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Enzymatic Preparation of Coniferaldehyde from Coniferyl Benzoate ex. Siam Benzoin

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ABSTRACT

Siam benzoin is a natural resin that contains up to 65% of coniferyl benzoate. Treatment of this material with an esterase followed by an alcohol dehydrogenase in a one-pot procedure gave 1.5 g/L coniferaldehyde in 36% molar yield. Several lipases and esterases were tested, but only pig liver esterase gave significant results. Various alcohol oxidases and alcohol dehydrogenases were screened for their ability to oxidize coniferyl alcohol to coniferaldehyde. Horse liver alcohol dehydrogenase coupled with a cofactor regeneration system was the most efficient of the enzymes tested.

Index Entries: Siam benzoin; coniferaldehyde; coniferyl benzoate hydrolysis; coniferyl alcohol oxidation; pig liver esterase; horse liver alcohol dehydrogenase; cofactor regeneration.

INTRODUCTION

Consumer demands for naturally produced and processed foods have revolutionized the methods used in food preparation. To be classified as natural, all ingredients must be of natural origin, and among these, flavorants are probably the most important for providing the right taste. This revolution has thus stimulated the search for biological methods which can be used to prepare "natural" flavoring materials. Of these,

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vanillin is one of the best known and most widely used. Although some of this material is still obtained from the original source (vanilla pods), significant amounts come from the sulphite liquors discarded from the Kraft paper process (*1*), or from the chemical modification of guaiacol (*2*).

Biologically, vanillin has been obtained from plant tissue cultures (*3*) and from the biotransformation of eugenol using various microorganisms (*4,5*). Coniferaldehyde, another potential precursor of natural vanillin, can be made from eugenol using *Arthrobacter* strain (*6*). The present work reports the enzymatic preparation of coniferaldehyde from Siam benzoin. This resin is a natural raw material obtained from Styrax trees such as *S. tonkinensis* and *S. benzoin,* a genus that is indigenous to Southeast Asia.

MATERIALS AND METHODS

Chemicals and Enzymes

Siam benzoin was purchased from C.V. Aroma, Medan, Indonesia. *Candida cylindracea* lipase (Type VII), *Rhizopus arrhizus* lipase (Type XI), wheat germ lipase (Type I), porcine pancreatic lipase (Type II), pig liver esterase (PLE), *Pichia pastoris* alcohol oxidase, horse liver alcohol dehydrogenase (HLADH), yeast alcohol dehydrogenase (YADH), and L-lactic acid dehydrogenase (LDH) (Type II from rabbit muscle) were purchased from Sigma Chemicals, St. Louis, MO. *Mucor miehi* esterase (esterase 3000) was obtained from Gist Brocades, Delft, Netherlands. Coniferaldehyde has been synthesized from ferulic acid (*7*). All other chemicals used were of reagent grade.

Purification of Coniferyl Benzoate

10 g of Siam benzoin was dissolved in 250 mL of ethyl acetate and filtered to remove insoluble material. The resulting solution was concentrated in vacuo to 15 mL and purified by flash chromatography on 800 g Florisil (100–200 mesh; Fluka, Buchs, Switzerland) in a 6×70 cm column eluted with hexane: diethylether (1:1).

Enzyme Assays

All enzymatic reactions were carried out at room temperature in magnetically stirred 100-mL flasks containing 50 mL of a 0.1*M* potassium phosphate buffer (pH 6.1–8.5). Samples were withdrawn at appropriate times and prepared for analysis by centrifugation (7500*g*, 5 min).

Analytical Methods

Coniferyl Benzoate

The quantitative determination of coniferyl benzoate was performed by HPLC using a C_{18} reverse phase column (5 μm, 250 mm×4, Macherey-Nagel Nucleosil, Düren, Germany), mobil phase water:acetonitrile

(50:50), flow rate 0.8 mL/min, room temperature, UV detection at 300 nm (Waters 490 UV detector). Samples were also analyzed by TLC after solvent extraction with diethyl ether on 0.2 mm silica gel plates (Merck Kieselgel, Darmstadt, Germany, 60 F_{254}) developed with hexane:ether (1:3).

Coniferyl Alcohol and Coniferaldehyde

After extraction with ethyl acetate and concentration, these two compounds were assayed by gas chromatography with a Hewlett-Packard 5890 gas chromatograph equipped with a Supelcowax 10 capillary column (30 m × 0.53 mm, Supelco, Bellefonte, PA) and a flame ionization detector. A 1 μL portion of the organic phase was analyzed after splitless injection. Nitrogen (15 mL/min) was used as the carrier gas. The temperature of the injector and detector was 250°C. The following temperature program was used: 140°C for 2 min, temperature ramp of 5°C/min, 230°C for 15 min. Cinnamic alcohol was used as internal standard. Under these conditions, the retention times (in minutes) were as follows: cinnamic alcohol, 17.7; coniferaldehyde, 23.0; and coniferyl alcohol, 27.8.

Acetaldehyde and Ethanol

These two compounds were estimated on a GS-Q megabore capillary column (30 m × 0.53 mm, J & W Scientific, Folsom, CA) fitted to a Varian 3700 gas chromatograph under the following conditions: 0.5 μL sample from centrifuged aqueous medium; splitless injection at 120°C; 18 mL/min nitrogen gas as carrier, FID detection at 280°C, temperature program from 110–240°C with a ramp of 10°C/min. *n*-Propanol was used as internal standard. Under these conditions, the retention times (in minutes) were as follows: acetaldehyde, 2.3; ethanol, 4.1; and *n*-propanol, 6.1.

RESULTS

Purification of Coniferyl Benzoate

The content of coniferyl benzoate in different lots of Siam benzoin varied from 15–70%. Flash chromatography on Florisil afforded this ester in a purified form that was suitable for enzymatic studies. Purification attempts on silica gel failed regardless of the solvent system employed, primarily because of *p*-coumaryl benzoate contamination. Four different benzoins are available: Siam, Sumatra, Penang, and Palembang. They are different both by their chemical composition and their natural occurrence (*8,9*).

Enzymatic Hydrolysis of Coniferyl Benzoate

Figure 1 shows the results of attempts to hydrolyze the ester bond of coniferyl benzoate using six commercially available lipases and esterases.

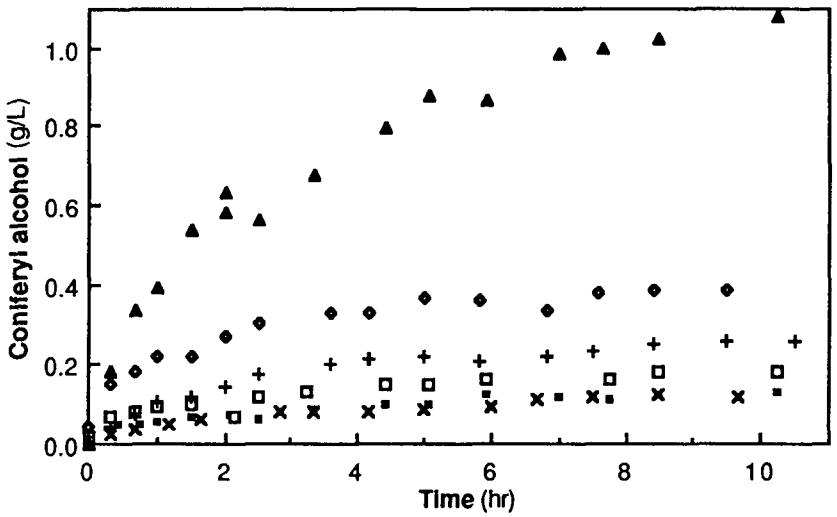


Fig. 1. Hydrolysis of coniferyl benzoate (25 m*M*) by various lipases and esterases. Reaction was carried out in water at 25°C, pH was maintained at 7.0 by 0.2*M* NaOH addition. ◆ PPL 4.2×10^5 U/L; x *Candida cylindracea* lipase 4×10^5 U/L; ■ wheat germ lipase 6.5×10^3 U/L; + *Rhizopus arrhizus* lipase 5.4×10^7 U/L; ▲ PLE 6.3×10^2 U/L; □ Gist Brocades esterase (U not determined).

This reaction is complicated by the fact that the substrate is practically insoluble in water, and furthermore, readily polymerizes in alkaline solution. Significant results were only achieved with PLE that gave 1 g/L coniferyl alcohol after 8 h incubation in buffered medium. Experiments with both miscible (10% v/v dioxane) and immiscible (10% v/v hexane, 5% v/v methylene chloride) solvents failed to improve the reaction. Under the conditions used, PLE had apparent K_m and V_{max} values of 24 m*M* and 5 mmoles/h respectively. It was also possible to perform the hydrolysis directly on unpurified milled Siam benzoin with this enzyme. Figure 2 shows the direct hydrolysis of Siam benzoin, 2.8 g/L of coniferyl alcohol was obtained after 8 h incubation of 16 g/L benzoin.

Enzymatic Oxidation of Coniferyl Alcohol

HLADH, YADH, and *Pichia pastoris* alcohol oxidase were tested for their ability to oxidize coniferyl alcohol to coniferaldehyde. The two alcohol dehydrogenases require NAD^+ as cofactor, whereas the oxidase does not. In order to establish the best reaction conditions, two different methods of cofactor regeneration were tried with each dehydrogenase. In one system, reduced cofactor was reoxidized with a second enzyme/substrate pair, namely lactate dehydrogenase/pyruvate. In the second system the dehydrogenase itself was used to regenerate the oxidized cofactor by catalyzing the reduction of a second substrate.

The results from these experiments are shown in Figs. 3 and 4. For the regeneration of NAD^+ with HLADH as oxidizing enzyme acetaldehyde, cyclohexanone, benzaldehyde, or acetone was used as second substrate.

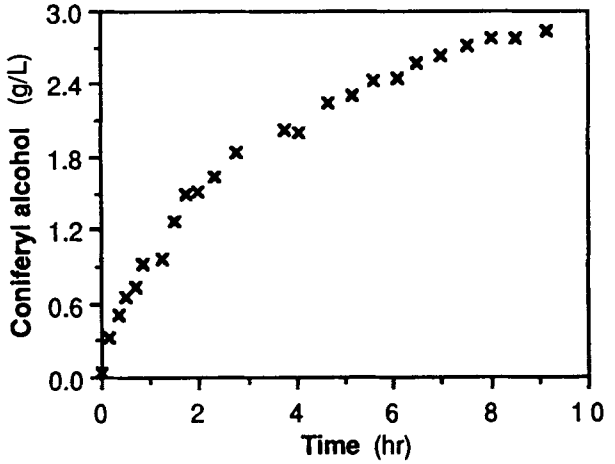


Fig. 2. Time course hydrolysis of Siam benzoin (16 g/L) in phosphate buffer (pH 7.5) by PLE 6.2×10^2 U/L.

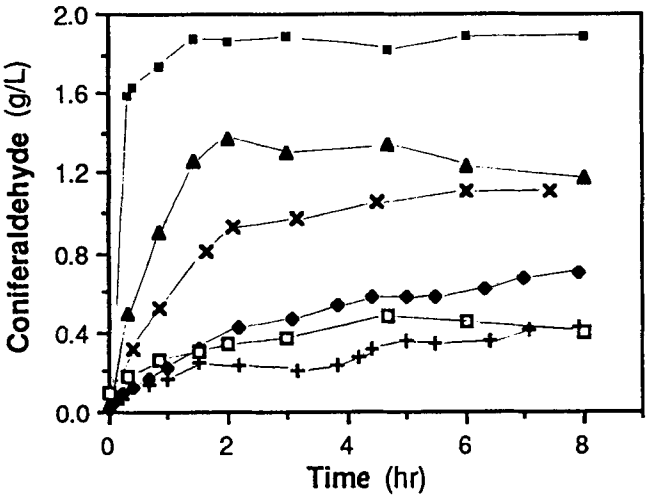


Fig. 3. Influence of various regenerating compounds on the coniferyl alcohol oxidation by HLADH with the following conditions: 12 m*M* coniferyl alcohol, 4 m*M* NAD, 45 m*M* regenerating molecule, 640 U/L HLADH (one unit of enzyme oxidases 1 μmole of ethanol/min at 25°C at pH 8.8), pH 8.1 and 25°C. ■ acetaldehyde; ▲ cyclohexanone; x benzaldehyde; ◆ pyruvate/LDH; □ acetone; + NAD alone.

Among these regeneration systems, acetaldehyde gave the best reaction rates and final concentrations of coniferaldehyde (1.8 g/L). The addition of NAD^+ in excess during the reaction had no stimulating effect. Coniferyl alcohol oxidation with YADH and the system lactate dehydrogenase/ pyruvate for cofactor regeneration gave similar results to those for HLADH. A similar pattern was also obtained when acetone was used as a second substrate. Acetaldehyde gave poor results in comparison with HLADH.

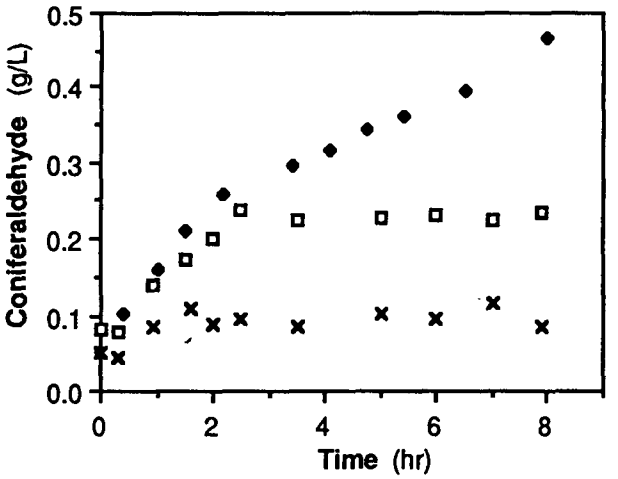


Fig. 4. Influence of different regenerating molecules on the coniferaldehyde oxidation by YADH (10^5 U/L) under the same conditions as described in Fig. 3. ◆ pyruvate/LDH; □ acetone; x acetaldehyde.

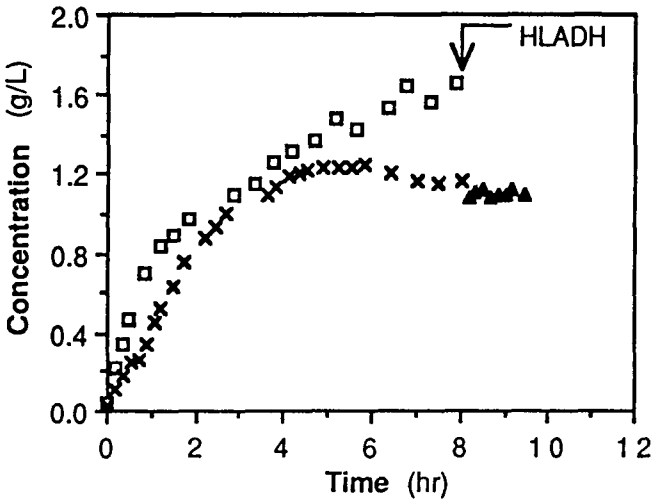
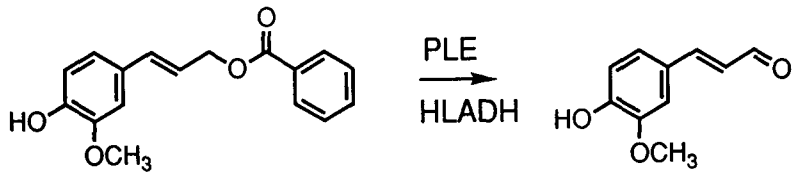


Fig. 5. Comparison between sequential or combined ester hydrolysis and alcohol oxidation of Siam benzoin (10 g/L) by PLE and HLADH under the following conditions: NAD 4 m*M*, acetaldehyde 45 m*M*, phosphate buffer pH 8.1, 25°C. □ alcohol produced by PLE (10,120/U/L); ▲ coniferaldehyde obtained by HLADH (640 U/L); x coniferaldehyde produced after simultaneous action of PLE and HLADH.

The *Pichia pastoris* alcohol oxidase, assayed to produce coniferaldehyde, showed no activity towards the alcohol even by using 10,000 U/L of enzyme and 15 m*M* of substrate.

One-Pot Reaction for Coniferyl Benzoate Hydrolysis and Coniferyl Alcohol Oxidation

Figure 5 shows the results of attempts to perform both the hydrolysis and oxidation reactions in one-pot (Scheme 1). PLE and HLADH were



Scheme 1. Ester cleavage by PLE and alcohol oxidation by HLADH to obtain coniferaldehyde from coniferyl benzoate.

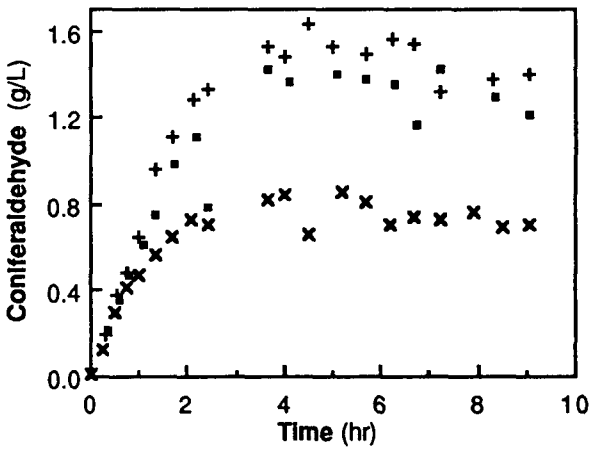


Fig. 6. Influence of HLADH concentration on coniferaldehyde production in a one-pot reaction. The following conditions were employed: Siam benzoin 10 g/L, NAD 4 m*M*, acetaldehyde 45 m*M*, PLE 10,120 U/L, phosphate buffer pH 8.1, 25°C, x 315 U/L; + 634 U/L; ■ 945 U/L.

tried both sequentially and simultaneously. In both cases, the hydrolysis was complete but not the oxidation. The increase of acetaldehyde concentration did not lead to further oxidation.

Figures 6, 7, and 8 show the results of trials to optimize the concentrations of HLADH, PLE, and Siam benzoin respectively. Under the conditions employed, HLADH activity was optimal between 640 and 945 U/L, PLE at 15180 U/L, and Siam benzoin at 10 g/L. Higher concentrations of NAD^+ or acetaldehyde than those stated did not give better yields of coniferaldehyde.

DISCUSSION

Enzymatic hydrolysis of benzoyl esters has rarely been described; some fungi can cleave the corresponding esters of simple sugars (*10*). In our case, both substrate and product have very low water solubility. A cosolvent might enhance solubility and substrate accessibility, but generally organic mediums promote esterification or transesterification and not hydrolytic reactions. In addition, an organic medium can

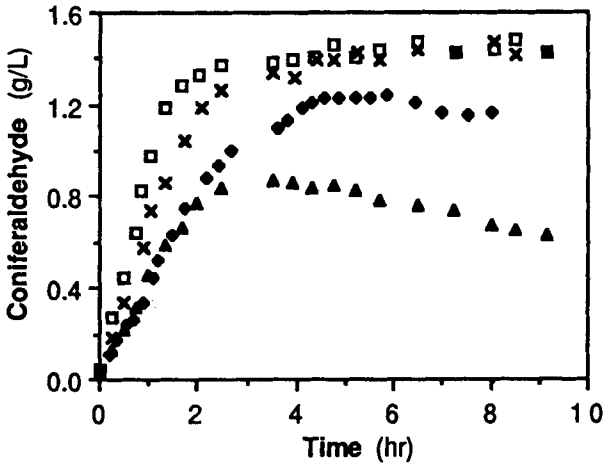


Fig. 7. Influence of PLE concentration on coniferaldehyde production in a one-pot reaction. ▲ 5060 U/L; ◆ 10,120 U/L; □ 15,180 U/L; x 20,240 U/L. Conditions as described in Fig. 6 except HLADH 640 U/L.

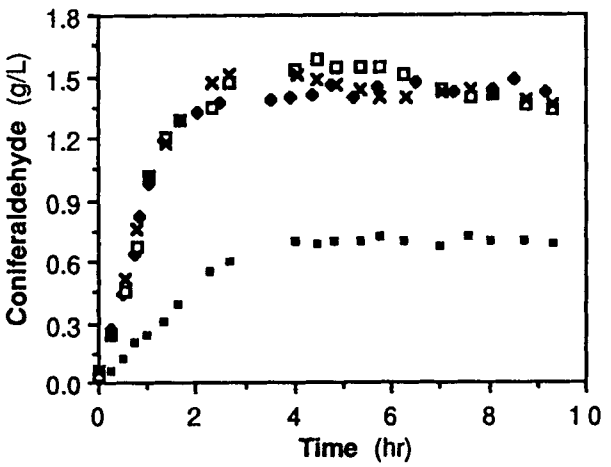


Fig. 8. Influence of Siam benzoin concentration on coniferaldehyde formation with the system PLE and HLADH. The following conditions were used: PLE 10,120 U/L, HLADH 640 U/L, NAD 4 m*M*, acetaldehyde 45 m*M*, phosphate buffer pH 8.1, 25°C. ■ 5 g/L; ◆ 10 g/L; x 15 g/L; □ 20 g/L.

drastically modify enzyme behavior (*11,12*). The pH must be neutral because of substrate and product instability (*8*). Among the many commercial lipases and esterases available, only the PLE gave significant hydrolysis (Fig. 1). The apparent K_m measured in buffered aqueous solution indicates a low affinity of the PLE toward the coniferyl benzoate that might be caused by the poor water solubility of the substrate. On the other hand, PLE is a very stable enzyme that does not require a cofactor and is relatively inexpensive (*13*). Direct hydrolysis was possible with

either crude Siam benzoin or purified coniferyl benzoate (Fig. 2) with a molar yield of 36%. No product or substrate inhibition and no side reactions were observed. The enzymatic oxidation of the coniferyl alcohol to the corresponding aldehyde has been extensively studied in plants where this step represents the final reaction for the biosynthesis of lignin precursors (*7,14,15*). In plants, this enzyme is called coniferyl alcohol dehydrogenase (CADH). It has been demonstrated that HLADH has a broad substrate specificity and is able to use cinnamic substrates (*16,17*). The two alcohol dehydrogenases, YADH and HLADH, were found to be active on the coniferyl alcohol (Fig. 3 and 4) but both require NAD^+ as cofactor.

Among the many possibilities described in the literature for NAD^+ regeneration, enzymatic reoxidation of NADH represents the most efficient and practical way. With electrochemical methods, the electrode is rapidly corroded and the reactants are unstable (*18,19,20*). Two different enzymatic systems can be used for NADH regeneration. First, a linked double enzyme system in which a second enzyme is used to reoxidize the cofactor, for example, pyruvate/lactate dehydrogenase (*21*) has been employed. The other system is a regeneration coupled to a second substrate reduced by the same enzyme (*21*).

These two systems have been tested with both HLADH and YADH, but in both cases the conversion of coniferyl alcohol to the aldehyde was at least four times lower for YADH than for HLADH (Fig. 3 and 4). The effect of cofactor regeneration was positive: without any NADH recycling, much lower coniferaldehyde concentrations were obtained (Fig. 3). Several hypotheses can be put forth to explain the differences of oxidation efficiency between the two alcohol dehydrogenases and the regeneration system employed: enzyme affinity for the substrate, competition between the substrate and the regenerating molecule, pyruvate reactivity, substrate or product inhibition for LDH, and ADH inhibition by pyruvate or lactate. HLADH coupled with acetaldehyde provided the highest coniferaldehyde production. The regenerating system pyruvate/LDH gave similar coniferaldehyde concentration independent of whether HLADH or YADH was used. For the YADH, the bi-enzymatic regeneration was the most efficient system.

Benzoate ester hydrolysis and coniferyl alcohol oxidation gave the same aldehyde concentration either by a stepwise reaction or by using a one-pot reaction (Fig. 5). In both cases, the oxidation of the alcohol was incomplete, only 70% conversion was obtained. Additional acetaldehyde or NAD^+ did not lead to higher coniferaldehyde concentration (Fig. 6). By increasing the NAD^+ concentration from 1–6 m*M* (45 m*M* acetaldehyde), no increase of the aldehyde concentration was observed above 2 m*M* of NAD^+. Similar experiments were performed to determine the optimal concentration of PLE and Siam benzoin. Above 15,180 U/L of PLE, no increase of coniferaldehyde concentration was produced in a one-pot reaction (Fig. 7). The influence of substrate concentration demonstrated that

optimal Siam benzoin concentration was around 10 g/L (Fig. 8). Optimization of all the reactants allowed a concentration of 8.3 m*M* (1.48 g/L) of coniferaldehyde to be achieved in 8 h, with an overall molar yield of 36%.

SUMMARY

Coniferaldehyde has been obtained from crude Siam benzoin by using pig liver esterase to hydrolyze the benzoate ester of coniferyl alcohol and horse liver alcohol dehydrogenase to oxidase the liberated alcohol. The oxidation step is combined with a cofactor recycling system using acetaldehyde as cosubstrate.

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