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ORIGINAL PAPER

# Novel metabolic pathway for salicylate biodegradation via phenol in yeast *Trichosporon moniliiforme*

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**Abstract** A novel metabolic pathway was found in the yeast *Trichosporon moniliiforme* WU-0401 for salicylate degradation via phenol as the key intermediate. When 20 mM salicylate was used as the sole carbon source for the growth of strain WU-0401, phenol was detected as a distinct metabolite in the culture broth. Analysis of the products derived from salicylate or phenol through reactions with resting cells and a cell-free extract of strain WU-0401 indicated that salicylate is initially decarboxylated to phenol and then oxidized to catechol, followed by aromatic ring cleavage to form *cis-cis* muconate.

**Keywords** Biodegradation · Metabolic pathway · Phenol · Salicylate · *Trichosporon moniliiforme*

**Abbreviations**

| | |
|---|---|
| CoA | Coenzyme A |
| HPLC | High-performance liquid chromatography |
| OD | Optical density |
| TLC | Thin-layer chromatography |
| HMBC | Hetero-nuclear multiple-bond connectivity |
| NMR | Nuclear magnetic resonance |

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## Introduction

Salicylate is a precursor of acetylsalicylate, which is widely used as a nonsteroidal anti-inflammatory and is better known as the analgesic aspirin (Jeffreys 2004). Salicylate has many physiological and biochemical effects, such as inducing pathogen resistance in plants (Lee et al. 1995). Since salicylate is also known to be a precursor of secondary metabolites and siderophores that act as iron chelators (Pelludat et al. 2003) and regulators of antibiotic efflux (Nair et al. 2005), it is considered as a key phenolic compound in several microorganisms.

Metabolic pathways in microorganisms and plants for the synthesis and degradation of salicylate have been the focus of much study. In microorganisms, salicylate is mainly synthesized from isochorismate in *Mycobacterium* spp. and *Pseudomonas* spp. (Adilakshmi et al. 2000; Gaille et al. 2002) or directly synthesized from chorismate in *Yersinia* spp. (Pelludat et al. 2003). For salicylate degradation in microorganisms, catechol and gentisate are known to act as common intermediates through the following two metabolic pathways: direct oxidation of salicylate to catechol followed by aromatic ring cleavage (Civilini et al. 1999); or direct oxidation of salicylate to gentisate followed by aromatic ring cleavage (Grund et al. 1992). In addition, the direct fission of salicylate (Hintner et al. 2001) and degradation after CoA addition (Ishiyama et al. 2004) have also been reported. In many plants, salicylate is mainly

synthesized from cinnamic acid through the following two metabolic pathways: side-chain decarboxylation of cinnamic acid to form benzoic acid followed by 2-hydroxylation to salicylate (Klambt 1962); or 2-hydroxylation of cinnamic acid to form *o*-coumaric acid followed by decarboxylation to salicylate (Chadha and Brown 1974). Occasionally, plants convert salicylate by glucosylation, esterification, and methylation to active components to induce pathogen resistance (Lee et al. 1995; Klick and Herrmann 1988; Andersen et al. 1988).

The biodegradation of phenolic compounds containing salicylate by *Trichosporon* spp. has been reported by several research groups (Gaal and Neujahr 1979; Caselli and Hanau 1994), and salicylate degradation via catechol as a key intermediate has been reported in *T. cutaneum* (Anderson and Dagley 1980). However, there have been no reports so far on the metabolic pathway for salicylate degradation via phenol.

In this paper, we report a novel metabolic pathway for salicylate degradation in the yeast *T. moniliiforme* WU-0401. We analyzed metabolites in a culture broth and the products derived from salicylate or phenol through reactions with resting cells and a cell-free extract of *T. moniliiforme* WU-0401 to find a novel metabolic pathway for salicylate degradation via phenol as the key intermediate.

## Materials and methods

### Materials

Salicylic acid, sodium salicylate, *m*-hydroxybenzoic acid, *p*-hydroxybenzoic acid, catechol, phenol, and 2,6-dichloroquinone-4-chloroimide (Gibbs reagent) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). All other chemicals used were commercially available and chemically pure grade.

### Isolation and cultivation of salicylate-degrading microorganisms

An SA medium (pH 6.0) was used for the isolation and cultivation of salicylate-degrading microorganisms. This medium contained the following: 0.8 g sodium salicylate, 0.5 g $K_2HPO_4$, 2.5 g $KH_2PO_4$, 0.5 g $NaNO_3$, 0.5 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4 \cdot 6H_2O$, 2 ml of a metal solution (Nakagawa et al. 2002) and 1 ml of a vitamin mixture (Nakagawa et al. 2002) in 1,000 ml of distilled water. Cell growth was turbidimetrically measured at 600 nm. Fifteen grams of agar per liter was added to the SA medium for the preparation of SA plates.

### Resting cell reaction

*T. moniliiforme* WU-0401 was cultivated for 48 h at 30°C in 500-ml Erlenmeyer flasks containing 200 ml of SA medium with reciprocal shaking at 120 strokes/min. After cultivation, the cells were harvested by centrifugation at 10,000*g* for 20 min at 4°C, washed twice with 50 mM $K_2HPO_4$-$KH_2PO_4$ buffer (pH 7.0), and suspended in 10 ml of the same buffer. For salicylate degradation, 70 µl of 1.0 M sodium salicylate solution was added to 1 ml of the cell suspension ($OD_{600}$ 40) in a 2-ml microcentrifuge tube. For phenol degradation, 50 µl of 0.5 M phenol solution was added to 1 ml of the cell suspension ($OD_{600}$ 40) in a 2-ml microcentrifuge tube. Each reaction was allowed to proceed for 24 h at 30°C, and 50 µl of 12 M HCl was added to stop the reaction. The reaction mixture was filtrated using a 0.20-µm PTFE membrane (Asahi Techno Glass Co., Tokyo, Japan), and the amounts of substrates and reaction products were measured by high-performance liquid chromatography (HPLC).

### Preparation and purification of reaction products from salicylate by resting cell reaction

To prepare the reaction products from salicylate, 376 mg of salicylate was added to 100 ml of a resting cell suspension ($OD_{600}$ 40) in 500-ml Erlenmeyer flasks. The mixture was incubated with shaking for 24 h at 30°C and then extracted with 200 ml of ethylacetate. The resulting extract was concentrated using rotary evaporation equipment and applied to a silica column chromatograph packed with Wakogel C-200 (Wako Pure Chemicals, Osaka, Japan). The elution was carried out with methanol-chloroform-ammonia (5:13:0.8 v/v/v). The fraction, including the product, was concentrated by rotary evaporation, and lyophilized, and then subjected to nuclear magnetic resonance (NMR) analysis (Sato et al. 2000).

### Preparation of cell-free extract of *T. moniliiforme* WU-0401

For the basal buffer, 50 mM $K_2HPO_4$-$KH_2PO_4$ buffer (pH 7.0) was used. *T. moniliiforme* WU-0401 was cultivated for 48 h at 30°C in 500-ml Erlenmeyer flasks containing 200 ml of SA medium with reciprocal shaking at 120 strokes/min. The cells in the 200-ml culture broth were harvested by centrifugation at 10,000*g* for 20 min at 4°C and then washed twice with the basal buffer (pH 7.0). The washed cells were frozen in liquid $N_2$ and ground into a fine powder using a mortar and pestle. The powdered cells were suspended in 10 ml of the basal buffer (pH 7.0). Cell debris was removed by centrifugation at 16,000*g* for 30 min at 4°C. The resulting supernatant was used as the cell-free extract. Protein concentrations were measured using the Coomassie Protein Assay kit (Pieace, IL, USA) with bovine serum albumin as the standard.

### Degradation of salicylate using cell-free extract of *T. moniliiforme* WU-0401

For the degradation assay, following standard reaction conditions were used, the mixture contained 100 μg of protein and 5 mM substrate in 50 mM $K_2HPO_4$-$KH_2PO_4$ buffer (pH 7.0) for a final volume of 0.5 ml in a 1.5-ml microcentrifuge tube. Each mixture was incubated with shaking at 30°C, and the reaction was stopped by the addition of 100 μl of 12 M HCl. The amounts of substrates and reaction products were measured by HPLC. One unit (U) of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 μmol of product per minute.

### Detection of phenolic compounds

Phenolic compounds in the reaction mixtures such as phenol, salicylate, and catechol were visually detected by the Gibbs assay, which was performed by adding 30 μl of 2.5 M $KHCO_3$ solution and 20 μl of 5.0 mM Gibbs reagent dissolved in ethanol solution for 50 μl reaction mixtures. The Gibbs assay was observed to produce blue, light blue, and brown mixtures with phenol, salicylate, and catechol, respectively. The Gibbs assay was mainly used to screen microorganisms degrading salicylate.

### Thin-layer chromatography (TLC) analysis

TLC was performed on silica gel 60 plates (E. Merck, Darmstadt, Germany) using the ascending method with methanol-chloroform (1:10 v/v) as the developing solvent. Spots were made visible by spraying with 2.5 M $KHCO_3$ and 5.0 mM Gibbs reagent solutions; this was followed by heating at 160°C.

### HPLC analysis

The amounts of salicylate, phenol, catechol, and *cis-cis* muconate were determined using HPLC (type LC-10A; Shimadzu, Kyoto, Japan) equipped with a Puresil C18 column (Waters, MA, USA) through a comparison with standard curves prepared with authentic compounds. The mobile phase was methanol-50 mM $Na_2HPO_4$-$NaH_2PO_4$ buffer (pH 7.0) (25:75 v/v), and the flow rate was 0.8 ml/min. The column temperature was 30°C. Compounds were spectrophotometrically detected at 270 nm.

## Results

### Screening and isolation of microorganisms degrading salicylate

Small amounts of 3,000 soil samples collected from various areas of Japan were suspended in 1 ml of distilled water and centrifuged at 1,500*g* for 5 min at 4°C to remove solid components. The resulting supernatant was diluted to the appropriate level with distilled water, and aliquots of 50 μl were inoculated into test tubes containing 5 ml of SA liquid medium and cultivated at 30°C for 6–8 days. Aliquots of turbid cultures with more than $OD_{600}$ 0.5 had transferred into 5 ml of fresh SA liquid medium. After four subcultivations, the culture filtrate was analyzed by the Gibbs assay and HPLC to confirm whether phenolic compounds had accumulated.

Among the 1,000 turbid cultures, the Gibbs assay revealed that phenol accumulated in two cultures derived from independent soil samples. Each culture was diluted to the appropriate level with distilled water and spread onto the SA medium agar plates. After cultivation at 30°C for 3–5 days, each single colony formed on the plate was isolated and inoculated again into the liquid SA medium. By repeated

cultivation in the liquid SA medium and single-colony isolation on the SA medium agar plates, strains showing stable growth at more than $OD_{600}$ 1.0 and accumulation of phenol in the liquid SA medium were selected. Two microorganisms designated as strains WU-0401 and WU-0501 were selected for further studies. WU-0401 showed a higher degradation yield for salicylate than WU-0501 (details not shown). Therefore, WU-0401 was focused on in this study.

## Conversion of salicylate to form phenol by resting cell reaction of strain WU-0401

TLC and HPLC was used to analyze metabolites produced by the degradations of salicylate and phenol through the resting cell reaction of strain WU-0401. TLC analysis revealed one product with an $R_f$ of 0.69 identical to that of an authentic phenol in salicylate degradation. Another product with an $R_f$ of 0.30 was identical to that of an authentic catechol in phenol degradation. The product from salicylate was extracted from the reaction mixture, purified by silica gel column chromatography, and subjected to NMR analysis. Ten milligrams of the product was obtained from the reaction mixture containing 376 mg salicylate as an initial substrate. As shown in Fig. 1, the 2-dimensional HMBC spectrum indicated that the isolated product was phenol.

## Identification of strain WU-0401

Strain WU-0401 formed wet colonies on YM medium agar plates and elongated hyphae around the colonies. The strain was observed to grow through budding during vegetative growth. WU-0401 formed true hyphae and arthroconidia but not pseudohyphae and sexual reproductive organs. Further taxonomical identification of WU-0401 was performed by Techno Suruga Co., Ltd. (Shizuoka, Japan); the 28S ribosomal DNA sequence of strain WU-0401 showed a 100% identity match to the type

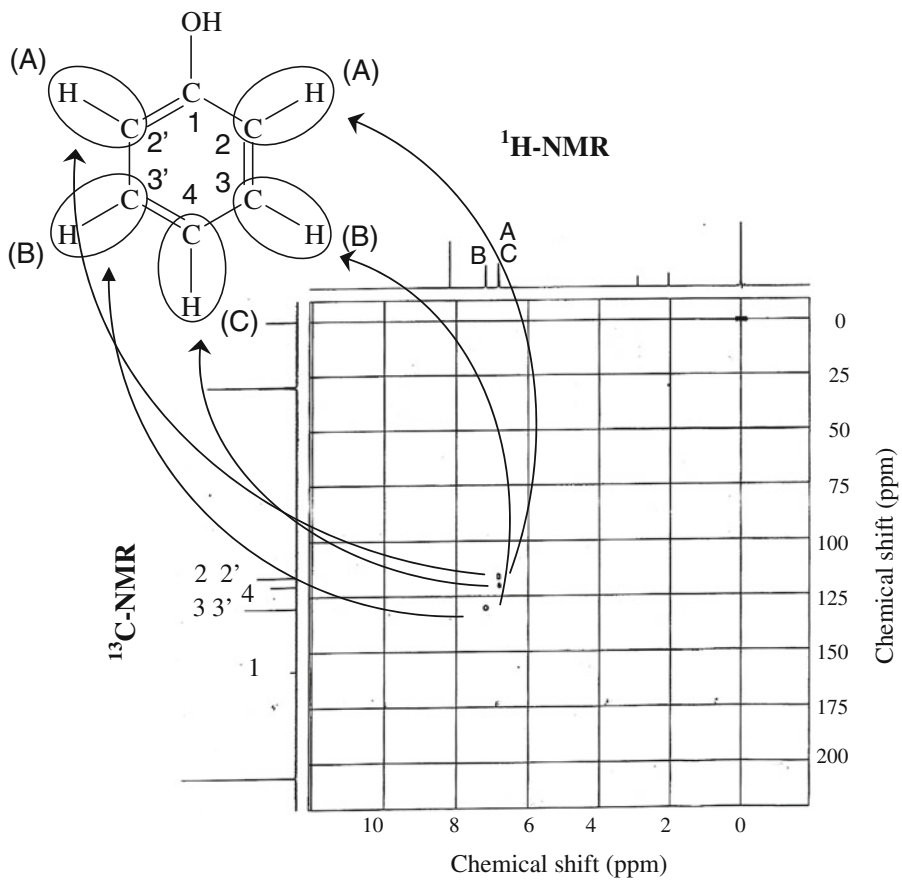


**Fig. 1** Two-dimensional HMBC spectrum analysis of the product from salicylate for the resting cell reaction of *Trichosporon moniliiforme* WU-0401. The analysis data show that the product was phenol

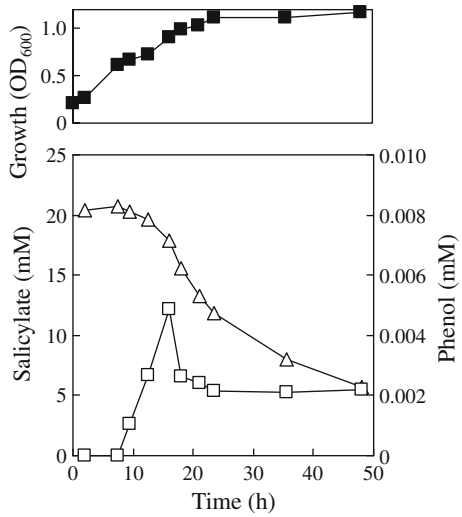


**Fig. 2** Time courses of salicylate degradation by growing cells of *Trichosporon moniliiforme* WU-0401. WU-0401 was cultivated in an SA medium containing 20 mM salicylate as the sole carbon source. Symbols: *open triangles*, salicylate; *open squares*, phenol; *closed squares*, growth ($OD_{600}$)

strain of *T. moniliiforme* CBS2467 (AF105392). Based on these results, strain WU-0401 was identified as *T. moniliiforme*. Strain WU-0501 was also identified as *T. moniliiforme*.

Because strains WU-0401 and WU-0501 were both identified as *T. moniliiforme*, we checked to confirm whether salicylate degradation activity would be detected in several strains closely related to the *T. moniliiforme* species: i.e., *T. moniliiforme* NBRC 1527, *T. cutaneum* NBRC 1198$^{T}$, and *T. asteroides* NBRC 0173. Because all three strains could grow in an SA medium, we prepared resting cells of these three strains to examine whether phenol would be produced through the degradation of salicylate. Activities for salicylate degradation and phenol production were detected in all three strains (data not shown). Therefore, these results indicate that the metabolic pathway for salicylate degradation via phenol may be widely distributed in strains closely related to *T. moniliiforme*.

## Degradation of salicylate

As shown in Fig. 2, the time course of salicylate degradation by growing cells of *T. moniliiforme* WU-0401 revealed phenol as a distinct metabolite in the culture broth. Using highly concentrated salicylate (more than 40 mM) as a substrate, the resting cell reaction was performed to detect other metabolites in addition to phenol. As shown in Fig. 3, the time courses of salicylate and phenol degradations by the resting cell reaction of *T. moniliiforme* WU-0401 revealed that salicylate is initially converts to phenol and then to catechol. *T. moniliiforme* WU-0401 degraded 40 mM salicylate and accumulated 10 mM phenol and 2 mM catechol from 70 mM salicylate after 96 h of the resting cell reaction (Fig. 3A). The resting cell reaction toward 40 mM salicylate was also performed: 31 mM salicylate was degraded and 6.3 mM phenol and 1.2 mM catechol accumulated (data not shown). This reaction showed a similar conversion rate as the resting cell reaction toward 70 mM salicylate. For the resting cell reaction

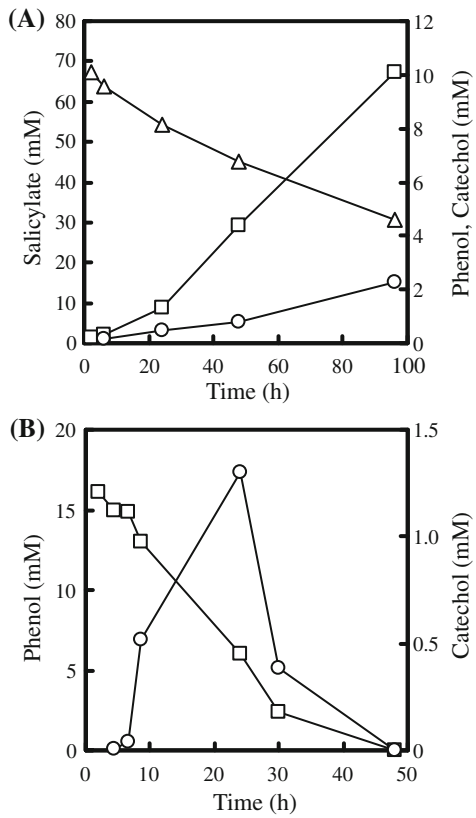


**Fig. 3** Time courses of (**A**) salicylate and (**B**) phenol degradations by resting cells of *Trichosporon moniliiforme* WU-0401 that were cultivated in an SA medium containing salicylate as the sole carbon source. Symbols: *open triangles*, salicylate; *open squares*, phenol; *open circles*, catechol

toward 20 mM phenol as a substrate, 1.3 mM catechol accumulated after 24 h before decreasing (Fig. 3B). These results suggest that phenol derived from salicylate is immediately converted to catechol, which is also degraded through the successive degradation pathway. Therefore, for experiments on growing cells, only a small amount (5 mM) of phenol accumulated in the culture for salicylate degradation. In contrast, when resting cells of *T. moniliiforme* WU-0401 were used after incubation in the boiling water for 10 min, no salicylate degradation or phenol production was detected in a resting cell reaction.

Cell-free extract reactions were performed to confirm the metabolic pathway of salicylate degradation in more detail. In the cell-free extract reaction toward 5 mM salicylate, 1.6 mM salicylate was degraded and 0.36 mM phenol accumulated after 24 h; no other product was detected by HPLC analysis. Reactions with cell-free extract in the presence and absence of NAD(P)H (5 mM) were also performed. In these reactions, phenol was converted from salicylate in approximately equal amounts regardless of the presence or absence of NAD(P)H; no other metabolite was detected. These results indicate that the cell-free extract of *T. moniliiforme* WU-0401 did not show salicylate hydoxylase activity converting salicylate to catechol or dihydroxybenzoate even in the presence of NAD(P)H. In the cell-free extract reaction toward 5 mM phenol, no catechol accumulated regardless of the presence or absence of NADH. In contrast, for the cell-free extract reaction toward 5 mM phenol in the presence of NADPH, 0.02 mM phenol was degraded and 0.02 mM catechol accumulated after 30 min; no other product was detected. No catechol accumulated in the absence of NADPH. In the cell-free extract reaction toward catechol, HPLC analysis detected a peak identical to that of the retention time for *cis-cis* muconate. Moreover, the production of *cis-cis* muconate from catechol was confirmed by spectrophotometric analysis (data not shown). Therefore, we characterized the metabolic pathway for salicylate degradation by *T. moniliiforme* WU-0401 as shown in Fig. 4.

Resting cells of *T. moniliiforme* WU-0401 cultivated in an LB medium did not show degrading activity of salicylate to phenol. In contrast, resting cells of *T. moniliiforme* WU-0401 cultivated in an LB medium supplemented with 20 mM salicylate showed degrading activity. When *T. moniliiforme* WU-0401 was cultivated in an SA medium containing glucose instead of salicylate as the carbon source, no salicylate-decarboxylation activity was detected in either the resting cells or cell-free extract. When *T. moniliiforme* WU-0401 was cultivated in SA medium containing phenol instead of salicylate as the carbon source, salicylate-decarboxylation activity was detected in the cell-free extract. The salicylate-decarboxylation activity in the cell-free extract prepared from salicylate-grown cells of *T. moniliiforme* WU-0401 was approximately 7 times higher than that of phenol-grown cells. Therefore, the expression of salicylate-degrading enzyme(s) seems to be induced in *T. moniliiforme* WU-0401 grown on salicylate as a carbon source.

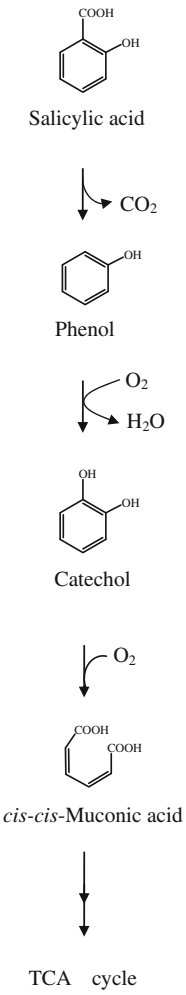


**Fig. 4** Proposed pathway of salicylate degradation by *Trichosporon moniliiforme* WU-0401. TCA cycle denotes the tricarboxylic acid (citric acid) cycle

## Discussion and conclusions

In this study, we show a novel metabolic pathway in *T. moniliiforme* WU-0401 for salicylate degradation via phenol as the key intermediate (Fig. 4). Salicylate is initially decarboxylated to phenol and then oxidized to catechol followed by an aromatic ring cleavage to form *cis-cis* muconate. Although many microorganisms degrade salicylate or phenol, this study is the first report to our knowledge on the microbial degradation of salicylate via phenol. Moreover, strains closely related to *T. moniliiforme* also degraded salicylate via phenol. These results indicate that such a metabolic pathway for salicylate degradation would be commonly conserved in strains closely related to *T. moniliiforme*.

For microbial salicylate-degradation, salicylate hydroxylase from *T. cutaneum* has been studied in detail as a key enzyme (Sze and Dagley 1984). Salicylate hydroxylase acts as a catalyst for oxidative decarboxylation of salicylate to catechol. *T. cutaneum* utilizes a wide range of aromatic compounds for growth. These restrictions may be eased by the ability of *T. cutaneum* to introduce a third hydroxyl group into the benzene nucleus when a dihydric phenol fails to serve as a ring-cleavage substrate, by extensive enzyme derepression using a few key metabolites, and by broadening the substrate specificities for certain enzymes (Anderson and Dagley 1980). Further investigation showed that salicylate hydroxylase belongs to this last category of enzymes (Sze and Dagley 1984). On the other hand, in cell-free extracts of *T. moniliiforme* WU-0401, salicylate was decarboxylated to phenol and then oxidized to catechol followed by aromatic ring cleavage (Fig. 4). These results indicate that *T. moniliiforme* WU-0401 degrades salicylate using the non-oxidative decarboxylation pathway.

In conclusion, we found a novel metabolic pathway in the yeast *T. moniliiforme* WU-0401 for salicylate degradation via phenol as the key intermediate. Currently, we have started to study enzymes related to salicylate degradation in *T. moniliiforme* WU-0401.

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