Microbial Hydroxylation of 5-Anilino-1,2,3,4-Thiatriazole

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Two hundred eighty-five fungi, including 100 basidiomycetes and 35 yeasts, 75 actinomycetes, and 40 bacteria were screened for their ability to convert 5-anilino-1,2,3,4-thiatriazole (AT) to 5-(p-hydroxyanilino)-1,2,3,4-thiatriazole (p-HT). Eleven cultures were found that formed p-HT, which was isolated and whose structure was determined. Aspergillus tamarii NRRL 3280 formed 8.6 g of p-HT/liter from 10 g of AT/liter (78.9% conversion) in shaken flasks and 4.57 g of p-HT/liter from 6 g of AT/liter (69.8% conversion) in 30-liter fermentors. Washed cells of A. tamarii NRRL 3280 also carried out this conversion. 5-(o-hydroxyanilino)-1,2,3,4-thiatriazole (o-HT) was identified as a second product formed by Aspergillus terreus NRRL 1960.

Materials and Methods

Cultures. Two hundred eighty-five fungi, including 100 basidiomycetes and 35 yeasts, 75 actinomycetes, and 40 bacteria were screened in this investigation for their ability to form p-HT from AT. All cultures were maintained on agar slants, transferred on a regular basis, and stored at 4°C.

Fermentation media. All screening and other fermentation studies were carried out in media which were different for each culture type. Ingredients were as follows (grams per liter). (i) Molds (excluding basidiomycetes): glucose monohydrate, 50.0; soybean flour, 5.0; yeast extract (Difco), 5.0; NaCl, 1.0; KH2PO4, 4.1; K2HPO4, 0.8; and deionized water to 1,000 ml were used. The pH was adjusted to 6.0. (ii) Basidiomycetes: the medium used for these cultures was identical to the one used for molds with the exception that malt extract was added at a level of 20.0 g/liter. (iii) Actinomycetes: glucose monohydrate, 50.0; soybean grits (meal), 5.0; yeast extract (Difco), 2.5; NaCl, 1.0; KH2PO4, 1.0; K2HPO4, 2.0; and deionized water to 1,000 ml were used. The pH was adjusted to 7.0. (iv) Yeasts: glucose monohydrate, 20.0; yeast extract (Difco), 5.0; peptone (Difco), 5.0; tryptone (Difco), 2.0; NaCl, 0.5; KH2PO4, 5.0; MgSO4.7H2O, 0.5; and deionized water to 1,000 ml were used. The pH was adjusted to 5.0. (v) Bacteria: glucose monohydrate, 10.0; yeast extract (Difco), 5.0; peptone (Difco), 3.0; tryptone (Difco), 2.0; NaCl, 0.1; KH2PO4, 2.0; K2HPO4, 4.0; MgSO4.7H2O, 0.5; and deionized water to 1,000 ml were used. The initial pH was adjusted to 7.0. With all of the media used, glucose monohydrate was sterilized and added separately.

Fermentation conditions. Initial screening studies with the 400 cultures for the desired product were carried out in 25-150-mm Pyrex tubes each closed with a Morton or Bellco stainless-steel enclosure and containing 10 ml of the appropriate medium. All tubes were autoclaved at 120°C and 15 lb/in2 for 35 min. Shaken tubes were inoculated with approximately 0.3 ml of an agar slant culture suspended in 6 ml of sterile nutrient broth. Tubes were incubated on a Gump rotary shaker (Blaw-Knox Co.) at 250 rpm and 28°C. Substrate (AT) was added in powdered form at a level of 0.1% (1 mg/ml) after 24 to 96 h of

Certain 5-(substituted)amino-1,2,3,4-thiatriazoles have been shown in our laboratories (H. G. Shoepke and L. R. Swett, U.S. Patent 3,265,576) to be potent agents in reducing blood pressure in cats and in both artificially induced neurogenic and renal hypertensive dogs. 5-Anilino-1,2,3,4-thiatriazole (AT) (Fig. 1) was one of the most active analogues in the series. Both aniline and p-aminophenol were proposed as possible mammalian metabolites of this drug. From the viewpoint of toxicity, p-aminophenol was the preferred metabolite. 5-(p-Hydroxyanilino)-1,2,3,4-thiatriazole (p-HT) (Fig. 1) was then proposed as a possibly more desirable antihypertensive agent. However, initial experiments to prepare this compound synthetically by reacting 4-p-hydroxyphenylthiosemicarbazide with nitric acid led to extensive degradation. Preparation of this drug by means of the microbial conversion of AT to p-HT was thus proposed. Some of the studies reported here have also been previously cited (R. J. Theriault and T. H. Longfield, U.S. Patent 3,592,735).
incubation, depending on the type of culture. Yeasts and bacteria were sampled at 120 h. Molds, actinomycetes, and basidiomycetes were sampled at 312 h. All whole culture samples were analyzed by ethyl acetate extraction and thin-layer chromatography.

All secondary screening and most other fermentation studies were conducted in 500-ml Erlenmeyer flasks containing 100 ml of appropriate medium and were closed with cotton plugs unless otherwise specified. All flasks were autoclaved at 120 °C and 15 lb/in² for 35 min. Flasks were incubated by suspending the surface growth of agar slant cultures in 6 ml of sterile nutrient broth. Three milliliters of the suspension was inoculated into each flask. Inoculated flasks were incubated on a Gump rotary shaker at 250 rpm and 28 °C. Unless otherwise indicated, the substrate (AT) was added at a level of 0.1% (1 mg/ml) in the nonsterile powdered form after 48 h of incubation on the shaker. Incubation was then continued, and flasks were sampled (10 ml) periodically between 120 and 312 h for ethyl acetate solvent extraction and thin-layer chromatography.

For the isolation of p-HT, 200 Erlenmeyer flasks (500 ml) each containing 100 ml of medium (i) and closed with cotton plugs were sterilized, cooled, and inoculated from agar slant cultures of Aspergillus tamarii NRRL 3280 as described. After 48 h of incubation on a Gump rotary shaker, substrate (AT) was added at a level of 0.1% (1 mg/ml). Incubation was continued on the shaker until the yield of conversion product (p-HT) was optimal (162 h).

5-(o-Hydroxyanilino)-1,2,3,4-thiatriazole (o-HT) (Fig. 1) was formed and isolated from shaken flask cultures of Aspergillus terreus NRRL 1960. Two hundred Erlenmeyer flasks (500-ml size) each containing 100 ml of medium (i), were inoculated with A. terreus NRRL 1960. After 48 h of incubation on the shaker, substrate (AT) was added at a level of 0.05% (0.5 mg/ml) to each flask. Incubation was continued on the shaker until the yield of desired product (o-HT) was at the maximum possible level. p-HT was produced as a secondary product by this culture. Flasks were harvested at 192 h.

The 30-liter fermentors used in these studies were identical to those reported by Thériault (4) and earlier by Friedland, Peterson, and Sylvester (2). Fermentors were assembled as previously described and normally charged with 15 liters of medium. The fermentors were autoclaved at 120 °C with 15 lb/in² for 1 h. After sterilization, the fermentors were cooled with sterile air and placed in operation after insertion of the bearings. Inoculum was usually 10% by volume from vegetative, shaken seed flasks of A. tamarii NRRL 3280 incubated at 28 °C for 72 h. Substrate (AT) was added from a 40% dimethyl sulfoxide (DMSO).

A. tamarii NRRL 3280 was grown in aerated shaken flasks as previously described [medium (i)]. After 48 h of incubation, flasks were removed from the rotary shaker and the cells were washed three times with 0.01 M (pH 6.0) phosphate buffer. The cells were then suspended in the same buffer at original volume in sterile 500-ml Erlenmeyer flasks closed with cotton plugs. AT was added at a level of 2.5 g/liter from a 40% DMSO solution, and the flasks were then incubated on a Gump rotary shaker (250 rpm) at 28 °C.

Preparation of AT. Phenylisothiocyanate (40.5 g) was dissolved in 150 ml of ethanol and cooled to 0 to 10 °C, and 9.6 g of 95% hydrazine was added dropwise with stirring. A precipitate formed, and the reaction mixture was stirred for 1 h at 0 °C. The precipitate was filtered off and suspended in 500 ml of 10% HCl (cooled in an ice bath), and 20.7 g of sodium nitrite in 150 ml of water was added dropwise to the precipitate suspended in 10% HCl. The mixture was stirred for 1 h at 10 °C. A precipitate which formed was filtered off and washed with cold water, and the product was recrystallized from methanol (37.2 g). The melting point of the product was 142 to 145 °C with decomposition. Analysis for C₉H₄N₂S: calculated C, 47.15; H, 3.40; N, 31.45; S, 18.00. Found: C, 47.43; H, 3.63; N, 31.30; S, 17.64 (by difference).

Thin-layer chromatography. Samples (10 ml) of the various fermentation broth were adjusted to pH 7.0 with NH₄OH and extracted with 4 vol of ethyl acetate. Ethyl acetate was removed under vacuum, the residue was redissolved in 2 ml of methanol, and 200 μl was applied on 20- by 20-cm glass plates coated with Merck Darmstadt silica gel GF-254 approximately 500 µm in thickness. The plates were then developed in methylene chloride: 95% aqueous methanol: water (85:15:1, vol/vol/vol) for 30 to 45 min, air-dried, viewed, and photographed with a 254-nm light by using a Polaroid Land camera with no. 107 film. Silica gel plates were then sprayed with one or more of the following detection reagents: (i) N-2, 6-trichloro-p-benzoquinoneimine reagent: Silica gel plates were sprayed with a 0.2 N aqueous solution of NaOH followed by a 0.4% methanolic solution of N-2,6-trichloro-p-benzoquinoneimine. The substrate and microbial conversion products appeared as black spots on a white to gray background after the plates had been exposed to a 254-nm light for 10 min. (ii) Phenols spray reagent: The plates were sprayed with aqueous 2 N H₂SO₄ followed by aqueous 1% FeCl₃-1% K₃Fe(CN)₆ (1:1). Phenolic products were revealed as intense blue spots on a pale yellow background. (iii) Sulfur spray reagent: The plates were first sprayed with a freshly prepared aqueous solution containing 3% sodium azide, 1% sodium iodide, and 1% starch, and then exposed to iodine.
vapors in a closed jar for 2 to 3 min. This procedure is a modification of one reported by Awe et al. (1). It revealed the substrate (AT) and related microbial conversion products as white spots on a purple background which later changed to brown.

To determine more definitively that the detected substances were derived from the substrate by microbial action, the developed silica gel plates were viewed with a 254-nm light. The UV-absorbing, apparent microbial conversion products were removed from the plates and extracted with 3.5 ml of methanol. Silica gel was centrifuged off, and the UV spectra of the supernatant fluid were determined (Cary model 14 recording spectrophotometer).

Quantitative determinations of p-HT were carried out by the following procedure. Ethyl acetate–extracted fermentation samples or product isolated in various stages of purification were appropriately diluted in methanol, and measured samples were applied on plates. After development in the previously described solvent system, the spots corresponding to p-HT (Rf = 0.55) were transferred to small test tubes. Each sample was then extracted three times with 2.5 ml of methanol. The combined centrifuged methanol extracts were then diluted to 8 ml with methanol. The UV spectrum of p-HT showed two maxima at 240 nm and 300 nm (A1 cm, λmax 300 nm = 450.5). The samples were read at the preferred maximum of 300 nm in a Beckman model DU spectrophotometer. Recovery of p-HT standards from silica gel plates averaged 94.1%.

Spectral analyses. Infrared (IR) spectra were determined with a Perkin Elmer model 421 spectrophotometer. Nuclear magnetic resonance (NMR) spectra were determined with Varian A-60 and HA-100 spectrometers. The NMR spectra were determined in deuterated chloroform (CDCl3) or deuterated DMSO ([CD3]2SO) with tetramethyl silane (Me4Si) as the internal standard. Mass spectra were determined with a mass spectrometer, Associated Electrical Industries model M.S.902.

Melting points. Melting points were determined with a Kofler Micro-Hot-Stage, melting-point apparatus with a calibrated thermometer.

Product isolations. p-HT product was isolated from a run of 200 shaken flasks inoculated with A. tamarii NRRL 3280 as previously described. The culture was harvested when the yield of product was optimal. The pooled harvested beer (18 liters) was adjusted to pH 7.0 with NH4OH and then extracted twice with 4 vol of ethyl acetate. The ethyl acetate extracts were combined, and the solvent was removed under vacuum. The residue was then dissolved in acetone, and the solution was added to a 6-cm diameter glass column packed to a height of 70 cm with Bio-Rad acid alumina (pH 4.1–4.8) in acetone. The column was developed, and the product was eluted with 1% methanol in acetone. Fractions (20 ml) were collected from the column, and the product was eluted over a range of 170 tubes. The column fractions containing the product were pooled, and the solvent was removed under vacuum. The residue obtained was dissolved in acetone, filtered through Whatman no. 50 paper, and again dried under vacuum. The resultant product residue was washed several times with small portions of acetone to remove trace impurities. The product was then dried under vacuum, yielding a white amorphous chromatographically pure solid (melting point 149–150 C).

p-HT was isolated from a run of 200 shaken flasks inoculated with A. terrestres NRRL 1980 and incubated as previously described. Culture was harvested at 192 h, and the pooled beer (17 liters) was adjusted to pH 7.0. One volume of acetone (17 liters) was added to the beer, and the mixture was extracted twice with 2 vol (34 liters) of ethyl acetate. The ethyl acetate was removed under vacuum, and the residue obtained was dissolved in chloroform and added to a 5.4-cm diameter glass column packed to a height of 70 cm with silica gel in chloroform. The products were developed initially with chloroform, collecting 20-ml fractions. p-HT was eluted from the column with chloroform. p-HT was eluted with 1% and 2% ethyl ether in chloroform. One hundred ninety fractions containing p-HT were pooled, and the solvent was removed under vacuum. p-HT was then crystallized by washing the residue with methylene chloride (melting point 122–123 C).

RESULTS AND DISCUSSION

In shaken tubes, 16 molds, 8 actinomycetes, and 5 basidiomycetes showed one or more major products when reacted with the phenols and N-2, 6-trichloro-p-benzoquinoneimine sprays on silica gel plates. When retested in shaken flasks, all of the cultures revealed the same types of products. Eleven of the cultures which formed varied amounts of what appeared to be the same microbial conversion product on silica gel plates (Rf = 0.55) were again grown in shaken flasks and are shown in Table 1. Silica gel plates revealed that all cultures again formed the same product (Rf = 0.55) and also gave a positive response when reacted with the phenol spray, N-2, 6-trichloro-p-benzoquinoneimine, and the sulfur spray. UV spectra of this product obtained from silica gel plates were qualitatively identical to AT. Thus, this product gave every indication of being substrate related, hydroxylated, and identical for all eleven cultures. Silica gel plates showed that A. tamarii NRRL 3280 gave the highest yield of this product and was later used for the isolation and confirmation of structure as p-HT. On the assumption that this product was the same for all eleven cultures, the yields in this experiment were calculated as p-HT. As the results indicate in Table 1, A. tamarii NRRL 3280 gave the highest yield (0.64 g/liter) or a 58.8% conversion. Aspergillus species NRRLA-13647 was second highest with 0.43 g/liter or a 39.5% conversion rate.

On the basis of these results, 200 flasks were
Table 1. Comparison of cultures producing p-HT from AT in aerated shaken flasks*

| Flask no. | Culture                        | Yield of p-HT | Molar conversion (%) |
|-----------|--------------------------------|---------------|----------------------|
| 1         | Aspergillus tamarii NRRL 3280  | 0.64          | 58.8                 |
| 2         | Aspergillus terreus NRRL 1960  | 0.03          | 2.7                  |
| 3         | Rhizopus stolonifer ATCC 10404 | 0.05          | 4.5                  |
| 4         | Penicillium sp. A-1 NRRL 3281 | 0.02          | 1.8                  |
| 5         | Penicillium sp. A-85 NRRL 3282| 0.02          | 1.8                  |
| 6         | Aspergillus sp. type NRRL A-13647 | 0.43       | 39.5                 |
| 7         | Nocardia Act-116 NRRL 3283     | 0.03          | 2.7                  |
| 8         | Streptomyces olivaceus NRRLB 1125 | 0.04       | 3.6                  |
| 9         | Boletinus pictus F-12 NRRL 3284 | 0.05       | 4.5                  |
| 10        | Coniophora puteana USDA Madison #515 | 0.02     | 1.8                  |
| 11        | Pleurotus candidus SS-31 NRRL 3285 | 0.01       | 0.9                  |

*Conditions were as follows: volume/flask, 100 ml/500-ml Erlenmeyer flask; medium, according to culture type as described; sterilization, 35 min at 120 C and 15 lb/in² pressure; aeration, cotton plug; agitation, Gump rotary shaker (250 rpm); incubation temperature, 28 C; substrate (AT), 0.1% (nonsterile powder) after 48 h of incubation.

inoculated with A. tamarii NRRL 3280 and incubated with substrate (AT). The run was harvested at the optimal age, and the product (p-HT) was isolated, as described, yielding a chromatographically pure white solid (melting point 149–150 C). Analysis for monohydroxylated 5-anilino-1,2,3,4-thiadiazole calculated for C₈H₇N₄O₅S: C, 43.30; H, 3.31; N, 28.85; O, 8.23; S, 16.51. Percentages found included C, 43.37; H, 3.57; N, 28.87; O, 8.19; S, 16.25. The UV spectrum of this product showed λₑₘₓ at 240 nm (ε = 7799) and 300 nm (ε = 8747). The Nujol mull IR spectrum showed increased absorption at 3,050 cm⁻¹ and 3,215 cm⁻¹ and was otherwise consistent with the proposed structure. The 60 MHz NMR spectrum showed a singlet at 11.18 which could be assigned to the NH proton. An additional singlet was observed at 9.445 which could be assigned to the OH proton. Both of these protons exchanged with D₂O. The four phenyl protons were split into an AA'BB' pattern centered at 7.10δ, confirming hydroxylation in the para position. The mass spectrum showed a molecular ion of 194.0270 (calculated for C₁₀H₉N₄O₅S = 194.0262). Thus, the structure of the major microbial conversion product formed by A. tamarii NRRL 3280 was established as p-HT. A synthetic method to yield the same product was developed later (R. J. Theriault and T. H. Longfield, U.S. Patent 3,592,735).

As seen in Table 1, A. terreus NRRL 1960 also formed p-HT but in very low yields. Silica gel plates revealed that this culture formed a second phenol-positive product of equal intensity and with a slightly higher Rₛ of 0.50. Two hundred 500-ml Erlenmeyer flasks containing medium 1 were inoculated with A. terreus NRRL 1960 and incubated with substrate (AT) as described. The flasks were harvested at 192 h, and the product was isolated yielding a small amount of chromatographically pure crystalline material (melting point 122–123 C). Analysis for monohydroxylated AT calculated for C₂H₄N₄O₅S: C, 43.30; H, 3.11; N, 28.85; O, 8.23; S, 16.51. Percentages found were C, 43.98; H, 3.10; N, 28.95; O, 8.38; S, 16.6. The NMR spectrum of this product showed a complex aromatic pattern which was consistent with a structure of o-HT.

To increase the yields of p-HT, A. tamarii NRRL 3280 and Aspergillus sp. NRRL-A-13647 were grown in shaken flasks comparing aeration, inoculum, and the utilization of substrate when dissolved in DMSO or dimethyl formamide (DMF) at maximum solubility. The approximate maximum solubility of the substrate (AT) was 40% in DMSO and 30% in DMF (Table 2). A. tamarii NRRL 3280 gave essentially a complete conversion of substrate (2 g/liter) to product (p-HT) when added in increments of 0.5 g/liter from a 40% DMSO solution or a 30% DMF solution at 24, 48, 72, and 96 h. Yields of p-HT with A. tamarii NRRL 3280 were uniformly higher than those obtained with Aspergillus sp. NRRL-A-13647. A. tamarii NRRL 3280 thus appeared to offer good potential for further increased yields of p-HT and was studied further in shaken flasks. Results obtained from adding still higher levels of substrate (AT) from a 40% DMSO solution are shown in Table 3. The highest yield of p-HT, obtained with slant inoculum, was 8.6 g/liter from 10 g of substrate per liter or a 78.9% conversion. The highest yield obtained with vegetative inoculum was 6.55 g/liter or a 60.1% conversion. The conversion of AT to p-HT was also carried out by washed cell preparations of
**Table 2. Comparison of p-HT yields by Aspergillus tamarii NRRL 3280 and Aspergillus sp. NRRL A-13647 in aerated shaken flasks**

| Flask no. | Culture               | Type of inoculum* | Flask closure† | Substrate (AT) added as:‡ | Yield of p-HT at 192 h (g/liter) | Molar conversion (%) |
|-----------|-----------------------|------------------|----------------|--------------------------|--------------------------------|----------------------|
| 1         | A. tamarii NRRL 3280  | AS               | CP             | DP                       | 1.04                           | 47.7                 |
| 2         | A. tamarii NRRL 3280  | AS               | CP             | DMSO                    | 1.95                           | 89.5                 |
| 3         | A. tamarii NRRL 3280  | AS               | CP             | DMF                     | 2.02                           | 92.7                 |
| 4         | A. tamarii NRRL 3280  | VI               | CP             | DP                       | 0.63                           | 28.9                 |
| 5         | A. tamarii NRRL 3280  | VI               | CP             | DMSO                    | 1.21                           | 55.5                 |
| 6         | A. tamarii NRRL 3280  | VI               | CP             | DMF                     | 1.61                           | 73.9                 |
| 7         | A. tamarii NRRL 3280  | VI               | MFG            | DP                       | 0.56                           | 25.7                 |
| 8         | A. tamarii NRRL 3280  | VI               | MFG            | DMSO                    | 1.04                           | 47.7                 |
| 9         | A. tamarii NRRL 3280  | VI               | MFG            | DMF                     | 1.35                           | 62.0                 |
| 10        | A. flavus sp. NRRL A-13647 | AS         | CP             | DP                       | 0.45                           | 29.6                 |
| 11        | A. flavus sp. NRRL A-13647 | AS         | CP             | DMSO                    | 0.46                           | 21.1                 |
| 12        | A. flavus sp. NRRL A-13647 | AS         | CP             | DMF                     | 0.40                           | 18.3                 |
| 13        | A. flavus sp. NRRL A-13647 | VI          | MFG            | DP                       | 0.31                           | 14.3                 |
| 14        | A. flavus sp. NRRL A-13647 | VI          | MFG            | DMSO                    | 0.62                           | 28.5                 |
| 15        | A. flavus sp. NRRL A-13647 | VI          | MFG            | DMF                     | 0.52                           | 23.9                 |

*Medium (i) was used. Except for substrate and aeration, conditions were as described in Table 1.
*Abbreviations: AS, agar slant; VI, 10%, 72-h, single-passage vegetative inoculum.
*Abbreviations: CP, cotton plug; MFG, milk filter gauge.
*All flasks received a total of 2.0 g at AT per liter which was added in increments of 0.5 g/liter after 24, 48, 72, and 96 h total incubation time. Abbreviations: DP, dry powder; DMSO, 40% DMSO solution; DMF, 30% DMF solution.

**Table 3. Effect of increased substrate levels on p-HT yields by Aspergillus tamarii NRRL 3280 in aerated shaken flasks**

| Flask no. | Inoculum* | Amt added at intervals† (g/liter) | Total (g/liter) | Substrate (AT) addition | Yield of p-HT (g/liter) after period of incubation |
|-----------|------------|----------------------------------|----------------|-------------------------|-----------------------------------------------|
|           |            |                                  |                | 120 h                  | 144 h                           | 168 h   | 192 h | 216 h |
| 1         | AS         | 1.0                              | 4.0            | 3.73                    | 3.35                           | 3.59    | 2.85  | 3.77  |
| 2         | AS         | 1.5                              | 6.0            | 4.34                    | 5.27                           | 5.76    | 5.10  | 5.40  |
| 3         | AS         | 2.0                              | 8.0            | 4.39                    | 4.12                           | 5.44    | 4.13  | 6.10  |
| 4         | AS         | 2.5                              | 10.0           | 5.44                    | 7.13                           | 7.64    | 7.45  | 8.60  |
| 5         | VI         | 1.0                              | 4.0            | 3.41                    | 3.18                           | 2.77    | 3.02  | 3.58  |
| 6         | VI         | 1.5                              | 6.0            | 4.58                    | 4.77                           | 4.67    | 4.23  | 5.05  |
| 7         | VI         | 2.0                              | 8.0            | 4.48                    | 5.84                           | 5.64    | 5.26  | 6.19  |
| 8         | VI         | 2.5                              | 10.0           | 5.88                    | 5.46                           | 6.41    | 5.36  | 6.55  |

*Fermentation medium (i) was used. Except for substrate, other conditions were as described in Table 1.
*Abbreviations: AS, agar slant culture; VI, 10%, 72-h, single-passage vegetative inoculum.
*Substrate (AT) was added in equal amounts at 24, 48, 72, and 96 h total incubation time.

A. tamarii NRRL 3280. Forty-eight-hour-old cells were washed with buffer and incubated with substrate (AT) at a level of 2.5 g/liter on a Gump rotary shaker. After 24 h of incubation, the substrate had been completely converted to p-HT. Acetone-dried cells and a cell-free extract prepared from washed cells with a Carver press, however, failed to yield any of the desired p-HT product.

Results obtained with A. tamarii NRRL 3280 in 30-liter fermentors are shown in Table 4. Various levels and ages of vegetative inoculum were compared at different substrate levels. With 7.5%, 72-h single passage, vegetative in-
TABLE 4. Microbial conversion of AT to p-HT by Aspergillus tamarii NRRL 3280 in 30-liter fermentors

| Fermentor no. | Vegetative inoculum | Substrate (AT) addition | Yield of p-HT (g/liter) after period of incubation |
|---------------|---------------------|-------------------------|-----------------------------------------------|
|               |                     | Amt added at intervals* (g/liter) | Total (g/liter) | 114 h | 138 h | 162 h |
| 1             | 7.5%, 72-h single passage | 1.0 | 4.0 | 2.72 | 2.72 | 3.36 |
| 2             | 7.5%, 72-h single passage | 1.5 | 6.0 | 3.26 | 3.96 | 4.57 |
| 3             | 7.5%, 72-h single passage | 1.0 | 4.0 | 1.39 | 2.14 | 3.47 |
| 4             | 2.5%, 72-h single passage | 1.0 | 4.0 | 2.58 | 2.73 | 3.23 |
| 5             | 2.5%, 24 h × 24 h × 24 h | 1.0 | 4.0 | 2.98 | 3.12 | 3.10 |
| 6             | 7.5%, 72 h × 96 h × 72 h | 1.0 | 4.0 | 2.19 | 2.10 | 2.05 |

* Conditions were as follows: medium (i): Antifoam, 0.005% Hodag F-1; charge volume, 15 liters; sterilization, 1.0 h at 120 C and 15 lb/in²; agitation blade angle, 90°; aeration, 0.75 vol/vol/min; agitation rate, 360 rpm; incubation temperature, 28 C.

* Substrate (AT) was added in four equal amounts after 20, 44, 68, and 82 h total incubation time from a 40% DMSO solution.

oculum, yields of p-HT were comparable to those obtained in shaken flasks under similar conditions (Table 3). The highest yield obtained in 30-liter fermentors was 4.57 g of p-HT per liter when 6 g of substrate per liter had been added (69.8% conversion). This study again demonstrates that hydroxylation of aromatic compounds can be achieved in good yields by microbial enzymes.

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