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Occurrence of fatty alcohol oxidase in alkane- and fatty-acid-utilising yeasts and moulds

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Abstract. Preparations of membrane fractions from 16 yeasts and three moulds were assayed for long-chain fatty alcohol oxidase (FAOD) activities after being grown on hexadecane or glucose and, in nine cases, on oleic acid. The enzyme was usually repressed in glucose-grown cells but in *Candida bombicola* ATCC 22214 and *Debaryomyces hansenii* NCYC 33 appeared to be constitutive. Highest activities occurred in *C. tropicalis* and *D. polymorphus* (about 0.8 unit/mg protein) grown on hexadecane. Growth of yeasts on oleic acid partially induced FAOD activity but not with the moulds. In two strains of *Yarrowia lipolytica* (DSM 3286 and CBS 2076) no activity of FAOD was found but this could have been due to the known photo-lability of the enzyme. FAOD from different species shared similar characteristics with respect to substrate specificity and pH optimum.

Introduction

The ability of a wide range of yeasts and moulds to utilize long-chain alkanes as growth substrates has long been established (see Watkinson and Morgan 1990). The pathway of oxidation is equally well-described, involving a sequence of oxidations at one of the two terminal C atoms thereby producing the fatty alcohol ($R-CH_2OH$), aldehyde ($R-CHO$) and finally fatty acid ($R-COOH$).

Until the mid 1980s, the conversion of the alcohol to the aldehyde was ascribed to NAD^+- or $NADP^+$-dependent dehydrogenases (Boulton and Ratledge 1984; Rehm and Reiff 1982). However, recent research has revealed the presence of an $NAD(P)^+$-independent fatty alcohol oxidase (FAOD) in species of alkane-utilizing yeasts such as *Candida maltosa* (Blasig et al. 1988), *C. tropicalis* (Kemp et al. 1988), *Torulopsis candida* (Il'chenko 1984) and *C. bombicola* (Hommel and Ratledge 1990) and also in an alkane-utilizing fungus, *Aspergillus flavipes* (Savitha and Ratledge 1991).

The purpose of the work reported here was to screen a diversity of alkane-utilizing micro-organisms for the presence of FAOD and to assess the likely ubiquity of FAOD. Bacteria, however, were not included as recent reports from this laboratory have identified only a number of discrete alcohol dehydrogenases but no alcohol oxidases (Broadway et al. 1993; Fox et al. 1992).

Materials and methods

Organisms and growth. The organisms and their sources are given in Table 1. They were grown in a basal culture medium consisting of ($g\,l^{-1}$): KH_2PO_4, 7.0; Na_2HPO_4, 2.0; $MgSO_4 \cdot 7H_2O$, 1.5; yeast extract, 1.5; diammonium tartrate, 1.0; $CaCl_2 \cdot 2H_2O$, 0.1; $FeCl_2 \cdot 4H_2O$, 0.008; $ZnSO_4 \cdot 7H_2O$, 0.001; in 1000 ml distilled water. The final pH was 6.5. The carbon source was either glucose (3%, w/v), hexadecane (4%, v/v) or oleic acid (4%, v/v).

Yeasts were grown in 500 ml medium in 2-l conical flasks and incubated at 30° C on a rotary shaker. Moulds were grown in 1-l vortex-aerated, stirred vessels maintained at 20° C.

Cell harvesting and disruption. Yeasts were harvested by centrifugation (3000 *g*, 15 min), resuspended in 10 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(2'-ethanesulphonic acid) (HEPES) buffer, pH 7.2, and disrupted by two consecutive passes through a French press, with cooling. The moulds were harvested by filtration and broken by grinding with an equal amount of clean sand in a chilled mortar using 10 mM HEPES buffer, pH 7.2, to suspend the paste. In each case, unbroken cells were removed by centrifugation (3000 *g*, 15 min). The supernatant was recentrifuged (90 min at 100000 *g*) and the resulting pellet resuspended in 10 mM HEPES buffer, pH 7.2. This represented the combined peroxisomal, mitochondrial and microsomal membrane fractions and was used for all determinations of FAOD activity.

Because of the known light sensitivity of the alcohol oxidase of *C. tropicalis* (Kemp et al. 1988, 1990) precautions were always taken to avoid unnecessary exposure of the cell-free extracts to daylight.

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Table 1. Fatty alcohol oxidase (FAOD) activities of membrane preparations from various yeasts and moulds grown on glucose hexadecane or oleic acid

Organism	Activity of FAOD (n mol·min^{-1}·mg^{-1} protein) after growth of cells on		
	Glucose	Hexadecane	Oleic acid
Yeasts			
Candida bombicola NRRL-Y-7506	17	148	ND
C. bombicola ATCC 22214	183	122	ND
C. maltosa ATCC 20275	0	301	ND
C. parapsilosis NCYC 458	35	71	ND
C. parapsilosis ATCC 20224	35	657	96
C. parapsilosis CBS 604	34	405	78
C. parapsilosis CBS 1954	29	304	ND
C. rugosa ATCC 20116	27	373	ND
C. tropicalis ATCC 20336	40	790	112
Debaryomyces hansenii[a] NCYC 33	158	220	ND
D. hansenii[b] CBS 8046	0	301	ND
D. polymorphus ATCC 20280	50	743	ND
D. polymorphus ATCC 20499	28	458	224
Yarrowia lipolytica CBS 2074	0	<10[c]	ND
Y. lipolytica DSM 3286	0	<10[c]	0[c]
Y. lipolytica CBS 2076	2	146	120
Fungi			
Cladosporium resinae ATCC 20495	0	82	18
Fusarium moniliforme IMI 58289	0	26	0
Penicillium soppii CBS 869.70	0	17	0

ND, not determined
[a] Supplied as *Saccharomyces cerevisiae*, re-classified as *Candida famata*, and now re-named as given
[b] Originally *Torulopsis candida*
[c] Activity is highly photo-labile

Enzyme assays. FAOD in the combined membrane fraction was assayed by the 2,2′-azino-di[3-ethylbenzothiazoline-(6)-sulphonic acid]/peroxidase method of Kemp et al. (1988).

Protein concentration was determined by the method of Bradford (1976).

Results and discussion

A number of yeasts had the ability to utilize alkanes as a carbon source (Table 1). All of the alkane-utilizing yeasts in this study were also able to utilize oleic acid as a carbon source, usually with an increased growth rate. For six cases, FAOD activity was demonstrated. Several moulds were also screened for the ability to utilize *n*-alkanes for growth and determinations of FAOD activity were made in three cases. The FAOD activities measured in membrane preparations are given in Table 1. FAOD was found in almost all of the yeasts capable of growing on *n*-alkanes.

It has been shown by Kemp et al. (1990) that FAOD is both thermolabile and photosensitive. As a result, the enzyme can be rapidly inactivated by exposure to heat or light. The degree of lability depends upon the organism from which the FAOD was isolated: the FAODs from *Yarrowia lipolytica* CBS 2074 and DSM 3286 were the most labile but that from strain CBS 2076 was relatively stable. Our best efforts to avoid exposure of the FAOD to light only gave low measurable activities with the two former strains. In addition, the assay method used has a limitation for measurement of low FAOD activities since it is a coupled assay system and therefore is dependent, to some extent, on the characteristics of the coupling enzyme (peroxidase) and the stability of the intermediate compound (hydrogen peroxide). Mercaptoethanol was not included in the assays for, although this can prevent autoxidation of enzymes, the peroxide produced by FAOD is easily reduced by thiol compounds. Activities of FAOD below 20 nmol·min^{-1}·mg^{-1} membrane protein were therefore difficult to measure accurately.

In the case of *C. maltosa*, *Debaryomyces hansenii* CBS 8046 and the strains of *Y. lipolytica*, FAOD was only detected after cells had grown on alkanes. There was no activity in glucose-grown cells. In other yeasts, however, FAOD activity was detectable in glucose-grown cells albeit at decreased levels compared to alkane-grown ones. However in *C. (Torulopsis) bombicola* ATCC 22214 and *D. hansenii* NCYC 33, FAOD appeared to be constitutive. Hommel and Ratledge (1990) have previously recorded high activities of FAOD in *C. bombicola* ATCC 22214 grown on glucose, although they concluded that there may be two FAOD enzymes present: one of these enzymes may be constitutive and the other inducible. There was a clear difference between this strain and the other strain of *C. bombicola* (NRRL 7506), which appeared conventional in the induction of FAOD activity by alkanes.

In those yeasts tested for FAOD after growth on oleic acid, the activity of FAOD was increased from the activity detected in glucose-grown cells but not to the same extent as found in alkane-grown cells. This suggests some form of partial or co-induction of the enzyme even though the metabolism of oleic acid would not involve the participation of an FAOD enzyme.

The growth of moulds on *n*-alkanes was slow. An improvement was achieved by substituting oleic acid for the hexadecane: however the activity of FAOD detected was significantly decreased with *Cladosporium resinae* and was only detected in *Fusarium moniliforme* and *Penicillium soppii* after being grown on hexadecane and not on oleic acid.

There is evidence that several of the FAOD activities detected are due to similar enzymes. The pH-activity profiles of FAOD from several sources show similar characteristics with pH optima at 9.0–9.2 (data not shown). Also, the relationship between the carbon chain length of the substrate is similar for FAOD from a variety of sources, with optimum activity occurring towards C_{10-12} alcohols (Fig. 1). An FAOD detected in *Aspergillus flavipes*, grown on an hexadecanol, has been reported by Savitha and Ratledge (1991). This enzyme also has an alkaline pH optimum and an optimum specificity towards decanol.

All of the FAOD species studied were photolabile to varying degrees.

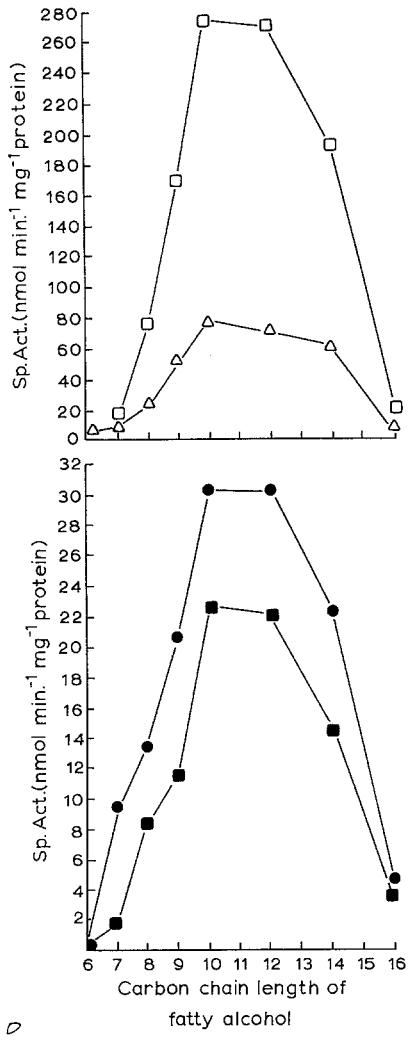

Fig. 1. Substrate specificities of the fatty alcohol oxidase activities towards fatty alcohols from hexanol to hexadecanol in membrane preparations from *Candida parapsilosis* CBS 604 grown on glucose (△) or hexadecane (□), *C. parapsilosis* ATCC 20224 grown on glucose (■) and *Debaryomyces polymorphus* ATCC 20499 also grown on glucose (●); Sp. Act, specific activity

Although it has only recently been characterised, it would appear that FAOD is a vital enzyme in the metabolic pathway for hydrocarbon assimilation in many alkane-utilizing yeasts and moulds. Although the ubiquity of this enzyme remains to be proved, the evidence gained from this study, bearing in mind the extreme photo-lability in some cases, would indicate that the enzyme could be common to all alkane-utilizing eukaryotes.

In two prokaryotes, *Acinetobacter calcoaceticus* and *Corynebacterium* 7E1C, the presence of an FAOD could not been detected and instead there appears to be a multiplicity of alcohol dehydrogenases to achieve oxidation of long-chain alcohols (Fox et al. 1992; Broadway et al. 1993). There is no evidence for a multiplicity of alcohol oxidases from this work or, to our knowledge, any previous study.

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