METABOLISM OF QUERCETIN BY A *PENICILLIUM* SP.

T. M. THOMAS[1] & J. R. L. WALKER

Department of Plant & Plant Microbial Sciences, University of Canterbury, Private Bag 4800, Christchurch, New Zealand

(Received 17 August 1993: accepted 9 December 1993)

ABSTRACT

Thomas, T.M. & Walker J.R.L. (1993). Metabolism of quercetin by a *Penicillium* sp. *New Zealand Natural Sciences* 20:63-70.

The metabolism of the flavonol, quercetin, by a *Penicillium* species isolated from local soil has been investigated. This *Penicillium* sp. exhibited maximum growth and degradation capability in static cultures at 25°C and at a substrate concentration of 2.5mM. In addition, this fungus could utilise kaempferol, quercitrin, rutin, dihydroquercetin, luteolin and fisetin. The products from the initial cleavage of quercetin were 2,4,6-trihydroxybenzoic acid and, probably, 3,4-dihydroxybenzoic acid. 2,4,6-Trihydroxybenzoic acid was metabolized further via pyrogallol. Subsequent ring cleavage of both aromatic intermediates was by *ortho* fission.

KEYWORDS: *Penicillium* sp - flavonoids - quercetin - metabolism *ortho* fission.

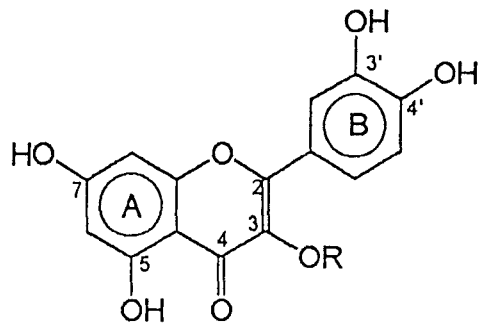
INTRODUCTION

The flavonol quercetin is widespread in higher plants, occurring predominantly as glycosylated derivatives (Harborne & Simmonds 1964; Harborne & Williams 1975; Britton 1983). However there have been relatively few studies of its degradation by micro-organisms most work having been done on its glycosides rutin and quercitrin (quercetin 3-monorhamnoside) since many microbes could not attack flavonoid aglycones, but could degrade their glycosylated derivatives. For example; naringin (naringenin 7-rhamnoglucoside) was hydrolysed to give prunin and naringenin but the latter was not metabolized further; this inability to cleave flavonoid aglycones was attributed to their relative insolubility (Cieglar *et al.* 1971). Structures of quercetin glycosides are shown in figure 1.

Micro-organisms were screened for their ability to grow on flavonoids by Westlake *et al* (1959, Westlake 1963) who found that *Aspergillus flavus* metabolised rutin and quercitrin to yield rutinose, 3,4-dihydroxybenzoic acid (DHBA, protocatechuic acid; from the B-ring) and 2,4,6-trihydroxybenzoic acid (THBA, phloroglucinol carboxylic acid; from the A-ring). The enzymes involved were studied extensively (Westlake & Simpson 1961; Westlake *et al.* 1961; Child *et al.* 1963; Simpson *et al.* 1963) and it was found that the first step was catalysed by quercetinase, a copper containing dioxygenase, which added oxygen across carbons 2 and 4 of the heterocyclic ring to produce an ester and carbon monoxide. Subsequently, several other fungi have been shown to degrade rutin and other flavonol derivatives via this pathway (Hattori & Nogchi 1959; Westlake *et al.* 1961; Westlake 1963, ; Barz 1971). This pathway appeared to be specific for flavonols and its specificity extended also to the nature of the sugar substituent(s); a specific glycosidase was induced according to the sugar(s) present.

A different pathway for quercetin breakdown was reported for a species of *Pseudomonas*; the first step being hydroxylation at C8 followed by *meta*-cleavage of the A-ring to yield eventually oxaloacetate and DHBA (Schultz *et al.* 1974; Barz & Weltring 1985). A similar A-ring hydroxylation was seen also in the degradation of taxifolin by a soil Pseudomonad (Jeffery *et al.* 1972). By contrast *Rhizobium loti* metabolized quercetin to phloroglucinol and DHBA by cleavage of the heterocyclic ring (Rao *et al.* 1991).

[1] Current address: School of Biochemistry, La Trobe University, Bundoora, Victoria 3083, Australia.



Quercetin R = H
Quercitrin R = rhamnose
Rutin R = rutinose

Figure 1. Structures of quercetin glycosides.

Once released from the parent flavonoid the smaller aromatic compounds can be broken down by *ortho*-(3-oxoadipate pathway) or *meta*-fission. The *meta*-fission pathway can also metabolize heterocyclic structures such as flavonoids (Chen & Tomasek 1991).

Despite its widespread occurrence in plants and soils the metabolism of the quercetin aglycone has not been studied extensively and was therefore the focus of this present investigation.

MATERIALS AND METHODS

Isolation and growth of flavonoid degrading micro-organisms

Enrichment culture techniques using a mineral salts medium (2 g l^{-1} KH_2PO_4, 1 g l^{-1} $(NH_4)_2SO_4$, pH 5.5) containing 0.5mM quercetin as the sole carbon source were used to select microbes capable of degrading flavonoids. After incubation for several days in the dark at 26°C, organisms were isolated using standard plating procedures and pure cultures were obtained by repeated subculture. Potato dextrose agar (PDA) was used for isolation and routine maintenance of micro-organisms. Shake cultures were grown on an orbital shaker at 300rpm and at 26°C.

Selection of isolate

Minimal salts medium (100ml) containing 0.7mM quercetin was inoculated with spore suspensions from the isolated microorganisms and also a range of fungi from the Departmental culture collection. Culture flasks (triplicate), either static or shaken, were incubated for six days and sampled daily to determine the residual concentration of quercetin. The isolate showing maximum rate of quercetin degradation was chosen for further study.

Table 1. Products of quercetin glycoside degradation by different micro-organisms.

Organism	Products			
	DHPhA[1]	DHBA	THBA	Phloroglucinol
Butyrivibrio sp C3	+			+
Eubacteria oxidoreductans	+			
Clostridium orbiscindens	+			+
Pseudomonas sp		+		
Rhizobium loti		+		+
Aspergillus flavus		+	+	
Pullularia sp.	+			+
Cryptococcus albidus & C.diffluens	+			

[1](DHPhA = 3,4-dihydroxyphenylacetic acid, DHPA = 3,4-dihydroxybenzoic acid, THBA = 2,4,6-trihydroxybenzoic acid)

FUNGAL REPLACEMENT CULTURE EXPERIMENTS

Flasks containing basal medium plus 2.5mM quercitrin or rutin were inoculated and incubated for four days. Mycelium from each flask was filtered on Whatman No.114 filter paper, washed with phosphate buffer (pH 5.5) and finally resuspended in 50ml phosphate buffer containing 2.5mM quercetin, quercitrin or rutin and incubated at 26°C for three days.

IDENTIFICATION OF METABOLIC INTERMEDIATES

Culture filtrates were extracted with ethyl acetate or n-butanol, the organic layer concentrated *in vacuo*, and the residue dissolved in 2ml methanol. These extracts were then applied to silica gel TLC plates developed in benzene:dioxane:acetic acid (BzDiAc, 90:24:4) and flavonoids detected by their fluorescence under long wave (360nm) UV light. Similarly, the formation of phenolic intermediate compounds was monitored chromatographically with the use of specific chromogenic spray reagents (Walker & Taylor 1983, Tillett & Walker 1990).

Bands of the concentrated methanolic extract were also applied to Whatman 3MM paper and developed in either BzAcW or BuAcW. After viewing the dried chromatograms under UV light (360nm), a narrow test strip was sprayed with diazotised p-nitroaniline. The remaining unsprayed zones were cut out, eluted with methanol, and analysed by UV spectroscopy (Walker & Taylor 1983, Tillett & Walker 1990).

Similarly, keto acid intermediates were isolated and chromatographed as their 2:4-dinitrophenylhydrazone (DNPH) derivatives (Walker & Taylor 1983, Tillett & Walker 1990).

UV SPECTROSCOPY

The concentration of quercetin in the culture medium was estimated spectrophotometrically; samples (0.5ml) of media were added to 4.5ml ethanol or methanol and their UV absorption spectra recorded with an HP8452A Diode Array Spectrophotometer. The characteristic bathochromic shift in alkaline solutions was used to confirm that quercetin was being analysed. Similar procedures were used for the estimation and identification of other flavonoids and phenolics. Bathochromic shifts after additions of sodium acetate and methanolic KOH were recorded.

RESULTS

ISOLATION OF QUERCETIN-DEGRADING MICRO-ORGANISMS.

Growth on quercetin in the enrichment cultures became apparent after three to four days incubation. Subsequent examination on PDA plates showed the majority of the organisms present to be fungi but also some yeasts and bacteria. Three fungi (A, B and C) predominated and were isolated into pure culture. A bacterium was also isolated. Cultures of *Fusarium solani, F. oxysporum* and *Phanerochaete chrysosporium* from the Departmental culture collection were also found to be capable of growth on quercetin.

Static cultures of fungal isolate B exhibited the highest rate of quercetin degradation (49% utilisation after 5 days) so it was chosen for further study. Colonies of this fungus on PDA plates produced slow-growing, fluffy white mycelia which eventually produced blue-green spores; it was identified as a species of *Penicillium*.

Subsequent growth experiments revealed that a substrate concentration of 2.5mM gave maximum quercetin degradation and highest growth rate. Similar experiments with other flavonoid substrates found that rutin, quercitrin, luteolin, dihydroquercetin and kaempferol were also readily metabolised by this *Penicillium* sp whilst galangin was only slightly degraded and cyanidin unaffected (Table 2). Structures of these compounds are exemplified in figure 2.

Incubation of the quercetin glycosides rutin and quercitrin with the *Penicillium* sp. resulted in an accumulation of the aglycone in the culture filtrate;

Table 2: Flavonoid substrates utilized by *Pencillium* sp.

Substrate	Approximate percentage degraded (4 days growth)
Quercetin	30
Quercitrin	100
Rutin	85
Dihydroquercetin	95
Kaempferol	100
Luteolin	95
Fisetin	65
Galangin	20
Cyanidin	0

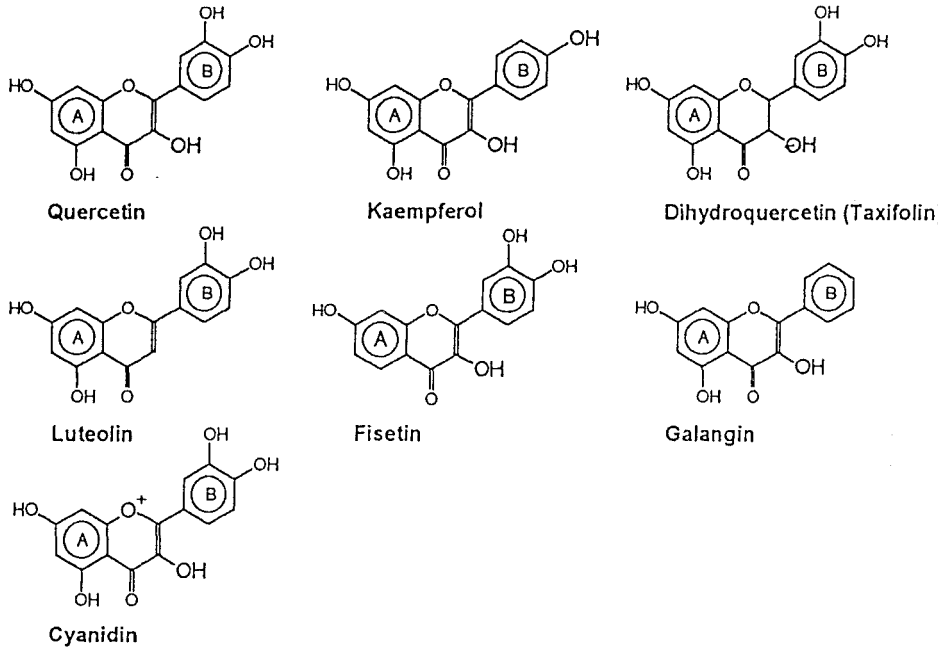


Figure 2.Flavonoid structures.

this suggested that breakdown of the quercetin might be the rate-limiting step.

REPLACEMENT CULTURE EXPERIMENTS

Quercetin metabolites could not be detected in growing cultures so replacement cultures, without added N-source, were employed to facilitate the accumulation of quercetin degradation products. These were sampled daily and the culture filtrates analysed for flavonoid and phenolic compounds by chromatography and UV spectroscopy. Using this system several phenolic compounds were detected and chromatography showed that THBA or phlorglucinol was produced from day 1 to day 3 whilst pyrogallol, and a blue fluorescent compound (unidentified), became obvious after day 3. Similar replacement experiments with putative phenolic intermediates showed that THBA was utilised faster than phloroglucinol or pyrogallol.

The band corresponding to the pyrogallol standard also yielded an identical UV spectrum. Neither spectrum changed when sodium acetate was added but after addition of KOH both solutions turned brown and showed similar bathochromic shifts.

The phloroglucinol/THBA spot was difficult to resolve but use of TLC and the BuAcW solvent system confirmed that it was the latter. It gave a spectrum comparable to that of authentic THBA; both spectra showed a slight change with sodium acetate and a dramatic bathochromic shift after addition of KOH.

ACCUMULATION OF KETO ACIDS

Replacement cultures containing 2.5 mM quercetin plus 5mM Na arsenite (an inhibitor of oxidative decarboxylation) were found to accumulate 3-oxo-adipic acid which suggests strongly that phenolic ring fission was via the *ortho*- fission pathway.

DISCUSSION

Growth and degradation of quercetin by microorganisms, including *Penicillium* sp. was generally slow and this may be related to the low solubility of the substrate. However the rate of degradation increased after several days once mycelial growth was apparent which suggests that the fungus required time to adapt and induce the degradative enzymes needed to produce more readily utilizable metabolites.

Static cultures supported the best growth and degradation characteristics for all organisms tested. This was in contrast to flavonoid decomposition by yeasts which required a high level of aeration and a nutritionally complex medium although glycosides were degraded in a simple synthetic medium (West-

lake & Spencer 1966). Similarly, *Pullularia fermentans* only metabolized quercetin when sucrose, rutinose or nucleic acids were present in the medium (Noguchi 1963); by contrast our *Penicillium* sp. degraded quercetin in a minimal salts media.

Utilization of other flavonoid compounds by our *Penicillium* sp. may reflect the structural specificity of the degradative enzymes; thus kaempferol was totally degraded whilst utilization of galangin (no B-ring -OH groups) and quercetin (two B-ring -OH groups) was comparatively low. From these results it seems that the pattern of B-ring hydroxylation was important since it is unlikely that subsequent degradation of the heterocycle cleavage product, thought to be DHBA, will be rate-limiting.

Absence of the A-ring C5 hydroxyl group, as in fisetin, decreased the extent of degradation whilst the ready utilisation of luteolin and dihydroquercetin suggests that the C3 hydroxyl group or the C2-C3 double bond were not important for recognition or degradation of these structures.

Addition of sugars to the C3 hydroxyl group of quercetin increased the percentage of substrate degraded since glycosylation enhanced water solubility. However both quercitrin and rutin are also only slightly soluble, so solubility was probably not the only reason for the observed increase in catabolism. Different sugar substuents may affect degadability since it appears that removal of the glycoside moieties usually preceded attack on the flavonoid aglycone (Westlake *et al.* 1959). Therefore, since quercetin accumulated in culture filtrates of *Penicillium* sp. grown on rutin or quercitrin, the first degradative steps must have involved removal of the sugars. This could lead to accumulation of flavonoid aglycones in the environment thus creating a significant role for flavonoid-metabolising microbes.

Quercitrin (3-rhamnoside) was degraded more rapidly than rutin (3-rutinoside); both compounds contain rhamnose but rutin also has a glucose group attached to the C3 hydroxyl and the *Penicillium* sp. glucosidase may be less active than its rhamnosidase. However, in *A.flavus*, rutinose was cleaved from rutin and was not degraded further (Westlake *et al.* 1959).

Metabolic intermediates

Putative intermediates in the quercetin breakdown pathway were identified in several ways. Pyrogallol was identified from its UV spectra and chromatographically from its Rf value and characteristic colour with diazotized *p*-nitroaniline.

The extra bands which appeared on chromatograms were separated and analyzed spectroscopically. It was thought that the yellow fluorescing band was an early cleavage product because its spectrum was very like that of quercetin and a similar, but slightly different, bathochromic shift was observed in KOH; this band was not characterised further.

THBA proved more difficult to identify since only low levels accumulated in the culture medium and it ran very close to phloroglucinol on chromatograms developed in BzAcW. The compound was also unstable, even when stored at 4°C. Phloroglucinol was also utilized by the quercetin-induced enzyme system whereas pyrogallol was only slowly removed from the medium over four days.

From these experimental results, a tentative pathway (Fig. 3) for quercetin break down by *Penicillium* sp. is proposed with the initial cleavage of the heterocylic ring yielding THBA, and probably DHBA also. Whilst the former was isolated from the culture medium, the latter could not be detected; probably because it is a commmon starting point for both *ortho-* annd *meta*-ring fission pathways and was metabolised as fast as it was formed.

Conversion of THBA to pyrogallol could proceed via either of the two routes shown. Pyrogallol may have been formed in a single step involving the loss of CO_2 and the C4 hydroxyl group or via a phloroglucinol intermediate but phloroglucinol could not be identified by UV spectrophotoscopy or chromatography; however it was slowly metabolised by the quercetin degrading enzyme system.

The initial products of quercetin cleavage are identical to those reported in earlier studies with *A. flavus* (Westlake *et al.* 1959; Westlake 1963) but these workers did not investigate their subsequent metabolism. Similar metabolites were reported also in studies with a *Pullularia* species, *Cryptococcus albidus* and *C. diffluens* (Westlake & Spencer 1966). Thus it appears that this flavonol-cleavage pathway is common in fungi.

Metabolism of 2,4,6-trihydroxybenzoic acid

Several studies have provided evidence for THBA breakdown by microorganisms. Schink and Pfennig (1982) found that the strict anaerobe *Pelobacter acidigallici* metabolised THBA, pyrogallol, phloroglucinol and gallic acid (3,4,5-trihydroxybenzoic

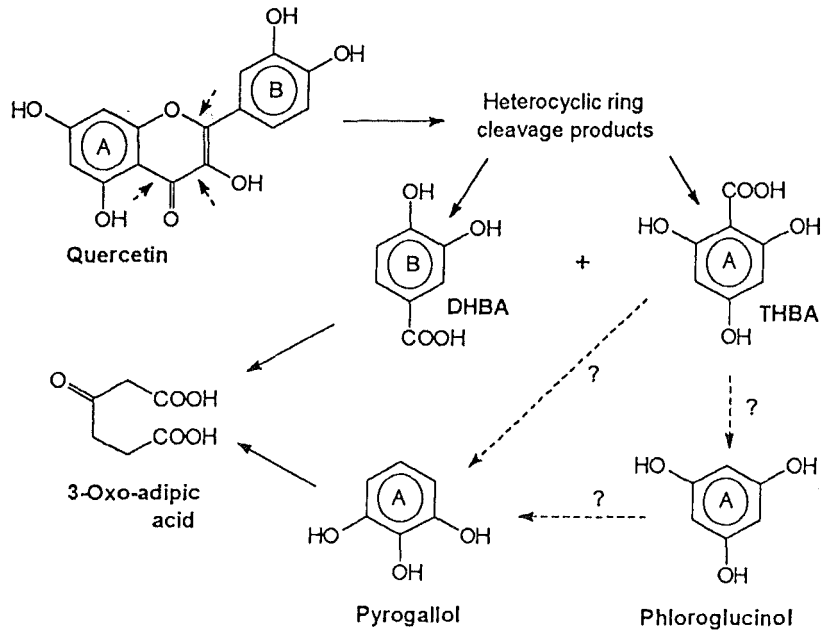


Figure 3. Tentative pathway for the metabolism of quercetin by a *Penicillium* sp.

acid) via phloroglucinol. THBA and DHBA were produced from catechin by the rhizosphere bacterium *Bradyrhizobium japonicum* (Hopper & Mahadevan 1991) and which converted THBA to phloroglucinol; the latter being metabolised via resorcinol to hydroxy-quinol and thence by *ortho*-fission to 3-oxo-adipic acid. This pathway for phloroglucinol metabolism was found also in *Penicillium simplicissimum* (Patel *et al.* 1990) whereas *Azotobacter vinelandii* converted phloroglucinol to resorcinol and thence to pyrogallol (Groseclose & Ribbons 1981). However, neither resorcinol nor hydroxyquinol were detected in our *Penicillium* sp. culture filtrates. Metabolism of phloroglucinol via pyrogallol, without detectable resorcinol intermediates, was reported in *Fusarium solani* (Walker & Taylor 1983).

Pyrogallol metabolism and mode of ring fission

The utilization of pyrogallol by microbes has not been studied in detail. Mills *et al.* (1981) compared growth of yeasts on aromatic substrates and found that none of the yeasts tested could grow on pyrogallol. A non-pigmented mutant strain of *Penicillium patulum* was found to produce large quantities of pyrogallol (Tanenbaum & Bassett 1958) whilst another *Penicillium* sp. accumulated pyrogallol when grown on gallotannin medium (Yoshida *et al.* 1982).

Anaerobic bacteria, such as *P. acidigallici*, *Pelobacter massiliensis* (Schnell *et al.* 1991) and *Eubacterium oxidoreducens* (Krumholtz & Bryant 1988) appear to convert pyrogallol to acetate via phloroglucinol. The pyrogallol-phloroglucinol isomerase of *E. oxidoreducens* was characterized and a conversion mechanism proposed (Krumholtz & Bryant 1988). Thus it is possible that this, or a similar phloroglucinol to pyrogallol interconversion, may operate in *Penicillium* sp; however, this would depend on favourable thermodynamics and enzyme kinetics for the reverse reaction.

Groseclose and Ribbons (1981) and Walker and Taylor (1983) demonstrated that pyrogallol could be cleaved by *meta* fission. However, in the present study, only evidence for *ortho* fission was encountered; thus it was concluded that pyrogallol and DHBA both underwent *ortho* fission. It is possible that, in our *Penicillium* sp., pyrogallol ring cleavage could be a rate-limiting step for quercetin break-

down if high concentrations of pyrogallol inhibited the enzymes involved early on in quercetin breakdown.

ACKOWLEDGEMENTS

The authors wish to thank Dr A. L. J. Cole for mycological advice, Mrs J. Healy for technical assistance and the University of Canterbury for provision of research facilities.

REFERENCES

Barz, W. (1971). Metabolism of aromatic compounds by *Fusarium oxysporum* Schlecht. *Archiv fur Mikrobiologie* 78: 341.

Barz, W. & Weltring, K.M. (1985) Biodegradation of aromatic extractives of wood. In *Biosynthesis and Biodegradation of Wood Components* (ed. T. Higuchi), pp 607-666. Academic Press, Florida.

Britton, G. (1983). The Biochemistry of Natural Pigments. pp 102-113, 124-127. Cambridge University Press, Cambridge.

Chen, C.M. & Tomasek, P.H. (1991). 3,4-Dihydroxyxanthone dioxygenase from *Arthrobacter* Sp Strain Gfb100. *Applied and Environmental Microbiology* 57: 2217-2222.

Child, J.J., Simpson, F.J. & Westlake, D.W.S. (1963). Degradation of rutin by *Aspergillus flavus* - production, purification and characterization of an esterase. *Canadian Journal of Microbiology* 9: 653-664.

Ciegler, A., Lindenfelser, L.A. & Nelson, G.E.N. (1971). Microbial transformation of flavonoids. *Applied Microbiology* 22: 974-979.

Groseclose, E.E. & Ribbons, D.W. (1981). Metabolism of resorcinylic compounds by bacteria: new pathway for resorcinol catabolism in *Azotobacter vinelandii*. *Journal of Bacteriology* 146: 460-466.

Harborne, J.B. & Simmonds, N.W. (1964). The Natural Distribution of the Phenolic Aglycones. In *Biochemistry of Plant Phenolics* (ed. Harborne), pp 77-128. Academic Press, New York.

Harborne, J.B. & Williams, C.A. (1975). Flavone and flavonoid glycosides. In *The Flavonoids* (ed. Harbourne, Mabry, Mabry), pp 376-441. Chapman and Hall, London.

Hattori, S. & Noguchi, I. (1959). Microbial degradation of rutin. *Nature* 184: 1145-1146.

Hopper, W. & Mahadevan, A. (1991). Utilization of catechin and its metabolites by *Bradyrhizobium japonicum*. *Applied Microbiology and Biotechnology* 35: 411-415.

Krumholz, L.R. & Bryant, M.P. (1988). Characterization of the pyrogallol-phloroglucinol isomerase of *Eubacterium oxidoreducens*. *Journal of Bacteriology* 170: 2472-2479.

Mills, C., Child, J.J. & Spencer, J.F.T. (1971). Utilization of aromatic compounds by yeasts. *Antonie van Leeuwenhoek* 37: 281-287.

Noguchi, N. (1963). Degradation of flavonols by *Pullularia fermentans* var. candida. *Botanical Magazine (Tokyo)* 76: 191-198.

Patel, T.R., Hameed, N. & Martin, A.M. (1990). Initial steps of phloroglucinol metabolism in *Penicillium simplicissimum*. *Archives of Microbiology* 153: 438-443.

Rao, J.R., Sharma, N.D., Hamilton, J.T.G., Boyd, D.R. & Cooper, J.E. (1991). Biotransformation of the pentahydroxy flavone quercetin by *Rhizobium loti* and *Bradyrhizobium* Strains. *Applied and Environmental Microbiology* 57: 1563-1565.

Schink, B. & Pfennig, N. (1982). Formation of trihydroxybenzenes by *Pelobacter acidigallica* gen. nov. sp. nov., a new strictly anaerobic, non-sporeforming bacterium. *Archives of Microbiology* 113: 195-201.

Schultz, E., Engle, F.E. & Wood, J.M. (1974). New oxygenases in the degradation of flavones and flavanones by *Pseudomonas putida*. *Biochemistry* 13: 1768-1776.

Simpson, F.J., Narasimhachari, N. & Westlake, D.W.S. (1963). Degradation of Rutin by *Aspergillus flavus*: The carbon monoxide producing system. *Canadian Journal of Microbiology* 9: 15-25.

Tillett,R. & Walker,J.R.L. (1990). Metabolism of ferulic acid by a *Penicillium* sp. *Archives of Microbiology* 154;206-208.

Walker, J.R.L. & Taylor, B.G. (1983). Metabolism of phloroglucinol by *Fusarium solani*. *Archives of Microbiology* 134: 123-126.

Westlake, D.W.S. (1963). Microbiological degradation of quercitrin. *Canadian Journal of Microbiology* 9: 211-220.

Westlake, D.W.S., Roxburgh, J.M. & Talbot, G. (1961). Microbial production of carbon monox-

ide from flavonoids. *Nature* 189: 510-511.

Westlake, D.W.S. & Simpson, F.J. (1961). Degradation of rutin by *Aspergillus flavus*. Factors Affecting Production of the Enzyme System. *Canadian Journal of Microbiology* 7: 33-44.

Westlake, D.W.S. & Spencer, J.F.T. (1966). Utilization of flavonoid compounds by yeast and yeast-like fungi. *Canadian Journal of Microbiology* 12: 165-174.

Westlake, D.W.S., Talbot, G., Blakley, E.R. & Simpson, F.J. (1959). Microbial decomposition of rutin. *Canadian Journal of Microbiology* 5: 621-629.

Yoshida, H., Tani, Y. & Yamada, H. (1982). Isolation and identification of a pyrogallol producing bacterium from soil. *Agricultural and Biological Chemistry* 46: 2539-2546.