Chapter 7
Genetics of Yeasts

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Introduction

The use of yeasts in biotechnology processes dates back to ancient days. Before 7000 BC, beer was produced in Sumeria. Wine was made in Assyria in 3500 BC, and ancient Rome had over 250 bakeries, which were making leavened bread by 100 BC. And milk has been made into Kefyr and Koumiss in Asia for many centuries (Demain, Phaff, & Kurtzman, 1999). However, the importance of yeast in the food and beverage industries was only realized about 1860, when their role in food manufacturing became evident.

Yeasts are used in many industrial processes, as they grow on a wide range of substrates and can tolerate extreme physico-chemical conditions. Today, the impact of yeasts on food and beverage production extends beyond the original and popular notions of bread, beer, and wine fermentations by *Saccharomyces cerevisiae* (Fleet, 2006; Querol, Belloch, Fernández-Espinar, & Barrio, 2003). In addition to *S. cerevisiae*, *S. bayanus*, and *S. pastorianus*, it is now well established that various species of *Hanseniaspora*, *Candida*, *Pichia*, *Metschnikowia*, *Kluyveromyces*, *Schizosaccharomyces*, and *Issatchenkia* can make a positive contribution in the manufacture of fermented foods, dairy, meats, cereals, coffee, and sauces. In the case of fermentation of meat sausages and maturation of hams, the most predominant and important yeasts are *Debaryomyces hansenii* (and the anamorph *C. famata*), *Yarrowia lipolytica*, and various *Candida* species.

Methods for Yeast Identification

The development of rapid and simple methods for the identification and characterisation of yeast strains is an essential tool for the meat industry. This is obvious, since yeasts in meat products can contribute to beneficial and detrimental aspects, and the

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identification of individual strains is often required to improve the microbiological quality of these foods.

Classical taxonomy is based predominantly on phenotypic characteristics, such as physiological traits and cell morphology. These approximations have been used in the identification of yeasts isolated from different meat products (Gardini et al., 2001; Núñez, Rodríguez, Córdoba, Bermúdez, & Asensio, 1996). However, nutritional characteristics have been shown to be highly variable as well as mutable, and genetic crosses have linked the characteristics to one or only a few genes, which in some cases could lead to an incorrect classification of a species or a false identification of strains (for a review see Boekhout & Robert, 2003; Fernández-Espinar, Martorell, de Llanos, & Querol, 2006). The conventional methodology for yeast identification requires the evaluation of some 60–90 tests and the process is complex, laborious, and time consuming. Besides, in the case of one of the most relevant yeast isolates in meat products like *Debaryomyces*, many of the results obtained with these tests are variable, thus making correct identification of the *Debaryomyces* species difficult. As an example, serious difficulties were experienced in identifying isolates assigned to *Debaryomyces*, due to the erroneous assimilation of D-xylose, and to a lesser extent, raffinose and L-arabinose (Metaxopoulos, Stavropoulos, Kakouri, & Samelis, 1996). In recent years, rapid kits for yeast identification have been developed to improve the conventional methods. However, these were designed initially for clinical diagnosis and their application is restricted to 40–60 yeast species of medical interest (Deák & Beuchat, 1996). Other methods based on the analysis of total cell proteins and long-chain fatty acids using gas chromatography have also been developed. However, the reproducibility of these techniques is questionable due to the fact that they depend on the physiological state of the yeast cells (Golden, Beuchat, & Hitchcock, 1994).

Recent progress in molecular biology has led to the development of new techniques for yeast identification (for a review see Boekhout & Robert, 2003; Fernández-Espinar et al., 2006). In the next sections we review the new methods that have been developed and their application to the rapid identification of meat yeasts in the industrial practice. The specificity of nucleic acid sequences has prompted the development of several methods for rapid species identification. The comparison of ribosomal RNA (rRNA) and its template ribosomal DNA (rDNA) has been used extensively in recent years, to assess both close and distant relationships among many kinds of organisms. In several hemiascomycetous yeasts, rRNA genes are located in a single genome region composed of 100–150 tandem repeats of a fragment of 9 Kb. The ribosomal genes (5.8S, 18S, and 26S) are grouped in tandem forming transcription units that are repeated in the genome between 100 and 200 times. In each transcription unit, two other regions exist, the internal transcribed spacers (ITS) and the external ones (ETS) that are transcribed but are not processed. In turn, the codifying units are separated by the IGS intergenic spacers, also called NTS. The gene 5S is not included in the previously described transcription unit but is found adjacent to the same repetition unit, in tandem, in the case of yeast. The sequences of the ribosomal genes 5.8S, 18S, and 26S, as well as the spacers ITS and NTS, represent powerful tools to establish phylogenetic relationships and identify species (Kurtzman & Robnett, 1998), due to the conserved sequences found there, as

well as their concerted evolution, i.e., the similarity between repeated transcription units is greater within the species than between units belonging to different species, due to mechanisms like unequal crossing-over or genetic conversion (Li, 1997).

Using the information contained in these regions, different methods have been developed to identify yeast species, as we will describe below.

Ribosomal RNA Gene Sequencing

One of these methods is based on the determination and comparison of the nucleotide sequences in these regions. The two most commonly used regions are those corresponding to the domains D1 and D2, located at the 5′ end of the gene 26S (Kurtzman & Robnett, 1998) and the gene 18S (James, Cai, Roberts, & Collins, 1997). The availability of these sequences in databases, especially in the case of the D1/D2 region of gene 26S, make this technique very useful to assign an unknown yeast to a specific species when the percentage of homology of its sequences is over or close to 99% (Kurtzman & Robnett, 1998).

Restriction Fragment Length Polymorphism of rDNA

With an industrial application in mind, other simpler identification methods based on PCR amplification of the regions of the ribosomal DNA and later restriction of the amplified fragment were developed. Guillamón, Sabate, Barrio, Cano, & Querol (1998) proposed a rapid and easy method for routine wine yeast identification, based on RFLPs of the 5.8S rRNA gene and the internal transcribed spacers (ITS1 and 2). Later on its use has been extended to a total of 191 yeast species (de Llanos, Fernández-Espinar, & Querol, 2004; Esteve-zarzoso, Belloch, Uruburu, & Querol, 1999; Fernández-Espinar, Esteve-Zarzoso, Querol, & Barrio, 2000) related with beverages and food including meat yeasts isolates. The amplified fragments and restriction profiles of these species with the enzymes *Hae*III, *Hin*fI, *Cfo*I, and *Dde*I are currently available "online" at the address http://yeast-id.com. The utility of the technique has been proved studying reference strains (Esteve-Zarzoso, Zorman, Belloch, & Querol, 2003; Fernández-Espinar et al., 2000; Ramos, Valente, Hagler, & Leoncini, 1998) and has been applied by numerous authors for yeast isolate identification in different foods and beverages, such as isolates pertaining to the genus *Debaryomyces*, *Yarrowia*, and *Candida* (Andrade, Rodríguez, Sánchez, Aranda, & Córdoba, 2006; Martorell, Fernández-Espinar, & Querol, 2005; Ramos et al., 1998).

PCR-DGGE Denaturing Gradient Gel Electrophoresis

Recently, a genetic fingerprinting technique based on PCR amplification and denaturing gradient gel electrophoresis or DGGE has been introduced into microbial

ecology (Muyzer, de Waal, & Uitterlinden, 1993). In PCR-DGGE, DNA fragments of the same length but with different sequences can be separated. The use of DGGE in microbial ecology is still in its infancy, but their future perspectives are promising (Muyzer & Smalla, 1998). Its application to yeast identification in foods and beverages is very recent (for a review see Fernández-Espinar et al., 2006), and only a few studies related with meat products or the yeast ecology of fermented sausages have been performed (Cocolin, Urso, Rantsiou, Cantón, & Comi, 2006; Rantsiou et al., 2005).

Yeast Biodiversity in Meat Products

The most important yeasts in meat products belong to the ascomycetous genera, *Debaryomyces*, *Pichia*, *Yarrowia*, and *Candida*, and the basidiomycetous genera, *Rhodotorula*, *Cryptococcus*, and *Trichosporon*. Fresh meat processing and storage causes the progressive replacement of basidiomycetous by ascomycetous yeasts. A predominance of *Candida* spp. occurs during spoilage of fresh meat, while meat salting, curing and fermentation are selective for *Debaryomyces* (Samelis & Sofos, 2003). Some important physiological and biochemical characteristics of meat yeasts and their interactions result in the selection of certain yeast species in specific meat products, like *Y. lipolytica* in fresh and spoiled poultry (Ismail, Deak, El-Rahman, Yassien, & Beuchat, 2000), and *D. hansenii* in cured dried and fermented meats (Durá, Flores, & Toldrá, 2004a, 2004b; Guerzoni, Lanciotti, & Marchetti, 1993). Consequently, yeasts in meat products have both spoiling and beneficial aspects.

Spoilage Yeasts in Meat

Yeasts have commonly been considered unimportant in meat spoilage because their low initial numbers and their slow growth rates at refrigeration temperatures prevent them from competing effectively with psychrophilic bacteria (Dillon, 1998; Nortje et al., 1990). Most yeasts, however, are more resistant than bacteria to several food related stresses, such as low water activity, low pH, high salinity, and chemical preservatives, while certain species are extremely psychrophilic (Fleet, 1990). Thus, opportunistic yeasts may become important spoilage agents in meat products when bacterial growth is retarded due to the inhibitory effects of the above stress factors (Samelis & Sofos, 2003).

The increasing consumer demand for less processed, more “natural” foods has led the food industries to commercialize new products, in which the use of lower concentration of preservatives, packaging in modified atmospheres, or new formulations occasionally permit the growth of yeasts. Yeast contributes a small, but permanent part of the natural microflora on meat. The ability of some yeast to grow at low temperature, high salt concentration, and under reduced oxygen tension

enables them to proliferate in refrigerated, cured and vacuum-packed meat and meat products (Deák & Beuchat, 1996). Meat spoilage caused by yeasts is mainly due to their lipolytic and proteolytic activities, although their action on carbohydrates and associated by-products of bacterial metabolism may also lead to the formation of compounds reducing the sensory quality of meat products, such as organic acids, alcohols, esters, and others (Dillon, 1998).

Numerous studies have been conducted in yeasts isolated from meat and meat products (for a review see Samelis & Sofos, 2003), and the yeasts most frequently isolated on spoiled meat are summarized in Table 7.1 (see Romano, Capece, & Jespersen, 2006; Samelis & Sofos, 2003; Selgas & García, 2007).

Beneficial Aspects of Yeast on Meats: Yeast Starter Cultures

Yeasts can also be considered habitual components of the micro-biota growing on fermented sausages and dry-cured hams, and their origin is mainly related to the environment and the meat used as raw material. In fermented meats, the lactic acid produced by bacteria and the low water activity resulting from the presence of salts or a dehydration process constitute a modified environmental factor that hinders the bacterial growth and favor the development of natural competitors. Thus, yeasts use all the nutrients and energy and grow quickly and easily (Dillon & Board, 1991). Yeasts can grow at pH, water activity, and temperature values usual in fermented sausages (Hammes & Knauf, 1994; Monte, Villanueva, & Domínguez, 1986). The presence of yeasts in fermented raw sausages has been studied less than bacteria and molds.

Sausages and dry-cured hams are traditional meat products obtained after several months of ripening. Proteolysis and lipolysis constitute the main biochemical reactions in the generation of flavor precursors, where the endogenous enzymes play the most important role (Toldrá, 1998). Yeasts and molds traditionally play an important role in sausages fermentation and in dry ham (Martín, Córdoba, Aranda, Córdoba, & Asensio, 2006; Martín, Córdoba, Benito, Aranda, & Asensio, 2003), and their contribution of the fungal population enzymes to proteolysis in minced dry-cured meat products, such as dry-cured sausages, is widely known.

The use of commercially available starters, mainly constituted of lactic acid bacteria and micrococci, may also produce an impoverishment of flavor and aroma and a loss of peculiar organo-leptic characteristics found in naturally fermented sausages. For this reason, in several European countries, artisanal sausages are still preferred by the consumer and are manufactured by relying on an unknown "factory flora" (Samelis, Stavropoulos, Kakouri, & Metaxopoulos, 1994). Lactic acid bacteria, micrococci, and coagulase-negative staphylococci have the most relevant role in the fermentative process and ripening, but also yeasts and molds can be involved as we explained before. Though there are several reports on the yeast populations in various meat products (Fung & Liang, 1990), studies on the yeast biodiversity in sausages are limited. The earliest studies on salami reported that *D. hansenii* was

Table 7.1 The most significant and commonly reported meat yeast species (Deáck & Beuchat, 1996; Martín et al., 2003, 2006; Romano, Capece, & Jespersen, 2006; Samelis & Sofos, 2003)

Fresh meats				Cured fresh and cooked meats		Dried and fermented meats		
Beef	Poultry	Lamb	Pork	Sausages	Frankfurters	Ham	Sausages and salamis	Dry-cured ham
C. albicans	*C. catenulata*	*C. glabrata*	*C. glabrata*	*C. catenulata*	*C. catenulata*	*C. catenulata*	*C. gropengiesseri*	*C. saitoana*
C. catenulata	*C. intermedia*	*C. zeylanoides*	*C. zeylanoides*	*C. intermedia*	*C. saitoana*	*C. saitoana*	*C. haemulonii*	*C. zeylanoides*
C. diddensiae	*C. parapsilosis*	*Cr. laurentii*	*Cr. laurentii*	*C. parapsilosis*	*C. zeylanoides*	*C. zeylanoides*	*C. intermedia*	*Cr. albidus*
C. intermedia	*C. rugosa*	*Cys. infirmominiatum*	*Cys. infirmominiatum*	*C. rugosa*	*Deb. hansenii*	*Deb. hansenii*	*C. kruisii*	*Cr. laurentii*
C. norvegica	*C. zeylanoides*	*Deb. hansenii*	*Deb. hansenii*	*C. saitoana*	*Tr. pullulans*	*Tr. pullulans*	*C. parapsilosis*	*Cr. humicolus*
C. parapsilosis	*Cr. laurentii*	*Rh. glutinis*	*Rh. glutinis*	*C. tropicalis*	*Y. lipolytica*	*Y. lipolytica*	*C. zeylanoides*	*Deb. hansenii*
C. rugosa	*P. membranifaciens*	*Rh. minuta*	*Rh. minuta*	*C. zeylanoides*			*Cit. matritensis*	*Deb. polymosphus*
C. sake	*Rh. glutinis*	*Rh. mucilaginosa*	*Rh. mucilaginosa*	*Cr. albidus*			*Cr. albidus*	*P. ciferii*
C. tropicalis	*Rh. minuta*	*Tr. pullulans*	*Tr. pullulans*	*Deb. hansenii*			*Cr. skinneri*	*P. holstii*
C. versatilis	*Sacch. exiguus*	*Y. lipolytica*	*Y. lipolytica*	*Dekkera spp.*			*Deb. hansenii*	*P. sydowiorum*
C. zeylanoides	*Tr. moniliforme*			*Gal. geotrichum*			*Deb. maramus*	*P. guillermondii*
Cr. albidus	*Tr. pullulans*			*I. orientalis*			*Deb. occidentalis*	*Rh. glutinis*
Cr. laurentii				*P. anomala*			*Deb.polymorphus*	*Y. lipolytica*
Cys. infirmominiatum				*P. guillermondii*			*Deb. vanrijiae*	
Deb. hansenii				*P. membranifaciens*			*G. geotrichum*	
P. burtonii				*Rh. minuta*			*H. uvarum*	
P. fermentans				*Rh. mucilaginosa*			*M. pulcherrima*	
P. membranifaciens				*Sporobolomyces spp.*			*P. farinosa*	
Rh. glutinis				*Tr. moniliforme*			*P. guillermondii*	
Rh. minuta				*Tr. pullulans*			*P. philogaea*	
Rh. mucilaginosa				*Y. lipolytica*			*Rh. minuta*	
Sacch. exiguus							*Rh. mucilaginosa*	
Tr. moniliforme							*Tr. beigelii*	
Tr. pullulans							*Sp. roseus*	
Y. lipolytica							*St. halophilus*	
							T. delbrueckii	
							Tr. pullulans	
							Y. lipolytica	
							Z. rouxii	

the most commonly isolated yeast. Most recently, several researches confirmed these results, but other yeast genera were found, such as *Candida*, *Pichia*, *Rhodotorula*, *Hansenula* (synonym of *Pichia*), and *Torulopsis* (synonym of *Candida*) (Dalton, Board, & Davenport, 1984; Grazia, Suzzi, Romano, & Giudici, 1989). On this basis, *D. hansenii* was used as a starter with positive effects on the development of a characteristic yeast flavor and stabilization of the reddening reaction. *D. hansenii* strains are presently used in starter preparations to be added to the sausages (Hammes & Knauf, 1994). The yeast *Yarrowia lipolytica*, the perfect form of *Candida lipolytica*, has also frequently been isolated from fresh beef (Dalton et al., 1984; Fung & Liang, 1990) and sausages (Viljoen, Dykes, Collis, & von Holy, 1993). Due to its lipolytic and proteolytic activity, this species can have a high technological potential (Sinigaglia, Lanciotti, & Guerzoni, 1994).

We can conclude that *D. hansenii* is the most frequently isolated yeast in fermented sausages and dry cured ham (Romano, Capece, & Jespersen, 2006; Samelis & Sofos, 2003; Selgas & García, 2007). The contribution of this yeast to the typical aroma of the products is based on their primary and secondary metabolites produced by the activity of lipases and proteinases, which are the key enzymes (Durá et al., 2004a, 2004b). In the next section we will give a detailed description of this yeast species.

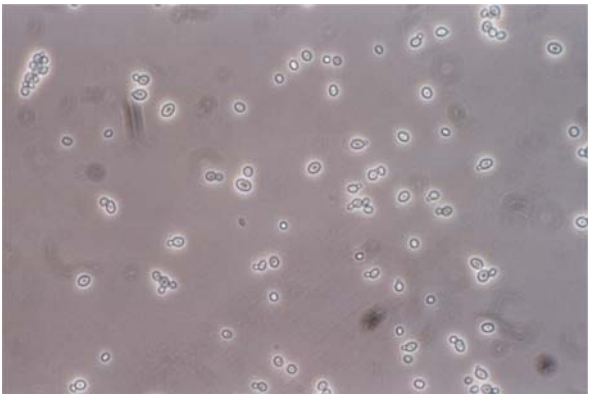
Physiological and Genetic Characteristics of *D.hansenii*

According to the last revision, the genus *Debaryomyces* comprises 15 species (Nakase, Suzuki, Phaff, & Kurtzman, 1998). Many representatives can be found in natural habitats such as air, soil, pollen, tree exudates, plants, fruits, insects, and faeces and gut of vertebrates (Barnett, Payne, & Yarrow, 2000). Nine of the *Debaryomyces* species, namely *D. carsonii*, *D. etchellsii*, *D. hansenii*, *D. maramus*, *D. melissophilus*, *D. polymorphus*, *D. pseudopolymorphus*, *D. robertsiae*, and *D. vanrijiae* have been found in a variety of processed foods, such as fruit juices and soft drinks, wine, beer, sugary products, bakery products, dairy products, and meat or processed meats. The presence of the *Debaryomyces* species in foods usually has no detrimental effects and in some cases is beneficial to the food. However, *D. hansenii* as well as other *Debaryomyces* species may also be responsible for food spoilage, causing off-odors and off-flavors. The best method for identification of this species is the PCR-RFLP of the intergenic spacer region (IGS) region of rDNA (Quiros et al., 2006).

Morpholgy and Physiology

Colonies on agar medium at 25°C are grayish white to yellowish, dull to shiny, and smooth to wrinkle. The two varieties within *D. hansenii* are distinguished by

Panel A.



Panel B.

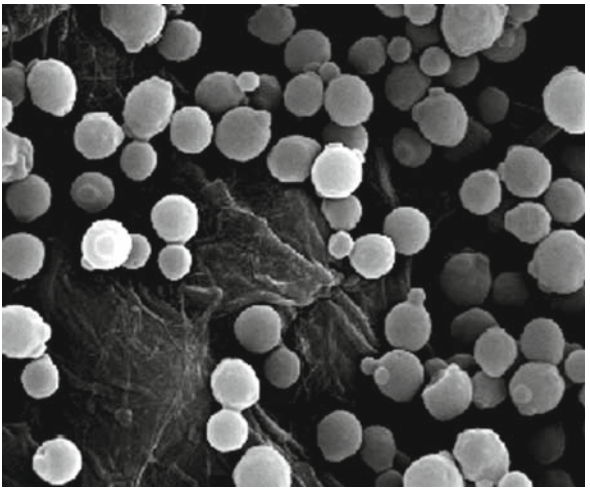


Fig. 7.1 Morphology of *Debaryomyces hansenii* strain PYCC 298. Panel A optic microscopy phase contrast (40X). Panel B, scanning electronic microscopy (JEOL JSM-6300). * Photo panel B, courtesy of Professor José Ramos, Department of Microbiology, University of Córdoba, Spain

maximum growth temperature. *D. hansenii* var. *hansenii* has a maximum growth temperature of 35°C while *D. hansenii* var. *fabryi* has a maximum growth temperature of 40°C and does not growth on cycloheximide. The microscopic morphology following 72 hours incubation at 25°C shows no pseudohyphae, the cells are spheroidal to short-ovoidal and single, in pairs or on short chains (see Fig. 7.1). Pseudo-mycelium is absent, primitive, or occasionally well developed (Nakase et al., 1998).

D. hansenii shows some remarkable biotechnological properties like the ability to grow at 10% NaCl or 5% glucose and assimilates a broad spectrum of carbon substrates. *D. hansenii* has a high chemo-stress tolerance, which means that high concentrations of many substrates can be used in the cultivation. It is characterized by its cryo and halo-tolerance (Prista, Almagro, Loureiro-Dias, & Ramos, 1997, Prista, Loureiro-Dias, Montiel, García, & Ramos, 2005), and its ability to metabolize lactic and citric acids. Such properties allow its development in all types of cheeses (Fleet, 1990) and different fermented meat products (Encinas, López-Díaz, García-López, Otero, & Moreno, 2000) contributing to the ripening process and the generation of aroma precursors by the lipolytic and proteolytic activities (Flores, Durá, Marco, & Toldrá, 2004; Leclercq-Perlat, Corrieu, & Spinnler, 2004; Martín et al., 2003; Petersen, Westall, & Jespersen, 2002).

D. hansenii is generally regarded as non-pathogenic (Swiss Agency for the Environment, Forests and Landscape [SAEFL], 2004); however, clinical isolates are not rare (Nishikawa, Tomomatsu, Sugita, Ikeda, & Shinoda, 1996) and there are reports of bone infection caused by *D. hansenii* (Wong et al., 1982). *D. hansenii* can cause subcutaneous abscesses, osteitis, and keratitis in both immunocompetent and immunocompromised patients (Mattsson, Haemig, & Olsen, 1999) and extrinsic allergic alveolitis (Yamamoto et al., 2002).

Genetic Characteristics

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. Vegetative reproduction is by multilateral budding. Heterogamous conjugation between the cell and its bud precedes ascus formation. The spores are spherical with a warty wall (Nakase et al., 1998). Conjugation between separate cells may also occur, thus making difficult the use of classical genetic techniques.

The genome of *D. hansenii* has been explored in the Genolevures project, and the genome sequence is available in http://cbi.labri.fr/Genolevures/about/GL1_genome.php).

The presence of several linear plasmids designated pDHL1 (8.4 kB), pDHL2 (9.2 kB) and pDHL3 (15.0 kB) [79] and pDH1A and B (Cong, Yarrow, Li, & Fukuhara, 1994) in *D. hansenii* has also been described. The stability of these plasmids seems to depend on the osmotic pressure (Breuer & Harms, 2006).

Chromosomal DNA

Electrophoretic karyotyping of *Debaryomyces* strains showed very divergent chromosomal profiles at both inter-specific (Martorell et al., 2005) and intra-specific levels (Corredor, Davila, Casaregola, & Gaillardin, 2003; Petersen, Westall, & Jespersen, 2002). These studies demonstrated that the chromosomal arrangement in *D. hansenii* strains is heterogeneous, showing variations in both the number and size of the chromosomal bands on a Pulsed field gel electrophoresis (PFGE) gel.

The number of chromosomal bands observed varies from five to ten, but the most common chromosome number was found to be six. However no data about chromosomal profiles in isolates of *D. hansenii* from meat products are available.

Tandem gene duplications are 5–10 times more frequent in *D. hansenii* than in other yeasts, but few duplicated blocks have been detected (Lepingle et al., 2000). 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 poly-ploidization event (Friedman & Hughes, 2001) are called duplicated blocks.

Transformation Systems

Several transformation systems have been developed for *Debaryomyces* (for a revision see Breuer & Harms, 2006). The first methods to be tested were designed for *Schwanniomyces occidentalis* cells. However, the halo-tolerance of *Debaryomyces* seemed too difficult for the transformation process, thus no specific and effective system for inserting the genes into the genome of *D. hansenii* was developed.

A transformation system that did effectively transform *D. hansenii* was also developed based on a plasmid that is integrated into the chromosomal DNA. The expression of heterologous genes in *Debaryomyces* has been tested using a bacterial gene coding for a NADPH-dependent acetoacetyl-CoA reductase (*phb*B).

In another study, six heterologous yeast expression vectors were constructed for *D. hansenii*, using heterologous promoters from *S. cerevisiae*. 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, except for the ADH2 gene promotor for alcohol dehydrogenase 2 from *S. cerevisiae* (Maggi & Govind, 2004).

Therefore, we can conclude that the genetic machinery and operational transformation methods required for efficient expression of the heterologous genes or whole pathways in *D. hansenii* are available. However, further investigation on this subject is recommended if secretory heterologous products are involved.

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