COMMUNICATION TO THE EDITOR

Yeast-Mediated Preparation of *l*-PAC in an Organic Solvent

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Abstract: The yeast-mediated acyloin condensation of benzaldehyde and pyruvic acid to form *l*-PAC occurs in a petroleum spirit solvent system at room temperature with moderate conversion (30%) and high enantioselectivity (86%ee) after 24 h. The addition of a small amount of ethanol (0.5% mL) to the reaction mixture inhibits the formation of the side product benzyl alcohol and increases the conversion to *l*-PAC. Conducting the reaction using ^{13}C-labeled pyruvate indicated that the pyruvate was incorporated into the *l*-PAC and that the excess pyruvate was converted into ethanol. Conducting the reaction at 5°C results in similar conversion but higher enantioselectivity. © 2002 John Wiley & Sons, Inc. *Biotechnol Bioeng* **77**: 827–831, 2002; DOI 10.1002/bit.10117
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INTRODUCTION

l-Phenylacetylcarbinol (*l*-PAC) is the intermediate in the preparation of the important vasopressor drugs, ephedrine and pseudoephedrine. The biotransformation process usually used for the production of *l*-PAC involves the yeast-mediated condensation of benzaldehyde with pyruvate (Hilderbrandt and Klavehn, 1934). Fermenting yeast is used for this reaction and the pyruvate is generated in situ by glycolysis of an appropriate carbohydrate source. It has been shown that the reaction involves decarboxylation of pyruvate to form acetaldehyde, which is then condensed with benzaldehyde to form *l*-PAC; both of these reactions are catalyzed by the enzyme pyruvate decarboxylase (PDC) (Crout et al., 1991). The major problem associated with the reaction is the concomitant yeast-mediated reduction of the benzaldehyde to benzyl alcohol, an unwanted by-product (Nikolova and Ward, 1991; Oliver et al., 1997). The benzaldehyde reduction is catalyzed by alcohol dehydrogenase enzymes present in the yeast. Efforts to completely prevent this reduction reaction have been largely hampered by the fact that active alcohol dehydrogenase enzymes are required for the conversion of glucose into pyruvate; inhibition of these enzymes prevents the production of pyruvate, and the acyloin reaction stops due to a lack of reagent (Oliver et al., 1997). In a reaction system in which the alcohol dehydrogenase enzymes have been inhibited, an alternative source of pyruvate is required. In the present study pyruvate is added to the reaction system. As with most reactions involving fermenting yeast, the isolation of the product from the aqueous reaction medium and its separation from the other metabolic by-products can be difficult and messy.

Recently, yeast-mediated reactions have been shown to proceed with high efficiency in organic solvent systems (Jayasinghe et al., 1993; Medson et al., 1997; Nakamura et al., 1991).The main advantage of the organic solvent system is that the product is readily isolated from the yeast mass by decantation. Nikolova and Ward (1992) reported some early work involving the use of immobilized yeast in hexane to prepare *l*-PAC. We now report our results concerning the factors that affect the yeast-mediated conversion of benzaldehyde to *l*-PAC in an organic solvent.

MATERIALS AND METHODS

Reagents

The baker's yeast (*Saccharomyces cerevisiae*) used in the reactions was Mauripan instant dry yeast supplied by Kerry Pinnacle Bakery Products, Sunshine, Australia, and it was stored at room temperature; no precautions were taken to exclude air or moisture, as reproducible results were obtained even after months of storage under these conditions. BDH AnalaR petroleum spirit (40–60°C) was used without purification; petroleum spirit is preferred to hexane as it is significantly cheaper, less toxic, and gave identical results. ^{13}C-labeled reagents were purchased from Cambridge Isotope Laboratories, Massachusetts. The pH 6 citrate buffer was prepared by mixing 0.1 *M* citric acid (9.5 mL) and 0.1 *M* trisodium citrate (40.5 mL) and diluting to 100 mL.

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Instrumental Techniques

Proton (^{1}H) NMR spectra were recorded at 300 MHz and carbon (^{13}C) at 75.43 MHz with a Bruker DPX300 spectrometer. The ^{1}H and ^{13}C NMR spectra refer to deuterochloroform or d_6-benzene solutions with tetramethylsilane (TMS) as the external reference (δ 0.00 ppm) unless otherwise stated.

Optical rotations were measured with an Optical Activity PolAAr 2000 AA series polarimeter.

Gas chromatographic analysis of products was performed on a Shimadzu GC-17A using a BP1 column, 0.25 mm ID, 15 m in length, and with a phase thickness of 0.25 μm. The oven temperature was held at 40°C for 3 min and was then increased at 8°C/min to 150°C.

Chiral gas chromatography was conducted using a Chiraldex G-TA (30 m × 0.25 mm) column with a phase thickness of 0.125 μm. The oven temperature was held at 95°C.

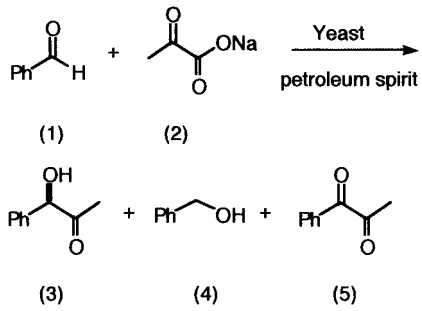
Procedure for the Yeast Reaction

This procedure is the result of optimizing the various parameters that affect the conversion of benzaldehyde to *l*-PAC (see Results and Discussion). Sodium pyruvate (2.5 g, 23 mmol), pH 6 citrate buffer (5 mL), ethanol (0.5 mL), and petroleum spirit (45 mL) were mixed and shaken at 20°C for 1 h. Baker's yeast (5 g) and benzaldehyde (0.12 g, 1 mmol) in petroleum spirit (5 mL) were added, and the reaction mixture was shaken at 20°C for 24 h. The aqueous buffer was fully absorbed by the yeast so that the reaction system was composed of hydrated yeast in an organic solvent; no aqueous phase was evident. The yeast tended to coagulate during the course of the reaction, but this did not adversely affect the conversion. Reactions conducted for longer periods showed no increase in conversion. Gas chromatographic analysis indicated a 30% conversion to *l*-PAC (**3**). Conversion is calculated by comparing the amount of product with starting material in the GC trace, and the values quoted represent an average of at least three reactions. The yeast was filtered off, and washed with diethyl ether, and the product purified via flash distillation (200°C/1 mm) to give *l*-PAC (0.024 g, 16% yield). Repeated washing with solvent yielded no additional product.

The *l*-PAC (**3**) was derivatized via the following method to determine the enantiomeric excess (ee). Dichloromethane (0.5 mL) and freshly distilled trifluoroacetic anhydride (0.5 mL) were added to a vial containing about 5 mg of *l*-PAC (**3**). The vial was capped and left at room temperature for 30 min. GC analysis indicated complete derivatization of the *l*-PAC after this period. Excess reagent was evaporated using a hotplate and dry dichloromethane added to the vial. The derivatized product was analyzed using chiral gas chromatography and showed an enantiomeric ratio of 93:7 (86% ee).

RESULTS AND DISCUSSION

The yeast-mediated acyloin condensation of benzaldehyde with pyruvic acid was carried out in petroleum spirit using a system comprising 5 g yeast/mmol benzaldehyde, 1 g sodium pyruvate, 45 ml petroleum spirit, 1 mL pH 6 citrate buffer/g yeast, 20°C, 24 h. This is based upon the system described by Medson et al. (1997) for yeast-mediated reduction reactions with the inclusion of pyruvate and 0.1 mL pH 6 citrate buffer/g yeast, in place of the water. Gas chromatographic analysis of this reaction mixture indicated 8% *l*-PAC (**3**), 21% benzyl alcohol (**4**), 1% 1-phenylpropan-l,2-dione (**5**), and 70% unreacted benzaldehyde. While the desired acyloin condensation reaction had taken place, reduction of beazaldehyde to benzyl alcohol and oxidation of *l*-PAC



Scheme 1

to the diketone (**5**) had also occurred (Scheme 1).

In an attempt to inhibit these oxidation and reduction reactions, ethanol was added to the reaction mixture. Conducting the yeast-mediated reaction in the presence of ethanol (0.5 mL) resulted in a mixture of benzaldehyde and *l*-PAC (88:12) and no trace of either benzyl alcohol or the diketone (**5**). Clearly the added ethanol had an inhibiting effect upon the oxidoreductase enzymes responsible for the formation of these side products (Salter and Kell, 1995).

Effect of Pyruvate

In an aqueous reaction system involving fermenting yeast, pyruvate is formed in situ from added carbohydrate, but the concentration of the pyruvate in the reaction is not easily measured and therefore cannot be readily manipulated. In order to determine the relationship between the pyruvate concentration and the conversion of benzaldehyde to *l*-PAC in petroleum spirit, the yeast-mediated reaction was conducted with varying amounts of pyruvate with 0.5 mL added ethanol (Fig. 1). Sodium pyruvate was dissolved in the citrate

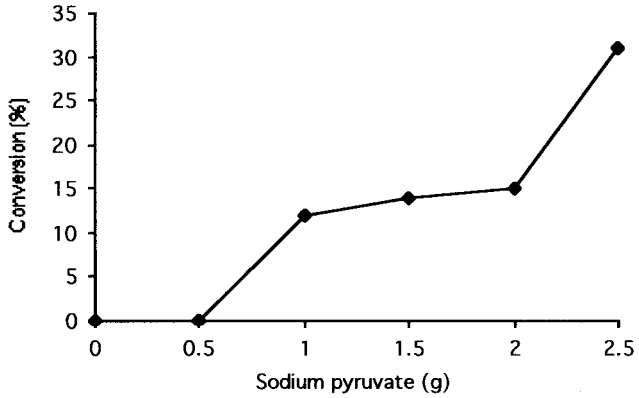


Figure 1. Effect of sodium pyruvate upon the production of *l*-PAC from the yeast-mediated acyloin condensation of benzaldehyde in the presence of ethanol.

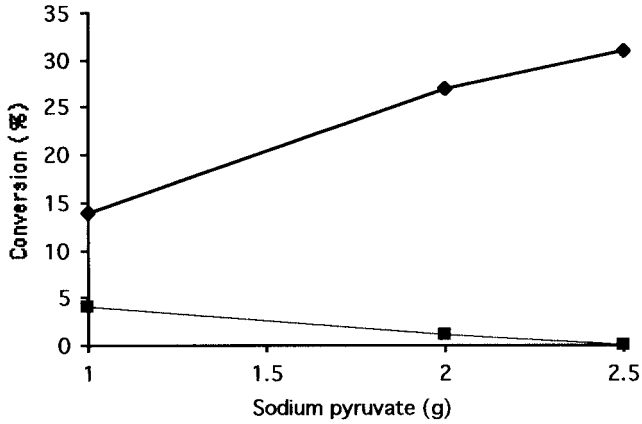


Figure 2. Effect of sodium pyruvate upon the production of *l*-PAC from the yeast mediated acyloin condensation of benzaldehyde with the pyruvate/citrate solution adjusted to pH 5.45; (◆) *l*-PAC (■) benzyl alcohol.

buffer, and the resultant solution was added to the reaction. It was found that no more than 2.5 g of sodium pyruvate could be dissolved in 5 mL of the citrate buffer, so higher amounts of pyruvate were not used.

l-PAC was not formed in the absence of pyruvate, indicating that the yeast does not contain residual pyruvate which could condense with the added benzaldehyde. As the amount of pyruvate was increased the conversion to *l*-PAC increased to a maximum of 31% at 2.5 g pyruvate. In all cases no side products were observed.

These results suggest that a large excess of pyruvate (2.5 g, 23 mmol) compared with benzaldehyde (1 mmol) is required for maximum conversion. The addition of this amount of pyruvate lowered the pH of the citrate solution, and it is possible that the increased conversion obtained in these reactions is due to the change in pH rather than increased amounts of pyruvate. Addition of 2.5 g of pyruvate to the citrate buffer resulted in a pH of 5.45. In order to determine whether the pH or the quantity of pyruvate was the more significant factor, the reactions were repeated and in each case the pH of the pyruvate/citrate solution was adjusted to pH 5.45 using ammonium acetate (Fig. 2).

The conversion of benzaldehyde to *l*-PAC was again shown to be influenced by the amount of pyruvate added to the reaction: the more pyruvate, the higher the conversion. With the pH of the pyruvate/citrate solution adjusted to 5.45 an increase in the production of *l*-PAC is observed. The effect was most pronounced when 2 g of pyruvate was added to the reaction: 27% compared with only 15% when the pH of the pyruvate/citrate solution was not adjusted. These results indicate that both the pH of the pyruvate solution and the concentration of pyruvate affect the amount of *l*-PAC produced.

It was interesting to note the apparent relationship between the amount of added pyruvate and the production of the unwanted side product benzyl alcohol. Small amounts of benzyl alcohol were formed when less than 2.5 g of pyruvate was added (Fig. 2). In order to examine this relationship further, the reaction was conducted in the absence of ethanol, in order to maximise the production of benzyl alcohol. Varying amounts of pyruvate were added to the citrate buffer and the pH adjusted to pH 5.45 (Fig. 3).

In the absence of ethanol, the greatest conversion of benzaldehyde to *l*-PAC (15%) occurred when only 2 g of pyruvate was added to the reaction system, compared to a maximum conversion of 31% with 2.5 g of pyruvate in the presence of ethanol. The ethanol therefore not only inhibits the formation of the side products but also results in an increased conversion to *l*-PAC. The addition of increased amounts of pyruvate also leads to a decrease in the amount of the side product, benzyl alcohol, produced in the absence of ethanol. With 1 g of added pyruvate 21% benzyl alcohol is formed, while the addition of 2.5 g of pyruvate completely eliminates the formation of this side product. These results indicate that the pyruvate concentration has a role to play in the inhibition of the reduction reactions.

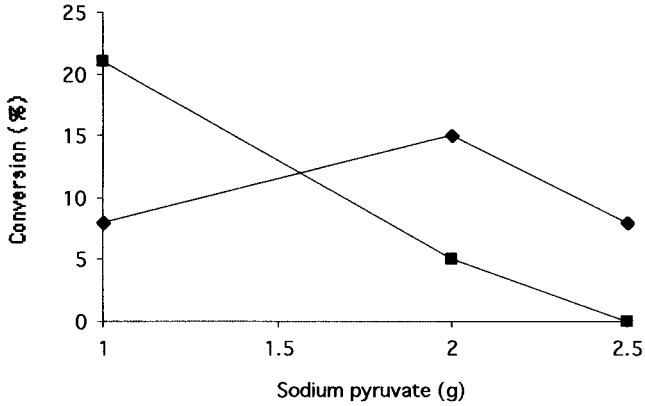
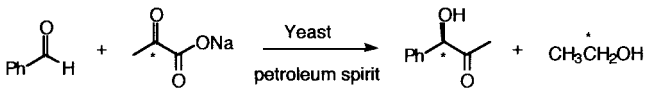


Figure 3. Effect of sodium pyruvate upon the production of *l*-PAC and benzyl alcohol from the yeast-mediated acyloin condensation of benzaldehyde in the absence of ethanol with the pyruvate/citrate solution adjusted to pH 5.45; (◆) *l*-PAC; (■) benzyl alcohol.

MECHANISTIC STUDIES

The influence of pyruvate upon the production of the side product benzyl alcohol was investigated by conducting a yeast-mediated reaction using sodium [2-^{13}C] pyruvate in the absence of ethanol. It was found that the ^{13}C label had been incorporated into *l*-PAC and ethanol (Scheme 2).



* ^{13}C label

Scheme 2

Analysis of the reaction mixture after 24 h using ^{13}C NMR spectroscopy indicated the presence of [1-^{13}C] ethanol, clearly indicating that some of the pyruvate had been converted into ethanol. Previous investigations into the yeast-mediated preparation of *l*-PAC in a fermenting system have shown that glucose is converted into pyruvate and then to acetaldehyde, which condenses with benzaldehyde to give *l*-PAC (Crout et al., 1991). Yeast also contains oxido/reductase enzymes, such as yeast alcohol dehydrogenase, which are capable of reducing acetaldehyde to ethanol. The reduction of acetaldehyde to ethanol involves the concomitant oxidation of NADH to NAD^+, as does the reduction of benzaldehyde to benzyl alcohol. In the reactions in an organic solvent there are no added nutrients to recycle NAD^+ back to NADH. The amount of NADH available in these reactions is therefore limited, and with both reactions consuming NADH at the same time, it is quickly exhausted. Increasing the amount of pyruvate added to the reaction results in an increase in the amount of ethanol formed, which has a 2-fold effect on the yeast system. First, the ethanol produced inhibits the reductase enzyme(s), and second, the production of ethanol from pyruvate consumes the NADH limiting the amount available to convert benzaldehyde to benzyl alcohol. Overall, this has the effect of decreasing benzyl alcohol formation without affecting the production *l*-PAC.

The solvent was removed from the reaction mixture and analysis of the concentrated sample using ^{13}C NMR showed a strong signal for the carbonyl group of *l*-PAC at 205.4 ppm, clear evidence that the C-2 from the ^{13}C-labeled pyruvate had been incorporated into the *l*-PAC.

Analysis of the product using ^{1}H NMR showed a distinct doublet for the CH at 5.1 ppm (J_{CH} = 3 Hz) due to coupling to the ^{13}C carbonyl group. No evidence of a singlet for this CH was observed indicating that the carbonyl carbon of the *l*-PAC formed in the reaction was completely ^{13}C labeled. The *l*-PAC is therefore formed exclusively from the added pyruvate, and there is no contribution from glucose or pyruvate already present in the yeast.

The addition of ethanol to the yeast-mediated reaction resulted in an increase in the amount of *l*-PAC produced, as well as a decrease in the production of benzyl alcohol. It is possible that the added ethanol could be oxidized to acetaldehyde, which could then undergo condensation with benzaldehyde to form *l*-PAC. [l-^{13}C]-Ethanol was added to the yeast-mediated reaction in petroleum spirit, in order to detect any *l*-PAC formed from ethanol. ^{13}C NMR analysis of the *l*-PAC isolated from this reaction showed no evidence of ^{13}C labeling. *l*-PAC is therefore formed exclusively from the added pyruvate, and although the addition of ethanol enhances the yield of *l*-PAC it is not a precursor to *l*-PAC. The increase in the amount *l*-PAC produced is probably due to inhibition of the enzymic reaction sequence which converts pyruvate to ethanol, resulting in an increase in the amount of available pyruvate.

Effect of Benzaldehyde

Studies of the acyloin condensation of benzaldehyde to *l*-PAC using fermenting yeast in an aqueous system indicated that the benzaldehyde has a toxic affect upon the yeast cells (Long and Ward, 1989). The concentration of benzaldehyde was shown to be a limiting factor in the production of *l*-PAC. The yield of *l*-PAC could be increased in an aqueous system by slowly adding the benzaldehyde (Netrval and Vojtisek, 1982) to the reaction mixture or by adding it in batches (Mahmoud et al., 1990a, 1990b). The slow addition of benzaldehyde to a yeast-mediated reaction in petroleum spirit gave only a limited conversion (7%) to *l*-PAC. Adding the benzaldehyde in two equal portions 6 h apart also resulted in a decreased conversion (14%) to *l*-PAC. In contrast to the reports of improved *l*-PAC formation in aqueous systems as a result of the slow addition of benzaldehyde, a decrease in the production of *l*-PAC is observed in an organic solvent system. This suggests that benzaldehyde does not have an inhibitory effect upon the formation *l*-PAC in an organic solvent system.

Effect of Temperature

It has been shown that the yeast reductase enzymes are slowly deactivated in the petroleum spirit reaction system; after 24 h very little residual reductase activity remains (Medson et al., 2001). Conducting yeast-mediated reduction reactions at reduced temperature (<10°C) prevented the enzyme deactivation and improved conversions were obtained. Shin and Rogers (1996) showed that the conversion of benzaldehyde to *l*-PAC using partially purified pyruvate decarboxylase (PDC) increased if the reaction was conducted at 4°C. The yeast-mediated conversion of benzaldehyde to *l*-PAC in petroleum spirit was carried out at 5°C for 24 h. Analysis

of the reaction mixture indicated a 24% conversion to *l*-PAC, slightly lower than that obtained at 20°C.

Enantioselectivity

The *l*-PAC formed from the yeast-mediated acyloin condensation of benzaldehyde using the optimum reaction conditions at 20 and 5°C was isolated and purified. The reaction at 20°C gave *l*-PAC in 16% yield and with 86% ee. The reaction conducted at 5°C gave a higher isolated yield (24%) and higher enantioselectivity (90% ee). Specific rotation measurements on the products indicated that in both cases *l*-PAC was the major enantiomer formed.

CONCLUSION

The yeast-mediated condensation of benzaldehyde with pyruvate has been shown to occur in petroleum spirit. Addition of a small amount of ethanol to the reaction mixture prevents the formation of the unwanted side product benzylalcohol, and a higher conversion to *l*-PAC is obtained. The increase in the conversion of *l*-PAC is solely due to inhibition of the reduction of benzaldehyde to benzyl alcohol, as the ethanol is not incorporated in the *l*-PAC. The added pyruvate is converted into ethanol, so a large excess of pyruvate is required. As with all yeast-mediated reactions in an organic solvent, the product is easily isolated from the reaction mixture by decanting; removal of the solvent gives a pure product. Conducting the yeast-mediated condensation reaction in petroleum spirit produces *l*-PAC, with high optical purity, as the sole product.

The yeast used in this study was Mauripan Instant Dry Yeast and was kindly provided by Kerry Pinnacle Bakery Products, Sunshine, Australia. Financial assistance provided by Polychip Pharmaceuticals Pty. Ltd (a wholly owned entity of Circadian Technologies Ltd) is gratefully acknowledged.

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