like detergent, food processing, brewing and pharmaceuticals. Since ancient times the enzymes and co-enzymes were used in the preparation of fermented foods, especially in oriental countries (Singh and Mittal, 2016). The manufacture of enzymes for use as drugs is an important facet of today's pharmaceutical industry (Cassileth, 1998).

The requirements for broiler chicken is increased due to increased human population. The biological treatments were most suitable method for removal of feathers in a clean way. Many microorganisms are able to produce keratinase. Microbial processing techniques generated usual products such as bio-fertilizer and animal feed. The study consequently endorsed appropriate information dissemination to agriculturalist and poultry feed maker about the public health prominence of proper poultry waste disposal. Hence, there is urgent need of new ways to deal with pathogens, especially multidrug resistant isolates. Against backdrop of this information, our investigation was focused on biological derivatives. Production of Keratinolytic enzyme from microbial agent serves as mitigation for the poultry waste as well as helping in arresting the antibiotic resistant bacterial population.

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