Chapter 5
Candida famata (Debaryomyces hansenii)

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Contents

Abstract *Debaryomyces hansenii* (teleomorph of asporogenous strains known as *Candida famata*) belongs to the group of so named 'flavinogenic yeasts' capable of riboflavin oversynthesis during starvation for iron. Some strains of *C. famata* belong to the most flavinogenic organisms known (accumulate 20 mg of riboflavin in 1 ml of the medium) and were used for industrial production of riboflavin in USA for long time. Many strains of *D. hansenii* are characterized by high salt tolerance and are used for ageing of cheeses whereas some others are able to convert xylose to xylitol, anti-caries sweetener. Transformation system has been developed for *D. hansenii*. It includes collection of host recipient strains, vectors with complementation and dominant markers and several transformation protocols based on protoplasting and electroporation. Besides, methods of multicopy gene insertion and insertional mutagenesis have been developed and several strong constitutive and regulatable promoters have been cloned. All structural genes of riboflavin synthesis and some regulatory genes involved in this process have been

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identified. Genome of *D. hansenii* has been sequenced in the frame of French National program 'Genolevure' and is opened for public access.

Keywords Riboflavin, *D. hansenii*, *C. famata*, flavinogenic, transformation, insertional mutagenesis

5.1 Introduction

Candida famata (teleomorph: *Debaryomyces hansenii*) is osmotolerant yeast able to grow in the presence of high concentrations of NaCl. The yeast tolerates 4 M of the salt, whereas growth of *Saccharomyces cerevisiae* is completely inhibited by 1.7 M NaCl (Onishi, 1963; Prista et al., 1997). Both *C. famata* and *D. hansenii* strains are able to overproduce riboflavin (vitamin B_2) in iron-deficient media (Gadd and Edwards, 1986; Shavlovsky and Logvinenko, 1988). Some *C. famata* mutants are the most flavinogenic organisms known (Heefner et al., 1988, 1992, 1993; Stahmann et al., 2000). Strains of *D. hansenii* and *C. famata* are found in habitats with high salinity levels, such as sea water, brines, salted food (cheeses, sausages) (Norkrans, 1966; Seiler and Busse, 1990; Lépingle et al., 2000). Ability of *D. hansenii* to grow in the presence of high NaCl concentrations resulted in a designation of the species as halotolerant (or, according to some authors: halophilic) yeast. Some *D. hansenii* strains are considered as potential producers of xylitol (Parajo et al., 1996; Roseiro et al., 1991). Osmotolerance of *D. hansenii* is advantageous for some biotechnological applications; it allows quasi-non-sterile production and high product/educt concentrations, conditions which should reduce production costs (Breuer and Harms, 2006). *D. hansenii* (*C. famata*) belongs to the monophyletic clade containing organisms that translate CTG as serine instead of leucine (Fitzpatrick et al., 2006). This chapter mainly is focused on the flavinogenic strains of the anamorph *C. famata*.

Complete sequence of *D. hansenii* genome has been published (http://cbi.labri.fr/Genolevures/elt/DEHA) and is available for public use (Dujon et al., 2004). It opens new opportunities for study and elucidation of molecular mechanisms of halotolerance and riboflavin overproduction in *D. hansenii* (*C. famata*) and using this yeast in basic and applied research.

Recently, the review on *D. hansenii* has been appeared (Breuer and Harms, 2006). This useful review contains the comprehensive data on many aspects of physiology, biochemistry, genetics and potential biotechnological applications of *D. hansenii*. However, in spite of the fact that the only industrial biotechnological application of *D. hansenii* to date is the use of the mutant strain of anamorph *C. famata* for riboflavin production (at Archer Daniels Midland Co. in USA), authors did not pay attention on this important aspect. They also did not mention on development of transformation system for *C. famata* and cloning structural and regulatory genes involved in riboflavin synthesis. Our review aims to fill these gaps.

5.2 History of Research, Phylogeny and Physiology

D. hansenii is studied actively as osmotolerant yeast since 1960-ies (Onishi, 1963; Norkrans, 1966, 1968) and, nowadays, obvious progress has been achieved in studying mechanisms underlying high salt tolerance of the yeast (Prista et al., 2005; Velkova and Sychrova, 2006). First articles on capability of *C. famata* strains to overproduce riboflavin were published at 1940-ies – 1950-ies (Tanner et al., 1945; Goodwin and McEvoy, 1959). Since then efficient approaches for improvement of *C. famata* riboflavin production were developed and very active overproducers were isolated (Heefner et al., 1988, 1992, 1993). A transformation system based on the *C. famata* strain L20105 (*leu2*) deficient in β-isopropylmalate dehydrogenase as a recipient and vectors containing the *S. cerevisiae LEU2* gene as a selective marker was developed for *C. famata* (Voronovsky et al., 2002; Abbas et al., 2006). Genomic fragments of the anamorph containing genes participating in the synthesis of riboflavin were cloned and sequenced (Dmytruk et al., 2004; Voronovsky et al., 2004). Recently, a method of insertional mutagenesis was applied successfully for *C. famata* and genes involved in positive regulation of riboflavin synthesis were identified (Dmytruk et al., 2006). Complete sequence of *D. hansenii* genome was published in July 2004 (http://cbi.labri.fr/Genolevures/elt/DEHA). All these achievements in the field of molecular biology of *D. hansenii* (*C. famata*) open new opportunities for study and clarification of metabolic peculiarities (halotolerance, riboflavin overproduction) of this yeast.

The species *D. hansenii* (anamorph: *C. famata*) is a haploid yeast that reproduces vegetatively by multilateral budding. Most *D. hansenii* strains mate very rarely and diploidize transiently by somatogamous autogamy to form asci containing generally a single spore (Kreger and Veenhuis, 1975; van der Walt et al., 1977). *D. hansenii* (*C. famata*) belongs to the monophyletic clade containing organisms that translate CTG as serine instead of leucine (Fitzpatrick et al., 2006). The species *D. hansenii* (Zopf) Lodder & Kreger comprises two varieties: *D. hansenii* (Zopf) Lodder & Kreger var. *hansenii* (anamorph: *C. famata* (Harrison) S.A. Meyer & Yarrow var. *famata*) and *D. hansenii* var. *fabryi* (Ota) Nakase & M. Suzuki (anamorph: *C. famata* (Harrison) S.A. Meyer & Yarrow var. *flareri* (Ciferri & Redaelli) Nakase & M. Suzuki) (Nakase and Suzuki, 1985; Nakase et al., 1998). The varieties of *D. hansenii* differ in the electrophoretic mobility of their glucose-6-phosphate dehydrogenases. They also differ in maximum growth temperatures: var. *hansenii* can grow at temperatures up to 35°C while var. *fabryi* is able to grow up to 39°C (Nakase and Suzuki, 1985). The varieties also differ in sequences of their 26S rDNA genes (Kurtzman and Robnett, 1997, 1998). All these differences were used for discrimination of the two varieties of *D. hansenii*. In addition, the pulse-field gel electrophoresis (PFGE) was used to discriminate between the two varieties (Corredor et al., 2003; Petersen and Jespersen, 2004). However, different results were obtained by the two groups of authors: Corredor et al. (2003) stated a marked chromosomal polymorphism in *D. hansenii* strains. This suggested that strains belonging to *D. hansenii* var. *fabryi* represent a different taxon from

D. hansenii var. *hansenii*. But, according to the other authors (Petersen and Jespersen, 2004), PFGE analysis did not result in a division of the two varieties to separate groups. So, further change and improvement of the taxonomic classification of *D. hansenii* varieties will be possible after development of new analytical methods.

D. hansenii (*C. famata*) has some remarkable physiological properties. The yeast can grow at concentrations of NaCl up to 2.5 M and tolerate 4 M of the salt (Prista et al., 1997; Lépingle et al., 2000). *D. hansenii* assimilates a broad spectrum of carbon substrates: glucose, galactose, sucrose, maltose, cellobiose, trehalose, raffinose, xylose, arabinose (Nakase et al., 1998). The optimal temperature for the yeast is 25–28°C, but growth at 5–10°C was also reported (Davenport, 1980). *D. hansenii* utilizes *n*-alkanes (Yadav and Loper, 1999). It has a poor growth in the absence of oxygen, therefore fermentation of glucose, galactose, sucrose, maltose, trehalose and raffinose by *D. hansenii* is weak (Nakase et al., 1998). The yeast does not utilize nitrate, but it can assimilate nitrite (Nakase et al., 1998). *D. hansenii* (*C. famata*) overproduces riboflavin in iron-deficient media (Gadd and Edwards, 1986; Shavlovsky and Logvinenko, 1988). Some of developed *C. famata* strains are the most flavinogenic organisms known (Heefner et al., 1988, 1992, 1993; Stahmann et al., 2000).

D. hansenii has a mitochondrial alternative oxidase (in addition to the cytochrome *c* oxidase) that is triggered by 1.5–2 M NaCl and can act as the terminal oxidase for an electron transfer chain that branches from the core pathway at the ubiquinone pool level. The alternative oxidase is insensitive to cyanide and antimycin A. The cyanide-resistant respiration pathway reduces the amount of energy from the cytochrome *c* pathway by diverting some of the electron flux to the alternative oxidase (Veiga et al., 2003a, b).

Generally the yeast *D. hansenii* (*C. famata*) is considered as non-pathogenic. There are many harmless strains of *D. hansenii* and *C. famata* involved in ripening of cheeses and sausages (Seiler and Busse, 1990; Saldanha-da-Gama et al., 1997). The industrial strain of *C. famata* involved in the production of riboflavin is also known (Stahmann et al., 2000). On the other hand, clinical isolates of *D. hansenii* and *C. famata* are not rare (Nishikawa et al., 1996). Hence, the possible pathogenicity of the yeast has to be investigated in more detail.

5.3 Mechanisms for the Halotolerance of the Yeast

A few peculiar characteristics provide the remarkable halotolerance of *D. hansenii* (*C. famata*); particularly, capability to keep glycerol at high concentrations inside the cells and less sensitivity of metabolism of the yeast to intracellular sodium concentrations are considered the most important among the characteristics.

The osmoregulation in *D. hansenii* has been studying for many years by many groups. Correlation between the intracellular polyol (glycerol and arabinitol) concentration and the salinity of the medium was established (Larsson et al., 1990).

^{13}C NMR studies showed that glycerol is the predominant end product when *D. hansenii* metabolizes glucose in the presence of 8% NaCl (Jovall et al., 1990). There was shown a key role of the enzyme glycerol-3-phosphate dehydrogenase (GPD) in the production of glycerol in *D. hansenii* (Adler et al., 1985). The enzyme was purified and characterized *in vitro* (Nilsson and Adler, 1990). The specific activity of *D. hansenii* GPD was doubled when the salinity of the medium increased to 1.4 M, but this stimulation was weaker than that observed in *S. cerevisiae* (Andre et al., 1991). The *GPD1* gene of *D. hansenii* (*DhGPD1*) was isolated and a high degree of homology with *GPD1* of *S. cerevisiae* was found (Thome, 2004). Immunoblot analysis and Northern blotting confirmed the previous observation on the role of GPD in osmoregulation (Thome and Trench, 1999). Recently, the significance of cell wall for the induction of *DhGPD1* gene and consequently for osmotic regulation in *D. hansenii* was shown (Thome, 2007).

It was established that enzymes leading to pyruvate, in particular glyceraldehyde-3-P dehydrogenase, were inhibited under the salt-induced stress in *D. hansenii* (Neves et al., 1997). It can explain the increased diversion of glycolytic flux towards the glycerol pathway in the yeast.

Glutamate in *D. hansenii* protects GPD against the toxic effect of salt (Nilsson and Adler, 1990). The increase of glutamate dehydrogenase activity in the yeast in the presence of salt was reported. Glutamate is suggested as the intracellular counter ion under high ionic strength (Alba-Lois et al., 2004).

Studies directed on identification of protein targets for Na^+ toxicity were performed. The *HAL2* gene was identified in *S. cerevisiae* as an important target of salt toxicity (Glaser et al., 1993). The overexpression of the gene significantly increased salt tolerance. The product of *HAL2* is a nucleotidase that hydrolyses 3′-phosphoadenosine-5′-phosphate (PAP) to AMP, recycling adenosine. A gene homologous to *HAL2* was identified in *D. hansenii* (Prista et al., 2005). Preliminary results suggested that, *in vitro*, Hal2p from *D. hansenii* was more salt-resistant than the corresponding protein from *S. cerevisiae* (Prista et al., 2005).

The only production of glycerol is not sufficient to provide high osmotolerance; the osmolyte has to be kept inside the cells at high concentrations. The osmoregulatory-active glycerol transport system was described for *D. hansenii* (Lucas et al., 1990). This transport system accumulates glycerol significantly (up to 150-fold) in the presence of 1M NaCl. Correlation between the maximum ratios of glycerol accumulation and the concentration of extracellular NaCl led to the hypothesis about involvement of sodium-glycerol symporter in the system. The data also suggested that potassium may be a co-substrate instead of sodium when the sodium concentration is low. Yeasts able to grow in the presence of 3M NaCl were able to accumulate glycerol in an energy-dependent process (Lages et al., 1999). There was shown for the range of yeasts (including *D. hansenii*) that accumulation of glycerol was reinforced by the presence of 1M NaCl (Lages et al., 1999).

The capability to maintain a high intracellular concentration of glycerol does not depend only on active transport of glycerol. It also depends on the permeability of the plasma membrane: the low permeability prevents glycerol leakage.

The permeability coefficient (ε) for glycerol diffusion in *D. hansenii* is lower compare to that in *S. cerevisiae* (Lages et al., 1999; Larsson et al., 1990; Prista et al., 2005). Searching in the Genolevures database for glycerol permeases (like the Fps1p of *S. cerevisiae*) or putative MIP channels for glycerol did not result in finding the corresponding *D. hansenii* homologues. This supports the view that *D. hansenii* is better equipped to retain than to release glycerol, opposite to *S. cerevisiae* (Prista et al., 2005).

The process of sodium efflux has been studied actively in *D. hansenii* since the sodium extrusion is a main way of defence for other yeasts during growth in the presence of salt. Particularly, two genes specifically involved in salt extrusion were cloned and characterized in *D. hansenii*. The genes code for Na^+-ATPases (Almagro et al., 2001). They were designated *DhENA1* and *DhENA2*. Northern analysis demonstrated the elevated expression of *DhENA1* gene in the presence of high NaCl concentrations. The gene *DhENA2* for its increased expression required high pH in addition to high sodium. The genes complemented NaCl-sensitivity of the *S. cerevisiae* mutant lacking the sodium efflux systems. Resulting transformants acquired sodium tolerance and ability to extrude the cation (Almagro et al., 2001). But the recovered tolerance was far from the tolerance level of *D. hansenii*. Thus, sodium extrusion alone is not sufficient to explain the high salt tolerance of *D. hansenii*.

Search in the Genolevures database found a range of *D. hansenii* genes that may play a role in Na^+ and K^+ efflux. The gene *DhNHX* coding for a putative protein mediating the transport of Na^+ into the vacuole was also found (Prista et al., 2005). Real significance of the genes in regulation of cation content and halotolerance in *D. hansenii* has to be tested by experimental studies.

5.4 Strains and Media

The wild-type *C. famata* strain VKM Y-9 (Russian Collection of Microorganisms, Poushchino, Russia) and its derivative, the leucine auxotroph *C. famata* L20105 (*leu2*, NRRL Y-30292) were used in our studies (A. Sibirny and A. Voronovsky; Institute of Cell Biology NAS of Ukraine, Lviv, Ukraine). Both strains overproduce riboflavin in iron-deficient media. For complete genome sequencing, the strain *D. hansenii* var. *hansenii* CBS767 was used. The sequenced strain also overproduces riboflavin in iron-deficient media (Voronovsky et al., 2004).

The *C. famata* strains were grown at 28°C in YPD (1% yeast extract, 1.5% peptone, 2% glucose) or minimal media, YNB (0.67% Difco yeast nitrogen base without amino acids, containing 2% glucose), or modified Burkholder medium (Voronovsky et al., 2002; Shavlovsky et al., 1978). Leucine at 40 mg $\times$ L^{-1} was added to minimal media for cultivation of the *leu2* mutant L20105. Iron-deficient media contained about 0.18 μM of iron. Iron was removed from the medium with 8-hydroxyquinoline as described earlier (Cowart et al., 1980). Iron supplemented media contained 7.2μM iron added as ammonium ferrous sulfate hexahydrate.

5.5 Genetic Data and Techniques

5.5.1 Transformation System for the Flavinogenic Yeast C. famata

5.5.1.1 Identification of *C. famata* Recipient Strains Deficient in *LEU2* gene

For development of *C. famata* transformation system, the collection of *C. famata* leucine auxotrophic mutants was isolated. Cells of wild-type strain VKM Y-9 were UV-irradiated and plated on the modified Burkholder medium containing L-leucine. 16 leucine auxotrophic mutants (Leu^-) of *C. famata* were isolated after testing 45,180 colonies. Identification of *leu2* mutants was carried out by spheroplast transformation of five randomly picked Leu^- mutants by plasmids harboring the *S. cerevisiae LEU2* gene coding for β-isopropylmalate dehydrogenase. Three Leu^- strains were transformed by the plasmids to Leu^+ colonies. These mutants were designated L203, L2012 and L20105. All three mutants were stable with a reversion frequency to prototrophy of less than 10^{-7}. Thus, there was concluded that the mutants L203, L2012 and L20105 have a deficiency in gene homologous to the *S. cerevisiae LEU2* gene encoding β-isopropylmalate dehydrogenase. The strain L20105 was used in further studies as a recipient in transformations using the *S. cerevisiae LEU2* gene as selective marker (Voronovsky et al., 2002; Abbas et al., 2006).

5.5.1.2 Cloning of *C. famata* Autonomously Replicating Sequences (ARS) and Obtaining Replicative Plasmids Providing Effective Transformation of This Yeast

Plasmids carrying heterologous autonomously replicating sequences (ARS) could not provide the high transformation frequency for *C. famata*. Efficient transformation is important factor for the host-vector system that is developed for gene cloning. A strategy of *C. famata* ARS cloning consisted in the isolation of this sequence from the yeast genome library constructed on a vector that does not contain any yeast plasmid replicator. The recombinant plasmid p19L2 (Voronovsky et al., 2002) was used for the library construction. The plasmid contains the *S. cerevisiae LEU2* gene as selectable marker. This plasmid transformed the *C. famata* mutant L20105 inefficiently. The gene library of *C. famata* wild-type strain VKM Y-9 was constructed on the basis of plasmid p19L2. The plasmid DNA of resulting library transformed the strain L20105 to leucine prototrophy with frequency six times higher than that with p19L2. Thus, the increased transformation frequency suggested the presence of ARS elements among inserts of the constructed gene library. For ARS cloning, plasmid DNA was isolated from *C. famata* transformants obtained after transformation with the gene library. First, total DNA was isolated from approximately 40,000 such transformants. This DNA was used for transformation of *E. coli* DH5α with following isolation of the plasmid DNA from 16 randomly picked separate bacterial clones. Electrophoretic analysis showed that the vectors can be divided into five groups by their sizes. One plasmid was picked from each group for further studies.

Restriction analysis of the plasmids was carried out and the results were compared to the restriction digest of the vector p19L2. The approximate length of the inserts was identified (from 0.25 to 6 kb). The frequency of spheroplast transformation by plasmids containing the inserts appeared to be, on average, 13 times higher than that obtained using the vector p19L2. Corresponding plasmid DNA was rescued through *E. coli* retransformation from yeast transformants obtained by insert-containing plasmids but not from transformants L20105/p19L2. High transformation frequencies and extrachromosomal status of insert-containing vectors suggested that the cloned fragments possess with the ARS function. These plasmids were named pCfARS1, pCfARS6, pCfARS11, pCfARS7 and pCfARS16. An ARS fragment of the smallest size of plasmid pCfARS16 (the CfARS16 insert) was sequenced (accession number AF435949 in GenBank database). The CfARS16 fragment had features characteristic for ARS elements. Namely, it had a region rich in adenine plus thymine (A + T) (more than 81%) and contained a 10-of-11-bp match to the *S. cerevisiae* ARS Consensus Sequence, ACS: (A/T)TTTAT(A/G) TTT(A/T) (Rowley et al., 1994; Voronovsky et al., 2002).

5.5.1.3 Optimization of Transformation Methods for *C. famata*

5.5.1.3.1 Spheroplast Transformation

C. famata spheroplast transformations were performed by the method described earlier for *P. pastoris* (Sreekrishna and Kropp, 1996) with some modifications. During spheroplast transformation, sucrose was used as an osmotic stabilizer instead of sorbitol, as there was found that sorbitol strongly reduces *C. famata* spheroplast viability. Important factor influencing the spheroplast transformation frequency appeared to be the purity of the enzyme used for cell wall hydrolysis. Lyticase (Sigma, 'crude') resulted in transformation frequency for the strain L20105 with an average of 1.44×10^3 transformants per μg DNA. Lyticase (Sigma, 'partially purified') resulted in more than 40 times higher transformation frequency (6.3×10^4 transformants per μg DNA). The other important factor influencing *C. famata* spheroplast transformation efficiency is the quality and/or supplier of PEG. We found that the best stock for transformation was PEG 3,350 (Merck, Germany) or PEG 3000 (Ferak, Germany). The use of PEG 3,350 Carbowax (Fisher, USA) was found to provide transformation frequencies 10 times lower than those of the PEGs mentioned above (Voronovsky et al., 2002; Abbas et al., 2006).

5.5.1.3.2 Electrotransformation

The *C. famata* strain L20105 (*leu2*) and plasmids pCfARS6 or pCfARS16 were used for optimization of the electrotransformation parameters for this yeast. Our protocol was based on two electrotransformation procedures previously described for *S. cerevisiae* (Becker and Guarente, 1991) and *H. polymorpha* (Faber et al., 1994). Electroporation was carried out with the Electro Cell Manipulator 600

(ECM600) from BTX, USA, using 2-mm cuvettes. The following important factors for electrotransformation efficiency were examined: significance of DTT treatment of cells, electric field strength, the cell culture age, and pulse duration. There was found out that the pretreatment of *C. famata* cells with DTT is essential for transformation. Omission of this step resulted in the total absence of transformants. Optimal field strength for electrotransformation of *C. famata* strain L20105 was determined. For this, electroporations of the strain under different field strengths were carried out. There was identified that the field strength of 11.5 kV cm^{-1} provided the highest transformation frequencies: 0.8–1 × 10^5 transformants μg^{-1} DNA. It is known that cells from exponential growth phase are the most suitable for highly efficient electrotransformation of yeasts (Becker and Guarente, 1991; Faber et al., 1994). Cells of strain L20105 from different stages of exponential growth were tested for their suitability for electrotransformation. Prior to electrotransformation, the cells were concentrated by centrifugation and resuspended in 1M sucrose (used as electroporation buffer) to achieve equal cell densities (approx. 4 × 10^9 cells ml^{-1}). The cells (0.2 ml per sample) were then transformed with 0.3 μg of plasmid. There was determined that the most efficient transformation frequency was achieved with cells of OD_{540} = 6 (2.7 × 10^7 cells ml^{-1}). The optimal pulse duration for electrotransformation of strain L20105 was also established. This parameter is determined by the timing resistance. For many yeast species, including strains of *S. cerevisiae*, *H. polymorpha*, *Pichia methanolica* and *Schizosaccharomyces pombe*, the optimal value of pulse duration during electroporation appeared to be 4–5 ms (Becker and Guarente, 1991; Faber et al., 1994). This pulse length was provided by the timing resistance of ~129 Ω. Therefore, the pulse duration of ~4.5 ms (129 Ω) was used during the determination of optimal field strength for electroporation of *C. famata* L20105. After that, the effect of different pulse lengths on electroporation of strain L20105 was investigated. There was established that the optimal pulse duration for *C. famata* appeared to be ~4.5 ms (129 Ω).

Thus, efficient transformation system for the flavinogenic yeast *C. famata* was developed. It is based on a mutant strain of *C. famata* L20105, which is defective in a gene homologous to the *S. cerevisiae LEU2* gene encoding β-isopropylmalate dehydrogenase, and on vectors containing the *S. cerevisiae LEU2* gene as a selective marker. For DNA transfer, two methods were optimized and used: spheroplast transformation and electroporation. The transformation system was successfully used for cloning of *C. famata* genes for riboflavin synthesis (see Sect. 5.5.3).

5.5.2 Insertional Mutagenesis of C. famata

5.5.2.1 Insertion Cassette, Transformation Frequency and Integration Events

Identification of regulatory mutations by functional complementation usually is difficult to fulfil due to similarity in the growth patterns of wild-type strains and derived mutants. Therefore, other methods for generation of mutations with subsequent

rapid identification of defective elements have to be applied in the cases. Insertional mutagenesis belongs to techniques providing significant improvements in the procedure for cloning and identification of regulatory genes. The method has been successfully used to tag genes in many fungi including different yeast species (Tilburn et al., 1990; Schiestl and Petes, 1991; Kuspa and Loomis, 1992; Kang and Metzenberg, 1993; Granado et al., 1997; Van Dijk et al., 2001).

Development of the method for insertional mutagenesis of *C. famata* was started with the construction of insertion cassette. The integrative plasmid pL2 was constructed as the cassette (Dmytruk et al., 2006). This plasmid consists of *S. cerevisiae LEU2* gene inserted into the bacterial vector pUC19. A range of restriction sites were reduced in the plasmid; this allowed using many different endonucleases for the recovery of genomic fragments. The unique site *Sal*I was used for linearization of the plasmid.

The *C. famata* strain L20105 (*leu2*) was transformed by the linearised DNA of plasmid pL2. Addition of the restriction enzyme *Sal*I to transformation mixtures increased transformation frequencies only slightly (up to 1.5 times). Stability of resulting pL2 transformants was tested. Twelve randomly selected Leu^+ transformants were picked up and cultivated by turns in minimal and non-selective rich media. In all cases transformants remained Leu^+ prototrophs, indicating stable integration of the vector pL2 into the *C. famata* genome. Random integration at different sites of linearized plasmid pL2 into genome of the recipient strain was shown by analysis of the *C. famata* transformants using Southern hybridization. The analysis also revealed that in 95% of the transformants a single molecule of the cassette was present in genome (Dmytruk et al., 2006).

5.5.2.2 Generation and Identification of *C. famata* Regulatory Mutants Unable to Overproduce Riboflavin

The *C. famata leu2* strain was transformed by the *Sal*I-linearised pL2 plasmid. Obtained approx. 3000 Leu^+ transformants were replica plated onto the ferrozine-containing (iron deplete) medium. Three mutants affected in riboflavin overproduction on the medium were selected. These mutants 101R, 4R and 1R were stable and used for further analysis. Study of riboflavin production by the isolated mutants in liquid medium showed that they are unable to overproduce riboflavin either in iron replete or iron deplete media. Therefore there was hypothesized that the mutants 101R, 1R and 4R have mutations in one or more regulatory genes required for riboflavin production.

In order to identify the sites of insertion of cassette pL2, the genomic DNA from selected mutants affected in riboflavin overproduction was isolated. Restriction enzymes, which do not cleave pL2, were used to isolate of the integrated plasmid together with flanking genomic sequences. Flanking fragments together with the vector were isolated from the mutants 101R, 1R and 4R by transformation with the self-ligated genomic DNA, digested with *Bam*HI, *Sac*I and *Hind*III, respectively, into *E. coli*. All rescued plasmids were amplified and sequenced.

Resulting sequences revealed that, in selected strains 101R, 4R and 1R, the insertional cassette disrupted the gene *RIB1* (GTP cyclohydrolase II) and the orthologs of *S. cerevisiae* genes *MET2* (homoserine O-acetyltransferase) and *SEF1* (putative transcription factor), respectively.

5.5.2.3 Confirmation of Gene Disruption

Confirmation that the observed phenotype is a result of insertion cassette integration, but not the secondary mutation occurring elsewhere in the genome, is an essential part of the study. For this purpose, the insertion mutants 101R, 4R and 1R were transformed with plasmids carrying the respective genes (*RIB1*, *MET2* and *SEF1*) isolated from wild-type strains. In the case of 101R mutant, the *C. famata RIB1* gene that was cloned and sequenced earlier was used (Dmytruk et al., 2004; Voronovsky et al., 2004). In two other cases (4R and 1R), the corresponding genes of *D. hansenii* CBS767 strain were used (homologs of *S. cerevisiae MET2* and *SEF1*; the sequences were taken from the Genolevures database). As the isolated insertion regulatory mutants did not contain auxotrophic selective markers, a dominant marker was used to select the transformants. There was found that growth of *C. famata* is inhibited by phleomycin (2.0 mg l^{-1}) in the rich YPD medium. The *Staphylococcus aureus ble* (*Sable*) gene confers resistance to bleomycin, phleomycin and zeocin. *C. famata* (as was mentioned in Sect. 5.5.2) uses an alternative genetic code in which the CUG codon (leucine) codes for serine. Fortunately, the *ble* gene does not contain CUG codons within its open reading frame (Semon et al., 1987). Therefore this gene was used as a dominant selection marker for transformant selection.

Plasmids that bear *CfRIB1*, *DhMET2* or *DhSEF1* genes were constructed on the base of the vector containing *Sable* gene driven with the *C. famata TEF1* promoter (Dmytruk et al., 2006). These plasmids were used for transformations of the corresponding 101R, 4R and 1R strains. Phleomycin resistant colonies were selected. Transformants were stabilized and verified by PCR using specific primers for the *Sable* gene. The genes *CfRIB1*, *DhMET2* and *DhSEF1* complemented corresponding mutations and the obtained transformants acquired the ability to overproduce riboflavin in the iron depleted medium. The functional complementation of the *C. famata* strains 101R, 4R and 1R by the genes *CfRIB1*, *DhMET2* or *DhSEF1*, is a sufficient evidence to assert that the observed phenotype of isolated mutants is a result of disruption of the genes *RIB1*, *MET2* and *SEF1*.

Some speculations can be put forward to explain the inability of mutants 101R, 4R and 1R to overproduce riboflavin. In the mutant 101R, integration of the cassette pL2 resulted in disruption of the *RIB1* gene (the first gene of the riboflavin synthesis pathway), cutting off 33 amino acids from the N-terminus. However the insertion did not result in riboflavin auxotrophy of the 101R mutant. The insertion created a hybrid ORF, in which the start cogon ATG originates from the bacterial part of the pL2 vector. Blast analysis showed that the catalytic domain of the GTP cyclohydrolase II is located in the C-terminus. Thus, insertion of the cassette led to

the separation of iron-regulated promoter from the ORF of *RIB1* gene, and the formation of a truncated version of Rib1p. Such alteration apparently led to the synthesis of small amounts of shortened Rib1p and resulted in the inability of the 101R strain to overproduce riboflavin in iron depleted media.

Analysis of the 4R mutant led to the identification of the *MET2* gene, which is involved in some way in the regulation of riboflavin synthesis and iron acquisition in *C. famata*. Disruption of the gene did not result in methionine auxotrophy. This implies that two alternative pathways for the formation of cystathionine via o-succinyl-L-homoserine and o-acetyl-L-homoserine exist in *C. famata*, as has been described for the yeast *Candida albicans* (http://www.genome.ad.jp/dbget-bin/get_pathway?org_name = cal&mapno = 00271) Contrary to *C. albicans*, in *S. cerevisiae* formation of cystathionine from homoserine occurs exclusively via o-acetyl-L-homoserine (Langin et al., 1986). The finding of *MET2* paralog (45% homology) in the *D. hansenii* genome could also be a possible explanation for the absence of methionine auxotrophy in the 4R mutant. The addition of methionine to the iron deplete medium partially restored the ability to oversynthesize riboflavin in the 4R mutant. Additionally, the 4R strain had two-fold increased intracellular concentration of iron during cultivation in iron deplete/replete media. This increased concentration of iron apparently caused the inability of the 4R mutant to overproduce riboflavin in the iron deplete medium. It can suggest that methionine shortage somehow activates iron transport into the cells of the 4R strain (*met2* mutant).

Disruption of the *SEF1* gene, encoding a potential transcription factor, was identified after analysis of the 1R mutant. Data concerning Sef1p functions are poor. The *Kluyveromyces lactis SEF1* and its *S. cerevisiae* homologue are able to suppress the mutation in Rpm2p, which is a protein subunit of yeast mitochondrial RNase P, an enzyme responsible for the 5′ maturation of mitochondrial tRNAs. DNA sequence analysis of the *K. lactis SEF1* gene revealed that it contained the Zn(2)-Cys(6) binuclear cluster motif found in a growing number of yeast transcription factors (Groom et al., 1998). Apparently *SEF1* also acts as a regulatory gene in riboflavin synthesis in *C. famata*. However, the mechanisms of such regulation remain to be elucidated (Dmytruk et al., 2006).

5.5.3 *Identified* C. famata *Genes Coding for Enzymes of Riboflavin Synthesis*

For cloning of *C. famata* genes involved in riboflavin synthesis, the riboflavin-auxotrophic mutants from the strain L20105 (*leu2*) were isolated. Their biochemical identification was carried out by identification of accumulated intermediates of riboflavin synthesis in the cultural medium of the mutants (Dmytruk et al., 2004). As a result, mutants deficient in GTP cyclohydrolase II (*rib1*), specific reductase (*rib2*), 6,7-dimethyl-8-ribityllumazine synthase (*rib5*), 3,4-dihydroxy-2-butanone-4-phosphate synthase (*rib6*) and riboflavin synthase (*rib7*) were isolated. All of them also carried the *leu2* mutation. The mutants were used as recipients for

transformation with the genomic DNA library of *C. famata* VKM Y-9 to clone corresponding genes by complementation of growth defects on the medium without leucine and riboflavin. The DNA fragments containing genes *RIB1, RIB2, RIB5, RIB6* and *RIB7* were isolated as a result of the cloning. Plasmids with these genes successfully complemented riboflavin auxotrophies of corresponding mutants of another flavinogenic yeast species, *Pichia guilliermondii* (Dmytruk et al., 2004; Abbas et al., 2006).

Cloned DNA fragments containing *C. famata RIB* genes were sequenced (Voronovsky et al., 2004).

The 1,763 bp *C. famata* DNA fragment (EMBL Accession No AJ810169) of plasmid pCR1Xb (Dmytruk et al., 2004) contains 1,029 bp open reading frame (ORF) encoding a protein of 343 amino acids with calculated molecular mass of 38.1 kDa. The derived amino acid sequence shows extensive homology to the GTP cyclohydrolase II sequences of yeasts, other fungi and prokaryotes. Data on comparisons of the amino acid sequences together with results on the functional complementation of *C. famata* and *P. guilliermondii rib1* mutations by the plasmid pCR1Xb are sufficient to indicate that the 1,029 bp ORF of 1,763 bp *C. famata* DNA fragment from the plasmid encodes the riboflavin synthesis enzyme GTP cyclohydrolase II (*RIB1* gene).

The 1,501 bp *C. famata* DNA fragment (EMBL Accession No. AJ810170) of plasmid pCR2-1 (Dmytruk et al., 2004) contains 786 bp ORF encoding a protein of 262 amino acids with calculated molecular mass of 29.1 kDa. The derived amino acid sequence shows homology to sequences of corresponding reductases of yeasts, other fungi and prokaryotes. Data on comparisons of the amino acid sequences together with results on the functional complementation of *C. famata* and *P. guilliermondii rib2* mutations by the plasmid pCR2-1 suggest that the 786 bp ORF of 1,501 bp *C. famata* DNA fragment encodes the riboflavin synthesis enzyme HTP reductase (*RIB2* gene).

The analysis of 1,465 bp *C. famata* DNA fragment (EMBL Accession No AJ810173) of plasmid pPR5 (Dmytruk et al., 2004) found a presence of 615 bp ORF (845–1,459; see the DNA fragment AJ810173 of EMBL Bank) containing the putative intron of 123 bp (888–1,010). The intron holds the 5′ (GTAAGT, 888–893) and 3′ (TAG, 1,008–1,010) splice sites and also the branch site (TACTAAC, 996–1,002) (Bon et al., 2003). The ORF without the intron is 495 bp and encodes a protein of 164 amino acids with calculated molecular mass of 18.2 kDa. The derived amino acid sequence shows extensive homology to the dimethylribityllumazine synthase sequences of yeast, other fungi and prokaryotes. Data on comparisons of the amino acid sequences, together with results on the functional complementation of *C. famata* and *P. guilliermondii rib5* mutations by the plasmid pPR5, demonstrate that the ORF of *C. famata* DNA fragment from the plasmid encodes the riboflavin synthesis enzyme dimethylribityllumazine synthase (*RIB5* gene).

The 1,333 bp *C. famata* DNA fragment (EMBL Accession No. AJ810171) of plasmid pF (Dmytruk et al., 2004) contains a 612 bp ORF encoding a protein of 204 amino acids with calculated molecular mass of 22.7 kDa. The derived amino acid sequence indicates extensive homology to the dihydroxybutanone phosphate

synthase sequences of yeasts, other fungi and prokaryotes. Data on comparisons of the amino acid sequences, together with results on the functional complementation of *C. famata* and *P. guilliermondii rib6* mutations by the plasmid pF, prove that the 612 bp ORF of 1,333 bp *C. famata* DNA fragment from the plasmid encodes the riboflavin synthesis enzyme dihydroxybutanone phosphate synthase (*RIB6* gene).

The 1,515 bp *C. famata* DNA fragment (EMBL Accession No. AJ810172) of plasmid pCR7 (Dmytruk et al., 2004) contains a 711 bp ORF encoding a protein of 237 amino acids with calculated molecular mass of 26.3 kDa. The derived amino acid sequence shows extensive homology to the riboflavin synthase sequences of yeasts, other fungi and prokaryotes. Data on comparisons of the amino acid sequences together with results on the functional complementation of *C. famata* and *P. guilliermondii rib7* mutations by the plasmid pCR7 are sufficient to assert that the 711 bp ORF of 1,515 bp *C. famata* DNA fragment from the plasmid encodes the riboflavin synthesis enzyme riboflavin synthase (*RIB7* gene).

Attempts to clone the *C. famata RIB3* gene encoding DRAP deaminase from the genomic DNA library by functional complementation of growth defect of corresponding auxotrophic mutant of *P. guilliermondii* were unsuccessful (available *rib3* mutants of *C. famata* were not applicable during these attempts because of their instability) (Dmytruk et al., 2004). Therefore, the gene was isolated as a homolog of corresponding yeast genes from genomic DNA of *D. hansenii* CBS767 after publication of complete genomic sequence of the strain. The homolog of yeast DRAP deaminase genes was isolated by PCR as a 2,569 bp DNA fragment containing the 1,821 bp ORF. The fragment was incorporated into the plasmid p19PR3 (Voronovsky et al., 2004). The isolated gene complemented riboflavin auxotrophy of *P. guilliermondii* strain *rib3* deficient in DRAP deaminase. Thus, high homology to DRAP deaminase genes of yeasts, other fungi and prokaryotes and capability of complementation of *P. guilliermondii rib3* mutation are sufficient to assert that the 1,821 bp ORF of 2,569 bp *D. hansenii* DNA fragment encodes the riboflavin synthesis enzyme DRAP deaminase (*RIB3* gene).

Thus, the *C. famata* (*D. hansenii*) genes for riboflavin synthesis encoding GTP cyclohydrolase II (*RIB1*), HTP reductase (*RIB2*), 6,7-dimethyl-8-ribityllumazine synthase (*rib5*), 3,4-dihydroxy-2-butanone-4-phosphate synthase (*rib6*), riboflavin synthase (*rib7*) and DRAP deaminase (*RIB3*) were cloned and identified. In addition, the involvement of *C. famata* (*D. hansenii*) genes homologous to *S. cerevisiae MET2* (homoserine O-acetyltransferase) and *SEF1* (putative transcription factor) in positive regulation of riboflavin synthesis was shown (see Sects. 5.5.2.2 and 5.5.2.3).

5.5.4 Development of a Promoter Assay System in C. famata

A system for analysis of promoter activities was developed for *C. famata,* based on the *K. lactis LAC4* gene encoding β-galactosidase as a reporter gene and *C. famata* mutant *lac4* unable for lactose utilization as a recipient strain (Ishchuk et al., 2008). The *E. coli* β-galactosidase gene *lacZ* could not be used in the system because of

difference in codon usage between the bacterium and *C. famata*. The *C. famata* mutant *lac4* was transformed with the plasmid containing analyzable promoters fused with the promoterless *LAC4* gene. Resulting transformants (unlike the mutant *lac4*) were able to utilize lactose as a sole carbon source. The promoter strength was estimated on the basis of β-galactosidase activity assayed in the transformants. Different promoters of *C. famata* and *D. hansenii* (a teleomorph of *C. famata*) were analyzed using this approach. The results showed an adequacy of the *K. lactis LAC4* gene for evaluation of promoter strength in *C. famata*.

C. famata and *D. hansenii* promoters *CfTEF1* (*C. famata* translation elongation factor 1A), *CfPGI1* (*C. famata* phosphoglucoisomerase), *DhPGK1* (phosphoglycerate kinase of *D. hansenii*), *DhPGI1* (*D. hansenii* phosphoglucoisomerase), *DhPHO5* (*D. hansenii* acid phosphatase) and *DhTPI1* (*D. hansenii* triosephosphate isomerase) were cloned by PCR using the genome database 'Genolevures' for *D.hansenii* (http://cbi.labri.fr/Genolevures/index.php). The *CfTEF1* and *CfPGI1* promoters were isolated by the inverse PCR. The primers were designed for isolation of central region of the *D. hansenii TEF1* and *PGI1* ORFs. Corresponding fragments of expected size were amplified using these primers with total DNA of *C. famata* VKM Y-9 as a template. These primers were used to design inverse primers complementary to corresponding *C. famata* genome regions. In such a way, the sequences of *C. famata* VKM Y-9 genomic fragments containing promoters and initial part of both *TEF1* and *PGI1* ORFs were isolated. Sequence comparison of the *TEF1* fragments of strains *C. famata* VKM Y-9 and *D. hansenii* CBS 767 showed 100% identity of initial ORF fragments but just partial homology for promoters. Sequence comparison of the *PGI1* genomic fragments of strains *C. famata* VKM Y-9 and *D. hansenii* CBS 767 showed again a very high level of identity (91%) for coding regions and much lower homology for analyzed promoter region.

Activities of promoters of the genes *CfTEF1*, *CfPGI1*, *DhPGK1*, *DhPGI1*, *DhPHO5* and *DhTPI1* were tested in *C. famata* using the *K. lactis LAC4* reporter gene. It is known that the promoter of yeast *TEF1* (translation elongation factor 1α) gene as well as promoters of the glycolytic genes *PGK1* (phosphoglycerate kinase), *PGI1* (phosphoglucoisomerase) and *TPI1* (triosephosphate isomerase) are strong and constitutive under many different conditions. The promoter for *PHO5* (acid phosphatase) gene is regulable and induced by limitation of phosphate. Plasmids bearing the mentioned above promoters fused with the *K. lactis LAC4* gene were introduced into the *C. famata lac4* mutant. Quantitative liquid β-galactosidase assays performed in the obtained transformants showed very low activities for the promoters *DhPGI1*, *DhPHO5* and *DhTPI1* in *C. famata* cells in spite of a high degree of relatedness between the *C. famata* and *D. hansenii* strains. Only one of the tested *D. hansenii* promoters, *DhPGK1*, showed relatively high β-galactosidase activity, which was comparable with that of the *CfPGI1* promoter. The promoters *DhPGK1* and *CfPGI1* displayed 3- and almost 4-times lower activities of β-galactosidase, respectively, compared to those expressed from the promoter *CfTEF1*. Thus, the promoter *CfTEF1* was found to be a strongest one among tested (Ishchuk et al., 2008).

The developed *LAC4* reporter system can be used as a potent tool for understanding the regulation of riboflavin synthesis, halotolerance and other processes in *C. famata* and *D. hansenii*.

5.6 Applied Aspects

This section describes current uses of *D. hansenii* (*C. famata*) in production of riboflavin, cheesemaking, meat fermentation, involvement of *D. hansenii* lytic enzymes in food industry (particularly in wine-making), the potential of the yeast in production of xylitol and some other chemicals.

5.6.1 Production of Riboflavin

The ability of *C. famata* (also known as *Candida flareri, Torulopsis candida*) to overproduce riboflavin in iron-deficient media is known for 60 years (Tanner et al., 1945). This species possesses the highest flavinogenic potential among other yeasts capable to overproduce riboflavin under iron limitation. Wild-type strains can accumulate in the medium near 600 μg ml^{-1} (Levine et al., 1949) whereas other flavinogenic yeasts accumulate 5–300 μg ml^{-1} and normally yeasts accumulate not more than 1–2 μg of riboflavin per ml (Sibirny et al., 2006).

Specially selected mutant of *C. famata* dep8, along with mutants of other microorganisms (bacterium *Bacillus subtilis,* mycelial fungus *Ahbya gossypii*) are used for industrial production of riboflavin (Stahmann et al., 2000). The *C. famata* riboflavin overproducing strain dep8 was isolated by classic multistep selection procedure (Heefner et al., 1988, 1992, 1993). On the first step of selection, cells of the wild-type strain *C. famata* NRRL Y245 mutagenized with nitrozoguanidine and adenine-deficient mutant was isolated which accumulated red pigment at high concentration of exogenous adenine. This suggested that mutant has impaired regulation of purine synthesis *de novo* (it is known that riboflavin is synthesized from purine precursor, GTP) (Shavlovsky and Logvinenko, 1988). Independently, the mutant was isolated from the wild-type strain which was resistant to riboflavin synthesis inhibition exerted by 5′-AMP. On the next stage of selection, mutants from first steps were hybridized by protoplast fusion. One of hybrid, named as GA18, showed 14 times increased riboflavin productivity relative to wild-type strain. After mutagenesis of GA18, several yellow colonies were picked up. The most stable of them, strain GA18Y8-6#2 was again mutagenized and plated on the medium with toxic glucose analog, 2-deoxyglucose. One most flavinogenic 2-deoxyglucose-resistant strain was again mutagenized and plated on the medium with toxic purine analog, 4-aminopyrazolo-(3,4-d)pyrimidine. The most flavinogenic strain, designated as ATCC 20755 was isolated after such selection step. From it, the new 2-deoxyglucose mutants were isolated, which served for several next rounds of selection for yellow

colonies on the medium with high riboflavin content and poor for nutrients. The best flavinogenic mutant, designated as dep8, was used for isolation of the mutants resistant to tubericidin, another purine structural analog. Several new mutants were isolated, however, they did not differ significantly from strain dep8 by their flavinogenic potential (Heefner et al., 1988, 1992, 1993; Sibirny et al., 2006).

Isolated strain accumulated during large-scale fermentation (volume of fermenter, 450 l, duration of fermentation, 200 h) around 20,000 μg riboflavin in 1 ml of the medium, which is comparable to riboflavin titers reached by other riboflavin overproducers (*B. subtilis, A. gossypii*) (Stahmann et al., 2000). The strain dep8, however, is not very stable and can revert to quite non-flavinogenic revertants (A. Sibirny, non-published observation). It is important to identify reverting locus/loci and the nature of evolved mutations. Depending on results of such studies, the strategy for strain stabilization can be developed. For fulfillment this work, methods of molecular genetics developed for *C. famata* can be used. One may assume that identified recently regulatory gene of positive control *SEF1* (Dmytruk et al., 2006) is important for riboflavin oversynthesis. If successful, the stable *C. famata* riboflavin overproducers can be superior to bacterial and fungal competitors. Indeed, the process based on the use of *B. subtilis* is sensitive to phagolysis whereas riboflavin overproduction by *A. gossypii* starts only after finishing the growth and during lysis of mycelium (Stahmann et al., 2000). The process based on *C. famata* is free from these drawbacks as yeasts are resistant to phagolysis and riboflavin synthesis occurs during growth and propagation of the producer. Further improvement of strains of industrial producer *C. famata* dep8 can be reached using developed methods for genetic manipulation of this species (Voronovsky et al., 2002; Dmytruk et al., 2006; Abbas et al., 2006). For example, structural genes of riboflavin synthesis can be amplified, GTP cyclohydrolase can be engineered to decrease or to block totally feedback inhibition exerted by FAD, genes of purine biosynthesis pathway can be overexpressed.

5.6.2 Cheese Production

D. hansenii (*C. famata*) is an essential species of microflora of many dairy products including cheeses. It is the common yeast species found in all types of cheese. *D. hansenii* is also present in brines of semi-hard and hard cheeses (Fleet, 1990). The prevalence of this yeast in cheeses and brines is a result of its peculiar properties: salt tolerance, ability to produce proteolytic and lipolytic enzymes that can metabolize milk proteins and fat, capacity to grow at low temperatures and low water activities (Fleet and Mian, 1987; Roostita and Fleet, 1996; Wyder and Puhan, 1999; Prista et al., 2005). In addition, there was shown a capability of *D. hansenii* 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 (Deiana et al., 1984; Fatichenti et al., 1983; Breuer and Harms, 2006). *D. hansenii* also modifies the cheese microenvironment

and thus supports some desired bacteria and/or fungi and protect cheeses from undesired carbohydrate fermentations (van den Tempel and Jacobsen, 2000; Breuer and Harms, 2006). The capacity of *D. hansenii* to assimilate lactate, citrate, lactose and galactose in addition to glucose is very important for cheese making: it places the yeast among favorable components of starter cultures for cheese production (Fatichenti et al., 1983; Welthagen and Viljoen, 1998).

D. hansenii is capable of synthesis of volatile acids and cheesy flavor compounds, namely methyl ketones with fruity, rose, cheesy, moldy, wine odors, and 2-phenylethanol (the faded-rose odor) (Arfi et al., 2002; Leclercq-Perlat et al., 2004). The capacity of *D. hansenii* and *K. marxianus* to govern the sensory properties of cheeses has been exploited in starter cultures containing these two species (Seiler and Busse, 1990). Like many cheese-ripening yeasts, *D. hansenii* can synthesize *S*-methylthioacetate, the most prevalent volatile sulphur compound in cheese. The yeast can also produce methional. This compound was found in some kinds of cheeses, for instance, in Cheddar and Camembert. Methional contributes to development of a strong Cheddar flavor (Ferreira and Viljoen, 2003). There was also found that *D. hansenii* capable to produce substantial amounts of another volatile sulphur compound: methylthiopropanal (Arfi et al., 2002).

D. hansenii possesses the peptidase activity. A peptidase with activity to β-casein-derived peptides, which significantly influenced proteolysis in cheese, was found in the yeast. There was also reported on capability of *D. hansenii* isolated from cheese to digest both α- and β-casein (Klein et al., 2002; Kumura et al., 2002; Leclercq-Perlat et al., 2000).

D. hansenii can produce pigments (Hansen et al., 2001; van den Tempel and Jacobsen, 2000). The production by this yeast of reddish-brown pigments involved in the brown surface discoloration of Portuguese ewes' cheese was reported (Carreira et al., 1998). The tyrosinase activity in *D. hansenii* was shown. It initiates oxidation of tyrosine to melanine (Nichol et al., 1996). The pigment production during ascospore formation that resulted in pigmented ascospores and brown color of cultures was also described (Nakase et al., 1998).

D. hansenii and some other yeasts involved in cheese ageing provide developing an appropriate bacterial surface flora by supplying with growth factors for the bacteria, such as vitamins and amino acids. Simultaneously, the yeasts, including *D. hansenii*, produce aroma components, lipolytic and proteolytic enzymes that govern the process of ripening (El Soda, 1986; Fleet and Mian, 1987; Guerzoni et al., 1993; Lépingle et al., 2000; Petersen and Jespersen, 2004). *D. hansenii* can grow both on the surface and inside of processed cheeses. The yeast was found in the curd or in the cheese interior, depending on the kind of cheese and composition of the involved starter culture (Deiana et al., 1984; Fatichenti et al., 1983; Ferreira and Viljoen, 2003; Fleet, 1990; Roostita and Fleet, 1996; van den Tempel and Jacobsen, 2000; Welthagen and Viljoen, 1998).

Thus, *D. hansenii* plays important and diverse roles in cheese production. No doubt, additional studies of the yeast and the process are needed to provide knowledge-based improvements of production efficiency and cheese quality.

5.6.3 Meat Fermentation

D. hansenii is the most common yeast species found in different sausages and minced beef (Dalton et al., 1984). There was shown that *Debaryomyces* could generate ammonia and several volatile compounds, alter the free amino acid contents of dry-cured sausages. Addition of *Debaryomyces* sp. as a starter culture generally modified the flavour profile of the sausages (Dura et al., 2004b). Effect of *Debaryomyces* spp. on production of volatile compounds during the ripening of dry-cured sausages by inhibiting the generation of lipid oxidation products and promoting the generation of ethyl esters, processes that contribute to the development of typical sausage aroma was also reported (Flores et al., 2004). A decrease of pH to 4.5 during the growth of *Debaryomyces* under dry-cured sausage processing conditions 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 (Dura et al., 2004b). Similar results were reported for the modification of flavour profiles of dry-cured ham (Martin et al., 2004). Thus, representatives of *Debaryomyces* spp. govern the sensory properties of the meat by producing volatile compounds.

A few enzymes of *D. hansenii* or *Debaryomyces* spp. involved in meat fermentation were described. Activities of prolyl aminopeptidase, arginyl aminopeptidase and glutaminase during the ripening of dry-cured sausages were reported (Bolumar et al., 2003a, b; Dura et al., 2004a). Nitrate, nitrite, glucose and ascorbic acid (curing agents), 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 at levels typically used in meat processing on the activity of glutaminase (Dura et al., 2004a).

Yeasts play not only a positive role in meat processing. There is a range of reports on identification of yeasts (including *D. hansenii*) in the surface slime of sausages (Lodder 1970; Breuer and Harms, 2006). Thus, the yeasts are involved in spoilage of sausages.

Current knowledge on the effect of *D. hansenii* on meat fermentation is far not sufficient. Further studies are needed for elucidation of the effect and use of this yeast in the meat fermentation.

5.6.4 Lytic Enzymes

Some lytic enzymes of *D. hansenii* are involved in wine production. Particularly, β-glucosidases from *Debaryomyces* can liberate monoterpenols from β-D-glucopyranoside, β-D-xylopyranoside, α-L-arabinofuranoside and α-L-rhamnopyranoside. This property is useful for a possible enzyme treatment as part of the processing of terpenol-containing juices, leading to an increase of flavor compounds in the wine (Yanai and Sato, 1999). Some non-conventional yeasts, including *Debaryomyces*,

exhibit significantly higher levels of β-glucosidase activity in the absence of glucose than *S. cerevisiae*, indicating that they have a stronger role in flavor development during wine production than baker's yeast (Charoenchai et al., 1997). The production of extracellular glucose-tolerant and thermophilic β-glucosidases in wine making by 48 yeast strains belonging to the genera *Candida, Debaryomyces, Kluyveromyces* and *Pichia* was explored (Saha and Bothast, 1996). The β-glucosidases of *Debaryomyces* strains had very low activity, but they were able to hydrolyse cellobiose without inhibition of the enzymes by glucose. Moreover, the enzymes had a high optimal temperature: 65°C. The intracellular β-glucosidase from *D. hansenii* was purified and analysed (Yanai and Sato, 1999). It was tested in the fermentation of Muscat juices. This enzyme has a significant tolerance to high concentrations of glucose in grape juices, in contrast to commercially available β-glucosidases of mycelial fungi, which are sensitive to glucose (Yanai and Sato, 1999).

D. hansenii along with other non-*Saccharomyces* wine yeasts possesses with esterase, one of the main enzymes involved in wine production (Besancon et al., 1995; Esteve-Zarsoso et al., 1998). The enzyme was isolated from *D. hansenii* and partially characterized (Besancon et al., 1995). The enzyme hydrolysed tributyrin and ethyl butyrate with maximal activity, methyl and ethyl esters of short fatty acids (C_2–C_5) rapidly and esters of longer-chain fatty acids (C_6–C_{14}) moderately quickly. Aliphatic and aromatic acetate esters were also hydrolysed by the esterase.

D. hansenii has an activity of superoxide dismutase (SOD), a metalloenzyme catalysing the dismutation of superoxide radicals. The yeast can be a source of the enzyme. SOD has important applications in medicine and food industry, including anti-inflammation, immune-response modulation, malignant tumor regression, radiation and chemotherapy protection, premenstrual syndrome, arthritis, and anti-ageing treatments, during the use of hyperbaric chambers, and against oxidative stress in general (Garcia-Gonzalez and Ochoa, 1999; Orozco et al., 1998). The SOD gene of *D. hansenii* was cloned and sequenced (Hernandez-Saavedra and Romero-Geraldo, 2001). Potentially, SOD production using *D. hansenii* is very competitive compared to current methods, due to ability of the yeast to grow in media with a wide range of cultivation parameters (Ochoa et al., 1995).

5.6.5 Production of Xylitol

D. hansenii is one of the best xylitol-producing yeasts (Parajo et al., 1997; Roseiro et al., 1991). The capability of the yeast to produce xylitol from D-xylose is studied actively for many years (Roseiro et al., 1991; Girio et al., 1994; Parajo et al., 1997). However, profitable industrial technology for production of xylitol using this yeast is still not developed.

Xylitol is used in the food industry as a sweetener and anti-caries tool. It is employed in the manufacture of sugar-free food for diabetics (Cruz et al., 2000a). Xylitol is formed as a metabolic intermediate of D-xylose fermentation, where D-xylose is converted into xylitol by xylose reductase (XR). The enzyme has broad

substrate specificity for aldehydes and aldol sugars. The overproduction of xylitol by *D. hansenii* was shown to be a result of combination of high NADPH-dependent XR activity and low xylitol dehydrogenase (XDH) activity (Converti and Dominguez, 2001). *D. hansenii* can convert xylose to xylitol as effective as other yeast representatives of xylitol producers: *C. guilliermondii*, *C. boidinii* and *C. parapsilosis* (Barbosa et al., 1988; Furlan et al., 1991). The level of oxygen supply is very important for conversion of D-xylose to xylitol by *D. hansenii*. The conversion cannot be performed under strictly anaerobic conditions, because of very low activity of XR in the absence of oxygen. It prevents assimilation of D-xylose by the yeast (Dominguez, 1998; Roseiro et al., 1991). On the other hand, under strictly aerobic conditions the reaction products are used for biomass production resulting in very little or absence of xylitol. High oxygen levels inhibit XR more than XDH, and thus affect xylitol production in *D. hansenii* (Girio et al., 1994). Therefore, the accumulation of xylitol is increased by limited aeration (Parajo et al., 1997; Converti and Dominguez, 2001). *D. hansenii* cultivated on oxygen-excess chemostat produced neither ethanol nor xylitol over the entire range of dilution rates tested (Nobre et al., 2002). On oxygen-limited chemostat the metabolism of the yeast changed substantially and, due to oxidative phosphorylation limitation, the cell biomass decreased and xylitol became the major extracellular product.

There are a few publications describing the capability of *D. hansenii* to grow in sugar mixtures. Particularly, according to the one article, growth on pentoses was slower than growth on hexoses, but the values obtained for biomass yields were very similar with the two types of sugars. Furthermore, when mixtures of two sugars were used, a preference for one carbon source did not inhibit consumption of the other (Nobre et al., 1999). It is important during fermentation of hydrolysates of hemicelluloses, which often consist of sugar mixtures. The other study showed a dependence of the growth of *D. hansenii* on the type of carbohydrate supplied (Tavares et al., 2000). According to the latter article, 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.

Dilute-acid hydrolysis of hemicellulose results in a range of byproducts (inhibitors), such as furfural and hydroxymethylfurfural, acetic acid, lignin degradation products, phenols. These compounds limit fermentation of resulting hydrolysates. Submission of the hydrolysates to detoxification treatments minimized inhibitory effects. Particularly, activated charcoal adsorption removed most of the lignin-derived compounds; evaporation was able to reduce the acetic acid concentration below the inhibition threshold. The combination of the treatments gave improvement of fermentation of the D-xylose solutions into xylitol (Converti et al., 1999). During growth of *D. hansenii* on undetoxified dilute-acid hydrolysate of brewery's spent grain, the yeast showed high biomass yields and productivity (Carvalheiro et al., 2004). Detoxification of the substrate with activated charcoal did not increase biomass yield and improved the volumetric productivity just slightly. Thus, detoxification was not useful in the study (Carvalheiro et al., 2004). The obtained results also suggest that *D. hansenii* (or some strains at least) during growth in some lignocellulosic hydrolysates has a resistance to fermentation inhibitors.

Optimization of xylitol production processes is very important for achievement of maximal productivity and yield of the final product. The importance of high cell concentrations for efficient xylitol production was shown. For instance, a low xylitol productivity (0.088 g $(l \times h)^{-1}$) and product yield (0.57 g g^{-1}) were reached when the starting cell concentration below 16 g biomass l^{-1} and D-xylose concentration above 70 g xylose l^{-1} were taken for fermentation. But, when initial cell concentration was increased to 50 g biomass l^{-1} at the same D-xylose concentration (>70 g xylose l^{-1}), the xylitol productivity amounted to 0.50 g $(l \times h)^{-1}$ and the yield was 0.73 g g^{-1} (Parajo et al., 1996). The high xylitol productivity (2.53 g $(l \times h)^{-1}$) was achieved in continuous fermentation of *D. hansenii* with maintaining the high cell concentration (with cell recycling at a dilution rate of 0.284 h^{-1}) (Cruz et al., 2000b). In addition to cell concentration, the substrate concentration is an important factor as well. High xylitol productivities (0.481–0.694 g $(l \times h)^{-1}$) and product yields (0.74–0.83 g g^{-1}) were reached during fermentation of *D. hansenii* at xylose concentrations between 90 and 200 g l^{-1} (Converti et al., 2002). The further increase of D-xylose concentrations (over 200 g l^{-1}) resulted in substrate inhibition. A study of effects of starting xylose concentration and oxygen mass flow rate on xylitol production from rice straw hydrolysate by *D. hansenii* found the optimal D-xylose concentration (71 g l^{-1}) and oxygen mass flow rate (4.1 mg O_2 s^{-1}) required to reach the maximal productivity (0.53 g $(l \times h)^{-1}$), product yield (0.71 g g^{-1}) and final xylitol concentration (42.2 g l^{-1}) (De Faveri et al., 2004).

Data presented on xylitol production by *D. hansenii* show that this process has been intensively studied. However, both the yeast and the process have to be further improved to provide a profitable industrial technology for production of the alditol.

In addition to xylitol, *D. hansenii* can synthesize other biotechnologically relevant products. Particularly, the *C. famata* strain is capable to produce D-arabinitol from D-glucose (Ahmed et al., 1999). The efficient conversion of glucose to arabinitol by *D. hansenii* (*C. famata*) can be used as a first step for production of D-xylitol from D-glucose (Mayer et al., 2002). Thiamine auxotrophs of *D. hansenii* accumulate pyruvic acid under thiamine limitation. Thus, potentially, the yeast can be used for production of the acid. *Debaryomyces* has a biotechnological advantage because it is capable to use inorganic ammonium as a sole nitrogen source (Yanai et al., 1994). Pyruvic acid is widely used in chemical, pharmaceutical and agrochemical industries. The acid is produced mainly by chemical method. But, the biotechnological production of pyruvic acid using the yeast *Candida glabrata* is also known (Li et al., 2001). The yield of pyruvic acid has to be improved significantly for *D. hansenii* to provide the industrially profitable process using the yeast.

5.7 Future Perspectives

C. famata (D. hansenii) possesses several interesting peculiarities, among which the highest productivity of riboflavin synthesis among all natural yeast isolates is apparently the most outstanding feature. Due to sophisticated scheme of classic

selection, very active industrial producer was obtained which accumulate more than 20 mg riboflavin per ml of the cultural broth. However, to compete with modern industrial riboflavin producers *B. subtilis* and *A. gossypii*, isolated on the base of molecular genetics methods, available industrial producer *C. famata* dep8 has to be improved using developed modern approaches of metabolic engineering. Strategies for strain improvement can include the amplification of structural genes of riboflavin synthesis (all genes or those limiting the process), protein engineering of GTP cyclohydrolase for decrease or total alleviation of feedback inhibition of this enzyme by FAD, increase in supplying the riboflavin synthesis with purine precursor, which can be achieved by overexpression the pathway of purine synthesis *de novo* and reactions from IMP to GMP as well as by impairment of allosteric inhibition of glycine phosphoribosylaminotransferase, the first enzyme of purine nucleotide biosynthesis pathway. Additionally, genetic stability of current producer has to be elevated. Riboflavin overproducers can be used as parental strains for construction of the producers of flavin nucleotides, FMN and FAD. As in *C. famata* riboflavin synthesis is not repressed by flavins, this organism can easier permit producers of flavin nucleotide relative to other riboflavin producers. However, question of toxicity of excess of flavin nucleotide content in the cell remains this possibility the open question.

Modern approaches of metabolic engineering and natural ability of *D. hansenii* to produce pentitols (xylitol and arabitol) opens possibility to construct efficient strains which will be able for efficient conversion of cheap glucose to xylitol or arabitol (with subsequent conversion of the last compound to xylitol using bacterial isomerase). As xylitol market is large and increasing, the corresponding work is highly desirable.

Much attention has been paid to natural tolerance of *D. hansenii* to high salt concentration. This feature promises potential benefit for many biotechnological processes as this yeast can be cultivated in semi-sterile conditions (Breuer and Harms, 2006). However, molecular mechanisms of high salt tolerance are far from elucidation and have to be studied in more details. Further development deserves ability of this species to produce some enzymes and toxins, to participate in cheese ripening and other processes.

To achieve these goals, researchers need to improve molecular genetic tools for studying this species. It is especially important to develop methods which provide homologous recombination, precise gene integration and knock out for *C. famata (D. hansenii)*.

5.8 Conclusions

C. famata (D. hansenii) represents the group of osmotolerant yeast strains capable of growing at high salt concentration (up to 4 mM NaCl). This yeast is natural overproducer of riboflavin, capable of xylitol, arabitol and industrially important enzyme production. Strains of this species are involved in ripening of cheeses and

in meat fermentation. Industrial riboflavin production based on *C. famata* strain dep8 exists. During last several years, much progress was achieved in molecular genetics study of *C. famata* (*D. hansenii*) due to complete sequencing genome of the type strain CBS767 and development of transformation system and methods of insertional mutagenesis for the strain VKM Y-9. These achievements are the pre-requisite for further development of existing process of riboflavin production as well as for creation the strains capable of production flavin nucleotides, xylitol from glucose and some industrial enzymes.

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