Synthesis of polyketides from low cost substrates by the thermotolerant yeast *Kluyveromyces marxianus*

Tami L. McTaggart, Danielle Bever, Shane Bassett, and Nancy A. Da Silva*

Department of Chemical and Biomolecular Engineering
University of California, Irvine, CA, USA

*Corresponding author

Tel: (+1)949-824-8288

E-mail: ndasilva@uci.edu

Key Words: *Kluyveromyces marxianus,* thermotolerance, xylose, polyketides, triacetic acid lactone

Abstract

Kluyveromyces marxianus is a promising nonconventional yeast for biobased chemical production due to its rapid growth rate, high TCA cycle flux, and tolerance to low pH and high temperature. Unlike *S. cerevisiae, K. marxianus* grows on low-cost substrates to cell densities that equal or surpass densities in glucose, which can be beneficial for utilization of lignocellulosic biomass (xylose), biofuel production waste (glycerol), and whey (lactose). We have evaluated *K. marxianus* for the synthesis of polyketides, using triacetic acid lactone (TAL) as the product. The 2-pyrone synthase (2-PS) was expressed on a CEN/ARS plasmid in three different strains, and the effects of temperature, carbon source, and cultivation strategy on TAL levels were determined. The highest titer was obtained in defined 1% xylose medium at 37°C, with substantial titers at 41°C and 43°C. The introduction of a high-stability 2-PS mutant and a promoter substitution increased titer 4-fold. 2-PS expression from a multi-copy pKD1-based plasmid improved TAL titers a further 5-fold. Combining the best plasmid, promoter, and strain resulted in a TAL titer of 1.24 g/L and a yield of 0.0295 mol TAL/mol C for this otherwise unengineered strain in 3mL tube culture. This is an excellent titer and yield (on xylose) prior to metabolic engineering or fed-batch culture relative to other hosts (on glucose), and demonstrates the promise of this rapidly growing and thermotolerant yeast species for polyketide production.

Introduction

The yeast *Kluyveromyces marxianus* has significant industrial potential due to its desirable growth characteristics and metabolism. *K. marxianus* is thermotolerant to 52°C (Banat et al., 1992), resistant to heat shock and temperature fluctuation (Matsumoto et al., 2018), and acid tolerant to pH 2.3 (Amrane and Prigent, 1998), all important attributes to both prevent contamination and reduce cooling costs in industrial fermentation. This yeast also boasts a very rapid doubling time of 45 mins in glucose medium (Groeneveld et al., 2009) and is considered the fastest growing eukaryote. It has the ability to grow on a wide range of carbon sources including xylose, glycerol, lactose, and many others (Lane and Morrissey, 2010). *K. marxianus* is commonly found in fermented dairy products, and has applications for production of fragrance and flavor molecules (Morrissey et al., 2015) enabled by GRAS (FDA, 2015) and QPS (Ricci et al., 2018) status in the United States and European Union, respectively. This yeast has also been observed to readily secrete enzymes, an attribute that has been utilized for simultaneous saccharification and fermentation of cellulose for ethanol production (Ballesteros et al., 2004; Tomás-Pejó et al., 2009). These traits make *K. marxianus* particularly suited to industrial production of enzymes such as pectinase (Espinoza et al., 1992), inulinase (Galindo-Leva et al., 2016; Kushi et al., 2000) and β-galactosidase (Rech et al., 1999; Zhou et al., 2013), PCV2 virus-like particles (Duan et al., 2018), as well as other chemicals such as ethyl acetate (Löbs et al., 2017; Löser et al., 2015), xylitol (Kim et al., 2015; Zhang et al., 2016) and 2-phenylethanol (Gao and Daugulis, 2009; Martínez et al., 2018).

Despite significant industrial advantages, *K. marxianus* has not been widely applied relative to other well-developed yeast hosts such as *Saccharomyces cerevisiae* due to a limited toolkit available for metabolic engineering. While low-copy plasmids have been developed (Ball et al., 1999; Iborra and Ball, 1994) from genomic centromere and autonomously replicating (CEN/ARS) sequences, there is no known native 2μ -like plasmid for multi-copy plasmid development. In the closely related *Kluyveromyces lactis*,

the multi-copy 2μ-like pKD1 plasmid (Falcone et al., 1986) has been studied extensively for expression of a range of heterologous enzymes (Cai et al., 2005; Hsieh and Da Silva, 1998; Spohner et al., 2016). However, pKD1-based vectors have been applied in only limited studies in *K. marxianus* and these vectors have been observed to be relatively unstable in both CBS 712 (21.4%) and CBS 6556 (35.1%). As in *K. lactis*, linearization of the pKD1 sequence at SphI did improve stability in *K. marxianus* strain BKM Y-719 to 72% (Bartkeviciute et al., 2000). An alternate multi-copy pDblet plasmid from *Schizosaccharomyces pombe* has also been used in CBS6556 with reported stability of 75% (De Souza and De Morais, 2000). Rapid development of CRISPR/Cas9 systems for *K. marxianus* (Juergens et al., 2018; Lee et al., 2018; Löbs et al., 2017; Nambu-Nishida et al., 2017) has recently expanded the ability to modify this yeast as a cell factory.

Metabolic studies of *K. marxianus* have demonstrated unique traits that are beneficial for waste substrate utilization including a range of sugar degradation enzymes and multiple significant and independent sugar transport systems (De Bruijne et al., 1988; Postma and Van den Broek, 1990). Metabolism of single sugars as well as mixed sugar feedstocks results in minimal by-product formation relative to other yeasts, including very little ethanol (Crabtree-negative) (Fonseca et al., 2013, 2007), which is desirable for product separation and yield. *K. marxianus* also has higher TCA-cycle and pentose phosphate pathway flux in glucose relative to *S. cerevisiae* (Blank et al., 2005). Although growth rate is highest in glucose, with significant catabolite repression of other sugar degradation pathways (Lertwattanasakul et al., 2011; Rodrussamee et al., 2011), the ability of *K. marxianus* to uptake xylose is notably high (Stambuk et al., 2003) and has been recently enhanced (Sharma et al., 2016). A weak xylose transporter has also been identified (Knoshaug et al., 2015). Increased biomass accumulation in xylose media relative to glucose is thought to be related to phosphoketolase activity (Evans and Ratledge, 1984). Recent metabolic models of *K. marxianus* also show xylose to be a promising substrate, especially after adjusting enzyme cofactor preferences and activity in the xylose reductase step (Pentjuss et al., 2017).

This organism's unique metabolism and ability to assimilate a wide range of substrates makes it a promising host for the synthesis of acetyl-CoA based products such as polyketides.

Polyketides are a diverse class of natural products of great importance due to their bioactive properties and structural diversity (Pfeifer and Khosla, 2001). Important applications include their use as therapeutics and more recently as precursors for conversion to biorenewable chemicals (Kraus et al., 2016; Shanks and Keeling, 2017). Yeast are particularly promising for the expression of Type I (iterative) and Type III fungal and plant polyketide synthases (Hashimoto et al., 2014), and for synthesis of polyketides built from acetyl-CoA and malonyl-CoA. Examples include dihydromonacolin L (the precursor to lovastatin) using the *Aspergillus terreus* lovastatin nonaketide synthase (LovB) (Ma et al., 2009), 6-methylsalicylic acid using the *Penicillium patulum* 6-methylsalicylic acid synthase (6-MSAS) (Kealey et al., 1998), and triacetic acid lactone (TAL) using the *Gerbera hybrida* 2-pyrone synthase (2-PS) (Cardenas and Da Silva, 2014; Xie et al., 2006). There has been significant attention on the synthesis of TAL as it can be converted into a wide range of high-value and commodity products (Chia et al., 2012). TAL is also a simple polyketide that requires expression of only one synthase enzyme and is easily assayed; it can thus be used as an effective and rapid indicator of strains with the high acetyl-CoA and malonyl-CoA pools needed for polyketide production. Extensive strain, pathway, and synthase engineering have been performed to increase TAL synthesis in *S. cerevisiae* (Cardenas and Da Silva, 2016, 2014; Saunders et al., 2015). More recently, high TAL titers have been achieved via engineering of the oleaginous yeast *Yarrowia lipolitica* (Markham et al., 2018; Yu et al., 2018), taking advantage of the native high flux through acetyl-CoA. In both yeast species, TAL was synthesized from glucose-based media.

K. marxianus holds significant potential for polyketide synthesis from a variety of carbon sources and under industrially favorable conditions due to its fast growth rate, thermotolerance, and acid tolerance. To demonstrate the promise of this yeast species, we evaluated both growth and TAL

production using eleven different carbon sources in three *K. marxianus* strains, and over a temperature range of 30°C to 43°C. We also improved the plasmid-based expression system and demonstrated the ability of this yeast to produce significant TAL titers in very minimal medium. The results clearly demonstrate the promise of *K. marxianus* for polyketide production on substrates such as xylose, glycerol, and lactose and over a large temperature range.

Materials and Methods

Strains and Plasmids

Escherichia coli strain DH5α (Invitrogen, Carlsbad, CA) was used for plasmid maintenance and amplification. The *K. marxianus* strains employed were CBS6556Δ*URA3* (Löbs et al., 2017), CBS 712 (ATCC 200963; ATCC®, Manassas, VA), and KM1Δ*URA3* (Pecota et al., 2007). For CBS 712, the *URA3* locus was disrupted as previously described (Pecota et al., 2007) resulting in CBS712Δ*URA3*. The Δ*ura3* strains were transformed using a standard *S. cerevisiae* lithium acetate method (Gietz et al., 1992) with the heat shock step performed at 45°C for 45 minutes. The Frozen-EZ Yeast Transformation II Kit (Zymo Research, Irvine, CA) was also used as it was found to be an efficient and rapid method for *K. marxianus* transformation.

To construct the *K. marxianus* CEN/ARS plasmid, a 1.2kb fragment containing a centromere and autonomously replicating sequence was PCR-amplified from CBS 712 genomic DNA and flanked with EcoRI restriction sites. The plasmid pXP842-2PS (Cardenas and Da Silva, 2014) which contains the *g2ps1* gene encoding 2-PS from *G. hybrida* fused to a HIS-tag (2PSHT) was digested with EcoRI to remove the *S. cerevisiae* 2μ origin and ligated to the *Km*CEN/ARS to generate pCA-A2PS. The native *K. marxianus PGK1* promoter was PCR amplified from the genomic DNA of CBS 712 and used to replace the *ScADH2p* in pCA-A2PS after digestion with AatII and SpeI, resulting in pCA-P2PS. These plasmids were digested at SpeI and XhoI restriction sites to replace the 2PS-HT with the 2-PSHT[C35S] mutant sequence (Vickery et al., 2018b). To develop the high-copy plasmid, pKD-P2PS, the *Kluyveromyces lactis* pKD1 sequence was isolated from

pSphI (Panuwatsuk and Da Silva, 2002) by SphI digestion and Gibson assembled with a PCR product of pCA-P2PS that removed the *Km*CEN/ARS and added 30 base pairs of homology to either end of pKD1. The multi-copy plasmid with the *ScADH2* promoter (pKD-A2PS) was constructed from pCA-A using the same Gibson primers used to construct pKD-P2PS but without the 2-PS gene, resulting in pKD-A(SpeXho). We replaced the SpeI and XhoI sites with NheI using Gibson assembly with overhangs in the *ADH2* promoter and *CYC1* terminator generating pKD-A. The 2-PS gene was PCR amplified and Gibson assembled with pKD-A to form pKD-A2PS. The plasmids constructed are shown in Table 1.

Plasmid recovery was performed using the GeneJet™ Plasmid Miniprep Kit (Thermo Scientific, Waltham, MA), and DNA sequence analysis confirmed the correct sequence of all PCR-amplified inserts (GeneWiz, South Plainfield, NJ; Eton Biosciences, San Diego, CA). Q5® Hot Start High-Fidelity DNA Polymerase, T4 DNA ligase, and deoxynucleotides were purchased from New England Biolabs (Ipswich, MA). Oligonucleotide primers were purchased from IDT DNA (San Diego, CA). All primer sequences are provided in Table S1.

Media and Cultivation

Luria-Bertani (LB) media with 150 mg/L ampicillin was used for *E. coli* culture. *K. marxianus* was cultivated in complex YP medium (1% Bacto yeast extract, 2% Bacto peptone), selective SC medium (0.67% yeast nitrogen base, 0.5% ammonium sulfate, 0.5% casamino acids, 100mg/L adenine-sulfate), and minimal selective S medium (0.67% yeast nitrogen base, 0.5% ammonium sulfate, 100mg/L adenine-sulfate), each supplemented with a variety of carbon sources. In all cases, an equimolar amount of carbon was used, resulting in supplements of 1.00 g dextrose, 0.95 g cellobiose, 1.00 g xylose, 0.95 g lactose, 1.00 g galactose, 0.95 g sucrose, 1.01 g sorbitol, 1.01 g mannitol, 1.02 g glycerol, 0.98 g acetate, or 0.98 g succinate per 100 mL of media. Media containing xylose are designated YPX, SXC, and SX. The adenine supplemented in the media had no effect on growth or TAL production (Figure S1).

K. marxianus was first inoculated from frozen glycerol stocks or plates into 3 or 5 mL medium in 15x125mm borosilicate glass culture tubes and cultivated at 37°C and 250 rpm overnight in an air incubator shaker (New Brunswick Scientific Co. Excella E25, Edison, NJ) or a gyratory water bath shaker (New Brunswick Scientific Co. Model G67D, Edison, NJ). Culture tubes were maintained at ~45° angle for the duration of incubation to enhance gas exchange. The overnight tube culture was used to inoculate subsequent tubes or flasks to an initial optical density (OD_{600}) of 0.1 (Shimadzu UV-2450 UV-Vis Spectrophotometer, Columbia, MD) in various carbon sources and cultivated at temperatures ranging from 30 to 43°C. A 500 µL sample was taken periodically to determine cell density and to measure TAL concentration in the medium. Maximum specific growth rate (μ_{max}) was determined by measuring the OD_{600} hourly during exponential growth (generally between 4 and 15 hours post-inoculation); μ_{max} values were calculated using a minimum of 4 points during exponential phase (Figure S2).

Plasmid Stability Analysis

Yeast strains harboring pKD1-based plasmids were cultivated on SXC plates, resuspended in water, and then inoculated to OD_{600} 0.05 in 3mL of SXC or YPX and grown for 48 hours at 37°C. Samples were taken in early exponential phase (OD_{600}=4), late exponential phase (OD_{600}=10), and stationary phase (48 h), diluted, and plated onto YPX medium. After 24 hours of growth at 37°C, 100 single colonies were streaked onto selective SXC plates for each of the three independent cultures (or ~300 colonies per time, per medium). The percent of plasmid-containing cells was determined as the number of colonies that grew on the selective plates divided by the total number of colonies transferred.

HPLC Assay

To measure TAL concentration, samples were centrifuged at 3500 rpm (2400 x g) for 5 min (Beckman Coulter Allegra X-22R Centrifuge, Brea, CA), followed by collection of the supernatants and storage at 4°C. The concentration of TAL was measured by HPLC using a Shimadzu HPLC system: LC-10AT

pumps (Shimadzu), UV–Vis detector (SPD-10A VP, Shimadzu), Zorbax SB-C18 reversed-phase column (2.1×150 mm, Agilent Technologies). Acetonitrile buffered in 1% acetic acid was used as the mobile phase, while HPLC grade water buffered in 1% acetic acid was used as the aqueous phase. A gradient program using a 95–85% Pump B gradient (H_2O with 1% acetic acid) provided an elution time of approximately 12 min (flow rate 0.25 mL/min, column temperature 25 °C).

Sugars and fermentation products were also analyzed from the medium via HPLC. Substances were detected using a RID detector (RD20-A, Shimadzu) eluting with 100% HPLC grade water in an Aminex HPX-42A column. Elution times were observed to be 26.6 min xylose, 23 min lactose, 26 min glucose, 28.7 min xylitol, 33.4 min ethanol, and 28 min glycerol (flow rate 0.4 mL/min, column temperature 85°C). Organic acids were quantified from supernatant via HPLC using an Aminex HPX-87H column and eluting with 5mM sulfuric acid in HPLC grade water. Peaks were measured both with a RID (RD20-A, Shimadzu) and a UV-Vis (SPD10-A VP, Shimadzu) detector. Elution times observed were 13.85 min succinic acid, 15.2 min lactic acid, and 18.1 min acetic acid. TAL is included in the broad peak at 23 min (flow rate 0.5 mL/min, column temperature 65°C).

Results and Discussion

Carbon substrate effects on growth and TAL production

K. marxianus is known to grow on a wide array of feedstocks. We thus first evaluated the effect of the carbon substrate on both growth and TAL synthesis. Strains CBS6556Δ*URA3*, CBS712Δ*URA3,* and KM1Δ*URA* were transformed with plasmid pCA-P2PS carrying a *K. marxianus* CEN/ARS sequence and the *K. marxianus PGK1* promoter for expression of the 2-PS gene. This *Km*CEN/ARS plasmid has very high stability in selective medium (ca. 90% after 48 h) (Table S2).

The three strains were cultivated at 37°C in selective SC medium containing eleven independent carbon sources (at equimolar carbon) for 48 h. Growth was observed on all substrates evaluated, with the highest final OD on xylose, and excellent growth on glucose, glycerol, galactose, sucrose, and lactose (Figure 1A). All three *K. marxianus* strains had the same growth profile on the various carbon sources. The excellent growth on xylose, glycerol, and lactose is noteworthy. These three substrates can be derived from waste biomass, biodiesel, or waste whey, respectively, and are often poorly metabolized by other yeasts such as *S. cerevisiae*, even after significant metabolic engineering (Kwak and Jin, 2017; Strucko et al., 2018; Zhou et al., 2012).

We also evaluated the effect of the eleven media on the synthesis of our model polyketide TAL. At 48 h, the extracellular TAL was quantified via HPLC. Cultivation in glycerol and xylose resulted in notably higher TAL titers (Figure 1B), with somewhat lower titers in sucrose, cellobiose, and lactose. The same general trends were observed for all three strains; however, significantly higher TAL levels were reached with strains CBS712Δ*URA3* and KM1Δ*URA* relative to strain CBS6556Δ*URA3*. Interestingly, glucose proved to be a poor carbon source for TAL synthesis, while xylose significantly outperformed all other substrates. Over 10-fold higher titers were observed in xylose relative to glucose. In addition, TAL synthesis in lactose was significantly higher than that in glucose or galactose. Specific production on acetate was comparable to xylose but growth and cell density were very low, indicating that acetate feeding may be a viable

strategy for TAL production without significant growth. Growth rate was significantly slower with xylose (0.25 h^{-1}) and lactose (0.52 h^{-1}) relative to glucose (0.66 h^{-1}), and less ethanol, glycerol, and organic acid byproducts were formed in selective xylose cultures of CBS712Δ*URA3* (Figure S.3). Increased byproduct formation in glucose relative to xylose has been previously observed, as well as reduced transcription of amino acid synthesis genes in xylose (Schabort et al., 2016) which may contribute to slower growth rates.

In *K. marxianus*, high-affinity sugar transport is known to be symport and introduce one H+ into the cytosol for every sugar molecule (Van Leeuwen et al., 1991); however, xylose transporters may be lower affinity than those for glucose, resulting in relatively slower uptake and growth (Stambuk et al., 2003). The minimal byproduct formation observed in xylose may contribute to the higher levels of TAL produced relative to glucose; however, these byproducts do not account for the significant difference in titers. Transcription analyses for *K. marxianus* report greater than 100-fold higher transcription of fatty acid and lipid catabolism genes in xylose relative to glucose as well as increased conversion of ethanol via the TCA cycle (Lertwattanasakul et al., 2015; Schabort et al., 2016); these may contribute to higher acetyl-CoA for conversion to TAL. While the full metabolism of *K. marxianus* in glucose and xylose is still not fully understood, important factors such as lipid degradation and accumulation, byproduct formation, cofactor balances, sugar transport and growth rate as well as putative phosphoketolase activity (Evans and Ratledge, 1984) may contribute to the improved growth and titers from xylose.

TAL was also found to be more detrimental to the growth of *K. marxianus* in xylose (Table S3) than to *S. cerevisiae* in glucose (Cardenas and Da Silva, 2014), influencing final cell density at titers of >1g/L. A reduction in growth rate was also observed, and was similar to that for *Y. lipolytica* in glucose (Markham et al., 2018). For both *Yarrowia* and *E. coli*, TAL toxicity did not prevent the late-phase accumulation of high TAL titers (Markham et al., 2018; Tang et al., 2013).

Temperature effects on growth and TAL production

The thermotolerance of *K. marxianus* is beneficial in an industrial setting to reduce cooling costs and contamination. We thus evaluated the effect of cultivation temperature on growth rate, final biomass, and TAL titer. The growth of sequenced strains CBS6556Δ*URA3* and CBS712Δ*URA3* at 30, 37, 41, and 43°C was first evaluated in rich YP medium containing glucose, glycerol, or xylose. Growth rate in glucose medium remained high despite temperature increases; however there was a marked decrease in final cell density with increasing temperatures (Table 2). In CBS6556Δ*URA3*, there is a large reduction in growth rate in xylose (by 70%) as temperature increases. Despite slower growth overall, temperature had little to no detriment to growth rate for CBS712Δ*URA3* when grown on xylose and glucose. In all cases, final OD_{600} was reduced as temperature increased. Interestingly, cell density in xylose matched or exceeded that of glucose and glycerol. The combination of CBS712Δ*URA3* and xylose resulted in the lowest decrease in final density at 43°C as well as the highest overall cell density observed at this temperature.

For further studies, we used *K. marxianus* CBS712Δ*URA3* in xylose medium because the CBS712 sequence is available, and the strain demonstrates high growth and TAL production on this carbon source (Figure 1B, Table 2). Strain CBS712Δ*URA3* transformed with plasmid pCA-P2PS was cultivated in 3 mL tube cultures in selective, defined SXC medium at 30, 37, 41, and 43°C. The 2-PS gene is known to be stable at these temperatures with a melting temperature of 60.7°C (Vickery et al., 2018). We found that using 3mL medium in tubes gave comparable growth and TAL production as baffled flasks (Figure S4). Both TAL titer and specific TAL production were similar at 37, 41, and 43°C, and 2-3-fold higher than at 30°C (Figure 2). It is worth noting that, unlike in rich medium, final OD does not decrease with increasing temperature in selective SXC (Table S4). Consistent production of TAL over a range of temperatures offers significant flexibility for industrial processes.

Improvements in expression system for TAL synthesis

To further increase TAL expression in *K. marxianus* CBS712Δ*URA3*, we evaluated the impact of a variant *g2ps1* gene and promoter strength. In our previous work on TAL synthesis in *S. cerevisiae*, we developed 2-PS mutants with higher *in vitro* and *in vivo* stability, enabling increased enzyme concentration and TAL synthesis (Vickery et al., 2018a). We replaced 2-PS with one of these mutants, 2PS[C35S], resulting in pCA-P[C35S], and measured TAL levels in SC medium supplemented with glucose, glycerol or xylose. As observed previously, TAL production was negligible in the glucose medium. However, in both glycerol and xylose, titers increased by 80% and 36%, respectively, for strains harboring 2-PS[C35S] relative to the wildtype 2-PS enzyme (Figure 3). This demonstrates the potential for further application of successful strategies developed for *S. cerevisiae* in *K. marxianus*.

Our initial studies used the *K. marxianus PGK1* promoter (P_{KmPGK1}) to control expression of the 2-PS gene on the *Km*CEN/ARS vector, resulting in yields of ~0.0014 mol TAL/mol carbon – in the same range as initial work in *S. cerevisiae* (Table S5). We replaced this promoter with the *S. cerevisiae ADH2* promoter (P_{ScADH2}) that we have used successfully for TAL expression in *S. cerevisiae*. Strain CBS712Δ*URA3* was transformed with pCA-P2PS or pCA-A2PS and cultivated for 48 h in SXC medium. A 2.2-fold improvement in TAL levels was observed when the alternate promoter P_{ScADH2} was employed (Figure 3). We then substituted the 2-PS variant (2-PS[C35S]) under this new promoter and repeated the experiment. Use of 2-PS[C35S] increased TAL titer by 57% relative to the wildtype 2-PS, and the combination of the more stable 2-PS with the stronger P_{ScADH2} increased TAL titer and yield by 3.3-fold relative to our initial system with the wildtype 2-PS and P_{KmPGK1}.

Interestingly, both P_{KmPGK1} and P_{ScADH2} resulted in similar TAL synthesis profiles, with P_{ScADH2} producing consistently higher titers throughout the batch culture (Figure S5). In our studies, TAL was detected as early as 5h with this promoter; however, TAL does not approach 1 g/L until stationary phase. This indicates that despite the toxicity effects of TAL (Table S3), the TAL produced should have minor

effects on final cell density and titer. The similar TAL synthesis profile observed for P_{KmPGK1} and P_{ScADH2} suggests that regulation may be similar for these promoters in this yeast. The native *ADH2* promoter has not been characterized in *K. marxianus.* Of the six constitutive promoters (*PGK*, *ADH1*, *TDH2* promoters from *K. marxianus* or *S. cerevisiae*) previously compared in *K. marxianus*, P_{KmPGK1} was the strongest during growth on all three carbon sources (xylose, glucose, and glycerol) tested, and the promoter that maintained the highest expression levels at elevated temperatures (Yang et al., 2015). It will be interesting to test the native *K. marxianus ADH2* promoter to determine the native regulation. In *K. marxianus*, native promoters have generally been found to be stronger than the *S. cerevisiae* versions (for the three promoters compared) (Yang et al., 2015). Replacing P_{ScADH2} with the native P_{KmADH2} may further improve polyketide levels.

Increases in gene copy number to improve TAL titer

All of our initial studies were performed using a *K. marxianus* CEN/ARS vector maintained at high stability but low copy number. Increasing copy number in *K. marxianus* (via a higher copy plasmid or gene integration) is necessary for higher TAL titers. Although no multi-copy plasmids are known for *K. marxianus*, pKD1-based plasmids from *K. lactis* have been successfully used in *K. marxianus* (De Souza and De Morais, 2000; Duan et al., 2018). For our studies, we replaced the *Km*CEN/ARS sequence on pCA-P2PS with the full pKD1 sequence (linearized at the native SphI site to improve stability (Bartkeviciute et al., 2000; Hsieh and Da Silva, 1998)), resulting in plasmid pKD-P2PS.

Strains CBS6556Δ*URA3* and CBS712*ΔURA3* were transformed with this new plasmid and cultivated in SXC medium for 48 h. Use of this higher copy plasmid (pKD-P2PS) increased TAL titers and yield by greater than 14.8-fold (Figure 4, Table S5) relative to the *Km*CEN/ARS based vector (pCA-P2PS). TAL titers of up to 0.89 g/L were observed from xylose in 3 mL tube cultures using an otherwise unengineered strain. This yield is also an improvement of 15-fold relative to initial studies of *S. cerevisiae* (with a similar expression system, growing on glucose) prior to metabolic engineering (Table S5).

Production was highest at 37°C (Figure S6); in contrast, titers were similar at 37°C, 41°C, and 43°C with the *Km*CEN/ARS plasmid (Figure 2).

The use of P_{ScADH2} in the CEN/ARS plasmid increased TAL levels; therefore, we replaced P_{KmPGK1} with P_{ScADH2} in the multi-copy pKD plasmid (pKD-A2PS) and repeated our experiment. In CBS712*ΔURA3*, we observed a 20% improvement in TAL titer, and a 2-fold improvement in specific titer when the *ADH2* promoter was used. Furthermore, in our most robust industrial strain KM1ΔURA3, we reached titers of 1.24 g/L with a yield of 0.0295 mol TAL/mol C from xylose in 3 mL culture. This is an excellent titer and yield relative to other yeast hosts; prior to metabolic engineering, the yield for *K. marxianus* on xylose approached those for the best engineered *S. cerevisiae* and *Y. lipolytica* strains on glucose (Table S5).

Given the ability of *K. marxianus* to grow on very minimal medium, we compared growth and TAL production for CBS712*ΔURA3*+pKD-P2PS in selective SXC and SX media at 37°C. In the very minimal SX medium, TAL titer was 30% lower and specific titer (g/L/OD) only 25% lower than in SXC (that contains casamino acids) (Figure S7). We measured the plasmid stability at various times during cultivation in SXC medium; for both strains, plasmid stability was greater than 80% after 48 hours (Table S6). Surprisingly, stability was also high in complex medium (55% in CBS712*ΔURA3* and 87% in CBS6556*ΔURA3*). The substantially higher TAL titers and the high plasmid stability demonstrates the promise of pKD1-based plasmids for polyketide production. In addition, the ability of *K. marxianus* to produce these titers in minimal xylose medium is advantageous for industrial production and downstream separation.

Conclusions

This study is the first to evaluate polyketide production in *K. marxianus*, achieving an excellent titer of 1.24 g/L TAL in test tube culture prior to metabolic engineering or bioreactor cultivation. Successful transfer of strategies effective in *S. cerevisiae* such as enzyme and strain engineering can be further explored to optimize *K. marxianus* as an industrial workhorse. Sustained production at elevated temperatures of 41 and 43°C, in true minimal medium, and on a variety of substrates, in particular xylose, demonstrates the promise of this rapidly growing, thermotolerant yeast species for sustainable and low-cost production of acetyl-CoA based polyketides.

Acknowledgements

This research was supported by the National Science Foundation: Grant No. CBET-1803677 and EEC-0813570 (through the Engineering Research Center CBiRC (Center for Biorenewable Chemicals)) and the Department of Energy/Office of Science: Grant No. DE-SC0019093. The authors thank Prof. Ian Wheeldon (University of California, Riverside) for strain CBS6556*ΔURA3*, Cesar Villavicencio for constructing pCA-P2PS, Tej Shah for performing introductory growth studies, and Ruben Fernandez-Moya for his helpful input and advice.

References

Amrane, A., Prigent, Y., 1998. Effect of culture conditions of Kluyveromyces marxianus on its autolysis, and process optimization. Bioprocess Eng. 18, 383–388. https://doi.org/10.1007/s004490050460

Ball, M.M., Raynal, A., Guerineau, M., Iborra, F., 1999. 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. J. Mol. Microbiol. Biotechnol.

Ballesteros, M., Oliva, J.M., Negro, M.J., Manzanares, P., Ballesteros, I., 2004. Ethanol from lignocellulosic materials by a simultaneous saccharification and fermentation process (SFS) with Kluyveromyces marxianus CECT 10875. Process Biochem. https://doi.org/10.1016/j.procbio.2003.09.011

Banat, I.M., Nigam, P., Marchant, R., 1992. Isolation of thermotolerant, fermentative yeasts growing at 52-degree C and producing ethanol at 45- degree C and 50-degree C. World J. Microbiol. Biotechnol. 8, 259–263. https://doi.org/10.1007/BF01201874

Bartkeviciute, D., Siekstele, R., Sasnauskas, K., 2000. Heterologous expression of the Kluyveromyces marxianus endopolygalacturonase gene (EPG1) using versatile autonomously replicating vector for a wide range of host, in: Enzyme and Microbial Technology. https://doi.org/10.1016/S0141-0229(00)00155-1

Blank, L.M., Lehmbeck, F., Sauer, U., 2005. Metabolic-flux and network analysis in fourteen hemiascomycetous yeasts, in: FEMS Yeast Research. https://doi.org/10.1016/j.femsyr.2004.09.008

Cai, X.P., Zhang, J., Yuan, H.Y., Fang, Z.A., Li, Y.Y., 2005. Secretory expression of heterologous protein in Kluyveromyces cicerisporus. Appl. Microbiol. Biotechnol. https://doi.org/10.1007/s00253-004-1834-9

Cardenas, J., Da Silva, N.A., 2016. Engineering cofactor and transport mechanisms in Saccharomyces cerevisiae for enhanced acetyl-CoA and polyketide biosynthesis. Metab. Eng. 36, 80–89. https://doi.org/10.1016/j.ymben.2016.02.009

Cardenas, J., Da Silva, N.A., 2014. Metabolic engineering of Saccharomyces cerevisiae for the production of triacetic acid lactone. Metab. Eng. 25, 194–203. https://doi.org/10.1016/j.ymben.2014.07.008

Chia, M., Schwartz, T.J., Shanks, B.H., Dumesic, J.A., 2012. Triacetic acid lactone as a potential biorenewable platform chemical. Green Chem. 14, 1850. https://doi.org/10.1039/c2gc35343a

De Bruijne, A.W., Schuddemat, J., Van den Broek, P.J.A., Van Steveninck, J., 1988. Regulation of sugar transport systems of Kluyveromyces marxianus: the role of carbohydrates and their catabolism. BBA - Biomembr. https://doi.org/10.1016/0005-2736(88)90104-6

De Souza, C.G., De Morais, M.A., 2000. The use of the replicating pDblet plasmid as a cloning vector with enhanced stability in Kluyveromyces marxianus. Biotechnol. Lett. https://doi.org/10.1023/A:1005624913974

Duan, J., Yang, D., Chen, L., Yu, Y., Zhou, J., Lu, H., 2018. Efficient production of porcine circovirus virus-like particles using the nonconventional yeast Kluyveromyces marxianus 2016.

Espinoza, P., Bárzana, E., García-Garibay, M., Ǵmez-Ruiz, L., 1992. Evaluation of Kluyveromyces marxianus for the production of lactase simultaneously to pectinase or inulinase. Biotechnol. Lett. https://doi.org/10.1007/BF01021058

Evans, C.T., Ratledge, C., 1984. Induction of xylulose-5-phosphate phosphoketolase in a variety of yeasts grown on d-xylose: the key to efficient xylose metabolism. Arch. Microbiol. 139, 48–52. https://doi.org/10.1007/BF00692711

Falcone, C., Saliola, M., Chen, X.J., Frontali, L., Fukuhara, H., 1986. Analysis of a 1.6-µm circular plasmid from the yeast Kluyveromyces drosophilarum: Structure and molecular dimorphism. Plasmid. https://doi.org/10.1016/0147-619X(86)90044-2

FDA, 2015. Microorganisms & Microbial-Derived Ingredients Used in Food (Partial List) [WWW Document]. Food Drug Adm. URL http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/MicroorganismsMicrobialDerivedIngredients/default.htm

Fonseca, G.G., de Carvalho, N.M.B., Gombert, A.K., 2013. Growth of the yeast *Kluyveromyces marxianus* CBS 6556 on different sugar combinations as sole carbon and energy source. Appl. Microbiol. Biotechnol. https://doi.org/10.1007/s00253-013-4748-6

Fonseca, G.G., Gombert, A.K., Heinzle, E., Wittmann, C., 2007. Physiology of the yeast Kluyveromyces marxianus during batch and chemostat cultures with glucose as the sole carbon source. FEMS Yeast Res. 7, 422–435. https://doi.org/10.1111/j.1567-1364.2006.00192.x

Galdieri, L., Zhang, T., Rogerson, D., Lleshi, R., Vancura, A., 2014. Protein acetylation and acetyl coenzyme a metabolism in Budding Yeast. Eukaryot. Cell. https://doi.org/10.1128/EC.00189-14

Galindo-Leva, L.Á., Hughes, S.R., López-Núñez, J.C., Jarodsky, J.M., Erickson, A., Lindquist, M.R., Cox, E.J., Bischoff, K.M., Hoecker, E.C., Liu, S., Qureshi, N., Jones, M.A., 2016. Growth, ethanol production, and inulinase activity on various inulin substrates by mutant Kluyveromyces marxianus strains NRRL Y-50798 and NRRL Y-50799. J. Ind. Microbiol. Biotechnol. https://doi.org/10.1007/s10295-016-1771-5

Gao, F., Daugulis, A.J., 2009. Bioproduction of the aroma compound 2-phenylethanol in a solid-liquid two-phase partitioning bioreactor system by Kluyveromyces marxianus. Biotechnol. Bioeng. https://doi.org/10.1002/bit.22387

Gietz, D., St Jean, A., Woods, R.A., Schiestl, R.H., 1992. Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res. 20, 1425.

Groeneveld, P., Stouthamer, A.H., Westerhoff, H. V., 2009. Super life - How and why “cell selection” leads to the fastest-growing eukaryote. FEBS J. 276, 254–270. https://doi.org/10.1111/j.1742-4658.2008.06778.x

Hashimoto, M., Nonaka, T., Fujii, I., 2014. Fungal type III polyketide synthases. Nat. Prod. Rep. 31, 1306–1317. https://doi.org/10.1039/C4NP00096J

Hasunuma, T., Sanda, T., Yamada, R., Yoshimura, K., Ishii, J., Kondo, A., 2011. Metabolic pathway engineering based on metabolomics confers acetic and formic acid tolerance to a recombinant xylose-fermenting strain of Saccharomyces cerevisiae. Microb. Cell Fact.

https://doi.org/10.1186/1475-2859-10-2

Hsieh, H.P., Da Silva, N.A., 1998. Partial-pKD1 plasmids provide enhanced structural stability for heterologous protein production in Kluyveromyces lactis. Appl. Microbiol. Biotechnol. 49, 411–416. https://doi.org/10.1007/s002530051191

Iborra, F., Ball, M.M., 1994. Kluyveromyces marxianus small DNA fragments contain both autonomous replicative and centromeric elements that also function in Kluyveromyces lactis. Yeast. https://doi.org/10.1002/yea.320101211

Juergens, H., Varela, J.A., de Vries, A.R.G., Perli, T., Gast, V.J.M., Gyurchev, N.Y., Rajkumar, A.S., Mans, R., Pronk, J.T., Morrissey, J.P., Daran, J.-M.G., 2018. Genome editing in Kluyveromyces and Ogataea yeasts using a broad-host-range Cas9/gRNA co-expression plasmid. FEMS Yeast Res. 1–16. https://doi.org/10.1093/femsyr/foy012

Kealey, J.T., Liu, L., Santi, D. V, Betlach, M.C., Barr, P.J., 1998. Production of a polyketide natural product in nonpolyketide-producing prokaryotic and eukaryotic hosts. Proc. Natl. Acad. Sci. U. S. A. https://doi.org/10.1073/pnas.95.2.505

Kim, J.S., Park, J.B., Jang, S.W., Ha, S.J., 2015. Enhanced Xylitol Production by Mutant Kluyveromyces marxianus 36907-FMEL1 Due to Improved Xylose Reductase Activity. Appl. Biochem. Biotechnol. 176, 1975–1984. https://doi.org/10.1007/s12010-015-1694-z

Knoshaug, E.P., Vidgren, V., Magalhães, F., Jarvis, E.E., Franden, M.A., Zhang, M., Singh, A., 2015. Novel transporters from Kluyveromyces marxianus and Pichia guilliermondii expressed in Saccharomyces cerevisiae enable growth on l-arabinose and d-xylose. Yeast. https://doi.org/10.1002/yea.3084

Kraus, G.A., Wanninayake, U.K., Bottoms, J., 2016. Triacetic acid lactone as a common intermediate for the synthesis of 4-hydroxy-2-pyridones and 4-amino-2-pyrones. Tetrahedron Lett. https://doi.org/10.1016/j.tetlet.2016.02.043

Kushi, R.T., Monti, R., Contiero, J., 2000. Production, purification and characterization of an extracellular inulinase from Kluyveromyces marxianus var. bulgaricus. J. Ind. Microbiol. Biotechnol. https://doi.org/10.1038/sj.jim.7000032

Kwak, S., Jin, Y.S., 2017. Production of fuels and chemicals from xylose by engineered Saccharomyces cerevisiae: A review and perspective. Microb. Cell Fact. https://doi.org/10.1186/s12934-017-0694-9

Lane, M.M., Morrissey, J.P., 2010. Kluyveromyces marxianus: A yeast emerging from its sister's shadow. Fungal Biol. Rev. https://doi.org/10.1016/j.fbr.2010.01.001

Lee, M.H., Lin, J.J., Lin, Y.J., Chang, J.J., Ke, H.M., Fan, W.L., Wang, T.Y., Li, W.H., 2018. Genome-wide prediction of CRISPR/Cas9 targets in Kluyveromyces marxianus and its application to obtain a stable haploid strain. Sci. Rep. 8, 1–10. https://doi.org/10.1038/s41598-018-25366-z

Lertwattanasakul, N., Kosaka, T., Hosoyama, A., Suzuki, Y., Rodrussamee, N., Matsutani, M., Murata, M., Fujimoto, N., Suprayogi, Tsuchikane, K., Limtong, S., Fujita, N., Yamada, M., 2015. Genetic basis of the highly efficient yeast Kluyveromyces marxianus: Complete genome sequence and transcriptome analyses. Biotechnol. Biofuels. https://doi.org/10.1186/s13068-015-0227-x

Lertwattanasakul, N., Rodrussamee, N., Suprayogi, Limtong, S., Thanonkeo, P., Kosaka, T., Yamada, M., 2011. Utilization capability of sucrose, raffinose and inulin and its less-sensitiveness to glucose repression in thermotolerant yeast kluyveromyces marxianus DMKU 3-1042. AMB Express. https://doi.org/10.1186/2191-0855-1-20

Löbs, A.K., Engel, R., Schwartz, C., Flores, A., Wheeldon, I., 2017. CRISPR-Cas9-enabled genetic disruptions for understanding ethanol and ethyl acetate biosynthesis in Kluyveromyces marxianus. Biotechnol. Biofuels 10. https://doi.org/10.1186/s13068-017-0854-5

Löser, C., Urit, T., Gruner, E., Bley, T., 2015. Efficient growth of Kluyveromyces marxianus biomass used as a biocatalyst in the sustainable production of ethyl acetate. Energy. Sustain. Soc. 5, 1–15. https://doi.org/10.1186/s13705-014-0028-2

Ma, S.M., Li, J.W.H., Choi, J.W., Zhou, H., Lee, K.K.M., Moorthie, V.A., Xie, X., Kealey, J.T., Da Silva, N.A., Vederas, J.C., Tang, Y., 2009. Complete reconstitution of a highly reducing iterative polyketide synthase. Science (80-.). https://doi.org/10.1126/science.1175602

Markham, K.A., Palmer, C.M., Chwatko, M., Wagner, J.M., Murray, C., Vazquez, S., Swaminathan, A., Chakravarty, I., Lynd, N.A., Alper, H.S., 2018. Rewiring *Yarrowia lipolytica* toward triacetic acid lactone for materials generation. Proc. Natl. Acad. Sci. 201721203. https://doi.org/10.1073/pnas.1721203115

Martínez, O., Sánchez, A., Font, X., Barrena, R., 2018. Bioproduction of 2-phenylethanol and 2-phenethyl acetate by *Kluyveromyces marxianus* through the solid-state fermentation of sugarcane bagasse. Appl. Microbiol. Biotechnol. 1–14. https://doi.org/10.1007/s00253-018-8964-y

Matsumoto, I., Arai, T., Nishimoto, Y.U.I., 2018. Thermotolerant Yeast Kluyveromyces marxianus Reveals More Tolerance to Heat Shock than the Brewery Yeast Saccharomyces cerevisiae 23.

Morrissey, J.P., Etschmann, M.M.W., Schrader, J., de Billerbeck, G.M., 2015. Cell factory applications of the yeast Kluyveromyces marxianus for the biotechnological production of natural flavour and fragrance molecules. Yeast. https://doi.org/10.1002/yea.3054

Nambu-Nishida, Y., Nishida, K., Hasunuma, T., Kondo, A., 2017. Development of a comprehensive set of tools for genome engineering in a cold- A nd thermo-tolerant Kluyveromyces marxianus yeast strain. Sci. Rep. 7. https://doi.org/10.1038/s41598-017-08356-5

Panuwatsuk, W., Da Silva, N., 2002. Evaluation of pKD1-based plasmid systems for heterologous protein production in Kluyveromyces lactis. Appl. Microbiol. Biotechnol. 58, 195–201. https://doi.org/10.1007/s002530100815

Pecota, D.C., Rajgarhia, V., Da Silva, N.A., 2007. Sequential gene integration for the engineering of Kluyveromyces marxianus. J. Biotechnol. https://doi.org/10.1016/j.jbiotec.2006.07.031

Pentjuss, A., Stalidzans, E., Liepins, J., Kokina, A., Martynova, J., Zikmanis, P., Mozga, I., Scherbaka, R., Hartman, H., Poolman, M.G., Fell, D.A., Vigants, A., 2017. Model-based biotechnological potential analysis of Kluyveromyces marxianus central metabolism. J. Ind. Microbiol. Biotechnol. https://doi.org/10.1007/s10295-017-1946-8

Pfeifer, B., Khosla, C., 2001. Biosynthesis of polyketides in heterologous hosts. Microbiol. Mol. Biol. Rev. https://doi.org/10.1128/MMBR.65.1.106-118.2001

Postma, E., Van den Broek, P.J.A., 1990. Continuous-culture study of the regulation of glucose and fructose transport in Kluyveromyces marxianus CBS 6556. J. Bacteriol. https://doi.org/10.1128/jb.172.6.2871-2876.1990

Rech, R., Cassini, C.F., Secchi, A., Ayub, M.A.Z., 1999. Utilization of protein-hydrolyzed cheese whey for production of ??-galactosidase by Kluyveromyces marxianus. J. Ind. Microbiol. Biotechnol. https://doi.org/10.1038/sj.jim.2900692

Ricci, A., Allende, A., Bolton, D., Chemaly, M., Davies, R., Girones, R., Koutsoumanis, K., Lindqvist, R., Nørrung, B., Robertson, L., Ru, G., Fernández Escámez, P.S., Sanaa, M., Simmons, M., Skandamis, P., Snary, E., Speybroeck, N., Ter Kuile, B., Threlfall, J., Wahlström, H., Cocconcelli, P.S., Peixe, L., Maradona, M.P., Querol, A., Suarez, J.E., Sundh, I., Vlak, J., Barizzone, F., Correia, S., Herman, L., 2018. Update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 7: suitability of taxonomic units notified to EFSA until September 2017. EFSA J. 16. https://doi.org/10.2903/j.efsa.2018.5131

Rodrussamee, N., Lertwattanasakul, N., Hirata, K., Suprayogi, Limtong, S., Kosaka, T., Yamada, M., 2011. Growth and ethanol fermentation ability on hexose and pentose sugars and glucose effect under various conditions in thermotolerant yeast Kluyveromyces marxianus. Appl. Microbiol. Biotechnol. https://doi.org/10.1007/s00253-011-3218-2

Saunders, L.P., Bowman, M.J., Mertens, J.A., Da Silva, N.A., Hector, R.E., 2015. Triacetic acid lactone production in industrial Saccharomyces yeast strains. J. Ind. Microbiol. Biotechnol. https://doi.org/10.1007/s10295-015-1596-7

Schabort, D.T.W.P., Letebele, P., Steyn, L., Kilian, S.G., Du Preez, J.C., 2016. Differential RNA-seq, multi-network analysis and metabolic regulation analysis of kluyveromyces marxianus reveals a compartmentalised response to xylose. PLoS One. https://doi.org/10.1371/journal.pone.0156242

Shanks, B.H., Keeling, P.L., 2017. Bioprivileged molecules: creating value from biomass. Green Chem. 19, 3177–3185. https://doi.org/10.1039/C7GC00296C

Sharma, N.K., Behera, S., Arora, R., Kumar, S., 2016. Enhancement in xylose utilization using Kluyveromyces marxianus NIRE-K1 through evolutionary adaptation approach. Bioprocess Biosyst. Eng. 39, 835–843. https://doi.org/10.1007/s00449-016-1563-3

Spohner, S.C., Schaum, V., Quitmann, H., Czermak, P., 2016. Kluyveromyces lactis: An emerging tool in biotechnology. J. Biotechnol. https://doi.org/10.1016/j.jbiotec.2016.02.023

Stambuk, B.U., Franden, M.A., Singh, A., Zhang, M., 2003. D-xylose transport by Candida succiphila and Kluyveromyces marxianus, in: Applied Biochemistry and Biotechnology - Part A Enzyme Engineering and Biotechnology. https://doi.org/10.1385/ABAB:106:1-3:255

Strucko, T., Zirngibl, K., Pereira, F., Kafkia, E., Mohamed, E.T., Rettel, M., Stein, F., Feist, A.M., Jouhten, P., Patil, K.R., Forster, J., 2018. Laboratory evolution reveals regulatory and metabolic trade-offs of glycerol utilization in Saccharomyces cerevisiae. Metab. Eng. https://doi.org/10.1016/j.ymben.2018.03.006

Tang, S.Y., Qian, S., Akinterinwa, O., Frei, C.S., Gredell, J.A., Cirino, P.C., 2013. Screening for enhanced triacetic acid lactone production by recombinant Escherichia coli expressing a designed triacetic acid lactone reporter. J. Am. Chem. Soc. 135, 10099–10103. https://doi.org/10.1021/ja402654z

Tomás-Pejó, E., Oliva, J.M., González, A., Ballesteros, I., Ballesteros, M., 2009. Bioethanol production from wheat straw by the thermotolerant yeast Kluyveromyces marxianus CECT 10875 in a simultaneous saccharification and fermentation fed-batch process. Fuel. https://doi.org/10.1016/j.fuel.2009.01.014

Van Leeuwen, C.C.M., Postma, E., Van den Broek, P.J.A., Van Steveninck, J., 1991. Proton-motive force-driven D-galactose transport in plasma membrane vesicles from the yeast Kluyveromyces marxianus. J. Biol. Chem.

Vickery, C.R., Cardenas, J., Bowman, M., Burkart, M.D., Da Silva, N.A., Noel, J.P., 2018a. A coupled *in vitro/in vivo* approach for engineering a heterologous Type III PKS to enhance polyketide biosynthesis in *Saccharomyces cerevisiae*. Biotechnol. Bioeng. 1–9. https://doi.org/10.1002/bit.26564

Vickery, C.R., Cardenas, J., Bowman, M.E., Burkart, M.D., Da Silva, N.A., Noel, J.P., 2018b. A coupled in vitro/in vivo approach for engineering a heterologous type III PKS to enhance polyketide biosynthesis in Saccharomyces cerevisiae. Biotechnol. Bioeng. 115, 1394–1402. https://doi.org/10.1002/bit.26564

Xie, D., Shao, Z., Achkar, J., Zha, W., Frost, J.W., Zhao, H., 2006. Microbial synthesis of triacetic acid lactone. Biotechnol. Bioeng. https://doi.org/10.1002/bit.20759

Yang, C., Hu, S., Zhu, S., Wang, D., Gao, X., Hong, J., 2015. Characterizing yeast promoters used in Kluyveromyces marxianus. World J. Microbiol. Biotechnol. 31, 1641–1646. https://doi.org/10.1007/s11274-015-1899-x

Yu, J., Landberg, J., Shavarebi, F., Bilanchone, V., Okerlund, A., Wanninayake, U., Zhao, L., Kraus, G., Sandmeyer, S., 2018. Bioengineering triacetic acid lactone production in Yarrowia lipolytica for pogostone synthesis. Biotechnol. Bioeng. https://doi.org/10.1002/bit.26733

Zhang, B., Zhang, J., Wang, D., Han, R., Ding, R., Gao, X., Sun, L., Hong, J., 2016. Simultaneous fermentation of glucose and xylose at elevated temperatures co-produces ethanol and xylitol through overexpression of a xylose-specific transporter in engineered Kluyveromyces marxianus. Bioresour. Technol. https://doi.org/10.1016/j.biortech.2016.05.068

Zhou, H., Cheng, J. sheng, Wang, B.L., Fink, G.R., Stephanopoulos, G., 2012. Xylose isomerase overexpression along with engineering of the pentose phosphate pathway and evolutionary engineering enable rapid xylose utilization and ethanol production by Saccharomyces cerevisiae. Metab. Eng. https://doi.org/10.1016/j.ymben.2012.07.011

Zhou, H.X., Xu, J.L., Chi, Z., Liu, G.L., Chi, Z.M., 2013. β-Galactosidase over-production by a mig1 mutant of Kluyveromyces marxianus KM for efficient hydrolysis of lactose. Biochem. Eng. J. 76. https://doi.org/10.1016/j.bej.2013.04.010

Table 1: List of plasmids.

Plasmid	Description
pCA-P2PS	CEN/ARS$_{KM}$, *PGK1p$_{KM}$-2PSHT-CYCt, URA3$_{SC}$*
pCA-P[C35S]	CEN/ARS$_{KM}$, *PGK1p$_{KM}$-2PSHT[C35S]-CYCt, URA3$_{SC}$*
pCA-A2PS	CEN/ARS$_{KM}$, *ADH2p$_{SC}$-2PSHT-CYCt, URA3$_{SC}$*
pCA-A[C35S]	CEN/ARS$_{KM}$, *ADH2p$_{SC}$-2PSHT[C35S]-CYCt, URA3$_{SC}$*
pKD-P2PS	PKD1$_{KL}$, *PGK1p$_{KM}$-2PSHT-CYCt, URA3$_{SC}$*
pKD-A	PKD1$_{KL}$, *ADH2p$_{SC}$-CYCt, URA3$_{SC}$*
pKD-A2PS	PKD1$_{KL}$, *ADH2p$_{SC}$-2PSHT-CYCt, URA3$_{SC}$*

Table 2. Maximum specific growth rates and final optical density (OD_{600}) for *K. marxianus* strains CBS712*ΔURA3* and CBS6556*ΔURA3* cultivated in YP media over a range of temperatures.

		CBS6556 ΔURA3				**CBS712ΔURA3**			
		30°C	37 °C	41 °C	43 °C	30 °C	37 °C	41 °C	43 °C
μ_{MAX}	GLUCOSE	0.78 ± 0.07	0.73 ± 0.03	0.65 ± 0.02	0.56 ± 0.04	0.52 ± 0.09	0.67 ± 0.10	0.66 ± 0.02	0.58 ± 0.02
	GLYCEROL	0.56 ± 0.16	0.68 ± 0.08	0.47 ± 0.03	0.26 ± 0.01	0.55 ± 0.06	0.39 ± 0.06	0.40 ± 0.02	0.32 ± 0.01
	XYLOSE	0.39 ± 0.05	0.40 ± 0.03	0.40 ± 0.03	0.12 ± 0.01	0.31 ± 0.08	0.28 ± 0.08	0.28 ± 0.05	0.28 ± 0.05
OD_{600}	GLUCOSE	37.6 ± 5.5	26.1 ± 1.5	20.0 ± 2.3	15.3 ± 1.0	39.8 ± 3.4	22.9 ± 1.5	14.6 ± 0.8	14.7 ± 1.0
	GLYCEROL	23.6 ± 0.5	30.8 ± 1.2	26.1 ± 0.8	18.4 ± 0.2	17.8 ± 0.8	11.6 ± 0.7	9.7 ± 0.25	4.7 ± 0.3
	XYLOSE	38.6 ± 2.4	33.2 ± 3.3	19.6 ± 1.7	15.5 ± 0.3	43.7 ± 3.3	44.6 ± 3.2	34.9 ± 1.6	26.4 ± 0.6

Values are listed as a mean ± standard deviation (n=3 biological replicates)

Figure Captions

Figure 1: Growth and TAL synthesis for three *K. marxianus* strains carrying the low-copy pCA-P2PS plasmid. Strains were cultivated for 48h at 37°C in 5mL SC medium with a range of carbon sources (at equimolar carbon, equivalent to 1% glucose). A) Growth (optical density at 600nm). B) TAL titer. C) Specific titer. Bars represent mean values ± standard deviation (*n*=3 biological replicates).

Figure 2: TAL production of strain CBS712*ΔURA3*+pCA-P2PS after 48h of culture in SXC medium over a range of temperatures. TAL titer: solid bars. Specific TAL: hatched bars. Bars represent mean ± standard deviation (n=3 biologial replicates).

Figure 3: Comparison of 2-PS enzymes and promoter strength on TAL titer. Strain CBS712*ΔURA3* transformed with pCA-P2PS or pCA-P[C35S] was cultivated at 37°C for 48h in SC medium with three different carbon sources. CBS712*ΔURA3* was also transformed with pCA-A2PS or pCA-A[C35S] (in both vectors, the *ADH2* promoter replaces the *PGK1* promoter) and cultivated at 37°C for 48h in SXC medium. Bars represent mean ± standard deviation (n=3 biological replicates).

Figure 4: Comparison of expression with low and high copy plasmids. Strains CBS6656*ΔURA3*, CBS712*ΔURA3*, and KM1*ΔURA3* were transformed with pCA-P2PS, pKD-P2PS, or pKD-A2PS and cultivated at 37°C for 48h in 3 mL SXC medium. TAL titer: solid bars. Specific TAL: hatched bars. Bars represent mean ± standard deviation (n≥3 biological replicates).

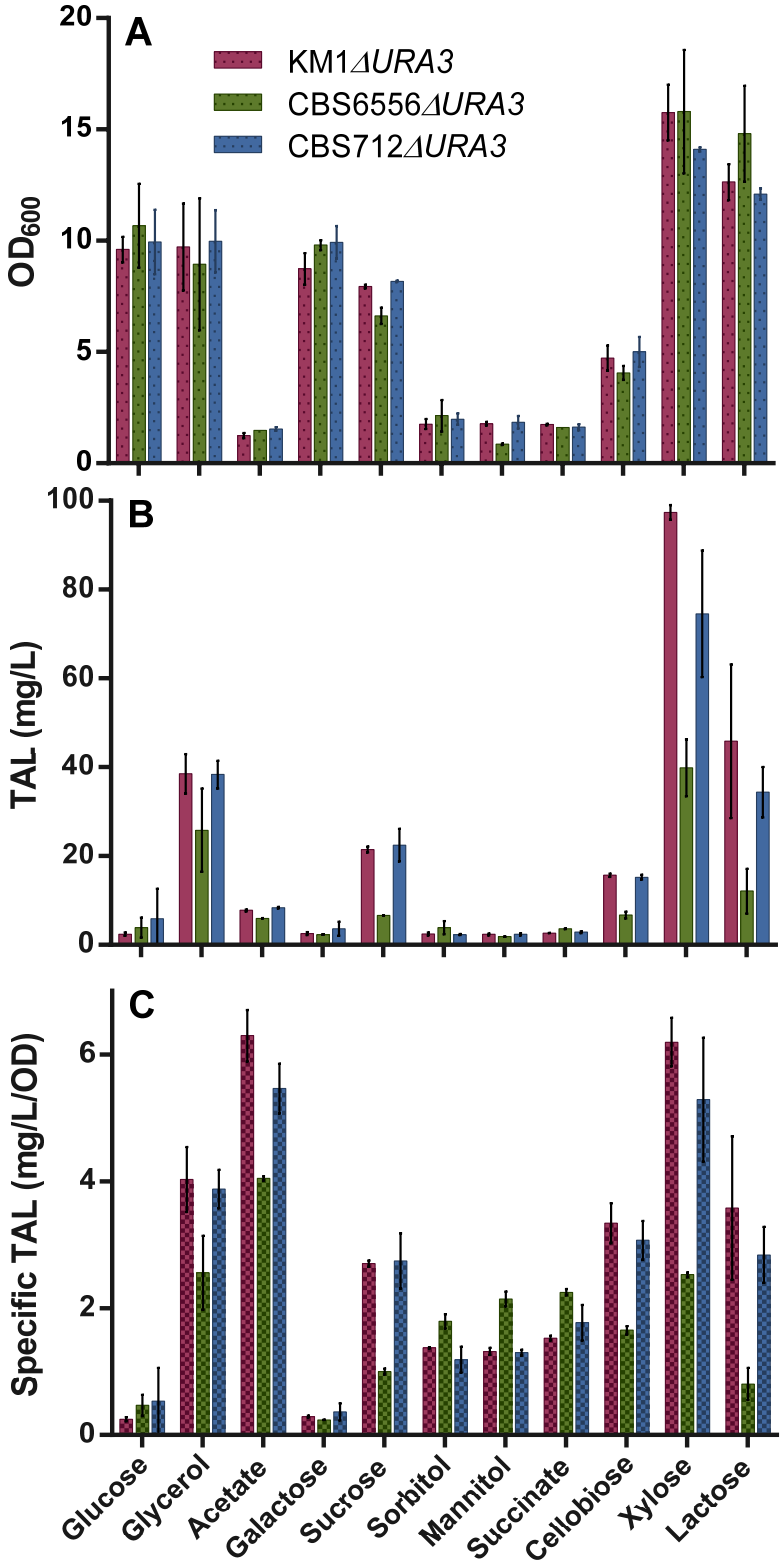

Figure 1: Growth and TAL synthesis for three *K. marxianus* strains carrying the low-copy pCA-P2PS plasmid. Strains were cultivated for 48h at 37°C in 5mL SC medium with a range of carbon sources (at equimolar carbon, equivalent to 1% glucose). A) Growth (optical density at 600nm). B) TAL titer. C) Specific titer. Bars represent mean values ± standard deviation (*n*=3 biological replicates).

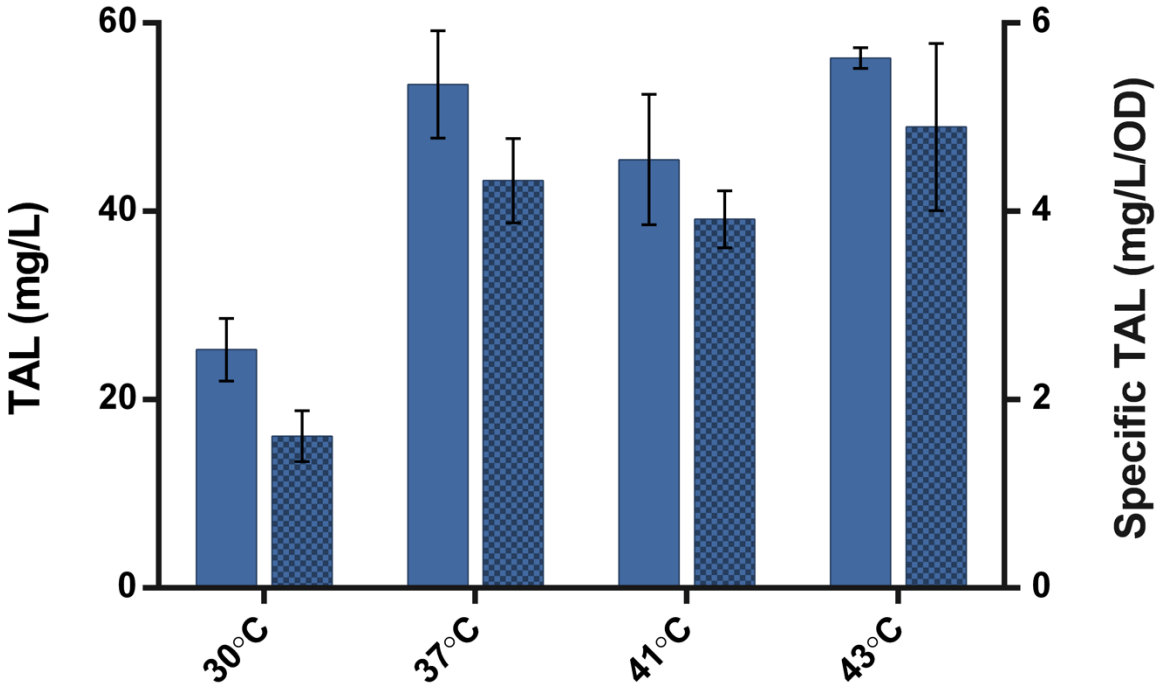

Figure 2: TAL production of strain CBS712*ΔURA3*+pCA-P2PS after 48h of culture in SXC medium over a range of temperatures. TAL titer: solid bars. Specific TAL: hatched bars. Bars represent mean ± standard deviation (n=3 biologial replicates).

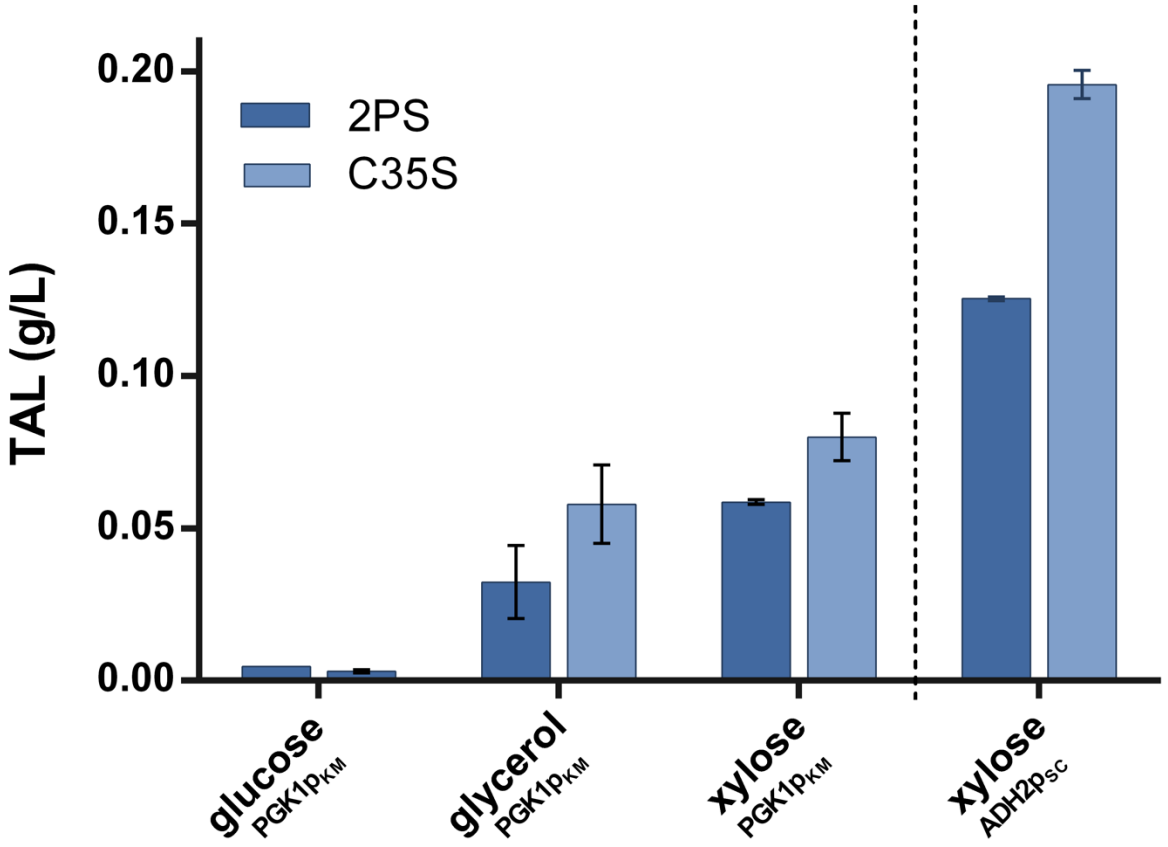

Figure 3: Comparison of 2-PS enzymes and promoter strength on TAL titer. Strain CBS712*ΔURA3* transformed with pCA-P2PS or pCA-P[C35S] was cultivated at 37°C for 48h in SC medium with three different carbon sources. CBS712*ΔURA3* was also transformed with pCA-A2PS or pCA-A[C35S] (in both vectors, the *ADH2* promoter replaces the *PGK1* promoter) and cultivated at 37°C for 48h in SXC medium. Bars represent mean ± standard deviation (n=3 biological replicates).

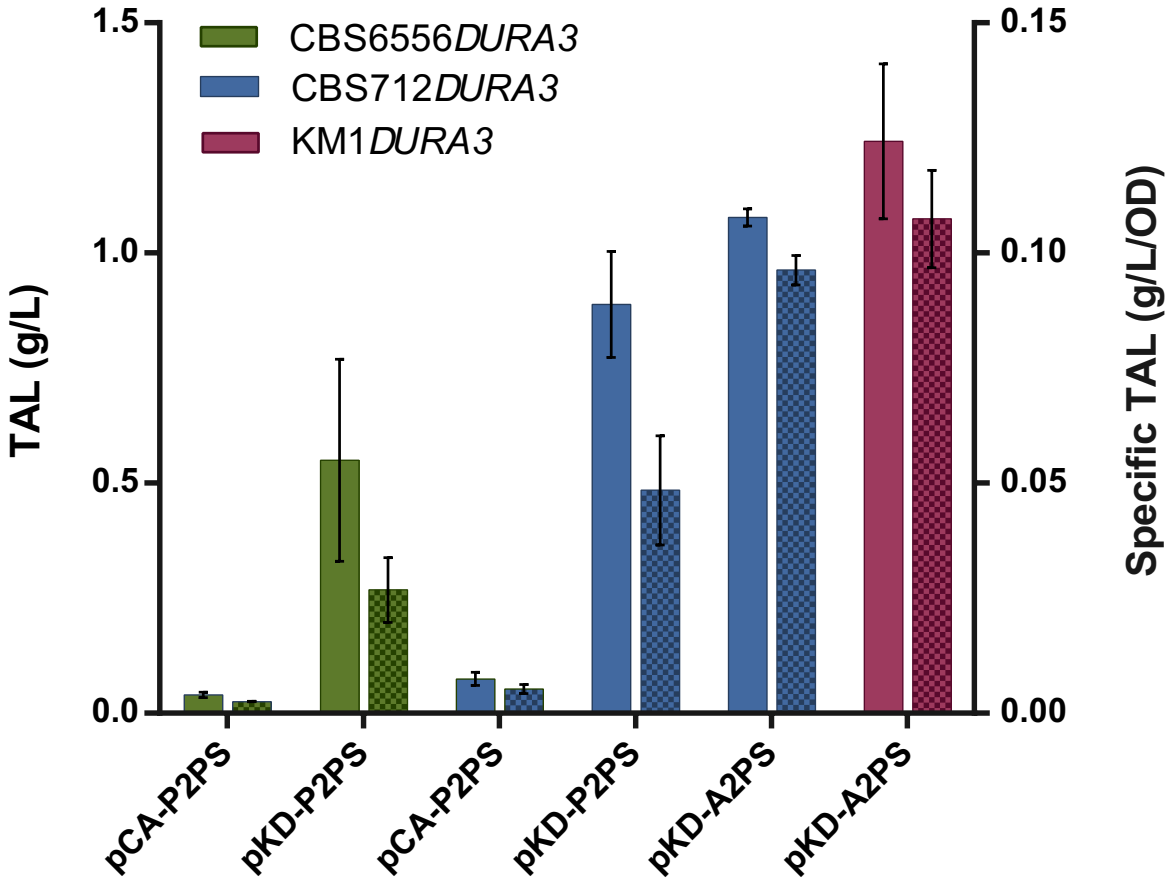

Figure 4: Comparison of expression with low and high copy plasmids. Strains CBS6656Δ*URA3*, CBS712Δ*URA3*, and KM1Δ*URA3* were transformed with pCA-P2PS, pKD-P2PS, or pKD-A2PS and cultivated at 37°C for 48h in 3 mL SXC medium. TAL titer: solid bars. Specific TAL: hatched bars. Bars represent mean ± standard deviation (n≥3 biological replicates).