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Isolation, Identification and Molecular Characterization of Tannase Producing *Klebsiella* sp., from the Rumen of Migratory Goats and Sheep

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ABSTRACT

Tannase, so called Tannin Acyl Hydrolase (TAH) (E.C.3.1.1.20) is one of the versatile biocatalyst with several industrial applications. It is solely responsible for the degradation of hydrolysable tannin thus surpassing the untoward effects of high concentration of tannin consumption in the gut of small ruminants. In the locality of Palampur the migratory goats and sheep have the unique property to resist excess amount of tannin consumed by them. This is because of their ruminal microflora. The concept of ruminal microflora can be used as Direct Fed Microbial (DFM) gaining momentum now-a-days. Thus in the present study attempts were made to explore new source of tannase producing microbes from the rumen of goats and sheep. Rumen samples were collected over several places of Palampur, then processed and finally the most tannin tolerable cultures were selected. Their genomic DNA were isolated and subjected for 16S rRNA sequencing to characterize the isolates. From the molecular characterization we identified *Klebsiella* sp. as a novel source of tannase from the rumen of these migratory animals. This is the first report of a *Klebsiella* sp. from the rumen of migratory small ruminants capable of degrading forage tannin.

Key words: Hydrolysable tannin, tannase enzyme, direct fed microbial, 16S rRNA amplification, molecular characterization

INTRODUCTION

Tannins are the one of the most abundant plant polyphenolics distributed widely among vascular plants as well as in pteridophytes, angiosperms and gymnosperms (Singh *et al*., 2003; Mueller-Harvey, 2006). Tannins being capable of forming reversible and irreversible complexes with proteins, often limits the use of nutritionally important forage trees, shrubs, legumes etc., as feeds for livestocks (Chavez-Gonzalez *et al*., 2012). Structurally tannins can be grouped as Condensed Tannins (CT) and Hydrolysable Tannins (HT). The HTs are made up of a carbohydrate core whose hydroxyl groups, are esterifies with phenolic acids (mainly gallic acid and hexahydroxydiphenic acid). The CTs or proanthocyanidins (PAs) are non-branched polymers of flavonoids units (flavan-3-ol, flavan-3,4-diol) and usually have a higher molecular weight. The

benefits or the bizarre of tannins to animals mostly depend on species of host, quantity of consumption and physiology of the animals consuming them (Hagerman and Butler, 1991). Beneficial effects of tannins include the use of tannins as an astringent, anti diarrheal, diuretic, anti inflammatory, antiseptic and anti-hemorrhagic agents and also to alleviate the poisoning caused by heavy metals (Rodriguez-Duran *et al*., 2011) and have defense mechanism against fungi, bacteria and viruses (Sung *et al*., 2012; Jana *et al*., 2014). Tannins present in the diet of ruminants interact with the biological macromolecules to form complexes that interfere with the digestion process of the nutrients i.e., render the feed ingredients less digestible suggesting the deleterious effects of it. Tannins significantly reduce the feed intake by livestock by various mechanisms (Belmares *et al*., 2004; Li *et al*., 2006). High concentration of tannins often leads to quality deterioration of beverages such as iced tea, beer, wine, fruit juices and coffee-flavored beverages and impose losses to food industry (Rodriguez-Duran *et al*., 2011). Livestock consuming tannin-rich diets (>5% w/v tannin) may develop a negative nitrogen balance and lose weight and body condition (Brooker *et al*., 1999). Pyrogallol, a product of hydrolysable tannins degradation by anaerobic bacteria in rumen is a potent hepatotoxin and nephrotoxin (Reed, 1995).

India is mainly an agriculture based country and population explosion creates havoc with regards to nutritional status among human as well as livestocks. So, livestock sector pay attention to make the unconventional feeds palatable and nutritious simultaneously. This can be possible by improving the nutritional value of the poor quality roughages through holistic approaches and make those feeds available for the farmer cost effectively. In Palampur area mostly the sheep and goats are reared in free range pattern i.e., the animals are of migratory type. These animals have the unique ability to withstand high concentration of forage tannin and this is mainly due to microbial degradation of tannins in the rumen itself. The ester bonds and depside linkages present in tannin are both probably cleaved in the rumen by microbes producing the enzyme tannase. Tannase, the hydrolase group of enzyme is solely responsible for the degradation of hydrolysable tannins and suppress its anti-nutritional activities (Bhat *et al*., 1998; Lu *et al*., 2009). A number of rumen bacteria such as *Eubacterium oxidoreducens*, *Streptococcus bovis*, *Syntrophococcus sucromutans* and *Coprococcus* sp. are involved in degradation of tannins (Tsai *et al*., 1976; Krumholz and Bryant, 1986a, b). Mechanism by which bacteria can overcome inhibitory effects of dietary tannins include tannin modification/degradation, dissociation of tannin-substrate complexes, tannin inactivation by high affinity binders, membrane modification/repair and metal ion sequestration (Singh *et al*., 2003; Goel *et al*., 2005; Smith *et al*., 2005). Apart from nullifying tannin negatives, tannase has greater application in feed, food and pharmaceutical industry (Beniwal *et al*., 2013). Being a novel tannase source, the rumen microbes degrading tannins can be used as feed supplements to overcome the tannin toxicity and improve rumen fermentation (Kohl *et al*., 2015). This study describes various strains of *Klebsiella* sp. from the rumen culture of migratory sheep and goat as a novel source of tannase.

MATERIALS AND METHODS

Chemicals: All the chemicals and reagents used were of high quality analytical grade. For media preparation individual components were purchased from HiMedia laboratories, Mumbai, India. Tannic acid, gallic acid, pyrogallol, EDTA, glycerol were purchased from Sigma (St. Louis, USA). Nuclease-free water was purchased from Ambion. The DNA markers and DNeasy Plant Mini Kit was from Qiagen, Valencia, CA, USA.

Isolation of tannic acid degrading cultures: Rumen contents were collected from slaughtered goats and sheep that are reared on conventional forages available locally in free range system in a sterile container flushed with carbon dioxide (CO_2). The contents were filtered through double layered musline cloth to obtain the rumen liquor, the base material of our study. Rumen liquors were inoculated to the milk dilution bottle containing the growth study medium fortified with 0.4% tannic acid (T-GSM). The composition of the medium (Table 1) was given by Russell (1987) with slight modification. The bottles were kept in incubation at 37°C for 1 day. Colonies showing a clear zone surrounding it were selected and sub cultured to another bottle containing the desired medium with an aim to obtain pure culture. The purity of the microbial culture was determined by simple microscopic examination. Once a pure culture was obtained it was maintained by sub culturing in T-GSM broth in tubes.

Forage tannin extraction: Tannin was extracted from the locally available forages by the method prescribed by Bade *et al*. (2010). About ten different forages were selected for tannin extraction and tannin was extracted from different parts like leaves, fruits, seed and fruit peels depending upon the forages (Table 2). The samples were dried and ground to powder form. The Soxhlet apparatus was used for extraction of tannins. First 100 g of each of the sample was defatted with hexane. The defatted samples were taken out from the Soxhlet and dried in hot air oven at 45°C. After drying the defatted contents were extracted with methanol for 15 cycles to completely extract the tannin contents. The methanolic extract was concentrated by evaporating methanol in vacuum evaporator.

Detection of tannase activity in the cultures and selection of elite isolates: The method described by Rodriguez *et al*. (2008) was used with little modification to detect tannase activity in cell-free cell lysate of our isolates. The isolates were grown for 72 h in GSM (pH, 5.5) containing filter sterilized galactose (0.2%, w/v) and tannic acid (0.4%, w/v). The cells were harvested by

Table 1: Composition of Growth Study Medium (GSM)

Components	Quantity
K_2HPO_4 (mg L^{-1})	290
KH_2PO_4 (mg L^{-1})	292
NH_4SO_4 (mg L^{-1})	480
NaCl (mg L^{-1})	480
$MgSO_4$, $7H_2O$ (mg L^{-1})	100
$CaCl_2$. $2H_2O$ (mg L^{-1})	64
Na_2CO_3 (g L^{-1})	4
L-Cysteine hydrochloride (g L^{-1})	6
Yeast extract (g L^{-1})	1
Trypticase (g L^{-1})	1
Galactose (20 mmole L^{-1})	3.6
Agar % (wherever required)	3

Table 2: List of different plant sources used for tannin extraction in the current study

Plant names (local names)	Scientific names	Part used for tannin extraction
Baheda	*Terminalia belerica*	Fruits
Arjun	*Terminalia arjuna*	Leaves
Herar	*Terminalia chebula*	Fruits
Tremal	*Ficus roxburghii*	Leaves
Safeda	*Eucalyptus* sp.	Leaves
Sea-buckthorn	*Hipophae* sp.	Leaves
Guava	*Psidium guajava*	Leaves
Tamarind	*Tamarindus indica*	Seeds
Pomegranate	*Punica granatum*	Outer covering of fruits

centrifugation (12 000×g, 15 min, 4°C) and were washed thrice with chilled phosphate buffer (5.0 mM, pH 6.5). The cells were disintegrated by sonication (LabSonic, B. Braun, Germany) in tubes kept in ice. About 2 mL of the filter-sterilized cell lysate was supplemented with tannic acid (0.4%) and incubated anaerobically (38.5±0.5°C, 48 h). Tannic acid metabolites were analyzed by the TLC method of Sharma *et al*. (1998). The tannase activities were detected by visual reading method (Osawa and Walsh, 1993).

For TLC the standard comprises of tannic acid, gallic acid, pyrogallol and resorcinol of 0.4% w/v concentration each. The solvent is a mixture of chloroform: ethyl acetate: acetic acid in 50:50:1 ratio. Tannase activity detection was performed on both the cell lysates of the culture and the culture itself. To T-GSM 50 µL of culture was added and incubated at 37°C and samples for TLC were collected at different time intervals like 8, 16, 24, 48, 72 and 96 h post incubation to observe the best degradation pattern. On each TLC plate 5 µL each of standard along with samples were taken and allowed to run inside TLC chamber containing solvent. After the run the plate was dried and placed inside the detection chamber. The isolates that have supreme power to withstand high concentration (>2%) of tannin were termed as elite isolates and were selected for molecular interventions.

Molecular characterization and phylogenetic analysis of isolates: Genomic DNA was extracted by using MO BIO ultraclean microbial DNA isolation kit (MO BIO Laboratories, Inc, 2746 Loker Ave West, Carlsbad, CA, USA). The 16S ribosomal DNA sequences obtained were analyzed by using the BioEdit software and aligned by ClustalW. The 16S rDNA sequences of our isolates were compared with the nucleotide sequences present in Genebank by using standard BLSTn search (Altschul *et al*., 1997) and conclusion was made about the species depending upon the result obtained. The Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6 (Tamura *et al*., 2013). Phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987) considering the 16S rDNA sequences of some of the known bacterial sources of tannase and our sequences. The correctness of the tree was evaluated by 1000 bootstrap resampling value.

RESULTS

Isolation of tannin degrading cultures: The morphology of microbial colonies obtained was diverse and different form each other. A total of 84 isolates (named G1-G84) were selected from goats and 62 isolates (named S1-S62) were isolated from sheep. The colonies were of circular to oval, dry and 1-2 mm in diameter. Figure 1 shows the colonies of bacterial isolates with clear zone of hydrolysis on T-GSM agar. Table 3 enlists the summery of bacterial isolates obtained from rumen.

Detection of tannase activity: All the sheep and goat isolates were able to tolerate at least 0.4% tannic acid. Only 44 isolates of sheep and goats were capable of showing degradation of tannic acid to pyrogallol through gallic acid as intermediate. Further, these isolates were subjected to increased concentration of tannins i.e., 2.0% w/v in GSM agar. A total of 14 isolates exhibiting tolerance to 2.0% (w/v) of tannins and were selected for further studies. Figure 2 indicates detection of tannase activity in different isolates by Thin Layer Chromatography (TLC). The forage tannin degradation by the isolates showed many variations. Figure 3 describes the forage tannin degradation by tannase activity of *Klebsiella oxytoca* strain G-25 (Goat isolate). Figure 4 describes the forage tannin degradation by tannase activity of *Klebsiella* sp. strain S-49 (Sheep isolate).

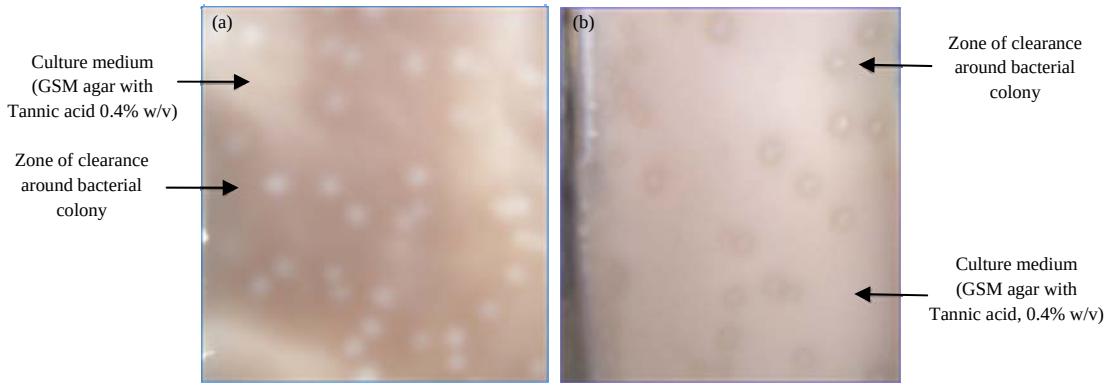

Fig. 1(a-b): Colonies of bacterial isolates with clear zone of hydrolysis on T-GSM agar

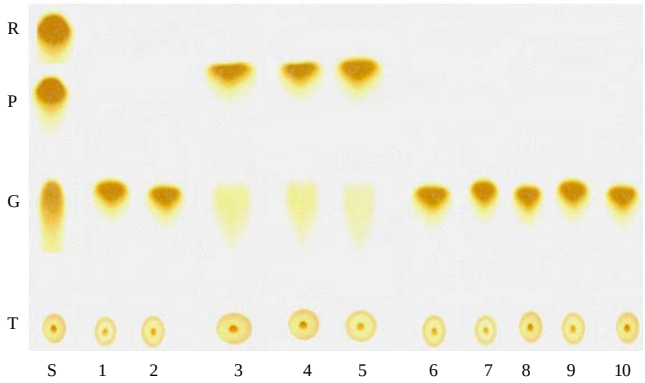

Fig. 2: Detection of tannase activity in different isolates by Thin Layer Chromatography (TLC). S: Standard, T: Tannic acid, G: Gallic acid, P: Pyrogallol, R: Resorcinol, 1: G20, 2: G23, 3: G25, 4: G40, 5: S49, 6: G33, 7: G37, 8: S40, 9: S43, 10: S45. (e.g., G20 means Goat isolate no. 20 and S40 means Sheep isolate no. 40)

Table 3: Summary of the bacterial isolates obtained from the rumen of goats

Morphological features	Total No. of the isolates	Level of tolerance to tannic acid (w/v) (%)	Degradation of forage tannins (Metabolites detected by TLC)
Gram negative cocci, scattered or arranged in chains	65	0.4-1.5	Gallic acid and pyrogallol
Gram negative bacilli, scattered, variable lengths	7	0.4-2.0	Gallic acid and pyrogallol
Gram positive, bacilli with thick and rounded ends	3	0.4-1.5	Gallic acid and pyrogallol
Gram positive curved bacilli, scattered or arranged in small groups	2	0.4	Gallic acid
Gram positive, small rods, scattered or arranged in small groups	4	0.4	Gallic acid
Gram negative, polymorphic bacteria	3	0.4	Gallic acid

Molecular characterization of isolates: The selected isolates were found to be Gram negative, non-motile and non-spore former. The isolates were catalase negative. The 16S rDNA sequencing result of two goat isolates and one sheep isolate confirmed that the isolates were of *Klebsiella* sp. Figure 5 describes phylogenetic (neighbor-joining) tree based on 16S rDNA sequences showing the relationship of our isolate (marked as pink triangle) with some of the related tannase producing bacteria. All bacteria name was written along with their accession number. Significant bootstrap probability values are indicated at the branching position. Number of substitution per nucleotide is 0.02. The 16S rDNA sequence of three isolates were submitted to GenBank under the accession nos; KM434222, KM434223, KM434224.

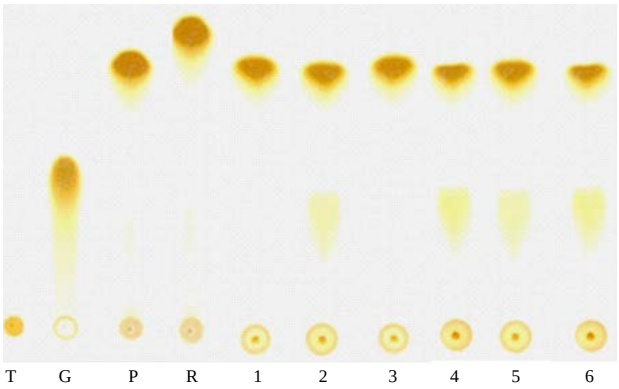

Fig. 3: Forage tannin degradation by *Klebsiella oxytoca* strain G-25. T: Tannic acid, G: Gallic acid, P: Pyrogallol, R: Resorcinol, (tannin extracts of) 1: Herar, 2: Arjun, 3: Tremal, 4: Baheda, 5: Sea-buckthorn and 6: Guava

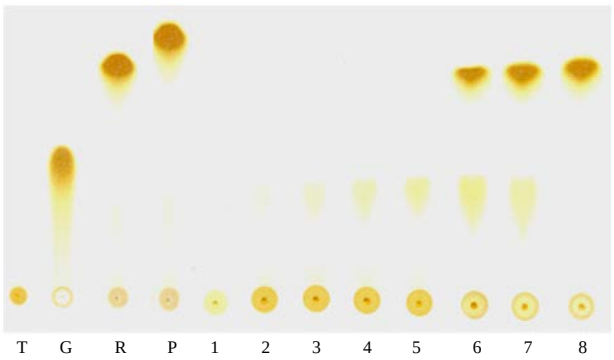

Fig. 4: Forage tannin degradation by *Klebsiella* sp. strain S-49. T: Tannic acid, G: Gallic acid, P: Pyrogallol, R: Resorcinol, (tannin extracts of) 1: Safeda, 2: Arjun, 3: Tamarind, 4: Baheda, 5: Sea-buckthorn, 6: Herar, 7: Guava and 8: Tremal

DISCUSSION

In the present scenario of population explosion, satisfying the nutritional hunger of both man and animal is really a challenging task. Thus craving for alternative feed sources and their rapid production through biotechnological intervention is the new rule. Rumen microbial flora is the richest repository of various microbes and their involvement in the feed and fodder improves the quality. Tannase is one of the most useful enzyme and ruminal microflora are a novel source for tannase. Treatment of feeds with tannase of rumen microbial origin improves the host ability to take on tannin rich diets (Raghuwanshi *et al*., 2014; Kohl *et al*., 2015). The conventional process of tannase production strikes several limit in the path of the efficient utilization of this enzyme. A major problem in the utilization of fungal strains for industrial applications was that degradation by fungi is relatively slow and it is also difficult to manipulate fungal strain genetically because of their complexity (Chowdhury *et al*., 2004). Also the fungal treatments can only be used as pre-feeding treatments which involves more cost and labor (Goel *et al*., 2015). So, the molecular intervention in this aspect is the light for the researcher. Thus possible new source of tannase helpful for decreasing the present gap exist in the field of tannase and tannin study.

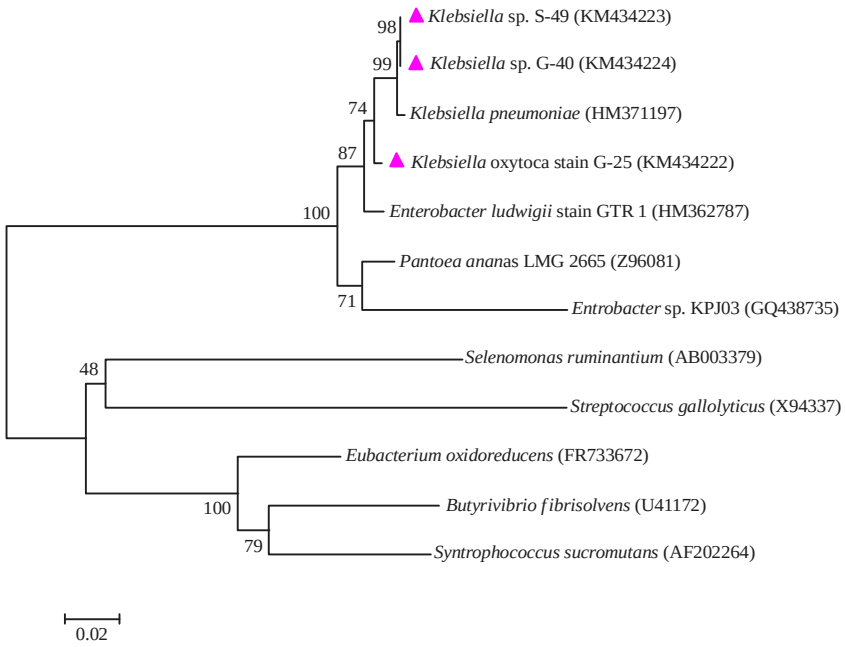

Fig. 5: Phylogenetic (neighbor-joining) tree based on 16s rDNA sequences showing the relationship of our isolate (marked as pink triangle) with some of the related tannase producing bacteria. All bacteria name was written along with their accession number. Significant bootstrap probability values are indicated at the branching position. Number of substitution per nucleotide is 0.02.

Isolation of tannin degrading cultures: It is well reported that tannase of bacterial origin can degrade tannic acid and natural tannins (Deschamps and Lebeault, 1984; Chowdhury *et al*., 2004). The GSM was preferred for studying tannic acid-degradation due to less precipitation of the medium after adding tannic acid (Singh *et al*., 2012). Thus T-GSM was used for initial screening of our isolates. The diverse nature of microbial colonies obtained may be due to the fact that goats and sheep in the region contain diverse populations of rumen microorganisms. Degradation of HTs by ruminal microbes is a feature of fore-stomach fermentation that is widely distributed among ruminants (McSweeney *et al*., 2001; Singh *et al*., 2012), thus giving zone of clearance in T-GSM. Besides, the Gram negative bacilli selected for present investigations, the Gram positive bacilli were also involved in degradation of tannic acid and tannins. But these isolates were tolerant to low levels of tannins, hence not studied further. These findings are similar to those showing that gut lactobacilli can degrade dietary tannins (Osawa *et al*., 2006). In addition, the isolates with diverse morphological features were able to degrade forage tannins and need further studies. The concept of HT being anti-nutritional or toxic to ruminants is well documented (Singh *et al*., 2003; Ephraim *et al*., 2005; Mueller-Harvey, 2006). The animals, from which rumen contents were collected in the present investigation, were apparently healthy and consumed tannin-containing forages efficiently. Earlier also, it is reported that migratory goats consuming forages containing anti-nutritional phytometabolites apparently did not suffer from any toxicity, which indicates that they may have developed mechanisms to overcome the negative effects of HT and may have evolved mechanisms to derive nutrients from the tanniniferous diets (Singh *et al*., 2012). This may be due to the establishment of tannin-tolerant bacteria like *E. ludwigii* GRT-1in their GI tract

(Singh *et al*., 2012). The goats and sheep may possibly have other tannin-degrading microbial species in their GI ecosystem as evidenced by the presence of phenotypically diverse range of isolates. Hence, it is speculated that certain gut microbes enable the goats and sheep to efficiently utilize the forages containing HTs and that residual tannins escaping microbial degradation may serve as anthelmintics as reported earlier (Waghorn and McNabb, 2003). However, the present work is a preliminary study and relevant to GI microbial degradation of HTs and the physiological role of their metabolites in these animals requires further investigations.

Detection of tannase activity: A number of gut bacteria have been reported to degrade HTs by means of synthesis of tannase. Tannase hydrolyses the ester and depsidic bonds of hydrolysable tannin, thus forming pyrogallol from tannic acid through gallic acid intermediate (Li *et al*., 2006: Aguilar-Zarate *et al*., 2014). This formed the basis for detecting tannase activity in TLC plates. The degradation profiles for the forage tannins followed almost same pattern in our isolates. They are equally capable of degrading forage tannins but with difference in the time to taken by them to convert the tannin to its products. This may be due to difference in nature and activity of the isolates, which needs to be validated. Sharma and John (2011) reported tannase from the Gram negative bacterium *Enterobacter* sp. which could be used to protect grazing animals against tannins. Goel *et al*. (2011) also reported tannase from *Enterobacter faecalis*. Singh *et al*. (2012) reported *Enterobacter luduwgii* strain GRT-1 from the rumen of migratory goats and sheep of Palampur and proposed the concept of using rumen anaerobes as DFM in livestock feeds. All these reports suggest tannase enzyme activity from anaerobic microbes. Tannase activity was reported in *Klebsiella* species from different sources (Deschamps *et al*., 1983; Jadhav *et al*., 2011; Sivashanmugam and Jayaraman, 2011; Pepi *et al*., 2013) but not from the rumen microbial culture. Information is scarce on molecular characterization of tannase of the gut microbial origin. To the best of our knowledge, the present report is the first report on tannase activity of *K. oxytoca* isolated from rumen of migratory goats and *Klebsiella* sp. in sheep which browse on tanniniferous foliages prevalent in North-Western Himalayan Region (NWHR). Further studies are warranted on polymorphism in tannase genes in rumen bacteria of migratory animals in NWHR. This will provide useful insights into genetic basis of synthesis and regulation of tannase for its utilization at commercial scales.

Molecular characterization and phylogenetic analysis of isolates: Till date, there are lot more documentation regarding sources of tannase. For constructing phylogenetic tree we only consider some of the bacterial sources as reported earlier. All those bacteria have their 16S ribosomal RNA (~1500 nucleotides) sequence present in NCBI. Recently discovered *Klebsiella* sp. C2A strain proposed by Pepi *et al*. (2013) has not taken into consideration as in NCBI it has partial 16S ribosomal RNA of ~700 nucleotides length only. Sivashanmugam and Jayaraman (2011) reported about *Klebsiella pneumoniae* MTCC 7162, isolated from tannery effluent. The phylogenetic tree was constructed by taking similar reported taxa as tannase producer means for the above mentioned *Klebsiella pneumoniae* MTCC 7162 we took *Klebsiella pneumonia*.

The rumen is a highly specialized fermentation vat containing a mixture of diverse nature of enzymes produced by bacteria, protozoa and fungi, with potential broad application in both research and industry. In this regards the rumen of small ruminants of Himachal surviving on tanniniferous diets can be a suitable source of tannase producing microbes. Further studies on molecular aspects of these microbes and metagenomics approaches will be helpful to enhance our existing knowledge.

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