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Degradation of Rutin by *Thermoactinomyces vulgaris* and Other Thermophilic Compost Isolates

Chao-Hsun Yang,* Yu-Chun Huang, and Cheng-Yu Chen

Department of Cosmetic Science, Providence University, 200 Chung-Chi Road, Shalu, Taichung, 43301, Taiwan

The mutagenic effects of rutin and quercetin have aroused the interest of many investigators. To develop the microbial degradation of rutin, a thermophilic actinomycete, which could hydrolyze rutin, was isolated from compost soil. The taxonomical characteristics of this thermophilic actinomycete were examined and identified as *Thermoactinomyces vulgaris* PU18-2. After cultivation of *T. vulgaris* PU18-2 in the rutin-CYC medium for 60 h, the culture filtrate had a rutin-degrading ability, but the cell-free extract did not. There was no quercetin, rutinose, rhamnose, and glucose accumulated in the rutin hydrolysate of the culture filtrate. Both α-rhamnosidase and β-glucosidase activities were not found in the culture filtrate of the *T. vulgaris* PU18-2 in the rutin-CYC medium. These results showed that the initial attack on rutin by the extracellular enzymes of *T. vulgaris* PU18-2 apparently was not through the glycosidase-mediated hydrolysis of glycosidic bond.

KEYWORDS: Rutin; quercetin; *Thermoactinomyces vulgaris*; degradation

INTRODUCTION

Flavonoids are a large group of natural polyphenols. Rutin, a kind of polyphenol, is a yellow or yellow-green-colored needle-shaped crystal that is found in buckwheat, citrus fruits, black tea, apple peels, and oats (*1*). Rutin is a glycoside comprised of the quercetin (3,5,7,3′,4′-pentahydroxyflavone) and the disaccharide rutinose (rhamnose and glucose). Rutin is normally metabolized into its aglycone, quercetin.

There are many reports concerned with the mutagenic effects of rutin and quercetin (*2*). Rutin has been linked with cellular mutation. Yu et al. reported that the mutagenic component in red wine was purified and identified as rutin (*3*). Wang et al. reported that quercetin was mutagenic in *Salmonella typhimurium* TA98 and TA100 strains (*4*). Quercetin showed mutagenic activity in the absence of liver-mediated metabolism; however, mutagenic activity was approximately tripled in the presence of liver microsomes (*5*−*7*). The mutagenicity of quercetin was of the same order of magnitude as that of *o*-aminoazotoluene and 4-aminobiphenyl with *S. typhimurium* TA98 and 3′-methyl-4-dimethylaminoazobenzene with *S. typhimurium* TA100 (*8*). Quercetin was also reported to be mutagenic to *Salmonella* TA102 tester strain in the Ames *Salmonella* microsome/mutagenicity assay in the presence of rat liver S9 mix (*9*). Quercetin has been demonstrated to have significant effects on DNA synthesis, lactate production, and cyclic adenosine 3′:5′-monophosphate levels in neoplastic cells (*10*). For quercetin, the metabolic pathway for activation to DNA-reactive species may include enzymatic and/or chemical oxidation of quercetin to quercetin *ortho*-quinone, followed by isomerization of the *ortho*-quinone to quinine methides. These quinine methides are suggested to be the active alkylating DNA-reactive intermediates. Recent results have demonstrated the formation of quercetin DNA adducts in exposed cells in vitro (*11*, *12*). Boots et al. also described that quercetin-induced toxicity is indeed caused by the formation of thiol-reactive oxidative products of quercetin (*13*).

In 1999, the International Agency for Research on Cancer (IARC) concluded that quercetin is not classified as carcinogenic to humans. In the United States and Europe, supplements of quercetin are commercially available (*14*). The dose levels in long-term animal studies at which no toxicologically significant adverse effects were reported support the addition of food-grade quercetin to foods at levels resulting in exposure estimates approximating the intakes of naturally occurring quercetin from the diet by consumers with a high fruit and vegetable intake (i.e., 200−500 mg/day) (*15*). However, the use of supplements, particularly antioxidant formulas and herbal mixtures that are commonly recommended in terms of gram rather than milligram doses, could result in exposure to potentially toxic levels (*16*).

It has been reported that quercetin and its glycoside, rutin, are degraded by microorganisms, especially fungi such as *Aspergillus flavus*, *Aspergillus niger* (*17*), *Penicillium decumbens* (*18*), and *Penicillium rugulosum* (*19*). Streptomycetes are widespread soil actinomycetes, which play important roles in the decomposition of biopolymers such as lignin, cellulose, hemicellulose, chitin, keratin, and pectin (*20*). The thermophilic actinomycetes are of particular interest because they produce a variety of thermostable enzymes involved in the degradation process (*21*−*25*).

Because rutin and quercetin are potentially mutagenic, it would be prudent to degrade these components from dietary sources before human consumption. The streptomycetes are reported to have a strong biodegrading capability for a variety of compounds; yet, their degradation potential of flavonoids has not been extensively investigated. The purpose of this study is to

*To whom correspondence should be addressed. Tel: +886-4-26311167. Fax: +886-4-26311167. E-mail: chyang@pu.edu.tw.

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screen potent strains for high rutin-degrading abilities. The mechanisms of biodegradation by the strains are also discussed. Results of this investigation have implications for food safety and human health.

MATERIALS AND METHODS

Samples and Microorganisms. About 100 agricultural waste compost soil samples collected in Taiwan were used as sources of thermophilic microorganisms. *Thermobifida alba* ATCC 27644, *Thermomonospora curvata* ATCC 19995, and *Laceyella sacchari* ATCC 14570 were obtained from Bioresource Collection and Research Center (Taiwan). *Thermobifida fusca* NTU22 was isolated and stored in our laboratory (*22*).

Materials. Czapek-dox powder, yeast extract, casamino acids, and agar were obtained from BD Diagnostic Systems (Sparks, MD). Inorganic salts, gelrite gellan gum (Gelrite) (*26*), rutin, quercetin, and all other chemicals were purchased from Sigma (St. Louis, MO). The chemical structures of rutin and quercetin are shown in **Figure 1**. Rutinose was purchase from Extrasynthese (Genay Cedex, France).

Isolation of Thermophilic Actinomycetes. Ten grams of soil sample collected in Taiwan was suspended in sterile water (pH 7.0) supplemented with 6% yeast extract and 0.05% sodium dodecyl sulfate and incubated at 65 °C for 30 min. Suitable dilutions were spread on CYC Gelrite plates consisting of 33.3 g of Czapek-dox powder, 2.0 g of yeast extract, 6.0 g of casamino acids, 11.0 g of Gelrite, and 0.1 g of $CaCl_2$ per liter of distilled water (pH 7.3). Actinomycete-like colonies were selected from the plate following incubation for 4 days at 65 °C. First, 70 strains of thermophilic actinomycetes were isolated on the CYC Gelrite plates. Then, the thermophilic actinomycetes were cultured in 50 mL of rutin-CYC medium consisting of 1.0 g of rutin, 33.3 g of Czapek-dox powder, 2.0 g of yeast extract, and 6.0 g of casamino acids per liter of distilled water (pH 8.0) in a 500 mL Hinton flask. Cultivation was conducted in a reciprocal shaker that was shaken (125 rpm) for 4 days at 50 °C. The culture broth was centrifuged at 10000*g* for 30 min at 4 °C, and then, the rutin was retained in the broth tested by high-performance liquid chromatography (HPLC).

Taxonomical Investigations. Identification was conducted mainly according to Bergey's Manual of Determinative Bacteriology (*20*). Amino acid and sugar analysis of the whole-cell hydrolysate was done as described by Konagata and Suzuki (*27*). Fatty acid esters were obtained from 40 mg of wet cells by saponification, methylation, and extraction by the method of Miller (*28*). The fatty acid methyl ester mixtures were separated by a 5890 series II gas chromatography (Hewlett-Packard, Wilmington, DE) with capillary column. For 16S rDNA sequencing, the genomic DNA of tested strain was amplified by polymerase chain reaction (PCR) (*29*). Purified PCR products were directly sequenced by using the MicroSeq 16S rDNA Gene Kit from Applied Biosystems (Foster City, CA). The sequence was manually aligned with other bacteria from the GenBank database.

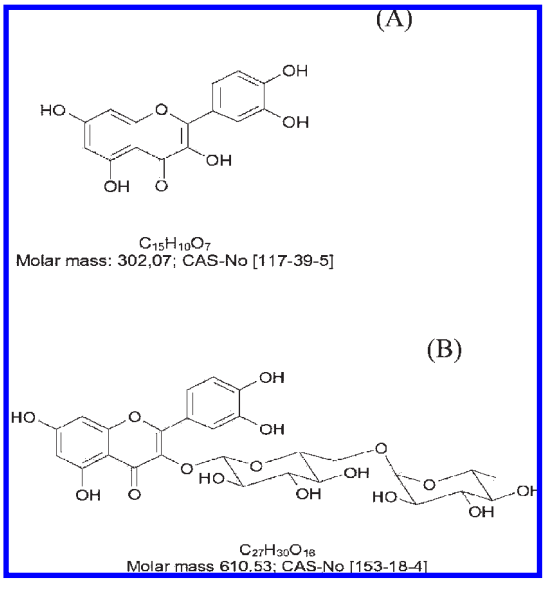

Figure 1. Chemical structure of rutin and quercetin: (**A**) quercetin and (**B**) rutin.

Biomass. Biomass (mg/L) was measured by dry cell weight estimation involving filtration of broth samples through predried and weighted #2 filter paper (Whatman Ltd., Maidstone, United Kingdom). The biomass was then washed, dried (at 60 °C oven), cooled in a desiccator, and weighed.

Preparation of Culture Filtrate and Cell-Free Extract. After cultivation of the strain in the rutin-CYC medium for 60 h, 100 mL of culture broth was centrifuged at 10000*g* for 30 min at 4 °C, and the supernatant was used as the culture filtrate preparation. The cells were harvested after centrifugation and washed with 2 volumes of 100 mM Tris-HCl buffer (pH 8.0). Wet cells were resuspended in 100 mL of 100 mM Tris-HCl buffer (pH 8.0). The suspension was disrupted with a Soniprep 150 sonicator (MSE Sci. Inc., Leicestershire, England) for 5 min at 50 W. The debris was removed by centrifugation at 12000*g* for 20 min. The supernatant liquid was used as a cell-free extract.

HPLC. The rutin and quercetin retained in the broth were determined by HPLC (Agilent 1100 series, United States) using a pre-packed column RT 250-4.6 Mightysil RP-18 GP 5 mm (4.6 nm × 250 nm, Kanto Chemical, Tokyo, Japan) column and 340 nm UV detector. A mobile phase composed of methanol:acetic acid:water (35:10:55) was used as the eluant at a flow rate of 1.0 mL/min. Rutinose, glucose, and rhamnose were also determined by HPLC using a Lichrospher NH_2 (4.5 nm × 250 nm, MetaChem Technologies Inc., Torrance, CA) column and refractive index detector. A mobile phase composed of acetonitrile and water (70:30) was used as the eluant at a flow rate of 1.0 mL/min.

Enzyme Activities Analysis. The α-L-rhamnosidase activity was determined using *p*-nitrophenyl-α-L- rhamnopyranoside (pNP-R) as the substrate (*18*). Fifty microliters of properly diluted sample was added in 200 μL of 1 mM substrate solution in 100 mM Tris-HCl buffer, pH 8.0. The release of *p*-nitrophenol was measured at 410 nm in a Microplate Spectrophotometer (μQuant, BioTek, Winooski, VT) at 37 °C. The δ-D-glucosidase activity was determined using *p*-nitrophenyl-β-D-glucopyranoside (pNP-G) as the substrate (*18*). Fifty microliters of properly diluted sample was added to 200 μL of 1 mM substrate solution in 100 mM Tris-HCl buffer, pH 8.0. The release of *p*-nitrophenol was measured at 410 nm in a Microplate Spectrophotometer (μQuant, BioTek) at 37 °C.

Statistical Analysis. All analytic measurements were performed at least in triplicate.

RESULTS AND DISCUSSION

From the approximately 100 soil samples, 70 strains of thermophilic actinomycetes were isolated first on CYC Gelrite plates at 65 °C. These isolated strains were then cultured in rutin-CYC plate for 4 days at 50 °C. This allowed for the selection of eight strains that showed superior ability to decompose rutin. Among these eight strains, strain 18-2 had the best rutin-degrading ability and was therefore selected for further analysis (**Figure 2**). When strain 18-2 was cultured in CYC medium with 1.64 mM rutin, we found that the rutin gradually reduced during the 48 h period following cultivation (**Figure 3**). Forty-eight hours after cultivation, rapid consumption of the rutin occurred during the growth of the culture.

The biological characteristics of strain 18-2 are summarized in **Table 1**. The whole-cell hydrolysate of strain 18-2 contains meso-diamino-pimelic acid but no diagnostic sugars. The fatty acid pattern of the strain contains mainly iso-C-4 and anteiso-C-4 fatty acids. **Figure 4** shows the scanning electron micrograph of strain 18-2. When these characteristics are compared with the descriptions in Bergey's Manual of Determinative Bacteriology, strain 18-2 resembles a number of thermophilic actinomycetes, especially the genus *Thermoactinomyces*. Comparing the 16S rDNA sequence with the GenBank database, the greatest similarity is found between strain 18-2 (accession number EU998647) and *Thermoactinomyces vulgaris*. Therefore, the thermophilic actinomycete strain 18-2 was identified as *T. vulgaris* PU18-2.

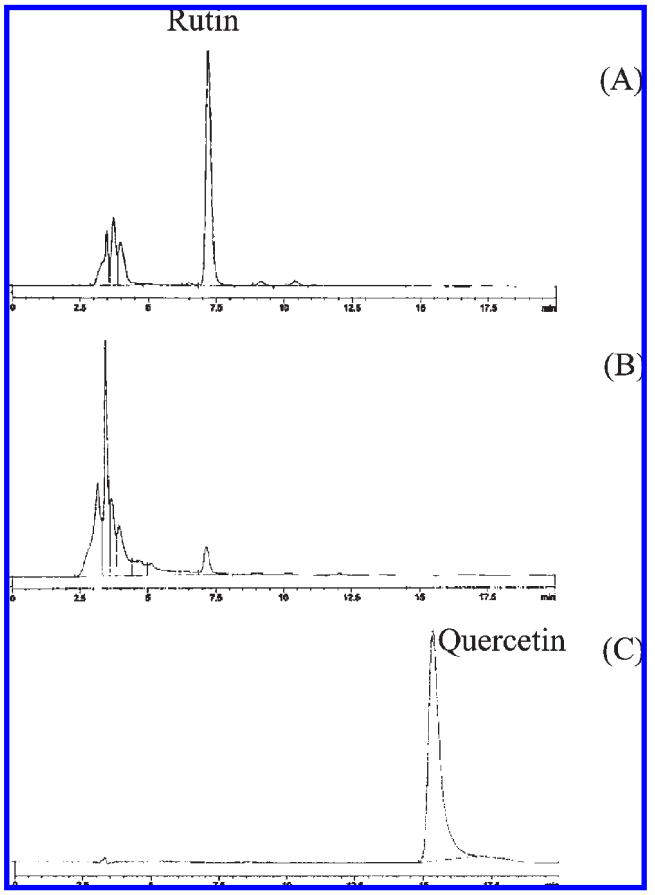

Figure 2. HPLC of rutin degradation by strain 18-2. (**A**) 1.64 mM rutin, (**B**) 1.64 mM rutin was degraded by strain 18-2 for 96 h at 50 °C, and (**C**) 1.64 mM quercetin.

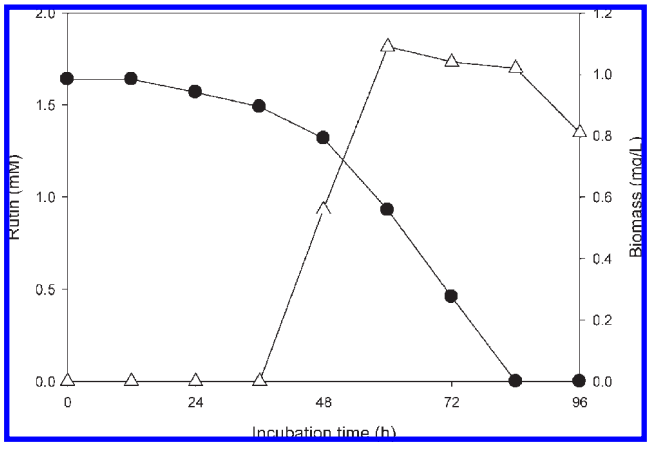

Figure 3. Time course for rutin degraded by strain 18-2. Rutin concentration, ●; and biomass, △. The reaction conditions were as follows: 100 mL of rutin-CYC medium in a 500 mL Hinton flask; shaking speed, 100 rpm; temperature, 50 °C; initial pH, 8.0; and culture time, 96 h. The rutin remaining in the broth was determined by HPLC.

The optimal cultural conditions in a 500 mL Hinton flask loading with 100 mL of rutin-CYC medium have been found to be as follows: shaking speed, 100 rpm; temperature, 50 °C; and initial pH, 8.0. After 60 h of cultivation with optimal conditions, the biomass of *T. vulgaris* PU18-2 was 1.09 mg/L (**Figure 3**). Similar results are obtained by applying these conditions to both CYC medium and rutin-CYC medium. The strain could not grow well in the mineral medium [1.0 g of yeast extract, 2.0 g of $(NH_4)_2SO_4$, 13.6 g of KH_2PO_4, 0.2 g of $MgCl_2$, and 11.0 mg of $CaCl_2$ per liter of distilled water at pH 8.0] with 1.64 mM rutin.

Table 1. Bacterial Characteristics of Strain 18-2[a]

characteristic	strain 18-2
colony color	white
growth temperature (°C)	30−55
decomposition of	
casein	+
adenine	−
xanthine	−
hypoxanthine	−
urea	+
esculin	+
L-tyrosine	−
starch	−
production of	
melanin	−
nitrate reductase	−

[a] Key: +, positive reaction; and −, negative reaction.

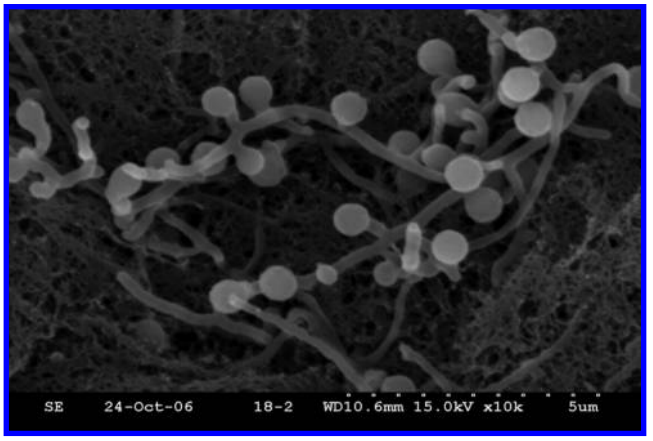

Figure 4. Scanning electron micrograph of strain 18-2.

After the cultivation of *T. vulgaris* PU18-2 in the rutin-CYC medium for 60 h, both the culture filtrate and the cell-free extract were prepared and used for the rutin-degrading ability test. The reaction mixture contained 1.64 mM rutin, 0.5 mL of culture filtrate or cell-free extract, and 100 mM Tris-HCl buffer (pH 8.0). The total reaction volume was 1.0 mL. After incubation for 2 h at 50 °C, the reaction was stopped by chilling the mixture in ice followed by centrifugation at 3000*g* for 1 min. The rutin remaining in the broth was determined. As shown in **Figure 5**, we found that the cell-free extract could not degrade rutin. The culture filtrate had the ability to degrade rutin in the first 6 h. After 6 h, the rutin was no longer degraded in the broth. This result suggests that *T. vulgaris* PU18-2 has secreted enzymes that degrade rutin in the broth and that the enzymes might be feedback inhibited by the hydrolysate in the reaction broth. The reaction broth was then further analysized by HPLC, and no quercetin, rutinose, rhamnose, and glucose accumulation was found. There was no α-rhamnosidase and β-glucosidase activity in the culture filtrate of the *T. vulgaris* PU18-2 in the rutin-CYC medium. From these results, we concluded that the initial attack on rutin by the extracellular enzymes of *T. vulgaris* PU18-2 was not hydrolysis of the glycosidic bond by glycosidase.

To understand if the rutin-degrading ability is a common property of thermophilic actinomycetes, we examined the strains collected in our laboratory and culture collections. As shown in **Figure 6**, only *T. vulgaris* PU18-2 and *L. sacchari* ATCC 14570 can degrade rutin. Other strains including *T. fusca* NTU22, *T. alba* ATCC 27644, and *T. curvata* ATCC 19995 cannot.

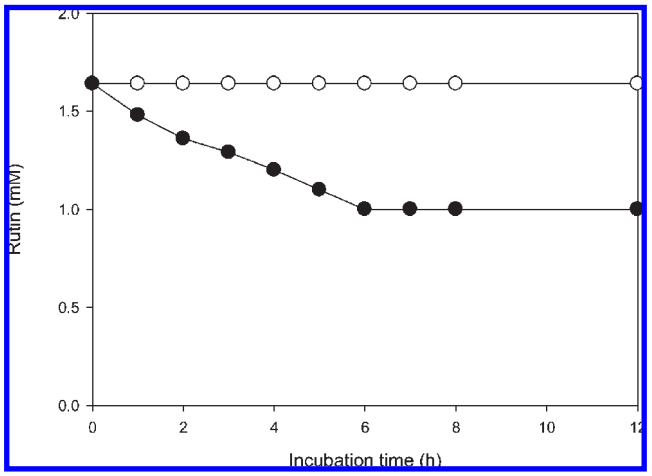

Figure 5. Time course of the rutin degradation by the culture filtrate or cell-free extract from strain 18-2. Culture filtrate, ●; and cell-free extract, ○. The reaction mixture consisted of 1.64 mM rutin, 0.5 mL of culture filtrate or cell-free extract, and 100 mM Tris-HCl buffer (pH 8.0). The total reaction volume was 1.0 mL. The reaction conditions were 50 °C for 12 h. The rutin remaining in the broth was determined by HPLC.

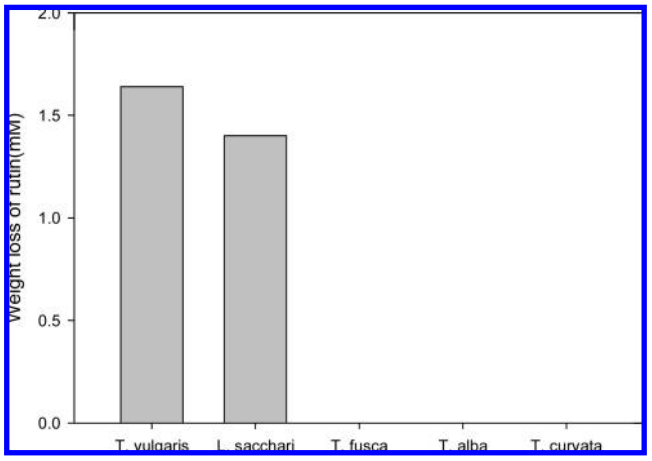

Figure 6. Comparison of the rutin-degrading ability of *T. vulgaris* PU 18-2 with other related species of thermophilic actinomycetes. The reaction conditions were as follows: 100 mL of rutin-CYC medium in a 500 mL Hinton flask; shaking speed, 100 rpm; temperature, 50 °C; initial pH, 8.0; and culture time, 96 h. The weight loss of rutin was determined by HPLC.

Quercetin and quercetin glycosides are absorbed from the small intestine of humans. Quercetin 4′-glucoside can be rapidly deglycosylated by cell-free extract from human small intestine and liver, but the enzyme from the extract cannot hydrolyze rutin (*30*). Keppler et al. found that rutin could be deglycosylated by the microflora in the cecum, and aglycone quercetin is absorbed from the cecum and colon (*31*).

There were two possible routes for the degrading of rutin. Rutin could be deglycosylated by glycosidase and then form phloroglucinol carboxylic acid by ring splitting enzyme and esterase (route I). Alternatively, rutin might be cleaved directly at the heterocyclic ring (route II) (*17*).

Almost all of the microorganisms tested degrade rutin with glycosidase first. *A. flavus* when grown on rutin excretes an inducible glycosidase, rutinase, which hydrolyzes rutin, and an inducible oxygenase, quercetinase, which oxidatively cleaves the heterocyclic ring of the aglycone, quercetin, to yield carbon monoxide and depside, 2-protocatechuoylphloroglucinolcarboxylic acid (*32*). Mamma et al. reported that *P. decumbens* could produce α-rhamnosidase and β-glucosidase (*18*). Both of them could degrade rutin to quercetin, rhamnose, and glucose. The quercetin might then be degraded by a monoxygenase (quercetinase). A β-rutinosidase, which specifically released the disaccharide rutinose from rutin, was isolated and purified from *P. rugulosum* IFO7242. The enzyme had a molecular mass of 245 kDa, a very low optimum pH of 2.2, and a remarkable specificity that the glycosidase could only hydrolyze rutin and isoquercitrin but not any other substrate like 4-nitrophenyl β-glucopyranoside and cellobiose (*19*). It should be concluded from these observations that there are two possible pathways for microorganisms to degrade rutin with route I. They can hydrolyze rutin by β-rutinosidase or by α-rhamnosidase and β-glucosidase. With route I, quercetin, rutinose, rhamnose, and glucose can be found in the broth of the rutin hydrolysate.

In this study, the culture filtrate of *T. vulgaris* PU18-2 in the rutin-CYC medium could hydrolyze rutin, but there was no quercetin, rutinose, rhamnose, or glucose in the reaction solution. There was also no α-rhamnosidase and β-glucosidase activity found in the culture filtrate. These results showed that the initial attack on rutin by the extracellular enzymes of *T. vulgaris* PU18-2 apparently was not the hydrolysis of the glycosidic bond by glycosidase. Our conclusion is that *T. vulgaris* PU18-2 degrades rutin through route II.

When microorganisms degrade rutin through route I, they produce quercetin in the broth. If quercetin is not further degraded, it could be a serious health threat. *T. vulgaris* PU18-2 degrades rutin through route II and can degrade rutin directly with no quercetin remaining in the broth. It therefore avoids potential mutagenic effects. Little is known about the ability of microorganisms to degrade rutin without glycosidase. Further work on the purification of rutin-degrading enzymes, as well as the enzymatic degradation of rutin and quercetin, is needed to clarify this research.

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