Journal Homepage: -www.journalijar.com
INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)
Article DOI:10.21474/IJAR01/4753
DOI URL: http://dx.doi.org/10.21474/IJAR01/4753
ISSN NO. 2320-5407

RESEARCH ARTICLE

SCREENING OF FEATHER DEGRADING BACTERIA AND ITS APPLICATION.

Ruhi Patrawala, Ria Odakayi, Sankalpa Palkar, Cyd Rodricks, Algamary Benny, Vivien Amonkar and *Pampi Chakraborty.
Department of Microbiology, St. Xavier's College-Autonomous, Mumbai-400001, Maharashtra, India.

Manuscript Info

Manuscript History

Received: 5 May 2017
Final Accepted: 7 June 2017
Published: July 2017

Key words:-
feather degrader, keratinolytic, bioremediation, *Bacillus*, cheap source of amino acid

Abstract

Environmental pollution is an issue of global concern and feather waste which is a byproduct of the growing poultry processing industry is an example of a pollutant not easily degraded by common proteolytic enzymes. However, feathers when acted upon by keratinolytic microbes are degraded to smaller peptides and amino acids which can be used as dietary supplements in animal feed. In the present study, feather degrading microorganisms isolated from poultry farm soil were initially screened for proteolytic activity of the organism on skim milk agar plates. The selected organism was subsequently grown on feather meal medium and biochemically, genotypically characterized and identified to be a *Bacillus* species. Significant keratinase activity was observed over a wide range of pH for six days, using the keratinase assay. The organism showed good bioremediation activity in broth as well as in soil with the crude feather. Further, it was confirmed that the organism can also degrade feathers using table sugar as a cheap carbon source. Optimum conditions for amino acid production were determined by the quantitative ninhydrin method which confirmed significant amino acid production, under both shaker and static conditions, after 96 hours of incubation at room temperature.

Abbreviations:-

Feather meal broth (FMB), Trichloroacetic acid (TCA)

Introduction:-

Large amounts of feather waste are discharged every year from the poultry processing industry with an estimated global annual discharge of millions of tons.[1] Feathers from poultry processing plants are the common source for the accumulation of more than 90% of keratinous proteins in the environment.[2] Discarded feather also causes various human ailments including chlorosis, mycoplasmosis and fowl cholera.[3] Feather waste has also been suggested to be a cause of H5N1 virus outbreaks.[4] The localized accumulation of feather waste around poultry processing sites creates a serious disposal problem leading to environmental pollution. A feather is mainly composed of 90% keratin protein which is highly resistant to proteases due to its molecular conformation. The structural amino acids are tightly packed in the α-helices (hairs) and β-sheets (feather) in the presence of cystine disulfide bonds, hydrogen bonds and hydrophobic interactions. Several different approaches have been used for disposing of feather waste, including land filling, burning and natural gas production.

Corresponding Author:-PampiChakraborty.
Address:-Department of Microbiology, St. Xavier's College-Autonomous,Mumbai-400001, Maharashtra, India.

Most feather waste across the world is land filled or burned which involves a huge expense and can cause contamination of air, soil and water. Since poultry waste usually includes feather waste which consists of keratin protein rich in amino acids, they can be used as potential dietary supplements in animal feedstuff.[5] Since chemical processing of feathers result in the loss of essential amino acids (Methionine, Lysine, Histidine), thus biotechnological processing of feathers for the production of feather meal is preferred.[6] Microbiological methods for hydrolysis of feather to soluble protein and amino acids represents an environment friendly approach which offers a cheap, and mild reaction condition that prevent loss of valuable amino acids and thus results in nutritionally upgraded feather meal. A vast variety of bacteria, actinomycetes and fungi are known to be keratin degraders.[7-9] The bacterial species frequently reported for enzyme production and characterization are Bacillus spp, Actinomycetes, *Vibrio* spp., *Chryseobacterium*sp., *Burkholderia*sp. and *Pseudomonas* sp.[10-15]

The current study is being done to screen organisms possessing keratinolytic activity for biodegradation of feather waste generated in poultry farms and industries, thereby helping in bioremediation; as well as checking their efficiency for breakdown of keratin to its constituent amino acids.

Materials and methods:-

Isolation and screening of feather degrading bacteria:-

Soil samples were collected from a poultry farm area in Mira Road, Maharashtra, India in sterile zip lock bags. A 1:100 dilution of the samples was spread plated on skim milk agar. Plates were then incubated at 37°C for 24 – 48 hours for observation of zones of clearance. The morphological characteristics of well isolated colonies showing zones of clearance were recorded and thereafter sub-cultured on sterile nutrient agar slants for further identification and characterization. One strain, which consistently showed the highest zone of clearance, was selected for further study.

Identification of the bacterial isolate:-

The isolate was identified by morphological, cultural and biochemical characteristics to its genus level. It was further confirmed by 16s rDNA sequencing.[16]

Preparation of defatted feather for enrichment of isolate:-

Defatted feathers were prepared for enrichment media preparation.[17] Briefly, the native chicken feathers were cut with scissors in to 3 to 4 cm long pieces and washed several times with tap water. Defatting of feather pieces was carried out by soaking them in a mixture of chloroform: methanol (1:1) for 2 days followed by chloroform: acetone: methanol (4:1:3) for 2 days. The feathers were finally washed several times with tap water to eliminate the solvent residues and dried at 60°C for 24 hours.

Enrichment of keratinolytic bacteria:-

Feather meal broth (FMB) [(g/L): NH4Cl, 0.5; NaCl, 0.5; K_2HPO4, 0.3; KH_2PO4, 0.4; $MgCl_2.6H_2O$, 0.1; Yeast extract/Glucose, 0.1 and Feather, 10; pH 7.5] containing defatted feathers (1%) was used for enriching the keratinolytic or feather degrading bacteria. Three types of feather meal broth were prepared, one flask had neither glucose nor yeast extract, whereas the other two flasks contained either 0.01% glucose or yeast extract. These flasks were then inoculated with 1.0 ml of the log phase culture having an O.D580nm =0.1 and incubated under shaker conditions at room temperature for 6 days. After incubation, O.D was measured at 580nm to study the growth of the organisms.

Keratinase assay:-

Keratinase activity was measured using 20mg of feather as the substrate, 1ml crude enzyme and 4ml of buffer (50mM Glycine-NaOH buffer, pH 10). The reaction mixture was incubated at 60°C for 1h and the reaction stopped with 4ml of 5% w/v trichloroacetic acid (TCA), followed by incubation at room temperature for 30min and centrifugation at 8000rpm for 10min. The Absorbance of the supernatant was measured at A_{280}. Similarly, a control reaction was set up with 1ml of 5% w/v TCA. One unit (U) of keratinase activity was expressed as1 µmol of tyrosine released per minute under the specific conditions. The keratinase assay was performed in FBM and glucose (0.01%) in shaker and static conditions. Three different pH (4.0, 7.0 and 10.0) were considered for growth condition optimization.

Optimization of Amino acid production:-
FMB containing 1% defatted feathers, adjusted to pH 4, 7 and 10 each and with static and shaker conditions were used for the purpose of optimizing amino acid production.1.0 ml of the culture suspension having an $O.D._{580nm}$ = 0.1 was used for inoculation. 5.0 ml of the culture broth was withdrawn every two days, over a period of 6 days and subjected to preliminary extraction of amino acids, in order to determine the ideal pH and time for amino acid production. This was achieved by carrying out the quantitative ninhydrin test for amino acids using the fractions collected. For quantitative estimation, a standard curve was prepared using asparagine solutions having a range from 10µg/ml to 100 µg/ml. 2.0 ml of 0.2% ninhydrin was added to 2.0ml of the sample and the reaction mixture boiled for 15 minutes. Thereafter 3.0 ml of ethanol was added and O.D measurements were taken at 530nm. The concentration of amino acids in the samples was determined using the standard graph. .

Paper chromatography:-
The culture supernatant of the previous experiment 'optimization of amino acid production' were collected and centrifuged at 5000rpm for 10 minutes. The supernatant, after further filtration using a syringe filter, was subjected to paper chromatography. Whatman's filter paper No. 1 was used as the stationary phase, while the mobile phase used was a solvent system containing butanol, glacial acetic acid and water in the ratio of 5:1:4, respectively. 1% solution of all the twenty amino acids was used as standards and 0.2% ninhydrin solution was used as the developing agent. The Rf value of the spots visualized in the sample lane were then compared with the Rf values of the standard amino acids, in order to enable identification of the amino acid present in the culture supernatant.

Bioremediation of feathers by the keratinolytic isolate:-
The feather degrading capability of the isolate was tested by inoculating the bacterial isolate in 100ml of the media containing 0.5g of native feathers. The liquid media used were FMB with glucose (0.01%), yeast extract (0.01%), without glucose or yeast extract and phosphate buffer saline (PBS). The media flasks were incubated under shaker conditions at room temperature for 10 days. Thereafter, the culture broth was filtered through pre-weighed Whatman's filter paper No.1. The residues of un-degraded feather present on the filter paper were then dried in a hot air oven at 40°C, overnight and their weights determined.

1.0 ml and 10 ml of the log phase culture having an $O.D._{580nm}$= 0.1, were inoculated in 50g of autoclaved garden soil, containing 0.25g of native feather (added to the soil before autoclaving). These were maintained in a 500ml flask under static conditions, in order to mimic the external environment. The flasks were sprinkled with 2.0 ml of sterile distilled water, every two days to maintain the soil moisture. This set up was kept for one month to observe feather degradation in soil.

Feather degradation efficiency using table sugar as a substrate:-
Effect of cheap carbon substrate was also studied in feather degradation by the isolate. Table sugar was sterilized with distilled water and added on the feather along with the organism. It was incubated for 10 days in a shaker condition at room temperature. Percentage degradation was calculated by determining the amount of undegraded feather as mentioned previously.

Statistical Analysis:-
All the statistical analysis described (One Way ANOVA and T-Test) have been done using Microsoft Excel and Sigmastat 3.5 software.

Results:-

Isolation and Identification of keratinase producing bacteria:-
Primary screening for feather degrading organisms was conducted using skim milk agar. One strain which showed large zones of clearance in consecutive subcultures was chosen for further study (Figure 1). Gram staining indicated gram positive short rods containing spores. The isolate was found to be strongly aerobic, catalase and oxidase positive. The morphological, cultural and biochemical characteristics indicated that the isolate belonged to the Genus *Bacillus.* The 16s rDNA sequencing results indicate similarity with *Bacillus subtilis*(Data not shown).

Enrichment of keratinolytic bacteria:-
The isolate was inoculated in three different types of Feather Meal Broth (FMB). After six days, absorbance was checked at 580nm for the inoculated broths. FMB containing Glucose showed the highest degradation of feathers followed by FMB containing Yeast Extract (Figure 2).

Effect of pH on keratinase activity:-
The keratinase enzyme was found to show higher activity at pH 7.0 and pH10.0 (Figure.3). The maximum keratinase production was observed at pH 10.0 with significantly high enzyme activity compared to pH 4, after six days, thereby indicating minimum keratinase production at pH 4.0. It was also observed that with the increasing time of incubation, the enzyme activity was also increased.

Optimization of amino acid production:-
The concentration of amino acids was determined by the quantitative ninhydrin method at different pH and aeration conditions. The highest concentration of amino acid was obtained at pH 7.0 under shaker conditions (Figure.4) after 96 hours**.** Also, as time increased, the amino acid concentration decreased, maybe due to the utilization of the amino acids by the organism itself.

Amino acid analysis:-
In order to identify the amino acids present in the culture broth, paper chromatography was performed (Figure 5). Our preliminary study could identify three spots in the culture supernatant sample. Based on Rf value, the amino acids present in the culture supernatant are suspected to be Asparagine, Histidine or Lysine for spot 1, Tyrosine or Proline for spot 2, and Phenylalanine, Tryptophan or Valine for spot 3.

Bioremediation of feather by the keratinolytic isolate:-
The feather degrading capability of the isolate was tested in order to find out its usefulness for bioremediation of native feathers. The bioremediation of feathers was carried out in both liquid media as well as soil. In case of liquid media, it was observed that FMB containg Glucose showed the highest degradation of feathers compared to other groups (Figure 6). The biodegradation of feathers was visually observed in the sterile soil sprinkled with 0.1ml and 1.0 ml of 1.0 $O.D._{580nm}$ feather degrading isolate suspension per gram of soil for one month (Figure 7).

Feather degradation efficiency using cheap source of substrate:-
Sucrose (table sugar) was used as a cheap source of carbon to determine the efficiency of the isolate to degrade feather. Percentage degradation of the feather was calculated and it was found that degradation takes place with table sugar as carbon source. Although, glucose in FMB proved to be most effective for feather degradation (Figure 8).

Discussion:-

Enzymes like keratinases have gained a lot of biotechnological importance for their ability to degrade highly rigid, cross-linked polymeric waste like feather.[18] These keratinases are usually produced by keratinolytic organisms which are present at sites either rich in or contaminated with keratin containing products. Poultry farms are ideal sampling sites for isolating keratinase producers which can degrade feathers.[19] Owing to their wide range of substrate specificity, keratinase has been identified to be capable of degrading not only keratin, but also non-fibrous proteins such as casein.[20-21] Thus, by using skim milk agar, a medium rich in casein, potential keratinolytic organisms can be identified based on the zone of clearance shown by the organism on the skim milk agar plates.

The isolate obtained was identified to be *Bacillus* sp from morphological, cultural and biochemical characteristics. Further, 16s rDNA sequencing also confirmed its highest similarity with *Bacillus subtilis* which has been found to be a very common keratinase producer in many studies.[10-13]

Previous literature states that Feather Meal Broth (FMB) containing 0.01% yeast extract and 1% feathers along with other essential salts (0.03- 0.05%) is a suitable medium for isolating keratinase producers. However, as it is known that yeast extract itself is a rich source of amino acids, this medium would not be suitable for the production of amino acids. Therefore, with an attempt to altering the medium composition, yeast extract was replaced by glucose in one medium whereas in another medium, both glucose and yeast extract were not added. Optimization studies indicated, FMB containing glucose, as the most suitable medium for enrichment as well as amino acid production. Thus, a suitable medium was identified for the enrichment and amino acid production for keratinolytic organisms. Keratinolytic activity was also determined by performing a keratinase assay.

It was evident from the present study that the organism exhibits keratinase activity over a wide range of pH at room temperature, which is important for industrial application. Our preliminary study on the amino acid analysis also showed similarity with a previous study.[22]Consistent with the results of enrichment and optimization, maximum

amount of crude feather degradation was observed in the medium containing glucose. The organism also showed feather degradation with table sugar as a cheap carbon source. In future, the isolate can also be coupled with other feather degrading organisms for quick synergistic degradation.

Figure1:- Colonies showing zone of clearance on Skim Milk Agar

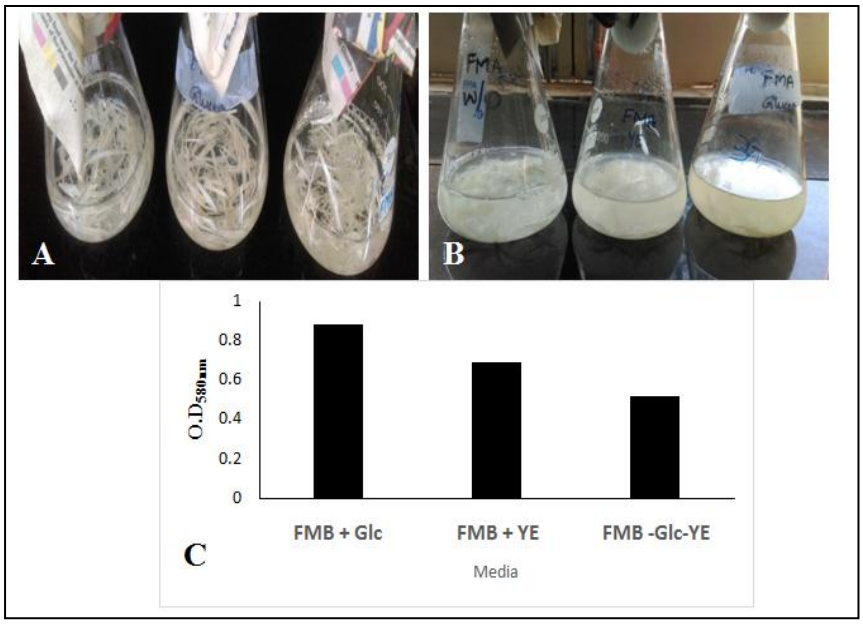

Figure 2:-Degradation of feather during enrichment of keratinolytic bacteria on Day 0 (A) and Day 6 (B) after inoculation in FBM and glucose, FBM and Yeast extract and only FBM as media (From left to right). Graphical representation of the $O.D_{580nm}$ measurement of the isolate is shown in the media after 6 days (C).

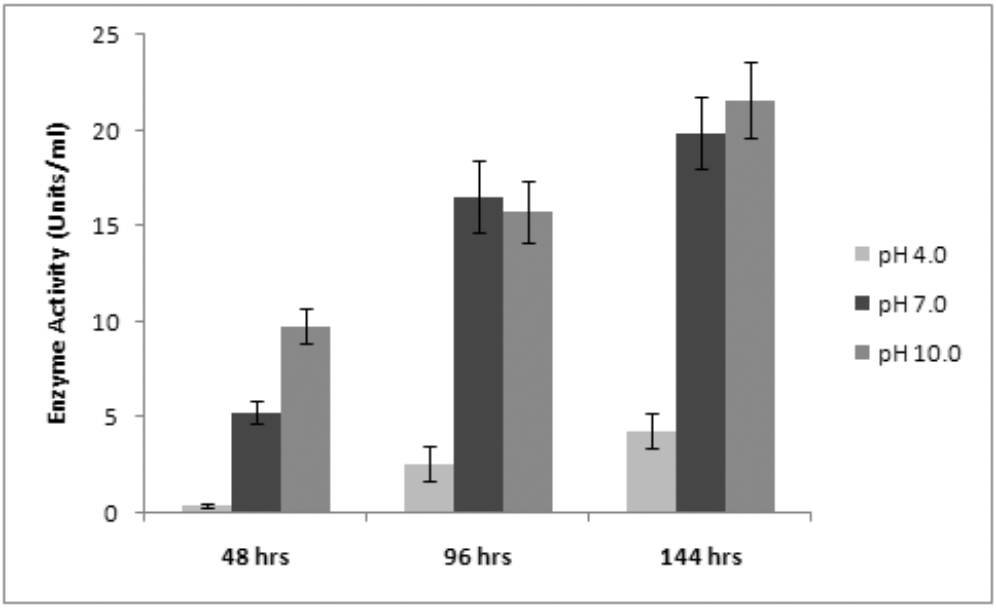

Figure 3:- The histogram represents the Effect of pH on keratinase activity. Data represent the mean ±SD of representative experiment.

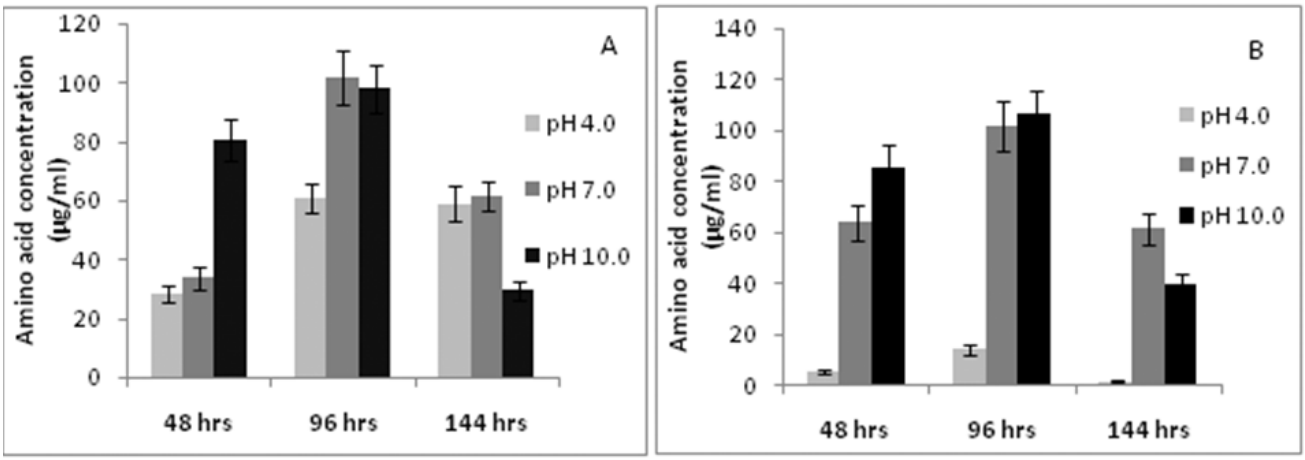

Figure 4:- Optimization of proteolytic activity in Static (A) and Shaking (B) conditions. Data represent the mean ±SD of one representative experiment.

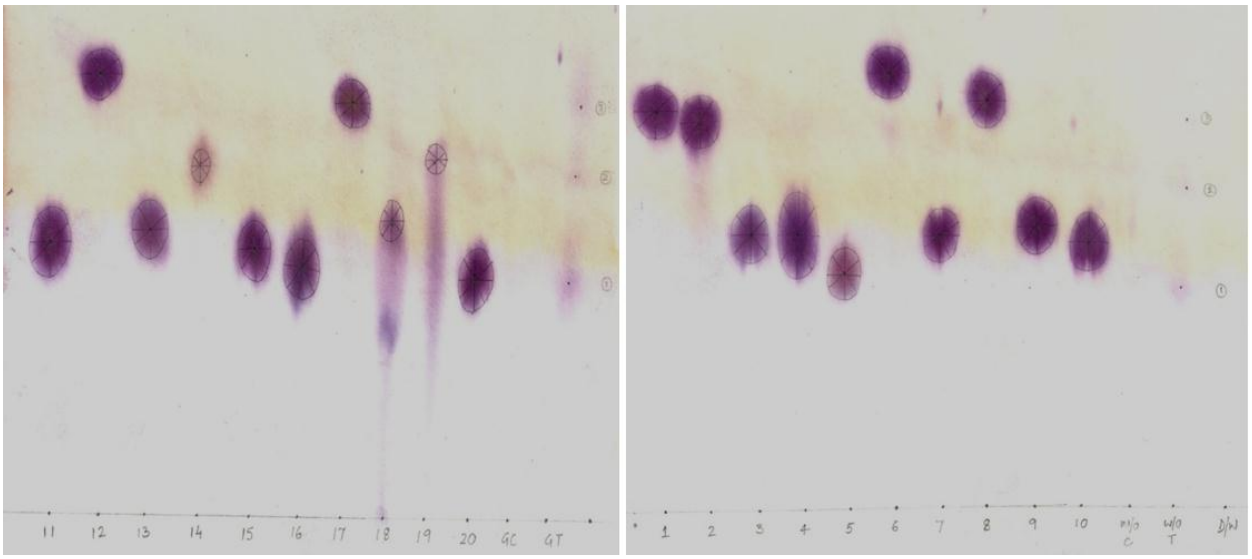

Figure 5:- Paper chromatography with culture supernatant; Spot 1 – Valine, Spot 2 – Methionine, Spot 3 – Aspartate, Spot 4 – Arginine, Spot 5 – Asparagine, Spot 6 – Leucine, Spot 7 – Glutamate, Spot 8 – Phenylalanine, Spot 9 – Alanine, Spot 10 – Threonine, W/O C – FMB ((-)Glc& (-)Yeast Extract) Media Control, W/O T – FMB ((-)Glc& (-)Yeast Extract) Culture Supernatant, And D/W – Negative Control. Spot 11 – Glutamine, Spot 12 – Isoleucine, Spot 13 – Glycine, Spot 14 – Proline, Spot 15 – Serine, Spot 16 – Histidine, Spot 17 – Tryptophan, Spot 18 – Cysteine, Spot 19 – Tyrosine, Spot 20 – Lysine, GC – FMB (With 0.01% Glucose) Media Control, And GT – FMB (With 0.01% Glucose) Culture Supernatant.

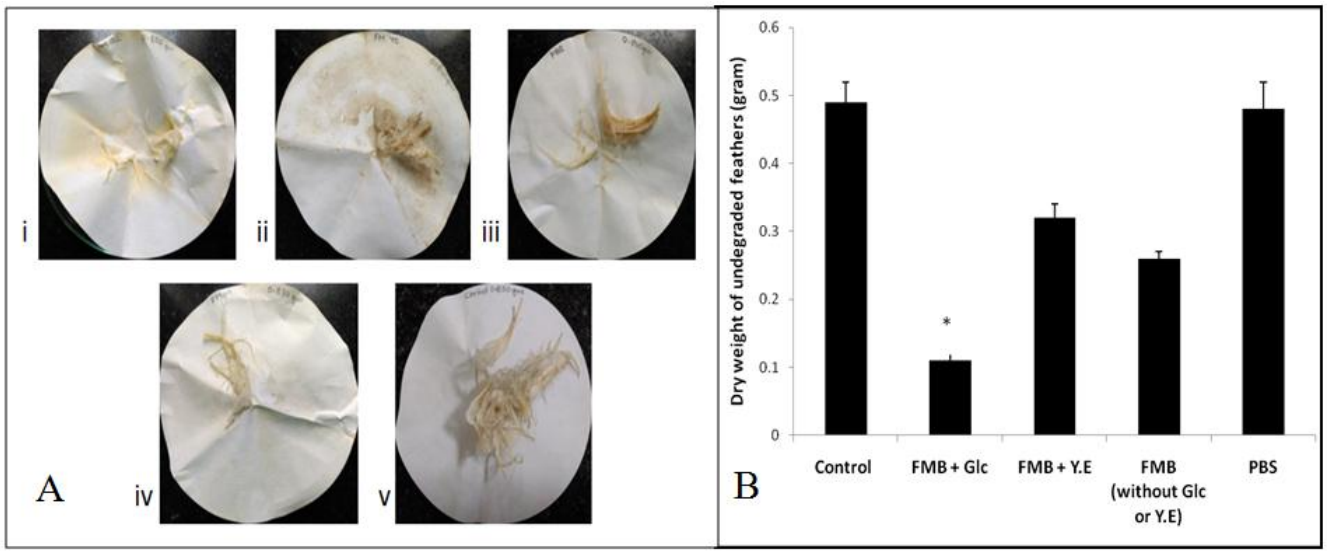

Figure 6:- A. The dry weight of undegraded feathers post inoculation is shown in various media – i. FMB + Glc(0.01%), ii. FMB + Y.E (0.01%), iii. PBS, iv. FMB, and v. Control. Here 'Control' is same amount of feather in FMB without the inoculam.

B. The histogram shows the graphical representation of dry weight of un-degraded feather. Data represent the mean ±SD of the representative experiment and * indicates the statistical significance.

Figure 7:-Bioremediation in soil containing - A.control (no culture), B. with 0.1ml of 1.0 $O.D_{580nm}$ feather degrading isolate suspension per gram of soil, and C with 1.0ml of 1.0 $O.D_{580nm}$ feather degrading isolate suspension per gram of soil.

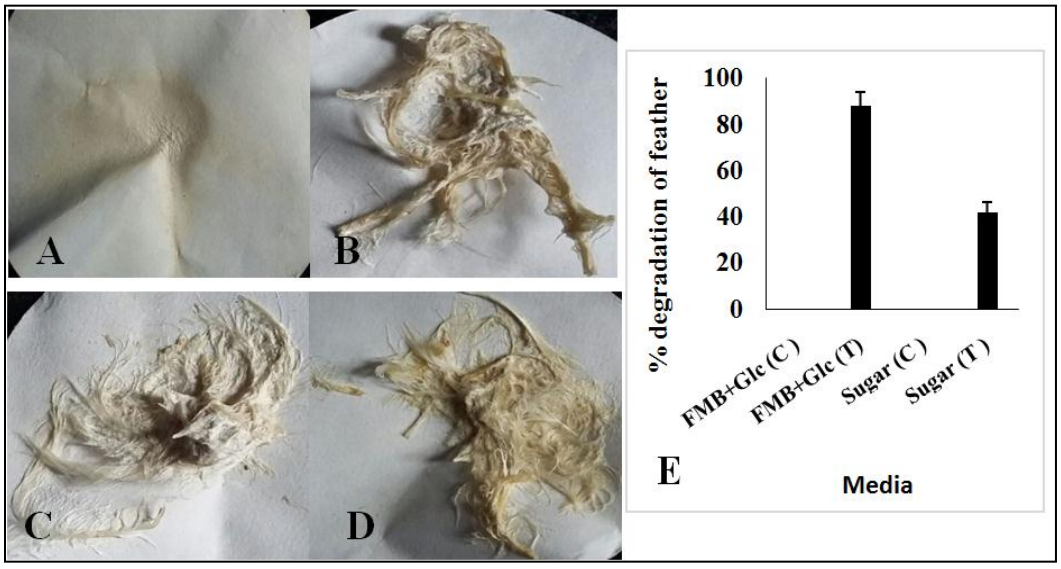

Figure 8:- Feather degradation efficiency of the bacterial strain using different substrates: Representative pictures of feather degradation A. With Glucose (Test) B. Without Glucose (C) With table sugar (Test) (D) Without table sugar. (E) graphical representation of the same experiment.

Acknowledgements:-

We are pleased to acknowledge Department of Microbiology, St. Xavier's College (Autonomous), Mumbai for providing us with all the materials required for research.

References:-

1. World Statistical Compendium for Raw Hides and Skins, Leather and Leather Footwear1998-2008, (Food and Agricultural Organization of the United Nations, Rome, Italy) 2001, 1-127.
2. El-Ayouty, Y. M., El-said, A., and Salama, A. M. **(**2012). Purification and characterization of a keratinase from the feather-degrading cultures of *Aspergillusflavipes*. *African Journal of Biotechnology*. **11**(9): 2313-2319.
3. Agrahari, S., and Wadhwa, N. **(**2010) Degradation of chicken feather a poultry waste product by keratinolytic bacteria isolated from dumping site at Ghazipur poultry processing plant. *Int J Poult Sci.* **9**(5): 482-489.
4. Mumford, E., Bishop, J., Hendrickx, S., Embarek, P. B., and Perdue, M. **(**2007). Avian influenza H5N1: Risks at the human-animal interface. *Food and nutrition bulletin*. **28**(2 suppl2): S357-S363.
5. Wang, X., and Parsons, C. M. **(**1997) Effect of processing systems on protein quality of feather meals and hog hair meals. *Poultry Science*. **76**(3): 491-496.
6. Sivakumar, T., Balamurugan, P., and Ramasubramanian, V. **(**2013) Characterization and applications of keratinase enzyme by *Bacillus thuringiensis* TS2. *Int J Future Biotechnol*.**2**(1): 1-8.

7. Vidhya, D., and Palaniswamy, M. **(**2013) Identification and characterization of a local bacterial strain with high keratinolytic activity from chicken feathers. *International Journal of Pharmacy and Biological Sciences* . **3**(3): 308-316.
8. Saber, W. I. A., El-Metwally, M. M., and El-Hersh, M. S. **(**2010) Keratinase production and biodegradation of some keratinous wastes by Alternariatenuissima and Aspergillusnidulans. *Research Journal of Microbiology*. **5**(1): 21-35.
9. Nagal, S., and Jain, P. C. (2010) Production of feather hydrolysate by Elizabethkingiameningoseptica KB042 (MTCC 8360) in submerged fermentation. *Indian journal of microbiology*. **50**(1): 41-45.
10. Mazotto, A. M., Coelho, R. R. R., Cedrola, S. M. L., de Lima, M. F. b., Couri, S., Paraguai de Souza, E., and Vermelho, A. B. **(**2011) Keratinase production by three bacillus spp. Using feather meal and whole feather as substrate in a submerged fermentation. *Enzyme research* 2011: 1-7.
11. Vigneshwaran, C., Shanmugam, S., and Sathish, K. T. **(**2010) Screening and characterization of keratinase from Bacillus licheniformis isolated from Namakkal poultry farm. *Researcher*. **2**(4): 89-96.
12. Tiwary, E., and Gupta, R. **(**2013) Rapid conversion of chicken feather to feather meal using dimerickeratinase from Bacillus licheniformis ER-15. *Journal of Bioprocessing &Biotechniques* 2012.
13. Lin, X., Lee, C.-G., Casale, E. S., and Shih, J. C. H. **(**1992) Purification and characterization of a keratinase from a feather-degrading Bacillus licheniformis strain. *Applied and Environmental Microbiology*.**58**(10): 3271-3275.
14. Deivasigamani, B., and Alagappan, K. M. **(**2008) Industrial application of keratinase and soluble proteins from feather keratins. *J Environ Biol*. **29**(6): 933-936.
15. Riffel, A., Lucas, F. o., Heeb, P., and Brandelli, A. **(**2003) Characterization of a new keratinolytic bacterium that completely degrades native feather keratin. *Archives of Microbiology*.**179**(4): 258-265.
16. Obeidat, M., Al-Zu'bi, E., and Otri, I. **(**2012) Rapid Detection of Bacterial Pathogens in Clinical Body Fluids by Nested PCR. *Biotechnology*. **11**(2): 81.
17. Kanchana, R. **(**2013) Utilization of biodegradable keratin containing wastes by enzymatic treatment. *Int J Pharm Biol Sci*. **4**(1): 117-126.
18. Gupta, R., and Ramnani, P. **(**2006) Microbial keratinases and their prospective applications: an overview. *Applied Microbiology and Biotechnology*. **70**(1): 21-33.
19. Raju, E. V. N., and Divakar, G. **(**2013) Screening and isolation of keratinase producing bacteria from poultry waste. *International Journal*. **2**(1): 70-74.
20. Savita, K., and Archana, P. **(**2014) Screening of keratinase producers from leather. Journal *of Environmental Research and Development***8**(3A): 639.
21. Kumar, G., Kartik, L., and Kv, B. R. **(**2010) Screening of extracellular keratinase producing bacteria from feather processing areas in Vellore, Tamil Nadu, India. *Journal of Scientific Research***2**(3): 559.
22. Saravanan, K., and Dhurai, B. **(**2012) Exploration on the amino acid content and morphological structure in chicken feather fiber. *Journal of Textile and Apparel, Technology and Management* 7(3).