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MINI-REVIEW

The yeast *Kluyveromyces marxianus* and its biotechnological potential

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Abstract Strains belonging to the yeast species *Kluyveromyces marxianus* have been isolated from a great variety of habitats, which results in a high metabolic diversity and a substantial degree of intraspecific polymorphism. As a consequence, several different biotechnological applications have been investigated with this yeast: production of enzymes (β-galactosidase, β-glucosidase, inulinase, and polygalacturonases, among others), of single-cell protein, of aroma compounds, and of ethanol (including high-temperature and simultaneous saccharification-fermentation processes); reduction of lactose content in food products; production of bioingredients from cheese-whey; bioremediation; as an anticholesterolemic agent; and as a host for heterologous protein production. Compared to its congener and model organism, *Kluyveromyces lactis*, the accumulated knowledge on *K. marxianus* is much smaller and spread over a number of different strains. Although there is no publicly available genome sequence for this species, 20% of the CBS 712 strain genome was randomly sequenced (Llorente et al. in FEBS Lett 487:71–75, 2000). In spite of these facts, *K. marxianus* can envisage a great biotechnological future because of some of its qualities, such as a broad substrate spectrum, thermotolerance, high growth rates, and less tendency to ferment when exposed to sugar excess, when compared to *K. lactis*. To increase our knowledge on the biology of this species and to enable the potential applications to be converted into industrial practice, a more systematic approach, including the careful choice of (a) reference strain(s) by the scientific community, would certainly be of great value.

Keywords *Kluyveromyces marxianus* · Yeast biotechnology · Yeast physiology · Yeast taxonomy

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Taxonomic history of the present species *Kluyveromyces marxianus*

Kluyveromyces marxianus was first described in 1888 by E. C. Hansen, which at that time was named *Saccharomyces marxianus* after Marx, the person who originally isolated this yeast from grapes (Lodder and Kreger-van Rij 1952). In their monograph, Lodder and Kreger-van Rij (1952) describe ten strains of *S. marxianus*, among which a particular strain labeled *Zygosaccharomyces marxianus*, which had been deposited at the Centraalbureau voor Schimmelcultures (CBS) in 1922 by H. Schnegg, was arbitrarily chosen as the type strain. This corresponds to the present CBS 712 strain. Some differences among the ten mentioned strains were already pointed out at that time, regarding the formation of pseudomycelium, and the capacities of assimilating and fermenting lactose. Rotting leaves of sisal, sewage of a sugar factory, and "Lufthefe" (aerated yeast) are other habitats from which the strains of *S. marxianus* had been isolated. Already in 1939, Sacchetti had observed that inulin is fermented by *S. marxianus* (Lodder and Kreger-van Rij 1952). Although it was already recognized at that time that *S. marxianus* and *Saccharomyces fragilis*, which had been isolated from kefir in 1909 by

Jörgensen, were very closely related, they were considered distinct species (Lodder and Kreger-van Rij 1952). In 1951, Luh and Phaff affirmed that *S. fragilis* "is the only yeast species capable of attacking pectin" (Lodder and Kreger-van Rij 1952). Yoghurt, soft cheese, a lung with tuberculosis, Koumiss (a beverage made of fermented mare's milk), a human lesion of tonsils and the pharynx, and feces are other habitats from which the 11 strains belonging to the *S. fragilis* species had been obtained from (Lodder and Kreger-van Rij 1952).

Mainly due to differences in spore and ascus morphology, in the capacity of fermenting and oxidizing different sugars, and in the occurrence of hybridization between strains, when compared to true *Saccharomyces* yeasts, there was a need to reclassify the former species *S. fragilis* and *S. marxianus*, besides *Saccharomyces lactis*, into a new taxon (van der Walt 1970). In 1956, van der Walt described the new genus *Kluyveromyces*, the type species of which was *Kluyveromyces polysporus* (van der Walt 1956). Later, it was found that the latter yeast had very similar properties to the three above-mentioned species, and consequently, they were all reclassified into the genus *Kluyveromyces*, which encompassed 18 species in the second edition of *The Yeasts, a taxonomic study* (Lodder 1970). Additional habitats from which strains had been isolated include Bantu Beer, milk of a mastitic cow, asthmatic expectoration, and maize meal (van der Walt 1970). Again, *Kluyveromyces fragilis* was considered closely related but still separate from *K. marxianus*, mainly due to the former's high capacity of fermenting lactose. Dairy products and human and animal lesions were the prevalent origin of strains in the *K. fragilis* taxon (van der Walt 1970).

In the third edition of *The Yeasts, a taxonomic study* (Kreger-van Rij 1984), the genus *Kluyveromyces* was divided into 11 species. On the basis of interfertility, the taxon *K. marxianus* was organized into seven varieties, which are able to readily hybridize (van der Walt and Johannsen 1984). Concomitantly, the former species *K. fragilis* and *K. lactis* disappeared.

In the most recent edition of *The Yeasts, a taxonomic study* (Kurtzman and Fell 1998), the chapter on the *Kluyveromyces* genus includes 15 species. The seven varieties within the *K. marxianus* species, proposed in the previous edition of the monograph, were eliminated by considering them as either independent species (e.g., *K. lactis* and *K. dobzhanskii*) or synonyms of *K. lactis* or *K. marxianus* (Lachance 1998).This is due to the examination of the genetic structure of populations, in combination with hybridization ability, as criteria for classification. Consequently, the former species or varieties *Kluyveromyces bulgaricus*, *K. cicerisporus*, *K. fragilis*, and *K. wikenii* could not be considered distinct from *K. marxianus* (Lachance 1998).

Since the biological concept of species cannot be applied to homothallic organisms, such as the majority of yeasts in the *Kluyveromyces* taxon, any classification is always based on arbitrary criteria, which have changed along time, as discussed above. Since the development of rapid and efficient gene sequencing tools, it became natural to utilize gene sequences as the criterion for the comparison and classification of microorganisms into the different taxa. Rather than performing single gene comparisons, the most recent reports on the taxonomy of *Kluyveromyces* yeasts employ multigene sequence analyses for elucidating the phylogeny of the different strains. Using this strategy, Kurtzman and Robnett (2003) showed that the species described by Lachance (1998) in the *Kluyveromyces* genus are actually distributed into six clades, indicating the polyphyly of this group of yeasts. This is mainly due to the previous criteria employed in classification, such as ascus morphology (in this particular case, ascus deliquescence), which are inadequate as phylogenetic descriptors (Kurtzman 2003; Kurtzman and Robnett 2003). It has been proposed that genera should be circumscribed according to the phylogenetically defined clades, rather than on phenotypic analyses (Kurtzman and Robnett 2003). As a result of this, the number of species in the *Kluyveromyces* genus decreased to six and the species *K. marxianus* is proposed as the conserved type species (Kurtzman 2003; Lachance 2007). The type species of the originally described *Kluyveromyces* genus (van der Walt 1956), namely, *K. polysporus*, has been reclassified into the newly proposed *Vanderwaltozyma* genus (Kurtzman 2003; Lachance 2007).

Biochemistry, metabolism, and physiology

It should be noted that the great majority of studies published on *K. marxianus* have not aimed at looking into its biochemistry, metabolism, or physiology. Most of the works that are publicly available explored potential applications of this organism (see "Biotechnological applications"), without investigating what takes place at the intracellular level. Typically, the yeast cells have been cultivated on a specific substrate, and measurements have been carried out in such a way that only the concentrations of a substrate and of a product, besides the cell concentration, are determined. In what concerns physiology, carbon balances are very rarely looked at, meaning that it is only possible to have a rough macroscopic picture of the cellular reactions and, hence, of the organism's physiology.

Since the 1970's, a number of studies has been published on biochemical and metabolic aspects of different *K. marxianus* strains (a summary is presented in Table 1). Some studies were actually aimed at identifying suitable classification methods for *K. marxianus*, which has always been a challenging task. These include the observation that, in contrast to *S. cerevisiae*, ergosterol is the only sterol

Table 1 Compilation of biochemical and metabolic studies performed with the yeast *K. marxianus*

Target of the study	Strain employed	Reference
Sterol composition	*K. fragilis* NCYC 100	Penman and Duffus (1974)
Subcellular localization of the enzyme alcohol dehydrogenase (ADH)	*K. fragilis* H32	Künkel and May (1976)
Measurement of heat evolution by microcalorimetry	*K. fragilis* NCYC 100	Beezer et al. (1979)
Characterization of the enzyme fructose-1,6-bisphosphatase	*K. fragilis* ATCC 10022	Toyoda and Sy (1984)
Lactose symporter	*K. marxianus* CBS 397; *K. marxianus* IGC 2902, CBS 712, IGC 2587, IGC 2671, IGC 3014, NRRL Y-1122, CBS 397	Van den Broek et al. (1987); Carvalho-Silva and Spencer-Martins (1990)
High- and low-affinity glucose transporters	*K. marxianus* IGC 2587	Gasnier (1987)
Regulation of four transport systems identified during the exponential and the stationary phases of batch growth on glucose	*K. marxianus* CBS 397	De Bruijne et al. (1988)
High- and low-affinity symporters of glucose and fructose	*K. marxianus* CBS 6556	Postma and Van den Broek (1990)
Proton-motive force-driven transport of galactose	*K. marxianus* CBS 397	van Leeuwen et al. (1991)
Transport of lactic acid	*K. marxianus* IGC 3014	Fonseca et al. (1991)
Mechanism of the enzyme UDP glucose 4-epimerase	*K. fragilis* ATCC 10022 (presumed, from one of the articles)	Mukherji and Bhaduri (1992); Bhattacharjee and Bhaduri (1992); Ray et al. (1995); Majumdar et al. (1998)
Absence of complex I NADH ubiquinone oxidoreductase	*K. marxianus* K5 (own collection)	Büschges et al. (1994)
Regulation of adenylate cyclase by Ras proteins	*K. marxianus* CBS 5795W	Verzotti et al. (1994)
Coenzyme Q system and the monosaccharide pattern of cell wall	*K. marxianus* (various strains)	Molnár et al. (1996)
Behaviour of *K. marxianus* during autolysis	*K. marxianus* CBS 397	Amrane and Prigent (1996)
Presence of killer activity	*K. marxianus* (isolated as a result of the work)	Abranches et al. (1997)
Glucose repression via Mig1p	*K. marxianus* SGE11 (Montpellier University)	Cassart et al. (1997)
composition of the cell wall	*K. marxianus* R157, 1586 (University of New South Wales)	Nguyen et al. (1998)
Presence of active efflux pumps involved in drug resistance	*K. marxianus* IGC 2671	Prudêncio et al. (2000)
Transport of malic acid via a symport mechanism	*K. marxianus* ATCC 10022, KMS3 (derivative of CBS 6556)	Queirós et al. (1998)
Identification and characterization of a cell-wall acid phosphatase	*K. marxianus* Y-610 (identical to ATCC 12424)	Yoda et al. (2000)
Capacity of using xenobiotic compounds as nitrogen source	*K. fragilis* UU1; *K. marxianus* IMB3	Ternan and McMullan (2000); Ternan and McMullan (2002)
Response of the NADP+-dependent glutamate dehydrogenase to nitrogen repression	*K. marxianus* CBS 6556	de Morais (2003)
Characterization of an amine oxidase	*K. marxianus* CBS 5795	Corpillo et al. 2003
The transport mechanism of xylose	*K. marxianus* ATCC 52486	Stambuk et al. 2003

This list does not include enzymes of industrial interest, which are separately listed in Table 2.

present in *K. marxianus*; the use of microcalorimetry for identification purposes; and the characterization of coenzyme Q and monosaccharide patterns of cell walls (Table 1).

In other cases, *K. marxianus* was simply used as the source of specific compounds, which were the actual focus of research (mainly enzymes). Examples of this kind of study include those on fructose-1,6-bisphosphatase, uridine diphosphate (UDP) glucose-4-epimerase, an acid phosphatase, an amine oxidase, protein phosphatases, carboxypeptidases, and aminopeptidases (Tables 1 and 2). A number of transport studies were also carried out, namely, on the transport of sugars (lactose, glucose, fructose, galactose, and xylose) and organic acids (lactic and malic acids) (Table 1). Finally, a few metabolic studies were carried out with the aim of characterizing some non-transport aspects of *K. marxianus*, such as the absence of complex I in the respiratory chain, the regulation of adenilate cyclase by Ras proteins, the functional characterization of Mig1p (involved in glucose repression), analysis of cell-wall composition, presence of efflux pumps and their role in drug resistance, and the regulation of the nicotinamide adenine dinucleotide phosphate (oxidized form) ($NADP^+$)-dependent glutamate dehydrogenase (Table 1). In many cases, these studies were performed in parallel with other yeasts, mainly with *S. cerevisiae*.

In terms of biochemical studies on enzymes that have industrial interest, *K. marxianus* has been used as a source of inulinase, β-galactosidase, β-glucosidase, and endopolygalacturonases (Table 2). Besides these, some less widespread enzymes with potential industrial application, such as protein phosphatases, carboxypeptidases, and aminopeptidases have also been investigated more recently (Table 2).

In the 1970s, physiological studies focusing on the influence of some common environmental factors on the growth of *K. marxianus* started to appear in the literature, as a reflection of the eventual interest in using this yeast for industrial applications. Chassang-Douillet et al. (1973) presented the first clear physiological comparison of *K. marxianus* and *S. cerevisiae*, carried out using synthetic media and demonstrating that the so-called glucose effect was absent in *K. marxianus*, as opposed to *S. cerevisiae*. Later studies reported on the effects of pH (de Sánchez and Castillo 1980), ethanol concentration (Bajpai and Margaritis 1982), and sugar concentration (Margaritis and Bajpai 1983) on the growth kinetics of *K. marxianus*. Importantly, *K. marxianus* started to be included in comparative biochemical and physiological studies on yeast in general, such as those related to catabolite repression (Eraso and Gancedo 1984), sensitivity toward toxins (Sukroongreung et al. 1984), and growth inhibition by fatty acids (Viegas et al. 1989).

The use of defined synthetic media combined with chemostat cultivations for quantitative physiological studies started around the 1990s, with works focusing on the regulation of respiration and fermentation and on the so-called Crabtree-effect in yeasts (van Urk et al. 1990; Verduyn et al. 1992). It was then quantitatively shown that *K. marxianus* presents a strong Crabtree-negative character, since no ethanol production was observed after a glucose pulse applied to respiring cells, in contrast to what is commonly observed with *S.*

Table 2 Studies on the biochemistry of enzymes of industrial interest performed with the yeast *K. marxianus*

Enzyme	Application	Strain	Reference
Inulinase	Production of fructose syrup from inulin-containing feed-stocks	*K. fragilis* ATCC 12424; *K. marxianus* CBS 6397, CBS 6556	Workman and Day (1984); Rouwenhorst et al. (1988, 1990a,b)
β-galactosidase	Reduction of lactose content in foods	*K. fragilis* (several strains); K. marxianus NCYC 111; *K. marxianus* ATCC 10022; K. marxianus IMB3; K. marxianus CBS 6556	Mahoney et al. (1975); Gonçalves and Castillo (1982); Bacci Júnior et al. (1996); Brady et al. (1995); Martins et al. (2002)
β-glucosidase	Hydrolysis of cellulosic materials	*K. fragilis* ATCC 12424	Raynal and Guerineau (1984); Leclerc et al. (1987)
Endopolygalacturonases	Reduce of viscosity in fruit processing products	*K. marxianus* CCT 3172; CCT 3172 (overproducing mutant); an unidentified NCYC isolate	Jia and Wheals (2000)
Protein phosphatases	modification of cheese-making qualities of caseins	*K. marxianus* (strain not indicated)	Jolivet et al. (2001)
Carboxypeptidases	reduction of bitter taste in protein-containing foods	*K. marxianus* (own isolate)	Ramírez-Zavala et al. (2004b)
Aminopeptidases	direct processing or aging of dairy and meat products	*K. marxianus* (own isolate)	Ramírez-Zavala et al. (2004a)

Only studies which focused on the biochemistry of enzymes are indicated here. For studies aiming at enzyme production, please refer to the section on "Biotechnologial applications"

cerevisiae and even with *K. lactis*, to a lesser extent (Kiers et al. 1998). This was later confirmed by Bellaver et al. (2004). Castrillo and Ugalde (1993) showed that when oxidoreductive metabolism sets in in *K. marxianus*, as a function of increasing glycolytic flux, the maximum respiratory capacity of the cells has not yet been achieved, which is in contrast with the situation in *S. cerevisiae*, in which the onset of respirofermentative metabolism coincides with the achievement of its maximum respiratory capacity. From these studies, *K. marxianus* was classified as facultatively fermentative and Crabtree-negative (van Dijken et al. 1993). It is important to note that it cannot grow under strictly anaerobic conditions and that the occurrence of ethanol formation is almost exclusively linked to oxygen limitation (Visser et al. 1990; van Dijken et al. 1993; Bellaver et al. 2004). More recently, Blank et al. (2005) showed that *K. marxianus* presents the highest tricarboxylic acid cycle flux during batch growth on glucose among the 14 hemiascomycetous yeasts studied within the Génolevures consortium (Souciet et al. 2000).

Other physiological studies report on various issues, such as flocculation (Fernandes et al. 1992, 1993), the influence of CO_2 on the survival of *K. marxianus* (Isenschmid et al. 1995), the influence of the specific growth rate on the morphology of the NRRLy2415 strain, which displays significant growth in pseudo-hyphal form (O'Shea and Walsh 2000), the effects of increased air pressure on the biomass yield of *K. marxianus* (Pinheiro et al. 2000), the response of *K. marxianus* to oxidative agents such as hydrogen peroxide (Pinheiro et al. 2002), and the macromolecular composition of *K. marxianus* cells as a function of the specific growth rate (Fonseca et al. 2007). The later data can be particularly useful for metabolic flux analysis studies.

One important aspect on the physiology of *K. marxianus* is the fact that significantly different growth parameters, such as μ_{max} and $Y_{x/s}$, have been reported not only for different strains within the species but also for the same strain when investigated in different laboratories (Fonseca et al. 2007).

From the data in Tables 1 and 2 (and also in Table 3), it can be observed that the number of strains that have been investigated is quite large, and many of them were not

Table 3 Biotechnologically relevant genes sequenced in *K. marxianus*

Gene/function	Strain	Reference
β-glucosidase	*K. fragilis* ATCC 12424	Raynal et al. (1987)
INU1/inulinase	*K. marxianus* ATCC 12424	Laloux et al. (1991)
LEU2/β-isopropylmalate dehydrogenase	*K. marxianus* CBS 6556	Bergkamp et al. (1991)
URA3/orotidine-5′-phosphate decarboxylase	*K. marxianus* CBS 6556	Bergkamp et al. (1993b)
PDC/pyruvate decarboxylase	*K. marxianus* ATCC 10606	Holloway and Subden (1993)
ADH1/alcohol dehydrogenase	*K. marxianus* ATCC 12424	Ladrière et al. (1993)
ABF1/a DNA binding protein	*K. marxianus* (strain not indicated)	Oberyé et al. (1993)
the *GAP* family/glyceraldehyde-3-phosphate dehydrogenases	*K. marxianus* ATCC 10022	Fernandes et al. (1995)
LAC4/β-galactosidase	*K. fragilis* (strains not indicated)	Huo and Li (1995)
MIG1/DNA-binding protein involved in glucose repression	*K. marxianus* SGE11 (Montpellier University)	Cassart et al. (1997)
EPG1/endopolygalacturonase	*K. marxianus* BKM Y-719	Šiekštelė et al. (1999)
PCPL3/purine-cytosine permease	*K. marxianus* ATCC 12424	Ball et al. (1999)
ADH2/alcohol dehydrogenase	*K. marxianus* ATCC 12424	Ladrière et al. (2000)
17% of the genome (1,300 genes by a partial random strategy)	*K. marxianus* CBS 712	Llorente et al. (2000)
Inulinase	*K. marxianus* (strain not indicated)	GenBank AF178979
URA3/orotidine-5′-phosphate decarboxylase	*K. cicerisporus* CBS 4857	Zhang et al. (2003)
QOR/NADPH quinone oxidoreductase	*K. marxianus* KCTC 7155	Kim et al. (2003)
URA 9/dihydroorotate dehydrogenase 2	*K. marxianus* NRRL Y-8281	GenBank AY444339
HIS3/imidazoleglycerol-phosphate dehydratase	*K. cicerisporus* CBS 4857	GenBank AY303539
OYE/old yellow enzyme	*C. macedoniensis* AKU 4588	Kataoka et al. (2004)
EPG1–2/endopolygalacturonase	*K. marxianus* CECT 1043	GenBank AY426825
FPS1/plasma membrane glycerol channel	*K. marxianus* IGC 3886	Neves et al. (2004)
β-galactosidase	*K. marxianus* (strain not indicated)	GenBank AY526090
Exoinulinase	*K. marxianus* IW 9801	GenBank AY649443
TPI1/triosephosphate isomerase	*K. marxianus* (strain not indicated)	GenBank AJ577476

Partial gene sequences deposited in public databases and sequences coding for RNA were not included. GenBank accession numbers were only given when there is no available article to be cited.

obtained directly from the main culture collections worldwide. If, on the one hand, this leads to an interesting metabolic diversity and to several potential applications, as described below in this review, it makes it difficult, on the other hand, to gain fundamental knowledge on the metabolism and physiology of this yeast. In this sense, it would be necessary that researchers started using a reduced number of strains (chosen from key culture collections), similarly to the way in which the *K. lactis* community has been using the CBS 2359 strain (Lachance 1998; Fukuhara 2006). This would allow the development of efficient molecular genetic tools for *K. marxianus* (probably starting with genome sequencing), which are the basis for performing systematic studies that will finally lead to a better understanding of the biology of this species. A possibility would be to choose one or two strains with characteristics that have given *K. marxianus* a clear advantage over other yeasts: thermotolerance, high growth rate, absence of fermentative metabolism upon sugar excess, and a broad substrate spectrum. For making this choice, an approach as the one reported by van Dijken et al. (2000) could be followed.

Recombinant DNA technology

As with any (potential) industrial organism, rational genetic manipulation is one of the most efficient ways of optimizing process yield and/or productivity. In some cases, the application of recombinant DNA (rDNA) technology may even become a prerequisite for a successful industrial process, either to increase product titer and/or purity to levels at which the process becomes economically feasible or to render the producing host capable of synthesizing a heterologous compound. This kind of activity has been well known as metabolic engineering, which is now a consolidated discipline (Stephanopoulos et al. 1998). rDNA technology is also an invaluable technique for genetic and physiological studies, which in turn are essential for increasing our understanding of *K. marxianus*.

Already more than two decades ago, transformation methods for inserting foreign DNA into *K. marxianus* have been developed. Das et al. (1984) constructed a plasmid called pGL2, containing the kanamycin resistance gene as a dominant selectable marker, and the *KARS2* autonomously replicating sequence of *K. lactis*. They showed that the transformation method of intact cells with alkali cations, originally developed for *Saccharomyces cerevisiae* by Ito et al. (1983), also worked in the strain *K. fragilis* C21. However, the transformation efficiency was rather low.

A breakthrough in molecular biology research of *Kluyveromyces* yeasts was the discovery of the pKD1 plasmid in the species *Kluyveromyces drosophilarum* (Falcone et al. 1986). The 4.8-kb, 1.65 μm pKD1 plasmid proved to have a similar organization but different sequences and host specificities, when compared to other already known plasmids, such as the 2 μ plasmid of *Saccharomyces* yeasts (Chen et al. 1986). In contrast to the latter, pKD1 can be maintained stable in *K. lactis*, but not in *S. cerevisiae*, in the absence of selective pressure (Bianchi et al. 1987). Later, it was shown that the insertion of the kanamycin resistance gene, the *URA3* gene of *S. cerevisiae*, a replication origin for *E. coli*, and the ampicillin resistance gene into pKD1 rendered a shuttle plasmid that could be transformed and maintained in *K. marxianus* strains CBS 6556 and CBS 712, though still with low-transformation efficiencies (Chen et al. 1989). This is in accordance with the fact that ARS and centromere sequences of *K. lactis* work in *K. marxianus* and vice versa (Das et al. 1984; Iborra and Ball 1994). Thus, pKD1-based plasmids have become the most common choice for inserting foreign DNA sequences into *K. marxianus* (Bergkamp et al. 1993b; Bartkevičiute et al. 2000; Zhang et al. 2003).

Iborra (1993) reported for the first time transformation efficiencies in the order of hundreds to thousands of transformants per microgram of DNA with *K. marxianus*, either with the lithium method (Ito et al. 1983) or using electroporation (Meilhoc et al. 1990). Similar results were obtained more recently by Zhang et al. (2003).

Besides requiring efficient vectors and transformation protocols, foreign gene expression also depends on the promoter and eventually a signal sequence for directing the synthesized protein into the extracellular environment, which usually facilitates downstream operations. For this purpose, Bergkamp et al. (1993a) used the promoter and prepro-signal sequence of the *INU1* (inulinase) gene to successfully direct heterologous expression and secretion of α-galactosidase in *K. marxianus*, with dramatically higher efficiencies when compared to the use of classical *S. cerevisiae* promoters, such as *PGK*.

With the *INU1* promoter, heterologous gene expression can be fine-tuned by choosing the appropriate carbon source. Another regulated promoter that was successfully used in *K. marxianus* is the tetracycline repressible promoter (Pecota and da Silva 2005).

Strong, constitutive promoters for driving heterologous gene expression have also been described, such as that of a purine-cytosine permease gene (Ball et al. 1999). Instead of fine-tuning foreign gene expression according to promoter strength or induction properties, Pecota et al. (2007) developed an insertion cassette that enables multicopy integration of a precise number of gene copies into *K. marxianus* with recycling of the selection marker.

Auxotrophic mutants of *K. marxianus*, for their use in transformation experiments, have been reported for leucine, uracil, histidine, or triptophane requirement (Bergkamp et

al. 1991, 1993b; Basabe et al. 1996; Hong et al. 2007). Dominant markers applicable for the selection of *K. marxianus* transformants include at least the kanamycin, the aureobasidin A, and the nurseothricin resistance genes (Das et al. 1984; Hashida-Okado et al. 1998; Goldstein and McCusker 1999; Steensma and Ter Linde 2001; Ribeiro et al. 2007). Recycling of the marker gene for multiple gene disruptions can be performed in *K. marxianus* in the same way as in *S. cerevisiae* and *K. lactis* with the Cre-*lox*P system (Güldener et al. 1996; Ribeiro et al. 2007).

A number of genes have been cloned and sequenced in different *K. marxianus* strains, and the most relevant ones are indicated in Table 3.

Biotechnological applications

When evaluating the yeast *K. marxianus* for biotechnological applications, it is impossible not to consider other most popular species, mainly *S. cerevisiae* and *K. lactis*. The former is probably the most employed biocatalyst in the biotechnological industry and a model organism in biological studies, whereas the latter has been chosen as a model Crabtree-negative, lactose-utilizing organism (Lachance 1998; Fukuhara 2006). The fact that several *K. marxianus* strains have obtained the generally-regarded-as-safe (GRAS) status, similarly to *S. cerevisiae* and *K. lactis* (Hensing et al. 1995) indicates that this aspect does not impose any disadvantage for the former, when compared to the latter yeasts, in terms of process approval by regulatory agencies. The fact that *K. lactis* was chosen by the scientific community as the model organism in the *Kluyveromyces* genus, and not *K. marxianus*, led not only to a much better understanding of its physiology (for reviews, see e.g., Schaffrath and Breunig 2000; Wolf et al. 2003; Breunig and Steensma 2003 and the whole issue no. 3, vol. 6 of FEMS Yeast Research), and to the full sequencing of its genome (Dujon et al. 2004) but also to the development of several applications, including the expression of more than 40 heterologous proteins (van Ooyen et al. 2006). This is due, to a great extent, to the fact that researchers have used, from the beginning, a very small number of *K. lactis* isolates (Fukuhara 2006), which has not been the case in the *K. marxianus* species.

The development of biotechnological applications with *K. marxianus* has been motivated by a number of advantages it has when compared to *K. lactis*. These include at least the fact that it can grow on a broader variety of substrates and at higher temperatures, its higher specific growth rates, and the lesser tendency to produce ethanol it has when exposed to sugar excess (Rouwenhorst et al. 1988; Steensma et al. 1988; Bellaver et al. 2004; see also "Biochemistry, metabolism, and physiology").

One very important aspect of the ecology of *K. marxianus* should be taken into account when considering its biotechnological utilization: Individuals have been isolated from an enormous variety of habitats (see "Taxonomic history of the present species *Kluyveromyces marxianus*").

The obvious consequence is that the metabolic diversity is broad, and hence, potential biotechnological applications of *K. marxianus* strains are manifold. A summary of the most explored applications with this yeast follows.

Although several yeasts have been reported for the production of aroma compounds, only a few of these can find industrial application due to their GRAS status (Medeiros et al. 2000, 2001). *Kluyveromyces* sp. produce aroma compounds such as fruit esters, carboxylic acids, ketones, furans, alcohols, monoterpene alcohols, and isoamyl acetate in liquid fermentation (Scharpf et al. 1986; Fabre et al. 1995). Of all these compounds, 2-phenyl ethanol (2-PE), with rose petals aroma, is the most important commercially (Welsh et al. 1989; Leclercq-Perlat et al. 2004). Natural 2-PE has a high-value (approximately US $1,000 kg^{-1}) serving a current world market of approximately 7,000 tons per annum (data from 1990; Etschmann et al. 2002). This alcohol presents sensorial characteristics that influence the quality of the wine, distilled drinks, or fermented foods. It is also found in fresh beer and is added to various industrial food products such as ice creams, bullets, non-alcoholic drinks, gelatines, puddings, and bubble gums (Wittmann et al. 2002). The influences of the carbon source (Fabre et al. 1998; Medeiros et al. 2000), aeration rate (Medeiros et al. 2001), media composition (Etschmann et al. 2004), and cultivation conditions (Etschmann and Schrader 2006) on the aroma production using *K. marxianus* were studied.

K. marxianus possesses the natural ability to excrete enzymes. This is a desired property for cost-efficient downstream processing of low- and medium-value enzymes (Hensing et al. 1994). The enzymes that hydrolyze pectic substances are known as pectic enzymes, pectinases, or pectinolytic enzymes (Wimborne and Rickard 1978). Pectinases are industrially used in the extraction and clarification of fruit juices (i.e., grape and apple; Schwan et al. 1997; Blanco et al. 1999). Other interesting applications are related to the maceration of vegetables, oil extraction, and formulation of animal feed using complex mixtures with cellulases to make the nutritional assimilation easier (Blanco et al. 1999). *K. marxianus* has considerable economic advantages over *Aspergillus* as an endo-PG source, even without genetic improvement of the strains (Harsa et al. 1993). Thus, the use of PGs from *K. marxianus* has attracted considerable interest (Garcia-Garibay et al. 1987b; Harsa et al. 1993; Donaghy and McKay 1994). Moreover, no other pectinolytic enzyme, besides PG, was reported to be secreted by *K. marxianus*

CCT 3172 in the culture media, which should facilitate the process to produce pure enzyme (Schwan et al. 1997).

In *K. marxianus*, the pectinolytic enzymes are only produced during exponential growth, but almost all PG is secreted in the start of the stationary phase (Schwan and Rose 1994; Schwan et al. 1997; Serrat et al. 2004). Among the cultivation parameters, dissolved oxygen was reported to be the key in the production of both biomass and endo-PG (Wimborne and Rickard 1978; Garcia-Garibay et al. 1987a). High rates and yields of biomass production require high oxygenation levels that, however, repress endo-PG induction (Wimborne and Rickard 1978). It was reported that *K. marxianus* exhibited pectolytic ability when it was grown without shaking and under anaerobic conditions, and no activity was found at high aeration rates (Barnby et al. 1990; Schwan and Rose 1994; García-Garibay et al. 1987a).

The effect of temperature was assessed on both growth and endo-PG production in combination with the effect of dissolved oxygen (Schwan and Rose 1994). Temperature was also reported to have no direct effect on the synthesis of this enzyme but influenced the growth rate and had an indirect effect due to changes in oxygen solubility (Cruz-Guerrero et al. 1999). Addition of pectin in an aerobic culture in a fermenter was reported to derepress the production of the enzyme (Garcia-Garibay et al. 1987a). However, whereas some authors did not find any effect of pectin addition to the medium in an endo-PG producing strain (Schwan and Rose 1994; Schwan et al. 1997), others reported the enhancement of pectinase production by this yeast when pectin was added (Wimborne and Rickard 1978; Lim et al. 1980; Cruz-Guerrero et al. 1999). *K. marxianus* CCT 3172 was able to break down pectin but required a usable source of carbon and energy to elaborate pectinolytic activity (Schwan and Rose 1994). It had a strong endo-PG activity between pH 4–6 with pH 5 as optimum (Schwan et al. 1997). Furthermore, the type of pectinase excreted by this strain was pointed as a feasible alternative to fungal production due to the lower broth viscosity, which can make downstream operations easier (Almeida et al. 2003a).

Lactose-intolerance can be circumvented by removing lactose from the diet or by converting this sugar into glucose and galactose with β-D-galactosidase (Rajoka et al. 2003). It was reported that only approximately 2% of the recognized yeast species are capable of fermenting lactose (Barnett et al. 1983), among which strains within the *Kluyveromyces* genus can be found. In a screening performed with yeast strains belonging to different genera, only two cultures of *K. fragilis* and *Ferrissia fragilis* showed β-galactosidase activity (Fiedurek and Szczodrak 1994). Lactose is considered the primary inducer of β-D-galactosidase synthesis (Furlan et al. 2000) and production of lactase by *K. marxianus* using cheese whey as a nutrient source has been investigated by several authors (Sonawat et al. 1981; Nunes et al. 1993).

During the stationary phase of growth, the β-D-galactosidase activity of *K. marxianus* CBS 712 and CBS 6556 remained approximately constant (Rech et al. 1999). In contrast, a reduction of β-D-galactosidase activity was reported by other authors in the stationary phase (Mahoney et al. 1975). The highest β-D-galactosidase activity of *K. marxianus* CBS 712 and CBS 6556 was reported to be at 37°C, decreasing quickly at temperatures above 40°C (Rech et al. 1999), while in *K. marxianus* IMB3, the enzyme is optimally active at 50°C (Barron et al. 1995a). On the other hand, the β-galactosidase of *K. marxianus* CBS 6556 is more stable than the corresponding enzymes of other strains, when stored at low temperatures, e.g., 4°C (Rech et al. 1999; Itoh et al. 1982; Brady et al. 1995). When different substrates were investigated for β-galactosidase production by *K. marxianus*, lactose supported the highest enzyme activities (Rajoka et al. 2003; Rajoka et al. 2004).

Inulinase is an enzyme that cleaves fructose molecules from inulin. Its expression is induced by inulin or sucrose, and the enzyme can be excreted to the culture medium or remain associated to the cell wall (Rouwenhorst et al. 1988; Barranco-Florido et al. 2001). *K. marxianus* has been widely studied for inulinase production, aiming at the production of fructose syrup from inulin (Cruz-Guerrero et al. 1995).

Pessoa and Vitolo (1999) obtained the highest inulinase activities with the *K. marxianus* DSM 70106 strain, using inulin as the carbon source. Growth of *K. marxianus* on sucrose also proceeds via the action of an extracellular inulinase (Hensing et al. 1994), which is repressed when growth is not sucrose-limited (Rouwenhorst et al. 1988; Parekh and Margaritis 1985; Grootwassink and Hewitt 1983).

K. marxianus has also been proposed as a source of: (1) oligonucleotides, used as flavour enhancers in food products; (2) oligosaccharides, used as prebiotics; and (3) oligopeptides, immuno stimulators added to dairy products that are released in the wort after whey protein proteolysis (Belem et al. 1997; Belem and Lee 1998, 1999). Recent studies have shown the potential of *K. marxianus* FII 510700 biomass as an alternative source to *S. cerevisiae* for yeast autolysates (Lukondeh et al. 2003a), alkali-insoluble glucans (Lukondeh et al. 2003c), and a natural bioemulsifier (Lukondeh et al. 2003b).

In the field of bioremediation, processes using *K. marxianus* were developed for the removal of copper ions (II) with molasses as a nutrients source (Aksu and Dönmez 2000). Lead (II) uptake by *K. marxianus* from contaminated molasses had negative effects on cell growth. Nevertheless, the decrease in biomass formation did not lead to decreased lead (II) uptake; on the contrary, the biosorption ability was

higher at higher initial lead (II) concentrations (Skountzou et al. 2003).

Ethanol production at elevated temperatures has received much attention because of the potential cost savings, which could be obtained by continuous evaporation of ethanol from the broth under reduced pressure (Hacking et al. 1984; Gough et al. 1996, 1997, 1998; Banat et al. 1998). This topic was recently reviewed for yeasts in general, including *K. marxianus*. The advantages described, besides the energy savings due to reduced cooling costs, were higher saccharification and fermentation rates, continuous ethanol removal, and reduced contamination (Banat et al. 1998). However, the temperature increase has a negative effect on ethanol yield and also reduces the cell viability (Anderson et al. 1986; Ballesteros et al. 1991). *K. marxianus* was reported to produce alcohol at temperatures above 40°C and to have a maximum growth temperature of 47°C (Anderson et al. 1986), 49°C (Hughes et al. 1984), or even 52°C (Banat et al. 1992). Lower ethanol tolerance was observed when *K. marxianus* was compared to *S. cerevisiae*, and this was correlated with the activity of the plasma membrane ATPase (Rosa and Sa-Correia 1992; Fernanda and Sa-Correia 1992). Hacking et al. (1984) screened yeast strains for their ability to ferment glucose to ethanol at high temperatures. The tolerance of all species seemed to decrease with temperature, but in general, *Kluyveromyces* strains were more thermotolerant than *Saccharomyces*, which in turn can produce higher ethanol yields. Anderson et al. (1986) compared *K. marxianus* strains isolated from sugar mills and CBS strains for ethanol production at high temperatures. The CBS strains produced the same ethanol amounts as the new isolates but with lower cell viability and higher cultivation time. Sakanaka et al. (1996) reported the fusion of a thermotolerant strain of *K. marxianus* with a high ethanol producing strain of *S. cerevisiae*; however, their fermentative capacity was severely impaired and the fusants' thermostability was lower than for either of the parental cells.

While Schwan and Rose (1994) reported that ethanol production in galactose-containing medium was not as high as when glucose was the carbon source, Duvnjak et al. 1987 found that galactose was a better carbon source for ethanol production than glucose; however, the strains employed in both works were different. The conversion of xylose into ethanol by *K. marxianus* was already reported some time ago (Margaritis and Bajpai 1982).

Different process strategies have been used for ethanol production with *K. marxianus*: batch cultures with elevated substrate concentrations (Grubb and Mawson 1993; Barron et al. 1996), fed-batch production (Ferrari et al. 1994; Gough et al. 1998; Love et al. 1996), continuous system (Love et al. 1998), membrane recycle bioreactors (Tin and Mawson 1993), two-stage fermentation (Hack et al. 1994; Banat et al. 1996), immobilization with β-galactosidase (Hahn-Hägerdal 1985), calcium-alginate-immobilized cells (Bajpai and Margaritis 1987a,b; Marwaha et al. 1988; Nolan et al. 1994; Riordan et al. 1996; Barron et al. 1996; Brady et al. 1996, 1997a,b, 1998; Ferguson et al. 1998; Gough and Mchale 1998), cells immobilized in poly(vinyl alcohol) cryogel beads (Gough et al. 1998), or in Kissiris, a mineral glass foam derived from lava (Nigam et al. 1997; Love et al. 1996, 1998), extractive fed batch cultures (Jones et al. 1993), simultaneous saccharification and fermentation processes with added enzymes (Barron et al. 1995b, 1996, 1997; Boyle et al. 1997; Nilsson et al. 1995; Ballesteros et al. 2002a,b, 2004; Kádár et al. 2004) or by cloning of heterologous cellulase genes (Hong et al. 2007), and the use of mixed cultures (Ward et al. 1995).

Cheese whey contains lactose and a protein fraction sufficiently rich in essential amino acids. Cheese whey cultivations with *K. marxianus* have been proposed, with promising results, as a means of reducing the pollution caused by this industrial waste stream (Ghaly and Singh 1989; Giec and Kosikowski 1992; Harden 1996; Aktas et al. 2005) and/or to produce single-cell protein (Giec and Kosikowski 1992; Ben-Hassan et al. 1992, Ben-Hassan and Ghaly 1995; Belem and Lee 1999; Schultz et al. 2006; Ghaly and Kamal 2004). Aerobic cultures of microorganisms in cheese whey can reduce up to 90–95% of its BOD (Grubb and Mawson 1993), resulting in bioingredients of high added value for the food industry (Belem et al. 1997).

Other potential applications of the yeast *K. marxianus*, which can be found in the literature include its use as baker's yeast (Caballero et al. 1995) and as an anticholesterolemic agent (Yoshida et al. 2004). The cellular components involved in the hypocholesterolemic activity of *K. marxianus* were further examined (Yoshida et al. 2005).

Last but not the least, *K. marxianus* has been investigated as a host for the production of heterologous proteins. In general, yeasts are capable of performing some post-translational modifications of proteins, such as glycosylation and/or other modifications required for optimal biological activity and stability (Hensing et al. 1995). *S. cerevisiae* has been the most commonly used yeast host for the production of heterologous proteins (Romanos et al. 1992; Gellissen and Hollenberg 1997; Porro et al. 2005). Nevertheless, this yeast has some drawbacks, such as its strong aerobic fermentation behavior and a tendency to hyperglycosylate secreted glycoproteins (Hensing et al. 1994). *K. lactis* has also been used for the production of heterologous proteins (van den Berg et al. 1990; Panuwatsuk and da Silva 2002; Bartkevičiute and Sasnauskas 2003; van Ooyen et al. 2006). *K. marxianus*, which is phylogenetically close to *K. lactis*, is supposed to have a similar capacity for synthesis and secretion of high molecular weight proteins (Wésolowski-Louvel et al. 1996). Some

examples showing that heterologous protein production in this yeast is possible have been reported in the literature (Bergkamp et al. 1993a; Bartkevičiute et al. 2000; Zhang et al. 2003). More recently, Pecota et al. (2007) successfully expressed lactate dehydrogenase activity in *K. marxianus*, using an integrative multi-copy system, resulting in lactate production by this yeast. Hong et al. (2007) expressed thermostable endo-β-1,4-glucanase, cellobiohydrolase, and β-glucosidase, also making use of an integrative system, generating a strain capable of converting cellulosic materials into ethanol. Although these studies demonstrate that the heterologous proteins expressed in *K. marxianus* were functional, the capacity of *K. marxianus* to perform post-translational modifications of heterologous proteins still remains to be investigated.

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