Aromatic Aldehydes as Substrates for Yeast and Yeast Alcohol Dehydrogenase

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The conversion of benzaldehyde to optically active L-phenylacetyl carbinol by yeast fermentation is a key step in the manufacture of L-ephedrine.[1] Typical fermentation raw materials are molasses, which provides a source of hexoses for glycolysis, and benzaldehyde. L-phenylacetyl carbinol formation is catalyzed by the pyruvate decarboxylase complex.[2] In the carboligase reaction, pyruvate is decarboxylated to active acetaldehyde which forms the carbinol product in the presence of benzaldehyde cosubstrate.[3,4] Quantitative conversion of benzaldehyde to phenylacetyl carbinol is never achieved and usually some of the benzaldehyde is reduced to benzyl alcohol.[5,6] Benzoic acid may also be produced at very low levels.[7]

It has previously been reported[8] that *Saccharomyces cerevisiae* can also catalyze the conversion of 3-methoxy-4-hydroxy benzaldehyde and 3,4-methylenedioxybenzaldehyde to the corresponding optically active acyloin compounds L(-)-3-methoxy-4-hydroxyphenylacetyl carbinol and L(-)-3,4-methylenedioxyphenylacetyl carbinol respectively. Under these conditions, side reactions involving formation of the corresponding substituted aromatic alcohols are likewise usually formed. Selected strains of *Aerobacter aerogenes* can also catalyze the conversion of 3,4-dimethoxybenzaldehyde to L(-)-3,4-dimethoxyphenylacetyl carbinol.[8]

In a recent study, the reactivity of yeast alcohol dehydrogenase towards a range of aldehydes was tested. Aromatic aldehydes such as benzaldehyde, *p*-anisaldehyde, *m*-tolualdehyde, and *p*-hydroxybenzaldehyde were found not to be substrates.[9]

We have investigated the conversion of aromatic aldehydes to L-acetyl aromatic carbinols and aromatic alcohols by *Saccharomyces cerevisiae* and also the conversion of aldehyde to corresponding alcohol by yeast alcohol dehydrogenase. One objective of the investigation was to evaluate the application of this bioconversion system for production of modified carbinols for use as precursors to synthesis of new ephedrine compounds with potential pharmacological activity. The second objective was to clarify the role of yeast alcohol dehydrogenase in production of unwanted by-products of this bioconversion. The reactions involved are illustrated in Figure 1.

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EXPERIMENTAL

Materials

Fresh pressed baker's yeast, *Saccharomyces cerevisiae* [dry wt of 30% (w/w)] was obtained from Irish Yeast Products (Dublin, Ireland). Purified yeast alcohol dehydrogenase (360 units/mg protein) and NADH were obtained from Sigma (Poole, Dorset, U.K.). Bacteriological peptone was supplied by Oxoid (Basingstoke, England). Gas chromatography (GC) column packing was obtained from

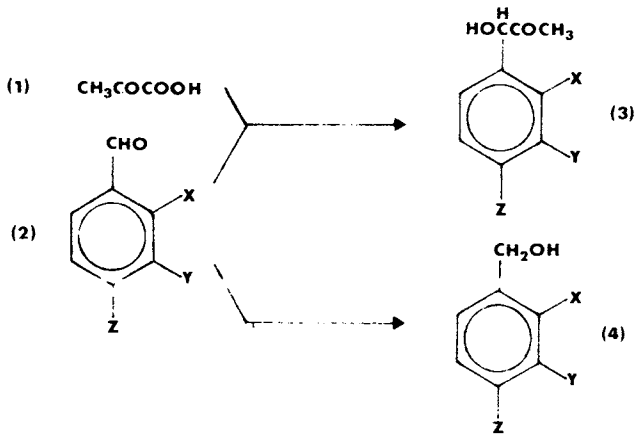

Figure 1. Bioconversion of aromatic aldehyde to L-acetyl aromatic carbinol and aromatic alcohol by *Saccharomyces cerevisiae*: (1) pyruvic acid, (2) aromatic aldehyde, (3) L-acetyl aromatic carbinol, and (4) aromatic alcohol.

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Phase Separations Ltd. (Clwyd, Wales, U.K.) A prepacked high-pressure liquid chromatography (HPLC) steel column was obtained from Waters Associates (Milford, MA). All other chemicals were of analytical or reagent grade.

Yeast Bioconversion Conditions

The fermentation medium contained bacteriological peptone, 6 g/L; sodium pyruvate, 61.7 g/L; and citric acid, 10.5 g/L, adjusted to pH 4.5. Fermentations were carried out in 250-mL Erlenmeyer flasks, containing 150 mL medium, inoculated with 4.5 g fresh baker's yeast. Flasks were incubated at 30°C on an orbital shaker set at 150 rpm. Following an equilibration period of 1 h, the fermentation was initiated by the addition of 2 g/L aromatic aldehyde. At 1, 2, 3, and 4 h intervals after fermentation initiation, aliquots equivalent to 2 g/L aromatic aldehyde were further added to each flask. After a fermentation period of 3 h an additional 2.25 g yeast was added per flask. After a fermentation period of 6 h cells were removed by centrifugation at 7500*g* and the clarified broth recovered for analysis.

Enzymatic Conversion of Aldehyde to Alcohol

The reaction mixture contained aromatic aldehyde, 4m*M*; NADH, 4m*M*; sodium phosphate buffer, 0.1*M*, pH 7.0; and purified yeast alcohol dehydrogenase, 7 mg/mL. Incubations were carried out at 25°C for 6 h. The reaction was then terminated by boiling and the denatured heat coagulated enzyme was removed by centrifugation. A control which contained no enzyme was included in each case.

Purification of L-Acetyl Aromatic Carbinols

Selected L-acetyl aromatic carbinols were extracted using a modification of the procedure reported by Smith and Hendlin.[3] The total fermentation broth was extracted with an equal volume of ether and the separated ether fraction was vacuum evaporated at 40°C to less than 10 mL. This concentrate was added to 100 mL ether and extracted with an equal volume of aqueous sodium carbonate to remove any acids present. The ether fraction was then extracted three times with an equal volume of aqueous 10% (w/v) sodium metabisulfate which forms a complex with the carbinol and aldehyde. The combined aqueous extract was washed with ether, 2 × 100 mL, to remove any alcohol present. Solid $NaHCO_3$ was added to the aqueous extract until CO_2 evolution ceased resulting in production of free carbinol and aldehyde. Both aldehyde and carbinol were then extracted into ether, 2 × 100 mL, and the ether extract dried with sodium sulfate and concentrated by vacuum evaporation.

Concentrated selected fractions, containing the carbinol and aldehyde, prepared by the above procedure, were finally separated by application to 40 × 4 cm silica gel columns. The L-acetyl aromatic carbinols, namely L-3-methylphenylacetyl carbinol, L-4-chlorophenylacetyl carbinol, and L-3-methoxyphenylacetyl carbinol, were eluted with the appropriate solvent systems chloroform, 50:50 ether:chloroform and 50:50 methanol:dichloromethane, respectively. Fractions containing the separated carbinols were combined and the purified carbinols recovered by vacuum evaporation.

Analysis

Colorimetry

L-Acetyl aromatic carbinols produced by fermentation and also recovered purified carbinols were estimated by the colorimetric method of Groger and Erge.[10] Acetyl benzoyl was used as a standard using the appropriate correction factor.

Gas Chromatography

Aromatic alcohols produced in the fermentation and solutions of purified carbinols were determined by gas chromatography. The GC analysis was carried out on a Perkin–Elmer model F17 gas chromatograph using a glass 6 ft long × 0.25 in. O.D. column packed with 30% silicone elastomer E301 on Chromosorb WHP 60-80. Oven temperature was maintained constant at 195°C, except for analysis of 3-methoxylbenzyl alcohol. In this case, a temperature program was used ranging from 155 to 195°C, increasing at a rate of 3°C/min. Standards and fermentation broth (10 mL) were extracted with ether (2 × 20 mL) and the ether extract made up to 20 mL. One-microliter volumes were injected. Peak areas were measured using a Carlo Erba SP 4720 integrator.

High-Pressure Liquid Chromatography

Aldehyde and alcohol concentrations in alcohol dehydrogenase samples were determined by high pressure liquid chromatography using a Waters Associates liquid chromatograph, containing a μBondapak TM/C18 reverse phase steel column (3.0 × 300 mm) with 40% (v/v) methanol in water as mobile phase. The flow rate was 1 mL/min and detection was by absorbance at 254 nm. Measurements were made before addition of the enzyme and at the end of the reaction.

^{1}HNMR and Optical Rotation Analysis

Purified acetyl aromatic carbinols were characterized by hydrogen-based nuclear magnetic resonance (^{1}HNMR) and specific optical rotation measurements. Structures were established by ^{1}HNMR as solutions in deuteriochloroform containing 1% TMS using a Perkin–Elmer R12B spectrometer. Optical rotations were measured on an Optical Activity AA-5 polarimeter. For optical activity measurements L-3-methoxyphenylacetyl carbinol and L-4-methylphenylacetyl carbinol were dissolved in ethanol. L-4-Chlorophenylacetyl carbinol was dissolved in chloroform.

RESULTS AND DISCUSSION

In a preliminary experiment, selected aromatic and aliphatic aldehydes were added to the fermentation medium. Following a 7-h fermentation, ether extracted cell free supernatants were qualitatively tested for the presence of acyloin product using ^{1}HNMR. Substituted aromatic aldehydes containing a CH_3, OCH_3, or Cl group substituted in the para position acted as substrates as did the aliphatic aldehydes, acetaldehyde and propionaldehyde. *p*-Nitrobenzaldehyde, cinnamaldehyde and salicylaldehyde did not appear to produce carbinol products.

The yields of L-acetyl aromatic carbinols and aromatic alcohols produced from benzaldehyde and substituted aromatic aldehydes containing a $-CH_3$, $-CF_3$, $-OCH_3$, or $-Cl$ in either the ortho, meta, or para ring position were determined after a 6-h fermentation. The results are presented in Table I. Highest carbinol yields were observed with benzaldehyde substrate. Aldehydes substituted in the ortho position were consistently poor substrates for carbinol production. Aldehydes with $-CH_3$ $-CF_3$, and $-Cl$ substituents located in the para position produced higher carbinol yields than their meta counterparts. The opposite was the case with $-OCH_3$ substituent. All substrates resulted in formation of aromatic alcohol. Where yields of carbinol were greater than 5 mg/mL levels of corresponding aromatic alcohols were relatively low, ranging from 3.5 to 14% (w/w) of carbinol yields.

Three of the acetyl aromatic carbinols were extracted, purified, and analyzed by ^{1}HNMR, by polarimetry and colorimetrically. The ^{1}HNMR data were consistent with the structures of the acetyl aromatic carbinols. Specific rotation values for L-3-methoxyphenylacetyl carbinol, L-4-chlorophenylacetyl carbinol, and L-4-methylphenylacetyl carbinol were $-115°$, $-185°$, and $-195°$ respectively. The purified compounds were assayed colorimetrically and by GC. The two sets of results were consistent and therefore validate the use of the colorimetric assay used in Table I for L-acetyl aromatic carbinol determination.

In a parallel experiment, the same group of aromatic aldehydes were incubated with NADH and purified yeast alcohol dehydrogenase. Corresponding aromatic alcohols were produced from all substrates tested (Table II). In general, poorest yields were observed with ortho substituted benzaldehydes. With the exception of CF_3 containing substrates, which produce low product yields, alcohol concentrations obtained from meta and para substituted benzaldehydes were 54–134% of the level produced from the benzaldehyde control. These results confirm the role

Table I. Conversion of aromatic aldehyde to L-acetyl aromatic carbinol and aromatic alcohol after a 6-h fermentation.

Substrate aromatic aldehyde	Product 1, L-acetyl aromatic carbinol	Yield (mg/mL)	Product 2, aromatic alcohol	Yield (mg/mL)	Location of ring substituents[a]		
					X	*Y*	*Z*
Benzaldehyde	L-phenylacetyl carbinol	10.1–10.2	benzyl alcohol	0.4	H	H	H
o-Toluaidehyde	L-2-methylphenylacetyl carbinol	2.0– 2.5	2-methylbenzyl alcohol	1.0	CH_3	H	H
m-Tolualdehyde	L-3-methylphenylacetyl carbinol	5.2– 6.2	3-methylbenzyl alcohol	0.4	H	CH_3	H
p-Tolualdehyde	L-4-methylphenylacetyl carbinol	5.4– 6.4	4-methylbenzyl alcohol	0.3	H	H	CH_3
2-Chlorobenz-aldehyde	L-2-chlorophenylacetyl carbinol	0.6– 0.7	2-chlorobenzyl alcohol	0.9	Cl	H	H
3-Chlorobenz-aldehyde	L-3-chlorophenylacetyl carbinol	2.1– 3.2	3-chlorobenzyl alcohol	0.6	H	Cl	H
4-Chlorobenz-aldehyde	L-4-chlorophenylacetyl carbinol	6.5– 8.0	4-chlorobenzyl alcohol	0.3	H	H	Cl
o-Anisaldehyde	L-2-methoxyphenylacetyl carbinol	0.8– 0.9	2-methoxybenzyl alcohol	0.9	OCH_3	H	H
m-Anisaldehyde	L-3-methoxyphenylacetyl carbinol	4.5– 5.7	3-methoxybenzyl alcohol	0.7	H	OCH_3	H
p-Anisaldehyde	L-4-methoxyphenylacetyl carbinol	1.2– 3.4	4-methoxybenzyl alcohol	0.6	H	H	OCH_3
α,α,α-Trifluoro-*o*-tolualdehyde	L-2-(trifluoromethyl)-phenylacetyl carbinol	0.2– 0.3	2-(trifluoromethyl)-benzyl alcohol	0.5	CF_3	H	H
α,α,α-Trifluoro-*m*-tolualdehyde	L-3-(trifluoromethyl)-phenylacetyl carbinol	0.3– 0.4	3-(trifluoromethyl)-benzyl alcohol	0.5	H	CF_3	H
α,α,α-Trifluoro-*p*-tolualdehyde	L-4-(trifluoromethyl)-phenylacetyl carbinol	0.5– 0.8	4-(trifluoromethyl)-benzyl alcohol	0.8	H	H	CF_3

[a] Refer to Figure 1.

Table II. Conversion of aromatic aldehyde to aromatic alcohol after a 6-h incubation with yeast alcohol dehydrogenase.

Substrate, aromatic aldehyde	Product, aromatic alcohol	Product yield (mmol/L)	Location of ring substituents[a]		
			X	*Y*	*Z*
Benzaldehyde	benzyl alcohol	2.4	H	H	H
o-Tolualdehyde	2-methylbenzyl alcohol	0.3	CH_3	H	H
m-Tolualdehyde	3-methylbenzyl alcohol	1.4	H	CH_3	H
p-Tolualdehyde	4-methylbenzyl alcohol	2.0	H	H	CH_3
2-Chlorobenzaldehyde	2-chlorobenzyl alcohol	1.3	Cl	H	H
3-Chlorobenzaldehyde	3-chlorobenzyl alcohol	2.0	H	Cl	H
4-Chlorobenzaldehyde	4-chlorobenzyl alcohol	2.8	H	H	Cl
o-Anisaldehyde	2-methoxybenzyl alcohol	0.6	OCH_3	H	H
m-Anisaldehyde	3-methoxybenzyl alcohol	3.0	H	OCH_3	H
p-Anisaldehyde	4-methoxybenzyl alcohol	3.3	H	H	OCH_3
α,α,α-Trifluoro-*o*-tolualdehyde	2-(trifluoromethyl)benzyl alcohol	[b]	CF_3	H	H
α,α,α-Trifluoro-*m*-tolualdehyde	3-(trifluoromethyl)benzyl alcohol	0.7	H	CF_3	H
α,α,α-Trifluoro-*p*-tolualdehyde	4-(trifluoromethyl)benzyl alcohol	0.7	H	H	CF_3

[a] Refer to Figure 1.
[b] Detected but not quantified.

played by yeast alcohol dehydrogenase in producing aromatic alcohols during acetyl aromatic carbinol production. The results obtained using benzaldehyde, *p*-anisaldehyde and *m*-tolualdehyde as substrates are at variance with the results of Bowen and co-workers.[9]

Typical reported yields of phenylacetyl carbinol produced by fermentation range from 4.5 to 10.2 mg/mL.[6,11,12] Our yield of 10.1 to 10.2 mg/mL obtained with benzaldehyde compares very favorably with these values. The yields obtained for other compounds in this study suggest that this is also an efficient system for bioconversion of ring substituted benzaldehydes to corresponding carbinols. The bioconversion of *p*-tolualdehyde by yeast has previously been observed.[13] We believe this is the first time synthesis of the other substituted acetyl aromatic carbinols has been reported.

This investigation illustrates the potential use of this and other microbial systems, containing enzymes having broad specificity, for production of a range of related optically active chemical intermediates. The concurrent production of alcohols underlines one of the major problems of cellular biotransformations, namely the tendency to produce unwanted by-products.

References

1. C. Hilderbrandt and W. Klavehn, DRP 548459 (1930).
2. V. Vojtisek and J. Netrval, *Folia Microbiol.*, **27,** 173 (1982).
3. P.F. Smith and D.J. Hendlin, *J. Baceteriol.*, **65,** 440 (1953).
4. D. Groger, H.P. Schmander, and K. Mothes, *Z. Allg. Mikrobiol.*, **6,** 275 (1966).
5. J.P. Voetz, E.J. Vandamme, and C. Vlerick, *Z. Allg. Mikrobiol.*, **13,** 355 (1973).
6. H. Becvarova, O. Hanc, and K. Macek, *Folia Microbiol.*, **8,** 165 (1963).
7. S.C. Agarwal, S.K. Basu, V.V. Vora, J.R. Mason, and S.J. Pirt, *Biotechnol. Bioeng.*, **29,** 783 (1987).
8. Merck and Co., USP 1,118,293 (1968).
9. W.R. Bowen, S.Y.R. Pugh, and N.J.D. Schomburgk, *J. Chem. Tech. Biotechnol.*, **36,** 191 (1986).
10. D. Groger and D. Erge, *Pharmazie*, **20,** 92 (1964).
11. K.G. Gupta, J. Singh, G. Sahni, and S. Dhawan, *Biotechnol. Bioeng.*, **21,** 1085 (1979).
12. J. Netrval and V. Vojtisek, *Eur. J. Appl. Microbiol. Biotechnol.*, **16,** 35 (1982).
13. M. Behrens and N. Ivanoff, *Biochem. Z.*, **169,** 478 (1962).