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ISOLATION OF TANNASE PRODUCING FUNGI FROM AIR BY IMPREGNATION METHOD AND ITS APPLICATION AS STAIN REMOVAL AGENT

Mahima Gunjal[1] Hitesh Gurav[2]

Department of Microbiology , Chitisak Samuha's S.S & L.S Patkar College of Arts & Science & V.P Varde College of Commerce.

ABSTRACT:

Tannase is a biocatalyst for tannin biodegradation. It is extensively used in food, beverage, food additives as well as in environmental pollution treatment. In the present study, tannin degrading fungi was isolated from air and further assessed for the production of tannin degrading enzymes. It was found that tannases are produced efficiently by *Aspergillus* sp. At neutral pH and 30C temperature. Tannase hydrolyzes esters and lateral bonds of tannins such as tannic acid releasing glucose and gallic acid whose production is done by solid state fermentation (SSF). A treatment formulation for teeth whitening containing tannase enzymes like whitening agents, toothpastes, powders, teeth gel and mouthwashes was made. Tannase was added in laundry detergent for coffee and tea stain removal.

Keyword: tannase, *Aspergillus* sp., SSF, teeth whitening, detergent.

INTRODUCTION:

Tannin acyl hydrolase commonly referred as tannase is a hydrolytic enzyme that acts on tannins. Tannins are naturally occurring plant polyphenolic compounds, present in a variety of plants utilized as food and feed. Tannins widely occur in common foodstuffs such as banana, strawberry, raspberry, blackberry, grape, mango, cashew nut, hazelnut and walnut etc. Drinks like wine, tea, coffee also contain these phenolic compounds. Tannins are considered nutritionally undesirable because they form hydrogen bonds in solutions that result in formation of tannin-protein complexes. These indigestible complexes inhibit digestive enzymes and affect the utilization of vitamins and minerals.They are also responsible for a bitter taste, which considerably reduces animal's feed intake too. Therefore, it is not advisable to ingest large quantities of tannins since they may constitute a risk of adverse health effects. (Beniwal et al., 2014)

Tea and coffee producing industries release caffeine and tannin rich effluent. Caffeine and tannin present in water bodies adversely affects humans, aquatic animals, soil and groundwater. It is reported that tannins can suppress the soil enzyme activity thus controlling ecosystem structure and processes. Tannin and caffeine also cause light yellow to dark brown discoloration in water. Tannins impart color and turbidity to the effluent and make it unsuitable for use in recreational purposes. Hence, it needs to be treated properly before discharge.(Mishra et al.,2016)

Tannase is an enzyme that hydrolyzes esters and lateral bonds of tannins, such as tannic acid, releasing glucose and gallic acid. These enzymes are naturally produced by ruminant animals, plants, and microorganisms such as filamentous fungi belonging to the genera *Aspergillus* and *Penicillium*.The genus *Aspergillus* is considered as the best producer, followed by Penicillium, both standing out as great decomposers of tannins.

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.

Isolation of microorganisms from the environment is the first step in screening for natural products such as secondary metabolites and enzymes. Potential microorganisms are isolated from a tannins-rich environment such as soil. Microorganisms can also be isolated from wastes, water, feces, pickles and beverages. (Aharwar et al.,2018) There are a number of tannins–degrading microorganisms, which are potential source of tannase.

Microorganisms used for tannase production	
Bacteria	*Bacillus pumilus*
	Bacillus polymyxa
	Corynebacterium sp
	Klebsiella pneumoniae
	Streptococcus bovis
	Selenomonas ruminantium
Yeast	*Candida* sp.
	Saccharomyces cerevisiae
	Mycotorula japonica
Fungi	*Aspergillus niger*
	Aspergillus oryzae
	Aspergillus japonicus
	Aspergillus gallonyces
	Aspergillus awamori
	Penicillium chrysogenum
	Rhizopus oryzae
	Trichoderma viride
	Fusarium solani
	Mucor sp.

Table 1: Microorganism used for tannase production

Filamentous fungi have the ability to degrade tannins as a sole source of carbon. Tannase production in commercial scale is through submerged cultures using filamentous fungi. Among fungi *Ascochyta, Aspergillus, Chaetomium, Mucor, Myrothecium, Neurospora, Rhizopus, Trichothecium, Penicillium, Fusarium and Trichoderma* were the most studied. Tannase has been produced by liquid surface, submerged and solid state fermentation, though production of tannase has been extensively carried out in a submerged fermentation system. Submerged fermentation involves the growth of the microorganism as a suspension in a liquid medium in which various nutrients are either dissolved or suspended as particulate solids in many commercial media. Since sterilization and process control are easier to engineer in Submerged Fermentation, hence it is being used at industrial level for tannase production. Alternatively, to minimize production costs, there is the solid-state fermentation (SSF). SSF is defined as the cultivation of microorganisms on moist solid supports, either on inert carriers or on insoluble substrates that can, in addition, be used as carbon and energy source. The fermentation takes place in the absence or near absence of free water, thus being close to the natural environment to which

microorganisms are adapted. SSF is a process in which substrates in a solid particulate state are utilized. The aim of SSF is to bring the cultivated fungi or bacteria into tight contact with the insoluble substrate and thus to achieve the highest substrate concentrations for fermentation.(Beniwal et al., 2014)

Applications of tannase:

Processing of tea, drinks and animal feed Tea cream formation expands the turbidity which is an issue in tea processing. Tannase reduces the tannin level in black tea that improves the color, flavor, strength, brightness, and antioxidant property. Tannase is used as a clarifying and de-bittering agent in refreshing drinks, fruit juices, wine and beer. During wine manufacturing, processing is required to increase its quality by enzymes like tannase. Animal feed is also being treated with tannase for the quality improvement and has been used as additives in animal feeds for nutritional improvement. *Aspergillus sp., Corynebacterium sp.,* and *Klebsiella sp*. are the microorganisms which are commonly used for gallic acid production. Gallic acid has anticancer, antibacterial, antiviral, antioxidant, antiinflammatory effect and anti melanogenic activity. The effluent of agro-industries and tanneries has a high amount of polyphenolic compounds which is harmful to the environment; however, treatment by tannase can lead to saving the aquatic environment. Fungal tannases are being used for polyphenolic compound reduction and decolorization in oil mill wastewater.

Tannase is used for stain removal from tea or coffee-stained clothes and teeth.

The present investigation is aimed at isolation of tannase producers, extraction and purification of the enzyme followed by its application as a stain remover.

LITERATURE SURVEY:

Tannin acyl hydrolase (E.C. 3.1.1.20) commonly referred as tannase, is a hydrolytic enzyme that acts on tannins. Tannase catalyzes the hydrolysis of ester and peptide linkages in hydrolysable tannins like tannic acid. The products of hydrolysis are glucose and gallic acid. Gallic acid finds applications in photography and printing inks, production of an antimicrobial drug trimethoprim, in manufacturing gallic acid esters, e.g., propyl gallate which is used as an antioxidant. As antioxidant gallic acid acts as an antiapoptotic agent and helps to protect human cells against oxidative damage. Gallic acid is also found to show cytotoxic activity against cancer cells, without harming normal cells. Besides this, gallic acid possesses a wide range of biological activities, such as antibacterial, antiviral, analgesic etc. Besides gallic acid production, the enzyme is extensively used in the food, feed, pharmaceuticals, beverage, brewing and chemical industries. The major uses of tannase are in the production of gallic acid, pyrogallol, propyl gallate, in wine-making, beer-chill proofing, production of instant tea by solubilization of tea cream and in the manufacture of coffee-flavored soft drinks. Tannase also finds use as a sensitive analytical probe for determining structures of gallic acid esters, in the pretreatment of animal feed additives, in the leather industry and in cleaning up of highly polluting tannery effluent. Tannases have also been used for the cleavage of polyphenolics such as dehydrodimer crosslinks present in cell wall of plants which is essential for plant cell wall digestibility(Beniwal et al., 2014)

Tannin 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. Many efforts have been made to remove tannin and caffeine from water bodies. The most common method of tannin treatment is based on the use of anion exchange resin. The tannase enzyme can be used for the degradation of tannin. 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. (Mishra et al.,2016)

Screening of tannase producing microorganisms is commonly performed using an agar plate assay method in which tannic acid is used as a substrate. Microorganisms degrade tannic acid, which makes a hydrolysis zone in the screening medium that gives the indication of tannase presence. However, detection of gallic acid presence

using the methods like reverse phase HPLC, spectrophotometry and visual reading methods is also used for the screening especially for bacteria (Aharwar et al.,2018)

Tannase has been produced by liquid surface, submerged and solid state fermentation, though production of tannase has been extensively carried out in a submerged fermentation system. Depending on the strain and the culture conditions, the enzyme can be constitutive or inducible, showing different production patterns.

Liquid state fermentation (LSF) involves the growth of culture on the surface of a liquid medium at a shallow depth and held in a suitable container.

Submerged fermentation involves the growth of the microorganism as a suspension in a liquid medium in which various nutrients are either dissolved or suspended as particulate solids in many commercial media. Tannase production has been extensively studied under submerged fermentation.

Tannases have pH and temperature optima ranging 4.0-8.0 and 30-70°C respectively, whereas stability optima are 3.0-8.0 pH and 20-90°C respectively. Tannases have the molecular weight range of 31-310 kDa furthermore, they might consist of single or more subunits. Bacterial tannases have a low molecular weight range from 31-90 kDa whereas fungal tannases have a high molecular weight range of 45-310 kDa. (Aharwar et al., 2016)

MATERIALS & METHODS:

(1) PREPARATION OF TEA AND COFFEE EXTRACT

Tea and coffee extracts were used as a source of tannin for the preparation of mycological media. Coffee and tea were weighed 1 g, 2 g, 3 g, 4 g and 5 g in order to make different concentrations of tannin extract. After this, a weighted amount of tea and coffee is collected in different conical flasks and 50ml distilled water is added to it. These flasks were boiled for 20 min in order to extract all tannin 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.

(2) MEDIA PREPARATION

Potato dextrose agar containing the tea and coffee extracts were prepared. The medium was pour plated for further isolation of microorganisms.

(3) ISOLATION

PDA medium containing tea and coffee extracts plates were left uncovered so that aeromicroflora can deposit on it. When fungal colonies appeared on the petri dishes, these isolated colonies were transferred to the fresh media till the colonies were obtained in pure form. For cultivation of fungi, petri dishes were incubated at room temperature 35± 2°C. The culture was observed daily for fungal growth.

(4) IDENTIFICATION OF FUNGI

The isolated fungi were identified after growth on tea extract and coffee extract agar medium by observing their macroscopic (color, 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.

(5) PROTEIN ESTIMATION

The protein content preparation was measured according to the method of Lowry et al. using bovine serum albumin as the standard.

(6) 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 an inoculating needle and placed in the center of petri dishes and then incubated at 37°C for 96 hours. After incubation, plates were taken out and flooded with grams 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

(7) 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 the 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 tannase was studied by assaying the enzyme after every 24 hours of incubation 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 varying temperatures i.e., 20°C, 30°C and 37°C.

(8) PRODUCTION OF TANNASE BY SOLID STATE FERMENTATION (SSF)

Five grams of tea and coffee were placed separately in flasks of 250 mL Erlenmeyer flasks and sterilized at 121°C for 30 min in flowing steam. The substrates were moistened with 5 mL of sterile salt solution containing 0.5% w/v of NH4NO3, 0.1% w/v MgSO4.7H2O, and 0.1% w/v NaCl, pH 5.0. Each vial was inoculated with 1 mL of isolated fungal solution. The contents were mixed and incubated at 30°C for 96 h . After this period, to each bottle was added 50 mL of distilled water containing 0.01% Tween 80, previously sterilized. Then the vials were shaken in a rotary shaker at 150 rpm for 10 minutes. Then the contents were filtered using Whatman number 1 paper filter and the filtrate was regarded as crude enzyme extract and packaged in conical vials and preserved at 4°C for later analysis.

(9) DETERMINATION OF THE ACTIVITY OF TANNASE

The tannase activity was determined colorimetrically at 520 nm. This method is based on the formation of a chromogen from gallic acid (released by the esterase activity of tannase) and rhodanine (2-thio-4-keto thiazolidine). To determine the gallic acid, 100µL crude enzymatic extract was incubated with (0.3 mM) tannic acid (10 mM, pH 5.5), sodium phosphate buffer for 30 min at 30°C. Then, 300µL of the methanolic solution of rhodanine (0.667% w/v rhodanine in 100% methanol) and 100µL of 500 mM KOH were added to the mixture which was diluted with 860 mL distilled water and incubated for 10 min at 30°C. After this period, samples were read in a colorimeter absorbance of 520 nm. Standard curve was performed using gallic acid in different concentrations.

(10) PURIFICATION OF TANNASE

The crude enzyme was precipitated employing ammonium sulfate at the saturation level of 75% concentration. Precipitated protein was collected by centrifugation at 10,000 rpm for 15 min at 4 ºC. The precipitate was re-suspended in a 0.1M citrate phosphate buffer (pH-5).

(11) APPLICATION OF TANNASE IN REMOVING TANNIN STAINS OF TEA AND COFFEE

Four small square pieces of clean cotton cloth were treated with tea extract and cotton extract (two each). The first piece was washed with tap water while the other was immersed in extracted tannase crude enzyme solution and left under observation.

RESULTS:

(1) ISOLATION OF FUNGI ON TEA AND COFFEE MEDIUM

Fungal colonies appeared on the PDA medium containing tea and coffee extracts that were left uncovered so that aeromicroflora can deposit on it.

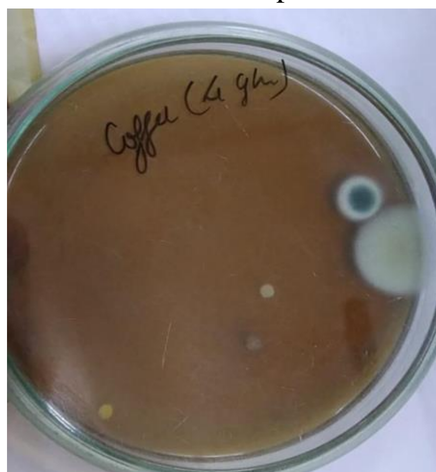

Fig 1: Isolation of fungi on PDA Medium containing coffee extract

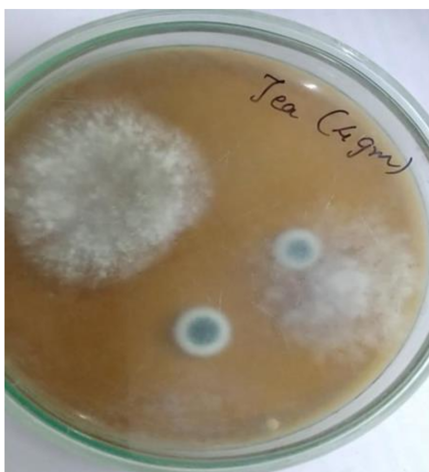

Fig 2: Isolation of fungi on PDA medium containing tea extract

(2) COLONY CHARACTERISTICS

ISOLATES OBTAINED	SIZE	SHAPE	COLOR	OPACITY	ELEVATION	CONSISTENCY
Coffee	20mm	circular	White with bluish black center	opaque	elevated	smooth
Tea	30mm	circular	White with bluish black center	opaque	elevated	smooth

Table 2: Colony characteristics of the isolated fungi

The fungi was identified to be of *Aspergillus sp.*

(3) RELATIVE ENZYME ACTIVITY

A small fragment of the fungal colony was picked up with the help of an inoculating needle and placed in the center of petri dishes and then incubated at 37°C for 96 hours. After incubation, plates were taken out and flooded with grams iodine instead of ferric chloride. Grams 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. The colony which was showing highest tannase producing ability on coffee and tea extract media was selected for further study.

Fig 3: 4% coffee extract PDA showed sharp distinct zone

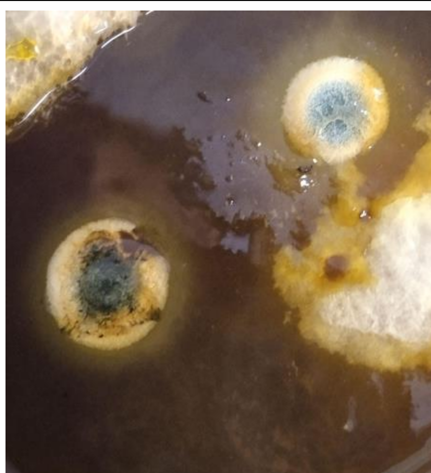
Fig 4: 4% tea extract PDA showed sharp distinct zone

Relative enzyme activity = zone of hydrolysis diameter
(1) 4% coffee extract PDA medium showed a zone of hydrolysis of 5mm.
(2) 4% tea extract PDA medium showed a zone of hydrolysis of 4mm.

(4) EFFECT OF TEMPERATURE AND pH ON TANNASE ACTIVITY
Tannase enzyme production was assessed in 4% tea and coffee containing medium because this medium is rich in tannin. 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.

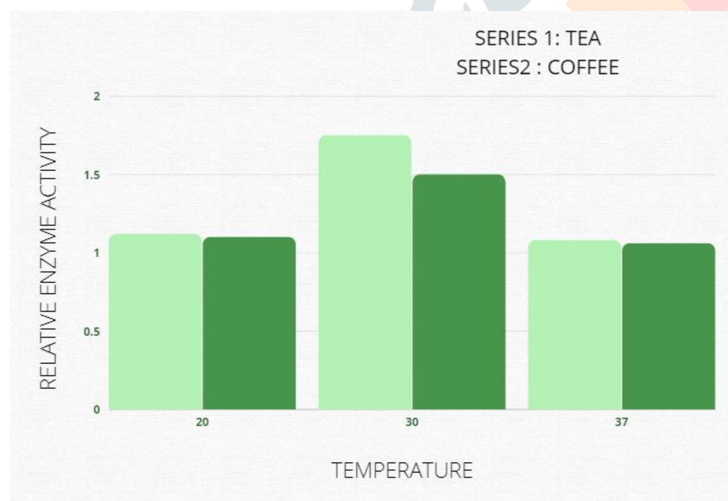

Fig 5: Relative enzyme activity of fungus *Aspergillus sp*. in tea and coffee extract containing medium at different temperatures

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. This shows that pH 7 is good for the growth of fungi and its enzymatic activity.

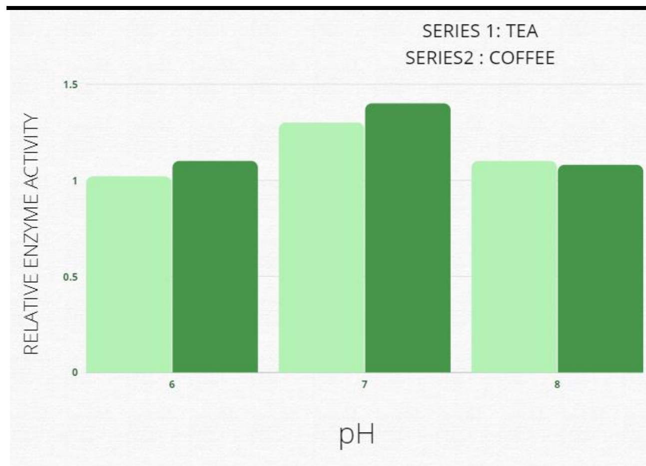

Fig 6: Relative enzyme activity of fungus Aspergillus sp. in tea and coffee extract containing medium at different pH.

(5)PRODUCTION OF TANNASE BY SOLID STATE FERMENTATION

The production of tannase was done by solid state fermentation. After 96 hours of incubation, fungal growth was observed in the fermentation flask.

Fig 7: Fungal growth in coffee fermentor

Fig 8: Fungal growth in tea fermentor

The final filtrate was regarded as the crude enzyme extract and packaged in conical vials and preserved at 4°C for later analysis.

Enzyme purification was achieved by ammonium sulfate precipitation and dialysis. Initially the crude enzyme was purified by precipitation with ammonium sulfate at the saturation level of 75%.

(6)DETERMINATION OF THE ACTIVITY OF TANNASE

The tannase activity was determined colorimetrically at 520 nm. Standard curve was performed using gallic acid in different concentrations. Tea and coffee tannase enzyme extracts were then plotted on the standard curve.

One unit of tannase activity (U) was defined as the amount of enzyme required to release one micromole of gallic acid per minute under the defined reaction conditions. Enzyme yield was expressed in U/mL.

CONCENTRATION OF GALLIC ACID (mg/ml)	O.D at 520 nm
Blank	0.00
10	0.10
20	0.16
30	0.22
40	0.30
50	0.42
Tea extract	0.20
Coffee extract	0.35

Table 3: Determination of tannase activity

29mg/ml of gallic acid was present in the tannase of tea extract, thereby the enzyme yield was 29U/ml.
34mg/ml of gallic acid was present in the tannase of tea extract , thereby the enzyme yield was 34U/ml.

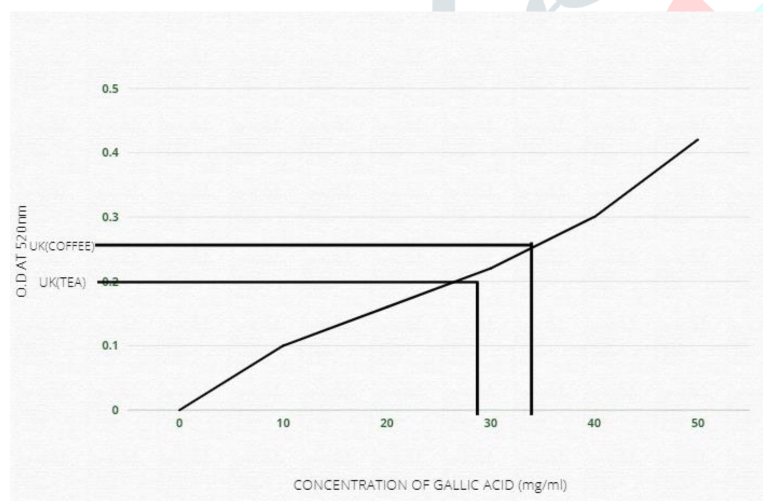

Fig 9: Determination of tannase activity

(7) APPLICATION OF TANNASE IN REMOVING TANNIN STAINS OF TEA AND COFFEE
Clean cotton cloth was treated with tea extract and cotton extract. The first piece was washed with tap water while the other was immersed in extracted tannase crude enzyme solution. It is obvious that tea and coffee stains removal was better in cloth pieces soaked in solution containing purified tannase when compared with control which was washed with tap water only. This is due to the activity of tannase on tannins. The results were also indicated that the purified *Aspergillus sp*. tannase can remove the tea and coffee stain and indicates that tannase from *Aspergillus sp* can be used in formulation of tannin stain remover or detergents.

CLOTH TREATED WITH TEA EXTRACT | CLOTH WASHED WITH TAP WATER | CLOTH SOAKED IN TANNASE EXTRACT

Fig 10: Application of tannase as tea stain remover

DISCUSSION:

In the present study, many fungal isolates were obtained on the tea and coffee extract containing media. These were transferred to fresh media for obtaining them in pure form. The pure fungal cultures were assessed for the tannase enzyme production. The strain Aspergillus sp. capable of producing tannase and able to grow on tea extract and caffeine extract agar was selected for the further studies.

In the next step, Aspergillus sp. was chosen for further analysis. The growing ability of these fungi was assessed on different concentrations of tea and coffee extract containing medium. Coffee and tea extract was prepared by boiling the water with tea and coffee for 15-20 min. It is reported that tannin and caffeine content in the extract is increased on increasing the boiling time. 6 mins time is sufficient to extract caffeine or tannin content. Therefore, boiling for 20 min extracted all tannin content from tea and coffee extract which gives high tannin containing media for fungal isolation.

The growth of *Aspergillus sp.* was not found to be affected by the tannin content of tea and coffee agar.

Relative enzyme activity was determined by the reaction of grams iodine only with non-hydrolyzed tannic acid and formed a dark brown complex. This gives a sharp distinct zone around the tannase producing fungal colonies. 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

(1) 4% coffee extract PDA medium showed a zone of hydrolysis of 5mm.

(2) 4% tea extract PDA medium showed a zone of hydrolysis of 4mm.

Further, tannase degrading enzyme production was assessed in different concentrations of tea extract containing medium because this extract is rich in both tannin and caffeine. 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. This reveals that enzymatic activities of Aspergillus sp. remain unaffected at all concentrations of and tannin.

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.

Filamentous fungi of the Aspergillus genus have been widely used for tannase production. Studies on tannase production by Aspergillus have been carried out on solid state cultures.Addition of carbon sources such as glucose, fructose, sucrose, maltose, arabinose to the culture medium at initial concentrations from 10 to 30 g/l improves tannase production by *Aspergillus niger.* Nitrogen requirements can be supplied by different organic and inorganic sources. Inorganic nitrogen can be supplemented as ammonium salts (sulfate, carbonate, chloride,

nitrate, monohydrated phosphate) or nitrate salts (sodium, potassium or ammonium). Other nutritional requirements such as potassium, magnesium, zinc, phosphate and sulfur are supplied as salts.
Enzyme purification was achieved by ammonium sulfate precipitation and dialysis. Tannases are enzymes used in different industrial applications as in food and pharmaceuticals. Many researches on tannase synthesis showed clearly that almost all species of Aspergillus are the predominant source of tannase for industrial production and
application.

CONCLUSION:

The present study showed the production of tannase from *Aspergillus* and confirmed its capability to produce an active tannase for degradation of tannin. Moreover, this enzyme can be used in different industrial and pharmaceutical uses. Good stability of pure tannase in a wide range of pH and temperature was obtained. *Aspergillus sp.* was found to be effective in degrading the tannin at neutral pH and 30°C temperature.These properties collectively point that the tannase enzyme obtained from *Aspergillus sp.* is a good candidate for removal of tannin stains of tea. Large scale production of tannase by using various recombinant engineering techniques will be tracked in the future study.

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