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ISOLATION AND CHARACTERIZATION OF TANNINOLYTIC BACTERIA FROM DIFFERENT VIRGIN LOCATIONS OF MAHARASHTRA

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Abstract: Tannase is a commercial important enzyme used to catalyze the hydrolysis of ester and depside bond of tannin rich material which has yielded industrially valuable products. Keeping the significance of this enzyme, it has been always isolated from tannin-rich source and showed its potential in degrading it. The present study deals with isolating bacteria producing tannase enzyme from non-tannin rich source. Six potential bacteria were isolated from virgin soils which had enzyme activity equally to reported bacteria. These bacteria were identified on basis of antibiotic sensitivity test and 16s rRNA. *Klebsiella pneumonia* isolated from garlic field soil showed 1.40 U/ml activity and *Enteroabacter cloacae*showed 1.23 U/ml which is more than reported. Hence this enzyme has potential in various food and pharmaceutical industries.

Keywords: *Klebsiella pneumonia, Enteroabacter cloacae,* Tannase, 16s rRNA.

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INTRODUCTION

Tannins are water-soluble phenolic secondary metabolites of higher plants. They are the fourth most abundant plant constituent, after cellulose, hemicellulose, and lignin[1],having a molar mass ranging from 300 to 3,000 Da, although molecules as large as 20,000 Da have been found.They are found to be as secondary metabolites in most of plants[2]. Tannins widely occur in common foodstuffs such as tea, strawberry, raspberry, blackberry, grape, mango, cashew nut, hazelnut, walnut, and so on. The ability of tannins to bind to proteins and other molecules has caused major problem in food industry as it biters the taste of fruit juices, ice tea, coffee-flavored beverages and thus leads to serious environmental pollution, hence implies the need for ecologically friendly degradation method for tannic compounds.[3] Furthermore, there is a need in the food industry to solve problems related to the binding of tannins to proteins, starch, and some other nutrients in livestock feeds since it not only affects the nutritional quality of the feed but also decreases digestibility Tannins are generally resistant to biodegradation but some microorganisms are resistant to tannins and have in due course of time developed enzymatic degradation of these by producing Tannase enzyme.[3][2]

Tannase, commonly referred as tannin acyl hydrolase (EC 3.1.1.20), it hydrolysis the depside bond between tannins and gallotannins to produce Gallic acid and glucose.[1]. Gallic acid formed during the degradation of tannic acid has varied applications in pharmaceuticals and food industry. It is used in production of anti-microbial drug Trimethoprim, used for producing propyll gallate which has applications in photography and printing inks, also propyll gallte is used as anti-oxidant in fats and oils.[4]. Gallic acid also has properties which have cytotoxic effect over cancer cells. [5]. Beside this tannase is used in manufacture of instant tea and acorn wine, used in clarification of beer and fruits juices and used improving grape wine.[6],[7]Tannase cleaves dehydrodimer (poly phenols) crosslink present in cell wall of plants, which is necessary for plant cell wall digestibility. [6]. It also used in treatment of effluent coming from oil-mill and leather industry.[7]. Tannase is obtained from microbial, animal and vegetal sources, but enzyme produced by microorganism is more stable and is amendable to genetic modification.[7][6]. Bacteria, yeast and fungi are major producer for tannase and these are mainly isolated from tannin rich forest, animals feces which feed on tannin rich plants may aerobically or anaerobically. Due the increased demand of this enzyme many companies have commercially produced it some are GmbH (Germany), Biocon (India), Sigma–Aldrich Co. (USA), ASA special enzyme, Wako Pure Chemical Industries, Ltd. (Japan), Kikkoman (Japan) and Novo Nordisk (Denmark).[7] mostly are from fungal source. But fungi are difficult for genetic manipulation hence there is as increase in isolation of new bacteria which can produced more enzyme.[6].

Keeping this in mind the present study aim to isolate this commercially important enzyme from bacteria which are found in virgin soil i.e those soils and water samples which not contaminated with tannin effluents or tannin rich samples.

I. MATERIALS AND METHOD:

2.1. Material and chemicals used:

Tannic acid, methyl gallate and rhodanine were obtained from Sigma Chemicals.Gallic acid and Flucanazole were obtained from SLR chemicals. Dehydrated media procured from Hi-Media, Mumbai was used as microbiology media.Other chemicals used for minimal media and mobile phase were of high analytical grade.

1.2. Isolation of Tannase producer and Colony characteristic:

Isolation of tannase producing bacteria was carried out as per the method of Osawa and Walsh (200) and R Kumar (2010) [8],[9][10][11][12]with slight modification. Soil samples were collected from varied location of India in sterile polypropylene bags fromTomato field (T), Grape field (GR), Garlic field (GA), Khopoli black soil (K) near the ghat area, Deonar Dumping ground water and soil (DNW and DNS), Panvel Express-Highway soil (PAN). These sampleswere stored at 4°C. One gram of soil samples were suspended in st 0.85% saline and were allowed to shake at 30°C/30min/180rpm, after which they were serially diluted and plated on nutrient agar plates containing 0.5% Tannic acid. The medium also contained 50µg/ml of flucanazole. The plates were incubated at 30°C till colonies were appeared. Singe colony was selected and again were grown on nutrient agar containing tannic acid 0.5% to purify them. The strains exhibiting maximum zone of clearance were considered for further tannase production and were characterized using systemic classification of microorganism's and 16S rRNA gene sequence homology.Colony morphology was studied by streaking them on nutrient agar, Gram nature of all the isolates were determined by Gram staining method using Hi-media kit and Lactose fermentor and non-fermentors were analyzed on Maconkeys agar plates.Glycerol stocks of the selected cultures were made and stored at -20°C.

1.3. Identification by 16s rRNA and Antibiotic Sensitivity test:

Genomic DNA was isolated using Alkaline lysis method and its purity was checked by Agarose Gel electrophoresis and Nandrop 2000 (Thermo Scientific). The universal primer pair 8F AGAGTTTGATCCTGGCTCAG (Turner et al. 1999) and 907R CCGTCAATTCMTTTRAGTTT (Lane et al. 1991) was used for amplification of 900bp region of 16s rRNA gene. A 50 µl reaction mixture included 50 ng of bacterial DNA as template, 10 mM Tris–HCl (pH 8.3), 2 mM $MgCl_2$, 100 µM of dNTPs, 200 ng of each primer and 1U Taq DNA polymerase (Thermo Fisher). The reactions were performed on a Thermal Cycler (Applied Biosystem Gene Amp 9700) and the reaction cycle

included an initial denaturation of 5 min at 96°C followed by 35 cycles of 45 seconds at 95°C, 45 seconds at 55°C and 1 min at 72°C with a final extension of 5 min at 72°C. The amplified product was visualized in 1% agarose gel stained with Ethidium Bromide and purified using QIAquick PCR purification kit (Qiagen). The Sequencing PCR was done according to manufacturer (Beckman Coulter). The amplified product was sequenced on Beckman coulter CEQ 8000 DNA sequencer.[1][13]Antibiotic sensitive test was done at LALA Ram Sarup Institute of Tuberculosis and Respiratory disease, New Delhi.

1.4. Growth curve, generation time and growth Rate:

The starter culture was inoculated at 2 %(v/v) into St. Luria Bertani broth and optical density was measured at regular intervals. Growth curve was plotted and Generation time (g) and growth rate (μ) were calculated using the formula: $g = [2.303 (\log OD_2 - \log OD_1)]/ t_2 - t_1$ and $\mu = 0.693/g$ [14]

1.5. Thin layer chromatography (TLC):

TLC was performed according to Sharma *etal*, 1999 method with slight modifications. Separation was achieved on pre-coated plate with silica gel 60 F_{254} (Merck). The plates were allowed to dry at 60˚C/ 1 hr. 5 µl of supernatant and standard were spotted on the TLC plate. The mobile phase was chloroform: ethyl acetate: formic acid: water (6:4:0.1:0.1) and detection was done using Iodine vapours. Standards used were Tannic acid and Gallic acid (1mg/ml)[15][16].

1.6. Gallic acidand Dry Cell mass estimation:

For studying the maximum Gallic acid production, the bacteria were grown in minimal media (MM)(KH_2PO_4 1g/l, K_2HPO_4 1g/l, $MgSO_4$ 1g/l, NH_4Cl 2g/l, $CaCl_2$ 0.02g/l, Glucose 5g/l)[17],[18],[19], inoculum was initially grown to mid-log phase in nutrient broth. Then the broth was centrifuged at (3000rpm/10mim/4˚C) and the pellet was washed twice with St. D/W then suspended in MM and 5% innoculum was added to the 100cm^3 of MM containing filter sterilized 1% tannic acid. The culture conditions30°C/ 180rpm/48hr. The samples were drawn at after every 12 hr intervals, were centrifuged at 10,000rpm/4°C/20min and stored at -20°C for further analysis of Gallic acid (by TLC and spectrophotometrically) and enzyme assay.Gallic acid produced during tannic acid biotransformation studies using culture supernatant wasestimated by the spectrophotometer method using GA (10-100 µg) as standard. The samples drawn also was used for dry cell mass as a measure to estimate increase in cell number.[4]

1.7. **Enzyme assay:**

Tannase activity was determined using a Rhodanine Assay specific for Gallic acid (Sharma etal, 2000). Rhodanine specifically reacts with Gallic acidto produce pink colour. Gallic acid was determined using the following enzyme assay: 250µl of culture supernatant was incubated with 250µl of 0.1M Methyl gallate and 250µl of citrate buffer pH 5.0 at 30°C/10min. After incubation 300µl of methanolic Rhodanine solution (0.667% w/v Rhodanine in 100% methanol) was added to reaction mixture. After 5min of incubation at 30°C, 200µl of 0.5M KOH was added. After incubation of 10min this solution was diluted to 5ml with D/W and absorbance was read at 520nmagainst standard of Gallic acid concentration. One unit of tannase activity was defined as the amount of enzyme required to release 1µmol of Gallic acid per minute under standard reaction conditions[16][20].

III RESULTS

3.1. Isolation of Tannase producers and Colony characteristics:

A total of 36 microorganisms were isolated from different locations of Maharashtra, of which Deonar Dumping soil and water, Garlic field soil was first time screened for the tannase producing microorganisms. Addition of tannic acidof 0.5% to nutrient agar forms a tannin-protein complex; cleavage of this complex by bacteria producing tannase forms a zone around the colonies. After a prolonged incubation of 3–4 days, a greenish brown zone around the colonies appears (Figure 1)[12][21]. The microorganism from Garlic field soil, grape soil, Deonar dumping water, tomato field and khapoli soil showed1.0 mm to 8.5 mm of zone clearance and browning of the medium, of which DNW3, GA2 and GR7 showed 8 mm, 5.3 mm and 6.3 mm of zone of clearance respectively.The addition of flucanazole in the medium inhibited the growth of fungus.Colony characteristic on nutrient agar revealed that the organism isolated were morphological different and all were Gram negative with shorts rods, bacilli. They were further recognized on basis of Maconkey agar which indicated that all were lactose fermenting bacteria which means belonging to Enterobactericeae sp.

3.2. Identification by 16s rRNA and Antibiotic Sensitivity test:

Based on 16s rRNA (Figure 2A and 2B) identification the cultures were identified as GA2 and DNW3 as *Klebsiella pneumoniae*, GR7, T6 and G3 as *Enterobacter cloacea* and K3 as *Acinetobacter baumannii*. From the antibiotic sensitivity test's result that was carried out against nineteen antibiotics (Table 1), which showed that GA2 was sensitive to eleven antibiotics and resistant to six, GR7 was sensitive to ten and resistant to five antibiotics, DNW3 was sensitive to ten and resistant to one antibiotic, T6 was sensitive to six and resistant to two, K3 sensitive to four and resistant to four and G3 was sensitive to four and resistant to four.

3.3 Growth curve, generation time and growth Rate:

These organisms were isolated from soil and they were induced against stressed condition of tannic acid, when any organism is subjected so such a condition they tend to change the growth pattern, hence it becomes important to study the growth curve so as to understand the midlog phase of these organism. Growth curve was performed to understand the adaptation and mechanism of growth, all the cultures were inoculated at the same OD_{620}. Growth curve was plotted and various phases of growth were observed as shown in Figure 3 and Table 2. Highest OD was achieved by *Klebsiella pneumoniae and Acinetobacter baumannii* in the shortest span of time i.e. 9 hour whereas G3 *Enterobacter cloacea* has the longest doubling time which is 3.41 hour.

3.4. Thin Layer Chromatography:

The formation of Gallic acid and propylgallol was observed over different time intervals. TLC showed formation of two spots (Figure 4) when compared with standards it revealed that the one spot is of Gallic acidand the other of propylgallol. The formation of Gallic acid starts from 12hour and then after 24 hour GA2 and GR7 forms both Gallic acid and propylgallol, whereas DNW3 and T6 after 24hour forms propylgallol.[16][15].

3.5. Gallic acid and Dry Cell mass estimation:

The degradation of tannic acid leads to production of Gallic acid and glucose, and this glucose is utilized by cell for its metabolism. Gallic acid present in medium sometime becomes toxic to cell and hence cell tries to metabolize into further products[7]. Degradation of tannic acid in the medium is monitored by increase in cell number and percentage of Gallic acid produced. Cell growth in case of tannic acid in medium cannot be monitored by measuring optical density due darkening of medium[17], hence increase in cell number is indicated by increase in DCM (Figure 5). The DCM of all the cultures are same 6^{th} hour but GA2 and GR7 increase after 12 hour to 3mg/l and 2mg/l. Even Gallic acid production starts beyond 6 hour and increase till 48 hour. GA2 shows maximum DCM after 24 hour i.e 20% of Gallic acid production till 48 hour which is 28 %, DNW3 shows minimum DCM after 24 hour with 7.39% of Gallic acid.(Figure 6).

3.6. Enzyme activity:

The organisms were grown in minimal media supplemented with 1% tannic acid. Initially there was no enzyme activity observed but after 6 hour there was substantial increase in the activity due to degradation of tannic acid, maximum activity was observed till 24 hour for all the organisms, which gradually decreased till 48 hour. GA2 showed highest tannase activity at 24hour of 1.40 u/ml and a total 140.43, followed by GR7 with 1.23 U/ml and DNW3 had lowest activity of 0.30 U/ml (Figure 7).

IV. DISCUSSION:

There are several Enterobaceriacae sp. reported which has ability to degrade tannins but these organism are isolated either from tannin contaminated areas or tannin rich forest, rumen or feces of goats oranimals which feed on tannin rich plant (Table 3). In the present study six tannase producing bacterial strains were isolated from different sources of which garlic field soil and Deonar dumping water were first reported, both the soil are not rich source for tannin contamination. The isolation of these organism were carried out 30°C whereas other reported bacterial strains were isolated at 37°C-45°C.[18]. The isolation was carried according by plate method but it was further confirmed to have tannase enzyme by growing them in minimal media supplemented with tannic acid (optimum 1%), glucose was added to medium as initial start for the metabolism. The enzyme activity of *klebsiella pneumoniae* isolate from garlic field has enzyme activity of 1.40 U/ml which is from non-tannin contaminated soil, *Enterobacter cloceae* isolated from grape field soil has enzyme activity 1.23 U/ml slightly more than reported i.e 0.6 U/ml. This activity is comparable to the other group of bacterial strain such as *Klebsiella pneumoniae* 3.46 U/ml by, Lactobacillus (0.3 U/ml), Bacillus sp. (1.03 U/ml) and Pseudomonas aeruginosa IIIB 8914 (13.65 and 12.90 U/ml using amla and keekar leaves (2% w/v) respectively under SmF)[17].

V. CONCLUSION:

Tannase producing enzyme were isolated from non-tannic acid rich source and the isolates were identified according to antibiotic sensitive and 16s rRNA. Further optimization of GA2 can increase threshold of enzyme activity and degradation of tannic acid yielding more amount of Gallic acid. Even more work can be done to analyze the gene sequence of enzyme producing bacteria and to clone in high expressing prokaryotic strains for more production of this enzyme. Even mutation of such enzyme can increase the production of this enzyme.

Conflict of Interest: Gaganjyot Kaur declares that she has no conflict of interest. Pratiksha Alagh declares that she has no conflict of interest.

Ethical approval: This article does not contin any studies with human participants performed by any of the authors.

List of tables:

Table 1: Antibiotic sensitivity test for all the cultures. R: Resistant, S: Sensitive and NT: Not tested.

SrNo.	Antibiotics	GA2	GR7	DNW3	T6	K3	G3
1	Amikacin	S	S	S	S	S	S
2	Gentamycin	S	S	S	S	S	S
3	Tobramycin	S	S	S	S	R	S
4	Ciprofloxacin	S	S	S	S	R	S
5	Cifotaxime	S	S	S	R	NT	R
6	Ciftazidime	R	S	S	NT	R	R
7	Imipenam	S	S	NT	NT	S	R
8	Meropenem	S	S	NT	NT	NT	R
9	levo floxacin	S	S	S	S	NT	NT
10	Pipracillin	R	NT	S	S	NT	NT
11	Tazobactum	S	NT	S	S	NT	NT
12	Amoxyelene	S	R	S	R	NT	NT
13	Mezlocillin	R	NT	NT	NT	S	NT
14	Ceptatoxime	NT	NT	NT	NT	R	NT
15	Ampicillin	R	R	R	NT	NT	NT
16	Cefazolin	S	R	NT	NT	NT	NT
17	Cefoxitin	R	R	NT	NT	NT	NT
18	Tetracyline	R	S	NT	NT	NT	NT
19	Cefuroxime	NT	R	NT	NT	NT	NT

Table 2: Shows Growth Rate and Generation time of the cultures GA2, GR7, DNW3, T6, G3 and K3

Culture Name	Growth Rate Hr^{-1}	Generation time Hr
GA2	0.30	2.35
GR7	0.26	2.68
T6	0.23	2.97
DNW3	0.31	2.22
G3	0.20	3.41
K3	0.35	1.95

Table 3: origin and Diversity of Tannase producing Bacteria (Jana et al., 2014)

Origin and Diversity of some tannase producing Enterobactericae sp.		
compost	*Enetrobactee cloacae*	Beniwal etal (2010)
forest litter and decaying bark of oak and pine	*Klebsiella pneumoniae*	Deschamps et al. (1983)
	Klebsiella planticola	Deschamps et al. (1983)
Tanner effluent	*Klebsiella pneumoniae*	Sivashanmugam and Jayaraman (2011)
Fish Gut	*Enterobacter asburiae*	Mandal and Ghosh (2013)
Goat feces	*Enterococcus faecalis*	Goel Etal (2011)
Rumen of Goats	*Enterobacter ludwigii*	Singh et al. (2012)
oil mill waste	*Klebsiella pneumoniae*	M Pepi etal (2013

Figure 1: (a) microorganism from Deonar Dumping Soil and water, (b) Microorganism from Garlic Field Soil, (c) Microorganism from Grape field soil.

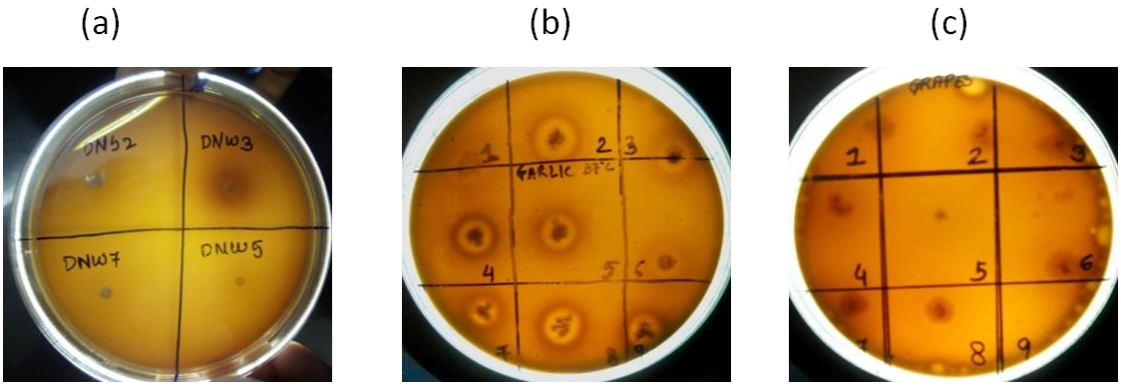


Figure 2(a): Genomic DNA isolation of culture on 0.7% agarose gel stained with 5 µg/µl ethidium bromide, Lane 1: standard 1 kb ladder, Lane 2: genomic DNA band and RNA at the base.

Figure 2(b): 16s rRNA amplification using 8F and 907R primers on 2% agarose gel stained with 5 µg/µl ethidium. Lane 1 shows standard 100bp ladder; lane 2 shows a 900bp amplicon specific to the 16s rRNA region.

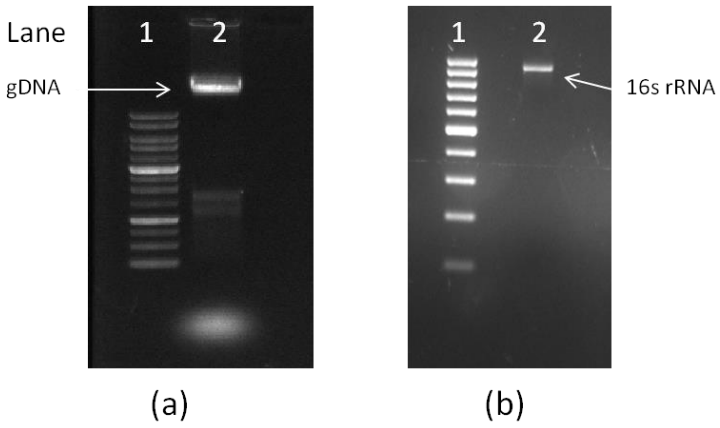


Figure 3: Growth curve of cultures: Shows the growth curve of the 6 cultures in St. LB over time, it explains the various phases of growth curve viz. Lag, log or exponential, stationary and death phase, cultures are named according to isolation source i.e GA2 and DNW3 as *Klebsiellapneumoniae*, GR7, T6 and G3 as *Enterobacter cloacea* and K3 as *Acinetobacter baumannii*.

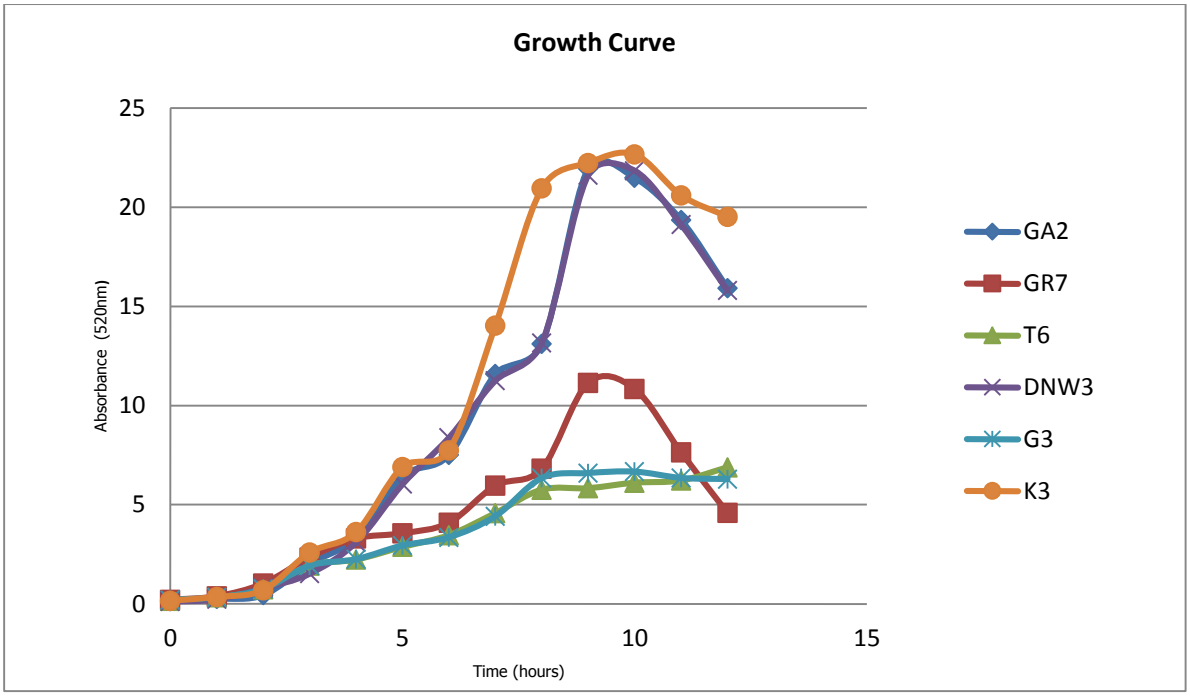


Figure 4: Mobile phase: Chloroform: Ethyl acetate: Water: Formic acid (4:6:0.1:0.1) (v/v/v/v), Sample loaded: 5ul, Developed with Iodine Lane 1:Gallic acid in MM, Lane 2: DNW3, Lane 3: T6, Lane 4: GA2, Lane 5 : GR7.

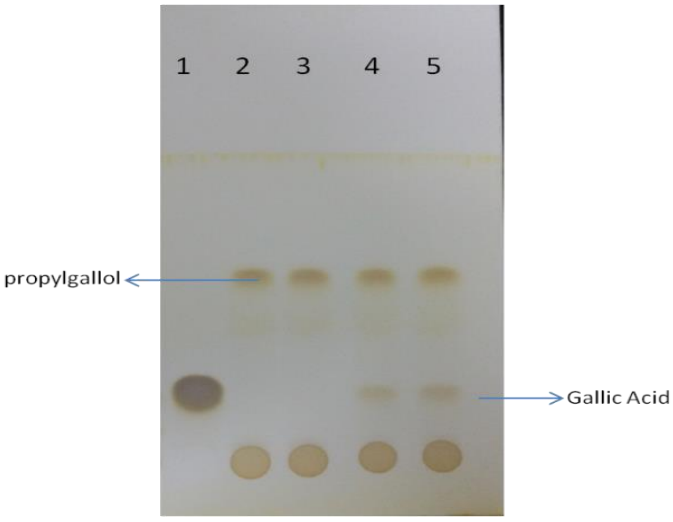


Figure 5: Shows the Dry Cell Mass (DCM) of the 6 cultures over various time interval, cultures were named according to isolation source i.e GA2 and DNW3 as *KlebsiellaPneumoniae*, GR7, T6 and G3 as *Enterobacter Cloacea* and K3 as *Acinetobacter baumannii*.

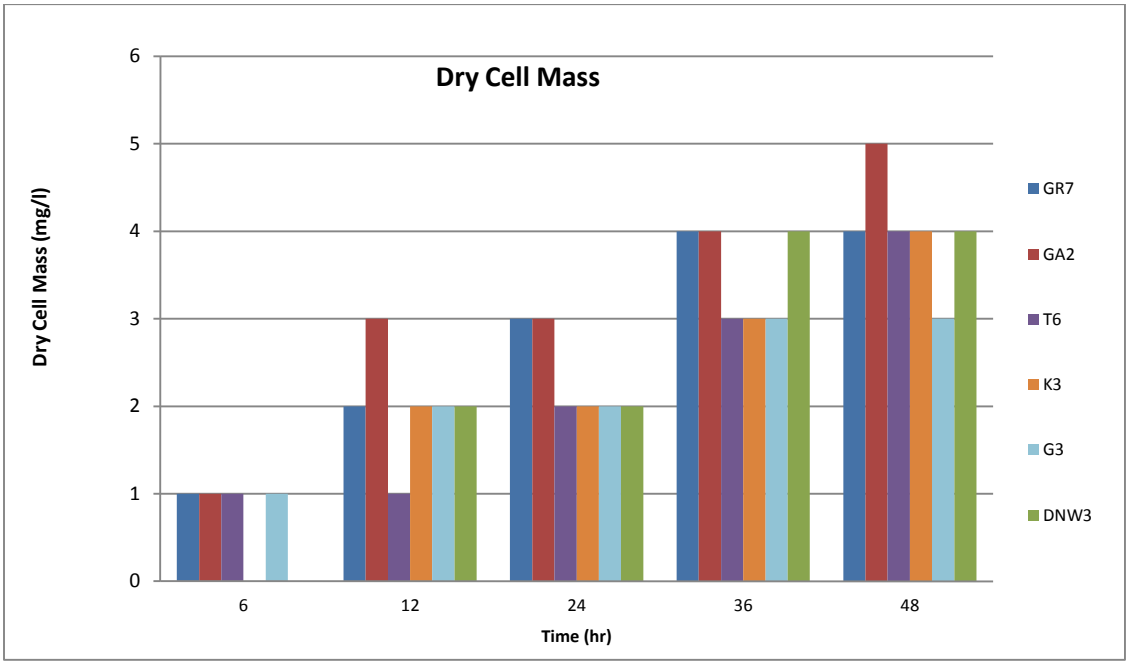


Figure 6: Production of Gallic acid of over 12 hour intervals for 6 cultures.

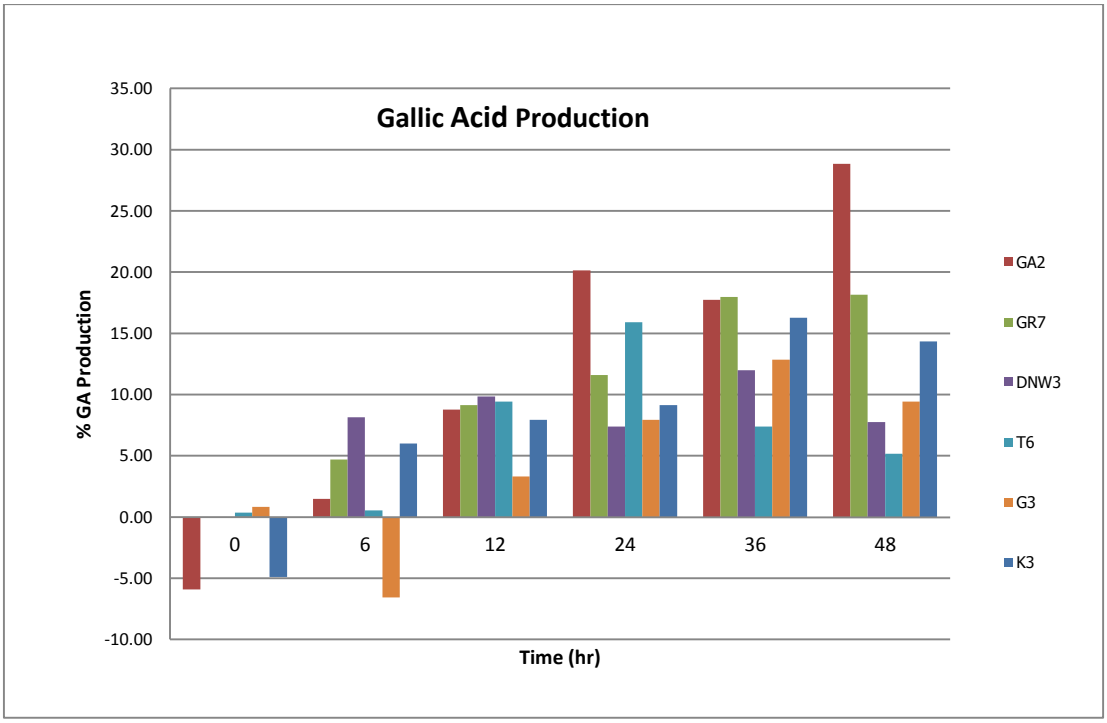
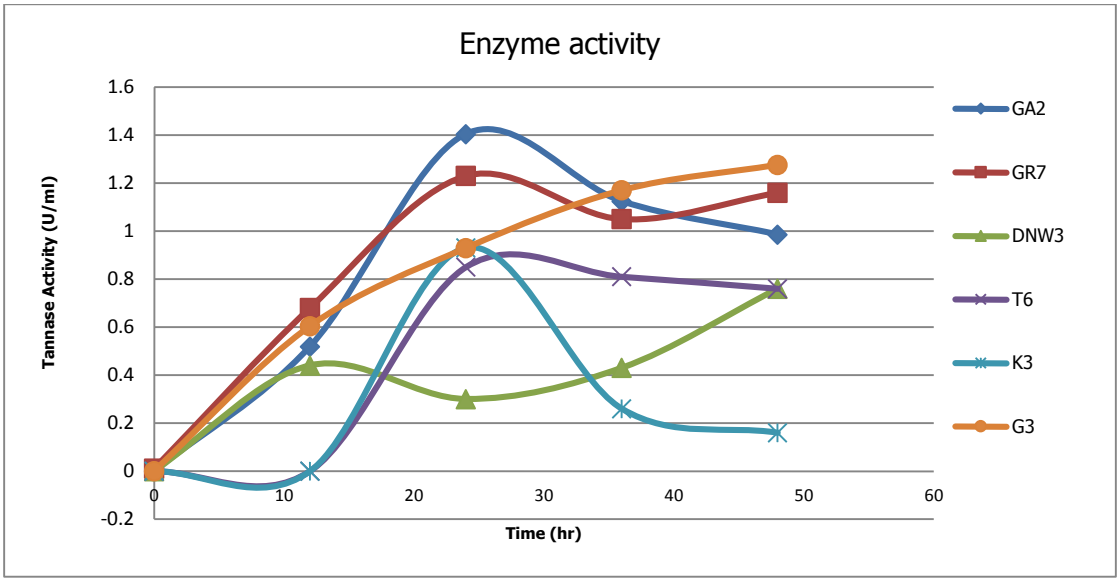


Figure 7: enzyme activity of isolates at various time interval



REFERENCES:

1. Chowdhury, S. P., Khanna, S., Verma, S. C., & Tripathi, A. K. (2004). Molecular diversity of tannic acid degrading bacteria isolated from tannery soil. Journal of Applied Microbiology, 97(Hillis 1985), 1210–1219.

2. Rodríguez-Durán, L. V., Valdivia-Urdiales, B., Contreras-Esquivel, J. C., Rodríguez-Herrera, R., & Aguilar, C. N. (2011). Novel Strategies for Upstream and Downstream Processing of Tannin Acyl Hydrolase. Enzyme Research, 2011, 1–20.

3. Chávez-González, M., Rodríguez-Durán, L. V., Balagurusamy, N., Prado-Barragán, A., Rodríguez, R., Contreras, J. C., & Aguilar, C. N. (2012). Biotechnological Advances and Challenges of Tannase: An Overview. Food and Bioprocess Technology, 5, 445–459.

4. Beniwal, V., Chhokar, V., Singh, N., & Sharma, J. (2010). Optimization of process parameters for the production of tannase and gallic acid by *Enterobactercloacae* MTCC 9125. J. Am. Sci., 6(8), 389–397.

5. Bajpai, B., & Patil, S. (2008). A new approach to microbial production of gallic acid. Brazilian Journal of Microbiology, 39, 708–711.

6. Beniwal, V., Kumar, A., Sharma, J., & Chhokar, V. (2013). Recent advances in industrial application of tannases: A review. Recent Patents on Biotechnology, 7, 228–233.

7. Jana, A., Halder, S. K., Banerjee, A., Paul, T., Pati, B. R., Mondal, K. C., & Das Mohapatra, P. K. (2014). Biosynthesis, structural architecture and biotechnological potential of bacterial tannase: A molecular advancement. Bioresource Technology, 157, 327–340.

8. Ueda, S., Nomoto, R., Yoshida, K., & Osawa, R. (2014). Comparison of three tannases cloned from closely related lactobacillus species: *L. Plantarum, L. Paraplantarum, and L. Pentosus*. BMC Microbiology, 14(1), 87.

9. Nishitani, Y., & Osawa, R. (2003). A novel colorimetric method to quantify tannase activity of viable bacteria. Journal of Microbiological Methods, 54, 281–284.

10. Nishitani, Y., & Osawa, R. (2005). Deceptive Halo Formation by Tannase-Defective Bacteria on Tannin-Treated Plate Media. Microbes and Environments, 20(2), 117–119.

11. Osawa, R., Kuroiso, K., Goto, S., & Shimizu, A. (2000). Isolation of tannin-degrading lactobacilli from humans and fermented foods. Applied and Environmental Microbiology, 66(7), 3093–3097.

12. Kumar, R., Kumar, A., Nagpal, R., Sharma, J., & Kumari, A. (2010). A novel and sensitive plate assay for screening of tannase-producing bacteria. Annals of Microbiology, 60, 177–179.

13. Wilson, P., Rojan, P., Kumar, P., & Sabu, T. (2010). Tannin Acyl Hydrolase Production by Citrobacter sp. isolated from Tannin rich Environment, using Tamarindus indica seed powder. Journal of Applied Sciences and Environmental Management, 13.

14. Kannan, N., Aravindan, R., & Viruthagiri, T. (2011). Effect of culture conditions and kinetic studies on extracellular tannase production by *Lactobacillusplantarum* MTCC 1407. Indian Journal of Biotechnology, 10(3), 321–328.

15. Goel, G., Kumar, A., Beniwal, V., Raghav, M., Puniya, A. K., & Singh, K. (2011). Degradation of tannic acid and purification and characterization of tannase from *Enterococcusfaecalis*. International Biodeterioration and Biodegradation, 65(7), 1061–1065.

16. Singh, B., Bhat, T. K., Sharma, O. P., Kanwar, S. S., Rahi, P., & Gulati, a. (2012). Isolation of tannase-producing *Enterobacterludwigii* GRT-1 from the rumen of migratory goats. Small Ruminant Research, 102(2-3), 172–176.

17. Sivashanmugam, K., & Jayaraman, G. (2011). Production and partial purification of extracellular tannase by *Klebsiellapneumoniae* MTCC 7162 isolated from tannery effluent. African Journal of Biotechnology, 10(8), 1364–1374.

18. Pepi, M., Cappelli, S., Hachicho, N., Perra, G., Renzi, M., Tarabelli, A., Heipieper, H. J. (2013). *Klebsiella* sp. strain C2A isolated from olive oil mill waste is able to tolerate and degrade tannic acid in very high concentrations. FEMS Microbiology Letters, 343(2), 105–112.

19. Karthikeyan Sivashanmugam. (2011). Media optimization for extra cellular tannase production by *Klebsiellapneumoniae* MTCC 7162 using response surface methodology. African Journal of Microbiology Research, 5(22), 3611–3615.

20. Sharma, S., Bhat, T. K., & Dawra, R. K. (2000). A spectrophotometric method for assay of tannase using rhodanine. Analytical Biochemistry, 279, 85–89.

21. Osawa, R., Walsh, T. P., & Osawalt, R. (1993). Visual Reading Method for Detection of Bacterial Tannase, Applied and Environmental Microbiology, 59(4), 1251–1253.