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

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Abstract: Tannins, the important recalcitrant natural polymers released along with tannery effluents limit their treatment and reuse. The currently employed treatment methods failed to degrade them completely. An attempt has been made specifically to treat tannin and remove associated color from the tannery effluent. The biomass of mango industry solid waste isolate Aspergillus candidus MTTC 9628 and the tannase enzyme from this organisms were found to degrade and thereby reduce the tannin content of the tannery effluent resulting in the decolourisation of the effluent. The results obtained after treatment with the immobilized tannase enzyme also showed considerable reduction in effluent tannin content and hence the color of the effluent. However, it is observed that the whole organisms treatment have a fundamental advantage over enzymatic treatment as they transform a broad range of compounds resulting decrease in Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD) and other physicochemical characteristic values. This present works reveals that these undegradeable tannins can be specifically removed by enzymatic treatment. Development of suitable treatment facility employing tannase enzymes could be adapted at laboratory and industrial level treatment thereby making it suitable for further use.

Keywords: Biological treatment, decolourisation enzymatic treatment, fungal degradation, tannase, waste water

INTRODUCTION

Awareness on environmental issues among the world community especially in developing countries has reached a level, which it has not seen so far. Extensive research and development works conducted in recent years enables the industrial, municipal, agricultural and commercial facilities to reduce their impact on environment. In particular, the implementation of stringent standard for the discharge of wastes into the environment had necessitated the need for the development of alternative processes for the production of
goods and for the treatment and disposal of wastes (Nicoll, 2001). The leather industry is one of the oldest and fast progressive industries worldwide. Skins and hides are tanned to achieve desired flexibility in the dried condition and to protect it from microbial attack or hydrolysis when moist (Muruganathan et al., 2005). The environment is always under constant and continued pressure from solid and liquid wastes emanating from the tanning industry. The tannery effluent wastes are ranked as high pollutants among all other industrial waste (Eye and Lawrance, 1971). The wastewater from vegetable tanning process imparts color and consists of non-biodegradable matter like tannin which persists for long (Dhaneswar, 1990). Tannins are widespread in the plant kingdom and found in the leaves, fruits, vegetables, bark and wood. Tannins are the fourth most abundant plant constituents after cellulose, hemicelluloses and lignin and composed of a very diverse group of oligomers and polymers. There is enormous potential for tannin by-products as they represent an important source of sugars, minerals, organic acid, dietary fibre and phenolics which have a wide range of action which includes antitumoral, antiviral, antibacterial, cardioprotective, immunosuppressant and antimutagenic activities if they are converted through a dependent manner. Industries using plant materials as raw and processing materials ultimately generate large amounts of waste materials containing tannin. Being recalcitrant in nature, disposal of these tannin containing materials have become a major environmental problem and calls forth appropriate technologies for efficient degradation and removal of them. Bio processing of these tannin rich residues employing suitable enzymes and efficient microbial whole cell systems may allow bio prospecting of them towards complete utilisation of them with their associated health benefits. Tannins are important constituents of not only vegetable tannery effluent but also of other organic industrial wastewaters such as olive oil mill effluent; wine vinasse; coffee pulp water; debarking wastewaters and composite wastewaters. Tannins used as antioxidants, stabilizers and as depressants in mineral processing operations, also find their way into the environment. Purification of tannery wastewaters containing tannins, sulfonates, ethoxylates, fatty acids, dyes, proteins and soluble carbohydrates has long been a problem owing to their persistence and toxicity. The tannery wastewater contains vegetable tannins, high amounts of protein, chlorides, trivalent chromium, nitrogen, phosphorous, sulphates and sulphides. The presence of color, oil and turbidity in them does not allow their intended use for domestic, industrial, recreational and any other purposes after treatment. Previously conducted study on the treated wastewater emanating from a common effluent treatment plant catering services to 152 tanneries in Chennai, India revealed the presence of residual organics, visible color due to undegraded tannins and dyestuffs (Sankar et al., 1997). Tannins are defined as polyphenols with molecular weight ranging from 500 to 20,000. Depending on the structure and action towards hydrolyte agents, tannins are classified into hydrolysable and condensed tannins. Hydrolysable tannins are characterized by sharp distribution of molecular weight around 2000. Condensed tannins are characterized by a wide molecular weight distribution, a flavonoid structure with mainly phenolic reactive groups (Muruganathan et al., 2005). Since most of the steps of the tannery manufacturing processes are carried out in aqueous environments, the presences of these compounds in wastewater are important (Lopez-Fiuza et al., 2003). Extensive exposure of skin to hydroxyl phenols may cause discoloration, local irritation, eczema or even death due to absorption. Hence it is important to remove this tannin compounds from wastewater (Muruganathan et al., 2005). Tannins being antimicrobial in nature inhibit the growth of microorganisms; resist microbial attack and remains as recalcitrant in the environment (Field and Lettinga, 1992a). Though destructive in nature, the biological systems often have difficulty in removing toxic pollutants to consistently low levels. Vegetable tannery
wastewater contain high concentration of recalcitrant organics and other chemicals that inhibit the activity of microorganisms during biological oxidation, so these biorefractory organics that are not removed by biological treatment requires tertiary or advanced waste water treatment for their removal (Panizza and Cerisola, 2004). Therefore, current conventional biological processes may not be able to improve water quality sufficiently enough to meet wastewater discharge criteria and reuse. Moreover, the conventional physico-chemical treatment methods such as coagulation or activated carbon adsorptions are insufficient for decolourisation and from the ecological point of view, these treatment techniques result in a phase transfer of pollutants and thus destructive treatment methods for the remediation of recalcitrant, toxic and or hazardous pollutants are currently under investigation. In an attempt to overcome some of the problems associated with chemical and biological treatment processes, recent research is focused on the solving environmental problems through the applications of pure enzymes that have been isolated from their processing organisms. Despite the antimicrobial properties of tannins, many microorganisms can grow and develop on tannin rich materials. They not only evolved tannin resistant but also utilizing mechanisms. A number of reviews on tannin biodegradation have appeared in the past, providing a general idea on the biodegradation of these polyphenols (William et al., 1986; Elhat et al., 1998). It is reported that the hydrolysis of tannins could be brought about with the help of tannase enzyme (Aguilar and Gutierrez-Sanchez, 2001). These enzymes catalyses the hydrolysis of bonds presents in the molecule of the hydrolysable tannins and gallic acid esters (Lekha and Lonsane, 1997). It plays an important role in the treatment of tannery effluents (Ipubu et al., 1968). A primary objective of any treatment system should be to minimize cost in order to make them economically viable one. Immobilization of enzymes has solved the limitations of enzyme use like their high cost, solubility in aqueous media and economical reuse (Souza, 2002). Since, the rate of introduction of recalcitrant tannin from tannery effluent into the environment is on the rise and it is becoming increasingly difficult to achieve an acceptable degree of removal of these pollutants using conventional chemical and biological processes, an attempt made by us, the biocompatible removal of tannins from the tannery effluent using tannase enzyme has been described in this study. The application of tannase enzyme on tannery effluent was carried out in two ways, by direct contact of enzymatic extract with the tannery effluent and by growing tannase producing fungal strains on tannin rich effluents.

MATERIALS AND METHODS

This one and half year study was conducted between June 2008 and November 2009.

Organism, Enzyme Production and Assay

Aspergillus candidus MTCC 9628, a known tannin acyl hydrolase (B.C.3.1.1.20) enzyme producing strain isolated from mango industry solid waste and deposited in microbial type culture collection centre (MTTC), India by Murugan and Gayathri (2008) was used in this study. The organism was maintained on Tannic Acid Agar (TAA) medium and subcultured regularly every two week. The enzyme tannase was produced by submerged fermentation using 3.5 L auto controlled bioreactor (Applikon B.V, A.C.Schiedem, Amsterdam, The Netherlands). The fermentation culture conditions were maintained as described elsewhere (Poursat et al., 1982).
Microbial Treatment of Tannery Effluent

The whole organism and enzymatic treatment of tannery effluent was carried out empirically as described by Abadulla et al. (2000). The mycelia were collected from the bioreactor by filtration under aseptic condition and washed three times with sterile distilled water. Samples of mycelia (2 g) were inoculated into the raw effluent and incubated for 5 days under aerated conditions. Raw effluent without mycelium serving as control was also maintained under the same conditions. The change in physico-chemical characters of the effluent by the metabolic activity of the fungus was confirmed by the dual experiments. Two sets of experimental setup were maintained one with the mycelia and the raw effluent and the other with the same along with antibiotics (10% v/v benzylate 100 mg L⁻¹, cyclohexamide 1000 mg L⁻¹, Streptomycin 300 mg L⁻¹), which inhibit the metabolism of fungi. Broth supernatant after centrifugation (5000 g x 10 min) was used as a source of enzyme. The enzyme was then concentrated with solid ammonium sulfate precipitation (80% saturation) and dialyzed in 0.2 M acetate buffer (pH 5.0) for 2 days. This partially purified enzyme was used for enzymatic treatment. The immobilization of the enzyme (10 mL mycelial extract) was carried out by entrapment in sodium alginate (Kierstan and Bucke, 1977). These alginate beads were added to the 250 mL of effluent taken in a 500 mL conical flask. Controls were prepared by heat inactivating the enzyme at 80°C. The flasks were incubated on a rotary shaker at 30°C C for 5 days. The wastewater samples in each experimental setup as well as controls were analyzed before and after the treatment period as per standard methods and the changes in physicochemical characters were determined. The BOD₅ was determined following Winkler’s method with azide modification (APHA/AWWA/WEF, 1998).

Analytical Methods

A colorimetric assay method based on the changes in the optical density of the specific substrate tannic acid was used for tannase assay (Mondal et al., 2001). The color of the effluent was determined with Spectronic 20 (Milton Roy Company, USA). The raw and treated effluents were centrifuged for 15 min at 5000 rpm and pH of the supernatant was adjusted to 7.6 using phosphate buffer (0.01 M) and absorbance read at 465 nm was compared to previously calibrated platinum-cobalt color standard. The physico-chemical characters and the tannin content of the effluent were determined according to standard methods (APHA/AWWA/WEF, 1998). The principle involved in the estimation of tannin is the development of a blue color on reduction of Folin-Phenol reagent by the aromatic hydroxyl groups present in tannins.

Statistical Analysis

All experiments were conducted in triplicate and values reported are the mean of the three replicates ± standard deviation. The statistical significance was done by t test using the software SPSS, Illinois, Chicago, USA.

RESULTS

The inevitable by-products of the leather manufacturing process released through effluent cause significant pollution unless treated in some way prior to their discharge. Wastewater samples from tannery characterized in terms of pH (11.7), Total Dissolved Solids (TDS) (6814), Total Soluble Solids (TSS) (3822), BOD(344), COD(3595), chloride (4699), tannin content (5655) and color (4159). The obtained values indicated that they are above than dischargeable limit. Hence, it is important to give destructive treatment to treat and
reduce the important parameters especially the tannins. The experimental tannin-degrading organism is found to produce (2.48 U mL⁻¹) extracellular and (0.83 U mL⁻¹) intracellular tannase enzyme. The results obtained after the treatment by mango industry solid waste isolate *Aspergillus candidus* MTTC 9628 and its tannase are statistically analyzed and are shown in Table 1. It was found that, there is a considerable reduction in the values of important parameters. The tannery effluent had color equivalent to 4250 U. The treatment of the effluent with the organism reduces the color to 2840 U. The pH value of the raw effluent was reduced from 11.4 to 8.7, TDS from 6814 to 5226 mg mL⁻¹, TSS from 3822 to 3558 mg mL⁻¹, BOD from 344 to 318 mg mL⁻¹ and the important pollutant tannin from 5655 to 3278 mg mL⁻¹. Significant reduction in the values of TDS, TSS, COD, chloride and tannin by the enzyme-produced organism was observed. However, at the same time, the direct enzyme treatment also found to have a significant impact on color, TDS, TSS and tannin content.

**DISCUSSION**

In nature, fungi do much of the dirty work. They are particularly efficient at degrading the major plant polymers, cellulose, lignin but they can also decompose a huge array of other organic molecules too. The filamentous fungi were recognized as good biodegraders because of their mycelial growth habit and battery of extracellular degradative enzyme production (Bennet *et al.*, 2002). Numbers of potential applications of fungi and their enzymes in waste water treatment have been identified which improves the quality of treated wastewater. Knudson (1913) was the first who reported the role of *Aspergillus niger* tannase enzyme in the degradation of tannic acid. Horse-radish peroxidase is one of the most studied enzymes in the treatment of aromatic wastes (Nicell *et al.*, 1993; Klibanov *et al.*, 1980). The ligninase enzyme produced by the fungus *Phanerochaete chrysosporium* has proved as a good potential for the degradation of environmentally persistent aromatics (Aitken, 1993). Ferrer *et al.* (1991) reported the use of horse-radish peroxidase and ligninase for the color removal of craft mill effluents. Both enzymes are found to be more efficient in the immobilized form than in the free form. Wastewater samples from tannery were characterized (Table 1) reveals their high pollutant load which is in agreement with those reported by (Prabhavathy and Sirshendu, 2010). These wastewaters when discharged into watercourse
will affect physical, chemical and biological characteristics of water and will deplete the dissolved oxygen, which in turn affects the aquatic organism, both plants and animals. The impact of excessive tannin levels commonly found in the vegetable tanning industry on environment would be severe. As the tannins enter the soil organic matter pool, they may affect several aspects of ecosystem functioning (Lorenz and Preston, 2002). They also reduce the rate of decomposition of soil organic matter by inhibiting the biodegradative enzymes of the attacking organism (Scalbert, 1991). These tannins found to inhibit the microbial activity and nitrogen availability in the soil possibly because of their protein binding properties (Kuiters, 1990; Schimel et al., 1996). Tannins released from plant litters are known to suppress the soil enzyme activity thus controlling ecosystem structure and processes (Joannis et al., 2007). Feasibility study on anaerobic degradation of three natural tannin extracts in Upflow Anaerobic Sludge Blanket (UASB) reactors using glucose as cosubstrate revealed that the tannin removal efficiency is low when reactor units were operated at tannin extract concentrations higher than 800 mg L$^{-1}$ (Lopez-Fiuza et al., 2003). Vijayaraghavan and Ramanujam (1999) also made similar observation when they operated an anaerobic filter with increasing concentrations of condensed tannin wastewaters, especially when low hydraulic retention times and high tannin concentrations were applied. Field et al. (1991) reported higher COD removal efficiencies from debarking wastewaters when the tannin fraction was eliminated (extracted with polyvinyl pyrrolidone) from reactor influent. All of these observations suggest the tannins typical recalcitrant behavior, slow biodegradable nature, their influence on the degradation of other substrates and significant persistence in the medium which is corroborated and evidenced by the results obtained by us. Hence, it is important to give destructive treatment to these tannins. Though the tannase enzyme is a ubiquitous enzyme of the microbial world, fungal tannases are quite versatile and efficient in degrading all types of hydrolysable tannins (Lewis and Starkey, 1969). The successful applications of anaerobic biological treatment for the removal of simple tannins have been reported previously (Field and Lettinga, 1992a). Since, tannins are the high molecular weight compounds, it necessitates its uptake into the cell limits their degradation by bacteria. In contrast, the fungal transformation/degradations are mediated mostly by exo-enzymes and therefore, the rate limiting membrane permeation of the substrate can be bypassed. In general, the treatment given by the organism is significant. It was also noticed that the treatment efficiency of the metabolically inactive organism is not a significant one. Tannin degradation in the leaf litter of forest soil was found to increase earthworm population (Satchell and Lowe, 1967). Therefore, it may also widen the possibility of treating tannery effluent sludges using vermicompost. The action of tannase on plant tannins is extremely important in the recycling of plant biomass since they inhibit the growth of a number of microorganisms, resist microbial attack and are recalcitrant to biodegradation (Field and Lettinga, 1992b). In recent years, electrochemical techniques have been applied both in the purification of drinking water and in the treatment of various industrial effluents. Study conducted by Muruganathan et al. (2005) employing electrofloatation and electro-oxidation techniques indicated the possibility of successful removal of commercial tannins from tannery waste water but these methods results in sludge accumulation and high power consumption where as the results obtained by this experiment reveals the destructive conversion of the tannin. Certain wastewater components may have negative effects on the enzymatic reaction. Comparatively it was found that this enzyme significantly acts on particular component tannin present in the effluent and reduced its content considerably. The results obtained is explained by specificity of the enzyme for their substrates which enables them to selectively remove target compounds from complex mixtures while using
chemical reagents with a high stoichiometric efficiency (Aitken, 1993). Earlier the tannase activity was known to have a number of commercial applications, including the preparation of instant tea, coffee flavoured soft drinks, beer, wine (Lekha and Lounsane, 1997) and also as an additive for detannification of feed to improve its quality (Murugan et al., 2005). The removal of tannins found in the coffee pulp by solid-state culture of tannase producing fungi make it suitable for animal feed (Aguilar et al., 2001). This isolate A. candidus MTCC 9628 is also found to produce high titer of both extracellular and intracellular enzyme during SSF culture using agricultural residue (Murugan and Gayathri, 2008) suggesting similar application possibilities. Comparatively, it was found that treatment with the enzyme-producing organism on tannery effluent was more significant than the treatment achieved by the enzymes directly. Statistically, it is noticed from the observed values that the efficiency of the enzyme-produced organism in the treatment of tannery effluent is more significant than the remaining. However, at the same time, the impact of tannase enzymes directly on tannins present in the raw effluent is highly significant when compared to the treatment with the whole organism. Despite the wealth of information available on the application of organisms, in particular, fungi to decolorize wastewater, the implementation of such remediation technologies remain a challenge. The reduction in color after addition of extract without mycelium indicated that the basis for color reduction in the effluent was tannin degradation rather than adsorption of tannin to the fungal mycelium. However demonstration of the biodegradation of tannin molecule requires analysis of the breakdown products. Similarly, when the metabolic activities of the organisms are inhibited; it was found that there is not much difference in the physico-chemical parameters of the raw effluent including tannin. The enzymatic treatment removes the color nearly to 55%. Since, the reaction products of the tannase enzyme, the glucose and gallic acid are non-toxic, more biodegradable one than the parent compound tannin, become more amenable to subsequent treatment. This is indicated by the increase in the BOD of the enzymatically treated effluent. Studies on the biodegradability of tannins can be extrapolated because of the diversity in the tannin structure, chemistry and availability in the industrial usage. The use of tannase enzyme rather than the fungi might expected to be more viable option from the point of view of the decolourisation of the effluent and the decolourisation process control at the industrial level.

CONCLUSION

This study has demonstrated that tannase enzyme based process can be used to treat tannery wastewaters to remove the residual recalcitrant tannins. Enzymatic treatment reduces the tannin concentration below the discharge limit. The biological treatment with the organism has a fundamental advantage over enzymatic systems i.e., their ability to simultaneously transform a broad range of compounds. Though the enzymatic treatment not results in the removal of a broad range of compounds from a waste stream, but accomplishes the transformation of tannin, the more problematic ones. Thus, this enzymatic treatment would be useful for the final polishing of a treated wastewater to remove color imparted by tannin or to meet the whole effluent toxicity criteria. However before their full potential can be realized, some major issues remain to be addressed. These include development of low cost technology to produce enzymes in quantities that are required at the industrial level, tolerance of the enzymes to the conditions prevailed in the wastewater treatment plant, toxicity and treatability of the incidental products must be investigated further.
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