MARIA FERNANDA DURÁN MENESES

# CONSTRUCTION AND PHENOTYPIC ANALYSIS OF *Kluyveromyces marxianus* CCT 7735 RECOMBINANT FLOCCULENT STRAINS FOR BIOETHANOL PRODUCTION

Dissertation presented to the Universidade Federal de Viçosa as part of the requirements of the Agricultural Microbiology's Postgraduate Program, to obtain the title of *Magister Scientiae*.

**VIÇOSA**
**MINAS GERAIS - BRASIL**
**2015**

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Durán Meneses, Maria Fernanda, 1989-
D948c Construction and phenotypic analysis of *Kluyveromyces*
2015 *marxianus* CCT 7735 recombinant flocculent strains for bioethanol production / Maria Fernanda Durán Meneses. - Viçosa, MG, 2015.
iv, 41f. : il. ; 29 cm.

Orientador: Denise Mara Soares Bazzolli.
Dissertação (mestrado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. *Kluyveromyces marxianus. 2.* Leveduras (Fungos).
3. Leveduras (Fungos) - Floculação. 4. Fermentação - Efeito da temperatura. 5. Etanol . I. Universidade Federal de Viçosa. Departamento de Microbiologia. Programa de Pós-graduação em Microbiologia Agrícola. II. Título.

CDD 22. ed. 579.562

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APROVADA: 29 de julho de 2015.

Miriam Teresinha dos Santos

Fábio de Ávila Rodrigues

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# SUMÁRIO

## RESUMO

DURÁN MENESES, Maria Fernanda, M. Sc., Universidade Federal de Viçosa, julho de 2015. **Construção e analises fenotípicas de linhagens floculantes de *Kluyveromyces marxianus* CCT 7735 para produção de bioetanol.** Orientador: Denise Mara Soares Bazzolli. Coorientador: Wendel Batista da Silveira.

Leveduras floculantes são usadas em processos industriais pois representam uma estratégia simples e econômica para recuperar as células a partir do mosto fermentado. No entanto, as leveduras só conseguem expressar o fenótipo floculante em condições extremas, nas quais, a integridade da população esteja sendo afetada. Neste trabalho, foi descrita a construção de novas linhagens de *Kluyveromyces marxianus* CCT 7735 capazes de expressar constitutivamente um fenótipo floculante a partir da integração de DNA linear correspondente aos genes *FLO1, FLO5, FLO9* e *FLO10* de *Saccharomyces cerevisiae* BY4700. Todas as linhagens recombinantes mostraram fenótipo floculante a temperaturas de 40°C e 45°C. As linhagens recombinantes de *K. marxianus* CCT 7735 *FLO1* e *FLO9* mostraram um perfil similar de produção de etanol quando comparadas com *K. marxianus* CCT 7735 tipo selvagem. Estas linhagens recombinantes possuem um fenótipo floculante estável que proporciona uma vantagem sobre o tipo selvagem em relação ao uso em sistemas de fermentação em temperaturas elevadas.

## ABSTRACT

DURÁN MENESES, Maria Fernanda, M. Sc., Universidade Federal de Viçosa, July 2015. **Construction and phenotypic analysis of *Kluyveromyces marxianus* CCT 7735 recombinant flocculent strains for bioethanol production.** Adviser: Denise Mara Soares Bazzolli. Co-adviser: Wendel Batista da Silveira.

Flocculent yeasts are used in industrial processes because is a simple and cost-effective strategy for cell recovery from fermentation mash. However, yeast only shows natural flocculent phenotype in extreme conditions, which makes it a complex phenotype. Here, we describe the construction of novel *Kluyveromyces marxianus* CCT 7735 strains expressing constitutive flocculent phenotype by linear DNA integration of *FLO1, FLO5, FLO9* and *FLO10* genes from *Saccharomyces cerevisiae* BY4700. All recombinant strains showed flocculation ability at 40°C and 45°C, *K. marxianus* CCT 7735 *FLO1* and *FLO9* strains showed similar ethanol production profile when compared to *K. marxianus* CCT 7735 wild type. These novel strains have special flocculent characteristics that provide an advantage over wild type for use in continuous ethanol fermentation systems at high temperatures.

# INTRODUÇÃO

Floculação em leveduras é definido como um processo reversível de agregação celular dependente de cálcio na qual a adesão das células resulta na formação de flocos que podem ser facilmente separados do mosto (Stratford, 1989; Nonklang et al., 2009). A adesão celular envolve glicoproteínas especificas chamadas floculinas, que interagem com receptores na parede celular da célula vizinha, resultando em floculação (Verstrepen and Klis, 2006). As interações mediadas por glicoproteínas da família Flo na levedura *Saccharomyces cerevisiae* são divididas em dois grupos: *Lectin-like* (genes *FLO1, FLO5, FLO9, FLO10*) que confere a habilidade de adesão entre células (floculação) e *sugar-insensitive* (gene *FLO11*) que confere habilidade para adesão a superfícies abióticas (Guo et al., 2000; Verstrepen and Klis, 2006). O uso de leveduras floculantes em processos de fermentação leva a uma fácil, econômica, rápida e inócua separação das células a partir do produto formado (Nonklang et al., 2009; Vallejo et al., 2012). Este fenótipo floculante confere resistência aos estresses oxidativo e por etanol (Smukalla et al., 2008) e além disso, as células organizadas em flocos mostram uma alta tolerância a inibidores da fermentação em procesos de produção de etanol de segunda geração como o furfural (Landaeta et al., 2013). Essas propriedades têm conduzido a pesquisas sobre a construção de linhagens floculantes a partir de leveduras não floculantes por métodos de transformação genética.

A levedura não-convencional *Kluyveromyces marxianus* tem recebido considerável atenção devido a sua capacidade nativa de utilização de um amplo espectro de substratos e também apresenta baixa tendência para produzir etanol se exposta a altas concentrações de açúcar (Fonseca et al., 2008). Outras características industrialmente relevantes desta levedura incluem a termotolerância, uma vez que, algumas linhagens são capazes de crescer a temperaturas superiores a 50°C (Morrissey et al., 2015); curto tempo de geração e alta taxa de crescimento inclusive em temperaturas elevadas (Zhang

et al., 2013) e produção eficiente de proteínas recombinantes (Lertwattanasakul et al., 2015). Em particular, a levedura não floculante *Kluyveromyces marxianus* CCT 7735 isolada a partir de um lacticínio no Brasil (Silveira et al., 2005), apresenta resistência a elevadas temperaturas tais como 45$^{o}$C (Costa et al, 2014) e alto fluxo fermentativo de lactose (Silveira et al., 2005), propriedade ausente em *Saccharomyces cerevisiae,* elevada taxa de crescimento em condições aeróbicas e hipóxias (Diniz et al., 2012) e capacidade de secretar baixo numero de proteínas quando cultivada em lactose, o que é uma propriedade desejável devido ao baixo numero de possíveis proteínas contaminantes no processo de purificação (Diniz et al., 2014). Essas caracteristicas e o fato de que o genoma de *K. marxianus* CCT 7735 tem sido recentemente sequenciado (Silveira et al., 2014) torna esta levedura uma alternativa viável como produtora de etanol, e portanto, conferir propriedades de floculação a *K. marxianus* CCT 7735 por integração de DNA linear tornaria esta linhagem útil em sistemas de fermentação visando uma via simples de separação das células a partir do mosto.

Técnicas de integração de DNA linear para expressão proteica em leveduras elimina os sistemas dependentes de vetores de integração (Nonklang et al. 2008). Este tipo de técnica foi pela primeira vez utilizado em *S. cerevisiae* baseada no princípio de que o sistema de reparo *Double-Strand Brick* em leveduras é estimulado por extremidades de DNA linear (Szostak et al, 1983), que por sua vez permitem a inserção de fragmentos de DNA linear no genoma da levedura, eliminando procedimentos como construção de vetores e suas variações (Court et al, 2002).

Integrações bem sucedidas de DNA linear têm sido reportadas em linhagens de *K. marxianus* (Nonklang et al. 2008; Nonklang et al. 2009; Abdel-Banat et al, 2010; Vallejo et al, 2012) demonstrando a capacidade de recombinação por integração de DNA linear, síntese e secreção de proteínas heterólogas nesta levedura.

No presente trabalho é reportada a obtenção e as análises fenotípicas de 4 novas linhagens floculantes de *K. marxianus* CCT 7735 para produção de etanol, aplicando-se a integração de DNA linear correspondente aos genes *FLO1, FLO5, FLO9* e *FLO10* de *Saccharomyces cerevisiae* sob o controle do promotor constitutivo *TDH3.*

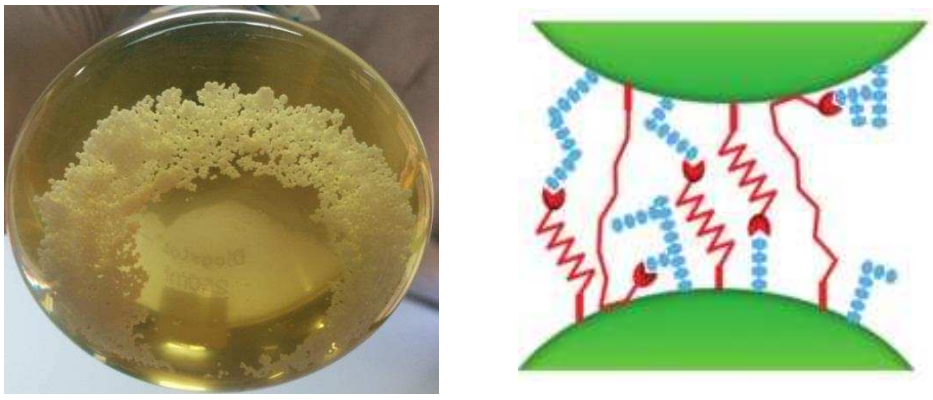
## REVIEW

The yeast flocculation phenomenon is employee in industrial ethanol production as an easy and cost-effective way to separate cells from the ferment produced in primary fermentation (Goossens and Willaert, 2010).

As reviewed by Verstrepen and Klis (2006), flocculation is conferred by specialized cell-surface proteins called 'adhesins' or 'flocculins' that interact with sugar residues in adjacent cells. The cell adhesion is a property that involves specific glycoproteins, called flocculins, interacting with carbohydrate receptors on the cell wall of neighbor cells, resulting in cell flocculation (Verstrepen and Klis, 2006). Yeast's flocculins have thee-domain structure: C-terminal domain, which contains a glycosylphosphati-dylinositol attachment (GPI-attachment) site that links covalently the flocculin to the cell wall (Groot et al., 2003); N-terminal (or lectin) domain contains a carbohydrate or peptide which links with sugar residues (Groes et al., 2002; Rigden et al., 2004); and the middle domain that contains a long and semi-rigid structure made of multiple serine and threonine repeats that are prone to *O*-glycosylation during the post-translational modification of the protein that might be stabilized by $Ca^{2+}$ ions (Verstrepen and Klis, 2006).

The interactions mediated by Flo glycoproteins family in yeast like *Saccharomyces cerevisiae* are divided into two adhesion phenotype groups: lectin-like (*FLO1, FLO5, FLO9* and *FLO10* genes) that confers cell to cell adhesion ability (Figure 1), and sugar-insensitive (*FLO11* gene) that confers adhesion ability to abiotic surfaces (Guo et al., 2000; Verstrepen and Klis, 2006). Lectin-like group also known as sugar-sensitive group is divided into two sub-categories, Flo1 which links mannose sugars and NewFlo which links several other sugars such as glucose and its oligomers (Sato et al., 2002). On the other hand, sugar-insensitive group is mediated by adhesins which link peptides or increase the cell-surface hydrophobicity, conferring hydrophobicity-based adhesion to abiotic components (Guo et al., 2000).

The importance of sugar-sensitive behavior in cell to cell interaction is supported by studies about the sugars inhibiting the flocculation competitively (Watanabe et al., 2008; Van Mulders et al., 2009; Matsuzawa et al., 2011; Sugiyama et al., 2015). Competitive inhibition conferred by sugars, it means, interaction between sugars on the medium with flocculin proteins on the cell surface, resulting in cell to cell adhesion only when all sugar on the medium is consumed (Van Mulders et al., 2009). This competitive inhibition prevents early or premature yeast flocculation which would avoid the impact of mass transfer limitation in heavily flocculent cells and reduce the risk to get incomplete fermentation of sugars to alcohol (Panteloglou et al., 2012). This information indicates that the flocculent phenotype in yeast cells should be achieved without affecting substrate consumption and product formation.



**A** **B**

**Figure 1.** Cell to cell adhesion ability.
(A) *Saccharomyces cerevisiae* RAK3981 strain were growth in liquid medium (YPD) and incubated overnight at 30°C with shaking at 180 rpm. Cells expressing *FLO9* flocculin showing strong cell-cell adhesion (flocculation) and sediment at the bottom of the flask. (B) Flocculin proteins (red) linked to sugars residues (blue) representing the interaction between two neighbor cells (El-Kirat-Chatel et al., 2015).

The genes encoding flocculins are not constitutively expressed in yeasts (Verstrepen et al., 2006). *S. cerevisiae* yeast grows in individual cell form and, only the changes on environmental conditions lead to modifications in the properties of cell wall (Bester et al., 2006). The flocculation is induced by stress condition because it might protect the cells in the middle of the flocs from the environment (Stratford, 1992) being controlled by nutritional shortage as carbon or nitrogen starvation and other environmental factors such as pH, high temperature and, osmotic, oxidative and ethanol stress (Claro et al., 2007; Ogata, 2012; Tofalo et al., 2014;). However, Claro et al. (2007) concluded in their work that not all kind of stress induce flocculation and each strain showed a different response, as well as, each *FLO* gene is able to produce different flocculation phenotypes and cell-surface properties (Halme et al., 2004).

Constant switching and adaptation of flocculation are crucial for cell survival in natural habitats, but, an unstable flocculation phenotype is a risky condition for use in industrial applications where is desirable the use of constant and predictable yeast (Verstrepen and Klis, 2006).

*Kluyveromyces marxianus* strains are considered yeasts of enormous potential for ethanol production because they exhibit the ability to ferment at high temperatures, to assimilate wide variety of substrate and they present high growth rate (Fonseca et al., 2008). Particularly, *Kluyveromyces marxianus* CCT 7735 has high temperature (45°C) resistance (Costa et al, 2014) and high flow lactose fermentation as reported by Silveira et al. (Silveira et al., 2005), maximum growth rate under aerobic and hypoxic conditions, 0.54 $h^{-1}$ and 0.28 $h^{-1}$ respectively (Diniz et al., 2012), great characteristics for its use in ethanol production (Fonseca et al., 2008). However, despite subjecting the yeast to various stress conditions, a flocculent phenotype of *K. marxianus* CCT 7735 has never been expressed naturally. Nonklang et al, (2009) characterized *K. marxianus* strains as non-flocculent yeast, because, even tested in condition which *S. cerevisiae* presents flocculent phenotype, *K. marxianus* did not.

Researches appointing to flocculent strains construction of *K. marxianus* has been done. Nonklang et al, (2009) demonstrated that *S. cerevisiae TDH3* promoter and *FLO* genes were functional in *K. marxianus* DMKU3-1042, conferring flocculation phenotype to this yeast and, Vallejo et al, (2012) generated inducible flocculation phenotype in *K. marxianus* CECT 11769 by expressing of *S. cerevisiae FLO5* gene. Thereby, conferring flocculation property to *K. marxianus* CCT 7735 makes this strain useful in fermentation systems, achieving a simple way to separate the cells from the wort and thus, it will contribute to save energy consumption through the process, the use of centrifuges won't be necessary.

Below, in table 1, is shown a summary of researches carried out on *S. cerevisiae* and *K. marxianus* strains to confer stable flocculent phenotype.

**Table 1**. Researches of flocculent phenotypes involving *S. cerevisiae* and *K. marxianus* strains for industrial processes.

| Organism | Strategy | Process | Reference |
|---|---|---|---|
| ***S. cerevisiae*** | ADH2 promoter controlled expression of the native FLO1 and FLO5 ORF | Beer and wine production | (Govender et al., 2010) |
| ***S. cerevisiae* Σ1278*b*** | Induction of FLO11 gene in *S. cerevisiae* | Exhibit differences in FLO regulation | (Fichtner et al., 2007) |
| ***S. cerevisiae* 6525** | Integration expression of *FLO1spsc* from yeast SPSC01 in *S. cerevisiae* | Ethanol production | (He et al., 2012) |
| ***S. cerevisiae* CEN.PK 113-7D** | Variants of the flocculation gene *FLO1* were transformed into *S. cerevisiae* | Biorefinery process on lignocellulosic raw material | (Westman et al., 2014) |
| ***K. marxianus* ATTC 10022** | Induction of *GAP1* gene in *K. marxianus* | Protein production | (Almeida et al, 2003) |
| ***K. marxianus* DMKU3-1042** | Induction of *S. cerevisiae FLO1, FLO5, FLO9* and *FLO10* genes in *K. marxianus* | Ethanol production | (Nonklang et al., 2009) |
| ***K. marxianus* CECT 11769** | Expression of *S. cerevisiae FLO5* gene under control of *K. marxianus* native EPG1 promoter. | Ethanol production | (Vallejo et al., 2012) |

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**CAPITULO 1**

# Construction and phenotypic analyses of *Kluyveromyces marxianus* CCT 7735 recombinant flocculent strains for bioethanol production

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*** Manuscript written according to *Applied Microbiology and Biotechnology Journal*.**

## ABSTRACT

Flocculent yeasts are used in industrial processes because is a simple and cost-effective strategy for cell recovery from fermentation mash. However, yeast only shows natural flocculent phenotype in extreme conditions, which makes it a complex phenotype. Here, we describe the construction of novel *Kluyveromyces marxianus* CCT 7735 strains expressing constitutive flocculent phenotype by linear DNA integration of *FLO1, FLO5, FLO9* and *FLO10* genes from *Saccharomyces cerevisiae* BY4700. All recombinant strains showed flocculation ability at 40°C and 45°C, *K. marxianus* CCT 7735 *FLO1* and *FLO9* strains showed similar ethanol production profile when compared to *K. marxianus* CCT 7735 wild type. These novel strains have special flocculent characteristics that provide an advantage over wild type for use in continuous ethanol fermentation systems at high temperatures.



**Key words:** *Kluyveromyces marxianus,* flocculation phenotype, ethanol fermentation.

## INTRODUCTION

Flocculation in yeast has been described as an asexual and reversible $Ca^{2+}$-dependent cellular aggregation in which adhesion of cells results in flocs, which can be easily separated from a fermentation mash (Stratford, 1989; Nonklang et al., 2009). The cell adhesion is a property that involves specific glycoproteins, called flocculins, interacting with carbohydrate receptors on the cell wall of neighbor cells, resulting in the cell flocculation (Verstrepen and Klis, 2006). The interactions mediated by Flo glycoproteins family in yeasts like *Saccharomyces cerevisiae* are divided into two adhesion phenotypes groups: lectin-like (*FLO1, FLO5, FLO9* and *FLO10* genes) that confers cell to cell adhesion ability (flocculation) and, sugar-insensitive (*FLO11* gene) that confers adhesion ability to abiotic surfaces (Guo et al., 2000; Verstrepen and Klis, 2006). In that way, the use of self-flocculation yeast in continuous fermentation processes leads to easy, cheap, fast and innocuous separation of the cells (Nonklang et al., 2009; Vallejo et al., 2012) from the newly fermented wort. This yeast phenotype also confers ethanol and oxidative stress resistance (Smukalla et al., 2008) and cells show a higher tolerance to fermentation inhibitors such as furfural (Landaeta et al., 2013). These properties have led to research on flocculent strains construction from non-flocculating yeast by transformation methods.

The nonconventional yeast *Kluyveromyces marxianus* has attracted considerable attention because of his native utilization of a broad variety of substrates and also presents lower tendency to produce ethanol if exposed to high sugar concentration (Fonseca et al., 2008). Other industrially-relevant features about this yeast include thermotolerance, some strains are able to grow at temperatures above 50 ºC (Morrissey et al., 2015), have short generation time and high growth rate even at elevated temperatures (Zhang et al., 2013), and have efficient recombinant protein production (Lertwattanasakul et al., 2015). In particular, the non-flocculent yeast *Kluyveromyces marxianus* CCT 7735 strain isolated from cheese factories in Brazil (Silveira et al., 2005), beyond its resistance to high temperature, such as 45ºC (Costa et al, 2014), shows high

fermentative flux of lactose (Silveira et al., 2005), property that is absent in *S. cerevisiae*; high growth rate under aerobic and hypoxic conditions (Diniz et al., 2012), and the fact of secrete low number of proteins when cultured in lactose, which is a desired point due to the low number of possible contaminant proteins in purification processes (Diniz et al., 2014). These capabilities and, the fact that *K. marxianus* CCT 7735 genome was sequenced recently (Silveira et al., 2014), turn this yeast into a viable alternative as an ethanol producer. Conferring constitutive flocculation properties to *K. marxianus* CCT 7735 by linear gene integrations turns it a useful strain in continuous fermentation systems that would provide a simple separation way of the yeast cells from wort.

Linear DNA (L-DNA) technics for protein expression on yeast eliminates the vector dependent system (Nonklang et al. 2008). The L-DNA type of recombination was first utilized in *S. cerevisiae* based on the genetic principle that the double-strand break repair system in yeast is stimulated by L-DNA ends (Szostak et al, 1983), which in time allows insertion of L-DNA fragments into yeast genome, without time-consuming procedures such as vector-insert construction and their variations (Court et al, 2002).

Successful L-DNA recombination, it means, L-DNA integration, has been reported in *K. marxianus* strains (Nonklang et al. 2008; Nonklang et al. 2009; Abdel-Banat et al, 2010; Vallejo et al, 2012) demonstrating the recombination capability by linear DNA integration, synthesis and secretion of heterologous proteins in this yeast.

Here, we report the successful construction of 4 novel flocculent *K. marxianus* CCT 7735 strains for industrial ethanol production. This was accomplished applying genetic engineering for L-DNA integration of *FLO1, FLO5, FLO9* and *FLO10 S. cerevisiae* genes under the control of the *TDH3* constitutive promoter.

## MATERIALS AND METHODS

### Yeast strains and growth conditions

*Saccharomyces cerevisiae* strains used in this work (*FLO* genes overexpression) were purchased from Department of Applied Molecular Bioscience, Yamaguchi University Graduate School of Medicine, Japan. All yeast strains used in this study are listed in table 1. Yeast cells were grown in YPD medium (1% yeast extract, 2% peptone, 2% glucose). Uracil dropout medium (2% glucose, 0.5% ammonium sulfate, 0.17% yeast nitrogen base and appropriate nutrients without uracil) (Nonklang et al. 2008) was used in transformant selection. YPL10 medium (10 g $L^{-1}$ yeast extract, 10 g $L^{-1}$ peptone, 100 g $L^{-1}$ lactose) and YPD10 medium (10 g $L^{-1}$ yeast extract, 10 g $L^{-1}$ peptone, 100 g $L^{-1}$ glucose) was used in fermentations assays. Yeast strains were grown in fresh YPD plates (2% agar) at 28ºC for 2 days before being used for transformations experiments. All media were sterilized by autoclaving at 121ºC for 20 min.

### Identification of *FLO* genes in the *K. marxianus* genome

We determined the *FLO* genes presence in *K. marxianus* CCT 7735 using the *K. marxianus* DMKU3-1042 annotated genome (NCBI BioProject: PRJDA65233) as a reference. *K. marxianus* DMKU3-1042 has three different type of flocculation genes: *FLO5, FLO9* and a Flo Suppression protein, then we used Blastn and Blastx alignments (Altschul *et al.,* 1997) between the flocculation genes present in *K. marxianus* DMKU3-1042 as a local database and the *K. marxianus* CCT 7735 genome (NCBI BioProject: PRJNA255779) as query, sequences with identities >95% and E-value=0 were defined as homologous genes.

**Table 1.** Yeast strains used in this study

| Strain | Description | Reference |
|---|---|---|
| ***S. cerevisiae*** | | |
| **RAK3977** | *MAT**a** ura3Δ0 URA3-TDH3p-FLO1* | Nonklang et al, 2009 |
| **RAK3979** | *MAT**a** ura3Δ0 URA3-TDH3p-FLO5* | Nonklang et al, 2009 |
| **RAK3981** | *MAT**a** ura3Δ0 URA3-TDH3p-FLO9* | Nonklang et al, 2009 |
| **RAK3983** | *MAT**a** ura3Δ0 URA3-TDH3p-FLO10* | Nonklang et al, 2009 |
| ***K. marxianus*** | | |
| **CCT 7735** | Wild-type | Silveira et al, 2005 |
| **CCT 7735 – *FLO1*** | Sc[*URA3-TDH3p-FLO1*] | This study |
| **CCT 7735 – *FLO5*** | Sc[*URA3-TDH3p-FLO5*] | This study |
| **CCT 7735 – *FLO9*** | Sc[*URA3-TDH3p-FLO9*] | This study |
| **CCT 7735 – *FLO10*** | Sc[*URA3-TDH3p-FLO10*] | This study |

## Gene Expression Analysis

We verified the expression of the *FLO* genes in *K. marxianus* CCT 7735 growing under ethanol stress conditions (ethanol 6%) using an RNA-seq dataset (unpublished data) from which we were able to retrieve the FoldChange values associated to the genes of interest. We conducted a scoped analysis focusing only in *FLO* genes that are present in the *K. marxianus* CCT 7735 genome. Expression were analyzed and visualized using the R programming language (R Core Team, 2015) and the "gplots" R package (Warnes et al., 2015).

## TF-binding sites prediction and Gene ontology

We predicted the Transcription Factor binding site (TF-bs) on the promoter region of each *FLO* gene found in *K*. *marxianus*, we assumed a promoter region of 1kb up-stream the ATG initiation codon, then we applied the TF-bs prediction available at the Yeastract server (Teixeira et al., 2014) to detect all the TF-bss (and TFs) on each one of the 9 promoters. As we obtained a large TFs dataset for the promoter regions, we designed an R computational framework based on Venn diagrams to determine the common TFs between *FLO5* promoters, *FLO9* promoters and *FLO5*+*FLO9* promoters.

As we were interested in the biological processes associated to the common TFs, we determined the Gene Ontology of the *FLO5*-*FLO9* common TFs using GOTermFinder (Boyle et al., 2004), with default parameters and SGD as annotation database. We visualized the GO results on ReviGO (Supek et al., 2011).

## Insertion of *FLO* genes from *S. cerevisiae* into *K. marxianus* CCT 7735

### Total DNA extraction of flocculating *S. cerevisiae*

DNA extraction of *S. cerevisiae* flocculent strains was carried out according to Braganca et al, (2014) with some modifications. Flocculating *S. cerevisiae* strains [RAK3977 (*FLO1*), RAK3979 (*FLO5*), RAK3981 (*FLO9*), RAK3983 (*FLO10*)] were grown overnight in 5 mL of YPD at 30ºC with shaking at 180 rpm. The cell mass was collected by centrifugation at 3000 x *g* for 5 min, resuspended in 0.2 mL lysis buffer (2% Triton X-100, 1% sodium dodecyl sulfate (SDS), 100 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA), 0.2 mL of PCI [phenol pH 6.7-chloroform-isoamyl alcohol (25:24:1)] and 0.3 g of glass beads were added. The mixture was vortexed for 2 min followed by centrifugation at 10,000 x *g* for 10 min. The supernatant was transferred into a new 1.5 mL microcentrifuge tube and mixture with 0.5 mL 90% ethanol was added and kept at -20ºC for 2 hours. The total DNA was separated for centrifugation at 14,000 x *g* for 10 min, washed with 70% ethanol and dried at room temperature. The DNA was dissolved in nuclease-free water and kept at -20ºC.

### Amplification the URA3-TDH3p-FLO fragments by PCR

An aliquot of 1 µL from genomic DNA extracted from flocculating *S. cerevisiae* strains *FLO1* (RAK3977), *FLO5* (RAK3979), *FLO9* (RAK3981) and *FLO 10* (RAK3983) was used for amplify *URA3-TDH3p-FLO* fragments, using primers pair FLO1-401/FLO1+5037c, FLO5-401/FLO5+3759c, FLO9-401/FLO9+4454c and FLO10-401/FLO10+3980c, designed by Nonklang et al. (2009). The PCR reaction was initialized at 94ºC for 2 min, followed by 35 cycles of 94ºC for 30 s, 60ºC for 45 s and 68ºC for 6 min. The reaction mixture (50 µL) contained 1X High Fidelity PCR Buffer, 0.2 mM of dNTP mixture, 2 mM $MgSO_4$, 0.2 µM of each primer and 1 U of Platinum® *Taq* High Fidelity (Invitrogen®).

### Yeast transformation conditions for linear DNA integration

Yeast transformations were performed by lithium acetate method, as described by Abdel-Banat (2010). The recombinant cells obtained were selected according to the flocculent phenotype on Minimal Medium (MM) without uracil. *URA3-TDH3p-FLO* fragments were amplified from flocculating *K. marxianus* CCT 7735 strains to confirm the correct integration of the fragment into *K. marxianus* CCT 7735 genome. The Total DNA extraction of flocculating *K. marxianus* were performed as described above with a unique modification: the incubation temperature used was 37ºC for 18 h. The confirmative amplification was realized using the reaction described previously.

## Phenotypic Analysis of the Recombinant Flocculent *K. marxianus*

### Aggregation assays

For visualization of flocculent *K. marxianus* CCT 7735 strains in scanning electron microscope (SEM), yeasts were collected from cultures in exponential growth phase and washed twice in PBS buffer (1x, pH 7.2) and 10 µL of this suspension was added over 10 µL of 0.1% (w / v) poly-L-lysine (Sigma-Aldrich, St. Louis, USA) previously spread on surface coverslip and kept at room temperature for 15 minutes. Subsequently, the coverslip was washed twice with PBS buffer and fixed overnight in 2.5% glutaraldehyde. The samples were again washed and subjected to sequential dehydration in alcohol concentrations of 70%, 80%, 90% and 100% (v / v) for 15 minutes at room temperature in each step. Immediately after the last stage of dehydration, coverslips were dried at critical point (Critical Point Dryer - CPD®, Bal-tec, model 030), immobilized in port specimens (stubs) and covered with 15 nm of gold using Sputtercoater (Balzers, model FDU 010). The images were captured by a Scanning Electron Microscope (LEO, model 1430 VP) operating at 20 kV. The analyze with scanning electron microscopy were performed at the Núcleo de Microscopia e Microanálise in the Federal University of Viçosa.

### Flocculation vortex test

For flocculation assays, one colony from each recombinant strain was grown in 15 mL tube, containing 5 mL of YNB medium without uracil with shaking. The culture tube was vortexed vigorously for 1 min and allowed to settle. The flocculation was measured by the amount of sedimenting cells present in the suspension after vortex. The test was carried out at 37ºC.

### Growth properties of flocculent *K. marxianus* CCT 7735

Flocculents *K. marxianus* CCT 7735 were streaked on YPD plates and incubated at 30, 45 and 51ºC for 2 days, and grown at 37°C on YP medium (1% yeast extract, 2% peptone) plate and broth, supplemented with 2% of different carbon sources (glucose, galactose, fructose, mannose, sucrose, raffinose, cellobiose, lactose, xylose, arabinose and glycerol).

### Fermentation by Recombinant *K. marxianus* strains

*K. marxianus* wild-type strain and flocculating *K. marxianus* CCT 7735 strains obtained were pre-cultured in 10 mL of YPD medium at 37ºC and 180 rpm for 16 h. One milliliter from each of the cultures was inoculated into 250 mL flask, containing 50 mL of YPD medium and incubated for 5 hours at 37ºC and 180 rpm. The cellular suspension was then adjusted to $OD_{600}$= 0,5 and inoculated into 50 mL Falcon tubes, containing 40 mL of YPL10 or YPD10 medium according to Vallejo et al. (2012). The cultures were grown at 30ºC, 40ºC and 45ºC in aerobic and hypoxic condition for 48 hours. Cell-free supernatants were collected by centrifugation at 10,000 x *g* for 5 min and filtered with 0,22 µm pore membrane. Glucose, lactose and ethanol were analyzed by HPLC (LC 20AT – Shimadzu), using a Rezex ROA - organic acids H+ 300x7,8 column eluted at 30°C with 0.005 M H2SO4 at a flow rate of 0.7 ml/min, and a refractive-index detector following the manufacturer's instruction.

# RESULTS

## Computational analyses of *FLO* genes expression

The *K. marxianus* CCT 7735 genome was recently sequenced by Silveira et al, (2014). This sequencing showed that *K. marxianus* CCT 7735 has three copies of *FLO5* gene (each copy in the chromosome 1, 2, and 8) and five copies of *FLO9* gene (one copy in the chromosome 4, 6, 7 and two copies in the chromosome 8).

Transcriptome analysis of *K. marxianus* CCT 7735 subject to stress of ethanol 6% for 4 hours (unpublished data) showed that there is a statistically significant increase in the expression of native *K. marxianus* CCT 7735 *FLO* genes. In general, we have obtained a significant increase (p-value < 0.05) in the *FLO* genes expression after 4 hours of ethanol (6%) stress (Figure 1). Specifically, *FLO9,* were highly expressed at this condition with $\log_2$(FoldChange) > 2 after 4 hours. It means that the yeast had at least 4 times of the quantity of *FLO9* transcripts when compared to non-ethanol growing condition. On the other hand, as we were expecting, the transcription of Flocculation Suppression gene has been down regulated (p-value < 0.05 and $\log_2$(FoldChange) < -2) after 4 hours of 6% ethanol stress. Interestingly, although the *FLO5* and *FLO9* genes were up-regulated and the *FLO* suppression gene was down-regulated *Kluyveromyces marxianus* CCT 7735 didn't show flocculent phenotype while growing under ethanol stress condition.

In the same way, we predicted the transcription factor binding-sites (TF-bs) on the promoter regions of each gene and we determined the common transcription factors (cTFs) of *FLO5* genes and then of *FLO9* genes (Figure 1). We have obtained the Gene Ontology of the cTFs, this shows a high content of TF-bs related to the cellular response to stimulus biological process, result that is in accord to the significant increase in the *FLO5* and *FLO9* expression under the ethanol stimulus as we related in the previous passage.

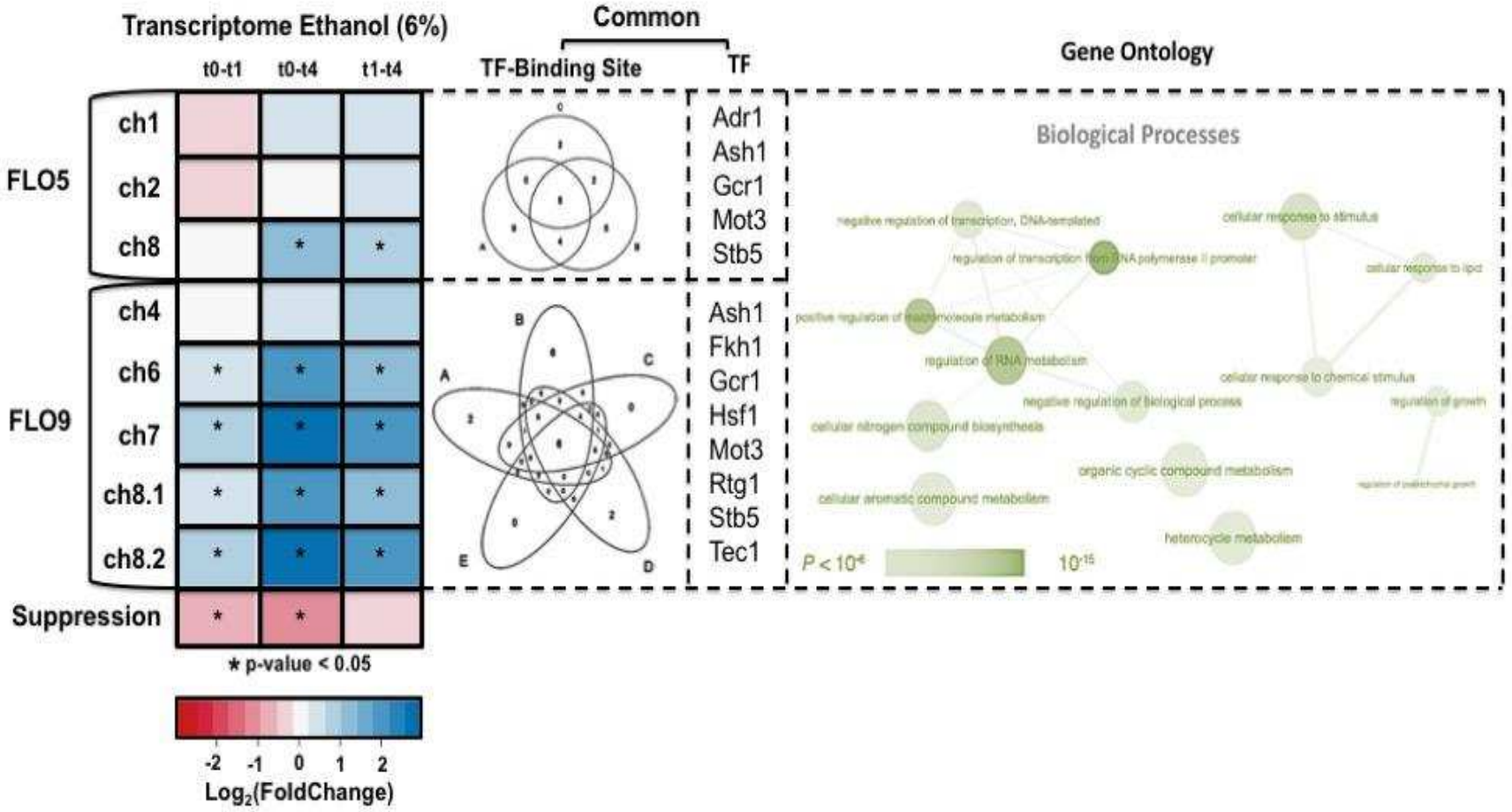


**Figure 1.** Expression of flocculation genes in *K. marxianus* CCT 7735 under ethanol stress conditions (6%) and the common transcription factors on the promoter regions of each gene (1000kb upstream of the ATG initiation codon).

**Insertion of *TDH3p-FLO* genes of *S. cerevisiae* into *K. marxianus* CCT 7735**

*URA3-TDH3p-FLO* fragments were amplified by PCR from genomic DNA of *S. cerevisiae* flocculent strains and directly transformed into *K. marxianus* CCT 7735. According with the sequences on the NCBI nucleotides database, *FLO1* (NM_001178230) has 4614 bp, *FLO5* (NM_001179342) has 3228 bp, *FLO9* (NM_001178205) has 3969 bp and *FLO10* (NM_001179892) has 3510 bp. The cassette *URA3-TDH3p* has 2000 bp (Nonklang et al, 2009)

*URA3-TDH3p-FLO* fragments were amplified by PCR from genomic DNA of *S. cerevisiae* flocculent strains and transformed into *K. marxianus* CCT 7735 by a lithium acetate method, as described by Abdel-Banat (2010). The cultures showed flocculation during cultivation (figure 2) indicating that *S. cerevisiae FLO* genes and the *TDH3* promoter were functional in *K. marxianus* CCT 7735 strain. After that, the fragments were amplified by PCR from *K. marxianus* CCT 7735 *FLO* strains, confirming the correct insertion of the fragments into *K. marxianus* genome (Figure 3).


**Figure 2.** *K. marxianus* CCT 7735 wild type, *FLO9* and *FLO10* respectively, photographed by SEM after cultured at 30°C.

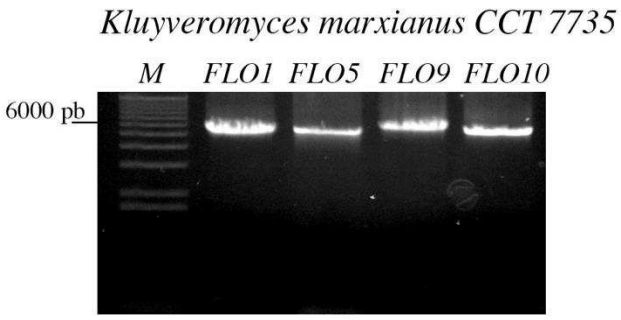


**Figure 3.** *URA3-TDH3p-FLO* fragments amplified from *K. marxianus* CCT 7735 FLO strains confirming correct insertion of the *S. cerevisiae FLO* genes.

To determine the degree of flocculation, was implemented the vortex test. One colony from each flocculent phenotype, as well as, one colony from wild-type, was inoculated overnight at 37ºC in uracil dropout medium with shaking (180 rpm), the sedimentation properties were analyzed. The *K. marxianus* CCT 7735 *FLO1, FLO5* and *FLO9* sedimented 30 minutes after vortex but, the *K. marxianus* CCT 7735 *FLO10* culture did not settle completely until 1 h (figure 4).

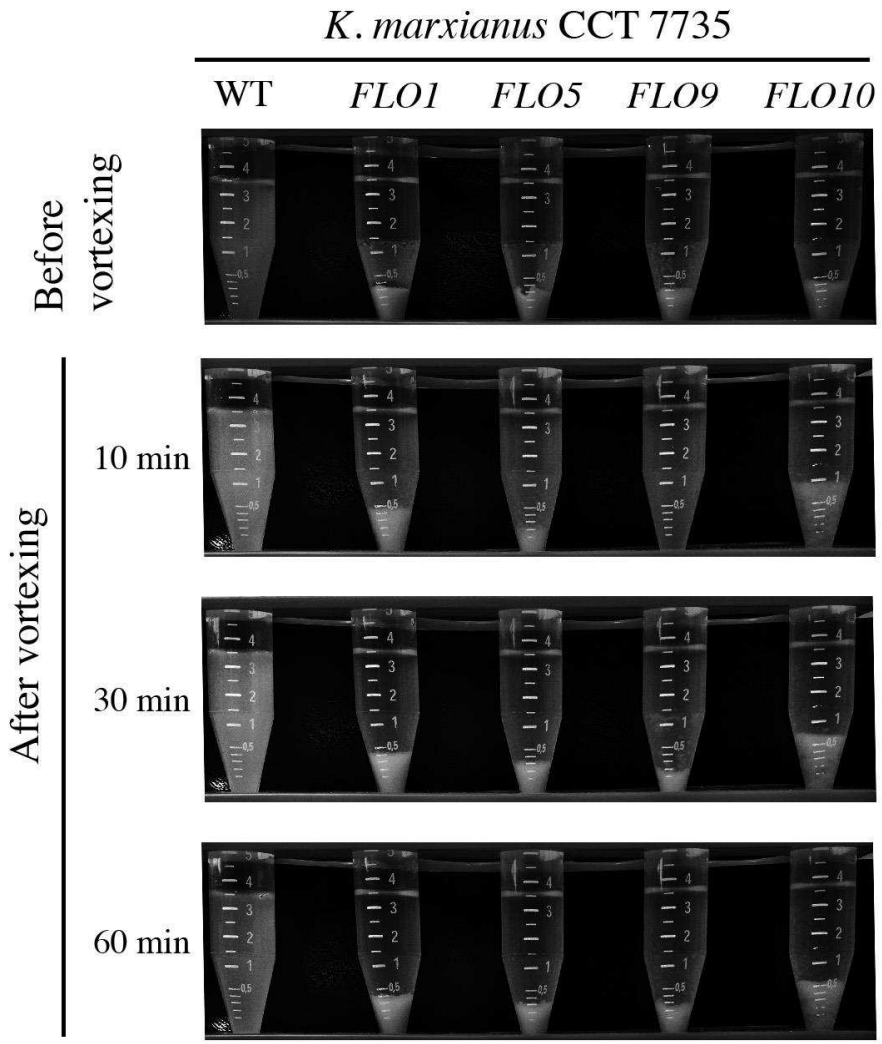


**Figure 4.** The *K. marxianus* CCT 7735 transformants were analyzed for flocculation phenotype before and after vortex.

## Growth properties of recombinant flocculent *K. marxianus* CCT 7735 strains

We have studied the thermotolerance, growth in different carbon sources and flocculation capability under different sugars by recombinant flocculent *K. marxianus* CCT 7735 strains constructed in this work [*K. marxianus* CCT 7735 *FLO1, K. marxianus* CCT 7735 *FLO5*, *K. marxianus* CCT 7735 *FLO9* and *K. marxianus* CCT 7735 *FLO10*] having the *K. marxianus* CCT 7735 wild type as control (figure 5).

The growth of these five strains was examined at 30ºC, 40ºC, 45ºC and 50ºC (figure 5A). All *K. marxianus* CCT 7735 strains have grown at temperatures between 30 and 40ºC, but in contrast with the wild type, the *K. marxianus* CCT 7735 *FLO5* is not able to growth at 45ºC and, *FLO1, FLO9* and *FLO10* are not able to growth at 50ºC.

We have also examined the utilization of a wide variety of carbon sources. All recombinant *K. marxianus* CCT 7735 strains as the wild type have grown in galactose, fructose, mannose, sucrose, glucose and, in contrast to *S. cerevisiae,* they were also able to grow in lactose, xylose, and glycerol. *K. marxianus* CCT 7735 is not able to grow in cellobiose or arabinose as the only carbon source (figure 5B).

The flocculent phenotype was evaluated in liquid medium with 2% of each carbon source (figure 5C). The result indicates that flocculation varies according to each recombinant *FLO* strain. In the case of *FLO1* strain, it shows aggregation after growing overnight under all the different carbon sources, except mannose. On the other hand, *FLO5*, *FLO9* and *FLO10* strains show aggregation only after 2 days, but in the case of glycerol aggregation occurs after the first day of growth.

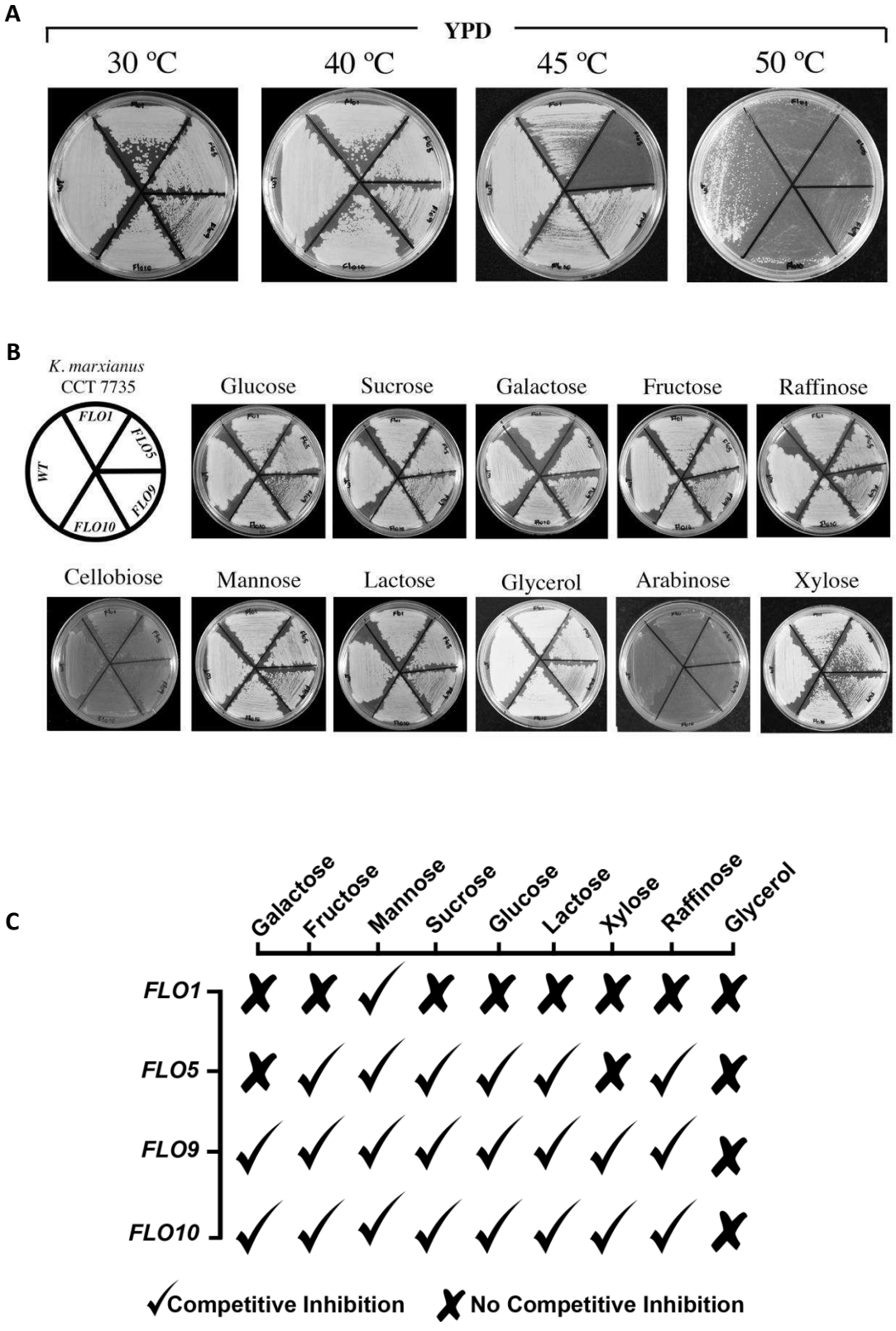


**Figure 5.** Growth properties of flocculent *K. marxianus* CCT 7735 strains.
(A) Strains of *K. marxianus* CCT 7735 were spread on YPD as described for panel A and incubated at indicated temperatures for 2 days. (B) Strains of *K. marxianus* CCT 7735 were spread on YP plates supplemented with 2% of the indicated sugars and incubated at 37ºC. (C) Recombinants *K. Marxianus* CCT 7735 were grown in YP broth supplemented with 2% of the indicated sugars and incubated at 37ºC.

To confirm the flocculation ability at different temperatures *K. marxianus* CCT 7735 *FLO* strains were incubated at 40°C and 45°C on YPD and YPL. The all *FLO* strains showed strongly flocculation at 40°C and despite having slow growth (data not show), the flocculent phenotype is maintained at 45°C (figure 6).

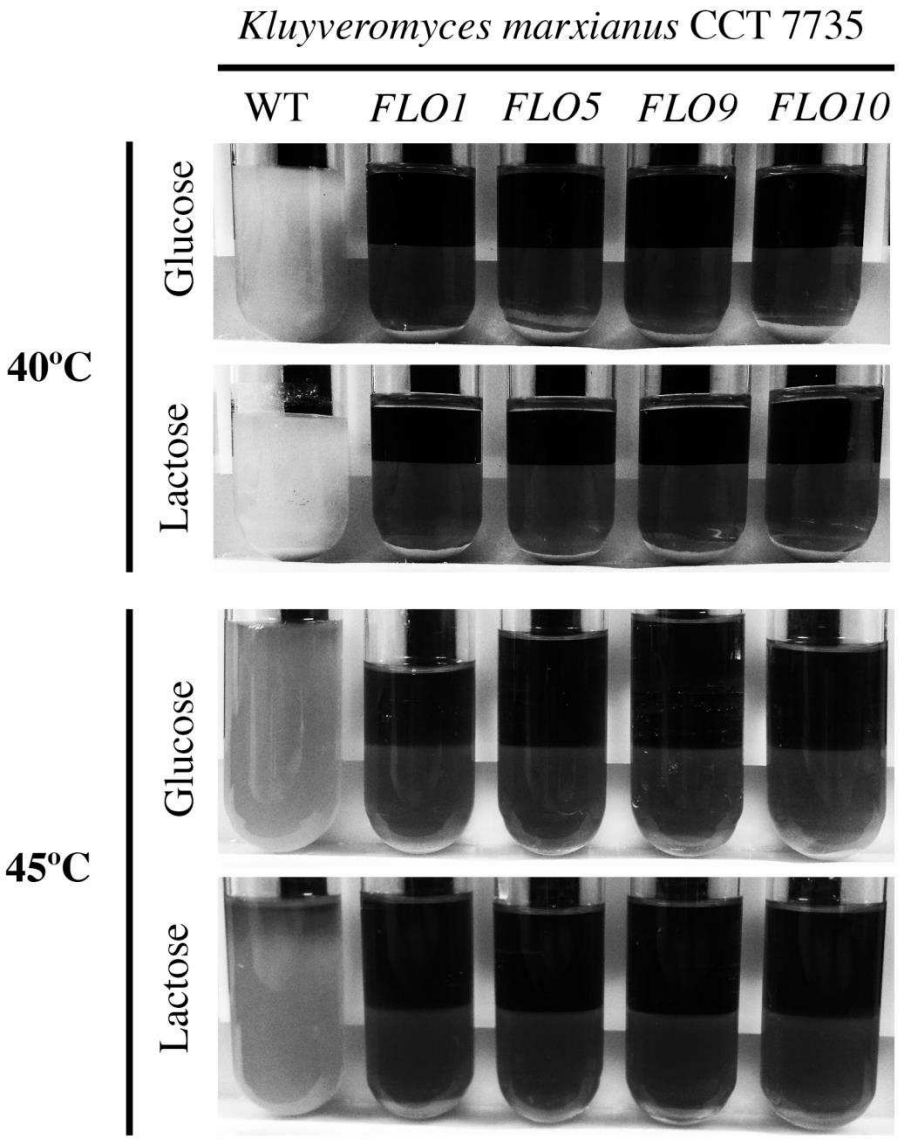


**Figure 6.** Flocculent *K. marxianus* CCT 7735 strains incubated overnight in YPD and YPL at indicated temperatures. The flocculation phenotype was observed without vortexing.

## Ethanol formation of *K. marxianus* CCT 7735 flocculent strains

The ethanol formation by flocculent *K. marxianus* CCT 7735 strains was compared to ethanol formation of *K. marxianus* CCT 7735 wild type. The fermentation was performed in aerobic and hypoxic conditions for 48 hours on YPD10 and YPL10 (figure 7 and figure 8 respectively) at 30, 40 and 45ºC.

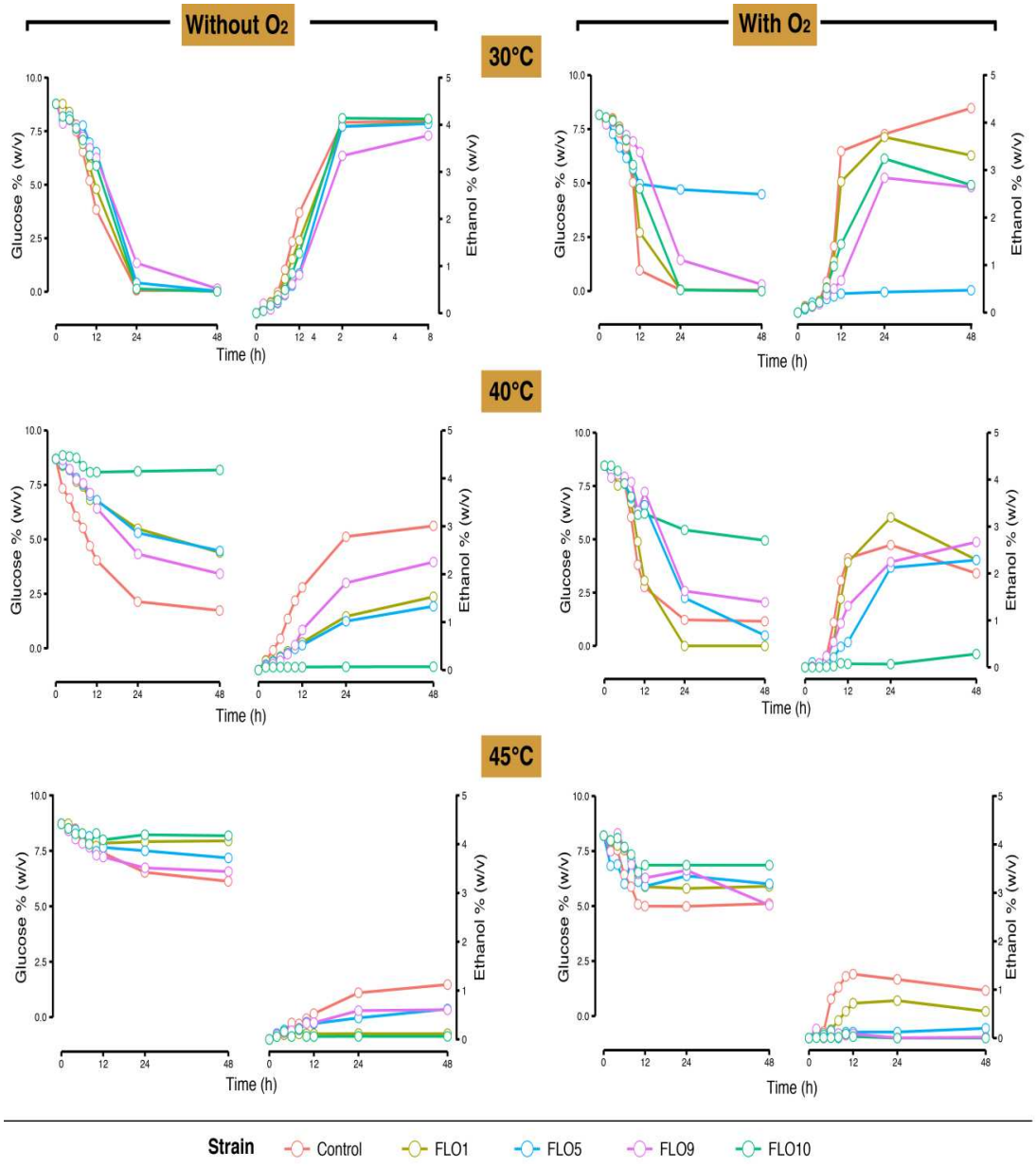


**Figure 7.** Ethanol formation by recombinant *K. marxianus* CCT 7735 *FLO* strains in YPD10 medium.
Glucose consumption and ethanol formation, without oxygen (left) and with oxygen (right) were analyzed in aerobic and hypoxic conditions at 30°C, 40°C and 45°C for 48 h.

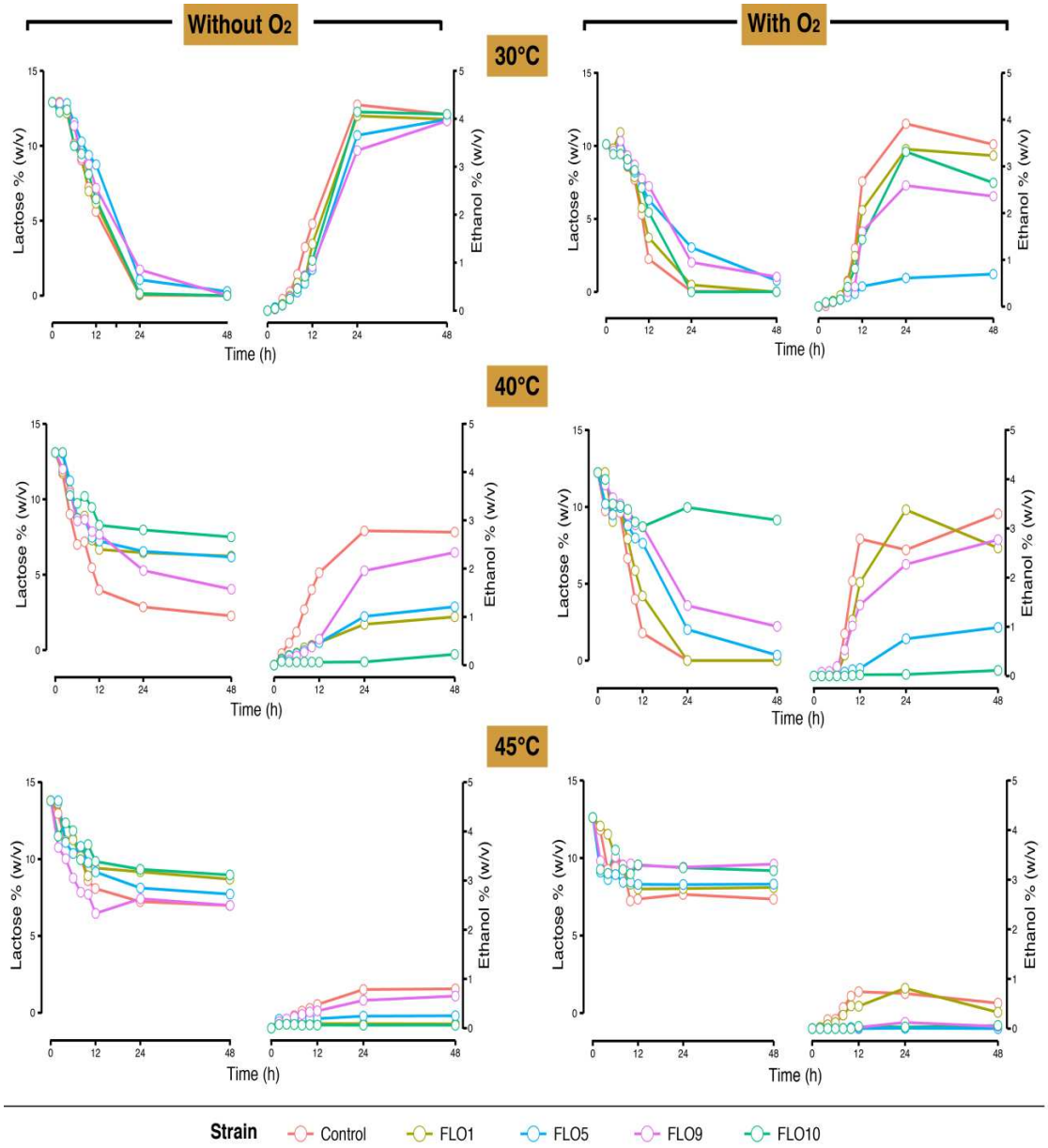


**Figure 8.** Ethanol formation by *K. Marxianus* CCT 7735 *FLO* strains on YPL10 medium.
Lactose consumption and ethanol formation, without oxygen (left) and with oxygen (right) were analyzed in aerobic and hypoxic conditions at 30 °C, 40 °C and 45 °C for 48 h.

The best condition for ethanol production in YPD and YPL for recombinant flocculent *K. marxianus* CCT 7735 was 30°C in hypoxic condition which in all cases the ethanol concentration reached 3,5% and 4% respectively. When fermentation was performed in YPD10 at 30°C in aerobic conditions, ethanol concentrations reached 3% for wild type and 2.5% for the

*FLO* strains except for the *FLO5* strain. The cells of *FLO5* strain grow slow in this condition, indicating that *FLO5* strain is oxygen sensitive.

When fermentation was performed at 40°C in hypoxic condition, *FLO9* strain have produced ethanol similarly to the wild type, reaching 2,5%. In aerobic conditions, the ethanol concentrations reached 2,5% in all cases except for *FLO10* strain. The cells of *FLO10* strain grow slow in this condition, indicating that *FLO10* strain is temperature sensitive. When fermentation was performed at 45°C in hypoxic and aerobic conditions, the maximum concentration of ethanol was 1%, that means that the *K. marxianus* CCT 7735 grow in glucose at 45°C but does not ferment well at this glucose concentration.

In YPL10 medium at 30°C in hypoxic was the best testing condition for recombinant flocculent *K. marxianus* CCT 7735, reaching 4% of ethanol concentration. In the aerobic conditions, *FLO1* strain presents ethanol concentration similar to the wild type (3,8%), *FLO5* presented ethanol concentration less than 1%, shown to be sensitive to oxygen. When fermentation was performed at 40°C in hypoxic condition, only *FLO9* strain showed ethanol concentration (2,5%) similar to the wild type. In aerobic conditions, *FLO1* and *FLO9* strains showed similar ethanol concentration to the wild type (2,5%), and, when fermentation was performed at 45°C in hypoxic and aerobic conditions, the maximum concentration of ethanol was 0,7%, that means that *K. marxianus* CCT 7735 grow in lactose at 45 °C but does not ferment well.

## DISCUSSION

The thermotolerant *K. marxianus* yeast strain is an attractive organism for industrial applications, mainly due to its ability to produce ethanol by fermentation at high temperature, assimilate wide range of carbon sources and its higher growth rate (Fonseca et al, 2008). Then, provide flocculation phenotype to this yeast favor separation of cells from the wort as we demonstrated with our results, this is a desirable property for commercial fermentation systems.

The *K. marxianus* CCT 7735 genome was recently sequenced by Silveira et al, (2014). This sequencing showed that *K. marxianus* CCT 7735 has three copies of *FLO5* gene and five copies of *FLO9* gene. Transcriptome analysis of *K. marxianus* CCT 7735 subject to stress of 6% of ethanol for 4 hours (unpublished data) showed that there is a statistically significant increase in the expression of native *K. marxianus* CCT 7735 *FLO* genes, in agreement with Alexandre et al, (2001) who indicated that high concentrations of ethanol increases expression of FLO genes in *S. cerevisiae,* however, in contrast to *S. cerevisiae* the *K. marxianus* CCT 7735 does not exhibit a flocculent phenotype. This information confirms the results of Nonklang et al, (2009), in that work is stated that 17 Thailand strains of *K. marxianus* were isolated and observed, they found that all of them were non-flocculent after different experimental procedures, with lack of genomic information they assumed that flocculation is not natural in *K. marxianus.* We are demonstrating through our analysis that, although *K. marxianus* CCT 7735 has significant increases on the transcription of its natural *FLO* genes, a flocculent phenotype never was perceived, based on that, we designed and developed a successful experiment of genetic engineering for provide flocculation phenotype to *K. marxianus* cells.

Through different genetic strategies, the flocculation has been widely studied in the model yeast *S. cerevisiae*, resulting in several flocculent strains. On the *K. marxianus* case, the early study made by Nonklang et al., (2009) demonstrated that *S. cerevisiae TDH3* promoter and *FLO* genes were functional in *K. marxianus* DMKU3-1042 conferring flocculation phenotype to

this yeast. On the other hand, Vallejo et al, (2012) generating inducible flocculation phenotype in *K. marxianus* CECT 11769 by expressing of *S. cerevisiae FLO5* gene. These results supports that the introduction of *S. cerevisiae* FLO genes is a useful approach for *K. marxianus* engineering and it is demonstrated by the construction of flocculent phenotypes in our present work. We have conferred constitutive flocculation in *K. marxianus* CCT 7735 by direct introduction of *S. cerevisiae FLO* genes under control of *TDH3*, functional promoter in *K. marxianus* strains.

The recombinant *K. marxianus* CCT 7735 *FLO* strains do not show the same phenotype that *S. cerevisiae* overexpressing *FLO* genes. *K. marxianus* CCT 7735 *FLO1* and *FLO5* show strong and stable flocculation at 37ºC and settle completely 30 minutes after vortex. *K. marxianus* CCT 7735 *FLO9* shows strong and stable flocculation too, but it settles completely only 60 minutes after vortex. *K. marxianus* CCT 7735 *FLO10* strain shows a weak flocculation since the cells were not completely settle 60 minutes after vortex. Our results are according to the results reported by Nonklang and collaborators (2009) who indicated that there are functional differences in these four *FLO* proteins.

On the other hand, an interesting fact is that *K. marxianus* CCT 7735 has the natural ability to assimilate and ferment xylose and lactose (Zhang et al., 2011; Silveira et al., 2014), both sugars cannot be fermented by the yeast usually utilized in the fermentation processes, *S. cerevisiae,* requiring genetically engineered strains to be able to harness sugar contents in possible substrates as cheese whey or lignocellulosic biomass (Guimarães et al., 2008; Matsushika et al., 2014). All recombinant *K. marxianus* CCT 7735 strains constructed in this work have the ability to assimilate galactose, fructose, mannose, sucrose, glucose, lactose, xylose, and glycerol, representing, for the last three carbon sources, an important advantage over *S. cerevisiae*. However, in contrast to *K. marxianus* DMKU3-1042 (Nonklang et al., 2008), the *K. marxianus* CCT 7735 strains are unable to assimilate cellobiose or arabinose as unique carbon source.

When inoculated in liquid medium, the flocculation by the *K. marxianus* *FLO* strains is differently inhibited depending on the sugar, while is not affected by glycerol. These results suggest that some sugars compete more effectively with flocculation as mentioned by Panteloglou et al., 2012. The importance of sugar-sensitive behavior in cell to cell interaction is supported by studies about the sugars inhibiting the flocculation competitively (Watanabe et al., 2008; Van Mulders et al., 2009; Matsuzawa et al., 2011; Sugiyama et al., 2015). Competitive inhibition conferred by sugars, it means interaction between sugars on the medium with flocculin proteins on the cell surface resulting in cell to cell adhesion only when all sugar on the medium is consumed (Van Mulders et al., 2009).

However, sugar competitive inhibition prevents premature flocculation, this is, the flocs formation before the fermentation end leading an incomplete fermentation (Kaur et al., 2012). Based on that, competitive inhibition provides two ideal situations for ethanol industry, first, fast sugar consumption, and second, flocculation only after the total sugar consumption (Verstrepen and Klis, 2006). Our *FLO* engineered strains have features that make them interesting strains for industrial ethanol production, they have affinity for a wide range of competitive sugars, this means, our recombinant strains achieve flocculation toward the end of fermentation and it facilitates their removal from the medium.

Ethanol production from fermentation of xylose and lactose by *K. marxianus* strains has been reported (Wilkins et al., 2008; Nonklang et al., 2008; Diniz et al., 2014; Gabardo et al., 2014). This is the first report confirming efficient ethanol production from lactose at 40ºC by *K. marxianus* CCT 7735 *FLO* strains. The lactose fermentation in hypoxic condition at 30ºC of all the *K. marxianus* CCT 7735 flocculent strains were similar to the wild-type, being that comparable with the performance of *K. marxianus* DMKU3-1042 from glucose at the same temperature (Nonklang et al., 2009).

*Kluyveromyces marxianus* CCT 7735 *FLO9* strain showed stable flocculation phenotype and ethanol formation ability at 40ºC in lactose comparable to *K. marxianus* CCT 7735 wild-type. Additionally, *K. marxianus*

CCT 7735 *FLO9* presents competitive inhibition by lactose, preventing cells flocculation before all lactose are convert into ethanol. This competitive inhibition is important as we described before, because flocculation will only occur when all the lactose is consumed, avoiding the risk of incomplete fermentation due to premature yeast flocculation.

In conclusion, the nonconventional *K. marxianus* CCT 7735 offers several advantages in ethanol production such as high growth rate, ability to ferment and use lactose among other sugars at high temperature, and now, exhibiting constitutive flocculation phenotype allows easy separation from the fermentation mash, turning the economy of the production process more profitable.

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## CONCLUSIONS

Insertion of *Saccharomyces cerevisiae FLO* genes and *TDH3* promoter generated successful flocculent phenotype in *K. marxianus* CCT 7735.

The flocculent phenotype of recombinant *K. marxianus* CCT 7735 *FLO1, FLO9* and *FLO5* were strong and stable at 40ºC, only *FLO10* showed weak flocculation properties.

All recombinant *K. marxianus* CCT 7735 constructed in this work have the ability to assimilate galactose, fructose, mannose, sucrose, glucose, lactose, xylose and glycerol and, additionally, *FLO1, FLO9* and *FLO10* strains were able to growth at 45ºC.

The best ethanol fermentation performance, from lactose, for all recombinant *K. marxianus* CCT 7735 flocculent strains, was under hypoxic condition at 30ºC reaching ethanol concentration to 4%.

*K. marxianus* CCT 7735 *FLO1* and *FLO9* strains showed stable flocculation characteristics and efficient lactose fermentation at 40ºC, presenting a similar ethanol production profile than wild type.