Chapter 7
Assimilation of Unusual Carbon Compounds

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Contents

Abstract Yeast taxa traditionally are distinguished by growth tests on several sugars and organic acids. During the last decades it became apparent that many yeast species assimilate a much greater variety of naturally occurring carbon compounds as sole source of carbon and energy. These abilities are indicative of a greater role of yeasts in the carbon cycle than previously assumed. Especially in acidic soils and other habitats, yeasts may play a role in the degradation of carbon compounds. Such compounds include purines like uric acid and adenine, aliphatic amines, diamines and hydroxyamines, phenolics and other benzene compounds and polysaccharides. Assimilation of purines and amines is a feature of many ascomycetes and basidiomycetes. However, benzene compounds are degraded by only a few ascomycetous yeasts (e.g. the *Stephanoascus/*

T. Satyanarayana, G. Kunze (eds.), *Yeast Biotechnology: Diversity and Applications,*
DOI: 10.1007/978-1-4020-8292-4_7, © Springer Science+Business Media B.V. 2009

Blastobotrys clade and black yeastlike fungi) but by many basidiomycetes, e.g. Filobasidiales, Trichosporonales, red yeasts producing ballistoconidia and related species, but not by Tremellales. Assimilation of polysaccharides is wide-spread among basidiomycetes.

Growth tests on these compounds separate *Trichosporon* species that otherwise are hardly distinguishable. Yeasts able to degrade phenolics can be applied for cresol removal from polluted soil and styrene removal from air by biofilters containing black yeast. Yeasts growing on polysaccharides may be a valuable source of hydrolytic enzymes that can be applied in food technology. Biodegradative abilities of yeasts inhabiting aerial plant surfaces and the fate of these yeasts during anaerobiosis and lactic acid fermentation are also dealt with.

Keywords Adenine, amines, benzene compounds, methanol, phenolics, polysaccharides, purines

7.1 Introduction

Yeast taxa traditionally are distinguished by their ability to produce ascospores, by cell wall structure and by morphology, e.g. the presence of budding cells, splitting cells and hyphae. These characteristics suffice for distinction of yeast genera, but at the species level physiological growth tests are required. These include ability to ferment sugars into ethanol and carbon dioxide and ability to utilize a great variety of naturally occurring organic compounds as sole sources of carbon and energy, and several nitrogenous compounds as sole nitrogen source. Additional distinguishing characteristics are maximum growth temperature, osmotolerance and vitamin requirement.

The organic compounds used in traditional growth tests include monosaccharides, disaccharides, trisaccharides, inulin and soluble starch, polyols and several organic acids. These growth tests are necessary for species description and identification, but do not give a complete picture of the degradative abilities shown by some yeast species. An example is methanol assimilation that until 1969 was generally believed not to be shown by any yeast species. Other studies revealed assimilation of alkanes, purines, C2 compounds, amines, diamines, hydroxyamines, benzene compounds and polysaccharides. In the following sections attention will be paid to assimilation of these unusual carbon compounds.

Yeasts in nature are found in many habitats, e.g. soil, or are associated with insects, mushrooms and animals, Men included, some being opportunistic pathogens. Another habitat rich in yeast species is the aerial surface of plants. The degradative abilities of these yeasts and their fate during anaerobiosis and lactic acid fermentation will also be dealt with.

7.2 Assimilation of C1 And C2 Compounds, Purines, Aliphatic Amines and Alkanes

7.2.1 C1 Compounds

Until 1969 when Ogata et al. (1969) isolated *Candida boidinii* from an enrichment culture on methanol it was generally believed that methanol could not support growth of any yeast species. Screening of the CBS yeast collection (Hazeu et al., 1972) showed that this character is exclusively shown by some ascomycetous yeast species. Sequencing of 26S rDNA nuclear bases pointed to close phylogenetic relationship of these *Candida* and *Pichia* species (Kurtzman and Robnett, 1998). Levine and Cooney (1973) isolated *Hansenula anomala*, later renamed *Pichia angusta*, a thermotolerant methylotrophic yeast. Methylotrophic yeasts metabolize methanol by an alcohol oxidase (Sahm and Wagner, 1973) that together with catalase is located in so-called peroxisomes (van Dijken et al., 1975). Detailed studies on the formation of these cell organelles were carried out by the group of Veenhuis in Groningen. For review see van der Klei and Veenhuis (2002). Production of single cell protein from methanol is feasible. It must be kept in mind that methylotrophic bacteria may give higher cell yields as they generate more energy from this substrate, due to activity of a specific methanol dehydrogenase rather than an alcohol oxidase. Methylotrophic yeasts respiring on methanol show a relatively high substrate constant for molecular oxygen, viz. 0.5-1.3 mg O_2 per litre rather than < 0.15 mg O_2 per litre when ethanol is respired (Middelhoven et al., 1976a). This implies that the maximum specific growth rate of yeast cultures on methanol can be attained only at oxygen concentrations near half air saturation. Some strains of *Candida boidinii* prefer nitrate to ammonium when growing in a methanol ammonium nitrate medium (Middelhoven et al., 1976a, b). These strains excrete substantial amounts of ammonia when growing in methanol potassium nitrate medium under oxygen-limited conditions, but not in well-aerated cultures or anaerobically (Middelhoven et al., 1976b). This nitrate reduction appears not to be linked to energy generation.

Different alcohol oxidases have been detected in methylotrophic yeasts (Szamecs et al., 2005). A detailed study of alcohol oxidase was presented by Ozimek et al. (2005). An alternative pathway of methanol catabolism starts with an alcohol dehydrogenase (Sakay et al., 1995). Methylformate is an intermediate and this pathway is particularly active at high concentrations of methanol and formaldehyde. Possibly it plays a part in detoxification of the latter.

Hexamethylenetetramine (urotropine) is a formaldehyde derivate. It is spontaneously formed in aqueous solutions of formaldehyde and ammonia. The compound is stable but subject to hydrolysis under acidic conditions. It does not support growth of yeasts when administered as carbon source, but 46 out of 60 yeast species tested readily assimilated urotropine as sole nitrogen source (Middelhoven and van Doesburg, 2007). These species include basidiomycetes and ascomycetes, methylotrophs as well as non-methylotrophs. Urotropine is not known to occur in nature, but spontaneous formation of small amounts in the cell from ammonia and formaldehyde is feasible.

7.2.2 Purines

Enrichment cultures on uric acid as sole carbon source, inoculated with soil, yielded an ascomycetous yeast identified as *Candida famata* (anamorph of *Debaryomyces hansenii*) and two basidiomycetes belonging to the genus *Trichosporon* (Middelhoven et al., 1983). In spite of its complex chemical structure, uric acid from a physiological viewpoint is a C2 compound as it is metabolized via glyoxylate, both in the ascomycete and in the basidiomycetous yeasts studied. From glyoxylate energy is generated and cell constituents are produced. The pathway of urate catabolism begins with urate oxidase by which allantoin is formed, that in three successive hydrolytic steps is converted into glyoxylate and two moles of urea (Middelhoven et al., 1983). Urate oxidase is located in peroxisomes (Middelhoven et al., 1983; Veenhuis et al., 1985). Several soil samples yielded yeast strains able to grow at the expense of uric acid (Middelhoven et al., 1985). These strains belong to some ascomycetous genera, e.g. *Candida, Stephanoascus* and *Arxula* and to basidiomycetous genera like *Cryptococcus* and *Trichosporon*. All the ascomycetous strains assimilated n-hexadecane as well. Yeastlike endosymbionts of the brown planthopper recycle uric acid stored in the insect's tissues (Sasaki et al., 1996).

Enrichment cultures on adenine as sole carbon source yielded some strains of *Stephanoascus ciferrii* (Middelhoven et al., 1985) and of a novel species, initially named *Trichosporon adeninovorans* (Middelhoven et al., 1984), later renamed as *Arxula adeninivorans* (van der Walt et al., 1990). The latter species assimilated adenine, uric acid, several aliphatic amines and amino acids (Middelhoven et al., 1991). In addition to these nitrogenous carbon compounds, several benzene compounds supported growth of *A. adeninivorans* (Middelhoven et al., 1991). The group of Kunze in Gatersleben detected many excreted hydrolytic enzymes in cultures of this industrially promising yeast species. A detailed genetic study was done by the group of Bode and Samsonova in Greifswald. These topics will be dealt with in other chapters of this book.

7.2.3 C2 Compounds

Enrichment of yeasts growing on glycollate as sole carbon source was unsuccessful. Similarly, enrichments from soil on allantoin were also unsuccessful, in spite of allantoin being the first intermediate of urate catabolism. Allantoin is an excellent nitrogen source for many yeasts, *Saccharomyces cerevisiae* included (Middelhoven, 1977; Middelhoven and Arkesteyn, 1981). Inoculation with a rotten mushroom resulted in isolation of *Cryptococcus allantoinivorans* sp. nov. (Middelhoven, 2005). This species belongs to the *Cryptococcus laurentii* complex. *C. allantoinivorans* was the only species of this group growing on allantoin. The other eight species studied assimilated one or more C2 compounds like ethanol, ethylamine, ethanolamine

and glycine. The pattern of C2 compound utilization shown by the type strains appeared to be species-specific (Middelhoven, 2005).

7.2.4 Amines

It has been known for a long time that amines support growth of yeasts when administered as sole nitrogen source. Van der Walt (1962) introduced a growth test on ethylamine as a diagnostic tool. Van Dijken and Bos (1981) showed that 60% of 461 yeast species utilized at least one primary amine as sole nitrogen source, but they failed to demonstrate assimilation as sole carbon source. Middelhoven et al. (1983, 1984, 1985) showed that several primary amines, diamines and hydroxyamines were assimilated as sole carbon source by many ascomycetous and basidiomycetous yeast species. The formula of Difco Yeast Nitrogen Base had to be amended for attaining complete substrate utilization. For this purpose, ammonium sulphate was omitted and the phosphate buffer concentration was increased tenfold.

Amines are oxidized to the corresponding aldehydes by amine oxidases (for review see Large, 1986) that are located in peroxisomes (Zwart et al., 1980, 1983). Yeasts produce different amine oxidases: methylamine oxidase is most active with short-chain primary amines, ethanolamine included (Zwart and Harder, 1983). Long-chain amines, benzylamine and isobutylamine are oxidized by benzylamine oxidase that may attack putrescine as well (Green et al., 1982). This type of benzylamine oxidase was detected in *Candida utilis* and *Pichia pastoris*. *Kluyveromyces* spp. and *Candida boidinii* produced a benzylamine oxidase that did not attack putrescine (Haywood and Large, 1981; Heath and Large, 1984). All of these amine oxidases showed pH optima of 7.0–7.5. The benzylamine oxidase of *Kluyveromyces marxianus* has been completely characterised and compared to other copper amine oxidases by Corpillo et al. (2003).

Middelhoven et al. (1986) grew five yeast species on amines as sole carbon source and screened cell-free extracts for amine oxidase activity, using primary amines, hydroxyamines and diamines of different chain lengths, isobutylamine and benzylamine as enzyme substrates. In cells of *Trichosporon cutaneum* (later renamed *T. domesticum*), grown on ethylamine or butylamine, maximum activities towards primary amines was found with propylamine and butylamine at pH 7.0, towards hydroxyamines at chain lengths C_4-C_6 (pH 7.0) and towards diamines at C_8-C_9 (pH 8.0). Cell-free extracts of *T. domesticum* grown on putrescine displayed little activity on primary and hydroxyamines, but attacked putrescine and cadaverine at pH 8.0. In putrescine-grown cells of the other basidiomycete studied, viz. *Cryptococcus laurentii*, putrescine was the preferred substrate, but significant activity on monoamines (C_3-C_4) and hydroxyamines (C_3-C_6) was also present, isobutylamine and benzylamine not being oxidized, all tested at pH 8.0. Putrescine-grown cells of three ascomycetes, viz. *Candida famata*, *Arxula adeninivorans* and *Stephanoascus ciferrii* were most active on diamines C_7-C_9 (pH 8.0) and hydroxyamines C_4-C_6. Of the monoamines tested, optimum chain lengths were C_4-C_{12} for *C. famata,* C_3-C_5

for *A. adeninivorans* and C_4-C_5 for *S. ciferrii*. The results presented by Middelhoven et al. (1986) show a greater variability of yeast amine oxidases than earlier studies. This appears from a higher pH optimum and a preference for long-chain diamines.

7.2.5 Hydrocarbons

Long-chain alkanes (C_{12}-C_{16}) are assimilated by many yeast species as sole carbon source (Markovetz and Kallio, 1964; Scheda and Bos, 1966; Hug and Fiechter, 1972). Bos and de Bruyn (1973) proposed growth tests on alkanes as a diagnostic tool. Unfortunately, growth may be very slow and is dependent on the methods used. Moreover, not all yeast species have been screened for this character. Middelhoven (2001) stated that the slant culture method of Markovetz and Kallio (1964) gives better results than growth tests in liquid media. Alkane-utilizing yeast cultures excrete emulsifiers that facilitate uptake of these hydrophobic liquids (Zinjada and Pant, 2002).

7.3 Phenolic and Other Benzene Compounds

7.3.1 General

Aromatic compounds received little attention by yeast taxonomists. This is regrettable as the benzene ring is a widely distributed unit of chemical structure in the biosphere. Some reports on phenol degradation appeared already in the late fifties. Zimmermann (1958) reported degradation of phenol by suspensions of some basidiomycetous yeast cells. Di Menna (1959) showed assimilation of lignin-related compounds like ferulic and vanillic acids by basidiomycetous yeasts isolated from the phyllosphere of pasture plants. To avoid growth inhibition due to toxicity of these compounds, she administered these at a low concentration, viz. 100 mg per litre mineral salts medium. Middelhoven (1993) applied the slant culture method, described by Middelhoven et al. (1991, 1992b, 2004) to demonstrate growth at the expense of toxic compounds. Sampaio (1999) carried out growth tests on aromatic compounds in liquid media, at 1 gram per litre. A comparison of these methods shows that more positive growth responses are seen with the slant culture method than in liquid medium. Sampaio (1994) isolated many basidiomycetous yeast strains from enrichment cultures on lignin-related benzene compounds, inoculated with plant leaves or soil. A comparison with type cultures learned that members of the *Trichosporon* and *Filobasidium* clades assimilated many benzene compounds, but members of the *Tremellales* and of the *Cystofilobasidium* clade generally did not. Middelhoven et al. (1992b) isolated yeasts from soil samples previously polluted

with some benzene compounds. Several basidiomycetes predominated, but *Schizoblastosporion starkeyi-henricii* was also among the isolates. Soil supplied with phloroglucinol yielded *Cryptococcus* spp., later reidentified as *Trichosporon porosum* (Stautz) (Middelhoven et al., 2001).

Middelhoven (1993) screened a yeast culture collection, isolates of Middelhoven et al. (1992b) included, representing about 20% of all species known at that time, for growth on phenol and 3-hydroxybenzoate. Fifteen ascomycetous yeast species and thirteen basidiomycetous yeast species and yeastlike fungi responded positively and were selected for further study. They were tested for growth on 84 benzene compounds, of which 63 supported growth of at least one yeast species. The black ascomycetous yeastlike fungus *Exophiala jeanselmei* assimilated 54 of these compounds. About 30 of these were assimilated by *Trichosporon* spp., *Rhodotorula* spp, *Leucosporidium scottii* and *Cryptococcus elinovii* and about 20 by *Arxula adininivorans* and the closely related *Stephanoascus ciferrii*. On the other hand, none of the *Saccharomyces* spp., *Kluyveromyces* spp. and *Pichia* spp. tested grew on any of these compounds. *Candida tropicalis* and *Debaryomyces hansenii* assimilated only phenol and dihydroxybenzenes.

Compounds not assimilated by any yeast strain tested could be classed in 3 groups. Compounds with more than one carbon side chain, such as xylenols and toluic acids, a branched side chain or with an amino group attached to the ring, such as anthranilic acid and 4-aminobenzoic acid. Repeated attempts to enrich yeasts on anthranilic acid were unsuccessful. This is contrary to the paper of Anderson and Dagley (1981) who described the catabolic pathway in *Trichosporon cutaneum*. Other compounds not assimilated by any yeast strain tested were 2-hydroxycinnamic acid and its internal lactone coumarin, umbelliferone, esculetin, 3,4,5-trimethoxycinnamic acid, benzaldehyde and para-quinone. Failure to grow on these compounds may be due to steric hindrance or toxicity.

7.3.2 Catabolism

Aerobic degradation of benzene compounds is achieved by ring fission. This may occur between two adjacent hydroxyl groups, e.g. in catechol, hydroxyhydroquinone, protocatechuate (3,4-dihydroxybenzoate) and homoprotocatechuate (3,4-dihydroxyphenylacetate), or next to a hydroxyl group in para (1,4) position, e.g. in gentisate (2,5-dihydroxybenzoate) or homogentisate (2,5-dihydroxyphenylacetate). Ring fission is catalyzed by dioxygenases. Ring fission substrates are formed by monooxygenases that introduce hydroxyl groups with concomitant oxidation of NAD(P)H. These pathways are common in aerobic microorganisms. Biochemical studies on yeasts were carried out by the groups of Halina Neujahr (Stockholm, Sweden) and S. Dagley (St. Paul, Minnesota). For review, see Middelhoven (1993) who studied the occurrence of cleavage enzymes in various phylogenetic groups. The catechol branch of the 3-oxoadipate pathway and its hydroxyhydroquinone variant were involved in phenol and resorcinol catabolism in ascomycetes as well

as in basidiomycetes. However, these two groups of yeasts showed characteristic differences in hydroxybenzoate catabolism. In the yeastlike fungus *Exophiala jeanselmei* and in basidiomycetes of the genera *Cryptococcus, Leucosporidium* and *Rhodotorula* the protocatechuate branch of the 3-oxoadipate pathway was induced by growth on 3- and 4-hydroxybenzoic acids. In three *Trichosporon* species and in all ascomycetous yeasts tested, 4-hydroxybenzoate was catabolized via protocatechuate and hydroxyhydroquinone. These yeasts were unable to cleave protocatechuate. 3-Hydroxybenzoic and 3-hydroxycinnamic acids were assimilated in ascomycetous yeasts via the gentisate pathway (Middelhoven et al., 1992a), but in basidiomycetes via protocatechuate.

Tannins are important plant constituents. Condensed tannins are covalently bound benzene compounds. Yeasts are unable to degrade these (Bhat et al., 1998). Hydrolysable tannins consist of glucose moieties esterified with substituted benzoic acids, e.g. gallic acid (3,4,5-trihydroxybenzoic acid). Some yeasts produce tannase that hydrolyses the ester bond (Bhat et al., 1998). Tannase of a *Candida* sp. has been purified by Aoki et al. (1976). Gallic acid supports growth of some *Trichosporon* sp., *Rhodotorula* sp. and *Leucosporidium scottii* (Middelhoven, 1993). Some *Trichosporon* spp. assimilate hydrolysable tannic acid (Middelhoven, 2004).

Cresol removal from polluted soil was speeded up by inoculation with *Rhodotorula* sp. cells (Middelhoven et al., 1992b), later described as *Rhodotorula cresolica* sp. nov. (Middelhoven and Spaaij, 1997). It was demonstrated that this yeast utilized ortho-cresol in competition with the soil microflora. Cox et al. (1996) used biofilters to remove styrene vapour from air. The black yeastlike fungus *Exophiala jeanselmei* is the active agent in these filters. Cinnamic acid is converted into styrene by growing cultures and cellfree extracts of *Cryptococcus elinovii*. (Middelhoven and Sollewijn Gelpke, 1995).

7.4 Polysaccharides

Growth tests on soluble starch and inulin are traditionally used for distinction of yeast species. Some yeasts are able to ferment these polysaccharides. Other polysaccharides did not receive much attention. Some hydrolytic enzymes excreted by yeasts that convert these polymers into low-molecular fragments are or were commercially available, e.g. xylanase from *Aureobasidium pullulans* (Leathers, 1989) and *Cryptococcus albidus* (Biely and Vrsanska, 1988). Most of the commercially available polysaccharide hydrolases are produced by filamentous fungi. A thermolabile xylanase is produced by *Cryptococcus adeliae* (Gomes et al., 2000).

In the taxonomic growth tests soluble starch is used. Some yeast species are also able to assimilate raw, unmodified starch. De Mot et al. (1984a) did a comparative study on starch degradation by ascomycetous species. Of 73 species tested, *Endomycopsis, Lipomyces, Pichia* and *Schwanniomyces* spp. were the most active. Another comparative study on starch assimilation was carried out by McCormack

and Barnett (1986). A thermostable α-amylase digesting raw starch is produced by a *Cryptococcus* sp. (Fuji et al., 1996) and by *Lipomyces starkeyi* (Punpeng et al., 1992). Wanderley et al. (2004) studied the α-amylase of *Cryptococcus flavus*. Debranching enzymes that hydrolyze 1,6 glucosidic bonds in amylopectin were produced by many yeast species. Highest activities were found with *Endomycopsis, Lipomyces, Filobasidium, Leucosporidium* and *Trichosporon* spp. grown on pullulan (poly-α-1,6-maltotriose) (de Mot et al., 1984b). Dextran (α-1,6-glucan) is another polymer of D-glucose, produced by *Leuconostoc mesenteroides* and some other bacteria. *Lipomyces starkeyi* produces an endodextranase (Webb and Spencer-Martins, 1983; Koenig and Day, 1988), but *Lipomyces lipofer* an exodextranase (Ramos and Spencer-Martins, 1983). A β-mannanase is produced by *Trichosporon cutaneum* CBS 5790 (Oda and Tonomura, 1996), later renamed *T. laibachii* of which it is the type strain. Kremnicky et al. (1996) screened many yeast species for mannanase production and found *Aureobasidium pullulans* and *Stephanoascus ciferrii* to be the best producers.

Pectic substances are complex structural polysaccharides of plant origin that contain a large proportion of partially methyl-esterified galacturonic acid residues linked by α-1,4-glycosidic linkages. These D-galacturonic acid residues present in the backbone of the pectin chain are interrupted by L-rhamnose units, to which arabinose and galactose residues can be attached. Roelofsen (1953) observed that yeasts belonging to the genera *Candida, Pichia, Saccharomyces* and *Zygosaccharomyces* attacked cell wall pectin. Many of these strains were isolated from fermenting cocoa. Salt-tolerant yeasts are also pectolytic and play a part in the softening of cucumbers pickled in brine (Bell and Etchells, 1956). Assimilation of pectic substances by yeasts during the dew-retting process of flax was reported by Wieringa (1956). A red yeast, initially named *Rhodotorula lini*, was responsible for this process. Frederiksen (1956) named it *Rhodotorula macerans.* Its present name is *Cryptococcus macerans*.

A review of pectolytic yeast species was given by Blanco et al. (1999). Endopolygalacturonase is the chief enzyme excreted into the growth medium (Luh and Phaff, 1951, 1953). In most species it is produced constitutively, but is subject to glucose repression. Several yeast species such as *Kluyveromyces* and *Saccharomyces* spp. are unable to grow on pectin or galacturonic acid (Schwan et al., 1997; Blanco et al., 1999). Hence, these yeasts must be involved in colonisation of plants and fruits. Tropical fruits are a habitat of many pectolytic yeasts (da Silva et al., 2005). Commercially available pectinases are produced by *Aspergillus niger*, but yeast enzymes could offer an alternative. Pectinases are used for softening of baby foods and for clarification of fruit juices. Large-scale production of endopolygalacturonase by *K. marxianus* has been reported by Almeida et al. (2004). It is also produced by *Debaryomyces hansenii* (da Silva et al., 2005), but *Stephanoascus smithiae* excreted an exopolygalacturonase. Pectin methylesterase was not secreted. Pectin lyase was produced by some strains of *K. wickerhamii*, *S. smithiae* and *Pichia anomala* (da Silva et al., 2005).

Middelhoven (2005) isolated yeast strains from a habitat rich in polysaccharides, viz. rotten wood. Several ascomycetous and basidiomycetous yeast species were

recovered and were tested for growth on polysaccharides. The ascomycetes assimilated none or a few, but the basidiomycetes tested assimilated soluble starch, pullulan, dextran, xylan, polygalacturonate, galactomannan and tannic acid or at least some of these. *Cryptococcus podzolicus* and *Trichosporon porosum* were the most active species. Previously, (Middelhoven, 2004) proposed growth tests on these polysaccharides for distinction of saprotrophic *Trichosporon* species. Pathogenic *Trichosporon* species generally do not assimilate these polysaccharides (Middelhoven, 2003). None of the yeasts tested was able to assimilate carboxymethyl cellulose, colloidal chitin, arabinogalactan and gum xanthan.

Some yeast species display hydrolytic activity towards polysaccharides but are unable to grow at the expense of the monomer. Gainvors et al. (1994) detected pectin-degrading enzymes in wine yeasts, but only one of 32 tested strains excreted these enzymes.

7.4.1 Immunology

Yeasts excrete heat-stable immunogenic polysaccharides that can be detected by enzyme-linked immunosorbent assays, ELISA (Middelhoven and Notermans, 1988; Middelhoven et al., 1998; Middelhoven and Notermans, 1993). The antigens of most ascomycetous yeast species were almost species-specific, but those of basidiomycetes gave cross reactions with many yeast species, ascomycetes included. This ELISA method permits specific detection of ascomycetous yeasts, even after their death. However, the preparation of specific antibodies is laborious. Hence, the technique became obsolete and was replaced with rDNA nuclear base sequencing and methods derived thereof. Nevertheless, the striking difference in behaviour of ascomycetes and basidiomycetes is interesting from a scientific viewpoint.

7.5 Yeasts Inhabiting Plants and Silage

7.5.1 Phyllosphere

The aerial surface of plants is covered by a thin layer of microorganisms. Ruinen (1956, 1961) introduced the term “phyllosphere”. This term is still used, though some authors prefer phylloplane to designate this intriguing habitat. The phyllosphere microflora of tropical foliage consists of bacteria, free-living nitrogen-fixing of the genus *Beyerinckia* included, and many yeasts (Ruinen, 1963, 1965), mainly *Cryptococcus* spp. and red basidiomycetes that produce ballistoconidia. These yeasts are able to decompose the plant’s cuticle, thus promoting leakage of nutrients from which the microflora benefits (Ruinen, 1965). Many of these yeasts produce, and even excrete, large amounts of lipids when grown in an unbalanced growth medium

containing an excess of sugars (Ruinen and Deinema, 1964; Stodola et al., 1967). Ballistoconidiogenous yeasts and their relatives were also detected on fresh maize foliage grown in a temperate climate Middelhoven and van Baalen, 1988). Sugar cane foliage is also inhabited by basidiomycetous yeasts (de Azaredo et al., 1998), of which *Cryptococcus albidus, C. laurentii* and *Rhodotorula mucilaginosa* were the most numerous; the ascomycete *Debaryomyces hansenii* was also present.

Plants growing in an arid climate, however, were inhabited by other yeasts as appeared from a study of 24 different plant species (Middelhoven, 1997). Ascomycetes predominated and about half of the isolates (22) were identified as *Debaryomyces hansenii*. Other ascomycetes were black yeastlike fungi like *Hormonema dematioides* (4 strains), *Hortaea werneckii* (1 strain) and 2 strains of a novel species *Hormonema schizolunatum* (Middelhoven and de Hoog, 1997). Basidiomycetes were represented by *Cryptococcus albidus* and *C. laurentii* (both by 4 strains) and by 5 red *Rhodotorula* spp., tentatively identified as *R. glutinis* and *R. mucilaginosa*. In addition to these true yeasts two yeastlike fungi of basidiomycetous affiliation were isolated, viz. an unidentified *Pseudozyma* sp. and a strain of *Cerinosterus cyanescens* that lacked the characteristic diffusible blue pigment (Middelhoven et al., 2000c).

Phyllosphere yeasts may protect the plant against infection by phytopathogenic fungi (Ruinen, 1961, 1963; Fokkema et al., 1979). Many of these yeasts attach to hyphae and conidia of these fungi (Allen et al., 2004). Phyllosphere yeasts and yeastlike fungi produce antibacterial compounds (McCormack et al., 1994). A monograph on the phyllosphere appeared some years ago (Lindow et al., 2002).

7.5.2 Biodegradation

Several phyllosphere yeast strains were screened for growth on plant constituents (Middelhoven, 1997). All of them readily assimilated lipid compounds, either hydrolytically or oxidatively or both. All strains grew on olive oil (*Hormonema* spp. excepted) and lecithin, indicating that these species may hydrolyze the cuticle. Assimilation of n-hexadecane was shown by all strains of *D. hansenii, R. glutinis, C. cyanescens and Pseudozyma* sp. This may be indicative of an oxidative attack of the cuticle that consists of long-chain aliphatic compounds.

The black yeastlike fungi, *Cryptococcus* sp. and *R. glutinis* assimilated xylan, pectin and starch. Disaccharides common in plants, like sucrose, maltose and cellobiose were assimilated by almost all strains tested.

Most strains assimilated protein, i.e. native casein, and several individual amino acids, lysine and methionine excepted. DNA and deoxyribose were no suitable carbon sources, but DNA and nucleic acid bases were assimilated as sole nitrogen sources, DNA and cytosine by all strains tested, the other bases giving variable results. RNA was assimilated as sole carbon and nitrogen source by almost all strains tested.

Phenolic compounds are important plant constituents. *D. hansenii* strains grew on phenol and hydroquinone, but not on more complex compounds. Some of these,

e.g. cinnamic acid and its hydroxy and methoxy derivatives, gallic and tannic acids supported growth of some *Rhodotorula* strains and of some yeastlike fungi. For more details, see Middelhoven (1997). The general conclusion is that the yeast flora of the phyllosphere benefits from many plant constituents. The latter are set free by mechanical damage and by leakage of the cuticle and cell wall that may be provoked by lipolytic activity of the yeast cells.

7.5.3 Maize Silage

The aerobic phyllosphere yeast flora drastically changes when plant material is compressed and stored under anaerobic conditions, as happens during ensiling. In maize silage the aerobic yeasts had vanished after two days, before lactic acid fermentation was complete, and were replaced with fermentative yeast species (Middelhoven and van Baalen, 1988). *Candida lambica, C. milleri* and *C. holmii* (anamorph of *Saccharomyces exiguus*) predominated this yeast flora. These species, along with *C. krusei* and *S. dairenensis*, had previously been demonstrated as dominant yeast species in an analysis of 13 different maize silages (Middelhoven and Franzen, 1986).

Another maize silage yielded a novel species, viz. *S. bulderi* (Middelhoven et al., 2000a), related to *S. exiguus*. It is able to ferment gluconolactone by a novel pathway for alcoholic fermentation (van Dijken et al., 2002) in which a NADP-dependent glucose dehydrogenase and enzymes of the pentose phosphate pathway participate.

Numbers of *C. lambica, C. milleri* and *C. holmii* increased during ensiling to approximately 10^7 per gram after 5 days, but gradually decreased to 10^4 after 4 months. Obviously, conditions in silage are adverse, also for these acid-tolerant yeasts. These are the principal agents responsible for aerobic deterioration of maize silage as appears from their increase to about 10^9 per gram after 4 days, at the expense of acetic and lactic acids. Some yeast species, e.g. *C. famata, Geotrichum candidum* and *Hansenula (Pichia) anomala* occurred in lower numbers. In addition to lactic acid and ethanol, these species assimilated minor products of bacterial sugar fermentation such as acetoin and butane-2,3-diol (Middelhoven and van Baalen, 1988).

7.5.4 Other Ensiled Crops

Whole-crop maize that was ensiled at 25°C or 30°C, rather than at 20°C as usual was inhabited by the same fermentative yeast species, but these were accompanied with ascomycetous non-fermentative fungi, i.e. *Exophiala jeanselmei* and *Verticillium psalliotae*, by the non-fermentative basidiomycete *Rhodotorula mucilaginosa* and the weakly fermentative ascomycete *Arxula adeninivorans* (Middelhoven et al., 1990). Other ensiled crops were inhabited by the same yeasts as present in maize silage at 20°C. These crops were beetroot foliage, grass, lucern, Jerusalem artichoke

foliage, green chicory foliage and hemp foliage. However, ensiled crops containing mustard oils were inhabited by other yeast species, i.e. *Rhodotorula minuta, Stephanoascus ciferrii, Candida famata* and *Trichosporon cutaneum* (later reidentified as *T. gracile*). These species were isolated from ensiled turnip foliage, rocket and leek.

7.6 Phylogeny and Distinction of Taxa

7.6.1 Phylogeny

In some cases a physiological character is only shown by a group of related species. A well-known example is growth on methanol that is a character of related ascomycetous species and not of other yeasts (Kurtzman and Robnett, 1998). Yeast species able to utilize adenine as sole carbon source are also found in only one clade. Phylogenetic analysis based on nuclear ribosomal base sequencing revealed close phylogenetic relationship of *Stephanoascus ciferrii* and *Arxula adeninivorans* (Kurtzman and Robnett, 1998). Several *Blastobotrys* species belong to the same clade of about 12 species. Middelhoven and Kurtzman (2003) showed that nearly all these species assimilated adenine as sole carbon source, a character not shown by other yeasts studied. In addition to adenine, these 12 species assimilated n-hexadecane, uric acid, isobutanol, L-leucine, L-isoleucine and putrescine. Some of these characteristics were also shown by other ascomycetes, but the 12 species of the *Stephanoascus/Blastobotrys* clade showed these all together, suggesting a relation between phylogeny and physiology.

Middelhoven and Kurtzman (2003) stated that yeast species of which respiratory deficient mutants are known do cluster in four clades according to the tree proposed by Kurtzman and Robnett, 1998). However, in a more recent phylogenetic analysis (Kurtzman and Robnett, 2003) these species were scattered over the tree. This is no surprise as several different mutations can lead to respiratory deficiency.

7.6.2 Trichosporon

The imperfect basidiomycetous genus *Trichosporon* is characterized by assimilation of many unusual carbon compounds. Growth tests on uric acid, ethylamine, L-4-hydroxyproline, tyramine and L-phenylalanine as sole source of carbon and nitrogen, and on quinate, 4-ethylphenol, 2,3-dihydroxybenzoate and orcinol as sole carbon source were used for distinction of pathogenic and suspected pathogenic species, able to grow at 37°C (Middelhoven, 2003) and an identification key based on these tests was provided. A key to saprotrophic *Trichosporon* species was provided by Middelhoven (2000b, 2004). In addition to the growth tests that distinguish

pathogenic species, some plant constituents such as xylan, polygalacturonate, galactomannan, tannic acid, phloroglucinol and orcinol were used as well.

Species of the genus *Trichosporon* cluster in four clades (Middelhoven et al., 2004), viz. the Ovoides, Cutaneum, Porosum and Gracile clades. The Ovoides clade contains most of the pathogenic species able to grow at 37°C and on uric acid, but not on polygalacturonate, quinate, phloroglucinol, melibiose and raffinose. The other three clades are variable for these characters, except the Porosum clade of which nearly all members assimilate polygalacturonate, quinate, phloroglucinol, melibiose and raffinose. The Gracile clade is negative for phloroglucinol and erythritol and variable for the other characteristics. Assimilation of dextran and tannic acid is exclusively shown by some species of the Porosum clade (Middelhoven, 2004).

References

Allen, T.W., Burpee, L.L. and Buck, J.W. 2004. *Can. J. Microbiol.* **50:** 1041–1048.

Almeida, C., Bránayik, T., Moradas, F.P. and Texeira, J. 2004. *Proc. Biochem.* **40:** 1937–1942.

Anderson, J.J. and Dagley, S. 1981. *J. Bacteriol.* **146:** 291–297.

Aoki, K., Shinke, R. and Nishimura, H. 1976. *Agric. Biol. Chem.* **40:** 79–85.

Bell, T.A. and Etchells, J.L. 1956. *Appl. Microbiol.* **4:** 196–201.

Bhat, T.K., Singh, B. and Sharma, O.P. 1998. *Biodegradation* **9:** 343–357.

Biely, P. and Vrsanska, M. 1988. *Meth. Enzymol.* **160:** 638–648.

Blanco, P., Sieiro, C and Villa, T.G. 1999. *FEMS Microbiol. Lett.* **175:** 1–9.

Bos, P, de Bruyn, J.C. 1973. Antonie van Leeuwenhoek 39: 99–107

Corpillo, D., Valetti, F., Scruffida, M.G., Conti, A., Rossi, A., Finassi-Agro, A. and Giunta, C. 2003. *Yeast* **15:** 369–379.

Cox, H.J.J., Magielsen, F.J. and Doddema, H.J. 1996. *Appl. Microbiol. Biotechnol.* **45:** 851–856.

da Silva, E.G., Borges, M. de-F., Medina, C., Piccoli, R.H., and Schwan, R.F., 2005. *FEMS Yeast Res.* **5:** 859–865.

de Azaredo, L.A.I., Gomes, E.A., Mendonça-Hagler, L.C. and Hagler, A.N., 1998. *Internat. Microbiol.* **1:** 205–208.

de Mot, R., Andries, K. and Verachtert, H., 1984a. *Syst. Appl. Microbiol.* **5:** 106–118.

de Mot R., van Oudendijck, E and Verachtert, H., 1984b. *Biotechnol. Lett.* **6:** 581–586.

Di Menna, M.E. 1959. *J. Gen. Microbiol.* **20:** 13–23.

Fokkema, N.J., den Houter, J.G., Kosterman, Y.J.C. and Nelis, A.L. 1979. *Trans. Br. Mycol. Soc.* **72:** 19–29.

Frederiksen, P.S. 1956. *Friesia* **5:** 234–239.

Fuji, I.E., Chino, M., Kato, M. and Iimura, Y. 1996. *Biochem. J.* **318:** 989–996.

Gainvors, A., Frézier, V., Lemarasquier, H., Lequart, C., Aigle, M., and Belarbi, A., 1994. *Yeast* **10:** 1311–1319.

Gomes, J., Gomes, I., and Steiner, W., 2000. *Extremophiles* **4:** 227–235.

Green, J., Haywood, G.W. and Large, P.J. 1982. *J. Gen. Microbiol.* –**128:** 991–996.

Haywood, G.W. and Large, P.J. 1981. *Biochem. J.* **199:** 187–201.

Hazeu, W., de Bruyn, J.C. and Bos, P. 1972. *Arch. Microbiol.* **87:** 968–969.

Heath, L.A. and Large, P.J. 1984. *FEMS Microbiol. Lett.* **22:** 15–19.

Hug, H. and Fiechter, A., 1972. *Arch. Microbiol.* **88:** 87–96.

Koenig, D.W. and Day, D.F. 1988. *Biotechnol. Lett.* **10:** 117–122.

Kremnicky, L., Slavikova, E., Mislovicova, D. and Biely, P., 1996. *Folia Microbiol.* **41:** 43–47.

Kurtzman, C.P. and Robnett C.J. 1998. *Antonie van Leeuwenhoek* **73:** 331–371.

Kurtzman, C.P. and Robnett C.J. 2003. *FEMS Yeast Res.* **3:** 417–432.

Large, P.J. 1986. *Yeast* 2-1-34
Leathers, T.D. 1989. *J. Ind. Microbiol. Biotechnol.* **4:** 341–347.
Levine, D.W. and Cooney, C.L. 1973. *Appl. Microbiol.* **26:** 982–990.
Lindow, S.E., Hecht-Poinar, E.I., and Elliott, V.J., 2002 Phyllosphere Microbiology, APS Press, St. Paul, Minnesota.
Luh, B.S. and Phaff, H.J., 1951. *Arch. Biochem. Biophys.* **33:** 213–227.
Luh, B.S. and Phaff, H.J., 1953. *Arch. Biochem. Biophys.* **48:** 23–37.
Markovetz, A.J., Kallio, R.E. 1964. *J. Bacteriol*. 87: 968–969
McCormack, A.K. and Barnett J.A. 1986. *Yeast* **2:** 109–115.
McCormack, P.J., Wildman, H.G. and Jeffries P. 1994. *Appl. Environm. Microbiol.* **60:** 927–931.
Middelhoven, W.J. 1977. *J. Gen. Microbiol.* **100:** 257–269.
Middelhoven, W.J. 1993. *Antonie van Leeuwenhoek* **63:** 125–144.
Middelhoven, W.J. 1997. *Antonie van Leeuwenhoek* **72:** 81–89.
Middelhoven, W.J. 1998. *Food Technol. Biotechnol.* **36:** 7–11.
Middelhoven, W.J. 2001. *Yeast, a News Letter for Persons interested in Yeast* **50:** 64–65.
Middelhoven, W.J. 2003. *Mycoses* **46:** 7–11.
Middelhoven, W.J. 2004. *Antonie van Leeuwenhoek* **86:** 329–337.
Middelhoven, W.J. 2005. *Antonie van Leeuwenhoek* **87:** 101–108.
Middelhoven, W.J. 2006. *Antonie van Leeuwenhoek* **90:** 57–67.
Middelhoven, W.J. Arkesteyn G.J.M.W. 1981. *Antonie van Leeuwenhoek* **47:** 121–131.
Middelhoven, W.J., Berends, J., Repelius, C. and Aert, A.J.M. van 1976b. *Eur.J. Appl. Microbiol.* **2:** 169–173.
Middelhoven, W.J., Berends, J, van Aert, A.J.M. and Bruinsma, D. 1976a. *J. Gen. Microbiol.* **93:** 185–188.
Middelhoven, W.J., Coenen, A., Kraakman, B. and Sollewijn Gelpke, M.D. 1992a. *Antonie van Leeuwenhoek* **62:** 181–187.
Middelhoven, W.J. and de Hoog, G.S. 1997. *Antonie van Leeuwenhoek* **71:** 297–305.
Middelhoven, W.J., de Jong, I.M. and Winter, M. de 1990. *Antonie van Leeuwenhoek* **57:** 153–158.
Middelhoven, W.J., de Jong, I.M. and Winter, M. de 1991. *Antonie van Leeuwenhoek* **59:** 129–137.
Middelhoven, W.J., de Kievit, H. and Biesbroek, A.L. 1985. *Antonie van Leeuwenhoek* **51:** 289–301.
Middelhoven, W.J. and Franzen, M.M. 1986. *J. Sci. Food. Agric.* **37:** 855–861.
Middelhoven, W.J., Guého, E. and de Hoog, G.S. 2000c. *Antonie van Leeuwenhoek* **77:** 313–320.
Middelhoven, W.J., Hoogkamer-te Niet, M.C., de Laat, W.T.A.M., Weyers, C. and Bulder, C. J.E.A. 1986. *Antonie van Leeuwenhoek* **52:** 525–536.
Middelhoven, W.J., Hoogkamer-te Niet, M.C. and Kreger-van Rij, N.J.W. 1984. *Antonie van Leeuwenhoek* **50:** 369–378.
Middelhoven, W.J. and Kurtzman, C.P. 2003. *Antonie van Leeuwenhoek* **83:** 69–74.
Middelhoven, W.J., Koorevaar, M. and Schuur, G.W. 1992b. *Plant and Soil* **145:** 37–43.
Middelhoven, W.J., Kurtzman, C.P. and Vaughan-Martini, A. 2000a. *Antonie van Leeuwenhoek* **77:** 223–228.
Middelhoven, W.J. and Notermans, S. 1988. *J. Gen. Appl. Microbiol.* **34:** 15–26.
Middelhoven, W.J. and Notermans, S. 1993. *Int. J. Food Technol.* **19:** 53–62.
Middelhoven W.J., Slingerland R.J., Notermans S. 1988. *Antonie van Leeuwenhoek* 54: 235–244
Middelhoven, W.J. and Sollewijn Gelpke, M.D. 1995. *Antonie van Leeuwenhoek* **67:** 217–219.
Middelhoven, W.J. and Spaaij, F. 1997. *Int. J. Syst. Bacteriol.* **47:** 324–327.
Middelhoven, W.J., Scorzetti, G. and Fell, J.W. 1999. *Can. J. Microbiol.* **45:** 686–690.
Middelhoven, W.J., Scorzetti, G. and Fell, J.W. 2000b. *Int. J. Syst. Evol. Microbiol.* **50:** 381–387.
Middelhoven, W.J., Scorzetti, G. and Fell, J.W. 2001. *FEMS Yeast Res.* **1:** 15–22.
Middelhoven, W.J., Scorzetti, G. and Fell, J.W. 2004. *Int. J. Syst. Evol. Microbiol.* **54:** 975–986.
Middelhoven, W.J. and Baalen A.H.M. van 1988. *J. Sci. Food Agric.* **42:** 199–207.

Middelhoven, W.J., Brink, J.A. and van den, Veenhuis, M. 1983. *Antonie van Leeuwenhoek* **49:** 361–368.
Middelhoven, W.J. and Doesburg, W. van 2007. *Antonie van Leeuwenhoek* **91:** 191–196.
Oda, Y. and Tonomura, K. 1996. *Lett. Appl. Microbiol.* **22:** 173–178.
Ogata, K., Nishikawa, H. and Ohsugi, M. 1969. *Agric. Biol. Chem.* **33:** 1519–1520.
Ozimek, P, Veenhuis, M. and van der Klei, I. 2005. *FEMS Yeast Res.* **5:** 975–983.
Punpeng, B., Nakata, Y., Goto, M. Teramoto, Y. and Hayashida, S. 1992. *J. Ferment. Bioeng.* **73:** 108–111.
Ramos, A. and Spencer-Martins, I. 1983. *Antonie van Leeuwenhoek* **49:** 183–190.
Ratledge, C. 1991. *Acta Biotechnol.* **11:** 429–438.
Roelofsen, P.A. 1953. *Biochim. Biophys. Acta* **10:** 410–413.
Ruinen, J. 1956. *Nature* **177:** 220–221.
Ruinen, J. 1961. *Plant and Soil* **15:** 81–109.
Ruinen, J. 1963. *Antonie van Leeuwenhoek* **29:** 425–438.
Ruinen, J. 1965. *Ann. Inst. Pasteur* **111:** 342–346.
Ruinen, J. and Deinema, M.H. 1964. *Antonie van Leeuwenhoek* **30:** 377–384.
Sahm, H. and Wagner, F. 1973. *Europ. J. Biochem.* **36:** 250–256.
Sakay, Y., Murdanoto, A.P., Sembering, L. Tanai, Y. and Kato, N. 1995. *FEMS Microbiol. Lett.* **127:** 229–234.
Sampaio, J.P. 1994. *Syst. Appl. Microbiol*. 17: 613–619
Sampaio, J.P. 1999. *Can. J. Microbiol*. 45: 491–512
Sasaki, T, Kawamura, M. and Ishikawa, H. 1996. *J. Insect Physiol.* **42:** 125–129.
Scheda, R. and Bos, P. 1966. *Nature* **211:** 660.
Schwan, R.F., Cooper, R.M. and Wheals, A.E. 1997. *Enz. Microbiol. Technol.* **21:** 234–244.
Stodola, F.H., Deinema, M.H. and Spencer, J.F. 1967. *Bacteriol. Rev.* **31:** 194–213.
Szamecs, B., Urban, G. Rubina, R. Kucsera, J. and Dorgai, L. 2005. *Yeast* **8:** 669–676.
Van der Klei, I.J. and Veenhuis, M. 2002. In: *Hansenula polymorpha Biology and Applications*. (ed. Gellissen, G.), Wiley-VCH, Weinheim, Germany, pp. 76–94.
Van der Walt, J.P. 1962. *Antonie van Leeuwenhoek* **28:** 91–96.
Van der Walt, J.P., Smith M. Yamada Y. 1990. *Antonie van Leeuwenhoek* **57:** 59–61.
Van Dijken, J.P. and Bos, P. 1981. *Arch. Microbiol.* **128:** 320–324.
Van Dijken, J.P., Veenhuis, M., Kreger-van Rij, N.J.W. and Harder, W. 1975. *Arch. Microbiol.* **102:** 41–44.
Van Dijken, J.P., Tuyl, A, van Luttik, M.A.H., Middelhoven, W.J. and Pronk, J.T. 2002. *J. Bacteriol.* **184:** 672–678.
Veenhuis, M., Hoogkamer-te Niet, M.C. and Middelhoven, W.J. 1985. *Antonie van Leeuwenhoek* **51:** 33–43.
Wanderley, K.J., Torres, F.A.G., Moraes, L.M.P. and Ulhoa, C.J. 2004. *FEMS Microbiol. Lett.* **231:** 165–169.
Webb, E. and Spencer-Martins, I. 1983. *Can. J. Microbiol.* **29:** 1092–1095.
Wieringa, K.T. 1956. *Neth. J. Agric. Sci.* **4:** 204–209.
Zimmermann, R. 1958. *Naturwissenschaften* **45:** 165.
Zinjada, S.S. and Pant, A. 2002. *J. Basic Microbiol.* **42:** 67–73.
Zwart, K. and Harder, W. 1983. *J. Gen. Microbiol.* **129:** 3157–3169.
Zwart, K., Veenhuis, M., Dijken, J.P. and Harder, W. 1980. *Arch. Microbiol.* **126:** 117–126.
Zwart, K.B., Veenhuis, M. and Harder, W. 1983. *Antonie van Leeuwenhoek* –**49:** 369–385.