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Biodegradation of Catechin

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Catechin is a major component of the condensed tannin, considered to be recalcitrant. A few microorganisms have developed mechanisms to degrade catechin. Catechin oxygenase, a key enzyme in the degradation of catechin, is present in fungi and bacteria. Initially catechin is degraded to phloroglucinol carboxylic acid and protocatechuic acid. Phloroglucinol carboxylic acid is degraded via phloroglucinol, resorcinol and hydroxyhydroquinone to form maleyl acetate. Maleyl acetate is then converted to β-ketoadipate. The metabolism of protocatechuic acids either through conversion to β-carboxy-*cis,cis*-muconate or conversion to catechol and then to *cis,cis*-muconate to form β-ketoadipate. Enzymology and molecular biology of catechin degradation will help to construct genetically engineered microbes for the efficient disposal of tannery wastes and related industrial pollutants.

Key Words: Biodegradation, Tannin degradation, Catechin, Oxygenase, β-ketoadipate pathway

Introduction

Successful evolution of plants from aquatic environment to land was achieved largely by massive formation of plant phenolic compounds (Croteau et al. 2000). Accounting for about 40% of organic carbon circulating in the biosphere, these phenolic compounds are primarily derived from phenylpropanoid, phenylpropanoid–acetate, and related biochemical pathways such as those leading to "hydrolysable tannins". Furthermore, it is their reassimilation back to carbon dioxide during biodegradation (mineralization) that presents the rate-limiting step in recycling biological carbon.

Of the secondary substances, tannins are the most abundant and widely distributed phenolic polymers found in higher plants and in many habitats including sewage sludge, forest litter and the rumen (Deschamps 1982, Field & Lettinga 1987, Field et al. 1988, Muller-Harwey et al. 1988). Tannins are defined as naturally occurring water soluble polyphenols of varying molecular weight, which differ from most other natural phenols in their ability to precipitate proteins from solutions (Spencer et al. 1988, Waterman 1998). The ability to complex with proteins and amino acids inhibit the organic matter degradation. Concentrations ranging from 325 to 3000 mg/L have been reported to be inhibitory to methanogenic bacteria (Northup et al. 1995). Concentrations of 1 – 2 % tannin reduce the overall decomposition of organic materials applied to soil (Field & Lettinga 1992). At a concentration higher than 10 mM tannins are literally toxic to their environment (Arunachalam 2001).

Based on their structure, vegetable tannins are classified into hydrolyzable and condensed tannins. Condensed tannins are more widespread in the plant kingdom than hydrolyzable tannins. Hydrolyzable tannins are characterized by having several gallic acid groups, or acids clearly derivable from gallic acid, united by ester linkage to central glucose residue (figure 1). Condensed tannins are polymers of flavan 3 ols (catechins) or flavan 3,4 diols or combination of both (figure 2). Of the two types of tannins, condensed tannins are more resistant to

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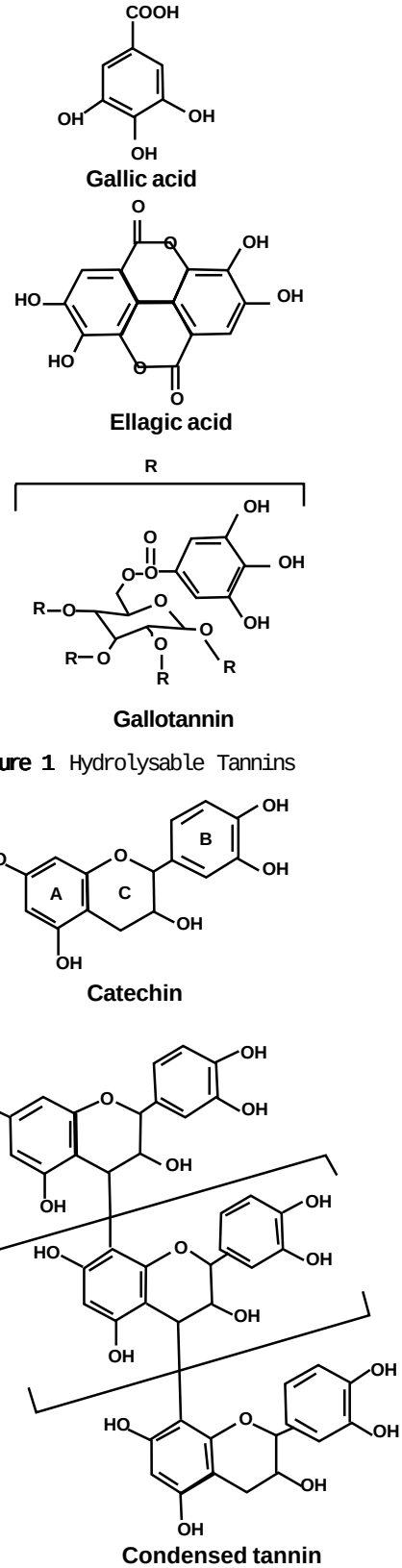

Figure 1 Hydrolysable Tannins

Figure 2 Condensed Tannins

microbial decomposition than hydrolyzable tannins (William et al. 1986) and are toxic to variety of microorganisms. For this reason, tannins generally retard the rate of decomposition of soil organic matter *via* inhibition of biodegradative enzyme of the attacking organism (Scalbert 1991).

Hydrolyzable tannins are readily cleaved by micro-organisms and so most of the reports available are on the degradation of hydrolysable tannins (Boominathan & Mahadevan 1985). Focus on the degradation of condensed tannins is limited and so the biology of degradation of these recalcitrant molecules is limited. Tannery wastes contribute to the pollution hazard in no small way since they have high proportions of putresible organic and inorganic wastes (Mukherjee 1992). Catechins and phenols are the major organic pollutants of tannery effluent and impart brown colour to receiving waters (Kumaran 1993). Due to the recalcitrant nature, catechins are highly resistant to microbial attack and cause pollution to our environment. They also cause "soil sickness" and are toxic to plants, animals and micro-organisms (Muthukumar et al. 1982, Bae et al. 1993, Jones et al. 1994, Odenyo & Osuji 1998). The toxicity of phenolic compounds in the environment fostered studies of micro-organisms that are able to tolerate and metabolise high level of these compounds (Allison et al. 1990, Huang et al. 1990, Osawa et al. 1993, Brooker et al. 1994, Nelson et al. 1995). The research team of Mahadevan from this centre investigated the bacterial and fungal degradation of catechin and the pathways for the degradation of catechin (Muthukumar et al. 1982, Arunakumari & Mahadevan 1984, Sambandam & Mahadevan 1993, Waheeta & Mahadevan 1997, Latha 1997, Arunachalam et al. 2003).

A number of reviews on hydrolyzable tannin biodegradation have appeared in the past which have provided a general idea of the biodegradation of these polyphenols (William et al.1986, Deschamps 1989, Field & Lettinga 1992, Saxena et al. 1995). A lot of work has been published on the industrial and agricultural applications of tannin biodegradation (Archambault et al. 1996,Hatamato et al. 1996, Selinger et al. 1996, Lane et al. 1997, Lekha & Lonsane 1997) and reviewed by several others (Scalbert 1991, Saxena et al.1995, Meselhy

et al. 1997, Waterman 1998, Wang et al. 2001) but no one revealed the mechanism and pathway for condensed tannin degradation.

To understand the mechanism of condensed tannin degradation, studying the degradation of their building unit, particularly catechin is very helpful. This review takes a holistic view of microbial degradation of catechin and the pathways involved in its catabolism. Besides, it focuses on the molecular basis of catechin degradation and cloning of genes involved in the degradative pathways.

Catechin

Catechin is a group that occupies an intermediary position in the tannin hierarchy as a family of catechin tannins. This is a combining element of hydrolyzable and condensed tannins (Bhat et al. 1998). Catechins are quite common in tropical shrub legumes (Muller-Harvey et al. 1987) and tea leaves (Graham 1992). They are structurally closely allied to leucoanthocynidines and are also optically active. (+) Catechin (figure 3-1) itself is the quercetin (figure 3-2)/cyanidin (figure 3-3) analogue. Catechins related to robinetin (figure 3-4), kaempferol (figure 3-5) and myricetin (figure 3-6) are also known. Collectively, catechins are distributed as widely as the flavan 3,4 diols and jointly, these two groups of reduced flavonols are the precursors of the economically important group of "condensed tannins".

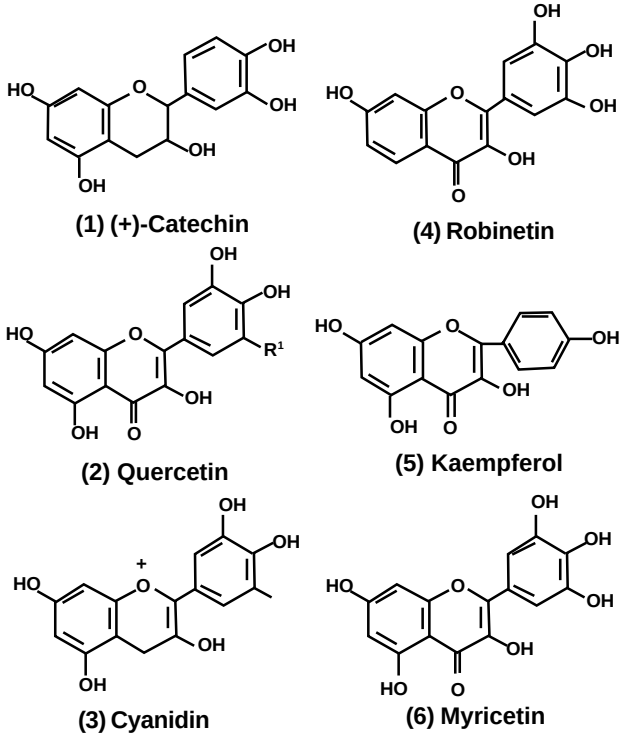

Figure 3 Catechin analogues and related groups

Catechin has two symmetric carbon atoms resulting in 4 optical isomers (+) catechin, (-) catechin, (+) epicatechin and (-) epicatechin. Of these, (+) catechin and (-) epicatechin occur in nature.

Degradation of Catechin

Tannins inhibit the growth of a number of microorganisms, resist microbial attack and are not readily degraded (Mahadevan 1991). However some wood rotting fungi, bacteria and yeasts are quite resistant to tannins, they grow and develop on them (Deschamps 1989). Most of the researchers concentrated on the degradation of the hydrolyzable tannins, which are easily hydrolyzed by either fungi or bacteria (Ganga et al. 1977, Suseela & Nandy 1985). Moulds such as *Aspergillus* and *Penicillium* grow on the surface of liquids of tannery pits and tannery wastes (Rajkumar & Nandy 1983).

Catechin Degrading Fungi

A few fungi degrade catechin and condensed tannin (table 1). Hathway and Seakins (1957) claimed that extracts of *Psalliota campestris* oxidized catechin as well as tannin extracts of *Acacia catechu* and *Ucaria gambier*. Lewis and Starkey (1969) reported that *Aspergillus fumigatus, A. niger, Penicillium frequentans, P. janthinellum* and *Fusarium* sp. decomposed catechin within 8 days. The growth of fungi closely correlated with the amount of catechin utilized. Degradation of wattle tannin was slower than that of catechin by soil microorganisms. No intermediate product of the decomposition of catechin was identified. From the Central Leather Research Institute, Chennai, Chandra et al. (1969) reported that *Aspergillus flavus, A. fumigatus, A. niger, A. teneus, Penicillium* sp. and *Streptomyces* sp. degraded catechin. *A. flavus* utilized 90% of catechin. Continuing their observations, Chandrakantha et al. (1973) found that *A. flavus* grew prolifically on catechin and degraded cashew apple tannin. Unfortunately these observations were not continued by the group.

Species of *Fusarium, Rhizoctonia, Cylindrocarpon* and *Trichoderma* are capable of degrading tannery waste constituents (Mahadevan & Muthukumar 1980). *Psalliota campestris* was found to oxidize catechin and

Table 1 *Catechin Degrading Fungi*

Fungi	Reference
Aspergillus flavus	Chandra et al. (1969) Chandrakantha et al. (1973)
A. fumigatus	Chandra et al. (1969)
A. niger	Lewis & Starkey (1969)
A. teneus	Chandra et al. (1969)
Calvatia gigantean	Galiotou-Panayotou and Macris (1986)
Chaetomium cupreum	Sambandam (1983)
Cladosporium sp.	Updegraff & Grant (1975)
Endothia parasitica	Elkins et al. (1978)
Fusarium sp.	Lewis & Starkey (1969), Updegraff & Grant (1975)
Penicillium adametzi-zaleski	Grant (1976)
P. frequantatus	
P. janthinellum	Lewis & Starkey (1969)
P. solitum	Bokadia et al. (1960)
Penicillium sp.	Chandra et al. (1969), Saxena et al. (1995)
Phlyctaena vagabunda	Lattanzio et al. (2001)
Psallitoa campestris	Hathway & Seakins (1957) Mahadevan & Sivaswamy (1985)
Rhizoctonia batadicola	Sambandam (1983)
Scropulariopsis sp.	Updegraff & Grant (1975)
Streptomyces sp.	Chandra et al. (1969)
Trichoderma sp.	Updegraff & Grant (1975)
T. viride	Sambandam (1983)

A. niger could degrade gallic acid (Mahadevan & Sivaswamy 1985). Collett (1992) isolated *Lentinus lepidus* by enrichment culture technique using the (+)- catechin as a carbon source. He concluded that fungal growth resulted from the degradation and utilization of the aromatic compound. This degradative ability suggests potential use of *L. lepidus* as a pretreatment for neutralizing phenolic pollutants of industrial origin. The pathway for the aromatic ring cleavage of protocatechuic acid by white rot fungus, *Pycnoporus coccineus*, was studied (Hayakawa et al. 1994). The mycelia adopted to protocatechuic acid solution and followed the intradiol ring cleavage pathway for tannin degradation. Subsequently, other workers also reported the utilization of catechin as sole carbon source by the species of *Aspergillus* and *Penicillium* (Saxena et al. 1995). The degradation of chlorogenic acid, (+)-catechin, (-)-epicatechin, phloretin glycosides and quercetin glycosides in fresh and stored apples by *Phlyctaena vagabunda* was reported (Lattanzio et al. 2001).

Yeasts are important fungal source for degradation studies. A number of yeasts were reported to degrade condensed tannins (wattle tannin). The strains isolated and studied were of *Candida guilliermondii, C. tropicalis* and *Torulopsis candida* (Otuk & Deschamps 1983, Vennat et al. 1986). Degradation of (+)-catechin and (-) -epicatechin in water- alcohol solutions was studied in the presence and absence of yeasts. On the basis of the results, degradation of the flavans by yeast was very slow and it slowly formed the browning products (Lopez-Toledano et al. 2002).

Catechin Degrading Bacteria

It is well known that tannins are toxic and bacteriostatic compounds making non-reversible reactions with proteins (Scalbert 1991). Nevertheless, some bacteria may degrade many phenolic compounds including the catechin degradation products like catechol and protocatechuic acid (Deschamps 1989, Gajendiran & Mahadevan 1990, Field & Lettinga 1992, Delneri et al. 1995, Paller et al. 1995).

Lewis and Starkey (1968, 1969) isolated three species of *Pseudomonas* from soil which utilized catechin and also reported that catechin was degraded within 12 days in forest soils and the carbon dioxide liberated was 27%. Deschamps et al. (1980) isolated catechin degrading *Bacillus, Staphylococcus* and *Klebsiella* by enrichment culture technique. These workers also isolated several bacterial strains by enrichment techniques which were capable of degrading hydrolyzable and condensed tannins including chestnut, wattle and quebracho commercial tannin extracts. Bacteria which degrade wattle tannin were identified as *Enterobacteraerogenes, E.agglomerans, Cellulomonas* and *Staphylococcus*.

Species of *Rhizobium*, such as *Rhizobium japonicum, R. leguminosarum, R. phaseoli, R. trifolii* and *Rhizobium* sp. utilized catechin as sole carbon source (Muthukumar et al. 1982, Gajendiran & Mahadevan 1988). Arunakumari and Mahadevan (1984) reported that *Pseudomonas solanacearum* utilized a spectrum of phenolic compounds such as tannic acid, catechin, myrobalan tannin, wattle

tannin, phenol, catechol, resorcinol, phloroglucinol, protocatechuic acid and variety of phenolic compounds. *P. solanacearum* isolated from the pseudo stems of banana was inoculated onto a medium containing one of the aromatic substrates: catechin, protocatechuic acid, catechol and hydroxyquinol. It degraded all the phenolic compounds and produced the corresponding enzymes involved in the degradation (Boominathan & Mahadevan 1994). The catabolism of catechin by *Bradyrhizobium* was investigated by Waheeta and Mahadevan (1991,1997). *Rhizobium* sp. isolated from the root nodules of *Arachis hypogea* utilized catechin as sole carbon source (Latha 1997). Bacteria isolated from tannery effluent utilized tannin up to 25 mg/100 mL. These isolates were identified as species of *Bacillus, Acinetobacter, Alcaligenes, Aeromonas* and *Pseudomonas* (Lakshmanperumalsamy 1993). Recently, degrada-tion of catechin by *Acinetobacter calcoaceticus* was investigated by Arunachalam et al. (2003).

Catechin Degrading Anaerobic Bacteria

Some animals have adapted to tannins through the synthesis of tannin binding salivary proteins, the presence of tannin resistant or tannin degrading ruminal/intestinal microbes, or other potential adaptations in the lower intestinal tract (Brooker et al. 1992, Odenyo & Osuji 1998). Formation of complexes of tannins with nutrients, especially proteins, has negative and positive effects on their utilization (Reed 1995, Meselhy et al. 1997).

Catechins generally reduce the nutritive value of forage through inhibitory effects on ruminal and intestinal functions. Tannins inhibit the activity of enzymes of rumen microbes (Mc Leod 1974, Martin & Akin 1988, Makkar et al. 1988, Bae et al. 1993, Meselhy et al. 1997). Current research on rumen microbial ecology has showed that tannin rich forage reduces the populations of fibre degrading *Ruminococcus* spp. and *Fibrobacter* spp. However, fungi, protozoa and proteolytic bacteria appeared to be less affected (Nelson et al. 1998). Apart from the synthesis of tannin binding salivary proteins, some animals have adapted to tannins through their association with tannin resistant or tannin degrading ruminal or intestinal bacteria (Brooker et al. 1992).

Some bacterial species have special mechanism to degrade the tannins in anaerobic environment. *Prevotella ruminicola* produced extra cellular material which may protect the organism from the effects of tannins (Jones et al. 1994). Biochemical studies have shown that *S. caprinus* metabolizes gallic acid to pyrogallol, although it does not metabolize pyrogallol and produces extracellular polysaccharides (EPS) in response to catechins in the growth medium. Induction of EPS appears to be a bacterial defence mechanism that permits the bacterium to maintain its populations when related species are dying (Brooker et al. 1994). Normally, rumen microbes have the capacity of degrading and detoxifying many incriminating and antinutritional factors into simpler and non-toxic constituents (Selinger et al. 1996, Osava et al. 2000). The rat-fecal microflora isolated by Groenwoud and Hundt (1984) anaerobically degraded the (+)-catechin.

Streptococcus caprinus and *S.gallolyticus* are found ubiquitously in the rumen of many animals browsing tannin rich forage legumes. Brooker et al. (1994) isolated *S. caprinus* from the ruminal contents of feral goats browsing on tannin-rich *Acacia* sp, which gave zones of clearing on tannin-protein agar medium. This rumen microbe was tolerant up to a concentration of 3% tannin (hydrolyzable or condensed).

Gram-positive cocci, isolated from the feces of Koalas and identified as *Streptococcus bovis* biotype I, formed a distinct clear zone on tannin-treated brain heart infusion agar, suggested the anaerobic degradation of tannin by this bacterium (Osawa 1990, Osawa et al. 1993). Substantial degradation and disappearance of labelled condensed tannins from *Desmodium intortum* in the gastrointestinal tract in sheep and goats was reported by Perez-Maldonado and Norton (1996). Anaerobic *Selenomonas* sp. isolated from enrichment culture of rumen microflora of sheep, goat and antilope was able to grow in media containing up to 6 g of condensed tannins per litre (Odenyo & Osuji 1998).

The denitrifying bacterium *Azoarcus anaerobicus* LuFRes 1 grew anaerobically with resorcinol, which is an intermediate of catechin degradation (Philipp & Schink 1998). Another intermediate of catechin degradation, hydroxy-hydroquinone was anaerobically

degraded by the *Desulfovibrio inopinatus*. It produces 2 mol of acetate and 2 mol of CO_2, with stoichiometric reduction of sulphate to sulfide (Reichenbecher et al. 2000).

Catabolic Pathway of Catechin Degradation

Information is now available on the tannin degradation pathway used by fungi and bacteria. Degradation mechanism of catechin by fungi is similar to the mechanism of bacterial degradation (Bhat et al. 1998). Initially catechin is degraded to phloroglucinol carboxylic acid and PCA by catechin oxygenase. The phloroglucinol carboxylic acid degradation proceeds through the formation of phloroglucinol, resorcinol and hydroxy-hydroquinone. Hydroxyhydroquinone is cleaved by hydroxyquinon-1,2-dioxygenase to form maleyl acetate. This substance is converted to β-ketoadipate.

The metabolism of PCA proceeds by two different pathways. These pathways finally converge with the formation of β-ketoadipate. In the first type of pathway, PCA is converted to β-carboxy *cis,cis* muconate and is transformed to β-ketoadipate. In the second pathway, PCA is converted to catechol, which is then cleaved to form *cis,cis* muconate and to β-ketoadipate. β-ketoadipate is used for the generation of acetyl CoA (figure 4).

In Fungi

The pathways of aerobic degradation of condensed tannins, phenolic monomers catechin and quercetin and their intermediates were thoroughly studied in *Penicillium* spp. (Patel et al. 1990) *Aspergillus* spp. and soil fungi like *Chaetomium cupreum* (Sambandam & Mahadevan 1993). The products of catechin degradation by *A. flavus* were identified as protocatechuic acid (PCA) and phloroglucinol carboxylic acid (figure 5) (Chandra et al. 1969). Sambandam and Mahadevan (1993) reported that the intermediates in the degradation of catechin by *C. cupreum* were catechol, PCA and phloro-glucinol carboxylic acid. Pyruvate and acetalde-hyde were the end products of catechin degradation (figure 6).

In Bacteria

Boominathan and Mahadevan (1984, 1994) reported that catechin was degraded by *Pseudomonas solanacearum* into phloroglucinol

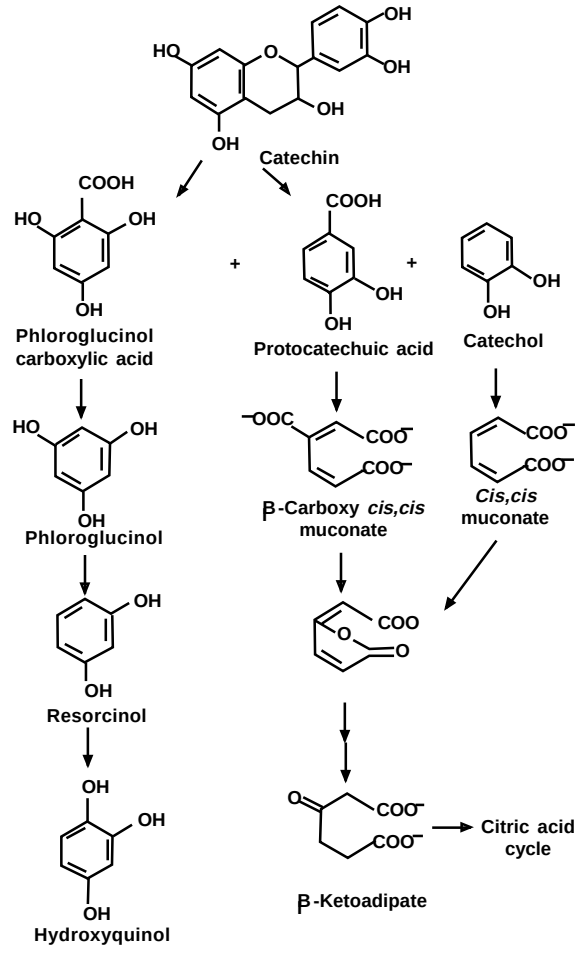

Figure 4 Catechin degradation in bacteria

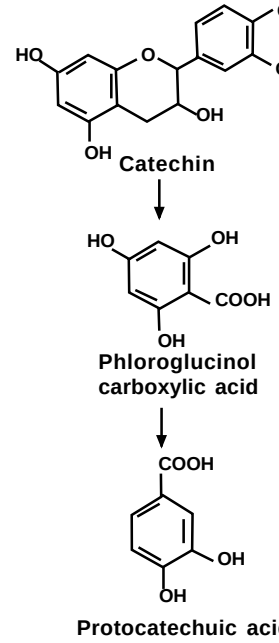

Figure 5 Catechin degradation by *Aspergillus flavus* (Chandra et al. 1969)

carboxylic acid, PCA, phloroglucinol, catechol, resorcinol and hydroxyquinol (figure 7). In *Bradyrhizobium* sp., phloroglucinol carboxylic acid, PCA, phloroglucinol, resorcinol and hydroxyquinol appeared as intermediates of catechin degradation. Catechin was cleaved into phloroglucinol carboxylic acid and protocatechuic acid by catechin oxygenase. Finally hydroxyquinol and PCA were ring opened through intradiol cleavage to form β-carboxy *cis,cis* muconic acid and maleylacetate, respectively (figure 8).

Rhizobium sp., degraded catechin into phloroglucinol carboxylic acid and protocatechuate (Gajendiran & Mahadevan 1988). Protocatechuate and phloroglucinol appeared from A and B rings of catechin. Further degradation of phloroglucinol carboxylic acid to phloroglucinol, resorcinol and hydroxyquinol was reported by Vasudevan and Mahadevan (1990). Protocatechuic acid was identified as an intermediate formed during the degradation of catechin by *Rhizobium* sp. (Latha 1997) and *Acinetobacter calcoaceticus* (Arunachalam et al. 2003).

Research findings of the catechin degradation revealed that the aerobic breakdown of catechin occurs through two pathways (Bhat et al. 1998). It is degraded to PCA and phloroglucinol carboxylic acid. Phloroglucinol carboxylic acid, by decarboxylation and scission of the aromatic rings by various oxygenases, finally forms β-ketoadipate, through intermediates like phloroglucinol, resorcinol, hydroxyhydroquinone and maleylacetate. PCA is converted to β-ketoadipate through β-carboxy-*cis,cis*- muconate and catechol pathways.

In Anaerobic Bacteria

In anaerobic system, the reductive breakdown of catechin proceeds through distinctly different pathway. The initial degradation product of catechin is diarylpropanol, which upon ring breakdown is transformed to acetate and phenylvalerolactone. The latter after converted to phenylpropionate derivatives may accumulate as such, or further converted to phenylacetate (Barz & Hosel 1975, Field & Lettinga 1989).

Certain members of the human colonic microflora have the ability to metabolize catechin and epicatechin. A study was carried out with model F344 rats associated with human total faecal flora

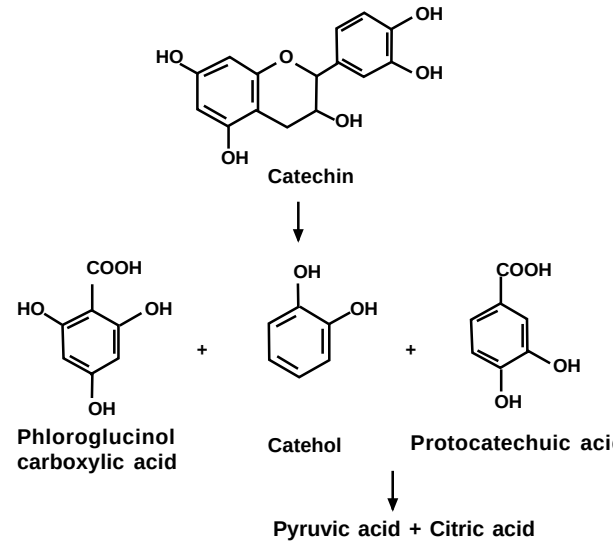

Figure 6 Catechin degradation by *Chaetomium cupreum* (Sambandam & Mahadevan 1993)

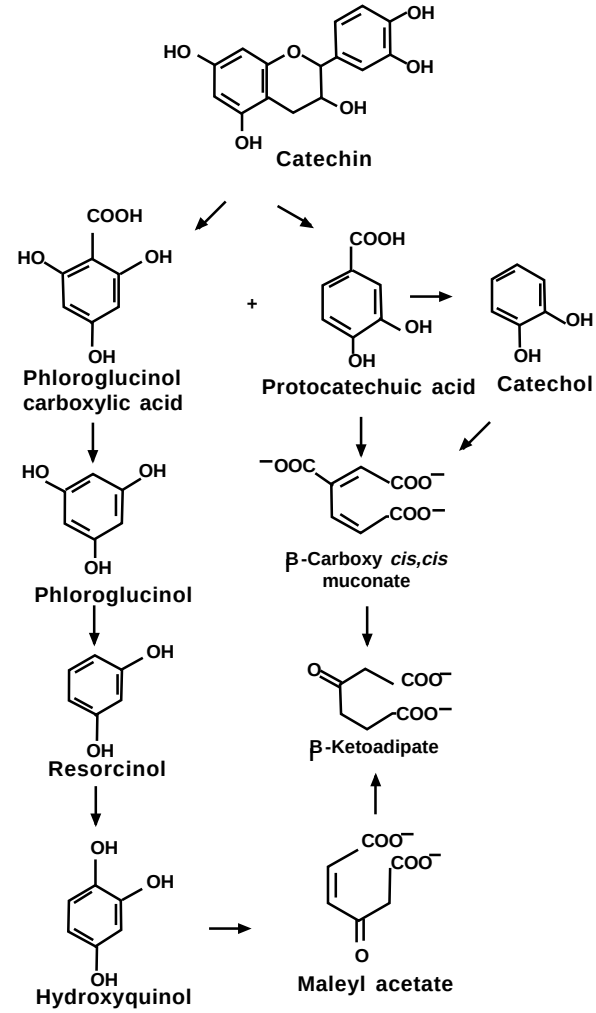

Figure 7 Catechin degradation by *Pseudomonas solanacearum* (Boominathan & Mahadevan 1994)

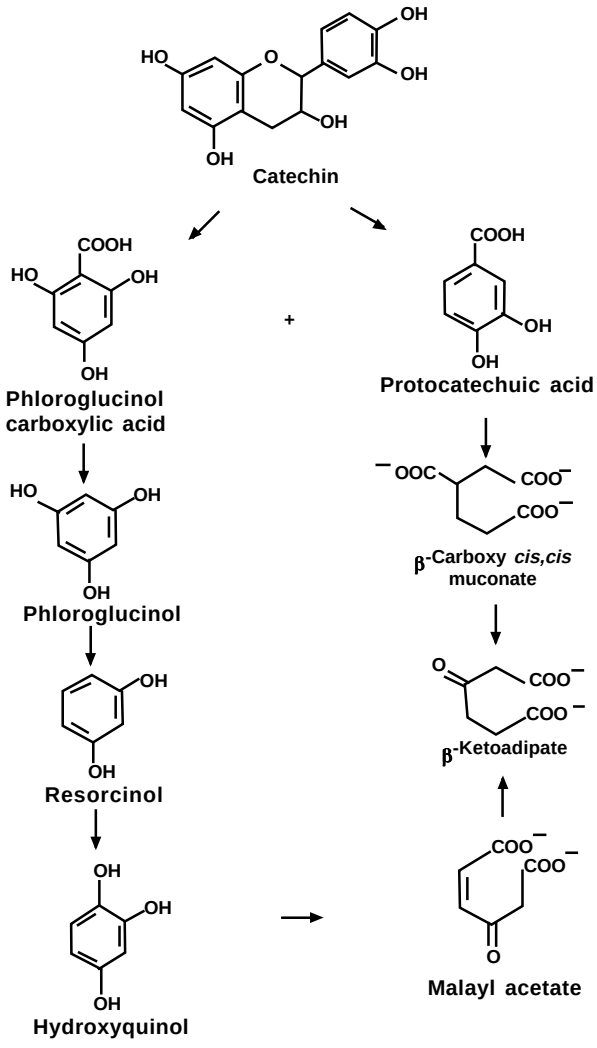

Figure 8 Catechin degradation by *Bradyrhizobium japonicum* (Waheeta & Mahadevan 1997)

(HF rats). Fresh faeces from HF rats receiving catechin were serially diluted in a catechin containing culture medium. A library of 57 isolates of catechin degrading bacteria were isolated. Of these 13 strains possessed high catechin degradation ability. According to the catabolic potential of the strain, 27% to 65% of the initial catechin (10 mm) was metabolized in 7 days. Two of three strains were identified as *Escherichia coli* and *Enterococcus faecalis* (Brezillon et al. 1998).

Degradation of catechin by rat faecal microflora showed the pattern of anaerobic degradation pathway, which differed from normal aerobic pathway (Groenwoud & Hundt 1984). Catechin underwent partial heterocyclic ring cleavage to 2-diarylpropan-2-ol metabolite. This is in contrast to the well known total heterocyclic ring cleavage of flavonoids. Catechin was converted to 1-(3-hydroxyphenyl)-3-(2,4,6-trihydroxyphenyl)-propan-2-ol. The diarylpropanol was converted to acid metabolites (figure 9).

On the other hand, the catechin analogue quercetin is broken into phloroglucinol and phenylacetate derivatives (Field & Lettinga 1992). Phloroglucinol is rapidly fermented in various anaerobic systems via the dihydrophloroglucinol to either acetate and butyrate (Krumholz & Bryant 1986) or acetyl CoA (Bruce & Schink 1992). The other intermediates of flavonoid decomposition by anaerobic bacteria generally accumulate in the media as derivatives of phenyl propionic or phenyl acetic acids, or other more simple phenolic intermediates such as catechol, phenol and p-cresol (Young 1984, Berry et al. 1987, Evans & Fuchs 1988). *Eubacterium ramulus*, an anaerobic bacterium was tested for its ability to transform the catechin analogs luteolin-7-glucoside, rutin, quercetin, kaempferol, luteolin, eriodictyol, naringenin, taxifolin and

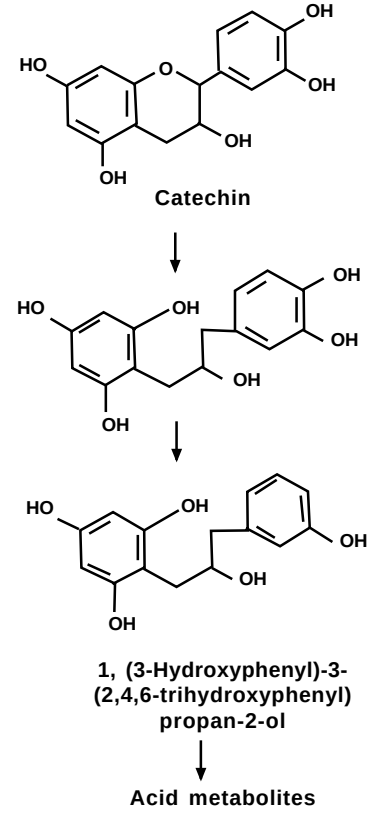

Figure 9 Anaerobic degradation pathway of catechin (Groenwoud & Hundt 1984)

phloretin to phenolic acids. But (+)-catechin was not degraded by this organism (Schneider & Blaut 2000).

Resorcinol, the product of anaerobic degradation of catechin, was found in *Azoarcus anaerobicus* LuFRes1 (Philipp & Schink 1998). In this organism, resorcinol was hydroxylated to hydroxyhydroquinone with nitrate or potassium ferricyanide as the electron acceptor. Hydroxy hydroquinone was further oxidized with nitrate to 2-hydroxy-1, 4-benzoquinone. The recently isolated sulfate reducer *Desulfovibrio inopinatus* oxidized hydroxyhydroquinone (HHQ) to 2 mol acetate and 2 mol CO_2 HHQ was reduced with NADH to dihydroxy hydroquinone. Dihydro hydroxy hydroquinone was converted stoichiometrically to acetate and to an unknown product (Reichenbecher et al. 2000).

Enzymology of Catechin Degradation

In many species of fungi and bacteria, enzymology of tannin degradation was mainly focused on hydrolyzable tannins (Bhat et al. 1998). Enzymes involved in degradation of condensed tannin were investigated by very few researchers.

Catechin oxygenase is the first enzyme that cleaves catechin by oxygenation. Chandra et al. (1989) reported that *A. flavus* produced extra cellular catechin oxygenase, which degraded catechin rapidly at *p*H 5.0 within 72 hr. Catechin oxygenase was isolated from *Chaetomium cupreum* and it was active in a *p*H range 2 to 8 and at 50^{0}C (Sambandam & Mahadevan 1993). Further, the Km value of the enzyme was 4 x10^7 M per litre with catechin as substrate. The molecular weight was 40 kDa. It was moderately active on wattle tannin, taxifolin and quercetin. Mannose, glucose, sucrose and maltose induced the production of catechin oxygenase by *C. cupreum* (Sambandam & Mahadevan 1993). It is constitutive and does not require any cofactor or metal ion for activity. The enzyme cleaves catechin, releasing catechol, protocatechuic acid and phloroglucinol carboxylic acid.

Galiotou Panayotou and Macris (1986) reported that the crude enzyme preparation from C*alvatia gigantia* showed maximum activity at *p*H 7.5-8.0 and at 40 to 45ºC and has Km value of 2.96 x10^5 mol per liter at *p*H 8.0. Galiotou Panayotou et al. (1988) purified catechin oxygenase from *C.gigantea*. Two isomeric forms, I and II with isoelectric points at 5.75 and 5.85 respectively of the enzyme were found. Each form was composed of two components, the molecular weight of which was 50.5 kDa (I_1 and II_1) and 49.5 kDa (I_2 and II_2 Enzyme extract from *Bradyrhizobium japonicum* showed maximum activity of catechin oxygenase, protocatechuate 3, 4-dioxygenase and hydroxyquinol 1,2-dioxygenase when grown on catechin (Waheeta & Mahadevan 1997). Induction of catechin oxygenase was highly specific to its substrate. Similarly cell extract of *P. solanacearum* grown on catechin, contained catechin oxygenase, catechol 1,2 dioxygenase and protocatechuate 3,4 dioxygenase indicating their inducible nature (Boominathan & Mahadevan 1994).

Selenomonas ruminantium K2 grows in the presence of catechins as sole carbon source and secretes a tannin inducible tannin acylhydrolase (Brooker et al. 1992). A number of other catechin resistant bacterial species, including *Lactobacillus, Butyrivibrio* and *Enterobacteriacae* have been isolated. Some organisms produce esterases that degrade hydrolysable tannins, but it is not known whether other mechanisms such as enzyme glycosylation exist.

Hydroxyquinol 1,2-dioxygenase from *Nocardioides simplex* 3E, the enzyme involved in aerobic biodegradation of a large class of aromatic compounds and condensed tannin was recently crystallized (Benvenuti et al. 1999). The enzyme is a homodimer composed of two identical subunits in a alpha2-type quaternary structure, with a molecular weight of about 65 kDa and contain catalytically essential Fe (III) ion. It catalyzes the intradiol cleavage of hydroxyquinol, which is an intermediate of catechin degradation.

Hydroxyquinol ring was cleaved by the *ortho* enzyme hydroxyquionol 1,2-dioxygenase. Induction of this enzyme by phloroglucinol carboxylic acid, phloroglucinol, resorcinol and hydroxyquinol revealed that it was substantially induced by resorcinol and hydroxyquinol. The order of induction was hydroxyquinol > resorcinol > phloroglucinol > phloroglucinol carboxylic acid (Boominathan & Mahadevan 1994).

Hydroxyhydroquinone reductase was isolated from *Desulfovibrio inopinatus* reduced the hydroxyhydroquinone to acetate (Reichenbecher et al. 2000).

It was present in the cell-free extract at 0.25 – 0.30 U (mg protein)$^{-1}$, with a *p*H optimum at 7.5. The enzyme was sensitive to sodium chloride, potassium chloride, EDTA and exhibited little sensitivity towards sulphydryl group reagents.

Other important oxygenases involved in tannin degradation are protocatechuate 3,4-dioxygenase (3,4-PCD) and catechol oxygenase. They are involved in the degradation of catechol and protocatechuic acid aromatic rings (Harwood & Parales 1996). Oxygenases that utilize both atoms of dioxygen in their substrates are known as dioxygenases (Harayama et al. 1991). In general, degradation proceeds in two phases. First, an aromatic compound is prepared for ring cleavage by a variety of ring modification reactions. The second phase of degradation includes ring fission and subsequent reactions leading to the generation of tricarboxylic cycle intermediates. Ring fission is catalyzed by dioxygenases and is termed *ortho*-cleavage when it occurs between the hydroxyl groups (intradiol cleavage) and *meta*-cleavage when it occurs adjacent to one of the hydroxyls (extradiol cleavage). *Ortho*-cleavage is commonly known as β-ketoadipate pathway. The latter name derives from the fact that β-ketoadipate is a key intermediate of the *ortho*-pathway.

Two key enzymes involved in this β-ketoadipate pathway are catechol 1, 2-dioxygenase and 3, 4-PCD. The chemistry and enzymology of the *ortho*-pathway have been studied primarily as they occur in *P. putida* (Ohlendorf et al. 1988). All nine enzymes catalyzing the conversion of PCA and catechol to tricarboxylic acid cycle intermediates have been purified and characterized, and in several cases, crystal structures are available (Harwood & Parales 1996). The best studied enzyme is 3,4-PCD from *A. vinelandii* (Durham et al. 1980), *P. putida* (Bull & Ballou 1981), *Rhizobium trifolii* (Chenn et al. 1984), *Norcardia erythropolis* (Kurane et al. 1984), *B. fuscum* (Whittaker et al. 1990, Earhardt et al. 1994a), *A. calcoaceticus* (Vetting et al. 1993). A *Bacillus* sp. isolated by Mashetty et al. (1996) showed the protocatechuate 3,4-dioxygenase and protocatechuate 4,5-dioxygenase activities in their cell free extracts. It followed both ortho and meta cleavage pathway for protocatechuate degradation.

Protocatechuate 3,4-dioxygenase catalyzes the intradiol cleavage of protocatechuate by incorporating two atoms of molecular oxygen to form β-carboxy *cis,cis* muconate. Enzyme activity requires the participation of a ferric ion (Ohlendorf et al. 1988, 1994).

Catechol 1,2-dioxygenase catalyzes the incorporation of oxygen to form *cis,cis*- muconate and resembles 3,4-PCD because it is a dimeric, ferric iron- containing enzyme (Nakai et al. 1995). Most catechol 1,2-dioxygenases studied are homodimers of identical subunits (Nakai et al. 1990). Conversion of 3-chlorocatechol by catechol 2, 3-dioxygenase in *Pseudomonas putida* GJ31 was identified by Mars et al. (1999). Detailed studies on catechol 1,2-dioxygenase was carried out in *A. calcoaceticus* (Patel et al. 1976), *Frateuria* sp. (Aoki et al. 1984), *P. putida* (Nakai et al. 1988) and *P. arvilla* (Nakai et al. 1990, Earhardt et al. 1994b). A catechol 1,2-dioxygenase has been purified to homogenity from *A. radioresistens* grown on phenol as the sole carbon and energy source (Briganti et al. 1997). It appears to be a homodimer, with a molecular mass of 78 kDa. The purified enzyme contains 0.96 iron (III) per unit and spectroscopic measurements suggest the presence of one high-spin iron (III) ion in an environment characteristic of intradiol cleaving enzymes.

Molecular Biology of Catechin Degradation

Studies on the genetics of tannin metabolism of microbes are very limited. A few reports are available on the genetics of PCA catabolism, which is a cleavage product of catechin.

Chakrabarthy (1972) first reported that degradative pathway associated with toluene is plasmid coded. On the involvement of plasmids in the dissimilation of aromatic substances, the researchers from our laboratory labeled them as "dissimilatory plasmids" (Balajee et al.1986).

Gajendiran and Mahadevan (1988) reported that a mega plasmid was involved in the degradation of aromatic substances in *Rhizobium* sp. Protocatechuate 3,4-dioxygenase activity and cloning of its genetic determinant from *B. japonicum* were reported by Podila et al. (1993). They identified a clone with a 2.5 kb insert from *B. japonicum* which codes for the PCD enzyme of the β-ketoadipate pathway.

The pcaD gene which codes for β-ketoadipate enol lactone hydrolase from *Agrobacterium tumefaciens* A348 was cloned into *E.coli* (Parke 1993, Parke et al. 2000). The pca structural genes are organised on two discrete regions about 4 kb apart in *A. tumefaciens.* These genes encode six enzymes that convert protocatechuate to citric acid cycle intermediates via β-ketoadipate in *A. calcoaceticus* and are present in a 11 kb genomic DNA fragment. This fragment was inserted in p*UC* 18 vector and transferred to *E. coli*. The induced cells formed the six pca gene products at levels 10 – 30 fold higher than in wild cells (Doten et al. 1987). The nucleotide sequence of pca IJFB and pca K is reported by Kowalchuck et al. (1994). In *P. cepacia,* 9.5 kb genomic DNA fragment was cloned into *E. coli*. It expressed the α and β subunits of the enzyme protocatechuate 3,4-dioxygenase (Zylstra et al. 1989a). This 9.5 kb fragment was sub cloned into the broad host range cloning vectors pRO2317, pRO2320 and pRO2321. The nucleotide sequence of the region encoding for protocatechuate 3,4-dioxygenase was determined (Zylstra et al. 1989b).

Boominathan and Mahadevan (1987) reported the presence of a very low mobility plasmid in *P. solanacearum* which was responsible for catechin catabolism. Cured cells neither utilized catechin nor its intermediates, except protocatechuic acid which was utilized significantly. Latha (1997) reported that catechin oxygenase genes are present in the genomic DNA of *Rhizobium* sp. and 10.3 kb DNA fragment of *Rhizobium* sp. was cloned in *E. coli*. The clone had the capacity to completely degrade catechin. In *Acinetobacter calcoaceticus*, catechin degradation was controlled by genomic DNA (Arunachalam 2001). A genomic library of A. calcoaceticus was constructed in p*UC* 19 vector with *Eco* RI. The clones were selected on catechin amended specific medium. To reduce the size of the insert and to be more precise, sub-cloning was done in p*UC* 19 with Hind III. The recombinant plasmid of the positive clone which showed growth on catechin amended specific medium was further characterized. It contained an insert of 3.2 kb. The insert was sequenced and was submitted to the GenBank under the accession number AF369935 (Benson et al. 2003). Based on the six frame analysis of this sequence three possible ORF's were deduced. The second and the longest ORF had a size of 1224 bp and extended from nt position 706 (ATG) to 1929 (TAA). To the best of our knowledge this is the first report on catechin oxygenase gene sequence from *A. calcoaceticus* (Arunachalam et al. 2003).

Iwagami et al. (2000) purified the protocatechuate 3,4-dioxygenase in *Streptomyces* sp. strain 2065. Moreover they cloned the pca GH genes and sequenced. These genes were formed to be part of a larger pca IJFHGBL gene cluster.

The nucleotide sequence of a 10 kb DNA segment containing the *A. calcoaceticus* cat genes has been reported earlier (Neidle et al. 1988, 1989). Hybridization between DNA fragments containing cat IJF and pca IJF suggested that they possessed region with similar nucleotide sequence (Doten et al. 1987, Shanley et al. 1986). A possible obstacle in the development of hybrid strains of *A. calcoaceticus* by the introduction of a metabolic pathway into the chromosome is genetic instability of the resulting recombinant strains. This was overcome by using new strategy by Jeong et al. (1996). They used the pobA gene as a chromosomal cloning site and inserted the catBCIJFD genes. Inserted cat genes were successfully expressed.

Future Perspective of Tannin Degradation Research

A microbial community reacts to the presence of xenobiotic organic chemicals in subtle ways depending on their structure and environment. The biological strategies played by them include co-metabolism, enzyme induction, transfer of metabolic plasmids, mutation leading to the evolution of enzymes with specificities and activities etc.

The work on the mechanism of hydrolyzable tannin degradation by different microorganism has resulted in our understanding of their biodegradation in natural environments (Field & Lettinga 1992, Lekha & Lonsane 1994, Saxena et al. 1995, Archambault et al. 1996, Lane et al. 1997).

There is also a need for increasing our knowledge about the biodegradation of condensed tannin, especially in mechanism, enzymology and genetic basis of degradation. This will lead to the overall understanding and industrial use of these tannins. It can facilitate application of

tannin-degrading enzymes or the genes encoding them in strategies for improved degradation of industrial pollutants (Field & Lettinga 1992, Hatamato et al. 1996, Selinger et al. 1996). It is appropriate to cite archaeological observations that tanned animal proteins in the form of skins and leathers persisted for extended periods under water logged conditions. Oak wood tannin might account for the recovery of Bronze Age oak coffins in Denmark and tannin might explain why samples of Roman leather were preserved in clay soil for 19 centuries although such leather readily underwent decomposition in a moist sandy soil. Flooding in fact creates anaerobic state in the soil which does not favour degradation of tannins by microbes as opposed to the observations in dry soil, where the toxic effect of tannins gradually reduced. Muthukumar (1980) after his extensive research on the effect of tannins on crop plants recommended the farmers in and around the tanneries where the soil is polluted with tannery effluents, to go for ground nut cultivation than rice as tannin degradation proceeds faster in the dry aerated soils than in the flooded rice soil and encouraged the use of urea as the fertilizer of choice since application of urea decreases the toxicity of tannins.

Concerted efforts are in progress to improve the efficiency of microbes to degrade the recalcitrant molecules in an eco friendly manner. Currently, attention is mainly focused on the construction of genetically engineered microbes (GEMs), which are capable of degrading a wide spectrum of aromatic pollutants in the environment. Knowledge on the molecular mechanism of catechin degradation is very helpful in the construction of GEMs to detoxify the tannery effluent polluted environment. This work is in an incipient stage and further studies have to be carried out to exploit the potentials of various microbes to combat other industrial pollutants.

There is little information on the bio-availability and metabolism of tannins in humans (Warden et al. 2001). Although there are links between flavonoid intake and protection against cardiovascular disease and cancer (Kavanagh et al. 2001), the evidence is not strong. Catechin affects collagen metabolism in various ways and offers anti-ulcer activity both in animal and human studies. This effect is due to catechin's ability to inhibit histidine decarboxylase, as enzyme responsible for the conversion of histidine to histamine which may have wider clinical application for allergic conditions and inflammation. Catechin is an inhibitor of procollagen and collagen biosynthesis. It has also been shown in a double blind human study to significantly reduce post operative edema. Osawa et al. (2000) reported the occurrence of lactobacilli capable of degrading hydrolysable tannins in human gut microflora and foodstuffs. Since humans do not rely entirely on tannin rich diets, the role played by these lactobacilli in human nutrition seems marginal. However, it is well known that many beverages and teas consumed by humans contain catechins with well known pharmacological activities (Chung et al. 1998). The presence of lactobacilli with distinct tannase activity in the human alimentary tract may thus have a significant effect on the medicinal properties of tannins. However, the evidences are not complete and more research is needed. In the worldwide search for microorganisms capable of degrading tannins, evaluation of rumen liquor from different sources and understanding their intricate association would offer clues about human gut biology and gut immunology.

Microbial ecology and phylogeny studies show that diverse populations of tannin tolerant bacteria can be isolated from feral livestock and wildlife. However, very little is known of these organisms, their relationship with other rumen bacteria or their mechanism of tannin resistance. Despite the fact that several catechin tolerant and some catechin degrading bacteria have been isolated from animals browsing tannin-rich forages, there is little direct evidence that these bacteria contribute to the animal's ability to utilize these feeds. However, the manipulations of rumen microflora appear to be a promising approach if exotic organisms can be isolated and do persist in the rumen and they can be good targets to fulfill the world-wide search for microbes capable of degrading tannins. More information on microbial interactions in the presence of tannins is necessary to understand and develop the potential for microbial alleviation of tannins to improve livestock productivity on tannin containing forages.

Indeed, the need of the hour is manifold. An agronomist is interested in selecting varieties which contain low tannin content coupled with enhanced nutritive value and protective action against insects and pathogens. The animal nutrition scientist is concerned with the antinutritional properties of tannins and how to minimize their deleterious effects on livestock. Those interested in human nutrition and health have to weigh up their potential antioxidant properties against possible harmful effects. The dream of environmentalists is to attain a clean and green land instead of a polluted and barren land. To a leather chemist, it is the binding of tannin and phenol that assumes greater importance. What ever be the specific interest and needs, the answer rests in circumventing the irreversible binding between tannin and protein.

The genetic design of novel metabolic routes could offer exciting possibilities for challenging the above problem. The design of regulated gene expression circuits that provide high levels of catalytic activity, even under abnormal environmental conditions that ordinarily repress expression of catabolic genes would be the future alternative.

In the present "omics" era, the rational alteration of relevant properties of proteins that qualitatively or quantitatively restrict catabolic activities through proteomics, the judicious assembly of gene block encoding selected catabolic traits through genomics, creation of novel metabolic routes and combination of routes through metabolomics and the design of tailored microorganisms exhibiting properties that contribute to most efficient process development through the tools of modern genetic engineering should be possible.

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