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Tannase from Novel Bacterial Isolates – Paved the Way of Industries

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Abstract

Bacteria capable of tolerate high levels of hydrolyzable and condensed tannins by producing extracellular tannase were isolated from different sources viz., tea garden soil, pine forest soil, ruminial fluid and sheep excreta. Based on morphological and biochemical identification using ABIS software these were identified as Klebsiella sp, Enterobacter sp, Staphylococcus sp and E. coli. Secondary screening revealed that genus belonging to Klebsiella and Enterobacter were the best enzyme producer (0.38Uml^{-1} and 0.30Uml^{-1}) and thus were identified using molecular methods. Nearly complete sequences of the small-subunit rRNA genes, which were obtained by PCR amplification and sequencing, were used for phylogenetic characterization which showed concordance results. The phylogenetic analysis based on the comparisons of the 16S rRNA gene sequences showed that the isolate Klebsiella was mostly related to Kleibsiella variicola KT261225 (with the sequence similarity of 98.33%) and isolate Enterobacter with Enterobacter hormaechei KT215540 (95.48%). This work paved the way for isolation of various tannin tolerant bacteria which is not restricted by climate, geography, or host animalis, although number of microbes vary according to tannin content of a particular area.

Keywords: Tannase, bacteria, isolation source, secondary screening, phylogenetic analysis

Introduction

The toxicity of phenolic compounds in the environment has fostered studies of bacteria that are able to tolerate and/or metabolize high levels of these compounds, particularly under anaerobic conditions[1,10,6,16,17]. Tannins are secondary polyphenolic compounds known primarily for their ability to bind to and precipitate proteins and other macromolecules. Soil is considered to be the normal habitat for most of tannin tolerant bacterium however, many others have also been isolated from various ecological niches, such as goat (Capra hircus) faeces[15] , rumnial fluid , fish[13], Antarctic sediment[11] and marine sea water[3]. The goal of the present study was to isolate and identify tannin-tolerant bacteria from various sources viz., tea garden soil, pine forest soil, cow (Bos indicus) ruminial fluid and sheep (Ovis aries) excreta by examining their phenotypic characteristics. The polyphasic analysis was performed for the bacteria which showed high tannase activity during secondary screening.

Our sampling site Solan and Palampur, situated in India within Himachal Pradesh province in between 30° 54' 14.256"North latitude; 77°5' 48.9948 East longitude with an elevation of 1,600 m and 32.12°N North latitude; 76.53° East longitude with an elevation of 1,470 m. With the help of sterile spatula soil samples were collected at a depth of 5cm from pine forest of Dr YSPUHF, Nauni and tea garden of CSKHPKV, Palampur. Ruminial fluid and sheep excreta was collected from Dr YSPUHF dairy in sterile vials All the samples were stored at 4°C before further use.1g

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of each soil sample and sheep excreta suspended in 10ml of sterile saline water (0.86% NaCl) and vortexed. A series of dilutions from 10^{-1} to 10^{-7} were made, these dilutions were used in the spread plate method. The resulting serial dilutions of soil and excreta samples (0.1 ml) were pipetted on different nutrient agar (HiMEDIA, India) media in triplicates. Swab culture of ruminial fluid spreaded on NA plates in triplicates. All culture media supplemented with 1% tannic acid (Merck Company–Germany). All the agar plates were incubated at 30Ú C for 48 h. Primary screening was done by well diffusion method in which sterile nutrient agar contains 1% tannic acid poured in sterile petriplates. Solidified agar punched with a six millimeters diameter wells. Wells filled with 100 ìL of bacterial suspension and blanks (sterile distilled water). The test was carried out in triplicate. The plates were incubated at 30 ± 2°C for 48 h. After incubation a dark zone formation around the microbial colonies indicates the hydrolysis of tannic acid. The highest activity assumed by the largest clear zone ratio[5]. Final screening was done by quantitative estimation of enzymes activity after producing them under submerged fermentation. To determine enzymatic activities, all the isolates were initially grown in nutrient broth for 48 h at 37°C. After incubation, 1000 ìl aliquots of each isolate (OD 660 nm = 1.06, approximately 10^7 $CFUml^{-1}$) dropped on selective media, TAB (Tannic acid broth) containing gl^{-1} potassium dihydrogen phosphate (KH_2PO_4) 0.5 g, dipotassium hydrogen phosphate (K_2HPO_4) 0.5 g, magnesium sulphate ($MgSO_4$) 2.0 g, ammonium chloride (NH_4Cl) 3 g, calcium chloride ($CaCl_2$) 1.0 g and 1% tannic acid, pH 5.5 for production of tannase. The inoculated flasks were incubated for 72 hours at 37°C in an orbital shaker incubator. The extracellular enzyme of each isolate was harvested by centrifuging at 10,000g for 10 min and the obtained supernatant was used for the estimation of tannase activity. Enzyme activity was assayed using rhodanine spectrophotometric method[22]. One unit (U) of the enzyme was defined as micromoles of gallic acid produced per minute by one ml of enzyme extract under optimum conditions. For physiological culture identification, isolates were grouped based on phenotypic characteristics such as colony colour, appearance, elevation, margin and texture on nutrient agar. Other characters such as Gram reaction (for cocci and bacilli detection) and cell arrangement were also recorded. The photos were slightly refined in sharpness and color tone with CorelDRAW X4. Routine biochemical tests such as indole; methyl red; Voges–Proskauer; citrate; the presence of oxidase and catalase; sugar utilization were assessed for each bacteria. Clustering of biochemical analysis was done with SXL software.The results obtained through morphological and biochemical tests were fed into ABIS (Advanced Bacterial Identification Software http://www.tgw1916.net/software.html) to identify the isolates at genus level. During secondary screening, the bacterial isolates which showed highest tannase activity were subjected to molecular analysis. For DNA extraction pure cultures of the target bacteria was grown overnight in liquid NB (Nutrient Broth) and bacterial genomic DNA was isolated as described by Sambrook et al. (1989)[21]. Partial ribosomal amplification was done with 16S eubacterial primers viz., 27f 5'GAGAGTTTGATC CTGGCTCAG3'and 1495r-5'CTACGGCTAC CTTGTTACGA3'for amplifying 16S rRNA genes in Bio Rad Amplification System. The PCR reactions were performed in a 50 ìL volume containing 5.0 ìl buffer, 2mM MgCl2, 0.2Mm each dNTPs, 0.25 ìM of each primer, 0.5 U Taq DNA polymerase and 10 ng of bacterial genomic DNA. Amplification was conducted under the following conditions: initial denaturation at 95 °C for 2 min followed by 29 cycles each consisting of 95 °C for 1 min, 50 °C for 2 min and 72 °C for 3 min with a final extension step at 72 °C for 7 min in an Eppendorf thermal cycler. The reaction products were analyzed on an agarose gel (1.2%) together with a 1 Kb DNA molecular weight marker (Geneii); the results were subsequently photographed. The target amplicon cut out, purified and sequenced at the IMTECH, Chandigarh, India. Consensus sequences, obtained from the comparison of both strands for each sequence, were compared using BLASTN[2] on the whole NCBI refseq genomic database (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) and on the DNA Data Bank of Japan (http://blast.ddbj.nig.ac.jp/). BioEdit program version 7.0 (http://www.mbio.ncsu.edu/bioedit/bioedit.html) was used for accurate alignment. Phylogenetic trees were inferred by using three algorithms namely, neighbor-joining[20], maximum-likelihood[7], and maximum-parsimony[8] methods using the BioEdit version 7.0[9] and MEGA 5[23] packages. Sequence divergences among strains were quantified using the Kimura-2-parameter distance model[12]. The topology of the obtained tree was evaluated by nonparametric bootstrap analysis[7] based on 1,000 resamplings. For treatment of gaps "Complete Deletion" option was chosen. The final phylogenetic tree was visualized with the Fig Tree program (http://tree.bio.ed.ac.uk/software/figtree/.''Concordance" was defined as detecting the same bacterium using molecular testing as were detected using culture testing for description of analysis.

Six bacterial isolates A, B, C, D, E and F obtained on culture medium (NA) supplemented with 1% tannic acid at 10^{-6} and 10^{-7} dilutions with following CFU's 3.56×10^8, 2.20×10^8, 2.21×10^7, 2.69×10^9, 4.10×10^7, 2.50×10^8 respectively. Bacteria capable of degrading /tolerating tannins have also been isolated from; goats (Capra hircus)[6,16] horses (Equus caballus)[18] and faeces of goat and sheep[15]. Extracellular enzyme activity of microorganisms on well diffusion agar plate showed a different size (mm) of zone formation by all isolates. The diameter of the brown halo of all the isolates showed the presence or absence of the tannase production (Fig. 1a). In the present study we obtained six isolates A, B, C, D, E and F which showed dark zone of hydrolysis on the TA plates with respective zone dia (mm) of 16 ± 0.37, 15 ± 0.38, 13 ± 0.45, 15 ± 0.43, 10 ± 0.57 and 11± 0.22 thus, confirming their ability to degrade tannic acid. During secondary screening, out of the 6 isolates, maximum enzyme activity was observed in A (0.38±0.026 Uml^{-1}) and D (0.30±0.032 Uml^{-1}). A significant differences ($p<0.05$) between tested isolates for tannase activity was found. Previously, Mohapatra et al. (2009)[14] reported Bacillus licheniformis to produce 0.356 Uml^{-1} of tannase in medium containing tannic acid-1.0, KH_2PO4-0.45, NH_4Cl-0.35, $MgSO_4$- 0.05 (%w/v). Beniwal et al. (2010)[4] reported 0.6 Uml^{-1} of tannase production by Enterobacter cloacae on MSM medium. Physiological characteristics viz., shape, surface, margin, elevation and pigmentation of the colonies were recorded. Among the gram negative organisms, five (A,B,C,D and F) were found to be bacilli and only one (isolate E) was found to be Gram positive cocci. The biochemical characterization of the isolates was done using various biochemical media. All of the gram-negative rods were facultative anaerobes, could utilize ammonia in the growth medium as a sole nitrogen source, and produced lactic acid as an end product. All isolates grew on a variety of sugars but was not able to utilize starch, or cellulose. Results in Fig 1b indicated that C, D, E had ornithine and arginine fermentation activities. Except A, and B, all C, D, E and F were not able to hydrolyze urea, did not produce hydrogen sulfide, and did not have tryptophan deaminase or oxidase activity.

ABIS identification revealed culture A and B as Klebsiella sp, C and D as Enterobacter sp, E as Staphylococcus and culture F as E.coli. Maximum tannase activity was observed in culture A and in culture D. Both the cultures were therefore subjected to molecular analysis. We chose to identify 16S rDNA sequences in the NCBI refseq genomic database with a 100% coverage of the query sequence and with a degree of identity 90%. The BLAST research, performed for all the sequences of the 16S rDNA gene, revealed valid identifications. Sequencing and homology queries in databases produce values e" 90% for maximum identity and E values near 0 for the Nucleotide Blast and DDBJ analysis. The identified homologies correspond to strains belonging to species of the genera Klebsiella (A) and Enterobacter (B). Consistent results were obtained in both consulted databases. Both genera were in complete concordance with culture bacterium. The obtained bacterial sequences were submitted to GeneBank (accession numbers Enterobacter hormaechei AN_1 KJ934724 and Klebsiella variicola AN_2 KJ934725). Peter et al. (2009)[19] used 16S F 5'CCGAATTCGTCGA CAACAGAGTTTGATCCT GGCTCAG 3'and 16S R 5'CCCGGGATCCAAGCTT ACGGCTACCTTG TTACGACTT 3' primers for amplification of 16S rrna gene for identification of Citrobacter. The analysis of 16S rRNA gene sequences indicated that both the sample strains (A and B) was placed in the phylogenetic lineage occupied by the genus Klebsiella and Enterobacter Strain A was clustered with Kleibsiella variicola BCD9 in the phylogenetic tree whereas strain B was clustered with Enterobacter hormaechei KT215540. The close relationship between the isolates was supported by different treeing algorithms, as well as by a high bootstrap value (Fig. 2a & 2b). Concurrent production of tannase from microbial isolates in large quantities in a short time by fermentation in cheap production medium will reduce the production cost, maintenance cost and man power. Bacterial isolates obtained here Enzyme producing industries are still looking for microbial isolates suitable to their needs of production on pilot scale.

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Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Disclosures

The manuscript does not contain clinical studies or patient data.

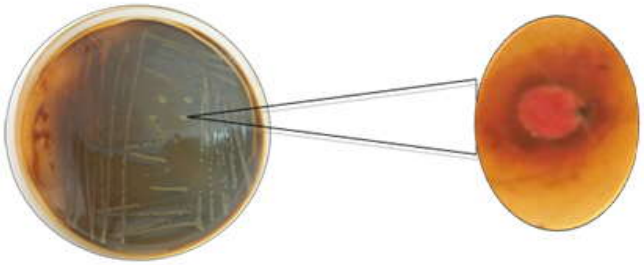

Fig 1a. Zone formation by isolate A in well diffusion method (right)

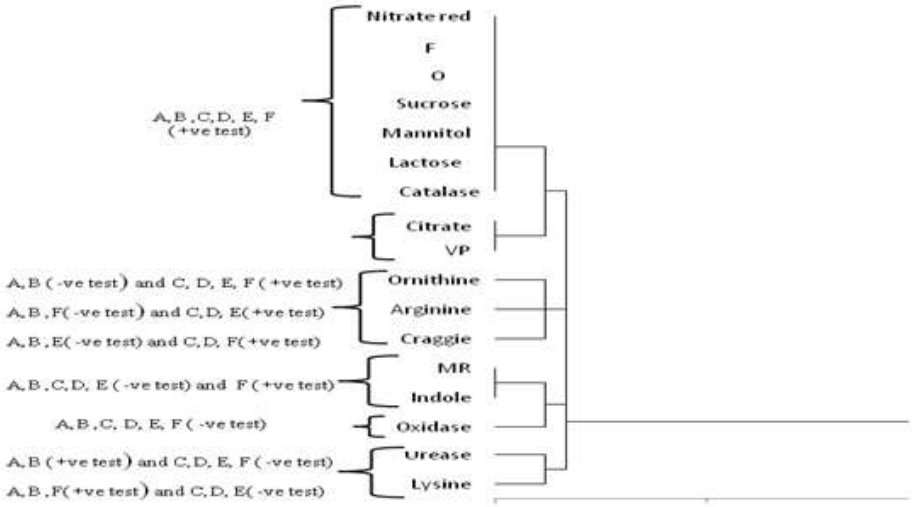

Fig. 1b Dendrogram of different biochemical test of six bacterial isolates based on rescaled distance

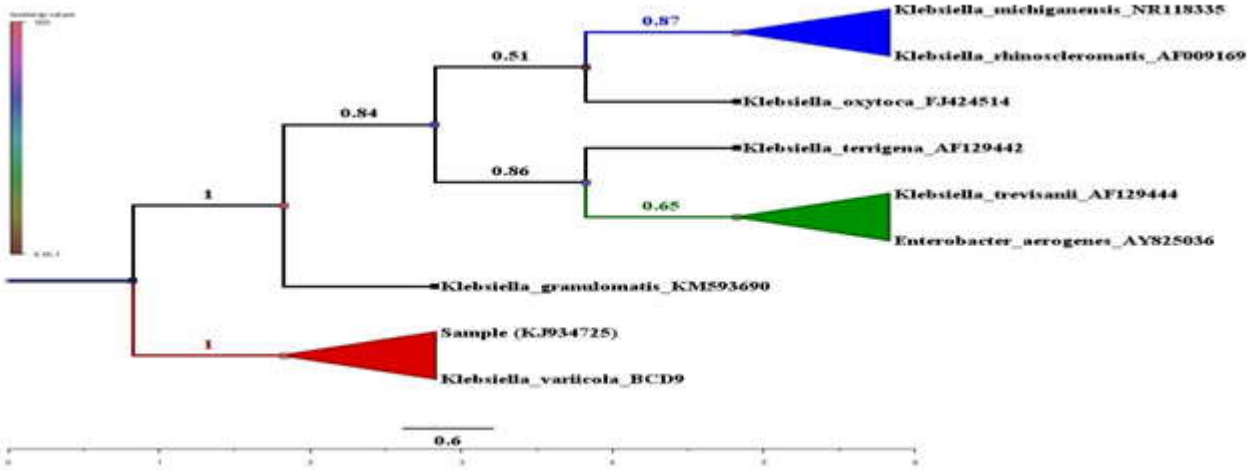

Fig. 2a Phylogenetic tree showing the taxonomic position of strain A (Sample KJ934725) within the genus Klebsiella varricola BCD9 strain. The tree was constructed from a Kimura two-parameter distance matrix and the neighbor-joining method. Percentage bootstrap values (> 50%, 1,000 replications) are given at branching points, and filled squares indicate that the corresponding nodes (groupings) were also recovered in maximum-parsimony and maximum-likelihood

trees. The legend represents scientific bootstrap values showing E-value range.

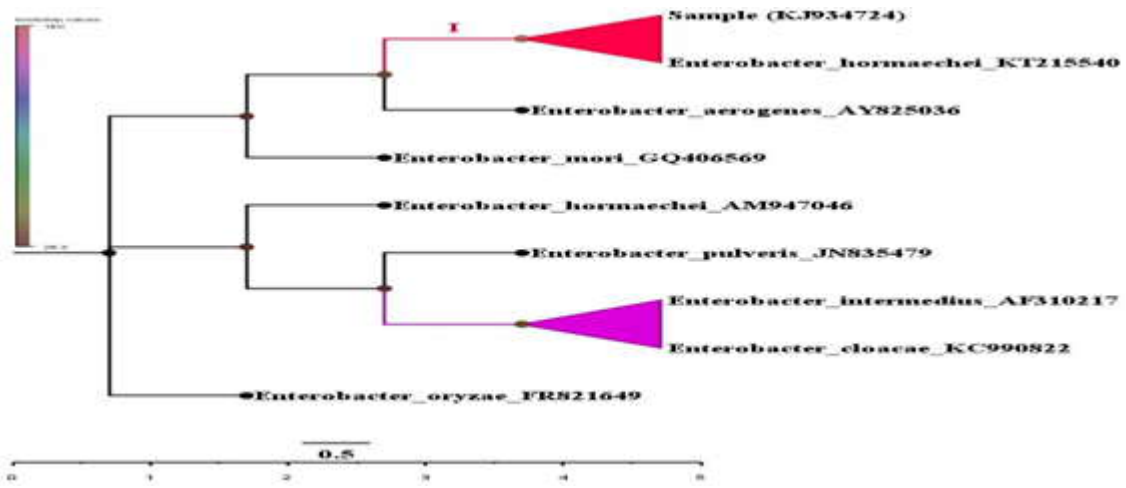

Fig. 2b Phylogenetic tree showing the taxonomic position of strain B (Sample KJ934724) within the genus Enterobacter hormaechei KT215540. The tree was constructed from a Kimura two-parameter distance matrix and the neighbor-joining method. Roman bootstrap values (e" 90%, 1,000 replications) are given at branching points, and filled circles indicate that the corresponding nodes (groupings) were also recovered in maximum-parsimony and maximum-likelihood trees. The legend represents scientific bootstrap values showing E-value range.

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