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Biocatalytic Production of Dihydrocoumarin from Coumarin by *Saccharomyces cerevisiae*

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Natural dihydrocoumarin, which is of great interest in the flavor industry, was biotechnologically produced from pure coumarin or tonka bean meal with *Pseudomonas orientalis*, *Bacillus cereus*, and various *Saccharomyces cerevisiae* strains. Coumarin was shown to be converted to melilotic acid, which yielded dihydrocoumarin upon distillation during purification. About 1.0 g/L product was obtained from 25 g/L tonka beans with *S. cerevisiae* within 147 h. This dihydrocoumarin thus fulfills all of the criteria of a natural raw material and can be used as a natural flavoring in accordance with U.S. and European Union regulations.

KEYWORDS: Biotransformation; dihydrocoumarin; *Saccharomyces cerevisiae*; coumarin; melilotic acid; *Bacillus cereus*; *Pseudomonas orientalis*

INTRODUCTION

Dihydrocoumarin (DHC), a compound found in *Melilotus officinalis* (sweet clover) (*1*, *2*) and *Dipteryx odorata* Willd. (tonka beans) (*3*, *4*), is added as a flavoring agent to a wide variety of foods, including soft drinks, yogurt, and muffins, and is used as a common fragrance in cosmetics, lotions, and soaps (*5*). DHC has the sweet herbaceous aroma of new-mown hay and can be found in concentrations as high as 100 ppm in such foods as gelatins, puddings, and frozen dairy products. On the basis of a reported annual volume of 3750 kg, the estimated daily per capita intake of DHC is 12 μg/kg (*5*).

All DHC on the market is still produced via chemical synthesis, mostly through hydrogenation of coumarin (COU) (*6*−*8*). Because chemical synthesis often uses environmentally unfriendly production processes such as heavy metal catalysts, it is desirable to switch to bioproduction. That consumers have developed a "chemophobia" attitude toward synthetic compounds, especially when related to food and products used in the home, makes the use of flavor compounds of biological origin even more attractive (*9*).

Because flavor chemicals are often only present in minor quantities in their natural plant and animal sources, isolation and formulation of these chemicals can be very expensive. An alternate natural flavor production method is based either on de novo microbial processes (fermentation) or on bioconversions of natural precursors using microorganisms or isolated enzymes (biocatalysis) (*9*).

The term natural flavor or natural flavoring was defined by the U.S. Food and Drug Administration (FDA) as the essential oil, oleoresin, essence or extractive, protein hydrolysate, distillate, or any product of roasting, heating, or enzymolysis, which contains the flavoring constituents derived from a spice, fruit or fruit juice, vegetable or vegetable juice, edible yeast, herb, bark, bud, root, leaf or similar plant material, meat, seafood, poultry, eggs, dairy products, or fermentation products thereof, the significant function of which in food is flavoring rather than nutritional [FDA, *Code of Federal Regulations*, 21, Section 101.22 (3)]. Similarly, the European Union limits natural flavor as chemical substances with flavoring properties that are obtained by distillation and solvent extraction or enzymatic or microbiological processes from material of vegetable or animal origin either in the raw state or after processing for human consumption by traditional food preparation processes [European Council Directive 88/388/EEC 1 (*2*) (b) (i)].

The heterocyclic compound COU is generally regarded as a natural precursor of DHC, and the degradation of COU has been studied in plant, animal, and microbial systems (*10*−*14*). It appears that there are two possible pathways for the conversion of COU. In the plant *Melilotus alba* it has been suggested that COU is first converted to DHC by hydrogenation, which is then converted to melilotic acid (MEA) by hydrolysis (**Figure 1**) (*11*). The fungus *Fusarium solani* and a *Pseudomonas* strain probably degrade COU by the same metabolic pathway (*15*, *16*). However, analyses of various metabolites in the urine of the rat and of the rabbit both fed on a diet of COU have suggested that *o*-coumaric acid (oCA) is an intermediate between COU and MEA (**Figure 1**) (*12*). The isolation of a highly specific NADH:*o*-coumaric acid oxidoreductase from *Arthrobacter* sp. confirmed the second pathway in this soil bacterium (*17*).

Recently, the natural abundance ^{2}H NMR spectra of extractive COU and of its dihydroderivative DHC produced by *Saccharomyces cerevisiae* reduction has been compared with synthetic

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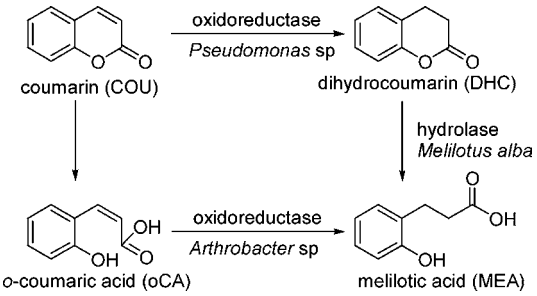

Figure 1. Degradation pathways of coumarin.

materials (*18*). The authors assumed that the preparation of DHC by enzymatic reduction of easily accessible COU might be economically interesting, because the material produced in this way is considered to be natural.

The present investigation was initiated to find microorganisms that could produce DHC from COU. Although a number of strains metabolized the substrate, quantitative transformation of COU was observed with only *S. cerevisiae*. The primary transformation product MEA was converted to DHC by distillation during the final purification step.

MATERIALS AND METHODS

Chemicals. Aromatic compounds, media, and solvents used in this study were purchased from Sigma-Aldrich Chemical Co., Deisenheim, Germany. All chemicals and solvents were of the highest purity available.

Microorganisms. Isolation and identification of *Pseudomonas orientalis* and *Bacillus cereus* Delaporte have been described previously (*19*). *S. cerevisiae* strains were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). *S. cerevisiae* (baker's yeast) was purchased at a local supermarket. Several microorganisms were isolated from soil, using COU as a sole source of carbon as described previously (*16*). The optical density of liquid media at 600 nm (OD600) was determined by UV mini 1240 (Shimadzu, Duisburg, Germany).

Biotransformation of Coumarin. The biotransformations were run over 6−13 days with microorganisms isolated from strawberry plants (*18*), microorganisms isolated from soil, and the following *S. cerevisiae* strains: DSMZ 2155, 70449, 70468, and 70487. The bacterial strains were maintained at −23 °C in Dworkin solution [2 g of $(NH_4)_2SO_4$, 4 g of KH_2PO_4, 6 g of Na_2HPO_4, and 0.2 g of $MgSO_4$ in 1 L of distilled water] supplemented with 25% w/v glycerol. The glycerol stocks of the bacteria and yeasts were resuspended in 60 mL of YPG medium (yeast extract 1% w/v, peptone from casein 2% w/v, glucose 2% w/v) and precultivated at 30 °C overnight at 180 rpm. A 10 mL aliquot of the preculture was diluted at a ratio of 1:10 with YPG medium and cultivated for 24 h at 30 °C. COU (50 g/L; 333 mmol/L) was added as a solution in absolute EtOH. Final concentrations of 0.1−5.0 g/L were tested. In a similar experiment tonka bean meal (25 g/L) was added to the medium as a source of COU. At specific time intervals, 1 mL samples were taken and analyzed directly by high-performance liquid chromatography−photodiode array detection (HPLC-DAD).

Dehydration of Melilotic Acid. One gram of MEA or 1 g of MEA obtained by bioconversion of COU with *S. cerevisiae* was distilled under vacuum with 10 mg of citric acid. The conversion rate determined with the reference material was >95% w/w, whereas the purity of the biotransformation product DHC determined by HPLC-DAD was >90%.

HPLC-DAD. For HPLC-DAD a Merck-Hitachi (Darmstadt, Germany) L-7100 HPLC gradient pump with a Merck-Hitachi L-7612 degasser, L-7200 autosampler, and L-7455 photodiode array detector was used including Merck-Hitachi HPLC-system manager software (version 4.0) for data acquisition and evaluation. A Eurospher 100-C18 column (length 250 mm × 4 mm i.d., particle size = 5 μm) (Knauer, Berlin, Germany) was employed. The LC parameters went from 5% v/v acetonitrile and 95% v/v water (acidified with 0.05% w/v formic acid) to 100% v/v acetonitrile and 0% v/v acidic water in 30 min, at which they were kept for 10 min before returning to 95% v/v water and 5% v/v acetonitrile in 5 min at a flow rate of 1.0 mL/min. Quantification of COU and its metabolites was performed using calibration curves determined with the references at 254 nm.

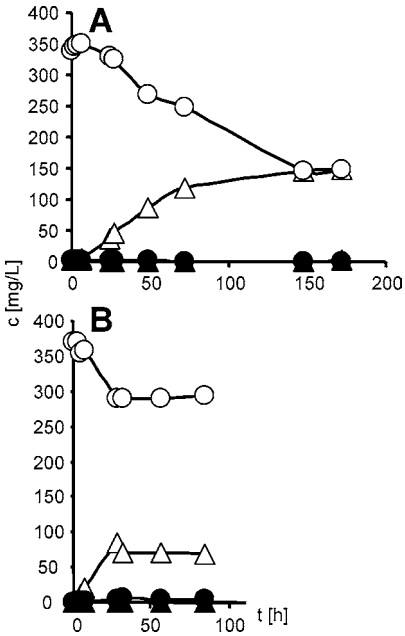

Figure 2. Biotransformation of coumarin by *B. cereus* (**A**) and *P. orientalis* (**B**). Coumarin (○) is metabolized to melilotic acid (△), dehydrocoumarin (▲), and *o*-coumaric acid (●). The values reported are mean values of duplicate experiments.

RESULTS

Screening for COU-Degrading Microorganisms. COU-degrading strains such as *Pseudomonas* sp., *Arthrobacter* sp., *Penicillium jenseni*, *Penicillium nigricans*, and *Fusarium solani* had already been obtained from soil samples by different groups (*15*−*17*, *20*). We decided to isolate microorganisms from soil according to a similar enrichment technique with COU as sole carbon source because DHC is one product of the COU degradation process and probably produced by a specific NADH:coumarin oxidoreductase (*16*).

Two of these strains and a number of bacterial strains that we had recently isolated from strawberry plants were tested for their ability to degrade the heterocyclic lactone in YPG medium (*18*). The strains that had been obtained from soil hardly converted the substrate, but significant metabolism of COU was observed with *B. cereus* Delaporte and *P. orientalis* isolated from strawberry leaves (**Figure 2**). Although MEA constituted the major product, which accumulated during the incubation period, minor amounts of oCA and DHC were also detected. More than 50% w/w of the fed COU was metabolized by *B. cereus* within 150 h. Because the strains that had been isolated from soil produced only 7 mg/L of MEA and undetectable levels of oCA and DHC, the experiments with these strains were discontinued and no efforts were put into their characterization. When YPG medium was replaced by a minimal salt medium, the growth rate of the bacterial strains greatly decreased, but the overall rate of COU metabolism was not affected (data not shown).

Biotransformation with *S. cerevisiae*. The hydrogenation of the α,β-unsaturated Massoi lactone (2-deceno-δ-lactone) to natural δ-decalactone by *F. solani* and *S. cerevisiae* strains has

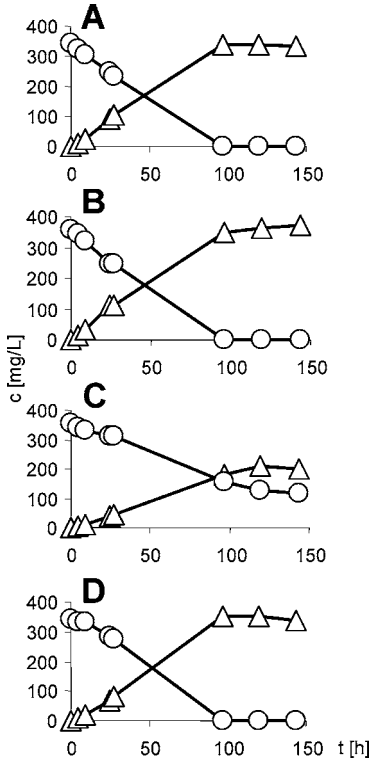

Figure 3. Biotransformation of coumarin by *S. cerevisiae* strains DSMZ 70449 (**A**), 2155 (**B**), 70468 (**C**), and 70487 (**D**). Melilotic acid (△) was detected as the major degradation product of coumarin (○) by HPLC-DAD. The values reported are mean values of duplicate experiments.

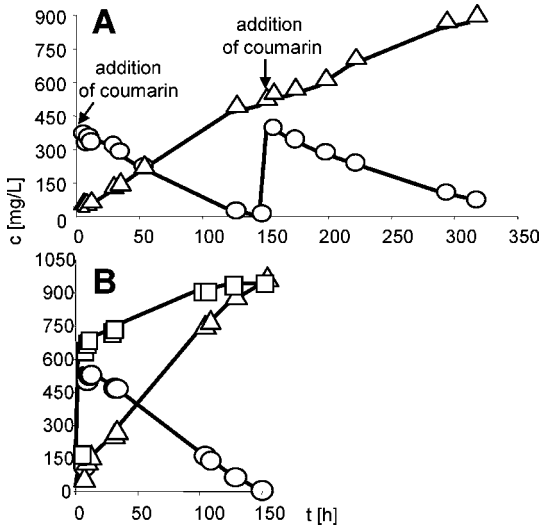

Figure 4. Biotransformation of coumarin (**A**) and tonka bean meal (**B**) by *S. cerevisiae* strain DSMZ 2155. Accumulation of melilotic acid (△) was observed even when an additional portion of coumarin (○) was added to the medium or when tonka beans were used as the source of coumarin. The total amount (□) of coumarin and melilotic acid increased during the biotransformation with tonka beans. The values reported are mean values of duplicate experiments.

been reported (*21*−*23*). Because *F. solani* is also able to degrade COU and the 2-deceno-*δ*-lactone and COU [lactone of (*Z*)-*o*-coumaric acid] display structural similarities, we tested the ability of a number of *S. cerevisiae* strains to hydrogenate the heterocyclic lactone. Three (DSMZ 70449, 2155, 70487) of the four *S. cerevisiae* strains investigated in this study completely converted the substrate to MEA, the hydrolysis product of DHC (**Figure 3**). The levels of the other metabolites detected by HPLC-DAD were negligible. Substrate concentrations up to 0.5 g/L were thoroughly transformed to the product, but higher levels of COU (1.0, 2.0, and 5.0 g/L) were toxic to the yeast, and the growth rate as well as the production rate of MEA decreased significantly (data not shown). Similar results were obtained when COU was converted with commercially available *S. cerevisiae* (baker's yeast).

Improvement of the Product Level and Biotransformation of Tonka Beans. To further increase the concentration of MEA, we added an additional amount of the substrate to the medium when the initial level of COU had been consumed by the yeast *S. cerevisiae* (**Figure 4A**). In this way toxic levels of COU (>0.5 g/L) were avoided during the biotransformation experiment. Because the concentration of MEA steadily increased to 0.9 g/L during the transformation process with no apparent effect on the yeasts, it can be concluded that the acid is less toxic to the yeast than its precursor. Comparable levels of the product were also obtained when commercially available *S. cerevisiae* (baker's yeast) was used.

It was the object of the present investigation to prepare DHC to high purity and good yield in an economical manner. Therefore, in addition to pure COU, COU-containing plant material was used as substrate for the biotransformation experiments with *S. cerevisiae*. COU is present in many plants, but it is found in high concentrations in sweet clover (*Melilotus officinalis*), sweet vernalgrass (*Anthoxanthum odoratum* L.), woodruff (*Galium odoratum*), bison grass (*Hierochloe odorata*), *Mikania glomerata* leaves, and the tonka bean (*Dipteryx odorata*) (*1*−*4*, *24*, *25*). Tonka bean meal was selected for its low cost and ready availability, prepared (25 g/L equivalent to 0.5 g/L free COU), and added to a YPG medium containing *S. cerevisiae* DSMZ 2155. At first, low levels of COU and MEA were detected by HPLC-DAD, but after 6 h, the level of COU had peaked (0.55 g/L). COU levels then declined for the duration of the experiment, whereas the concentration of MEA increased steadily until the end of the experiment, reaching >1.0 g/L. After 122 h, the sum of the extracted phenylpropanoids peaked at 1.1 g/L.

Generation of DHC from MEA. In a control experiment MEA was distilled under vacuum with citric acid. HPLC-DAD analysis of the product confirmed the total transformation (>95% w/w) to DHC. Therefore, to complete the biotransformation, MEA obtained by yeast-catalyzed catabolism of COU was quantitatively converted into DHC (>90% purity) by distillation in the presence of a catalytic amount of citric acid.

DISCUSSION

Although a number of the enzymes that are involved in the degradation of the heterocyclic lactone COU have been characterized and although the degradation pathways in bacteria, fungi, and yeasts are still not known in detail, MEA appears to be a common intermediate in the different pathways discussed (*16*, *17*). In accordance with published data we obtained COU-degrading strains from soil samples but found that two bacterial strains isolated from strawberry plants converted the substrate more effectively (**Figure 2**). Aside from minor levels of the primary products DHC and oCA, MEA constituted the major product in our biotransformation experiments, which agrees with previous results (**Figure 1**) (*16*). Due to the occurrence of the intermediates DHC and oCA, it is conceivable that both degradation pathways operate in *P. orientalis* and *B. cereus*. We observed that DHC is almost completely hydrolyzed within

72 h at pH values ranging from 3 to 6 (data not shown). Thus, accumulation of DHC in the medium (pH 4.5−6.5) is improbable.

An almost quantitative conversion of COU to MEA was achieved with a number of *S. cerevisiae* strains (**Figure 3**). Because the hydrogenation of COU [lactone of (*Z*)-*o*-coumaric acid] resembles the hydrogenation of Massoi lactone (2-decen-5-olide) (*21*), a process improvement similar to the biocatalytic production of natural δ-decalactone from 2-deceno-δ-lactone by *S. cerevisiae* should be possible. Recently, it has been shown that DHC produced by the *S. cerevisiae* reduction from COU cannot be differentiated from DHC prepared by chemical hydrogenation by stable isotope characterization (*18*).

When large (up to 5 L) batches of yeast were run repeatedly with COU added successively to maintain nontoxic levels, concentrations of up to 1 g/L of MEA were obtained within 312 h. However, when tonka bean meal (25 g/L; COU content 2% w/v) was used as a source for COU, >1 g/L MEA was produced within 122 h, less than half the time needed by the fed culture (**Figure 4**). It appears that COU is continuously released from the tonka bean meal, whereby the concentration of COU only briefly exceeds the toxic level for the yeast of 0.5 g/L. The maximum level of MEA (>1 g/L) even exceeded the concentration of free COU in the tonka beans at the beginning of the biotransformation experiment (2% w/v or 0.5 g of COU/L), which had been calculated by HPLC-DAD analysis of a tonka bean hexane extract (**Figure 4**). This prompts the speculation that the *O*-glucoside of oCA, melilotoside, constitutes an additional pool of COU in tonka beans that can release the precursor of MEA during the bioprocess (*26*−*28*). The normal procedure for liberating COU from tonka beans involves soaking for 24 h and drying, whereby a fermentation process takes place. The COU content following this procedure may be as high as 10% w/w (*29*). Recently, a supercritical fluid CO_2 extraction procedure was proposed to reduce the COU content of tonka bean extracts (*29*). We suggest that a tonka bean extract devoid of COU can also be obtained by fermentation with *S. cerevisiae*.

Here, we describe a bioprocess for the production of natural DHC from COU by *S. cerevisiae*. Tonka bean meal can be used without any pretreatment as an inexpensive natural source for the substrate. The DHC obtained through the conversion of COU by *S. cerevisiae*, followed by the distillation of the primary product MEA in the presence of natural citric acid, fulfills all of the criteria of a natural raw material and for this reason can be used as a natural flavoring in accordance with U.S. and European Union regulations.

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