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Purification, characterization and application of extracellular Tannase by *Klebsiellsa pneumoniae* isolated from Goat rumen

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ABSTRACT

Several bacteria were isolated from the partially digested contents of the goat rumen. The bacteria were screened for production of enzyme tannase. One bacterium was found to efficiently utilize tannic acid as a sole carbon source, which was selected as the best tannase producer and utilized for the further study. The bacterium was found as pale yellowish in colour by morphological identification in nutrient agar plates, Upon Gram Staining, it was found to be Gram positive rods. It showed various biochemical characteristics related to the genus *Klebsiella*. It was further subjected to 16S rRNA sequencing. Blast analysis revealed that the bacteria was closely related to *Klebsiella pneumoniae*. The crude tannase enzyme was produced from the bacteria and it was purified by Ion exchange and gel filtration chromatography. The specific activity of the crude and purified tannase was found to be 26.1 and 68.2 U/mg respectively. The enzyme had optimal activity at the temperature of 30°C and pH 5. The addition of calcium, magnesium and manganese were found to increase the enzyme activity. The enzyme activity was influenced by the addition of several detergents. Upon, the use of solid substrates, pomegranate peel was found to be one of the best substrates for high enzyme production. The purified tannase was also found to successfully clarify pomegranate juice in 1 h.

Keywords: Goat rumen, *Klebsiella pneumoniae*, tannase, pomegranate juice clarification.

INTRODUCTION

Tannins are naturally occurring water-soluble polyphenol compounds found in various parts of the plant body, such as leaves, barks, stems and fruits. Tannin is abundant in natural plants such as monocots, tea, coffee, sorghum, berries, nuts, pomegranates, legumes, some herbs and species such as cloves and cinnamon, palm kernel, phyllanthus emblica (amla) and other plant or plant-based products used for human consumption (Bhat *et al*., 1998). Tannin is categorised into two major classes: (a) Hydrolyzable

tannin and (b) Condensed tannin. Most of these occur in condensed form (Bhat *et al*., 1998). Tannin is known for its antimicrobial properties and is resistant to microbes to protect plant bodies. Despite having antimicrobial activity, tannin is used as a nutrient compound or substrate for certain microbes that use it with the aid of a hydrolytic enzyme called Tannase.

Tannase is also known as tannin acyl hydrolyase (E.C.3.1.1.20) which catalyzes the hydrolysis of esters and depside bonds in hydrolysable tannins that produce glucose and gallic acid as products. Tannase is an inducible enzyme produced by a variety of micro-organisms. Research over the past 140 years has led to the discovery of a greater variety of tannase producers. Bacteria, fungi and yeast are the most prominent producers. Few animals have also been found to be tannase producers. These microbes are primarily isolated from tannin-rich forests, animal faeces that feed on tannin-rich plants can be aerobic or anaerobic. Much of the research work was carried out using the filamentous fungi of *Aspergillus* and *Penicillium*, although other bacterial species, including *Lactobacillus plantarum*, *L.paraplantarum* and *L. Pentosus* is also estimated to produce tannase. Fungi show greater behaviour for the degradation of hydrolysable tannins. However, the relatively slow growth rate and genetic complexity of the fungal strain is a major problem at industrial level. On the other hand, the growth rate of the bacteria is very high and can be easily controlled at the genetic level. The bacteria are also capable of surviving under intense temperature conditions, which can prove to be a possible source of thermostable tannase. In addition, bacterial tannins can also degrade and hydrolyse naturally occurring tannins and tannic acids very effectively. However, studies on the production of tannase by bacteria are very limited.

The enzyme has potential uses in the treatment of tannery effluent and pre-treatment of tannin containing animal feed (Aguilar *et al*., 2007; Murugan and Al-Sohaibani, 2010). Gallic acid formed during tannic acid degradation has varied applications in the pharmaceutical and food industries. It is used in the production of antimicrobial drug trimethoprim, used for the manufacture of propyl gallate for use in photography and printing inks, and propyl gallate is also used as an antioxidant in fats and oils. Gallic acid also has properties that have a cytotoxic effect on cancer cells. Tannase enzyme was used to avoid phenol-induced turbidity in wine (Koichi and Tokuji, 1972). Coffee flavoured soft drinks (Suzuki, 1973), beer and fruit juice clarification (Massechelin and Batum, 1981), polyphenol stabilisation (Giovanelli, 1989) and as a flexible analytical probe to determine the structure of naturally occurring gallic acid esters.

Tannin forms insoluble protein complexes. This activity plays a significant role in the non-ruminants (Rabbit, Poultry, etc.) industry. In many dietary and endogenous protein complexes, tannins, as well as digestive enzymes, interfere with normal digestion, leading to high-quality protein leakage from the body. Tannin has also been known to interfere with the absorption of iron. Tannin also affects the mucosal lining of the gastrointestinal tract. The use of tannase as an ingredient in animal feed would increase the digestibility of the food (Lekha and Lonsane, 1997).The enzyme has potential uses in the pre-treatment of tannin containing animal feed (Aguilar *et al*., 2007; Murugan and Al-Sohaibani, 2010).

Gut flora, or gut microbiota, or gastrointestinal microbiota, is the complex community of microorganisms that live in the digestive tracts of humans and other animals, including insects. Some human gut microorganisms benefit the host by fermenting dietary fiber into short-chain fatty acids (SCFAs), such as acetic acid and butyric acid, which are then absorbed by the host. Intestinal bacteria also play a role in synthesizing vitamin B and vitamin K as well as metabolizing bile acids, sterols, and xenobiotics. Many gastrointestinal bacteria from domesticated adoptive and wild animals have been found to produce tannase. Several species of these bacteria have been isolated from feces of koalas, goats, cows and humans (Sabu *et al*., 2006; Nishitani *et al*. , 2004; Goel *et al* . , 2005).

The present study focussed on the isolation of tannase producing bacteria from the gut particles of goat fed with the grass. The bacteria capable of producing the enzyme was isolated, the crude enzyme was produced and its enzyme activity was determined. The isolated bacterium was identified to be *Klebsiella pneumoniae*. The crude enzyme was purified and characterized for its optimal temperature, pH, carbon and nitrogen sources. The effect of inhibitors and detergents on the was assayed. The activity in the presence of various solid substrates were analysed. The purified enzyme was subjected to pomegranate juice clarification.

MATERIALS AND METHODS

The chemicals used in the present study were purchased from Merck and Sigma Aldrich, India and the Culturing Media were purchased from HiMedia laboratories, India.

Sampling and Isolation of bacteria from goat gut particles

Ten samples of partially digested goat gut particles were collected from slaughter house in a sterile airtight container. Samples were enriched in nutrient broth containing 0.1% tannic acid for 24 h and then it was streaked in nutrient agar containing 1% tannic acid. The plates were incubated for 48 to 72 hrs at 37ºC. The bacterial culture which grew in the plate was maintained as slants and stored at 4 ºC for further use.

Screening of bacteria for tannase production

The bacterial culture (1%) was suspended in 50 mL minimal medium containing k_2HPO_4: 0.5g/l, KH_2PO_4: 0.5 g/l, $MgSO_4$: 2.0 g/l, $CaCl_2$: 1.0 g/l and NH_4Cl: 3.0 g/l, supplemented with 1% tannic acid (pH 5.5). The flasks were incubated for 72 h at 37ºC on rotary shaker at 150rpm. The medium was noticed for a change in pale yellow to dark brown color, to confirm the tannase production (Osawa and T. P. Walsh, 1993)

Swarm plate assay

1X minimal medium containing monopotassium phosphate (15.0 g/l), sodium chloride (2.5 g/l), disodium phosphate (33.9 g/l), ammonium chloride (5.0 g/l), 0.2% magnesium sulphate and 0.01% calcium chloride, supplemented with 1% tannic acid and 0.05% tetrazolium chloride was used for the assay. Wells were punctured in the plates using gel puncture and bacteria was inoculated into the wells and incubated at temperature for 37ºC 72 h (Osawa and T. P. Walsh, 1993).

Thin layer chromatography

TLC was performed according to the method of (Sharma *et al.,* 1999). Pre-coated plate with silica gel 60 F25 plate was used for separation. The TLC plates were allowed to dry at 60 ºC for 1 hr. 5 µl of sample and standard tannic acid (1mg/ml) were spotted on the TLC plate. Chloroform: ethyl acetate: formic acid: water was used as mobile phase in the ratio of (6:4:0.1:0.1) and the plate was detected by using iodine vapors.

Identification of bacteria

Morphological and Biochemical identification

The shape, color and texture of the bacteria was identified by visual observation. The structure of bacteria was identified by Gram's staining. The following biochemical tests were performed for bacterial identification: Methyl red test, Indole production test, Vogus Proskauer test, Citrate utilization test, Starch hydrolysis, Catalase production, Nitrate reduction test and Hydrogen sulfide test.

Molecular Identification of bacteria by 16S rDNA sequencing

DNA was extracted from the bacterial cultures grown in Brain heart infusion agar for overnight culture. DNA was harvested from 1 loop of bacterial colony inoculated in 200 µL of Lysis buffer and the mixture was kept incubated at 70 °C for 10 min. The whole lysate was centrifuged at 6000 rpm for 2 min. After centrifugation the supernatant of extracted DNA is used for PCR amplification. For identifying the isolated bacterium by sequencing by sequencing the primers 16S27F Forward-5-AGA GTT TGA TCC TCG CTC AG-3' and 16S1492R Universal Reverse-5'-TAC CTT GTT ACG ACT-3' were used. A total volume of 25 µL reaction mixture contained the following components: Master mix: 12 µL, 16S27F specific Primer-Forward: 2 µL, 16S1492R specific Primer-Reverse: 2 µL, Genomic DNA: 5 µL (100ng), Water, Nuclease free: 4 µL. The Reaction mix was mixed gently and spun down briefly. The PCR protocol consisted of denaturation at 95 °C for 2 min and subsequent 24 cycles of denaturation at 95 °C for 30 seconds, annealing at 51 °C for 1 min, and extension at 72 °C for 2 min, Final Extension at 72 °C for 10 min. The presence of PCR products was determined by electrophoresis of 2 µL of the reaction product in a 1.6% agarose gel with 1XTBE buffer and 100bp ladder as the molecular marker, followed by Ethidium Bromide staining. The remaining PCR products where given for sequencing (Bar Code biosciences, Bangalore, India). which were purified by the PCR purification kit as recommended by the manufacturer.

Crude enzyme production

The bacteria was inoculated into 50 mL of tannic acid broth in an Erlenmeyer flask and incubated at 37ºC for 72 h. The culture was centrifuged at 6000 rpm for 20 min. The supernatant was obtained as crude enzyme.

Estimation of tannase activity

The tannase activity was determined by modified spectrophotometric method of (Sharma *et al., 2000).* Tannic acid was used as the substrate. The principle of this assay is based on the formation of chromogen between gallic acid (released by the action of tannase on tannic acid) and rhodanine (2-thio-4-ketothiazolidine). Gallic acid was prepared as the standard in appropriate concentrations. Crude enzyme was used for the assay. All the assays were performed in triplicates. One unit of tannase activity is defined as the amount of enzyme required to liberate 1µM of gallic acid/ min under defined condition. Enzyme activity was expressed as U/min.

Determination of specific activity of the enzyme

Concentration of the enzyme (mg/mL) was determined (Markwell *et al*., 1978). Specific activity of the enzyme was determined as U/mg.

Enzyme purification:

Ammonium sulphate precipitation

Ammonium fractionation of the crude enzyme was done at 80% saturation levels. The crude enzyme was added with ammonium sulphate under constant stirring at 4 ºC. Then mixture was incubated at 4 ºC for 3 hrs for protein to be precipitated. Precipitated proteins were separated by centrifugation at 8000 rpm at 4 ºC for 20 min. The separated proteins were then dissolved in 0.05M citrate buffer pH 5 (Jana, Arjit *et al*., 2014).

Dialysis

The crude enzyme was then subjected to dialysis against 0.05M citrate buffer, pH 5. It was dialysed over night at 4 ºC and the buffer was changed several times. (Pingoud *et al* ., 2005).

Enzyme purification by DEAE Cellulose chromatography

DEAE-cellulose A50 (4×10 cm) column was used for the purification and it was equilibrated with 0.05M citrate buffer, pH 5 at 4 ºC. The dialyzed enzyme solution was passed on to the column Citrate buffer was used to remove unbound proteins. Elution was carried out at 4 ºC with a linear gradient (0.5 – 2M) of sodium chloride (Chhokar *et al*. 2010).

Gel filtration chromatography

A sephadex G-150 column was equilibrated with 0.1 M citrate buffer, pH 5.0 and the crude enzyme was added to the column. The citrate buffer is added to remove the unbound proteins and the purified proteins are eluted by adding a linear gradient of sodium chloride (0.5 to 2 M). 1 mL of protein fractions was eluted and it was subjected to tannase assay (Anitha and Arunkumar, 2013).

Determination of specific activity of the enzyme, Purification fold and yield percentage

Concentration of the enzyme (mg/mL) was determined. Specific activity of the enzyme was determined as U/mg. Purification fold of the enzyme was calculated as final specific activity/ initial specific activity. Yield (%) was determined as (final total units/ initial total units)×100.

SDS PAGE Analysis

Molecular weight of the purified tannase enzyme was determined by SDS PAGE analysis by the method of (Laemmli *et al*., 1970). Enzyme sample was separated by SDS-PAGE in 12% separation gel and the gel was stained by coomassie brilliant blue staining. Molecular markers (Fermantas. Pvt .Ltd) ranging from 20 to120 KDa were used for analysing the molecular weight of the enzyme.

Effect of temperature on purified tannase

Effect of temperature was estimated by incubating the purified enzyme in 20°C, 30°C, 40°C, 50°C and 60°C and Rhodanine assay was performed. Total activity and relative activity of the purified tannase were estimated.

Effect of pH on purified tannase

Effect of pH was estimated by incubating the purified enzyme in various buffers at pH 4,5,6,7,8 and 9 and Rhodanine assay was performed. Total activity and relative activity of the purified tannase were estimated.

Effect of metal ions on purified tannase

Effect of metal ions was estimated by incubating the purified enzyme in various buffers with calcium chloride, potassium chloride, zinc sulphate, magnesium chloride, Manganese chloride and ferric chloride (1mM) and Rhodanine assay was performed. Total activity and relative activity were estimated.

Effect of detergents on purified tannase

Effect of detergents was estimated by incubating the purified tannase enzyme in various buffers with

sodium dodecyl sulphate, Tween 80 and CTAB (0.5%) and Rhodanine assay was performed. Total activity and relative activity were estimated.

Solid state fermentation

5 g of different substrates like Tea powder, Wheat bran, dried pomegranate peel and Commercial green fodders were taken in a 250 mL conical flasks. The substrates were autoclaved for 20 min at 121 ºC at 15 lbs. Under aseptic conditions, 1 mL of cell suspension (18 – 20h old) were inoculated with the sterilized solid substrates after cooling. After 72 h of fermentation, crude enzyme was extracted from the by adding 50 mL of 0.05 M citrate buffer (pH 5). The crude enzyme was subjected to Rhodanine assay to find out the total enzyme activity.

Application for crude tannase in pomegranate juice clarification

Pomegranate juice was collected, filtered and used freshly for the assay. 1 mL of purified tannase was mixed with 10 mL of the filtered juice. The control tube was added with 1 mL of distilled water instead of the enzyme. The test tubes were incubated up to 1 hour at 37ºC. The tubes were boiled at 60 ºC for 5 min to deactivate the enzyme. The juice was observed for clarification (Rout and Banerjee, 2006).

Statistical Analysis

All the assays were performed in triplicates and the values are expressed as mean ± SD.

RESULTS AND DISCUSSION

Isolation of tannase producing bacteria

The bacteria which was able to grow in nutrient agar supplemented with 1% tannic acid was considered as tannase producers and it was maintained in nutrient agar for further screening. Rumen contents is the richest source of various microbes and they are involved in the utilizing feed and fodder there by improving the quality of the feed. Tannase is one of the most useful enzyme and rumen microflora are a novel source for tannase. Treatment of feeds with tannase enzyme of rumen microbial origin improves the digestive ability of the animals (Raghuwanshi *et al*., 2014; Kohl *et al*., 2015).

Screening of bacteria for tannase production

All the bacteria from the nutrient agar were grown in minimal media supplemented with tannic acid. Change of medium color to dark brown color was observed which indicated the production of tannase enzyme. Upon the efficient color change, one bacteria was selected and studied for further assays (Figure 1).

Swarm plate assay

The production of tannase was confirmed by swarm plate assay by decolorisation of the media with utilization of tannic acid as a sole carbon source. After 24 h, chemotaxis was detected by the movement of the cells from the centre and zone of clearance was observed (Figure 2). Bacteria inoculated in the center of a nutrient-rich plate fortified with less than 0.3 per cent agar can absorb nutrients locally, produce a nutrient gradient and chemotax up the gradient through the pores of the agar. For this assay, tannic acid is applied to the medium as the key source of carbon and the culture is applied to the well. Organisms which display movement and growth in a semi-solid medium containing tannic acid are those which have a high tolerance for the compound. (Tripathi *et al*., 2016)

Thin layer chromatography

Pure gallic acid was used as a standard and it is compared with the test sample to detect the presence of gallic acid. R_f value of standard gallic acid was 1.0 and the R_f value of test sample was 0.73. This shows that tannase produced by the microbes converted tannic acid in to gallic acid and glucose (Figure 3). Similar results have been obtained in previous studies by Tripathi *et al*., 2016.

Identification of bacteria

The bacterium was round shaped and pale yellowish in colour (Figure 4). It was maintained in nutrient media. The tannase producing bacterium was subjected to Gram's staining. It was observed in microscope under 10x and 40x magnification. The results showed that it is a Gram-positive rod-shaped bacterium (Figure 5). The results for biochemical analysis are produced in table 1. PCR amplification of the bacterium using a universal 16S r DNA primers showed the amplification product of 1.5Kb (Figure 6).

Sequencing and blast analysis

Nucleotide Blast analysis of the obtained sequence using n Blast was performed. The obtained sequence showed 99% identity to *Klebsiella pneumonia*. The sequence was submitted to NCBI and the obtained accession number was MK691698.

Figure 1

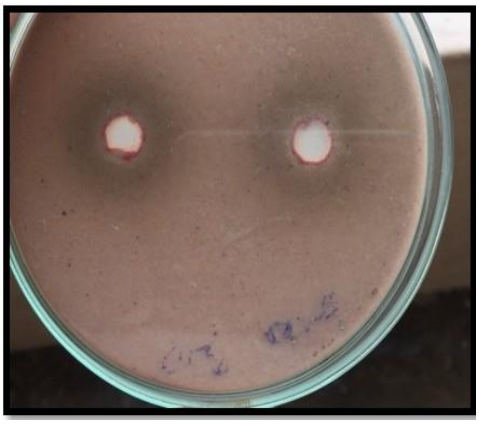

Figure 2

Figure 3

Figure 1: Bacterium grown in minimal media with tannic acid. Formation of dark brown color of the medium indicates the tannase production

Figure 2: Swarm plate assay. Zone of clearance was observed around the well showing utilization of tannic acid

Figure 3: Thin Layer Chromatography. Lane 1 : Spot observed for standard gallic acid, Lan 2: Spot observed for bacteria.

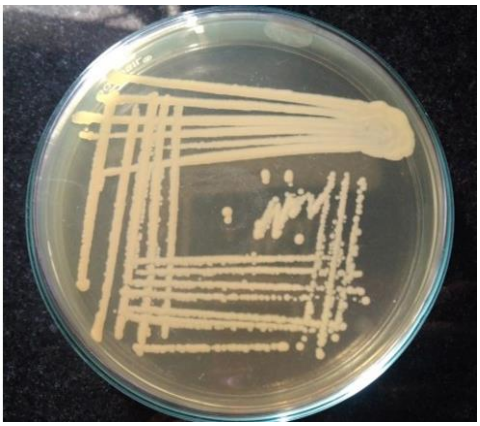

Figure 4

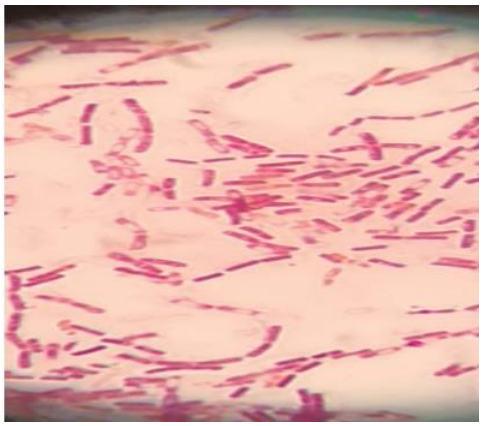

Figure 5

Figure 4: Tannase producing bacterium maintained in nutrient agar medium

Figure 5: Gram staining of tannase producing bacteria. The bacteria was viewed under 40x magnification

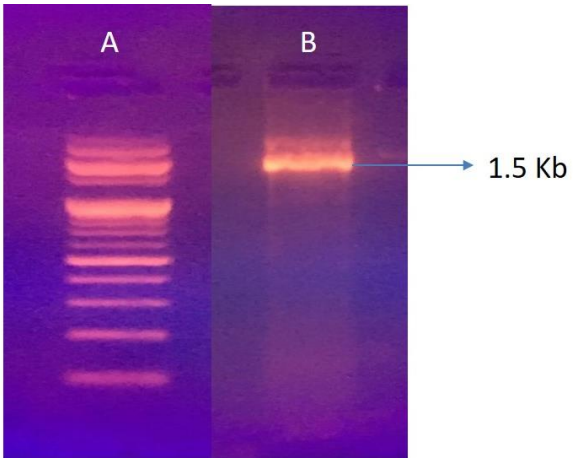

Figure 6: Agarose gel electrophoresis of PCR product of tannase producing bacteria using 16s rDNA primers Lane A: 100bp marker, Lane B: 1.5kb amplified gene.

Sharma and John (2011) reported tannase from the Gram negative bacterium *Enterobacter sp.* which could be used to protect grazing animals which are feeding high tannins. Singh *et al*. (2012) reported *Enterobacter luduwgii* strain GRT-1 from the gut of migratory goats and sheep. Tannase activity was reported in *Klebsiella* species from various sources (Jadhav *et al*., 2011; Sivashanmugam & Jayaraman, 2011; Pepi *et al*., 2013). Also, Tannases from *Klebsiella oxytoca,* isolated from migratory goats was reported by Sahu *et al*., (2015).

Tannase activity and concentration

Tannase activity was determined by Rhodanine assay specific for gallic acid. Indication of pink colour in the tubes presence of gallic acid production. Absorbance was read by spectrometrically at OD_{520} nm and graph was plotted and activity was determined. The total activity of tannase was found to be 47.9 U. Protein concentration was determined by using a BSA as a standard Dark blue colour indicates the presence of protein in the sample. Absorbance was read by spectrometrically at OD_{660} nm and graph was plotted.The concentration of tannase was found to be 1.8 mg/mL (Table 2).

Highest enzyme activity (47.9 U/min) was observed after 72 h of inoculation. After 72 hr of growth the tannase activity was decreased and remained stable thereafter. Study by Rodriguez *et al*., (2008) on *Bacillus sphaericus* showed higher tannase activity after fermentation period of 96 h and Jana *et al.,* (2013) reported that *Bacillus subtilis* produced higher activity after 72 h, which is in correspondence with the present report.

Enzyme purification and determination of specific activity of enzyme, Yield % and Purification fold

Tannase was precipitated at 80% saturation with ammonium sulphate to remove some of the non-enzyme proteins. The precipitate obtained after ammonium sulphate precipitation was further dialyzed with 0.1 M citrate buffer (pH 5) in order to remove the ammonium sulphate from the precipitate. Dialyzed sample was further purified by DEAE Cellulose column. The purified enzyme was eluted as fractions with a linear gradient of 0.5 -2 M NaCl. The partially purified tannase was further purified by Sephadex G-150 column. The enzyme was eluted with a linear gradient of 0-2 M NaCl. The specific activity of crude and purified enzyme was found to be 26.1 and 68.2 respectively (Table 2). The yield of the purified tannase was found to be 56.1% with 2.6 times purification fold.

Earlier study on tannase purification showed that use of ion exchange columns in the beginning and gel filtration technique in the last gave highly purified fractions (El-Toukhy *et al*. 2013). In a study by Mahapatra and Banerjee (2009), tannase was purified from the bacteria with 2.33 fold purification with 79.06% recovery. Similar results were also produced in reports by Abdal *et al*., (2020).

Table 1. Biochemical tests for the tannase producing bacteria

Biochemical tests	Bacterial charecteristics
Catalase test	Positive
Oxidase test	Negative
Methyl Red test	Negative
Voges prausker test	Positive
Indole production test	Negative

Table 2. Calculation of specific activity, Yield%, purification fold of crude and purified tannase

Sample	Protein concentration (mg/ml)	Total activity (U/Min)	Specific activity (U/mg)	Yield %	Purification fold
Crude enzyme	1.8 ± 0.8	47.9 ± 0.9	26.1	100	0
Purified enzyme	1 ± 0.7	68.2 ± 1.1	68.2	56.1	2.6

Molecular weight determination by SDS PAGE

Upon SDS PAGE analysis, a single band of ~70KDa was obtained after purification indicating the monomeric nature of enzyme (Figure 7). Several authors have reported the monomeric nature of bacterial tannases ranging from 46 KDa to 90 KDa (Kumar *et al*., 2015). In a previous report by Sivashanmugam and Jayaraman (2010), a tannase was isolated and partially purified from *Klebsiella pnemoniae* MTCC 7126 which obtained a single band of 46.5K Da upon SDS PAGE analysis.

Effect of Temperature for purified tannase

Tannase production by the bacteria was found increasing in the range of 30 and 40ºC and significantly reduced at 50ºC. An optimum temperature for the enzyme was found to be 30ºC (Figure 8). The maximum tannase production (47.9 U/Min) was obtained at 30ºC and the results are in accordance with Selwal *et al*., 2010, who reported that maximum tannase activity produced by *Pseudomonas aeruginosa* was observed at an incubation temperature of 37ºC. Similar results were obtained for bacterial tannases from *Citrobacter freundii* (Kumar at al., 1999). Optimum temperatures in the range of 30ºC to 40 ºC was earlier obtained for many bacterial tannases (Mondal *et al*., 2001, Sabu *et al*., 2006 etc.,).

Effect of pH for purified tannase

The pH of the extracellular tannase was increasing over pH of 5 and 6 whereas the production was found to decrease substantially at pH 8.0. The optimum pH of the tannase enzyme was found to be pH 5.0 (Figure 9).

Similar results were reported by Ayed & Hamadi (2002). In case of bacterial cultures, Selwal *et al*., (2010) has reported an optimum pH of 5.5 for *Pseudomonas aeruginosa.* Tripathi *et al*., 2016, reported an optimum pH of 6 for tannase obtained from *Bacillus megaterium* isolated from tannery effluent.

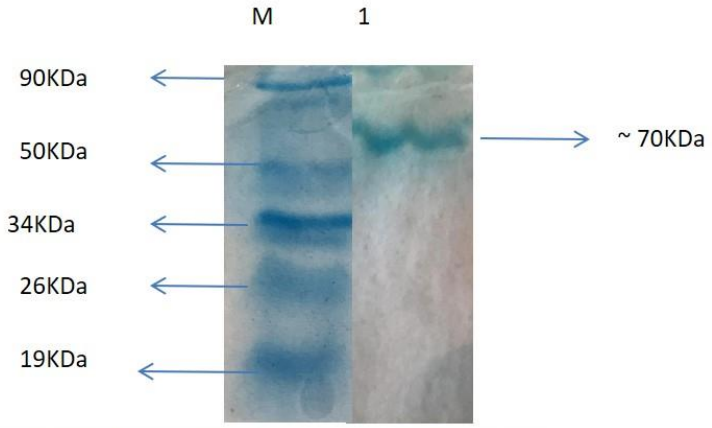

Figure 7: SDS PAGE Analysis of purified Tannase enzyme: Lane M: Molecular Weight Marker 20KDa to 120KDa (FermentasPvt.Ltd.,), Lane 1 : Purified Tannase (~70KDa)

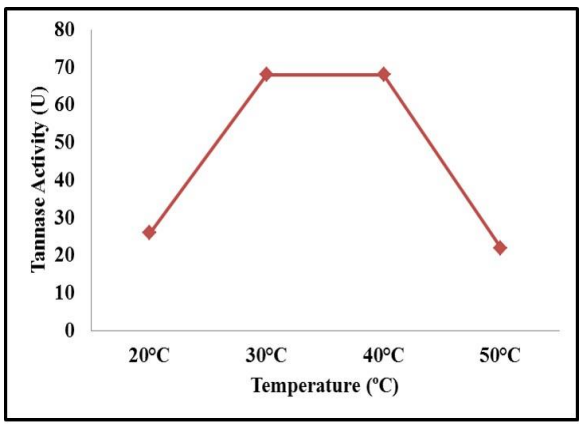

Figure 8

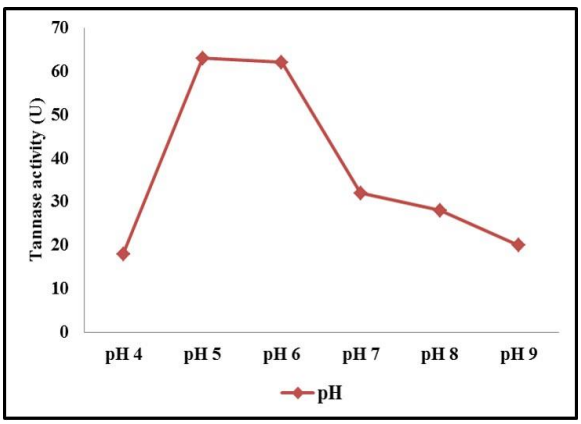

Figure 9

Figure 8: Effect of Temperature on purified tannase enzyme

Figure 9: Effect of pH on the purified tannase enzyme

Effect of metal ions on purified tannase

The purified tannase was subjected to various metal ions to analyze the activation or inhibitory effects on the enzyme. The addition of calcium, magnesium and manganese was found to increase the enzyme activity, while other metals had significant loss of the tannase activity (Table 3). The presence of Ca^{2+}, Mg^{2+} and Mn^{2+} was earlier reported in several studies to have stimulatory effects on tannase (Hossam, 2008 and Kumar *et al*., 2015).

Effect of detergents for purified tannase

Detergents play as significant role in enzyme catalyzed reactions. Upon addition of the different detergents SDS was about to slightly inhibit the enzyme activity by 96.12%, Tween 80 greatly influenced the decrease in activity by 28.89% and CTAB moderately inhibited the activity by 68.92% (Table 4). The inhibition may be due to the disturbances in the hydrophobic interactions of the enzyme. The inhibitory effects of the tannase activity with respect to addition of detergents was discussed earlier in previous reports (Beniwal *et al*., 2013, Jana *et al*., 2013).

Solid state fermentation

Tannase enzyme was extracted by utilizing various agro-industrial residues and commercial green fodders by solid state fermentation and their tannase activity was estimated. The results are recorded in table 5. The maximum activity of 54.32 U was obtained for the substrate pomegranate peel, indicating the high tannin content present in the peel.

Pomegranate juice clarification

The purified enzyme successfully clarified the pomegranate juice within a period of 1 h (Figure 10). This was possible because, the tannase produced by the bacteria was able to degrade the tannin content present in the juice. In 2006, Rout and Banerjee reported that the tannase coproduced by *Aspergillus* and *Rhizopus oryzae* aided in the clarification of tannin rich pomegranate juice.

Table 3. Effect of various metal ions on the purified tannase

Metal ions (1mM)	Total activity (U/min)	Relative activity (%)
Control	61.5 ± 0.4	100
Calcium chloride	62.1 ± 0.7	100.97
Potassium chloride	57.3 ± 1.2	93.17
Magnesium chloride	65.5 ± 1.5	106.5
Manganese chloride	68.9 ± 0.9	112
Zinc sulphate	44.2 ± 0.9	71.86
Ferric chloride	44.8 ± 0.8	72.84

Table 4. Effect of various detergents on the purified tannase

Detergents (0.5%)	Total activity (U/min)	Relative activity (%)
Control	60.5 ± 1.4	100
SDS	58.2 ± 1.1	96.12
TWEEN 80	17.3 ± 0.9	28.89
CTAB	41.7 ± 0.9	68.92

Table 5. Solid state fermentation for determination of the tannase activity.

Solid Substrates	Total Activity (U/min)
Tea Powder	48.23 ± 0.7
Wheat Bran	36.47 ± 0.3
Pomogranate peel	54.32 ± 0.7
Green Fodder 1	32.52 ± 1.2
Green fodder 2	34.70 ± 1.2

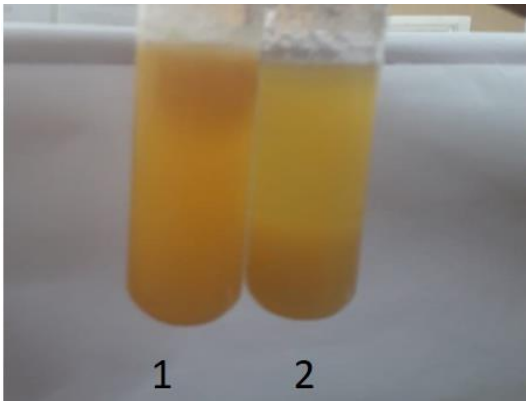

Figure 10: Pomogranate juice clarification by the purified tannase enzyme

Tube 1: Juice mixed with distilled water (Control) Tube2: Juice mixed with 1ml of purified tannase enzyme (Test)

CONCLUSION

In the present study, *Klebsiella pneumoniae* was isolated from goat rumen contents and the organism was able to produce tannase enzyme, which could effectively degrade natural tannins. The paper also reports the effects of physicochemical parameters on the obtained tannase enzyme. The yield of the enzyme produced was reasonable when compared to already tannases. The tannase enzyme could be used for clarification of fruit juices rich in tannin content. The enzyme effectively utilizes the tannin contents present commercial fodder given for ruminants, so that it can be supplemented along with them. In future, the tannase gene of *Klebsiella pneumoniae* could be cloned and expressed in a new host in order to improve its activity and stability. Though a variety of tannases have been reported in the past, only very few reports are available on the production of tannase enzyme from goat rumen. Thus, this study would pave a way for further researches on isolation of various enzymes from rumen contents of ruminants.

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Conflict of interest and declaration

The authors declare that there is no conflict of interest. The article has not been published or sent for publication anywhere.

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