

Review

***Kluyveromyces marxianus*: Current state of Omics studies, strain improvement strategy and potential industrial implementation**

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Abstract: Bioethanol has been considered as an excellent alternative to fossil fuels since it importantly contributes to the reduced consumption of the crude oil and to the alleviation of environmental pollution [1]. Up to now, the baker yeast *Saccharomyces cerevisiae* is the most common eukaryotic microorganism used in ethanol production. The inability of *S. cerevisiae* to grow on pentoses, however, hinders its effective growth on plant biomass hydrolysates, which contain large amounts of C₅ and C₁₂ sugars. The industrial-scale bioprocessing requires high temperature bioreactors, diverse carbon sources, and the high titer production of volatile compounds [2]. These criteria indicate that the search for alternative microbes possessing useful traits that meet the required standards of bioethanol production is necessary. Compared to other yeasts, *Kluyveromyces marxianus* has several advantages over the others, e.g. it could grow on a broad spectrum of substrates (C₅, C₆ and C₁₂ sugars) [3], tolerate to high temperature, toxin [4,5] and a wide range of pH values [6], and produce volatile short-chain ester [2]. *K. marxianus* also shows a high ethanol production rate at high temperature and is a Crabtree-negative species [7]. These attributes make *K. marxianus* a promise as an industrial host for the biosynthesis of biofuels and other valuable chemicals.

Keywords: Bioethanol; *Kluyveromyces marxianus*; Omics technologies; gTME, and CRISPR-Cas9

Introduction

Saccharomyces cerevisiae plays an extremely important role for millennia in food and beverage productions and is the most studied yeast species [8]. The importance of *S. cerevisiae* in bioethanol production is unquestionable as it is the most common microorganism being used in the 1st generation bioethanol production from sugar or starch crops [9]. Besides the well-known yeast *S. cerevisiae*, however, the demand for other non-conventional yeasts which possess advantageous characters such as thermotolerance or pentose metabolism for industrial application is continuously rising. The thermotolerant yeast *K. marxianus* has many good traits to be used as cell factory in food and biotechnology [10]. These advantages include the fastest growth rate (with the maximum growth rate of 0.80 h⁻¹) among any eukaryotic microbes [11,12], the ability to assimilate a wide range of sugars (e.g., glucose, lactose, galactose, xylose, inulin, and arabinose), thermo- (up to 52°C) and toxin (furaldehyde) tolerance, wide range of pH values (pH 2.5-9), high ethanol yield at elevated temperatures, production of value-added aromatic chemicals (e.g., 2-phenylethylethanol and 2-phenylethyl acetate), and secretion of lytic enzymes [6,8]. This review aims to focus on the latest progress in Omics studies of *K. marxianus*, especially the recent transcriptomic and proteomic studies of *K. marxianus* grown on specific substrates (e.g., Jerusalem artichokes)

or in stress conditions (heat, ethanol stress or toxic compounds). The review also updates the current state of *K. marxianus* strain improvement using advanced molecular biology techniques. The third part of the review emphasizes the role of *K. marxianus* as a complementary microbe in microbial co-culture system and also highlights the use of *K. marxianus* in different configurations of substrate hydrolysis and fermentation for bioethanol production.

Omics studies in *K. marxianus* upon stress conditions

Contrary to the conventional yeast *S. cerevisiae*, *K. marxianus* cannot tolerate high ethanol concentration. Under ethanol stress, e.g., 6% (v/v) ethanol, Diniz *et al.* [15] found that the metabolic flow through the central metabolic pathways was impaired. Genes encoding heat shock proteins were upregulated and ribosome biogenesis-related genes were down regulated, indicating the harmful effect of ethanol on *K. marxianus* growth and cell proliferation machinery. Upon ethanol stress, *S. cerevisiae* increases the degree of unsaturated fatty acids and ergosterol of plasma membrane to maintain the membrane stability [16]. In *K. marxianus* CCT 7735, however, genes encoding unsaturated fatty acid biosynthesis were downregulated at high ethanol concentration [15]. In addition, the fatty acid profile of *K. marxianus* showed that the degree of unsaturated fatty acid did not increase upon ethanol stress. This finding is in agreement with the study of Alvim *et al.* [17] as they found that after 12 h of ethanol exposure, the concentration of ergosterol decreased compared to that of 1 h, 4 h, and 8 h after ethanol exposure. Similarly, in the study of Wang *et al.* [18], various genes encoding fatty acid and ergosterol metabolism were downregulated under multiple inhibitors stress such as phenols, furfural, HMF, and acetic acid. These consistent findings might explain differences in ethanol tolerance capability between *S. cerevisiae* and *K. marxianus*. In the work of Fu *et al.* [19], in contrast, at high temperature (45°C), genes related to lipid metabolism of the plasma membrane were upregulated and *K. marxianus* DMKU3-1042 also produced more ergosterol than it did in the normal condition. Differences in the expression patterns of lipid metabolism-encoding genes and/or ergosterol profiles between the studies of Diniz *et al.* [15], Wang *et al.* [18], Alvim *et al.* [17], and Fu *et al.* [19] might be explained by the differences in *K. marxianus* strains used in these experiments (e.g., CCT 7735, YHJ010, CCT 7735, and DMKU3-1042, respectively) since they used the same method [20] to measure ergosterol content. The downregulation of genes involved in central carbon metabolism are consensus between ethanol stress [15,17], high temperature [19], and mixed inhibitors stress [18] (Table 1). In 1995, Piper stated that many changes induced by ethanol stress were similar to those triggered by heat stress and the synergistic effects of heat and ethanol stresses were recorded [21]. Intriguingly, these present reports reconfirmed Piper's statement as various genes related to central carbon metabolic pathways were found to exhibit the low expression levels upon heat or ethanol exposure (Table 1).

Industrially relevant substrate loading caused overflow metabolism and growth cessation in *Hungateiclostridium thermocellum*, a robust thermophilic, cellulolytic bacterium [22,23]. This phenomenon also happens to *K. marxianus* when growing at high concentration of inulin-containing materials like Jerusalem artichokes. In the study of Gao *et al.* [24], two modules of treatments were used to investigate the effects of inulin loadings and aeration conditions on sugar consumption and ethanol fermentation of *K. marxianus* Y179. The 1st module consists of treatment with 230 g/L inulin, with micro-aeration by oxidation-reduction potential (ORP) controlling at -130 mV and the yeast sample was collected at 36 h (230-130 mV-36) vs. treatment with 230 g/L inulin, without aeration and the sample was collected at 72 h (230-N-72). Similarly, the 2nd module composes the treatment with low substrate loading 120 g/L inulin, without aeration (120-N-24) vs. 230-N-72. In the 1st module, micro-aeration condition (230-130 mV-36) promoted inulin consumption and ethanol fermentation. In the 2nd module, genes related to ethanol metabolism and transcriptional factors were upregulated in 120-N-24, thus suggesting the inhibitory effect of high inulin loading on *K. marxianus* metabolism. Accordingly, gene *HXK1* encodes hexokinase, gene *GLK1* encodes glucokinase and gene *INU1* encodes inulinase were upregulated in 230-

130 mV-36 and 120-N-24 relative to those in 230-N-72. In a previous study [25], under carbon deprivation (when ethanol is the sole carbon source in the medium), the gene *GPM1* encoding phosphoglycerate mutase in *S. cerevisiae* was upregulated and ethanol was used to generate ATP (through oxidative phosphorylation) and sugar phosphates for nucleotide biosynthesis, cell wall construction and storage carbohydrates biosynthesis. Therefore, the function of *GPM1* gene is inferred to be associated with respiratory growth on non-fermentable substrates like ethanol. This gene might be a good candidate for further gene silencing strategy to prevent the consume of ethanol as a substrate, thus enhancing ethanol productivity. The *GPM1* gene in *K. marxianus* was found to be downregulated in low inulin loading (120-N-24) and micro-aeration (230-130 mV-36), suggesting the abundance of carbon source and the favored growth condition for *K. marxianus*. The high expression levels of *PDC1* gene encoding pyruvate decarboxylase and *ADH3* encoding alcohol dehydrogenase in 230-130 mV-36 assumed the carbon flux towards fermentative pathways, which enhance the respiration and regulate reduction reactions. Glycerol-encoding gene *KmGPD1* was upregulated in high inulin concentration to maintain the high cell viabilities during ethanol fermentation. Moreover, two reactive oxygen species (ROS) stress-related genes thioredoxin reductase *TRXR* and glutathione peroxidase *GPX* were upregulated in 230-130 mV-36 treatment, suggesting their important roles in helping cells defend better against ROS damages. Altogether, the regulation patterns of key genes in this study indicated that the micro-aeration in high substrate loading system is suitable for ethanol fermentation using inulin as the starting material.

In summary, the rapid development of Omics technologies helps to gain insight into transcriptomic and proteomic profiles of *K. marxianus* in response to stress conditions such as high temperature, high ethanol concentration or furfural, phenol inhibitors. Based on the gene expression patterns and/or protein abundance upon these harsh circumstances, best candidate genes could be selected for further detailed study or metabolic engineering to develop industrially relevant phenotypes.

Table 1: A brief overview of Omics studies in *K. marxianus*

| Omics Technologies | Growth conditions | Important findings | Sources |
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| RNA-seq, HiSeq 2000 system (Illumina, San Diego, CA 92121, USA) | <p>Yeast strain: Y179</p> <p>Treatments:</p> <ul style="list-style-type: none"> - 120 g/L inulin without aeration (120-N-24) - 230 g/L inulin without aeration (230-N-72) (control) - 230 g/L inulin with aeration (ORP -130 mV) (230-130 mV-36) <p>Differentially expressed genes (DEGs) analysis:</p> <ul style="list-style-type: none"> Module 1: 230-130 mV-36 vs. 230-N-72 Module 2: 120-N-24 vs. 230-N-72 | <p>Result 1: Module 1 had 1840 DEGs, module 2 had 2658 DEGs</p> <p>Conclusion 1: Inulin concentrations had greater effect on transcriptome profiles than aeration and hypoxic condition.</p> <p>Result 2: More genes related to ethanol metabolism and transcriptional factors upregulated in 120-N-24 relative to 230-N-72</p> <p>Conclusion 2: High inulin loading inhibited yeast metabolism</p> <p>Downregulated genes in 230-130 mV-36: <i>GPM1</i></p> <p>Downregulated genes in 120-N-24: <i>GPM1</i></p> <p>Upregulated genes in 230-130 mV-36:</p> <ul style="list-style-type: none"> - Central carbon metabolic pathways: <i>INU1</i>, <i>HXXK1</i>, <i>GLK1</i>, <i>MDH1p</i>, <i>PDC1</i>, <i>ADH3</i>, <i>GPD1p</i>, <i>TRXR</i>, <i>GPX</i>, <i>KMALLA2475</i>, <i>TPO1</i>, <i>HSP31</i> <p>Upregulated genes in 120-N-24:</p> | [24] |

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| | | <p>- Central carbon metabolic pathways: <i>INU1, HXK1, GLK1</i></p> <p>Upregulated genes in 230-N-72: <i>PDC1, MIG1, ATG8</i></p> | |
| <p>RNA-seq, (SOLiD 5500 XL sequencer, Thermo Fisher Scientific, Waltham, USA)</p> | <p>Yeast strain: CCT 7735</p> <p>Treatments: High ethanol exposure 6% (v/v)</p> <p>DEGs analysis: Module 1: 1 h vs. 0 h Module 2: 4 h vs. 0 h Module 3: 1 h vs. 4 h</p> | <p>Downregulated genes in ethanol treatment:</p> <p>- Unsaturated fatty acid and ergosterol biosynthesis: <i>FEN1, SUR4, FAS1, SCS7, KLMA-40623, ERG25, ERG3, SUR2, OLE1, KLMA_20527, KLMA_10244, KLMA_20392</i></p> <p>- Central carbon metabolic pathway: <i>RAG5, GLK1, RAG2, FBA1, GAP3, GAP1, PGK, GPM1, ENO, PYK1, LAT1, PYC2, ACO2b, LSC2</i></p> <p>- Leloir pathway: <i>GAL1, GAL7, GAL10</i></p> <p>- Fermentation pathway: <i>LAT1, ACS2, ADH, ADH1, ADH2, ADH3, ADH4b</i></p> <p>- Translation initiation factors: <i>eIF3a, eIF3e, eIF5A</i></p> <p>Upregulated genes in ethanol treatment:</p> <p>- Central metabolic pathway: <i>ZWF, KLMA70303, PYC2</i></p> <p>- Heat shock protein: <i>HSP26, HSP60, HSP78</i></p> | [15] |
| <p>RNA-seq, Illumina HiSeq 4000 instrument (Illumina, San Diego, CA 92121, USA)</p> | <p>Yeast strain: YHJ010</p> <p>Treatments: Mixed inhibitors (0.7 g/L furfural + 0.7 g/L HMF + 3 g/L acetate acid + 0.28 g/L phenols (4-hydroxybenzaldehyde, syringaldehyde, catechol and vanillin, 0.07 g/L each)</p> <p>DEGs analysis: Mixed inhibitors treatment vs. Control (without stress)</p> | <p>Downregulated genes in mixed inhibitors:</p> <p>- Central carbon metabolism: <i>HXK1, GND1, PGI1, PFK1, PFK2, FBA1, TPI1, TDH1, TDH3, PGK1, GPM1, GPM2, ENO1, PYK1, PDC, ADH2, DAK1</i></p> <p>- Fatty acid and ergosterol metabolism: <i>OLE1, SCS7, FAS2, DUG3, LipA, ERG25, LTA4H, ERG1, ATH1, ERG20</i></p> <p>- B1 & B6 metabolism: <i>KMAR_30698, KMAR_30699, KMAR_30041, KMAR_20540, KMAR_40549, KMAR_30339</i></p> <p>- Transporters: <i>KMAR_50344, KMAR_10529, KMAR_10514, KMAR_10360, KMAR_10458, KMAR_10759, KMAR_20313, KMAR_70169, KMAR_20003, KMAR_70277, KMAR_30323, KMAR_40422, KMAR_60332, KMAR_50593</i></p> <p>- Transcription factors: <i>KMAR_40216, KMAR_40526, KMAR_70129, KMAR_10730, KMAR_60223</i></p> <p>Upregulated genes in mixed inhibitors:</p> <p>- Central carbon metabolism: <i>FBP1, TDH2, ADH3, ADH4, ADH6, ALD6, GUT2, MAE1, CIT1,</i></p> | [18] |

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| | | <p><i>ACO1, ACO2, IDH1, IDH2, KGD1, KGD2, SDH1, SDH2, SDH3, SDH4, MDH2, PCK1, ICL1, MLS1, GDH1</i></p> <p>- Transcription factors: <i>KMAR_30570, KMAR_50272, KMAR_30474, KMAR_30246, KMAR_60382, KMAR_50274, KMAR_40048</i>)</p> <p>- Mitochondrial respiratory chain: <i>ND1L, SDH1, SDH2, SDH3, SDH4, QCR1, QCR2, QCR9, RIP1, CYT1, ATP1, ATP16, ATP14, ATP6C</i></p> <p>- ROS detoxification: <i>KMAR_70075, KMAR_20527, KMAR_40107, KMAR_80342, KMAR_40185, KMAR_50400</i></p> <p>- Transporters: <i>KMAR_80370, KMAR_30579, KMAR_80266, KMAR_50347, KMAR_20602, KMAR_70126, KMAR_10531, KMAR_40029, KMAR_50130, KMAR_80409, KMAR_60406, KMAR_10004, KMAR_40093, KMAR_10790, KMAR_20248, KMAR_40425, KMAR_60075, KMAR_30642, KMAR_30337, KMAR_40188, KMAR_40156, KMAR_70262, KMAR_10802, KMAR_80400, KMAR_40340, KMAR_20004, KMAR_30588, KMAR_70319</i></p> | |
| <p>RNA-seq, HiSeq 4000 system (Illumina Inc., San Diego, CA 92121, USA)</p> | <p>Yeast strain: DMKU3-1042</p> <p>Treatments: High temperature 45°C</p> <p>DEGs analysis: 45°C-14h vs. 30°C-14h 45°C-22h vs. 30°C-22h</p> | <p>Downregulated genes at 45°C vs. 30°C:</p> <p>- Central carbon metabolic network: <i>GLK1, RAG2, PFK1, GPD1, FBA1, TDH1, TDH3, RHR2, TPI1, PGK, ADH2, GPM1, PDX1, LAT1, ACS2, ALD4, CIT1, MDH1, MDH3, FUM1, LSC2, ACO2b, IDP1, KGD1</i></p> <p>- BCAA biosynthesis: <i>LEU1, LEU2, LEU4, SDL1, ILV3, ILV6</i></p> <p>Upregulated genes at 45°C vs. 30°C:</p> <p>- Mitochondrial respiratory chain: <i>COX5A, COX7, COX12, RIP, QCR2</i></p> <p>- Glycerol and acetic acid generation: <i>GPD2, ALD6</i></p> | [19] |
| | <p>DEGs analysis: 45°C-16h vs. 45°C-14h 45°C-18h vs. 45°C-14h 45°C-20h vs. 45°C-14h 45°C-22h vs. 45°C-14h</p> | <p>Downregulated genes at 45°C (16, 18, 20, 22 h vs. 14 h):</p> <p>- Central carbon metabolic network: <i>HXXK, ZWF, GPD1, FBA1, TDH1, TDH3, PGK, ADH1, ADH2, PDC1, ENO, ALD6, MDH1, MDH2, MDH3, SDH1, LSC2, KGD1</i></p> <p>Upregulated genes at 45°C (16, 18, 20, 22 h vs. 14 h):</p> | |

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| | | <p>- Central carbon metabolic network: <i>GLK1, RAG2, FBP1, GPD2, PFK1, RHR2, TPI1, GPM1, GPM3, PYK1, ADH3, ADH4b, ALD4, LAT1, PDX1, ACS2, FUM1, ACO2b, IDP1</i></p> | |
| <p>MALDI-TOF/TOF (Ultraflex III, Bruker, Daltonics, Bremen, Germany)</p> | <p>Yeast strain: CCT 7735</p> <p>Treatments: High ethanol exposure 6% (v/v)</p> <p>Protein abundance analysis: 1 h and 4 h after ethanol exposure vs. 1 h and 4 h (absence of ethanol) (control)</p> | <p>Less abundant at 1 h (ethanol stress) vs. control</p> <p>- Central carbon metabolism: <i>Enolase_8, Enolase_9, Triosephosphate isomerase_2, Triosephosphate isomerase_3, Phosphoglycerate mutase 1_3, NAD(P)H-dependent D-xylose reductase_1, Pyruvate kinase, Fructose-bisphosphate aldolase_1, Phosphoglycerate kinase, Transaldolase_1, Transaldolase_2, Triosephosphate isomerase_1</i></p> <p>- Heat shock proteins: <i>HSP SSA3_9, HSP SSA2</i></p> <p>- Translational proteins: <i>40S ribosomal protein S14, 40S ribosomal protein S18</i></p> <p>More abundant at 1 h (ethanol stress) vs. control</p> <p>- Central carbon metabolism: <i>Enolase_1, Enolase_2, Enolase_4, Enolase_5, Enolase_6, Enolase_7, Enoate reductase 1_1, Enoate reductase 1_3, Enoate reductase 1_4, Hexokinase, Phosphoglycerate mutase 1_2, Phosphoglycerate mutase 1_4, Malate dehydrogenase, Alcohol dehydrogenase 1, Alcohol dehydrogenase 2_2</i></p> <p>- Heat shock proteins: <i>HSP SSA3_1, HSP SSA3_2, HSP SSA3_3, HSP SSA3_4, HSP SSA3_5, HSP SSA3_6, HSP SSA3_7, HSP SSA3_8</i></p> <p>Less abundant at 4 h (ethanol stress) vs. control</p> <p>- Central carbon metabolism: <i>Phosphoglycerate kinase, Fructose-bisphosphate aldolase_1, Enolase_8, Enolase_9, Transaldolase_1, Triosephosphate isomerase_1, Enolase_1, Enolase_2, Enolase_3, Enolase_4, Enolase_5, Enoate reductase 1_2</i></p> <p>- Heat shock proteins: <i>HSP SSA2, HSP104</i></p> <p>More abundant at 4 h (ethanol stress) vs. control</p> <p>- Central carbon metabolism: <i>Enolase_6, Enolase_7, Enoate reductase 1_1, Enoate reductase 1_3, Enoate reductase 1_4, Fructose-bisphosphate aldolase_2, Transaldolase_2, NAD(P)H-dependent D-xylose reductase_1</i></p> <p>- Heat shock proteins: <i>HSP78, HSP26</i></p> | [17] |

Abbreviation of gene/enzyme in the Table 1: RAG5, Hexokinase; RAG2, Glucose-6-phosphate isomerase; FBA1, Fructose-bisphosphate aldolase; GAP3, Glyceraldehyde-3-phosphate dehydrogenase 3; GAP1, Glyceraldehyde-3-phosphate dehydrogenase 1; PGK, Phosphoglycerate kinase; GPM1, Phosphoglycerate mutase 1; GPM2, Probable phosphoglycerate mutase YOR283W; GPM3, Phosphoglycerate mutase 3; ENO, Enolase; PYK1, Pyruvate kinase; LAT1, Acetyltransferase component of pyruvate dehydrogenase complex; PYC2, Pyruvate carboxylase; ACO1, Aconitate hydratase; ACO2b, Aconitate hydratase; LSC2, Succinyl-CoA ligase subunit β ; PFK1, Phosphofructokinase 1; GPD1, Glycerol-3-phosphate dehydrogenase 1; GPD2, Glycerol-3-phosphate dehydrogenase 2; TDH1, Glyceraldehyde-3-phosphate dehydrogenase 1; TDH2, Glyceraldehyde-3-phosphate dehydrogenase 2; TDH3, Glyceraldehyde-3-phosphate dehydrogenase 3; RHR2, Glycerol-3-phosphatase 1; TPI1, Triose phosphate isomerase; PDX1, Pyruvate dehydrogenase; ACS2, Acetyl-CoA synthetase 2; ALD4, Aldehyde dehydrogenase; CIT1, Citrate synthase; MDH1, Malate dehydrogenase 1; MDH2, Malate dehydrogenase 2; MDH3, Malate dehydrogenase 3; FUM1, Fumarate hydratase; IDP1, Isocitrate dehydrogenase 1; KGD1, 2-oxoglutarate dehydrogenase E1 component; KGD2, Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex; GAL1, Galactokinase; GAL7, Galactose-1-phosphate uridylyltransferase; GAL10, Bifunctional protein; ADH, Alcohol dehydrogenase; ADH1, Alcohol dehydrogenase 1; ADH2, Alcohol dehydrogenase 2; ADH3 Alcohol dehydrogenase 3; ADH4b, Alcohol dehydrogenase 4; ADH6, NADP-dependent alcohol dehydrogenase 6; ZWF, Glucose-6-phosphate 1-dehydrogenase; KLMA_70303, 6-phosphofructo-2-kinase; PYC2, Pyruvate carboxylase; ALD6, Magnesium-activated aldehyde dehydrogenase; COX5A, Cytochrome c oxidase polypeptide 5A; COX7, Cytochrome c oxidase subunit 7; COX12, Cytochrome c oxidase subunit 6B; RIP, Cytochrome b-c1 complex subunit Rieske; QCR2, Cytochrome b-c1 complex subunit 2; LEU1, 3-isopropylmalate dehydratase; LEU2, 3-isopropylmalate dehydrogenase; LEU4, 2-isopropylmalate synthase; SDL1, L-serine dehydratase; ILV3, Dihydroxy-acid dehydratase; ILV6, Acetolactate synthase small subunit; BCAA, Bacterial branched-chain amino acid biosynthesis, HXK, Hexokinase; PDC1, Pyruvate decarboxylase 1; SDH1, Succinate dehydrogenase 1; SDH2, Succinate dehydrogenase 2; SDH3, Succinate dehydrogenase 3; SDH4, Succinate dehydrogenase 4; FBP1, Fructose 1,6-bisphosphatase; HXK1, Hexokinase; GND1, 6-phosphogluconate dehydrogenase; PGI1, Glucose-6-phosphate isomerase; PFK1, 6-phosphofructokinase subunit alpha; PFK2, 6-phosphofructokinase subunit beta; PGK1, Phosphoglycerate kinase; ENO1, Enolase 1; DAK1, Dihydroxyacetone kinase 1; GUT2, Glycerol-3-phosphate dehydrogenase; MAE1, NAD-dependent malic enzyme; IDH1, Isocitrate dehydrogenase [NAD]; IDH2, Isocitrate dehydrogenase [NAD]; PCK1, Phosphoenolpyruvate carboxykinase [ATP]; ICL1, Isocitrate lyase; MLS1, Malate synthase 1; GDH1, NADP-specific glutamate dehydrogenase; NDI1, Rotenone-insensitive NADH-ubiquinone oxidoreductase; SDH1, Succinate dehydrogenase 1; SDH2, Succinate dehydrogenase 2; SDH3, Succinate dehydrogenase 3; SDH4, Succinate dehydrogenase 4; QCR1, Cytochrome b-c1 complex subunit 1; QCR2, Cytochrome b-c1 complex subunit 2; QCR9, c reductase complex; RIP1, Cytochrome b-c1 complex subunit Rieske; CYT1, Cytochrome c1; ATP1, ATP synthase subunit alpha; ATP16, ATP synthase subunit delta; ATP14, ATP synthase subunit H; ATP6C, v-Type proton ATPase subunit C; OLE1, Acyl-CoA desaturase 1; SCS7, Inositolphosphorylceramide-B C-26 hydroxylase; FAS2, Fatty acid synthase subunit alpha; DUG3, Probable glutamine amidotransferase DUG3; LipA, Lipoyl synthase; ERG25, c-4 Methylsterol oxidase; LTA4H, Leukotriene A-4 hydrolase; ERG1, Squalene monoxygenase; ATH1, Vacuolar acid trehalase; ERG20, Farnesyl pyrophosphate synthetase; INU1, Inulinase1; GLK1, Glucokinase 1; GPD1p, Glycerol-3-phosphate dehydrogenase; TRXR, Thioredoxin reductase; GPX, Glutathione peroxidase

Advanced techniques in *Kluyveromyces marxianus* strain improvement

K. marxianus can transport various types of sugar such as glucose [26], lactose [27], fructose [28], galactose [29], xylose [30], cellobiose and arabinose [6], organic acids such as lactic acid [31] and malic acid [32] into the

cells. However, the ability of *K. marxianus* to digest cellobiose, was still very poor [6]. To improve the capability of metabolizing cellobiose for *K. marxianus* KY3, Chang *et al.* [6] transformed a rumen fungal β -glucosidase gene from *Neocallimastix* sp. W5 into its genome. Consequently, the transformant *K. marxianus* KY3-NpaBGS strain was able to use cellobiose better and produced approximately 1 g/L ethanol when growing on YP medium supplemented with 20 g/L cellobiose. In contrast, *K. marxianus* SSSJ-0, a native kefir yeast strain that possesses β -glucosidase enzyme, could only use cellobiose for cell growth, but was unable to convert cellobiose into ethanol.

Lignocellulosic biomass is an abundant and renewable resource for the production of biofuels and other value-added compounds [33]. Therefore, many efforts have been made to combine the high ethanol productivity and the robust lignocellulose degradability into a single host cell for a consolidated bioprocessing (CBP). In this concept, the *K. marxianus* KY3 was engineered to be an artificial cellulolytic microbe with five cellulase genes including two exoglucanases (from *Trichoderma reesei*), two endoglucanases (from *Aspergillus niger*) and one β -glucosidase (from *Neocallimastix patriciarum*) transformed into the yeast genome using the Promoter-based Gene Assembly and Simultaneous Overexpression (PGASO) technique [34,35]. In addition, to facilitate the import of cellodextrin into the cells, a fungal cellodextrin transporter gene from the red bread mold *Neurospora crassa* was selected for genetic transformation. Consequently, ethanol productivity of the recombinant *K. marxianus* KR7 strain in YP medium with 10% (w/v) Avicel as the sole carbon source was \sim 0.6 g/L. These foreign genes functioned properly in the host cell, reflecting via cellulolytic enzyme assay, cellodextrin transport, cellobiose digestion and the ethanol production. Although the conversion of Avicel to ethanol was not that efficient, the PGASO method proved its potential for practical applications as it could assemble multiple exogenous genes into *K. marxianus* genome in one single step to facilitate enzyme combinations or to construct *de novo* desired pathways in *K. marxianus* host cell [34].

H. thermocellum cellulosome, the nature's largest cellulolytic machinery, accounts for the fastest growth rate of any bacterium on crystalline cellulose [36]. A cellulosomal enzyme contains a type I dockerin, which could interact with the type I cohesin of the central nonenzymatic scaffolding subunit CipA via type I dockerin-type I cohesin interaction. Due to the Lego-like architecture of cellulosome, each scaffolding subunit CipA, with nine type I cohesins on its structure, can carry simultaneously nine different cellulosomal enzymes. In turn, CipA, with its type II dockerin, enables the interaction with one of three surface anchoring proteins SdbA, Orf2p, or OlpB via type II dockerin-type II cohesin modules. Since the anchoring protein OlpB has seven type II cohesins, the interaction between CipA-OlpB can accommodate up to $9 \times 7 = 63$ cellulosomal enzymes in a single cellulosome complex. Up to now, several research groups have been sought to design cellulosome microbes that can express a full size of cellulosome structure instead of some individual cellulosomal genes called mini-cellulosomes [37–42]. Recently, the group of Anandharaj *et al.* [43] succeeded in developing an engineered *K. marxianus* host that can express a full size of *H. thermocellum* cellulosome on its cell surface. The engineered yeast, with its *de novo* powerful cellulosome, could efficiently degrade Avicel and phosphoric acid-swollen cellulose (PASC) to produce 3.09 g/L and 8.61 g/L of ethanol, respectively. This result could be recorded as the highest ethanol productivity of any constructed yeast cellulosome thus far [43].

The Thioredoxin/Thioredoxin reductase (Trx/TrxR) system is widely present in yeast mitochondria and plays important roles in protecting yeast from ROS damages [24]. In the study of Gao *et al.* [24], they found that the gene encoding thioredoxin reductase (*KmTrxR*) in *K. marxianus* was upregulated under high substrate loading and aerobic conditions. To confirm the protective functions of *K. marxianus* Trx/TrxR system in other yeasts, two genes *KmTRX* and *KmTrxR* were transformed into the *S. cerevisiae* 280 host cell to create the *KmTRX* overexpression strain, the *KmTrxR* overexpression strain, and the double *KmTRX-KmTrxR* overexpression strain. The results showed that although the overexpression of a single *KmTRX* gene in *S. cerevisiae* had adverse effect

on the host cell, the overexpression of *KmTrxR*, in contrast, aided the host cell tolerate to lignocellulose-derived inhibitors such as acetic and formic acids. Moreover, the double overexpression of two genes *KmTRX* and *KmTrxR*, with their synergistic effects, could improve ethanol productivity, and shorten the lag phase of *S. cerevisiae* cell under the inhibitory effects of mixed chemicals such as acetic and formic acids and furfural (FAF) [44]. Also in the study of Gao *et al.* [24], the *KmTPX1* gene, which encodes peroxiredoxin, was found greatly upregulated under aerobic conditions and high inulin concentration. The gene *KmTPX1* is homologous to *Tsa1p* gene in *S. cerevisiae*, which is involved in redox reactions to remove excess ROS like peroxides, to regulate the concentration of peroxides to protect cells from DNA damage and cell death [45]. Taking advantage of their previous finding, Gao *et al.* [46] constructed an overexpression vector which contained *KmTPX1* gene and transformed it into *S. cerevisiae* cell. As expected, the overexpression of *KmTPX1* in the transformant *S. cerevisiae* strain helped the yeast tolerate better to both oxidative stress and inhibitory compounds released from the degradation of lignocellulose. Consequently, the enhanced tolerance of *S. cerevisiae* to oxidative stress and furfural led to the overall higher rates of glucose consumption and ethanol fermentation in the transformant *KmTPX1* strain compared with the control.

Based on the stress-related transcription factor (TF) profiles in *S. cerevisiae* in a prior study [47], Li *et al.* [48] performed a protein-protein BLAST to determine the stress-related TFs in *K. marxianus*. Subsequently, they carried out the genetic transformation of exogenous stress-related TF derived from *K. marxianus* DMKU3-1042 into *S. cerevisiae* TSH3 cell to enhance the thermotolerance, growth and ethanol productivity of *S. cerevisiae* TSH3. As a consequence, at elevated temperature (43°C) and 104.8 g/L glucose, the transformant KmHSF1 and KmMSN2 *S. cerevisiae* strains yielded the final ethanol concentrations of 27.2 ± 1.4 g/L and 27.6 ± 1.2 g/L, respectively, much higher than the control with 18.9 ± 0.3 g/L ethanol. When looking into details, the transcriptomic profiles of these transgenic *S. cerevisiae* strains revealed that the *KmHsf1* gene improved ethanol production by regulating transporter-related genes in the host cell to limit the excessive ATP consumption and by promoting glucose uptake whereas the *KmMsn2* gene might aid in regulating glucose metabolism and glycolysis/gluconeogenesis. In addition, *KmMsn2* promoted the host cell tolerate better to high temperature by regulating genes involved in lipid metabolism, thereby changing membrane fluidity. These above studies exemplify excellently a straightforward procedure from transcriptomic or proteomic studies to the selection of candidate genes for genetic transformation or other technologies for the improvement of microbial biofuel microorganisms.

Recently, in the study of da Silveira *et al.* [49], ethanol-tolerant *K. marxianus* CCT 7735 strains were developed using the Adaptive laboratory evolution (ALE) strategy [50]. Briefly, hundreds of generations of *K. marxianus* were exposed to 4% (v/v) ethanol and the trained yeast cells were considered "ethanol tolerant" when a significant increase (> 50%) in the specific growth rate was observed. In the evolved ethanol-tolerant *K. marxianus* ETS4 strain, the intracellular amine/amide compounds and organic acids abundance were higher than those in its parent strain P4 under ethanol stress. The membrane fatty acid and ergosterol, an important sterol in yeast membranes which is responsible for ethanol tolerance trait [51], were more abundant in the evolved strain ETS4 than in the P4 strain. This phenotype was in accordance with a INDEL mutation in the upstream region of the coding sequence (CDS) detected in the *RR1* gene which is involved in the positive regulation of ergosterol biosynthesis. Likewise, two genes *KLMA_10136* and *PXA*, which are associated with lipid metabolic process, had mutations as follows: INDEL in the upstream region of *KLMA_10136* CDS and INDEL in the downstream region of *PXA* CDS, respectively. Additionally, the accumulations of valine and metabolites of the TCA cycle such as isocitric acid, citric acid, and cis-aconitric acid were recorded only in the ETS4 strain when exposed to ethanol. This might contribute to an increase in ethanol tolerance of the evolved strain.

The TATA-binding protein (TBP) Spt15, one of the components of the general factor RNA polymerase II (RNA Pol II) transcription factor D (TFIID), is the most common target of yeast for global transcription machinery engineering (gTME) technique [52,53]. This technique could induce the global perturbations of the transcriptome through mutagenesis of key proteins that regulate the global transcriptome, thereby improving complex phenotypes quickly and effectively [53]. In the study of Li *et al.* [54], the *SPT15* gene was subjected to error-prone PCR, cloned into an expression vector and then, pooled recombinant plasmids were transformed into *K. marxianus* to construct a random mutagenesis library in its cells. The results of mutant screening under 6% (v/v) ethanol stress showed that two mutant strains M2 and M10 demonstrated faster growth rates than others. Regarding ethanol productivity, M2 strain performed better compared with M10 and control strain (i.e., M2 produced 57.29 ± 1.96 g/L ethanol, which was 23.74% and 22.05% higher than those of M10 and the control, respectively). Moreover, M2 strain also tolerated to high ethanol concentration better than M10 and the control e.g., its ethanol inhibition concentration (EIC) value was 57 g/L, much higher than that of M10 and the control with 46 and 47 g/L, respectively. As a global transcriptome regulator, a Non-synonymous (Non-Syn) mutation (Lys was substituted by Glu³¹) in the *Spt15* gene could influence the expression patterns of hundreds of genes including those involved in the central carbon metabolism, amino acid transport, long-chain fatty acid biosynthesis and MAPK signaling pathway (upregulated) and also ribosome biosynthesis, translation and protein synthesis (downregulated). In this perspective, the gTME method could be used for the improvement of other complex phenotypes such as furfural tolerance or thermotolerance in *K. marxianus*.

Despite several advantageous traits for industrial applications, however, genetic engineering approaches for *K. marxianus* strain improvement have been still limited since the genome-editing tools and stable heterologous expression systems for this yeast species have not well-established yet [55]. In the study of Löbs *et al.* [2], CRISPR-Cas9 system, which was adapted from *Streptococcus pyogenes*, was used to create functional disruptions to alcohol dehydrogenase (ADH) and alcohol-O-acetyltransferase (ATF) genes in *K. marxianus*. The study aimed to investigate the metabolic pathways which are involved in the ethyl acetate and ethanol biosynthesis. In industry, ethyl acetate is used as a solvent and as flavor and fragrance compound and its worldwide demand is ~ 1.7 million tons per year [56]. The data from Löbs *et al.* report showed that the knockout of *KmAtf* gene reduced the production of ethyl acetate by 15%, whereas the disruption of *KmAdh2* gene almost entirely abolished the production of ethanol, resulting in the accumulation of acetaldehyde. The data obtained from *KmADH2* and *KmATF* knock-out strains indicated the fundamental role of *KmAdh2* gene in ethanol production in both aerobic and anaerobic conditions. In regard to ethyl acetate biosynthesis, *KmADH2* played a role in providing ethanol as a substrate for the reaction of *Atf*-catalyzed condensation with acetyl-CoA. Since the disruption of *KmAtf* gene only reduced a little amount of ethyl acetate, it suggested that probable alternative metabolic routes might take responsibility for the biosynthesis of ethyl acetate in *K. marxianus*.

Mono-, Co-culture systems and other fermentation process configurations in bioethanol production using *K. marxianus*

Many fermentation approaches have been widely investigated to improve the productivity of bioethanol, thereby reducing the cost of industrial operation [4,5,34,43,54,57–79] (Table 2). A compatible co-culturing strategy could leverage the useful features from different microbes, thereby improving the productivity relative to monocultures [80]. Since an ideal microbe for consolidated bioprocessing (CBP) still remains to be found, the co-culture of engineered microorganisms, which confer newly advantageous genetic traits on microbes, would be a good approach for biofuels production. As there are numerous studies that have been published, we just took few examples to clarify this concept. In the study of Ho *et al.* [81], a recombinant cellulosomal *Bacillus subtilis* which carried eight genes from *H. thermocellum*, namely one scaffolding protein gene (*cipA*), one cell-surface anchoring gene (*sdbA*), two exoglucanase genes (*celK* and *celS*), two endoglucanase genes (*celA* and *celR*), and

two xylanase genes (*xynC* and *xynZ*) was cultured with a recombinant *K. marxianus* KY3-NpaBGs carrying a β -glucosidase gene from rumen fungus in the YP medium supplemented with 20 g/L Napier grass as the sole carbon source. At 42°C, the ethanol productivity of the dual-microbe co-culturing yielded 3.28 g/L, indicating the potential of *K. marxianus* as a complementary partner for bioprocessing. In this dual *K. marxianus*-*B. subtilis* system, the engineered *B. subtilis* was responsible for cellulolytic hydrolysis via its complex heterologous cellulosomal enzymes and the engineered *K. marxianus*, in turn, helps to convert the resultant cellobiose into glucose via secretory β -glucosidase. In the study of Guo *et al.* [57], they used cheese whey powder (CWP), a by-product of cheese industry, which contains high concentration of lactose and other essential nutrients for co-culturing *S. cerevisiae* and *K. marxianus*. As *S. cerevisiae* cannot ferment lactose but *K. marxianus* can, the co-culturing strategy was applied to make use of the carbon source and nutrient availability in CWP to produce ethanol. In addition, the mixed and alginate-immobilized cells produced higher ethanol yield and productivity relative to the free cell cultures. To enhance ethanol productivity and thermotolerance of yeast cells, the immobilized cocultures of *K. marxianus* DMKU 3-1042 and *S. cerevisiae* M30 on thin-shell silk cocoons (TSC) and alginate-loofa matrix (ALM) were carried out by Eiadpum *et al.* [58]. At high temperatures (range of 40-45°C), both monoculture and coculture performed better than the monoculture of *S. cerevisiae* in producing ethanol. TSC and ALM functioned as yeast cell carriers and might protect cells from adverse conditions like high concentration of inhibitors or elevated temperatures [82]. On average, TSC-immobilized cell system yielded 16% higher ethanol production than ALM-immobilized cell system. This might be due to the high biocompatibility, high mechanical strength, light weight, high surface area, and proper porous structure of TSC that provided a convenient growth environment for yeast cells to live and to produce ethanol [82]. However, in a mixed culture, the cells-cells interaction between different strains is an important issue that should be taken into consideration. Differences in growth rates, nutrient uptake rates and secreted metabolites might be probable factors that affect cell viability [83]. In addition, killer toxins and extracellular proteases synthesized by yeasts may be another matter of mixed fermentation as these toxic compounds might function against their coculture partners [84]. As both *S. cerevisiae* and *K. marxianus* could produce killer toxins [84], they might exclude each other in specific circumstances. In Lopez *et al.* study [83], the viability loss of *K. marxianus* was recorded in mixed culture conditions. Moreover, in the direct contact mixed culture, *S. cerevisiae* was also unfavorably affected.

Table 2: Monoculture, co-culture of *K. marxianus* with other microbes and other fermentation processes for bioethanol production

| Strain | Growth condition | Theoretical ethanol yield (%) | Ethanol yield (g eth/ g sugar) | Maximum ethanol (g/L) | Sources |
|--|---|-------------------------------|--------------------------------|-----------------------|---------|
| Monoculture and direct fermentation | | | | | |
| UFV-3 | Aerobic: 30°C, 250 rpm, whey permeate (240 g/L lactose) | - | 0.35 | 57 | [59] |
| | Hypoxia: 30°C, 40 rpm, whey permeate (170 g/L lactose) | - | 0.53 | 76 | |
| | Anoxia: 30°C, whey permeate (170 g/L lactose) | - | 0.51 | 80 | |
| DMKU 3-1042 | 37°C, sugarcane juice (22% total sugars) | 77.5 | - | 8.7 | [60] |

| | | | | | |
|---|---|--------------|--------------|-------------|------|
| KD-15 | 30°C, 90 rpm, 60 h, saccharified flour mixed with cheese whey (99.3 g/L glucose, 59.4 g/L lactose) | - | 0.45 ± 0.027 | 71.4 ± 2.6 | [61] |
| | 30°C, 90 rpm, 60 h, saccharified potato tubers mixed with cheese whey (137 g/L glucose, 19.1 g/L lactose) | - | 0.44 ± 0.05 | 69.1 ± 3.9 | |
| <i>Kluyveromyces</i> sp. IIPE453 | 45°C, pH 4.5, 16 h, pretreated sugarcane bagasse pith (40 g/L total sugar) | 88 | - | 17.4 | [62] |
| DMB3-7 | 30°C, 40 g/L xylose, 96 h | - | 0.187 ± 0.01 | 6.9 | [63] |
| - | 30 ± 2°C, enzyme-hydrolyzed henequen leaf juice (74.4 ± 3.29 g/L reducing sugar) | 80.04 ± 5.29 | - | 16.5 ± 0.56 | [4] |
| Engineered TATA-binding protein Spt15 strain | 45°C, 100 rpm, 200 g/L glucose | - | - | 58 | [54] |
| UFV-3 | 48°C, 100 rpm, 10 g/L glucose | - | 0.4 ± 0.01 | - | [64] |
| PW | 30 ± 1°C, pH 6, 4% (w/v) NaCl, 10% (v/v) molasses | 39.1 | - | 7.92 | [65] |
| OFF1 | 30°C, <i>A. angustifolia</i> juice (140 g/L reducing sugar) | - | 0.38 | 52.27 | [5] |
| - | 30°C, 24 h, 100 rpm, pomegranate peels (100 g/L ~ 16.83 g/L reducing sugar) | 83.1 | 0.48 | 7.2 | [66] |
| Coculture | | | | | |
| <i>K. marxianus</i> (isolated from the henequen plant) & <i>S. cerevisiae</i> (commercial strain) (25% Km/75% Sc) | 35 ± 2°C, henequen leaf juice + molasses (69.4 g/L total sugar) | - | - | 41.2 | [67] |
| <i>K. marxianus</i> TY-3 & <i>S. cerevisiae</i> AY-5 | 30°C, alginate-immobilized cells, cheese whey powder (100 g/L total sugar) | - | 0.43 | 41.8 | [57] |
| <i>K. marxianus</i> DMKU 3-1042 & <i>S. cerevisiae</i> M30 | 37°C, thin-shell silk cocoon-immobilized cells (IC-TSC), sugarcane juice or blackstrap molasses (220 g/L total sugar) | - | 0.41 | 81.4 | [58] |

| | | | | | |
|--|---|------|------|-------|------|
| | 40°C, thin-shell silk cocoon-immobilized cells (IC-TSC), sugarcane juice or blackstrap molasses (220 g/L total sugar) | - | 0.43 | 77.3 | |
| Other process configurations | | | | | |
| IMB3 | SSF: 45°C, 168 h, hydrothemolysis pretreated switchgrass 8% (w/v) + 0.7 mL Accellerase 1500/g glucan | 86.3 | | 22.5 | [68] |
| β -glucosidase-producing strain YG1027 | SSF: 45°C, air ventilation (3 L/min), 100 g/L cellobiose, 48 h | 51 | | 29.5 | [69] |
| CECT 10875 | SSF: 42°C, 72 h, 150 rpm, 50 mM sodium citrate buffer + 15 FPU cellulase/g substrate + 15 IU β -glucosidase/g substrate | 10.8 | 0.06 | 10.8 | [70] |
| | PSSF: Pre-saccharification 50°C, 8 h, 150 rpm, + 15 FPU cellulase/g substrate + 15 IU beta-glucosidase/g substrate, followed by SSF, 42°C, 72 h | 10.7 | 0.05 | 10.7 | |
| | LSSF: 50°C, 8 h, 150 rpm + 10 IU laccase/g substrate, followed by SSF, 42°C, 72 h | 69.2 | 0.35 | | |
| | LPSSF: Pre-saccharification 50°C, 8h, 150 rpm, + 10 IU laccase/g substrate + 15 FPU cellulase/g substrate + 15 IU beta-glucosidase/g substrate, followed by SSF 42°C, 72 h | 70.9 | 0.36 | 10.7 | |
| K213 | PSSF: pretreated carrot pomace, 50°C, 84 h, 15 FPU Accellerase TM 1000/g dry carrot pomace + 52.3 U pectinase/g dry carrot pomace, followed by SSF with 10% (w/v) resultant carrot pomace, 15 FPU Accellerase™ 1000/g dry carrot pomace + 52.3 U pectinase/g dry carrot pomace, 42°C, pH 5, 680 rpm | - | 0.18 | 18 | [71] |
| <i>K. marxianus</i> UFV-3 | PSSF: 8% (w/v) pretreated sugarcane bagasse, 50°C, 72 h, 15 FPU cellulase/g substrate, 180 rpm, followed by SSF, 37°C | - | 0.28 | 22.62 | [72] |
| <i>S. cerevisiae</i> CAT-1 | PSSF: 8% (w/v) pretreated sugarcane bagasse, 50°C, 72 h, 15 FPU cellulase/g substrate, 180 rpm, followed by SSF 42°C | - | 0.29 | 22.84 | |

| | | | | | |
|--|--|--------|-----------|------------|------|
| Km UOFS Y-2791 Sc UOFS Y-0528 | SHF: Pretreated slurry of <i>O. ficus-indica</i> cladode + 15 FPU cellulase/g substrate + 15 IU beta-glucosidase/g substrate + 100 U pectinase/g substrate, 50°C, 300 rpm, 48 h. SHF: non aeration (<i>S. cerevisiae</i> 35°C, 36 h; <i>K. marxianus</i> , 40°C, 48 h) | - | 0.4; 0.42 | 19.6; 19.5 | [73] |
| | SSF: Pretreated slurry of <i>O. ficus-indica</i> cladode + 15 FPU cellulase/g substrate + 15 IU beta-glucosidase/g substrate + 100 U pectinase/g substrate, non-aeration (<i>S. cerevisiae</i> 35°C, 36 h; <i>K. marxianus</i> 40°C, 48h) | 70; 64 | - | 20.6; 19.3 | |
| CCT 7735 | SSF: 39.5°C, 72.5 rpm, pH 5.05, 72 h, 22.5 FPU cellulase/g substrate, saccharified sugarcane bagasse (80 g/L) + ricotta whey 5% (w/v), hipoxia | - | - | 49.65 | [74] |
| K213 | SHF: NaOH/H ₂ O ₂ -pretreated water hyacinth, 52.29 FPU/g substrate, 50°C, incubated 3 days, 150 rpm. | - | 0.13 | 6.41 | [75] |
| | SSF: 42°C, 20 mL fermentation medium, 52.29 FPU cellulase, 1 g NaOH/H ₂ O ₂ -pretreated water hyacinth | - | 0.16 | 7.34 | |
| KR9 (glycoside hydrolase from <i>A. niger</i> , <i>T. reesei</i> , <i>N. patriciarum</i>) | 37°C, 200 rpm, saccharified rice straw (~60 g/L glucose) | 90 | - | 50 | [76] |
| CCT 7735 | PSSF: Pre-saccharification of pretreated elephant grass (16%, w/v) + 60 FPU cellulase/mL substrate, 50°C, 72 h, gentle agitation, followed by SSF, 38°C, pH 4.8, 50 rpm | - | - | 45.5 | [77] |
| Consolidated bioprocessing (CBP) | | | | | |
| Engineered <i>K. marxianus</i> (<i>T. reesei</i> endoglucanase, <i>A. aculeatus</i> β -glucosidase) | 48°C, 10 g/L β -glucan, 12 h | 92.2 | 0.47 | 4.24 | [78] |
| Engineered <i>K. marxianus</i> KR5 | 37°C, 120 rpm, 2% (w/v) cellobiose, 168 h | 93 | - | 8.5 | [34] |

| | | | | | |
|--|--|------|-----------------|----------------|------|
| (<i>T. reesei</i> endoglucanase, exoglucanase, cow rumen fungus beta-glucosidase) | 37°C, 120 rpm, 2% (w/v) β -glycan, 168 h | 74 | - | 5.4 | |
| Inulinase-producing strain Y179 | Aeration (0.025 vvm): 33°C, pH 4.7, 250 rpm, Jerusalem artichoke tuber meal (210 g/L total sugars), 48 h | 77.1 | 0.4 \pm 0.01 | 75.6 \pm 1.6 | [79] |
| | Without aeration: 33°C, pH 4.7, Jerusalem artichoke tuber meal (210 g/L total sugars), 84 h | 86.9 | 0.45 \pm 0.01 | 83.1 \pm 1.5 | |
| Engineered <i>K. marxianus</i> (C. <i>thermocellum</i> largest cellulosome complex OlpB) | 1% (w/v) Avicel, 37°C, 300 rpm | - | - | 3.09 | [43] |
| | 1% (w/v) PASC, 37°C, 300 rpm | - | - | 8.61 | |

Abbreviation: LSSF, Laccase treatment, Simultaneous Saccharification and Fermentation; LPSSF, Laccase treatment, Pre-saccharification, Simultaneous Saccharification and Fermentation; PSSF, Pre-saccharification, Simultaneous Saccharification and Fermentation; SSF: Simultaneous Saccharification and Fermentation; IU, International Unit; FPU, Filter Paper Unit; SHF, Separate Hydrolysis and Fermentation

Conclusion

The non-conventional yeast *K. marxianus* has been proved to be a promising eukaryotic microbe for bioethanol production and other food and environmental applications. Although having several useful traits which are suitable for bioethanol production at industrial scale, its genetic drawback such as the sensitivity to high concentration of ethanol or the incapability of growing on polysaccharides should be improved to meet the demand of industrial fermenting yeast strain. In addition, despite a lot of efforts have been deployed for constructing a robust *K. marxianus* strain appropriate for CBP, the ethanol productivity of these current engineered strains was still modest. Up to now, the highest ethanol concentration produced by an engineered *K. marxianus* is only 8.61 g/L, too low for any practical consideration.

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