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STUDIES ON SUGARS CONSUMPTION AND LIPIDS PRODUCTION IN YEASTS ISOLATED FROM CHILEAN VALDIVIAN FOREST

(Estudios sobre el consumo de azucares y la producción de lípidos en levaduras aisladas del bosque Valdiviano Chileno)

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Dedicada a mi hijo Nicolás Ignacio Vera Valdés

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RESUMEN

Los microorganismos oleaginosos son capaces de acumular lípidos en altos porcentajes al interior de sus células. Ellos han sido extensamente estudiados y propuestos como una alternativa para la producción de oleoquímicos y biocombustibles. La producción de lípidos microbianos, llamados aceites unicelulares (SCO), depende de varios parámetros de crecimiento, entre ellos, la elección del sustrato es muy importante. Para considerar una producción de SCO a escala industrial, el sustrato debe ser abundante, de bajo costo adquisitivo y adecuado para la fermentación por microorganismos.

La biomasa lignocelulósica ha sido evaluada y propuesta como una estupenda alternativa de sustrato para producción de SCO, principalmente por su alto contenido de azúcares fermentables (hexosas y pentosas). En este contexto, el presente trabajo tiene como objetivo exhibir los principales aspectos relacionados con el cultivo de microorganismos oleaginosos, creciendo en los azucares más abundantes en la biomasa lignocelulósica como sustrato. Inicialmente se realiza una revisión y discusión de los trabajos científicos actuales en la producción de SCO, de tal forma de contribuir al desarrollo futuro en un proceso sostenible. En segundo lugar, se estudió el crecimiento y metabolismo de tres cepas de levaduras aisladas de madera en descomposición. Ellas demostraron crecer eficientemente y al mismo tiempo acumular lípidos a partir de los principales monosacáridos contenidos en la madera de *Pinus spp*. y *Eucalyptus spp*. que incluye glucosa (Glc), xilosa (Xyl) y manosa (Man). Este estudió permitió contribuir a los antecedentes científicos en el comportamiento de microorganismos frente a mezclas de azucares. Se concluye que los microorganismos capaces de asimilar simultáneamente los principales azúcares lignocelulósicos y convertirlos eficientemente en biomasa oleaginosa, presentan un potencial biotecnológico que merece ser investigado más a fondo. Con los antecedentes de este trabajo se proponen realizar nuevas investigaciones utilizando hidrolizados lignocelulósicos para la producción de SCO y continuar contribuyendo al desarrollo de sus aplicaciones, las cuales han comenzado a cobrar mucho interés científico e industrial.

ABSTRACT

Oleaginous microorganisms are able to accumulate high percentages of lipids inside their cells. These have been extensively studied and proposed as an alternative for oleochemicals and biofuels production. The production of microbial lipids, known as unicellular oils (SCO), depends on several growth parameters, among them, the election of the substrate is especially important. In order to consider an industrial scale production of SCO, the substrate must be abundant, low in cost and suitable for fermentation by microorganisms.

Lignocellulosic biomass has been evaluated and proposed as a great alternative for SCO production, mainly due to its high content of fermentable sugars (hexose and pentoses). In this context, the present work aims to show the main aspects related to the cultivation of oleaginous microorganisms, growing in the most common sugars from lignocellulosic biomass as substrate. Initially, a review and a discussion of the current scientific work is realized, in order to contribute to the future development of a sustainable process. Secondly, the growth and metabolism of three yeasts strains isolated from decay wood were studied. These were shown to grow efficiently and at the same time to accumulate lipids using the main monomers contained in the wood of *Pinus spp*. and *Eucalyptus spp*., which include glucose (Glc), xylose (Xyl) and mannose (Man). The study allows to contribute to the scientific background on the behavior of microorganisms against mixtures of sugars. We concluded that the microorganisms able to assimilate simultaneously the main lignocellulosic sugars and to convert efficiently them into oily biomass, have a biotechnological potential that deserves to be further investigated. The background of this work proposes to carry out new research using lignocellulosic hydrolysates for the production of SCO and to contribute to the development of its applications, which have started to generate an important industrial and scientific interest.

INTRODUCCIÓN GENERAL

Microorganismos oleaginosos

Los microorganismos oleaginosos tienen la habilidad de consumir diversos sustratos carbonados y en condiciones ambientales específicas transformarlos en ácidos grasos (AG). Ellos pueden acumular energía química en forma de lípidos también conocidos como Single cell-oil (SCO), los cuales se almacena en cuerpos lipídicos con estructuras conservadas tanto en eucariontes como procariontes y representan sobre el 20% del peso seco celular (% p/p) [1, 2]. En las últimas décadas los SCO se han convertido en productos muy atractivos para la industria, y han sido reconocidos como una alternativa a los biocombustibles, los productos oleoquímicos y los aceites comestibles [3,4].

Entre los microorganismos oleaginosos se encuentran diversas especies de microalgas, hongos (filamentosos y levaduras), bacterias y protistas [5,6,7]. Especialmente se han destacado las levaduras y se han propuesto como los candidatos más prometedores para la producción de SCO. Las levaduras oleaginosas cuentan con interesantes aplicaciones biotecnológicas dada por la composición química de los SCO que acumulan, los cuales comúnmente contienen ácidos grasos (AG) saturados y monoinsaturados de las familias C16 y C18 y algunos ácidos grasos poliinsaturados con un número restringido de dobles enlaces, como el ácido linoleico (C18:2, n-6) y el ácido α-linolénico (C18:3, n-3). Este perfil lipídico es similar al de los aceites vegetales que se consideran como materia prima en la industria química, incluida la industria del biodiésel [7,8].

Para que la producción de los lípidos microbianos sea eficaz, es necesario determinar condiciones adecuadas para el crecimiento de los microorganismos, dentro de ellas, se encuentran las condiciones ambientales de cultivo y la composición de la materia prima, siendo esta última un factor fundamental en la respuesta metabólica de los microorganismos [9].

Biomasa lignocelulósica

La biomasa lignocelulósica representa una materia prima fascinante para producir compuestos valiosos, ya que es un sustrato renovable, abundante y económico, compuesto por un alto porcentaje de azúcares fermentables (hexosas y pentosas) [10]. Su estructura y composición química consiste principalmente en una red de celulosa, hemicelulosas (que juntas forman la holocelulosa) y lignina y en menor cantidad también están presentes el almidón, la pectina, los extractos y las cenizas [11]. El contenido de cada uno de ellos varía dependiendo de la especie, los tejidos y la edad de las células de las plantas [12].

La celulosa es el polímero más abundante en la biomasa vegetal, representando entre el 34 y 50% de la biomasa seca en las especies de madera blanda y el 41 y 50% en las especies de madera dura, mientras que en los residuos agrícolas es aproximadamente entre el 17 y 42% [12, 13]. Su estructura está compuesta por moléculas de glucosa que se unen entre sí formando cadenas lineales de glucosa [11]. Por otro lado, las hemicelulosas son heteropolisacáridos amorfos y ramificados formados por una cadena principal y otras laterales de monosacáridos. La cadena principal está compuesta por unidades iguales (homopolímero) o diferentes (hetreropolímero) y son más cortas en longitud que la celulosa. Las hemicelulosas en maderas representan aproximadamente el 18-35% [14,15], mientras que en la biomasa agrícola representa entre el 12 y el 37% [16]. Finalmente, la lignina es un polímero complejo y estable compuesto de diferentes tipos de lignina, guayacil (G), siringil (S) y p-hidroxifenil (H), derivados de los alcoholes coniferilo, sinapilo y p-cumarilo, respectivamente. En las maderas blandas la lignina predominante es el guayacil, en las maderas duras el guayacil y siringil en una proporción de 1:1 a 1:3, mientras que las hierbas están presentes todos los monómeros de lignina. La lignina representa entre el 19 y el 35% de la madera y entre el 3,5 y el 30% en herbáceas [14,16,17] y se forma principalmente en los tejidos vasculares proporcionando resistencia mecánica a las plantas.

Los diversos monosacáridos de la celulosa y las hemicelulosas son adecuados para

la fermentación por microorganismo, por lo que su obtención es muy preciada y diversos procesos han sido desarrollados para dejarlos a disposición. Sin embargo, la estructura que forman los azucares junto a la lignina le confiere un carácter altamente recalcitrante a la biomasa difícil de eliminar.

La recalcitrancia está dada en primer lugar, por las moléculas de celobiosa (dímero de glucosa) que se unen a través de grupos hidroxilos por medio de enlaces de hidrógeno para formar microfibras de celulosa, que a su vez, se unen mediante fuerzas de Vander Waals para formar macrofibrillas que poseen regiones altamente estables (regiones cristalinas) [11]. Las macrofibrillas además se unen a las hemicelulosas, y las hemicelulosas a la lignina formando una red que controla la permeabilidad, mejora las propiedades mecánicas y protege a las células de los microorganismos y de la degradación química, siendo el principal factor de la recalcitrancia de la biomasa.

Para eliminar esta recalcitrancia y acceder a los azucares fermentables se requiere de tratamientos previos químicos, físicos, biológicos o combinados, que permitan liberar los azucares [18]. Sin embargo, estos tratamientos también generan una serie de subproductos que pueden inhibir el crecimiento microbiano y/o la lipogénesis [19].

Para superar los obstáculos asociados al uso de biomasa lignocelulósica en la producción de SCO se han propuesto diversos enfoques. Entre ellos, por ejemplo, se considera el desarrollo de técnicas de pretratamientos, respetuosas con el medio ambiente, que generen menos compuestos inhibidores y que alcancen los niveles más altos de azucares fermentables [19]. Así como también, se propone el uso de técnicas biotecnológicas que permitan obtener microorganismos capaces de crecer y producir lípidos eficientemente, utilizando sustratos lignocelulósicos [20-23]. Ambos enfoques continúan su desarrollo y se espera continuar con investigaciones para obtener procesos sustentables.

Biomasa lignocelulósica como sustrato para producción de SCO

El consumo de los azúcares lignocelulósicos por microorganismos oleaginosos y no

oleaginosos se han estudiado en detalle en varias levaduras, incluida la especie de *Yarowia lipolytica y Saccharomyces cerevisiae*, las cuales se han utilizado como modelo para indagar sobre el metabolismo de hexosa y pentosas [24, 25]. Particularmente lo que sucede es una serie de eventos que comienzan con el transporte de los azucares al interior de las células, luego las azucares son metabolizadas por diferentes enzimas hasta piruvato en una etapa final de la glicólisis. El piruvato luego entra en la mitocondria y participa en el ciclo del ácido tricarboxilico (TCA) proporcionando energía y precursores anabólicos a la célula. Sin embargo, cuando la fuente de nitrógeno se agota en el medio de crecimiento, el ciclo del TCA se altera a nivel de la enzima isocitrato deshidrogenasa (ICDH) y el citrato se excreta al citoplasma, donde mediante la enzima ATP- citrato liasa (ATP:CL) es convertido en acetil-CoA, que es el precursor universal de los AGs y en oxalacetato [26].

El mecanismo de asimilación en levaduras creciendo en mezclas de azucares se ha descrito frecuentemente como un proceso catabólico secuencial, es decir, donde normalmente existe consumo de un azúcar preferido, generalmente glucosa, la cual activa mecanismos de represión catabólicos de otros azucares por un período determinado de tiempo. Los mecanismos de represión producen una etapa de retardo del crecimiento conocido como comportamiento diauxico [27,28]. El cual representa un gran inconveniente cuando hay múltiples azúcares (pentosas y hexosas) presentes en el medio de crecimiento, como en el caso del hidrolizado lignocelulósico, provocando un aumento significativo del tiempo de cultivo [29].

El desarrollo de esta tesis se fundamentó en el estudio de levaduras aisladas de madera en avanzado estado de descomposición del bosque Valdiviano Chileno, con cualidades naturales para crecer en sustratos con alto contenido de azucares lignocelulósicos y sintetizar SCO a partir de ellos. Un esquema general de este proceso se presenta en la Fig. Resumen. Considerando los antecedes, la tesis doctoral presenta una visión integral de los aspectos fundamentales que afectan el proceso de producción de SCO utilizando hidrolizados lignocelulósicos y se estudia particularmente el crecimiento de levaduras y su producción de lípidos en cultivos con mezclas de los azucares más comunes presentes en la biomasa lignocelulósica.

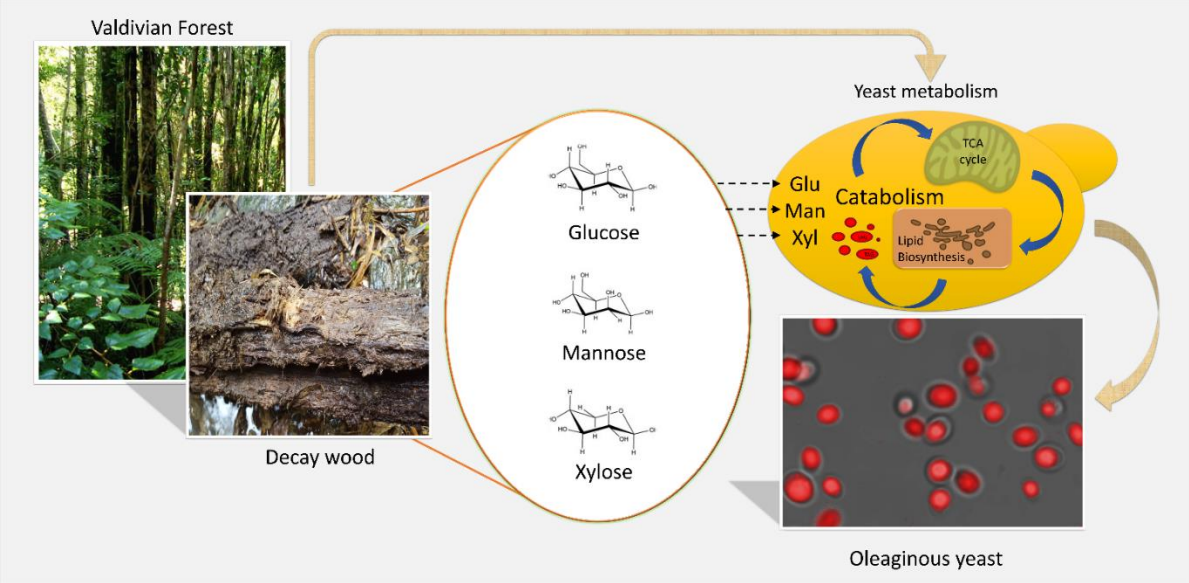

Fig. Resumen. Esquema global del trabajo de tesis doctoral

HIPÓTESIS

Las hexosas y pentosas más abundantes de la biomasa lignocelulósica, como son glucosa, manosa y xilosa, pueden ser consumidos simultáneamente y transformados hasta lípidos de almacenamiento (Single cell oil, SCO) mediante el metabolismo de tres cepas de levaduras aisladas de madera en descomposición del bosque Valdiviano. Además, el cultivo de las levaduras en una mezcla de azucares a mayor temperatura produce rendimientos superiores de lípidos que cultivos con un solo tipo de azúcar y menor temperatura.

OBJETIVO GENERAL

Estudiar el crecimiento y lipogénesis de levaduras aisladas de madera en descomposición, durante su cultivo en mezclas de hexosas (glucosa y manosa) y pentosa (xilosa) más comunes presentes en la biomasa lignocelulósica, y evaluar la capacidad de consumir los azucares simultáneamente a diferentes temperaturas de crecimiento.

OBJETIVOS ESPECÍFICOS

1. Evaluar el crecimiento y la producción de lípidos de tres levaduras aisladas de madera en descomposición en cultivos con glucosa, manosa y xilosa a dos temperaturas de crecimiento (20 y 28º C).
2. Evaluar el crecimiento y la producción de lípidos de las tres levaduras cultivadas en una mezcla de glucosa, xilosa y manosa a 20 y 28º C.
3. Estudiar las actividades de las enzimas claves en el metabolismo y lipogénesis de las tres levaduras cultivadas en mezclas de glucosa, xilosa y manosa y demostrar su consumo simultáneo.

CAPÍTULO I: LIGNOCELLULOSIC BIOMASS TO MICROBIAL OIL. A REVIEW [1]

Gabriela Valdés, Regis Teixeira Mendonça, George Aggelis

1.1. ABSTRACT

The oleaginous microorganisms are able to accumulate lipids in high percentages, have been widely studied as an alternative for producing oleochemicals and biofuels. The production of microbial lipid, known as Single-cell oil (SCO), depends on several growth parameters, including the nature of the carbon substrate, which must be consumed efficiently and converted in storage lipid. The substrates considered for large scale applications should be abundant and low cost of acquisition. Among others, lignocellulosic biomass is a promising renewable substrate alternative, containing high percentages of fermentable sugars (hexose and pentoses). However, it is also highly recalcitrant and therefore it requires specific pretreatments in order to release its assimilable components. The main disadvantage of pretreatment is the generation of several inhibitory by-products in the microbial metabolism. In this review, we discuss the main aspects related to the cultivation of oleaginous microorganisms using lignocellulosic biomass as substrate, hoping to contribute to the development of a sustainable process for SCO production in the near future.

Keywords: Oleaginous microorganism, microbial lipids, Lignocellulose pretreatments, enzymatic hydrolysis, lignocellulose by-products

[1] El contenido de este capítulo fue enviado como artículo Review a la revista Applied Sciences y se encuentra en revisión. 2020.

1.2. INTRODUCTION

The development of new products to meet the demands of the world's population is currently focused on processes that preserve environment healthy. Single Cell Oil (SCO) is one of the new products that has been recognized in the last decade as an alternative to biofuels, oleochemicals and edible oils [3,4,30–35]. SCOs are lipids produced from oleaginous microorganisms and have similar chemical characteristics to plant lipids. One of the major advantages of oleaginous microorganisms over vegetable crops is their reduced need for soil and shorter growing periods [36].

Effective production in SCO requires the correct selection of growth condition for microorganisms, in order to convert the carbon substrate into storage lipids. Considering the perspective of commercial applications, special attention should be paid to the substrate acquisition cost, because it represents 40-80% of the total production cost [9]. The lignocellulosic biomass is a great option because is a renewable and abundant substrate, containing important amounts of fermentable sugars [11,37]. However, due to the high recalcitrant structure of the lignocellulosic biomass, special chemical, physical or biological pretreatment is necessary [38,39], in which many by-products generated may be inhibitors to microbial growth and/or lipogenesis [19]. Several approaches to overcome inhibitory effects of the by-products of lignocellulose pretreatment and increase lipid productivities have been proposed. These include pretreatment techniques that generate fewer inhibitory compounds or a detoxification system able of reducing their concentration [41]. In addition, genetic and metabolic engineering techniques [22,43], evolutionary adaptation [21,23] and specialized studies in the microbial consortium [10,46-48] are proposed, for new approaches for the biotechnological valorization of lignocellulose biomass. However, the level of technology achieved so far is still insufficient to support cost-effective production of SCO.

The main proposal of this paper is to review recent research on the pretreatment of lignocellulosic biomass and to provide an overview of the growth of oleaginous

microorganisms and lipid production on lignocellulosic substrates.

1.3. OLEAGINOUS MICROORGANISMS

1.3.1. Description and main oleaginous species

oleaginous microorganisms share the common characteristic of being able to accumulate chemical energy in the form of lipids. The metabolism of these organisms is adapted to convert, under specific growth conditions, the carbon substrate into storage lipids that are stored in their cytoplasm as lipid bodies with preserved structures in prokaryotes and eukaryotes [2]. In eukaryotes, and rarely in prokaryotes, the lipid bodies are made up of neutral lipids, mainly triacylglycerols (TAG) [7].

Oleaginous microorganisms can be found among species of microalgae, fungi (filamentous and yeasts), bacteria and protists. Microalgae strains associated with *Chlorella, Scenedesmus, Chlamydomonas, Nannochloropsis, Chlorococcum, Isochrysis, Cylindrotheca, Tetraselmis, Auxenochlorella, Botryococcus* species are referenced to have excellent lipid production ability [5,33,49–54]. Among bacteria the known species are *Rhodococcus,* Streptomyces, *Nocardia, Mycobacterium, Dietzia or Gordonia* [6,55–57]. Representative oleaginous species of filamentous fungi, Mucorales and yeasts belong to *Mortierella, Microsphaeropsi, Fusarium, Candida, Meyerozyma, Rhodotorula, Rhodosporidium, Pichia, Cryptococcus, Lipomyces, Trichosporon* and *Yarrowia* [5,8,23,58–76].

The microbial consortia with the participation of microalgae and bacteria [77–80] or microalgae and yeast species [81–83] have been reported as a strategy to increase lipid production [47,81]. Although consortia have shown that synergism occurs between different microorganisms, allowing increased accumulation of lipids, further studies are needed to determine the individual contributions of each during the lipid production process. Since the physiology of oleaginous microorganisms varies among species [84–87], the ability to accumulate lipids is influenced by different factors that are species specific.

1.3.2. Environmental conditions that promote lipid synthesis

Nutrients concentration in culture medium such as nitrogen and carbon is decisive to lipid production [33,59,88–90]. In heterotrophs, usually a high C:N molar ratio is needed to achieve an important lipid accumulation [31,33,67,91,92]. As well as, nature of the nitrogen source plays a critical role. In some cases, ammonium nitrogen favors lipid accumulation [93], while in others, organic nitrogen is preferred for lipogenesis [31,33,94,95]. In addition, nutrient such as iron and magnesium are also especially important, as their absence inhibits the lipids oxidation [91]. The temperature, pH, dissolved oxygen, and culture agitation are other important variables to consider [91,96]. Studies have shown low production of unsaturated FA at temperatures above 30ºC [67] and positive lipid production under neutral and basic pH conditions [7]. On the other hand, oxygen dissolution is related to a suitable agitation (of 200 to 300 rpm) while, the lipids decrease at lower agitation, and the metabolism is diverted to extracellular products such as alcohols (e.g. ethanol, mannitol, arabitol and 2,3-butanediol) [67,96].

In autotrophs, photosynthesis is favored in nitrogen rich media providing reducing potential for fatty acid biosynthesis [97]. In many species, lipid accumulation is favored in phosphate limited conditions [30,98]. Other parameters considered in microalgae are the pH, which affect nitrogen uptake. Specifically, the dissociation of NH_4^+ to NH_3 occurs at pH> 8 thus, at lower pH values, nitrogen exists as NH_4^+ ions which is preferred by microalgae over NH_3 [99,100]. The temperature effect is relative and depends in physiology [101]. Additionally, the lipid content increases with light intensity, CO_2 concentration and oxidative stress caused by salinity [102–104].

Recently, heterotrophs and a autotrophs have been evaluated according to the effect of reactive oxygen compounds (ROS) or free radicals generated in aerobic metabolism, including superoxide (O_2^-), hydroxyl (OH^-), perhydroxyl (HO_2^-) and the alkoxy radical (RO^-), and non-radical forms such as hydrogen peroxide (H_2O_2) and oxygen (O_2). Their accumulation usually results in peroxidation of lipids and

denaturation of proteins and DNA. However, at balanced levels lipid synthesis is enhanced [105,106]

The right setting of environmental parameters to improve lipid synthesis is evident [67,21,107]. However, these remain complex and more experimental data and models are needed to optimize the process.

1.3.3. Sugar conversion into SCO and regulatory mechanisms in sugar assimilation

It has been reported that some oleaginous microorganisms are capable of consuming hexose and pentose and converting them into lipids. The preferred sugars are hexose such as glucose [70,108-110], although some species are efficient at converting pentoses such as xylose [70,92,111]. Sugar compounds such as cellobiose and molasses have also been reported to be consumed [70,92].

The efficient and simultaneous use of hexose and pentose to improve the production of SCO from lignocellulose biomass is highly appreciated. However, many microorganisms consume sugar sequentially and when grown in a mixture of sugars have shown the classic phenomenon known as diauxic growth [112,113]. This behavior is attributed to two mechanisms that may or may not occur together. One is the repression of catabolic carbon, in which the preferred sugar causes repression in the transcription of enzymes for the catabolism of a second sugar. The other mechanism is allosteric competition from transporters, in which the transporters of the preferred sugar inhibit the transporters of the other sugars. Both phenomena result in inefficient consumption of biomass and low rates of lipid accumulation [28].

On the other hand, simultaneous consumption of hexose and pentose has been reported in some strains, with a lipid productivity ranging from 0.15 to 0.26 g/g when glucose and xylose are consumed [29,70,116–119] and 0.18 g/g for simultaneous consumption of glucose and mannose [120]. In addition, Newly *Rhodosporidium*, *Pseudozyma* and *Meyerozyma guilliermondii*, *Scheffersomyces coipomensis* and

Sugiyamaella paludigena strains showed simultaneous consumption of three sugars, glucose, xylose and fructose [121], glucose, xylose and arabinose [122] and glucose, mannose and xylose [26]. The study of the metabolism of these advanced microorganisms is essential to advance in the efficient use of lignocellulosic biomass. In this context, it has been recently documented that isolated strains of decay wood can simultaneously consume hexose and pentose, presenting the expression of diverse enzymes that participate in the metabolism of sugars and happened in absence of regulatory mechanisms, such as catabolic repression [26].

Metabolism of hexose and pentoses or other hydrophilic compounds for SCO biosynthesis is known as de novo synthesis and takes place in the late stationary phase of growth. The process begins when nitrogen is depleted in the media producing a decrease in the concentration of adenosine monophosphate (AMP), and consequently the Krebs cycle is deregulated. Citric acid begins to accumulate within the mitochondria until it reaches a critical value and then is secreted into the cytosol. Citric acid from the cytosol is converted to oxaloacetate, acetyl and malonyl CoA, precursors of fatty acid (FA) synthesis. Fatty acids are then esterified with glycerol to generate structural (phospholipids, sphingolipids, etc.) and reserve (TAG) lipids through a complex of different enzymes, comprising acyltransferases and desaturases [32,91].

However, if carbon stocks are exhausted, the opposite phenomenon occurs, known as β-oxidation. TAGs are degraded to acyl CoA and acetyl CoA through an enzymatic complex composed of various oxidases, lipases and acyl CoA hydrolases [8] promoting energy to the cells for their growth and maintenance.

Theoretical yields for glucose consumption and similar sugars produced 1.1 mol acetyl CoA from 100 g of glucose (0.56 mol) and a maximum theoretical value of 0.32 g/g SCO. Similarly, 1.2 moles of acetyl from 100g xylose (0.66 moles) produce 0.34 g/g SCO [7]. However, under real culture conditions, the lipid yield rarely exceeds 0.24 g/g from glucose and 0.23 g/g from xylose [20,120]. Hence, investigations concerning, key

enzymes and genes involved in lipid synthesis have increasing. Among enzymes studied are ATP citrate lyase [26,43,124–125], malic enzyme [125], acetyl-CoA carboxylase, fatty acid synthase complex, glycerol 3-phosphate dehydrogenase, acyltransferases, lipases and acyl-CoA oxidases [8,33,126].

The maximum production of lipids from sugar is possible if the environmental variables have an optimal configuration, otherwise, the lipid production machinery turns to the production of other compound as organic acids, esters, sugar alcohols, ethanol, lactic acid, malic acid, citric acid, among others [70,127,128].

1.3.4. SCO composition

The term "SCO" extends to all microorganism lipids [1]. Although, various oleaginous microorganisms are able to storage lipids. Most of them are composed of fatty acids with 16 or 18 carbon atoms forming saturated, mono, di or polyunsaturated fatty acid (PUFAs). Including palmitic (C16: 0), palmitoleic (C16: 1), stearic (C18: 0), oleic (C18: 1), linoleic (C18:2) and α-linolenic and γ-linolenic (GLA) (C18:3) acid [4,8,70,95,129]. Similar to FAs from soybean, rapeseed, sunflower, and palm oil [8,130,131]. Microalgae species also accumulate long chain fatty acids with higher unsaturation numbers such as docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), arachidonic acid (ARA) [30,50,54,132]. To a lesser extent, lipids are free and polar fatty acids (e.g., monoacylglycerols, diacylglycerols, and sterile ester), sterols, and polar fractions (e.g., phospholipids, sphingolipids, and glycolipids) that mainly make up cell membranes [7]

The industrial applications for each microorganism is limited to its FAs chemical composition. Microalgae and protists are studied to produce biodiesel with similar physical and chemical properties to petroleum diesel [50,54,85,132]. Microalgaes are also used to produce cosmetics, infant formula additives and animal feed [50,51]. Yeast and bacteria are especially suitable to biodiesel because produced mainly C16 and C18 FA with one or two unsaturation optimal to biodiesel production [3,90,133].

1.4. LIGNOCELLULOSIC BIOMASS AS SUBSTRATE FOR SCO PRODUCTION

1.4.1. Importance of lignocellulosic biomass as raw material

The use of lignocellulose biomass promises a production of SCOs economically viable and sustainable [10,134]. Lignocellulosic biomass represents a fascinating raw material for producing valuable compounds because it is a renewable, abundant and economical substrate [12,135]. It is composed of a high percentage of fermentable sugars suitable for the production of biofuels or oleochemicals [10,12,19,39]. Most research has been focused on terrestrial lignocellulosic biomass; however, the use of marine lignocellulosic biomass from micro and macro algae has also been considered [14]. Structure and chemical composition

Lignocellulosic biomass provides mechanical support and tolerance to biotic and abiotic stresses in plants. The structure consists mainly of a network of cellulose, hemicelluloses (which together form the holocellulose) and lignin polymers and in less quantity starch, pectin, extractives and ashes are also present [13,136-138]. The content of each depends on the species, tissues and age of the cells [14].

Cellulose is the most abundant polymer, representing 34-50% of the dry biomass in softwood species and 41-50% in hardwood species, while in agricultural residues it is approximately 17-42% [14]. It is an amphiphilic and linear homopolymer composed of more than 10,000 glucose molecules linked by glycosidic bonds (β - 1-4). Specifically, between two glucose molecules, the second unit rotates around the C1-C4 axis, forming a dimer known as a cellobiose that binds to each other forming linear glucose chains [11].

Hemicelluloses are amorphous, branched heteropolysaccharides formed by a main chain and other lateral ones. The main chain is composed of equal (homopolymer) or different (heteropolymer) sugar units and is shorter in length than cellulose. The side chains are short and linked mainly by glycosidic bonds β- (1,4) and occasionally by β- (1,3), which are easily hydrolysable. Sugars can be hexose (glucose, mannose and

galactose), pentose (xylose and arabinose), uronic acid and deoxyhexose (rhamnose and fucose) [11]. Hardwoods (angiosperms) contain mainly glucuronoxylans, while softwoods (gymnosperms) contain mainly galactoglucomannans, which represent approximately 18-35% of the lignocellulosic biomass [14,15]. In agricultural biomass it represents between 12 and 37% [14] and the main hemicellulose is xylan.

Lignin is the most abundant organic polymer after cellulose, representing 19-35% of the lignocellulosic biomass of woods and 3.5-30% of grasses [14,16,17]. It is mainly formed in vascular tissues and provides mechanical resistance to xylem. The macromolecule is classified into three types of lignin, guaiacyl(G), syringyl (S) and p-hydroxyphenyl (H), derived from alcohols, coniferyl, sinapyl and p-coumaryl, respectively. In softwoods the predominant lignin is guaiacyl, in hardwoods guaiacyl and syringyl in a ratio of 1:1 to 1:3, while grasses present all lignin monomers [141].

In addition, other biomolecules are also present in the cell wall of plants in smaller quantities. Compounds such as tannins, phenols and lignans are mostly connected to lignin and represent between 1% and 5% of lignocellulosic biomass [11]. It is also possible to find pectin and pectin compounds such as galacturonans, galactans and arabics. Starch and proteins are also present, although they decrease as plants age. Free amino acids or alkaloids contribute to the nitrogen content and inorganic components (Ca, K, Mg, Mn, Na, P and Cl) and trace elements (Ba, Al, Fe, Zn, Si, Pb, Ni, V, Co, Ag and Mo) are essential for growth [11].

Among the highly diverse compounds in the lignocellulosic biomass, most of them can be metabolized by oil microorganisms, but not all of them will lead to an optimal production of lipids. For this reason, it is essential to analyze the effect and maximum tolerance of the different lignocellulosic compounds on the oleaginous microorganisms in order to advance in the production process. In this sense, there is still much to be experimented.

1.4.2. Recalcitrance of lignocellulosic biomass

Recalcitrance of lignocellulosic biomass is linked to physical and chemical factors of the bonds among polymers. Firstly, cellobiose molecules are bound through hydroxyl groups by means of hydrogen bonds to form cellulose microfibers, with a hydrophobic interior and a hydrophilic exterior. These, in turn, are joined by Vander Waals forces to form macrofibrils with highly recalcitrant regions (crystalline regions) [11,142]. The abundance of crystalline regions changes according to the type of plant, even among species of the same genus [13]. Macrofibrils are bind to hemicelluloses, and hemicelluloses to lignin forming a stable structure. Lignin at the molecular level controls permeability, improves the mechanical properties of the cells and protects them from microorganisms and chemical degradation, being the main factor in the recalcitrance of the biomass.

To eliminate recalcitrance and obtain fermentable monosaccharides, it is necessary to destabilize the cellulose, hemicellulose and lignin matrix. The amorphous regions of the cellulose and the branches of the hemicelluloses provide a certain degree of flexibility and susceptibility to decomposition and are the key points to begin dismantling, actually and has been carried out through the application of various pretreatments [142,143].

1.5. DIRECT CONVERSION OF LIGNOCELLULOSIC BIOMASS INTO FERMENTABLE SUGARS

In nature, degradation of lignocellulosic material can be mediated mainly by decomposing animals, insects and fungi. Insects such as wasps, ants and termites, animals such as beavers, rats and woodpeckers, and mollusks from aquatic environments perform mechanical degradation, while decaying fungi perform enzymatic degradation [141,144]. Wood decay fungi have been extensively studied. They are a diverse group of species belonging mainly to the Basidiomycota and Ascomycota divisions [90]. They are saprophytes and are capable of penetrating and colonizing the interior of plants through their hyphae, entering through the pores or through the secretion of enzymes that cause the rupture of the cell walls [11, 141].

Decomposition of wood by fungi is typically classified into three types: mild rot, brown rot and white rot. Brown rot is commonly brown and crumbly and is degraded by non-enzymatic and enzymatic systems. Brown fungi produce cellulases but not enzymes that degrade lignin. However, they have a non-enzymatic mechanism that results in the modification of lignin and a slow depletion of lignin from wood in the process of decomposition known as the chelation-mediated Fenton system (CMF). [144,145]. White rot fungi are the only organisms capable of degrading all wood and lignin-degrading enzymes are present. This capacity for secretion of non-specific extracellular and ligninolytic cellulolytic enzymes allows the transformation of a great variety of recalcitrant compounds whose extracellular nature allows fungi to access non-polar and insoluble compounds [146–148]. However, the mechanisms on how to degrade lignin are not fully understood

Soft rot fungi produce cavities and erosion in the cell walls of wood. Under aerobic conditions, these fungi can completely mineralize the lignin and polysaccharides in the wood (cellulose, hemicelluloses) into CO_2 and H_2O, in addition to causing wood rot [149]. These natural mechanisms have slow degradation rates, however some technologies based on natural processes have been applied to degrade lignocellulosic biomass, such as biopulping, biodegradation, degradation of resins and dyes, among others [147,150,151].

1.6. BIOMASS PRETREATMENT TO DECREASE RECALCITRANCE

To obtain fermentable sugars from lignocellulosic biomass, it is necessary to apply previous processes that include mechanical methods, pretreatments and enzymatic hydrolysis. First, it is necessary to reduce the size of the particles to decrease the degree of polymerization and increase the surface area and porosity of the biomass to the action of reagents. This can be done through a mechanical process, which includes processes such as cutting or grinding (the size varies from meters to centimeters), coarse grinding (from cm to mm, from cm to 500 µm), intermediate micronization (from cm to 100 µm), fine grinding (<100 µ). These are generally obtained by compression

friction or shearing [11,152]. It has been reported that these processes can increase carbohydrate production in further treatments, improving yield and enzymatic hydrolysis rate by 5-25% and 23-59%, respectively, compared to untreated biomass [90]. After the mechanical process, a pretreatment is applied that allows to reduce the recalcitrance of the biomass and, in some cases, to hydrolyze a significant percentage of sugars. However, they usually focus on increasing the accessible surface of cellulose and hemicelluloses to the enzymes that will be used in enzymatic hydrolysis [19].

Pretreatments are diverse and have been widely studied in productive, technical, economic and environmental terms [18,154–157]. In this context, chemical pretreatments as well as acid, alkali and acid combined with alkalis have been the most preferred and patented techniques in recent years, compared to other methods. These represent advantages associated with the use of highly commercial compounds, flexibility of application at the industrial level and high final yields of fermentable sugars [143,155,156], recommended pretreatments are steam explosion and treatment with Organosolv. Steam explosion, also known as autohydrolysis (AH), is the most widely used physicochemical pretreatment method, has been described as an economical and environmentally friendly method and reduces the concentration of toxic compounds in hydrolysates compared to other pretreatments such as acids [20,155,156]. On the other hand, pretreatment with organosolventes, although more expensive than the other processes mentioned, may provide some valuable by-products that allow the sustainability of the process. In a biorefinery, organosolventes pretreatment is one of the best options, as it produces more than one valuable product (e.g., bioethanol and lignin) in the same process [136,159].

The selection of the pretreatment and its working conditions is fundamental for the successful completion of the stage of enzymatic hydrolysis and subsequent fermentation. The selection of the conditions depends largely on the nature and chemical composition of the substrate (Hartwood, softwood or herbaceous) and they are closely related to the amount of by-products that they generate and that can

interfere with the hydrolysis and the fermentation of the sugars. Below is a description, advantages, and disadvantages of the most recommended processes (acid, alkaline, acid-alkaline, steam explosion and organosolv) and a discussion of the yields and effects of these pretreatments on enzymatic hydrolysis. Table 1 shows the most frequent values of the working conditions and yields of the different pretreatments mentioned applied to different lignocellulosic substrates.

1.6.1. Principal pretreatments used

Acid

Acid pretreatments are effective in breaking the lignocellulosic matrix in the glycosidic bonds between lignin and hemicellulose by solubilizing most of the hemicelluloses (commonly over 90% w/w of the dry biomass), also reducing some of the cellulose and removing part of lignin (Table 1). These are suitable for removing lignin in hardwoods and agricultural residues, however the difficulty of removing lignin from softwoods has been well documented and is due to the structural characteristics of the lignin behavior of softwoods [15,160]

The acids used are diverse and can be both inorganic (e.g. phosphoric, nitric, hydrochloric and sulphuric acids) and organic (e.g. acetic, citric, maleic and oxalic acids) [15, 155,156], with sulphuric acid (H_2SO_4) being the most commonly used. Acid pretreatment can be wet acid using concentrated (> 30% p/w) or diluted (<30% p/v) acid, or dry diluted acid which has been recently documented [155,156,161].

Concentrated acid is a process that allows to obtain a high content of fermentable sugars (>50%) with low energy costs since it is carried out at low temperature (< 100 ^{o}C), even at room temperature. The disadvantages of using concentrated acids are the corrosion equipment that increases investment costs, the reagents are highly dangerous and require investment in safety issues and cause a partial degradation of hemicellulose to by-products that accumulate in the liquid waste as organic acids, furfural and HMF mostly. The report show total values even over 13 g/L [162]. These have been reported as inhibitors of enzymatic hydrolysis and fermentation

Table 1.1 Condition and yield of main lignocellulosic biomass pretreatments

							After hydrolysis															
							Solid (% w/w, 100g)				Liquid (% w/w, 100g)				By-products (g/L)						HE %w/w		
Pretreat	Substrate	Reagent	% v/v	S:L ratio	T °C	t min	Total	G	H	L	Total	G	H	L	F	HMF	PC	FA	AA	LA	G	H	Ref
DA	S. bagasse	HCl	2.5	1:15	121	45					21	4.0	16		0.12	0.61							[184]
DA	Rice Bran	H_2SO_4	3	1:8	90	360					50	43	7		0.03	0.3							[185]
DA	Rice straw	H_2SO_4	1	1:10	180	1		59	1.9	21		5	14		1.6	0.2			1.6		70		[173]
DA	Rapeseed	H_3PO_4	3	1:12	200	15	48	48	0	47		4	10		8.0	2.6		0.6	5.5		83		[198]
DA	Corncob	H_2SO_4	2.2		170	30	56	34	4.4	14											88		[199]
DA	Rice straw	$C_2H_2O_4$	5.0		136	31	60	24	22	5											43		[200]
DA	Palm trunk	H_2SO_4	3	1:5	180	40	47	52	ND	37											79		[201]
DA	S. bagasse	$C_2H_2O_4$	3.6	1:20	120	15			3.3			5	93	80							92	96	[202]
DA	Artichoke	H_2SO_4	5	1:10	121	60	60	42	1.9	27		7	83		0.3	0.2	1.1	0.4	3.3		58		[203]
DA	Artichoke	HNO_3	5	1:10	121	60	66	77	1.3	21		15	97		0.04	0.01	0.5	1.2	3.2		89		[203]
DA	Cotton	$C_4H_4O_4$	1.6	1:10	130	45	77	39	2.3	17		1.1	84	88	0.8	0.22	0.1		1.0		68		[204]
DA	*Eucalyptus*	H_2SO_4	0.75	1:20	160	10			17	30		13	90		1.4	0.13			2.0		76		[205]
DA	Poplar	H_2SO_4	0.05		240	10		0		0		50		100									[160]
DA	Pine	H_2SO_4	0.05		240	10		0		65		70	95	30							70		[160]
DA	Softwood	$C_2H_2(CO)_2O$		1:4	180	30				47		16	19		0.9	2.4	5.3		2.3		57		[15]
DA	Wheat straw	H_2SO_4	2	1:10	121	60						14	82		0.44	0.05			4				[92]
DA	Corn stover	H_2SO_4	1	1:10	160	10									1.3	2.6	2.9		2.3				[92]
DA	Rice grass	H_2SO_4	2	1:10	121	60	85	38	6	36											45		[190]
DA	Cassava	H_2SO_4	0.01	1:20	180	20		55	17	18		7.6	39		0.05	0.02		0.76	0.4		70		[176]
ATS	Cassava	H_2SO_4	0.05	1:20	190	10		60	6	21		2.5	58		0.12	0.02		0.13	0.2		73		[176]
ATS	*Populus*	H_2SO_4	65	1:15	35	180						36	19		0.5	0.04		2.7	1.9	1.6			[162]
ATS	*Pinus sp.*	H_2SO_4	73	1:15	53	60						44	17		0.2	0.05		6.8	3.5	1.9			[162]
ATS	*Pinus sp*	HCl	3	1:10	65/80	108/240		38	14	42		38	54										[165]
DDA	Corn stover	H_2SO_4	2.5	2:1	185	1440		39	21												85		[161]
Alk	Corn stover	NaOH	2	1:10	121	20											6.4		5.3				[92]

Alk	Corn stover	NaOH	5	1:10	60	1440	74	28	7.6	3.3		19	65	81						99		[171]
Alk	Poplar	NaOH	5	1:10	120	1440	88	42	8.1	23		5	46	21						80		[171]
Alk	Silver grass	$Ca(OH)_2$	10	1:10	25	8 W	72	98	37	77	28	2.4	63	23						60	50	[206]
Alk	Silver grass	NH_3	3	1:10	25	8 W	71	93	53	90	29	6.1	47	9.8						45	10	[206]
Alk	Napiergrass	$Ca(OH)_2$	10	1:10	25	8 W	68	92	32	86	32	8	68	2.8						65	45	[206]
Alk	Napiergrass	NH_3	3	1:10	25	8 W	65	91	55	90	35	9	45	10						70	30	[206]
Alk	Rice straw	$Ca(OH)_2$	10	1:10	25	8 W	70	96	30	49	31	4.2	71	51						45	40	[206]
Alk	Rice straw	NH_3	3	1:10	25	8 W	66	92	55	54	34	8	45	46						50	10	[206]
Alk	Hardwoods	NH_4			140	60	95													100	98	[207]
A/A	corn stover	HCl/NH_3	1/13	1:10	120/130	40/40	42	85	-	14		1	83				0.9			72		[208]
A/A	Corn stover	HCl/ $Ca(OH)_2$	1/0.1	1:10	120/60	40/12h	55	92	6	75		8	92	25						78	97	[209]
A/A	Corncobs	H_2SO_4/ NaOH	1	1:10	120/60	60/120	62	75	8	3.2		4	89	88						93		[210]
A/A	Corn stover	H_2SO_4/ NaOH	0.5/2	1:10	180/80	1/ 60	62	83	4	5.4		14	71	89	0.9	ND			2.8	98	76	[168]
SE	Sugarcane	H_2O		1:10	121	120						61										[20]
SE	rice husk	H_2O		1:10	121	120						63										[20]
SE	*E. globulus*	H_2O	-	8:1	200	30	69	37	ND	62					1.3	2.2		0.6	2.4	73		[158]
SE	*P. radiata*	H_2O	-	10:1	200	90	67	50	0.7	47					0.9	2.0		0.4	0.8	10		[158]
OS	Palm fronds	Ethanol/ H_2SO_4	60/ 1.5	1:10	200	60	85	90	30	57		5	70	43				0.7	4	95	78	[211]
OS	Cordgrass	Ethyl acetat-ethanol- H_2O-H_2SO_4	37-25-38-0.4	1:10	140	20								51			4.0					[211]
OS	Switchgrass			1:10	140	20								59			6.0					[211]
OS	Corn stover			1:10	140	20								68			2.5					[211]
OS	Poplar	Ethanol/ H_2SO_4	50/ 1	1:10	140	10	61	46		13		7.7		39	<0.1	<0.1			2.4			[169]
OS	Poplar	Ethanol/ NaOH	50/ 1	1:10	140	10	73	59		16		0.3		27	<0.1	0			4.6			[169]

Abbreviations: DA, diluted acid; ATS, acid two step; DDA, dry diluted acid, Alk, alkali; A/A, acid/alkali; SE, steam explosion; OS, organosolv; S:L, solid – liquid ratio; W, weeks; T, temperature; t, time; G, glucose o glucan; H, hemicelluloses; L, lignin; EH, enzymatic hydrolysis; F, furfural; PC phenyl compounds; AA, acetic acid; FA, formic acid; LA; levulinic acid.

Strategies have been developed to reduce the inhibitor concentration, such as the use of two stages acid where a less concentrated acid is first applied to extract the hemicellulose with a low degradation of them and then more concentrated to destructure the cellulose [162,163]. However, the disadvantages have made them less attractive processes to implement and, in this sense, diluted acid pretreatments are preferred.

Diluted acid pretreatment is less aggressive, more environmentally friendly and less costly given the lower acid requirements [143]. In addition, it allows a large part of the sugars to be recovered from the hemicellulose at the end of the process, usually with a lower total concentration (g/L) of inhibiting compounds (Table 1). In addition, it is possible to recover glucose and a highly hydrolysable cellulose solid that usually has over 50% digestibility (Table 1). The disadvantage is that they require higher temperatures than treatments with concentrated acids, increasing energy costs. Glucose yields in diluted acid pretreatments have been optimized to reduce the use of subsequent enzymatic hydrolysis, using more than one stage of the process, alternating high temperatures for short or low retention periods and long retention periods [114,165]. They can also use solid catalysts during pretreatment (e.g., carbon-based; zeolites, ion exchange resins) that can be recycled and reused several times. These improve the yields of fermentable sugars and leave a cellulose that is easily hydrolysable, obtaining glucose yields of more than 91% after enzymatic hydrolysis [156,166].

On the other hand, pretreatment with dry diluted acid has been proposed as an alternative to the use of less diluted acid. In general, both the raw material and the product are solid. The biomass is even used at more than 70% w/w with respect to the diluted acid, which is impregnated and fully absorbed by the biomass [161]. After enzymatic hydrolysis, sugar yields similar to those obtained with diluted acid pretreatment are obtained. One of its advantages is that it produces very low concentrations of furfural and HMF in the pretreatment (< 0.1 and up to 0.90%) compared to those formed in other pretreatments such as acid pretreatment [156,161].

However, dry diluted acid pretreatment requires special equipment to improve impregnation, more stages in the process and few studies have been achieved.

Alkali

Alkali pretreatment allows a dismantling of the lignocellulose and decreases the degree of polymerization and crystallinity of the cellulose. It is effective in dismantling the lignin and solubilizing a part of the hemicellulose (commonly >40% w/w) and generates negligible degradation of the cellulose (Table 1). During alkali pretreatment, the first reactions are the dissolution and saponification of the intermolecular ester and ionic bonds that cross the hemicelluloses and other components, and the porosity of the lignocellulosic biomass increases with the disintegration of these bonds. In addition, hydrolysis of glycosidic bonds and acetyl groups may occur, breaking down polysaccharides and enhancing subsequent enzymatic hydrolysis [154,167]. The most used bases are sodium, potassium and calcium hydroxide and ammonium [154,167]. In general, alkali pretreatment is more effective on hardwoods, the crops and agricultural residues with low lignin content than on softwoods with high lignin content [155]. It is considered low cost compared to other pretreatments and its main disadvantage is the formation of salts that are difficult to remove and low monomeric sugar yields [143,154]. The digestibility in the solid fraction is frequently more than 45% (Table 1).

Sequential Acid-alkali

Some researchers have also tested the combination of both acid and alkaline pretreatment processes for significant recovery of reducing sugars. Commonly, the process involves performing hydrolysis using a dilute acid [19], the solids are extracted and washed and then treated with dilute alkali. The first acidic stage allows the recovery of high contents of hemicelluloses that are usually above 70% w/w and the alkaline stage allows the extraction of a high percentage of lignin frequently >80% w/w (Table 1). Compared to diluted acid pretreatment, the proposed combined pretreatment minimizes the generation of by-products [168]. The main disadvantage is

that a mayor investment in equipment is required for the develop in two stages. The number of stages increases and therefore more processing time is needed [143].

Steam explosion

The pretreatment consists of treating the biomass at high pressure using water vapor and at high temperature for a short period of time and then quickly depressurizing, which generates a disruption of the biomass with the partial elimination of the lignin [19,90]. During the process, organic compounds such as acetic acid are generated, generating acetyl groups that allow an autohydrolysis of hemicellulose and a partial designation, increasing the exposure of the cellulose for a later enzymatic hydrolysis, being recognized as one of the most efficient processes with these biomasses [19,155]. Also, it can be used in two stages for enhanced recovery of fermentable sugars, first under milder conditions to recover hemicellulose, and then, under more severe conditions to destabilize the cellulose and recover higher percentages of glucose [154]. The advantages are a lower environmental impact than acid and alkaline processes and a lower production of toxic compounds for subsequent hydrolysis or for microorganisms [155]. On the other hand, it is less effective for softwoods as they have a low concentration of acetyl groups and it is necessary to use catalysts. In this case, chemical compounds such as SO_2, H_2SO_4 and CO_2 have been used to impregnate the wood before pretreatment [90,155]. Nevertheless, this has the disadvantages of requiring more time for impregnation and generating greater formation of inhibitors and greater degradation of sugars [154,114]. Treatment pressure has a direct effect on the severity and yields of reducing sugars, with values of over 60% w/w [20].

Organosolv

The main principle of organosolv pretreatment is the treatment of lignocellulosic biomass with an organic solvent in order to separate the lignin fractions in the liquid fraction and obtain a high content of cellulose in solid form. Pretreatment generates excision of lignin bonds, especially a-O-aryl ether bonds with carbohydrates, a solid

phase consisting mainly of cellulose and hemicellulose and solvation of lignin fragments [136]. It is achieved in different organic solvents (ethanol, methanol, acetic acid, formic acid, acetone, glycerol or phenol) whose concentrations generally vary from < 1% w/w to over 80% w/w [136] and is applied with or without catalysts (sulphuric acid, magnesium chloride, or sodium hydroxide). Treatment with ethanol is the most attractive and used method due to low cost of the solvent and with good yields of extracted lignin [136,159]. The organosolv pretreatment in hardwood and herbaceous with aqueous solutions can generate acetic acid from the acetyl xylan groups, which in turn catalyzes the degradation of hemicelluloses, the dissolution of lignin and stimulates the hydrolysis of cellulose [159]. Contrary the softwoods do not contain xylan, so pretreatment is done using catalysts [114,136]. The advantages are can be applied to hard and soft woods and the solvents can be recovered by more than 90% easily and generate little environmental effect [136]. The main disadvantage is the danger of ignition by concentrate solvents, and the generation of various inhibitory residues for enzymatic hydrolysis [159]. The main degradation products are g-type lignin derivatives, such as guaiacol (C6), vanillin (C6C1), vanillic acid (C6C1) and ferulic acid (C6C3), and S-type derivatives such as syringaldehyde (C6C1) and syringic acid (C6C1) [155].

1.6.2. Optimization of principal pretreatments

In general, the pretreatment conditions do not allow to obtain 100% of the sugars present in the lignocellulosic biomass. On the contrary, they leave a variable percentage of non-hydrolysed cellulose fibres and/or a percentage of degraded sugars that cannot be used in enzymatic hydrolysis or fermentation. In this context, studies have shown that combined pre-treatments improve the yield of sugars after enzymatic hydrolysis [138,143,154,167]. Work has been done, for example, on combined treatments of alkalis with ionic liquids (IL) and microwaves. [170] IL with steam explosion [158] alkalis with oxidation [118,171] acid with steam exploitation [172], among others, improving sugar yield even by more than 100% compared to the application of a single pre-treatment. Table 2 presents the main advantages and disadvantages of the alternative treatments that can be used in combination with the

preferred treatments. It is also possible to use catalyst compounds that are impregnated before or during pre-treatment and improve the dismantling of the cellulose. This results in shorter chains that are easily hydrolyzed and less degradation of cellulose and hemicellulose monosaccharides [16,90,156,166]. However, these should be used with care so as not to generate a negative effect, such as an increase in by-products that inhibit hydrolysis and fermentation, and therefore further studies are needed.

Table 1.2. Feature of different pretreatment utilized to improve the conventional pretreatment (acid, alkali, acid/alkali, steam explosion and organosolv)

Pretreatment	Advantage	Disadvantages	Action mechanism	Work condition	References
Ionic liquids (IL)	Applied with alkali pretreatment Environmentally friendly Decreases crystallinity and increases porosity Up to 90% fermentable sugars are obtained after enzymatic hydrolysis	Expensive liquids IL interfere with enzymatic hydrolysis	Division of the β-O-4 lignin bond followed by dipole-ion formation. Cellulose is destabilized, and hemicelluloses are dissolved. Composed of organic cations and small inorganic or organic anions, linked by a strong ionic bond. Commonly used are imidazonium salts, AMIMCl (1-Allyl-3-methylimidazonium chloride) and BMIMCl (1-butyl-3-methylimidazolium chloride)	80-160º C 3-50 % solid 30min-8h 60-80 % w/w	[38,138,154, 157,181, 212]
AFEX	Partial disruption of the fibers leaving short cellulose chains and disrupt lignin Solid 99% recovered Low inhibitor concentration Removes acetyl groups by deacetylation Herbaceous biomass and agricultural residues are high susceptible Lignin removed > 85% Up to 95% fermentable sugars are obtained after enzymatic hydrolysis	Hardwood low susceptible Not suitable for softwoods To need between 1 and 2 kg of ammonia per kg of biomass (dry weight) which increases the costs Residual lignin generates unspecific bonds in the enzymatic hydrolysis	Alkaline reagent is impregnated into the biomass, pressure is applied, and it quickly depressurizes Anhydrous liquid or gaseous ammonia	60-200º C 10-50 % solid 5-60 min >100 % w/w	[38, 114,138,154 ,212, 213]
Ammonia recycled percolation (ARP) and Soaking in aqueous ammonia (SAA)	Significant degree of delignification in hardwood and herbaceous woods Solids 99% recovered Used to preserve most of the glucan and xylan	High cost due to the solvent	Solubilize hemicelluloses and lignin	140-210º C 10-90 min 5-15 % w/w	[114,154]
CO_2 explosion.	Efficient to removing lignin in hard and softwoods and to dissolving cellulose and hemicellulose No inhibitory compounds have been reported Low-cost pretreatment Acceptable environmental impact More cost-effective treatment than AFEX and less toxic than steam explosion	Lower yield than steam and AFEX explosion	Supercritical fluid, reacting with the moisture in the substrate to form carbonic acid that contributes to the degradation of the biomass	31-250º C 20-60 min 5-15 % w/w	[90,114,154,213]
Hot water	Reduces the size of particles Effective for solubilizing hemicelluloses as oligomers not require catalysts, chemical products or corrosion-resistant materials Lignin removed > 73% Up to 95% fermentable sugars are	Inhibitors can be produced requires a high demand for water and energy	Consists in cooking the lignocellulosic biomass	121-240º C 10-20 % solid 4-60 min	[37,90,114,154,,214, 217]

	obtained after enzymatic hydrolysis Inhibitor compounds in low concentrations compared to acid treatments.				
Oxidative (Wet oxidation	Used with alkaline solution (NaOH) reducing inhibiting products Combination with stem exploitation the conversion of cellulose and hemicellulose is increased Removes hemicelluloses and lignin (between 50-70%). Xylan from hardwoods and herbaceous are affected Lignin removed > 60% Up to 95% fermentable sugars are obtained after enzymatic hydrolysis	Mannans from softwoods are low affected Cellulose is not affected Possibility of non-selective oxidation causing loss of hemicellulose and cellulose components Large amount of acids and chemical compounds are generated High temperatures and pressure and oxidizing agents are costly	When the biomass is suspended in water, the oxide agents produce electrophilic substitution chain reactions that divide the aromatic lignin nuclei or the bonds between the alkyl and aryl groups Mainly degrades lignin by attacking aromatic ring. Oxide agents such as oxygen (O2), hydrogen peroxide (H2O2) or peracetic acid, ozone (O3)	25-195º C 10-20 min 1-2% w/v	[90,154,213]
Microwave pretreatment	Applied to acids and alkalis and steam exploitation Low cost Short reaction times Change ultra-structure of cellulose by degrading lignin and hemicelluloses Homogeneous heating of the reaction mixture Improve the recovery yields of glucose, xylose and total sugar by 13-27%, 17-25%, and 20-21%, respectively	Increase the formation of inhibitors	Consists of radiating energy that accelerates the molecules, which begin to friction and quickly increase the temperature generating physical, chemical or biological reactions	150-180º C 3 min 1-2% w/v	[215,216]

1.7. INHIBITOR COMPOUNDS

The by-products from pretreatment of lignocellulosic biomass have a principal problem to efficient enzymatic hydrolysis and fermentation of sugars. Studies have shown that small concentrations of compounds derived from pretreatment can inhibit growth and lipid accumulation in oleaginous microorganisms [19,92,173]. The most abundant and common inhibitory compounds are furan compounds such as furfural and HMF generated in acidic media from pentose- and hexose dehydration respectively [19,174,175]. By-products from lignin are lesser extent, as well as vanillin, syringaldehyde and 4-hydroxybenzaldehyde from the guaiacyl group (G), syringyl group (S) and hydroxyphenyl group (H), respectively [19,92,110]. Furfural and 5-HMF derivatives from pretreatments have concentration ranges between >0.1-13 g/L and 0.1-4.3 g/L, respectively [14] and appear to be especially toxic to microorganisms at concentrations between 0.3 and 5 g/L (Table 3). Furthermore, the effects of high inhibitor concentrations on the enzymatic hydrolysis of cellulose were noticeable at approximately 2.0 g/L furfural, 5.0 g/L HMF, and 200 mmol/L carboxylic acid [176]. Weak acids have also been considered inhibitors. The most formed acids are acetic, formic and levulinic acid. Acetic acid is formed through the deacetylation or acid hydrolysis of the acetyl groups in hemicelluloses; levulinic acid is the terminal product of glucose and mannose oxidation; formic acid has two pathways of formation, one is the terminal product of xylose oxidation and the other is the product of glucose and mannose oxidation. These are produced mainly during acid, alkali and organosolv pretreatment [70,110,177]. They are commonly produced at range of 0.1-5 g/L (Table 1). Also, when the acid concentration is raised to umbral percentage, the total sugar concentration decreased due to the decomposition of hexoses to 5-hydroxymethyl furfural (HMF) and pentoses to furfural

However, acetic acid has been used as a co-substrate or substrate for lipid production by various oleaginous microorganisms, e.g. *C. curvatus* ATCC 20509*, M. isabellina* ATCC 42613*, R. toruloides* AS 2.1389 and *Y. lipolytica* MUCL 28849. Poontawee et al. [119] showed than addition of acetic acid at concentrations in the range of 0.1 to

0.25 g/L improves lipid accumulation (6.2 to 6.3 g/L of lipids) compared to the control (5.9 g/L of lipids).

Other inhibiting compounds reported are aromatic hydroxycinnamic acids such as vanillic, ferulic, p-coumaric and benzoic acids, associated with lignin, these compounds are more commonly derived from herbaceous lignin than in hardwood and softwood. Sugar alcohols such as sorbitol, mannitol and adonitol have also been reported [70]. The effect of inhibitory compounds on microorganisms includes the reduction of biological factors and enzymatic activities, DNA decomposition, inhibition of protein and RNA synthesis. They can also penetrate the cell membrane in an undissociated form inhibiting the formation of products, causing pH imbalances, and ultimately inhibiting growth or causing cell death [70]

Some microorganisms are susceptible to by-products from pretreatment of lignocellulosic biomass. However, some studies have reported that, at appropriate concentrations, some microorganisms can assimilate the compounds and degrade them, some even completely [178–181]. The table 3 present the percentage of inhibition of different by-products in growth and lipids production of diverse oleaginous microorganism. To reduce the concentration of inhibitors it is possible to perform technician detoxification. Among them, recovery can be done by nanofiltration separation [182] or, as recently reported, by immobilization with the bacterium *Bordetella sp*. which has proven to be able to selectively assimilate HMF and furfural [177,183]. Studies on the detoxification of residual fluid after pre-treatment have shown that they reduce the concentration of 5-HMF and furfural and phenolics compounds in 21-74% and 24-42% and in 28.2%, respectively [184,185]. However, this increases the cost of production and results in the loss of fermentable sugars [185].

On the other hand, it is necessary to emphasize that work has been done on the recovery of by-products such as HMF and furfural, which can be used as high value products helping to give sustainability to the processes.

Table 1.3. By-products from pretreatment that Inhibit growth and lipids in different oleaginous microorganism

	Furan compounds						Phenol compounds									Weak acids									
	Furfural			5-HMF			Vanillin			Syringaldehyde			4-HB			Formic			Acetic			Levulinic			
	g/L	Inhibition (% w/w)		g/L	Inhibition (% w/w)		g/L	Inhibition (% w/w)		g/L	Inhibition (% w/w)		g/L	Inhibition (% w/w)		g/L	Inhibition (% w/w)		g/L	Inhibition (% w/w)		g/L	Inhibition (% w/w)		
Microorganism	S	X	L	S	X	L	S	X	L	S	X	L	S	X	L	S	X	L	S	X	L	S	X	L	Ref.
C. curvatus	1	-	62	3	-	7.9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	[92]
T. dermatis	4	-	59	2	-	22	1.2	71	80	1	88	98	1.5	100	100	4	100	-	>9	30	57	10	27	54	[92]
T. cutaneum	1	40	30	2	NH	NH	2	26	21	-	-	-	1.5	42	38	5	38	7	5	25	59	10	NH	NH	[177]
R. toruloides	-	-	-	-	-	-	2	100	-	2.2	16	-	1.2	100	-	4	40	-	-	-	-	-	-	-	[218]
R. toruloides	2	70	-	-	-	-	2	50	-	-	-	-	-	-	-	4	50	-	15	50	-	-	-	-	[208]
L. starkeyi	1.4	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.9	100	-	-	-	-	[219]
T. cutaneum	0.5	65	91	5	35	25	2	40	40	2.5	20	20	2	50	50	6	18	26	25	50	34	10	50	50	[180]
T. mycotoxinivorans	1	47	-	2.5	15	-	-	-	-	-	-	-	-	-	-	-	-	-	2	0	-	-	-	-	[60]
R. fluviale	0.3	75	87	2	75	0	0.5	92	0	-	-	-	-	-	-	0.5	100	100	1	72	97	-	-	-	[119]

Abbreviations: S, substrate; X, biomass, L, lip

1.8. ENZYMATIC HYDROLYSIS

The enzymatic deconstruction of the residual lignocellulose after pre-treatment is the last step before fermentation. The most commonly used enzymes after pretreatment of lignocellulosic biomass are cellulases and hemicellulases, which convert cellulose and hemicellulose into pentose and hexose monomers, many of which are currently commercialized [168,186,187]. Cellulases represent a group of at least 15 protein families and some subfamilies that are mainly divided into the endo- and exo-glucanases types and β-glucosidases. Endoglucanases attack the amorphous and low crystallinity regions of cellulose leaving a reducing and non-reducing end, while the exo-glucanases attack the free ends and release cellobiose molecules and β-glucosidases break the β-1,4-D links of the cellobiose, releasing glucose molecules. On the other hand, hemicellulases achieve a complete degradation of hemicelluloses and are formed by a diverse group of enzymes, e. g. Xylanases that degrade xylan have endo-β-1,4-xylanase that produces xylo-oligosaccharides and β-xylanase that breaks down xylan to produce xylose. Other accessory enzymes include α-glucuronidase acetylxylan esterase, ferulic acid esterase, α-galactosidase and α-L-arabinofuranosidase which help hydrolyze the hemicellulose side chains [173]. The synergistic action of cellulases and hemicelluloses increases the conversion rate of cellulose and hemicellulose into free sugars.

The process of enzymatic hydrolysis can be complex because numerous characteristics make it very susceptible. Factors affecting the activities of enzymes include high concentrations of fermentable sugars in the medium, degradation products from chemical or physical pre-treatment [188], residual lignin and residence time. They increase the non-specific and irreversible binding of enzymes mainly to lignin. In general, after pre-treatments, softwood is more recalcitrant than hardwood in enzymatic hydrolysis, due to the high residual lignin content, compared to hardwood. Therefore, they require relatively higher enzyme loads for enzymatic hydrolysis.
Increased enzyme specificity and activity along with reduced cost of enzymes is one of the main challenges of enzymatic hydrolysis. Currently, work has been done to

increase the digestibility of cellulose and decrease non-specific functions by adding non-ionic bases or surfactants such as polyethylene glycol and sorbitol ester and polyethoxylated, Tween-80, Tween-20, dodecyl benzenesulfonic acid, Triton X-100 and PEG 4000, which have proven to be highly effective [67,90,155,189].

On the other hand, sequences that encode cellulases have been cloned in yeasts, bacteria, plants and other fungi to understand their action and create new sources of production [190,191]. In addition, interesting studies have been developed on enzymes of phytopathogenic microorganisms with the capacity to degrade the cell walls of plants, such as *Fusarium verticillioides*, *Pycnoporus sanguineus* and *Chrysoporthe cubensis* [22]. The results are successful saccharification and simultaneous fermentation, without the use of external enzymes. Therefore, the study of hydrolytic enzymes remains a broad and interesting area of study to develop in pretreatments to improve the yields of sugars.

1.9. SCO FROM LIGNOCELLULOSIC BIOMASS

Several researchers have studied the development of oleaginous microorganisms in lignocellulosic hydrolysates, using appropriate growth conditions (C:N ratio, temperature, agitation speed, among others) that stimulate lipid production, according to the microorganism and the substrate used as a carbon source. Table 4 presents a compilation of the oleaginous microorganisms that have presented an accumulation of lipids greater than 20% w/w while growing in both detoxified hydrolysates (DH) and non-detoxified hydrolysates (NDH) obtained from different sources of plant carbon. The results have shown a diverse biomass production (g/L), percentage of lipids (% w/w) and lipid concentration (g/L) in the range of 5.6-54, 21-75 and 2.4-12 respectively.

DH substrates can be a good alternative for growth and lipid production, and it is important to add that strategies have been developed to recover the co-products obtained from the production of lipids such as proteins, amino acids, carbohydrates, carotenoids, glycerol and alcohols, and it is considered that their application and

commercialization in different approaches can guarantee the processes of production of ODS [70,128,175,192-194]. However, it is known that DH can make the process more expensive and a reduction in sugar content.

On the other hand, many microorganisms have shown to be able to produce a high concentration of lipids in NDH. In this way, the study of *Cryptococcus*, *Lipomyces*, *Rhodotorula*, *Rhodosporidium*, *Trichosporon*, *Mortierella*, *Meyerozyma*, *Pichia* strains are an interesting option to produce SCO by lignocellulosic biomass on an industrial scale. Most studies have focused on the use of yeast fungi, which are able to grow rapidly and obtain maximum productivity in less than 180 hours. In general, sugars obtained from agricultural residues by pre-treatment with weak acids have clearly been the trend in pre-treatments for the growth of the mentioned oil microorganisms, with maximum lipid yields above 60% w/w [60,129,195,196]. For example, Poontawee et al. [118] showed that an unconventional yeast, *Rhodosporidiobolus fluvialis*, obtained high biomass production from an alkaline/oxidative pre-treatment with lipid yields of up to 75% w/w. On the contrary, Ananthi and others [20] have shown that different conventional oleaginous microorganisms growing on hydrolyzed agricultural biomass, pre-treated with steam explosion, obtain lipid yields not exceeding 40% w/w, which, of course, are not bad results, but could be feasibly improved using an acid or alkaline pre-treatment.

Although the results on lipid productivity have been promising, the effects of biomass on growth are, in most research results, something that should be improved to increase process productivity. The main disadvantages of growth are due to the by-products of biomass pre-treatment, which inhibit the growth of microorganisms.

Some strains of oil-bearing microorganisms such as, *Candida, Rhodosporidium, Cryptococcus* and *Trichosporon* and the new strains of *Wickerhamomyces* and *Vanrija*, *Rhodococcus* have been shown to tolerate and even degrade by-products derived from pretreatments [56,61,92,118,119,180,181,196] for example *T. cutaneum*, has shown that it can transform 3g/L of furfural and 3g/L of HMF into the corresponding

alcohols (furfuryl alcohol and HMF alcohol), completely degrade 15g/L of formic acid and 10g / L of acetic acid [180]. However, the percentages of growth inhibition are evident (Table 3) and it is necessary to continue experimenting to create productive processes that allow a greater use of plant biomass with better results in the growth of microorganisms.

In this context an applied strategy that has confirmed the improvement of cell growth has been the application of lignosulfonates derived from alkali treatment with sulfites. Sodium lignosulfonate is known to be a good surfactant, which could have a positive influence on microbe growth. According to the experimental results, the concentration of cell biomass increased in *Thraustochytrium aureum* ATCC 34304by 10% when 1.5 g / l of sodium lignosulfonate (derived from alkaline treatment lignin) was added. Moreover, the addition of Tween 80, a non-ionic surfactant, to the culture medium significantly improved the growth and lipid buildup [197].

On the other hand, enzymes involved in the degradation of Furfural and HMF derived from sugars and lignin-derived compounds have been identified. They are mostly dehydrogenase and aldehyde reductase enzymes that are present in microorganisms [180]. However, more metabolic studies are required to understand the physiology of oleaginous microorganisms against lignocellulose biomass degradation compounds and thus obtain advances in productivity.

Finally, a modern strategy has been to use recombinant microorganisms, such as the yeast R. mucilaginosa BIS11 and R. toruloides BIS18, which have showed to be able to efficiently consume acetic acid, 4-HBA and PCH, and in turn achieve a consumption of between 70 and 100% of the glucose of different lignocellulosic hydrolysates [110].

1.10. OUTLOOK

The conversion of lignocellulosic biomass to SCO could be a promising technology, although the process still has several challenges and limitations. Based on the review,

chemical pre-treatments are a promising alternative for obtaining fermentable sugars from lignocellulosic biomass. They are applied preferably using diluted acids and variable residence times depending on the species. The most used conditions are above 121º C up to 1 hour of residence. The results of the treatment with acids and the subsequent enzymatic hydrolysis allow a high recovery of pentoses and hexoses. However, the by-products it generates interfere with the subsequent fermentation by oil microorganisms, reducing the yield of biomass and lipids. Future efforts should concentrate, on the one hand, on obtaining acid pre-treatments combined with other chemical or physical processes that have been shown to reduce by-products and improve the yield of sugars. On the other hand, the identification of inhibitor-tolerant microorganisms with a high growth capacity would greatly reduce the limitation of acid pre-treatment or other pre-treatments.

Finally, through the application of biotechnological tools, the ability of microorganisms to convert lignocellulosic biomass into SCO can be improved and is considered a promising alternative for obtaining oil production processes that can contribute to meeting the needs of the population, such as food and biofuels.

Table 1.4. Growth and lipids production of different oleaginous microorganism cultivated in lignocellulosic hydrolysates

Microorganism	Strain	biomass	pretreatment	System		NDH/DH	S(g/L)	X (g/L)	L %(w/w)	L (g/L)	L (g/g)	Refe.
Trichosporon dermatis	CH007	corncob	DA	batch	flask	DH	42	17	40	7	0.16	[220]
Cryptococcus curvatus	ATCC 20509	Wheat straw	DA	batch		NDH	29	17	34	5.8		[221]
Cryptococcus curvatus	ATCC 20509	Wheat straw	DA	batch		DH	21	16	27	4.2		[221]
Lipomyces starkeyi	ATCC 12659	Wheat straw	DA	batch		NDH	29	15	31	4.6		[221]
Lipomyces starkeyi	ATCC 12659	Wheat straw	DA	batch		DH	21	13	29	3.7		[221]
Rhodosporidium toruloides	ATCC 10788	Wheat straw	DA	batch		DH	21	9.9	25	2.4		[221]
Rhodotorula glutinis	ATCC 204091	Wheat straw	DA	batch		NDH	29	14	25	3.5		[221]
Rhodotorula glutinis	ATCC 204091	Wheat straw	DA	batch		DH	21	12	21	2.4		[221]
Trichosporon dermatis	32903	Corncob	DA	batch		NDH	71		24	7.5	0.1	[92]
Trichosporon dermatis	32903	Corncob	DA	batch		DH	73		45	11	0.16	[92]
Cryptococcus sp.	SM5S05	Corncob	DA	batch	flask	NDH	40	13	60	7.6	0.13	[195]
Trichosporon cutaneum	Ch002	Corncob	DA	batch	flask	DH	46	22	36	7.9		[206]
M. isabellina	ATHUM 2935	Rice hull	DA	batch	flask	NDH	26	5.6	64	3.6	0.21	[129]
Trichosporon cutaneum	ACCC 20271	Corncob	DA	batch	flask	NDH	49	38	32	12	0.1	[222]
Cryptococcus curvatus	ATCC 20509	Corn stover	DA	batch	flask	NDH	53	11	61	6.9		[196]
Trichosporon cutaneum	AS 2.571	Corn stover	DA	batch	Bior 3L	DH	60	19	39	7.6	0.15	[29]
Trichosporon dermatis	CH007	Corncob	DA	batch	flask	NDH	60	24	40	9.8	0.16	[223]
Trichosporon fermentans	CICC 1368	Rice straw	DA	batch	flask	DH	35	29	40	12		[224]
Mortierella isabellina	ATCC42613	Corn stover	DA	batch	flask	NDH	31	14	34	4.8		[58]
Lipomyces tetrasporus	NRRL Y-11562	Cornstover	DA	batch	flask	NDH	123	54	53	29	0.15	[60]
Lipomyces kononenkoae	NRRL Y-7042	Cornstover	DA	batch	flask	NDH	123	48	59	28	0.22	[60]

Rhodosporidium toruloides	NRRL Y-1091	Cornstover	DA	batch	flask	NDH	123	43	61	26	0.19	[60]
Yarrowia lipolytica	Po1g	Sugarcane bagasse	DA	batch	flask	DH	21	11	59	6.7		[183]
Yarrowia lipolytica	Po1g	Rice bran	DA	batch	flask	DH	30	11	48	5.2		[76
Trichosporon cutaneum	ACCC 20271	Corn stover	DA	batch	Bior 20L	DH	73-130			46-8.1		[180]
Rhodosporidiobolus fluvialis	DMKU-SP314	Sugar cane	DA/OX	batch	flask	NDH	36	21	67	14		[118]
Rhodosporidiobolus fluvialis	DMKU-SP314	Sugar cane	DA/OX	batch	Bior 2L	NDH	36	24	75	18		[118]
Mortierella isabellina	ATCC42613	Corn stover	Alk	batch	flask	NDH	30	11	29	2.5		[127]
Trichosporon mycotoxinivorans	S 2	Paddy straw	Alk			NDH	35	14	35	7.3		[70]
Rhodosporidium paludigenum	KM281510	Corncob	Alk	batch	flask	NDH	100	23	70	16		[67]
Rhodosporidium paludigenum	KM281510	Corncob	Alk	batch	Bior 3L	NDH	100	28	70	20	0.21	[67]
Rhodosporidium paludigenum	KM281510	Corncob	Alk	fed batch	Bior 3L	NDH	100	36	70	25	0.28	[67]
Trichosporon dematis	32903	Corncob	Alk	batch		NDH	67		28	6.8	0.1	[92]
Trichosporon dematis	32903	Corncob	Alk	batch		DH	131		56	20	0.19	[92]
Rhodococcus toruloides	DSMZ 4444	Corn stover	A/A	batch		NDH	110	36	59		0.19	[225]
Rhodococcus toruloides	DSMZ 4445	Corn stover	A/A	Fed batch		NDH	110	54	59		0.29	[225]
Rhodotorula glutinis	CGMCC 2.703	Corncob	A/A	batch	Bior 5L	NDH	42	15	36	5.5	0.13	[226]

Rhodotorula glutinis	CGMCC 2.703	Corncob	A/A	fed batch	Bior 5L	NDH	42	75	47	34	0.15	[226]
Meyerozyma guilliermondii	G5-MK414782	Sugarcane bagasse	SE	batch		NDH	60	6.1	38	2.3	0.05	[20]
Meyerozyma guilliermondii	G5-MK414782	Rice husk	SE	batch		NDH	60	6.5	37	2.4	0.04	[20]
Pichia kudriavzevii	G9-MH000699	Sugarcane bagasse	SE	batch		NDH	20	6.2	31	1.9	0.1	[20]
Pichia kudriavzevii	G9-MH000699	Rice husk	SE	batch		NDH	20	8.2	24	1.9	0.1	[20]
Pichia manshurica	G10-MH279643	Sugarcane bagasse	SE	batch		NDH	20	8.2	24	1.9	0.09	[20]
Pichia manshurica	G10-MH279643	Rice husk	SE	batch		NDH	21	6.5	28	1.8	0.09	[20]
Pichia kudriavzevii	SY2-MF926445	Sugarcane bagasse	SE	batch		NDH	60	6.1	30	1.9	0.04	[20]
Pichia kudriavzevii	SY2-MF926445	Rice husk	SE	batch		NDH	51	8.4	29	2.4	0.04	[20]
Candida albicans	SY3-MG996750	Sugarcane bagasse	SE	batch		NDH	20	6.2	31	1.9	0.1	[20]
Candida albicans	SY3-MG996750	Rice husk	SE	batch		NDH	21	8.3	22	1.8	0.09	[20]
Rhodotorula mucilaginosa	SY4-MH279637	Sugarcane bagasse	SE	batch		NDH	20	6.7	30	2	0.1	[20]
Rhodotorula mucilaginosa	SY4-MH279637	Rice husk	SE	batch		NDH	20	8	24	2	0.1	[20]
Rhodococcus opacus	DSM 1069	Loblolly pine	OS	batch	flask				27			[227]

Abbreviations: DA, diluted acid; Alk, alkali; A/A, acid/alkali; SE, steam explosion; OX, oxidative; OS, organosolv; S, substrate; X, biomass; L, lipids; NDH, no detoxified hydrolyzed; DH, detoxified hydrolyzed, Bior, Bioreactor

CAPÍTULO II. PATTERNS OF LIGNOCELLULOSIC SUGAR ASSIMILATION AND LIPID PRODUCTION BY NEWLY ISOLATED YEAST STRAINS FROM CHILEAN VALDIVIAN FOREST [2]

Gabriela Valdés, Regis Teixeira Mendonça, Carolina Parra, George Aggelis

2.1. ABSTRACT

Three yeast strains were isolated from decaying wood of Chilean Valdivian forest and identified as *Meyerozyma guilliermondii*, *Scheffersomyces coipomensis*, and *Sugiyamaella paludigena*. These strains were able to efficiently grow on the major monomers contained in *Pinus* spp. and *Eucalyptus* spp. wood that includes glucose (*Glc*), xylose (*Xyl*), and mannose (*Man*), showing at 28 °C higher uptake rates for *Man*, and in some cases for *Glc*, than for *Xyl*, used as single carbon sources. Nevertheless, in cultures performed on sugar mixtures, the strains displayed a notable preference for *Glc*. Additionally, in sugar mixtures, the absence of regulatory mechanisms in sugar assimilation (e.g., catabolic repression) was observed and documented when the activities of several enzymes involved in sugar assimilation (i.e., phosphoglucose isomerase, phosphomannose isomerase, and xylulokinase) were determined. The activity of the key enzymes involved in the onset of lipid accumulation (i.e., NAD+-*ICDH*) and in fatty acid (FA) biosynthesis (i.e., *ATP:CL*) indicated a significant accumulation of storage lipids (i.e., up to 24%, w/w) containing oleic and palmitic acids as the major components. The present paper is the first report on the potential of *M. guilliermondii*, *S. coipomensis*, and *S. paludigena* as oleaginous yeasts. We conclude that the new isolates, being able to simultaneously assimilate the major lignocellulosic sugars and efficiently convert them into oily biomass, present a biotechnological potential which deserve further investigation.

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[2] Valdes G, Mendonça RT, Parra C, Aggelis G (2020) Patterns of Lignocellulosic Sugar Assimilation and Lipid Production by Newly Isolated Yeast Strains From Chilean Valdivian Forest. *Applied Biochemistry and Biotechnology*.
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Keywords: *Meyerozyma guilliermondii; Scheffersomyces coipomensis; Sugiyamaella paludigena;* Lignocellulosic sugars; Single cell oil.

Abbreviations: *ATP:CL,* ATP-citrate lyase; FA, Fatty acid; *Glc,* Glucose; *Man,* Mannose; *NAD+-ICDH,* NAD+-dependent isocitrate dehydrogenase; *PGI,* Phosphoglucose isomerase; *PMI,* Phosphomannose isomerase; PUFA, Polyunsaturated fatty acid; SCO, Single cell oil; TCA, Tricarboxylic acid; *XK,* Xylulokinase; *Xyl,* Xylose.

2.2. INTRODUCTION

Among oleaginous microorganisms, yeasts are the most likely candidates for lipid (single cell oil, SCO) production with interesting biotechnological applications. SCO derived from yeasts usually contains saturated and monosaturated fatty acids (FAs) of C16 and C18 families and some polyunsaturated fatty acids (PUFAs) with a restricted number of double bonds, such as linoleic acid (C18:2, n−6) and α-linolenic acid (C18:3, n−3). This FA profile is similar to that of common plant oils that are considered as a raw material in the chemical industry, including the biodiesel industry [7,8,33]. The oleaginous yeasts are not able to naturally synthesize PUFAs of commercial interest, contrary to fungi (Zygomycetes) and microalgae. However, due to their morphology and enhanced biochemical arsenal, yeasts are preferred for industrial PUFA production over fungi, after genetic manipulation [31].

Various carbon substrates, including glycerol [72,228], industrial sugars [108], and lignocellulosic residues [88,221,224,229,230], have been considered as feedstock for SCO production. Particularly, lignocellulosic biomass is considered as a promising substrate due to its low cost, availability, and variety of fermentable sugars [88,229]. For instance, wood of commercially important plant species, such as *Pinus* spp. and *Eucalyptus* spp., have glucose (*Glc*), mannose (*Man*), and xylose (*Xyl*) as the major monomers released from cellulose and hemicellulose after hydrolysis [37,40].

The metabolism of lignocellulosic sugars has been studied in detail in several yeasts, including the non-oleaginous species *Saccharomyces cerevisiae* used as a model for hexose, but not for pentose, fermentation [86]. In this yeast, transporter proteins, located at the interface of the cell membrane, are used to transport multiple types of sugars, often with preference for one [25]. Likewise, some specific putative transporters of hexoses and pentoses were proposed for the model oleaginous yeast *Yarrowia lipolytica* [24]. Intracellularly, hexoses are phosphorylated on the sixth carbon atom through hexokinase reaction and then converted by specific isomerases, such as phosphoglucose isomerase (*PGI*) for *Glc* and phosphomannose isomerase (*PMI*) for *Man*, into fructose-6-phosphate (F-6-P) (Fig. 1) [231,232]. Nevertheless, the last step has not yet been fully elucidated in *Y. lipolytica* [86]. Pyruvate, the end product of glycolysis, enters the mitochondrion and participates in the TCA cycle providing energy and anabolic precursors to the cell. However, when the nitrogen source is exhausted in the growth medium, TCA cycle is disturbed at the level of isocitrate dehydrogenase (*ICDH*) and citrate is excreted to the cytoplasm in exchange with malate. Citrate in the cytoplasm is cleaved by the ATP- citrate lyase (*ATP:CL*) into acetyl-CoA, which is the universal precursor of FAs and oxaloacetate (Fig. 1).

According to the above-mentioned model, which is widely accepted for oleaginous micro- organisms, the inhibition of *ICDH* is the event that signals the onset of lipogenesis. Further- more, the activity of *ATP:CL* is of high importance for organisms that utilize citrate as a donor of acetyl CoA in the cytoplasm. High activity of this enzyme correlates with lipid accumulation in *Y. lipolytica* and other oleaginous microorganisms [64]. On the other hand, *Xyl* is poorly metabolized by *S. cerevisiae*, while *Y. lipolytica* can utilize this sugar during adaptation or starvation periods [86]. *Scheffersomyces* (*Pichia*) *stipitis* was described as a yeast competent to efficiently metabolize *Xyl*, and therefore, it serves as a model for *Xyl* assimilation [233]. *Xyl* metabolism in yeast commonly proceeds through the so-called oxidoreductase pathway, in which *Xyl* is successively converted to xylitol, xylulose, and xylulose-5-phosohate (Fig. 1), which can be further converted into acetate, eventually through the pentose phosphate pathway, and then to the acetyl-CoA [86].

Most yeasts growing on mixtures of sugars catabolize them sequentially, demonstrating usually a preference for *Glc*, sugar that is able to activate catabolite repression mechanisms associated with a lag period due to metabolic shifts occurring into the microbial cell [27, 28,113,234]. From a technological point of view, these physiological phenomena constitute a major drawback when multiple sugars (pentoses and hexoses) are present in the growth medium, such as in the case of lignocellulosic hydrolysate, provoking a significant increase to the cultivation time [29]. For this reason, genetic engineering tools have been utilized as a strategy to improve the conversion of lignocellulosic biomass into lipids [28,29,111,116,120,119,196,235,]. Moreover, identifying strains capable of efficient/simultaneous utilization of hexoses and pentoses is of obvious interest and worthy of investigation.

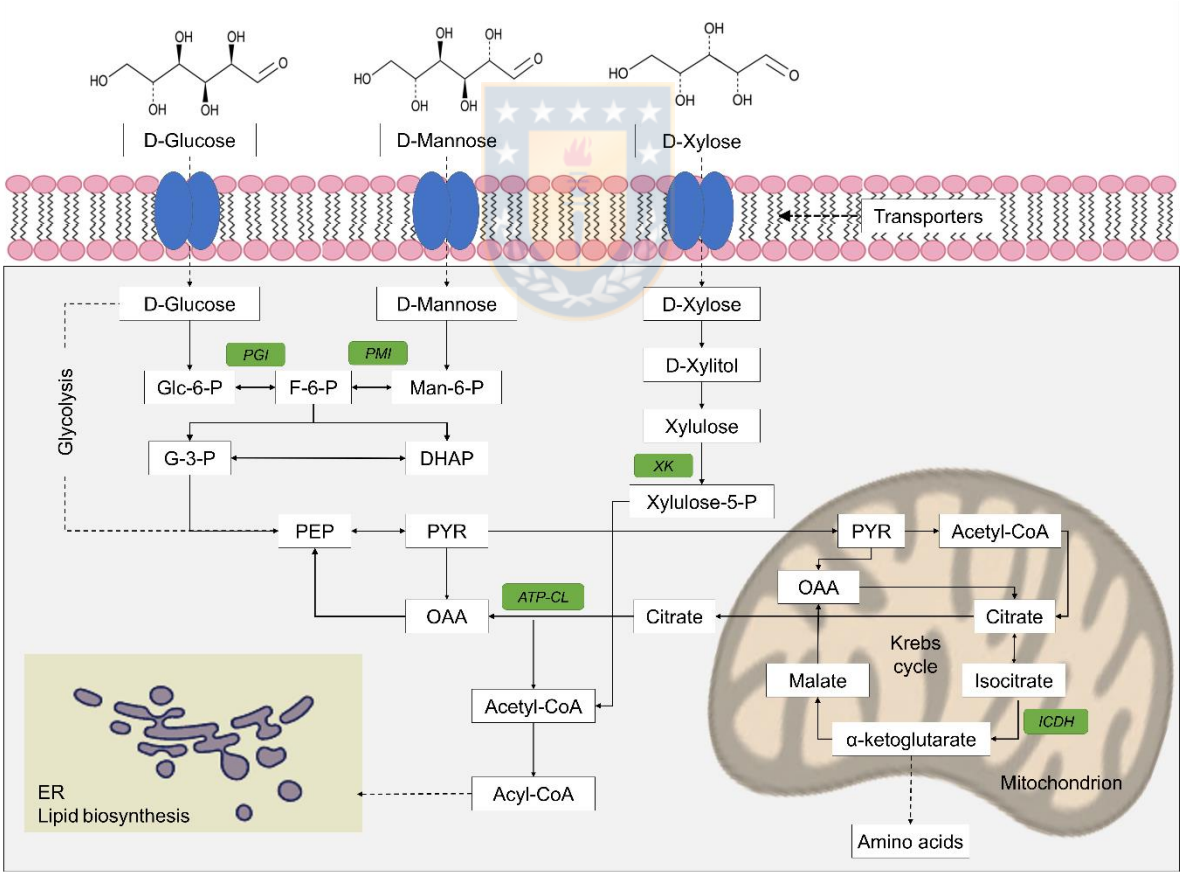

Fig. 2.1. Simplified scheme of biochemical pathways of glucose, mannose, and xylose catabolism and principal enzymes involved in lipid biosynthesis. The key enzymes considered in this study are highlighted in green. ICDH, NAD+-dependent isocitrate

dehydrogenase; PGI, phosphoglucose isomerase; PMI, phosphomannose isomerase; XK, xylulokinase; ATP:CL, ATP citrate lyase

2.3. MATERIAL AND METHODS

2.3.1. Yeast strains and phylogenetic analysis

Three yeast strains were isolated from decaying wood found in the Valdivian Rainforest Ecoregion located in Chile (35° to 48° S). The strains were maintained in 30% (v/v) glycerol solution at − 80 ± 1 °C and regenerated on potato dextrose agar (PDA) (Conda, Madrid, Spain). The strains were identified using their ITS rRNA regions through PCR-amplified with ITS1 and ITS4 primers (forward ITS 1–5′ TCC GTA GGT GAA CCT GCG G 3′ and reverse ITS 4–5′ TCC TCC GCT TAT TGA TAT GC 3′) and were directly sequenced [236]. The sequences obtained were compared with references using BLAST search and registered in GenBank, and phylogenetic tree was inferred using the Neighbor-Joining method [237], and the evolutionary distances were computed using the Maximum Composite Likelihood method [238]. Evolutionary analyses were conducted in MEGA X [239].

2.3.2. Culture conditions

Pre-cultures were performed on media containing the following (g/L): yeast extract 3 (Conda); glucose 10 (AppliChem, Darmstadt, Germany); peptone 5 (HiMedia, Mumbai, India); and malt extract 3 (Sigma-Aldrich, Steinheim, Germany), which were incubated in a rotary shaker (Zhicheng ZHWY 211C, Shanghai, China) at 28 ± 1 °C and 180 rpm.

Cultures were performed in 250 mL Erlenmeyer flasks containing 50 mL of medium with the following composition (g/L): yeast nitrogen base 1.7 (Conda); yeast extract 0.75 (Conda); and ammonium sulphate 0.5 (Merck, Darmstadt, Germany). The sugars D-glucose (AppliChem), D-xylose (Sigma-Aldrich), or D-mannose (Sigma-Aldrich) at 40 g/L or a mixture of them at 40 g/L each (in total 120 g/L) were used as carbon sources. The flasks were sterilized at 121 °C for 20 min and inoculated with 10^8 cells, originated

from a 48 h pre- culture. All cultures were incubated in a rotary shaker (Zhicheng ZHWY 211C) at agitation rate of 180 rpm and temperature of 20 ± 1 °C (which is the maximum average temperature of the natural ecosystem of the strains) or 28 ± 1 °C (which is the optimal growth temperature of the most oleaginous yeasts of the Candida group, in which the new isolates were placed).

2.3.3. Analytical methods

Culture density and biomass determination

The number of cells in the culture was determined under light microscope (Carl Zeiss, GmbH, Gottingen, Germany) using a Neubauer chamber. For dry cell mass determination, culture samples were filtered through previously dried Whatman 0.2 µm membrane, washed three times with distilled water, dried at 80 °C for 10 min, and weighed. The results were expressed as gram of dry biomass per liter of growth medium (*X*, g/L).

Sugar determination

D-glucose, D-xylose, and D-mannose were determined during microbial growth using an HPLC (Ultimate 3000 Dionex, Germering, Germany) equipped with RI and UV–Vis detectors and an HPX-87P Bio-Rad column (Sigma-Aldrich). An aliquot of 300 µL was withdrawn from the culture and filtered through Whatman 0.2 µm membrane before being injected into the chromatograph. Water was used as a mobile phase at 0.6 mL/min flow rate. Oven temperature was 85 °C.

Lipid extraction and purification

Cells were harvested by centrifugation (15,000 rpm, 15 min, 4 °C) for the culture using a Heraeus centrifuge (Biofuge Stratos, Osterode, Germany), washed twice with cold distilled water, dried at 80 °C until constant weight, and then gravimetrically determined. Yeast lipids were extracted from dry biomass according to the modified method of Folch et al. [240] in chloroform:methanol (Fisher Scientific, Loughborough, United Kingdom) solution (2:1 v/v) under reflux for 4 h. The extracts were filtered through Whatman paper no. 1 and washed with a KCl (Sigma-Aldrich) 0.88% (w/v)

solution. The chloroform phase was dried with anhydrous Na_2SO_4 (Honeywell Fluka, Seelze, Germany), the solvent was evaporated under vacuum using a Rotavapor R-20 device (BUCHI, Flawil, Switzerland), and the total lipids were gravimetrically determined and expressed as percentage of dry biomass, *L*/*X*% (w/w).

Fatty acid composition and estimation of desaturase activity

The FA moiety of total lipids was converted through transesterification into methyl esters. The transesterification was performed in a two-stage reaction using CH_3O^--Na+ and CH_3OH/HCl according to the AFNOR [241] method. The FA methyl ester mixture was analyzed through a gas chromatography apparatus (Agilent Technologies 7890 A, Wilmington, USA) equipped with a flame ionization detector and a HP-88 (60 m × 0.25 mm) column (J&W Scientific, California, USA). Helium was used as a carrier gas at a flow rate of 1 mL/min. Injection temperature was 250 °C, oven temperature 200 °C (isotherm), and FID temperature 280 °C. Peaks of methyl esters were identified using as reference authentic standards.

The activities of Δ7, Δ9, and Δ12 desaturases were estimated by using the ratios C16:1Δ7/ C16:0, C18:1Δ9/C18:0, and C18:2Δ9,12/C18:1Δ9, respectively, according to Fakas et al. [242]. The total desaturase activity was calculated using the following equation:

$$\textbf{Total desaturase activity} = \frac{\mathbf{1x}\sum\textbf{monoene} + \mathbf{2x}\sum\textbf{diene}}{\sum\textbf{FA}}$$

Microscopy

Yeast morphology, with emphasis to the ability of strains to form pseudomycelia, was studied as described in [243]. Briefly, slides covered by a thin layer of PDA were placed onto rods inside sterilized Petri dishes and inoculated with yeast cells by dragging a bacteriological loop bringing cells on the slide. In order to establish a lower oxygen concentration environment, which induces dimorphism, a sterilized coverslip was placed on the inoculum. Approximately 3 mL of sterile glycerol 20% (v/v) solution was

added in the Petri dishes, and the cultures were incubated at 28 °C for 48 h. Microscopic observations of intact yeast structures were performed directly on the slides using an optical microscope Carl Zeiss (GmbH, Gottingen, Germany), equipped with a video camera (Exwave HAD, Sony, Tokyo, Japan).

Intracellular lipid bodies were visualized after Nile red fluorescence staining. Briefly, 1 mg/ mL of Nile red in acetone was mixed with yeast culture at a ratio of 1:10 (v/v), and the samples were kept in dark at room temperature for 12 h. The stained cells were harvested, washed three times with distilled water, and re-suspended in sodium phosphate (50 mM, pH 6.8) buffer. A droplet of the suspension from each sample was placed on a slide with a drop of glycerol. The samples were observed using an inverted confocal spectral microscope (Leica TCS SP8, Wetzlar, Germany) with a 63×/1.4NA oil objective and fluorescence filter over and an emission spectral range of 549 to 602 nm.

Determination of enzyme activities

Initially, cell-free extracts were prepared. Specifically, the cells were harvested by centrifugation and washed twice with 50 mM Na_2PO_4/KH_2PO_4 (AppliChem) buffer (pH 7.5). The wet biomass was weighted and then re-suspended in a 30 mM Na_2PO_4/KH_2PO_4 pH 7.5 buffer, containing 1 mM DTT (Sigma-Aldrich), 250 mM sucrose (Merck), and 1 mM benzamidine (Honeywell Fluka), at a ratio of 1 mL buffer per 0.5 g wet biomass. The cells were ruptured at 0–4 °C by Sonics Vibra cell CV 188 sonicator (Newtown, CT, USA). The samples were exposed three times to 90 W sonic bursts for 2 min, with a 2 min break between them, and one additional 90 W burst for 1 min at the end. The disrupted cells were centrifuged at 24,000*g* at 4 °C, the pellet was discarded, and the supernatant was filtered through Whatman 0.2 µm membrane to remove solidified lipids and the remaining cell debris [244].

The activities of key enzymes involved in sugar and lipid metabolism were determined in the cell-free extracts. Specifically, the selected enzyme activities involved in sugar metabolism were those of phosphoglucose isomerase (*PGI*) (EC 5.3.1.9) determined

according to Bergmeyer et al. [245], phosphomannose isomerase (*PMI*) (EC 5.3.1.8) determined according to Gracy and Noltmann [246], and xylulokinase (*XK*) (EC 2.7.1.17) determined according to Bunker et al. [247]. The selected enzymes activities involved in the first stages of lipid production (FA biosynthesis) were NAD+-dependent isocitrate dehydrogenase (*ICDH*) (NAD+-*ICDH*, EC 1.1.1.41) determined according to Kornberg [248] and ATP-citrate lyase (*ATP:CL*) (*ATP:CL*, EC 4.1.3.8) determined as describe by Srere [249].

Enzyme activities were reported as the conversion of 1 µmol of substrate into specific product per min, expressed as unit per milligram of soluble protein (U/mg protein) and unit per milligram of dry biomass (U/mg biomass). Soluble protein was determined according to Lowry et al. [250].

Statistical analysis

Three independent cultures were performed for each yeast. Two-sample *t* test analysis for mean values was calculated at the significance level of null hypothesis ($p \leq 0.05$). The modeling on single sugar media was performed using the results of three cultures, and chi- squared test ($\chi 2$) was used to represent the correlation factor between the predicted and experimental data. The enzyme activities were determined in duplicate at incubation time of t = 30, 56, and 122 h for *M. guilliermondii*, t = 30, 82, and 140 h for *S. coipomensis*, and t = 50, 100, and 146 h for *S. paludigena*. Significance of differences was evaluated by the one-way analysis of variance ANOVA followed by a Bonferroni post-hoc test. The significance level of null hypothesis was $p \leq 0.05$.

2.4. RESULTS AND DISCUSSION

Molecular identification and morphological features of the newly isolated strains

The yeast strains were isolated from a decay wood of the Chilean Valdivian Forest, which is an ecoregion part of the Neotropical ecozone characterized by dense understories of bamboos and ferns and dominated by evergreen angiosperm trees with some deciduous specimens, though conifer trees are also common. Its temperature in

the summer can climb to 20.5 °C (69 °F), while during winter, it drops below 7 °C (45 °F) [251]. The strains were molecularly identified as *Meyerozyma guilliermondii*, *Scheffersomyces coipomensis*, and *Sugiyamaella paludigena* with 100% similarity (GenBank access codes KX609758, KX609756, and KX609757, respectively). All strains were deposited to the Collection of Agricultural University of Athens (ACA-DC) and, *Meyerozyma guilliermondii* was deposited to the Chilean Collection of Microbial Genetic Resources (RGM) too, obtaining strain numbers ACA-DC 5397 for *Meyerozyma guilliermondii*, ACA-DC 5395 (RGM 2933) for *Scheffersomyces coipomensis*, and ACA-DC 5396 for *Sugiyamaella paludigena*.

The phylogenetic relationships of the newly isolated yeast strains with species of their respective clades are illustrated in Fig. S1. The tree showed a close relationship between *M. guilliermondii* and *Scheffersomyces* clade and the closeness of both with *Sugiyamaella* clade. Besides, the three yeasts are genetically related more to *Saccharomyces* than to *Yarrowia*. Strains of *M. guilliermondii* have been isolated from samples of rotting wood from the National Park of Serra do Cipó, Brasil [252] and other environmental samples [253,254]. Species of *Scheffersomyces* and *Sugiyamaella* have been found in association with insects (such as lignicolous beetles), wood, and rotting wood [255,256]. Specifically, strains of *Sugiyamaella paludigena* have been isolated from high-moor peat [257] and the rhizosphere of white spruce [258]. Phylogenetically, the newly isolated strains are related to *Candida* species. *M. guilliermondii* is the teleomorph of *Candida guilliermondii* [153], while *Scheffersomyces coipomensis* and *Sugiyamaella paludigena* have been related to asexual members of *Candida* species [139,140,255,153].

These yeasts, similarly, to ascomycetous yeasts belonging to *Saccharomyces*, *Debaryomyces*, *Yarrowia*, etc., growing under specific conditions, are able to form pseudomycelia and some of them true mycelia [123]. However, the new isolates produced single cells organized in loose/unstable structures (*M. guilliermondii*), or elongated cells forming rudimental pseudomycelial structures (*S. coipomensis* and *S. paludigena*). Specifically, undisturbed yeast cultures grown under conditions that

favored dimorphic growth, observed under light microscope, revealed the formation of subglobose to elongated ellipsoidal cells, and the ability of the strains to grow forming pseudomycelial loose structures after 24 h of incubation for *M. guilliermondii* and *S. coipomensis* (Fig. 2a and b, respectively) or 48 h for *S. paludigena* (Fig. 2c). These features are suitable for organisms with an industrial perspective, since mycelium-forming yeast-like organisms cause difficulties in oxygen/mass transfer when grown in large-scale bioreactors [115,243]. In addition, single cells are considered easily manipulated and presumably metabolically more active than mycelia.

The strains cultivated under fully aerobic and lipogenic conditions (i.e., in high C/N ratio media containing 1.7 g of yeast nitrogen base, 0.75 g of yeast extract, and 0.5 g of ammonium sulphate as nitrogen source, and a mixture of *Glc*, *Man*, and *Xyl* at 40 g/L each as carbon sources) formed subglobose to ellipsoidal cells, less than 10 µm in length (Fig. 3). Asci and ascospores were not observed. Storage lipid within the yeast cells usually accumulated in the form of one large and round lipid droplet, especially in *S. coipomensis* (Fig. 3b), the size of which ranged from 0.2 to 4 µm approximately, similarly to lipid droplets observed in the oleaginous yeast *Rhodosporidium kratochvilovae* [264]. Besides, some elongated ellipsoidal or clavate cells of *M. guilliermondii* and *S. paludigena* were inclined to form two lipid droplets located in the polar region of the cells (Figs. 3a and c). It has been reported that the number and the size of lipid droplets significantly varied between *Candida* species [115]. For instance, the presence of one or two small lipid droplets, one at each pole of the cell, was observed in *Candida ethanolica* and *Candida boidinii* strains while many irregular lipid droplets appeared in *Candida parapsilosis* and *Candida pararugosa* strains. In contrary, *C. metapsilosis* produced only one voluminous spherical LB close to the cell membrane.

Yeast growth evaluation on single sugar media

The strains were cultivated on the major sugars that are present in hydrolysates of hardwood and softwood, i.e., *Glc*, *Man*, and *Xyl*, at 20 and 28 °C. The parameters of growth were determined by fitting the following model, which is based on the Monod's

equation, on the experimental data:

Biomass production:

$$\frac{dX}{dt} = \mu_{max} \frac{S}{K_s + S} X$$

Substrate consumption:

$$-\frac{dS}{dt} = \frac{1}{Y_{X/S}} \mu_{max} \frac{S}{K_S + S} X$$

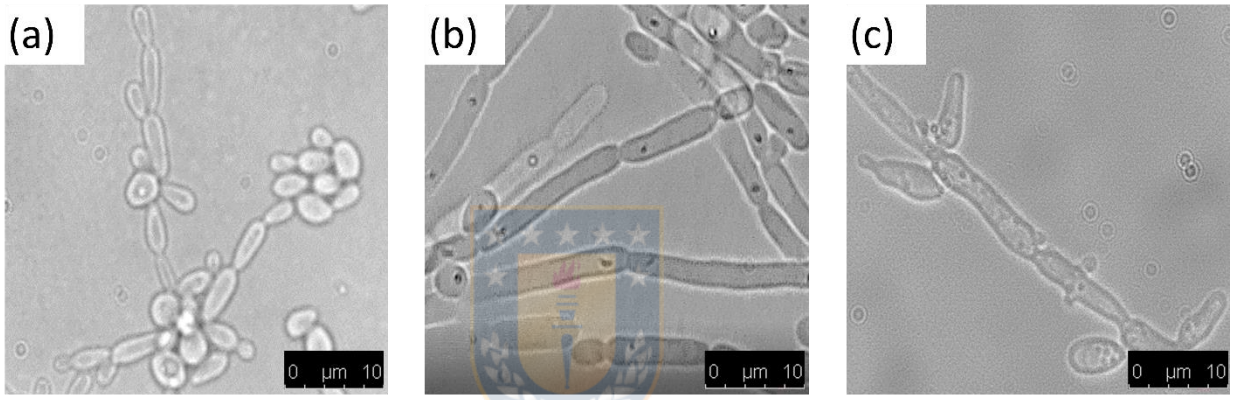

Fig. 2.2. Intact cultures of *M. guilliermondii* (a), *S. coipomensis* (b), and *S. paludigena* (c) performed on PDA media placed on slides and covered with a cover, visualized under light microscope (400×)

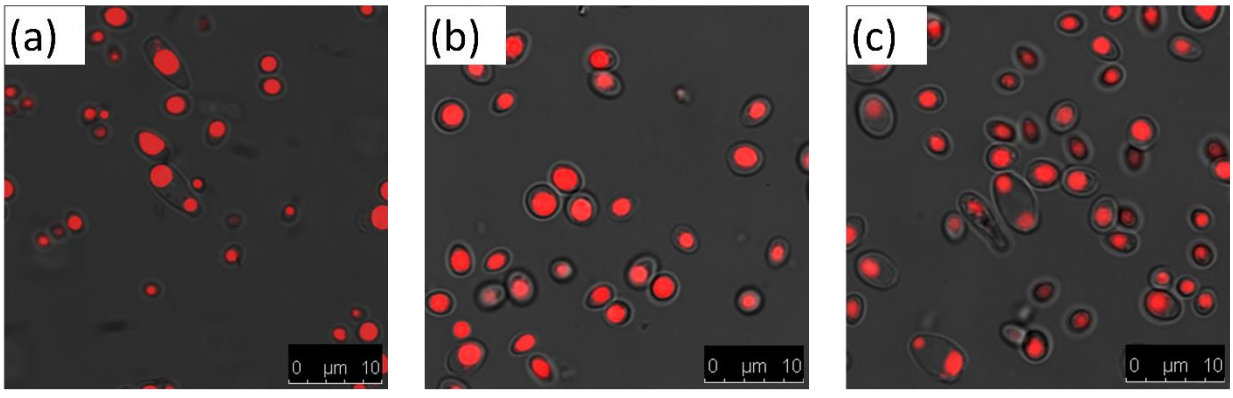

Fig. 2.3. Lipid droplets within the yeast cells of *M. guilliermondii* (a), *S. coipomensis* (b), and *S. paludigena* (c) visualized under confocal microscope (630×) using

fluorescent filter after Nile red staining

Where *X* is the biomass concentration (g/L), *S* the sugar concentration (g/L), μ_{max} the maximum specific growth rate (h−1), *K*s the saturation constant (g/L), and $Y_{X/S}$ the biomass yield (gram of biomass produced / gram of sugar consumed).

The model was fitted to the experimental data using Aquasim software, Version 2.1d (Fig. 4). The calculated (*C*) and predicted (*P*) kinetics parameters were overall similar to each other, demonstrating a high accuracy of the model in describing yeast growth (Table 1). Considering the growth curves, we note that the strains entered the stationary phase in different fermentation times, ranging from 40 to 160 h. In all growth conditions, *M. guilliermondii* totally consumed sugars reaching the stationary phase after 50 to 90 h of incubation (Fig. 4a). *X* production was favored when *Glc* and *Xyl* were used as substrates at 28 °C, while in *Man*- containing medium *X* production did not present significant differences at 20 or 28 °C ($p < 0.05$) (Table 1). μ_{max} was higher when *Man* was used as carbon source. Except for the growth on *Xyl* at 20 °C, *S. coipomensis* displayed a similar kinetics behavior with *M. guilliermondii* (Fig. 4b, Table 1). However, this yeast growing on *Xyl* at 20 °C presented a very long lag phase followed by a slow growth, while significant substrate quantities remained unconsumed in the growth medium. As a consequence, relatively low biomass production was observed at 20 °C when compared with that of 28 °C. The *S. coipomensis* μ_{max} was always higher at 28 °C than at 20 °C, obtaining in the case of *Glc* and *Man* very satisfied values. The *S. paludigena* was the yeast that growing in media containing *Glc* and *Xyl* as carbon sources in both temperatures presented the longer lag phase (Fig. 4c). Besides, *Glc* was totally consumed in both 20 and 28 °C temperatures, while important quantities of *Xyl* remained unconsumed after 160 h of fermentation. Instead, in media containing *Man* as carbon source at 20 and 28 °C, *S. paludigena* presented a short lag phase, grown faster than on *Glc* and *Xyl* and totally consumed *Man* in both temperatures. *X* production was comparable to the previous strains, except for the cases of *Glc* at 20 °C and *Xyl* at 28 °C, where *X* production was rather limited (Table1).

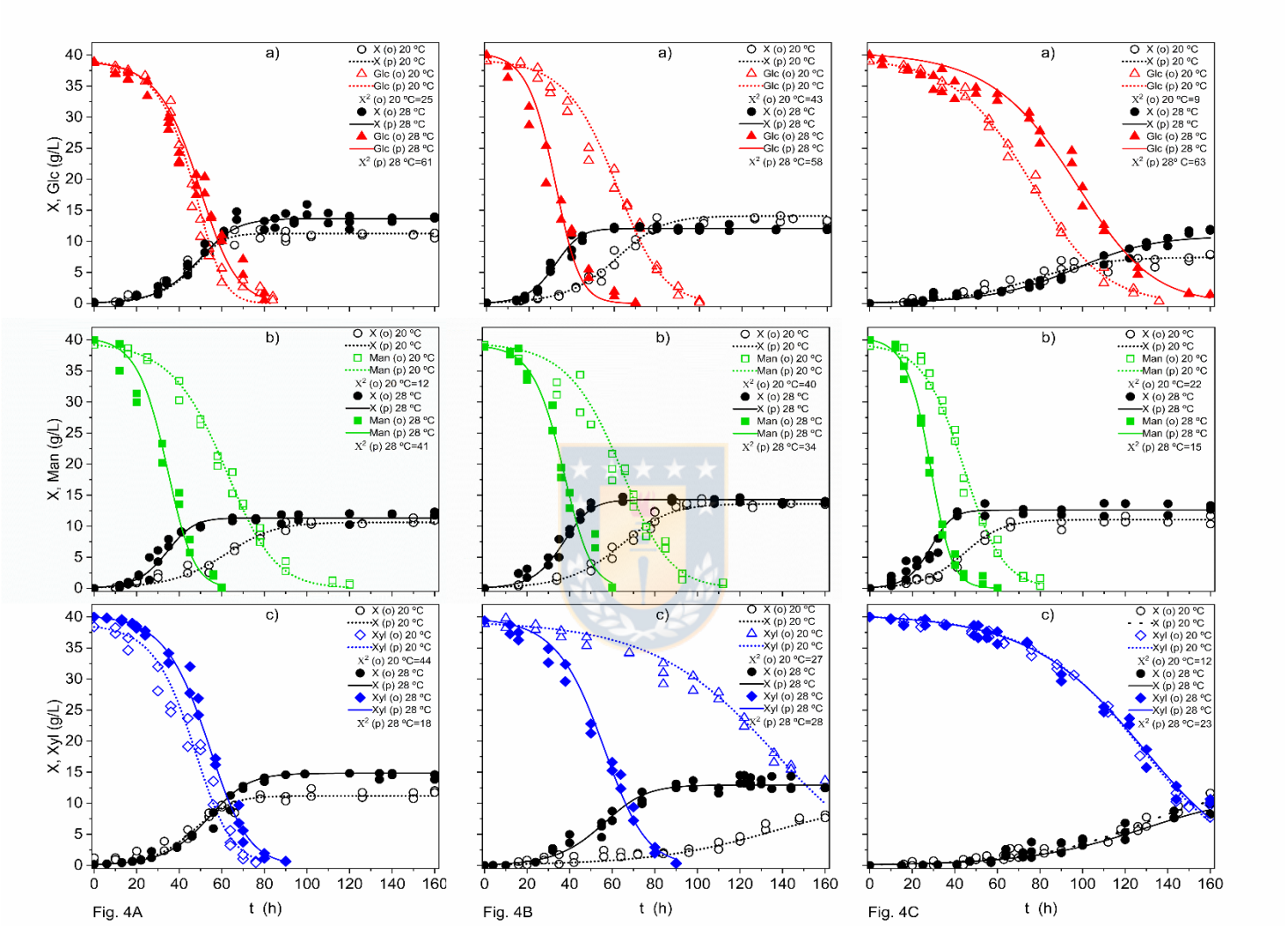

Fig. 2.4. Biomass (*X*, g/L) production and sugar (g/L) consumption (i.e., a glucose [*Glc*]; b mannose [*Man*]; c xylose [*Xyl*]) by *M. guilliermondii* (a), *S. coipomensis* (b), and *S. paludigena* (c) growing at 20 and 28 °C. Points and lines represent experimental data and model predictions, respectively, for biomass and sugar concentration

The *M. guilliermondii* and *S. paludigena* presented higher specific consumption rate (q_{Smax}, h−1) for *Man* at 28 °C, while both *Man* and *Glc* were rapidly taken up by *S. coipomensis* that exhibited high q_{Smax} values for these sugars (Table 1).

Strains of *M. guilliermondii* were able to assimilate common hexoses, disaccharides, polyols, organic acids [153], and *Xyl* [253,254]. Likewise, *S. coipomensis* and *S. paludigena* have been reported as good consumers of hexoses and *xylose* [139,253, 256]. The yeast strains studied in the current paper assimilated *Glc*, *Man*, and *Xyl* with different rate and at different extent, with *Man* being rapidly taken up by the three strains, as indicated by the values of the parameter q_{Smax}. The high uptake rate for *Man* may be explained by the high yeast requirements for this sugar consisting the major building block of the cell wall polysaccharides. Actually, the yeast wall, which represents 30 to 50% (*w*/w) of cell dry mass, consists of polysaccharides up to 90% (w/w), mainly glucans and mannans and a small portion of chitin [45,68]. Some of these polysaccharides contain *Man* in high percentages than other sugars [120].

Lipid production and fatty acid composition on single monosaccharide media

The *M. guilliermondii* cultivated in *Glc*-containing media at both temperatures and in *Man*- containing media at 28 °C accumulated lipids in significant percentages (Table 1). Lower lipid accumulation was achieved when *Xyl* was used as substrate. The *S. coipomensis* mostly accumulated around 18% (w/w) of lipids, except for *Man* at 28 °C, in which high lipid accumulation (i.e., 24%, w/w) occurred, and for *Xyl* at 28 °C, where only 10% (w/w) of lipids were accumulated inside the yeast cells. The *S. paludigena* accumulated lipid in higher amounts at 28 °C than 20 °C, which was satisfying when *Glc* and *Man* were employed as carbon sources (Table 1). Oleic acid (18:1Δ9) was the major FA in all lipids produced, followed by palmitic acid (16:0) (Table 2). In the lipids produced by *M. guilliermondii* and *S. coipomensis*, palmitoleic acid (16:1Δ9) participated in non-negligible percentages, contrary to linoleic acid (18:2Δ6,12), which was detected in limited amounts, especially in the lipids of *M. guilliermondii*. The *S. paludigena* lipids are relatively rich in linoleic acid, the percentages of which are often higher to those of palmitoleic acid.

Table 2.1. Growth of *Meyerozyma guilliermondii*, *Scheffersomyces coipomensis*, and *Sugiyamaella paludigena* in media containing glucose (*Glc*), mannose (*Man*), or xylose (*Xyl*) as carbon source at 20 and 28 °C and their kinetics parameters

Yeast	S	T (ºC)	S (g/l)		X (g/l)		$Y_{X/S}$ (g/g)		μ_{max} (h^{-1})		q_{Smax} (h^{-1})		L/X (%, w/w)	$Y_{L/S}$ (g/g)
			C	P	C	P	C	P	C	P	C	p	C	C
M. guilliermondii	*Glc*	20	38.4±0.4	38.9	10.9±0.6	11.3	0.28±0.02	0.28	0.23	0.22	0.82±0.11	0.79	18.0±1.3	0.05±0.01
		28	38.2±0.8	37.8	13.6±1.0	13.7	0.36±0.03	0.35	0.30	0.32	0.83±0.15	0.91	19.2±1.9	0.07±0.01
	Man	20	38.6±0.2	39.0	10.8±0.4	10.6	0.28±0.01	0.27	0.19	0.24	0.69±0.07	0.89	19.9±1.1	0.06±0.01
		28	39.9±0.1	39.5	11.3±0.7	11.3	0.29±0.02	0.28	0.39	0.43	1.36±0.15	1.54	16.4±0.5	0.05±0.01
	Xyl	20	37.9±0.5	37.7	11.3±0.6	11.2	0.30±0.02	0.29	0.29	0.33	0.97±0.12	1.14	12.4±1.7	0.04±0.01
		28	39.4±0.1	39.4	14.5±0.4	14.8	0.37±0.01	0.35	0.25	0.24	0.68±0.05	0.69	13.4±1.5	0.05±0.01
S. coipomensis	*Glc*	20	38.9±0.2	38.2	13.2±0.6	14.1	0.35±0.02	0.35	0.27	0.26	0.78±0.08	0.74	18.1±0.6	0.06±0.01
		28	39.9±0.1	40	12.1±0.3	12.1	0.30±0.01	0.29	0.42	0.47	1.39±0.06	1.62	17.9±0.6	0.05±0.00
	Man	20	38.2±0.2	38.8	13.4±0.9	13.6	0.35±0.03	0.34	0.23	0.25	0.67±0.12	0.74	17.2±1.4	0.06±0.01
		28	38.8±0.1	38	14.0±0.5	14.3	0.37±0.01	0.36	0.46	0.44	1.25±0.08	1.22	24.2±1.1	0.09±0.01
	Xyl	20	25.3±0.6	28.9	7.5±0.4	7.7	0.26±0.02	0.26	0.10	0.11	0.39±0.08	0.42	18.5±0.7	0.05±0.01
		28	39.1±0.1	38.4	13.3±0.8	12.9	0.35±0.02	0.32	0.24	0.28	0.69±0.10	0.88	10.5±1.8	0.04±0.01
S. paludigena	*Glc*	20	38.6±0.3	37.9	7.7±0.2	7.4	0.20±0.01	0.19	0.28	0.31	1.38±0.07	1.63	7.8±0.1	0.02±0.00
		28	38.6±0.1	39.1	11.7±0.2	10.6	0.30±0.01	0.27	0.13	0.15	0.43±0.03	0.56	18.7±2.1	0.06±0.01
	Man	20	38.6±0.9	38.3	10.8±0.7	11.1	0.28±0.02	0.27	0.29	0.34	1.03±0.16	1.23	8.17±0.6	0.02±0.00
		28	39.9±0.1	40	12.7±0.8	12.6	0.32±0.02	0.31	0.54	0.54	1.70±0.17	1.74	21.6±1.7	0.07±0.01
	Xyl	20	30.2±1.4	33	11.1±0.6	10.3	0.34±0.03	0.30	0.13	0.12	0.39±0.09	0.40	8.10±0.7	0.03±0.01
		28	30.0±0.4	32.4	8.5±0.8	9.0	0.26±0.03	0.27	0.10	0.12	0.38±0.14	0.44	10.9±4.4	0.03±0.02

T, temperature; *S*, substrate (sugar) consumed; *X*, biomass; $Y_{X/S}$, biomass yield on substrate consumed; μ_{max}, maximum specific growth rate; q_{Smax}, maximum specific substrate consumption (=μ_{max} / $Y_{X/S}$); *L/X*, lipid content in dry biomass; $Y_{L/S}$, lipid yield on substrate consumed; *P* is predicted and *C* is calculated parameter of growth

The unsaturation index in the lipids produced seemed to be unaffected by the incubation temperature (Table 2). However, with few exceptions, the desaturation of palmitic and stearic acids to their monounsaturated derivatives was favored at 28 °C. On the other hand, with exception of *S. paludigena*, the desaturation of the oleic acid to linoleic acid remains at low levels. This conversion in *S. paludigena* was favored at 20 °C, especially when *Glc* was employed as carbon source.

Simultaneous uptake of sugars and the activity of key enzymes involved in sugar metabolism and fatty acid biosynthesis

The strains cultivated in a mixture of *Glc*, *Man*, and *Xyl* were able to efficiently grow in both 20 and 28 °C, simultaneously consuming the three sugars without exhibiting a diauxic growth behavior (Fig. 5, Table 3). However, a clear preference for *Glc* was observed, which was more impressive at 28 °C than at 20 °C.

The *M. guilliermondii* cultivated at 28 °C reached the stationary phase after 100 h of incubation (Fig. 5a and Table 3). Among the sugars, *Glc* was preferentially consumed and exhausted almost totally while reduced amounts of *Man* and *Xyl* were assimilated. During the exponential growth phase (at t = 30 h), high activities of phosphoglucose isomerase (*PGI*) were recorded, suggesting an intense catabolism of *Glc* (Fig. 1, Table 4). Instead, low activities of phosphomannose isomerase (*PMI*) and xylulokinase (*XK*), involved in *Man* and *Xyl* catabolism, respectively, were observed at this phase of growth. All the above-mentioned enzymes remained active as growth proceeded, presenting reduced activities in late stationary phase. *ICDH* activity was low during all growth phases indicating citrate availability in the cytosol for acyl-CoA synthesis. The activity of *ATP:CL*, enzyme involved in citrate cleavage to acetyl-CoA and oxaloacetate in the cytosol, was active in the first growth steps, including exponential and early stationary phases, but drastically decreased in the late stationary phase (Table 4). The lipid synthesis in this strain attained 22.4 ± 0.2% (w/w) in dry cell mass (Table 3).

Table 2.2. Fatty acid composition of total lipids produced by the yeast strains growing under different culture conditions and estimation of desaturase activity

			Fatty acid composition (% w/w) of total lipids								Desaturase activities
Strain	S	T ºC	16:0 palmitic	$16:1^{\Delta 9}$ palmitoleic	18:0 stearic	$18:1^{\Delta 9}$ oleic	$18:2^{\Delta 9,12}$ linoleic	C16:1/ C16:0	C18:1/ C18:0	C18:2/ C18:1	$\frac{1x\sum monoene + 2x\sum diene}{\sum FA}$
M. guilliermondii	*Glc*	20	22.1±0.8	9.6±0.5	2.7±0.2	63.8±1.5	1.1±0.4	0.43±0.04	23.9±2.4	0.02±0.01	0.76±0.03
		28	18.6±2.3	12.4±1.8	2.3±0.8	62.6±3.2	3.0±0.5	0.67±0.18	26.7±9.9	0.05±0.01	0.81±0.06
	Man	20	20.9±0.5	11.0±0.1	2.6±0.1	62.8±0.5	1.2±0.1	0.53±0.02	23.8±1.1	0.02±0.00	0.76±0.01
		28	20.2±0.4	13.3±0.6	2.3±0.2	60.8±1.0	1.5±0.9	0.66±0.04	26.3±2.6	0.02±0.01	0.77±0.03
	Xyl	20	18.8±0.9	12.4±0.5	1.7±0.2	65.1±0.5	0.8±0.2	0.66±0.06	37.3±4.6	0.01±0.00	0.79±0.01
		28	19.8±0.2	15.4±0.1	2.2±0.4	61.0±1.4	1.2±0.1	0.78±0.01	27.7±5.7	0.02±0.00	0.79±0.02
S. coipomensis	*Glc*	20	14.7±0.8	7.9±0.4	2.4±0.4	68.0±0.6	6.5±0.1	0.54±0.06	28.2±4.9	0.10±0.00	0.89±0.01
		28	16.3±2.2	11.2±1.1	2.3±0.3	64.4±2.5	5.0±2.0	0.69±0.16	27.7±4.1	0.08±0.03	0.86±0.08
	Man	20	16.0±0.7	7.8±0.7	2.7±0.2	68.4±0.6	3.2±0.8	0.48±0.06	25.5±2.1	0.05±0.01	0.83±0.03
		28	18.9±1.8	11.6±2.6	2.7±0.8	62.6±3.6	2.8±1.1	0.61±0.19	23.1±8.5	0.04±0.02	0.80±0.08
	Xyl	20	17.3±0.6	8.2±0.5	2.5±0.4	65.4±0.5	4.2±0.5	0.48±0.05	26.4±4.5	0.06±0.01	0.82±0.02
		28	16.7±0.8	11.5±1.2	2.0±0.3	59.5±1.4	9.0±0.4	0.69±0.10	29.3±4.9	0.15±0.01	0.89±0.03
S. paludigena	*Glc*	20	21.7±1.5	3.0±0.2	17.1±0.9	43.1±0.1	10.9±0.3	0.14±0.02	2.5±0.14	0.25±0.01	0.68±0.01
		28	17.1±1.8	10.1±0.4	3.2±0.2	64.4±0.9	4.2±0.8	0.59±0.09	19.8±1.5	0.07±0.01	0.83±0.03
	Man	20	17.5±0.9	2.4±0.6	4.6±0.5	62.5±0.5	11.4±0.3	0.14±0.04	13.6±1.6	0.18±0.01	0.88±0.02
		28	12.6±2.3	9.2±1.1	6.7±0.5	62.9±1.7	6.5±1.5	0.73±0.22	9.3.1±0.9	0.10±0.03	0.85±0.06
	Xyl	20	17.7±1.4	2.7±0.5	5.9±0.4	59.0±0.4	13.8±0.3	0.15±0.04	10.1±0.8	0.23±0.01	0.89±0.02
		28	11.8±0.2	*tr*	6.1±0.1	61.0±0.6	21.1±0.4	*tr*	9.9±0.3	0.35±0.01	1.03±0.01

Abbreviations as in Table 1; tr traces (<0.1)

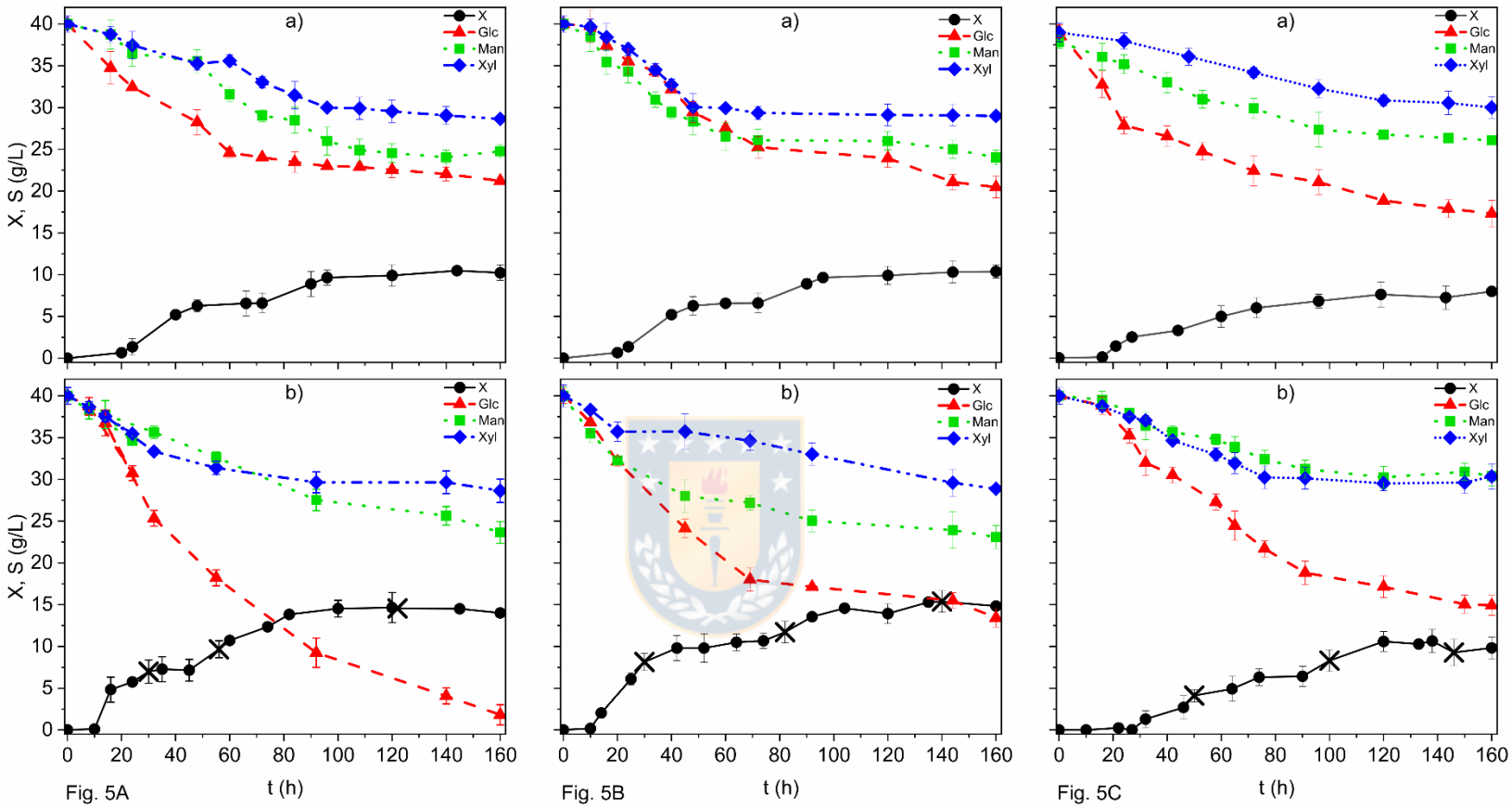

Fig. 2.5 Biomass (*X,* g/L) production and sugar (g/L) consumption by *M. guilliermondii* (a), *S. coipomensis* (b), and *S. paludigena* (c) growing at (a) 20 and (b) 28 °C in a mixture of *Glc*, *Man*, and *Xyl*. X marks in panels a (b), b (b), and c (b) indicate sampling for determination of enzyme activities

The *S. coipomensis*, alike to *M. guilliermondii*, presented the maximum biomass production at 28 °C (Fig. 5b). At this incubation temperature, substrate consumption reached the maximum value, while the preferably assimilated sugar was *Glc* (Table 3). Regarding catabolic activities, we note that both *PGI* and *PMI* presented high activities during the exponential growth phase, which significantly declined later in the stationary growth phase (Table 4). Low *XK* activities were detected in the exponential growth phase, which further declined as growth proceeded. The detected activities of all three enzymes involved in *Glc*, *Man*, and *Xyl* catabolism indicated simultaneous consumption of these sugars. Furthermore, the strains presented low *ICDH* activity, indicating a potential for lipid accumulation, and high *ATP:CL* activity in the exponential phase, promoting the lipid accumulation, which reached the value of 24.3 ± 0.5% (w/w) (Table 3).

Contrary to *M. guilliermondii* and *S. coipomensis*, *S. paludigena* showed no significant differences at 28 and 20 °C (at $p < 0.05$) in biomass production and substrate consumption (Fig. 5c). However, similarly to the previous strains, *S. paludigena* presented a preference for *Glc* consumption than for *Man* and *Xyl* (Table 4). Maximum activity of *PGI* was detected in the exponential phase while both *PMI* and *XK* activities were low. In this case, the activities of the above-mentioned enzymes implicated in sugar catabolism decreased over time. As for lipogenic activity, increased *ICDH* activities were recorded, and this fact, in parallel with the low *ATP:CL* activity, which decreased over time (Table 4), predicts low lipid accumulation. Accordingly, this yeast accumulated lipids in low percentages. The biomass yield on substrate consumed ($Y_{X/S}$) was around 0.2 g/g, while that of lipid ($Y_{L/S}$) was around 0.05 g/g for *M. guilliermondii* and *S. coipomensis* and 0.03 g/g for *S. paludigena* (Table 3).

From the above-mentioned results, we find out a simultaneous assimilation of *Glc*, *Man*, and *Xyl*, although a clear preference for *Glc* over *Man* was observed while *Xyl* uptake rate was low. Despite the above substrate specificity, diauxic growth was not observed as indicated by the absence of any lag period within the growth curves, which is typical when sugars are sequentially consumed [27,113]. Furthermore, in the sugar mixture

medium, the activities of key enzymes involved in the catabolism of *Glc*, *Man*, and *Xyl*, i.e., *PGI*, *PMI*, and *XK*, respectively, were recorded in all growth phases, which further strengthen the conclusion that concomitant consumption of the above sugars occurred in the strains under investigation. Likewise, *Lipomyces starkeyi* strain cultivated in a mixture of *Glc* and *Man*, simultaneously consumed these hexoses [120]. It was reported that in yeasts the hexose transporters commonly tend to be less/not specialized, as generally one transporter is capable of transporting multiple types of hexoses [86]. The simultaneous assimilation of hexoses and pentoses is highly desired in microbial fermentations performed on a variety of substrates, such as the mixture of sugars derived from lignocellulosic biomass, as this feature is likely to lead to a shorter and more productive process [28]. A similar behavior of simultaneous sugar (hexoses and pentoses). uptake has been described in *Trichosporon cutaneum* [29], *Pseudozyna hubeiensis* [122], *Rhodosporidium kratochvilovae* [264], *Y. lipolytica* [184], *Lipomyces starkeyi* [196], and *Cryptococcus curvatus* [61,116], among others. On the contrary, catabolic repression mechanisms are present in *L. starkeyi* [44], *Trichosporon fermentans* [224], *Rhodosporidium fluviale* [119], and *Rhodosporidium toruloides* [111], growing in mixtures of pentoses and hexoses.

Lipid accumulation in oleaginous yeasts is related to low *ICDH* activities, which induce the outflow of citrate from mitochondria to cytoplasm, and high *ATP:CL* activities, which generate acetyl-CoA from citrate [1,42,64, 244]. These prerequisite conditions are fulfilled in the investigated yeast strains, as the activity of *ICDH* remained low during growth, while *ATP:CL* activity was high, especially in the early stationary phase for *M. guilliermondii* and S. *coipomensis* and in the exponential phase for *S. paludigena*. The *M. guilliermondii* and *S. coipomensis* cultivated on the mixture of sugars at 28 °C accumulated lipids > 20% (w/w), while *S. paludigena* was able to grow and accumulate lipids at both 20 and 28 °C but at lower extent. In some cases, the carbon substrate also affected lipid accumulation. However, it should be noted that lipid accumulation was not high when compared with other oleaginous yeasts, such as *T. cutaneum* [29], *Y. lipolytica*, *Cryptococcus curvatus*, *Rhodotorula glutinis*, *Rhodotorula toruloides* [116,44], and *L. starkeyi* [116, 120, 196].

Table 2.3. Growth of the yeast strains cultivated in a sugar mixture at 20 and 28 °C and their kinetics parameters

		S (g/l)							
Strain	T (ºC)	*Glc*	*Man*	*Xyl*	Total	X (g/l)	$Y_{X/S}$ (g/g)	L/X (% w/w)	$Y_{L/S}$ (g/g)
M. guilliermondii	20	18.8±1.0	15.2±1.3	11.3±1.5	45.3±3.8	10.2±0.9	0.23±0.04	18.2±0.3	0.04±0.01
	28	38.2±2.2	16.3±2.3	11.4±2.4	65.9±6.9	14.0±0.8	0.21±0.03	22.4±0.2	0.05±0.01
S. coipomensis	20	19.5±1.9	16.0±1.5	11.0±1.5	46.5±4.9	10.4±0.8	0.21±0.04	18.1±0.1	0.04±0.01
	28	26.6±2.1	16.9±2.4	11.1±1.5	54.7±6.0	14.8±0.3	0.19±0.03	24.3±0.5	0.06±0.01
S. paludigena	20	21.8±2.4	14.8±1.1	10.0±2.4	46.6±5.9	8.0±0.4	0.17±0.02	15.4±0.3	0.02±0.01
	28	26.1±2.2	10.6±2.2	10.2±2.5	46.9±6.9	9.8±0.4	0.21±0.04	16.2±0.2	0.03±0.01

Abbreviations as in Table 1.

Table 2.4. Activities of key enzymes in the cell-free extract of the yeast strains growing on a sugar (glucose, mannose, and xylose) mixture at 28 °C during growth (i.e., in the exponential, early stationary, and late stationary phases—see X marks in Fig. 5b)

		Enzymes involved in sugar catabolism						Enzymes involved in lipid biosynthesis			
		PGI		*PMI*		*XK*		*NAD+-ICDH*		*ATP_CL*	
Strain	T (h)	Units/mg of protein	U/mg biomass	Units/g of protein	U/mg biomass	Units/g of protein	U/mg biomass	Units/g of protein	U/mg biomass	Units/g of protein	U/mg biomass
M. guilliermondii	30	52.41 ±0.51^{a1}	27.37 ±1.26^{a1}	4.98 ±0.25^{a2}	2.97 ±0.56^{a2}	13.52 ±0.03^{a3}	6.97 ±0.04^{a3}	<0.01^{a4}	<0.01^{a4}	6.77 ±0.21^{a5}	3.49 ±0.36^{a5}
	56	27.39 ±1.23^{b1}	10.93 ±0.9^{b1}	3.27 ±0.54^{b2}	1.57 ±0.08^{b2}	6.91 ±0.01^{b3}	2.53 ±0.02^{b3}	<0.01^{a4}	<0.01^{a4}	7.98 ±0.12^{a5}	2.93 ±0.17^{a5}
	122	16.31 ±1.15^{c1}	7.08 ±0.34^{c1}	3.09 ±0.33^{b2}	1.36 ±0.02^{b2}	8.03 ±0.02^{b3}	3.55 ±0.03^{b3}	<0.01^{a4}	<0.01^{a4}	1.37 ±0.23^{b5}	0.61 ±0.27^{b5}
S. coipomensis	30	329.63 ±4.35^{a1}	210.12 ±3.54^{a1}	339.18 ±4.87^{a2}	165.68 ±5.34^{a2}	22.05 ±0.11^{a3}	10.77 ±0.16^{a3}	<0.02^{a4}	<0.01^{a4}	10.18 ±0.05^{a5}	5.57 ±0.13^{a5}
	82	227.35 ±6.85^{b1}	141.01 ±6.12^{b1}	290.43 ±6.54^{b2}	123.36 ±2.87^{b2}	15.80 ±0.11^{b3}	6.71 ±0.04^{b3}	<0.05^{a4}	<0.02^{a4}	17.99 ±0.60^{c5}	9.39 ±0.09^{c5}
	140	234.95 ±2.34^{b1}	139.59 ±1.05^{b1}	295.08 ±5.34^{b2}	94.84 ±0.98^{c2}	16.93 ±0.05^{b3}	5.44 ±0.02^{b3}	<0.01^{a4}	<0.01^{a4}	6.15 ±0.73^{b5}	1.97 ±0.04^{b5}
S. paludigena	50	216.22 ±5.65^{a1}	125.64 ±1.26^{a1}	6.68 ±0.09^{a2}	4.52 ±0.02^{a2}	7.91 ±0.03^{a3}	5.36 ±0.08^{a3}	0.36 ±0.01^{a4}	0.60 ±0.02^{a4}	17.14 ±0.76^{a5}	12.06 ±1.25^{a5}
	100	112.35 ±4.12^{b1}	75.32 ±0.47^{b1}	4.68 ±0.11^{b2}	2.47 ±0.07^{b2}	5.01 ±0.02^{b3}	2.68 ±0.014^{b3}	1.91 ±0.03^{a4}	1.11 ±0.02^{a4}	8.24 ±0.16^{b5}	4.35 ±0.75^{b5}
	146	79.98 ±1.76^{c1}	63.26 ±1.67^{c1}	2.46 ±0.17^{c2}	1.40 ±0.01^{c2}	3.38 ±0.01^{c3}	1.93 ±0.09^{c3}	<0.04^{a4}	<0.02^{a4}	5.58 ±0.23^{c5}	1.17 ±0.20^{c5}

Data are presented as mean values from three replications. Analysis was performed through Bonferroni post hoc test. Different letters indicate statistically significant differences at $p \leq 0.05$. Numbers referred to different enzyme. 1: *PGI*, phosphoglucose isomerase; 2: *PGM*, phosphomannose isomerase; 3: *XK*, xylulokinase; 4: *ICDH*, NAD+ -dependent isocitrate dehydrogenase; 5: *ATP:CL*, ATP citrate lyase

Table 2.5. Fatty acid composition of total lipids produced in yeasts growing in sugar mixture at 20 and 28 ºC and estimation of desaturase activities.

		Fatty acid composition (% w/w) of total lipids							Desaturase activities	
Strain	T ºC	16:0 palmitic	$16:1^{\Delta 9}$ palmitoleic	18:0 stearic	$18:1^{\Delta 9}$ oleic	$18:2^{\Delta 9,12}$ linoleic	C16:1/ C16:0	C18:1/ C18:0	C18:2/ C18:1	$\frac{1x\sum monoene + 2x\sum diene}{\sum FA}$
M. guilliermondii	20	19.4±1.1	11.3±0.5	2.5±0.7	61.5±1.5	2.9±0.4	0.58±0.06	24.5±7.4	0.05±0.01	0.79±0.03
	28	18.0±1.4	11.3±0.8	1.8±0.2	64.9±1.7	2.7±0.3	0.63±0.09	36.2±5.0	0.04±0.01	0.82±0.03
S. coipomensis	20	14.1±0.2	6.5±0.1	3.4±0.1	66.0±0.8	7.7±2.0	0.46±0.01	19.5±0.8	0.12±0.03	0.88±0.05
	28	16.8±0.7	11.0±1.9	2.4±0.7	60.4±4.4	9.1±1.2	0.65±0.14	25.6±7.9	0.15±0.02	0.90±0.06
S. paludigena	20	21.7±1.5	3.0±0.2	17.1±0.9	43.1±0.1	10.9±0.3	0.14±0.00	2.5±0.14	0.25±0.01	0.68±0.01
	28	17.7±1.8	tr	2.0±0.3	73.3±0.9	5.0±0.8	tr	36.7±6.0.0	0.07±0.01	0.83±0.03

Abbreviations as in Table 1 and 2.

Besides the fact that the culture conditions used in the current paper were not optimized, the low lipid production could be related to the relatively low uptake rate of the carbon source resulting in prolonged fermentation time. Low uptake rate of the carbon source may induce lipid degradation for maintenance energy generation or for further cell proliferation, provided that all essential nutrients are available in the growth medium [7,23].

Oleic acid was the predominant FA in yeast lipids with percentages ranging between 43 and 73% (w/w), followed by palmitic acid (Table 5). In *M. guilliermondii*, palmitoleic acid was also synthesized in significant amounts, contrary to stearic and linoleic acids, the concentration of which was less than 3% (w/w) in the total lipids. In contrary, *S. coipomensis* was able to synthesize both palmitoleic and linoleic acids in non-negligible concentrations. The FA composition of *S. paludigena* lipids significantly varies depending on the incubation temperature. In particular, the lipids produced at 28 °C contained oleic acid in very high concentration and stearic acid in low concentration, contrary to those produced at 20 °C which were relatively rich in stearic acid and poor in oleic acid. In these lipids, palmitoleic acid was present in low percentages, while linoleic acid was synthesized in non-negligible percentages, especially at 20 °C. The unsaturation index values seem to be unaffected by the incubation temperature. However, the desaturation of the saturated FAs, especially that of stearic acid, was favored at 28 °C. Except for *S. paludigena* growing at 20 °C, the desaturation of oleic to linoleic acid was low. The lipids produced by the strains under investigation, containing oleic acid in high concentration followed by palmitic and palmitoleic/linoleic acids, showed the common FA composition encountered in ascomycetous oleaginous yeasts [29,75,115,116,196].

2.5. CONCLUSIONS

The strains of *M. guilliermondii*, *S. coipomensis*, and *S. paludigena* isolated from the Valdivian Forest were able to efficiently grow on the major building blocks of polysaccharides contained in *Eucalyptus globulus* and *Pinus radiata* (i.e., hardwoods

and softwoods, respectively), showing higher uptake rates for *Man* (and sometimes for *Glc*) than for *Xyl*, used as single carbon sources. In cultures performed on sugar mixtures, the strains simultaneously assimilated *Glc*, *Man*, and *Xyl*, displaying a notable preference for *Glc*. The strains, especially those of *M. guilliermondii* and *S. coipomensis*, were able to accumulate storage lipids up to 24% (w/w) in dry cell mass, which were rich in oleic and palmitic acids. Further research work concerning the biotechnological valorization of lignocellulosic sugars may include the optimization of yeast metabolism using laboratory evolution strategies towards maximization growth efficiency and lipid accumulation.

2.6. SUPPLEMENTARY INFORMATION

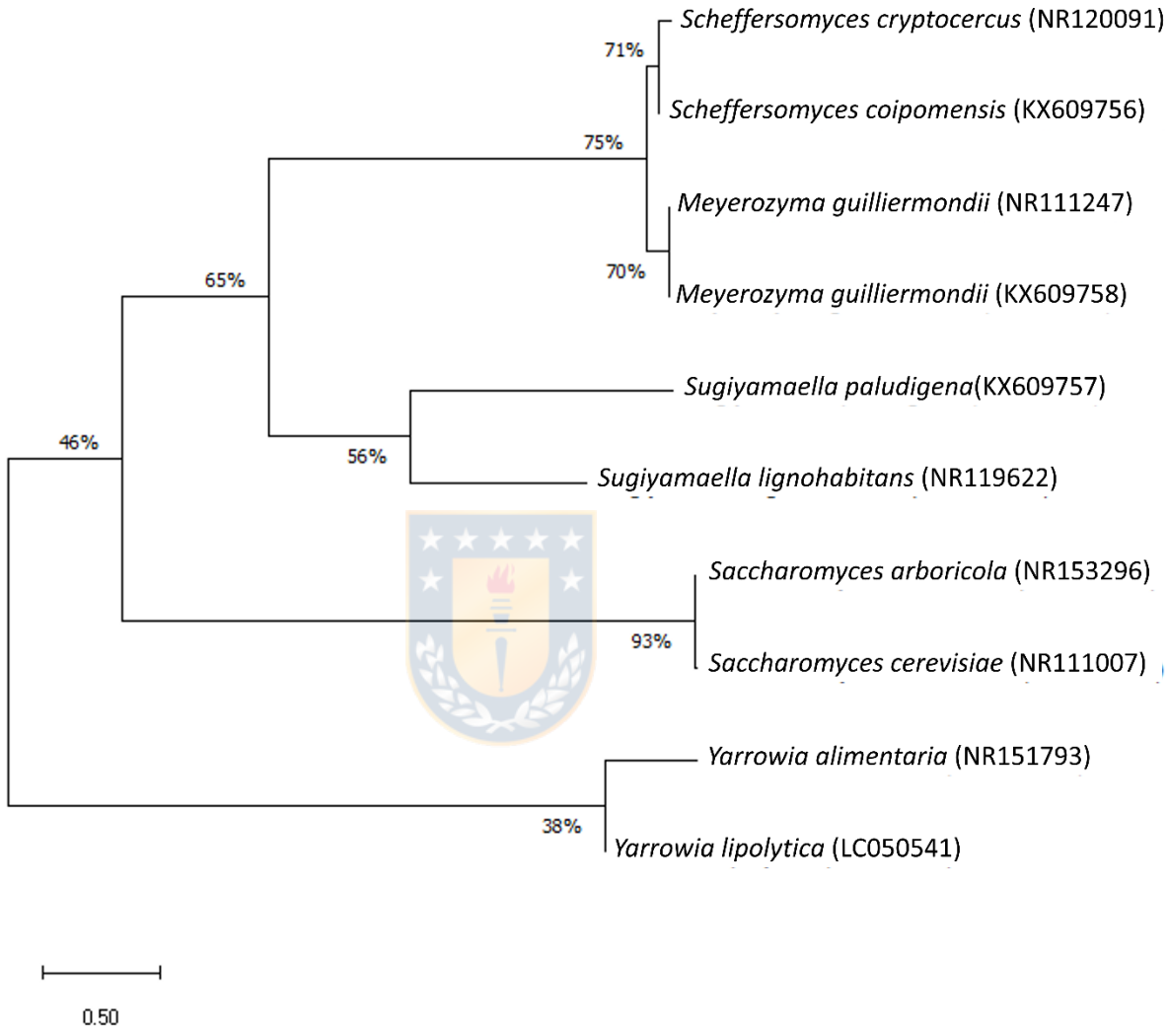

Fig. S1

DISCUSIÓN GENERAL

Varios investigadores han estudiado el desarrollo de microorganismos oleaginosos en hidrolizados lignocelulósicos obtenidos de pretratamientos de la biomasa vegetal y subsecuente hidrolisis enzimática, utilizando condiciones de crecimiento adecuadas (relación C:N, temperatura, velocidad de agitación, entre otras) que estimulan la producción de lípidos. Los resultados han mostrado una producción de biomasa (g/L), porcentaje de lípidos (% p/p) y una concentración de lípidos (g/L) diversos en el rango de 5,6-54, 21-75 y 2,4-12 respectivamente (Resultados presentados en la Tabla 1.1). En general, los azúcares obtenidos de residuos agrícolas mediante pretratamiento con ácidos débiles han sido claramente la tendencia de los últimos años. El uso de estos pretratamientos para el cultivo de microorganismos oleaginosos, han alcanzado rendimientos de lípidos superiores al 60% p/p [60,129,195,196].

En general los sustratos no detoxificados derivados de los pretratamientos pueden ser una buena alternativa para el crecimiento y la producción de lípidos, además, podrían contribuir en la recuperación de coproductos como lípidos, proteínas, aminoácidos, carbohidratos, carotenoides, glicerol y alcoholes que pueden considerarse para la comercialización, lo que favorecería enormemente en la estabilidad del proceso de producción de SCO [70,128,175]. Sin embargo, también se ha reportado que el proceso de detoxificación implica pérdida de azucares fermentables y encarecimiento del proceso, por lo que su aplicación debe ser rigurosamente estudiada.

La mayoría de los estudios de aplicación de sustratos lignocelulósico como materia prima para la producción de SCO se han centrado en cultivos de levaduras. Estas se han considerado microorganismos aventajados frente a otros microorganismos oleaginosos por su rápido crecimiento y altas productividad en menos de 180 horas [20, 118]. Sin embargo, aunque los resultados en la productividad de los lípidos han sido prometedores, los efectos negativos de la biomasa pretratada en el crecimiento y producción de lípidos deben mejorar para obtener procesos sustentables.

Por otro lado, el estudio metabólico de levaduras creciendo en mezclas de

monosacáridos más comunes de la lignocelulosa en condiciones aeróbicas y hipogénicas, como es el caso de las tres levaduras estudiadas en esta tesis (*Meyerozyma guilliermondii, Scheffersomyces coipomensis, y Sugiyamaella paludigena*), demostraron ser capaces de crecer eficientemente consumiendo simultáneamente los tres azúcares, esto se evidenció con la identificación de actividades enzimáticas claves en el metabolismo de los azucares analizados (glucosa, manosa y xilosa). A pesar de que no presentaron un comportamiento de crecimiento diauxico, se observó de igual forma una clara preferencia por la glucosa. Los resultados también demostraron un carácter oleaginoso de los microorganismos, siendo *S. coipomensis*, al igual que *M. guilliermondii*, los microorganismos que presentaron mayor producción de biomasa y lípidos [26]. Los resultados son congruentes con el estudio de otras levaduras cultivadas en más de un monosacárido [86,210].

La asimilación simultánea de hexosas y pentosas es muy deseada en las fermentaciones microbianas de diversos sustratos, como la mezcla de azúcares derivados de la biomasa lignocelulósica, ya que es probable que esta característica dé lugar a un proceso más corto y productivo [28].

En cuanto a los perfiles lipídicos de las levaduras estudiadas predominan el ácido oleico, seguido del ácido palmítico mostraron la composición común de los ácidos grasos encontrados en las levaduras oleaginosas ascomicetes, las cuales poseen un importante interés para la producción principalmente de biodiesel [25, 26].

CONCLUSIÓN GENERAL

Las conclusiones de este trabajo establecen que:

1. Las cepas de levaduras aisladas de madera en descomposición identificadas como *Meyerozyma guilliermondii*, *Scheffersomyces coipomensis* y *Sugiyamaella paludigena* demostraron crecer eficientemente en los principales monosacáridos presentes en la biomasa lignocelulósica, como son glucosa y manosa (hexosas), y xilosa (penosa), siendo en la mayoría de los casos más eficiente a 28º C que a 20º C.

2. Las levaduras oleaginosas cultivadas en mezclas de azúcares tienen la capacidad de asimilar simultáneamente hexosas (glucosa y manosa) y pentosa (xilosa), mostrando una notable preferencia por glucosa.

3. Las cepas, especialmente las de *M. guilliermondii* y *S. coipomensis*, cultivadas en una mezcla de monosacáridos fueron capaces de acumular mayor cantidad de lípidos de almacenamiento (22 y 24%, p/p de la biomasa seca, respectivamente) ricos en ácidos oleico y palmítico cuando fueron cultivadas a 28º C que a 20º C.

4. *M. guilliermondii* y *S. coipomensis* acumularon mayor cantidad de lípidos cuando se cultivaron en una mezcla de hexosas y pentosas que en cultivos con xilosa, sin embargo, los cultivos con glucosa y manosa alcanzaron porcentajes de lidios incluso, en algunos casos, iguales a los obtenidos de los cultivos en mezcla de monosacáridos

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