Genetic and Biochemical Analysis of the Ability of *Saccharomyces cerevisiae* to Decarboxylate Cinnamic Acids

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Decarboxylation of ferulate to 4-vinylguaiacol is associated with the production of a phenolic off-flavour in beer. The ability (Pof⁺) of non-brewing strains of *Saccharomyces cerevisiae* to carry out this reaction has been assigned, by tetrad analysis, to a single nuclear gene. This gene (*POF1*) is dominant in heterozygous diploids and segregates independently from *MATa/MATα*, *lys2* and *DEX1*. Therefore, elimination of the Pof⁺ phenotype from strains intended for brewing is feasible by either mutation or genetic segregation. Since ferulate was decarboxylated by cell-free supernatants derived from Pof⁺ strains, but not by similar fractions from Pof[−] strains, *POF1* encodes production of a cellular decarboxylase. Strains carrying *POF1* also decarboxylated coumarate and cinnamate *in vivo*, but with caffeate, 4-hydroxybenzoate, 4-hydroxyphenylacetate, styrylacetate, mandelate or phenylpropionate as substrates, the corresponding decarboxylation products were not detected. *POF1* may confer resistance to inhibitory effects of naturally occurring cinnamates on yeast growth.

INTRODUCTION

Cereal grains are rich in hydroxycinnamic acids (Wall *et al.*, 1961; Steinke & Paulson, 1964; Slonimski, 1980). In brewing, these compounds (e.g. ferulic and *p*-coumaric acids) are released when malted barley is extracted with hot water, and, if subsequently decarboxylated, can give rise to phenolic off-flavours in beer (Brumsted *et al.*, 1965; Halcrow *et al.*, 1966; Dadić *et al.*, 1971; Maule & Thomas, 1973; Wackerbauer *et al.*, 1977; Ryder *et al.*, 1978). Micro-organisms able to decarboxylate ferulate to 4-vinylguaiacol include non-brewing strains of *Saccharomyces* sp. or yeasts of other genera (Halcrow *et al.*, 1966; Wackerbauer *et al.*, 1977; Gramatica *et al.*, 1981), and enterobacteria (Finkle *et al.*, 1962; Lindsay & Priest, 1975). In contrast, typical brewing strains of yeast have little or no ability to carry out this reaction (Brumsted *et al.*, 1965; Thurston & Tubb, 1981).

The rare-mating technique (Gunge & Nakatomi, 1972; Spencer & Spencer, 1977) is a potential means of constructing novel strains of yeast for brewing. For example, Tubb *et al.* (1981*a*) mated dextrin-fermenting (Dex⁺) haploids with a polyploid lager yeast in an attempt to construct strains suitable for the production of low-carbohydrate beers. However, initial hybrids inherited the unwanted ability of the non-brewing parent to produce 4-vinylguaiacol. A genetic analysis of this Pof⁺ phenotype (Pof = phenolic off-flavour) has been carried out as a preliminary to constructing hybrid brewing strains which are Dex⁺ but Pof[−]. In addition we have sought the biochemical nature of the difference between Pof⁺ and Pof[−] strains, and the substrate specificity of decarboxylation *in vivo*.

METHODS

Strains and genetic techniques. The strains of yeast used in this work are described in Table 1. Growth, sporulation and general genetic procedures were as described by Sherman & Lawrence (1974). The methodology of Mortimer & Hawthorne (1975) was used in testing for linkage of genes.

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Table 1. *Strains of Saccharomyces cerevisiae used*

Strain	Genotype/description	Pof ⁺ /Pof ⁻	Source/reference
NCYC 1324	Polyploid lager strain (syn. <i>Saccharomyces uvarum</i>)	—	National Collection of Yeast Cultures (NCYC)
NCYC 361	Dextrin-fermenting strain (syn. <i>Saccharomyces diastaticus</i>)	+	NCYC
NCYC 177	Californian wine strain	+	NCYC
NCYC 77	Baking strain	+	NCYC
DCL 'M'	Distillery strain	+	Distillers Company Ltd; commercially available
BRG 520	Hybrid strain unsuitable for brewing	+	Tubb <i>et al.</i> (1981 <i>a</i>); strain RDH2
BRG 536*	α <i>DEX1</i>	+	Meiotic segregant of NCYC 361
BRG 530	α <i>DEX</i>	+	Meiotic segregant of NCYC 361
BRG 526	<i>a lys2</i>	—	Goodey (1980); strain SA
BRG 161D	<i>a lys2 DEX1</i>	—	Meiotic segregant from the cross BRG 536 $\times$ BRG 526
BRG 162C	<i>a DEX1</i>	+	Meiotic segregant from the cross BRG 536 $\times$ BRG 526
BRG 194C	α <i>his3 lys2 DEX1</i>	+	Meiotic segregant from the cross LL-20 $\times$ BRG 161D
LL-20	α <i>leu2 his3</i>	+	Storms <i>et al.</i> (1980)

* The single *DEX* gene in BRG 536 and its derivatives is allelic with that from strain J132b (Erratt & Stewart, 1978), and therefore is assigned *DEX1*.

Scoring of phenotypes. A Pof⁺ phenotype was assigned either on the basis of the production of 4-vinylguaiacol from ferulate or that of styrene from cinnamate (see below). The latter method was more convenient for scoring a large number of tetrads because styrene could be detected in culture media with complete reliability by its typical pungent aroma. A Dex⁺ phenotype was assigned on the basis of production of amyloglucosidase as described by Searle & Tubb (1981).

Assay for decarboxylation of ferulate and other substrates in vivo. Cultures (10 ml) of BRG 520 (Pof⁺) and NCYC 1324 (Pof⁻) were grown at 25 °C in a medium containing (l⁻¹): 50 g glucose, 10 g yeast extract, 10 g bacto-peptone, 0.1 g ferulic acid or other potential substrate (see Table 3), and 10 ml ethanol. Substrates were dissolved in ethanol and added to the basal medium prior to inoculation; control cultures with no substrate were also grown. After 4 d, culture supernatants were extracted and analysed by gas-liquid chromatography for the presence of decarboxylation products (Thurston & Tubb, 1981). Peaks appearing only on chromatograms produced from cultures of the Pof⁺ strain grown with a substrate were identified by reference to pure compounds (where available); 4-vinylphenol was identified by mass spectrometry. Where appropriate, concentrations of decarboxylation products were calculated as described previously (Thurston & Tubb, 1981), using the following calibration factors: 4-vinylguaiacol, 1.7; 4-vinylphenol, 1.7; styrene, 0.33. Factors for 4-vinylguaiacol and styrene were determined by subjecting known concentrations of the pure compounds to extraction and gas chromatography; that for 4-vinylphenol was assumed to be identical to that for 4-vinylguaiacol and, therefore, is only an approximation.

Preparation and assay of sub-cellular fractions. Cultures (100 ml) were grown to stationary phase in glucose/yeast extract/peptone medium (see above). Cells were harvested by centrifugation and resuspended with their own weight of glass beads (200 mesh) in 10 ml chilled phosphate buffer (120 mM, pH 6.5). This suspension was vortexed for 8 min, interspersed with periods of cooling on ice. Large cell debris, unbroken cells and glass beads were pelleted by low-speed centrifugation (3000 g; 10 min) and the cell-free supernatant decanted. To locate decarboxylase activity, ferulic acid (0.1 g l⁻¹, final concentration) was added to (i) the cell-free supernatant obtained after disrupting cells, (ii) the cell debris resuspended in phosphate buffer (10 ml) and (iii) the culture supernatant. In all cases, the amount of 4-vinylguaiacol produced after 1 h at 25 °C was measured as indicated above. Protein contents of preparations were determined using the biuret procedure (Herbert *et al.*, 1971).

Chemicals. The majority of hydroxycinnamic acids, related compounds and, where available, their decarboxylated derivatives were obtained either from BDH or from Sigma. The exceptions were ferulic acid, 4-vinylguaiacol (Oxford Organic Chemicals, Brackley, Northants) and styrylacetate (Aldrich Chemical Co.).

RESULTS

Genetic analysis

Strain NCYC 361 was chosen as an example of a wild-type which was both Dex⁺ and Pof⁺. This strain sporulated readily but only 5% of spores were viable. Six such meiotic segregants

Table 2. Decarboxylation of ferulate by cell-free extracts

Stationary phase cells were disrupted and cell-free supernatants prepared and assayed as described in Methods.

Strain	Ferulate decarboxylase activity	
	[μ g 4-vinylguaiacol produced (mg protein) ⁻¹]	
BRG 536	0.11	
LL-20	0.12	
BRG 520	0.11	
BRG 526	0.0	
NCYC 1324	0.0	

were examined: four were Dex⁺ (containing one or more *DEX* genes), two were Dex⁻, but all were Pof⁺. Strains BRG 530 and BRG 536 (α *DEX* Pof⁺; Table 1) were chosen for further analysis. The genetically-marked Pof⁺ and Pof⁻ strains (LL-20 and BRG 526, respectively) were selected from a collection of haploid strains. Of 16 strains tested from diverse origins, BRG 526 was the only Pof⁻ strain obtained.

Heterozygous diploids were constructed by mating BRG 526 with either BRG 536 or BRG 530, and by mating LL-20 with BRG 161D. A diploid tested from each mating was Pof⁺, indicating the dominant nature of this characteristic. Tetrads resulting from sporulation of these diploids were dissected and the segregation of markers scored. In all of 13 tetrads from BRG 526/BRG 536, 12 tetrads from BRG 526/BRG 530 and 10 tetrads from BRG 161D/LL-20, Pof⁺:Pof⁻ segregated in a ratio of 2:2. Therefore, in strains BRG 536, BRG 530 and LL-20, the Pof⁺ phenotype is inherited as a single dominant gene, *POF*. Since *POF*:*pof* segregated 4:0 in all of 8 tetrads from a further cross, BRG 162C $\times$ BRG 194C, it follows that BRG 536 and LL-20 possess the same *POF* gene, namely *POF1*. No evidence for linkage between *POF1* and *MATa*/*MAT α* , *lys2* or *DEX1* was obtained.

Biochemical difference between Pof⁺ and Pof⁻ strains

Sub-cellular fractions were prepared from three Pof⁺ strains and two Pof⁻ strains and assayed for their ability to decarboxylate ferulate. In no case was activity detected in the culture supernatant or in the pellet obtained after low-speed centrifugation of disrupted cells. The production of 4-vinylguaiacol was specific to cell-free supernatants obtained from Pof⁺ strains (Table 2). Therefore, *POF1* is required for the production of a cellular decarboxylase. In other words, the biochemical basis of the Pof⁻ phenotype examined here is a lack of decarboxylase activity, and not for example, failure to transport ferulate into the cell.

Substrate specificity of decarboxylation *in vivo*

The association of *POF1* with the ability to decarboxylate ferulate to 4-vinylguaiacol is important in the context of off-flavours arising in brewery fermentations. However, the biological significance of *POF1* may be associated with other decarboxylation reactions. Since activities obtained in crude extracts with ferulate as substrate were rather low (Table 2), examination of a range of substrates was feasible only by assaying for decarboxylation *in vivo*. In such circumstances, the complete range of substrates for the decarboxylase may well be masked by effects on transport of substrates into, or products out of, the cell. However, in addition to 4-vinylguaiacol (from ferulate) the decarboxylation products of cinnamate and coumarate were detected (Table 3). In terms of overall yield of product, substrate specificity was in the following order: ferulate > cinnamate > coumarate. No evidence was obtained for the decarboxylation of caffeate, phenylpropionate, mandelate, 4-hydroxyphenylacetate, styrylacetate or 4-hydroxybenzoate. In addition, neither the production of phenylacetaldehyde from phenylpyruvate, nor 4-hydroxyphenylacetaldehyde from 4-hydroxyphenylpyruvate, was detected. However, *Saccharomyces* strains can reduce a wide range of carbonyl compounds to the corresponding alcohol (Peppard & Halsey, 1981). Therefore, if formed, phenylacetaldehyde and 4-hydroxyphenylacetaldehyde are likely to be further metabolized to β -phenylethanol and tyrosol respectively. Production of these alcohols from the acids added to the culture medium was not measured,

Table 3. *Compounds tested as substrates for decarboxylation in vivo by Saccharomyces cerevisiae BRG 520*

Substrate*	Decarboxylation product(s) sought	Rf†	Concn of product (mg l ⁻¹)
Ferulate (4-hydroxy-3-methoxycinnamate)	4-Vinylguaiacol	0.77	16.0
Coumarate (4-hydroxycinnamate)	4-Vinylphenol	0.53	4.3
Caffeate (3,4-dihydroxycinnamate)	Dihydroxystyrene	NM	ND‡
Cinnamate	Styrene	0.10	5.7
4-Hydroxyphenylpyruvate	Hydroxyphenylacetaldehyde	NM	ND‡
Phenylpyruvate	Phenylacetaldehyde	0.16	ND
Mandelate (α -hydroxyphenylacetate)	{ Benzaldehyde	0.11	ND
	{ Benzyl alcohol	0.17	ND
Phenylpropionate (hydrocinnamate)	Ethylbenzene	0.06	ND
4-Hydroxyphenylacetate	<i>p</i> -Cresol	0.19	ND
4-Hydroxybenzoate	Phenol	0.12	ND
Styrylacetate (4-phenylbut-3-enoate)	β -Methylstyrene	NM	ND‡

ND, Not detected.

NM, Not measured because pure compound was unavailable.

* All substrates were added to give a final concentration of 0.1 g l⁻¹.

† Rf, Retention factor relative to eugenol (internal standard).

‡ Based on an absence of any detectable difference in the chromatograms obtained from cultures grown with and those grown without substrate.

since β -phenylethanol and tyrosol are formed by decarboxylation of aromatic amino acids in Pof⁻ strains (Äyräpää, 1965; Szlavko, 1973; Thurston & Tubb, 1981).

DISCUSSION

The evidence presented demonstrates that a single dominant gene, *POF1*, enables *Saccharomyces cerevisiae* to decarboxylate ferulate to 4-vinylguaiacol, a compound responsible for a phenolic off-flavour in beer. Therefore, elimination of the Pof⁺ phenotype from breeding programmes with brewer's yeast is highly feasible either by mutation or by genetic segregation. Using the latter approach, we have described elsewhere (Tubb *et al.*, 1981*b*) the construction of dextrin-fermenting brewing strains which produce palatable beer.

Of wider significance to brewing is the fact that the Pof⁺ character can be eliminated with ease from the well-characterized, genetically-marked haploids used in fundamental studies on yeast genetics. This should now encourage use of this considerable genetic reserve for the development of brewing yeast by hybridization, either in the conventional way (Johnston, 1965; Emeis, 1971; Anderson & Martin, 1975; Gjermansen & Sigsgaard, 1981) by rare-mating (Tubb *et al.*, 1981*b*), or by protoplast fusion (Russell & Stewart, 1979; Freeman, 1981). Since representative baking, distilling and wine strains have been shown to be Pof⁺ (Table 1), breeding yeasts for other applications does not appear to require removal or inactivation of *POF* genes.

The cytoplasmic genetic make-up of haploid or polyploid strains of *Saccharomyces* can be manipulated independently of nuclear genotype. This process is called cytoduction (see Tubb *et al.*, 1981*b*) and usually involves a cross where one parent is defective in nuclear fusion (i.e. Kar⁻; Conde & Fink, 1976). The assignment of Pof⁺ to a single, dominant nuclear gene provides a marker which can be scored to distinguish cytoductant (Pof⁻) from hybrid (Pof⁺) progeny in crosses between *kar POF* haploids and Pof⁻ brewing strains. Such an assignment also dismisses a previous suggestion that Pof⁺ might be inherited cytoplasmically (Tubb *et al.*, 1981*a*).

However, there is evidence (Tubb *et al.*, 1981*b*) that replacing the mitochondrial genome of a Pof⁺ strain can alter the amount of 4-vinylguaiaicol formed from ferulate during fermentation.

The *POF1* decarboxylation system described here appears to be specific for certain cinnamic acids. However, unlike the decarboxylase from *Klebsiella aerogenes* (Finkle *et al.*, 1962), the yeast enzyme acts on cinnamate itself and, therefore, cannot require substrates to have a particular substitution (e.g. *p*-hydroxyl) in the benzene ring. A similar conclusion can be drawn from the results of Gramatica *et al.* (1981). These authors reported decarboxylation *in vivo* of ferulate, coumarate, *p*-methoxycinnamate and 3,4-dimethoxycinnamate. However, using stationary phase cells from only one strain, they did not detect formation of styrene from cinnamate, a reaction predicted to occur from earlier observations by Chen & Peppler (1956). Caffeic acid is a substrate for the decarboxylase from *K. aerogenes* (Finkle *et al.* 1962), but in agreement with Gramatica *et al.* (1981), we were unable to detect decarboxylation of this substituted cinnamate by *S. cerevisiae* *in vivo*; possibly it is not transported into the yeast cell.

Some naturally-occurring cinnamates have been shown to possess antimicrobial activity. Gupta & Banerjee (1976) identified ethyl *p*-methoxycinnamate as the principal antifungal agent from the East Indian plant, *Curcuma zedoaria*; more recently, Baranowski *et al.* (1980) found that caffeate, *p*-coumarate and ferulate increased the lag phase or reduced the growth rate of a Champagne strain of *S. cerevisiae* in a complex medium. Therefore, the ability to decarboxylate these compounds may well confer some resistance to their inhibitory effects.

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