Chapter 1

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Tannin acyl hydrolase (E.C.3.1.1.20) is commonly referred as tannase. Teighem discovered this unique enzyme in 1867 (Teighem, 1867) in an experiment of formation of gallic acid into an aqueous solution of tannins, where grew two fungal species later identified as *Penicillium glaucum* and *Aspergillus niger* (Lekha and Lonsane 1997). Tannase enzyme catalyzes the hydrolysis of ester bond and depside bond present in hydrolysable tannins to form glucose and gallic acid (Fig 1.1). Tannins are used as the natural substrate for tannase enzyme. Also tannase enzyme attack gallic acid methyl esters, but it possesses high specificity towards the acyl moiety of the substrate. Tannic acid hydrolysed to form tannase and this enzyme used for the production of gallic acid (3,4,5-trihydroxy benzoic acid) and it found to be the natural phenolic substrate for the tannase enzyme.

![Hydrolysis of tannase by tannic acid](image)

Fig 1.1 Hydrolysis of tannase by tannic acid
The significance and utility of tannase enzymes has prompted great research from 1867 onwards resulting in many publications and many patents in this area of research (Beniwal et al. 2013). Both tannases and gallic acid, their hydrolytic products find various industrial applications. Tannase is widely used in manufacture of instant tea and acorn wine, clarification of beer and fruit juices, manufacture of coffee flavored soft drinks, and improvement in flavor of grape wine, and as an analytical probe for determining the structure of naturally occurring gallic acid esters (Seth and Chand 2000). Tannase has also been applied for cleavage of poly phenolics such as dehydrodimer cross links present in the cell wall of plants, which is necessary to plant cell wall digestibility. Moreover, it is incorporated into the manufacture of high grade leather (Barthomeuf et al. 1994) and is used to clean up the tannery effluents released from leather industry (Belmares et al. 2004). However, the practical use of this enzyme is at present limited due to insufficient knowledge about its properties, optimal expression, and large-scale application.

Tannase can be obtained from tannin rich plant and animal tissues, but for its industrial production, microbial source are preferred. (Ayed and Hamdi, 2002; Belmares et al. 2004). Tannase is produced as a membrane bound or intracellular enzyme. Fungal tannases have a better activity in degrading hydrolysable tannins, whereas yeast tannases degrade tannic acid better and has a lower affinity for naturally occurring tannins (Deschamps et al. 1983). On the other end of the spectrum, bacterial tannase can degrade and hydrolyse natural tannin and tannic acid very efficiently (Deschamps et al. 1983, Lewis and Starkey, 1969). Tannins inhibit the growth of many microorganisms, but there are species that have
developed mechanisms to degrade and use them is as sole carbon source. These mechanisms include the production of tannase. (Banerjee and Pati 2007). Tannase is mostly produced by fungi from the *Aspergillus* and *Penicillium* species and lactic acid bacteria. Plants contain 5-20% tannin by weight. *Aspergillus* sp. capable of growing on tannic acid medium as sole carbon source might definitely produce tannase for its survival. “According to Van Diepeningen et al. (2004), black *Aspergillus* sp. can utilize more tannic acid than non black *Aspergillus* sp. Although tannase production by *Aspergillus* can occur in the absence of tannic acid, *A. niger* tolerates tannic acid concentrations as high as 20% without having a deleterious effect on both growth and enzyme production” (Van Diepeningen et al. 2004; Cruz-Hernandez et al. 2006).

Bacterial cultures are also considered for the production of extracellular tannase to degrade tannins, thus releasing gallic acid and glucose. “Deschamps et al. (1983) showed that strains of *Bacillus pumilus, B. polymyxia, and Klebsiella planticola* were able to produce extracellular tannase with chestnut bark as the sole source of carbon. The most abundant group of bacteria able to degrade tannins is found in the gastrointestinal track of ruminants” (Deschamps et al. 1983).

Tannins are a complex group of polyphenolic compounds that are present in many plants. Tannins belongs to second major positions among other natural products. As they do not possess any direct influence in plant metabolism, they are strongly regarded as a plant secondary metabolite. (Haslam E. 1989). Tannins are wide spread in the plant kingdom, and are found in the leaves, fruits, bark and
wood. They occur in many edible fruits and vegetables and are often considered nutritionally undesirable because they form complexes with protein, starch and digestive enzymes and cause a reduction in nutritional value of food (Chung et al. 1998). The usual complexation of proteins with tannic acid and naturally occurring tannins to form water insoluble complexes inactivate the enzymes (Haworth et al. 1985). Some other case tannin present in the diet of ruminants affects their growth and milk production. The concentration of tannin is high in beverages like cold tea, beer and wine, coffee flavoured drinks etc. And it forms like a precipitate during the production because of the interaction with other molecule present in the beverages. These changes can be resolved by either decrease or remove tannin by using chemical or enzymatic treatment (Belmares et al. 2004; Lekha and Lonsane, 1997).

Hydrolysable tannins are polyphenolic plant constituents derived from mono- to pentagalloyllated β-D-glucopyranose. Hydrolysable tannins are composed of esters of gallic acid (gallotannins) or ellagic acid (ellagitannins) with a sugar core, which is usually glucose (Bhat et al. 1998). They can occur in wood, bark, leaves, fruits and galls (Mueller-Harvey, 2001). Major commercial hydrolysable tannin sources are Chinese gall (Rhussemialata), sumac (Rhuscoriaria), Turkish gall (Quercusinfectoria), tara (Caesalpiniaspinosa), myrobalan nuts (Terminaliachebula) and chestnut (Castaneasativa) (Bhat et al.1998). Hydrolysable tannins are readily hydrolyzed chemically by acidification or biologically by tannase. Gallic acid can be produced by the microbial hydrolysis of tannic acid by tannase. Mainly Aspergilli have been used for hydrolysis of tannic acid to yield gallic acid (Mondal et al. 2001; Seth and Chand,
2000) among bacteria *Klebsiella pneumonia* and *Corynebacterium* sp. have been reported to produce gallic acid from crude extract of taragallotannin (Deschamps and Lebeault, 1984). Various groups have reported the gallic acid production from myrobalan (Mukherjee and Banerjee, 2004), tara (Pourrat et al. 1985), sumac (Pourrat et al. 1987), gall nuts (Regerat et al.1989), Chinese tannins (Kar et al. 1999), teri pod (*Caesalpinia digyna*) (Kar et al.1999; Mukherjee and Banerjee, 2004) and sake cake (Kawakubo et al. 1993).

Gallic acid (3,4,5-trihydroxy benzoic acid), a phenolic compound and the mono metric unit of the gallotannins and complex tannins presents in many plants either as a free molecule or as part of tannic acid molecule ions. The main application area is in the manufacture of the anti bacterial agent trimethoprim, a broad spectrum antibiotic. However, it is utilized in the production of trimethoxy benzaldehyde, which is used in ink industry, dye industry, leather industry and most importantly pharmaceutical industry. In pharmaceutical industry 3, 4, 5 trimethoxy benzaldehyde is converted to trimethoprim. Though technological advances have introduced a number of antibiotics in markets, trimethoprim is still very significant. In combination with sulphonamides, it is highly effective against many drug resistant species of bacteria. It is very important to have an economical indigenous technology for its commercial production.

In spite of the immense potential for the practical utility of tannase in a wide range of industries, the real use of this enzyme is at present limited owing to the high production cost. Hence, processes are to be developed for their economically feasible production. In industrial level tannase is mainly produced by *Aspergillus* species under Solid state fermentation (SSF).
Vegetable residues such as coffee wastes, grape wastes, cashew wastes, wheat bran, rice bran etc. supplemented with tannic acid were used as substrates for tannase production employing SSF. The utilization of agro-industrial wastes, on one hand, provides alternative substrates and, on the other hand, helps to solve pollution problems by eliminating the need for disposal of the wastes. The nature of the substrate employed is the most important factor affecting fermentation processes, and its selection depends upon several factors mainly related to cost and availability and, thus, may necessitate the screening of several agro-industrial residues (Rodriguez couto and Sanroman 2006). Several studies have reported interesting advantages of tannase production by solid state culture (SSC) compared with that produced by Submerged culture (Belmares et al. 2004). The SSF allows the construction of more compact reactors with less energy requirement and causing less damage to the environment (Lekha and Lonsane, 1997; Viniegra-Gonzalez et al. 2003).

The use of a sequential experimental design strategy is a constructive tool for process optimization. Optimization through response surface methodology (RSM) is now widely used to evaluate and understand the interactions between different physiological and nutritional parameters (Puri et al. 2002). This includes factorial design and regression analysis which helps in evaluating the effective factors and building blocks to study interactions and select optimum conditions of variables for a desired response. This multivariate approach enables the statistical elucidation possibilities and checks the relative significance of several contributing factors (Dilipkumar et al. 2011). RSM is widely in various biotechnological experiments such as the optimization of media, process
conditions, etc. (Mannan et al. 2007 and Pan et al. 2008). Statistical optimization allows the interaction among possible influencing parameters to be evaluated with a limited number of experiments (Rodriguez et al. 2008).

Anacardium occidentale L. (Cashew) is a multipurpose tree of India that grow up to 15m high. Cashew trees produce many resources and products. The cashew products have several applications in food as well as medicine. The bark and leaves of the cashew trees are used for medical purpose and the cashew nut considered for the edible food product. Nowadays, cashew shell cakes (cashew industrial waste) are used in the fuel or thermal applications. CNSL (cashew nut shell liquid), presents in cashew nut used medicinally and industrially for the plastic and resin industries for its phenol content. Anacardic acids are the most valuable compound from CNSL. It has tremendous application in pharmaceutical and cancer studies. In addition to this, other by products could be used for the preparation of microbial enzymes like Pectinase, Cellulase and Tannase.

The cashew shell is about 0.3 cm thick, having a soft feathery outer skin and a thin hard inner skin. Between these skins is the honeycomb structure containing the phenolic material known as CNSL. Inside the shell is the kernel wrapped in a thin skin known as the testa. It is the outer skin of cashew kernel containing about 25% f tannin material and 11% of non-tannin material. Cashew testa provides a cheap source of material with comparatively high tannin content. Tannins act as the sole source of carbon for tannin degrading microorganisms and to degrade the tannins microbes produces tannase.
Commercial production of tannase is carried out by fermentation of substrates which contains tannins. *Aspergillus niger* is one of the most important fungi exploited for the production of tannase for industrial applications. Tannase has been shown to be an inducible enzyme, therefore tannase is only expressed in the presence of its substrate or a substrate analogue, such as tannic acid or its end product e.g., gallic acid (Haslam and Tanner, 1970). The novelty of the present work lies in the use of a new agro substrate cashew testa by an *Aspergillus* isolate *Aspergillus niger* CEPC 11 for optimum production of tannase. Considering the great importance of this biomass and the potentiality for development of new eco-friendly compounds, this work deals with the isolation and molecular characterisation of Tannase enzyme from cashew testa by using *Aspergillus niger* CEPC 11 with the following objectives.

**Specific objectives of the study**

1. Screening and identification of microbes for the production of Tannase using cashew testa as a Substrate.

2. Optimization of bioprocess variables for the production of Tannase using *Aspergillus niger* MTCC 5898 by Response surface Methodology.

3. Purification and characterization of tannase from *Aspergillus niger* CEPC 11.

4. Genetic characterisation of Tannase from *Aspergillus niger* and structural elucidation by Molecular docking

5. Studies on the application of *Aspergillus niger* Tannase