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TO IMPROVE THE QUALITY OF BLACK TEA BY TANNASE ENZYMATIC TREATMENT USING ENTEROBACTER CLOACAE

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ABSTRACT

The word tannin is very old and reflects a traditional technology. "Tanning" (waterproofing and preserving) was the word used to describe the process of transforming animal hides into leather by using plant extracts from different plant parts of different plant species. Plant parts containing tannins include bark, wood, fruit, fruit pods, leaves, roots, and plant galls. Tannins are phenolic compounds that precipitate proteins. Tannase hydrolyses tannic acid completely to gallic acid and glucose through 2, 3, 4, and 6, tetragalloyl glucose and two kinds of monogalloyl glucose. Tannase has catalytic activity to remove gallic acid moieties from tannins and the polyphenols from tea extracts which results in coldwater soluble products. The treatment of tea with tannase enhances the natural levels of epicatechin and gallic acid which in turn favours the formation of epicatechin flavic acid.

KEYWORDS: Black tea, Tanin, Tannase enzyme, Gallic acid, Enterobacter cloacae.

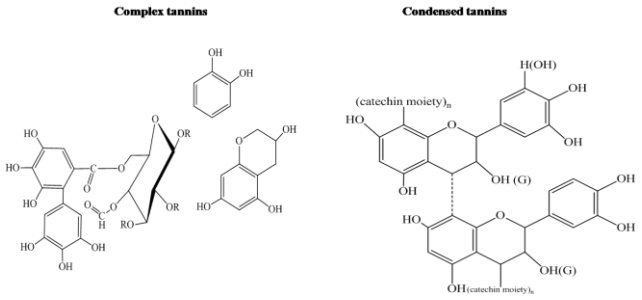
INTRODUCTION

Tannase was discovered accidentally during the extraction of gallic acid from soluble tannins (Lekha *et al*., 1993). Tannins are polyphenolic compounds of varying molecular weights and widely occur in the plant kingdom. Also, tannins are the fourth most abundant plant constituent after cellulose, hemicellulose and lignin. Generally, tannins are accumulated as secondary metabolites in the bark, leave and stem, but do not play any direct role in plant metabolism. However they have important role in plant immunity and protect them from microbial attacks (Aguilar *et al*., 2001). Although tannase is present in plants, animals, and microorganisms, it is produced in substantial amounts by the latter. Tannase is produced by bacteria, yeasts and fungi. Filamentous fungi of the *Aspergillus, Penicillium* genus and bacteria of the *Bacillus* and *Lactobacillus* genus have been investigated for tannase

production. Gallic acid, one of the structural constituents of some hydrolysable tannin, such as tannic acid, has been reported as an inducer of tannase synthesis under submerged fermentation, while it represses tannase synthesis under solid-state fermentation. It is observed that few of the microorganisms utilize tannins as substrates for growth and produce various secondary metabolites which are industrially important.

Tannase is used in the manufacture of instant tea, wine, beer, coffee, flavored soft drinks where it is used to eliminate water insoluble precipitates. Tannins are naturally occurring water soluble polyphenols with molecular weight ranging from 0.3-5 kDa. They are classified into three groups, hydrolysable tannins which consist of polyhydric alcohol esterifies with gallic acid, condensed tannins formed from monomeric flavan-3-ol and recalcitrant to hydrolysis and catechin gallates which occupy an intermediate position sharing the properties of hydrolysable and condensed tannins (Bhat *et al*., 1998). Tannins are in fact antimicrobial agents, and most of the microorganisms cannot tolerate its polyphenolic nature. Only a few microorganisms can degrade tannic acid and utilize it as nutrient. Microbial production of tannase, their production and industrial applications have been extensively studied (Aguilar *et al*., 2007; Lekha and Lonsane., 1997). In spite of these wide applications, studies on tannase production by bacteria are very obscure. The problem of pollution of waste water and soil from the tannery effluent is a serious environmental threat especially in the developing countries.

Industries consume large quantities of water and are therefore a source of considerable colour pollution. Colour is contributed by phenolic compounds such as tannins, lignins (2–3%) and organic colourants (3–4%) and with a maximum contribution from dye and dye intermediates, which could be sulphur/mordant/reactive/cationic/ dispersed/azo/acid/vat dye. In the present study, tannery soil was chosen as probable source for the isolation of tannin-degrading bacteria as they had a history of either discharges of tannin-containing effluents or disposal of tannin-rich woods resulting in leaching of tannin into the soil. Investigation on the production and molecular characterization of bacterial strains from the effluents will not only unravel potential tannase producers but also help in discovering novel enzymes that can meet the industrial demand. Therefore, we have analysis tannery effluent degradation tannase producing bacteria and then investigated for the physical and chemical conditions under which optimal amount of tannase are produced.



Gallic acid synthesis from tannic acid using *E. cloacae* tannase in relation to time

Tannin Acyl Hydrolase (E.C. 3.1.1.20) is commonly referred to as tannase which catalyzes the hydrolysis of ester bond and depside bond present in hydrolysable tannins to form glucose and gallic acid tannase catalyzes the breakdown of hydrolysable tannins such as tannic acid, methyl gallate, ethyl gallate, n-propylgallate, and isoamyl gallate. It is well known that tannase hydrolyses the ester bonds of tannic acid, although tannic acid is known to denature proteins. Tannins have been shown to be the natural substrate for the tannase enzyme which also attacks gallic acid methyl esters. But it possesses high specificity towards the acyl moiety of the substrate. The tanning process is almost wholly a wet process that generates very large amounts of wastewater. Certain streams are hyper saline, such as the pickling and the chromium tanning effluents or the soak liquor generated by the soaking of hides and skins that can contain as much as 80g/l of NaCl. Biological treatment process is usually most efficient for degrading pollutants occurring in wastewater. Refractory and toxic compounds limited their applicability. In such cases combinations of chemical oxidation process may improve the overall efficiency and efficacy.

Natarajan *et al*. (2009) treated tea leaves with tannase enzyme, either continuously or batch wise, for the production of instant tea powder. This offered many benefits for the resulting convenience beverage product with better acid stability, color, clarity, cold water solubility, flavor and higher yield. Many researchers focused on tannase production by *Penicillium atramentosum* KM under SSF and its applications in wine clarification and tea cream solubilization. A newly isolated tannase-yielding fungal strain identified as *Penicillium atramentosum* KM was used for tannase production under solid-state fermentation (SSF) using different agro residues like amla (*Phyllanthus emblica*), ber (*Zyzyphus mauritiana*), jamun (*Syzygium cumini*), jamoa (*Eugenia cuspidate*) and keekar (*Acacia nilotica*) leaves. Among these substrates, maximal Extracellular tannase production i.e. 170.75 U/gds and 165.56 U/gds was obtained with jamun and keekar leaves respectively at 28ºC after 96 h. A substrate to distilled water ratio of 1:2 (w/v) was found to be the best for tannase production.

Supplementation of sodium nitrate ($NaNO_3$) as nitrogen source had enhanced tannase production both in jamun and keekar leaves. Applications of the enzyme were studied in wine clarification and tea cream solubilization. It resulted in 38.05% reduction of tannic acid content in jamun wine, 43.59% reduction in case of grape wine and 74% reduction in the tea extract after 3 h at 35°C.

Hamdy and Fawzy (2012) emphasized economic production of tannase by *Aspergillus niger* Van Tiegh adopting different fermentation protocols and possible applications. They demonstrated the ability of *Aspergillus niger* to utilize *Ficus nitida* leaves and crude tannic acid extract to produce tannase. Applicability of the research findings was illustrated through suggesting the possibility of using the fermentation residue of *Fargesia nitida* obtained from SSF in animal feed. The haze formation in tea is due to coacervation of tea flavanoids, consisting mainly of epicatechin, epicatechin gallate, epigallocatechin and epigallocatechin gallate. Tea polyphenols form hydrogen bonds with caffeine which leads to cream formation. Consumers would prefer clear products, so the compounds forming haze should be removed to get a product free of turbidity and chemicals used as clarifiers. Tannase has catalytic activity to remove gallic acid moieties from tannins and the polyphenols from tea extracts which results in coldwater soluble products. The treatment of tea with tannase enhances the natural levels of epicatechin and gallic acid which in turn favours the formation of epicatechin flavic acid which is responsible for bright reddish colour of tea with good cold water solubility and colour.

MATERIAL AND METHODS

Enzymatic treatment of black tea to improve the quality of tea

5.4. Preparation of tea infusion

For this, CTC tea and Kangra orthodox black tea (5.0 g each) were mixed with 100.0 ml of boiling water (reverse osmosis) in 250 ml beaker separately. Both the samples were incubated at 85°C for 20 min in a water bath. The tea infusions obtained in each case were filtered through Whatman filter paper No. 1 and the filtrate was analyzed to determine contents of the individual catechins. In all further experimental studies, both tea infusions will act as control, i.e., without enzyme. The pH of the tea infusion was 5.0 ± 0.2.

Enzymatic biotransformation

Evaluation of tea cream formation

Evaluation of antioxidant activity

Treatment of tannery effluent

Experimental Setup

The experiments were carried out on a lab scale coagulation and flocculation unit with a pretreatment filtration step. They showed schematic of the process and equipment that was employed in this work (Fig.2).

Fig. 2: Raw tannery effluent collection station I, Permit tannery effluent collection station II.

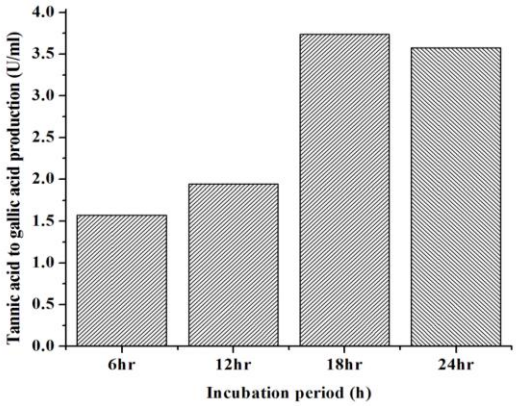
5.10. Collected Tannery effluent

The study area is Ranipet (Ranipettai) suburb town is an major industrial area located in Wallajah taluk, Vellore district, Tamil Nadu at 79°19′– 79°22′ E longitude and 12°53′–12°57′ N latitude and is 114 Km west of Chennai (Fig.2). It is situated at a distance of 3.5 km from river Palar and adjoining Chennai- Bangalore-Chittoor Highway (NH-4). It is one of the biggest exporting centers of tanned leather in India and discharging their effluents on the open land and surrounding water bodies. The effluent samples were collected from the all stages of tanning processing viz., soaking, liming, deliming, pickling, chrome tanning and retanning. The effluent was collected in polythene containers of 2 to 10liters capacity and were brought to the laboratory with due care and was stored at 20°C for further analysis. Chemicals used for the analysis of spent liquor were analytical grade reagents. The physical and chemical characteristics of tannery effluents parameters viz. pH, total alkalinity, total acidity, COD, BOD, total solids (TS), total dissolved solids (TDS), total suspended solids (TSS), chlorides and sulfides were analyses as per standard procedures.

RESULTS

The enzyme tannase (tannin acyl-hydrolase E.C 3.1.1.20) catalyses both hydrolytic and synthetic reactions under different conditions. The enzyme carries out hydrolytic reactions for the production of gallic acid from tannic acid/natural tannins and under conditions of low water activity and synthetic reactions such as synthesis of methyl, propyl, butyl, amyl and ethyl gallate. This enzyme has shown potential applications in pharmaceuticals, food processing, animal feed improvement, in the production of instant tea, as clarifier in beer & wine industry and in the degradation of effluents containing tannins. Tannase have also been exploited in the detergent formulations as stain remover for tannins.

Gallic acid acts as an antioxidant and helps to protect human cells against oxidative damage. It was found to show cytotoxicity against cancer cells, without harming healthy cells. Gallic acid is used as a remote astringent in cases of internal haemorrhage. It is also used to treat albuminuria and diabetes. It can also be used as a starting material in the synthesis of the psychedelic alkaloid mescaline. Conventionally, gallic acid is produced by acid hydrolysis of tannins, but this process releases a large amount of toxic effluent that causes environmental hazards.



Gallic acid synthesis from tannic acid using *E. cloacae* tannase in relation to time

Enzymatic treatment of black tea (CTC and Kangra orthodox) using tannase to improve the quality of tea

Tea (*Camellia sinensis*) is the second most widely consumed non-alcoholic beverage and is rich in polyphenolic compounds, known as tea flavonoids. Black tea contains several polyphenols, including epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG) and epicatechin (EC). These flavonoids or polyphenolic compounds (also known as catechins) possess strong antioxidant properties. The tannase mediated

biotransformation of tea infusion (CTC and Kangra orthodox) improves the quality of tea in relation to reduction in tea cream formation. The reduction will help to produce a turbidity free, cold water soluble instant tea or tea extract. Furthermore, on tannase treatment, an increase in an antioxidant activity was also observed due to certain flavonoids present in tea that show potential health benefits against cardiovascular diseases and cancer. There was no significant change in the volatile compounds that thereby resulted in an improved color, brightness, strength and flavor of tea.

Enzymatic biotransformation

The filtrate obtained from tea infusion of black tea, i.e., CTC tea and Kangra orthodox tea samples (as mention in Materials and Methods), was used as substrates (100 ml each) and each sample was incubated with 100 mg of tannase at 30°C for 1 h. The hydrolysis process was stopped by placing the reaction in an ice bath for 15 min. Now, this enzyme treated tea infusion (reaction) and untreated tea infusion (control) was used for the evaluation of tea cream formation, estimation of catechins/flavonoids, evaluation of antioxidant activity, determination of volatile compounds and evaluation of quality of tea.

DISCUSSION

Tanning industry is one of the most polluting industries and tanning is the art of converting animal skins into lesthure. It is considered that tanning industry in India is one of the oldest and stands among the five top export oriented industry (Dhaneshwar, 1990). In tanning process, vegetable and chrome tanning are the two major process involved. Estimated water consumption is 78 M3/tone of hide processed. So the tannery effluent, which is discharged by various processes from soaking to finishing, contains pollutants and chemicals. Tannase, a key enzyme in the degradation of hydrolysable tannins, is present in a diverse group of microorganisms (Bhat *et al*., 1998). Biodegradation using white rot fungi *Phanerochaete Chiysosporium* is reported as an effective treatment solution for wastewaters contaminated with phenolic compounds (Manimekalai and Swaminathan, 2000). Chromium is the major hazardous component of tannery effluents. Tannery effluents, when released either on land or into water bodies, are known to affect denizens of ecosystem. Chromium is highly toxic to fish and other aquatic life and interferes with the natural purification system (Manivasakam, 1987). These compounds have a range of effects on various organisms from toxic effects on animals to growth inhibition of microorganisms. Some microbes are however, resistant to tannins, and have developed various mechanisms and pathways for tannin degradation in

their natural milieu. The microbial degradation of condensed tannin is, however, less than hydrolysable tannins area both aerobic and anaerobic environment. A number of microbes have also been isolated from gastrointestinal tract of animals, which have the ability to break tannin - protein complexes and degrade tannins, especially hydrolysable tannins.

The tannery effluent when discharged into stream causes deoxygenating effect. The wastewater contains high amount of organic content and high value of BOD, COD, TSS, TDS and other important parameters. In the present study, raw tannery effluent from vegetable tanning industry was collected and their physicochemical characters are tabulated. The physico-chemical values of tannery effluent clearly indicate its toxic nature (Dhaneshwar, 1990; Viswaranjan & Somnath, 2002). Since the effluent showed higher BOD, COD, TDS, TSS and tannin values, an attempt has been made for the biodegradation of these effluents by different fungal isolates. Viswaranjan & Somanath (2002) studied the toxicity of tannery effluents on some aquatic animals and the characters of the tannery effluent reported by Sujatha and Gupta (1996) clearly showed the toxic nature of the effluent. The treatment of tannery wastewater by different physical and chemical methods that lead to lot of environmental problems. Hence, in the present study an attempt has been made to reduce the pollution load of the tannery effluent by biodegradation method using fungal forms. Tannins are defined as naturally occurring water-soluble polyphenols of varying molecular weight. Many works has been made on the biodegradation of various industrial effluents by microorganisms. In the present an attempt has been made to isolate the fungal forms from tannery effluent discharged soil using standard method. The tannase isolates such as *Aspergillus, Fusarium, Penicillium* and *Trichoderma* were, identified and pure cultured for the improvement of black tea.

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