



## OPTIMIZATION OF TANNASE PRODUCTION BY *KLEBSIELLA PNEUMONIAE* STRAIN AMAR ISOLATED FROM SOIL OBTAINED FROM TEA PLANTATION

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### ABSTRACT

Tannase is among the most versatile enzymes that hydrolyse tannins which is the second most abundant secondary metabolites of plants. They act as biocatalysts in a wide range of bioconversion reactions that are a key step in various industries like food and pharmaceuticals. In the current study, the tannase producing bacteria was isolated from tea plantation soil sample collected from Assam, and identified as *Klebsiella pneumoniae* amar (NCBI accession number LC469777) based on the morphological, cultural, biochemical and 16s rRNA sequence analysis. The optimized cultural and physicochemical parameters for tannase production by *K. pneumoniae* amar was obtained when 2% v/v culture of 0.7 O.D<sub>540nm</sub> was inoculated in K-MMB medium containing 1% tannic acid, 0.12% ammonium chloride, 1% glucose (1%), 0.05% MgSO<sub>4</sub>, 0.01% KCl, 0.1% K<sub>2</sub>HPO<sub>4</sub> and pH 3.5; and incubated at 30°C for 96h under shaker (150rpm) condition. The enzyme activity of the crude tannase enzyme produced in our study was further analysed in presence of several metals, organic acids and enzyme inhibitors. It was found to be maximum at room temperature (28°C) and pH 5.5. All the metal ions tested in our study i.e. Ca<sup>2+</sup>, Fe<sup>2+</sup>, Cu<sup>2+</sup>, Zn<sup>2+</sup> inhibited the tannase activity, but complete inhibition was observed by Hg<sup>2+</sup> whereas Mg<sup>2+</sup> showed least inhibition. Similarly, all the enzyme inhibitors and organic acids inhibited tannase activity. In addition to optimization of enzyme production and activity, the application of tannase as the debittering agent in pomegranate juice was also studied where it showed 50% clarity of the fruit juice.

**KEYWORDS:** versatile enzymes, metabolites, tea plantation, tannase activity.

### INTRODUCTION

Tannins are a complex secondary metabolite of plants that are mainly responsible for plant defense against infections caused by microbes. The tannins occur mainly in 2 major forms i.e., hydrolysable or condensed (Proanthocyanidins). In addition, the intermediate group of tannins also occurs that shares the characteristics of hydrolysable tannins and condensed tannins (Aguilar *et al.*, 2007; Li *et al.*, 2006). They are the second most abundant group of polyphenols after lignins and have a molecular weight of over 500 kDa upto 20,000 kDa (Bhat *et al.*, 1998). They form irreversible or strong complexes with protein, starch, cellulose, minerals as well as digestive enzymes including pectinase, amylase, lipase, protease, cellulase and -galactosidase (Griffiths, 1986). Hence it widely used as an antinutrient in management of metabolic disorders like obesity and diabetes (Smeriglio *et al.*, 2017). The breakdown products of tannins such as gallic acid, glucose and some galloyl esters is also of commercial importance as they find applications in industries like leather, pharmaceutical and food (Singh and Kumar, 2019). The enzyme used for hydrolysis of tannins i.e. tannase (tannin acyl hydrolase, EC3.1.1.20) catalyzes the ester bonds present in gallotannins, complex tannins and gallic acid esters to release gallic acid as the major product (Yao *et al.*, 2014). The gallic acid is among the most important substrate used in preparation of food preservatives (i.e., gallates and pyrogallol, propyl gallate), pharmaceuticals (i.e., antibacterial drugs), fruit juices (debittering), beer,

wine and bioactive polyphenolic compounds (García-Nájera *et al.*, 2002; García-Conesa *et al.*, 2001; Duenas *et al.*, 2007). It also finds major application in treatment of wastes produced by tanneries and leather industries that are rich in tannins and pose a serious threat to the environment (Van de Lagemaat and Pyle, 2001; Gammoun *et al.*, 2006; Singh and Kumar, 2019). Besides, the cosmetic industries use tannase to eliminate the turbidity due to natural ingredients (Singh and Kumar, 2019). Other uses of tannase include the production of developers used in photography, printing inks and laundry detergents (Beniwal *et al.*, 2013).

The conventional production of gallic acid required acid hydrolysis of synthetic tannic acid. This process suffers from major disadvantages like high production cost, low yields and low purity of products. Moreover, it leads to the production of toxic byproducts (Bajpai and Patil, 2008). For this reason, the green approach for microbial production of tannase is empirically acknowledged and persuasively encouraged for economical and environmental friendly outcomes. Subsequent to the discovery of tannase enzyme in 1867 (Aguilar *et al.*, 2007), much research was focused on fungal production and characterization of the same (Bhat *et al.*, 1998; Bradoo *et al.*, 1997). However, it is associated with slow growth of culture and hence low yield. Moreover, it has lower protein, nucleic acid and methionine content in the end product as compared to bacterial production of tannases. Hence the discovery of rarely occurring bacterial

tannase in 1983 (Deschamps *et al.*, 1983), commended pioneering applications of the same in food and pharmaceutical industries. The major advantage of bacterial culture is the high growth rate and ease of manipulation at genetic level. The bacteria are also capable of living under extreme temperature conditions and therefore they prove to be potential source of thermostable tannase (Parmjit *et al.*, 2016). Despite these advantages, unfortunately, bacterial tannases also suffer from limitations of scale up processes to meet the industrial demands.

In general, now we know that tannase can be obtained from plants, animals as well as microorganisms; the most important source being microbiological. This is because the microbial enzymes are more stable as compared to those from other sources. The microbial tannases are mostly inducible and extracellular enzymes. Currently, they are effectively produced by submerged fermentation and solid-state fermentation processes (Zhang *et al.*, 2019; Gurung *et al.*, 2013). Recently, much efforts is taken to improve the production of tannase. These efforts mainly include isolation of novel isolates, development of novel fermentation systems, optimization of culture conditions, strain improvement by genetic manipulations and advanced approaches for product recovery (Raveendran *et al.*, 2018; Sena *et al.*, 2014; Jian *et al.*, 2014; Banerjee and Mahapatra, 2012; Beniwal *et al.*, 2010; Belmares *et al.*, 2004).

Given the multi million dollars market value, and the limited number of commercially available enzymes (Li *et al.*, 2012), the propitious nature of this field is unquestionable. Hence, novel tannase producing bacterial isolates needs to be isolated from nature and optimised for increased production of the enzyme. The current study was carried out with an objective to screen tannase producing bacteria from soil and optimize the cultural and physicochemical conditions for its production and activity. In addition, the potential application of crude tannase enzyme in debittering of pomegranate juice was also studied.

## MATERIALS AND METHODS

### Sample collection

The soil samples used in our study was collected from tea plantation located in Assam. The samples were collected in sterile containers and transferred to laboratory for processing.

### Enrichment, isolation and screening of tannase producers

The soil sample (1g) was suspended in 10ml of sterile phosphate buffer (pH 7.2), mixed thoroughly and allowed to stand for 30mins. The enrichment of tannase producing organisms was done by inoculating 1ml of the above suspension in 50ml of M9 minimal liquid medium containing 0.5% glucose and 1% of tannic acid. The enrichment broth was incubated at 30°C on shaker (150rpm) for 7 days. The screening of tannase producing bacteria was done by spot inoculating the cultures on M9 medium containing 1% tannic acid and incubating it at 37°C for 48h. The isolates were then subjected to FeCl<sub>3</sub> test and the one producing a clear zone around the spot inoculated culture indicated the production of tannase.

### Tannase assay

The crude extract of tannase enzyme was prepared by centrifugation of the culture medium at 5000rpm (4°C) for 10mins. The clear supernatant was used as a crude enzyme for the UV spectrophotometric enzyme assay. In this method, the enzyme activity is determined by the hydrolysis of the ester bonds of tannic acid. To carry out the assay, 0.5ml of crude enzyme was added to 2ml of 0.35% (w/v) tannic acid dissolved in 0.05M citrate buffer (pH 5.5) solution. To the remaining reaction mixture, 4ml of ethanol solution was added to stop the reaction. The absorbance of the mixture was noted immediately (t<sub>1</sub>) after addition of ethanol and after 10mins of incubation at 37°C (t<sub>2</sub>), on UV spectrophotometer (UV-Vis spectrophotometer 117, Systronics, India) at 310nm. The enzyme activity was calculated by using the following formula.

$$\text{Enzyme activity (U/mL)} = 114 \times \frac{\text{Change in Absorbance (a}_2 - \text{a}_1)}{\text{Difference in time (t}_2 - \text{t}_1)}$$

One unit of tannase activity was defined as the amount of enzyme required to hydrolyse 1µmol of ester in 1min per ml under assay conditions (Iibuchi *et al.*, 1968).

### Identification of the tannase producing bacteria

The potential isolate showing maximum production of tannase enzyme was identified preliminarily by morphological, cultural and biochemical tests. The strain was confirmed by 16S rRNA gene sequence analysis. PCR based 16S rRNA gene amplification and sequencing of the isolated bacterium was carried out using universal primers at Sai Biosystems Private Limited, India.

### Optimization of tannase production by the potential bacterial isolate

Most microbial tannases are extracellular in nature. Its production as well as activity is greatly influenced by nutritional and physico-chemical factors such as pH, incubation temperature, incubation time, agitation and dissolved oxygen concentration, nitrogen and carbon

sources, and inorganic salts. Hence optimization of the tannase production process was carried out by applying one factor at a time (OFAT) approach, where one of the parameters is studied by applying variables while keeping the others constant. All of the experiments were carried out in triplicate, and the mean values with standard deviation was reported.

In this study, firstly the optimum nutrient medium was determined for tannase production. For this purpose, the different nutrient medium used and their composition are stated below.

**Medium A:** Luria Bertani broth containing 0.5% w/v tannic acid (Wilson *et al.*, 2009).

**Medium B:** Bushnell and Hans's medium containing (in g/L) KH<sub>2</sub>PO<sub>4</sub> (1), MgSO<sub>4</sub>·7H<sub>2</sub>O (0.2), NH<sub>4</sub>NO<sub>3</sub> (1), K<sub>2</sub>HPO<sub>4</sub> (0.5) and 0.1% tannic acid (Issa, 2016).

**Medium C:** Media composition in g/L [K<sub>2</sub>HPO<sub>4</sub> (0.5), KH<sub>2</sub>PO<sub>4</sub> (0.5), MgSO<sub>4</sub> (2.0), CaCl<sub>2</sub> (1.0), NH<sub>4</sub>Cl (3.0) and 1% tannic acid, pH 5.5] (Kumar *et al.*, 2015).

**Medium D:** Media composition in g/L [K<sub>2</sub>HPO<sub>4</sub> (0.5), KH<sub>2</sub>PO<sub>4</sub> (0.5), MgSO<sub>4</sub> (0.5), NH<sub>4</sub>Cl (1.0), CaCl<sub>2</sub> (0.1), glucose (5.0), 1% tannic acid, pH 6.0] (Raghuwanshi *et al.*, 2011).

**Medium E:** Media composition in g/L [NH<sub>4</sub>Cl (1.0), KH<sub>2</sub>(PO<sub>4</sub>)<sub>2</sub> (3.0), NaHPO<sub>4</sub> (4.4), NaCl (0.5), glucose (2.0), MgSO<sub>4</sub> (0.02%), 1% tannic acid, pH 6.0] (Sarang *et al.*, 2016).

**Medium F:** Tannic acid broth (TAA) containing (in g/L) peptone (5.0), NaCl (5.0), Yeast extract (1.5), Beef extract (1.5), tannic acid (5.0), pH 5 (Brahmbhatt and Modi, 2015).

**Medium G:** Nutrient broth supplemented with 2% w/v tannic acid.

**Medium H:** Media composition in g/L [NaCl (10.0), KH<sub>2</sub>PO<sub>4</sub> (0.25), NH<sub>4</sub>Cl (1.0), Na<sub>2</sub>BO<sub>7</sub> (2.0), FeCl<sub>3</sub> (0.0125), CaCl<sub>2</sub> (0.06), MgCl<sub>2</sub> (0.05), Yeast extract (10mg), pH 7] (Makkar *et al.*, 1993).

**Medium I:** Media composition in %w/v [NaNO<sub>3</sub> (0.6), KCl (0.5), MgSO<sub>4</sub> (0.05), K<sub>2</sub>HPO<sub>4</sub> (0.05), KH<sub>2</sub>PO<sub>4</sub> (0.05), tannic acid (1) (Girdhari and Peshwe, 2017).

**Medium J:** Media composition in %w/v [NaNO<sub>3</sub> (3), KCl (0.5), MgSO<sub>4</sub>.7H<sub>2</sub>O (0.5), KH<sub>2</sub>PO<sub>4</sub> (1), FeSO<sub>4</sub>.7H<sub>2</sub>O (0.01) tannic acid (5%) (Girdhari and Peshwe, 2017).

**Medium K:** MMB Media composition in %w/v [glucose (1), NH<sub>4</sub>NO<sub>3</sub> (0.12), MgSO<sub>4</sub>.7H<sub>2</sub>O (0.05), KCl (0.01), KH<sub>2</sub>PO<sub>4</sub> (0.1), tannic acid (1) (Das Mohapatra *et al.*, 2009).

The major factor for the expression of tannase activity is the carbon source, since most tannases are induced in the presence of tannic acid. However, their production is also significantly influenced by other carbon sources, such as raffinose, mannose, mannitol, glycerol, sucrose, lactose, maltose, glucose, fructose, arabinose, starch, galactose and xylose. Besides carbon sources, the different nitrogen sources in the medium also influence the tannase production in the medium. The inorganic nitrogen sources like sodium nitrate, ammonium nitrate, potassium nitrate, ammonium sulfate, ammonium chloride, urea and ammonium monohydrated phosphate was used in our study. Optimization studies using 0.5% of the above carbon sources and 0.03% nitrogen sources was carried out. The effect of various concentration of tannic acid (0.5%- 5% with interval of 0.5%), carbon source (0.2%, 0.4%, 0.5%, 0.8%, 1%, 1.5% and 2%) and nitrogen source (0.03%, 0.06%, 0.09%, 0.12%, 0.15%, 0.18%, 0.21%, 0.24%, 0.27% and 0.30%) was also studied (Kumar *et al.*, 2015; Sarang *et al.*, 2016; Muslim, 2017; Selwal *et al.*, 2011).

The other varying physico-chemical parameters optimised in our study included the incubation period (24h, 48h, 72h,

96h, 120h, 144h and 168h), initial optical density of test isolate (O.D<sub>530nm</sub> 0.1, 0.2, 0.3, 0.4, 0.5), inoculum size (5%-25%), temperature (20°C, 30°C, 40°C, 50°C, 60°C, 70°C, 80°C), pH (3-8), concentration of NaCl (1 to 10%) and aeration i.e., static or shaking (150rpm) condition (Kumar *et al.*, 2015; Sarang *et al.*, 2016; Selwal *et al.*, 2011; Sabu *et al.*, 2006; Battestin and Macedo, 2007; Anburaj, 2015).

#### Optimization of conditions for tannase activity

The characterization of tannase activity was also carried out using OFAT modification of the parameters. For each optimization process, the crude enzyme extract was incubated with pure tannin, after adjusting the parameters. The studied parameters included temperature (20°C, 30°C, 40°C, 50°C, 60°C, 70°C, 80°C), pH (3-10.5), metal ion concentration (1-5mM salt solutions of Ca<sup>2+</sup>, Mg<sup>2+</sup>, Fe<sup>2+</sup>, Cu<sup>2+</sup>, Mn<sup>2+</sup>, Zn<sup>2+</sup>, Na<sup>2+</sup>, Pb, Cd<sup>2+</sup>, Co, Fe<sup>2+</sup>, Ni, Ba<sup>2+</sup>, Hg, K<sup>+</sup>), inhibitors/ activators other than metal ions (1mM EDTA, 1mM Tween 80 and 0.5% SDS), organic solvents (1% v/v acetone, ethanol, methanol, n-butanol, isopropanol and glycerol). The buffers used during optimization of pH included sodium acetate (pH 3.5-5.5), citrate-phosphate (pH 5.5-7.0), tris-HCl (pH 7.0-8.5) and Gly-NaOH (pH 8.5-10.5) (Jimenez *et al.*, 2014).

#### Potential application of tannase in juice industry

Fresh fruit juices were prepared after washing the fruits thoroughly with water and homogenizing in a blender. The juices were then filtered through cheese cloth and stored at 4°C until use. One ml fruit juice was mixed with 1ml of tannase and incubated at 30°C with gentle shaking up to 30min. The test tube was then placed in water bath at 50°C for 10min to deactivate the enzyme. A 1ml aliquot of fruit juices was taken from each test tube at different time intervals and their tannin content was measured using Folin Denis method, that detects the decrease in amount of tannic acid content after treatment with crude tannase enzyme (Rout and Banerjee, 2005).

## RESULT AND DISCUSSION

### Enrichment, isolation and screening of tannase producing bacteria

Two bacterial isolates were obtained after enrichment of the medium and screening for tannase producers in our study. These isolates were maintained on M9 medium with 1% tannic acid agar slants at 4°C.

### Tannase assay for selection of potential isolate giving maximum tannase activity

Table 1 represents the tannase activity of different isolates. It can be clearly observed that MMB1 showed maximum tannase activity and hence it was used for further optimization studies.

**TABLE 1:** Tannase activity of different isolates

Isolate	Enzyme activity (U/ml)			
	Value 1	Value 2	Value 3	Average
MMB 1 Mucoid	2.62656	2.62656	2.63112	2.62808
MMB 2 White	0.45144	0.44688	0.46056	0.45296

Similar to our study, other researchers have also used soil samples, previously, for isolation of tannase producers. A novel tannase producing bacterial strain, identified as *Klebsiella pneumoniae* KP715242, was isolated from rhizospheric soil of Acacia species. On optimization with computational methods i.e., central composite design using response surface methodology, a 3.25-fold increase in tannase production was achieved (Kumar *et al.*, 2015). Another study reported the use of fungal system isolated from soil samples from different places to produce gallic acid from banana flower stalk and coir pith. The study suggested that agricultural wastes containing tannin could be considered as alternative substrates for gallic acid production. Krishnaswamy *et al.* (2016) identified halotolerant co-cultured bacteria, *Bacillus amyloliquefaciens* and *Bacillus polyfermenticus*, from soil samples near tannery industry which degraded 92% tannin as compared to *B. amyloliquefaciens* alone.

#### Identification of the isolate

The isolated mucoid colony was subjected to 16srRNA identification and was identified as *Klebsiella pneumoniae*. The nucleotide sequence analysis of the isolate was done at BlastN site on NCBI server (<http://www.ncbi.nlm.nih.gov/BLAST>) and corresponding sequences were downloaded. The identified isolate was deposited as *Klebsiella pneumoniae amar* at National Centre for Biotechnology Information (NCBI) with an accession number LC469777.

Many strains of *Klebsiella* have been previously reported to produce extracellular tannase. Few examples include *K. pneumoniae* KP715242 (Kumar *et al.*, 2015), *K. pneumoniae* MTCC 7162 (Sivashanmugam and Jayaraman, 2011), *K. pneumoniae* (Belmares *et al.*, 2004) and *K. planticola* (Belmares *et al.*, 2004; Deschamps *et al.*, 1983).

#### Optimization of tannase production by *Klebsiella pneumoniae amar*

The optimized cultural and physicochemical parameters for tannase production by *K. pneumoniae amar* is presented in Fig. 1-12. The optimized culture condition for the growth of *K. pneumoniae amar* to effectively produce tannase enzyme was obtained when 2% v/v culture of 0.7 O.D<sub>540nm</sub> was inoculated in MMB medium (pH 3.5) containing 1% tannic acid, 0.12% ammonium chloride, 1% glucose (1%), 0.05% MgSO<sub>4</sub>, 0.01% KCl and 0.1% K<sub>2</sub>HPO<sub>4</sub>, and incubated at 30°C for 96h under shaker (150rpm) condition.

The optimization of cultural parameters i.e the media composition including various carbon and nitrogen sources was carried out as the first step in our experimental set up. The maximum tannase production was observed when MMB media containing 1% glucose and 1% tannic acid was used as carbon source (Fig. 1). Studies with individual carbon sources also confirmed 1% glucose (Fig. 2 and 3) and 1% tannic acid (Fig. 4) to be optimum for production of tannase. The optimum nitrogen source was found to be 0.12% ammonium chloride (Fig. 5 and 6). Further increase in nitrogen and carbon sources showed considerable decrease in tannase production. This may be due to the osmotic stress created in the medium on

increased sugar concentration, and/or accumulation of undesired metabolites (Selwal *et al.*, 2011). The presence of glycerol and urea showed minimum tannase production by *K. pneumoniae amar* (Fig. 3 and 5). In a study carried out by Raghuvanshi *et al.* (2011), the tannase production by *Bacillus sphaericus* was supported by galactose, maltose, sucrose, dextran and xylitol in the presence of 1.5% tannic acid and 0.5% NH<sub>4</sub>Cl. However, 2.0% galactose showed maximum enzyme production of 5.83 IU/ml, which was upto 1.4 fold higher than other carbon sources. It further showed an increase in tannase production with increasing concentration NH<sub>4</sub>NO<sub>3</sub> in basal medium containing tamarind seed powder. Comparatively low concentrations (0.1-0.2%) of glucose, lactose and sucrose stimulated tannase production in *A. japonicus* and *B. licheniformis* KBR6. However, higher concentrations (0.3-0.5%) of these sugars significantly repressed tannase production by these microbes (Bradoo *et al.*, 1997; Das Mohapatra *et al.*, 2009). The stimulatory effect of low concentration of glucose on tannase production is also reported by other workers (Beverini and Metche, 1990; Goncalves *et al.*, 2012; Ayed and Hamdi, 2002; Lagemaat and Pyle, 2005; Banerjee and Pati, 2007). They further reported catabolite repression at high concentration of sugars, inspite of the presence of inducer.

Similar to our study, *S. marcescens* and *A. japonicus* showed optimum tannase production in presence of 0.1% ammonium chloride (Sarang *et al.*, 2016; Bradoo *et al.*, 1997). A 1-1.5% tannic acid concentration was also reported to be optimum in *B. licheniformis* and *Lactobacillus plantarum* (Mondal and Pati, 2000; Ayed and Hamidi, 2002).

In contrast to our study, the tannase production by *Pseudomonas aeruginosa* IIB 8914 was suppressed on the addition of carbon sources to basal minimal media containing amla and keekar leaves. However, it showed considerable yield of tannase in presence of NH<sub>4</sub>NO<sub>3</sub> (Selwal *et al.*, 2010). Similarly, Sabu *et al.* (2006) reported the inhibition of tannase production by *Lactobacillus* sp. ASR-S1 on supplementing the medium with 1% carbon sources. Fathy *et al.* (2017) reported tannase production of 1714.7 U/g dry substrate by *K. marxianus* NRRL Y-8281 under solid state fermentation using olive cakes. In another study, tannase production by *A. niger* PKL104 was not affected in the presence of readily utilizable carbon or nitrogen sources (Lekha and Lonsane, 1994). Another study reported optimum tannase production by *Streptomyces* sp. SKA1 growing on agro-waste *Azadirachta indica* (neem) leaves in presence of 1% starch and 0.03% casien (Girdhari and Peshwe, 2017). Cavalcanti *et al.* (2017) reported optimum tannase production from *A. niger* in medium containing 2% tannic acid and devoid of nitrogen source, in 24h. The best condition for tannase production by *Aspergillus* sp. GM4 was observed under solid-state fermentation using a medium containing different vegetable leaves (mango, jamun and coffee), agricultural residues (coffee husks, rice husks and wheat bran), and 1.53% tannic acid at 1.53% and 2.71% potassium nitrate concentrations, as carbon and nitrogen source respectively, on incubation of 48h.

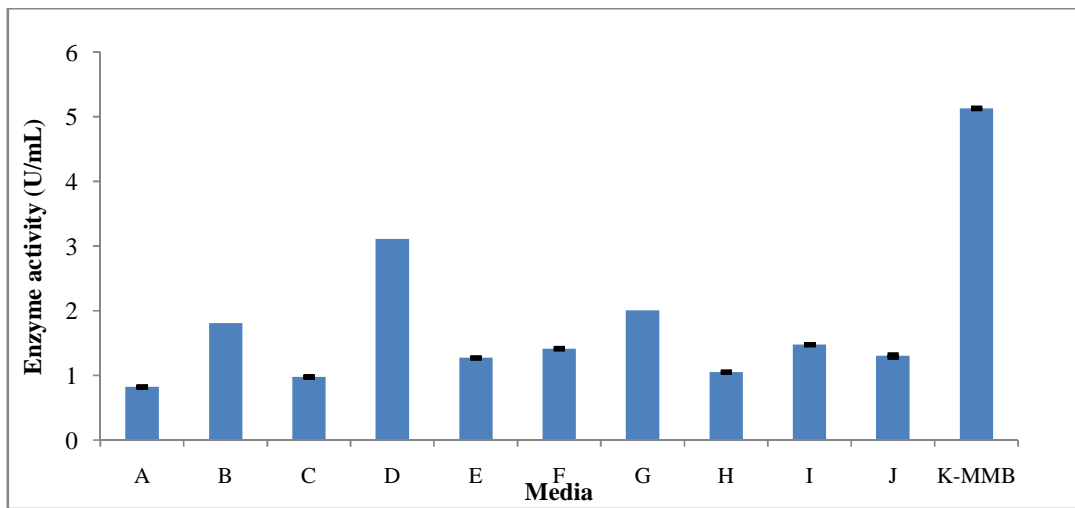


FIGURE 1: Effect of media on tannase production

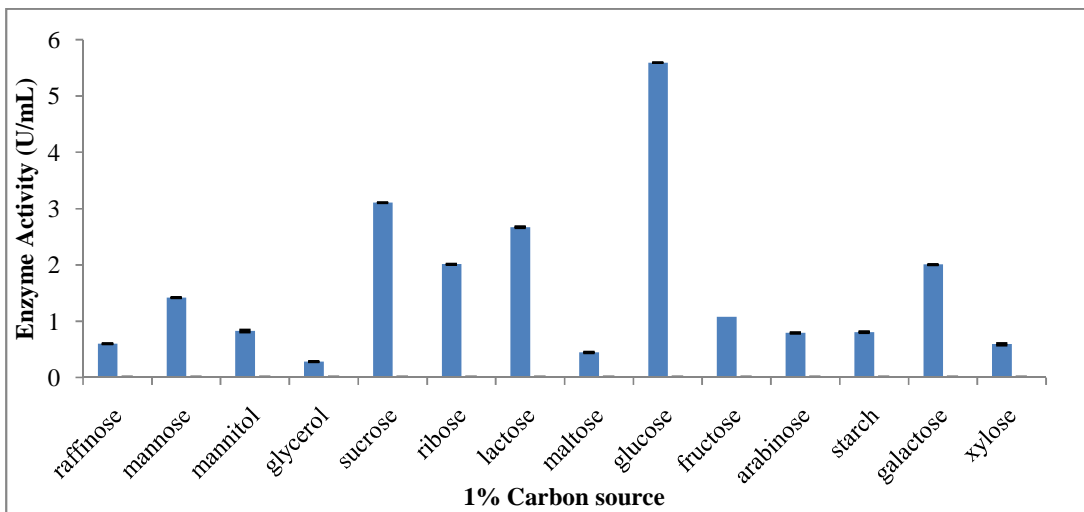


FIGURE 2: Effect of carbon source on tannase production

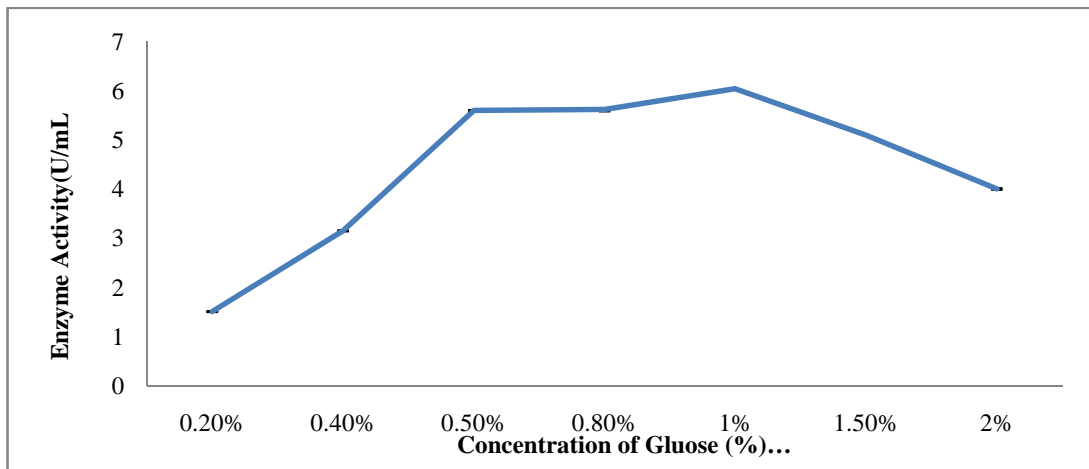
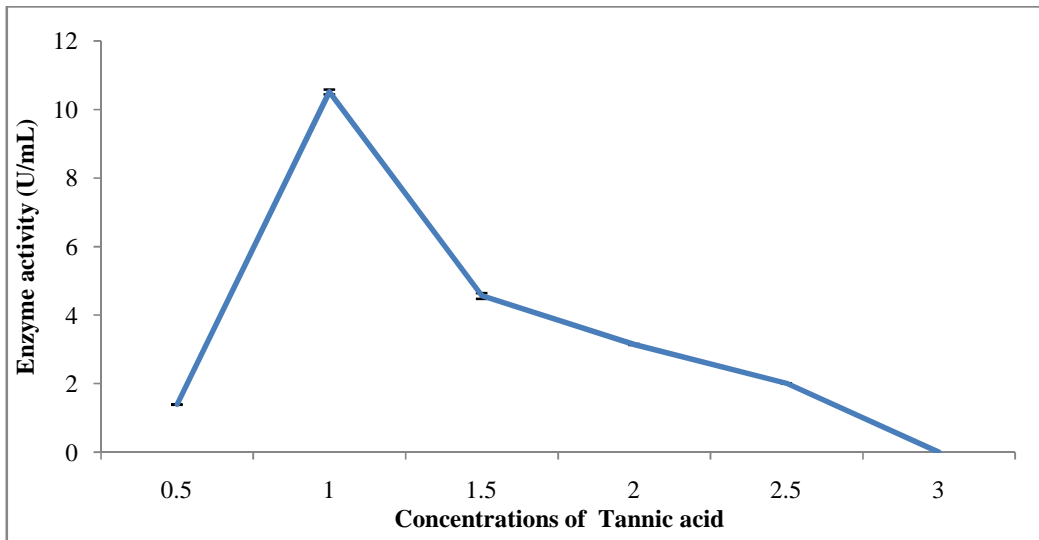
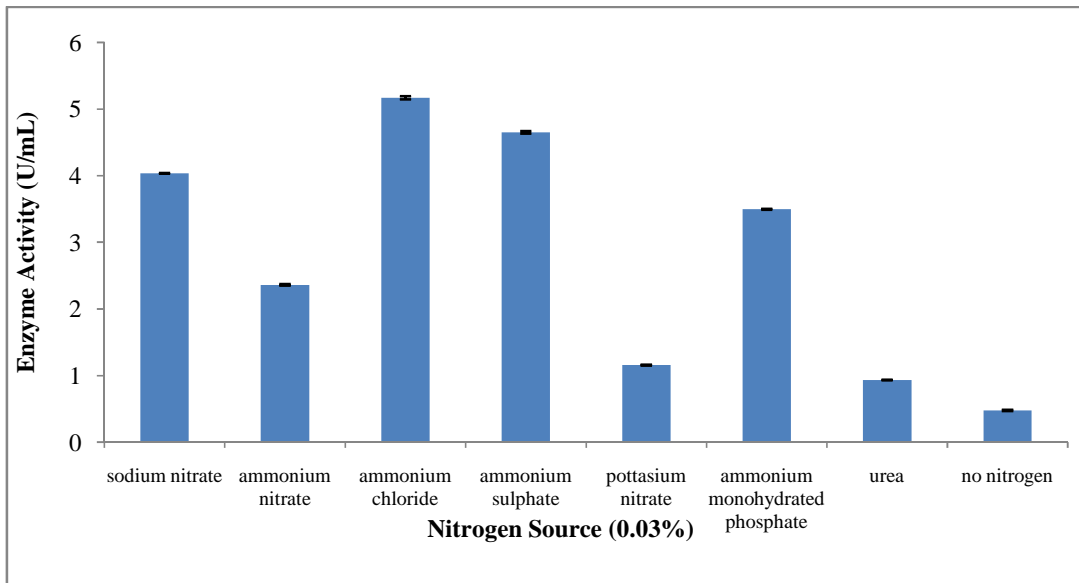


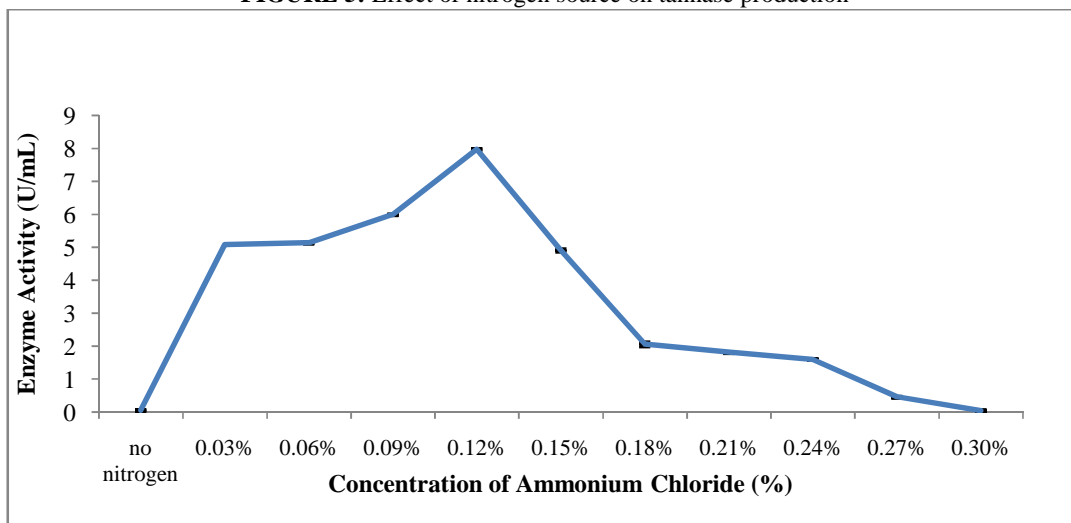
FIGURE 3: Effect of glucose concentration on tannase production



**FIGURE 4:** Effect of concentration of tannic acid on tannase production



**FIGURE 5:** Effect of nitrogen source on tannase production



**FIGURE 6:** Effect of ammonium chloride concentration on tannase production

The physicochemical parameters of an experimental set up have immense importance since it dictates the optimum growth of the isolates which in turn leads to its optimum exploitation for production and recovery of enzymes. The pH and temperature affects enzyme production by influencing factors like solubility, ionization and stability of substrates and its ionization to make it available for bacterial growth (Muslim *et al.*, 2015). Aeration increases the availability of dissolved oxygen in the medium that promotes bacterial growth. An optimal inoculum level is necessary for maintaining the balance between proliferating biomass and available nutrients to obtain maximum enzyme yield. A lower enzyme yield at higher inoculum level could result due to faster consumption of nutrients. In the current study, maximum production of enzyme was observed in 24h (5.13U/ml, Fig. 7) at pH3.5 (8.52U/ml, Fig. 8), 2% inoculum size (9.83U/ml, Fig. 9), 0.7O.D<sub>530nm</sub> (10.358U/ml, Fig. 10), shaker condition (10.39U/ml, Fig. 11) and 30°C (10.45U/ml, Fig. 12).

In the current study, the incubation period significantly affected the enzyme production. *K. pneumoniae* amar showed tannase production within 24h (3.15U/ml) and reached a maximum in 96h, after which it declined considerably. Similar results were obtained by Kumar *et al.* (2015) who reported optimum tannase production by *K. pneumoniae* KP715242 in 91.34h. The subsequent decline in tannase production after reaching a peak may most probably be due to the depletion of nutrients in the medium. However, it may also be due to the competitive inhibition of tannase enzyme. Other possibility is the microbial cell death or enzyme denaturation/ degradation due to accumulation of gallic acid produced on hydrolysis of tannins (Fathy *et al.*, 2017; Muslim, 2017). A shorter optimum incubation period of 24h, 30h and 48h is reported for tannase producing bacteria i.e., *Erwinia Carotovora* (Muslim *et al.*, 2015), *P. aeruginosa* IIB 8914 (Selwal *et al.*, 2010) and yeast *K. marxianus* NRRL Y-8281 (Fathy *et al.*, 2017) respectively. Among the fungal isolates, *Streptomyces* sp. SKA1 showed optimum tannase production in 120h (Girdhari and Peshwe, 2017). Other researchers studied optimization of tannase

production by *A. foetidus* MTCC 3557 and *A. japonicus* and reported 97h and 92h to be ideal for these cultures respectively (Mohan *et al.*, 2013; Abdel-Nabey *et al.*, 2011).

In another study, *Streptomyces* sp. SKA1 showed optimum tannase production when 6% inoculum was added to the medium adjusted at pH7 and incubated at 30°C for 120h (Girdhari and Peshwe, 2017). Kumar *et al.* (2015) reported maximum production of tannase by *K.pneumoniae* KP715242 at 34.97°C, pH5.2, 2% inoculum size, 103.34rpm agitation speed and 91.34h of incubation time. *K. pneumoniae* MTCC 7162 also showed similar parameters and produced optimum amount of tannase at 37°C, pH7.5 and 100 rpm (Sivashanmugam and Jayaraman, 2011). The optimum pH and temperature for maximum tannase production from *A. foetidus* MTCC 3557 was reported to be 5.5 and 35.5°C respectively (Mohan *et al.*, 2013). Similarly *A. fumigatus* showed increased production of tannase at 30°C and pH4 (Cavalcanti *et al.*, 2017). Fathy *et al.* (2017) reported optimum tannase production by *K. marxianus* NRRL Y-8281 under conditions of 45°C, pH 6.0, 20% inoculum size and an initial moisture level of 35% when maintained for 48h. *E. Carotovora* isolated from spoiled tomatoes showed maximum tannase production of 61.03U/ml at pH5 (Muslim *et al.*, 2015). Similarly, the acidic pH was reported to be optimum for tannase production by *L. plantarum* MTCC 1407 and *K. pneumoniae* MTCC 7162 in submerged fermentations (Natarajan, 2009; Sivashanmugam and Jayaraman, 2011). *P. guelpinii* URM 7114 showed 11.4 times higher productivity of tannase in submerged fermentation compared to solid-state fermentation (Sena *et al.*, 2014). Although an important parameter, agitation and very high rate of aeration has shown reduction in tannase production by *A. pullulans* DBS66 and *Serratia* sp. (Banerjee and Pati, 2007; Sarang *et al.*, 2016). An inoculum size of 1-2% have been found to be optimum for *L. lactis* (Varsha *et al.*, 2014), *P. aeruginosa* IIB 8914 (Selwal *et al.*, 2010), *B. licheniformis* KBR6 (Mondal and Pati, 2000) and *B. licheniformis* KBR6 (Das Mohapatra *et al.*, 2009).

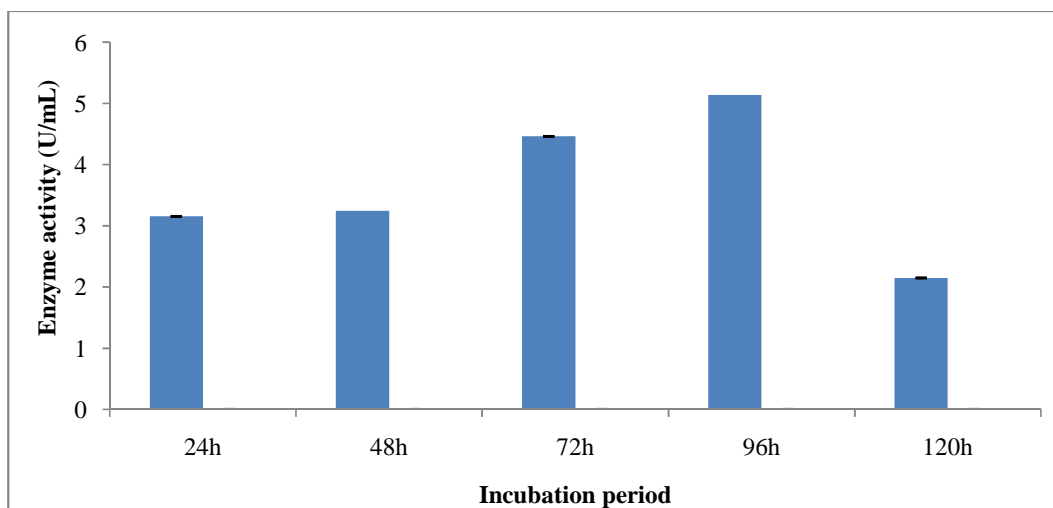
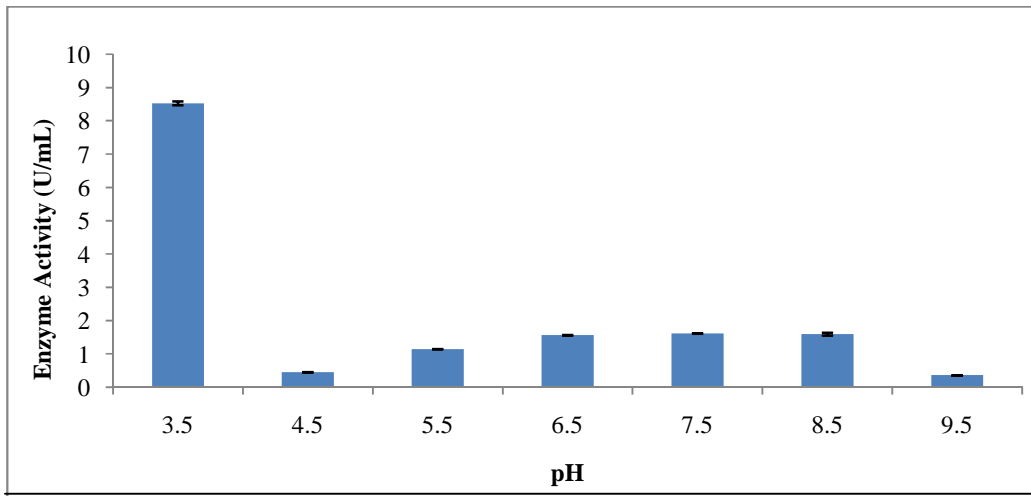
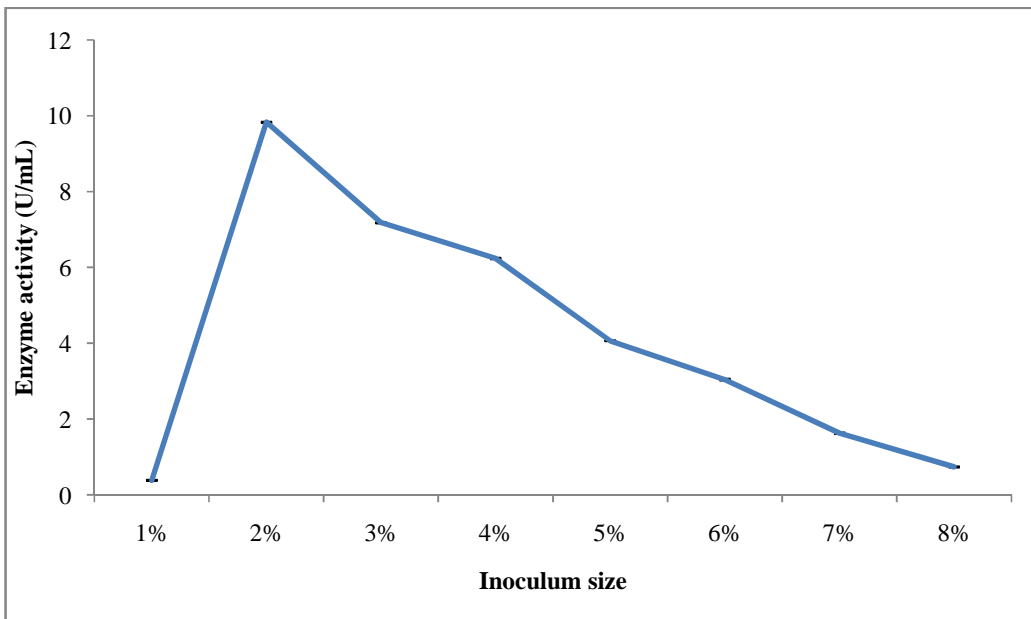


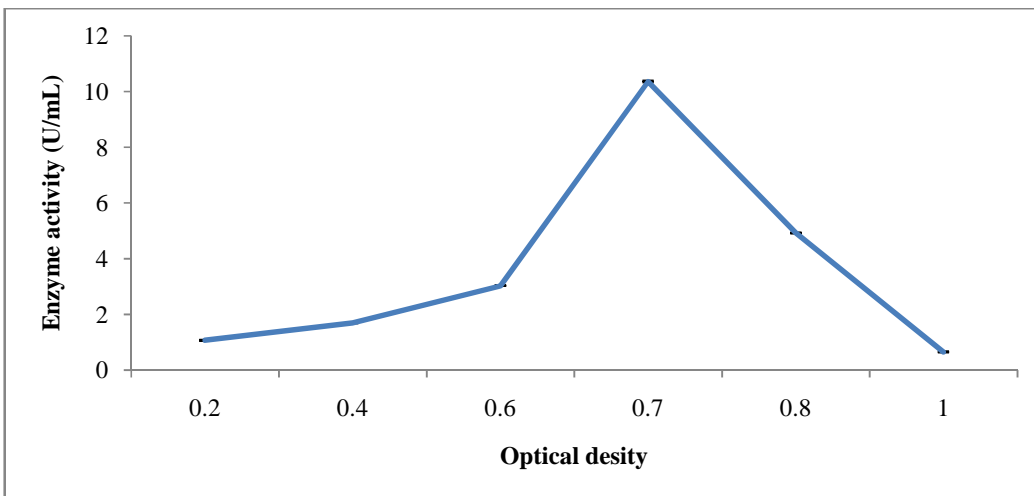
FIGURE 7: Effect of incubation period on tannase production



**FIGURE 8:** Effect of pH on tannase production

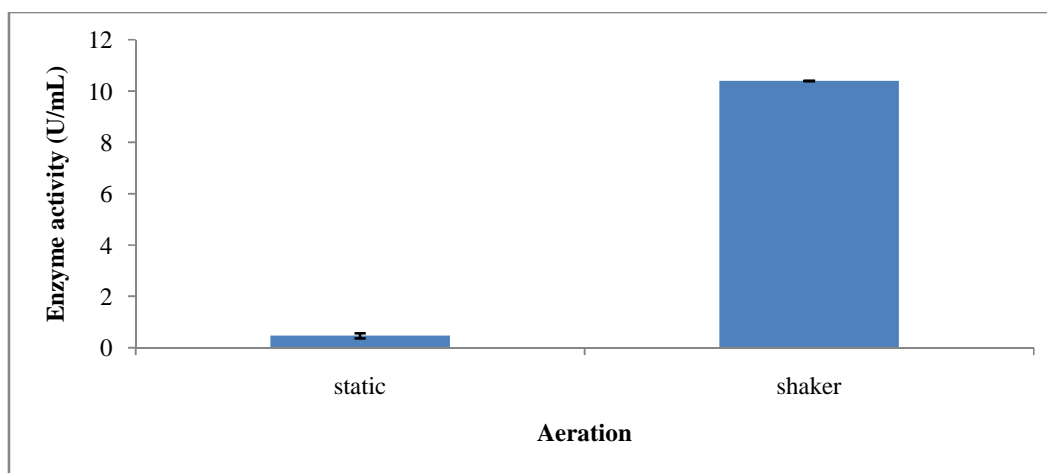


**FIGURE 9:** Effect of inoculum size on tannase production

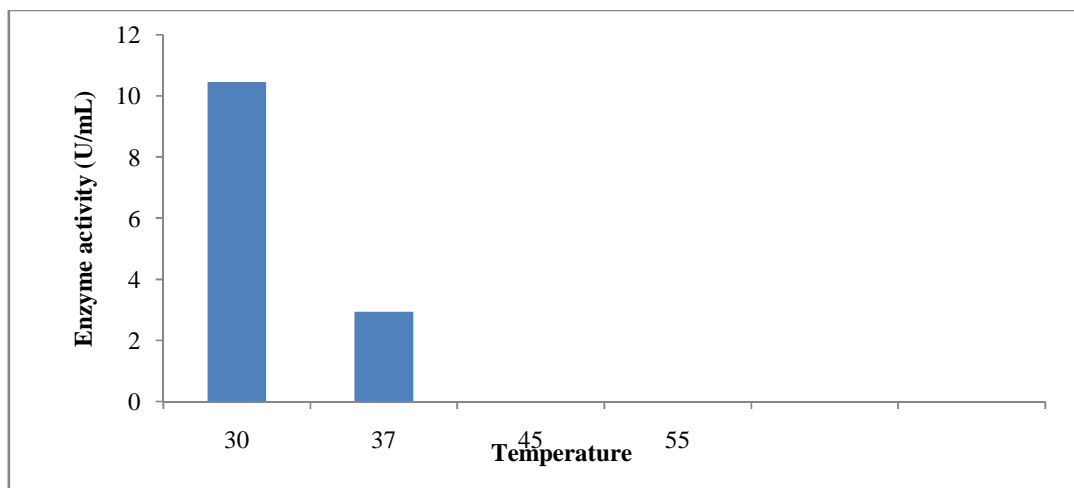


**FIGURE 10:** Effect of optical density on tannase production





**FIGURE 11:** Effect of aeration on tannase production



**FIGURE 12:** Effect of temperature on tannase production

The effect of various metal ions and inhibitors were checked on tannase enzyme activity. The residual enzyme activity in the presence of metal ions such as  $\text{Ca}^{2+}$  (58.62%),  $\text{Mg}^{2+}$  (107.44%),  $\text{Fe}^{2+}$  (37.7%),  $\text{Cu}^{2+}$  (13.7%),  $\text{Zn}^{2+}$  (9.61%) and  $\text{Hg}^{2+}$  (0%) was observed. All the metal ions inhibited the tannase activity but the maximum inhibition was observed by  $\text{Hg}^{2+}$  and the least inhibition observed by  $\text{Mg}^{2+}$  (Fig. 13). Significant reduction of tannase activity in the presence of inhibitors such as EDTA (20%), SDS (11.9%) and Tween 80 (74.2%) was also observed (Fig. 14). Further studies demonstrated negligible tannase activity in the presence of solvents (Fig. 15) such as acetone (0.96%), ethanol (0.40%), butanol (5.1%) and glycerol (3.40%). All the inhibitors and solvents inhibited the tannase activity. The optimum pH (Fig. 16) and temperature (Fig. 17) for tannase activity was found to be 5.5 and room temperature (28°C).

A study identified 3 tannase producing strains of *Klebsiella* (*K. variicola* PLP G-17 LC, *K. variicola* PLP S-18 and *K. pneumoniae* PLP G-17 SC) isolated from rumens of migratory gaddi goats which exhibited optimal tannase activity at 40°C and pH 6.0 (Sharma *et al.*, 2017). The optimum enzyme activity of tannase produced by

*Staphylococcus lugdunensis* was found at 40°C and pH7. Further characterization revealed that metal ions such as  $\text{Zn}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Fe}^{3+}$  and  $\text{Mn}^{2+}$  inhibited tannase activity and SDS supported the same at lower concentrations. Among the organic solvents tested, in general, the non-polar organic solvents increased, and polar solvents inhibited, the tannase activity (Amballa and Anbalagan, 2016). An endophytic fungi isolated from jamun *P. guepinii* produced thermostable extracellular tannase under submerged, slurry-state and solid-state fermentations. The tannase activity was found to be highest in presence of  $\text{Ca}^{2+}$  at pH6 and 30°C. It was also found to be resistant to tested chelators and detergents. Moreover, it showed only marginally inhibition by certain cations including  $\text{Mg}^{2+}$  and  $\text{Mn}^{2+}$  (Sena *et al.*, 2014).

Among other isolates, pathogenic *S. marcescens* b9 showed better enzyme activity (63U/ml) in nutrient broth supplemented with ber leaves and incubated at pH5.5 and 37°C for 72h. The tannase activity was affected by some detergents and metal ions like  $\text{Cu}^{+2}$ ,  $\text{Co}^{+2}$  and  $\text{Fe}^{+3}$  (Muslim, 2017). Kivanc and Temel (2019) reported optimum tannase activity of *L. brevis* A6X at 37°C, pH 5.0 and presence of  $\text{Ca}^{+2}$ ,  $\text{Zn}^{+2}$  and  $\text{K}^{+}$  ions. Further, it was

reported that the presence of surfactant (Tween 80), inhibitor (DMSO) and denaturing agent (urea) did not have any effect on tannase activity; whereas metal ions like  $Mg^{+2}$ ,  $Hg^{+2}$  and  $Zn^{+2}$  significantly reduced tannase activity. A thermostable tannase enzyme was obtained from *E. coli* in another study. This enzyme maintained 100% of its activity after prolonged incubations at different temperatures between 22°C and 55°C (Tomas-Cortazar *et al.*, 2018). In another study, *L. plantarum* showed optimum tannase activity at 30°C and pH6 in presence of  $Ca^{2+}$  ions (Jiménez *et al.*, 2014). *P. aeruginosa* IIB 8914 gave maximum tannase activity with  $MgSO_4 \cdot 7H_2O$  and  $HgCl_2$  when grown in amla (13.44 U/ml) and Keekar leaves (12.79U/ml) respectively (Selwal *et al.*, 2010). A stimulatory effect of  $Mg^{+2}$  was reported on tannase production in *Verticillium* sp. P9 (Kasieczka-Burnecka *et al.*, 2007), *A. foetidus* and *Rhizopus oryzae* (Kar *et al.*, 2003). In contrast, divalent cations inhibited tannase production in *A. japonicus* (Bradoo *et al.*, 1997). The cation  $Ca^{2+}$  inhibited the activity of tannase produced by *Penicillium verrucosum* (Bhoite and Murthy, 2015). In another study, the chelator EDTA disodium salt (1 mM), inhibited the tannase obtained from *Paecilomyces variotii*

(Kar *et al.*, 2003), whereas no inhibition by EDTA was observed in the case of the tannase from *A. flavus* (Yamada *et al.*, 1968) and yeast (Aoki *et al.*, 1976). Similarly, Tween 80 (1%) and Tween 60 (0.05-1.0%) caused an inhibition of the lipase activity obtained from *Pseudomonas* sp. KWI-56. It also showed inhibition in presence of 0.03-0.7% Sodium Lauryl Sulphate (SLS) and 0.03-0.5% Triton X-100 (Kar *et al.*, 2003). EDTA acts as a chelating agent and primarily influences the interfacial area between the substrate and enzyme, thus preventing contact between the two and resulting in decreased activity (Silva Lopes *et al.*, 2002; Jinwal *et al.*, 2003). Similarly, the inhibition due to Tween 80 and Tween 20 can be explained on the basis of their chemical composition. Tween 80 is predominantly composed of oleic acid (70%) while Tween 20 consists of lauric acid (20%). The predominance of these acids causes a decrease in tannase activity. The inhibition due to SLS may be due to the combined effect of factors such as the reduction in the hydrophobic interactions and denaturation of proteins (Kar *et al.*, 2003).

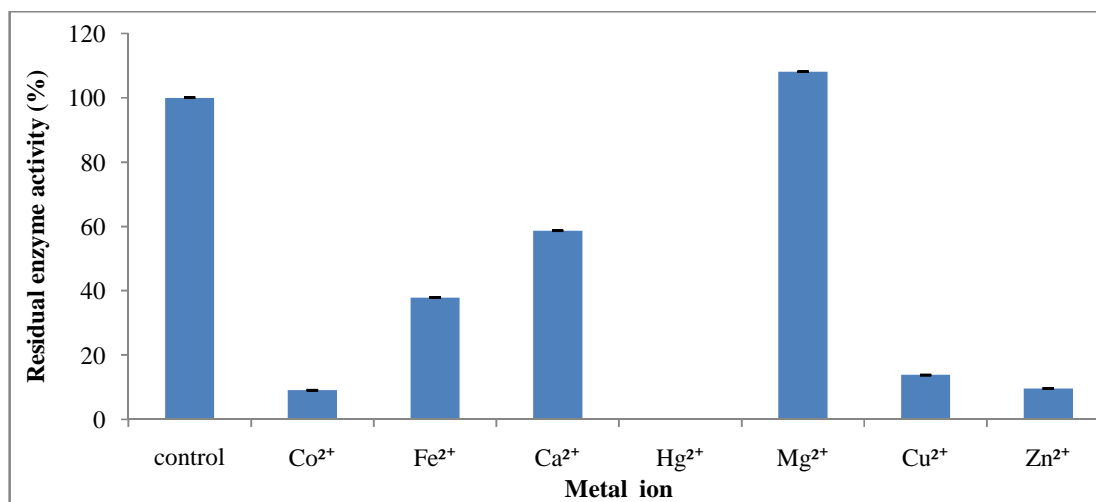


FIGURE 13: Effect of metal ion on tannase activity

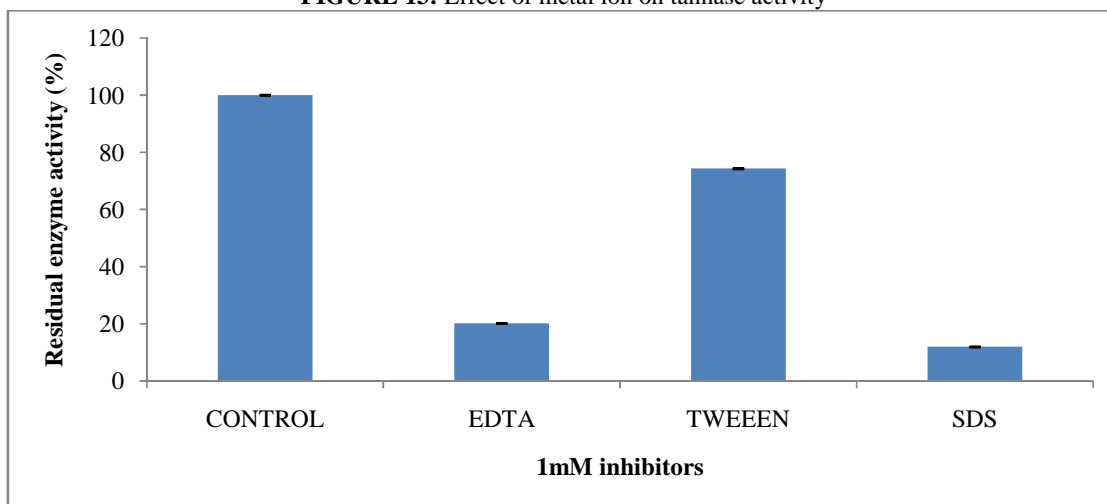
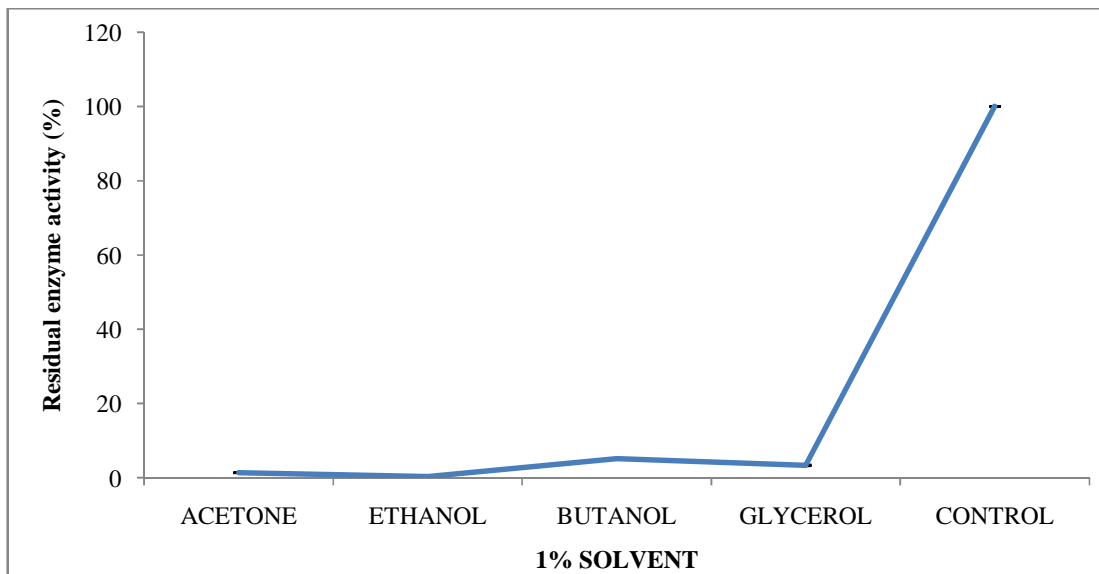
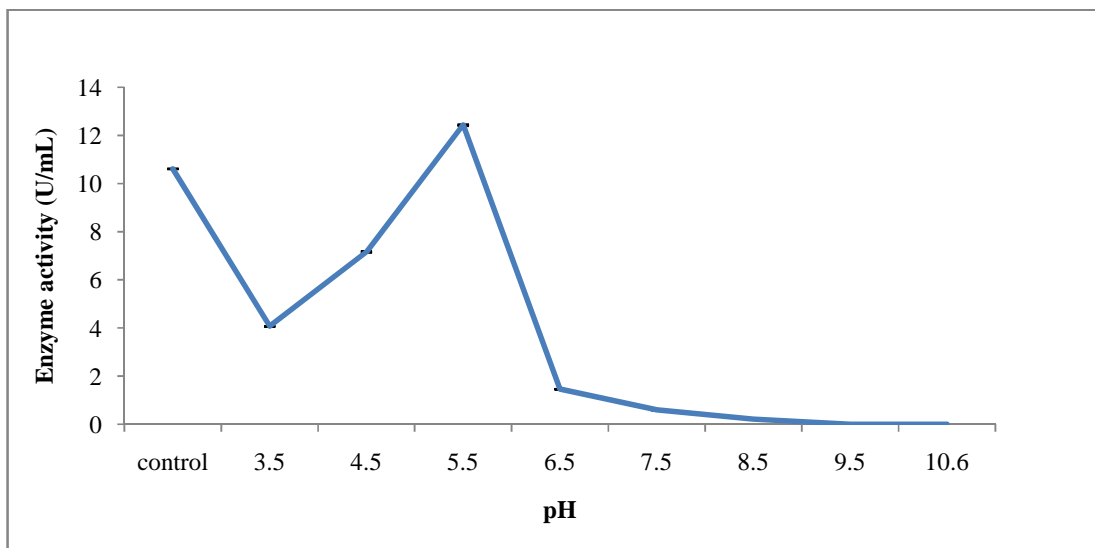


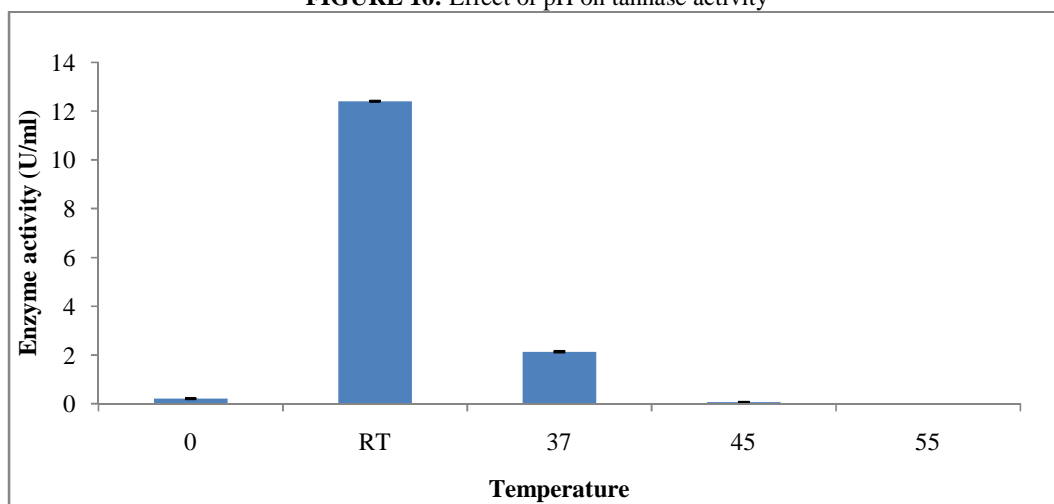
FIGURE 14: Effect of enzyme inhibitors on tannase activity



**FIGURE 15:** Effect of solvents on tannase activity



**FIGURE 16:** Effect of pH on tannase activity



**FIGURE 17:** Effect of temperature on tannase activity

### Application of tannase in debittering of pomegranate juice

The properties of crude tannase enzyme observed in our study showed that it has protein precipitation activity and thus, can be used in juice and wine industries. Hence the application of crude tannase as a debittering agent was evaluated using pomegranate fruit juice. The enzyme showed a 50% reduction in tannin content after 30mins of incubation at 30°C with 1ml of crude tannase. The raw press fruit juices have a cloudy appearance and sediment formation mainly due to the presence of tannic acid. This also gives a bitter and astringent taste to the juices upon storage. In addition tannins interfere with the absorption and digestion of nutrients from food samples. The enzymatic treatment of fruit juices to reduce the bitterness improves the quality of the finished products without compromising the quality. The anti-oxidant potential of berries and other fruits promotes a health by fighting common diseases.

### CONCLUSION

The test bacteria *K. pneumoniae* amar gave a considerable yield (upto 10.5U/ml) of tannase enzyme under optimum conditions. However, they were sensitive to metal ions, organic acids and other enzyme inhibitors. Further attempts can be made to adapt the enzyme activity to conditions favourable for its commercialization. Moreover, a wider substrate range, preferably agrowastes, can be used to study the production and activity of tannase.

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