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Câmpus de São José do Rio Preto

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

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Dissertação apresentada como parte dos requisitos para obtenção do título de Mestre em Microbiologia, junto ao Programa de Pós-Graduação em Microbiologia, do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de São José do Rio Preto/SP.

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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.

Àqueles que apesar de todas as adversidades sempre acreditaram que eu seria capaz de atingir meus objetivos.

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I am most grateful to my advisor, supervisor, and mentor, Dr. Ronivaldo Rodrigues da Silva, who taught me the basics of microbiological laboratory and scientific research and showed me the way to become an independent thinker. He has been both patient and lenient when things have been arduous, but has always encouraged me to complete the work to the fullest.

For this work to see the light, I thank my committee members, Profª. Drª. Tatiane Beltramini Souto and Profª. Drª. Gabriela Salvador de Amo, for their intellectual guidance, efforts, and support throughout this process.

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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 Profª. Drª. 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*.

2 GENERAL BIBLIOGRAPHIC REVIEW

2.1 Global population growth and the increasing demand for food and energy

The world's population has surpassed 7 billion people. According to the United Nations (DESA, 2013), and a study published in the journal Science by the University of Washington (GERLAND et al., 2014), it is highly possible that we will have 9.6 billion Earthlings by 2050, and roughly 11 billion or more by 2100.

This population explosion forces us to confront some of the world's most pressing environmental problems, among other concerns, including climate change, energy demand, food security, and freshwater (UNITED NATIONS NEW YORK, 2019). Producing enough food to sustain an ever-increasing population seems to have become what the 2014 World Food Prize Symposium calls "the greatest challenge in human history." (THE WORLD FOOD PRIZE, 2014). However, seed breeding, soil nutrient replenishment (such as chemical and organic fertilizers), irrigation, mechanization, and other innovations may help the food supply keep pace with population growth. Thus, technological advancements in all areas (such as agriculture, energy, water use, manufacturing, disease control, information management, transportation, communications) would help to keep production ahead (ALEXANDRATOS, 2012).

Furthermore, it is becoming clear that the more affluent humanity becomes, the more energy and natural resources, usually carbon-based fuels such as coal, oil, and gas, are consumed. And it is noticeable in consumption behavior that includes almost everything, from higher protein foods such as meat and dairy products, to more consumer goods, enormous buildings, more vehicles, and even more aviation (Popul. Summit World’s Sci. Acad., 1993).

Nevertheless, regarding natural resources, and according to studies, humanity is living beyond its means. An ongoing Global Footprint Network study shows that we are currently using an average of 1.7 planets to provide the resources we use and to absorb our waste. And by 2050, we will need 3 planets if things remain the same (GLOBAL FOOTPRINT NETWORK, 2021). This means that we are consuming much more resources than the capability of the planet to reproduce and recover.

Consequently, this unconscious consumption in general causes an acceleration of the depletion of non-renewable resources like fossil fuel, which is the major source of energy these days. This fuel, which notwithstanding that it has begun to be exhausted, has also been used with such intensity that it has caused many misfortunes on the planet, such as global warming,

environmental pollution, climate change, and water scarcity. Besides being relatively expensive and with unstable prices due to several factors, including economic and political ones.

The world nowadays is confronted with three crucial issues: high energy demand, a significant depletion of non-renewable energy resources, and higher levels of environmental pollution both locally and globally (DEMIRBAS, 2009b), which show the urgent need to search for alternative renewable energy sources that are environmentally friendly and cheaper.

2.2 Energy: importance, types and sources

The World Bank's previous Vice President, Rachel Kyte, announced in an interview, "It is energy that lights the lamp that lets you do your homework, that keeps the heat on in a hospital, that lights the small businesses where most people work. Without energy, there is no economic growth, there is no dynamism, and there is no opportunity." (WORLD BANK, 2013).

Energy is a fundamental part of almost all aspects of life. It is one of the most dominant cornerstones in nations' development and economic growth and promotes living standards and human welfare. Providing sufficient and affordable energy is a prerequisite to having an advantage in global competition. Since all the current indicators confirm the imminent depletion of fossil fuels, searching for sustainable and renewable sources of energy is crucial for sustainable development and production processes (TÜRKOĞLU; KARDOĞAN, 2018).

Diversification of energy sources and increasing its efficiency has become one of the main factors indicating the development and leadership position of a nation globally. In the search for the cheapest, fastest, and most environmentally-friendly ways of meeting the nation's energy needs, expanding the energy resources isn't enough anymore, but its efficiency has started to be considered too, which means utilizing less energy to provide the same product or service (PATTERSON, 1996).

Energy resources are ordinarily fossil, nuclear, and renewable energy resources. Conventional energy based on coal, gas, and oil is the dominant source nowadays. Nevertheless, its lousy impacts on the environment have prompted the world to try to reduce its excessive use, as well as to search for alternative solutions that meet the increasing need for energy without having the same harmful impact on the environment (KUMAR, 2020).

Nuclear energy has been identified as one of the reasonable options too. However, the panic and fear that it has brought over the years only increases the uncertainty about its future. Three nuclear disasters have occurred in the past 30 years in Russia, the United States, and Japan. The impact of such a terrifying incident is so huge that, regardless of the all-safety preparations provided by the nuclear equipment operators and suppliers, environmental

protection associations are pressing to force governments to seek safer alternatives (AZARPOUR et al., 2013).

Renewable energy is gaining important fame as the future resource of energy because of its green nature, its renewability, and its ability to be used endlessly. These resources can be effective in mitigating climate change, greenhouse effects, and global warming (BILGILI; KOÇAK; BULUT, 2016). Other valuable outcomes are also noticeable alongside energy security, such as job creation, which could increase job opportunities, improve health care, and promote life standards. That would reflect positively on social bond creation and community development if proper usage of the renewable energy system was achieved (ZEB et al., 2014).

According to predictions, the world's energy demand will grow by up to five times its current consumption in the future. Currently, three-fourths of that demand is fulfilled by fossil fuels. Various renewable energy resources, like hydropower, wind, solar, biomass, tidal energy, biofuel, etc., provide 15–20% of the entire world's energy (KUMAR, 2020).

These resources reduce price swings and fossil fuel imports and dependence. However, there are several flaws as well. Each resource has its own limitations that must be considered. Seasonal variations, length of day and night, sun and moon position, demographic and geo-restrictions, technical and technological constraints, and other factors all contribute to power generation change and instability (EVANS; STREZOV; EVANS, 2009).

Other barriers, like political, institutional, administrative, fiscal, social, economic, industrial, distributional, and informational, play an essential role in the repression of these kinds of energy resources (YAZDANIE; RUTHERFORD, 2010).

2.3 Different renewable energy types; advantages and disadvantages.

2.3.1 Solar Energy

The abundance of solar energy makes it a transcendent new power. According to much research, solar energy may become the most likely source of energy in the future (REHMAN; HALAWANI, 1997). It is often called the alternative energy source to fossil fuel sources. It may be considered today's best solution for a decentralized energy supply in several parts of the world (COUNCIL, 2008;"Welcome to Solar Heat Europe - Solar Heat Europe", 2019).

Being the most expensive source of renewable energy At the moment, solar power meets only a small portion of the world's energy needs (AZARPOUR et al., 2013). Because photovoltaic (PV) cells have a low efficiency in converting light energy directly into electricity, their contribution to electricity supply is considered negligible when compared to other sources

of energy, and a large portion of the solar radiation energy would be dissipated as heat (REN21, 2019).

Manufacturing these PV cells isn't cheap either, which adds another problem to their use. Besides that, chemicals utilized in its fabrication are potentially harmful to human health (INTERNATIONAL ENERGY AGENCY, 2013).

Some other factors, such as energy storage, geographical location, duration of daylight, season, as well as local landscape and weather, are, in many cases, parts and circumstances that would lead to a drop in the amount of usable energy (REHMAN; GHORI, 2000).

2.3.2 Wind Energy

Wind energy is a clean, low-cost, and abundant type of energy. Its energy conversion system is more cost-effective compared to other renewable energy conversion systems. As a result, it can provide immense economic benefits in areas with high wind densities (WEN; ZHENG; DONGHAN, 2009).

But it has some disadvantages, such as the significant amount of land required for the installation of wind turbines and other related wind energy facilities. On the other hand, the wind is intermittent and periodic, which would be a further addition to the concern about using this type of energy. Storage of the produced energy could be a possible issue, too, as storage units suffer from reliability and efficiency problems. Not to mention the climate change concern that could have a long-term impact on wind patterns (REN, 2010).

2.3.3 Hydropower Energy

Hydropower energy appears to be a potential renewable energy source. Approximately 20% of the world's electricity is generated by hydropower (2,700 TWh/yr) and represents the most significant source of supply for 55 countries globally (KAYGUSUZ, 2004). It is the main Brazilian energy matrix that handles around 65% of the generated electrical energy (EIA, 2021). Hydropower energy stands out for its accessibility, consistent and reliable technology when compared to other renewable energy sources (YÜKSEL, 2009).

Yet, the concept of harvesting energy from water flow may appear so straightforward that its consequences for the environment and inhabitants are frequently neglected. The social and environmental impact assessments receive insufficient attention (BENT; ORR; BAKER, 2002). Climate change vulnerability is a principal problem for this type of energy. For example, the rain shortage season may have an impact on stream level and flow, which will drastically reduce the amount of available energy produced by the hydroelectric dam (MORIARTY;

HONNERY, 2012). Failure of dam structures could be the worst condition. It affects not only the local biota but also the residents living nearby (AZARPOUR et al., 2013).

Various other problems are also raised, such as deforestation, change in water quality and hydrology, dam construction-related implications, and greenhouse gas emissions resulting from the decomposition of biomass carried and accumulated by the water flow over time (SOVACOOL; BULAN, 2011).

2.3.4 Geothermal Energy

Geothermal energy is the energy produced and stored beneath the Earth's surface. It referred to the internal structure of the Earth as its source. Humans have used this kind of energy since the age of ancient Rome in the public bath. Nowadays, it is used for power generation and heating purposes. It is affordable, efficient, and environmentally friendly (GLASSLEY, 2010).

Some disadvantages vary from the risk of land subsidence, inconvenience to local inhabitants, to the worst situation, induced by an earthquake (MORIARTY; HONNERY, 2011).

2.3.5 Biomass Energy

Biomass energy, which is produced from organic materials, is considered an environmentally-friendly energy source because of its much lower greenhouse gas emissions when compared to fossil fuels. Biomass energy is also recognized as a low-cost energy when compared to other types of energy, and that makes it more useful and economically efficient. However, it cannot meet the global need for energy due to the shortage of biomass supplies of different kinds (HONNERY; MORIARTY, 2007).

Not to mention other concerns such as the risks of food security (MAKARIEVA; GORSHKOV; LI, 2008), direct and indirect land-use change (SCHUBERT, 2009), biodiversity loss (HENNENBERG et al., 2010), water source availability (DE FRAITURE; GIORDANO; LIAO, 2008), and agricultural product costs (TILMAN et al., 2009) that must be taken into account when using this energy.

In general, the major challenge for renewable energy is to increase capacity to meet rising energy demand. With less than 20% of the total global energy contribution, it is still far from other energy sources. More reliable solutions should be searched for to meet the immense need for clean and renewable energy (AZARPOUR et al., 2013).

2.4 Biofuel

Biofuel is a renewable energy source that utilizes organic (bio-based) materials that can be used to replace petroleum-based fuels (biomass energy). It includes a diverse variety of fuels that arise from biomass. This term refers to solid biofuels such as biochar, which is charcoal produced from plant matter, liquid biofuels such as bioethanol, biodiesel, and biomethanol, and gaseous biofuels such as biogas, bio-syngas, and biohydrogen (BALAT, 2008; KONG et al., 2008).

The most common biofuels are bioethanol, which is produced from sugar-rich crops like sugarcane, wheat, and corn, and bio-diesel, which is produced from oil-rich seeds such as soya and palm oil, which are produced from traditional food crops that require good agricultural land to grow. These sugars and oils found in arable crops are used to make first-generation biofuels that can be extracted easily using conventional technology methods. However, second-generation biofuels that are made from lignocellulosic biomass such as woody crops, agricultural and forestry residues or wastes, are much harder to extract (DEMIRBAS, 2008a, 2008c, 2008d, 2009a; DEMIRBAS; DINCER, 2008).

The need for alternate technologies to produce biofuel that could potentially help achieve certain goals that include energy security, respect for environmental concerns, foreign exchange savings, some diversity in the fuel sector, adding market value to these agricultural residues, and not competing against food production and supply (BALAT, 2009; DEMIRBAS, 2009c). By developing and improving biotechnologies, we can expand the spectrum of biomass that we can use in the production of biofuels (AZARPOUR et al., 2013).

The warm climate and hydroelectrical availability at a relatively low cost help in reducing the production costs for ethanol in countries like Brazil, which is probably the lowest-cost producer in the world (DEMIRBAS, 2007, 2008b, 2008d; JONKER et al., 2015).

2.5 Lignocellulosic biomass

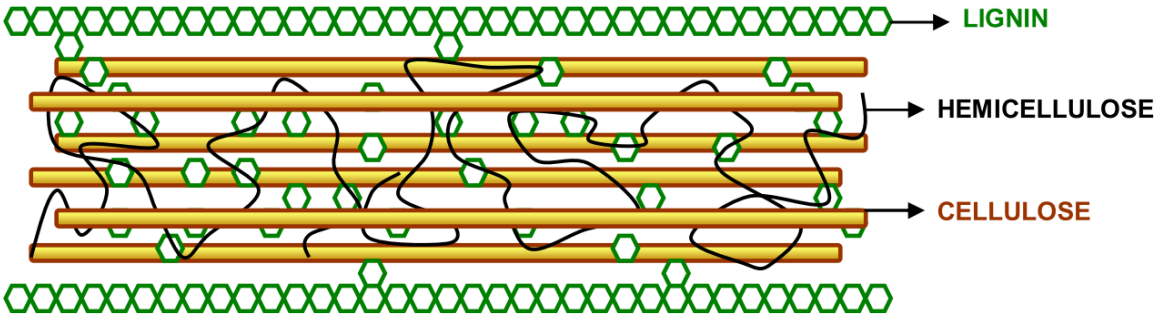
The most abundant bio-renewable resource in the world is lignocellulosic biomass (ZHOU et al., 2011). It is cheap, widely distributed, and readily available all over the world, and it is also recognized as a sustainable carbon source (LIN et al., 2018). These characteristics make it a great potential for obtaining products of economic value (TAARNING et al., 2011). Currently, this material is being explored in various biotechnology fields, such as the production of enzymes or biocatalysts (PERIYASAMY et al., 2018), biofuels, and other chemical products (NIE et al., 2018).

The principal component of plant biomass is the cell wall of plant cells (SOREK et al., 2014), which is formed mainly by three distinct elements: cellulose, hemicellulose, and lignin (Figure 1) (WALIA et al., 2017). The ratio of these three components is very relative and depends on many factors, such as the source of the biomass, type of plant tissue, and response to environmental conditions (KNOX, 2008; SERRANO-RUIZ; LUQUE; SEPÚLVEDA-ESCRIBANO, 2011; TAIZ et al., 2014).

The dominant part of lignocellulosic biomass is cellulose, which is also the most abundant polysaccharide in nature (SOMERVILLE, 2006). It is a linear homopolymer that has units of D-glucose joined by β (1→4) glycosidic bonds, among different orientations, with the general formula $(C_6H_{10}O_5)_n$ (STELTE et al., 2011). β-glucose dimers are called cellobiose and are considered the basic unit of the cellulose polymer (POLETTO; JUNIOR, 2015). Its structure comprises extensive networks of intramolecular and intermolecular hydrogen bonds that strongly bind glucose units to form microfibrils (ARO; PAKULA; PENTTILÄ, 2005; SOMERVILLE et al., 2010).

Depending on the organization and compaction of microfibrils, cellulose has two differentiated regions: crystalline and amorphous regions (Figure 2). The crystalline portions of cellulose are notably more stable and less susceptible to degradation by enzymatic action than the amorphous portions because of their higher organization and compression (MATHEWS; PAWLAK; GRUNDEN, 2015). The high compaction of glucose molecules also gives cellulose a high molecular mass, which results in its insolubility in water (BRANDT et al., 2013).

Figure 1 - Representation of lignocellulose structure showing cellulose, hemicellulose and lignin fractions.



Source: (MUSSATTO; TEIXEIRA, 2010).

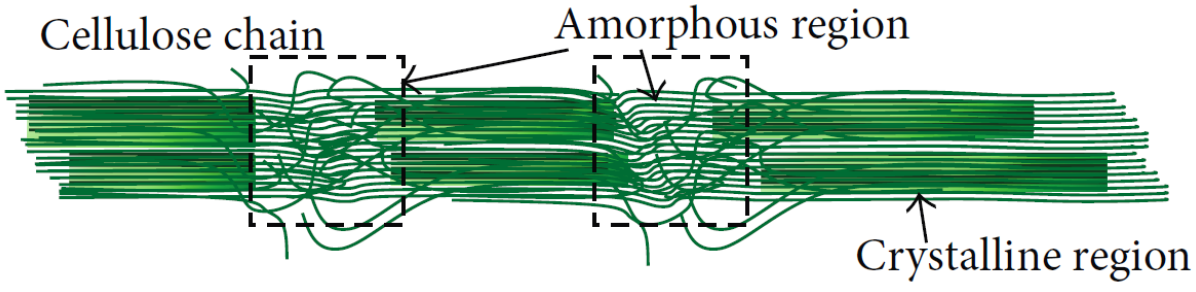
Hemicellulose represents 20 to 35% of the lignocellulosic biomass and is considered the second most abundant polysaccharide in nature (LUO et al., 2012). Its amorphous nature allows it to be hydrolyzed more easily than cellulose. Hemicellulose is a highly diverse

heteropolysaccharide composed of various types of sugars such as pentose (arabinose, xylose), hexoses (mannose, glucose, galactose, rhamnose), acetylated sugars, and uronic acids (α-D-galacturonic acid) (GÍRIO et al., 2010; ISIKGOR; BECER, 2015). It's commonly classified based on the predominant sugar residue present in the main chain. Xylan is the most prevalent type of hemicellulose, which has a main chain formed by xylose monomers joined via β bonds (1→4) (TERRONE, 2017). Therefore, xylose represents over 80% of the hemicellulose's structure (BIAN et al., 2012). And that's why xylose is one of the most abundant sugars on Earth.

Lignin is the most prominent polymer on Earth besides cellulose. It is an amorphous three-dimensional polymer that grants rigidity and resistance to the plant cell wall, which increases its integrity and promotes resistance to microbial attacks, mechanical damage, enzymatic degradation, oxidation, and seals the conducting vessels of the plant to ensure their protection (BROWN; CHANG, 2014; SANTOS et al., 2012). Lignin is almost based on phenylpropanoid units interconnected by stable C-C and C-O bonds (BOURBONNAIS; PAICE, 1988) and derived from the oxidative polymerization of hydroxycinnamoyl alcohol derivatives. The proportion of these derivatives results in different types of lignin (VOGT, 2010).

The robustness and recalcitrance of lignocellulosic biomass are associated with many factors, such as the cellulose crystallinity level, links between hemicellulose and lignin, and lignin composition. Thus, the delignification process is fundamental to facilitate the access of cellulolytic and hemicellulolytic enzymes during the saccharification process (LI et al., 2015).

Figure 2 - Different regions of cellulose.



Source: adapted from (LEE; HAMID; ZAIN, 2014).

2.6 Agro-industrial wastes

Annually, humanity produces massive quantities of agro-industrial waste. According to estimates, the global production of lignocellulosic biomass is approximately 3-5 gigatons per year (MURALI; SRINIVAS; AHRING, 2017). Latin America produces over 500 million tons/year of agro-industrial by-products and residues, and Brazil is responsible for more than half of this total (SOUZA; SOUZA; SANTOS, 2007).

For the most part, these residues are released into the environment without proper control procedures, making them a relevant cause of environmental pollution and having a harmful impact on different forms of life (SADH; DUHAN; DUHAN, 2018). Even though this type of waste is biodegradable, it demands minimal time to be degraded, and due to the high level of human activity on the planet, it becomes so hard for these wastes to be recycled naturally (BÔAS; ESPOSITO, 2000).

Lignocellulosic biomass refers to agricultural, agro-industrial, and urban waste (YANG et al., 2015). Bioconversion of lignocellulosic biomass wastes has become an essential issue nowadays due to the variety of sugars and aromatic derivatives that can be obtained and used as raw material for the employment of noble materials such as sugars, which give value to residues considered to be waste materials (LIGUORI; AMORE; FARACO, 2013).

These residues are composed of rich nutrients such as proteins, sugars, and minerals, besides holding an abundant and inexpensive amount of carbohydrates. Consequently, agro-industrial residues should not simply be considered as "waste" but as raw materials for the formation and development of different products with high added value (CARVALHO et al., 2002, 2005; SADH; DUHAN; DUHAN, 2018). Using these raw materials reveals the possibility of adding value to agricultural residues, diminishes the cost of production, and does not compete with the food supply (ROBAK; BALCEREK, 2018).

Sugarcane bagasse, rice husk, corncob, sawdust, rice straw, and wheat bran are some examples of residues generated in Brazilian agricultural activity, which are rich in pentoses and hexoses (MURUGAN et al., 2011; SADH; DUHAN; DUHAN, 2018; WIZANI et al., 1993).

Table 1 - Partial chemical composition of some Brazilian common agro-industrial wastes.

Agro-industrial wastes	Chemical composition (% w/w)			References
	Cellulose	Hemicellulose	Lignin	
Sugarcane bagasse	30.2	56.7	13.4	(EL-TAYEB et al., 2012)
Rice straw	39.2	23.5	36.1	(EL-TAYEB et al., 2012)
Corn stalks	61.2	19.3	6.9	(EL-TAYEB et al., 2012)
Sawdust	45.1	28.1	24.2	(MARTIN et al., 2012)
Soya stalks	34.5	24.8	19.8	(MOTTE et al., 2013)
Wheat straw	32.9	24.0	8.9	(MARTIN et al., 2012)
Orange peel	9.21	10.5	0.84	(RIVAS et al., 2008)
Coffee skin	23.77	16.68	28.58	(BALLESTEROS; TEIXEIRA; MUSSATTO, 2014)

Source: Modified from (SADH; DUHAN; DUHAN, 2018).

2.7 Inhibitors derived from hydrolysis of lignocellulosic biomass

Use of lignocellulosic biomass for biofuel production might help alleviate energy, food, and environmental crises. To take advantage of lignocellulosic feedstocks in producing biofuels and other commodities through biochemical conversion, it needs to pass through pretreatment processes to disrupt the close inter-component covalent or non-covalent bonds between constituents of the plant cell wall (YANG; WYMAN, 2008). And improve the susceptibility of available sugars to be used for saccharification and fermentation (JÖNSSON; MARTÍN, 2016).

Pretreatment processes are required to remove lignin, release hemicellulose carbohydrates, reduce cellulose crystallinity, and increase biomass porosity. Physical, chemical, physicochemical, and biological processes make up the majority of pretreatment operations today. Microorganisms are exposed to a variety of inhibitors during the biomass pretreatment and biofuel fermentation processes. These inhibitors suppress microbial growth, lowering biofuel yields and raising fermentation costs (LEE et al., 2017; WANG; SUN; YUAN, 2018).

Solubilization and degradation of hemicellulose and lignin are the main reasons for the formation of most inhibitors (Figure 3) (JÖNSSON; ALRIKSSON; NILVEBRANT, 2013). These inhibitors include aliphatic carboxylic acids, furans, and phenolic compounds. Also, the pretreatment solvents like ionic liquids, ethanol, and methanol would remain in the biomass slurries and inhibit microbial growth (LI et al., 2013; LYU et al., 2017; OLIET et al., 2002).

2.7.1 Furan aldehydes inhibitors

Furfural and hydroxymethylfurfural (HMF) are common furan aldehydes derived from the degradation of pentose and hexose, respectively (CHHEDA; ROMÁN-LESHKOV; DUMESIC, 2007; PARK et al., 2011).

The responsible pretreatment processes involved in the generation of furan aldehydes are acid-based techniques, such as acid hydrolysis, acid pretreatment, sulfite pulping, and hydrothermal techniques (AKOBI; HAFEZ; NAKHLA, 2016). Dehydration of the pentoses and the uronic acids produced by hydrolysis of the hemicelluloses results in 2-furaldehyde, commonly referred to as furfural, while dehydration of hexoses results in 5-hydroxymethyl-2-furaldehyde, henceforth referred to as HMF. Their concentrations in lignocellulose hydrolysates vary according to biomass type and pretreatment severity (PAN et al., 2016).

Yeast cell growth is slowed down and ethanol output is reduced when furan aldehydes are present in the medium. Furan aldehyde toxicity generally causes dose-dependent inhibition of glycolytic and fermentative enzymes (including pyruvate, acetaldehyde, and alcohol dehydrogenases) as well as the development of single-strand breaks in double-stranded DNA (HADI; SHAHABUDDIN; REHMAN, 1989). Furthermore, furan aldehydes may promote the rerouting of the cell's energy flux to damage repair while decreasing intracellular ATP and NAD(P)H levels (ALMEIDA et al., 2007).

Furfural might cause an accumulation of reactive oxygen species (ROS), which damage mitochondria, vacuole membranes, actin cytoskeleton, and nuclear chromatin, among other things (ALLEN et al., 2010). Furthermore, the toxicity of acetic acid and phenols could be boosted in the presence of furfural (ZALDIVAR; MARTINEZ; INGRAM, 1999).

2.7.2 Aliphatic acids inhibitors

Acetic acid, formic acid, and levulinic acid are the most common aliphatic acids detected in lignocellulose hydrolysate (ZHANG et al., 2016). Acetic acid is produced by the hydrolysis of acetyl groups in hemicellulose and lignin (LEE et al., 2017). Levulinic acid is formed during HMF degradation (ULBRICHT, 1984), while formic acid arises from HMF and furfural further degradation because of instability in the dehydrative medium under extreme pretreatment conditions, such as a long reaction time, high temperature, and acid concentration (DANON; VAN DER AA; DE JONG, 2013; FENGEL; WEGENER, 1989).

It has been shown that yeasts' specific growth rates and biomass yield can be reduced in the presence of acetic acid in media (PAMPULHA; LOUREIRO-DIAS, 2000). Also, acetic acid was able to enter yeast cells via facilitated diffusion by the aquaglyceroporin Fps1p

(MOLLAPOUR; PIPER, 2007). Thus, when the diffusion rate of aliphatic acids exceeds the efflux rate, the pH drops irreversibly, and the cellular functions stop, resulting in cell death (VAN DER POL et al., 2014).

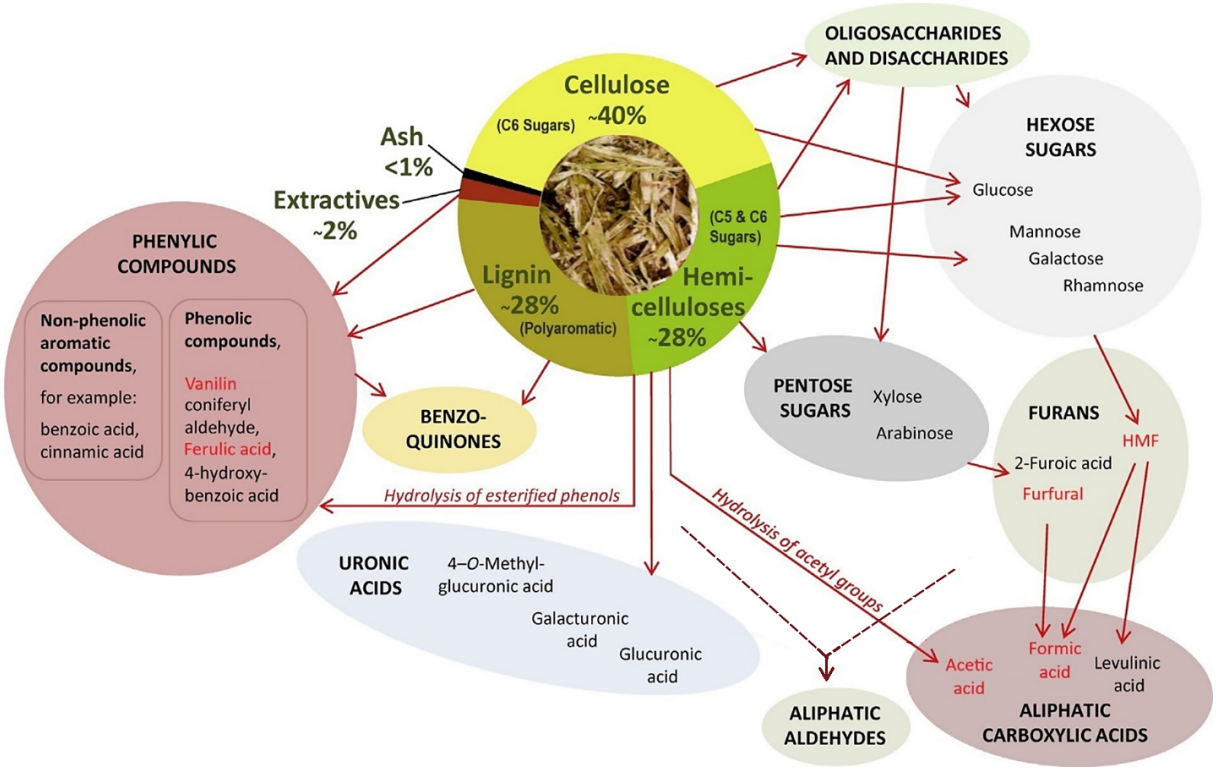
2.7.3 Phenolic compounds inhibitors

Phenolic compounds, including acids (ferulic acid, vanillic acids, 4-hydroxybenzoic acid, and syringic acid), alcohols (guaiacol, catechol, and vanillyl alcohol), and aldehydes (vanillin, syringic aldehyde, and 4-hydroxylbenzaldehyde), were mainly developed during the degradation of lignin and hemicellulose in biomass pretreatment using acid-based, hydrothermal, alkaline, and oxidative methods. The types of phenolic compounds depend on the sort of biomass and pretreatment conditions (DU et al., 2010; JÖNSSON et al., 1998; LARSSON et al., 1999; MARTÍN et al., 2002, 2007; MITCHELL; TAYLOR; BAUER, 2014).

Phenolic compounds could increase the lag phase and decrease the fermentation production of ethanol, lactic acid, and xylitol (CHEN et al., 2017; WANG et al., 2017; ZHANG et al., 2012). These compounds can penetrate the membrane, increasing its fluidity and damaging the cellular membrane by hydrophobicity, resulting in the leakage of intracellular elements, a change in protein-to-lipid ratio, and attenuating the function of selective barriers and enzyme matrices (KEWELOH; WEYRAUCH; REHM, 1990).

Phenolic compounds of lower molecular weight have higher toxicity levels because they could diffuse into the cell more rapidly and increase the inhibition severity of glucose assimilation (LIN et al., 2015). Moreover, they could increase the level of reactive oxygen species (ROS), which results in cytoskeleton damage, DNA mutagenesis, and programmed cell death (IBRAHEEM; NDIMBA, 2013).

Figure 3 - Degradation products from lignocellulosic biomass as a result of acidic pretreatment.



Source: adapted from (JÖNSSON; MARTÍN, 2016).

2.8 Xylose

Xylose ($C_5H_{10}O_5$), or wood sugar, is a monosaccharide of the aldopentose type that makes up the principal chain of Xylan, the most frequent variety of hemicellulose in plant cell walls, present in grass and hardwood plants (TERRONE, 2017). It is, without a doubt, the most abundant pentose sugar in lignocellulosic biomass and the second most abundant of all sugars after glucose, forming about 25% of the total dry weight of some forest and agricultural residues (MORI et al., 2015).

Xylose is an attractive sugar because it can be converted to ethanol, furfural, xylitol, organic acids, and other bio-products. Its fermentation is an essential step to improve the yield of ethanol production and other bio-products of biotechnological concern from lignocellulosic biomass (AMOAH et al., 2019; MARTINS et al., 2018).

Xylose-fermenting microorganisms are found amongst bacteria, yeast, and filamentous fungi. Anaerobic bacteria ferment pentoses (DIEN; COTTA; JEFFRIES, 2003; JEFFRIES; JIN, 2004). However, they are already hindered at low sugar and ethanol concentrations. Furthermore, significant by-product formation occurs during ethanolic fermentation, reducing ethanol yield. Although some natural yeast strains, such as *Pichia stipitis* CBS 6054, can turn

xylose into ethanol with a respectable yield and productivity, chemicals formed during the pretreatment and hydrolysis of the lignocellulose material inhibit these yeasts. Filamentous fungi are too slow for a competitive industrial process, despite their ability to tolerate inhibitors (KOMESU et al., 2020).

Nevertheless, xylose is not as easily fermented as glucose (WEN et al., 2016). Native strains of the yeast *S. cerevisiae* are generally incapable of fermenting pentoses since the xylose-to-ethanol conversion requires particular membrane transporters and specific enzyme expression, which do not exist or are not expressed by these strains (MCMILLAN, 1993). Still, some yeasts can use xylose as a carbon source, which indicates the existence of a transport system and intracellular enzymes to metabolize it (VAZ, 2020).

Therefore, efforts have primarily been directed at obtaining recombinant strains of bacteria and yeast that can handle lignocellulose fermentation processes and meet the industrial criteria (HAHN-HÄGERDAL et al., 2006). However, larger cell size, thicker cell walls, better growth in acidic pH, less rigorous nutritional requirements, and better contamination resistance give yeasts an edge over bacteria in industrial fermentations (JEFFRIES, 2006).

2.8.1 Xylose assimilation and metabolism in yeasts

Yeasts are chemoorganotrophic microorganisms that employ a wide variety of organic substances as carbon sources, including hexose and pentose sugars, glycerol, and sorbitol, among many others. Nevertheless, various species differ in their ability to assimilate these molecules and how they metabolize them, whether through respiratory or fermentation pathways, in the course of generating energy for their vital processes (MARTINS, 2011).

From this perspective, the discovery of xylose-fermenting yeast in the early 1980s was regarded as a turning point in biotechnology, allowing this sugar to be more effectively used. Many xylose-fermenting yeasts have been reported since then (JEFFRIES, 1981; SCHNEIDER et al., 1981; SLININGER et al., 1982).

The yeast genera most frequently mentioned as xylose assimilators via oxidative or fermentative pathways include *Brettanomyces*, *Candida*, *Clavispora*, *Kluyveromyces*, *Meyerozyma*, *Pachysolen*, *Pichia*, and *Schizosaccharomyces* (CHANDRAKANT; BISARIA, 1998; GONG et al., 1981; MCMILLAN, 1993). Between 2005 and 2018, around 85 research papers have been published using *Scheffersomyces stipitis* specie in pentose fermentation. Other species are investigated on a less regular basis, such as *Spathaspora arborariae* (CUNHA-PEREIRA et al., 2011; HICKERT et al., 2014), *Pichia guilliermondii* (FAN et al., 2013), *Hansenula polymorpha* (VORONOVSKY et al., 2009), *Rhodotorula* sp (MARTINS, 2011),

Pichia ofunaensis (KURTZMAN, 1998; MARTINS, 2011; ROBERT; SMITH, 2011) and *Trichosporon multisporum* (GUAMÁN-BURNEO et al., 2015; MARTINS, 2011).

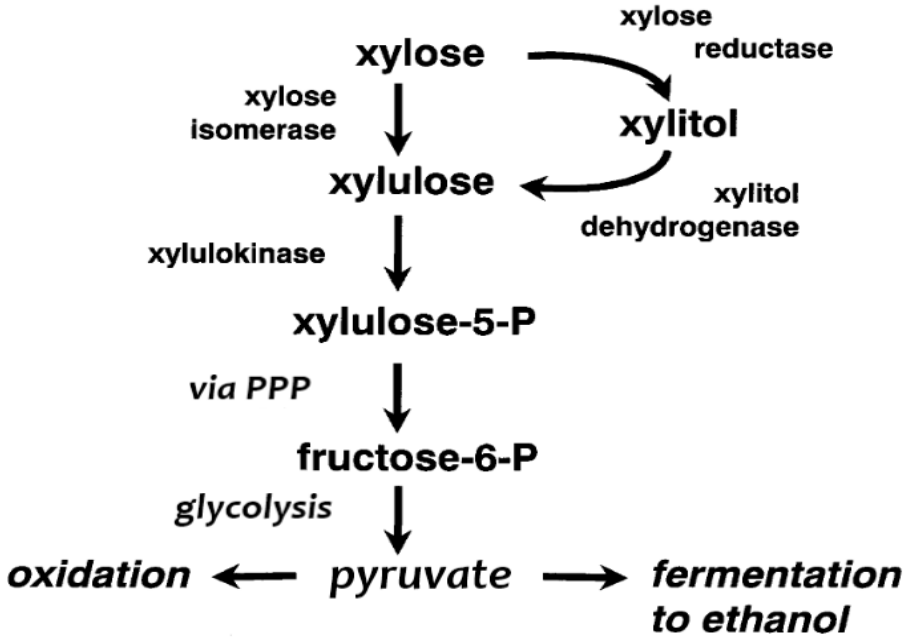
Scheffersomyces (Pichia) stipitis, *S. (Candida) shehatae*, *Pachysolen tannophilus*, *Rhodotorula* sp., and *Pichia guilliermondii* have all been described as yeasts capable of converting xylose to ethanol. Still, some are mentioned in the literature as being better for achieving higher levels of alcoholic fermentation yields from xylose, while others are not. However, none has achieved a reasonable yield level for industrial uses until now (BAJWA et al., 2011; BALAT; BALAT; ÖZ, 2008; MARTINS, 2011; MCMILLAN, 1993; WEBB; LEE, 1990).

The initial steps of xylose metabolism may follow two pathways (Figure 4): microorganisms that have xylose isomerase enzyme (prevalent in bacteria) convert D-xylose into D-xylulose, which is easily converted into xylulose-5-phosphate, that enters the Pentose-Phosphate pathway (PPP), generating fructose-6-phosphate, which, through glycolysis, is converted into pyruvate.

The second pathway (prevalent in yeasts, filamentous fungi, and other eukaryotes) of D-xylose is catalyzed by the xylose reductase enzyme, generating xylitol, which, through xylitol dehydrogenase, is converted into xylulose-5-phosphate, which also follows via PPP, and then, via glycolysis, is converted into pyruvate. In the absence of oxygen, yeasts reduce pyruvate to ethanol and CO_2 (JACKSON; NICOLSON, 2002; JEFFRIES, 2006).

The xylose reductase enzyme, which converts xylose into xylitol, can use NADPH and NADH, with a preference for NADPH. While xylitol dehydrogenase, which produces xylulose from xylitol, depends on NAD^+. According to the cell's redox balance (the balance between NADPH and NAD^+), xylitol can be released into the external medium, resulting in low ethanol output. In this manner, the cell's redox potential can determine the extracellular product obtained in the xylose fermentation process (MONTEIRO, 2020).

Figure 4 - Metabolic pathways for xylose catabolism.



Source: adapted from (JACKSON; NICOLSON, 2002).

2.8.2 Xylose transport in yeasts

Molecules' transport across the membrane and its related biochemical aspects are a fundamental part of any organism's physiology. The facilitated and active transport systems described in prokaryotes and/or eukaryotes are: 1) facilitated diffusion (passive transport, no energy needed); 2) active transporters using electrochemical energy, redox or light potential, coupled to ions or by ATP hydrolysis; 3) transport of solutes (ions, protons, small hydrophobic molecules) mediated by pore-forming channels across the membrane (CONDE et al., 2010).

The assimilation of pentoses and high-yield microbial growth using these sugars as the only carbon source assumes the existence of an efficient transport system across the membrane that does not limit the catabolic pathway. D-xylose transport in yeast is accomplished via low and high affinity systems. Among these systems are the facilitated diffusion and the symporter mechanism linked to proton cotransport and depend on the nutritional status of the yeast. For example, *Rhodotorula glutinis* has at least one low and one high-affinity transport system for D-xylose. The high-affinity system is repressed when yeast grows in xylose and derepressed in starvation. Both systems are totally repressed by glucose, indicating that they are the same ones that transport this sugar (KILIAN; VAN UDEN, 1988).

On the path to achieving second-generation ethanol, many recent studies have focused on genetically engineering *S. cerevisiae* to produce ethanol from xylose as well (MOON et al., 2013). Several xylose-specific membrane transporters and enzymes found in yeasts that assimilate this pentose and produce ethanol more efficiently have been cloned and expressed (SHARMA et al., 2018).

However, xylose transport is restricted in the presence of glucose through competitive inhibition when both of them are available. Yeasts use glucose first and then xylose, performing a diauxic growth and comprising two lag phases (LEE et al., 2002). Therefore, for decades, the barrier for researchers has been the inhibition of xylose and other pentose assimilation in the presence of glucose within ethanol-producing strains, which prevents co-fermentation of both sugars (JEFFRIES; JIN, 2004).

Most metabolic engineering approaches focus on internal cellular pathways, such as replacing low-capacity enzymes with more active versions or even inserting genes to create new pathways or optimize those that are not very expressive in the native microorganism (MATSUSHIKA; SAWAYAMA, 2010). However, the proteins involved in the transmembrane transport system of pentoses haven't been explored sufficiently, and few tools have been developed to modify them (BETTIGA et al., 2009).

Although successful engineering works on *S. cerevisiae* with the expression of up to six key xylose metabolic enzymes (XI, XK, TAL, TKL) (KUYPER et al., 2005) have increased xylose consumption or fermentation, xylose transport still requires optimization to achieve a sufficient sugar flux to maintain the optimized cytoplasmic pathway.

Several studies have focused on improving pentose transport in *S. cerevisiae* via expression of heterologous transporters (SILVA et al., 2019; DU; LI; ZHAO, 2010; LEANDRO; SPENCER-MARTINS; GONÇALVES, 2008; SALOHEIMO et al., 2007; WEIERSTALL; HOLLENBERG; BOLES, 1999).

Therefore, despite some successful investigations describing the production of ethanol from xylose fermentation by engineered strains of *S. cerevisiae*, the yield achieved by these engineered strains is similar to that of xylose-fermenting species. The use of xylose is slow and only occurs after glucose depletion, thus limiting the economic viability of industrial fermentations to produce ethanol (FARIA et al., 2014; HECTOR et al., 2013).

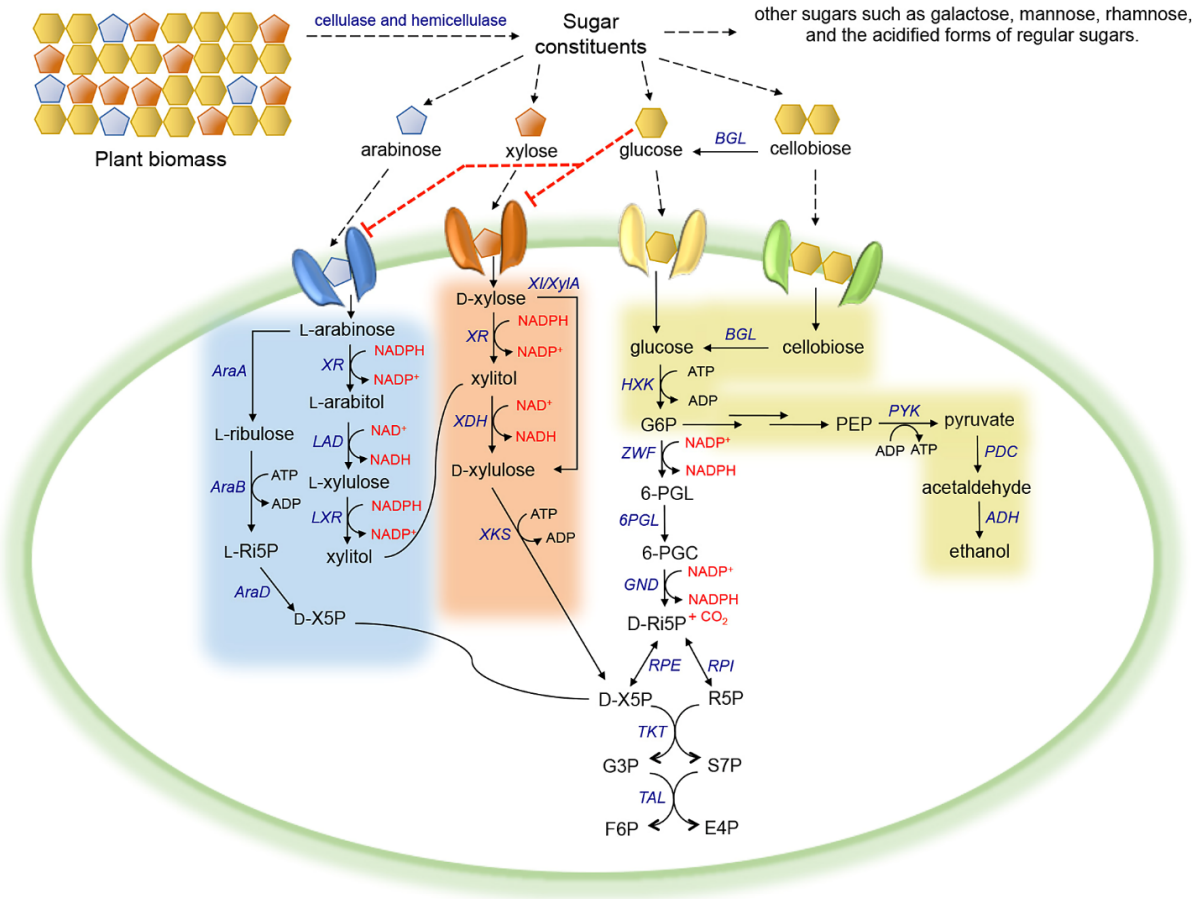
Yeasts known to assimilate xylose efficiently have served as a source of genes for metabolic engineering of *S. cerevisiae*. Alternatively, these yeasts can also be used to obtain products with high added value, such as the production of glycolipid biosurfactants (FARIA et al., 2014), as well as the production of xylitol and arabitol (GÍRIO et al., 2000). In addition, the

production of alternative compounds, like organic acids (CHEN; ZHU; XIA, 2014; YE et al., 2013; ZHAO et al., 2013), lipids for biodiesel production (BABAU et al., 2013; GAO et al., 2013; IVERSON et al., 2013), isobutanol (MONDALA et al., 2013) and hydrogen (BRAT; BOLES, 2013).

Consequently, investigations that consider evaluating the capacity of xylose consumption by other yeasts are of exceptional contribution to this area of biotechnology since these yeasts can be explored in-depth for a greater understanding of the mechanisms of xylose assimilation and, in future studies, enable heterologous expression of more specific transporters for xylose without competitive inhibition by glucose.

Figure 5 shows an idealized virtual *S. cerevisiae* and explains how it is vital to look for specific pentose transporters, which, when combined with its already efficient D-glucose transporters, will enhance the organism's ability to metabolize pentoses and hexoses found in lignocellulosic material.

Figure 5 - Yeast with ideal properties for fermenting plant biomass pentose and hexose sugars.



Source: (GAO; PLOESSL; SHAO, 2019).

2.9 Biotechnological applications

About 200 value-added compounds can be obtained from lignocellulosic biomass, which is therefore benefitted by its high availability and low cost. Nonetheless, producing these chemicals with increased selectivity and yields at reasonable prices and on industrial scales remains a huge challenge to be overcome (AREVALO-GALLEGOS et al., 2017; KUMAR et al., 2018a; MORGAN et al., 2017).

The pentoses derived from the plant biomass with the higher levels of oxygen present in the biomass make it more suitable for the production of plenty of these value-added chemicals, such as biobutanol, biohydrogen, biopolymer, 2, 3- butanediol, 1, 3- propanediol, organic acids, xylitol, furfural, single-cell protein, and amino acids, among many others (KUMAR et al., 2018b).

Xylitol ($C_5H_{12}O_5$) is a polyol with a high sweetening capacity and fewer calories when compared to sucrose. Because of its anti-diabetic, antioxidant, and anti-carcinogenic properties, it is applied in the food and dental products industries (DASGUPTA et al., 2017). ALBUQUERQUE et al. (2014) discussed its production from corncob by *Candida tropicalis*.

Bioethanol is a clean non-petroleum liquid fuel that reduces CO_2 and greenhouse gas emissions. Bioethanol is used as a transportation fuel in Brazil, the USA, and some other places in the world. And as an additive to gasoline to improve its consumption and burning process. Many fungal and bacterial species, including *Fusarium*, *Rhizopus*, *Neurospora*, and *Klebsiella*, *Bacillus*, and *Aeromonas*, have been reported as ethanol producers from pentose (CHANDEL; SINGH; VENKATESWAR RAO, 2010; HILL et al., 2006; KIM et al., 2009; KUNDU; LEE, 2016).

2,3-Butanediol (2,3-BD) is a significant chemical organic compound. Its toxicity level compared to other alcohols is low. 2,3-BD is applied in various industries such as agrochemicals, pharmaceuticals, plasticizers, perfumes, etc. (LEE; LAVOIE, 2013; NANDA et al., 2015). KIM et al. (2014) reported the xylose conversion to 2,3-BD by engineered *S. cerevisiae.*

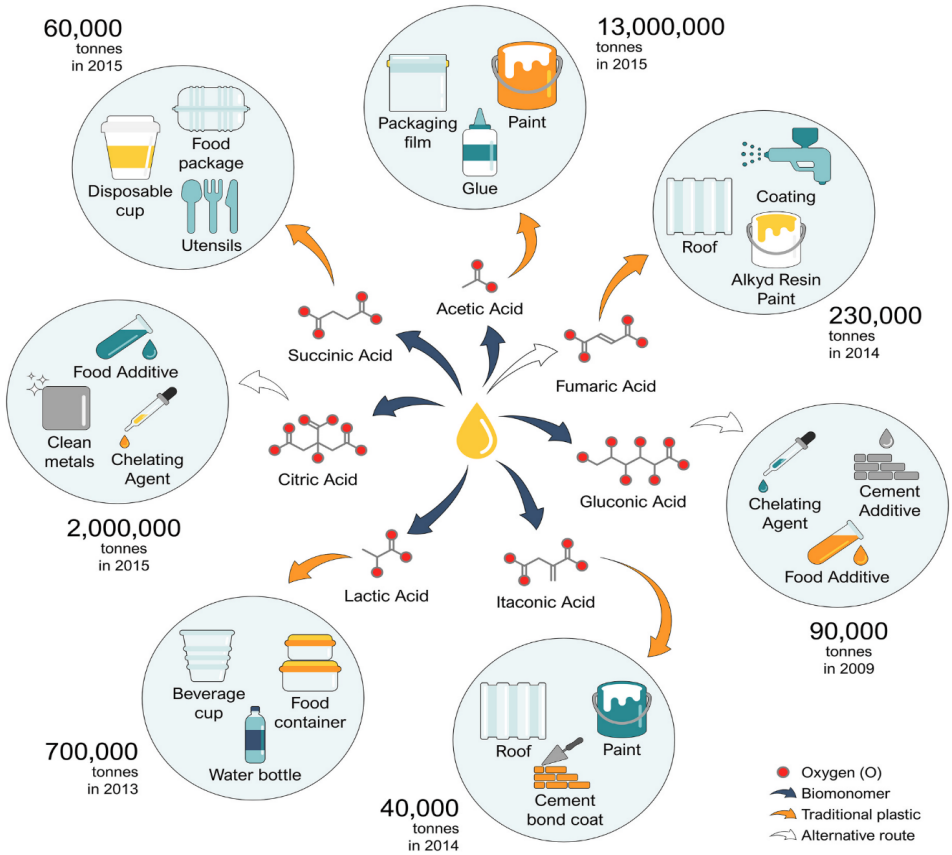
Organic acids such as lactic acid, acetic acid, citric acid, itaconic acid, fumaric acid, gluonic acid, succinic acid, xylonic acid, among others, are very important chemical products with several industrial applications in food, cosmetics, textiles, pharmaceuticals, chemicals, etc. They can be generated by many microorganisms, including bacteria, fungus, yeast, cyanobacteria, and algae. Each biocatalyst of these has outperformed the others in one or more ways, such as a broader substrate range, higher yield and productivity, fewer nutritional needs,

or enhanced purity (ABDEL-RAHMAN; TASHIRO; SONOMOTO, 2013; CARVALHEIRO; DUARTE; GÍRIO, 2008; CHANDEL; SINGH; VENKATESWAR RAO, 2010; MUSSATTO et al., 2010). *Glucanobacter oxydans* converts non-glucose sugars present in the biomass into sugar acids. These non-glucose mixed sugar acids (xylonate, arabonate, mannonate, and galactonate) exhibited cement retarding setting properties (YAO; HOU; BAO, 2017).

As environmental awareness has grown in recent years, the need for biopolymers that degrade has also increased. The possibility of producing bio-based polymer materials from lignocellulosic biomass organic acids to substitute petroleum-based plastics makes the process much more viable economically and environmentally, which has increased the interest in these organic acids lately (MAGALHÃES JÚNIOR et al., 2021; SINDHU et al., 2013). Figure 6 shows selected organic acids, their main applications in the polymer industry, and their production volumes.

Other products, like furfural, which are used in the production of different chemicals and solvents, amino acids, single-cell protein, and yeast extract are compounds with intriguing applications in various industries like animal feed, additives, flavor enhancers, cosmetic ingredients, pharmaceuticals, and others (KUMAR et al., 2018b).

Figure 6 - Some Organic acids produced from lignocellulosic biomass hydrolysates and their main applications.



Source: (MAGALHÃES JÚNIOR et al., 2021).

3 OBJECTIVES

3.1 General Objective

Explore the possibility of future improvement and potential biotechnological application of two pentose-assimilating yeasts isolated from the environment.

3.2 Specific Objectives

Evaluate the yeasts' tolerance to furfural, HMF, ferulic acid, vanillin, acetic acid, and formic acid.

Evaluate the ethanol production capacity during alcoholic fermentation in the YEPX medium.

4 MATERIALS AND METHODS

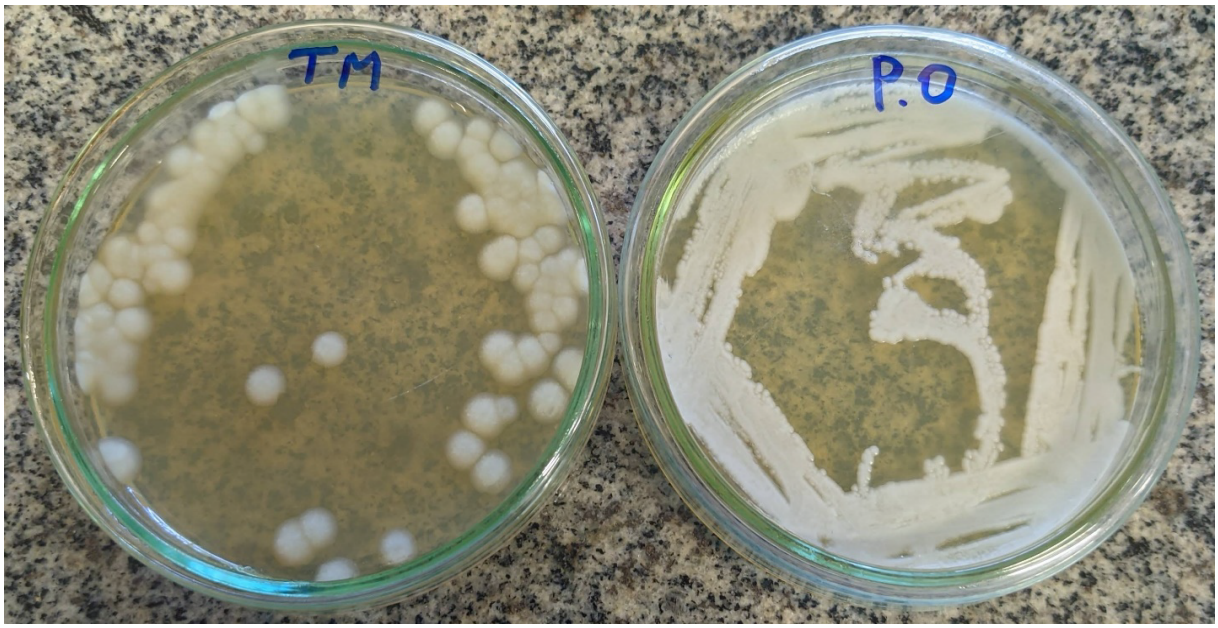
4.1 Microorganisms

In this work, strains of the yeasts *P. ofunaensis* 1A-14 and *T. multisporum* 1A-10 (Figures 7 and 8), were isolated from the environment and stored in the working collection of the Laboratory of Biochemistry and Applied Microbiology, IBILCE/UNESP, in São José do Rio Preto (VAZ et al., 2021).

The species identification was carried out by researchers from the Microbiology Laboratory of the Center for the Study of Social Insects (CEIS) under Prof. Dr. Fernando Carlos Pagnocca's coordination, through sequencing of the rDNA D1/D2 domains (PAGNOCCA et al., 2008).

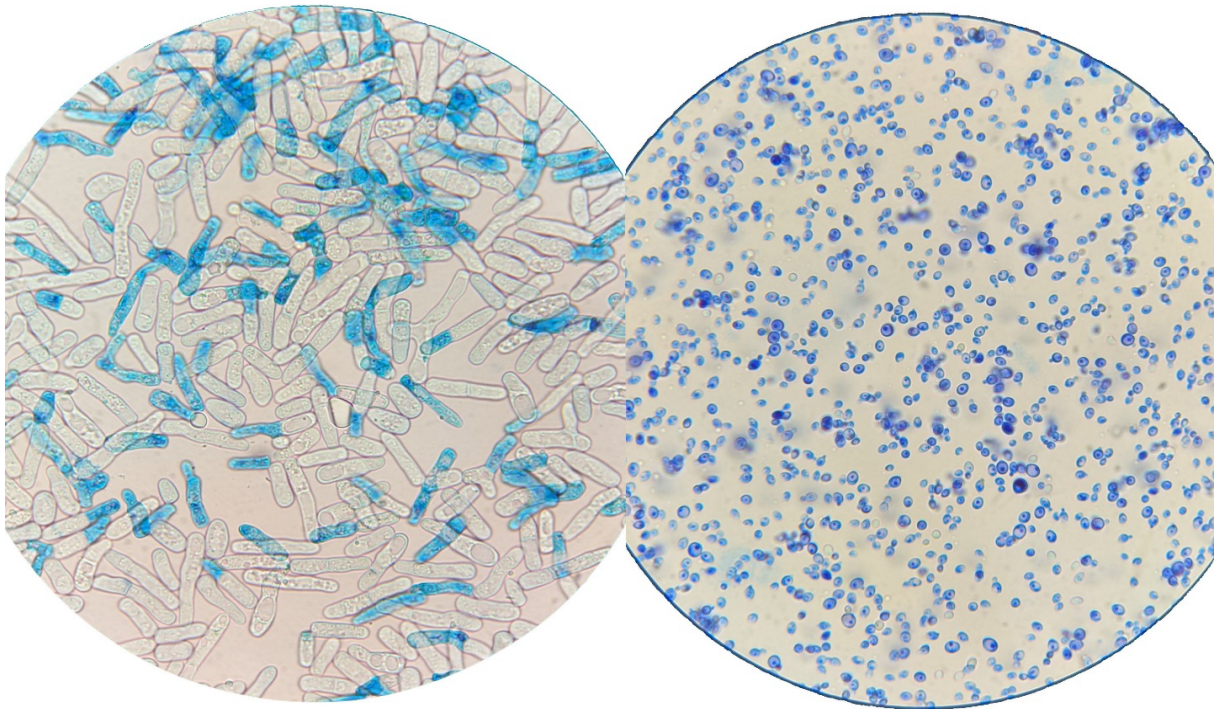
The conservation of pure cultures was carried out for storage at -80 °C. Glass beads washed with nitric acid and sterilized were added to the yeast suspension, which was kept overnight in glycerol (15%) in the freezer at -20 °C and then transferred to a freezer at -80 °C.

Figure 7 - *P. ofunaensis* (right) and *T. multisporum* (left) yeasts in a Petri dish.



Source: Own Authorship.

Figure 8 - *P. ofunaensis* (right) and *T. multisporum* (left) yeasts under microscope.



Source: Own Authorship.

4.2 Strains cultivation and quantification of growth in xylose

Depending on the experiments carried out by Vaz (2020), which tested the most suitable growth conditions for yeasts *P. ofunaensis* and *T. multisporum* in different temperatures (28 º, 32 º, 36 ºC) and different pH levels (4.5, 6.5, 8.5) in the culture media, the results of these experiments showed that the most proper conditions for the growth of these two yeasts are a 28 ºC temperature and pH level of 4.5, so in this work, these same conditions have been applied for tolerance test experiments.

4.2.1 The pre-inoculum preparation

Cells of *P. ofunaensis* and *T. multisporum* were removed from the stock in a freezer at -80 ºC and grown in a petri dish containing 30 mL of YEPD with the following composition (g/L): yeast extract (10.0), peptone (20.0), glucose (20.0) and agar (15.0), for 48 hours at 28 ºC. Subsequently, one colony was resuspended by gently scraping the culture surface on the plate of each species in 100 mL of liquid YEPD medium contained in 250 mL of Erlenmeyer, for 24 hours at 28 °C under 150 rpm agitation.

The pre-inoculum was centrifuged at 10,000 xg for 10 minutes at 4 °C. The supernatant was discarded, then the pellet was resuspended in 200 mL of sterile distilled water and centrifuged under the same conditions mentioned above. Then the supernatant was discarded, and the biomass was resuspended in 20 mL of sterilized distilled water. This cell suspension was used to inoculate the culture medium.

4.2.2 Cultivation of microorganisms in media containing xylose and glucose

To assess the xylose's best growth condition, the submerged yeast culture was performed in a 250 mL Erlenmeyer containing 100 mL of YEPX medium with the following composition (g/L): yeast extract (10.0), peptone (20.0) and xylose (20.0), at 28 ºC and 150 rpm for 96 hours, with pH adjusted to 4.5 using ortho-Phosphoric acid 85% (H_3PO_4). For comparison purposes, the microbial growth in medium containing glucose 20 g/L (YEPD) and base YEP (20 g/L peptone and 10 g/L yeast extract) was also evaluated under the same growth conditions. All culture media were inoculated with cell suspension to initiate growth with an optical density (O.D.) of 0.1 at 600 nm. All cultivations were carried out in duplicate (n=2).

4.2.3 Growth quantification, pH measurement, and sugar assimilation of culture media containing xylose

Aliquots of 1.5 mL were taken every 4 hours for 12 hours a day, for a total time of 96 hours for the experiment. The samples were shaken, and the optical density (O.D.) was measured in a spectrophotometer at 600 nm in triplicate (n=3). The results were expressed in base 10 log. After that, the samples were centrifuged at 8,000 rpm for 10 min to discard the biomass.

Finally, the pH of the medium and the consumption of sugar were evaluated. pH was measured at every point for the first 12 hours and after that, every 12 hours.

The evaluation of sugar assimilation by the yeasts *P. ofunaensis* and *T. multisporum* in the YEPX media was performed through the measurement of reducing sugars using the DNS method (3,5-dinitrosalicylic acid) (MILLER G.L., 1959). The tests were carried out in triplicate for each culture replicate using 10 µL of the supernatant from the centrifuged medium, diluted in 90 µL of water and 100 µL of DNS, conducted by a thermal cycler for 10 minutes at 95 ºC, followed by cooling at 4 ºC for 1 minute, and then measured in a spectrophotometer at 540 nm.

4.2.4 Standardization of the inoculum

The fermentation was carried out in 125 mL Erlenmeyer flasks containing 25 mL of YEPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) for 24 hours at 28 °C with agitation of 150 rpm. During the fermentation time, dilutions of the culture were made, and absorbances were measured at 600 nm. After that, the same suspensions were centrifuged at 10,000 xg for 15 minutes, with the supernatant discarded. Then, the cells were suspended in distilled water and centrifuged again. Finally, the cell pellet was kept in an oven at 60 ºC until it reached a constant weight.

Dependent on these data, a calibration curve was constructed, plotting the absorbance values representing the corresponding cell density and dry weight, obtaining a linear regression equation. This equation was used to estimate the cell mass to be used for inoculum and monitor cell growth during cultivation in fermentation media.

The determination of cell viability was monitored using the erythrosine B staining method and counted in a Neubauer chamber. Viable cells were colorless, while non-viable cells were red (KAMILOGLU et al., 2020).

4.2.5 Yeast tolerance test to different inhibitor compounds

The inhibitor concentrations applied in these tolerance tests depend on the amount of these inhibitors formed during the pretreatment processes of lignocellulosic biomass. Pacheco et al. (2021) reported a furfural concentration of 0.20 g/L in a sugarcane bagasse hydrolysate pretreated with diluted sulfuric acid. Deacetylated corn stover with sulfuric acid pretreatment analyzed by Yang et al. (2018) had 0.17 g/L. HMF concentrations were reported at 0.02 g/L by Pacheco et al. (2021) and 0.12 g/L in sugarcane bagasse pretreated hydrothermally by Zetty-arenas et al. (2019), who also showed the presence of 0.18 g/L of formic acid in the same sample.

In spruce hydrolysate, Adeboye et al, (2014) found 0.043 to 0.045 g/L of ferulic acid and 0.036 g/L of vanillin, while Yang et al. (2018) found 0.09 g/L of vanillin in deacetylated corn stover. Cola et al. (2020) counted the existence of ferulic acid (0.26 mM - 0.29 mM) and acetic acid (74.14 mM - 137.89 mM) in the liquor of two samples of sugarcane bagasse pretreated by a steam explosion (STEX) with dilute phosphoric acid.

Therefore, tested concentrations correspond to those that would be verified in pretreatment processes of lignocellulosic hydrolysates. In the YEPX medium, yeast tolerance was tested for furfural, HMF, ferulic acid, vanillin, acetic acid, and formic acid. The compounds and their tested concentrations are listed in table 2. As a control, YEPX medium was used but no inhibitor was added. The incubation and growth evaluation were carried out as previously described.

Table 2 - Analyzed inhibitor compounds and tested concentration range.

Inhibitor	Concentrations ($g.L^{-1}$)					References
Furfural	0.192	0.480	0.960	1.441	1.921	(PACHECO et al., 2021)
HMF	0.252	0.630	1.261	1.891	2.522	(PACHECO et al., 2021)
Vanillin	0.152	0.304	0.456	0.760	1.521	(YANG et al., 2018)
Ferulic acid	0.097	0.194	0.388	0.582	-	(COLA et al., 2020)
Acetic acid	1.201	2.402	3.603	4.804	-	(COLA et al., 2020)
Formic acid	0.460	0.920	1.380	1.841	-	(ZETTY-ARENAS et al., 2019)

4.2.6 Yeast tolerance test to combined inhibitor compounds

After individual assessment of each inhibitor's effect on the yeasts' performance in growth and xylose consumption aspects, the impact of these inhibitors was tested once more,

but this time concurrently, as a way of simulating a liquid hydrolyzate that results from the pretreatment processes in which these six inhibitors, among others, might be produced.

The culture media were prepared using the following inhibitors' concentrations: 5 mM furfural, 5 mM HMF, 2 mM ferulic acid, 2 mM vanillin, 20 mM acetic acid, and 10 mM formic acid, which were almost the least tested concentrations individually of these inhibitors.

4.3 Ethanol production assay

For ethanol production evaluation, alcoholic fermentation was assayed in 125 mL Erlenmeyer flasks adapted for alcoholic fermentation, closed with a valve containing 5 mL of sodium metabisulfite solution at 1 g/L to confirm that no oxygen could enter. 25 mL of saline culture medium, composed of KH_2PO_4 (2.0 g/L), $(NH_4)_2SO_4$ (2.0 g/L), $MgSO_4 \cdot 7H_2O$ (1.0 g/L), urea (0.3 g/L), $CaCl_2$ (0.3 g/L), yeast extract (10.0 g/L), and xylose (50.0 g/L) without inhibitor compounds (MARTINS et al., 2018). The tests were carried out in duplicate at 28 ºC and 150 rpm. The initial cell inoculation was 0.5 O.D. at 600 nm, while the initial pH was 4.5. Two fermentation flasks of each yeast were collected at 24, 48, 72, and 96 hours of fermentation, analyzed for their cellular growth, residual sugar, and pH, and then preserved frozen at -20 ºC.

4.4Ethanol identification and quantification

The detection and quantification of ethanol were performed by a capillary column BD-EN14103 of 30 meters in length, 0.32 mm in diameter, and 0.25 µm in film thickness coupled to PerkinElmer Clarus 480 Gas Chromatography. The initial temperature was 40 °C, and after that, the heating rate was increased by 15 °C/minute up to 150 °C. The total runtime was 7 minutes and 33 seconds. The injector temperature was 200 °C, and the detector temperature was 250 °C.

Samples with 5 mL of fermentation media were prepared using headspace vials. After that, the samples were heated for 10 minutes at 40 °C. Then a gas-tight 500 µL syringe was used to inject 100 µL of the vapor in splitless mode. The flow rates of H_2 and O_2 (compressed air) were 45 mL/min and 450 mL/min, respectively. The carrier gas used in this experiment was N_2, with a flow rate of 1,7 mL/min.

The quantification was performed with a calibration curve constructed from the analysis of (ethanol:water) solutions in different proportions (0.05, 0.1, 0.2, 0.5, 1.0%, w/w).

5 RESULTS & DISCUSSIONS

5.1 Furan aldehydes inhibitors

5.1.1 Furfural effect on cellular growth and xylose consumption

Yeasts *P. ofunaensis* (Figures 9A and 10A) and *T. multisporum* (Figures 9B and 10B) were able to grow and consume xylose in fermentative YEPX culture media containing furfural in several concentrations. Table 3 shows, in a condensed form, tested concentrations, achieved optical densities (OD), xylose consumption, and pH data gathered after 96 hours of fermentation of YEPX culture media containing furfural as inhibitor.

Table 3 - Optical density and pH of YEPX culture media containing furfural after 96 hours of incubation and sugar consumed during fermentation by *P. ofunaensis* and *T. multisporum*.

Specie	Furfural Concentration	Final **OD**	Final **pH**	Sugar Consumption
P. ofunaensis	0 mM	6.48	4.67	100% (84h)
	2 mM	6.63	4.55	100% (84h)
	5 mM	7.59	4.59	100% (84h)
	10 mM	7.22	4.66	100% (84h)
	15 mM	6.71	4.64	100% (96h)
	20 mM	2.29	4.54	43% (96h)
T. multisporum	0 mM	3.40	6.19	50% (96h)
	2 mM	3.07	6.01	42% (96h)
	5 mM	2.86	5.95	40% (96h)
	10 mM	2.63	6.03	42% (96h)
	15 mM	1.57	6.15	41% (96h)
	20 mM	0.10	4.65	15% (96h)

Source: own authorship.

The yeast *P. ofunaensis* in the fermentative culture media containing concentrations 2 and 5 mM of furfural exhibited similar behavior to the medium without inhibitor (control), it quickly moved from the lag to the exponential phase, where it showed an accelerated increase in the growth rates during the first 24 hours, followed by a continuation of growth yet with lower rates during the second 24 hours. At 48 hours after the beginning of fermentation, the growth rates moderately stabilized and entered the stationary phase, which continued until the end of the assay after 96 hours.

The medium with a 10 mM concentration took the yeast about 12 hours to move from the lag phase to the exponential phase when the growth rates continued to rise quickly, and they moved to the stationary phase 48 hours after the initiation of the fermentative process. It finished the assay with growth rates close to the control and the other two previous concentrations. At a 15 mM medium concentration, the yeast needed 36 hours to move from

the lag phase to the exponential phase, when the growth rates started to rise rapidly until the 72nd hour of the experiment. But after that, it continued to grow at a slower pace.

While the culture medium with the 20 mM concentration required about 76 hours to move from the lag to the exponential phase, the growth rates started to increase significantly after 80 hours from the start of the fermentative process, which continued to rise until the end of the experiment after 96 hours.

The yeast *T. multisporum* in the fermentative culture media containing a concentration of 2 mM of furfural exhibited a slight decrease in behavior compared to the medium without the inhibitor (control). It quickly moved from the lag to the exponential phase, where it showed an accelerated increase in the growth rates during the first 32 hours, followed by a continuation of growth yet with lower degrees when its rates moderately stabilized and entered the stationary phase, which continued until the end of the assay after 96 hours.

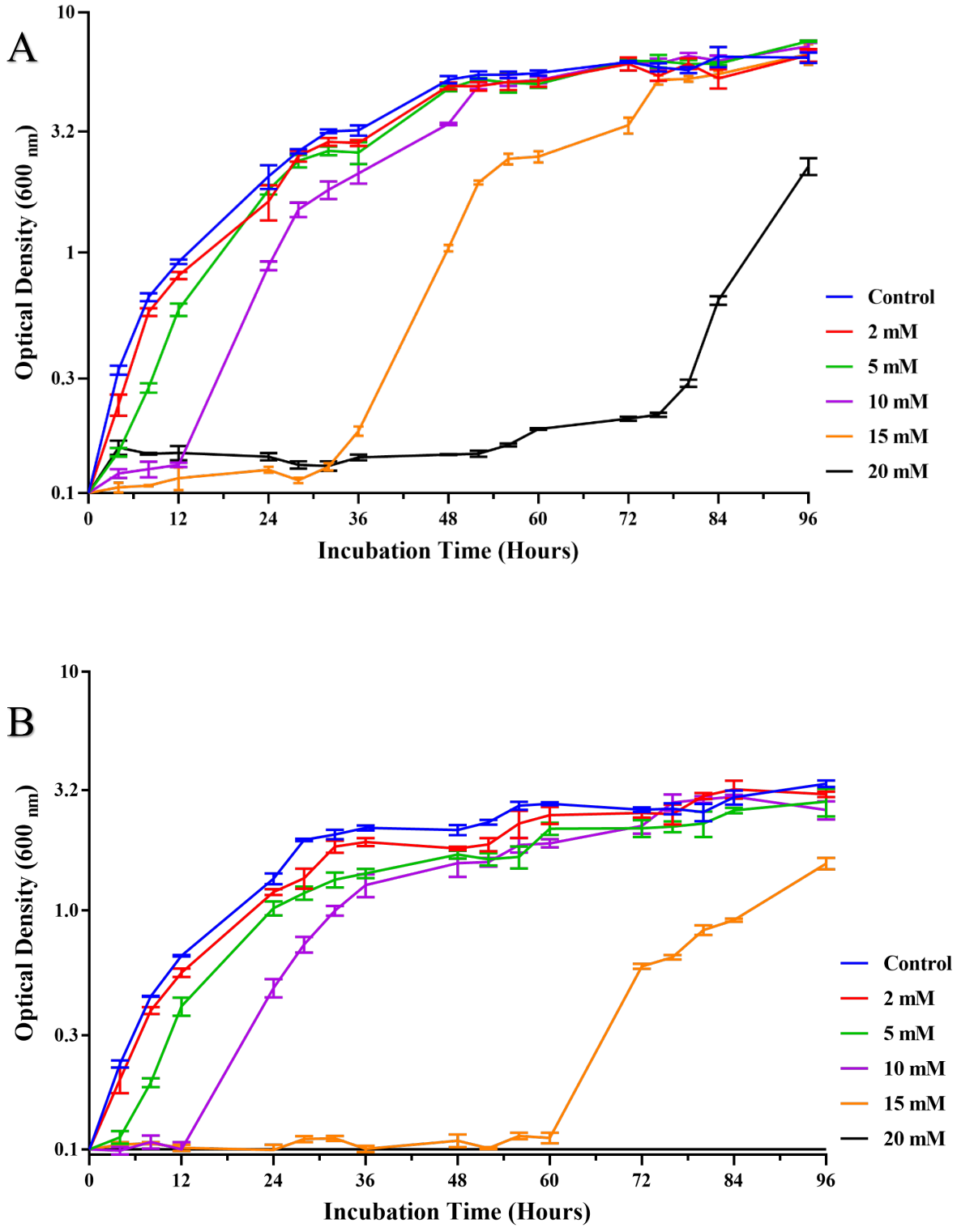
The medium with a 5 mM concentration took the yeast about 4 hours to move from the lag phase to the exponential phase, when the growth rates continued to rise quickly and moved to the stationary phase 32 hours after the initiation of the fermentative process. It finished the experiment with growth rates slightly behind the control and the previous concentration. At the 10 mM medium concentration, the yeast required about 12 hours to move from the lag phase to the exponential phase, when the growth rates started to rise rapidly until the 36th hour of the assay. After that, it continued to grow at a slower pace.

With the culture medium containing the 15 mM concentration, about 60 hours were needed to move from the lag to the exponential phase. Then the growth rates accelerated significantly after that, which continued to rise until the end of the assay, which took 96 hours. *T. multisporum* has not grown at the highest tested concentration (20 mM).

P. ofunaensis showed great xylose assimilation, depleting all the sugar in the media, including concentrations of 2 to 10 mM of the inhibitor, during 84 hours from the beginning of the fermentation assay, and consuming 43% of the xylose in the medium with the highest concentration (20 mM furfural) after 96 hours. On the other hand, *T. multisporum* had a lower assimilation capacity when exposed to furfural. In concentrations between 2 mM and 15 mM, the yeast was able to tolerate the presence of the compound, consuming about 41% of the sugar in the culture media.

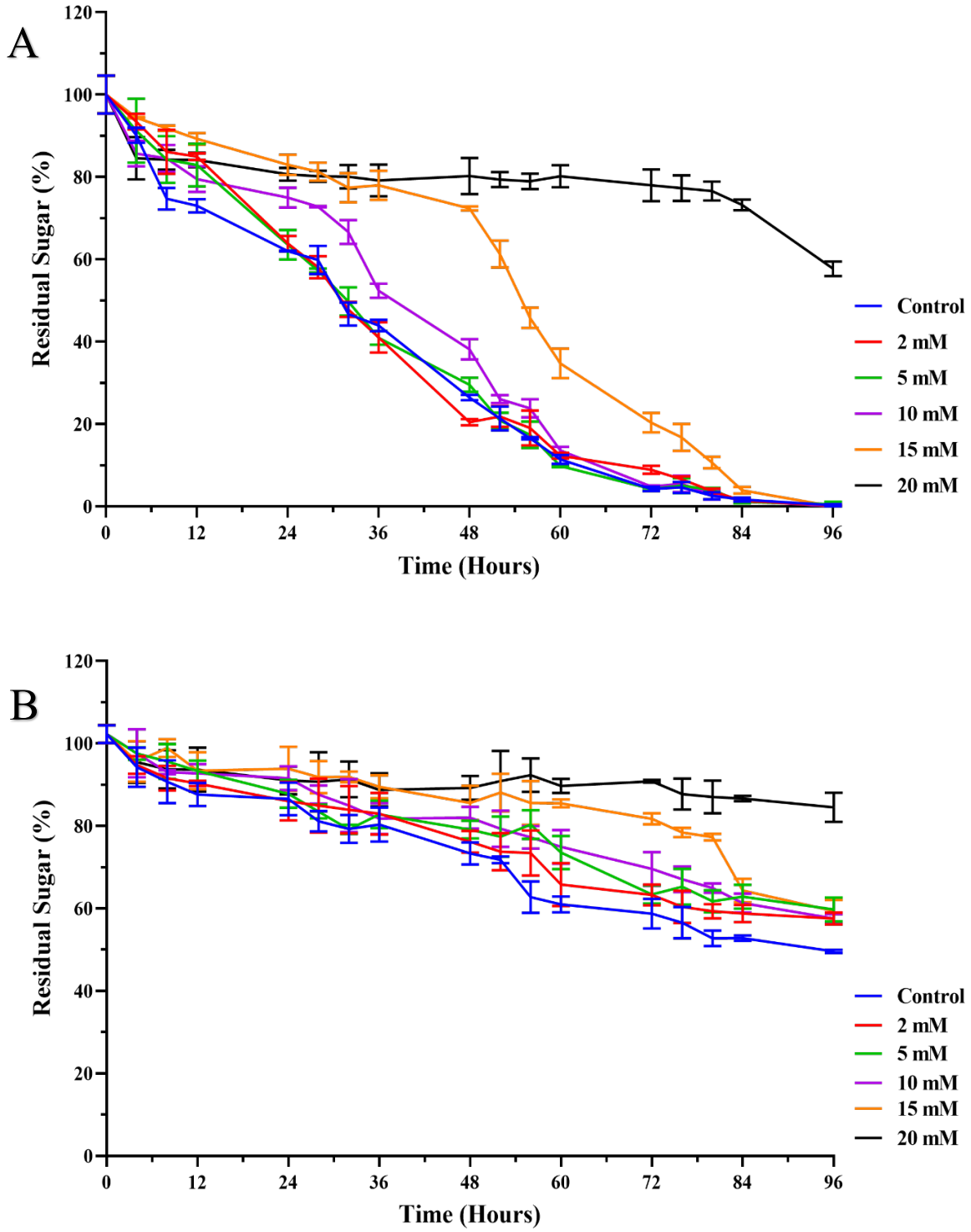
When comparing the pH variations of the two yeasts' culture media (Figures 11A and B), *P. ofunaensis*' cultivation shows no considerable fluctuations. The medium's pH varies from 4.5 to 5 and then returns to decrease again at the end of the 96 hours. On the other hand, *T. multisporum* shows an increase in pH, from pH 4.5 to 6 at the end of 96 hours.

Figure 9 - Cellular Growth in culture media containing furfural inhibitor (2-20 mM) for 96 h at 28 °C and 150 rpm, *P. ofunaensis* (A) and *T. multisporum* (B).



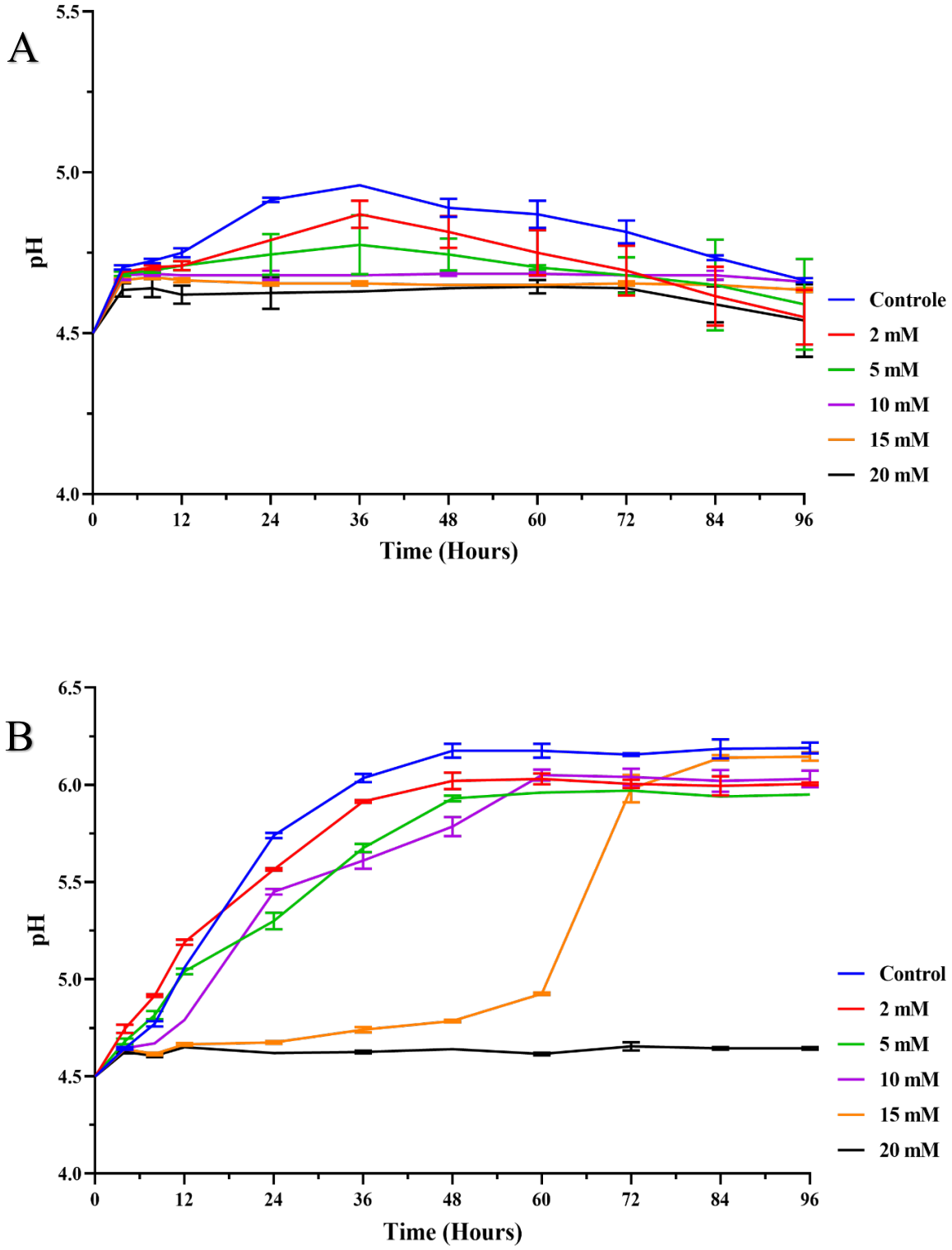
Source: own authorship.

Figure 10 - Xylose assimilation in culture media containing furfural inhibitor (2-20 mM) for 96 h at 28 °C and 150 rpm, *P. ofunaensis* (A) and *T. multisporum* (B).



Source: own authorship.

Figure 11 - pH variation in culture media containing furfural inhibitor (2-20 mM) for 96 h at 28 °C and 150 rpm, *P. ofunaensis* (A) and *T. multisporum* (B).



Source: own authorship.

5.1.2 HMF effect on cellular growth and xylose consumption

Yeasts *P. ofunaensis* (Figures 12A and 13A) and *T. multisporum* (Figures 12B and 13B) were able to grow and consume xylose in fermentative YEPX culture media containing HMF in several concentrations. Table 4 shows, in a condensed form, the tested concentrations, the optical density, xylose consumption, and pH data gathered at the end of fermentation of YEPX culture media containing HMF in different concentrations.

Table 4 - Optical density and pH of YEPX culture media containing HMF after 96 hours of incubation and sugar consumed during fermentation by *P. ofunaensis* and *T. multisporum*.

Specie	HMF Concentration	Final **OD**	Final **pH**	Sugar Consumption
P. ofunaensis	0 mM	6.85	4.47	100% (84h)
	2 mM	6.62	4.45	100% (84h)
	5 mM	6.68	4.46	100% (84h)
	10 mM	6.51	4.42	100% (84h)
	15 mM	6.32	4.30	100% (84h)
	20 mM	6.46	4.27	100% (84h)
T. multisporum	0 mM	5,10	6.57	54% (96h)
	2 mM	4.94	6.54	48% (96h)
	5 mM	4,88	6.31	53% (96h)
	10 mM	4.83	6.28	50% (96h)
	15 mM	4,44	6.38	44% (96h)
	20 mM	4,24	6.28	44% (96h)

Source: own authorship.

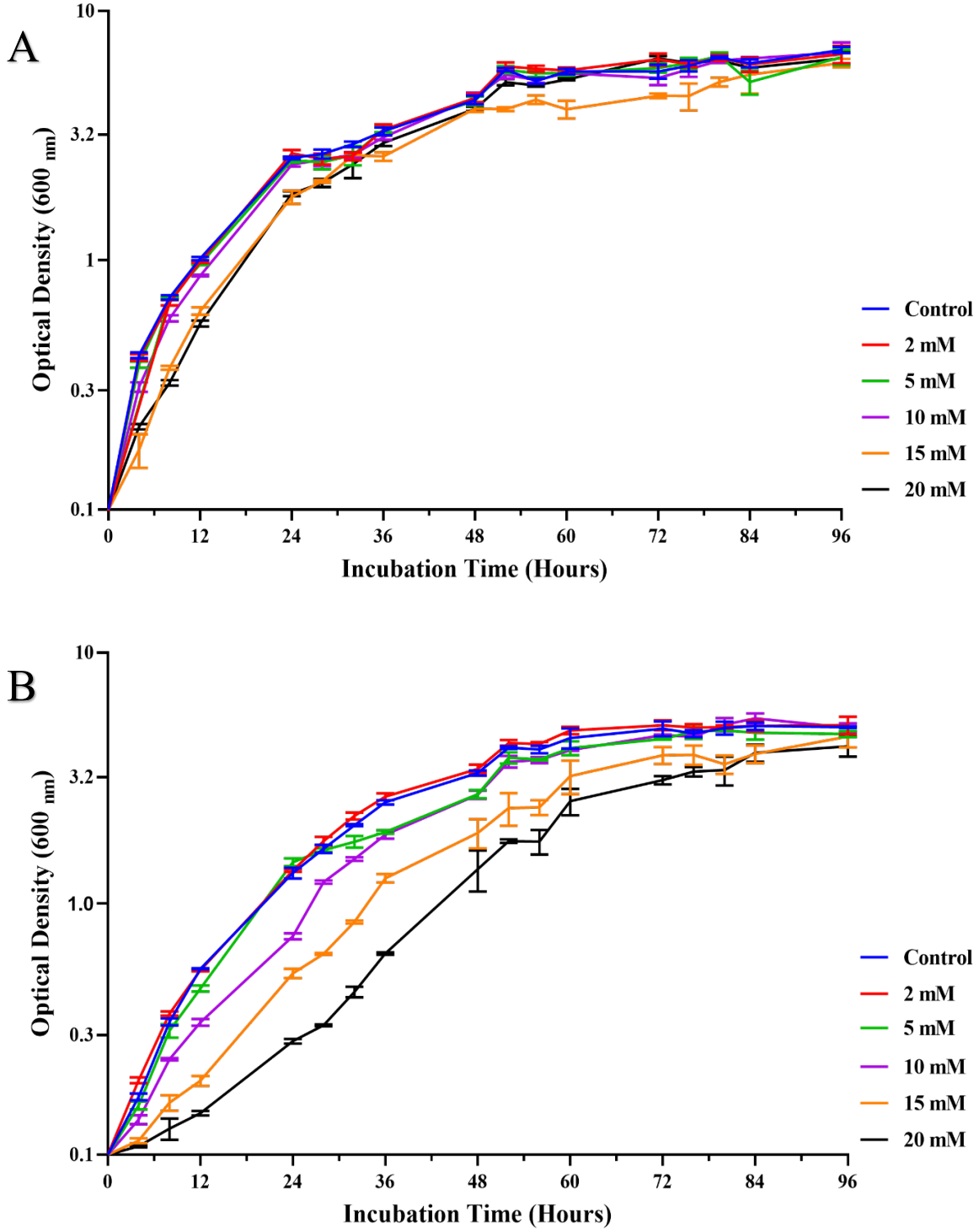
Unlike furfural, figure (12A) shows that HMF does not cause an extensive lag phase. The cellular growth profile for the yeast *P. ofunaensis* in all tested concentrations represented similar behavior to that of the control culture medium, with a minor delay in the highest two concentrations. The accelerated yeast growth began even before the first sample point reading (4 hours) after the inoculation started.

Likewise, *T. multisporum* suffered little in the presence of this inhibitor. The fermentative culture media containing concentrations of 2, 5, and 10 mM of HMF exhibited a similar growth profile to control, with a minor decrease in the 10 mM concentration, where 52 hours were needed to reach the stationary phase, which continued until the end of the assay. The higher two concentrations showed a late response compared to the rest, which required more time to move from the lag to the log phase, followed by a continuation of growth until 60 hours, when growth rates moderately stabilized and entered the stationary phase, which continued until the end of the assay after 96 hours.

As in furfural, *P. ofunaensis* showed excellent xylose consumption, depleting all the sugar in the media in all concentrations during 84 hours from the beginning of the fermentation assay. Alternatively, *T. multisporum* is the species with the lower assimilation capacity. At concentrations between 2 mM and 10 mM, the yeast was able to consume about 50% of the sugar in the culture media during 96 hours, while in the presence of the two higher tested concentrations (15 and 20 mM), the yeast displayed less performance, with a consumption of about 44% of the xylose in the culture media at the end of 96 hours.

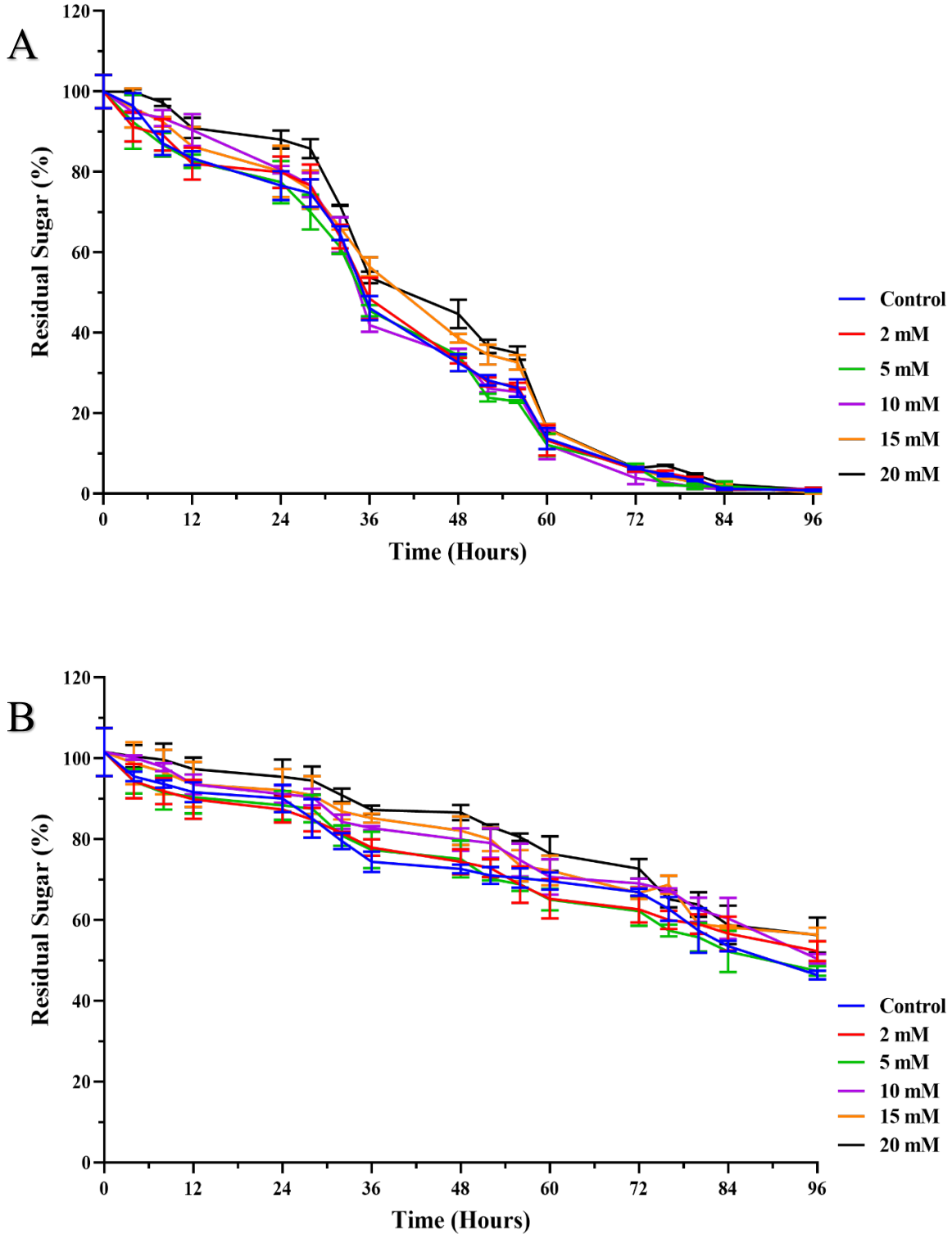
When comparing the pH variations of the two yeasts' culture media (Figures 14 A and B), *P. ofunaensis* cultivation shows no considerable variations. The medium's pH varies from 4.5 to 4.75 and then returns to decrease again at the end of the 96 hours. It's noticeable that the higher the inhibitor concentration, the lower the pH reaches at the end of the assay. At the end of 96 hours, *T. multisporum* shows an increase in pH of about 6 to 6.5.

Figure 12 - Cellular Growth in culture media containing HMF inhibitor (2-20 mM) for 96 h at 28 °C and 150 rpm, *P. ofunaensis* (A) and *T. multisporum* (B).



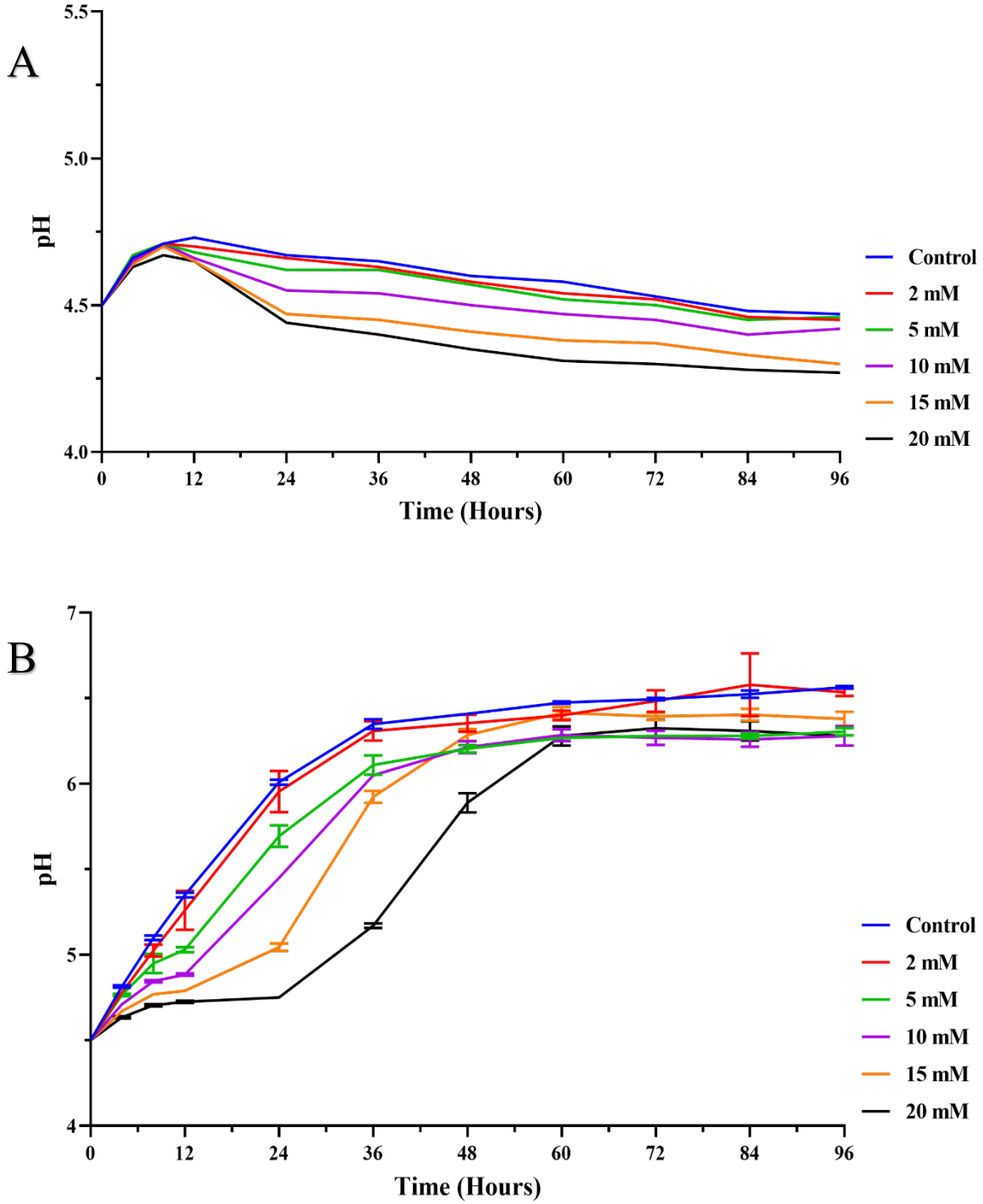
Source: own authorship.

Figure 13 - Xylose assimilation in culture media containing HMF inhibitor (2-20 mM) for 96 h at 28 °C and 150 rpm, *P. ofunaensis* (A) and *T. multisporum* (B).



Source: own authorship.

Figure 14 - pH variation in culture media containing HMF inhibitor (2-20 mM) for 96 h at 28 °C and 150 rpm, *P. ofunaensis* (A) and *T. multisporum* (B).



Source: own authorship.

5.1.3 Discussion

P. ofunaensis displayed a better performance than *T. multisporum* in the presence of furfural inhibitor in all tested concentrations, as it was able to grow in all of them, while *T. multisporum* could not do so since it did not tolerate the highest tested concentration (20 mM), the one that *P. ofunaensis* was able to grow in, even after a lag phase that lasted for about 72 hours. As for the rest of the concentrations, both yeasts were able to grow in them, even with different performance between the two yeasts, which was always in favor of *P. ofunaensis*.

Also, *P. ofunaensis* was able to deplete all the sugar available in the medium except in the highest concentration, while *T. multisporum* yeast could not consume more than 42% of the sugar in the medium in the lowest concentration (2 mM).

P. ofunaensis showed a slight increase in pH that continued until the cells reached the stationary phase, and then it appeared to acidify the media. As for *T. multisporum*, the alkalinity of the culture medium kept increasing during cellular growth. It can be suggested that *T. multisporum* uses facilitated diffusion to uptake xylose, while *P. ofunaensis* uses sugar/H^+ symporters for D-xylose, similar to that exhibited by *P. guilliermondii* (SILVA et al., 2020). As *P. ofunaensis* starts to transport this pentose jointly with H^+, the extracellular pH increases slightly, but tends to decrease as this sugar is consumed. Sugar/H^+ symports are directly influenced by the plasma membrane H^+-ATPase (FERREIRA et al., 2013). The reduction in extracellular pH may also be related to the secretion of metabolites such as organic acids (MURAKAMI et al., 2011).

Monteiro (2020) tested the growth of the yeasts *Sporidiobolus pararoseus* U3 and *Pichia terricola* G20 in YEPD medium containing furfural. The cultures took place in 96-well plates and were left to incubate for 72 hours in a SpectraMaxplus 384 spectrophotometer, which kept the temperature at 29 °C and took O.D. measurements at 600_{nm} automatically every hour.

S. pararoseus U3 was able to tolerate the lowest tested concentration only (0.04 g/L furfural) and was able to increase its optical density by nearly two and a half times, but could not grow in the rest, which makes it more sensible than *T. multisporum* and *P. ofunaensis*. As for *P. terricola* G20, it was able to grow in all tested concentrations. Its growth has more than quadrupled at the lowest tested concentration, tripled at the second, and more than doubled at the third. These results show its clear superiority in the ability to tolerate the presence of furfural

inhibitor in high concentrations over yeasts *T. multisporum* and *S. pararoseus* U3, and an equivalent performance with *P. ofunaensis* in terms of tolerating the highest concentration.

Sitepu et al. (2014) tested 48 different yeasts in a saline medium that contains glucose as the sole carbon source in the presence of furfural as an inhibitor with two different concentrations (0.5 and 1.0 g/L), where all cultures were incubated in a roller drum at room temperature (23 °C). Relative growth was visually scored at 3, 7, 10, and 14 days after inoculation. Half of them were able to grow in the weakest concentration, but 18 of those showed a growth delay or a lag phase before they could move on. The matter differed considerably with doubling the furfural concentration, where the number of yeasts that were able to tolerate this concentration was reduced to only 7, and one of them showed a delay in growth.

This study included the yeasts *T. coremiiforme*, *T. dermatis*, and *T. guehoae*, which belong to the same genus as *T. multisporum*. All of these yeasts could not grow in the higher tested concentration (1.0 g/L), but they could tolerate the lower one (0.5 g/L), although they all showed a growth delay, where it took them some time to move on to the log phase, this makes these yeasts more sensitive to furfural than *T. multisporum,* which showed tolerance to this inhibitor at about 1.5 g/L.

Regarding the performance of the two yeasts in the presence of HMF, both yeasts could grow in all tested concentrations, with an advantage for *P. ofunaensis* since it was able to grow in all concentrations like that of control, while with *T. multisporum*, the inhibitor influenced its growth, the higher the HMF concentration, the lower the yeast performance.

P. ofunaensis exhausted all the available sugar in the media within 84 hours at all concentrations. Still, for *T. multisporum*, it could provide nearly half the performance compared to *P. ofunaensis*, as it consumed about 53% of the sugar in the medium at best.

Among the *S. pararoseus* U3 and *P. terricola* G20 yeasts that Monteiro (2020) tested with HMF inhibitor in three different concentrations (0.1, 2.5, and 5.0 g/L), and under the same conditions mentioned previously, *S. pararoseus* U3 was able to grow only at the lowest concentration and triplicate its growth rate, which again makes it more sensitive than *P. terricola* G20, *P. ofunaensis*, and *T. multisporum*. While *P. terricola* G20 was able to tolerate the first concentration, doubling its growth rate, but its growth in the second concentration was not that good, with just about a 25% increase from its initial inoculum, yet it did not grow in

the higher one, which makes *P. ofunaensis* and *T. multisporum* superior in terms of tolerance to this inhibitor as they showed better growth rates in the second concentration.

The Sitepu et al. (2014) experiment, which tested HMF at three concentrations (0.5, 1.0, 2.0 g/L) under the same conditions mentioned previously, showed that all the yeasts in the study were able to withstand the lowest tested concentration without exception. Only one of them showed a growth delay. At the middle concentration, 44 yeasts were able to grow at this concentration, even though 13 of them showed a delay in growth and needed some time to move to the log phase. At the higher tested concentration, the number of yeasts that could tolerate this concentration decreased to 38, of which 23 yeasts needed some time to move from the lag to the log phase.

Likewise, both yeast *T. coremiiforme* and *T. guehoae* could tolerate all concentrations tested, though *T. guehoae* showed a growth delay at the highest concentration tested. This performance puts them on par with *T. multisporum.* While *T. dermatis* managed to grow only in the lowest concentration, which makes it the least performer among all these yeasts in the presence of HMF.

5.2 Phenolic compounds inhibitors

5.2.1 Ferulic acid effect on cellular growth and xylose consumption

Yeasts *P. ofunaensis* (Figures 15A and 16A) and *T. multisporum* (Figures 15B and 16B) show the ability to xylose assimilation and cellular growth in fermentative YEPX culture media containing ferulic acid in several concentrations. When exposed to the inhibitor, both species were able to grow and assimilate pentose in all-tested concentrations. Table 5 shows, in a condensed form, the tested concentrations, the optical density, xylose consumption, and pH data gathered at the end of fermentation of YEPX culture media containing ferulic acid as inhibitor.

Table 5 - Optical density and pH of YEPX culture media containing ferulic acid after 96 hours of incubation and sugar consumed during fermentation by *P. ofunaensis* and *T. multisporum*.

Specie	Ferulic Acid Concentration	Final **OD**	Final **pH**	Sugar Consumption
P. ofunaensis	0 mM	7.23	4.42	100% (84h)
	0.5 mM	6.61	4.39	100% (96h)
	1 mM	5.75	4.39	100% (96h)
	2 mM	5.33	4.28	100% (96h)
	3 mM	3.10	4.29	84% (96h)
T. multisporum	0 mM	5.17	6.59	49% (96h)
	0.5 mM	5.02	6.50	45% (96h)
	1 mM	4.78	6.42	45% (96h)
	2 mM	4.57	6.39	42% (96h)
	3 mM	4.67	6.28	40% (96h)

Source: own authorship.

As with HMF, ferulic acid did not have a powerful impact on the two yeasts. The cellular growth profile for the yeast *P. ofunaensis* in all tested concentrations represented similar behavior to that of the control culture medium, with some pause in the highest concentration. The accelerated yeast growth began even before the first sample point reading (4 hours) after the inoculation started. The growth rates began to settle after 24 hours, and then the yeast entered the stationary phase and continued until the end of the assay.

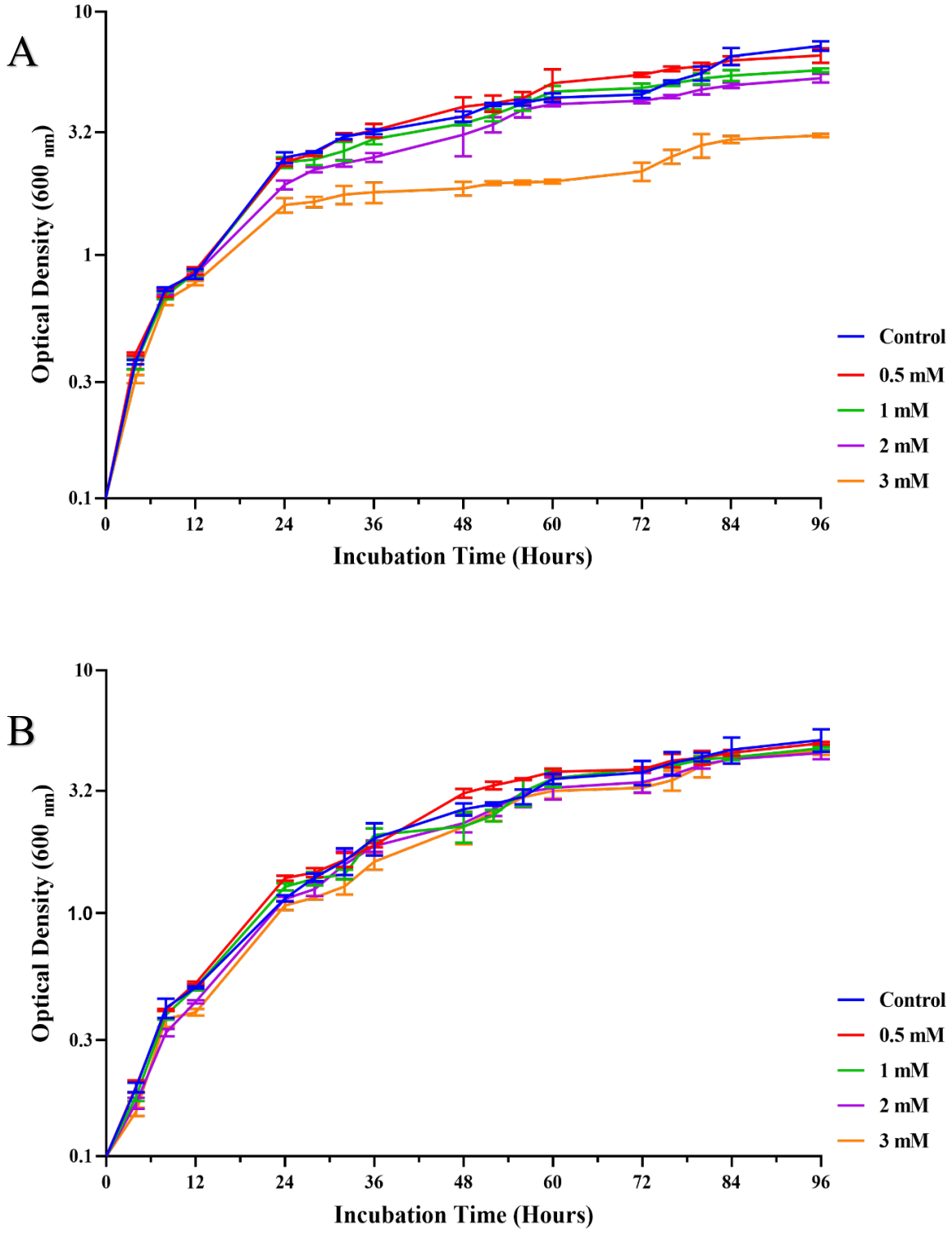
Moreover, the presence of this inhibitor had a minimal effect on *T. multisporum*. The fermentative culture media in all tested concentrations of ferulic acid displayed a similar growth profile to control. After 48 hours, the yeast reached the stationary phase, and the growth rates moderately stabilized, which continued until the end of the assay after 96 hours.

Unlike the previous two inhibitors, *P. ofunaensis* showed less xylose consumption in the presence of ferulic acid, depleting all the sugar in the media in all concentrations during 96 hours from the beginning of the fermentation assay, except for the highest concentration, in which the yeast consumed only 84% of the sugar available in the culture medium in 96 hours.

Additionally, *T. multisporum* in the first two concentrations (0.5 and 1 mM) was capable of consuming about 45% of the sugar existing in the culture media through 96 hours, but this consumption decreased with 2 mM concentration (42%) and 3 mM concentration (40%) at the end of 96 hours.

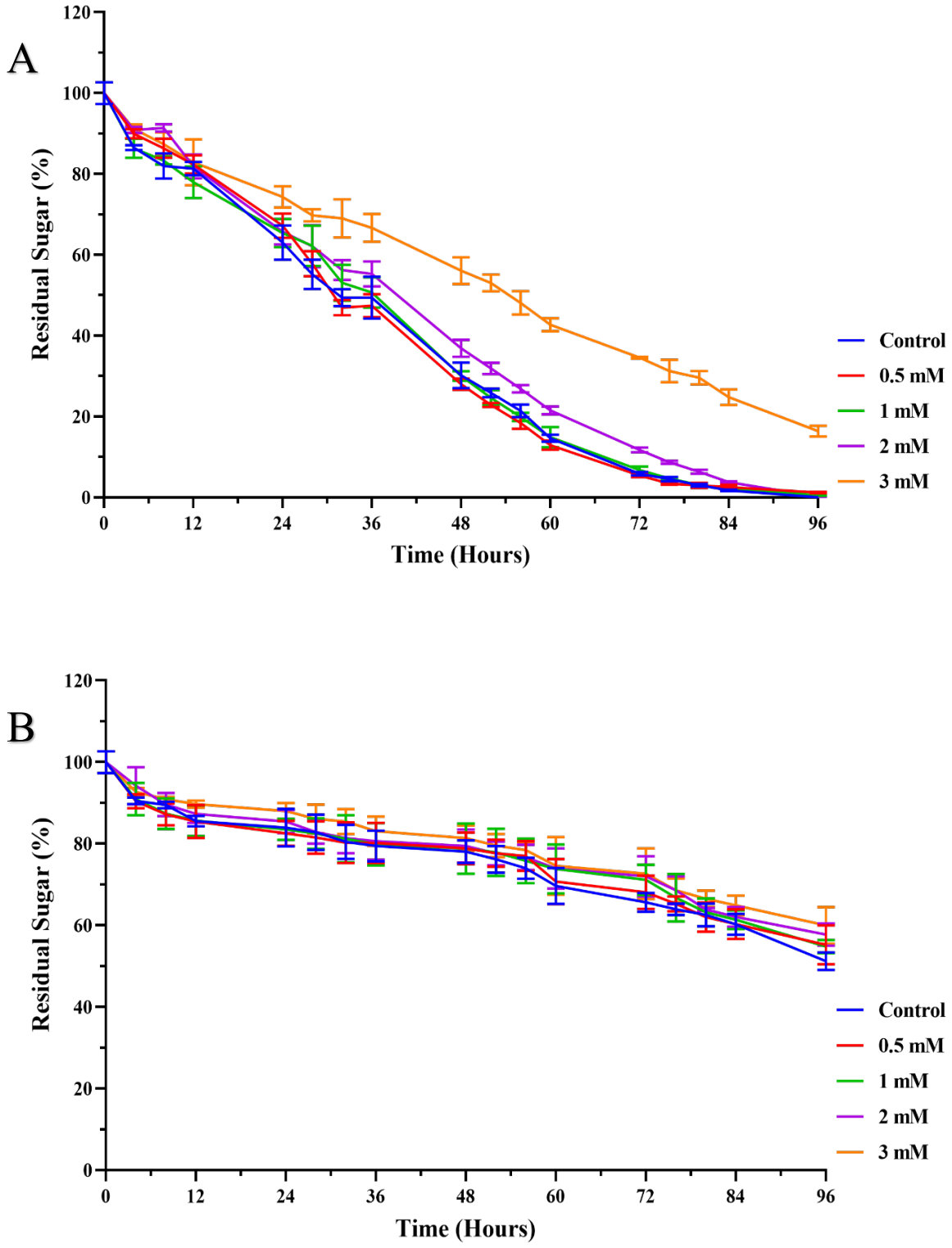
The pH variation graphs for *P. ofunaensis* and *T. multisporum* during the assay in culture media containing ferulic acid are presented in the figures 17A and 17B, respectively. The pH readings of *P. ofunaensis*' culture media didn't show any significant change. It rose during the first 12 hours of the assay and barely reached 4.7. After that, scores started to decrease slightly and stabilized after 60 hours from the beginning and continued until the end of the assay. The highest two concentrations scored a bit lower than the others. With similar behavior to HMF, *T. multisporum* showed a considerable increase in pH scores for all concentrations, reaching about 6.5 at the end of 96 hours. The higher the inhibitor concentration, the lower the pH score achieved.

Figure 15 - Cellular Growth in culture media containing ferulic acid inhibitor (0.5-3 mM) for 96 h at 28 °C and 150 rpm, *P. ofunaensis* (A) and *T. multisporum* (B).



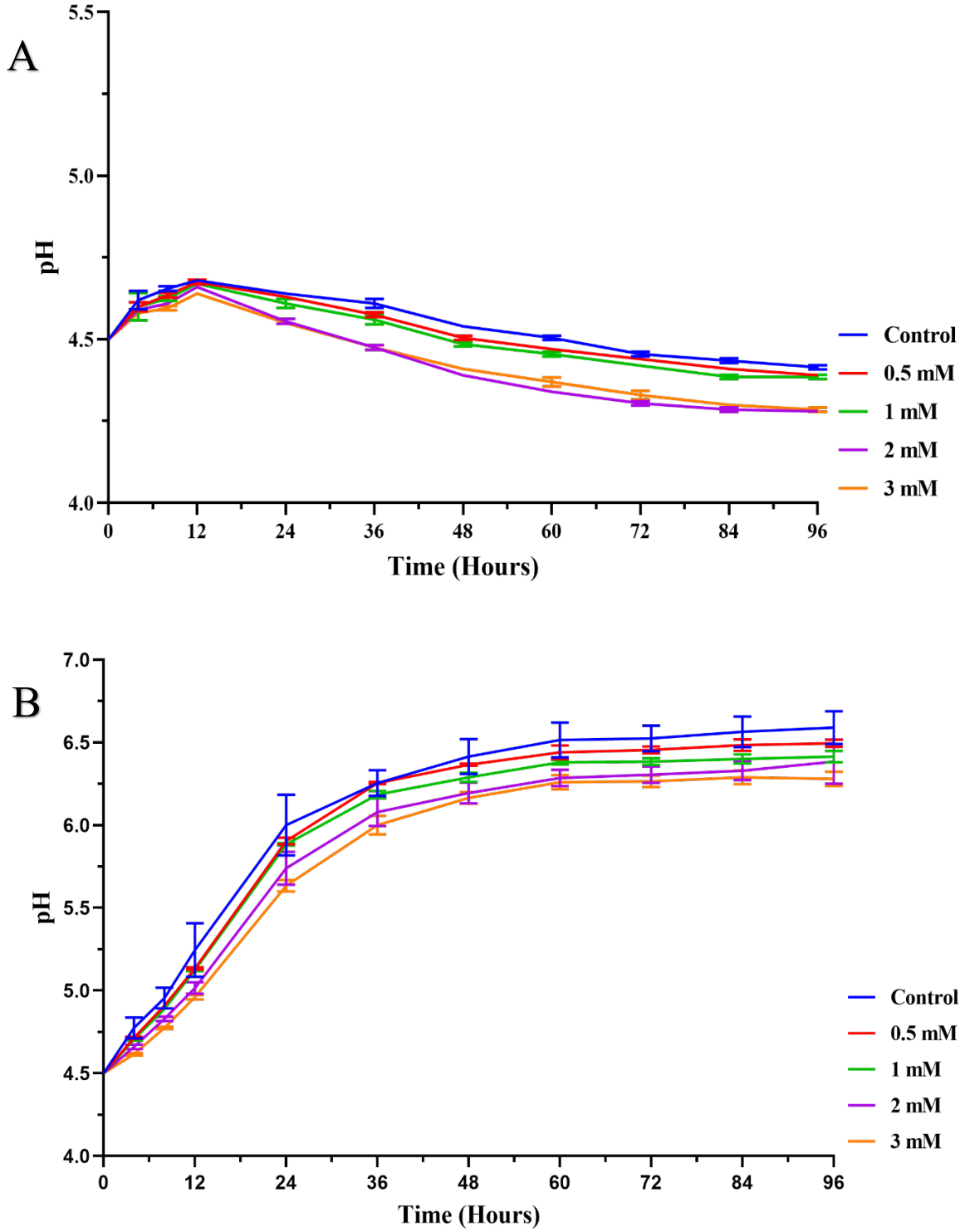
Source: own authorship.

Figure 16 - Xylose assimilation in culture media containing ferulic acid inhibitor (0.5-3 mM) for 96 h at 28 °C and 150 rpm, *P. ofunaensis* (A) and *T. multisporum* (B).



Source: own authorship.

Figure 17 - pH variation in culture media containing ferulic acid inhibitor (0.5-3 mM) for 96 h at 28 °C and 150 rpm, *P. ofunaensis* (A) and *T. multisporum* (B).



Source: own authorship.

5.2.2 Vanillin effect on cellular growth and xylose consumption

Yeasts *P. ofunaensis* (Figures 18A and19A) and *T. multisporum* (Figures 18B and19B) show the ability to xylose assimilation and cellular growth in fermentative YEPX culture media containing vanillin in several concentrations. When exposed to the inhibitor, *P. ofunaensis* was able to grow and assimilate pentose in all-tested concentrations, while *T. multisporum* could not grow in the highest concentration of the inhibitor (10 mM). Table 6 shows, in a condensed form, the tested concentrations, the optical density, xylose consumption, and pH data gathered at the end of fermentation of YEPX culture media containing vanillin in the tested concentrations.

Table 6 - Optical density and pH of YEPX culture media containing vanillin after 96 hours of incubation and sugar consumed during fermentation by *P. ofunaensis* and *T. multisporum*.

Specie	Vanillin Concentration	Final **OD**	Final **pH**	Sugar Consumption
P. ofunaensis	0 mM	8.78	4.35	100% (84h)
	1 mM	6.69	4.37	100% (84h)
	2 mM	7.13	4.35	100% (84h)
	3 mM	6.57	4.31	100% (84h)
	5 mM	6.93	4.28	100% (84h)
	10 mM	5.28	4.32	92% (96h)
T. multisporum	0 mM	6.84	6.85	50% (96h)
	1 mM	4.54	6.89	40% (96h)
	2 mM	4.13	7.18	31% (96h)
	3 mM	3.66	7.56	23% (96h)
	5 mM	1.74	7.26	11% (96h)
	10 mM	0.11	4.74	4% (96h)

Source: own authorship.

P. ofunaensis in the culture media with the lowest two concentrations of vanillin (1 and 2 mM) displayed similar behavior to the medium without inhibitor (control) at the beginning of the fermentation. It showed an accelerated increase in the growth rates during the exponential phase in the first 24 hours, after which the growth speedup decreased, and the yeast then shifted to the stationary phase, preserving a growth profile similar to the control assay. For the concentrations of 3 mM and 5 mM, the yeast showed similar behavior to the previous ones, but with slower growth rates due to the higher inhibition ability. After moving to the stationary phase and until the end of the experiment, all these inhibitor concentrations showed somewhat similar effects on the yeast.

The lag phase continued for about 32 hours in the culture medium with the highest concentration (10 mM), then the growth rates started to increase and entered the log phase. After 72 hours, the stationary phase started and continued until the end of the assay.

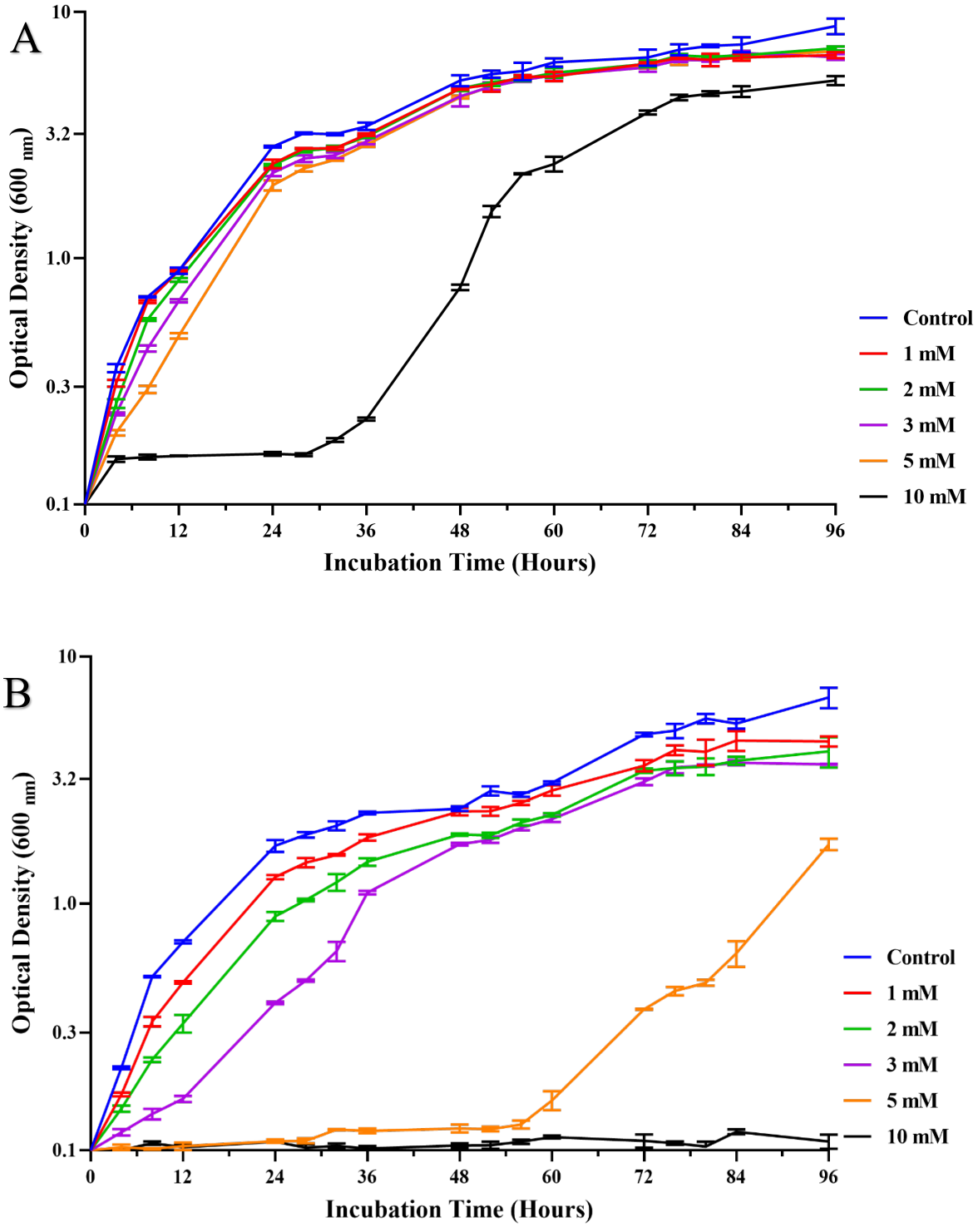
The situation was a little different for the yeast *T. multisporum.* It was clear to observe that the higher the inhibitor concentration, the slower the growth rate. The concentrations of 1, 2, and 3 mM displayed growth steps similar to control, with a slight delay that would rise with the inhibitor concentration increase, and started the stationary phase after about 36 hours and until the end of the assay. At the penultimate tested concentration (5 mM), the yeast needed about 60 hours to start the lag to the log phase, which continued until the end of the experiment. The highest tested concentration (10 mM) of vanillin was able to inhibit the growth of *T. multisporum* during the 96 hours.

At the end of 84 hours, *P. ofunaensis* was able to deplete all the sugar in the media in all concentrations of the inhibitor except the highest one. Yet it needed 96 hours to consume 92% of the xylose in the culture medium with the highest tested concentration (10 mM).

However, *T. multisporum* is a species with a weaker ability to assimilate xylose in general. It showed a gradient of consumption inversely proportional to the inhibitor concentration. The yeast was able to tolerate the presence of vanillin at 1, 2, 3 and 5 mM, consuming 40%, 31%, 23%, and 11% of the sugar that exists in the culture medium, respectively. But at the highest tested concentration (10 mM), the yeast consumed just 4% of the available sugar in the culture medium.

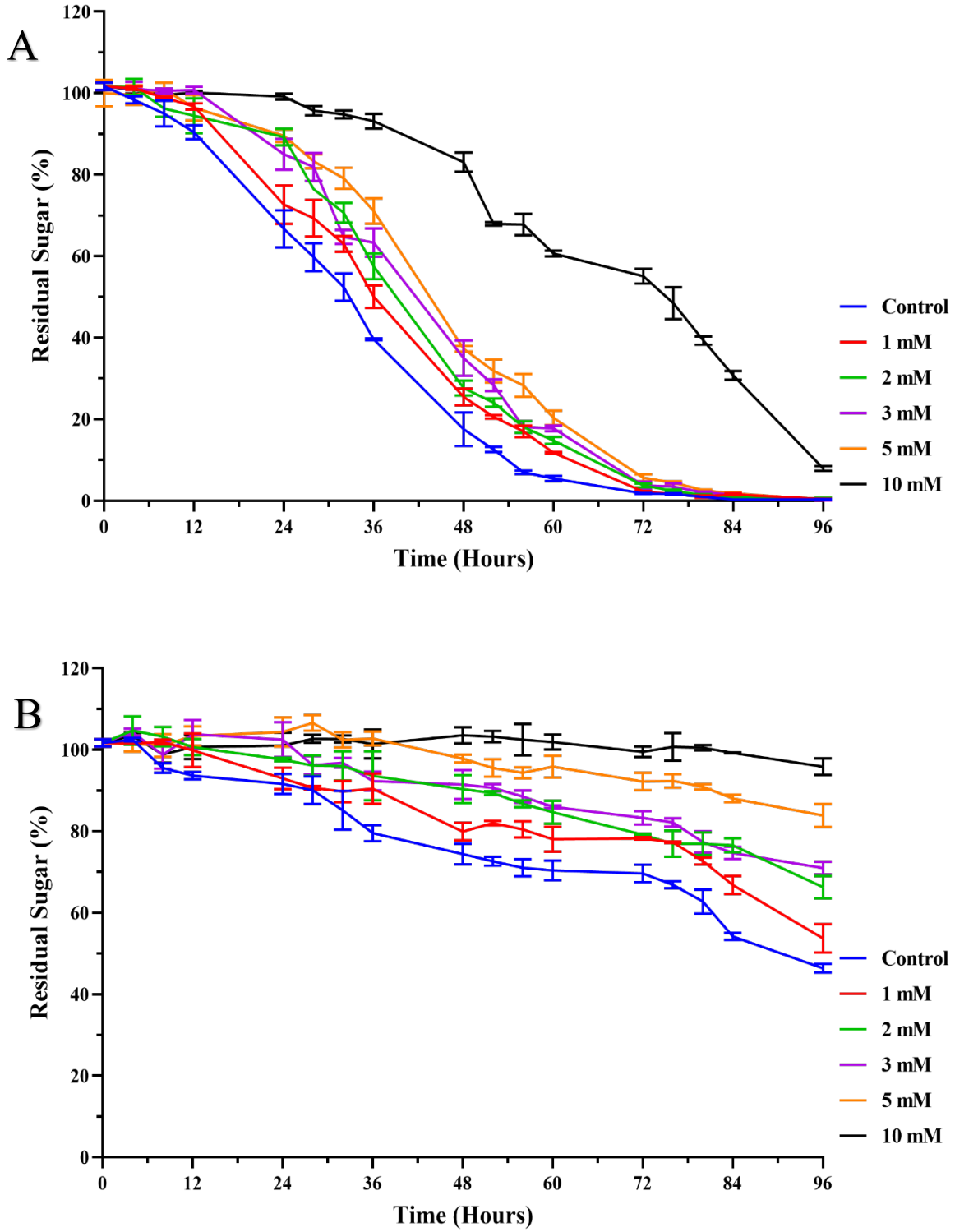
We measured the pH variation in the culture of *P. ofunaensis* (Figure 20A) and *T. multisporum* (Figure 20B) in media containing vanillin. *P. ofunaensis* kept the same performance with no considerable fluctuations. The medium's pH increased a little to reach about 4.75 during the first 12 hours and then returned to decrease again at the end of the 96 hours. On the other hand, *T. multisporum* showed a considerable increase in pH, which was directly proportional to the inhibitor concentration that continued until the end of 96 hours.

Figure 18 - Cellular Growth in culture media containing vanillin inhibitor (1-10 mM) for 96 h at 28 °C and 150 rpm, *P. ofunaensis* (A) and *T. multisporum* (B).



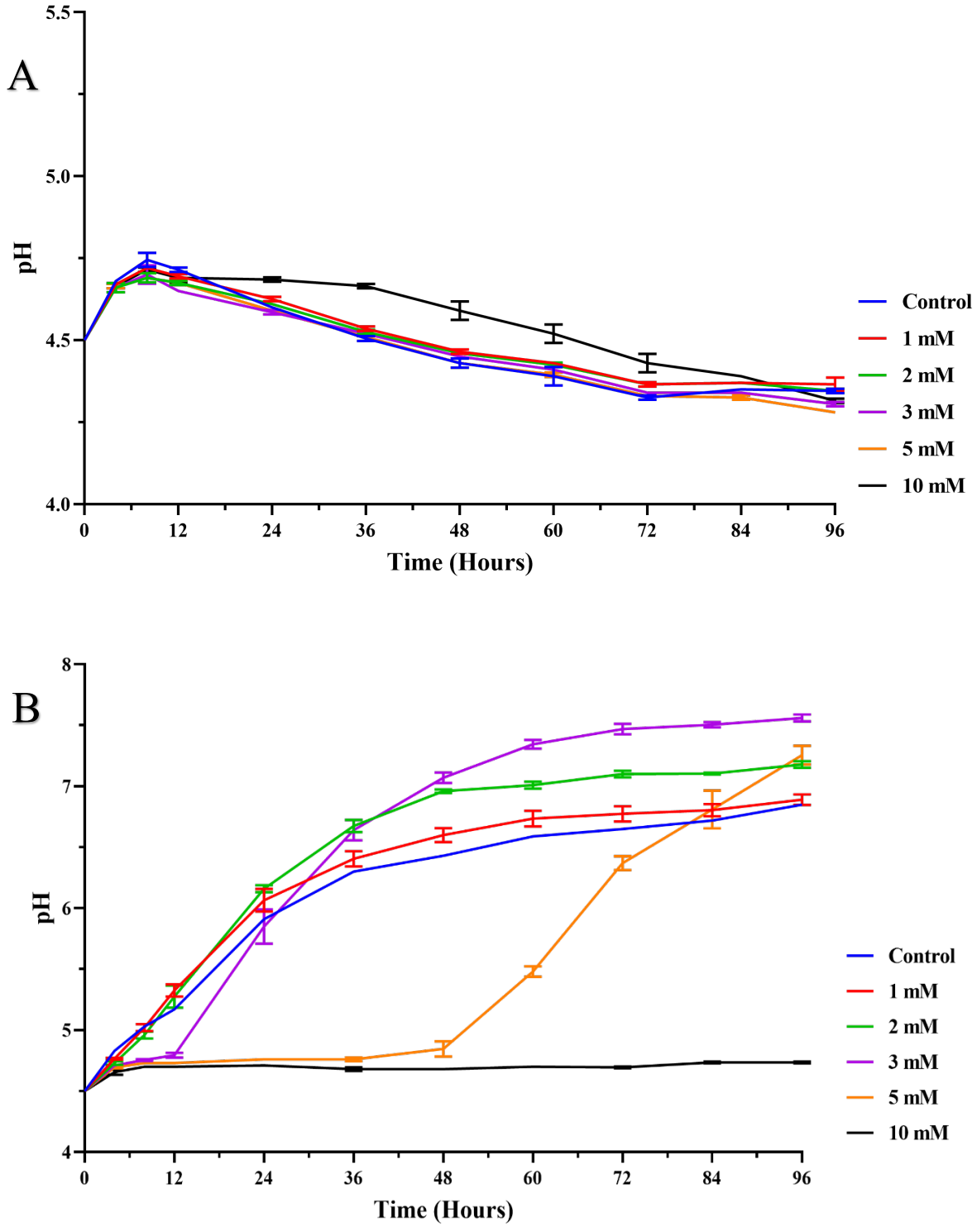
Source: own authorship.

Figure 19 - Xylose assimilation in culture media containing vanillin inhibitor (1-10 mM) for 96 h at 28 °C and 150 rpm, *P. ofunaensis* (A) and *T. multisporum* (B).



Source: own authorship.

Figure 20 - pH variation in culture media containing vanillin inhibitor (1-10 mM) for 96 h at 28 °C and 150 rpm, *P. ofunaensis* (A) and *T. multisporum* (B).



Source: own authorship.

5.2.3 Discussion

Both *P. ofunaensis* and *T. multisporum* were able to tolerate the presence of ferulic acid inhibitor and grow in all tested concentrations. *P. ofunaensis* needed about 24 hours to move on to the stationary phase, and the effects of higher concentrations started to appear, assimilating just 84% of sugar at the end of the assay in this medium compared to the rest.

Still, *T. multisporum* presented a similar performance in all concentrations, and it took about 48 hours to transfer to the stationary phase, but the consumption of sugar in the higher concentration medium was about 5% less than the medium with a lower concentration and about 10% less than the control.

Again, the pH variations in the media during the growth of *P. ofunaensis* and *T. multisporum* were similar to those with the previous inhibitors evaluated (furfural and HMF), which can be proposed the same explanation mentioned previously.

In a cultivation of *Candida guilliermondii,* Pereira et al (2011) tested the inhibition effect of ferulic acid with three different concentrations (0.2, 0.4, and 0.6 g/L) in a semi-defined buffered culture medium containing xylose (80.0 g/L) and glucose (15.0 g/L) for 96 hours at 30 °C, 200 rpm.

The presence of ferulic acid in the fermentation medium had an effect on yeast cell growth at all tested concentrations, with a 15% reduction in cellular growth in the presence of the lowest tested concentration, a 23% decrease in the presence of the second concentration, and a 30% decrease in the presence of the highest one. On the other hand, ferulic acid did not affect xylose consumption at all of the tested amounts, as the yeast was able to consume about 87% of the xylose present in the medium after 96 hours.

Both *P. ofunaensis* and *T. multisporum* were able to grow on the same tested concentrations as *C. guilliermondii*, with better performance in cellular growth by *T. multisporum* and *P. ofunaensis*. The sugar consumption at the higher tested concentration was better in *C. guilliermondii* (87%), followed by *P. ofunaensis* (84%), and finally *T. multisporum* (40%).

In the experiment conducted by Yang et al. (2018) in saccharified slurries from deacetylated corn stover to determine the effect of ferulic acid at seven different concentrations, ranging between 0.009 and 0.9 g/L, on the growth of three types of bacteria, namely *C. necator*,

E. coli, and *R. opacus*, all tested bacteria showed the ability to grow in all concentrations, but with differences in the performance of each.

C. necator showed an increase of 8% in the growth rate in the lowest tested concentration and a decrease of 11% in the highest one. As for *E. coli*, ferulic acid had a stimulatory effect. The higher the inhibitor's concentration, the higher the growth rate, which reached about 20% compared to the control sample. For *R. opacus*, the inhibitor caused a growth decline of between 3% and 7% regardless of its concentration, which means there is no relation between the inhibitor's concentration and growth inhibition rates.

At the tested concentrations, vanillin was more intense than ferulic acid on the growth of the two yeasts, and this is clear in the highest concentration of vanillin 10 mM, as *P. ofunaensis* needed 24 hours to move to the log phase, while *T. multisporum* could not grow at the highest tested concentration and spent approximately 60 hours to reach the log phase at the 5 mM concentration of this inhibitor.

P. ofunaensis was able to deplete the sugar present in the medium in all concentrations within 96 hours, except for the highest concentration, where it consumed 92% of the sugar available in the medium. As for *T. multisporum*, its consumption ranged between 4% at the highest concentration and 40% at the lowest concentration of vanillin.

The inhibitory effect of phenolic compounds is generally determined by the presence and functionality of functional side groups (such as methoxy groups) and unsaturated bonds in the compound's structure (ADEBOYE; BETTIGA; OLSSON, 2014). For example, ferulic acid has two carbon atoms sharing a double bond, which links the carboxylic group to the aromatic ring.

Monteiro (2020) tested the tolerance of *S. pararoseus* U3 and *P. terricola* G20 yeasts to vanillin inhibitor at different concentrations (0.04, 1.0, 2.0 g/L) during a 72-hour experiment in YEPD media in 96-well plate static fermentation, as explained previously. *S. pararoseus* U3 was able to tolerate the lowest concentration of vanillin, where it almost increased four times its growth. *P. terricola* G20 was able to tolerate the two higher concentrations, albeit weakly compared to the lowest one, as it just increased two times its growth rates at the higher two concentrations and five times at the lower one, which makes it superior to both *S. pararoseus* U3 and *T. multisporum*, as both could not tolerate the second tested concentration. For *P. ofunaensis*, we did not test the higher concentration of vanillin (2.0 g/L) to find out if it could tolerate it.

Yang et al. (2018) measured the ability of three bacteria to tolerate vanillin inhibitor with seven different concentrations ranging between 0.002 and 0.2 g/L in saccharified slurries from deacetylated corn stover. At any concentration, vanillin could not completely inhibit the growth of all bacteria, but the *C. necator* showed a decrease in the growth level with an increase in the inhibitor's concentration. The growth rate decreased by 8% at the concentration of 0.05 g/L, which rose to 32% at 0.1 g/L, reaching nearly 42% at the highest concentration (0.2 g/L).

E. coli showed different performance as the growth rates increased with the rise in the vanillin's concentration, which reached about 6% in the highest concentrations. *R. opacus* also had a positive interaction with this compound in the lower concentrations, showing an increase of 5% in growth rates, but the highest concentration harmed the growth rates and showed a decrease of 8% compared to the control. It is possible to observe a relatively similar effect of vanillin at the highest tested concentrations in both *C. necator* and *T. multisporum* as it drastically reduces their growth rates compared to control. Although for *E. coli*, its vanillin tolerance was higher than *T. multisporum* at 0.2 g/L.

5.3 Aliphatic acids inhibitors

5.3.1 Acetic acid effect on cellular growth and xylose consumption

Yeasts *P. ofunaensis* (Figures 21A and 22A) and *T. multisporum* (Figures 21B and 22B) show the ability to xylose assimilation and cellular growth in fermentative YEPX culture media containing acetic acid in several concentrations. Table 7 shows, in a condensed form, the tested concentrations, the optical density, xylose consumption, and pH data gathered at the end of fermentation of YEPX culture media containing acetic acid in the tested concentrations.

Table 7 - Optical density and pH of YEPX culture media containing acetic acid after 96 hours of incubation and sugar consumed during fermentation by *P. ofunaensis* and *T. multisporum*.

Specie	Acetic Acid Concentration	Final **OD**	Final **pH**	Sugar Consumption
P. ofunaensis	0 mM	7.37	4.5	100% (84h)
	20 mM	7.17	4.74	100% (96h)
	40 mM	6.27	5.24	87% (96h)
	60 mM	2.90	5.59	43% (96h)
	80 mM	0.10	4.44	21% (96h)
T. multisporum	0 mM	6.88	6.77	64% (96h)
	20 mM	5.63	6.76	48% (96h)
	40 mM	0.10	4.45	19% (96h)
	60 mM	0.10	4.38	19% (96h)
	80 mM	0.10	4.36	16% (96h)

Source: own authorship.

The concentration of 20 mM of acetic acid in the culture medium had no significant effect on *P. ofunaensis*, which showed similar growth to the control with just a 0.2 difference in the final optical density in 96 hours. The 20 mM concentration, along with control, started to grow immediately after the inoculation and entered the stationary phase within 48 hours. After 12 hours in the lag phase, the yeast in the 40 mM acetic acid culture medium shifted to the log phase and started to grow, and 72 hours were needed to reach the stationary phase. That was not the case for the cells in the medium with 60 mM of this inhibitor, which spent half the time of the experiment (48 hours) in the lag phase. After that, the growth started to accelerate until the end of the assay. The yeast could not grow in the culture medium with the highest concentration (80 mM). For *T. multisporum*, the case was totally different as the yeast could not tolerate the presence of this inhibitor and showed no growth at all tested concentrations but

the lowest one (20 mM), which required 8 hours to move from the lag to log phase, but also kept a growth profile parallel to that without the inhibitor (control).

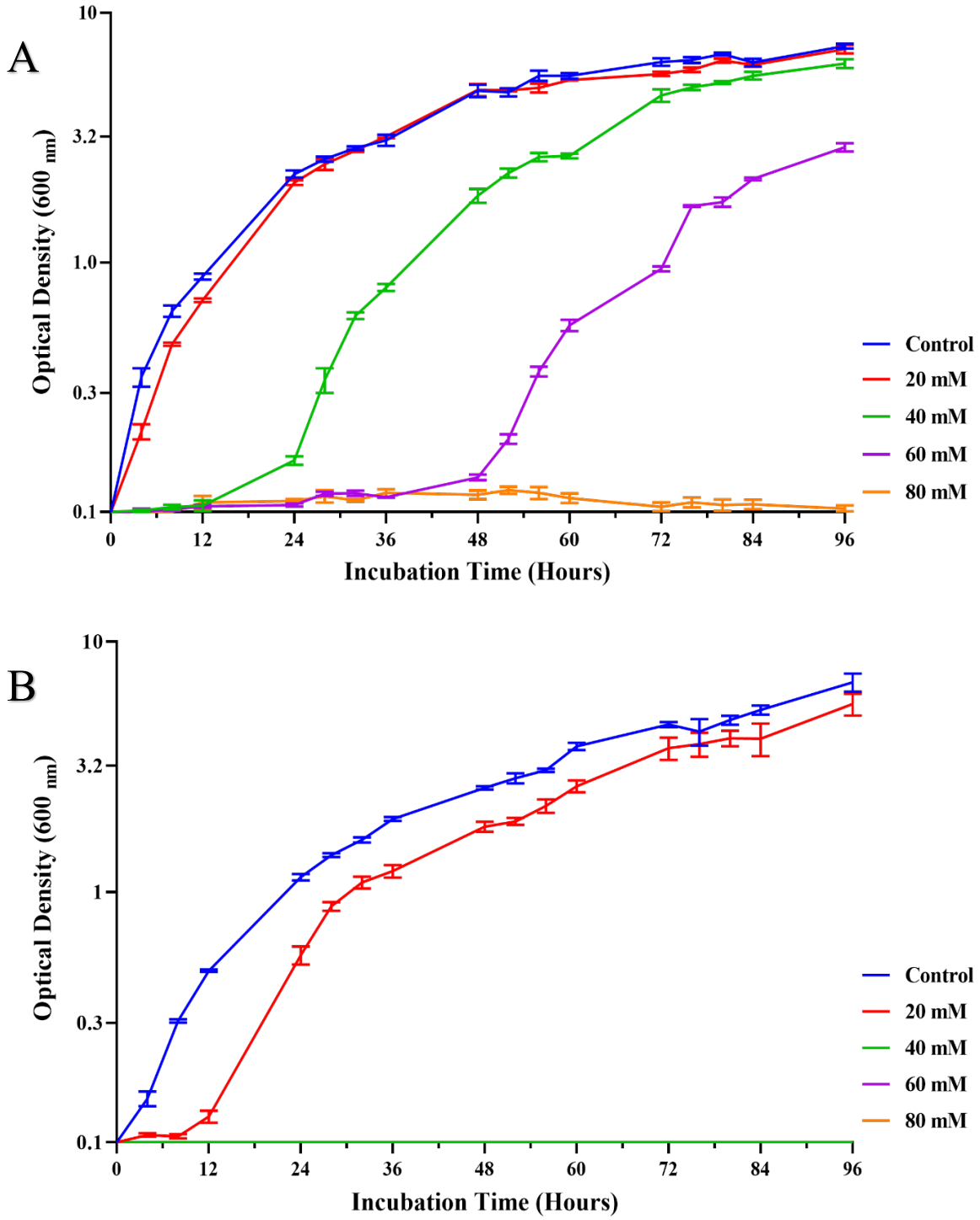
At 96 hours cultivation, *P. ofunaensis* was able to deplete all the sugar in the culture medium containing 20 mM of acetic acid. None of the rest of the culture media with other concentrations showed 100% consumption even in 96 hours. Just 87% of the xylose in the culture medium with 40 mM of inhibitor was consumed. This percentage decreased with 60 mM to reach 43%, while at 80 mM of inhibitor, it barely passed 20%.

Nevertheless, *T. multisporum* consumed just 48% of the available xylose in the culture medium with a 20 mM acetic acid in 96 hours, which is 16% less than the sugar consumed by the control culture. The rest of the tested concentrations (40, 60 and 80 mM) could not pass a 19% of sugar consumption, with the lowest percentage result (16%) being at the highest concentration.

The pH variation graphs of *P. ofunaensis* and *T. multisporum* during cultivation in media containing acetic acid are shown in the figures 23A and B, respectively. This time the pH performance was very different for *P. ofunaensis*. The control kept the same profile behavior as other experiments, while other culture media showed totally different ones. The pH in the culture medium with a 20 mM of acetic acid passed 5.0 within 36 hours and then began to decrease progressively. After 24 hours, the pH in the 40 mM culture medium began to increase, reaching around 5.5 before it was lower again after 72 hours. The medium with 60 mM held the initial pH for 48 hours before it rose and kept this behavior until the end of the experiment. While in the highest concentration (80 mM), there is a tiny decrease at the beginning. After that, it held the pH score until the end.

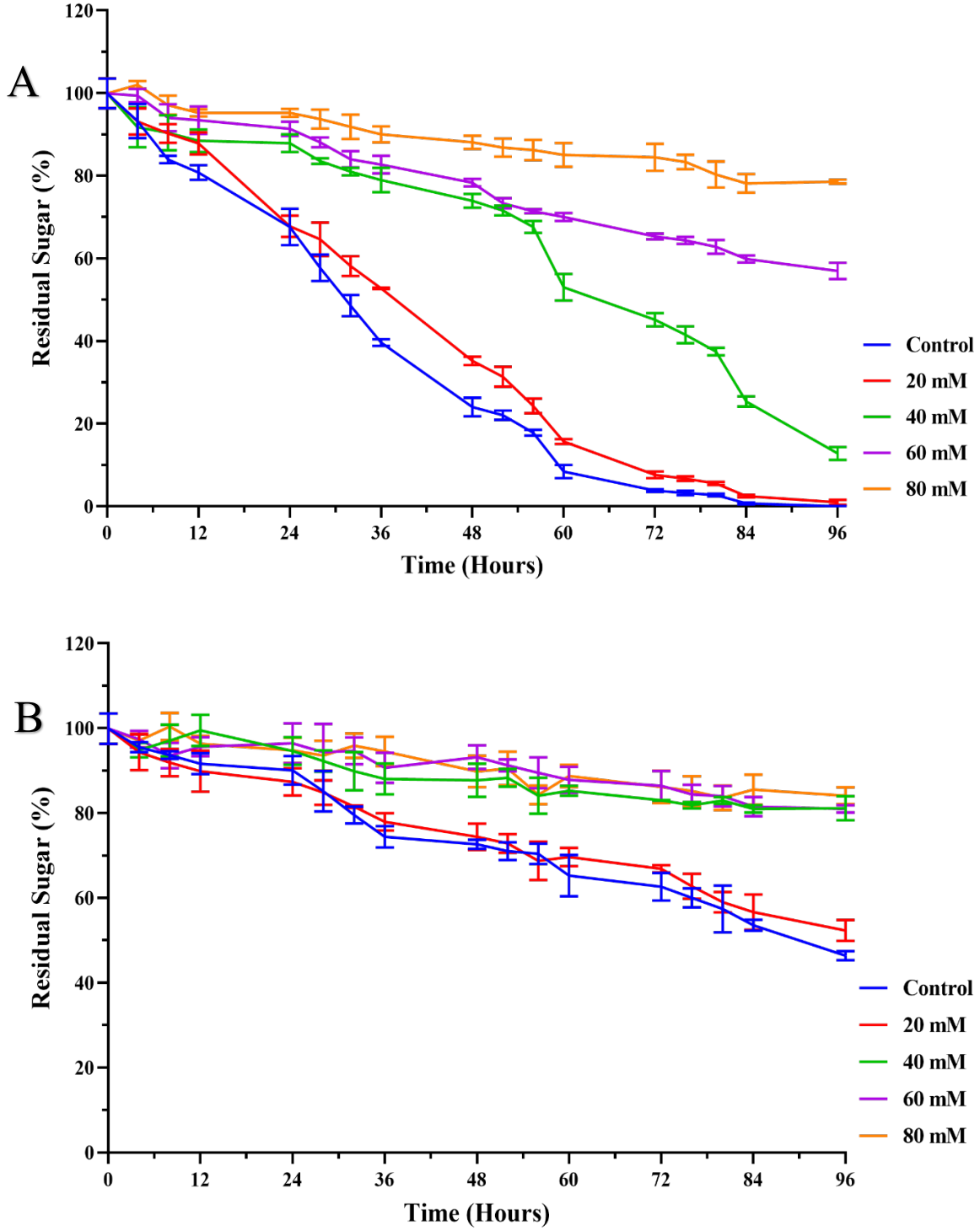
While with *T. multisporum*, only the control assay and medium with 20 mM showed a pH variation. In the medium containing 20 mM acetic acid, the scores started to rise after about 12 hours and kept reaching up to hit the 6.5 score. The rest of the culture media showed no considerable change in pH during the experiment.

Figure 21 - Cellular Growth in culture media containing acetic acid inhibitor (20-80 mM) for 96 h at 28 °C and 150 rpm, *P. ofunaensis* (A) and *T. multisporum* (B).



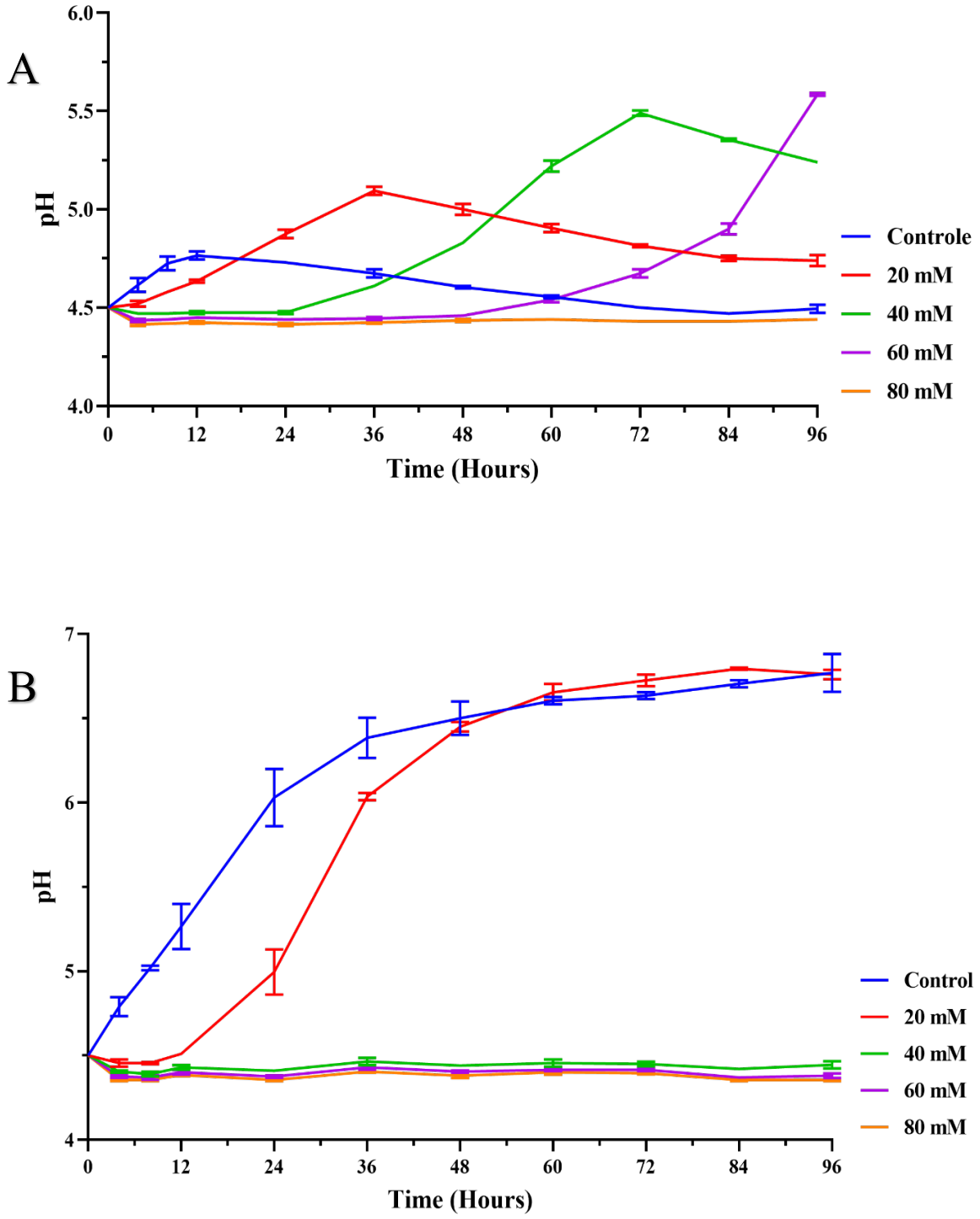
Source: own authorship.

Figure 22 - Xylose assimilation in culture media containing acetic acid inhibitor (20-80 mM) for 96 h at 28 °C and 150 rpm, *P. ofunaensis* (A) and *T. multisporum* (B).



Source: own authorship.

Figure 23 - pH variation in culture media containing acetic acid inhibitor (20-80 mM) for 96 h at 28 °C and 150 rpm, *P. ofunaensis* (A) and *T. multisporum* (B).



Source: own authorship.

5.3.2 Formic acid effect on cellular growth and xylose consumption

Yeasts *P. ofunaensis* (Figures 24A and 25A) and *T. multisporum* (Figures 24B and 25B) show the ability to xylose assimilation and cellular growth in fermentative YEPX culture media containing formic acid in several concentrations. When exposed to this acid, both species were able to grow and assimilate pentose in all-tested concentrations. Table 8 shows, in a condensed form, the tested concentrations, the optical density, xylose consumption, and pH data gathered at the end of fermentation of YEPX culture media containing formic acid in different concentrations.

Table 8 - Optical density and pH of YEPX culture media containing formic acid after 96 hours of incubation and sugar consumed during fermentation by *P. ofunaensis* and *T. multisporum*.

Specie	Formic Acid Concentration	Final **OD**	Final **pH**	Sugar Consumption
P. ofunaensis	0 mM	7.29	4.38	100% (84h)
	10 mM	7.29	4.79	100% (96h)
	20 mM	6.92	5.26	100% (96h)
	30 mM	6.92	5.60	100% (96h)
	40 mM	6.47	5.77	93% (96h)
T. multisporum	0 mM	4.55	6.37	59% (96h)
	10 mM	4.69	7.28	54% (96h)
	20 mM	4.53	7.70	43% (96h)
	30 mM	3.80	7.92	40% (96h)
	40 mM	3.68	7.97	40% (96h)

Source: own authorship.

Formic acid did not cause an extensive lag phase. The cellular growth profile for the yeast *P. ofunaensis* at 10 mM of this acid expressed similar behavior to that of the control culture medium and a minor delay in the attendant two concentrations (20 and 30 mM). At the highest concentration tested (40 mM), the yeast spent 8 hours in the lag phase, followed by an accelerated growth until the 48th hour. After that, the yeast started the stationary phase.

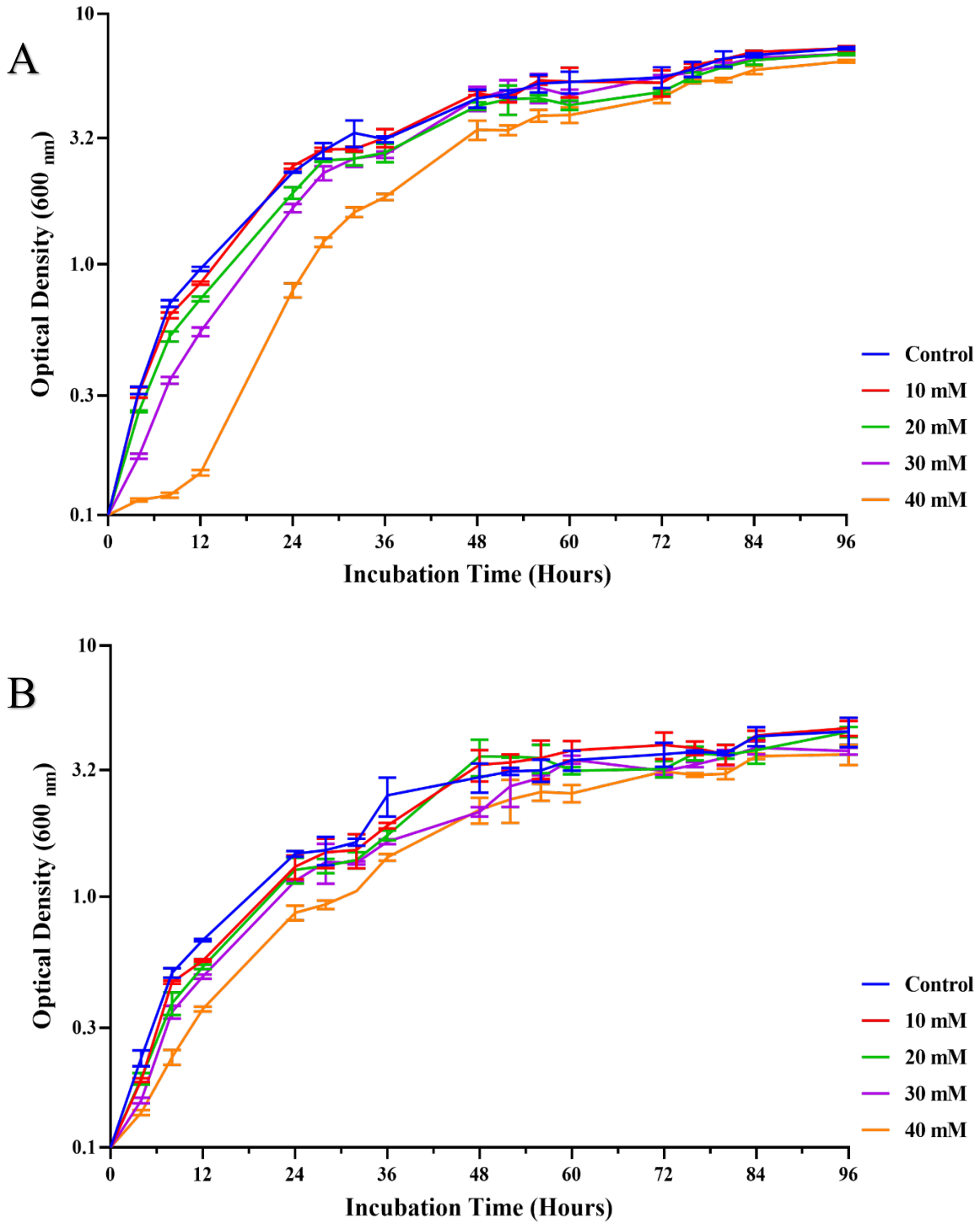
Furthermore, *T. multisporum* was able to tolerate the presence of the formic acid. At the beginning of the log phase, each fermentative culture media concentration exhibited a similar growth profile to the control assay, with a small difference compared to the medium containing 40 mM of this acid. The yeast took 48 hours to reach the stationary phase, which continued until the end of the assay.

P. ofunaensis showed excellent xylose consumption, depleting all the sugar in the media before 96 hours at all concentrations, except the highest one, in which the yeast was able to consume 93% of the sugar in 96 hours. In contrast, in the medium containing 10 mM of formic acid, *T. multisporum* consumed 54% of the xylose at the end of 96 hours. This percentage dropped to 43% for the medium with 20 mM and about 40% for the 30 mM and 40 mM of formic acid, all in 96 hours.

The pH variation graphs of *P. ofunaensis* and *T. multisporum* during cultivation in media containing formic acid are shown in the figures 26A and B, respectively. Also, in the formic acid experiments, the pH variation of the media was unusual for *P. ofunaensis*. The control maintained the same profile behavior as the previous studies, while other culture media showed totally different scores. The pH in the culture medium with a 10 mM concentration passed 4.7 after 60 hours and then manifested a negligible decrease. At 12 hours after the inoculum, the pH of the medium with 40 mM formic acid began to increase, crossing pH 5.5 in 60 hours, and kept rising until the end of the 96 hours. Culture media with 20 mM and 30 mM of formic acid continued to ascent gradually for 84 hours, then showed a tendency to stabilize at the end. The explicit observation at the end of the experiment is that the higher the inhibitor concentration, the higher the pH reading.

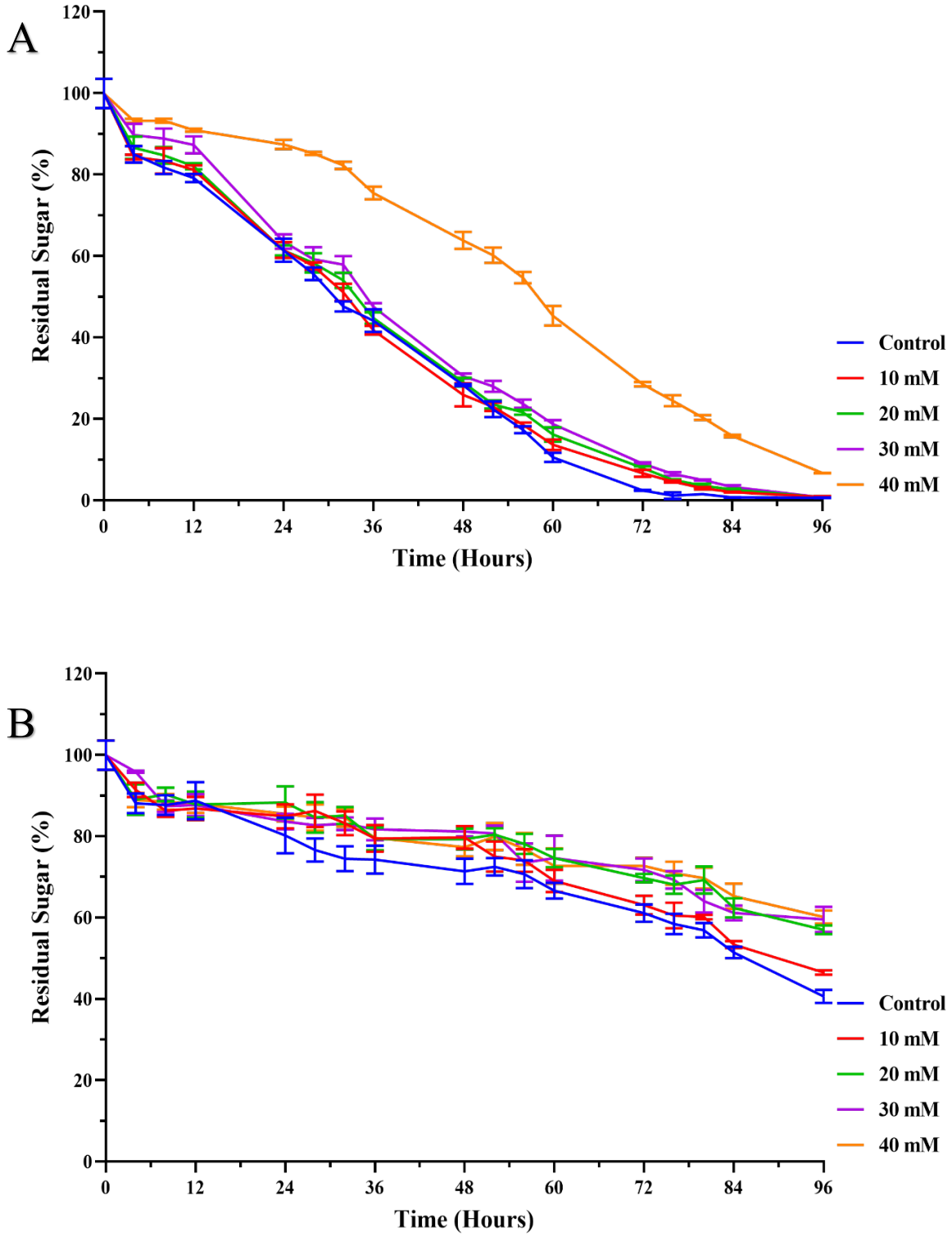
While with the *T. multisporum*, the yeast increased the alkalinity of the culture media in all concentrations, almost reaching pH 8.0 in culture media containing 30 mM and 40 mM of formic acid, while pH of the media with 10 mM and 20 mM formic acid passed 7.0 and 7.5, respectively. It was also clear that the higher the inhibitor concentration, the higher the pH reading.

Figure 24 - Cellular Growth in culture media containing formic acid inhibitor (10-40 mM) for 96 h at 28 °C and 150 rpm, *P. ofunaensis* (A) and *T. multisporum* (B).



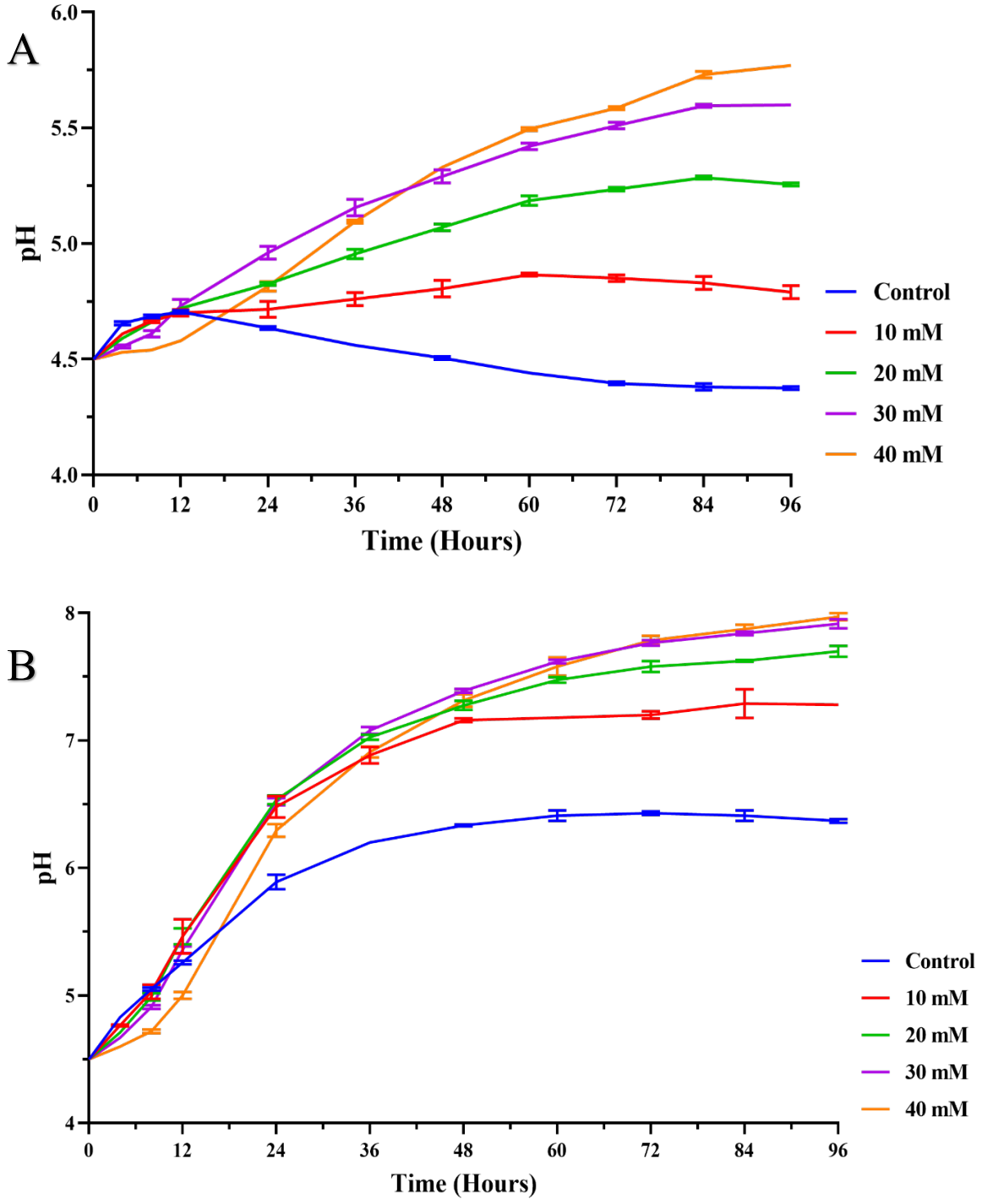
Source: own authorship.

Figure 25 - Xylose assimilation in culture media containing formic acid inhibitor (10-40 mM) for 96 h at 28 °C and 150 rpm, *P. ofunaensis* (A) and *T. multisporum* (B).



Source: own authorship.

Figure 26 - pH variation in culture media containing formic acid inhibitor (10-40 mM) for 96 h at 28 °C and 150 rpm, *P. ofunaensis* (A) and *T. multisporum* (B).



Source: own authorship.

5.3.3 Discussion

Acetic acid is the most harmful inhibitor of the growth of both yeasts. *T. multisporum* tolerated only the weakest concentration (20 mM). Even so, it took about 8 hours to move to the log phase. *P. ofunaensis* was unable to grow in the highest concentration (80 mM), but it was able to grow at the other concentrations tested, though it took up to 48 hours to enter the log phase at a 60 mM concentration. Sugar consumption was also affected by this effect. *T. multisporum* could not consume over 48% of the sugar in the unique concentration that was tolerated, while *P. ofunaensis* depleted all the available sugar only in the lowest tested concentration and within 96 hours.

Organic acids derived from the lignocellulosic biomass pre-treatment are weak acids because of their pK_a values (4.25 for acetic acid and 3.75 for formic acid). They dissociate by entering the cell via the plasma membrane, increasing the intracellular pH, which forces the cell to export protons, spending ATP in return. This mechanism is responsible for the slower growth rate shown by cells exposed to weak acids (CASPETA; CASTILLO; NIELSEN, 2015; COLA et al., 2020; PAMPULHA; LOUREIRO-DIAS, 1990). The consumption of these weak acids by the cells might be the reason for the increase in pH at the beginning, while the release of protons by cells is the reason for acidifying the media after that.

In his experiment on *C. guilliermondii*, Pereira et al (2011) tested the inhibition effect of acetic acid with three different concentrations (0.8, 1.1, and 2.6 g/L) on cellular growth and xylose consumption under the same conditions mentioned previously. The presence of acetic acid in the fermentation medium had no effect on yeast cell growth at 0.8 g/L and 1.1 g/L, whereas the xylose consumption was only significantly affected at 2.6 g/L of acetic acid. Under this concentration value, cell growth and xylose consumption were reduced by 30% and 13%, respectively, compared with the control.

P. ofunaensis was able to grow on the same tested concentrations as *C. guilliermondii*, with a better performance in cellular growth by *P. ofunaensis* than *C. guilliermondii*. *T. multisporum* showed the lowest growth among these three yeasts, as it could grow only at 20 mM acetic acid (1.2 g/L). At the highest tested concentration, sugar consumption was similar in *C. guilliermondii* (86%) and *P. ofunaensis* (87%), while *T. multisporum* just consumed 19%.

Monteiro (2020) tested different concentrations (0.3, 8.0, and 15.0 g/L) of acetic acid on *S. pararoseus* U3 and *P. terricola* G20 yeasts at the same conditions mentioned previously. Both of them tolerated the lowest inhibitor concentration, duplicating their growth, but they

failed to withstand the other two concentrations. The range of tested concentrations by Monteiro (2020) is much higher than the ones we tested, which makes it impossible to compare between the yeasts. All four yeasts were able to grow at the lowest tested concentration.

Sitepu et al. (2014) tested only one concentration of acetic acid, 2.5 g/L, under the same conditions mentioned previously. Only 12 yeasts could tolerate this concentration, and one of these yeasts showed a delay in growth. *T. dermatis* and *T. guehoae* also failed to tolerate this concentration. Only *T. coremiiforme* was able to grow in the presence of acetic acid at this level, which makes it more tolerant than *T. multisporum.*

The two yeasts were able to tolerate all the selected concentrations of the formic acid inhibitor, although the higher concentration affected the performance of the *P. ofunaensis* as it took about 8 hours for the yeast to enter the log phase stage.

On the other hand, *T. multisporum* did not show this clear lag phase in the higher concentration. However, it could not drain over 54% of the sugar in the initial medium, and this percentage decreased to 40% in the highest one. *P. ofunaensis* exhausted sugar in all tested concentrations except for the highest one, depleting about 93% by the end of the experiment.

As in the acetic acid experiment, *P. ofunaensis* raised the alkalinity of the media in direct proportion to the inhibitor's concentration. *T. multisporum* showed the same behavior as well.

Monteiro (2020) tested the effect of formic acid at three concentrations (0.1, 2.5, and 5.0 g/L) in the cultivation of *S. pararoseus* U3 and *P. terricola* G20, at the same conditions mentioned previously. Both yeasts could tolerate just the first tested concentration and did not tolerate the other two. The range of tested concentrations by Monteiro (2020) is much higher than the ones we tested. This prevents the possibility of comparisons between yeasts. This result shows a similar performance between *S. pararoseus* U3, *P. terricola* G20, and *P. ofunaensis* and *T. multisporum* in the ability to grow in the presence of formic acid in this concentration range.

5.4 The combined impact of the tested inhibitors on the two yeasts

After individual assessment of each inhibitor's effect on the yeasts' performance in growth and xylose consumption aspects, the impact of these inhibitors was tested once more, but this time concurrently, as a way of simulating a liquid hydrolyzate that results from the pretreatment processes in which these six inhibitors, among others, might be produced.

The culture media were prepared using the following inhibitors' concentrations: 5 mM furfural, 5 mM HMF, 2 mM ferulic acid, 2 mM vanillin, 20 mM acetic acid, and 10 mM formic acid. In this experiment, neither of the two yeasts was able to tolerate the presence of these mixed chemicals.

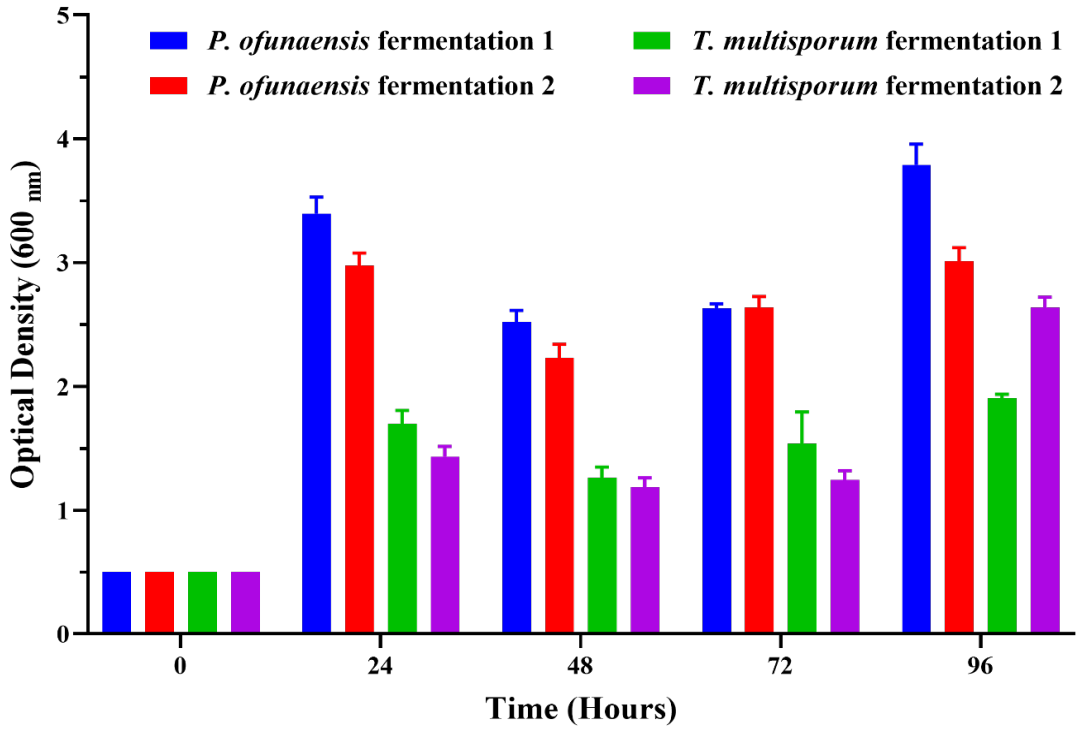
5.5 Ethanol production, identification and quantification

An anaerobic fermentation assay was performed in duplicate using a saline culture media for ethanol production, identification, and quantification assessment. Figure 27 shows the cellular growth for both yeasts.

Even though the experiment started with five times the amount of initial inoculum (O.D 0.5 at 600_{nm}), and the culture media did not have any inhibitors and contained two and a half times the amount of xylose (50 g/L) compared to the aerobic assays, the growth level of the two yeasts was lower than the previous. This is expected for facultative anaerobic organisms, such as yeasts, which under aerobic conditions tend to develop higher biomass.

Cell growth was greater in the first 24 hours of fermentation than at 48 and 72 hours, especially for *P. ofunaensis*. Perhaps this was due to the oxygen present in the media at the beginning of the assay, and then after 96 hours of cultivation the yeasts reached their highest growth.

Figure 27 - Anaerobic cultivation of the yeasts *P. ofunaensis* and *T. multisporum* in saline culture media for 96 h at 28 °C and 150 rpm.



Source: own authorship.

At 24 hours, *P. ofunaensis* reached about O.D 3.39, while *T. multisporum* attended the O.D 1.53. The yeasts, *P. ofunaensis* and *T. multisporum* showed little change in their growth between 48 and 72 hours; *P. ofunaensis* ranged around O.D 2.60 while *T. multisporum* held its scores around 1.45. At the end of the assay, after 96 hours, *P. ofunaensis* touched O.D 3.7, while the best that *T. multisporum* reached was O.D 2.63. In other words, *P. ofunaensis* and *T. multisporum* increased seven and five times their cellular growth, respectively.

The xylose consumption was relatively proportional to cellular growth. *P. ofunaensis,* which grew more, has also consumed more xylose. In the 24h fermentation samples, the xylose consumption was around 20% ± 4%, while the 48h and 72h samples consumption was about 21% and 22%, respectively. However, the highest consumption was in the 96h samples that reached 25%. On the other hand, *T. multisporum* wasn't able to consume much xylose, assimilating around 10% to 14% of the available sugar during the whole assay (Figure 28).

Regarding the pH of the samples, the behavior of *P. ofunaensis* was quite constant during the whole assay, as it maintained the pH around 4.5 without any significant change from

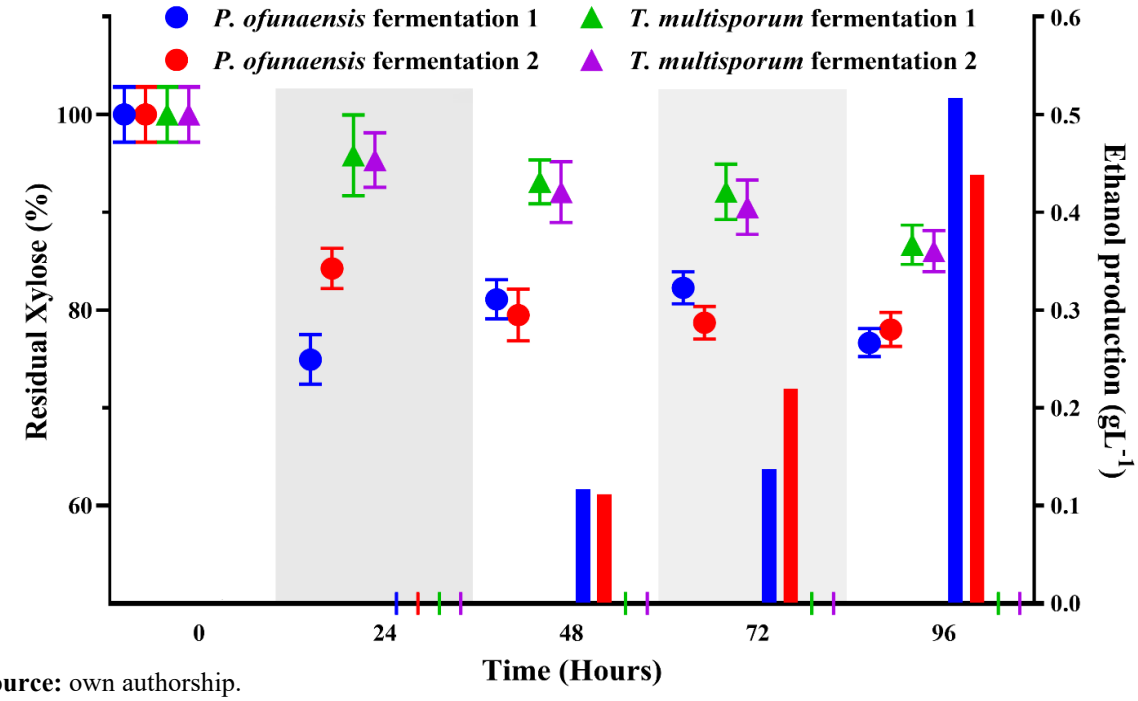
the initial pH. Whereas *T. multisporum* was less conservative and kept its repeating conduct, increasing the pH of the culture medium (Figure 29).

As for ethanol production by yeasts, *P. ofunaensis* fermentation exhibited an unsatisfactory amount for application in larger-scale processes, generating a maximum of 0.51 g/L in the culture medium at 96 hours. *T. multisporum,* on the other hand, was not able to produce any amount of ethanol (Figure 28).

Martins (2011) evaluated the cellular growth, xylose consumption, and ability to produce ethanol in an alcoholic fermentation under quite similar conditions that we tested, but by adding 10% of xylose instead of 5%, temperature of 32 °C instead of 28 °C, and for 120 hours instead of 96 hours, for two yeasts; *Candida shehatae* BR6-2AY and *Rhodotorula* sp. G10.2.

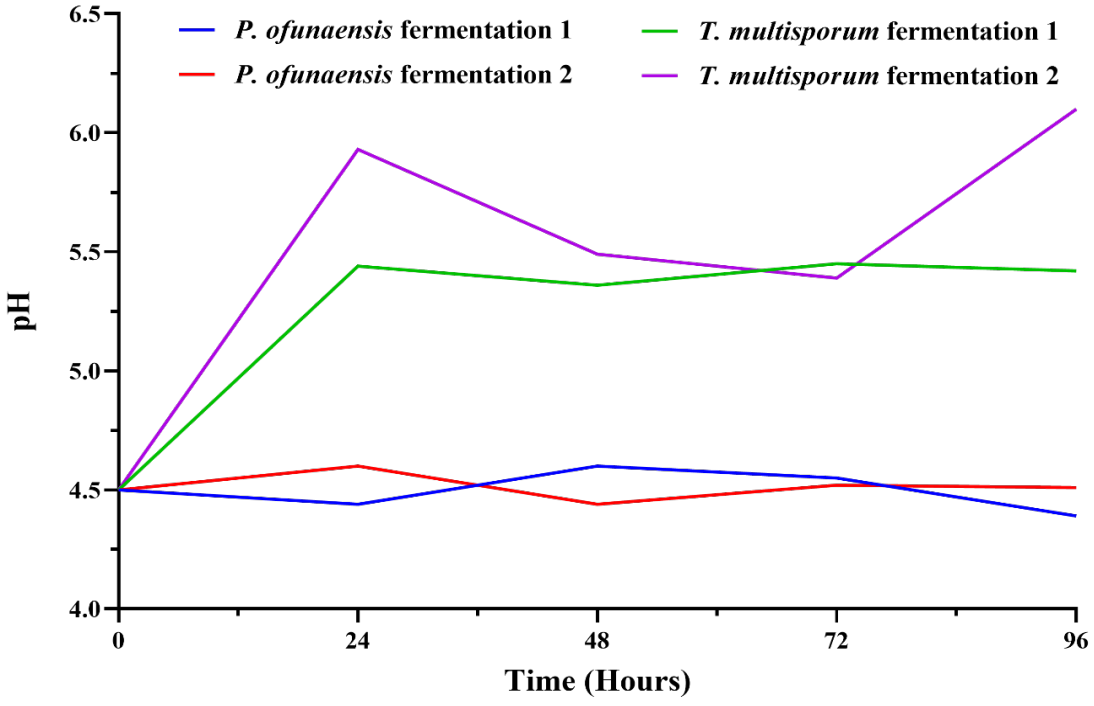
Regarding cell growth, *C. shehatae* BR6-2AY was roughly able to triplicate its cellular mass during the anaerobic fermentation, while *Rhodotorula* sp. G10.2 duplicated its cellular growth within 96 hours. *C. shehatae* BR6-2AY sugar consumption was less than *Rhodotorula* sp. G10.2 consumption, they consumed 69.7 and 64.2% of xylose, respectively. In 96 hours of fermentation, *C. shehatae* BR6-2AY was able to produce around 2.6 g/L of ethanol, while *Rhodotorula* sp. G10.2 produced about 3.1 g/L.

Figure 28 - Xylose consumption (symbols) and ethanol production (bars) during anaerobic cultivation of the yeasts *P. ofunaensis* (circle) and *T. multisporum* (triangle) in saline culture media for 96 h at 28 °C and 150 rpm.



Source: own authorship.

Figure 29 - pH variation of the saline media during anaerobic cultivations of the yeasts *P. ofunaensis* and *T. multisporum* for 96 h at 28 °C and 150 rpm.



Source: own authorship.

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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