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Screening of Keratinolytic Bacteria from the Feather Dumping Site of Sivakasi

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Abstract: The aim of this study was to screening and isolation of keratin degrading microorganism from chicken feather dumping site. Three different feather dumping soils samples were taken from Sivakasi area for this study. These three different soils were serially diluted and plated for screening of efficient protease producing microorganism. The zone of protease activity of the isolates determined on skim milk agar medium varied from 5 to 20 mm. Among them, one strain TS2 were produced higher level of zone of 20 and by its protease production. Keratinase assay was carried out by inoculating the eleven isolates individually in feather minimal medium with keratin as a sole source of carbon and nitrogen. Further, Azokeratin assay was performed with 24 h culture supernatant for TS2. Maximum keratinase specific activity was observed for TS2 (41±0.5 U mL^{-1}). Further, the selected strain TS2 was identified by various physical, biochemical characters and it was confirmed *Bacillus thuringiensis* TS2 by 16S rDNA Sequences.

Key words: Feather waste, keratin, keratinase, 16S rRNA

INTRODUCTION

A myriad of microorganisms, mostly fungi and bacteria, have been identified and reported to utilize keratin, by many authors (Rai *et al*., 2010). Keratin is degradable by some species of saprophytic and parasitic fungi (Anbu *et al*., 2005), actinomycetes (Lin *et al*., 1995), some *Bacillus* spp. (Lin *et al*., 1992), *Streptomyces pactum* DMS 40530, (Kumar and Takagi, 1999) *Streptomyces fradiae* ATCC 14544 (Williams *et al*., 1990). Observing natural degradation and parasitization of keratinous substrates, as well as duplication of the process under laboratory conditions, suggest the feasibility of biotechnological processing for improving the utilization of feather as animal feed protein. The application of microbial technology for feather processing holds the following nutritional significance. First, culturing of the microorganisms and keratinase activity may result in a modification of the structure of feather keratin. This may alter its resistance to digestive enzymes of the consuming animals (Elmayergi and Smith, 1971).

Biotechnological approaches involving microorganisms and their enzymes appear a conceptually appropriate processing technology. However, there is no compendious literature on the prospects for industrial applications of keratinolytic microorganisms, especially with emphasis on their production of keratinases, properties of keratinases, mechanism (s) and limitations of keratinolysis. Therefore, we reviewed recent information on microbial keratinolysis is reviewed in order to stimulate the application of the biotechnology in feather processing as animal feedstuff. The upgrading of feather nutritional value should yield a high-protein feedstuff that may greatly spare the use of soybean and fish meal in livestock diets. Furthermore, bioconversion of feather will predictably benefit the poultry industry, man and the environment. It was mainly focused on the screening and isolation of keratin degrading microorganism from chicken feather dumping site.

MATERIALS AND METHODS

Collection of sample: The soil sample was collected from the feather dumping site at Sivakasi, Tamil Nadu, India. Soil sample was collected by using sterile scalpel, at 4 cm depth and transferred to sterile polythene bag. Then this sample was used for further microbiological analysis.

Serial dilution method (Cappuccino and Sherman, 1999): The soil sample was serially diluted i.e., making 10 fold dilutions. One gram of soil sample was mixed with 99 mL of sterile distilled water in the conical flask, which

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gave a dilution procedure was performed up to 10^{-10} of dilutions. The diluted samples were used for the isolation of bacterial species.

Isolation of bacterial species: The sterilized plate with nutrient agar medium was prepared and marked with respective dilutions. With the help of sterile pipettes, 0.1 mL of the diluted sample was kept on Petri plate rotator and with the help of sterilized L-rod, the sample was evenly spread. The plates were incubated at 37°C for 24 h.

Screening on skim milk agar: Skim milk agar medium was sterilized at 121°C for 15 min at 15 lbs pressure. The isolates were streaked on the medium. The zone formed around the colonies due to production of caseinase enzyme, was considered as positive result. The organisms screened with skim milk agar medium were sub cultured by growing the bacterium in nutrient broth medium at 37°C for 24 h.

Screening and selection of keratinolytic bacterial strains: Skim milk plates were prepared for primary screening of bacteria that produced proteases. The selected protease producing bacteria were subsequently grown in feather consisted of (g L^{-1}); NaCl-0.5; $MgCl_2.6H_2O$ -0.1; $CaCl_2$-0.06; KH_2PO_4-0.7; K_2HPO_4-1.4 and feather- 1 g; pH of the medium adjusted to 7.0 (using 1 N NaOH) in which feather were the only sources of carbon and nitrogen (Lin *et al*., 1995). Cultures were grown at 37°C at 120 rpm for 24 h. Keratinolytic strains that completely broke down feathers in the medium were selected.

Enzymatic hydrolysis of azokeratin (keratinase assay): This procedure tested the keratinolytic activity of keratinase on azo-keratin. To begin the process, 5 mg of azo-keratin was added to a 1.5 mL centrifuge tube along with 0.8 mL of 50 mM potassium phosphate buffer (pH 7.5) at 37°C for 1 h with constant agitation (900 rpm). This mixture was agitated until the azo-keratin was completely suspended. A 0.2 mL aliquot of supernatant of crude enzyme was added to the azo-keratin, mixed and incubated for 15 min at 50°C with shaking. The reaction was terminated by adding 0.2 mL of 10% trichloroacetic acid (TCA). The reaction mixture was filtered and analyzed for activity.

The absorbance of the filtrate was measured at 450 nm with a UV-160 spectrophotometer. A control sample was prepared by adding the TCA to a reaction mixture before the addition of enzyme solution. The unit of keratinase activity was defined as a 0.01 unit increase in the absorbance at 450 nm as compared to the control after 15 min of reaction (Burtt and Ichida, 1999).

Identification of feather degrading bacteria by morphological characterization: Efficient feather degrading bacteria was identified based on the morphological, cultural and biochemical characteristics. Biochemical test was performed in KBOO2 Hi Assorted ™ Biochemical Kit. Carbohydrate fermentation test was performed in KBOO9 Hi Carbohydrate ™ Kit (HiMedia). 16S ribosomal RNA (rRNA) gene sequencing.

PCR on the extracted DNA was performed on 100 Volume. Oligonucleotide primers with specificity for eubacterial 16S rRNA genes, primers 16S rDF (CGCTGGCGGCAGGCTTAACA); 16S rDR (CCAGCCGCAGGTTCCCCT) were used to amplify the 16S rRNA gene fragments with template DNA originating from bacteria. The following conditions were used for DNA amplification: 35 cycles consisting of denaturation at 94°C for 0.5 min, annealing at 55°C for 1 min and extension at 72°C for 1.5 min followed by a final extension at 72°C for 3 min. Amplified PCR products of proper size (about 1500 base pair fragment) were confirmed by gel electrophoresis of 1 L samples through a 1% horizontal agarose gel electrophoresis containing 0.5 g mL^{-1} ethidium bromide. Gels were viewed under UV light and photographed.

PCR products were purified using QIA quick Spin columns (Qiagen Inc., Chatsworth, CA). A Perkin Elmer 377 DNA sequencer, in combination with Dye deoxy Terminator Cycle sequencing Kit (Perkin, Foster City, CA) was used for sequencing the purified PCR products by the help of MWG bio informatics centre. Nucleotide sequencing was compared with sequences in the National Centre for Biotechnology Information (NCBI). Gen bank database using the BLASTn program and Ribosomal Database Project (RDP) database using the sequence matching program.

Phylogenetic analysis: Identical 16S rDNA sequence were recognized by analysis of phylogenetic trees and manual comparisons, in which sequence with more than 90% similarity were defined as identical, and these sequences were used for further phylogenetic analysis as Operational Taxonomic Unit (OUT). All sequences were submitted to GenBank for preliminary analysis using the program BLAST (http://www.ncbi.nlm.nih.gov/BLAST) to identify putative close phylogenetic relatives. Distance based evolutionary trees were constructed using the neighbor joining algorithm.

RESULTS

Isolation and screening of keratinolytic bacteria from feather dumping soil: Three different feather dumping soils samples were taken from Sivakasi area for this study these three different soils were serially diluted and plated for screening of efficient protease producing

Fig. 1: Isolation of protease producing microbes from feather dumping soil using skim milk agar

Fig. 2: Selection of protease producing microbes from feather dumping soil using skim milk agar

microorganism. After 24 h incubation the plates were kept at 37°C. Out of nineteen isolates eleven strains exhibited protease activity were further confirmed by skim milk agar and therefore they were used for further study. They were designated as namely TS1, TS2, TS6, TS9, TS11, TS12, TS13, TS14, TS15, TS17 and TS19. The zone of protease activity of the isolates on skim milk agar medium varied from 5 to 20 mm (Fig. 1). Among them strain TS2 produced higher level of zone of 20 mm by its protease production.

Keratinase activity of bacterial isolates: Keratinase assay was carried out by inoculating the eleven isolates individually in feather minimal medium with keratin as a sole source of carbon and nitrogen. Initially the cultures were selected based on the feather degradation ability in the medium. Among 11 isolates four of them were positively degrade the feather in the medium. Further, Azokeratin assay was performed with 24 h culture supernatant. Based on the keratin assay experiment isolate TS2 strain showed higher for keratinase activity. Maximum keratinase specific activity was observed for TS2 produced (41±0.5 U mL^{-1}). TS2 was selected for further analysis (Fig. 2).

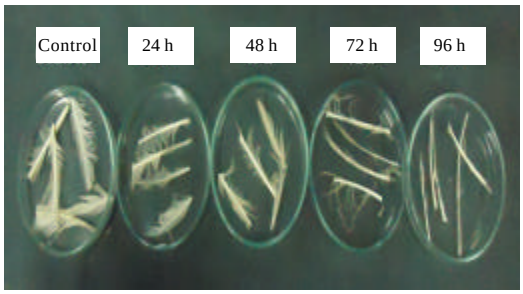

Fig. 3: Feather degradation efficiency of the isolates (TS2) at various time intervals

Table 1: Morphological and biochemical characteristics of TS2

Cultural characters	TS2
Morphology	
Colony property	On nutrient agar, colonies are circular, smooth round, waxy, slight yellow to white and mucoid, produces no pigment
Spores staining	Ellipsoidal and cylindrical, central subterminal, swelling the sporangium
Gram's staining	Gram positive rod
Motility	Motile
Biochemical characters	
Indole production test	+
Methyl red test	+
Voges-Proskauer test	+
Catalase	+
Gelatin liquefaction	+
Caseinase	+
Starch hydrolysis	+

+: Positive result

Effect of various incubation times of bacterial isolates for keratinase production: Selected microbes were inoculated into feather minimal medium for feather degradation at different time intervals. This experiment was carried out in Feather minimal medium supplemented with feather as substrate. After inoculation, it was periodically checked at different time intervals of 24, 48, 72 and 96 h at 37°C with 250 rpm. The maximum activity was observed for TS2 in 96 h (Fig. 3).

Identification of strains based on biochemical and cultural characteristics: The selected strain TS2 was identified by various physical, biochemical and molecular characterization analysis. The strain was Gram positive, rod shaped, endospore forming, and motile bacteria. According to Bergey's manual of Determinative Bacteriology, the selected microbes were identified as *Bacillus* TS2 sp. All the carbon utilizing and biochemical studies were tested using KB0002 Hi assorted TM Biochemical kit and KB009 Hicarbohydrate kit and results were presented (Table 1-3).

Identification of strains based on 16S rDNA gene: The 16S rDNA gene of the *Bacillus* sp. strains TS2 was amplified using Polymerase Chain Reaction (PCR) with the

Table 2: Biochemical results for TS2 by KBOO2 Hi Assorted™ biochemical kit

Biochemical tests	TS2 strain
Citrate utilization	+
Lysine decarboxylase	+
Ornithine	+
Decarboxylase	-
Urease	-
Phenyl alanine	+
Deamination	-
Nitrate reduction	+
H_2S production	-
Glucose	+
Adonitol	-
Lactose utilization	+
Arabinose utilization	+
Sorbitol	-

+: Positive, -: Negative

Table 3: Carbohydrate fermentation tests for TS2 by KBOO9 Hi carbohydrate ™ kit

Carbohydrate	TS2 strain
Lactose	+
Xylose	+
Maltose	+
Fructose	-
Dextrose	+
Galactose	+
Raffinose	-
Trehalose	+
Mellibiose	-
Sucrose	+
L-arabinose	+
Mannose	+
Inulin	+
Sodium gluconate	+
Glycerol	+
Salicin	-
Glucosamine	+
Inositol	-
Sorbitol	-
Manitol	+
Adonitol	-
α methyl D-glucoside	-
Ribose	+
Rhamnose	-
Cellobiose	+
Melezitose	-
α-methyl D-mannoside	-
Xylitol	-
ONPG	+
Esculin	-
D-arabinose	+
Citrate	-
Malanate	+
Sorbose	-

+: Positive, -: Negative

help of 16S rDNA Universal primers. The amplified product was checked in 0.7% Agarose gel showed the expected size of 1.5 kb. The amplified fragment was eluted and both the strands were sequenced. The sequences were compared against 16S rDNA sequences available in the RDP database sequence analysis revealed that the strains were phylogenetically closely related to the genus *Bacillus*. Blast analysis of the 16S rDNA sequence of TS2 isolate revealed that the selected isolates showed maximum similarity of 92% with *Bacillus thuringiensis*.

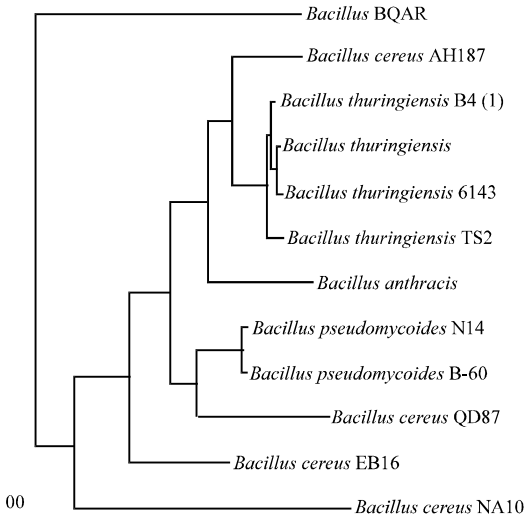

Fig. 4: Phylogenetic analysis of TS2 (*Bacillus thuringiensis*) and some bacterial strains

The Phylogenetic relationship was obtained using neighbor joining by pair wise comparison among the 16S rDNA gene sequence of selected TS2 isolate with species. The dendrogram was constructed for their Phylogenetic relationship and it revealed that the isolate TS2 it grouped with in the *Bacillus thuringiensis* cluster confirmed that it is belongs to *Bacillus thuringiensis*. The 16S rRNA gene sequences of the isolates had been submitted to the GenBank under the accession number FJ377887 for *Bacillus thuringiensis* TS2. Further it was confirmed by 16S rDNA sequencing analysis (Fig. 4).

DISCUSSION

Three different feather dumping soils samples were taken from Sivakasi area for this study. Out of nineteen isolates eleven strains exhibited protease activity were further confirmed by skim milk agar and therefore they were used for further study. They were designated as namely TS1, TS2, TS6, TS9, TS11, TS12, TS13, TS14, TS15, TS17 and TS19. Among them strain TS2 produced higher level of zone of 20 mm by its protease production. Selected microbe was inoculated into feather minimal medium for feather degradation at different time intervals, the maximum activity was observed for TS2 in 96 h. The selected strain TS2 was identified by various physical, biochemical and molecular characterization analysis. The strain was Gram positive, rod shaped, endospore forming,

and motile bacteria. According to Bergey's manual of Determinative Bacteriology, the selected microbes were identified as *Bacillus* spp. Screening of the strain was very similar to that of the work done by Lin *et al.* (1995), where the seven proteolytic strains of *Bacillus* species isolated from canola meal compost; five feather hydrolyzing strains have been identified as *Bacillus licheniformis*. Arasu *et al.* (2009) have reported the prevalence of *Bacillus* sp. among 13 feather degrading strains screened. Eight strains of *Bacillus* obtained from natural composting sample have shown efficient degradation of wastes and decrease in the feather protein from 13.6 to 1.92%.

In the present study, *Bacillus thuringiensis* TS2 showed maximum keratinase activity at 96 hrs of growth at 37°C. In another study, Williams *et al.* (1990) demonstrated that *Bacillus licheniformis* PWD-I degrades intact feather completely at 50°C in 10 days. Lal *et al.* (1996) recorded the degradation of child scalp hair by *Bacillus subtilis* and *Bacillus licheniformis* strains after 14 days. Suntornsuk *et al.* (2005) observed that the *Bacillus licheniformis* FK14 is also to show and degrade feather at 50°C in 5 days. Giongo *et al.* (2007) reported that keratinolytic *Bacillus* sp. isolated from Brazilian Amazon basin shows considerable degradation after 72 h of incubation. Recently Ionata *et al.* (2008) isolated *Clostridium sporogenes* from solfataric muds which degraded native feather in 7 days. Hence we used *Bacillus thuringiensis* TS2 as an efficient feather degrading strain.

Radha and Gunasekaren (2008) reported that the *Bacillus licheniformis* MKU produced maximum keratinase after 48 h of growth. Similarly Lin *et al.* (1995) observed maximum level of keratinase production between 48 and 60 h growth of *Bacillus licheniformis* strains. El-Refaia *et al.* (2005) found maximum keratinase activity in *B. pumilus* F49 after 48 h of growth. However, Gupta and Ramnani, (2006) reported increased keratinase activity at 72 h of growth by *Bacillus licheniformis* RGI. *Microbacterium* sp. kr10 produces maximum keratinase activity in raw feather medium at 36 h coinciding with the end of exponential phase (Thys *et al.*, 2004).

Phenotypic characterization of any microorganism is the first step towards its identification and taxonomic classification. However, many of the microbes from the same genus share overlapping phenotypic characters and therefore, they are difficult to distinguish only on the basis of phenotypic tests (Rai and Mukherjee, 2010; Rai *et al.*, 2010).

Over the years, determination of the phylogenetic relationship or identification of bacteria based on 16S rDNA sequencing (ribotyping) has been widely accepted. On the basis of phylogenetic analysis the AS-S10-II may thus classified as a species of the genus *Brevibacillus*. Furthermore, Menaquinone-7 isolated from strain AS-S10-11 was also reported to be major quinone in *B. limnophilus* sp. nov. *B. ginsengisoli* sp. nov. (Suzuki *et al.*, 2006) and *B. panacihumi* sp. nov. On the basis of the data obtained from phenotypic, chemotaxonomic and Phylogenetic analyses, the strain AS-S10-11 could not be classified under a known species of the *Brevibacillus* and the name of this new strain was proposed as *Brevibacillus* sp. Strain AS-S10-11.

The 16S rRNA gene of the *Bacillus* spp. strain TS2 was amplified using Polymerase Chain Reaction (PCR) with the help of 16S rDNA Universal primers. 16S rDNA sequences available in the RDP database sequence analysis revealed that the strains were phylogenetically closely related to the genus *Bacillus*. The dendrogram was constructed for their phylogenetic relationship and it revealed that the isolate TS2 it grouped with in the *Bacillus thuringiensis* cluster Blast analysis of the 16S rDNA sequence of TS2 isolate revealed that the selected isolates showed Maximum similarity of 92% with *Bacillus thuringiensis*. The 16S rRNA gene sequences of the isolates had been submitted to the GenBank under the accession number FJ377887 for *Bacillus thuringiensis* TS2. *Bacillus thuringiensis* TS2 was considered as the potential keratinolytic strain. The most effective keratin degrading strains in the *Bacillus* sp. belongs to *Bacillus licheniformis* (Manczinger *et al.*, 2003).

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