Screening and Isolation of Tannin and Caffeine Degrading Microbes

Shailaja Mishra, Kanika Dudi, Shubhi Chawla and Shweta Kulshreshtha

Amity Institute of Biotechnology, Amity University Rajasthan, Jaipur

***Abstract*: Tannins and caffeine are recalcitrant compounds and persist for longer time in the environment after discharge. These pose harmful effects on the living beings and therefore need to be treated properly before discharge. In the present paper, tannin and caffeine degrading fungi was isolated from air and further assessed for the production of tannase and caffeine degrading enzymes. It was found that tannase and caffeine degrading enzymes are produced efficiently by Aspergillus sp. at neutral pH and 30°C temperature. After detailed study of optimized conditions, this fungus can be used for removal of tannin and caffeine in future.**

Keywords: tannin, caffeine, tea extract, coffee extract, microbes

I. INTRODUCTION

The quality of environment is at risk due to industrial pollution which poses negative impact on animals, plants, human beings, environment and climate. In the present era, importance of industries cannot be ignored and therefore, there is a need to treat industrial wastes and effluent before discharge.In this paper, we have focused on one of the most recalcitrant compounds that is – tannin and caffeine. Tannin is widespread in leaves, fruits, vegetables, bark and wood and therefore, released from various industries based on the processing of these materials [1]. There are many industries which are discharging tannin and caffeine rich effluent to the nearby water bodies [2]. Olive oil industrial effluent, leather processing industries, tea processing industrial effluent, tea leave dumping sites, debarking effluent and composite effluents possess high amount of tannin [1]

Tannins used as antioxidants, stabilizers and as depressants in many food and leather processing industries. Tannins impart colour and turbidity to the effluent and make it unsuitable for use in recreational purposes. Caffeine (1,3,7-trimethylxanthine), a natural alkaloid present mainly in tea and coffee products. Caffiene is released during decaffeination process and has been suggested as an environmental pollutant [3]. Tea and coffee producing industries release caffeine and tannin rich effluent [4]. A huge amount of water is used in the process of converting raw fruits of coffee plant into the finished coffee product. Caffeine is extracted from green coffee beans in the form of by-product during the process of decaffeination. In the decaffeination process, ethyl acetate is used as solvent which can be extracted and recycled. However, 100% caffeine content is not removed during decaffeination [5] and after decaffeination caffeine containing water is discharged to nearby area or water bodies in the form of untreated effluent.

Discharging of effluent causes pollution of water which not only affects the human beings but also the aquatic life. Caffeine and tannin present in water bodies adversely affects humans, aquatic animals, soil and ground water [6-7]. It is reported that tannins can suppress the soil enzyme activity thus controlling ecosystem structure and processes [8]. Tannin and caffeine also causes light yellow to dark brown discoloration in water. Tannin causes aesthetic problems because these may make water non-potable. Hence, it needs to be treated properly before discharge [9]. Many efforts have been made to remove tannin and caffeine from water bodies [10-11, 3]. The most common method of caffeine and tannin treatment is based on the use of anion exchange resin. The anion resin is sensitive to hardness and therefore, unable to treat hard water. To make it suitable for use, a water softner is added during the pretreatment stage. The water softener extends the life of the anion resin and increases tannin absorption. This anion exchange resin system also changes the chloride, alkanity and sulphate levels of the treated water. The problem associated with this technology is- tannin makes a coat on the iron filters, cation exchange filters and neutralizing filters and can interfere with equipments used to treat water problems. This leads to the malfunctioning of treatment devices. Therefore, a process is needed to treat caffeine and tannin containing effluent before send it to the filtration process. In this pretreatment step microbial filters can be introduced as pre-treatment step.

Till today, many biological processes have been developed for the removal of tannin [8, 12-13]. These processes are reported to degrade the tannin and reducing the COD of the effluent but are not effective at higher concentration of tannin. The biodegradation of vegetable effluent with tannin content occurred inefficiently due to the inhibition of activated sludge process by tannin content. Besides, degradation process is slow because tannin is recalcitrant compound. Hence, it is important to find out microbial inoculum that is able to degrade tannin at higher concentration.

The tannase enzyme can be used for the degradation of tannin [1]. It is also reported that treatment with tannase enzyme is less effective compared to microbial culture having tannase enzyme because microbes can be adapted to high concentration of tannin while enzymes can be inactivated. Keeping this in mind, we aimed at isolation and screening of tannin and caffeine degrading fungi which can degrade tannin and caffeine at higher concentration and in very less time.

II. METHODOLOGY

A. *Preparation of tea and coffee extract*

Tea and coffee extract contain tannin and caffeine, respectively. Therefore, tea and coffee extracts were used as a source of tannin and caffeine for the preparation of mycological media.At first, coffee and tea were weighed 1 g, 2 g, 3 g, 4 g and 5 g in order to make different concentrations of caffeine and tannin extract. After this, weighed amount of tea and coffee is collected in different conical flasks and 50ml distilled water was added to it. These flasks were boiled for 20 min in order to extract all tannin and caffeine content to the aqueous solution. The solution was stirred continuously during the boiling. After 20 min, the amount of water left in the flask was 20 ml. This was further used in the preparation of tea and coffee containing media.

B. *Media preparation*

A mycological media was prepared using the mentioned composition: Dextrose 2gm, Potatoes infusion 200g, Agar-Agar 15g, Tea extract 1ml, 2ml, 3ml, 4ml, and 5ml or coffee extract were added and make up the solution up to 100ml using distilled water. All contents were mixed properly and put in autoclave at 15psi and 121°C for sterilization. Autoclaving was done for 15-20min. Sterile media were kept overnight for sterility testing. After that, it was used for pouring in petridishes.

C. *Isolation*

Coffee and tea extracts were left uncovered so that aeromicroflora can deposit on it. When fungal colonies appeared on the petridishes, these were isolated on the fresh media having different concentration of tea and coffee extracts. These isolated colonies were transferred on the fresh media till the colonies obtained in pure form. For cultivation of fungi, petridishes were incubated at room temperature 35± 2°C. The culture was observed daily for the fungal growth.

D. *Identification of Fungi*

The isolated fungi were identified after growth on tea extract and coffee extract agar medium by observing their macroscopic (colour, texture, appearance, and diameter of colonies) and microscopic (microstructures) characteristics.Smears of the isolated fungi were prepared in lactophenol cotton blue and examined with the 40x objectives of a compound binocular microscope for microscopic appearance.

E. *Relative enzyme activity*

Isolated samples were obtained in pure form on tea and coffee extract agar medium. A small fragment was picked up with the help of inoculating needle and placed in the center of petridishes and then incubated at 37°C for 96 hours. After incubation, plates were taken out and flooded with gram's iodine instead of ferric chloride. Gram's iodine reacted only with non-hydrolyzed tannic acid and formed a dark brown complex. This gives a sharp distinct zone around the tannase producing fungal colonies even in cases of low levels of tannase production. The colony which was showing highest tannase producing ability on coffee and tea extract media, was selected for further study. The relative enzyme activity was calculated by using the following formula:

Relative enzyme activity = zone of hydrolysis/colony diameter

F. *Effect of Temperature and pH on Tannase Activity*

The selected fungus was tested for assessing the effect of temperature and pH on tannase enzyme production. Three media were prepared by varying the pH of medium. The pH of the medium was adjusted 6, 7 and 8 using hydrochloric acid and sodium hydroxide, respectively. The effect of pH on the production of tannasewas studied by assaying the enzyme after every 24 hours ofincubation period in the culture medium. To observe the effect of temperature fungus was inoculated on tea extract and coffee extract agar media and incubated at varyingtemperatures *i.e.*, 20°C, 30°C and 37°C. The whole experiment was run in triplicates.

G. *Statistical analysis*

The mean was taken for all triplicates and standard deviation was calculated using microsoft excel. The values were compared using one way ANOVA test at 5% significant level by using Origin 50 software. Regression matrix was used to find out the most significant values.

III. RESULT AND DISCUSSION

In the present study, many fungal isolates were obtained on the tea and coffee extract containing media which are shown in figure 1. This figure is revealing that different fungi were obtained on different concentration. Colony and morphological characteristics reveals that these fungi are- *Curvularia* sp., *Aspergillus* sp., *Bipolaris* sp., and *Paecilomyces*sp. These were transferred on fresh media for obtaining them in pure form. The pure fungal cultures were

assessed for the tannase and caffeine degrading enzyme production. The strain*Aspergillus* sp. capable of producing both enzyme and able to grow on tea extract and caffeine extract agar was selected for the further studies.

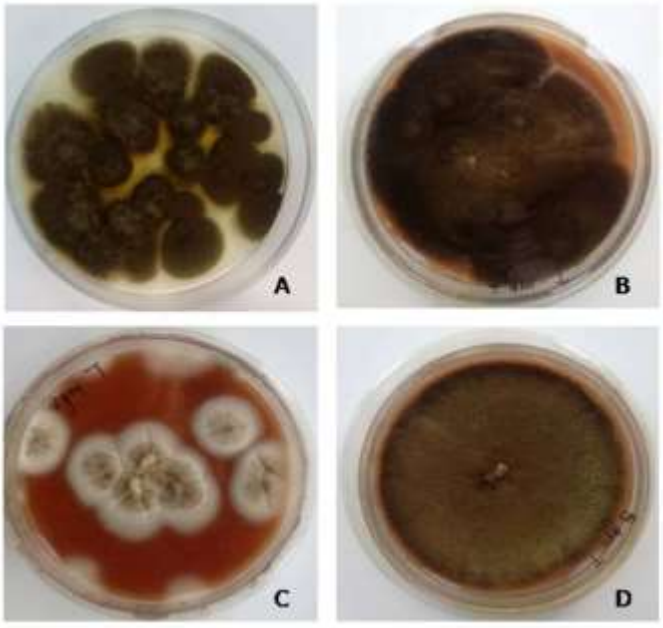

Fig. 1. fungal colonies appeared on tannin and caffeine containing media. Maximum discoloration of the medium can be observed in figure 1A compared to B, C and D.

In the next step, *Aspergillus* sp. was chosen for the further analysis. The growing ability of these fungi was assessed on different concentration of tea and coffee extract containing medium. In the previous studies, coffee pulp was used as sole source of carbon in the Tea extract was prepared by boiling the water with tea or coffee for 15-20 min. It is mentioned earlier that tannin and caffeine content in the extract is increased on increasing the boiling time [14]. According to Rehman et al. [14], 6 mins time is sufficient to extract caffeine or tannin content. Therefore, boiling for 20 min extracted all tannin and caffeine content from tea and coffee extract which gives high tannin and caffeine containing media for fungal isolation.The growth curve was drawn after taking readings at every 24 hrs interval.The growth curve reveals that *Aspergillus* sp. is able to grow at high concentration of tea and coffee extract. A significant increase in the size of the colonies each day was observed after incubation. This shows that growth of the fungi is not affected by the increased concentration of tannin and caffeine which is present in tea and coffee extract medium, respectively (Figure 2 and 3).

The growth of *Aspergillus* sp. was not found to be affected by the tannin and caffeine content of tea and coffee agar. Further, tannase and caffeine degrading enzymes production was assessed in different concentration of tea extract containing medium because this extract is rich in both tannin and caffeine [15-17]. After incubating the plates for 72 hrs at 20°C, 30°C and 37°C, relative enzyme activity was calculated and it was found that *Aspergillus* sp. showed best growth and enzymatic activity at 30°C at all the concentrations (figure 4). This reveals that enzymatic activities of *Aspergillus* sp. remain unaffected at all concentration of caffeine and tannin.

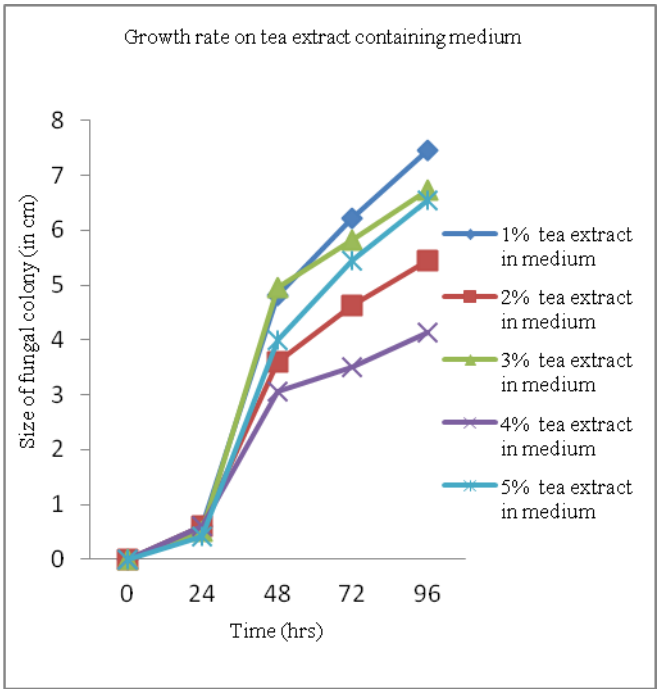

Fig. 2. Growth curve of fungus *Aspergillus* sp. on tea extract containing medium.

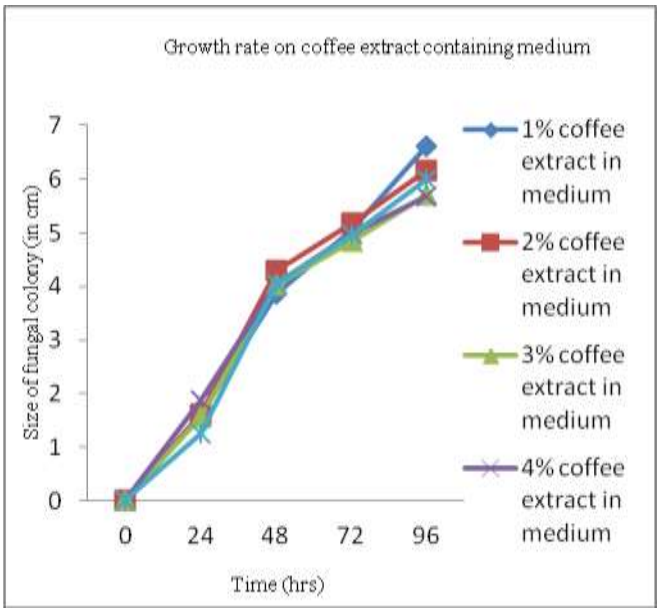

Fig. 3. Growth curve of fungus Aspergillus sp. on coffee extract containing medium.

The pH of tea extract agar was varied as 6, 7 and 8 and growth was observed regularly. After 72 hrs of incubation, relative enzyme activity was observed as shown in figure 5. This shows that pH 7 is good for the growth of fungi and its enzymatic activity. Different concentration of the tea extract in agar was not found to be affected by varying pH.*Aspergillus* sp. was found to grow at neutral pH with significant enzymatic activities. This was found in contrast with the previous reports in which tannase was found to be produced at acidic pH by *Aspergillus tamari* [18].

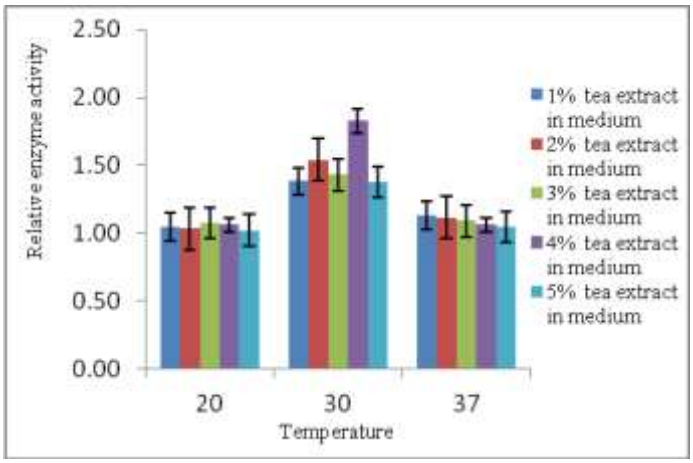

Fig. 4. Relative enzyme activity of fungus Aspergillus sp. in tea extract containing medium at different temperatures.

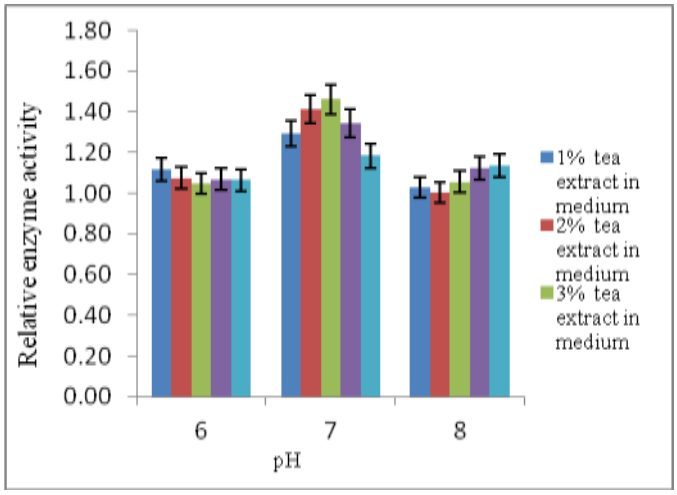

Fig. 5. Relative enzyme activity of fungus Aspergillus sp. in coffee extract containing medium at different pH.

Many strains have been isolated from various sources and found to degrade tannin. A tannin-degrading Bacillus sp. AB1 was isolated from a garden soil by enrichment. This organism was able to utilize 1% (w/v) tannic acid-a gallotannin at 30°C and pH below 4.5 in a defined mineral medium [11]. Another bacterial strain, *Klebsiella pneumoneae* was isolated and characterized for tannin degradation. It is reported that this strain is able to degrade 98% tannin in 72 hrs and pH of the medium turned acidic after degradation [19]. In another study, novel potential tannin degrading fungi *Penicillium* sp. CFR303 was isolated from coffee by-products. The potent tannin degrader can degrade 66.5% tannin was identified as *Penicillium verrucosum* (Bhoite and Murthy). Our results were found to be in agreement with the previous studies in which tannase production was reported to be optimum at 30°C. The isolated *Aspergillus* sp. was found to be better at neutral pH in our study. However, tannase degradation strains were found to be effective at acidic pH. It is also reported that pH changed to acidic range during tannin degradation. *Paecilomyces variotii* was isolated and found to produce optimally at 40 to 65ºC and pH 4.5 to 6.5 [21]. In previous reports, it is reported that temperature and pH optima were 60°C and 6.0, respectively for the production of tannase by *Aspergillus niger* GH1 strain when methyl 3,4,5-trihydroxybenzoate (MTB) was used as substrate [22]. In contrast to this, our isolate was found to produce it at 30°C and neutral pH.

The detailed study of this *Aspergillus* sp. isolate will be helpful in controlling the pollution caused by tannin and caffeine.

IV. CONCLUSION

In the present study, *Aspergillus* sp. was found to be effective in degrading the tannin and caffeine at neutral pH and 30°C temperature. The tannin and caffeine degradation potential of this isolate could be exploited in future for the production of tannase, improvement of livestock production and also detoxification of industrial effluents.

REFERENCES

[1] K. Murugan, A. Saleh, and Al-Sohaibani, "Biocompatible removal of tannin and associated color from tannery effluent using the biomass and tannin acyl hydrolase (e.c.3.1.1.20) enzymes of mango industry solid waste isolate Aspergillus candidus MTTC 9628," Research Journal of Microbiology, vol.5, pp.262-271, 2010.

[2] I.J. Buerge, T. Poiger, M.D. Muller, and H.R. Buser, "Caffeine, an anthropogenic marker for wastewater contamination of surface waters," Environ. Sci. Technol., vol. 37, pp. 691-700, 2003.

[3] V. Lakshmi and N.Das, "Removal of caffeine from industrial wastewater using Trichosporonasahii", J. Environ. Biol., vol.34(4), pp.701-708, Jul. 2013.

[4] T.K. Bhat, B. Singh, and O.P. Sharma, "Microbial degradation of tannins--a current perspective", Biodegradation, vol.9(5), pp.343-57, 1998.

[5] R.R. McCusker, B. Fuehrlein, B.A. Goldberger, M.S. Gold, and E.J. Cone ," Caffeine content of decaffeinated coffee," J. Anal. Toxicol., vol.30(8), pp.611-613, Oct 2006.

[6] J.A. Field, and Letting, " Toxicity of tannic compounds to microorganisms, Plants Polyphenols: Synthesis, Properties, Significance" Basic Life Sci., vol. 55, pp. 673-69, 1992.

[7] K.T. Chung, T.Y. Wong, C.I. Wei, Y.W. Huang, and Lin, " Tannins and human health, A review," Crit. Rev. Food Sci. Nutr., vol.38, pp.66, 1998.

[8] G.D. Joanisse, R.L. Bradley, C.M. Preston and A.B. Munson, " Soil enzyme inhibition by condensed litter tannins may drive ecosystem structure and processes' The case of Kalmia angustifolia" New Phytol., vol. 175, pp. 535-546, 2007.

[9] Q.A. Edwards, S.M. Kulikov, and L.D. Garner- O'Neale, "Caffeine in surface and wastewaters in Barbados, West Indies", doi: 10.1186/s40064-015-0809-x, Feb 2015.
[10] T.K. Bhat, B. Singh, and O.P. Sharma, "Microbial degradation of tannins--a current perspective", Biodegradation, vol.9(5), pp.343-57, 1998.
[11] M.O. Ilori, S.A. Adebusoye, O.O.Amund, and B.O. Oyetoran , "A study of tannic acid degradation by soil bacteria", NJ Environ Biol. Pak J Biol Sci., vol.10(18), pp.3224-7, 2007.
[12] K.Vijayaraghavan and T.K. Ramanujam,"Effect on chloride and condensable tannin in anaerobic degradation of tannery wastewaters," Bioprocess Eng., vol.20: pp.499-503, 1999.
[13] G.Lofrano, S. Meric, G.E. Zengin, and D.Orhon, "Chemical and biological treatment technologies for leather tannery chemicals and wastewaters,"Sci. Total Environ., doi:10.1016/j.scitotenv.2013.05.004, pp. 461-462, Sep 2013.
[14] S.Ur-Rehman, K. Almas, N. Shahzadi,N. Bhatti and A. Saleem, "Effect of Time and Temperature on Infusion of Tannins from Commercial Brands of Tea," International Journal Of Agriculture and Biology, pp.1560–8530, 2002.
[15] H. Savolainen ,"Tannin content of tea and coffee", J. Appl. Toxicol., vol.12(3), pp.191-2, 1992.
[16] S.L.Feng , J.M.Tang, and J. Fan, "Study on the kinetic fluorimetric determination of tannins in tea," Guang Pu Xue Yu Guang Pu Fen Xi.,vol.23(2), pp.322-4, Apr 2003.
[17] R.L.Chen, C.H.Lin, C.Y.Chung, and T.J.Cheng ,"Determination of tannin in green tea infusion by flow-injection analysis based on quenching the fluorescence of 3-aminophthalate", J. Agric. Food Chem., vol.53(22), pp. 8443-8446, Nov 2005.
[18] M.Chávez-González, L.V.Rodríguez-Durán, N. Balagurusamy , A.P.PradoBarragá, R. Rodríguezn, J.C.Contreras, and C.N. Aguilar ,"Biotechnological advances and challenges of tannase: an overview", Food Bioprocess. Technol., vol.5, pp. 445-459, 2012.
[19] A.Tahmourespour , N.Tabatabaee, H. Khalkhali, I. Amini,"Tannic acid degradation by Klebsiella strains isolated from goat feces", Iran J. Microbiol., vol.8(1), pp.14-20, Feb 2016.
[20] R.N. Bhoite and S. P. Murthy, "Biodegradation of coffee pulp tannin by Penicillium verrucosum for production of tannase, statistical optimization and its application", Food and Bioproducts Processing, vol. 94, pp. 727–735, Apr 2015.
[21] V.Battestin and G.A.Macedo, "Tannase production by Paecilomyces variotii". Bioresource Technology, vol. 98, pp. 1832-7. 2007.
[22] M.Mata-Gomez, L.V.Rodriguez, E.L. Ramos, J Renovato, M.A. Cruz-Hernandez, R. Rodriguez, J.Contreras, C.N. Aguilar, "A novel tannase from the xerophilic fungus Aspergillus niger GH1. J Microbiol Biotechnol, vol. 19, pp 987-96, Sep 2009.