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Biotechnological potential of the yeast *Kluyveromyces marxianus*

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Thesis presented to obtain the degree of Doctor in Science: Area: Agricultural Microbiology

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versão revisada de acordo com a Resolução CoPGr 6018 de 2011

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SUMÁRIO

RESUMO

Potencial biotecnológico da levedura *Kluyveromyces marxianus*

A fermentação alcoólica em escala industrial para produção de etanol a partir da cana-de-açúcar continua sendo um desafio para manter o melhor rendimento e relação custo-benefício. Apesar dos avanços, aplicações industriais mais recentes, como a produção de bioetanol em altas concentrações de açúcares e em altas temperaturas, demanda novos estudos sobre o metabolismo das leveduras. Assim surgiu a motivação para o estudo das aplicações biotecnológicas de leveduras não convencionais, como a levedura *Kluyveromyces marxianus*, que se destaca por apresentar alta tolerância a temperaturas elevadas e capacidade de crescimento celular acelerado nestas condições, incluindo a facilidade de crescer em um maior número de substratos e com um metabolismo eficiente e veloz. Diante do objetivo de avaliar o potencial biotecnológico de *K. marxianus*, determinando seu perfil fermentativo e de resposta a estressores, consumo de açúcares e crescimento, foram utilizadas as linhagens IZ 1339 (isolada de Drosophila) e FT 146L (isolada de bioprocessos). As características de crescimento em microplaca, consumo de açúcares em fonte única de carbono (glicose, sacarose, xilose, lactose e maltose) e misturas binárias (gli/xil, gli/lac, sac/lac e mal/lac), bem como o perfil de crescimento sob fontes de estresse (salino, etanólico e ácido) foram explorados, sendo obtidos curvas de crescimento e parâmetros cinéticos. Em seguida, o perfil cinético de ambas as linhagens de *K. marxianus* foi avaliado em um sistema não-invasivo de amostragem, comparando seus perfis de crescimento, incluindo a velocidade máxima específica, e sua performance em meio contendo caldo de cana. Finalmente, ensaios fermentativos foram conduzidos em escala de shaker com reciclo de inóculo, e em biorreator. Os parâmetros fermentativos e de rendimento foram avaliados, fornecendo dados como consumo de açúcar e produção de etanol. Sendo assim, é possível concluir que *Kluyveromyces marxianus* possui características positivas para aplicação industrial em substratos comumente utilizados no Brasil, como caldo de cana-de-açúcar e melaço.

Palavras-chave: Leveduras não convencionais, Fermentação alcoólica, Fatores estressantes, Caracterização

ABSTRACT

Biotechnological potential of the yeast *Kluyveromyces marxianus*

Alcoholic fermentation on an industrial scale for ethanol production from sugarcane remains a challenge to maintain the best yield and cost-benefit ratio. Despite advances, more recent industrial applications, such as bioethanol production at high sugar concentrations and at high temperatures, demand further studies on yeast metabolism. There is the motivation for the study of the biotechnological applications of unconventional yeasts, such as the yeast *Kluyveromyces marxianus*, which stands out for presenting high tolerance to high temperatures and accelerated cell growth capacity in these conditions, including the facility to growing on a larger number of substrates and with an efficient and fast metabolism. In order to evaluate the biotechnological potential of *K. marxianus*, determining its fermentative profile and its response to stressors, sugar consumption and growth, the strains IZ 1339 (isolated from Drosophila) and FT 146L (isolated from bioprocesses) were used. The growth characteristics in microplate, consumption of sugars in single carbon source (glucose, sucrose, xylose, lactose and maltose) and binary mixtures (glu/xyl, glu/lac, suc/lac and mal/lac), as well as the growth profile under stress sources (saline, ethanol and acid) were explored, with growth curves and kinetic parameters obtained. Then, the kinetic profile of both *K. marxianus* strains was evaluated in a non-invasive sampling system, comparing their growth profiles, including the specific maximum velocity, and their performance in sugarcane juice medium. Finally, fermentation assays were carried out on a shaker scale with inoculum recycling, and in a bioreactor. The fermentation and yield parameters were evaluated, providing data such as sugar consumption and ethanol production. Therefore, it is possible to conclude that *Kluyveromyces marxianus* has positive characteristics for industrial application in substrates commonly used in Brazil, such as sugarcane juice and molasses.

Keywords: Unconventional yeasts, Alcoholic fermentation, Stress factors, Characterization

THESIS STRUCTURE

This thesis opens with a brief introduction and the justification of the research problem followed by three chapters and the authors final considerations. The chapters are structured in the format of drafted manuscripts, in preparation for submission to peer-reviewed journals relevant to the research field.

After the introduction and justification for the study, Chapter one, entitled "Biochemical and physiological characterization of *Kluyveromyces marxianus* strains" explores the traits of two *K. marxianus* strain regarding fermentative capacity, growth on different sugars as carbon sources, growth in the presence stress factors and cell protein content. We expect that this work to contribute to the understanding of the physiology and growth patterns of two *K. marxianus* strain isolated from distinct backgrounds.

Chapter two, entitled "Parallelized biomass monitoring of two distinct *Kluyveromyces marxianus* strains in shake flask cultivation" explores the growth profile of the strains on a non-invasive, parallelized cell growth monitoring system (Cell Growth Quantifier – CGQ, Aquila Biolabs), as well as monitoring sugar consumption, aiming to better understand the differences in metabolism of two strains cultivated under the same conditions.

Chapter three, entitled "Ethanol production from sugarcane juice and molasses by *Kluyveromyces marxianus* strains" approaches fermentative assays in bench-scale and in a bioreactor, utilizing a mixture of sugarcane juice and diluted molasses as medium, in order to obtain the fermentative profile of the two *K. marxianus* strains, evaluating sugar consumption, ethanol production, productivity and yield, as well as organic acids production.

Lastly, the thesis is concluded by the author's final considerations and future perspectives.

1. INTRODUCTION AND JUSTIFICATION

It is known that yeasts, a heterologous group of eukaryotic fungi, have a significant impact on biotechnological, industrial, medical and food sectors, in fact, yeasts have been a part of our lives from the rise of civilization (BASSO et al., 2008). Among this diversity, *Saccharomyces cerevisiae*, a model organism, has a prominent position, as it is widely studied and employed in the biotechnological field. Looking beyond *S. cerevisiae*, there is a lot to explore when it comes to non-conventional yeasts and their diverse traits (MADEIRA-JR; GOMBERT, 2018; WALKER; BASSO, 2020).

Kluyveromyces marxianus stands out amongst these non-conventional yeasts as a species with many industrial applications. Increasing demands for innovations in biologically synthesized molecules for food and drugs, and for environmental applications, creates opportunities to explore the potential of *K. marxianus*. The only drawback is the limited knowledge of the physiology and genetics of this species; however, this tendency is about to change, with many research groups taking interest in this yeast and publishing exploratory studies, exploring it as an interesting alternative do diversify the product portfolio for sugarcane-based biorefineries (KARIM; GERLIANI; AÏDER, 2020; LANE; MORRISSEY, 2010; MADEIRA-JR; GOMBERT, 2018).

Physiology and metabolism of *K. marxianus*

Kluyveromyces marxianus is a homothallic yeast, which reproduces predominantly by budding, widely exploited for their ability to metabolize lactose; very close phylogenetically to *Kluyveromyces lactis* (FONSECA et al., 2008; GABARDO; RECH; AYUB, 2012). This yeast possesses a high capacity to convert substrate into biomass, in addition to the faster specific growth rate observed among eukaryotes (GROENEVELD; STOUTHAMER; WESTERHOFF, 2009).

The morphology of this species can be affected by the specific growth rate, in the middle range (between 0.15 h^{-1} and 0.4 h^{-1}) cells have a rounded appearance characteristic of yeasts, while in larger or smaller ranges, cells may have a filamentous/pseudohyphae appearance. This feature can be exploited for industrial application, as it is known that filamentous cells have a greater capacity to secrete proteins (ROCHA; ABRAHÃO-NETO; GOMBERT, 2011).

As *K. marxianus* is not a model organism, there is a consensus that this species has great potential for biotechnological application, but there is difficulty comparing available data on this microorganism due to the lack of comprehensive studies on its metabolic and physiological diversity (ROCHA; ABRAHÃO-NETO; GOMBERT, 2011).

Some authors have described specific aspects of K. marxianus metabolism, such as the ability to form biomass and produce ethanol from cheese whey, formation of β-galactosidase and inulinase in cells, ergosterol being the only sterol present in the cell, differently from *S. cerevisiae*, and the composition of its cell wall (BÜSCHGES et al., 1994; CORPILLO et al., 2003; NGUYEN; FLEET; ROGERS, 1998; POSTMA; BROEK, VAN DEN, 1990; QUEIROS et al., 1998; RAMÍREZ-ZAVALA et al., 2004; STAMBUK et al., 2003; TERNAN; MCMULLAN, 2000).

The metabolism of *K. marxianus* is described as respiro-fermentative. Nonklang et al. (2008) compared the fermentative performance of a strain of *K marxianus* opposed to a strain of *S. cerevisiae* and observed that *K. marxianus* is an interesting alternative for the production of ethanol at 45ºC, having the advantage over *S. cerevisiae* of the ability to use cellobiose, xylose, xylitol, arabinose, glycerol and lactose, thus presenting an opportunity not only for the production of ethanol from conventional matrices but for the expansion of technologies, such as second-generation ethanol.

Although *K. marxianus* is, generally, classified as Crabtree negative, unlike *S. cerevisiae*, both species carry the genes necessary to produce ethanol by fermentation under specific conditions, and it should be noted that there are conflicting reports in the literature regarding *K. marxianus*' Crabtree status (LANE; MORRISSEY, 2010; MERICO et al., 2007).

It is also known that growth parameters such as μ_{max} (maximum specific growth rate) and $Y_{x/s}$ (substrate conversion factor) present different values, not only between different strains, but also between the same strains evaluated in different laboratories. It is due to the high rate of polymorphism that makes it susceptible to mutations if there is no care in the handling and storage of cells.

Different values of maximum growth rate are presented for *K. marxianus*, such as 0.69 h^{-1} at 33ºC utilizing glucose as a carbon source, and 0.86 h^{-1} at 40ºC in the same conditions (ROUWENHORST et al., 1988). Hoekstra et al. (1994) stated that the μ_{max} value could reach 1.1 h^{-1} and Fonseca et at. (2013) observed that growth rates vary depending on the sugar used as a carbon source, ranging between 0.39 and 0.49 h^{-1}.

It is worth noting that such experiments were carried out under different conditions with different strains, and that strain conservation is crucial to ensure consistent results with this yeast, given that *K. marxianus* presents a high level of intraspecific polymorphism, being highly susceptible to mutations that result in rapid evolutionary changes that are reflected in performance. Given this characteristic, it is difficult to compare experimental data between different authors, as the same experimental conditions with the same strains cannot always be reproduced (FONSECA; BARBOSA DE CARVALHO; GOMBERT, 2013).

Differently from S. cerevisiae, *K. marxianus* has the ability to use xylose, xylitol, cellobiose, lactose and arabinose, both in solid and liquid medium, being able to ferment glucose between 30 and 45ºC, reaching ethanol production yield and consumption of glucose similar to S. cerevisiae at temperature of 45ºC. It is also remarkable for its ability to use substrates such as sugar cane, corn, molasses, surpluses from dairy production, all of which are of great industrial relevance (RADECKA et al., 2015).

Undoubtedly, this yeast species has several interesting characteristics, with emphasis on thermotolerance, ability to cultivate and ferment different substrates, in addition to its rapid growth rate, especially when compared to *Saccharomyces cerevisiae* (FONSECA et al., 2008). These characteristics are relevant for industrial application.

Biotechnological applications

Considering that *K. marxianus* strains were isolated from the most diverse habitats, a wide metabolic diversity was observed, as well as a significant number of intraspecific polymorphisms. Therefore, different strains may have more advantageous applications, such as enzyme production, intracellular protein, aromatic compounds, ethanol from different matrices, including high temperature, reduced lactose content in foods, bioingredients from cheese protein, bioremediation, among other applications (FONSECA et al., 2008).

The vast majority of strains of *K. marxianus* are considered safe for industrial application for human consumption, as is *S. cerevisiae*, although it is much less documented than the model yeasts *S. cerevisiae* and *K. lactis* (HENSING et al., 1995). *K. marxianus* received the Qualified Presumption of Safety (QPS) and GRAS status in European Union and United States, respectively (KARIM; GERLIANI; AÏDER, 2020).

There is a strong motivation for the industrial application of *K. marxianus* based on the advantage that this species has over *K. lactis*, including the ability to grow on a greater number of substrates and its efficient metabolism. The fact that strains of this yeast have been isolated from the most diverse substrates makes its ecology complex, and demonstrates the ease of adaptation and metabolic plasticity of this species (FONSECA; BARBOSA DE CARVALHO; GOMBERT, 2013; MADEIRA-JR; GOMBERT, 2018).

In particular, there is interest in the application of *K. marxianus* for the production of ethanol at high temperature, due to the potential for cost savings through the constant evaporation of ethanol produced under reduced pressure (NONKLANG et al., 2008; SINGH, D.; NIGAM; BANAT, 1998). The ideal temperature for ethanol production by *S. cerevisiae* is 25 - 37ºC, therefore existing a demand for the use of a thermotolerant organism (AMORIM et al., 2011).

In addition to this advantage, there are also savings in cooling costs, especially in tropical countries where the average daily temperature is elevated and high saccharification and fermentation rates. In addition to the reduction of bacterial contamination due to high temperatures, taking into account that most industrial contaminants are mesophilic, developing between 15 - 37°C (ANDERSON et al., 1986; BANAT et al, 1992; NONKLANG et al., 2008; RADECKA et al., 2015).

This is a very advantageous feature, considering that the ethanol production process in Brazil is still very susceptible to contamination by bacteria, which have a direct impact on productivity. Ethanol production by *K. marxianus* has been reported at temperatures above 40ºC, and cell growth up to 47ºC, or even 52ºC (ANDERSON; MCNEIL; WATSON, 1986; MADEIRA-JR; GOMBERT, 2018; NONKLANG et al., 2008; RADECKA et al., 2015; SINGH, D.; NIGAM; BANAT, 1998).

Ethanol production at 45ºC was observed for all strains evaluated, which confirms the thermotolerance capacity of this species (NONKLANG et al., 2008). This value represents a significant increase compared to Brazilian fermentation conditions, which corroborates the hypothesis that *K. marxianus* is an unconventional yeast that offers great metabolic attractions for the replacement of *S. cerevisiae*, being a promising candidate for the production of ethanol.

Different processes for the production of ethanol by *K. marxianus* have been proposed, such as batch fermentation with high concentration of substrates, fed batch, continuous system, use of bioreactors with membrane cycle, two-stage fermentation, among other experimental techniques (FONSECA et al., 2008; FONSECA; BARBOSA DE CARVALHO; GOMBERT, 2013).

Nonklang et al. (2008) also report that the tests for ethanol production with *K. marxianus* were carried out under very specific experimental conditions, being necessary to evaluate the yeast under the same conditions used for *S. cerevisiae*, in order to compare the data obtained and thus define it as a substitute viable on a large scale.

Sugarcane juice has 12 to 17% total reducing sugars, of which 90% are sucrose and 10% are glucose and fructose. Molasses from sugar production, which is also used as a substrate for ethanol production, has 45-60% total reducing sugars, of which approximately 50-55% are sucrose and 40-45% are glucose and fructose. Yeasts can directly ferment glucose and fructose, but for sucrose fermentation to occur, enzymatic conversion must occur, in order to obtain glucose and fructose, this conversion takes place through the enzyme invertase (EIADPUM; LIMTONG; PHISALAPHONG, 2012).

The first-generation fuel ethanol production process in Brazil has become a robust and sustainable industrial process, which has achieved high yields over decades of practice

and innovations, but fermentation yield values have remained stable over the last twenty years (DELLA-BIANCA; GOMBERT, 2013). Although this yield is high, investments to improve the process are justifiable, taking into account the large volume of ethanol produced in the country.

For this to be achieved, innovative solutions will be needed, among the alternatives is the production of ethanol at high temperatures (45-48ºC). Brazil is a tropical country, where the average annual temperature is generally high (24-37ºC) (INMET, 2018), which makes it suitable for ethanol production at high temperatures, and as *S. cerevisiae* is not the most adapted yeast for fermentations above 40ºC, an alternative yeast would be necessary. In addition to thermotolerance, the ability to grow under anaerobic conditions, high yield in the conversion of sugar into ethanol, and the ability to withstand pH below 2.5 resulting from acid treatment for cell recycling, characteristic of the Brazilian ethanol production, such traits are found in *K. marxianus* (MADEIRA-JR; GOMBERT, 2018).

The implementation of a high temperature ethanol production system in a tropical country like Brazil could save US$ 90,000 per year on a production of 30,000-kL, if the operating temperature was from 35ºC to 45ºC. This would imply the total removal of the cooling system and the reduction of operating costs compared to the traditional operating system utilizing *Saccharomyces cerevisiae* (MADEIRA-JR; GOMBERT, 2018).

In this context, it is necessary to expand exploratory studies of the yeast *K. marxianus*, an organism with characteristics of interest to the industry and promising application. A study on the physiological and fermentative behavior of this yeast is needed for comparative purposes with commercially used Saccharomyces strains, specifically in the Brazilian scenario.

This study is based on the need of the applied biotechnology sector, seeking to obtain not only the fermentative profile, but also information on the physiology and metabolism of the yeast, generating data of interest to the sugar-energy industry, which needs innovative resources in order to maintain productivity and profitability to satisfactory levels, considering that *K. marxianus* has a lot of untapped potential for the production of ethanol in an optimized process.

Aiming to study the biotechnological potential of *K marxianus* yeast, this work presents a biochemical characterization of two strains in Chapter 1. The same strains were submitted to kinetic studies (Chapter 2) and fermentation assays in bench and bioreactor scale (Chapter 3). With these studies it is expected to expand the knowledge about *K. marxianus* and perspectives for biotechnological applications.

1.1 Aims

1.1.1 Hypothesis

The yeast *Kluyveromyces marxianus* is a viable alternative for production of compounds linked to biomass production and/or fermentation, using the substrates characteristic of the Brazilian ethanol production industry, such as sugarcane juice and molasses.

1.1.2 Objectives

This study aims to evaluate the biotechnological potential of the yeast *Kluyveromyces marxianus* by contributing to a better understanding of the behavior of this unconventional yeast, which has characteristics of interest to the ethanol industry and whose potential remains unexplored. This will be achieved by exploring metabolic, kinetic and fermentative traits of two *Kluyveromyces marxianus* strains.

1.1.3 Specific aims

- Obtain the bench-scale and bioreactor fermentation profile
- Obtain the physiological profile and assess physiological responses to stressors (different factors and concentrations)
- Obtain the assimilation profile of several sugars as a single source of carbon in growth

References

AMORIM, H.V. et al. Scientific challenges of bioethanol production in Brazil. Applied Microbiology and Biotechnology. **Applied Microbiology and Biotechnology**, 2011. V. 91, p. 1267-1275. Available in: < https://link.springer.com/article/10.1007/s00253-011-3437-6>

ANDERSON, P.J.; MCNEIL, K.; WATSON, K. High-efficiency carbohydrate fermentation to ethanol at temperatures above 40°C by *Kluyveromyces marxianus* var. *marxianus* isolated from sugar mills. **Applied and Environmental Microbiology**, 1986. v. 51, n. 6, p. 1314–1320. Available in: <http://www.ncbi.nlm.nih.gov/pubmed/ 16347089>. Accessed at: 30 set. 2018.

BASSO, L.C. et al. Yeast selection for fuel ethanol production in Brazil. **FEMS Yeast Research**, 2008. v. 8, p. 1155–1163.

BÜSCHGES, R. et al. NADH: Ubiquinone oxidoreductase in obligate aerobic yeasts. **Yeast**, 1994. v. 10, n. 4, p. 475–479. Available in: <http://www.ncbi.nlm.nih.gov/pubmed/ 7941733>. Accessed at: 20 out. 2018.

CORPILLO, D. et al. Induction and characterization of a novel amine oxidase from the yeast *Kluyveromyces marxianus.* **Yeast**. Available in: <http://www.ncbi.nlm.nih.gov/pubmed/ 12673620>. Accessed at: 21 set. 2018.

DELLA-BIANCA, B.E.; GOMBERT, A.K. Stress tolerance and growth physiology of yeast strains from the Brazilian fuel ethanol industry. **Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology**, 2013. v. 104, n. 6, p. 1083–1095. Available in: <http://www.ncbi.nlm.nih.gov/pubmed/24062068>. Accessed at: 21 set. 2018.

EIADPUM, A.; LIMTONG, S.; PHISALAPHONG, M. High-temperature ethanol fermentation by immobilized coculture of *Kluyveromyces marxianus* and *Saccharomyces cerevisiae*. **Journal of Bioscience and Bioengineering**, 2012. v. 114, n. 3, p. 325–329. Available in: <https://www.sciencedirect.com/science/article/pii/S1389172312001351>. Accessed at: 17 jul. 2019.

FONSECA, G.G. et al. The yeast *Kluyveromyces marxianus* and its biotechnological potential. **Applied Microbiology and Biotechnology**. Available in: <http://www.ncbi.nlm.nih.gov/pubmed/18427804>. Accessed at: 21 set. 2018.

FONSECA, G.G.; BARBOSA DE CARVALHO, N.M.; GOMBERT, A.K. Growth of the yeast *Kluyveromyces marxianus* CBS 6556 on different sugar combinations as sole carbon and energy source. **Applied Microbiology and Biotechnology**, 2013. v. 97, n. 11, p. 5055–5067. Available in: <http://www.ncbi.nlm.nih.gov/pubmed/ 23435899>. Accessed at: 21 set. 2018.

GABARDO, S.; RECH, R.; AYUB, M.A.Z. Performance of different immobilized-cell systems to efficiently produce ethanol from whey: Fluidized batch, packed-bed and fluidized continuous bioreactors. **Journal of Chemical Technology and Biotechnology**, 2012. v. 87, n. 8, p. 1194–1201.

GROENEVELD, P.; STOUTHAMER, A.H.; WESTERHOFF, H.V. Super life - how and why 'cell selection' leads to the fastest-growing eukaryote. **FEBS Journal**, 2009. v. 276, n. 1, p. 254–270. Available in: <http://doi.wiley.com/10.1111/j.1742-4658.2008.06778.x>. Accessed at: 1º ago. 2019.

HENSING, M.C.M. et al. Physiological and technological aspects of large-scale heterologous- protein production with yeasts. **Antonie van Leeuwenhoek**, 1995. v. 67, n. 3, p. 261–279. Available in: <http://www.ncbi.nlm.nih.gov/pubmed/7778895>. Accessed at: 21 set. 2018.

HOEKSTRA, M.F. et al. Budding and fission yeast casein kinase I isoforms have dual-specificity protein kinase activity. **Molecular biology of the cell**, 1994. v. 5, n. 8, p. 877–86. Available in: <https://www.molbiolcell.org/doi/10.1091/mbc.5.8.877>. Accessed at: 1º ago. 2019.

INMET. INMET - Instituto Nacional de Meteorologia. 2018. Available in: <http://www.inmet.gov.br/portal/index.php?r=clima/normaisClimatologicas>. Accessed at: 17 jul. 2019.

KARIM, A.; GERLIANI, N.; AÏDER, M. *Kluyveromyces marxianus*: An emerging yeast cell factory for applications in food and biotechnology. **International Journal of Food Microbiology**, 2020. v. 333, n. May, p. 108818. Available in: <https://doi.org/10.1016/j.ijfoodmicro.2020.108818>. Accessed at: 17 jul. 2021.

LANE, M.M.; MORRISSEY, J.P. *Kluyveromyces marxianus*: A yeast emerging from its sister's shadow. **Fungal Biology Reviews**, 1 fev. 2010. v. 24, n. 1–2, p. 17–26. Available in: <https://www.sciencedirect.com/science/article/abs/pii/S1749461310000035>. Accessed at: 1º ago. 2019.

MADEIRA-JR, J.; GOMBERT, A. Towards high-temperature fuel ethanol production using *Kluyveromyces marxianus*: On the search for plug-in strains for the Brazilian sugarcane-based biorefinery. **Biomass and Bioenergy**, 2018. v. 119, n. September, p. 217– 228. Available in: <https://doi.org/10.1016/j.biombioe.2018.09.010>. Accessed at: 17 jul. 2019.

MERICO, A. et al. Fermentative lifestyle in yeasts belonging to the *Saccharomyces* complex. **FEBS Journal**, 2007. v. 274, n. 4, p. 976–989. Available in: https://doi.org/10.1111/j.1742-4658.2007.05645.x. Accessed at: 1° ago. 2019.

NGUYEN, T.H.; FLEET, G.H.; ROGERS, P.L. Composition of the cell walls of several yeast species. **Applied Microbiology and Biotechnology**, 1998. v. 50, n. 2, p. 206–212. Available in: <http://www.ncbi.nlm.nih.gov/pubmed/9763691>. Accessed at: 21 set. 2018.

NONKLANG, S. et al. High-temperature ethanol fermentation and transformation with linear DNA in the thermotolerant yeast *Kluyveromyces marxianus* DMKU3-1042. **Applied and Environmental Microbiology**, 2008. v. 74, n. 24, p. 7514–7521. Available in: <http://www.ncbi.nlm.nih.gov/pubmed/18931291>. Accessed at: 21 set. 2018.

POSTMA, E.; BROEK, P.J.A. VAN DEN. Continuous-culture study of the regulation of glucose and fructose transport in *Kluyveromyces marxianus* CBS 6556. **Journal of Bacteriology**, 1990. v. 172, n. 6, p. 2871–2876. Available in: <http://www.ncbi.nlm.nih.gov/pubmed/2160928>. Accessed at: 21 set. 2018.

QUEIROS, O. et al. Isolation and characterization of *Kluyveromyces marxianus* mutants deficient in malate transport. **Yeast**, 1998. v. 14, n. 5, p. 401–407. Available in: <http://www.ncbi.nlm.nih.gov/pubmed/9559548>. Accessed at: 21 set. 2018.

RADECKA, D. et al. Looking beyond *Saccharomyces*: The potential of non- conventional yeast species for desirable traits in bioethanol fermentation. **FEMS Yeast Research**, 2015. v. 15, n. 6, p. fov053. Available in: <https://academic.oup.com/femsyr/article-lookup/doi/10.1093/femsyr/fov053>. Accessed at: 19 set. 2018.

RAMÍREZ-ZAVALA, B. et al. Purification and characterization of a lysine aminopeptidase from *Kluyveromyces marxianus*. **FEMS Microbiology Letters**, 2004. v. 235, n. 2, p. 369–375. Available in: <http://www.ncbi.nlm.nih.gov/pubmed/15183887>. Accessed at: 21 set. 2018.

ROCHA, S.N.; ABRAHÃO-NETO, J.; GOMBERT, A.K. Physiological diversity within the *Kluyveromyces marxianus* species. **Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology**, 2011. v. 100, n. 4, p. 619–630.

ROUWENHkRST, R.J. et al. Production, distribution, and kinetic properties of inulinase in continuous cultures of *Kluyveromyces marxianus* CBS 6556. **Applied and Environmental Microbiology**, 1988. v. 54, n. 5, p. 1131–1137. Available in: <http://www.ncbi.nlm.nih.gov/pubmed/16347626>. Accessed at: 21 set. 2018.

SINGH, D.; NIGAM, P.; BANAT, I.M. Ethanol production at elevated temperatures and alcohol concentrations: Part II–Use of *Kluyveromyces marxianus* IMB3. **World Journal of Microbiology & Biotechnology**, 1998. v. 14, n. 6, p. 823-834. Available in: <http://link.springer.com/10.1023/A:1008852424846>. Accessed at: 21 set. 2018.

STAMBUK, B.U. et al. D-xylose transport by *Candida succiphila* and *Kluyveromyces marxianus*. **Applied Biochemistry and Biotechnology**, 2003. v. 106, p. 255–264. Available in: <http://www.ncbi.nlm.nih.gov/pubmed/12721449>. Accessed at: 21 set. 2018.

TERNAN, N.G.; MCMULLAN, G. The utilization of 4-aminobutylphosphonate as sole nitrogen source by a strain of *Kluyveromyces fragilis*. **FEMS Microbiology Letters**, 2000. v. 184, n. 2, p. 237–240. Available in: <https://academic.oup.com/femsle/ article-lookup/doi/10.1111/j.1574-6968.2000.tb09020.x>. Accessed at: 21 set. 2018.

WALKER, G.M.; BASSO, T.O. Mitigating stress in industrial yeasts. **Fungal Biology**, 2020. v. 124, n. 5, p. 387–397. Available in: <https://doi.org/10.1016/j.funbio.2019.10.010>.

2. CHAPTER 1. BIOCHEMICAL AND PHYSIOLOGICAL CHARACTERIZATION OF *Kluyveromyces marxianus* STRAINS

Abstract

Yeasts have been a reliable cell factory in biotechnology for a long time, apart from the widely studied *Saccharomyces cerevisiae;* there is a lot of untapped potential in non-conventional yeasts. One of these alternatives is *Kluyveromyces marxianus*, a food-grade yeast with several beneficial traits, such as thermotolerance, rapid growth rate and capability to assimilate several substrates, making it appealing to applications in biotechnology and food industries. This work aimed to characterize the physiological and biochemical profile of two different *K. marxianus* strains, isolated from distinct backgrounds. Fermentative capacity was evaluated and was positive for both strains under the assayed conditions. Response to growth under stress factors was evaluated, first of plating assays, followed by growth on microplates in growth medium supplemented with stress factors, both strains managed to grow under most conditions assayed, only being hindered by the elevated concentrations. Cell growth and sugar assimilation were also evaluated, showing a clearer picture of how both strains of *K. marxianus* grow in single sugars and binary mixtures as carbon sources, and how they growth rates are.

2.1 Introduction

Yeasts are unanimously utilized in biotechnology for the production of food, beverages and chemical compounds of industrial interest. *Saccharomyces cerevisiae*, a model organism, is particularly well-known and explored, but when it comes to yeasts, there is a wide diversity to be explored in the field of non-conventional species (LANE; MORRISSEY, 2010).

Kluyveromyces marxianus is a homotallic, hemiascomycetous yeast, phylogenetically related to *S. cerevisiae* and a sister species to the more well-known *Kluyveromyces lactis* (KARIM; GERLIANI; AÏDER, 2020; KURTZMAN; FELL, 1998). Due to its association with food, *K. marxianus* obtained GRAS (Generally Regarded as Safe) and QPS (Qualified Presumption of Safety) in the United States and European Union, respectively (FONSECA et al., 2008; KARIM; GERLIANI; AÏDER, 2020).

As of recently, *K. marxianus* became focus of research due to its beneficial traits that deem it exceptionally suitable for industrial application. This yeast is capable of utilizing a broad range of substrates, including lactose and inulin, thermotolerance in ranges up to 52ºC in some strains, production of single cell protein and aroma compounds (CARVALHO et al., 2004a; FONSECA et al., 2008; MADEIRA-JR; GOMBERT, 2018; RADECKA et al.,

2015). In addition, it has a higher growth rate highest growth rate among eukaryotes and the capability to produce fuel ethanol via fermentation (EIADPUM; LIMTONG; PHISALAPHONG, 2012; FONSECA et al., 2008; GABARDO; RECH; AYUB, 2012; MEHMOOD et al., 2018; SAINI et al., 2018; SINGH; NIGAM; BANAT, 1998).

K. marxianus, like *S. cerevisiae*, is a respiro-fermentative yeast, able to generate energy through oxidative phosphorylation or by fermentation, producing ethanol. Although *K. marxianus* is generally classified as Crabtree negative, unlike *S. cerevisiae*, both species carry the genes necessary to produce ethanol by fermentation under specific conditions, and it should be noted that there are conflicting reports in the literature regarding *K. marxianus*' Crabtree status (LANE; MORRISSEY, 2010; MERICO et al., 2007).

It was reported in several studies that *K. marxianus* strains possesses different tolerance levels to the different stressing conditions commonly found in the industrial process, such as lower pH, elevated temperature, severe oxygen restraint condition, and higher concentration of ethanol; which is very beneficial for future industrial applications (DINIZ et al., 2017; FONSECA et al., 2008, 2013; ILLARIONOV; LAHTVEE; KUMAR, 2021; WALKER; BASSO, 2020)

At last, *K. marxianus* is known for strain variability among the species, such as the ability to carry out respiration and fermentation simultaneously, producing both ethanol and biomass (MERICO et al., 2007; ROCHA; ABRAHÃO-NETO; GOMBERT, 2011). These variations explain why some, but not all, strains of *K. marxianus* are effective ethanol producers and are resistant to multiple stress factors commonly found in industrial processes (HONG et al., 2007; NONKLANG et al., 2008). This knowledge of specific strain's physiology is important when selecting them for targeted industrial application, and, as mentioned by Rocha; Abrahão-Neto; Gombert (2011), there is a need to bridge the knowledge in physiological diversity for *K. marxianus*.

The aim of this study was to evaluate the biochemical and physiological characteristics of two *Kluyveromyces marxianus* strains, highlighting their growth pattern and responses to stress factors.

2.2 Material and Methods

2.2.1 Yeast strains and maintenance conditions

Two *Kluyveromyces marxianus* strains were utilized: strain IZ 1339 (*Kluyveromyces marxianus* isolated from a drosophilid) (GOMES et al., 2003; LEAL et al., 2008), kindly provided by Prof. Dr. Luiz Humberto Gomes (ESALQ), and strain FT 146L (isolated from ethanol production), kindly provided by Fermentec Ltda (Piracicaba, SP, Brazil). *S. cerevisiae* strain CAT-1 (LNF Latino Americana) was used as a reference.

Strains were routinely maintained at 4°C on YPDA medium (10 $g.L^{-1}$ yeast extract; 10 $g.L^{-1}$ peptone; 20 $g.L^{-1}$; glucose; 18 $g.L^{-1}$ agar), and the stocks (cryotubes containing skim milk as a cryoprotectant) were stored a -80°C.

2.2.2 Inoculum preparation

An inoculation loop full of yeast cells from strains IZ 1339 and FT 146L was transferred to 5 mL of YPD medium (10 $g.L^{-1}$ yeast extract; 10 $g.L^{-1}$ peptone; 20 $g.L^{-1}$; glucose), incubated at 30ºC for 24 h. Cells were collected by centrifugation, washed and resuspended in 1 mL of saline solution 0.85 %.

This solution was used as an inoculum in all experiments.

2.2.3 Total cell protein concentration by Bradford protein assay

Erlenmeyer flasks (125 mL) containing 20 mL of YPSuc medium (10 $g.L^{-1}$ yeast extract; 10 $g.L^{-1}$ peptone; 20 $g.L^{-1}$ sucrose) were inoculated with an inoculum, prepared as described in item 2.2.2.

The cultivation was conducted at 30°C 24 h. After that, protein extracts were obtained at 4 and 24 h.

Obtaining protein extracts

Yeast cells were centrifuged, washed 2x with saline solution and, subsequently, transferred to Eppendorf tubes. The supernatant of centrifugation was collected and labeled Extracellular Extract (EE).

In order to obtain the Intracellular Extract (IE), yeast cell pellets were washed twice with Acetate Buffer 0.1 M; resuspended in $CaCO_3$ 0.15 M solution (VILELA et al., 1973 apud BUZATO; BROGGI; CELLIGOI, 1999/2000). Yeast cells were then hydrolyzed in Bain- Marie at 40ºC for 3 h. After the hydrolysis, the cells were once again centrifuged, and the supernatant collected, characterizing the Intracellular Extract (IE).

Total protein concentration

Total protein concentration was obtained by carrying out the Bradford Protein Assay. To Eppendorf tubes, 20 µL of each extract (IE and EE) and 1000 µL of Bradford solution (Bio Rad) were added, and homogenized. After 5 min of pause, the absorbance readings were performed at ABS_{595nm}. The total protein concentration was calculated using a calibration curve established from known quantities of (0.1 to 1.4 mg/mL protein) of bovine albumin serum.

2.2.4 Studies of *K marxianus* on stress plates

The *K. marxianus* strains IZ 1339 and FT 146L were cultivated in Petri dishes, in a medium containing stress factors, in order to improve understanding on the physiology of *K.marxianus* strains when exposed to major stress factors faced by yeast in the industrial fuel ethanol production.

Osmotic conditions

YPD medium was supplemented with NaCl in different concentrations (0.2– 2.0 M), according Table 1.

Yeast cells were cultivated as previously described in item 2.3.2. Plates were inoculated with 100 µL of cell suspension (cell concentration of 10^{-8} - 10^{-9}) for both strains, utilizing the drop plate method (HERIGSTAD; HAMILTON; HEERSINK, 2001). Plates were incubated at 35ºC during 48 h; this experiment was performed in triplicates.

Ethanol resistance

Ethanol in the concentrations (0 – 30%, v/v) was added to YPD medium. YPD medium was cooled down to room temperature before adding the aforementioned ethanol concentrations, in order to avoid evaporation. This method was adapted from Della-Bianca and Gombert (2013).

Table 1- Concentration of stress factors added to YPD 2% glucose medium

Stress Factor	**Concentration of stress factors**					
NaCl (M)	0.0	0.3	0.6	1.0	1.5	2.0
Ethanol (%, v/v)	0.0	12	15	20	30	

Experiments were carried out in duplicates; M = molarity

2.2.5 Growth kinetics on microplates

Growth curves for *K. marxianus* strains IZ 1339 and FT 146L were obtained in a microplate reader (Tecan Infinite 200). For this, an inoculum was previously prepared in YPD medium, incubated at 30ºC for 24 hours. The cell suspension was then centrifuged and adjusted to 0.1 ABS_{600}. From this cell suspension, a 25 µL aliquot was inoculated in the 96 well microplate. Readings were performed every 30 min after brief agitation (15 sec), Optical Density (OD) was measured at 620 nm.

Growth on different sugars as carbon sources

In order to evaluate the growth pattern of the *K. marxianus* strains in different carbon sources, 25 µL of the cell suspension was added to 50 µL of YNB 6.7g.L^{-1} medium (Yeast Nitrogen Base), prepared in accordance with the manufacturer's instructions. The medium was supplemented with 25 µL of different carbon sources, individually or in binary combinations.

The different sugars (glucose, sucrose, xylose, lactose and maltose) were sterilized separately and added at a final concentration of 20 g.L^{-1} (as the sole carbon source). Binary combinations of sugars were also studied (Glu/Xyl 10 g.L^{-1} each) and Glu/lac, Suc/lac, Xyl/lac and Mal/Lac (12.5 and 7.5 g.L^{-1}, respectively). Lactose was utilized in smaller concentrations in order to observe whether it could improve the metabolism of the strains when combined with other carbon sources. Each carbon source was studied in triplicates.

S. cerevisiae CAT-1 strain was used as a reference, in a different sucrose concentration (30 g.L^{-1}), prepared under the same conditions.

Effect of stress factors on strain growth kinetics

The YPD medium was supplemented with stress factors commonly found, such as: NaCl at different concentrations (0 - 2 g/L) and acetic acid (0 - 0.4 M), as shown in Table 2.

For the ethanolic stress study, YPD medium was supplemented with ethanol at concentrations 0 to 15% (v/v). This method was adapted from Della-Bianca and Gombert (2013), and was carried out in duplicates.

For the assays, YPD medium (75 µL) and stress factor was added to the 96 well microplate, plus 25 µL of the cell suspension previously prepared.

Table 2 - Concentration of stress factors added to YPD medium

Stress Factor	**Concentration**				
NaCl (g/L)	0	0.5	1.0	1.5	2.0
Acetic acid (M)	0	0.05	0.1	0.2	0.4
Ethanol (%, v/v)	0	5.0	10	12.5	15

Experiments were carried out in duplicates; M = molarity

Calculation of physiological parameters

The maximum specific growth rate was obtained by plotting the natural logarithm of absorbance against time in the exponential growth phase. The slope of the straight line obtained by linear regression represented the μ_{max}. (RAGHAVENDRAN *et al.*, 2017).

Physiological parameters were calculated following the methods of Stroppa et al. (2009), where μ_S (specific rate of substrate consumption) = μ_{max} (h^{-1}) / $Y_{x/s}$.

2.2.6 Fermentative capacity

Fermentative capacity was determined through the standard fermentation test in the Durham tube, based on inspection for visible gas formation trapped inside the tubes. Standard test tubes were filled with 10 mL of medium and inoculated with 50 µL of yeast cells previously grown in YPD medium plate (MOREIRA et al., 2015).

Two media were utilized in the assay, YPSuc and sterile/filtered sugarcane juice (SCJ) in the following concentrations (total soluble solids): 12, 22 e 30ºBrix.

For the inoculum, yeast strains IZ 1339 and FT 146L were cultivated in YPD medium for 24 h, by transferring three inoculation loops full of yeast cells previously grown in YPDA. Afterwards, a 10 mL aliquot of the media was transferred to an Erlenmeyer flask containing 100 mL of sterile and filtered sugarcane juice at 6ºBrix, incubated in a shaker at 30ºC during 24 h, under constant agitation. The medium was then centrifuged, the yeast cells were washed and resuspended in saline solution, and the cell density was adjusted to 0.1 OD (ABS_{600nm}).

After preparing the experiments, the test tubes containing the inverted Durham tubes were incubated during 48 hours. The combinations of temperature and media concentration were determined utilizing factorial experimental design 2^2 (Table 3 and 4).

Table 3 – Values and reach levels of independent variables

Independent variables	**Symbols**	**Reach**		
		-1.00	0.00	1.00
TSS (°Brix) of YPSuc e SCJ medium	X_1	12	21	30
Temperature (°C)	X_2	30	35	40

Table 4 – Application of factorial experimental design 2^2

Experiments	**X_1**	**X_2**	**Independent variables**	
			TSS (°Brix) initial of media	Temperature (°C)
1	-1	-1	12	30
2	+1	-1	30	30
3	-1	1	12	40
4	+1	+1	30	40
5	0	0	21	35
6	0	0	21	35
7	0	0	21	35

Following the 48-h period, signs of fermentation such as CO_2 bubbles trapped inside the Durham tubes, surface bubbles and alcoholic odor were observed

Statistical analysis

Factorial design experiments were used to analyze the influence of the total soluble solids and temperature on the fermentative capacity of two *K. marxianus* strains.

The effects of total soluble solids and temperature on the fermentative capacity were examined through a two-way analysis of variance (ANOVA) using software R (R Core Team, 2020) and Excel.

2.3 Results and Discussion

K. marxianus strains IZ 1339 and FT 146L were submitted to biochemical and physiological assays to evaluate the potential of biotechnological applications of unconventional yeasts.

2.3.1 Total protein cell concentration

The total protein concentration present in the yeast cell for strain IZ 1339 and FT 146L is described in Figure 1. Yeast cells were sampled at 4 and 24 hours of cultivation.

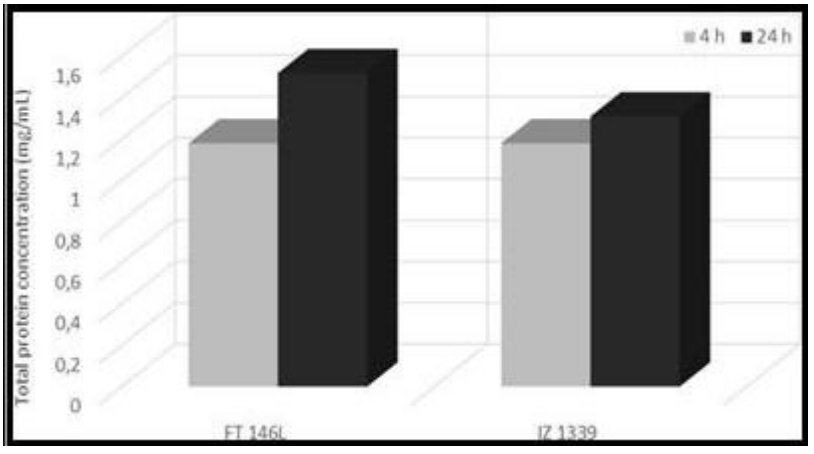

Figure 1 - Total protein concentration present in *K. marxianus* strains IZ 1339 and FT 146L; growth conditions = 24h at 30ºC in YPD medium

Both strains had the same total protein concentration at 4 hours of cultivation (1.2 mg/mL), however, strain FT 146L had increased concentration at the end of the 24-hour cultivation period (1.5 mg/mL), compared to strain IZ 1339 (1.3 mg/mL).

Yeast biomass can be utilized in the form of nutritional supplements as a protein source, as well as the production of yeast extract to supplement new fermentative processes. Knowing the characteristics of each species enables the optimization of the amount of protein obtained.

The most frequently used yeasts for the production of SCP (single cell protein) are *Saccharomyces cerevisiae*, *Kluyveromyces marxianus*, *K. lactis*, *Candida utilis*, *Yarrowia lipolitica* and *Picha pastoris*, with the first three of them receiving the status of generally recognized as safe (GRAS) for human consumption by the US Food and Drugs Administration (FONSECA et al., 2008; GARCÍA-GARIBAY et al., 2014).

SCPs are known as a dietary supplement, protein-rich foods or food ingredients, produced by pure of mixed cultures of microorganisms, such as yeast, microalgae, mushrooms or bacteria, utilized for human and animal consumption (RITALA et al., 2017). *K. marxianus* is known for its high multiplication rate and high protein content (30–80% protein in terms of dry weight) (KARIMI et al., 2018).

This yeast is considered a reliable producer of extracellular protein due to its capacity to grow on various low-cost substrates, such as whey and molasses (FONSECA et al., 2008; HENSING et al., 1995). Due to having a higher specific growth rate than that of *S. cerevisiae*, *K. marxianus* is an interesting alternative for SCP production, showing maximum biomass yield of 4.8 g/L/h in an aerobic continuous fermentation of 2.5% fructose medium (KIM; TAK; MOON, 1998).

This method of quantification was chosen in order to determine whether the *K. marxianus* strains were likely candidates to produce protein extracts for animal nutrition. Fonseca et al. (2008) highlights the production of single-cell protein as one of the several different biotechnological applications for *K. marxianus*.

It is clear from the results that the conditions employed were not the most suitable for biomass production in order to obtain SCP, as there was not a significant increase of protein content in the biomass. Further studies are required to investigate other growth media and conditions better suited to induce biomass growth.

2.3.2 Response of *K. marxianus* strains under stress factors

The *K. marxianus* strains IZ 1339 and FT 146L were cultivated in Petri dishes containing solid medium with added stress factors, in order to improve understanding on the physiology of *K. marxianus* strains when exposed to major stress factors faced by yeast in the industrial fuel ethanol production. Although the conditions employed are not the exact ones encountered in the industrial environment, the approach to evaluate individual stress factors in order to obtain clearer results was suggested by Ivorra et al. (1999). Della-

Bianca & Gombert (2013) conducted one of the first systematic analysis of stress tolerance of industrial strains from the Brazilian fuel ethanol industry, evaluating S. cerevisiae strains, and their methodology was adapted for this study.

Stress response to saline stress

The growth parameters after incubation of yeast strains IZ 1339 and FT 146L in YPD supplemented with different NaCl concentrations are shown in Table 5.

Table 5 – Colony growth of *K. marxianus* strains in media under saline stress conditions

	IZ 1339		FT 146L	
[NaCl] (M)	**Growth**	**Size (mm)**	**Growth**	**Size (mm)**
0.0	normal	15	normal	15
0.3	normal	15	normal	15
0.6	normal	15	normal	15
1.0	normal	10	normal	10
1.5	reduced	5.0	reduced	10
2.0	reduced	5.0	reduced	5.0

Growth conditions: YPD medium, 35ºC for 48 hours.
[NaCl] = molar concentration of NaCl; Size = colony size measured in mm; Values on the table are means of triplicates

K. marxianus strains were not affected by increased NaCl concentration up to 0.6 M (equivalent to 35.1 g/L); the colonies of both strains presented the same growth. In concentrations above 1 M, there is a gradual decrease in the size of the colony. This can be an indicative of saline stress inhibiting yeast growth. Remarkably, there was colony growth, albeit reduced, in all NaCl concentrations evaluated.

In *S. cerevisiae*, reduction of colony size was observed in addition to 0.5 M of NaCl, and did not show any growth under the addition of 1 M NaCl (DELLA-BIANCA; GOMBERT, 2013).

The results obtained in this study suggest that the two *K. marxianus* strains had a much higher threshold of tolerance to saline stress.

The study conducted by Posas et al. (2000) determined that yeast cells response to saline stress induces the expression of a large number of genes, suggesting that this adaptation requires the adaptation of many cellular aspects, depending on concentration and time of exposure. A possible industrial application of yeast under osmotic shock is to isolate stress tolerant strains for fermentation process (WALKER; BASSO, 2020).

Studies have shown an increase in fermentation performance by yeast previously exposed or grown on hyperosmotic media (e.g., supplemented with high NaCl concentrations). However, most of these studies have been conducted on S. cerevisiae (TRAINOTTI; STAMBUK, 2001), thus, making it necessary to evaluate such conditions on K. marxianus.

Stress response to high ethanol concentrations

The growth parameters after incubation of yeast strains IZ 1339 and FT 146L in YPD medium supplemented with different ethanol concentrations are shown in Table 6.

Table 6 - Colony growth of *K. marxianus* strains in media under ethanolic stress conditions

[Ethanol] (%, v/v)	**IZ 1339**		**FT 146L**	
	Growth	**Colony size (mm)**	**Growth**	**Colony size (mm)**
0	Normal	15	normal	15
12	Normal	13	normal	15
15	reduced	5.0	reduced	5.0
20	reduced	5.0	reduced	5.0
30	reduced	5.0	reduced	5.0

Growth conditions: YPD medium, 35ºC for 48 hours. [Ethanol] = percentage of ethanol added to YPD mediumValues on the table are means of triplicates

The ethanol concentration range used in this study was chosen based on the alcoholic levels found in the Brazilian industrial ethanol production and above (DELLA-BIANCA; GOMBERT, 2013).

In this cultivation utilizing ethanol as the sole carbon source, it was observed regular growth for the Reference (YPD medium), but for the ethanol concentration of 12% there was a decrease in colony size for strain IZ 1339. For the concentration 15% ethanol (v/v) forward, both strains both strains presented the effects of ethanolic stress in the form of reduced colony size. However, no complete inhibition of growth was observed in any of the strains assessed.

Studies carried utilizing *S. cerevisiae* strains showed that both laboratory strains can be just as tolerant as industrial strains, however, it is more common to find industrial strains with a higher ethanol tolerance, due to the conditions they are exposed to (BRAVIM et al., 2010; DELLA-BIANCA; GOMBERT, 2013). In the study performed Della-Bianca & Gombert (2013), the *S. cerevisiae* strains did not grow on ethanol concentrations of 17.5 or 20%, unlike what we observed for both *K. marxianus* strains.

Keeping in mind that strains IZ 1339 and FT 146L were cultivated on plates containing ethanol as the main carbon source (no glucose added), and this condition activates fully respiratory metabolism. Growth in this particular condition may be an indicative of oxidative stress tolerance, since ethanol primarily targets membranes, increasing their fluidity and permeability, leading to a decrease in viability (BLEOANCA et al., 2013).

Carrasco, Querol and del Olmo (2001) found one wine strain of *S. cerevisiae* capable of growing in 15% ethanol after 15 days. Studies on *K. marxianus* show that most strains have a reduced tolerance to ethanol in comparison to *S. cerevisiae*, as its growth is strongly inhibited by an ethanol concentration higher than 6%. This happens because of the low membrane stability and the down-regulation of gene-encoding enzymes of the ergosterol biosynthesis pathway under high ethanol stress (DINIZ et al., 2017).

Nonetheless, techniques including pre-treatment, cocultures and evolutionary engineering have improved *K. marxianus* strains resistance to ethanol, with some particular strains presenting increased tolerance levels to higher ethanol concentration (GÜNEŞER et al., 2016; MADEIRA-JR; GOMBERT, 2018; SAINI; BENIWAL; VIJ, 2017), which is a very desirable trait for future industrial applications.

2.3.3 Yeast response to different sugars as carbon sources during growth kinetics

First of all, the yeasts *S. cerevisiae* CAT-1 and *K. marxianus* IZ 1339 and FT 146L were cultured in sucrose-containing medium (Figure 2). The CAT-1 and IZ-1339 strains presented similar growth profiles, with lag phase, log phase and stationary phase. The decline phase is not perceived in these cultivation conditions. On the other hand, the FT 146L strain presented a very different growth profile between the two of them. In this particular condition, *S. cerevisiae* CAT-1 produced more biomass when compared to *K. marxianus* strains.

Strains were cultivated in YNB (Yeast Nitrogen Base without amino acids) medium, often utilized to determine carbon source assimilation, as it contains all the essential nutrients and vitamins needed for yeast cultivation, to be added the desired carbon source (ROBERTS; KALTENBACH; RUDOLF, 2020).

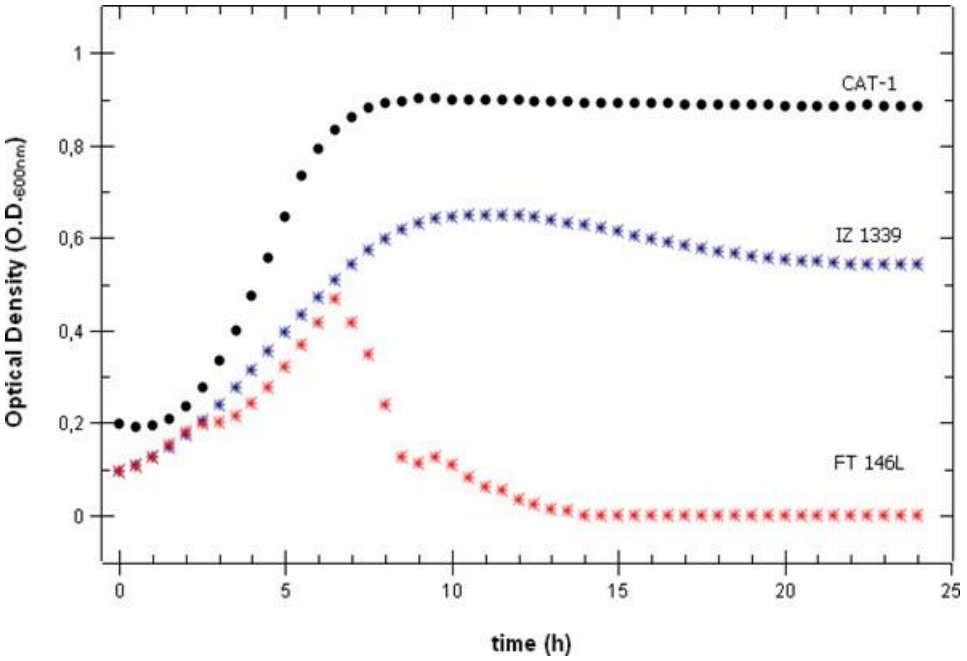

Figure 2. Cultivation of *K. marxianus* IZ 1339 (blue); FT 146L (red), and *S. cerevisiae* CAT-1 (black) in YNB sucrose-containing medium. Growth was evaluated at 30ºC for 24 hours in a Tecan microplate reader, under agitation.

Microorganisms generally have the following phases during growth in glucose and other sugars: “Lag” phase – also known as latency phase, where the microorganisms adapt to the environment, showing no increase in biomass. Followed by the Exponential, or Log phase – where microorganisms present constant growth rate, and nutrients are abundantly present in the medium; Stationary phase – where the microorganisms’ growth rate beings to slow down due to substrate and O_2 depletion and accumulation of metabolism inhibitors, and population numbers remain constant. Finally leading to the Death phase – where the number of non-viable cells progressively exceeds the viable ones (WANG et al., 2015).

Growth response from K. marxianus on isolated sugars

Growth response from *K. marxianus* strains IZ 1339 and FT 146L was determined on different sugars as carbon sources, in order to understand the physiology of sugar consumption in strains of distinct backgrounds (lab-isolated and isolated from bioprocess) we evaluated them on both isolated and binary combinations. The growth curves of *K. marxianus* strains in medium containing different sources of sugar are presented in Figure 3.

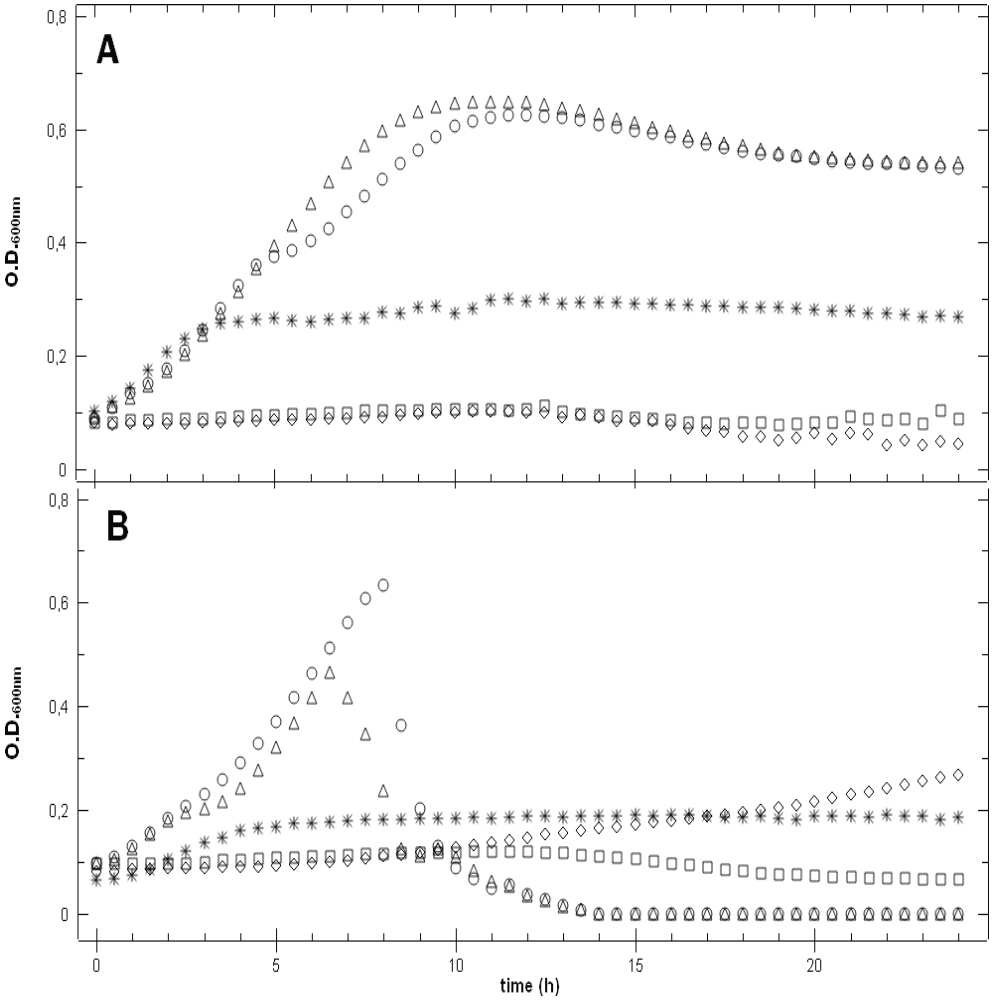

Figure 3- Cultivation of *K. marxianus* (A) IZ 1339; (B) FT146L strain in YNB medium containing 2% (m/V): (○) glucose, (Δ) sucrose, (□) xylose, (◊) lactose or (✳) maltose. Growth was evaluated at 30ºC for 24 hours in a Tecan microplate reader, under agitation.

Strain IZ 1339 presented a typical growth profile in the presence of glucose, or sucrose.

The growth curves indicate that strain IZ 1339 exhibited a more expressive growth when cultivated in the presence of Glucose or Sucrose. In the medium containing Sucrose this strain presented superior maximum OD (OD_{max}) of 0.650 at 10.5 h, when compared to the cultivation containing Glucose, that presented OD_{max} of 0.626 at 11.5 h.

K. marxianus strain FT146L presented distinct characteristics when compared to strain IZ 1339. One of the key points was the lower production of biomass in general, albeit reaching the exponential phase faster. Cultivation in the medium with Glucose presented OD_{max} of 0.633 in 8 h, whereas Sucrose also reached OD_{max} faster, in 6.5 h of cultivation.

The physiological parameters obtained during the exponential phase of growth for *K. marxianus* IZ 1339 and FT 146L are presented in Table 7.

Table 7– Physiological parameters obtained for *K. marxianus* during the exponential growth phase indifferent carbon sources

Yeast strain	Carbon Source	$O.D._{max}$	Time for $O.D._{max}$ (h)	μ_{max} (h^{-1})	μ_S (OD/h)	$Y_{x/s}$	X_{max} (OD/l)
IZ1339	Glucose	0.626	11.5	0.31	4.19	0.07	0.325
	Sucrose	0.650	10.5	0.25	3.17	0.08	0.395
	Xylose	0.113	12.5	0.03	9.92	0.00*	0.095
	Lactose	0.102	11	0.02	10.76	0.00*	0.086
	Maltose	0.301	11.5	0.28	5.31	0.05	0.248
FT146L	Glucose	0.633	8	0.22	1.62	0.135	0.561
	Sucrose	0.466	6.5	0.30	8.46	0.035	0.196
	Xylose	0.121	10.5	0.03	15.8	0.002	0.099
	Lactose	0.267	24	0.08	6.60	0.012	0.142
	Maltose	0.190	15	0.27	8.82	0.031	0.147

Growth conditions: YNB medium; initial substrate concentration 20 g/L; 30ºC for 24 hours.

$O.D._{max}$ = maximum optical density μ_{max} = maximum specific growth rate, μ_S = specific rate of substrate consumption, $Y_{x/s}$ = biomass yield on substrate, X_{max} = maximum biomass concentration,
***** = values under 0.001

With the exception of strain FT 146L reaching $O.D._{max}$ at 8 h in Glucose and 6.5 h for Sucrose, strain IZ 1339 reached $O.D._{max}$ at a faster rate for the other sugars evaluated, particularly for Lactose, at 11 h. Both strains had a comparable $O.D._{max}$ rate for glucose and sucrose, decreasing growth for the other carbon sources.

Growth response from K. marxianus on binary sugar combinations

The growth curves of *K. marxianus* strains in medium containing binary sugar combinations are presented in Figure 4.

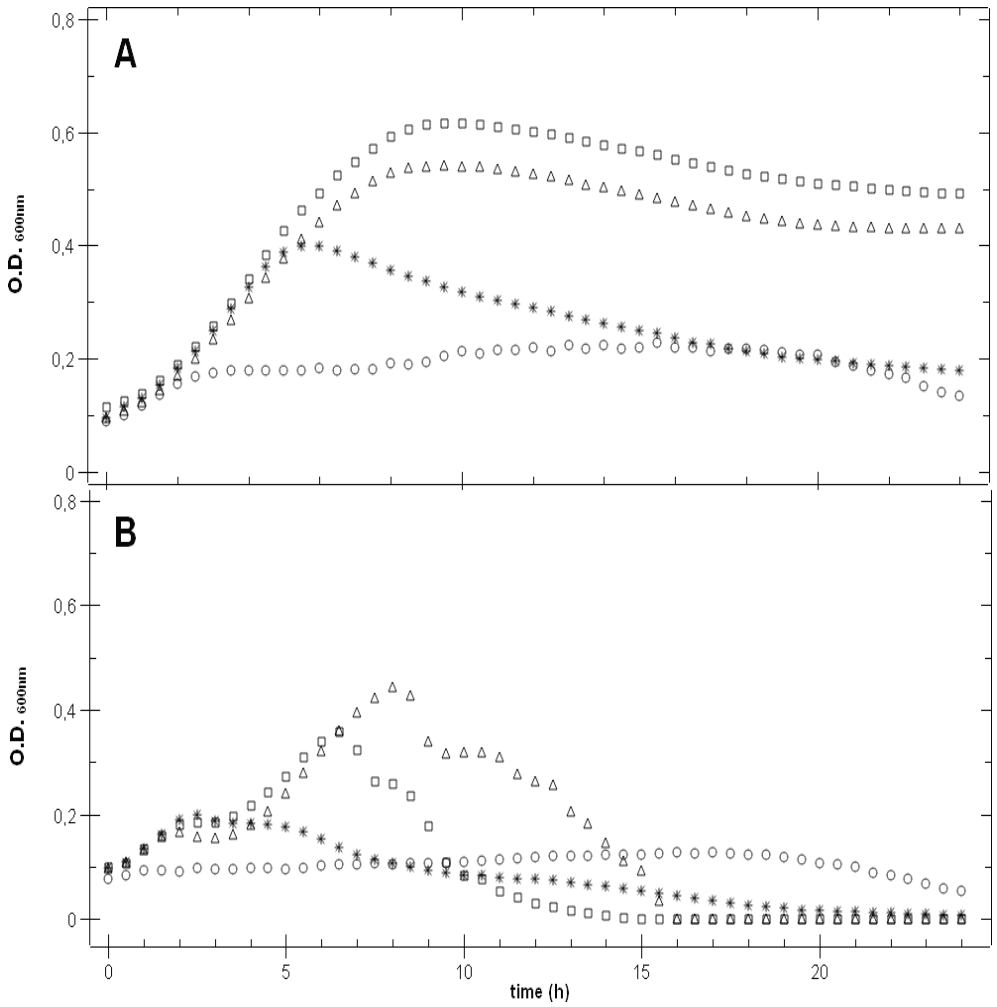

Figure 4- Cultivation of *K. marxianus* strain (A) IZ 1339 and (B) FT146L in YNB medium binary sugar combinations (m/V): (✳) Glu/Xyl (10 $g.L^{-1}$ each), and (□) Glu/lac, (Δ) Suc/lac, and (○) Mal/Lac (12.5 and 7.5 $g.L^{-1}$, respectively). Growth was evaluated at 30ºC for 24 hours in a Tecan microplate reader, under agitation.

For strain IZ 1339, the combination of Glucose and Xylose (10 $g.L^{-1}$ each) presented OD_{max} of 0.399. While this OD_{max} value was not as expressive when compared to other sugars utilized, this value was reached at 5.5 h of cultivation, faster than any carbon source herein evaluated.

Binary combination of Glucose and Lactose and Sucrose and Lactose also presented increased growth curves, which matches the ability of the yeast *K. marxianus* to metabolize lactose, unlike *S. cerevisiae*.

Cultivation in the medium supplemented by the binary sugar combinations presented distinct growth patterns, which is compatible to previously published data (FONSECA *et al.*, 2008; KARIM; GERLIANI; AÏDER, 2020) that show that *K. marxianus* does not have anyspecific nutritional needs, being a very versatile yeast, which is interesting in from an industrial point of view.

The physiological parameters obtained during the exponential phase of growth for *K. marxianus* FT 146L and IZ 1339 are presented in Table 8.

Table 8- Physiological parameters obtained for *K. marxianus* during the exponential growth phase utilizing binary sugar combinations

Yeast strain	Carbon Source	O.D.$_{max}$	Time for O.D.max (h)	μ_{max} (h^{-1})	μ_S (OD/h)	$Y_{x/s}$	X_{max} (OD/l)
IZ1339	Glucose/Xylose	0.399	5.5	0.32	4.06	0.08	0.287
	Glucose/lactose	0.618	9.5	0.25	2.97	0.08	0.426
	Sucrose/lactose	0.543	9.5	0.27	3.77	0.07	0.344
	Maltose/lactose	0.218	18	0.20	6.95	0.03	0.175
FT146L	Glucose/Xylose	0.199	2.5	0.26	8.15	0.032	0.199
	Glucose/lactose	0.358	6.5	0.20	3.47	0.058	0.358
	Sucrose/lactose	0.444	8	0.28	3.66	0.076	0.360
	Maltose/lactose	0.127	17	0.03	10.1	0.003	0.120

O.D.$_{max}$ = maximum optical density μ_{max} = maximum specific growth rate, μ_S = specific rate of substrate consumption, $Y_{x/s}$ = biomass yield on substrate, X_{max} = maximum biomass concentration

Comparing all of the carbon sources herein evaluated (Table 7 and 8), the higher maximum specific growthy rate (μ_{max}) was observed in the cultivation supplemented with the combination of Glucose/Xylose (0.32 h^{-1}), followed by Glucose 2% (0.31 h^{-1}), which demonstrates that the presence of Xylose did not negatively affect this strain's growth. Similar behavior was observed in the study of Fonseca et al. (2013).

The binary sugar combinations containing Lactose also presented satisfactory growth (0.25 e 0.27 h^{-1}). Exceptionally, growth supplemented only by Xylose 2% or Lactose 2% as a carbon source did not present significant growth rates (0.03 and 0.02 h^{-1}, respectively). This indicates that while, these particular sugars did not hinder the growth of strain IZ 1339, they also did not enhance it.

Remarkably, the cultivation containing Glucose and Xylose (1%, 1%) reached OD_{max} at only 2.5 h. Even though this binary sugar combination did not result in abundant biomass growth, it is important to explore the relation of Xylose in the metabolism of *K. marxianus*, because the presence of this monosaccharide sugar obtained from the hydrolysis of hemicellulose, found in sugarcane fibers, had a stimulating effect on the growth rate.

The behavior observed for strain FT 146L was considerably distinct, presenting higher μmax values in the medium supplemented with the binary combination of Sucrose/Lactose (0.28 h^{-1}).

Followed by Maltose (0.27 h^{-1}) and the combination of Glucose/Xylose (0.26 h^{-1}). This strain seemed to favor the binary sugar combinations as carbon sources.

Several studies have shown the maximum specific growth rate of *S. cerevisiae* and *K. marxianus* yeasts, as shown in the Table 9.

Table 9 - Maximum specific growth (μ_{max} h^{-1}) rates observed in yeast strains in different substrates and sugar concentrations

Strain	Substrate	Concentration (g/L)	μ_{max} (h^{-1})	Reference
S. cerevisiae - CAT1	molasses	80	0.37	Cabral (2020)
	molasses	150	0.31	
S. cerevisiae spp.	glucose	40	0.38 – 0.67	Oliveira et al. (2004)
S. cerevisiae spp.	sugar cane juice	100	0.24 – 0.40	Stroppa et al. (2009)
S. cerevisiae spp.	molasses + yeast extract	40 + 2.5	0.14 – 0.32	Amorim et al. (1996)
S. cerevisiae TKPK10.5.1	YPD (glucose)	40	0.15	Riyanti et al. (2020)
	sweet sorghum juice (sucrose)	98	0.67	
	sorghum straw (glucose+xylose)	5.39 + 12.05	0.83	
	rice straw (glucose+xylose)	2.9 + 8.4	0.70	
K. marxianus CBS 6556	glucose	10	0.49	Fonseca et al. (2013)
	fructose	10	0.42	
	sucrose	10	0.43	
	lactose	10	0.40	
	galactose	10	0.41	
K. marxianus ATCC 26548	glucose	10	0.56	Fonseca et al. (2007)
K. marxianus CBS 6556	glucose	10	0.44	Bellaver et al. (2004)
	lactose	10	0.44	
K. marxianus IZ 1339	glucose	20	0.31	Present work
	sucrose	20	0.25	
K. marxianus FT 146L	glucose	20	0.22	
	sucrose	20	0.30	

According to Table 7, 8 and 9, the μ_{max} values obtained by strain IZ 1339 (0.31 h^{-1} for glucose; 0.25 h^{-1} for sucrose, both 20 g/L) are comparable to those obtained by *S. cerevisiae* strain CAT-1 cultivated in molasses in higher sugar concentrations (0.37 h^{-1} for 80 g/L; 0.31 h^{-1} for 150 g/L) (CABRAL, 2020), being also comparable to other *S. cerevisiae* strains (AMORIM; BASSO; ALVES, 1996; OLIVEIRA et al., 2004; RIYANTI et al., 2020; STROPPA et al., 2009). All of which were obtained in higher sugar concentrations, with the exception of those cultivated in a glucose+xylose medium, which showed higher μ_{max} values.

Strain FT 146L has a similar μ_{max} value range, performing better on sucrose (20 g/L) reaching 0.30 h^{-1}, compared to 0.22 h^{-1} for glucose (20 g/L). This indicates that both *K. marxianus* strains had a performance comparable to largely utilized commercial *S. cerevisiae* strains, while utilizing less sugar to do so.

However, the *K. marxianus* strains referenced in Table 9 had an average of 0.44 h^{-1} on different sugars, all in the concentration of 10 g/L (BELLAVER et al., 2004; FONSECA et al., 2007; FONSECA; BARBOSA DE CARVALHO; GOMBERT, 2013), while the strains utilized in this study averaged 0.18 h^{-1} on different sugars, all in the concentration of 20 g/L. On average, *K. marxianus* strains maximum specific growth rate ranges from 0.15 h^{-1} to 0.4 h^{-1}, presenting a high heterogeneity among members of the genus (ROCHA; ABRAHÃO-NETO; GOMBERT, 2011). This physiologic diversity has been a common aspect described in recent works (FONSECA et al., 2008; KARIM; GERLIANI; AÏDER, 2020; LANE; MORRISSEY, 2010) that describe *K. marxianus* strains, and therefore, worth further exploring.

2.3.4 Effect of stress factors during growth kinetics

Even though the conditions reproduced in microculture are not identical to the ones found in bioprocesses, the evaluation of the stress factors in each individual strain is essential to comprehend yeast's response to such factors.

Amongst the most notable stress factors that yeasts are exposed to during fermentation, it is important to highlight oxidative stress, hyperosmolarity caused by high concentration of sugar in the wort. In addition, ethanolic stress, caused by increased ethanol concentration in the fermented wort, which negatively affects nitrogen absorption, and acetic acid stress, caused by acid washes during the cell recycling process. Understanding the effects of stress factors in the viability of yeast cells has already been proposed by Ivorra et al. (1999), and explored by Della-Bianca and Gombert (2013), Saini et al. (2017), Diniz et al. (2017).

Yeast strains employed in bioprocesses are also exposed to conditions such as toxins present in the substrate (potassium, aluminum, cadmium, iron, phenols, etc) that can cause stress; therefore, it is expected that these yeasts present more resistance than lab-isolated strains (DELLA-BIANCA; GOMBERT, 2013).

In order to contribute to the understanding of the physiology of *K. marxianus* strains of distinct backgrounds (lab-isolated and isolated from bioprocess) we evaluated their tolerance towards particular stress factors faced by yeasts in the industrial ethanol production on a scaled-down system. This study follows the models employed by Ivorra et al. (1999) and Della-Bianca and Gombert (2013), who explored the effects of stress factor faced by S. cerevisiae.

Growth under osmotic and saline stress

K. marxianus strains were cultivated in YPD medium under osmotic or saline stress conditions. The growth curves obtained are presented in Figures 5 and 6, respectively.

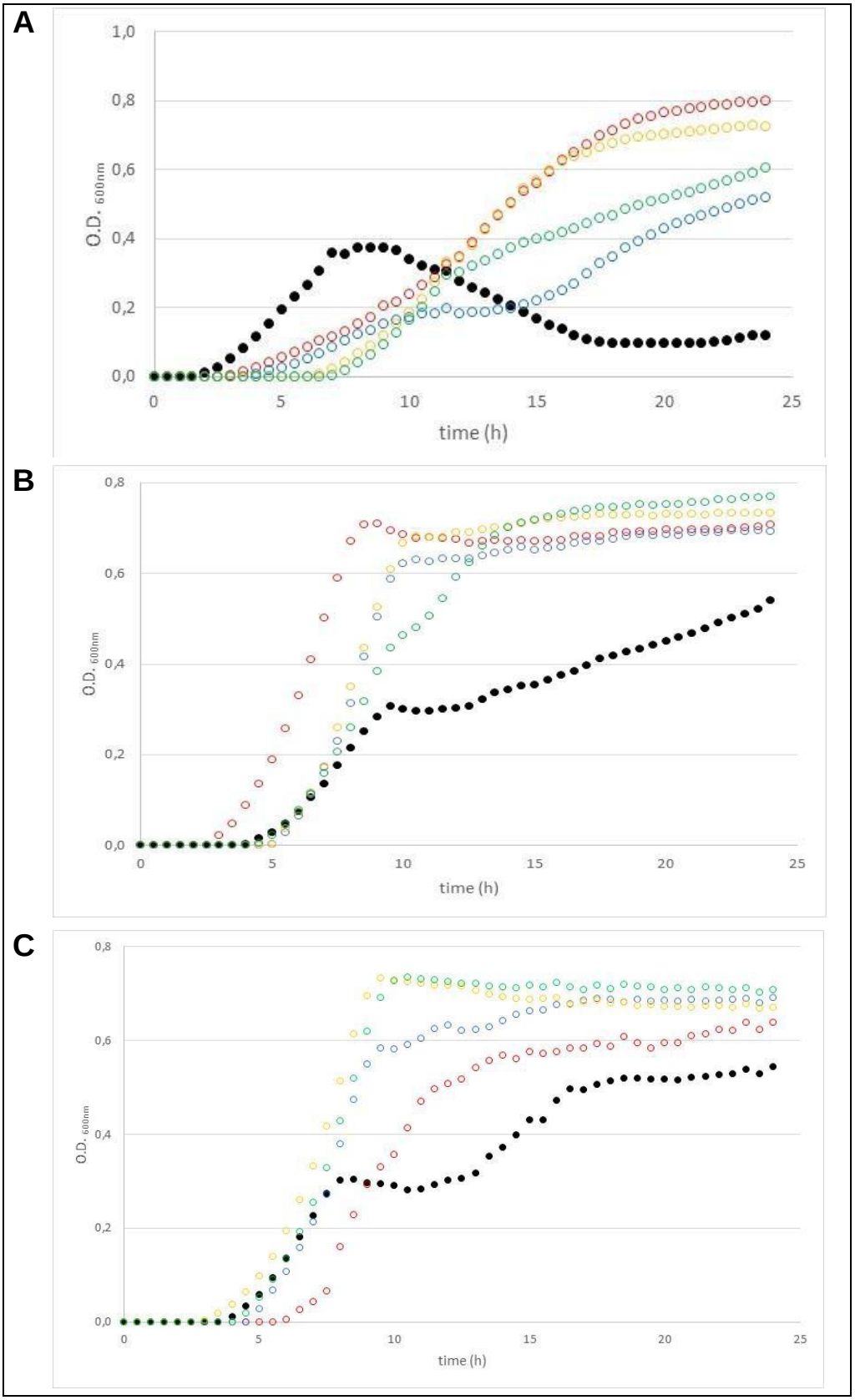

Figure 5- Cultivation of *K. marxianus* strain (A) IZ 1339; (B) FT146L and (C) *S. cerevisiae* CAT-1 in YPD medium supplemented with NaCl in the following concentrations: (●) 0.0 g/L; (○) 0.5 g/L; (○) 1.0 g/L; (○) 1.5 g/L and (○) 2.0 g/L. Growth was evaluated at 30ºC for 24 hours in aTecan microplate reader, under agitation.

K. marxianus strains IZ 1339 showed growth in all NaCl concentrations studied. However, at concentration 0.5 g/L the strain obtained higher O.D., followed closely by 1.5 g/L (Figure 5 A).

The growth curve for strain IZ 1339 for the concentration of NaCl 1.0 g/L suggest the occurrence of a diauxic shift, a change in metabolism where glucose consumption fuels glycolytic fermentation, then shifts to respiration by ethanol import upon glucose depletion. This shift is likely shown in strain IZ 1339 as the growth curve primarily suggests the consumption of glucose present in the growth medium, followed by a period of stabilization and, subsequently, another growth period, likely promoted by the ethanol present in the medium.

The diauxic shift may occur in aerobic fermentations with different carbon sources, taking place in two stages. Initially, the yeast cells consume the more readily available carbon source. Glucose is initially consumed with the production of ethanol, biomass, carbon dioxide and other byproducts, such as acetic acid and glycerol. After the consumption of glucose, yeast cells go through a transition, where they consume the produced ethanol as a source of carbon, then producing more biomass and carbon dioxide (OHLMEIER et al., 2004).

As for the control (0 g/L NaCl), the growth curve presented a decline in the middle, around 9 H of growth, a phenomenon that we could not quite explain.

None of the concentrations were growth limiting, although depending on the saline concentration, the lag phase increases. However, the presence of a longer lag phase can suggest that the metabolism for the strain IZ 1339 had more difficulties adapting.

It is possible to speculate whether the presence of NaCl stimulated a metabolic pathway in strain IZ 1339, causing it to produce more biomass that the Reference (0 g/L of NaCl). This particular condition is worth of further exploration.

Strain FT 146L presented a more significant growth curve for the concentration NaCl 0,5 g/L (Figure 5 B). In all concentrations studied, the growth of FT146 was better than the Reference.

This *K. marxianus* strain had its growth limited in the concentration 1.5 g/L, presenting the least significant growth curve. The concentration 2.0 g/L was an inhibitor, presenting minimal growth, as also observed for strain IZ 1339.

The *Saccharomyces cerevisiae* strains studied by Della-Bianca and Gombert (2013) did not grow when the medium was supplemented with 1.0 g/L NaCl, which is in agreement with what was observed in previous studies (BLOMBERG, 1997), being this concentration considered an inhibitor for *S. cerevisiae*.

Although the cited studies were carried out in solid medium, it is possible to draw a

comparison and suggest that the strains of K. marxianus FT 146L and IZ 1339 presented a superior performance.

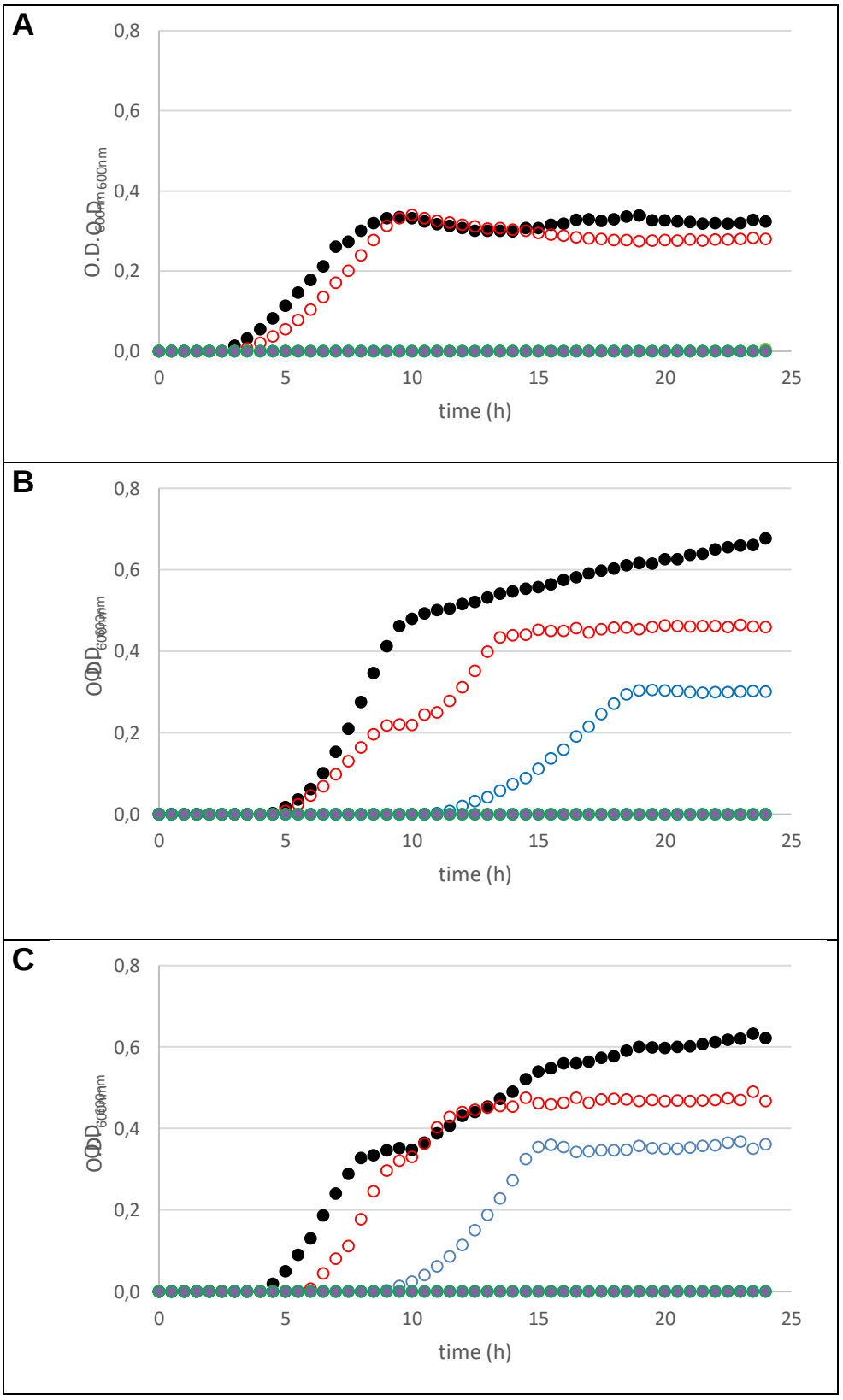

Figure 6 - Cultivation of *K. marxianus* strain (A) IZ 1339; (B) FT146L and (C) *S. cerevisiae* CAT-1 inYPD medium complemented with Acetic acid in the following concentrations, (M): (●) 0.0 M; (○) 0.05 M; (○) 0.10 M; (○) 0.20 M and (○) 0.40 M. Growth was evaluated at 30ºC for 24 hours in a Tecan microplate reader, under agitation.

K. marxianus strain IZ 1339 only presented growth in the Reference (0 M of acetic acid) and 0.05 M (Figure 6 A), both curves being similar in growth performance. All of the other concentrations evaluated (0.1; 0.2 and 0.4 M) presented no growth, meaning that the acetic acid present in the culture medium acted as an inhibitor.

A similar growth pattern was observed for strain FT 146L (Figure 6 B), where the more pronounced growth curve was obtained in the Reference (0 M of acetic acid), followed by concentration 0.05 M. Diverging from strain IZ 1339, there was growth under the addition of 0.1 M of acetic acid, albeit slowed down, presenting a too long lag phase. The concentrations (0.2 e 0.4 M) prevented growth, acting as inhibitors.

As observed, both *K. marxianus* strains were affected by the addition of increased concentrations of acetic acid to the culture medium. Yeasts, in particular *Saccharomyces cerevisiae*, are capable of withstand the presence of weak organic acids, however, the prolonged exposure makes difficult for the cell to recover its internal pH, resulting in greater energy expenditure, causing growth impediment (ULLAH et al., 2012).

Once again, lab-isolated strains present more susceptibility to acetic acid stress compared to strains isolated from bioprocesses.

Both in previous studies and in the results observed it is possible to claim that none of the strains displayed tolerance to all stress factors evaluated, this is probably related to the fact that each strain was isolated from a distinct environment.

Previous studies (ALBERS; LARSSON, 2009; BRAVIM et al., 2010) show that there is no correlation to ploidy and stress tolerance in yeast strains, the tolerance does not vary between haploid and diploid individuals.

As remarked by Della-Bianca & Gombert (2013), tolerance to heath and low pH stresses are what distinguishes fuel ethanol strains from the others, indicating that these two conditions exert the most selective pressure on yeast cells. Nevertheless, it is important to mention that industrial media and conditions are hardly, if at all, reproducible.

Ethanolic stress

Tolerance to ethanolic stress for *K. marxianus* strains IZ 1339 and FT 146L can be seen in Figure 7.

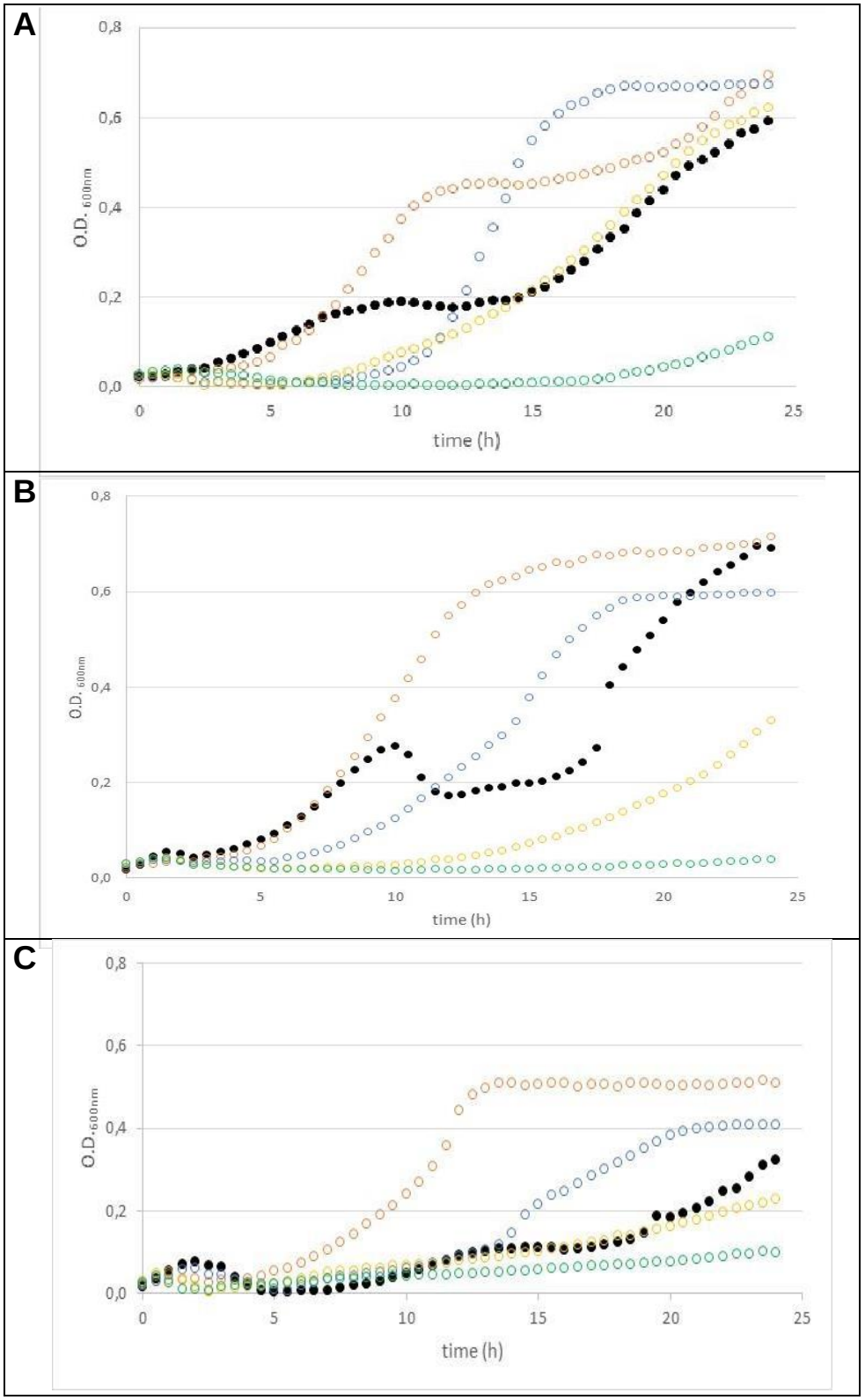

Figure 7- Cultivation of *K. marxianus* strain (A) IZ 1339; (B) FT146L and (C) *S. cerevisiae* CAT-1 in YPmedium complemented with Ethanol in the following concentrations, (%, v/v): (●) 0.0; (○) 5.0; (○) 10.0; (○) 12.5; (○) 15.0. Growth was evaluated at 30ºC for 24 hours in a Tecan microplate reader, under agitation.

Strain IZ 1339 showed increased growth curves for concentrations 5 and 12,5 % (v/v), followed by concentration 15% (v/v), presenting a slight decrease in the growth curve, as it appears the yeast cells took longer to adapt to the culture medium.

Notably, the concentration 10% (v/v) had average performance when compared to the higher concentrations employed in this experiment, which suggests the strain IZ 1339 has the ability to better adapt to higher ethanol concentrations.

Strain FT 146L presented a similar growth curve pattern between all the ethanol concentrations utilized in the experiment. Concentration 5% (v/v) allowed the yeast cells to adapt and grow faster, followed by 12.5 and 10% (v/v), respectively. It is noteworthy that, although adaptation took longer than the other concentrations, 15% (v/v) yielded the more significant growth rate out of all concentrations.

Silveira et al. (2020) identified accumulation of valine and metabolites of the citric acid cycle (isocitric acid, citric acid, and cis-aconitic acid) in the *K. marxianus* strain ETS4, which was subjected to ethanol stress. The accumulation of said metabolites may be important to increase ethanol tolerance.

The ethanol concentrations herein evaluated are based on the possible levels obtained during industrial ethanol production. Della-Bianca and Gombert (2013) observed that lab-isolated strains present more susceptibility to ethanolic stress than strains isolated from bioprocesses, the results obtained in this study are compatible to their findings. It is important to point out that tolerance to higher ethanol concentrations added to culture medium is different than the situation where the ethanol is produced by the yeast cells after the fermentative process, however, it is possible to draw comparison in order to understand the influence of this particular stress factor.

Determination of maximum specific growth rate:

Maximum specific growth rate was obtained according Fallahzadeh et al. (2010) and Della-Bianca and Gombert (2013) where (μ_{max}) (h^{-1}) is determined by the linear regression of the points **lnX vs t**, being lnX the Natural logarithm of the OD_{620nm} readings for the *K. marxianus* strains, and t being the time in hours. For this parameter, we chose to calculate the maximum specific growth rates of the stress factor concentrations that showed more expressive growth, the concentrations are indicated in Table 10.

Table 10 – Maximum specific growth rate values for *K. marxianus* strains IZ 1339 and FT 146L

	μ_{max} (h^{-1})	
	IZ 1339	**FT 146L**
YP (reference)	0.294	0.279
YP + Ethanol 5%	0.286	0.374
YP + Ethanol 10%	0.431	0.280
YP + Ethanol 12.5%	0.468	0.124
YP + Ethanol 15%	-	0.177
YPD (reference)	1.109 / 1.015	0.719 / 0.607
YPD + NaCl 0.5 g/L	0.536	0.960
YPD + NaCl 1.0 g/L	0.584	0.722
YPD + NaCl 1.5 g/L	0.450	0.808
YPD + NaCl 2.0 g/L	0.527	0.680
YPD + Acetic acid 0.05M	0.645	0.580
YPD + Acetic acid 0.10M	-	0.575

Growth conditions = 24 hours, 30º C, readings performed every 30 minutes

Maximum specific growth rate (μ_{max}) determined by the relation of biomass production vs time shows that, for strain IZ 1339, none of the stress factors caused a significative decrease in growth rate when compared to the Reference (YPD medium). The same can be said for the ethanol concentrations, as it does not cause major inhibiting effects on *K. marxianus* strain IZ 1339.

As for strain FT 146L, there was a considerable decrease in growth rate in the presence of Ethanol 12.5% (v/v), reaching 0.124 h^{-1} compared to 0,28 h^{-1} for the Reference. Remarkably, the addition of NaCl (0.5 g/L) had the effect of increasing the maximum specific growth rate, reaching 0.72 h^{-1}, compared to 0.72 h^{-1} for the reference.

While most *K. marxianus* exhibit a low tolerance to ethanol in elevated concentrations, the stress responses linked to ethanol are still poorly understood. In the study conducted by Alvim et al. (2019), *K. marxianus* CCT 7735 seems to exhibit an immediate reaction in which the metabolism is immediately impaired, followed by an adaptive response.

Rocha et al. (2011) describes the importance of studying the intraspecific diversity in physiology for *K. marxianus* in order to understand how the parameters varies within the species, and to be able to pinpoint each strain's strongest characteristics for future industrial application.

From the calculation of μ_{max} from both strains subjected to stress factors and the Reference (YPD medium), it is possible to calculate the growth inhibition factor (ULLAH et al., 2012), where the growth inhibition factor = **100 x [1- (μ_{max} stress) / (μ_{max} reference)],** presented in Table 11.

Table 11 – Growth inhibition factor caused by stress factor treatments evaluated for *K. marxianus* strains IZ 1339 and FT 146L

Strains	Condition	Growth inhibition factor (%)
IZ 1339	Ethanol 12.5%	-59.2
	NaCl 1.5 g/L	59.1
	Acetic acid 0.05M	41.4
FT 146L	Ethanol 12.5%	57.8
	NaCl 2.0 g/L	3.0
	Acetic acid 0.05M	17.1

Growth conditions = 24 hours, 30º C, readings performed every 30 minutes, values present inpercentage (%)

It is possible to observe, combined with the μ_{max} values, that the presence of some stress factors acted as inhibitors, mainly Ethanol 12.5% (v/v) for the strain IZ 1339, exerting 46.7% of inhibition when compared to the same strain grown in YPD medium (Reference); followed by NaCl 0.5 g/L. However, strain FT 146L was only inhibited by 42.1% in the presence of NaCl 0.5 g/L when compared to the reference.

Interestingly, some stress factors not only were not inhibited, but apparently had their growth rate stimulated by their presence, such as Ethanol 12.5 % and Acetic acid 0.05 M; strain FT 146L presented μ_{max} values of 0.60 and 0.52 h^{-1}, respectively, compared to 0.38 h^{-1} obtained in the reference. This effect presents itself in the negative inhibition factor.

2.3.5 Fermentative capacity

Fermentative capacity of the yeast strains was determined through observation of the presence of displaced CO_2, a byproduct of fermentation, trapped inside the Durham tubes, alongside the presence of bubbles in the media surface and pleasant alcoholic odour. The results were observed after 48 hours of fermentative activity (Table 12).

Table 12 – Fermentative capacity observed in *K. marxianus* strains growth in different conditions

Strain	Exp	TSS (°Bx)	T(°C)	YPSuc			SCJ		
				FC	Odour	CO_2	FC	Odour	CO_2
IZ 1339	1	12	30	+	Alcoholic	0	+	Alcoholic	5
	2	30	30	+	Alcoholic	20	+	Alcoholic	20
	3	12	40	+	Alcoholic	5	+	Alcoholic	5
	4	30	40	+	Alcoholic	10	+	Alcoholic	10
	5	21	35	+	Alcoholic	5	+	Alcoholic	10
	6	21	35	+	Alcoholic	10	+	Alcoholic	10
	7	21	35	+	Alcoholic	10	+	Alcoholic	10
		TSS (°Bx)	**T(°C)**	**FC**	**Odour**	**CO_2**	**FC**	**Odour**	**CO_2**
FT 146L	1	12	30	+	Alcoholic	10	+	Alcoholic	5
	2	30	30	+	Alcoholic	10	+	Alcoholic	10
	3	12	40	+	Alcoholic	5	+	Alcoholic	10
	4	30	40	+	Alcoholic	10	+	Alcoholic	10
	5	21	35	+	Alcoholic	10	+	Alcoholic	20
	6	21	35	+	Alcoholic	20	+	Alcoholic	10
	7	21	35	+	Alcoholic	5	+	Alcoholic	20

FC = fermentative capacity; CO_2 = height in mm of CO_2 trapped inside Durham tubes.
Values on the table are means of triplicates; Experiment = experiments organized according to Table 4

Strain IZ1339 had the best performance in the experiment conducted at 30°Bx/30°C, in both media analyzed. The increase in temperature negatively affects the detachment of CO_2. Comparing the obtained results with experiments, which were performed at 35ºC e 40°C, there was no noteworthy difference in performance.

Experiment two, performed at 30ºC and 30ºBrix concentration, showed slight increase in the size of the trapped CO_2 bubbles for both media. Experiment 1, performed at 30ºC / 12º Brix in concentration showed no production of trapped CO_2 bubbles for YPSuc medium, nonetheless, there were visible CO_2 bubbles at the surface, and the presence of alcoholic odour.

Strain FT 146L showed a similar performance to strain IZ 1339, producing trapped CO_2 bubbles in all evaluated conditions. It is worth mentioning that there was a slight increase in CO_2 production in the experiment 5, 6 and 7 (35º C, 21º Brix) for MCC medium.

Both strains presented signs of fermentative activity in all conditions assessed. Remarkably, both strains managed to perform even at 40ºC, confirming the thermotolerant trait described for *K. marxianus* (CARVALHO et al., 2004; LANE; MORRISSEY, 2010; PAULINO DE SOUZA et al., 2018). The Durham fermentation test is a reliable method of screening to select yeast strains based on their desired application and substrate consumption (CHAROENSOPHARAT et al., 2015; DÍAZ-VERGARA et al., 2017).

These results confirm the aptitude of both strains to be utilized for bench-scale fermentations, in order to determine their ethanol yield.

The effect of total soluble solids and temperature in the fermentative capacity of *K. marxianus* strain IZ 1339 and FT 146L were examined through a two-way analysis of variance (ANOVA). Results for strain IZ 1339 are presented in Figure 8. As observed, there was not a significant variability among the factors analyzed.

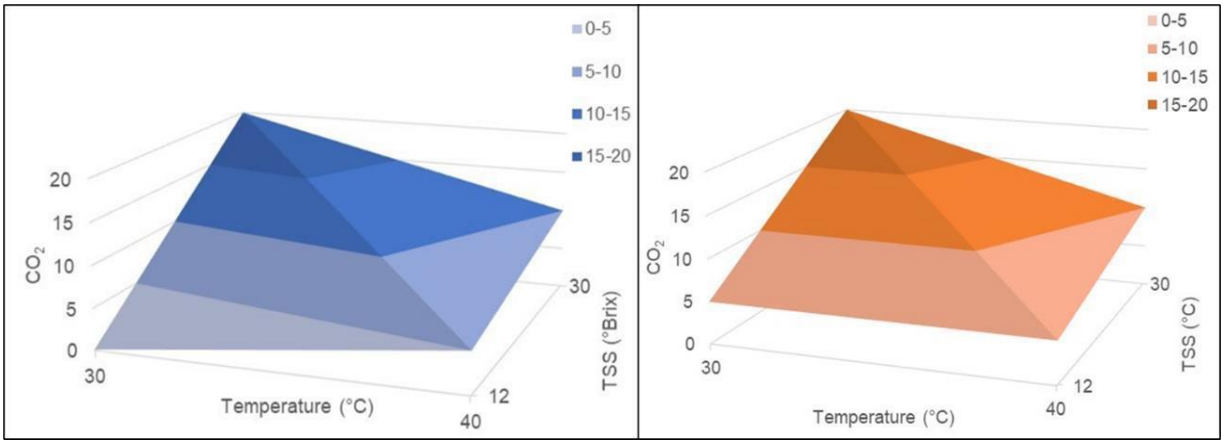

Figure 8 – Response surface methodology for fermentative capacity of *K. marxianus* strain IZ 1339 inYPSuc medium (left) and SCJ (sugarcane juice) (right)

YPSuc medium showed slight variability, however, temperature 30ºC and total soluble sugar concentration 30ºBrix were the best condition for fermentation in strain IZ 1339.Results for strain FT 146L are presented in Figure 9.

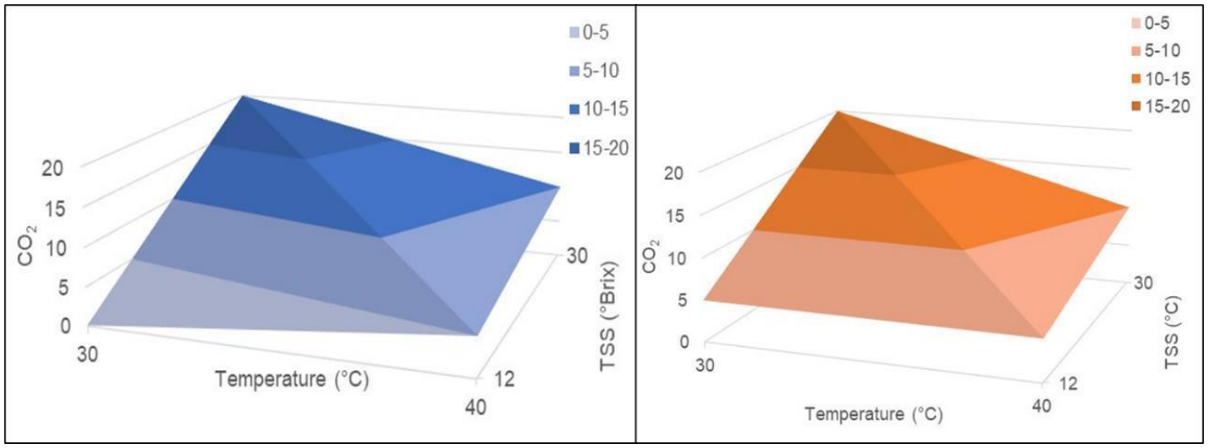

Figure 9 - Response surface methodology for fermentative capacity of *K. marxianus* strain FT 146L in YPSuc medium (left) and SCJ (sugarcane juice) (right)

Likewise, strain FT 146L seemed to favor the condition of temperature 30ºC and total soluble sugar concentration 30ºBrix, but there was not a large variability between the conditions.

References

ALBERS, E.; LARSSON, C. A comparison of stress tolerance in YPD and industrial lignocellulose-based medium among industrial and laboratory yeast strains. **Journal of Industrial Microbiology and Biotechnology**, 2009. v. 36, n. 8, p. 1085–1091. Available in: <https://academic.oup.com/jimb/article/36/8/1085/5993595>. Accessed at: 16 jun. 2022.

ALVIM, M.C.T. et al. Ethanol stress responses of *Kluyveromyces marxianus* CCT 7735 revealed by proteomic and metabolomic analyses. **Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology**, 2019. v. 112, n. 6, p. 827– 845.

AMORIM, H.V; BASSO, L.C.; ALVES, D.M.G. **Processos de produção de álcool: controle e monitoramento**. 2. ed. ed. Piracicaba: Piracicaba Fermentec-Fealq- Esalq, 1996.

BELLAVER, L.H. et al. Ethanol formation and enzyme activities around glucose-6-phosphate in *Kluyveromyces marxianus* CBS 6556 exposed to glucose or lactose excess. **FEMS Yeast Research**, 2004. v. 4, n. 7, p. 691–698. Available in: <http://www.ncbi.nlm.nih.gov/pubmed/15093772>. Accessed at: 21 set. 2018.

BLEOANCA, I. et al. Relationship between ethanol and oxidative stress in laboratory and brewing yeast strains. **Journal of Bioscience and Bioengineering**, 2013. v. 116, n. 6, p. 697–705.

BLOMBERG, A. The osmotic hypersensitivity of the yeast *Saccharomyces cerevisiae* is strain and growth media dependent: Quantitative aspects of the phenomenon. **Yeast**, 1997. v. 13, n. 6, p. 529–539.

BRAVIM, F. et al. Biotechnological properties of distillery and laboratory yeasts in response to industrial stresses. **Journal of Industrial Microbiology and Biotechnology**, 1 out. 2010. v. 37, n. 10, p. 1071–1079. Available in: <https://academic.oup.com/jimb/article/37/10/1071/5993888>. Accessed at: 16 jun. 2022.

BUZATO, J.B.; BROGGI, R.L.; CELLLGOI, M.A.P.C. Imobilização de invertase de *Saccharomyces cerevisiae* em esferas de ácido algínico. **Semina: Ciênc. Biol. Saúde.** 2000, v. 20/21, n. 2, p. 33-37. Available in: http://dx.doi.org/10.5433/1679-0367.1999v20n2p33. Accessed at: 21 set. 2018.

CABRAL, G.B**. Fermentação alcoólica de melaço com alta concentração de açúcar: efeito da esterilização do mosto e tratamento ácido da levedura**. Universidade de São Paulo Escola Superior de Agricultura “Luiz de Queiroz” - ESALQ/USP, 2020. n. Tese, 85 p..

CARRASCO, P.; QUEROL, A.; OLMO, M. DEL. Analysis of the stress resistance of commercial wine yeast strains. **Archives of Microbiology**, 2001. v. 175, n. 6, p. 450–457.

CARVALHO, A.S. et al. Effects of various sugars added to growth and drying media upon thermotolerance and survival throughout storage of freeze-dried *Lactobacillus delbrueckii* ssp. *bulgaricus*. **Biotechnology Progress**, 2004a. v. 20, n. 1, p. 248–254.

CARVALHO, A.S. et al. Relevant factors for the preparation of freeze-dried lactic acid bacteria. **International Dairy Journal**, 2004b. v. 14, n. 10, p. 835–847.

CHAROENSOPHARAT, K. et al. Ethanol production from Jerusalem artichoke tubers at high temperature by newly isolated thermotolerant inulin-utilizing yeast *Kluyveromyces marxianus* using consolidated bioprocessing. **Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology**, 2015. v. 108, n. 1, p. 173–190.

DELLA-BIANCA, B.E.; GOMBERT, A.K. Stress tolerance and growth physiology of yeast strains from the Brazilian fuel ethanol industry. **Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology**, 2013. v. 104, n. 6, p. 1083–1095. Available in: <http://www.ncbi.nlm.nih.gov/pubmed/24062068>. Accessed at: 21 set. 2018.

DÍAZ-VERGARA, L. et al. Encapsulated whey–native yeast *Kluyveromyces marxianus* as a feed additive for animal production. **Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment**, 2017. v. 34, n. 5, p. 750–759. Available in: <https://doi.org/10.1080/19440049.2017.1290830>.

DINIZ, R.H.S. et al. Transcriptome analysis of the thermotolerant yeast *Kluyveromyces marxianus* CCT 7735 under ethanol stress. **Applied Microbiology and Biotechnology**, 2017. v. 101, n. 18, p. 6969–6980.

EIADPUM, A.; LIMTONG, S.; PHISALAPHONG, M. High-temperature ethanol fermentation by immobilized coculture of *Kluyveromyces marxianus* and *Saccharomyces cerevisiae*. **Journal of Bioscience and Bioengineering**, 2012. v. 114, n. 3, p. 325–329. Available in: <https://www.sciencedirect.com/science/article/pii/S1389172312001351>. Acesso em: 17 jul. 2019.

FALLAHZADEH, V.; AHMADZADEH, M.; SHARIFI, R. Growth and pyoverdine production kinetics of *Pseudomonas aeruginosa* 7NSK2 in an experimental fermentor. **Journal of Agricultural Technology**, 2010. v. 6, n. 1, p. 107–115. Available in: <http://www.ijat-rmutto.com>.

FONSECA, G. G. et al. Physiology of the yeast *Kluyveromyces marxianus* during batch and chemostat cultures with glucose as the sole carbon source. **FEMS Yeast Research**, 2007. v. 7, n. 3, p. 422–435. Available in: <http://www.ncbi.nlm.nih.gov/pubmed/17233766>. Accessed at: 21 set. 2018.

FONSECA, G.G. et al. The yeast Kluyveromyces marxianus and its biotechnological potential. **Applied Microbiology and Biotechnology**. Available in: <http://www.ncbi.nlm.nih.gov/pubmed/18427804>. Accessed at: 21 set. 2018.

FONSECA, G.G.; BARBOSA DE CARVALHO, N.M.; GOMBERT, A.K. Growth of the yeast *Kluyveromyces marxianus* CBS 6556 on different sugar combinations as sole carbon and energy source. **Applied Microbiology and Biotechnology**, 23 jun. 2013. v. 97, n. 11, p. 5055–5067. Available in: <http://www.ncbi.nlm.nih.gov/pubmed/23435899>. Accessed at: 21 set. 2018.

GABARDO, S.; RECH, R.; AYUB, M.A.Z. Performance of different immobilized-cell systems to efficiently produce ethanol from whey: Fluidized batch, packed-bed and fluidized continuous bioreactors. **Journal of Chemical Technology and Biotechnology**, 2012. v. 87, n. 8, p. 1194–1201.

GARCÍA-GARIBAY, M. et al. Single Cell Protein: Yeasts and Bacteria. **Encyclopedia of Food Microbiology: Second Edition**. [S.l.]: Elsevier Inc., 2014, p. 431–438.

GOMES, L.H. et al. Presence of the yeast *Candida tropicalis* in figs infected by the fruit fly *Zaprionus indianus* (dip.: Drosophilidae). **Brazilian Journal of Microbiology.** 2003. v. 34, p.5-7. Available in: https://doi.org/10.1590/S1517-83822003000100002. Accessed at: 10 dez. 2022.

GÜNEŞER, O. *et al.* Volatile metabolites produced from agro-industrial wastes by Na-alginate entrapped *Kluyveromyces marxianus*. **Brazilian Journal of Microbiology**, 1 out. 2016. v. 47, n. 4, p. 965–972.

HENSING, M.C.M. *et al.* Physiological and technological aspects of large-scale heterologous-protein production with yeasts. **Antonie van Leeuwenhoek**, 1995. v. 67, n. 3, p. 261–279. Available in: <http://www.ncbi.nlm.nih.gov/pubmed/7778895>. Accessed at: 21 set. 2018.

HERIGSTAD, B.; HAMILTON, M.; HEERSINK, J. How to optimize the drop plate method for enumerating bacteria. **Journal of Microbiological Methods**, 1 mar. 2001. v. 44, n. 2, p. 121–129.

HONG, J. et al. Construction of thermotolerant yeast expressing thermostable cellulase genes. **Journal of Biotechnology**, 15 jun. 2007. v. 130, n. 2, p. 114–123.

ILLARIONOV, A.; LAHTVEE, P.-J.; KUMAR, R. Potassium and sodium salt stress characterization in the yeasts *Saccharomyces cerevisiae*, *Kluyveromyces marxianus*, and *Rhodotorula toruloides*. 2021. Available in: <https://doi.org/10.1128/AEM>. Accessed at: 4 jul. 2022.

IVORRA, C.; PÉREZ-ORTÍN, J. E.; DEL- OLMO, M. An inverse correlation between stress resistance and stuck fermentations in wine yeasts. A molecular study. **Biotechnology and Bioengineering**, 1999. v. 64, n. 6, p. 698–708.

KARIM, A.; GERLIANI, N.; AÏDER, M. *Kluyveromyces marxianus*: An emerging yeast cell factory for applications in food and biotechnology. **International Journal of Food Microbiology**, 2020. v. 333, n. May, p. 108818. Available in: <https://doi.org/10.1016/j.ijfoodmicro.2020.108818>.

KARIMI, S. et al. Use of organic wastes and industrial by-products to produce filamentous fungi with potential as aqua-feed ingredients. **Sustainability (Switzerland)**, 2018. v. 10, n. 9.

KIM, J. K.; TAK, K. T.; MOON, J. H. A continuous fermentation of *Kluyveromyces fragilis* for the production of a highly nutritious protein diet. **Aquacultural Engineering**, 1998. v. 18, n. 1, p. 41–49.

KURTZMAN, C.; FELL, J. The Yeasts (Fourth Edition). 1998. n. 4, p. 1076.

LANE, M. M.; MORRISSEY, J. P. *Kluyveromyces marxianus*: A yeast emerging from its sister's shadow. **Fungal Biology Reviews**, 1 fev. 2010. v. 24, n. 1–2, p. 17–26. Available in:<https://www.sciencedirect.com/science/article/abs/pii/ S1749461310000035>. Accessed at: 1º ago. 2019.

LEAL, G.A. et al. Fermentation of cacao (*Theobroma cacao* L.) seeds with a hybrid Kluyveromyces marxianus strain improved product quality attributes. **FEMS Yeast Research**, 2008. v. 8, n. 5, p. 788–798.

MADEIRA-JR, J.; GOMBERT, A.K. Towards high-temperature fuel ethanol production using *Kluyveromyces marxianus*: On the search for plug-in strains for the Brazilian sugarcane-based biorefinery. **Biomass and Bioenergy**, 2018. v. 119, n. September, p. 217– 228. Available in: <https://doi.org/10.1016/j.biombioe.2018.09.010>.

MEHMOOD, N. et al. *Kluyveromyces marxianus*, an attractive yeast for ethanolic fermentation in the presence of imidazolium ionic liquids. **International Journal of Molecular Sciences**, 16 mar. 2018. v. 19, n. 3, p. 887. Available in: <http://www.mdpi.com/1422-0067/19/3/887>. Acesso em: 19 set. 2018.

MERICO, A. et al. Fermentative lifestyle in yeasts belonging to the *Saccharomyces* complex. **FEBS Journal**, fev. 2007. v. 274, n. 4, p. 976–989.

MOREIRA, C.S. et al. Análise dos parâmetros morfofisiológicos de linhagens de leveduras industriais com potencial biotecnológico para a produção de etanol. 2015. p. 55–63. Available in: <http://dx.doi.org/105902/2179460X18107>.

NONKLANG, S. et al. High-temperature ethanol fermentation and transformation with linear DNA in the thermotolerant yeast *Kluyveromyces marxianus* DMKU3-1042. **Applied and Environmental Microbiology**, 15 dez. 2008. v. 74, n. 24, p. 7514–7521. Available in: <http://www.ncbi.nlm.nih.gov/pubmed/18931291>. Acesso em: 21 set. 2018.

OHLMEIER, S. et al. The yeast mitochondrial proteome, a study of fermentative and respiratory growth. **Journal of Biological Chemistry**, 6 fev. 2004. v. 279, n. 6, p. 3956–3979.

OLIVEIRA, E.S. et al. Fermentation characteristics as criteria for selection of cachaça yeast. **World Journal of Microbiology and Biotechnology 2004 20:1**, fev. 2004. v. 20, n. 1, p. 19–24. Available in: <https://link.springer.com/article/ 10.1023/B:WIBI.0000013286.30695.4e>. Accessed at: 29 jun. 2022.

PAULINO DE SOUZA, J. et al. Improvement of Brazilian bioethanol production – Challenges and perspectives on the identification and genetic modification of new strains of Saccharomyces cerevisiae yeasts isolated during ethanol process. **Fungal Biology**, 2018. v. 122, n. 6.

POSAS, F. et al. The transcriptional response of yeast to saline stress. **Journal of Biological Chemistry**, 2000. v. 275, n. 23, p. 17249–17255.

RADECKA, D. et al. Looking beyond Saccharomyces: The potential of non- conventional yeast species for desirable traits in bioethanol fermentation. **FEMS Yeast Research**, 2015. v. 15, n. 6, p. fov053. Available in: <https://academic.oup.com/femsyr/article-lookup/doi/10.1093/femsyr/fov053>. Accessed at: 19 set. 2018.

RAGHAVENDRAN, V. et al. A simple scaled down system to mimic the industrial production of first-generation fuel ethanol in Brazil. **Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology**, 2017. v. 110, n. 7, p. 971– 983.

RITALA, A. et al. Single cell protein-state-of-the-art, industrial landscape and patents 2001-2016. **Frontiers in Microbiology**, 2017. v. 8, n. OCT.

RIYANTI, E.I. et al. Potential use of *Saccharomyces cerevisiae* TKPK 10.5.1 isolated from several sources for ethanol production using various sugar sources. **AIP Conference Proceedings**, 2020. v. 2260.

ROBERTS, T.M.; KALTENBACH, H.M.; RUDOLF, F. Development and optimization of a defined high cell density yeast medium. **Yeast**, 2020. v. 37, n. 5–6, p. 336–347.

ROCHA, S. N.; ABRAHÃO-NETO, J.; GOMBERT, A. K. Physiological diversity within the *Kluyveromyces marxianus* species. **Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology**, 2011. v. 100, n. 4, p. 619–630.

SAINI, P. et al. Response and tolerance of yeast to changing environmental stress during ethanol fermentation. **Process Biochemistry**, 2018. v. 72, n. July, p. 1–12.

SAINI, P.; BENIWAL, A.; VIJ, S. Physiological response of *Kluyveromyces marxianus* during oxidative and osmotic stress. **Process Biochemistry**, 1 maio. 2017. v. 56, p. 21–29.

SILVEIRA, F.A. DA et al. Assessment of ethanol tolerance of *Kluyveromyces marxianus* CCT 7735 selected by adaptive laboratory evolution. **Applied Microbiology and Biotechnology**, 2020. v. 104, n. 17, p. 7483–7494. Available in: <https://doi.org/10.1007/s00253-020-10768-9>. Accessed at: 4 jul. 2022.

SINGH, D.; NIGAM, P.; BANAT, I. M. Ethanol production at elevated temperatures and alcohol concentrations: Part II–Use of *Kluyveromyces marxianus* IMB3. **World journal of microbiology & biotechnology**, 1998. v. 14, n. 6, p. 823–834. Available in: <http://link.springer.com/10.1023/A:1008852424846>. Accessed at: 21 set. 2018.

STROPPA, C.T. et al. Parâmetros cinéticos de linhagens de levedura isoladas de alambiques mineiros. **Ciência e Agrotecnologia**, 2009. v. 33, n. spe, p. 1978–1983.

TRAINOTTI, N.; STAMBUK, B.U. NaCl stress inhibits maltose fermentation by Saccharomyces cerevisiae. **Biotechnology Letters**, 2001. v. 23, n. 20, p. 1703–1707.

ULLAH, A. et al. Quantitative analysis of the modes of growth inhibition by weak organic acids in *Saccharomyces cerevisiae*. **Applied and Environmental Microbiology**, 2012. v. 78, n. 23, p. 8377–8387.

WALKER, G.M.; BASSO, T.O. Mitigating stress in industrial yeasts. **Fungal Biology**, 2020. v. 124, n. 5, p. 387–397. Available in: <https://doi.org/10.1016/j.funbio.2019.10.010>.

WANG, L. et al. Bacterial growth, detachment and cell size control on polyethylene terephthalate surfaces. **Scientific Reports 2015 5:1**, 14 out. 2015. v. 5, n. 1, p. 1–11. Available in: <https://www.nature.com/articles/srep15159>. Accessed at: 15 jun. 2022

3. CHAPTER 2. PARALLELIZED BIOMASS MONITORING OF TWO DISTINCT *Kluyveromyces marxianus* STRAINS IN SHAKE FLASK CULTIVATION

Abstract

Kluyveromyces marxianus, a non-conventional yeast, carries traits deemed suitable for industrial applications, such as ethanol production, exhibiting advantages over *Saccharomyces cerevisiae* in terms of growth rate and thermotolerance. Non-invasive parallel monitoring of biomass in shake flask cultures allows for efficient microorganism characterization, providing much-needed and accurate data on these strains through continuous sampling. Therefore, this study aimed to assess the behavior of two *K. marxianus* strains during continuous shake flask cultivation. Strain IZ 1339 exhibited a constant, however, slower growth pattern when compared to strain FT 146L, which grew constantly up until the 12 h, after that the strain presented flocculation, affecting the quality of the readings. Strain IZ 1339 also had a higher OD_{max} value when compared to FT 146L, nevertheless, their growth rate was similar, showing that both strains had a satisfactory performance in both concentrations of molasses. Non-invasive monitoring makes it possible to accompany the growth pattern of the strains, indicating that both *K. marxianus* strains perform well when grown in a sugarcane molasses medium. This feature makes these *K. marxianus* strains an interesting non-conventional alternative to *S. cerevisiae* when it comes to industrial application.

Keywords: Cell growth, shake flask, cell growth quantifier, *Kluyveromyces marxianus*

3.1 Introduction

Kluyveromyces marxianus is a homotallic, hemiascomycetous yeast observed to have potential and many beneficial traits for industrial applications (KARIM; GERLIANI; AÏDER, 2020), such as bioethanol production from both sugarcane and cheese whey, protein derived from biomass, enzyme production such as inulinase and β-galactosidase, pharmaceutical compounds (LANE; MORRISSEY, 2010), aromatic compounds and food-grade proteins, due to its Qualified Presumption of Safely (QPS) and GRAS status in European Union and United States, respectively (KARIM; GERLIANI; AÏDER, 2020).

Some of the traits that makes this yeast a promising candidate for biotechnological application is thermotolerance, high growth rates, and broad range of substrates (FONSECA et al., 2008). *K. marxianus*, like *S. cerevisiae*, is a respiro-fermentative yeast. Although *K. marxianus* is generally classified as Crabtree negative, it does carry the genes necessary for ethanol productions, and will veer towards the fermentative lifestyle under certain conditions, questioning the Crabtree status of this species (LANE; MORRISSEY, 2010).

This diversity in measurements in not primarily based on manual error, but the physiological differences of strains used in different studies. Strain preservation, origin, manipulation from stock to growth medium, all play a major role in the physiological diversity of this yeast known to present high levels of intraspecific polymorphism (BELLOCH et al., 1998; FONSECA et al., 2007).

This divergence is also explained by the intraspecific variations and the fact the most studies utilize one single strain as the representative of the species. It can be concluded that *K. marxianus* is capable of carrying out simultaneous fermentation and respiration, and the shift between these pathways is strain specific (LANE; MORRISSEY, 2010).

This metabolic shift responsible for the Crabtree effect results from multiple related factors, and these may not express themselves equally for all strains, creating a spectrum between Crabtree negative and Crabtree positive, which explain why some, but not all *K. marxianus* strains are effective ethanol producers (HONG et al., 2007; LANE; MORRISSEY, 2010; NONKLANG et al., 2008).

As for *K. marxianus*, there are conflicting data regarding maximum specific growth rate, particularly due to differences in experimental conditions and the intraspecific variation displayed by this species (KARIM; GERLIANI; AÏDER, 2020). The untapped biotechnological potential of *K. marxianus* serves as guide for future developments, such as genetics, evolutionary engineering and other physiological and molecular tools for *K. marxianus* (KARIM; GERLIANI; AÏDER, 2020).

However, in order to better explore the biotechnological potential of a yeast strain, it is essential to understands its metabolism and response to growth medium and other factors, such as temperature, pH, sugar consumption and biomass concentration, even more so in the case of production of compounds whose titer are linked to biomass production (FONSECA et al., 2007).

Monitoring the growth of cultures in shake flasks has been traditionally carried out by manual sampling and offline biomass analysis, however, this process is insufficient for modern bioprocess monitoring, due to low data density, invasive sampling and lack of parallelization. Non-invasive parallelized biomass monitoring of cultures in shake flask under agitation allows the characterization of microorganisms in a precise and efficient way, providing high data density and accuracy (BRUDER et al., 2016).

In order to characterize the growth profile of two *K. marxianus* strains, this study evaluated growth in shake flasks under continuous agitation through online, automated biomass monitoring system, aiming to better understand the differences in metabolism of two strains cultivated under the same conditions.

3.2 Material and Methods

3.2.1 Microorganisms and substrate

Two *Kluyveromyces marxianus* strains were utilized: strain IZ 1339 (native strain isolated from Drosophila) (GOMES et al., 2003; LEAL et al., 2008), kindly provided by Prof. Dr. Luiz Humberto Gomes (ESALQ/USP), and strain FT 146L (isolated from ethanol production), kindly provided by Fermentec Ltda (Piracicaba, SP, Brazil).

Strains were inoculated on Petri dishes containing YPDA medium (10 $g.L^{-1}$ yeast extract; 10 $g.L^{-1}$ peptone; 20 $g.L^{-1}$ glucose; 18 $g.L^{-1}$ agar), and, subsequently transferred to cryotubes containing skim milk as a cryoprotectant for maintenance at -80°C.

Sugarcane molasses, a by-product of the sugar industry, utilized in this study was provided by Sugar and Ethanol Industries from the region of Piracicaba, São Paulo, Brazil. The molasses was diluted to the desired concentrations and sterilized at 121º C, 1 atm, during 15 min. Aliquots were stored at -20°C.

3.2.2 Study of growth profile of *K. marxianus*

Cultivations were carried out utilizing sterile sugarcane molasses (SCM), diluted to 8 and 15 ºBrix (M8 and M15, respectively). Both strains were previously cultivated in YPD medium, and the cell suspension was adjusted to $O.D._{600}$ 1,6. Subsequently, 1 mL of the cell suspension was inoculated in 50 mL of M8 and M15 in an Erlenmeyer flask (250 mL).

Biomass growth was monitored online and non-invasively by the CGQ dispositive (“Cell Growth Quantifier”, Aquila Biolabs), readings were performed at approximately every 4 sec. The experiments were carried out in duplicates, at 30°C during 24 h. Both yeast strains were also cultivated in YPD medium, which was utilized as reference.

The CGQ (Cell Growth Quantifier) method has the advantage of high data density and non-invasive sampling, thus, eliminating possible manual errors, sampling biases, sedimentation and equipment calibration. For both strains, graphs were obtained, detailing backscatter and maximum growth rate (h^{-1}).

The measurement of cell density by backscattering takes place through light radiated by an LED located at the base of the equipment, which interacts with the cells and is then reflected back by a photodiode, which converts the light into an electrical signal. This method allows the reading of higher cell densities, in the range of 0.1 to 150 $O.D._{600}$, without the need for any dilution (BRUDER et al., 2016).

3.2.3 Parameters analysis

After the 24 h period of growth, the samples cultivated in M8 and M15 were centrifuged at 2046 g for 3 min (NT-815, Novatecnica), and the supernatant was collected

for analysis. Parameters were determined at 0 and 24 h of cultivation.

The pH was determined through a digital pH meter (LUCA-210, Lucadema). Total Acidity (acetic acid g/L) was determined by the titratable total acidity method (BRASIL, 1986).

Residual sugars were determined through DNS method (MILLER, 1959) in order to determine sugar consumption.

3.3 Results and Discussion

In order to evaluate the biomass data and strain-specific characteristics, strain IZ 1339 and FT 146L were grown on diluted sugarcane molasses (M8 and M15).

The growth profile of the strains (Figure 1) shows that strain IZ 1339 exhibited a distinct growth pattern in the Reference (cultivated in YPD medium). Adaptation took around 6.5 h, followed by the initial rapid growth phase, which then shift to a much slower growth. This behavior is similar to that observed by Bruder et al. (2016) in *Saccharomyces cerevisiae*, where the authors attribute this growth patter to the positive Crabtree-effect, the rapid growth phase is associated with ethanol formation, followed by the typical metabolic shift to respiratory ethanol metabolization. Similar behavior can be observed for the Reference in Figure 1, for both repetitions, in strains cultivated in glucose (superior left and right).

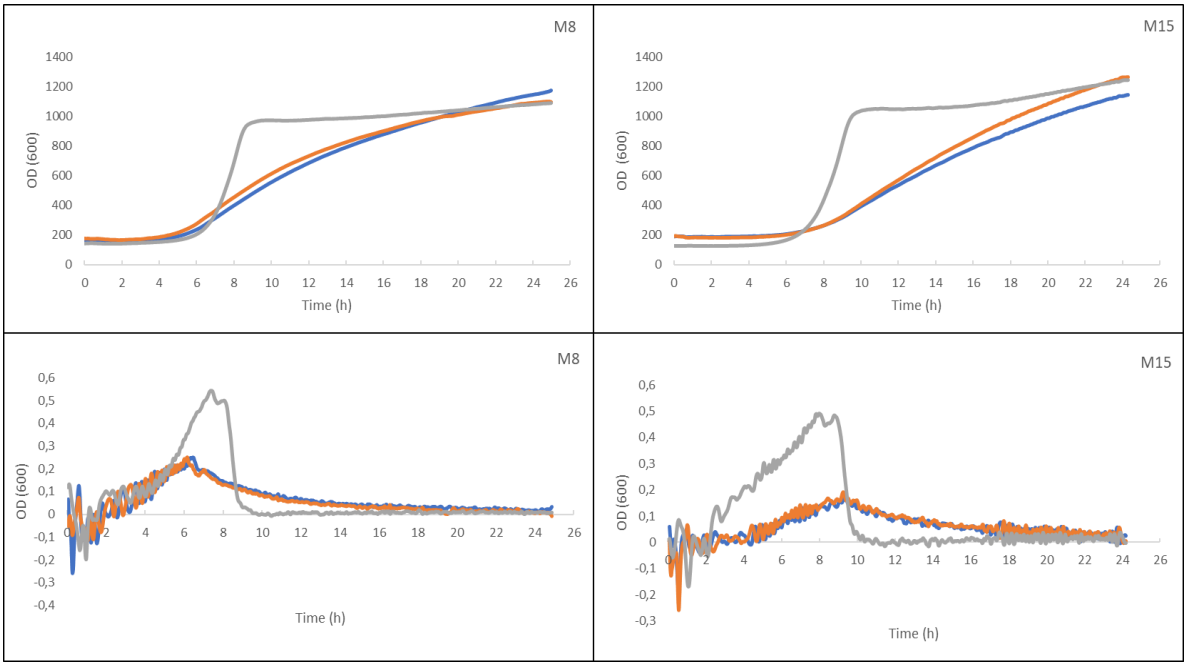

Figure 1 – Growth of strain IZ 1339 in sterile molasses 8º Brix (M8) (left), 15° Brix (M15) (right) and YPD 2% (Reference), at 30°C during 24 h. Experiments performed in duplicates. Sup. Growth rate by backscattering, Inf. Maximum specific growth rate.

As for the growth in molasses, there was not an evident rapid growth phase. The adaptation period was similar to the Reference, around 6 to 7 h, followed by a slower growth curve. In terms of cell density as determined by backscattering, both molasses concentrations (M8 and M15) and the Reference were fairly similar.

Maximum specific growth rate for strain IZ 1339 in M8 (Figure 1, bottom row) presented a peak, related to the maximum growth rate recorded, around 6 h of cultivation (0.24 h^{-1}). The Reference displayed a higher growth rate, probably due to the exponential growth phase (0.54 h^{-1}). Fonseca et al. (2013) observed growth rates of 0.39 and 0.49 h^{-1} utilizing 10g/L of supplemented carbon source.

When grown in M15, strain IZ 1339 also presented smaller growth rates when compared to growth in M8, reaching higher values around 9 h of cultivation at 0.17 h^{-1}, as opposed to 0.49 h^{-1} observed in the Reference (Figure 1, left).

As for strain FT 146L, growth in the Reference medium (2% glucose) presented a similar pattern to strain IZ 1339, with a rapid growth phase followed by a slower growth, much as described by Bruder et al. (2016). The one notable difference for strain FT 146L was that M15 yielded a higher biomass concentration, over 1200 (Figure 2, top row).

Growth in M8 presented a constant growth curve up until 15 h, starting to decline shortly after, unlike the Reference, which remained stable. The maximum growth rate graph (Figure 2, bottom row) showed abnormal peaks at the beginning of cultivation, after 18 h. This probably occurred because the strain presented flocculation, which makes it difficult to accurately read the cell density.

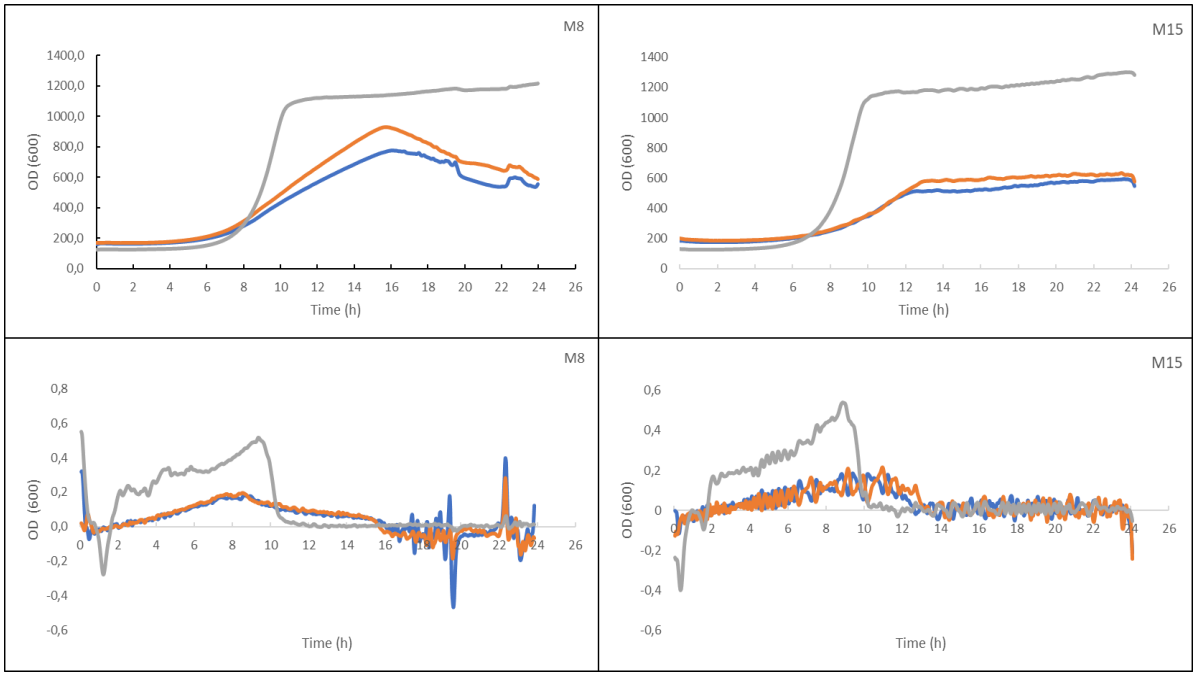

Figure 2 - Growth of strain FT 146L in sterile molasses 8º Brix (M8) (left), 15° Brix (M15) (right) and YPD 2% (Reference), at 30°C during 24 h. Experiments performed in duplicates. Sup. Growth rate by backscattering, Inf. Maximum specific growth rate.

For industrial applications, such as the production of enzymes, flocculation is a desirable trait in *K. marxianus*, as a means to obtain higher cell density, therefore increasing productivity in bioreactor operations. Flocculation is a mechanism that occurs in some yeast strains as a result of non-sexual aggregation of single cells into a multicellular mass, which then sediments at the bottom of the medium. The mechanism behind flocculation is correlated by cell wall proteins (ALMEIDA et al., 2003; VERSTREPEN et al., 2003).

Growth in M15 presented a notably small growth rate and cell concentration when compared to M8 and the Reference, even though the growth curve was more stable throughout the 24 h of cultivation. Cell density for both media was smaller than strain IZ 1339 in both concentrations assayed.

Growth rates for M15 also presented abnormal peaks in the reading, due to the flocculent behavior, even though the peaks have a more uniform pattern, which indicates a more constant maximum growth rate throughout the 24 h period, averaging 0.18 h^{-1}. It is worth noting that the Reference was grown in YPD, a complex medium that provides all nutrients necessary for yeast growth, the sugar, vitamins, minerals and amino acids present in the medium act as carbon and nitrogen sources.

The *K. marxianus* strains were also cultivated on molasses, a raw byproduct of the production of sugar and ethanol, consisting of 75–85% total solids, 30–36% sucrose, 10–17% fructose + glucose, 10–16% ash, and minor varying compositions of oligosaccharides, polysaccharides, organic acids, proteins, and nitrogen compounds (CARIOCA; LEAL, 2019). Therefore, there are notable differences in the composition, and mainly, available sugars to stimulate growth.

It is worth noting that while the Reference was grown in YPD medium containing glucose, the duplicates for M8 and M15 had to hydrolyze the sucrose present in the medium, which would explain the slower growth curves when compared to the Reference.

Overall, strain IZ 1339 presented a more constant growth pattern, and higher growth rate/biomass concentration compared to strain FT 146L. Table 1 shows the average maximum specific growth rate for each strain and media concentration, as well as the Reference.

Table 1 – Average values of maximum specific growth rates μ_{max} (h^{-1}) presented by strains IZ 1339 and FT 146L in M8, M15 and reference YPD 2%

Medium	Strain	
	IZ 1339	**FT 146L**
YPD 2%	0.54	0.54
M8	0.25	0.18
M15	0.18	0.19

M8 – sterile molasses 80 g/L; M15 – sterile molasses150 g/L; study was conducted in duplicates

From the average growth rate (h-1) values, it is possible to infer that the Reference provided better conditions for both strains to grow, as for the molasses in both concentrations; there were not significant variations in growth rate values. Strain IZ 1339 had a higher biomass concentration and average growth rate for M8, however, strain FT 146L had higher average growth rate for M15, despite having a lower concentration of biomass.

From a biotechnological standpoint, strain IZ 1339 seems to be more adapted for biomass production in this particular condition, while strain FT 146L grows at a faster rate, adapting more easily to the growth medium.

Maximum specific growth rate (μ_{max} h^{-1}) of 0.56 was obtained during batch cultivations by Fonseca et al. (2007), utilizing glucose as the sole carbon source at 10 g/L, in a complex mineral medium, supplemented for growth optimization. However, there are sparse and conflicting data regarding maximum specific growth rate for *K. marxianus*, due to the intraspecific variation and the distinct conditions assayed (KARIM; GERLIANI; AÏDER, 2020). Fonseca et al. (2007) highlights the diversity of measurements are not based on measurement errors, but the physiological differences of strains used in different studies. It is possible to speculate that strain preservation, origin, and manipulation play a major role in this physiological diversity. *K. marxianus* in known to present high levels of intraspecific polymorphism, and may be prone to high mutation rates that result in rapid and unexpected evolution during the propagation process (BELLOCH et al., 1998).

3.3.1 Experimental variables

After 24 h of cultivation, the supernatant was obtained by centrifugation. The parameters of the supernatant were evaluated for pH, total titrated acidity and residual sugar concentration (Table 2).

Table 2 – Post-cultivation parameters analysis values for strains IZ 1339 and FT 146L in both media

Samples	T (h)	RS (g/L)	TRS (g/L)	Consumed sugar (%)	Acidity (g/L)	pH
M8	0	7.33	80.62	*	0,62	5.61
IZ1339 M8	24	24.35	46.70	42.1	3.62	3.86
FT146L M8	24	18.32	39.81	50.6	2.25	4.19
M15	0	14.95	135.77	*	1.17	5.52
IZ1339 M15	24	89.98	74.12	45.4	4.27	4.20
FT146L M15	12	13.65	82.44	39.3	2.49	4.92
FT146L M15	24	111.35	132.23	2.61	3.03	4.03

M8 – sterile molasses 80 g/L; M15 – sterile molasses150 g/L; Acidity = concentration of acetic acid (g/L); T = time of sampling; RS = reducing sugars; TRS = total reducing sugars

It is possible to observe that neither of the strain was able to consume all of the sugar content in the medium, whether to produce biomass or, likely, to produce ethanol. Strain FT 146L was able to consume half of the sugar present in M8, while strain IZ 1339 consumed 42% (33.9 g/L out of 80 g/L). As for M15, there was even more residual sugars left at the end of the growth period, with strain IZ 1339 consuming 45.4 % (74.1 g/L), as opposed to strain FT 146L, which consumed 39.3 % (53.33 g/L) at the 12 h of cultivation.

On average, both *K. marxianus* strains consume around 45% of total sugars present in the growth medium. It is worth noting that strain FT 146L presented flocculation midway through the cultivation period, around the 12h mark, which is why the readings around 24h are not as accurate. Because growth in CGQ cannot be interrupted for external sampling, an experiment was done again in order to sample total soluble sugars and other parameters describe in Table 2.

The behavior herein observed for strain FT 146L could be triggered due to fructose or the total sugars inhibiting growth and causing flocculation. This could also occur due to this strain being suffering mutations throughout the generations, causing unstable behavior (KARIM; GERLIANI; AÏDER, 2020; LANE; MORRISSEY, 2010).

Korkoutas et al. (2002) produced wine utilizing *K. marxianus* strain IMB3 and noticed that, while the final product had good quality and reached the desired ethanol concentration, there was relatively high content of residual sugars, presumably due to a combination of cell density and temperature, which require further exploration. Plessas et al. (2008) utilized *K. marxianus* strain IFO 288 to produce lactic acid from cheese whey, with initial sugar concentration of 36 g/L, and observed 0.4 g/L of residual sugars

concentration of 36 g/L, and observed 0.4 g/L of residual sugars after the fermentation. The outcome of sugar consumption can vary depending on the employed conditions, which is why it is essential to understand a particular strain behavior and metabolism.

As for total acidity, no condition demonstrated a significant increase, being the highest concentration M15 for strain IZ 1339, at 4.27 g/L, a regular byproduct of fermentation. Acetic acid production under fermentative conditions is linked to glycerol formation via redox balancing (EGLINTON et al., 2002), also, aeration and sugar content are also responsible for the increase of organic acids during fermentation, such as acetic acid, produced by yeast metabolic activity (LEE et al., 1999).

3.4 Conclusion

Characterizing a strain via growth-based methods provides essential data to understand sugar consumption and biomass production. The CGQ method for online biomass monitoring proved to be a valuable tool regarding growth rates and biomass data with high resolution and non-invasive sampling. It was possible to infer that both *K. marxianus* strains had distinct behavior and diverging growth patterns when cultivated under the same conditions.

References

ALMEIDA, C. et al. Acquisition of flocculation phenotype by *Kluyveromyces marxianus* when overexpressing GAP1 gene encoding an isoform of glyceraldehyde-3- phosphate dehydrogenase. **Journal of Microbiological Methods**, v. 55, n. 2, p. 433–440. 2003.

BELLOCH, C. et al. Inter- and intraspecific chromosome pattern variation in the yeast genus Kluyveromyces. **Yeast**, v. 14, n. 15, p. 1341–1354. 1998.

BRASIL. Portaria nº 76 de 26 de novembro de 1986. Dispõe sobre os métodos analíticos de bebidas e vinagre. **Diário Oficial da União**, 1986. p. Brasília.

BRUDER, S. et al. Parallelized online biomass monitoring in shake flasks enables efficient strain and carbon source dependent growth characterization of *Saccharomyces cerevisiae*. **Microbial Cell Factories**, v. 15, n. 1, p. 1–14. 2016.

CARIOCA, J.O.B.; LEAL, M.R.L.V. Ethanol production from sugar-based feedstocks. **Comprehensive Biotechnology**. [S.l.]: Elsevier, 2019, p. 24–34.

EGLINTON, J.M. et al. Decreasing acetic acid accumulation by a glycerol overproducing strain of *Saccharomyces cerevisiae* by deletion the ALD6 aldehyde dehydrogenase gene. **Yeast**, v. 19, n. 4, p. 295–301. 2002

FONSECA, G.G. et al. Physiology of the yeast *Kluyveromyces marxianus* during batch and chemostat cultures with glucose as the sole carbon source. **FEMS Yeast Research**, v. 7, n. 3, p. 422–435. 2007. Available in: <http://www.ncbi.nlm.nih.gov/pubmed/17233766>. Accessed at: 21 set. 2018.

FONSECA, G. G. et al. The yeast *Kluyveromyces marxianus* and its biotechnological potential. **Applied Microbiology and Biotechnology,** v. 79, n. 3, p. 339-354. 2008. Doi: 10.1007/s00253-008-1458-6. Available in: <http://www.ncbi.nlm.nih.gov/ pubmed/18427804>. Accessed at: 21 set. 2018.

FONSECA, G.G.; BARBOSA DE CARVALHO, N.M.; GOMBERT, A.K. Growth of the yeast *Kluyveromyces marxianus* CBS 6556 on different sugar combinations as sole carbon and energy source. **Applied Microbiology and Biotechnology**, v. 97, n. 11, p. 5055–5067. 2013. Available in: <http://www.ncbi.nlm.nih.gov/pubmed/23435899>. Accessed at: 21 set. 2018.

GOMES, L.H. et al. Presence of the yeast *Candida tropicalis* in figs infected by the fruit fly *Zaprionus indianus* (dip.: Drosophilidae). **Brazilian Journal of Microbiology.** 2003. v. 34, p.5-7. Available in: https://doi.org/10.1590/S1517-83822003000100002. Accessed at: 10 dez. 2022.

HONG, J. et al. Construction of thermotolerant yeast expressing thermostable cellulase genes. **Journal of Biotechnology**, v. 130, n. 2, p. 114–123. 2007

KARIM, A.; GERLIANI, N.; AÏDER, M. *Kluyveromyces marxianus*: An emerging yeast cell factory for applications in food and biotechnology. **International Journal of Food Microbiology**, v. 333, n. May, p. 108818. 2020. Available in: <https://doi.org/10.1016/ j.ijfoodmicro.2020.108818>.

LANE, M.M.; MORRISSEY, J.P. *Kluyveromyces marxianus*: A yeast emerging from its sister's shadow. **Fungal Biology Reviews**, v. 24, n. 1–2, p. 17–26. 2010. Available in: <https://www.sciencedirect.com/science/article/abs/pii/S1749461310000035>. Accessed at: 1º ago. 2019.

LEAL, G.A. et al. Fermentation of cacao (*Theobroma cacao* L.) seeds with a hybrid *Kluyveromyces marxianus* strain improved product quality attributes. **FEMS Yeast Research**, v. 8, n. 5, p. 788–798. 2008.

LEE, P.C. et al. Succinic acid production by *Anaerobiospirillum succiniciproducens*: Effects of the H_2/CO_2 supply and glucose concentration. **Enzyme and Microbial Technology**, v. 24, n. 8–9, p. 549–554. 1999.

NONKLANG, S. et al. High-temperature ethanol fermentation and transformation with linear DNA in the thermotolerant yeast *Kluyveromyces marxianus* DMKU3-

1042. **Applied and Environmental Microbiology**, v. 74, n. 24, p. 7514–7521. 2008. Available in: <http://www.ncbi.nlm.nih.gov/pubmed/18931291>. Accessed at: 21 set. 2018.

VERSTREPEN, K.J. et al. Yeast flocculation: What brewers should know. **Applied Microbiology and Biotechnology,** v. 61, n. 3, p. 197-205. 2003. Available in: < https://link.springer.com/article/10.1007/s00253-002-1200-8>

ZAGO, E.A. et al. **Métodos analíticos para o controle da produção de álcool e açúcar**. Piracicaba: Fealq, 1996.

4. CHAPTER 3. ETHANOL PRODUCTION FROM SUGARCANE JUICE AND MOLASSES BY *Kluyveromyces marxianus* STRAINS

Abstract

Taking into consideration the need for biotechnological advances in biofuel production, it is important to explore other processes other than the consolidated 1G ethanol produced by *Saccharomyces cerevisiae*. In this scenario, non-conventional yeasts are an interesting alternative. Amongst them, *Kluyveromyces marxianus* stands out as a candidate for application, due to its particular traits, such as thermotolerance, ability to consume a broad range of substrates and fast growth rate. Fermentations were carried out firstly on shake flask scale with cell recycling, followed by fermentation in a bioreactor. Variables such as sugar consumption, pH, total acidity, ethanol production, yield and productivity were determined in order to understand the outcome of the fermentative assays. The substrate consisted of a mix of sugarcane juice and diluted molasses, with a final sugar concentration of 222.2 g/L. For the shake flask scale fermentations, strain IZ 1339 produced 17.3 g/L of ethanol, with a yield of 16.93% and productivity of 0.58 g/L.h, while strain FT 146L produced 3.9 g/L of ethanol, with a yield of 3.82% and productivity of 0.13 g/L.h. For the bioreactor fermentations, strain IZ 1339 produced 63.01 g/L of ethanol, with a yield of 63.01% and productivity of 1.34 g/L.h, while strain FT 146L produced 30.7 g/L of ethanol, with a yield of 30.04% and productivity of 0.85 g/L.h.

Keywords: Fermentation, ethanol production, *Kluyveromyces marxianus*, bioreactor

4.1 Introduction

Saccharomyces cerevisiae yeast has been utilized for millennia in the production of food and beverages, and is currently the microorganism more utilized in the production of first-generation fuel ethanol from sugarcane or starchy raw materials, and it is, undoubtedly, the most studied yeast species (RADECKA et al., 2015).

Due to diverse factors, *S. cerevisiae* became a model organism for taxonomic and genetic research for eukaryotic cells, being the first eukaryotic microorganism whose genome was completely sequenced (GOFFEAU et al., 1996).

Millennia of evolution and coexistence with the human routine made *S. cerevisiae* capable of growth under aerobic and anaerobic conditions, tolerating high concentrations of ethanol, however, more recent industrial applications, such as the production of bioethanol, pose challenges for the metabolism of this yeast, with the introduction of new cytotoxic stressors and inhibitors, such as ethanolic, osmotic and thermal stress (RADECKA et al., 2015).

There is a motivation to study the biotechnological applications of unconventional yeasts, that is, non-*Saccharomyces* yeasts. These yeasts have a great potential for

superior tolerance to stress and inhibitors. Such yeasts evolved independently from *S. cerevisiae* and, therefore possess unique defense mechanisms that are not present in this yeast, taking into account that the need for innovation in ethanol production has pushed *S. cerevisiae* to its performance limit (SOUCIET et al., 2009; TAYLOR et al., 2012).

In this scenario, the yeast *Kluyveromyces marxianus* stands out. This species has many attractive traits to be employed in food and biotechnology, such as thermotolerance, being able to grow in temperatures up to 52 ºC, the ability to assimilate a broad range of substrates, such as glucose, lactose, galactose, xylose, inulin, and arabinose, tolerance to a wide range of pH (2.5-9), production of ethanol in elevated temperatures, production of added-value compounds such as aromatic chemicals (2-phenylethylethanol and 2-phenylethyl acetate, among others), secretion of enzymes of industrial interest (HA-TRAN; NGUYEN; HUANG, 2020; KARIM; GERLIANI; AÏDER, 2020; LANE; MORRISSEY, 2010).

Ethanol production by *K. marxianus* strains has been explored in a broad range of substrates and temperatures, whether as a sole inoculum or in a co-culture system (ARORA et al., 2015; HA-TRAN; NGUYEN; HUANG, 2020; RADECKA et al., 2015), with strains proving to be promising alternatives for bioethanol production, among other food and environmental applications. However, studies on substrate consumption and process optimization are still needed in order to better understand this yeast and its behavior during ethanol production. The present study aimed to evaluate fermentation of two *K. marxianus* strains isolated from distinct backgrounds in bench-scale and bioreactor, as well as the parameters obtained post-fermentation.

4.2 Material and Methods

4.2.1 Microorganisms

Two *Kluyveromyces marxianus* strains were utilized: strain IZ 1339 (native strain isolated from the gut microbiome of Drosophila) (GOMES et al., 2003; LEAL et al., 2008), kindly provided by Prof. Dr. Luiz Humberto Gomes (ESALQ/USP), and strain FT 146L (isolated from ethanol production), kindly provided by Fermentec Ltda (Piracicaba, SP, Brazil).

Strains were inoculated on Petri dishes containing YPDA medium (10 $g.L^{-1}$ yeast extract; 10 $g.L^{-1}$ peptone; 20 $g.L^{-1}$ glucose; 18 $g.L^{-1}$ agar), and, subsequently transferred to cryotubes containing skim milk as a cryoprotectant for maintenance at -80°C.

4.2.2 Fermentation medium

Sugarcane juice (75%) plus molasses (25%), diluted to 20ºBrix (initial sugar concentration 200 g/L) was utilized as fermentation medium. Molasses was diluted to the desired concentration and sterilized at 121º C, 1 atm, during 15 min. Sugarcane juice was pasteurized in process adapted from Kunitake et al. (2014), consisting of constant heating to a temperature of 85 °C, followed by removal after 30 s, and cooling to 8 °C.

4.2.3 Shake flask fermentation

Shake flask scale fermentation assays were conducted in Erlenmeyer flasks (250 mL), with working volume of 100 mL. Inoculum concentration was 10^8 to 10^9 cells/mL, which is equivalent to 0,9 a 1,0 OD_{600} (SAINI; BENIWAL; VIJ, 2017).

Fermentation was carried out at 30ºC for 30 h, in duplicates. Each fermentation consisted of three cycles, using inoculum recycling.

Inoculum was previously cultivated in YPD medium at 30 ºC for 15 h, under constant agitation. Subsequently, cells were centrifuged, washed and inoculated in fermentation medium 8 ºBrix (sugarcane juice and molasses) during 4 h. After the period of adaptation, cells were once again centrifuged, washed and resuspended in 5 mL of saline solution 0.9 %, followed by OD_{600} adjustment to the desired concentration. An aliquot of 2 mL was then transferred to the Erlenmeyer flask containing 20 ºBrix medium.

4.2.4 Bioreactor fermentation

Bioreactor fermentation assays were conducted in a Minifors 2 bioreactor (Infors HT) with working volume of 2L, utilizing a closed system batch fermentation. Fermentation was carried out at 30ºC for 48 h for strain IZ 1339 and for 36 h for strain FT 146L. Time discrepancy was due to the bioreactor operator's availability.

Inoculum was prepared as described in the bench-scale fermentation, and subsequently transferred to the bioreactor. Two fermentations were carried out, one for each strain of *K. marxianus*. Samples were taken at T (h) 0; 6; 12; 24; 30; 36, totaling 6 samples (7 samples for strain IZ 1339). The use of the bioreactor allowed the execution of a fermentation cycle with greater control of the defined parameters, such as temperature, aeration and sample removal without opening the fermenter flask, thus avoiding possible contamination.

4.2.5 Analysis experimental variables

Shake flask fermentation variables:

For the shake flask scale fermentations, samples were collected at the end of each cycle, at 30 h. The pH was determined through a digital pH meter (LUCA-210, Lucadema). Total Acidity (acetic acid g/L) was determined by the titratable total acidity method (BRASIL, 1986). Total sugars were determined through DNS method (MILLER, 1959) in order to evaluate sugar consumption.

CO_2 detachment was measured by weighting the fermentation flasks at the beginning and final time of fermentation (T30 h), after agitation in order to dissipate gas, analyzing the difference in mass. Cell viability was determined by direct counting in a Neubauer chamber, using differential staining of cells by methylene blue solution under microscope.

Ethanol production was determined through distillation of a 25 mL aliquot of the samples on a bench-top distiller (Tecnal). Distilled samples were analyzed in a digital densimeter (DDM2910, Rudolph) in order to determine ethanol concentration. The yield in grams of ethanol per gram of initial total reducing sugar was calculated considering the theoretical yield 0,511 $g_{ethanol}/g_{ART}$ as 100%. The calculation of productivity, in relation to the ethanol produced, expresses the average speed of production.

Bioreactor variables:

As for the samples from the bioreactor fermentation assays, pH and cell viability were determined as described in the item above. The concentration of total soluble sugars dissolved in the medium was measured in ºBrix in a digital refractometer (HI96800, Hanna).

Concentrations of the three sugars (sucrose, glucose and fructose) were combined and reported as total sugar concentration. Total sugar concentration plus glycerol was quantified with Ion Chromatography (930 Compact IC Flex, Metrohm). The detector used was the Amperometric Detector 1 (930 Compact IC Flex 1), with a Metrosep Carb 1 - 150/4.0 column, recording time of 10 min and 1,000 mL/min of monitored flow. The pressure used was 7.04 MPa at 35.0°C. The eluent used in the analysis was composed of NaOH 200mM (2 L). Samples were diluted 500x to fit the standard curve. The wort with a concentration of 20ºBrix used as a reference was diluted 700x.

Organic acids derived from fermentation (acetic, propionic, butyric, lactic, aconitic and succinic) were quantified with Ion Chromatography (930 Compact IC Flex, Metrohm). The detector used was the Conductivity Detector 1 (930 Compact IC Flex 1), with a Metrosep Organic Acids - 250/7.8 column, recording time of 25 min and 0.400 mL/min of monitored flow. The pressure used was 4.73 MPa at 40 °C. The eluent used in the analysis

was composed of 0.5 mM sulfuric acid (56uL) + 15% acetone (300mL). Samples were diluted 10x to fit the standard curve. The wort with a concentration of 20ºBrix used as a control was diluted 700x.

4.2.6 Calculations of fermentative parameters

The fermentation yield calculation was performed according to the methodology of Cruz (2019), considering the theoretical yield of 0.511 g_{etanol}/g_{TS} as 100%, it was determined by the equation:

$$\mathbf{Y_{P/S} = C_{ethanol\ f} / (0{,}511 \times C_{TS\ i}) \times 100}$$

Where:

Y = yield of ethanol formed in relation to the initial TS (%); $C_{ethanol\ f}$ = ethanol concentration (g/L) at the end of fermentation; C_{TSi} = initial total sugar concentration (g/L).

Furthermore, it was possible to calculate the productivity, in relation to the ethanol produced, by the equation:

$$\mathbf{Pr_{ethanol} = C_{ethanol\ f} / t}$$

Where:

$P_{Rethanol}$ = ethanol productivity ($g/L.h^{-1}$);

$C_{ethanol\ f}$ = ethanol concentration (g/L) at the end of fermentation; t = final fermentation time (h).

4.3 Results and Discussion

K. marxianus strain IZ 1339 and FT 146L were submitted to fermentative assays in a sugarcane/molasses medium, in different conditions.

4.3.1 Shake flask scale fermentation

The parameters found in the in the shake flask scale fermentations carried out in three cycles for *K. marxianus* strains IZ 1339 and FT 146L are described in Table 1.

Table 1 - Fermentation parameters from shake flask scale fermentation assays for *K. marxianus* strains IZ 1339 and FT 146L

IZ 1339									
Sample	**pH**	**Viability (%)**	**Total acidity(g/L)**	**CO_2 (g/L)**	**TSS (g/L)**	**Consumed sugar (%)**	**Ethanol (g/L)**	**Yield (%)**	**Productivity (g/L.h)**
T0	5.23	100	*	*	222.2	*	0	*	*
Cycle 1 (1)	5.04	98	3.91	3.9	131.83	41	6.6	6.46	0.22
Cycle 1 (2)	5.05	98	3.91	3.8	155.87	30	6.6	6.46	0.22
Cycle 2 (1)	5.06	96	4.21	2.1	148.40	33	7.1	6.95	0.24
Cycle 2 (2)	5.04	96	10.26	3.0	155.93	30	17.3	16.9	0.58
Cycle 3 (1)	5.15	95	9.60	1.3	155.18	30	16.2	15.8	0.54
Cycle 3 (2)	5.08	95	2.61	3.8	154.43	31	4.4	4.31	0.15

FT 146L									
Sample	**pH**	**Viability (%)**	**Total acidity (g/L)**	**CO_2 (g/L)**	**TSS (g/L)**	**Consumed sugar (%)**	**Ethanol (g/L)**	**Yield (%)**	**Productivity (g/L.h)**
T0	5.22	100	*	*	222.2	*	0	*	*
Cycle 1 (1)	3.60	97	6.88	4.2	76.84	65	1.8	1.76	0.06
Cycle 1 (2)	3.58	97	7.06	3.9	66.29	70	3.9	3.82	0.13
Cycle 2 (1)	5.18	96	1.49	1.5	140.11	37	3.4	3.33	0.11
Cycle 2 (2)	5.16	96	1.41	4.1	166.48	25	5.0	4.89	0.17
Cycle 3 (1)	5.09	96	1.46	0.3	191.34	14	3.9	3.82	0.13
Cycle 3 (2)	5.06	96	1.44	2.5	213.18	4	2.8	2.74	0.09

CO_2 (g) = CO_2 detachment expressed in g/L; Yield (%) = Fermentation yield expressed in percentage; Productivity (g/L.h) = fermentation productivity expressed in g/L.h; TSS = total soluble sugars. Fermentation was carried out at 30ºC for 30 h, in duplicates

The pH in the fermentation by strain IZ 1339 did not present any significant decrease, while strain FT 146L promoted a decrease in pH only in the first cycle, dropping from 5.22 to
3.6. The pH in sugarcane juice usually varies between 5.2 and 6.8 (SOBRINHO; SILVA; CEREDA, 2011). Kamal (2020) describes a correlation of decreased pH and decreased ethanol yield when the value drops below 5.5, also leading to increased residual sugars in the medium, this can be observed in strain FT 146L, which managed to consume only 32.5
% of sugar present in the medium (average value of the duplicates), as opposed to strain IZ 1339, which averaged 64.5 % of sugar consumption in the first cycle. According to Gibson et al. (2007), wort pH can decrease by up to two units during the fermentation process.

Cell viability values (%) were stable for both strains, with no significant drop in viability being observed, meaning that there was no critic stress upon the strains, that managed to survive until the end of the fermentation period.

As for Total Acidity, strain IZ 1339 present higher values among the three fermentative cycles when compared to strain FT 146L, which presented elevated acidity only in the first cycle. According to Zoecklein (1999) a fermentation in must with 28 °Brix can present up to 1.7 g/L of acetic acid. Tonoli (2017) found 1.27 g/L under similar conditions; this tendency was only observed in strain FT 146L.

CO_2 detachment observed at the end of each fermentation cycle was lower than 1 g for all cycles, in both strains. This low value may have relation to the small working volume utilized (100 mL) and the incomplete fermentation. For aerobic metabolism, the sucrose molecule is oxidized in the presence of oxygen, generating carbon dioxide (CO_2) and water, while in anaerobic metabolism, sucrose is oxidized in the absence of O_2, generating ethanol and CO_2 (LEHNINGER, 2002).

Sugar consumption for strain IZ 1339 averaged around 30% of the total sugar concentration (222.2 g/L), this rate of consumption remained constant throughout the three fermentative cycles. Strain FT 146L, on the other hand, had an average consumption of 67.5% on the first fermentative cycle, declining consumption for the following cycles. While strain IZ 1339 consumed less sugar present in the medium as a whole, the stable behavior it present through fermentation can be explored in optimized conditions, such as co-cultures or longer fermentations, since stability is a desirable trait for yeasts (HA-TRAN; NGUYEN; HUANG, 2020). It is possible to speculate that strain FT 146L was more susceptible to mutations along the cycles, causing it to become more unstable and, therefore, consume less available sugars, even though cell viability did not decrease and remained stable, since it is known that *K. marxianus* is prone to polymorphisms (FONSECA et al., 2007; KARIM;

GERLIANI; AÏDER, 2020).

Ethanol production in the conditions applied was not significant in both *K. marxianus* strains. Strain IZ 1339 averaged 9.7 g/L of ethanol in the three fermentative cycles, with a slightly more elevated yield, particularly in cycles 2 and 3. While strain FT 146L averaged 3.5 g/L of ethanol in the same conditions and had a less significant yield, the values remained stable between the three cycles, this can be an interesting trait to be further explored under optimized conditions. Productivity ($g/L.h^{-1}$ of ethanol) did not reach 1 g/h for any of the strains evaluated.

It is important to note that these results are often compared to results obtained by fermentations performed by *Saccharomyces cerevisiae*, where the ethanol obtention process is much more elucidated and the outcome is more predictable. The fermentative process described in this study has not yet been optimized. Ethanol producing units in Brazil often utilize a mix of sugarcane juice and diluted molasses to conduct their fermentations, and said molasses can go through pre-treatment or enrichment, if needed, in order to maximize yield.

4.3.2 Bioreactor fermentation

In order to better comprehend the behavior of the two *K. marxianus* strains during fermentation, they were submitted to fermentative assays in a bioreactor, in a closed batch system with controlled parameters. Table 2 describes the parameters observed in the two fermentation assays carried out in a bioreactor, for *K. marxianus* strains IZ 1339 and FT 146L.

Table 2 - Fermentation parameters from bioreactor fermentation assays for *K. marxianus* strains IZ 1339 and FT 146L

IZ 1339								
T (h)	**pH**	**TSS (°Brix)**	**Viability(%)**	**TS (g/L)**	**Consumed sugar (%)**	**Ethanol (g/L)**	**Yield (%)**	**Productivity (g/L.h)**
0	5.23	20.6	100	226.48	*	0	0	0
6	4.8	19.5	100	217.88	3.8	3.9	3.82	0.65
12	4.55	19	100	191.90	15.3	12.4	12.13	1.03
24	3.92	16.5	97	198.60	12.3	35.2	34.44	1.47
30	3.88	15.4	96	180.66	20.2	49.4	48.34	1.65
36	3.88	13.5	72	157.23	30.6	48.8	47.36	1.36
48	3.87	8.4	70	64.55	71.5	64.4	63.01	1.34

FT 146L								
T (h)	**pH**	**TSS (°Brix)**	**Viability(%)**	**TS (g/L)**	**Consumed sugar (%)**	**Ethanol (g/L)**	**Yield (%)**	**Productivity (g/L.h)**
0	5.15	20.9	100	222.20	*	0	0	0
6	4.9	20.4	100	219.13	1.4	3.4	3.33	0.57
12	4.73	19.9	100	198.61	10.6	8.2	8.02	0.68
24	4.3	17.8	97	163.66	26.3	19.5	19.08	0.81
30	4.19	17.3	95	154.79	30.3	25	24.46	0.83
36	4.17	17	69	160.76	27.6	30.7	30.04	0.85

TSS = total soluble solids dissolved in the medium; TS = total sugar is the sum of glucose, fructose and sucrose; Yield (%) = Fermentation yield expressed in percentage; Productivity (g/L.h) = fermentation productivity expressed in g/L.h

As shown on Table 2, pH decreased slightly for both strains during the fermentative cycle, slightly more so for strain IZ 1339, this decrease in pH agrees with the data described by Gibson et al. (2007), as the pH in the wort is expected to drop one or two units during the fermentation process. The TTS (ºBrix) measurement was taken as a complementary measure to monitor fermentation, as the measurement decreases due to the conversion of sugars into ethanol (WALKER et al., 2021). This gradual decrease can be observed in both fermentations, but more significantly, for strain IZ 1339, TTS reduction for the fermentation in strain FT 146L was more modest. Even so, the values indicate the presence of residual sugars at the end of the fermentation process.

For sugar consumption, strain IZ 1339 had an initial concentration of total sugars (the sum of glucose, fructose and sucrose) of 226.48 g/L, at 36 h this concentration decreased to 157.23 g/L, with 30.6 % of consumed sugars, as this fermentation was carried out until 48 h, the total sugar concentration decreased to 64.55 g/L, a consumption of 71.5 % of total sugars.

Strain FT 146L started fermentation with an initial concentration of total sugars of 222.2 g/L, it is important to note that this fermentative cycle was conducted until 36 h due to the bioreactor operator's availability. At 36 h the total sugar concentration decreased to 160.76 g/L, a consumption of 27.60 %. Remarkably, at 24 h, this strain had already consumed 26.3 % of the total sugar concentration, another evidence of this strains' stable behavior that needs further exploring.

Ethanol production observed for strain IZ 1339 increased significantly during the fermentation process, reaching 64.4 at 48h, with a yield of 63.01 % and productivity of 1.34 g/L.h. It is interesting to observe that productivity rate remained fairly stable after the 12 h period, even though ethanol yield only showed a significant increase at the 30-h mark.

For strain FT 146L, ethanol production was more modest, reaching 30.7 g/L at 36 h, with 30.0 % yield. After 24 h, productivity remained considerably stable, averaging 0.80 g/L.h. This behavior suggests, once again, that strain FT 146L requires more time to adapt to the medium, and optimized conditions in order to explore its stable performance. In terms of ethanol production and productivity, both strains had a similar performance at the 6-h mark, but diverged during the fermentative process.

Limtong et al. (2007) produced ethanol at high temperatures from sugarcane juice (220 g/L, supplemented) and obtained 7.60 % (w/v) of ethanol at 30 ºC, with a productivity of 1.06 g/L.h, and 6.43 % (w/v) of ethanol at 40 ºC, with a productivity of 1.41 g/L.h, the fermentations were carried out for 72 and 48 h, respectively. Strain IZ 1339 reached a higher productivity at 48 h, with no supplementation added, and strain FT 146L came close at 36 h.

These results show the capability of this yeast to efficiently convert sugar into ethanol under the right conditions, whether the medium is supplemented or not. Several works have shown different *K. marxianus* strains performances in media containing glucose, xylose, sugarcane juice, among others (Table 3).

Table 3 – Ethanol production ($g.L^{-1}$) obtained by *Kluyveromyces marxianus* strains in different substrates and sugar concentrations

Strain	Substrate	Sugar Concentration (g/L)	Temp (ºC)	Ethanol production (g/L)	Productivity (g/L/h)	Yield	Reference
K. marxianus BUNL-21	YPXyl (xylose)	20	30	2.91	*	*	Nitiyon et al. (2016)
K. marxianus DMKU3-1042	YPXyl (xylose)	20	30	1.71			
K. marxianus DBKKU Y-102	Jerusalem artichoke tuber (inulin)	230	37	90.5	1.67	*	Charoensopharat et al. (2015)
		250	37	93.4	1.70	*	
K. marxianus NIRE-K1	Salt medium + glucose	100	37	39.1 (16h)	2.45	*	Arora et al. (2015)
K. marxianus NIRE-K3	Salt medium + glucose	100	37	43.2 (12h)	3.60	*	Arora et al. (2015)
K. marxianus UFV-3	Whey permeate (lactose)	170	30	80	1.33	*	Silveira et al. (2005)
K. marxianus Y179	YP medium + Inulin	232	30	93	*	*	Gao et al. (2015)
		227	30	98	*	*	
K. marxianus DMKU 3-1042 In shaking flask cultivation	Sugarcane juice supplemented	220	37	87.0 (72h)	1.45	77.5*	Limtong et al. (2007)
		220	40	67.8 (72h)	1.13	60.4*	
K. marxianus IZ 1339 In shaking flask cultivation	Sugarcane juice (75%) plus molasses (25%)	200	30	17.3 (30h)	0.58	16.9	Present work
K. marxianus IZ 1339 In bioreactor	Sugarcane juice (75%) plus molasses (25%)	200	30	64.4 (48h)	1.34	63.01	

Table 3 shows fermentations performed by diverse *K. marxianus* strains under a broad range of substrates and sugar concentrations, from supplemented media to different carbon sources, such as xylose, inulin and lactose. It is important to note that there was significant variability in the results obtained by the mentioned studies. This divergence is explained by the intraspecific variations and the fact the most studies utilize one single strain as the representative of the species. *K. marxianus* strains are capable of performing simultaneous fermentation and respiration, and the shift between these pathways depends on the strain's particular characteristics (LANE; MORRISSEY, 2010).

Remarkably, the results obtained by this study, particularly strain IZ 1339, were comparable to the results obtained by Limtong et al. (2007), but in a shorter fermentation period (48 h), and no supplementation added in the medium. Perhaps strain FT 146L would reach the desired yield in similar conditions, a longer fermentation process with added supplementation, or even in a co-culture with *S. cerevisiae*, taking advantage of its stable behavior of ethanol productivity, as described by Ha-Tran et al. (2020).

The results obtained by strain IZ 1339 and FT 146L are within the range of studies cited on Table 3 that utilized glucose as the main carbon source, even though the conditions employed vary. As observed, the metabolic plasticity of *K. marxianus* is greater than that of

S. cerevisiae, making it possible to modulate the metabolic outcomes depending on the cultivation and fermentation conditions.

Lastly, it is important to consider that the difference in yield from the shake flask scale fermentation to the bioreactor one may be due to the controlled agitation employed in the bioreactor, creating a more suitable environment for both strains to consume sugar and produce ethanol. In the shake flask fermentation, even though the Erlenmeyer flasks were sealed, the seal is not as efficient as in the bioreactor, which may have contributed to the evaporation of ethanol, as observed in vat fermentations.

Sugar consumption vs. Ethanol and glycerol production for strain IZ 1339 are described in Figure 1, where the decrease in total sugar concentration (g/L) is opposed to an increase in ethanol concentration (g/L) during the 48-h fermentative cycle. Glycerol, one of the most important metabolites produced by yeasts during fermentation, induces cellular adaptation mechanisms to maintain integrity and viability in the medium, under stressing conditions (MASCARENHAS SANTOS et al., 2020). For both strains, glycerol production was first detected at 6 h, as a result from the adaptation process in the fermentative medium.

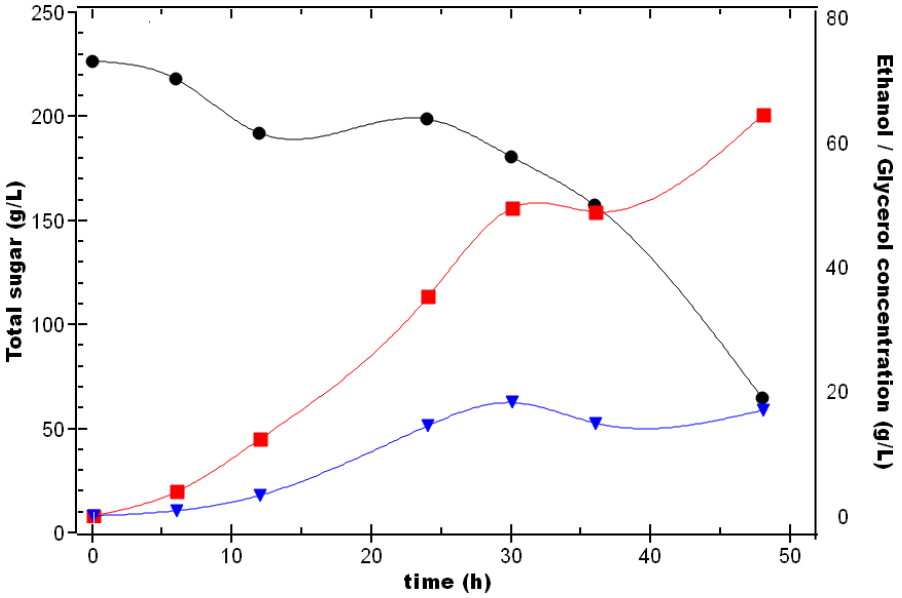

Figure 1 - Sugar concentration (black), ethanol concentration (red) and glycerol concentration (blue) obtained by strain IZ 1339 in a bioreactor fermentation

Strain FT 146L shows production of ethanol (g/L) and glycerol (Figure 2), while also showing the decreased sugar consumption during the 36-h fermentation period. The presence of glycerol is a sign that this strain did adapt to the medium, however, it could need more time in order to sufficiently convert sugar into ethanol and increase the fermentative yield.

The higher the substrate concentration, more glycerol will be produced, the high osmolarity of the extracellular environment leads to the depletion of intracellular water and, in order for this not to occur, the cell metabolism starts to produce and retain glycerol, thus allowing osmotic balance within the cell and cell death (CORDIER et al., 2007; HERSEN et al., 2008).

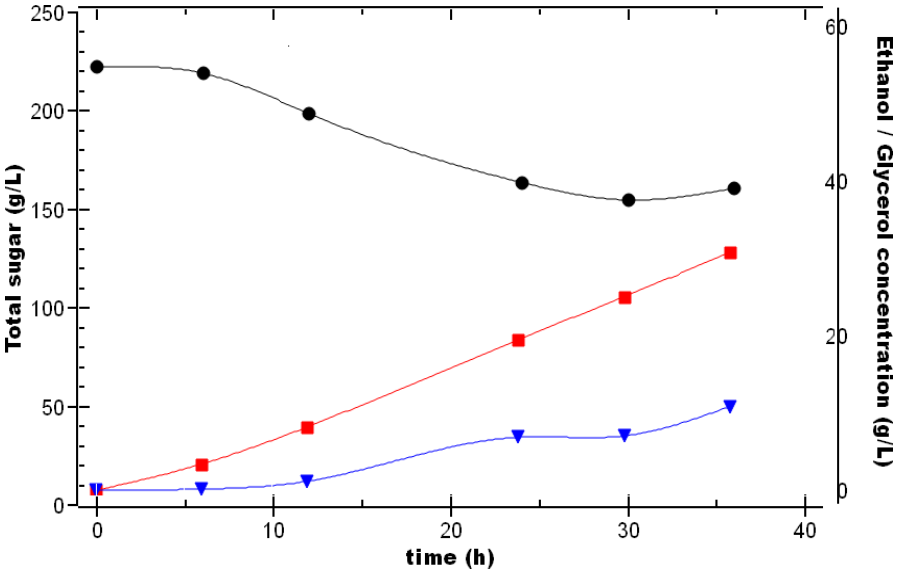

Figure 2 - Sugar concentration (black), ethanol concentration (red) and glycerol concentration (blue) obtained by strain FT 146L in a bioreactor fermentation

It is possible to observe that both strains IZ 1339 (left) and FT 146L (right) had very different sugar consumption profiles. As observed in Figure 3, strain IZ 1339 seems to be hydrolyzing sucrose outside of the cell wall, a behavior similar to that of *S. cerevisiae*, while strain FT 146L seems to be suffering an inhibition that makes it unable to hydrolyze sucrose, this inhibition can happen to enzymes outside of the cell wall, or perhaps a sugar transporter having its effect blocked. The elevated glycerol concentrations, particularly observed in strain IZ 1339, may be caused by biomass production.

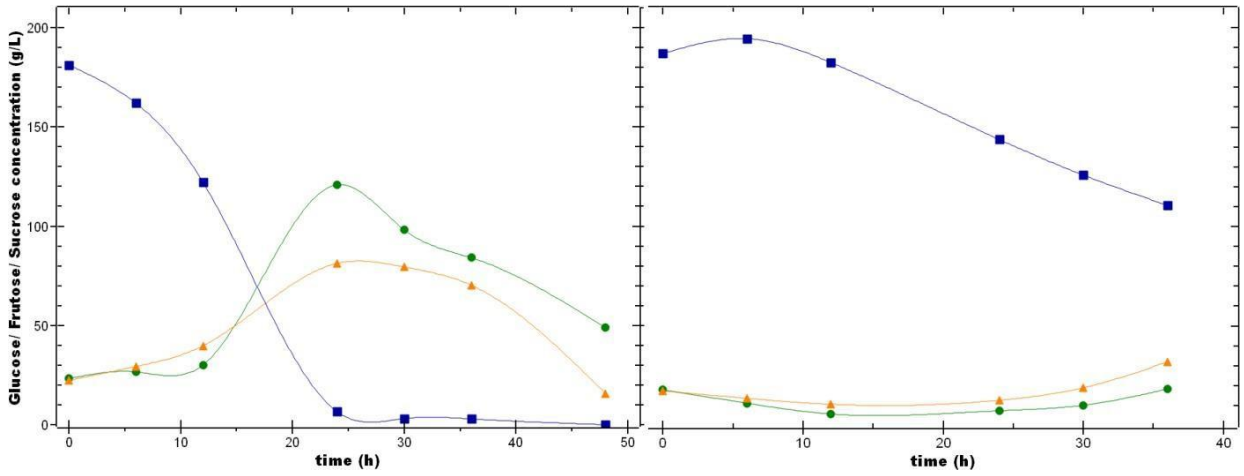

Figure 3 – Concentrations of sucrose (blue); fructose (yellow); glucose (green) concentration (g/L) in the fermentation medium. Strain IZ 1339 (**left**); Strain FT 146L (**right**)

Commonly, particularly with *S. cerevisiae*, invertase hydrolyzes sucrose outside of the cell wall, consuming glucose and leaving fructose out until all of the glucose in consumed. One possible hypothesis worth further exploration is that *K. marxianus* has the ability consume both glucose and fructose simultaneously.

Karim et al. (2020) mentions that due to the advantages in metabolism, *K. marxianus* is a feasible alternative to *S. cerevisiae* for ethanol production, but mentions the need for studies regarding process optimization. Saratale et al. (2017) explored a co-culture of *S. cerevisiae* and *K. marxianus* and managed to obtain 21.12 g/L of ethanol, with 88 % of sugar consumption, the substrate utilized was enzymatic hydrolysates (50 g/L, 30 ºC).

Nigam et al. (1998) explored the production of ethanol in elevated temperatures utilizing strains of *K. marxianus* isolated from soil in India (BANAT; NIGAM; MARCHANT, 1992), and obtained relatively high ethanol concentrations: 5.7 to 7.0% (w/v) at 45 °C and 5.0 to 5.5% (w/v) at 50 °C when growing on 14.0% (w/v) glucose. Their isolates fermented diluted molasses containing 16.0% (w/v) total sugars, producing 5.6 to 6.0% (w/v) ethanol concentrations.

Banat (1992), however, did not explore whether ethanol was obtained from reduced sugar content or the sucrose content in the fermentation medium. Fleming et al. (1993) explored this and reported that *K. marxianus* was able to produce a thermostable cell-associated enzyme with high affinity for sucrose, compare to the enzymes produced by *S. cerevisiae*.

Madeira-Jr and Gombert (2018) explored production of ethanol at high temperatures from *K. marxianus* strains in a synthetic medium containing 10 g/L of sucrose, and obtained a satisfactory ethanol yield, however, there was residual sugars in the medium. Strain UFV-3 displayed good fermentation performance during the first cycle of a miniaturized 1G fuel ethanol production system between 34 and 41 °C, but ethanol production decreased in the following cycles. The authors mention that this study had an exploratory character, and further investigation and optimization of the fermentative process are required, however, all results point to promising ethanol producing applications. The same can be said about the results obtained in this study.

Organic acids derived from fermentation

Organic acids such as lactic, acetic, aconitic and succinic, were determined during the fermentation process from *K. marxianus* in sugarcane juice/molasses, in controlled conditions (bioreactor). The concentrations of organic acids were determined in g/L with the help of Ion Chromatography. Figure 4 shows the concentrations of Acetic acid, Lactic acid, Aconitic acid and Succinic acid detected in the fermentation carried out by *K.*.

marxianus IZ 1339.

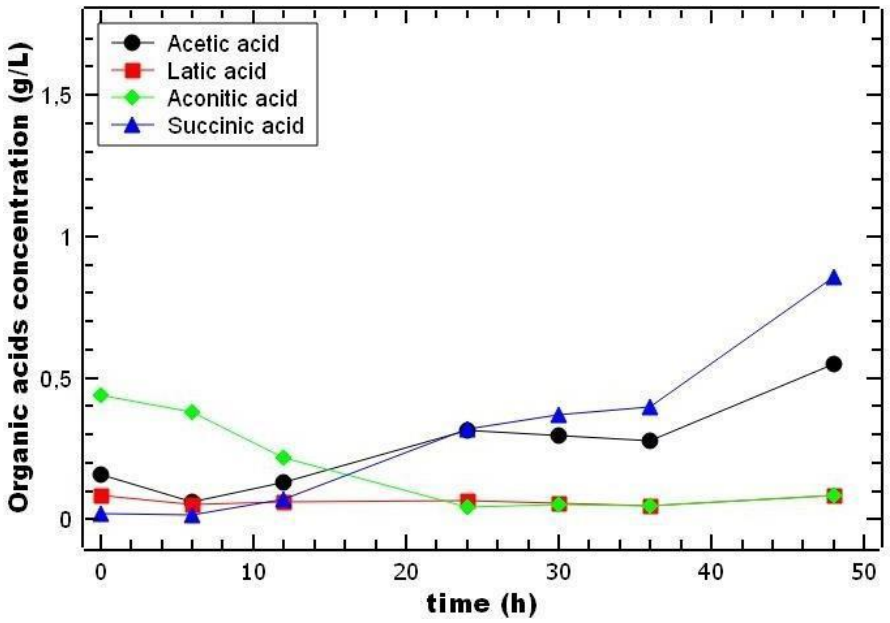

Figure 4 - Concentration (g/L) of organic acids detected in the fermentation by *K. marxianus* IZ 1339

Acetic acid, Lactic acid, Aconitic acid and Succinic acid concentrations detected in the fermentation carried out by *K. marxianus* FT 146L were shown at Figure 5.

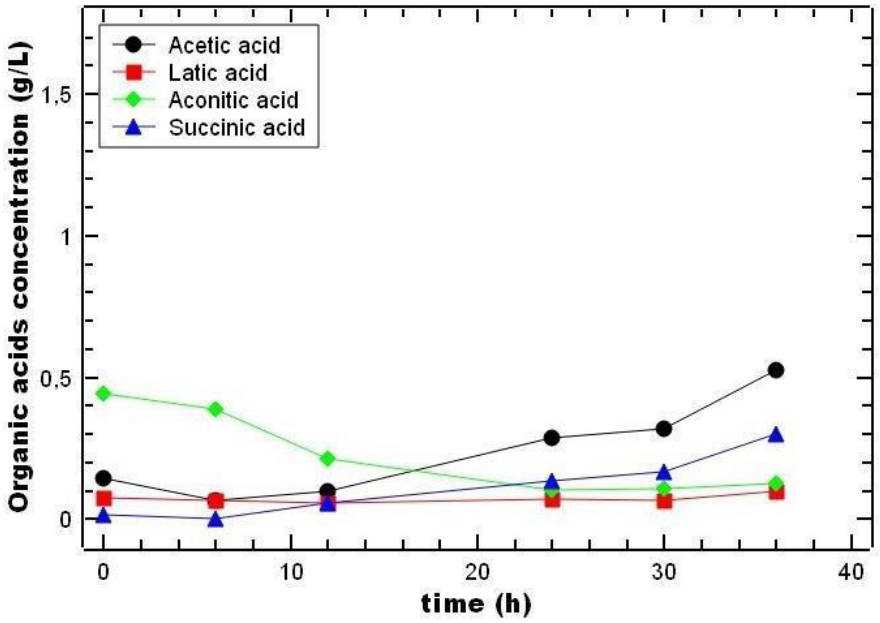

Figure 5 - Concentration (g/L) of organic acids detected in the fermentation by *K. marxianus* FT 146L

Propionic and butyric acids were not detected in any of the fermentations. For acetic acid, an increase was observed at the end of the fermentation process for both strains, strain FT 146L had an initial concentration of 0.031g/L and increased to 0.524 g/L at the end of the fermentation. Strain IZ 1339 started with a higher concentration of 0.159 g/L, ending with 0.548 g/L. Acetic acid, as a by-product of fermentation, can be used to produce plastics, solvents, herbicide, paints and resins (DIONISI; SILVA, 2016).

As for Lactic acid, it showed little variation throughout the process, with a modest increase at the end for strain FT 146L, going from 0.076 g/L to 0.099 g/L while for strain IZ 1339, concentration slightly decreased at the end of the fermentative process, from 0.084 g/L to 0.082 g/L. Lactic acid can be used for plastics and food ingredient production (DIONISI; SILVA, 2016).

Aconitic acid started at similar concentrations for both fermentative processes, 0,455 g/L for strain FT 146L and 0.438 g/L for strain IZ 1339. Strain IZ 1339 consumed more aconitic acid during the fermentation, ending at 0.082 g/L at 48 h, as opposed to 0,125 g/L for strain FT 146L. It is remarkable that both *K. marxianus* strains consumed aconitic acid, as this is a characteristic worth further exploration, seeing that such a trait is not usually observed in yeasts.

Aconitic acid is the main acid in sugarcane; its concentration may vary according to the variety of the sugarcane, maturation time and environmental conditions, and is responsible for the titratable acidity of the sugarcane. The average concentration in the broth is approximately 1% and in the molasses from 3 to 7% (results expressed on a dry basis) (HONIG, 1953).

Lastly, succinic acid concentration increased in both fermentation processes, more expressively for strain IZ 1339, going from 0.019 g/L to 0.857 g/L. Strain FT 146L had a smaller production of this acid, ending the process at 0.229 g/L. Succinic acid is a secondary product of fermentation, as are glycerol and secondary alcohols, being the main acid formed by yeasts. This organic acid is a dicarboxylic acid produced as an intermediate of the tricarboxylic acid (TCA) cycle during aerobic respiration; it is also one of the byproducts of anaerobic metabolism, and one of the organic acids responsible for the increase in titrable acidity during fermentation (SONG 2006). With the exception of succinic and acetic acids, both fermentations had a similar pattern of production of organic acids.

4.4 Conclusion

Despite not performing fermentations on optimized conditions, this study utilized an exploratory approach on ethanol production from *K. marxianus* strains, in order to aid the understanding on how this yeast perform in conditions similar to a Brazilian ethanol producing unit. Strain IZ 1339 had a superior yield, while strain FT 146L had a stable behavior throughout the fermentative process, a characteristic that is worth further exploration. It is important to consider that, for both strains, fermentation required more time, also the impact of stressors should be investigated in order to improve yield.

References

ARORA, R. *et al.* A new search for thermotolerant yeasts, its characterization and optimization using response surface methodology for ethanol production. **Frontiers in Microbiology**, 2015. v. 6, n. SEP.

BANAT, I. M.; NIGAM, P.; MARCHANT, R. Isolation of thermotolerant, fermentative yeasts growing at 52 ºC and producing ethanol at 45ºC and 50ºC. **World Journal of Microbiology & Biotechnology**, 1992. v. 8, n. 3, p. 259–263. Available in:

<http://www.ncbi.nlm.nih.gov/pubmed/24425473>. Acesso em: 21 set. 2018.

BRASIL. Portaria nº 76 de 26 de novembro de 1986. Dispõe sobre os métodos analíticos de bebidas e vinagre. **Diário Oficial da União.**, 1986. p. Brasília.

CHAROENSOPHARAT, K. et al. Ethanol production from Jerusalem artichoke tubers at high temperature by newly isolated thermotolerant inulin-utilizing yeast *Kluyveromyces marxianus* using consolidated bioprocessing. **Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology**, 2015. v. 108, n. 1, p. 173–190.

CORDIER, H. et al. A metabolic and genomic study of engineered *Saccharomyces cerevisiae* strains for high glycerol production. **Metabolic Engineering**, jul. 2007. v. 9, n. 4, p. 364–378.

CRUZ, M.L. **Avaliação de condições operacionais na fermentação alcoólica VHG empregando diferentes cepas de *Saccharomyces cerevisiae***. Universidade Federal de Uberlância - Faculdade de Engenharia Química, 2019. p. 119. Available in: <http://dx.doi.org/10.14393/ufu.te.2019.321>.

DIONISI, D.; SILVA, I. Production of ethanol, organic acids and hydrogen: an opportunity for mixed culture biotechnology? **Reviews in Environmental Science and**

Biotechnology, 2016. v. 15, n. 2, p. 213–242.

FLEMING, M. et al. Studies on the growth of a thermotolerant yeast strain, *Kluyveromyces marxianus* IMB3, on sucrose containing media. **Biotechnology Letters**, 1993. v. 15, n. 12, p. 1195–1198.

FONSECA, G.G. et al. Physiology of the yeast *Kluyveromyces marxianus* during batch and chemostat cultures with glucose as the sole carbon source. **FEMS Yeast Research**, 2007. v. 7, n. 3, p. 422–435. Available in: <https://academic.oup.com/femsyr/article/7/3/422/523745>.

GAO, J. et al. Transcriptional analysis of Kluyveromyces marxianus for ethanol production from inulin using consolidated bioprocessing technology. **Biotechnology for Biofuels**, 14 dez. 2015. v. 8, n. 1, p. 115. Available in: <http://www.biotechnologyforbiofuels.com/content/8/1/115>. Accessed at: 21 jan. 2019.

GIBSON, B.R. et al. **Yeast responses to stresses associated with industrial brewery handling**. [S.l.]: [s.n.], 2007. v. 31.

GOFFEAU, A. et al. Life with 6000 genes. **Science**, 25 out. 1996. v. 274, n. 5287, p. 546–567. Available in: <http://www.ncbi.nlm.nih.gov/pubmed/8849441>. Accessed at: 21 set. 2018.

GOMES, L.H. et al. Presence of the yeast *Candida tropicalis* in figs infected by the fruit fly *Zaprionus indianus* (dip.: Drosophilidae). **Brazilian Journal of Microbiology.** 2003. v. 34, p.5-7. Available in: https://doi.org/10.1590/S1517-83822003000100002. Accessed at: 10 dez. 2022.

HA-TRAN, D.M.; NGUYEN, T.T.M.; HUANG, C.C. *Kluyveromyces marxianus*: current state of omics studies, strain improvement strategy and potential industrial implementation. **Fermentation**, 2020. v. 6, n. 4, p. 1–19.

HERSEN, P. et al. Signal processing by the HOG MAP kinase pathway. **Proceedings of the National Academy of Sciences of the United States of America**, 2008. v. 105, n. 20, p. 7165–7170.

HONIG, P. **Principles of Sugar Technology**. [S.l.]: Elsevier Pub. Co, 1953.

KAMAL, M. et al. Correlation between pH with saccharification and ethanol fermentation of sugarcane juice industrial wastes by genotypic hybrids of *Sacccharomyces cerevisiae* as pH determining factor. **Journal of Agricultural Chemistry and Biotechnology**, 2020. v. 11, n. 7, p. 209–218. Available in: <www.jacb.mans.edu.eg>. Accessed at: 11 set. 2022.

KARIM, A.; GERLIANI, N.; AÏDER, M. *Kluyveromyces marxianus*: An emerging yeast cell factory for applications in food and biotechnology. **International Journal of Food**

Microbiology, 2020. v. 333, n. May, p. 108818. Available in: <https://doi.org/10.1016/j.ijfoodmicro.2020.108818>.

KUNITAKE, M. et al. Effect of pasteurization temperature on stability of an acidified sugarcane juice beverage. **Ciência e Agrotecnologia**, 2014. v. 38, n. 6, p. 554–561.

LANE, M.M.; MORRISSEY, J.P. *Kluyveromyces marxianus*: A yeast emerging from its sister's shadow. **Fungal Biology Reviews**, 1 fev. 2010. v. 24, n. 1–2, p. 17–26. Available in <https:// www.sciencedirect.com/science/article/abs/pii/ S1749461310000035>. Accessed at: 1º ago. 2019.

LEAL, G.A. et al. Fermentation of cacao (Theobroma cacao L.) seeds with a hybrid *Kluyveromyces marxianus* strain improved product quality attributes. **FEMS Yeast Research**, 2008. v. 8, n. 5, p. 788–798.

LEHNINGER, A.L. Lehninger princípios de bioquímica. - 3. ed. / 2002 - Livros. **São Paulo: Sarvier**, 2002. Available in: <http://bibcentral.ufpa.br/arquivos/135000/ 135400/19_135457.htm>. Acesso em: 11 set. 2022.

LIMTONG, S.; SRINGIEW, C.; YONGMANITCHAI, W. Production of fuel ethanol at high temperature from sugar cane juice by a newly isolated *Kluyveromyces marxianus*. **Bioresource Technology**, 2007. v. 98, n. 17, p. 3367–3374.

MADEIRA-JR, J.; GOMBERT, A. Towards high-temperature fuel ethanol production using *Kluyveromyces marxianus*: On the search for plug-in strains for the Brazilian sugarcane-based biorefinery. **Biomass and Bioenergy**, 2018. v. 119, n. September, p. 217– 228. Available in: <https://doi.org/10.1016/j.biombioe.2018.09.010>.

MASCARENHAS SANTOS, M. Do S. et al. Glycerol and ethanol accumulation profile of the yeast FT-858 on saccharine substrates. **Ciência e Natura**, 2020. v. 42, p. e52. Available in: <https://periodicos.ufsm.br/cienciaenatura/article/view/35612/html>. Accessed at: 12 set. 2022.

MILLER, G.L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. **Analytical Chemistry**, mar. 1959. v. 31, n. 3, p. 426–428. Available in: <http://pubs.acs.org/doi/abs/10.1021/ac60147a030>. Accessed at: 2 ago. 2019.

NITIYON, S. et al. Efficient conversion of xylose to ethanol by stress-tolerant *Kluyveromyces marxianus* BUNL-21. **Springer Plus**, 2016. v. 5, n. 1, p. 1–12.

RADECKA, D. et al. Looking beyond Saccharomyces: The potential of non- conventional yeast species for desirable traits in bioethanol fermentation. **FEMS Yeast Research**, 1 set. 2015. v. 15, n. 6, p. fov053. Available in: <https://academic.oup.com/femsyr/ article-lookup/doi/10.1093/femsyr/fov053>. Accessed at: 19 set. 2018.

SAINI, P.; BENIWAL, A.; VIJ, S. Physiological response of Kluyveromyces marxianus during oxidative and osmotic stress. **Process Biochemistry**, 1 maio. 2017. v. 56, p. 21–29.

SARATALE, G. D. et al. Solid state fermentative lignocellulolytic enzymes production, characterization and its application in the saccharification of rice waste biomass for ethanol production: An integrated biotechnological approach. **Journal of the Taiwan Institute of Chemical Engineers**, 2017. v. 76, p. 51–58.

SINGH, D.; NIGAM, P.; BANAT, I.M. Ethanol production at elevated temperatures and alcohol concentrations: Part II–Use of *Kluyveromyces marxianus* IMB3. **World Journal of Microbiology & Biotechnology**, 1998. v. 14, n. 6, p. 823–834. Available in: <http://link.springer.com/10.1023/A:1008852424846>. Accessed at: 21 set. 2018.

SOBRINHO, V. Dos S.; SILVA, V.C.F. Da; CEREDA, M. P. Fermentation of sugar cane juice (*Sacharum officinarum*) cultivar RB 7515 by wild yeasts resistant to UVC. **Journal of Biotechnology and Biodiversity**, 2011. v. 2, n. 1, p. 13–21.

SOUCIET, J.L. et al. Comparative genomics of protoploid Saccharomycetaceae. **Genome Research**, 2009. v. 19, n. 10, p. 1696–1709. Available in: <http://www.ncbi.nlm.nih.gov/pubmed/19525356>. Accessed at: 21 set. 2018.

TAYLOR, M.P. et al. Understanding physiological responses to pre-treatment inhibitors in ethanologenic fermentations. **Biotechnology Journal**. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22331581>. Accessed at: 21 set. 2018.

TONOLI, F.C. **Adaptação de leveduras para fermentação com alto teor alcoólico**. Dissertação de Mestrado. Escola Superior de Agricultura “Luiz de Queiroz”, 2017. Universida. Piracicaba.

WALKER, G.A. et al. Monitoring site-specific fermentation outcomes via oxidation reduction potential and uv-vis spectroscopy to characterize “hidden” parameters of pinot noir wine fermentations. **Molecules**, 2021. v. 26, n. 16. Available in:

<https://doi.org/10.3390/molecules26164748>.

ZOECKLEIN, B.W. et al. **Wine Analysis and Production**. [S.l.]: Springer US, 1999.

5. AUTHOR'S FINAL CONSIDERATIONS AND FUTURE PERSPECTIVES

In conclusion, our intentions to explore the biotechnological potential of two distinct strains of *Kluyveromyces marxianus* are considered successful. This work had a largely exploratory approach, and while some results were corroborated by previously published studies, we managed to offer a new perspective, bringing forth answered questions and furthering the available knowledge on this yeast species. Even though we were unable to explore further fermentative studies, testing optimized conditions, the results obtained were successful.

There is room to explore, such as studies in thermotolerance, fermentation under stressing conditions and one particularly interesting characteristic noted during our studies: the ability these two strains have to produce pleasant aromatic compounds, which we would very like to explore in the future.

The main lesson from this study was to consider more than just the known applications of yeast, knowledge which we guide by our experience working with *Saccharomyces cerevisiae*, and to explore results and applications from this non-conventional yeast that offers so much potential. Overall, this study can be considered successful, as the explorative results in characterization are plentiful and diverse.