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Mohammed Anas Zaiter

Assessment of tolerance to inhibitors derived from plant biomass hydrolysis and xylose consumption by yeasts isolated from the environment

São José do Rio Preto
2022

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São José do Rio Preto
21 de fevereiro de 2022

DEDICATION

To my mother, Safaa, for her never-ending desire to provide a better future for her children,

To my father, Tayser, for being the voice of reason and supporting me on my path,

To my wife, Alaa, for standing by my side on the journey of life,

To my daughters, Safa and Mariam, for giving me a reason to keep going,

To my keenly supportive brothers and sister, for their love and affection,

Without you all, I wouldn’t be the person I am today.

Thank you.

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seria capaz de atingir meus objetivos.

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RESUMO

A tendência por esgotamento das reservas de petróleo e os problemas ambientais decorrentes do uso de combustíveis de origem fóssil têm evidenciado a necessidade por fontes alternativas de energia sustentáveis e baratas. Isso impulsionou o uso de etanol como combustível líquido, especialmente em países como EUA e Brasil. Contudo, para suprir esta crescente demanda por combustíveis, no Brasil extensas áreas de terras são ocupadas por cana-de-açúcar, e têm competido com outras culturas vegetais, especialmente para suprimento da cadeia de alimentos. Como alternativa, nos últimos anos têm se intensificado estudos para produção de etanol a partir da biomassa vegetal residual, como bagaço de cana-de-açúcar e outros subprodutos da agroindústria, uma vez que este material é subaproveitado e rico em hexoses (C_6) e pentoses (C_5). Para que a hidrólise enzimática da biomassa vegetal resulte em açúcares fermentescíveis, são necessárias etapas de pré-tratamento deste material, os quais tendem a gerar compostos inibidores de crescimento microbiano. Desta forma, leveduras resistentes a estes inibidores e capazes de co-fermentar açúcares C_5 e C_6 são amplamente investigadas. Diferentemente da levedura *Saccharomyces cerevisae*, algumas espécies não-*Saccharomyces* têm se mostrado capazes de assimilar pentoses, mas ainda são muito pouco exploradas, frente a diversidade de espécies na natureza. Investigar leveduras fermentadoras de xilose, assim como suas tolerâncias a inibidores do crescimento microbiano, pode suportar futuros estudos de engenharia metabólica para capacitação da linhagem industrial *S. cerevisiae.* Portanto, este trabalho propôs-se a avaliar a capacidade das leveduras *Pichia ofunaensis* e *Trichosporon multisporum* para assimilar xilose, e suas tolerâncias a alguns compostos inibitórios, bem como suas capacidades para produção de etanol a partir de xilose. As leveduras foram cultivadas em meio YEPX, pH 4,5 contendo, separadamente, os inibidores hidroximetilfurfural (HMF), furfural, ácido ferúlico, ácido acético, ácido fórmico e vanilina, cultivadas a 28 ºC e 150 rpm por 96 h. A levedura *P. ofunaensis* apresentou crescimento em todas as concentrações destes inibidores e foi capaz de consumir completamente a xilose presente nos meios de cultivo, exceto em 20 mM de furfural, onde houve consumo de 40% da xilose, e entre 80 a 90% em maiores concentrações de ácido ferúlico (3 mM), ácido fórmico (40 mM) e vanilina (10 mM). Em presença de ácido acético 80 mM, a levedura não cresceu, e nas concentrações de 60 mM e 40 mM consumiu 20% e 90% da xilose presente nos meios, respectivamente. A levedura *T. multisporum,* em presença de furfural, exibiu crescimento até 15 mM e consumiu 30% da xilose. Em meio com ácido ferúlico, HMF e ácido fórmico, houve crescimento em todas as concentrações e consumo de até 40% (ácido ferúlico e HMF) e 30% (ácido fórmico) da xilose. Com vanilina, observamos crescimento até 5 mM deste inibidor e máximo consumo de 30% da xilose, e em meio contendo ácido acético a levedura não apresentou crescimento a 20 mM, exibindo consumo de até 40% de xilose em concentrações inferiores. A levedura *P. ofunaensis* se mostrou mais tolerante aos compostos considerados potencialmente tóxicos, exibindo melhor crescimento e consumo de xilose em comparação a levedura *T. multisporum*. Furfural e ácido acético se mostraram os compostos mais prejudiciais ao crescimento das leveduras. Por fim, na fermentação alcoólica, detectamos a produção de etanol apenas no cultivo da levedura *P. ofunaensis*, com máxima produção de 0,51 g/L após 96 h de cultivo em meio salino contendo 50 g/L de xilose.

Palavras-chave: Biomassa Lignocelulósica, Bioetanol, Ácidos Orgânicos, Fermentação, Leveduras, Pentose, Xilose.

ABSTRACT

Oil reserves are being depleted at an alarming rate, while the environmental problems arising from fossil fuels usage have highlighted the need for sustainable and cheap alternative energy sources, which has boosted the use of ethanol as a liquid fuel, especially in countries like the USA and Brazil. However, to meet this growing demand for combustibles, extensive land areas in Brazil have been occupied by sugarcane and have competed with other vegetable crops, especially for supplying the food chain. Alternatively, in recent years, studies have been intensified for ethanol production from residual plant biomass, such as sugarcane bagasse and other agro-industrial residues, since this material is underutilized and rich in hexoses (C_6) and pentoses (C_5). For enzymatic action in the degradation of plant biomass, resulting in fermentable sugars, pre-treatment steps of this material are necessary, which tend to generate compounds that inhibit microbial growth. Thus, yeast resistance to these inhibitors and the capability of co-fermenting C_5 and C_6 sugars are widely investigated. Unlike the yeast *Saccharomyces cerevisiae*, some non-*Saccharomyces* species can assimilate pentoses, but they are still very little explored, given the diversity of species in nature. Investigating xylose-fermenting yeasts, as well as their tolerances to microbial growth inhibitors, may support future studies of metabolic engineering to enable the industrial strain *S. cerevisiae*. Therefore, this work aimed to evaluate the capacity of *Pichia ofunaensis* and *Trichosporon multisporum* yeasts to assimilate xylose and their tolerances to some inhibitory compounds, as well as their capability to produce ethanol from xylose. Yeasts were cultivated in YEPX medium, pH 4.5, containing, separately, the inhibitors hydroxymethylfurfural (HMF), furfural, ferulic acid, acetic acid, formic acid, and vanillin, cultivated at 28 ºC and 150 rpm for 96 h. The yeast *P. ofunaensis* grew at all concentrations of these inhibitors and was able to completely consume the xylose present in culture media, except in 20 mM furfural, in which 40% of xylose was consumed, and between 80 and 90 % in higher concentrations of ferulic acid (3 mM), formic acid (40 mmM), and vanillin (10 mM). In the presence of 80 mM acetic acid, the yeast did not grow, and at the concentrations of 60 mM and 40 mM, it consumed 20% and 90% of the xylose present in the media, respectively. The yeast *T. multisporum*, in the presence of furfural, exhibited growth up to 15 mM and consumed 30% of the xylose. In media with ferulic acid, HMF, and formic acid, there was growth at all concentrations and consumption of up to 40% (ferulic acid and HMF) and 30% (formic acid) of xylose. With vanillin, we observed growth up to 5 mM of this inhibitor and maximum consumption of 30% of xylose, and in a medium containing acetic acid the yeast did not grow at 20 mM, exhibiting consumption of up to 40% of xylose at lower concentrations. The yeast *P. ofunaensis* was more tolerant to compounds considered potentially toxic and showed better growth rates and xylose consumption compared to the yeast *T. multisporum*. Furfural and acetic acid proved to be the most harmful compounds for yeasts growth. Finally, in the alcoholic fermentation, we detected ethanol production only in the yeast *P. ofunaensis* cultures, with a maximum output of 0.51 g/L after 96 h of cultivation in a saline medium containing 50 g/L of xylose.

Keywords: Bioethanol, Fermentation, Lignocellulosic Biomass, Organic Acids, Pentose, Yeasts, Xylose.

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LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

2,3-BD	2,3-Butanediol
ATP	Adenosine triphosphate
DNS	3, 5 - Dinitrosalicylic Acid
h	Hours
HMF	Hydroxymethylfurfural
M	Molar
mL	Milliliter
mm	Millimeter
mM	Millimolar (10^{-3} mol L^{-1})
nm	Nanometer
O.D.	Optical density
pH	Potential of hydrogen
rpm	Rotations per minute
STEX	Steam explosion
xg	Centrifugal force
YEP	Yest Extract & Peptone
YEPD	Yest Extract, Peptone & Dextrose
YEPX	Yest Extract, Peptone & Xylose

CONTENTS

1 Introduction

According to United Nations, the world population has exceeded 7 billion people. Overpopulation, along with the rapid development of civilized lifestyles, has contributed to the rise in energy demand, as global consumption has grown by around 50% in the last twenty years, and carbon-based fuels such as coal, oil, and gas have been the principal sources.

This puts us in front of three crucial problems: high energy demand; significant depletion of non-renewable energy resources; and environmental concerns such as global warming, and pollution, which show the urgent need to search for alternative renewable, environmentally friendly, and cheaper sources of energy.

One of these sources is lignocellulosic biomass. It is an abundant bio-renewable resource, is inexpensive, broadly available, and has been identified as a sustainable carbon source. The use of this biomass points to the opportunity to add value to agro-industrial residues and does not compete with the food supply at the same time. This energy has much lower gas emissions compared to fossil fuels and lower costs compared to other renewable energy types.

This lignocellulosic biomass is composed of three main parts: cellulose, which is the dominant part of the lignocellulosic biomass, is a linear homopolymer that has D-glucose units; hemicellulose, representing 20 to 35% of the lignocellulosic biomass, is a highly diverse heteropolysaccharide, composed of several types of hexoses and pentose, and xylan is the most predominant type of hemicellulose, which has a main chain formed by xylose monomers; and lignin is an amorphous three-dimensional polymer that gives rigidity and resistance to the cell wall.

Xylose is an important sugar derived from the hydrolysis of plant biomass and can constitute up to 25% of the total dry weight of some forest and agricultural residues, which denotes the importance of its use in fermentation processes. However, the yeast *S. cerevisiae* is unable to efficiently co-assimilate glucose and xylose. Thus, prospecting for non-*Saccharomyces* yeasts capable of assimilating xylose may provide a better knowledge of the xylose transport mechanisms in the cell and offer support for the metabolic engineering of *S. cerevisiae* to assimilate it.

In the laboratory of biochemistry and applied microbiology - Unesp, São José do Rio Preto, SP, Brazil, a research group under the supervision of Prof^{a}. Dr^{a}. Eleni Gomes, keeps prospecting for microorganisms capable of fermenting xylose and producing ethanol and other valuable products. This work is a continuation of what a previous master's student (VAZ, 2020) had done to study the two yeasts, *P. ofunaensis* and *T. multisporum*.

6 CONCLUSIONS

The yeast *P. ofunaensis* exhibited more tolerant abilities toward potentially harmful chemicals than *T. multisporum*. Acetic acid had the most potent inhibitory effect, while HMF had the weakest inhibitory effect on yeast growth. The yeast *T. multisporum* had a poorer tolerance for the various inhibitors investigated, and its performance in terms of cellular growth and sugar consumption is considerably lower than that of *P. ofunaensis*.

Despite low ethanol production and low tolerance to the inhibitors tested, the yeast *P. ofunaensis* was able to consume all of the xylose in the YPX medium at pH 4.5, implying the possibility of using xylose/H^+ symporters, which have been described as transporters with higher affinity for D-xylose. These findings support the continuation of research on xylose transporters.

In addition, this study can be a starting point for future projects expanding to a broader panel of xylose-assimilating yeast species that would be useful for the development of more robust industrial yeast strains able to utilize a broader range of the sugars present in the lignocellulosic hydrolyzate and tolerate higher levels of inhibitors derived from different pretreatment processes.

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