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John P. Morrissey, M. M. W. Etschmann, Jens Schrader, Gustavo M. de Billerbeck

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Special Issue Article

Cell factory applications of the yeast *Kluyveromyces marxianus* for the biotechnological production of natural flavour and fragrance molecules

John P. Morrissey[1]*, Maria M. W. Etschmann[2], Jens Schrader[2] and Gustavo M. de Billerbeck[3,4,5,6]
[1]*School of Microbiology, University College Cork, Ireland*
[2]*DECHEMA Research Institute, Biochemical Engineering, Frankfurt am Main, Germany*
[3]*Université de Toulouse, INSA, UPS, INP, LISBP, 135 Avenue de Rangueil, Toulouse, France*
[4]*INRA, UMR792 Ingénierie des Systèmes Biologiques et des Procédés, Toulouse, France*
[5]*CNRS, UMR5504, Toulouse, France*
[6]*INP-ENSAT, Avenue de l'Agrobiopole, Castanet-Tolosan Cedex, France*

**Correspondence to:*
J. P. Morrissey, School of Microbiology, University College Cork, Ireland.
E-mail: j.morrissey@ucc.ie

Abstract

Kluyveromyces marxianus **is emerging as a new platform organism for the production of flavour and fragrance (F&F) compounds. This food-grade yeast has advantageous traits, such as thermotolerance and rapid growth, that make it attractive for cell factory applications. The major impediment to its development has been limited fundamental knowledge of its genetics and physiology, but this is rapidly changing.** ***K. marxianus*** **produces a wide array of volatile molecules and contributes to the flavour of a range of different fermented beverages. Advantage is now being taken of this to develop strains for the production of metabolites such as 2-phenylethanol and ethyl acetate. Strains that were selected from initial screens were used to optimize processes for production of these F&F molecules. Most developments have focused on optimizing growth conditions and the fermentation process, including product removal, with future advancement likely to involve development of new strains through the application of evolutionary or rational engineering strategies. This is being facilitated by new genomic and molecular tools. Furthermore, synthetic biology offers a route to introduce new biosynthetic pathways into this yeast for F&F production. Consumer demand for biologically-synthesized molecules for use in foods and other products creates an opportunity to exploit the unique potential of** ***K. marxianus*** **for this cell factory application. Copyright © 2014 John Wiley & Sons, Ltd.**

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Keywords: ***Kluyveromyces marxianus*****; cell factory; synthetic biology; natural flavour and fragrance compounds**

Introduction to *Kluyveromyces marxianus*

Kluyveromyces marxianus is the type species of the genus *Kluyveromyces*, which, following several reclassifications, currently contains six described species (Kurtzman, 2003; Lachance, 2007). In the scientific literature, *K. marxianus* has also been described by synonyms, notably *K. fragilis* and *Saccharomyces keyfr*. Although yeasts in the genus may be isolated from diverse habitats, including insects and fruit, they are best known because of their association with fermented dairy products, such as cheeses and kefir. The frequent isolation of *K. marxianus* from dairy products, and its long history of safe use, means that this yeast has GRAS (US) and QPS (EU) status (http://www.efsa.europa.eu/en/efsajournal/pub/3449.htm). As well as this approval for food use, *K. marxianus* possesses traits that render it attractive for certain biotechnological applications (Fonseca *et al.*, 2008;

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Lachance, 2011; Lane and Morrissey, 2010). First is the production of β-galactosidase and inulinase, enzymes that allow the use of lactose and inulin, respectively, as a carbon source. Lactose assimilation is quite rare in yeasts but is possible in *K. marxianus* (and *K. lactis*) because of the presence of the genes *LAC12* and *LAC4*, encoding a lactose permease and a β-galactosidase, respectively. Inulin is a heterogeneous polymer of fructose joined by β(2,1) glycosidic bonds and is a common plant storage carbohydrate. The capacity to use inulin (encoded by the *INU1* gene) as a sole carbon source is one of the diagnostic tools used to identify *K. marxianus* strains. The ability of *K. marxianus* to grow on wastes from the cheese (lactose) and plant-processing (inulin) industries is important for the biotechnological applications of this yeast. A second important, industrially relevant trait is thermotolerance, with all *K. marxianus* strains able to grow at 44°C and some able to tolerate growth at temperatures >50°C. High-temperature fermentations reduce cooling costs and also prevent the growth of many contaminating bacteria, most notably coliforms. Third, *K. marxianus* exhibits a remarkably fast growth rate, with generation times as low as 45 min on rich medium (Groeneveld *et al.,* 2009). Fourth, *K. marxianus* is a respiro-fermentative yeast capable of generating energy by either respiration or fermentation. In the latter case, ethanol is a by-product and this has led to various applications in the biofuels sector (Abdel-Banat *et al.,* 2010a; Guimaraes *et al.,* 2010).

Kluyveromyces marxianus and flavours in natural fermented products

Although *K. marxianus* can be isolated from diverse sources, it is best known because of its association with naturally fermented milk products, such as kefir and cheese (Gao *et al.,* 2012). In these cases, the product is produced by the combined activity of a consortium of bacteria and yeasts. These natural products have a wide diversity of flavour compounds, with yeasts likely to play a role in the production of particular esters, ketones, aldehydes and alcohols (Fabre *et al.,* 1995; Plessas *et al.,* 2008). The term 'flavour' is used to describe the full sensory profile of food and beverages, comprising both smell and taste sensations. Although there are many studies reporting the isolation and identification of *K. marxianus* in fermented milk products, much of what is described is anecdotal and postulated and, in fact, there are few robust data demonstrating the precise role of *K. marxianus* in modulating the flavours of these products. Interestingly, studies that have addressed the flavour contribution of yeasts in mixed fermentations have found that co-culturing yeasts with bacteria seems to enhance flavour production by yeasts, and although the mechanism is not known, it is suggestive of metabolic interactions between the different microbial species growing in a consortium (Martin *et al.,* 2001; Plessas *et al.,* 2008). One recent study of yeast association with flavours in sheep and goat cheese links *K. marxianus* to molecules such as 2-phenylethanol, ethyl decanoate, benzaldehyde and 2,3-butanediol (Padilla *et al.,* 2014). Indeed, while not specific to *Kluyveromyces*, there is a growing recognition of the contribution of non-*Saccharomyces* yeasts to the flavours of fermented beverages (Jolly *et al.,* 2014). Much of what we know about yeast-based production of flavours comes from studies with *Saccharomyces* (Cordente *et al.,* 2012; Pires *et al.,* 2014; Swiegers and Pretorius, 2005), and it is likely that broadly similar metabolic pathways are present in other yeast species. The majority of flavour molecules are derived from central anabolic or catabolic pathways in the cell by the action of modifying enzymes (Figure 1). The same pathways that give rise to desirable flavours are also involved in the synthesis of off-flavours, such as diacetyl and 2,3-butanediol, in some fermented beverages. An important note of caution must be sounded, however, when attempting to extrapolate from *S. cerevisiae* to *K. marxianus*, as the evolutionary history of the two yeasts is very different. First, *S. cerevisiae* underwent a whole-genome duplication c100 MYA that created duplicate copies of all genes (although many have since been lost) (Wolfe and Shields, 1997), and second, optimization of *S. cerevisae* metabolism for alcoholic fermentation and the subsequent domestication imposed very different selective pressure on the *S. cerevisiae* genome (Dashko *et al.,* 2014; Hagman *et al.,* 2013; Piskur *et al.,* 2006; Scannell *et al.,* 2007). Thus, *S. cerevisiae* is a powerful resource to provide starting points for studying *K. marxianus* genes, proteins, pathways and networks,

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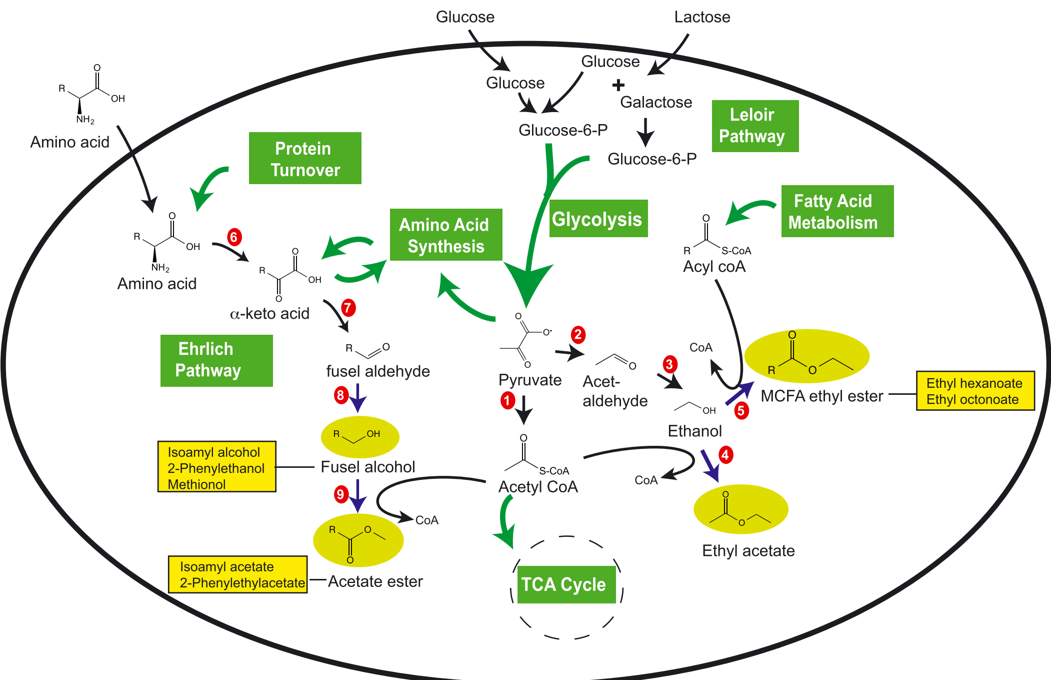

Figure 1. Metabolic pathways for synthesis of aroma and flavour molecules. Molecules with flavour characteristics are derived from normal anabolic and catabolic processes in the cell. Abbreviated versions of how these processes that lead to the formation of classes of metabolite are shown, along with examples cited in the main text. Reactions are depicted as unidirectional, although many are reversible. Important enzymatic steps are indicated by the key, as follows: 1, pyruvate dehydrogenase; 2, pyruvate decarboxylase; 3, alcohol dehydrogenase; 4, alcohol acyl transferase; 5, acyl-coenzymeA:ethanol *O*-acyltransferase-keto acid decarboxylase; 6, amino acid transaminase; 7, α-keto-acid decarboxylase; 8, oxidoreductase; 9, alcohol acyl transferase. In *S. cerevisiae*, several of these reactions can be carried out by multiple enzymes, but limited data are available for *K. marxianus*

but the two yeast species cannot be considered to be equivalent in all aspects. Only a few flavour compounds have been studied in significant detail in *K. marxianus* and these are listed in Table 1 and described below.

Higher alcohols

As well as ethanol, which is derived from the decarboxylation of pyruvate, yeasts synthesize a range of so-called 'higher alcohols' (also described as 'fusel alcohols'). It was first proposed and demonstrated by Ehrlich in 1906 that higher alcohols are synthesized from catabolism of amino acids, and the pathway leading to their formation bears his name (Hazelwood *et al.,* 2008). This (Ehrlich) pathway proceeds in three steps: first, the amino acid is transaminated to create an *α*-keto acid; next, this *α*-keto acid is decarboxylated to an aldehyde; and finally, the aldehyde is reduced to an alcohol or oxidized to a fusel acid, depending on the redox status of the cells (Figure 1, reactions 6, 7 and 8). Higher alcohols may in turn be acted on by alcohol acetyl transferases (AATases) to yield acetate esters (Figure 1, reaction 9). In addition to their synthesis via the Ehrlich pathway, *α*-keto acids are intermediates in the biosynthesis of certain amino acids and therefore constitute a link between anabolic and catabolic processes. Depending on metabolic fluxes, unbalanced amino acid synthesis can also contribute to synthesis of flavour molecules. Many of the enzymes involved in amino acid metabolism have been identified in *S. cerevisiae*, but there is little detail available from other yeasts. Various branched-chain and aromatic amino acids can serve as the starting point for synthesis of different volatile molecules, but only 2-phenylethanol (2-PE) has been the subject of detailed study in *K. marxianus*. 2-PE is widely used in the flavour industry because of its pleasant rose-like aroma and sweet taste. It is also an

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Table 1. *Kluyveromyces* spp. strains used as cell factories for the bioproduction of 2-phenylethanol, 2-phenylethyl acetate and ethyl acetate

Flavour compound	Flavour note	Strain[a]	Production	Productivity[b] (mg/l/h)	Other information	Reference
2-Phenylethanol (2-PE)	Rose	CBS 600	10200 mg/l in 30 h	330	Fed-batch and ISPR[c] w. PPG 1200	Etschmann and Schrader, 2006
		CBS 600	20380 mg/l in 50 h	408	Semi-continuous reactor w. ISPR	Gao and Daugulis, 2009
		CBS 600	5600 mg/l in 39 h	144	With ISPR and optimized medium	Etschmann *et al.*, 2004
		CBS 600	3470 mg/l in 15 h	231	With ISPR by pervaporation	Etschmann *et al.*, 2005
		CBS 397	3050 mg/l in 67 h	45	Bioproduction in molasses w. ISPR	Etschmann *et al.*, 2003
		K. lactis DSMZ 70793	1270 mg/l in 33 h	38	Bioproduction in molasses w. ISPR	Etschmann *et al.*, 2003
		CBS 6556	770 mg/l in 76 h	10	Cultivation on grape must of Riesling	Garavaglia *et al.*, 2007
			850 mg/l in 24 h	35	Without ISPR	Fabre *et al.*, 1998
			400 mg/l in 120 h	3	Fed-batch w. glucose as carbon source (*de novo* biosynthesis)	Fabre *et al.*, 1995
		K. marxianus [d]	1300 mg/l in 72 h	18	Evolved *K. marxianus* overexpressing *aroG*^{fbr} from *Klebsiella pneumoniae*	Kim *et al.*, 2014b
		K. marxianus [d]	1000 mg/l in 72 h	14	*K. marxianus* overexpressing *ARO10* and *ADH2* genes from *S. cerevisiae*	Kim *et al.*, 2014b
2-Phenylethyl acetate (2-PEA)	Rose, fruit-like	CBS 600	2300 mg/l in 30 h	80	Fed-batch and ISPR w. PPG 1200	Etschmann and Schrader, 2006
		CBS 600	4000 mg/l in 17 h	235	With ISPR by pervaporation	Etschmann *et al.*, 2005
		KY3	435 mg/l in 192 h	2	Higher 2PEA accumulation than *K. marxianus* CBS600 and CBS6432	Chang *et al.*, 2014
Ethyl acetate	Fruit	DSM 5422	17960 mg/l in 18 h	998	Medium produced from whey permeate (*de novo* biosynthesis) RY[e] =46%, 0.24 g EA/g lactose	Loser *et al.*, 2013
		DSM 5422	21470 mg/l in 36 h	596	Media produced from whey permeate (*de novo* biosynthesis) RY[e] = 56%, 0.29 g EA/g lactose	Urit *et al.*, 2013a
		K. marxianus		700	RY[e] = 27%, 0.14 g EA/g lactose Continuous culture on diluted whey permeate supplemented with ethanol	Kallel-Mhiri and Miclo, 1993

[a]Unless specified, all strains are *K. marxianus.*
[b]Overall productivity = mass produced/unit reaction volume/unit time).
[c]ISPR, *in situ* product removal.
[d]Genetically engineered yeast strain.
[e]RY, relative yield (= absolute yield/maximum theoretical yield for the substrate under consideration). RY values are from Loser *et al.* (2014).

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important 'fragrance', a term which is used to describe a single volatile compound with commercial use as an odorant, for example in the perfume industry. Many microbially produced volatiles can be used as both flavour and fragrance compounds. Naturally, 2-PE can be extracted from rose petals, but the bulk of commercial production is via chemical synthesis. It is also synthesized by a range of bacteria and yeasts and these have been identified as possible sources of natural 2-PE for the food industry (Etschmann *et al.,* 2002). It has been known for some time that some strains of *Kluyveromyces* are quite good producers of 2-PE, and a number of studies have addressed the physiological basis of 2-PE synthesis by *K. marxianus*. In addition, optimization strategies have been employed to enhance production with a view to developing commercial production of 2-PE in this yeast (Table 1). The first systematic efforts focused on screening *K. marxianus* strains to identify those with good potential for 2-PE production (Etschmann *et al.,* 2003; Fabre *et al.,* 1995), and on then exploring how nutrition affected levels of 2-PE in the cell (Etschmann *et al.,* 2004; Fabre *et al.,* 1998). Detailed analysis of physiology and metabolism established the pathways for 2-PE production in *K. marxianus*. 2-PE is derived from phenylpyruvate via the catabolism of phenyalanine (Phe) in the Ehrlich pathway (Etschmann *et al.,* 2002). 2-PE can be further metabolized to 2-phenylethylacetate (2-PEA) by a trans-esterification reaction. Natural levels of 2-PE are quite low because of balanced metabolism in the yeast cell, but addition of exogenous L-phenylalanine (L-Phe) to the growth medium, especially as a sole nitrogen source, leads to greatly increased levels of 2-PE (Etschmann *et al.,* 2002; Etschmann *et al.,* 2005; Fabre *et al.,* 1998). A detailed study of L-Phe metabolism and fluxes using ^{13}C labelling found that just over 73% was metabolized via the Ehrlich pathway, with the remainder metabolized either via the cinnamate pathway (22%) or used for biomass generation (4%) (Wittmann *et al.,* 2002). Although it is possible to achieve high conversion of L-Phe to 2-PE, overall yield is limited by the toxicity of 2-PE to *K. marxianus*. The development of strategies for *in situ* product removal (ISPR) of 2-PE during the fermentation has facilitated significantly higher production levels. Thus, although 2-PE is toxic to *K. marxianus* at 2 g/l, it was possible to achieve final overall concentrations of $>$10 g/l and 20 g/l using liquid–liquid (Etschmann and Schrader, 2006) or solid-phase (Gao and Daugulis, 2009) ISPR methods, respectively. Summarizing the progress with *K. marxianus*, it can be seen that the maximum overall concentration of 2-PE increased from 400 mg/l to 20.4 g/l and the productivity from 3 mg/l/h to 408 mg/l/h over the course of 14 years of research and development. These gains were largely achieved through process-engineering strategies (see Table 1). As mentioned previously, other yeast species have also been studied for the bioproduction of natural 2-PE, namely *S. cerevisiae*, *Clavispora lusitaniae*, *Yarrowia lipolytica*, *Kloeckera saturnus*, *Hansenula anomala*, *Pichia anomala*, *P. membranaefaciens*, *Pichia fermentans* and *Z. rouxii* (Table 2). Among them, the yeast *S. cerevisiae* R-UV3 achieved a higher productivity than the best *K. marxianus* strain CBS 600 (900 vs 408 mg/l/h). The *S. cerevisiae* process was a continuous culture system, whereas the *K. marxianus* system was fed-batch, but in both cases ongoing product removal was the key important feature. This is an area where further developments are likely. For example, adsorption of 2-PE onto polymeric resins has been investigated with good results with *S. cerevisiae* (Carpiné *et al.,* 2013; Hua *et al.,* 2010; Mei *et al.,* 2009). The results can probably be transferred easily to *K. marxianus*, provided there is biocompatibility of the adsorber material. Future process optimization and new reactor concepts (Mihaľ *et al.,* 2012a, 2012b, 2013), as well as strain selection or engineering, are likely to further enhance the prospects for commercial production of 2-PE in *K. marxianus*.

Acetate esters

Acetate esters are very significant contributors to flavours in all yeast-based fermented foods and beverages, most notably beer and wine (Pires *et al.,* 2014). Their formation involves the transfer of an acetate group from acetyl-CoA to the hydroxyl group of an alcohol to create the particular acetate ester, depending on the substrate alcohol (Figure 1, reactions 4 and 9). The enzyme catalysing this reaction is an alcohol acetyltransferase (AATase), PFAM PF07247.7, with the best studied being the two paralogous genes in *S. cerevisiae*, *scATF1* and *scATF2* (Verstrepen *et al.,* 2003). Genetic analysis in *S. cerevisiae* indicates that these genes are

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Table 2. Other yeast species used as whole cell biocatalysts for the bioproduction of 2-phenylethanol and ethyl acetate

Flavour compound	Flavour note	Yeast	Production	Productivity[a] (mg/l/h)	Other information[b]	Reference
2-Phenylethanol	Rose	*Saccharomyces cerevisiae* R-UV3	56700 mg/l in 63 h	900	Fed-batch and continuous ISPR[c] process	Wang *et al.*, 2011
		Saccharomyces cerevisiae Giv 2009	24000 mg/l in 93 h	258	Concentration in the organic phase	Stark *et al.*, 2002
		Saccharomyces cerevisiae P-3	6600 mg/l in 39 h	169	ISPR w. adsorption resin	Hua *et al.*, 2010
		Saccharomyces cerevisiae BD	6170 mg/l in 24 h	257	ISPR w. adsorption resin	Mei *et al.*, 2009
		Saccharomyces cerevisiae [d]	6100 mg/l in 72 h	85	*ald3D ARO80 ARO9 ARO10*	Kim *et al.*, 2014a
		Clavispora lusitaniae DSMZ 70102		81	Bioproduction in molasses w. ISPR	Etschmann *et al.*, 2003
		Saccharomyces cerevisiae Giv 2009	2350 mg/l in 48 h	49		Stark, 2001
		Yarrowia lipolytica NCYC3825	1980 mg/l in 95 h	21		Celinska *et al.*, 2013
		Kloeckera saturnus CBS 5761	1700 mg/l in 24 h	71		Albertazzi *et al.*, 1994
		Hansenula anomala CBS 110	1700 mg/l in 24 h	71		Albertazzi *et al.*, 1994
		Saccharomyces cerevisiae NCYC 739	1500 mg/l in 24 h	63		Albertazzi *et al.*, 1994
		Pichia anomala DSMZ 70130	1260 mg/l in 33 h	38	Bioproduction in molasses w. ISPR	Etschmann *et al.*, 2003
		Pichia membranaefaciens CBS 637	1030 mg/l in 28 h	37	Bioproduction in molasses w. ISPR	Etschmann *et al.*, 2003
		Saccharomyces cerevisiae DSMZ 70487	950 mg/l in 47 h	20	Bioproduction in molasses w. ISPR	Etschmann *et al.*, 2003
		Zygosaccharomyces rouxii CBS 5717	1010 mg/l in 33 h	31	Bioproduction in molasses w. ISPR	Etschmann *et al.*, 2003
		Pichia fermentans L-5	506 mg/l in 16 h	32		Huang *et al.*, 2001
		Pichia fermentans L-5	453 mg/l in 16 h	28		Huang *et al.*, 2000
		Saccharomyces cerevisiae NCYC 739	250 mg/l in 28 h	9	Bioproduction in molasses w. ISPR	Etschmann *et al.*, 2003
		Zygosaccharomyces rouxii	152 mg/l			Aoki and Uchida, 1990
Ethyl acetate (EA)	Fruit	*Candida utilis*	15000 mg/l			Armstrong and Brown, 1994
		Candida utilis ATCC 9950	2800 mg/l in 24 h	117	RY[e]= 40%, 0.39 g EA/g ethanol	Armstrong *et al.*, 1984
		Candida utilis NRC 2721			RY = 55%, 0.27 g EA/g glucose	Williams *et al.*, 1988
		Pichia anomala			RY = 76%, 0.73 g EA/g ethanol	Bol *et al.*, 1987
		Pichia anomala			RY = 36%, 0.18 g EA/g glucose	Tabachnick and Joslyn, 1953
		Zygosaccharomyces rouxii			RY = 22%, 0.21 g EA/g ethanol	Yong *et al.*, 1981
		Lachancea kluyveri			RY = 16%, 0.08 g EA/g glucose	Moller *et al.*, 2002
		Williopsis saturnus			RY = 4%, 0.02 g EA/g glucose	Davies *et al.*, 1951

[a]Overall productivity = mass produced/unit reaction volume/unit time.
[b]Unless specified, production is by bioconversion.
[c]ISPR, *in situ* product removal.
[d]Genetically engineered yeast strain.
[e]RY, relative yield (= absolute yield/maximum theoretical yield for the substrate under consideration). RY values are from Löser *et al.* (2014).

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responsible for the formation of the majority of acetate esters in the cell, although in their absence some smaller esters are still synthesized, indicating that unidentified genes also play some role in ester formation. The most important esters produced by *K. marxianus* are 2-phenylethyl acetate (2-PEA), isoamyl acetate and ethyl acetate, synthesized from 2-PE, isoamyl alcohol and ethanol, respectively (Figure 1, reactions 4 and 9). The levels of acetate esters produced under any particular set of growth conditions will be determined by three parameters: availability of the substrate alcohol; acetyl-CoA levels in the cytoplasm; and expression and activity of the alcohol acetyltransferase(s). The conditions that give rise to elevated levels of higher alcohols (discussed above) thus also provide the circumstances in which elevated levels of acetate esters are possible. Expression levels of AATase are certainly important and, in *S. cerevisiae*, variable expression is known to be an important factor in determining the final ester profile following fermentation (Verstrepen *et al.,* 2003). Despite the importance of 2-PEA and isoamyl acetate, there has been little research in *K. marxianus* on production of these metabolites, other than as by-products of higher alcohol synthesis. The highest yields and productivities have been obtained with *K. marxianus* strain CBS 600 in production processes largely the same as for 2-PE synthesis (Table 1). A recent patent describes a new *K. marxianus* strain, KY3, that was reported to accumulate more 2-PEA than strains CBS600 or CBS6432, when cultivated on glucose medium supplemented with 2.4 g/l L-Phe and incubated at 30°C under 'slightly anaerobic' conditions (Chang *et al.,* 2014). Insufficient data are available, however, to allow robust comparison of all the strains and their production traits.

In contrast to 2-PEA, there has been some very detailed research focusing on the production of ethyl acetate, driven mainly because, as well as influencing flavour, ethyl acetate is an important industrial solvent. These studies have sought to develop an ethyl acetate production process from whey permeate, using *K. marxianus* as the production organism (Loser *et al.,* 2014). It is important to note that the metabolic origin of ethyl acetate is quite different and this metabolite is not derived from amino acid metabolism (Figure 1, reaction 4). Since ethanol is the substrate for ethyl acetate, conditions (notably oxygen status) that increase ethanol accumulation also favour ethyl acetate production. Formation of ethyl acetate itself is an aerobic process (Kallel-Mhiri and Miclo, 1993; Urit *et al.,* 2013b) and precise control of oxygenation is required to optimize ethyl acetate production. Interestingly, however, the regulation of its biosynthesis is more complex and studies have also found that nutrition, growth rates and, especially, activity of the TCA cycle have a profound effect on formation of this ester. The most significant results showed that limiting the metals iron (Fe) and/or copper (Cu) enhanced the production of ethyl acetate (Loser *et al.,* 2012). The hypothesis is that certain enzymes in the TCA cycle are dependent on Fe (aconitase, succinate dehydrogenase) or Cu (electron transport, complex IV) and limiting these elements impairs the TCA cycle. In addition, Fe is also needed for the recycling of NADH to NAD^+ in the mitochondrion. This dual effect of Fe limitation is postulated to reduce the flux of acetyl CoA into the TCA cycle and thereby increase its availability to the AATase enzyme(s) (Loser *et al.,* 2012). Although this work focused exclusively on ethyl acetate, the conclusions should be directly transferable to other esters that are synthesized by the same enzyme, and it may be possible to enhance the production of esters such as isoamyl acetate and 2-PEA by manipulating acetyl CoA levels, for example by restricting TCA activity.

Extrapolating from *S. cerevisiae*, it is postulated that the majority of acetate esters in *K. marxianus* are synthesized by the single *kmATF* gene. Although *kmATF* is clearly orthologous to *scATF2*, it remains to be experimentally demonstrated that it is functionally equivalent to either *scATF1* or *scATF2*. The best data come from heterologous expression studies, where it was found that *K. lactis* Atfp and *S. cerevisiae* Atf2p had a similar AATase activity but, given that this was <10% of the activity of *S. cerevisiae* Atf1p, it is questionable whether this is the true biological activity of the Atf2p enzyme (Van Laere *et al.,* 2008), which, in *S. cerevisia*e, is also involved in sterol metabolism (Tiwari *et al.,* 2007). A similar conclusion was reached in a recent study that compared flavour production in a number of *K. marxianus* strains (Gethins *et al.,* 2014). Some older literature also reports that ethyl acetate may be formed by the reverse activity of esterases (Kallel-Mhiri and Miclo, 1993; Plata *et al.,* 2003), therefore it is important to be aware that other possible biosynthetic

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routes may occur. It is also reported that other yeasts, such as *Candida utilis* (syn. *Lindnera jadinii*), *Pichia anomola* (syn. *Wickerhamomyces anomalus*), *Zygosaccharomyces rouxii*, *Lachancea kluyverii* and *Williopsis saturnus* can synthesize ethyl acetate (Table 2), but differing methodologies preclude comprehensive comparison to *K. marxianus* across all production parameters (Loser *et al.,* 2014).

Strategies to develop *K. marxianus* as a natural F&F production platform

Despite the intrinsic potential of *K. marxianus* for commercial flavour and fragrance production, there are relatively few relevant patents using *K. marxianus* for the production of defined F&F compounds. The most common product with two patents is 2-PE (Chang *et al.,* 2014; Etschmann *et al.,* 2011), followed by one of each for the cheese-like flavour methionol (Leslie and Quan, 2007) and the sweetening steviol glycosides (Lehmann *et al.,* 2013). *K. marxianus* is more frequently used in starter cultures for the fermentation of various substrates, such as milk, cocoa fruits and grape juice (Brandsma and Meijer, 2008; De and Camu, 2007; Mahdavi, 2003; Meijer *et al.,* 2014; Preston and Murphy, 2011; Sommer and Nielsen, 2006). It is also used for the production of various enzymes. Due to its background as a dairy yeast, the application of *K. marxianus* for the production of lactase is obvious (Harju *et al.,* 2008; Swaaf *et al.,* 2007), but it can also be used to manufacture epoxide hydrolase (Botes *et al.,* 2006), sulphhydryl oxidase (Maat *et al.,* 1993) or xylanase for use in flavour production. Biomass, which can be conveniently produced with *K. marxianus* because of its high growth rate and broad substrate spectrum (De Boer *et al.,* 1996; Fieldhouse *et al.,* 2009; Lewandowski, 2011), is often further processed to the natural flavour enhancer yeast extract (Monch and Stute, 2002; Oriol and Kaid, 2009; Potman and Wesdorp, 1995).

The main reason why there are not more examples of application in industry is that the levels of volatile metabolites produced by wild-type *K. marxianus* strains under standard culture conditions are insufficient for commercial production, and therefore commercial development needs innovation at multiple levels. Strategies that can be implemented for the development of *K. marxianus* cell factories for F&F production are summarized in Figure 2. In effect, there are two parallel approaches to developing optimized strains that have high production levels of the desired metabolite: identification and improvement of wild-type strains; or application of genetic engineering to design tailored strains. The former approach relies on non-GM methodology and seeks to exploit the large diversity of *K. marxianus* that can be found in culture collections. Screening these collections to identify natural variants with higher levels of production is the classical approach, and this has been the first step in most of the studies already discussed in this review (Etschmann *et al.,* 2003; Fabre *et al.,* 1995; Loser *et al.,* 2011). Screening

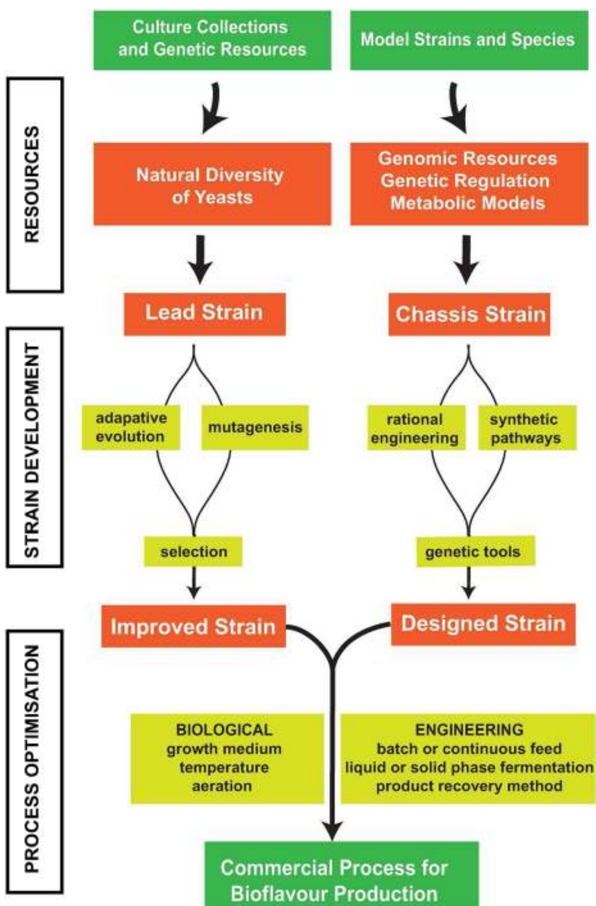

Figure 2. Strategies for developing a commercial bioflavour production process. The strategy can be divided into three phases: accessing of resources; strain development; and process optimization. Alternative 'non-GM' and 'GM' strategies for strain development are presented

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is particularly relevant for *K. marxianus,* which appears to be quite a physiologically variable species (Lane *et al.,* 2011; Rocha *et al.,* 2011b). One limitation with this approach is the fact that most cultured *K. marxianus* diversity is found in proprietary strain collections, which limits systematic screening for relevant phenotypes. Once lead strains are identified, they can be further developed through classical mutagenesis and selection or through adaptive evolution and evolutionary engineering, which takes advantage of inherent knowledge of biosynthetic routes and pathways. Although these methods have not yet been implemented with *K. marxianus* for F&F strains, they have been widely applied with industrial strains of *S. cerevisiae* for many different applications. The main challenge with these non-GM methods is identification of the appropriate conditions that will allow the selection of over-producing strains.

The alternative strategy to strain development is to apply molecular methods, both via what is now 'traditional' genetic engineering and using the newer synthetic biology tools that are becoming available. The knowledge gained from studies of model yeasts and strains, and the development of molecular tools, are the key resources that facilitate this designer approach to strain development. An understanding of genes and pathways, of metabolic fluxes and of regulatory steps enables researchers to carry out rational engineering to overcome bottlenecks, remove unnecessary reactions and maximize flux towards the metabolite of interest. There are many examples from *S. cerevisiae*, where these approaches have been successful at modifying flavour profiles (Cordente *et al.,* 2012; Pretorius *et al.,* 2012; Swiegers and Pretorius, 2005). The major restriction to implementation of rational/metabolic engineering in *K. marxianus* has been the lack of sufficient molecular tools, but this limitation is rapidly being overcome. In recent years, several tools have been developed for *K. marxianus* and this is facilitating molecular approaches, both to understand pathways in the yeast and to engineer new ones (Table 3). Some of these tools were recently applied to engineer a *K. marxianus* strain for 2-PE production (Kim *et al.,* 2014b). This study involved overexpressing two genes of the Ehrlich pathway, *ARO10* and *ADH2* (Figure 1, reactions 7 and 8), and a phenylalanine biosynthetic gene, *aroG*^{fbr}. Although the levels of 2-PE production are much lower than in previous studies (Table 1), this work demonstrates the potential of engineering, as levels in the engineered strain were five-fold greater than in the parental strain. It should also be noted that this strain produced 2-PE *de novo* – without the addition of L-Phe to the medium, and so differs from the whole-cell biocatalyst of other studies. Engineering studies have also recently been applied to improved productivity of *S. cerevisiae* by engineering similar steps of the pathway (Kim *et al.,* 2014a) and deletion of the *Saccharomyces cerevisiae* gene *ARO8*, encoding an aromatic amino acid transaminase, also enhances phenylethanol production from glucose (Romagnoli *et al.,* 2014). It is also worth noting the importance of genomics, comparative genomics and comparative physiology. A genome sequence of *K. marxianus* is also available (Jeong *et al.,* 2012) and this will allow the identification of the key genes involved in flavour production. It will be equally important, however, to also compare the physiology and metabolism of *K. marxianus* with other yeasts, as we already know that the species has unique and distinct features. The development of global-scale models of metabolism is likely to contribute significantly to this area. Synthetic biology offers an interesting alternative, as this philosophy centres on the creation of novel pathways by combining genes that encode the requisite enzymatic reactions. Thus, *K. marxianus* could be engineered to produce flavour and fragrance compounds beyond the current spectrum of the capacity of this yeast species. The first synthetic *K. marxianus* cell factory strain was recently described, and the approach that was taken shows the potential of the methodology (Cheon *et al.,* 2014). There have also been recent examples applying synthetic biology to producing F&F compounds in *S. cerevisiae*, notably vanillin (Hayden, 2014) and β-ionone (Beekwilder *et al.,* 2014).

The non-GM vs GM strategies that can be applied mirror developments with *S. cerevisiae*, where the non-GM approach is extensively used by the beverage industry because of regulatory and consumer issues with genetic engineering, whereas the rational/designer approaches are favoured in the pharmaceutical and white biotechnology sectors. There is of course cross-over, as the lessons learned from nature certainly inform

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Table 3. Development of molecular tools for engineering *K. marxianus*

Genome sequences	
Genome sequence of the thermotolerant yeast *Kluyveromyces marxianus* var. *marxianus* KCTC 17555	Jeong *et al.*, 2012
Genomic exploration of the hemiascomycetous yeasts: 12. *Kluyveromyces marxianus* var. *marxianus*	Llorente *et al.*, 2000
Promoters and vectors	
Characterization of *Saccharomyces cerevisiae* promoters for heterologous gene expression in *Kluyveromyces marxianus*	Lee *et al.*, 2013
Evaluation of the tetracycline promoter system for regulated gene expression in *Kluyveromyces marxianus*	Pecota and Da Silva, 2005
Construction of efficient centromeric, multicopy and expression vectors for the yeast *Kluyveromyces marxianus* using homologous elements and the promoter of a purine–cytosine-like permease	Ball *et al.*, 1999
Markers and genetic integration	
Non-homologous end joining-mediated functional marker selection for DNA cloning in the yeast *Kluyveromyces marxianus*	Hoshida *et al.*, 2014
Deletion of a KU80 homolog enhances homologous recombination in the thermotolerant yeast *Kluyveromyces marxianus*	Choo *et al.*, 2014
Simultaneous integration of multiple genes into the *Kluyveromyces marxianus* chromosome	Heo *et al.*, 2013
Identification of auxotrophic mutants of the yeast *Kluyveromyces marxianus* by non-homologous end joining-mediated integrative transformation with genes from *Saccharomyces cerevisiae*	Yarimizu *et al.*, 2013
Random and targeted gene integrations through the control of non-homologous end joining in the yeast *Kluyveromyces marxianus*	Abdel-Banat *et al.*, 2010b
Sequential gene integration for the engineering of *Kluyveromyces marxianus*	Pecota *et al.*, 2007
Application of the Cre–LoxP system for multiple gene disruption in the yeast *Kluyveromyces marxianus*	Ribeiro *et al.*, 2007
Transformation system for prototrophic industrial yeasts using the *AUR1* gene as a dominant selection marker	Hashida-Okado *et al.*, 1998
Construction of recombinant strains	
A biosynthetic pathway for hexanoic acid production in *Kluyveromyces marxianus*	Cheon *et al.*, 2014
Biosynthesis of 2-phenylethanol from glucose with genetically engineered *Kluyveromyces marxianus*	Kim *et al.*, 2014b
Heterologous expression of a thermophilic esterase in *Kluyveromyces* yeasts	Rocha *et al.*, 2011a
Heterologous expression of glucose oxidase in the yeast *Kluyveromyces marxianus*	Rocha *et al.*, 2011a
Cloning and characterization of *Kluyveromyces marxianus HOG1* gene	Qian *et al.*, 2010
High-temperature ethanol fermentation and transformation with linear DNA in the thermotolerant yeast *Kluyveromyces marxianus* DMKU3-1042	Nonklang *et al.*, 2008
Construction of thermotolerant yeast expressing thermostable cellulase genes	Hong *et al.*, 2007
Construction of flocculent *Kluyveromyces marxianus* strains suitable for high-temperature ethanol fermentation	Nonklang *et al.*, 2009
Expression of an α-galactosidase gene under control of the homologous inulinase promoter in *Kluyveromyces marxianus*	Bergkamp *et al.*, 1993

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metabolic engineering, with the term 'inverse engineering' used to describe the process by which traits identified in (experimentally) selected strains are introduced into genetically-clean host strains (Hirosawa *et al.,* 2004; Saerens *et al.,* 2010). It is worth noting that the requirement for 'natural' metabolites does not preclude genetic-engineering approaches for strain improvement as, unlike yeasts used in brewing or wine-making, there is no GM material in the final product (the fragrance or flavour compound) and thus there are no labelling requirements. This is analogous to the microbial platforms currently used to produce most vitamins for use in supplements.

The final, and often most challenging, step in cell factory development is taking a high-producing strain and developing a viable production process. Some of the biological and engineering parameters that need to be considered are listed in Figure 2. Effective process optimization is a knowledge-led process where insights gained from fundamental studies on metabolism, gene expression and protein activity are used to guide experimental approaches. This also needs to consider what feedstocks are available, and the capacity of *K. marxianus* to utilize lactose and other sugars is important in this regard. Once growth conditions are established, it becomes a process-engineering challenge, where issues as to type of fermentation, methods for recovering product and life cycle assessment all come into play. Examples of how both biological and engineering approaches have been applied to develop *K. marxianus* for the production of 2-PE and ethyl acetate are described above.

Concluding comments

Across a wide range of cell factory applications, *K. marxianus* is emerging as a genuine competitor to *S. cerevisiae* and, in many cases, may be a superior option. Specifically for flavours and fragrances, the published data indicate that higher yields and productivities can be achieved in *K. marxianus*. There are, however, significant knowledge gaps and much of what has been achieved was through systematic and empirical approaches. To move to the next level – commercial production platforms – it is necessary to integrate the different aspects of strain and process development. It is especially important to fill the knowledge gaps around genes, pathways, enzymes and regulation, so that we have a comprehensive understanding of how *K. marxianus* produces relevant metabolites. This information can then be used to inform strain development. In the future, the newer methods of strain engineering and synthetic biology will be combined with traditional skills of process development and optimization, in a holistic approach for new cell factory applications of *K. marxianus* in the commercial production of natural flavours and fragrances.

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