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Review

Debaryomyces hansenii — an extremophilic yeast with biotechnological potential

Uta Breuer* and Hauke Harms
UFZ — Centre of Environmental Research Leipzig-Halle, Department of Environmental Microbiology, Permoserstrasse 15, D-04318 Leipzig, Germany

*Correspondence to:
Uta Breuer, UFZ — Centre of Environmental Research Leipzig-Halle, Department of Environmental Microbiology, Permoserstrasse 15, D-04318 Leipzig, Germany.
E-mail: uta.breuer@ufz.de

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Abstract

We illuminate the ecological, physiological and genetic characteristics of the yeast *Debaryomyces hansenii* in the view of our belief that this metabolically versatile, non-pathogenic, osmotolerant and oleaginous microorganism represents an attractive target for fundamental and applied biotechnological research. To this end, we give a broad overview of extant biotechnological procedures using *D. hansenii*, e.g. in the manufacture of various foods, and propose research into the heterologous synthesis of a range of fine chemicals. Copyright © 2006 John Wiley & Sons, Ltd.

Keywords: ***Debaryomyces hansenii*; yeast; osmotolerant; oleaginous; biotechnological applications**

Contents

Introduction

Saccharomyces cerevisiae was the first yeast to be investigated in the early years of biochemistry and most of our knowledge of heterologous expression in eukaryotes was obtained from studies of this organism. *S. cerevisiae* was clearly not suitable for all biotechnological purposes. Significant efforts were made to investigate other yeasts in order to gain insights into an expected wealth of biochemical pathways and the genetic diversity of yeasts. For example, interest was aroused in extremophilic yeasts, including *Debaryomyces* spp. which proved to be genetically and biochemically interesting yeasts with considerable biotechnological promise [12,156]. *Debaryomyces* spp. are osmotolerant and can grow in media containing up to 4 M NaCl, whereas growth of *S. cerevisiae* is restricted to media containing less than 1.7 M NaCl. [139] *D. hansenii* can be found in many habitats with low water activity, such as sea water, from which it was initially isolated, cheese, meat, wine, beer, fruit and soil [11,40,133] as well as in high-sugar products [177]. Its osmotolerance is highly advantageous for some biotechnological applications because it allows quasi-non-sterile production and high product/educt concentrations, conditions which should reduce production costs dramatically, e.g. in the agro-food sector. Unfortunately, *D. hansenii* is also known to be the causal agent of the spoilage of brine-preserved foods, such as gherkins.

D. hansenii is one of the lipid-accumulating, 'oleaginous' yeasts. Oleaginous yeasts can accumulate lipids to concentrations up to 70% of their dry biomass [156] and their metabolism is clearly dominated by pathways that contribute to lipid metabolism. The extreme capacity of *D. hansenii* to synthesize, accumulate and store lipids could be advantageous for the biotechnological production of both natural and artificial products.

D. hansenii is a highly heterogeneous, and thus versatile, species — as shown by the phenotypic

differences between strains, such as variations in their ability to assimilate and ferment various carbon sources, the expression of different lipase and protease activities and their highly diverse optimal growth conditions [146]. According to the present taxonomy, two varieties of *D. hansenii* are distinguished, *D. hansenii* var. *fabryi* and *D. hansenii* var. *hansenii*, with different properties, e.g. their maximum growth temperatures.

This literature review introduces the physiology, genetics and ecology of *Debaryomyces* species, particularly *D. hansenii*, before discussing current biotechnological applications of the genus in detail. We also attempt to show that the genus *Debaryomyces* has only been studied superficially and that further investigation is needed to exploit its immense potential as a tool for tailored biotechnological production, as an object for fundamental scientific investigation and as a potent cell factory.

Phylogeny, ecology, physiology and molecular biology

Phylogeny

The genus *Debaryomyces* Lodder et Kreger-van Rij Nom. Cons. (Figure 1) is described in detail by Nakase *et al*. [125]. All *Debaryomyces* species are perfect, haploid yeasts that reproduce vegetatively by multilateral budding. A pseudomycelium is absent, primitive or occasionally well developed. The sexual reproduction proceeds via heterogamous conjugation, i.e. the conjugation of two cells of different form or size, here a mother cell and a bud. This conjugation generally leads to a short diplophase followed by meiosis and ascospore formation [65]. The asci contain one to four spherical, globular, ovoidal or lenticular smoothy or warty ascospores. Isogamous conjugation also occurs [125].

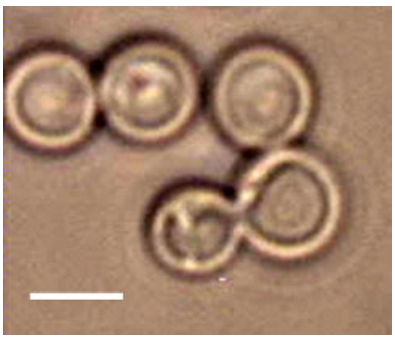

Figure 1. *Debaryomyces hansenii* H158 asci with one ascospore after 6 weeks on YEPD agar at 30 °C. Magnification ×1125. Bar = 2.5 μm

Members of the genus *Debaryomyces* are characterized physiologically by their weak or non-existent fermentation capacities, as well as their inability to assimilate nitrate and chemotaxonomically by their expression of coenzyme Q-9 [94]. The detailed description of the species *D. hansenii* (Zopf) Lodder & Kreger-van Rij can be read in Nakase *et al*. [125] The ability to grow at 10% NaCl or 5% glucose deserves special mention, since it is used to discriminate *D. hansenii* from other ascomycetous yeasts.

The species *D. hansenii* (Zopf) Lodder & Kreger-van Rij [112] comprises two varieties: *D. hansenii* (Zopf) Lodder & Kreger-van Rij var. *hansenii* [anamorph: *Candida famata* (Harrison) S. A. Meyer Yarrow var. *famata*] and *D. hansenii* var. *fabryi* (Ota) Nakase & M. Suzuki [anamorph: *Candida famata* (Harrison) S. A. Meyer & Yarrow var. *flaveri* (Ciferri & Redaelli) Nakase & M. Suzuki]. Zopf [195] published the first description of var. *hansenii* and Ota [141] that of var. *fabryii*. Besides the sequence divergences of their 26S rRNA genes [96,97], differences in the electrophoretic mobility of their glucose-6-phosphate dehydrogenase and their maximum growth temperatures (var. *hansenii* can only grow in temperatures up to 35 °C while var. *fabryi* grows up to 39 °C) have been used to discriminate between the two varieties of *D. hansenii* [124]. Corredor *et al*. [36] strongly advocated the use of pulsed-field gel electrophoresis (PFGE), a technique that can separate especially long strands of DNA by length, for accurate discrimination of *D. hansenii* strains. Using this tool for karyotype analysis, they found that strains of *D. hansenii* differ markedly at the level of genomic organization. The observed chromosomal length polymorphism of *D. hansenii* var. *hansenii* and *D. hansenii* var. *fabryii* seems to result from variations in their numbers of repeated sequences or chromosomal rearrangements. Strains belonging to *D. hansenii* var. *fabryii* actually represent a different taxon from *D. hansenii* var. *hansenii*, as suggested by a number of previous authors [124,148]. Conversely, PFGE analysis by Petersen and Jespersen [146] did not

result in a division of the two *D. hansenii* varieties into separate groups, although the authors confirmed that PFGE has very high discriminative power for typing *D. hansenii* strains. Romano *et al*. [158] found that the two groups could be separated using a combination of mt (mitochondrial) DNA RFLP and RAPD (random amplification of polymorphic DNA) analysis with appropriate primers. The taxonomic classification of both the two varieties and the genus *Debaryomyces* in general seems likely to change in the near future as further analytical methods are developed and applied.

Ecology

D. hansenii is an osmo-, halo- and xerotolerant yeast. It is known to be a contaminant of various foods with low water activity, as are *Pichia guilliermondi, Yarrowia lipolytica* and *Candida parapsilosis. D. hansenii* is found in hypersaline waters, such as the salterns on the Atlantic coast in Namibia and the Great Salt Lake (Utah). It can be cultivated in media with up to 25% NaCl or 18% glycerol. [23] *D. hansenii* can also cause diseases, as reported by Van Uden and Fell [181] and Wong *et al*. [188], or is associated with infections [125]. Knowledge of this ability of *D. hansenii* is important in order to diagnose and prevent their development, as will be discussed later in this review.

D. hansenii is able to synthesize or tolerate several toxins. Toxin synthesis could be an interesting subject for biotechnological research, to establish economically viable processes for the production of the toxins themselves or to facilitate the cultivation of *D. hansenii*, since such toxins may limit or prevent the growth of unwanted organisms. As early as 1993, Gunge and co-workers reported that a toxin (myocin) secreted by *Debaryomyces* killed various yeast species. The activity of this toxin was only demonstrated in the presence of salts such as NaCl or KCl. [79] Furthermore, the killer phenotype was not associated with the pDHL plasmids [68,79], despite previous suggestions that the gene encoding the toxin could be located on one of the three pDHL plasmids. The pDHL1 plasmid is known to carry a DNA-fragment encoding a protein that has some similarities to the α-subunit of the *Kluyveromyces lactis* killer toxin (see section on Molecular biology, below). *D. hansenii* reportedly produces a killer toxin that has stable activity against pathogenic yeasts at 37 °C. Yeast killer toxins with stable activity at human body temperature could have medical applications. For instance, the use of concentrated purified toxin preparations in therapies against pathogenic yeasts is quite conceivable [24]. The lethal activity of this killer toxin was increased in the presence of NaCl in the medium used for assaying the killing action in studies by Llorente *et al*. [109] and Marqina *et al*. [116] Marquina and co-workers investigated optimal conditions for toxin production and showed that the presence of proteins in complex culture media, with non-ionic detergents and additives such as dimethylsulphoxide, stimulated production of the killer toxin significantly. The toxin secretion pattern was found to be a growth-related response, and titres peaked in the early stationary phase [116]. Santos *et al*. [163] identified the (1–6)-ß-D-glucans as the cell wall-binding site for this killer toxin. *D. hansenii* has been shown to be resistant to the killer toxins produced by *Trichosporon cutaneum* and *C. zeylanoides* [136]. The ability of *Debaryomyces* to synthesize toxins and to withstand toxins from other yeasts should be investigated in more detail, especially for a further medical application of these toxins.

Another very interesting observation was that *D. hansenii* has high tolerance to chlorine dioxide (ClO_2), a powerful biocide [152]. This resistance could be used to cultivate the yeast in non-sterile media containing up to 0.3 mg/l ClO_2 to control unwanted microorganisms. *D. hansenii* is highly resistant to the antimicrobial agent penconazol and intermediately resistant to the antimicrobial agents benomyl and cycloheximide. In addition, the minimum inhibitory concentrations (MICs) of fluconazole and amphotericin B are higher for *Debaryomyces* than for the pathogenic yeast *Candida albicans*, while those of 5-fluorocytosine are similar. These resistances could be mediated by active efflux proteins (AEP) transporting the drugs out of the cell. [151]

Physiology

The description of the physiology of *D. hansenii* (Zopf) Lodder and Kreger van Rij [112] is summarized by Nakase *et al*. [125] The yeast shows some remarkable properties. In addition to its ability to grow at 10% NaCl or 5% glucose, *Debaryomyces* assimilates a broad spectrum of

Table 1. Assimilation of substrates by *D. hansenii*

Glucose	+	*N*-Acetyl-D-glucosamine	V
Galactose	+	Methanol	—
L-Sorbose	V	Ethanol	+/W
Sucrose	+	Glycerol	+
Maltose	+	Erythritol	V
Cellobiose	+	Ribitol	+
Trehalose	+	Galacitol	V
Lactose	V	D-Mannitol	+
Melibiose	V	D-Glucitol	+/W
Raffinose	+	α-Methyl-D-glucoside	+
Melezitose	V	Salicin	+/W
Inulin	V	D-Gluconate	+/W
Soluble starch	V	DL-Lactate	V
D-Xylose	+	Succinate	+
L-Arabinose	+/W	Citrate	V
D-Arabinose	V	Inositol	—
D-Ribose	V	Hexadecan	V
L-Rhamnose	V	Nitrate	—
D-Glucosamine	V	Nitrite	V
2-Keto-D-gluconate	+	5-Keto-D-gluconate	V
Saccharate	—		

+, positive; W, weak; V, variable; — , negative.
After Nakase *et al.* [125]

carbon substrates, as Table 1 shows. In contrast to other biotechnologically utilized yeasts, such as *Candida albicans, Pichia angusta* (formely *Hansenula polymorpha*) or *Pichia pastoris, D. hansenii* readily utilizes *n*-alkanes [190] and assimilates melibiose, raffinose, soluble starch and inositol. *Yarrowia lipolytica*, another biotechnologically important yeast, assimilates *n*-paraffins, but does not grow in 10% NaCl or 5% glucose [93]. *D. hansenii* has high chemostress tolerance, which means that high concentrations of many substrates can be used in the cultivation. The yeast shows poor growth in the absence of oxygen [177], and in the presence of glucose *D. hansenii* can co-metabolically oxidize naphthalene and benzo[*a*]pyrene [28].

Fermentation of glucose, galactose, sucrose, maltose, raffinose and trehalose by *D. hansenii* is weak and lactose fermentation not observed. This is in accordance with the poor anaerobic growth of *D. hansenii*. While nitrate is not utilized, the yeast is known to assimilate nitrite [125]. *D. hansenii* is reported to grow optimally at 20–25 °C, but growth between 5 °C and 10 °C and even below 0 °C has also been reported [40]. Van den Tempel and Jacobsen [179] tested the growth of *D. hansenii* as functions of temperature and water activities. They found that at 10 °C the yeast is capable of growing at pH 4.0–6.0 in water activities (a_W) up to 0.99. Investigations in the authors' laboratory show that *D. hansenii* is able to grow over the pH range 3.0–10 (results not shown).

Besides cytochrome *c* oxidase, *D. hansenii* has a second oxidase that can act as the terminal oxidase for an electron transfer chain that branches from the core pathway at the ubiquinone pool level, as described by Veiga and co-workers [182]. This alternative oxidase is insensitive to cyanide and antimycin A, but sensitive to salicylhydroxamic acid. The role of the cyanide-resistant respiration pathway that is very common in yeasts, and its contribution to energy conversion, was elucidated in *D. hansenii*. It has been thought to be involved in the fine adjustment of the cells' energy provision under various physiological conditions. It reduces the amount of energy from the cytochrome *c* pathway by diverting some of the electron flux to the alternative oxidase [182].

D. hansenii is generally regarded as non-pathogenic [172]. However, clinical isolates are not rare [129] and there are reports of bone infection caused by *D. hansenii* [188]. Mattsson *et al*. [119] identified feral pigeons as carriers of *D. hansenii*, and assumed this yeast to be a cause of subcutaneous abscesses, osteitis and keratitis in both immunocompetent and immunocompromised patients. In addition, Yamamoto and co-workers [191] reported a case of extrinsic allergic alveolitis in a 65 year-old female induced by this yeast. Therefore, this yeast's possible pathogenicity should be investigated in more detail.

Molecular biology

The molecular biology of *D. hansenii* is poorly established. Most strains are haploid, mate very rarely and diploidize transiently by somatogamous autogamy to form asci [180].

The genome of *D. hansenii* has been explored in the Génolevures project [107]. In this project the genomes of *Saccharomyces cerevisiae* and 14 other yeast species, representing the various branches of the hemiascomycetous class, are being compared. *Saccharomyces sensu stricto* (*S. bayanus* var. *uvarum), Saccharomyces sensu lato* (*S. exiguous, S. servazzii, Zygosaccharomyces rouxii, S. kluyveri), Kluyveromyces thermotolerans,* the genus *Kluyveromyces* (*K. lactis, K. marxianus* var. *marxianus)* and the distantly related species *Pichia*

angusta, Debaryomyces hansenii var. *hansenii, P. sorbitophila, Candida tropicalis* and *Yarrowia lipolytica* have been investigated. Among the hemiascomycetes, *Debaryomyces hansenii* was selected for complete sequencing because it is halotolerant, related to *C. albicans* and other pathogenic yeasts, and is often found in food such as salted dairy products. The Génolevures investigation, a large-scale comparative genomics project across the evolutionary range of the hemiascomycetous yeast phylum, should allow useful comparisons with other phyla of multicellular organisms, especially the distribution of species-specific and class-specific genes, the distribution of genes among functional families, rates of divergence and the mechanisms of chromosome shuffling. This study is not the first in this regard and continues explorations of the molecular systematics of ascomycetous yeasts by Kurtzman and Robnett [97] and of basidiomycetous yeasts and yeast-like fungi by Fell *et al*. [59].

Chromosomal DNA

In a PFGE-based study of the genetic diversity and chromosome polymorphism among strains of *D. hansenii*, Petersen and Jespersen [146] demonstrated that the chromosomal arrangement of *D. hansenii* strains is heterogeneous (with variations in both the number and size of chromosomal bands on a PFGE gel), showing chromosome polymorphism. The number of chromosomal bands observed varied from five to ten, the most common chromosome number was found to be six. For strains with six chromosomes the total genome size varied (9.4–12.6 Mb). The study included type strain, other strains from culture collections and strains isolated from Danish surface-ripened cheeses.

Codon usage by *D. hansenii* deviates from the universal genetic code. The CUG codon (usually encoding leucine) is used as a codon for serine, which is read by the special, single copy tRNA-Ser (CAG), as already described for 10 species of the genus *Candida* [171]. The non-universal usage of the leucine CUG codon was associated with the molecular phylogeny of these 10 *Candida* species. They formed distinct clusters on molecular phylogenetic trees, using neighbour-joining and maximum likelihood methods. Some of the investigated *Candida* species are teleomorph species and the teleomorph of *C. famata* is *D. hansenii*. The deviation from the universal genetic code is therefore not rare but widely distributed in the hemiascomycetous yeast group [171]. The Génolevures study [107] revealed some unique features of *D. hansenii*, which has the largest number of potentially co-transcribed tRNA gene pairs (in which the distance separating the two genes is shorter than the minimal 5′ sequence required for transcription, suggesting simultaneous transcription of the two genes) of all investigated yeasts. This yeast uses a 43-tRNA set, in contrast to *C. glabrata, K. lactis* and *S. cerevisiae* — which use exactly the same 42-tRNA set. The total number of tRNA types in *D. hansenii* is 43 and it has a total complement of 205 tRNA genes [51]. Furthermore, *D. hansenii* seems to have the highest coding capacity of all the yeasts examined in the Génolevures study, amounting to 79.2% of the genome, with a putative number of 6906 detected CDs (coding sequences). It is also the yeast with the most redundant genome, with an overall redundancy of 49.2%; compared to 40% in *S. cerevisiae*. Gene redundancy is here defined as the presence of genes representing conserved gene families that were present in the organisms' last common ancestor as well as gene families that have emerged or disappeared since specification [110]. Tandem gene duplications are 5–10 times more frequent in *D. hansenii* than in other yeasts, but few duplicated blocks have been detected [107]. Tandem gene duplications refer to head-to-tail repeats of directly adjacent homogeneous genes or gene groups, whereas homologous blocks of genes (up to 250 contiguous genes) on different chromosomes, which duplicated simultaneously as a result of a polyploidization event [66], are called duplicated blocks.

The genetic characteristics of *D. hansenii* could be potentially useful in biotechnological applications, due to their flexibility. On the other hand, their relative complexity may complicate the control of biotechnological processes.

Plasmids of *D. hansenii*

Two research groups have shown the presence of linear plasmids in *D. hansenii*, designated pDHL1 (8.4 kB), pDHL2 (9.2 kB) and pDHL3 (15.0 kB) [79] and pDH1A and B [32]. The stability of the plasmids seems to depend on the osmotic pressure. They were entirely cured when the cells were grown in NaCl-free culture medium, but they

were stably maintained in media containing salts, sorbitol or glycerol at varying concentrations (the *D. hansenii* plasmids are stable in growth media containing >0.3 M NaCl, >0.3 M KCl, >0.7 M sorbitol or >1 M glycerol) [79]. Fukuda *et al.* [67] linked the salt dependency of the plasmids' stability to the growth temperature. They found that the plasmids were stably maintained in cells growing at 25 °C without osmotic pressure, while growth at temperatures between 30 °C and 35 °C at osmolarities lower than 0.3 M NaCl resulted in the gradual loss of the plasmids. This suggests the involvement of stress proteins and/or high glycerol levels in the stabilization process [67]. The linear plasmids are of special interest both for models of DNA replication and as possible endogenous vectors for heterologous genes.

Fukuda and co-workers [68] found a pDHL1-encoded protein with similarity to the α-subunit of the *Kluyveromyces lactis* killer toxin, but they did not detect any killer activity (toxin activity against various yeast species) in *D. hansenii.* The pattern of plasmid-curing suggests that pDHL3 plays a key role in the replication of the *D*. plasmids and Southern hybridization experiments have revealed extensive homology between specific regions of pDHL1 and pDHL2, whereas pDHL3 appeared to be unique [79].

Transformation systems

Several transformation systems have been developed for *Debaryomyces*. The first to be tested was a method that was designed for *Schwanniomyces occidentalis* cells, but proved to be successful with *S. cerevisiae, Schizosaccharomyces pombe, Klyuveromyces lactis* and *Hansenula polymorpha* [44,88]. There seemed to be good reasons for believing that the system would also be able to transform *Debaryomyces*, due to the high degree of similarity between *Schwanniomyces* and *Debaryomyces* [95]. However, the halotolerance of *Debaryomyces* seemed to complicate the transformation, for reasons discussed below in the section on Halotolerance. Thus, no effective system for inserting genes into the genomes of *D. hansenii* and *D. polymorphus* were available at that time, although Ricaurte and Govind [157] were able to transform *Debaryomyces* with an autonomically replicating plasmid.

Later a transformation system that did effectively transform both *D. hansenii* and *D. polymorphus* was developed based on a plasmid which is integrated into the chromosomal DNA [92]. Using this strategy, *Arxula adeninivorans* — and subsequently *D. polymorphus, D. hansenii, S. cerevisiae, Y. lipolytica* and *H. polymorpha* — were successfully transformed [174,185].

In a first attempt to demonstrate the capacity of the newly developed transformation system for expressing heterologous genes in *Debaryomyces*, a bacterial gene coding for a NADPH-dependent acetoacetyl-CoA reductase (*phb*B) was used. Further investigations are under way in the authors' laboratory [19,20,21].

In another investigation, six heterologous yeast expression vectors were constructed for *D. hansenii* using five inducible (regulated) heterologous promoters from *S. cerevisiae* and an endogenous gene promoter to assess the protein production efficiency in this yeast. The expression parameters of proteins in *D. hansenii* seemed to be similar to those in *S. cerevisiae*, with transcription being controllable by almost all of the *S. cerevisiae* and *D. hansenii* inducible promoters tested (CYC1, isocytochrome C1 from *S. cerevisiae*; GPD1, glycerol 3-P-dehydrogenase 1 from *S. cerevisiae*; GPD1d, glycerol 3-P-dehydrogenase 1 from *D. hansenii*; HSP12, heat shock protein 12 from *S. cerevisiae*; and SME1, protein kinase 1 from *S. cerevisiae*), except for the ADH2 gene promotor for alcohol dehydrogenase 2 from *S. cerevisiae* [115]. Heterologous gene expression is a complex, multistep process that is influenced by many factors, e.g. host physiology at high cell densities, promoter induction strength and the stability of the heterologous protein. Therefore, further investigation is recommended if, for instance, secretory heterologous products are involved. One can conclude that the genetic machinery and operational transformation methods required for efficient expression of heterologous genes or whole pathways in *D. hansenii* are available.

Promising characteristics of *Debaryomyces* for biotechnological applications

Oleaginicity

Some microbial species, including *D. hansenii*, produce and accumulate high amounts of lipids. In

analogy to oil-bearing plant seeds these microorganisms have been termed oleaginous [80]. The capacity to accumulate lipids is found in less than 30 of about 600 yeast species [155]. There is no official definition of oleaginous microorganisms, but operationally microorganisms capable of accumulating more than 20–25% of their biomass as oil are regarded as oleaginous. For yeasts, moulds and eukaryotic algae, but not bacteria, a biochemical definition of oleagenicity based on the presence of the enzyme ATP : citrate lyase (EC 2.3.3.8) has been proposed [154,155]. This enzyme generates acetyl-CoA, the key substrate for fatty acid biosynthesis, in the cytoplasm from citrate. Oleaginous microorganisms transport citrate out of the mitochondria into the cytoplasm via a malate–citrate translocase system, and cleave citrate by ATP-citrate lyase to form acetyl-CoA:

$$\text{Citrate} + \text{CoA} + \text{ATP} \longrightarrow \text{acetyl-CoA} + \text{oxaloacetate} + \text{ADP} + \text{Pi}$$

Non-oleaginous organisms do not possess the enzyme and rely on less effective means of producing acetyl-CoA in the cytoplasm. The ATP-citrate lyase is undoubtedly crucial for the lipid accumulation but not the only enzyme controlling and directing the metabolism towards storage lipids. Malate dehydrogenase [oxaloacetate-decarboxylating; NADP(+); malic enzyme E.C. 1.1.1.40) seems to control the activity governing the extent of lipids which are accumulated in oleaginous microorganisms [155]:

$$\text{Malate} + \text{NADP}^{+} \longrightarrow \text{pyruvate} + \text{NADPH} + \text{CO}_2$$

The first investigators to analyse the lipids of *D. hansenii* were Merdinger and Devine [120]. They showed that neutral lipids and phospholipids comprised 67% and 33% of the organism's total lipids, respectively. The neutral lipids, which are present as microdroplets, were almost entirely composed of triacylglycerols. The fatty acyl groups of both the neutral and phospholipids are usually conventional plant-like entities in the following order of relative abundance: oleic (18 : 1) > palmitic (16 : 0) > linoleic (18 : 2) = stearic acid (18 : 0) [156]. Some variations occur between various yeast strains. Ergosterol, together with smaller amounts of stigmasterol and another unidentified sterol, were also found in the cells, as were saturated hydrocarbons containing 16–39 carbon atoms, C22 being the most prevalent. The major phospholipid was phosphatidylcholine, followed by phosphatidylinositol, phosphatidylethanolamine, phosphatidylserine, phophatidylglycerol and cardiolipin. The overall fatty acid composition of the phospholipids was only slightly changed when *D. hansenii* was grown at high NaCl concentrations. Similarly, the fatty acid profiles of the individual phospholipids were only slightly affected by increases in salinity, the most notable change being an increasing proportion of polyenoic C18 acids in cardiolipin. Increases in salinity caused increases in the proportion of phosphatidylserine, while those of phophatidylglycerol, phosphatidyl-ethanolamine, and phosphatidylinositol decreased. Only minor changes were observed in the relative abundance of phosphatidylcholine and cardiolipin [178].

Yeasts do not possess rare fatty acids that could be of commercial interest. Hence, microbial oils will not be able to compete with cheap plant seed oils unless very cheap substrates or waste substrates can be identified. However, yeasts can produce other potentially useful lipids that are usually not found in plants, including glycolipid surfactants, such as a sophortose lipid produced by *Candida bombicola*, and carotenoids, e.g. astaxanthin from *Phaffia rhodozyma* [156]. It is possible that yeast-based biotechnological production of such lipids could be commercially competitive with their chemical production, if the organisms can be induced or manipulated to produce them cost-effectively. Genetic modification may pave the way to such syntheses, as will be illustrated in detail later.

Halotolerance

D. hansenii is notably extremophilic, due to its osmo- and xerotolerance as well as its halotolerance (Table 2) [9,40]. These features have prompted many investigations on *D. hansenii*, and its growth dependency on high salt regimes has been repeatedly shown [40,76,150,176]. These investigations have shown that *D. hansenii* grows faster in the presence of up to 1 M NaCl or KCl than in lower concentration of salts. *Debaryomyces* can grow in a wide range of water activities, and

Table 2. Osmotolerant and osmophilic yeasts [111,177]

Candida apicola
C. etchellsii
C. famata
C. glabrata
C. guilliermondii
C. lactis-condens
C. magnoliae
C. parapsilosis
C. tropicalis
C. versatilis
Citeromyces matritensis
Debaryomyces hansenii
Hanseniaspora guilliermondii
Hyphopichia burtonii
Issatchenkia orientalis
Kluyveromyces thermotolerans
Pichia angusta
P. anomala
P. farinosa
P. guilliermondii
P. membranaefaciens
P. ohmeri
Schizosaccharomyces octosporus
Sz. pombe
Torulaspora delbrueckii
Zygosaccharomyces_bailii
Z. bisporus
Z. microellipsoides
Z. roux

natural habitats for that yeast include sea water and habitats such as meat, wine, beer, cheeses, fruits and soil. [11] According to a widely accepted definition by Kushner [98], one can distinguish slight halophiles (many marine organisms; optimal growth at about 3% w/v NaCl), moderate halophiles (optimal growth at 3–15% w/v salt), extreme halophiles (optimal growth at 25% w/v NaCl; halobacteria and halococci) and borderline extreme halophiles (which require at least 12% w/v salt). According to these definitions *D. hansenii* can be classified as moderately halophilic, because this species grows optimally at 3–5% w/v salt (unpublished studies in the authors' laboratory).

Since it is one of the most common yeasts in salty environments and can tolerate salinity levels up to 25%, *D. hansenii* is an important model organism for salt tolerance studies, and its mechanism of osmotolerance has been studied in detail. [23,82,133]

A major component of the mechanisms that allow halophilic organisms to maintain their osmotic balance and to grow in high-salt environments is the production and accumulation of substances named compatible solutes [22]. In fungi, the alditols glycerol, mannitol, arabinitol and erythritol are the principal organic solutes used to adjust the internal osmotic potential as a prerequisite for survival and growth [15]. The dominant solute in growing cells of *D. hansenii* was found to be glycerol, while arabinitol was the main solute found in stationary phase cells in studies by Nobre and Da Costa [132] and Larsson and Gustafsson [101]. These observations are consistent with other investigations showing that the concentration of glycerol declines when cells enter the stationary phase [84]. Salt stress induces changes in two key parameters related to the yeast's energy metabolism (the ATP pool and the heat production rate) and enhances glycerol production in *D. hansenii*. This yeast has the ability to regulate its alditol metabolism in response to high salt concentration and thus optimize its growth [101]. There are also reports indicating that trehalose is used as a compatible solute in *Debaryomyces* (see below). Unlike in some bacteria, amino acids play no role in the osmoprotection of *D. hansenii* [2]. As early as 1985, Adler and co-workers described links between osmoregulation and glycerol metabolism in a glycerol non-utilizing mutant of *D. hansenii*. Their study indicated that glycerol was synthesized via dihydroxyacetone phosphate, which was reduced to glycerol 3-phosphate, followed by dephosphorylation to glycerol. [1] The intracellular glycerol concentration increased with the solute concentration of the growth medium, while accumulation of arabinitol was less pronounced [101,102,132]. At low salinity, arabinitol was the most prominent intracellular solute throughout the growth cycle [2,132]. The yeast's ability to synthesize two different solutes under different growth conditions may be important for its practical application. In addition, *D. hansenii* produces more trehalose than *S. cerevisiae* [76]. Gonzalez-Hernandez and co-workers [76] found that under saline stress (2.0 and 3.0 M salts) *D. hansenii* accumulated more glycerol than trehalose, whereas the opposite held true under moderate NaCl stress, leading to the suggestion that trehalose serves as a reserve carbohydrate, as it does in other microorganisms.

Unlike *S. cerevisiae, D. hansenii* has the capacity to regulate its glycerol metabolism under hyperosmolaric conditions [101]. It also has superior transport capacities, with twice as many amino acid and

carbohydrate transporters and more genes involved in osmo-sensing than *Saccharomyces* species [84].

As a rule, sodium and potassium ions seem to be very important in the mechanisms involved in maintaining osmobalance. The halotolerance mechanisms of *D. hansenii* include the accumulation of higher concentrations of either K^+ or Na^+ than in *S. cerevisiae*, indicating that haloadaptation does not involve the capacity to extrude sodium ions, as assumed by Norkrans and co-workers [134,135], but probably an intrinsic resistance to their toxic effects [150]. Further evidence supporting the hypothesis that *D. hansenii* has such intrinsic resistance unknown in other yeasts [77], was provided by Gonzalez-Hernandez *et al*. [75].

Norkrans and Kylin found that although *Debaryomyces* accumulated large concentrations of Na^+, it rapidly extruded Na^+ in exchange for K^+ when incubated in the presence of K^+, leading them to suggest the involvement of an Na^+/K^+ antiporter [134,135]. This putative K^+/Na^+ exchanger does not seem to be the main efflux mechanism, because effluxes of both cations were also observed in the absence of the other ion [134,135,176]. The proposed cation channel was thought to be unspecific, since it was found to promote effluxes of both cations with very similar kinetic parameters [75]. Gonzalez-Hernandez and co-workers also observed non-competitive inhibition of K^+ uptake by Na^+ uptake, and vice versa, while other investigators found that Na^+ substituted for K^+, but only when K^+ was scarce [150]. In addition, Gonzalez-Hernandez *et al*. [75] postulated the existence of a uniporter that is responsible for the transport of either cation to the interior of the cell, with higher affinity for K^+ than for Na^+, when *D. hansenii* is incubated with the other cation. Armstrong and Rothstein [8] had previously described a similar uniporter in *S. cerevisiae*.

A mechanism for the transport of Na^+ and K^+ in *D. hansenii* has been proposed, in which an AP type H^+-ATPase, similar to that encoded by the *PMA*1 gene of *S. cerevisiae*, putatively energizes the plasma membrane and generates a transmembrane potential and a pH gradient by massive proton efflux [75,169,176]. The membrane potential was proposed to drive the uptake of Na^+ or K^+ via one or more uniporters. Na^+ might also be slowly expelled in *D. hansenii* by another P-type ATPase, encoded by *ENA1*, in a manner that appears to be independent of the membrane potential, [5] and an electroneutral exchanger similar to that encoded by *NHA*1 in *S. cerevisiae* [75,149]. The *DhENA*1 and *DhENA*2 genes of *D. hansenii* are similar to genes encoding Na^+-ATPases in *S. cerevisiae*, and appear to be involved in Na^+ extrusion. They also exhibit high homology to the corresponding *ENA* genes of *Schwanniomyces occidentalis* and *Zygosaccharomyces rouxii* [84]. *DhENA*1 is expressed in the presence of high Na^+ concentrations, while the expression of *DhENA*2 also requires high pH values according to Almagro *et al*. [5], who suggested that the proteins encoded by the *ENA* genes in *D. hansenii* do not cause Na^+ extrusion, unlike those in *S. cerevisiae*, but play an important role in maintaining balanced levels of intracellular cations and ionic homeostasis in the cell.

Maintenance of osmotic balance in *D. hansenii* has been proposed to involve the activity of several transporters under salt stress conditions (see above). However, views about the transporters involved have repeatedly changed in the past, resulting in some confusion. Therefore, an attempt is made here to briefly describe the key transporters (Figure 2). The first detailed investigation concerning the transporters was presented in 1990 [114]. For *D. hansenii* growing exponentially on glucose medium containing sodium chloride, it was proposed that the osmotic balance is maintained by coupling sodium and glycerol gradients of opposite signs across the plasma membrane through a sodium–glycerol co-transport mechanism. According to this hypothesis, the transmembrane sodium gradient is the force that drives active glycerol transport, which maintains the endogenous glycerol gradient and thus osmotic balance. The symporter also purportedly accepts potassium ions instead of sodium ions as co-substrates for glycerol transport, while glycerol uptake in the presence of extracellular sodium chloride is accompanied by proton uptake, and the sodium gradient is maintained by sodium-proton antiport activity [114].

Further investigations focused on the influence of salt stress on the regulation of enzyme synthesis. It was shown that rapid transcriptional upregulation of glycerol 3-phosphate dehydrogenase (GDP) occurred in response to salt challenges [84,114,175]. The specific activity of several glycolytic enzymes was inhibited and, in agreement with the GDP stimulation, a general glycolytic flux deviation during salt stress towards glycerol as the major compatible solute occurred [126]. In order

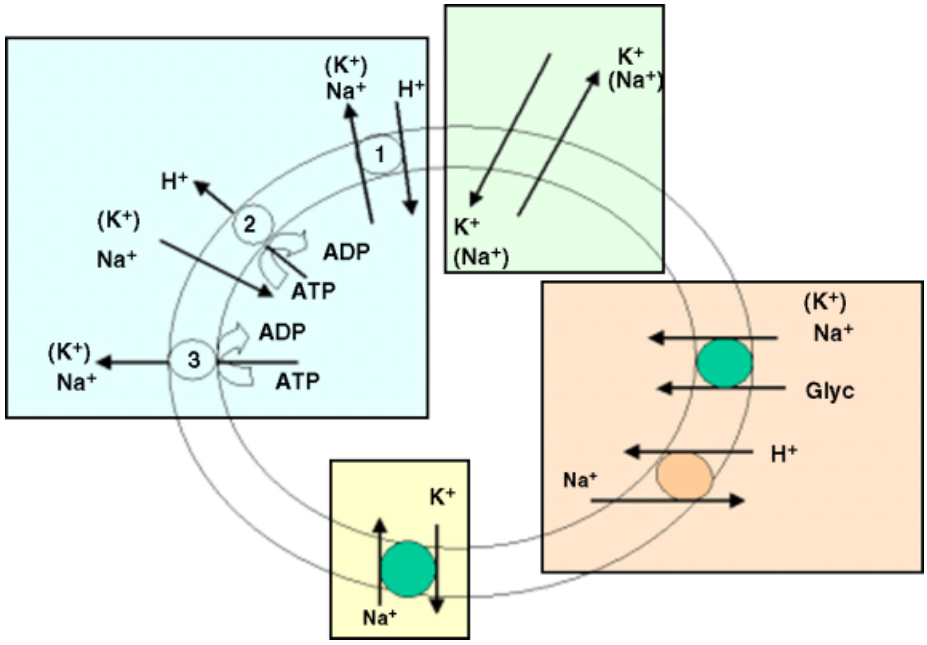

Figure 2. Model showing important transport pathways for maintaining osmotic balance in *D. hansenii*. Right box, transporters proposed by Lucas *et al.* [114] Middle box, transporters proposed by Prista *et al.* [150] Left box, transporters proposed by Serrano [169], Thome-Ortiz *et al.* [176] and Gonzalez-Hernandez *et al.* [75] 1, *Nha1* exchanger; 2, AP-type H^+-ATPase similar to *Pma1* gene; 3, AP-type ATPase similar to *Ena1* gene. Lower box, transporters proposed by Norkrans [134] and Norkrans and Kylin [135]

to overcome the negative effects of salt stress, the expression of genes encoding enzymes of the central metabolic pathways can also be modulated to increase transcription and enzyme synthesis. In *D. hansenii* the activity of NADP-glutamate dehydrogenase (NADP-GDH) was found to be five-fold higher in the presence of 1 M NaCl than in salt-free media [4]. However, amino acids did not seem to be involved in the process of xero-resistance, because glutamate was not accumulated when *D. hansenii* was grown in the presence of NaCl. The increased NADP-GDH activity under these conditions suggested that higher enzyme activity was needed to counteract the inactivation resulting from the accumulated ions. The increased enzyme activity in this case could be attributed to amplified enzyme synthesis [4]. Whether this is only true for this enzyme or reflects a general mechanism involved in osmoadaptation needs further investigation. Guerrero and co-workers [77] investigated the hitherto unknown mechanism of the increased enzyme synthesis and demonstrated that the salt-dependent regulation of the studied genes involved mechanisms that are present in *D. hansenii* but not in *S. cerevisiae*. They showed that expression of the *DhGDH1*-encoded $NADP^+$-glutamate dehydrogenase was increased when *D. hansenii* was grown in the presence of high salt concentrations, while that of *DhGLN1* (encoding glutamine synthetase) was reduced.

The osmotolerant yeast *D. hansenii* could be also very important as a parental strain in protoplast fusion experiments. This technique can be used to obtain genetically modified industrial yeast strains of *Saccharomyces cerevisiae* with increased tolerance to stress factors such as elevated concentrations of salts [113]. In future studies it would be interesting to investigate whether the increased tolerance to salts affects or changes the production of secondary metabolites (e.g. esters or higher alcohols) or the ability to metabolize organic acids [62].

In addition, there is a growing interest in making plants resistant to salinity in order to increase the area of cultivable land. In regions with extremely arid climates, it has been impossible to cultivate robust and fast growing forage plants to date. The development of halotolerant or halophilic plants should solve this problem. Expressing the genes

conferring salt resistance in *D. hansenii* in plants could be an effective strategy and could make a substantial contribution to reducing hunger in the world [168].

We suggest that the ability to withstand high concentrations of salt, and grow at high rates in their presence, makes *D. hansenii* very valuable for biotechnological applications. *D. hansenii* can be cultivated without stringent sterility measures (due to the yeast's ability to withstand high salt concentrations in the medium the possibility of a *Debaryomyces* cultivation contaminated with unwanted organisms is reduced) and can use cheap salt waste products as substrates, e.g. salt-containing glycerine/water mixtures resulting from the trans-esterification of rapeseed oil. In addition it can withstand high educt and product concentrations occurring in the process (i.e. it has high chemostress tolerance) and, thus, can be highly productive (data from the authors' laboratory, not shown). Further potential applications stem from its ability to produce compatible solutes of commercial interest.

Applications in biotechnological processes

In this section, current uses of *D. hansenii* in production processes such as the synthesis of dairy products, fermentation of meat products and formation of lytic enzymes of commercial interest will be introduced.

Dairy products

Yeasts are important components of the microflora of many dairy products. They can also be involved in the spoilage of foods that are highly acidic and/or have low water activity [63,86]. Thus, it is very important to consider the various influences of *D. hansenii* on food products.

D. hansenii is a common species in all types of cheese, including soft cheeses and the brines of semi-hard and hard cheeses [61]. Deak and Beuchat [41] reported its frequent occurrence in dairy products, while Seiler [166] and Viljoen and Greyling [183] indicated that it is prevalent in cheese brines, cheese quark, yoghurt and fruit preparations. Key properties of the genus *Debaryomyces* include its salt tolerance, its ability to produce proteolytic and lipolytic enzymes that can metabolize milk proteins and fat, and its capacity to grow at low temperatures and low water activities (a_W), which are also proposed reasons for its prevalence [14,40,63,123,159,177,189]. Moreover, *D. hansenii* has been found to inhibit the germination in cheese brines of undesired microorganisms, such as *Clostridium butyricum* and *C. tyrobutyricum*, by out-competing them for nutrients and producing antimicrobial metabolites, both extra- and intracellular [43,58]. Welthagen and Viljoen [186] and Laubscher and Viljoen [103] suggested that *D*. hansenii's ability to multiply in cheese, together with its capacity to assimilate lactate, citrate, lactose and galactose, favour this organism as a component of starter cultures for cheese production. Its abilities to utilize acetate as a sole source of carbon, to assimilate (but not ferment) lactose and to assimilate glucose while fermenting it to a limited extent [58] are also very important for cheese manufacturers. In addition, metabolic activities of *D. hansenii* have been found to modify the microenvironment in cheese to the benefit of some desired bacteria and/or *Penicillium roqueforti* and protect the cheese against undesired carbohydrate fermentations [179,192].

Arfi *et al*. [7] found that the yeast synthesized volatile acids, alcoholic and cheesy flavour compounds and carbonyl compounds. The spectrum of volatile compounds in a cheese medium that had been deacidified by *D. hansenii* was analysed by Leclerq-Perlat *et al*. [104] Methyl ketones with fruity, floral (rose), mouldy, cheesy or wine odours, and 2-phenylethanol (which has a faded-rose odour) were identified. Pure *D. hansenii* also has a faded-rose odour that could be explained by a high concentration of 2-phenylethanol. The ability of *D. hansenii* and *Kluyveromyces marxianus* to influence the sensory properties of cheeses has already been exploited in starter cultures comprising these two species [167].

D. hansenii and many cheese-ripening yeasts can synthesize *S*-methylthioacetate, the most prevalent volatile sulphur compound in cheese, and to a lesser extent methional, which is found, for instance, in Cheddar and Camembert. It thus contributes to the development of a strong Cheddar flavour [60], which likely results from the enzymatic decarboxylation of 4-methylthio-2-oxobutyric acid (KMBA) via the Ehrlich pathway. The cited authors found that KMBA can accumulate in *D. hansenii* as

a result of L-methionine trans-amination by L-methionine aminotransferase, suggesting the possible occurrence of decarboxylase activity in the organism. In addition, Arfi *et al*. [7] found that, in comparison to the other cheese-ripening yeasts *Geotrichum candidum, K. lactis, S. cerevisiae* and *Y. lipolytica, D. hansenii* was able to produce volatile sulphur compounds, especially substantial amounts of methylthiopropanal.

During growth in milk, *D. hansenii* produces only small amounts of free amino acids (predominantly glutamic acid, glycine, arginine, proline and alanine) and free fatty acids [160]. In addition, the yeast metabolized small amounts of succinic acid and produced small amounts of ethanol (<5 g/l) and lactic acid during the surface-ripening of Danish cheese [147].

Van den Tempel and Jacobsen [179] investigated the enzyme contents of various strains of *D. hansenii*, finding phosphatases, C4-esterases, C8-esterase-lipases, leucine arylamidases, valine arylamidases, aminopeptidases, ß-galactosidases as well as α- and ß-glucosidases. In addition, intrapeptidase activity was found in *Brevibacterium linens* and *D. hansenii* by Leclercq-Perlat *et al*. [105] Klein and co-workers [89] confirmed this result and found a peptidase in *D. hansenii* with activity towards ß-casein-derived peptides, which significantly influenced proteolysis in cheese. Kumura *et al*. [91] also reported casein digestion (both α- and ß-casein) by *D. hansenii* isolated from cheese.

Although Leclercq-Perlat *et al*. [105] did not observe any pigment production in *D. hansenii* after 28 days of incubation at 10 °C, it reportedly produced pigments in studies by van den Tempel and Jacobsen [179] and Hansen *et al*. [81], while Nichol *et al*. [128] found tyrosinase activity, which is known to initiate tyrosine oxidation to melanine in *D. hansenii*. In addition, Carreira *et al*. [25] described the production by *D. hansenii* of reddish-brown pigments of unknown nature, involved in the brown surface discolouration of Portuguese ewes' cheese. Pigments were not only observed during the formation of dairy products; Nakase *et al*. [125] described pigmented ascospores responsible for the brown colour of cultures.

In several types of surface-ripened cheeses, e.g. Limburger, Tilsiter, Port Salut, Trappist, Brick and the Danish Danbo, the development of an appropriate bacterial surface flora (with lactic acid and aerobic bacteria) had been shown to depend on the metabolism of lactic acid by yeasts, in particular *D. hansenii,* as described earlier. It has been suggested that *D. hansenii* might provide important growth factors for the bacteria, such as vitamins (lactoflavone, thiamine, pantothenic acid, nicotinic acid, folic acid and biotin) and amino acids [164], along with aroma components and lipolytic and proteolytic enzymes that contribute to the ripening process [31,56,63,78,107,146]. The ability of yeasts to supply growth factors had previously been demonstrated by La Riviére [100] and Lenoir [106].

Another potentially important consideration is the preferred location of *Debaryomyces* in cheese. First investigations by Ikemiya and Yasumi [85] on yeast spoilage of cheese showed that *D. hansenii* grows well on the surface of processed cheese, but Marth [117] found *Debaryomyces*, amongst other species, inside surface-ripened cheeses. Further investigations showed that *Debaryomyces* activity can be found in either the curd or the interior of the cheese, depending on the kind of cheese and the composition of the starter culture involved [43,58,60,61,117,159,179,186].

One can conclude that *Debaryomyces* plays important, although quite diverse, roles in cheese making. To increase production efficiencies and cheese quality through knowledge-based process management, it is imperative to obtain more insight into these processes.

Meat fermentation

D. hansenii was the most common yeast among 383 isolates from samples of unsulphited or sulphited sausages, skinless sausages and minced beef examined by Dalton *et al*. [39], indicating its importance for meat products. The involvement of *Debaryomyces* in meat fermentation has been known for a long time; as early as the mid-1960s Rankine [153] briefly described unspecified *Debaryomyces* strains as producers of hydrogen sulphite, for instance.

In one study *D. hansenii* was found to have little effect on the production of volatile compounds responsible for, or involved in, aroma formation in garlic-flavoured fermented sausages and model minces [138], but other authors found that the yeast was able to produce volatile compounds. Dura and co-workers [53] showed that *Debaryomyces* can generate ammonia and several volatile compounds,

alter the free amino acid contents of dry-cured sausages, and that addition of *Debaryomyces* sp. as a starter culture generally modifies their flavour profile [53]. In addition, Flores *et al*. [64] reported that *Debaryomyces* spp. can have important effects on the generation of volatile compounds during the ripening of dry-fermented sausages by inhibiting the generation of lipid oxidation products and promoting the generation of ethyl esters, processes that contribute to the development of a typical sausage aroma.

The synthesis of volatile compounds from branched-chain amino acids is negatively affected by the presence of salt and influenced by a number of other variables, according to Dura *et al*. [53] For instance, a reduction of pH to 4.5 increased the yield of alcohols and aldehydes, while the transition from exponential to stationary growth phase diminished alcohol and aldehyde production but increased acid generation. Martin *et al*. [118] described similar results for the modification of flavour profiles of dry-cured ham. The cited authors found that as well as producing volatile compounds yeasts influenced the sensory properties of the meat. A study by Dura and co-workers [54] supported these results, finding that during the manufacture of dry fermented sausages with *Debaryomyces* sp., sarcoplasmic proteins were not affected, whereas the degradation of myofibrillar proteins was accelerated at the beginning of the drying stage.

There are several reports concerning *D. hansenii* enzymes that may be involved in meat fermentation, the first of which was published by Bolumar and co-workers [17], who identified and purified a prolyl aminopeptidase. In another study concerning exopeptidase activity during the ripening of a fermented sausage, they purified and characterized a further (arginyl) aminopeptidase [18]. Dura and co-workers [52] described a glutaminase that is active during the processing of dry-cured sausages. The curing agents nitrate, nitrite, glucose and ascorbic acid, which inhibit growth of the pathogen *Clostridium botulinum* and are responsible for the pink colour associated with cured meats and for particular meat flavours, had no significant effect on the enzyme activity at levels typically used in meat processing [52]. However, earlier results published by Fleet [62] showed that utilization of the curing agents sodium nitrite and sodium nitrate by *D. hansenii* had implications for the microbiological stability and safety of processed meats. Direct comparison of the two sets of observations is difficult, because Fleet did not analyse the effects of curing agents and microbial stability on any enzyme activities.

Yeasts are not always useful during the manufacture of meat. Almost a century ago Kühl [90] described yeast spoilage in sausage-type meats, *Debaryomyces* being the main causal agent, and recovered white colonies consisting of yeasts from the surface slime of dried sausages. Later, Mrak and Bonar [122] identified yeast cultures of *D. guilliermondii* var. *nova-zeelandicus* (subsequently identified as *D. hansenii* [111]) in the surface slime on Wiener sausages. Drake *et al*. [49] also isolated these species from surface slimes of Frankfurters. However, Dalton and co-workers [39] found only minor quantities of yeasts (including *Candida, Debaryomyces, Rhodotorula* and *Pichia* sp.) in cured meat products, and concluded that they were not involved in their spoilage.

Little is known about the effect of *Debaryomyces* on meat fermentation, and further investigations would be required to exploit *D. hansenii* in meat processing.

Fine chemicals

D. hansenii can synthesize biotechnologically relevant products, as shown by its involvement in cheese ripening and meat manufacture. As an osmotolerant yeast, *D. hansenii* produces compatible solutes such as D-arabinitol, which could be commercially attractive if it can be done cost-effectively. D-Arabinitol is produced after the growth phase in batch culture, simultaneously with the excretion of riboflavin [6].

Thiamine auxotrophs of *D. hansenii* have been shown to accumulate pyruvic acid under thiamine limitation, and although the yields were considerably lower than those obtained using the yeast *Candida glabrata*, which is already being exploited biotechnologically, *Debaryomyces* has a biotechnological advantage in its ability to use inorganic ammonium as a sole nitrogen source [193]. Pyruvic acid can be widely used in the chemical, pharmaceutical and agrochemical industries and the biotechnological production of this acid could be a viable alternative to the current chemical method, because it is a relatively cheap, one-step procedure

that gives more efficient conversion of the carbon substrate [108].

D. hansenii can form pentadiene from potassium sorbate, albeit at a lower rate than *Zygosaccharomyces rouxii*. The sorbate degradation accompanied by production of pentadiene may be considered as a new mechanism of yeast resistance to the preservative sorbate. The most generally accepted mechanism for a resistance against weak acids consists in the transport of the non-dissociated form into the yeast cell, the dissociation in the neutral pH cytoplasm, and the transport of protons by an ATP-dependent proton pumping system out of the cell in order to maintain an appropriate internal pH [27].

Use of *D. hansenii* as a cell factory for producing fine chemicals is obviously in its infancy. However, the authors see great opportunities in this field for developing new processes and identifying new products.

Lytic enzymes

Lytic enzymes of *D. hansenii* have been, and will continue to be, exploited in the food industry. Due to their growing importance they will be discussed in more detail in this section. Yeast proteases could be used to remove protein hazes from beer and wine [62], and extracellular hydrolytic yeast enzymes have been investigated due to their importance in wine fermentation [29,170].

Ahearn *et al*. [3] investigated 68 strains of *D. hansenii* because of its association with food spoilage, and found only four strains with extracellular proteolytic activity, whereas Lagace and Bisson [99] found little or no extracellular protease activity in *D. hansenii*.

Saha and Bothast [162] explored the production of extracellular glucose-tolerant and thermophilic ß-glucosidases in wine making by 48 yeast strains belonging to the genera *Candida, Debaryomyces, Kluyveromyces* and *Pichia*. These glycosidases were shown to be able to hydrolyse cellulose to glucose, which is important in a commercial process for the production of fuel alcohol. Although the ß-glucosidases of *Debaryomyces* strains had almost 100-fold lower activity than the enzyme of *Pichia kodamae*, they were able to hydrolyse cellobiose without inhibition of the enzymes by glucose. Moreover, the enzymes had a relatively high optimal temperature of 65 °C. An intracellular ß-glucosidase from *D. hansenii* has already been purified, analysed and tested in the fermentation of Muscat juices [194]. This enzyme has an advantageous tolerance to high concentrations of glucose in grape juices, in contrast to commercially available ß-glucosidases of mycelial fungal origin, which are sensitive to glucose. ß-Glucosidases from *Debaryomyces* can liberate monoterpenols from ß-D-glucopyranoside, ß-D-xylopyranoside, α-L-arabinofuranoside and α-L-rhamnopyranoside. This property might be useful for a possible enzyme treatment as part of the processing of terpenol-containing juices, leading to an increase of flavour compounds in the wine [194]. Non-conventional yeasts, such as *Debaryomyces*, exhibited significantly higher levels of ß-glucosidase activity in the absence of glucose than *Saccharomyces cerevisiae*, indicating that they would have a stronger role in flavour development during wine production than baker's yeast [29].

Besancon *et al*. [13] isolated and partially characterized an esterase (EC 4.1.1.1) from a *D. hansenii* strain. This enzyme has also been found in other non-*Saccharomyces* wine yeasts and is one of the main enzymes involved in wine making [57]. The monomeric enzyme hydrolysed tributyrin and ethyl butyrate with maximal activity, methyl and ethyl esters of short fatty acids (C_2–C_5) very rapidly and esters of longer-chain fatty acids (C_6–C_{14}) moderately quickly. Aliphatic and aromatic acetate esters were also hydrolysed.

D. hansenii is a potential source of superoxide dismutase (SOD) (EC 1.15.1.1), a metalloenzyme catalysing the dismutation of superoxide radicals. SOD has important applications in medicine and the food industry, including anti-inflammation, immune-response modulation, malignant tumour regression, radiation and chemotherapy protection, premenstrual syndrome, arthritis, and anti-ageing treatments, during the use of hyperbaric chambers, and against oxidative stress in general. [70,140] SOD production using *D. hansenii* is very competitive compared to current methods, due to its ability to grow, as halotolerant yeast, either in sea- or freshwater-formulated media, with a wide range of cultivation parameters [137]. Hernandez-Saavedra and Romero-Geraldo [83] first described the cloning of the SOD gene of *D. hansenii*.

Yeast species with antifungal activity, including *Cryptococcus laurentii*, *D. hansenii* and *Candida*

sp. have been isolated from fruits (see also section on Plasmids of *D. hansenii*) and examined for their potential utility as biocontrol agents of fruit spoilage fungi [62]. Droby *et al*. [50] demonstrated the possibility of such biocontrol by showing that the addition of *D. hansenii* to grapefruits resulted in significant inhibition of spore germination and hyphal growth of *Penicillium digitatum*. In biocontrol, the competition for nutrients might play a substantial role. Payne and Bruce [145] showed that *D. hansenii* has applicability as a short-term biological control agent against fungal spoilage (visual degradation by surface growth of moulds and staining fungi) of sawn *Pinus sylvestris* timber. However, when considering the technical development and application of *D. hansenii* as a biocontrol agent, one should keep in mind that some clinical isolates of *D. hansenii* are described, although the yeast is usually non-pathogenic.

More detailed information about enzymes in *Debaryomyces* is required to realize their potential in biotechnological processes. More important enzymes could and will be found in species of this non-conventional yeast if more research is done.

Alditol production

The following section deals with *Debaryomyces* as a traditional 'cell factory' of alditols. The ability of *Debaryomyces hansenii* to produce xylitol from commercial D-xylose and wood hydrolysates, generating high xylitol : ethanol ratios (>4) in the process, has been exploited for several decades [72,74,142,144,161]. Although *D. hansenii* is one of the best xylitol-producing yeasts [144,161], we are not aware of commercial xylitol production using this yeast. As an osmotolerant yeast, *D. hansenii* might also be very attractive for the production of other alditols [2].

Xylitol is used in the food industry for its high sweetening power, anti-caries properties and tolerance by diabetics. For these reasons, xylitol has been employed in the manufacture of sugar-free confections and food [37]. The pentitol xylitol is formed as a metabolic intermediate of D-xylose fermentation, in which D-xylose is converted into xylitol by aldose reductase, commonly called xylose reductase (XR, Figure 3). This enzyme has broad substrate specificity for aldehydes (aliphatic and aromatic) and aldol sugars.

Xylose–xylitol bioconversion by the halotolerant yeast *D. hansenii* has been intensively studied [47,71,131,161]. The overproduction of xylitol by *D. hansenii* was found to be the result of a combination of high NADPH-dependent XR activity and low xylitol dehydrogenase (XDH) activity [33]. The bioconversion yields of *Debaryomyces* were found to be similar to, or higher than, those of other typical xylitol producers, such as *Candida* sp. B-22 [30], *C. guilliermondii* [10,121], *C. boidinii* [184], and *C. parapsilosis* [69]. The concentration of dissolved oxygen also plays a significant role in the conversion of D-xylose into xylitol, which cannot be performed under strictly anaerobic conditions, due to the very low NADH-linked XR activity in the absence of oxygen preventing the D-xylose being assimilated by *Debaryomyces* [45,161]. On the other hand, under strictly aerobic conditions the reaction products are used in biomass production and the rate of xylitol synthesis is very low; the NADH formed in the XDH-catalysed reaction can be reoxidized by the respiratory chain, but xylitol is consumed for cell growth. Furthermore, high oxygen levels inhibit XR activity more than XDH activity, and thus affect xylitol production in *D. hansenii* [74], favouring cell growth and thereby improving the yield of ATP. The accumulation of xylitol is therefore enhanced by limited aeration [33,142], when just sufficient oxygen is provided to satisfy NADH regeneration for the second oxidative step, and thus NADPH is almost solely used to reduce D-xylose, leading to xylitol accumulation [10].

Debaryomyces cultivated under xylose-limited chemostat (oxygen-excess) conditions produced neither ethanol nor xylitol over the entire range of dilution rates tested in a study by Nobre *et al*. [130] Under oxygen-limited chemostat (xylose-excess) conditions the yeast metabolism changed dramatically and, due to oxidative phosphorylation limitation, the cell yield decreased and xylitol became the major extracellular product, along with minor amounts of glycerol.

D. hansenii synthesizes a range of products, depending on the substrate used. The yeast produced xylitol and arabinitol as well as ethanol from pentose sugars, while it formed ethanol in significant amounts only from glucose in experiments described by Girio *et al*. [71] It utilized both glucose/arabinose and xylose/arabinose mixtures with a simultaneous sugar consumption pattern. The

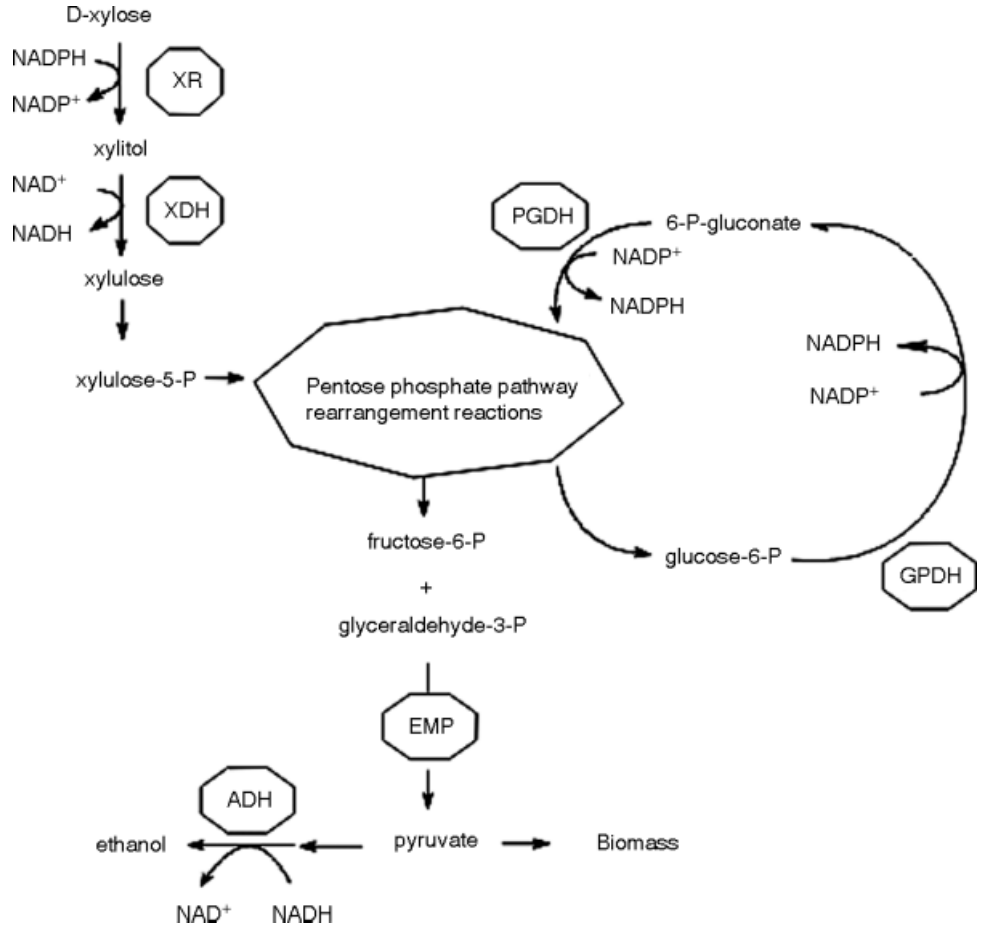

Figure 3. Simplified scheme for D-xylose metabolism by *D. hansenii* (after Giorio *et al.* [74])

arabinitol volumetric activity increased four-fold after the addition of low amounts of xylose to an arabinose medium. Conversely, glucose addition had no effect on arabinitol production. D-Xylose induced the first two xylose-catabolic enzymes in glucose- and xylose-grown *D. hansenii* (NADPH-linked XR and NAD^+-linked XDH). D-Glucose caused total inhibition of XDH, whereas XR was only partially repressed [71]. The NAD^+-linked XDH of *D. hansenii* has a low K_m value for xylitol; low enough to assure the fast oxidation of xylitol. This low K_m for xylitol could be the reason for its efficient xylitol production, but the higher K_m value of XDH for NAD^+ in *D. hansenii* compared to those of other xylose-fermenting yeasts seems to be the main reason for its higher xylitol yields [73].

Nobre *et al*. [131] found that *D. hansenii* grew more slowly on pentoses than on hexoses, although the biomass yields were very similar on both types of sugars. Furthermore, *Debaryomyces* used both sugars to similar extents in sugar mixtures and a preference for one carbohydrate did not inhibit the consumption of the other. This could be important, since the usual substrates for xylose fermentation are hydrolysates of hemicelluloses, which often consist of sugar mixtures. In contrast to the similar utilization pattern of sugars by *D. hansenii* observed by Nobre *et al*. [131] and Girio *et al*. [71], Tavares and co-workers [173] found that the type of carbohydrate supplied influenced the growth of *Debaryomyces*. In their study, D-glucose inhibited or retarded the utilization of D-xylose, and the yeast preferentially assimilated the sugars in a substrate mixture in the order D-glucose, D-mannose, D-xylose. D-galactose might be used concurrently with, before or subsequent to D-xylose [173].

Inhibitors that are either present in the raw material or are produced during chemical processing, such as furfural and hydroxymethylfurfural (generated by the degradation of sugars), acetic acid (liberated from the acetyl groups of the raw materials), lignin degradation products, compounds derived from wood extractives (primarily composed of phenolic compounds), and inhibitors derived from the metals or minerals in wood, soil or hydrolysis equipment limited the fermentation of the hemicellulose hydrolysates [34]. The hydrolysates were submitted to several detoxification treatments to minimize the inhibitory effects. Activated charcoal adsorption, for example, removed most of the lignin-derived compounds, and evaporation was able to reduce the acetic acid concentration below the inhibition threshold. The combination of the treatments applied allowed efficient fermentation of the D-xylose solutions into xylitol. When *D. hansenii* grew on a complex treated substrate (spent grain from a brewery that had been subjected to dilute-acid hydrolysis) without any previous detoxification stage and utilized the constituent sugars glucose, xylose and arabinose as well as furfural, hydroxymethylfurfural and acetic acid, the yeast showed high biomass yields and productivity [26]. Detoxification of the medium with activated charcoal resulted in a similar biomass yield and only a slight increase in the volumetric productivity. Therefore, detoxification seemed to be unimportant in the cited study.

D. hansenii exhibited yeast–mycelium dimorphism in continuous fermentation with xylose made from acid hydrolysates of barley bran in study by Cruz *et al*. [37] The lower the dilution rate, the earlier the yeast–mycelium transition occurred. Low aeration caused the transition from oval cells to hyphae, and further increases in dissolved O_2 concentration resulted in recuperation of the oval shape. Xylitol was the major fermentation product, regardless of the morphological form, whereas the production of ethanol increased with yeasts growing as hyphae and under oxygen limitation.

Much attention has been paid to optimizing yeast-based xylitol production processes, both batch and continuous [33,161]. Maintaining high cell concentrations, e.g. by immobilization or continuous fermentation in combination with cell recycling, can increase fermentation productivities. For instance, xylitol production rates (xylitol productivity, q_P) of 2.53 g/l/h have been attained in continuous fermentation of *D. hansenii* with cell recycling at a dilution rate of 0.284/h [38]. In contrast, continuously fermenting *D. hansenii* cells entrapped in calcium alginate beads with charcoal-treated wood hydrolysates (15.5 g D-xylose/l) as substrates showed only a q_P of 0.91 g/lh, but this was still higher than the productivity of *C. guilliermondii* under the same conditions ($q_P = 0.58$ g/lh) [46]. High cell concentrations are an important factor for an efficient xylitol production. With a starting cell concentration below 16 g biomass/l and a D-xylose concentration above 70 g xylose/l, a q_P and a product yield ($Y_{P/S}$) of only 0.088 g/lh and 0.57 g/g, respectively, were reached. With a initial cell concentration of 50 g biomass/l and the same D-xylose concentration (>70 g xylose/l) the q_P was 0.50 g/lh and the $Y_{P/S}$ was 0.73 g/g [143]. As well as the cell density, the substrate concentration is also an important factor. Converti *et al*. [35] observed substrate inhibition at D-xylose concentrations higher than 200 g/l, whereas at concentrations between 90 and 200 g/l D-xylose *Debaryomyces* gave satisfactory $Y_{P/S}$ (0.74–0.83 g/g) and q_P (0.481–0.694 g/lh). A statistical investigation of the effects of starting xylose concentration and the oxygen mass flow rate on xylitol production from rice straw hydrolysate identified the optimum D-xylose concentration (71 g/l) and oxygen mass flow rate (4.1 mg O_2/s) required to maximise the q_P (0.53 g/lh), the $Y_{P/S}$ (0.71 g/g) and the final xylitol concentration (42.2 g/l) [42].

Besides xylitol, *D. hansenii* like most of the ascomycetous yeasts could synthesize alkali-soluble glucans and alkali-insoluble glucans associated with chitin. These polysaccharides could have enormous industrial and medical applications. The alkali-soluble glucans could be used as thickening agents, fat substitutes or sources of dietary fibre [55,165] in the food processing and the cosmetics industries [48]. Furthermore, they have antitumour activity [16], stimulate the immune system [87,187] and can lower the serum cholesterol level [127]. Nguyen *et al*. [127] investigated several yeast species for their cell wall contents. Although *Debaryomyces* had the highest chitin content of the chosen yeasts, unfortunately this yeast synthesized the lowest levels of alkali-soluble glucans and the highest levels of alkali-insoluble glucans. Therefore, more investigations on these features are required. The above discussion shows that

xylitol production by *D. hansenii* has already been intensively investigated and applied as an efficient biotechnological process. However, the producer itself and/or the process could be further improved using genetically modified strains.

Future prospects

This review describes the current ways in which *Debaryomyces* as an osmotolerant, oleaginous and non-pathogenic yeast with a broad spectrum of carbon substrates and a very attractive microorganism for both fundamental and applied biotechnological research, including possible process control applications, is used as a catalyst and as a producer of many valuable substances. However, the authors are convinced this yeast has great further potential in both biotechnological procedures and environmental sciences.

The following advantages make *Debaryomyces* an attractive potential producer. The first is its ability to tolerate and grow at high rates in high concentrations of salt (allowing the yeast to be cultivated without stringent sterility measures and the use of cheap salty waste products as substrates). The second remarkable property of *Debaryomyces* is the wide substrate spectrum and the ability of the yeast to withstand chemostress. For instance, the yeast can grow in high concentrations of ethanol, a renewable raw material, which could be important for designing eco-friendly and sustainable processes. In addition the yeast synthesizes compatible solutes. These substances have already been successfully used to protect cells and other biological structures in extreme stress conditions.

The *Debaryomyces* toxins could be used as therapeutic agents in medicine against pathogenic yeasts and as biopreservatives in food fermentations with sodium chloride to control spoilage yeasts. Moreover, large-volume processes could be facilitated by allowing growth solely of desirable microorganisms. Furthermore, heterologous genes or whole pathways of interest could be efficiently expressed in *D. hansenii*, exploiting its amenable genetic machinery. Its use of an alternative genetic code offers further potential. The spectrum of relevant products could thus be expanded, e.g. unusual fatty acids or tailor-made lipids could be produced. *D. hansenii* can also produce attractive fine chemicals, such as secondary end-products of cheese ripening or pyruvic acid and riboflavin, in addition to the compatible solutes glycerol and arabinitol. In addition to its ability to produce xylitol, which has already been intensively investigated, *D. hansenii* can synthesize further polysaccharides, providing potential alternatives to existing processes or enter new applications in the food processing, the cosmetics industry and medicine. All of these substances would be attractive biotechnological products if they could be produced competitively. They could be valuable alternatives to chemical syntheses, offering not only low costs but also the scope to fabricate new products. Besides the above-mentioned products, the potential use of yeast enzymes for biotransformations could be interesting fields of research.

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