Universidade do Minho
Escola de Engenharia

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Yarrowia lipolytica biomass production from wood shavings: potential application on bioglues

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Trabalho realizado sob a orientação da
Professora Doutora Isabel Maria Pires Belo
e
Doutora Marlene Alexandra da Silva Lopes

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Yarrowia lipolytica biomass production from wood shavings: potential application on bioglues

RESUMO

A biomassa de levedura é rica em macromoléculas como proteínas, lípidos, hidratos de carbono e minerais, o que a torna valiosa para diversas aplicações industriais. Recentemente, foi descoberto o seu potencial no desenvolvimento de biocolas, uma alternativa ecológica aos adesivos convencionais derivados do petróleo. Entre as leveduras que podem ser aplicadas, destaca-se *Yarrowia lipolytica*, uma espécie não convencional reconhecida pela sua versatilidade metabólica e capacidade de crescer em hidrolisados lignocelulósicos. Assim, este estudo investigou o potencial de produção de biomassa de levedura a partir de hidrolisados de aparas de madeira, um subproduto lignocelulósico da indústria madeireira, numa perspetiva de desenvolvimento de economia circular neste setor.

Neste trabalho, foram avaliados dois pré-tratamentos: pré-tratamento hidrotérmico (PH) e pré-tratamento hidrotérmico com ácido diluído (PHAD). No caso do PHAD, a influência do tempo, da concentração de ácido e da carga de sólidos foi otimizada através de um planeamento fatorial Box–Behnken. Neste pré-tratamento foi obtido um sólido com 63 % de celulose, o qual foi posteriormente submetido a hidrólise enzimática com Cellic CTec3, originando um hidrolisado rico em glucose (57 $g \cdot L^{-1}$). Este hidrolisado foi utilizado como substrato para produção de biomassa de *Y. lipolytica* em frascos Erlenmeyer e em biorreator agitado. No biorreator a operar em descontínuo, obteve-se um rendimento de biomassa de 0.27 $g \cdot g^{-1}$. A biomassa de levedura obtida apresentava 33 % de proteína, 11 % de cinzas, 6 % de lípidos e 11 % de hidratos de carbono. Esta biomassa foi sujeita a um processo de autólise realizado a 55 °C durante 96 horas, tendo sido obtido um lisado que, após liofilização tinha um alto teor de proteína (45 %, p/p).

Os resultados obtidos demonstraram o potencial de valorização das aparas de madeira para a produção de biomassa de *Y. lipolytica* e a sua possível conversão em lisados ricos em proteínas, representando um passo promissor no desenvolvimento de biocolas.

Palavras-chave: aparas de madeira, biocolas, biomassa de levedura, biorreator, *Yarrowia lipolytica.*

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ABSTRACT

Yeast biomass is rich in valuable macromolecules such as proteins, lipids, carbohydrates and minerals, making it suitable for diverse industrial applications. Recently, it has also shown potential in the development of bioglues, an eco-friendly alternative to conventional petroleum-based glues. Among the yeasts with underexplored potential, *Yarrowia lipolytica* stands out due its metabolic versatility and ability to grow on lignocellulosic hydrolysates. This study investigated the potential of production of yeast biomass from wood shavings (WS), a lignocellulosic by-product of the wood industry, with potential for bioglue formulation.

In this study, two pretreatments were evaluated: hydrothermal pretreatment (HP) and hydrothermal dilute acid pretreatment (HDAP). For HDAP, the influence of the time, acid concentration and solid loading were optimized using a Box–Behnken factorial design. After HDAP, the solid fraction contained 63 % cellulose and was subjected to enzymatic hydrolysis with Cellic CTec3, resulting in a glucose-rich hydrolysate (57 $g \cdot L^{-1}$). The hydrolysate was then used as substrate for the production of *Y. lipolytica* biomass in Erlenmeyer flasks and in a stirred-tank bioreactor. In batch bioreactor experiment, a biomass yield of 0.27 $g \cdot g^{-1}$ was achieved. The yeast biomass contained 33 % protein, 11 % ash, 6 % lipids, and 11 % carbohydrates. The biomass was then subjected to an autolysis process at 55 °C for 96 hours, resulting in a lysate that, after lyophilization, exhibited a high protein content (45 %, w/w). These results demonstrated the potential of valorizing WS for the production of *Y. lipolytica* biomass and the possible conversion into protein-rich lysates, representing a promising step toward bioglues development.

Key words: bioglues, bioreactor, wood shavings, yeast biomass, *Y. lipolytica.*

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LIST OF NOMENCLATURES AND ABBREVIATIONS

ATP	Adenosine triphosphate
BGL	β–glucosidase
C/N	Carbon/nitrogen ratio
CAGR	Compound annual growth rate
CFU	Colony-forming unit
CoA	Coenzyme A
DNS	3,5-dinitrosalicylic acid
EDGE	Ethylene glycol diglycidyl ether
EDTA	Ethylenediamine tetraacetic acid
EFSA	European Food Safety Authority
EPS	Exopolysaccharides
FAS	Fatty acid synthase
FDA	Food and Drug Admnistration
FPU	Filter paper units
FTIR	Fourier transform infrared spectroscopy
GRAS	Generally recognized as safe
GUR	Glucose consumption rate
HDAP	Hydrothermal acid pretreatment
HMF	5-hydroxymethylfurfural
HP	Hydrothermal pretreatment
HPLC	High-performance liquid chromatography
LCCs	Lignin-carbohydrate complexes
$[\log R_0]$	Severity factor
NADPH	Nicotinamide adenine dinucleotide phosphate
NCYC	National collection of yeast cultures
OD	Optical density
PMSF	Phenylmethylsulfonyl fluoride
RI	Refractive index
rpm	Rotations per minute

RSM	Response surface methodology
SmF	Submerged fermentation
TN	Total nitrogen
TOC	Total organic carbon
UV	Ultraviolet light
vvm	Volume of air per volume of medium per minute
VOCs	Volatile organic compounds
WS	Wood shavings
WSH	Wood shavings hydrolysates
WSH-HDAP	Wood shavings hydrolysate derived from hydrothermal dilute acid pretreatment
WSH-HP	Wood shavings hydrolysate derived from hydrothermal pretreatment
YPD	Yeast extract, peptone, dextrose medium
$Y_{X/S}$	Biomass yield ($g \cdot g^{-1}$)
ρ	Density of solution ($g \cdot L^{-1}$)
μ	Specific growth rate (h^{-1})
λ	Wavelength (nm)

1. State Of The Art

1.1. *Yarrowia lipolytica*

Yarrowia lipolytica is a yeast from the class *Ascomycetes* and subclass *Hemiascomycetes* (Madzak, 2015). It is an oleaginous yeast capable of accumulating more than 20 % of its dry cell weight as lipids, primarily stored as intracellular triacylglycerols (Dias et al., 2023; Lopes et al., 2018). This yeast is also considered extremotolerant, as it can thrive in environments rich in hydrophobic substrates (e.g., n-alkanes, fats) (Fickers et al., 2005), marine and hypersaline habitats (Katre et al., 2012), and can also be found in fermented dairy products (e.g., cheese and yogurt) (Kazemi et al., 2025).

As a strict aerobe, oxygen concentration is a critical parameter that limits the growth of *Y. lipolytica* (Kerssemakers et al., 2023). Higher dissolved oxygen levels during fermentation have been reported to improve biomass yield, lipid accumulation, and citric acid production (Miranda et al., 2024; Xin-xin et al., 2018). This yeast can tolerate high salt concentrations (Andreishcheva et al., 1999), pH fluctuations between 3.5 and 8.0 (Madzak, 2021) and exposure to organic solvents (Walker et al., 2019). Its optimal growth temperature ranges between 25 °C and 30 °C (Madzak, 2021).

Y. lipolytica is a dimorphic yeast, capable of reversibly alternating between two distinct morphological forms (typical yeast oval cells or filamentous hyphae), depending on environmental conditions (Barth and Gaillardin, 1997). The morphology of *Yarrowia lipolytica* is modulated by multiple environmental factors, including pH, temperature, dissolved oxygen concentration, carbon source, and mechanical stress (Timoumi et al., 2018). Under reduced dissolved oxygen conditions, *Y. lipolytica* forms extremely long filaments (Timoumi et al., 2017) (**Figure 1**).

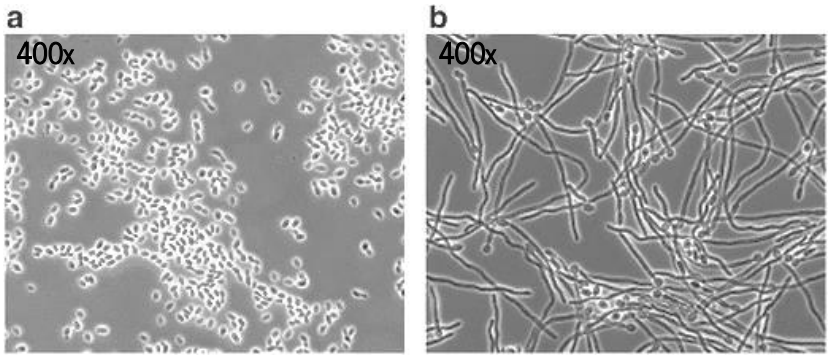


Figure 1: Morphological forms of *Yarrowia lipolytica* W29 grown in oxygen-rich media as (**a**) oval cells and under oxygen-limited conditions as (**b**) filamentous hyphae (Timoumi et al., 2018).

Y.lipolytica is a non-pathogen microorganism recognized as GRAS (Generally Recognized As Safe) by the U.S. Food and Drug Administration (FDA) (Barth and Gaillardin, 1997), enabling its safe application in the pharmaceutical and food industries. In 2010, the European Feed Manufacturers' Federation approved the use of *Y. lipolytica* as a fodder yeast (Drzymała et al., 2020). Furthermore, in 2019, the European Food Safety Authority (EFSA) assessed the safety of *Y. lipolytica* yeast biomass for use in dietary supplements intended for the general population above three years of age (Jach and Malm, 2022).

Y. lipolytica biomass is a valuable source of nutrients, rich in lipids, proteins, peptides, essential amino acids (e.g., lysine and methionine), polyunsaturated fatty acids, vitamins, and trace minerals (Carsanba et al., 2023; Jach et al., 2021). *Y. lipolytica* has been extensively used in diverse biotechnological processes, such as the production of lipids (Dias et al., 2024; Dias et al., 2025), enzymes (Costa et al., 2022; Miranda et al., 2024), organic acids (Carsanba et al., 2019), sweeteners (Paulino et al., 2021) and supplements (Carsanba et al., 2023; Dias et al., 2024; Drzymała et al., 2020). Reported studies focusing in *Y. lipolytica* biomass production in large-scale fermentation (bioreactors) include the cultivation of this yeast on waste bread, achieving 62 $g \cdot L^{-1}$ of biomass with a yield of 0.45 $g \cdot g^{-1}$ (Carsanba et al., 2023); on raw glycerol, resulting in 21 $g \cdot L^{-1}$ of biomass and a yield of 0.51 $g \cdot g^{-1}$ (Juszczyk et al., 2019); and on lignocellulosic hydrolysate, which produced 66 $g \cdot L^{-1}$ of biomass with a yield of 0.39 $g \cdot g^{-1}$ (Dias et al., 2025). Despite these promising results, large-scale biomass production of *Y. lipolytica* still faces several challenges, including the optimization of the C/N ratio, the reduction of by-products accumulation such as citric acid, and the control of excessive foam formation (Carsanba et al., 2023).

1.1.1. Carbon sources used by *Y. lipolytica*

Yarrowia lipolytica has emerged as a highly promising microbial platform for sustainable biotechnological applications due to its exceptional metabolic versatility, enabling the assimilation of a broad spectrum of carbon sources, including both hydrophilic and hydrophobic substrates.

Y. lipolytica demonstrates a remarkable ability to degrade hydrophobic carbon sources, including animal fats (Kamzolova et al., 2005; Lopes et al., 2018; Papanikolaou et al., 2003), waste cooking oils (Lopes et al., 2019) and n-alkanes (Fukuda, 2023; Miranda et al., 2024). In addition, this yeast metabolizes hydrophilic substrates such as glucose and other sugars, and acetic acid, which are readily accessible from renewable feedstocks and efficiently produced at an industrial scale (Lazar et al., 2014; Papanikolaou et al., 2006).

The growing interest in *Y. lipolytica* arises from its remarkable ability to valorize a wide range of low-cost and renewable carbon sources, particularly those derived from industrial, agricultural, and household residues, converting them into valuable bioproducts (Silva et al., 2025). Examples include the utilization of fatty acids derived from food waste, olive mill wastewater, waste cooking oils, crude glycerol from biodiesel production, and lignocellulosic hydrolysates obtained from agro-industrial by-products (**Table 1**).

Table 1: Examples of non-refined substrates used by *Yarrowia lipolytica* strains.

Carbon sources	Strain	Mode of operation	References
Food wastes	K57	Batch	Carsanba et al. (2023)
	NCYC 2904	Batch/ Two-stage batch	Pereira et al. (2023)
	W29	Batch	Lopes et al. (2018)
Olive mill wastewater	ACA-YC 5033 and LMBF Y-46	Batch	Sarris et al. (2023)
	ACA-YC 5033	Batch	Sarris et al. (2017)
	W29	Fed-batch	Gonçalves et al. (2012)
Waste cooking oils	ATCC 20177	Batch	Mitrea et al. (2022)
	W29	Batch	Lopes et al. (2019)
	SWJ-1b	Batch	Liu et al. (2015)
Crude glycerol	NCYC 2904	Fed-batch/ Two-stage batch	Pereira et al. (2023)
	W29	Batch	Ferreira et al. (2022)
	SKY7	Batch	Mathiazhakan et al. (2016)
	A101	Batch	Dobrowolski et al. (2016)
Eucalyptus bark hydrolysates	W29	Batch	Dias et al. (2025)
	W29	Batch/Two-stage repeated batch	Dias et al. (2024)
Olive tree prunings hydrolysates	NCYC 2904	Batch	Dias et al. (2025)

Table 1: Continuation.

Carbon sources	Strain	Mode of operation	References
Wheat straw hydrolysates	ATCC 46483	Batch	Caporusso et al. (2023)
	ATCC 20460	Batch	Yu et al. (2011)

1.1.2. Sugars metabolism in *Y. lipolytica*

Most yeast species, including *Saccharomyces cerevisiae* and *Yarrowia lipolytica*, are naturally capable of utilizing C6 sugars (hexoses) such as glucose as primary carbon sources for growth and metabolism. **Figure 2** illustrates the pathways involved in sugar metabolism in *Y. lipolytica*. Glucose is directly transported into the cell through specific hexose transporters and phosphorylated by hexokinase to form glucose-6-phosphate, which subsequently enters the glycolytic pathway to generate ATP and metabolic intermediates (Naifeh et al., 2023). Additionally, glucose-6-phosphate can be redirected into the pentose phosphate pathway to produce NADPH and biosynthetic precursors (Stincone et al., 2014). Xylose metabolism typically occurs through the action of two enzymes, xylose reductase and xylitol dehydrogenase, which sequentially convert xylose into xylulose before it enters the pentose phosphate pathway (Ryu et al., 2016). Also, some disaccharides can be used for *Y. lipolytica*. For cellobiose utilization, two main routes have been described in yeasts. In the first route, cellobiose is hydrolyzed extracellularly by a secreted β-glucosidase into glucose, which is subsequently transported into the cell (Rooyen et al., 2005). In the second route, cellobiose is directly transported across the plasma membrane by a cellodextrin-specific transporter and subsequently hydrolyzed intracellularly by BGL to release glucose (Nakamura et al., 2008).

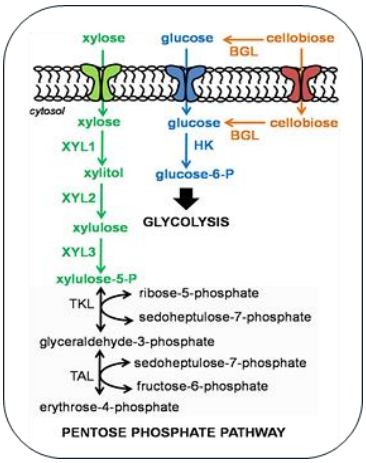


Figure 2: Overview of degradation pathways of glucose (blue), xylose (green), and cellobiose (orange) in *Y. lipolytica*., with a simplified pentose phosphate pathway (adapted from Ryu et al., 2016). XYL1, xylose reductase; XYL2, xylitol dehydrogenase; XYL3, xylulose kinase; TKL, transketolase; TAL, transaldolase; BGL, β – glucosidase.

The ability of *Y. lipolytica* to assimilate C5 sugars (pentoses), particularly xylose and cellobiose and their mixtures with glucose remains limited and not yet fully understood (Ryu et al., 2016). Sequential sugar consumption, where glucose is utilized preferentially before xylose has been reported for *Y. lipolytica* and other yeasts cultivated on lignocellulosic hydrolysates (Dias et al., 2023). The limited utilization of these mixed sugars is primarily attributed to either catabolite repression caused by glucose, where the presence of glucose suppresses the expression or activity of enzymes involved in the metabolism of alternative carbon sources (Ryu et al., 2016) or allosteric competition among sugar transporters (Poontawee et al., 2017).

Recent studies have focused on engineering *Y. lipolytica* to enhance its ability to utilize pentose sugars by developing heterologous xylose and cellobiose degradation pathways from *Pichia stipitis* and *Neurospora crassa* (Ryu et al., 2016; Tai, 2012; Zhang et al., 2023), along with the overexpression of genes encoding key enzymes of the xylose pathway (Ledesma-Amaro et al., 2016) . These metabolic modifications could enable efficiently assimilation of mixed sugars and facilitate the production of valuable biofuels and biochemicals from renewable carbohydrate-rich sources (Ledesma-Amaro et al., 2016).

1.2. Wood by-products

Wood is a highly valuable material due to its renewable, biodegradable nature and mechanical strength, and thermal properties (Besserer et al., 2021). The wood industry encompasses a wide range of products that serve critical roles across construction, manufacturing, and consumer goods sectors

(Salima and Johanssonb, 2016). Wood is widely used in construction (lumber, plywood), interior products (furniture, flooring, cabinetry) (Wegner et al., 2010), pulp and paper, renewable energy (pellets, briquettes, charcoal) (Rimantho et al., 2023), and advanced products such as cellulose-based textiles (Kallio, 2021) and bioplastics (Chen et al., 2022).

Globally, the wood and timber products market was valued at approximately 993 billion dollars in 2024 and is projected to grow at a compound annual growth rate (CAGR) of about 4.7 % in the coming years, with Asia remaining the key producer and consumer (Research, 2024). However, rising demand for wood is increasingly associated with the challenge of managing wood waste and by-products. In 2022, approximately 50 million tons of wood waste were generated in the European Union, of which only 40 % – 50 % were recycled or reused, while the remainder was incinerated or sent to landfill (Eurostat, 2024).

Wood waste is divided into two main categories: industrial waste, generated within the production process, and final waste, produced after the use of wood-based products (Besserer et al., 2021). Focusing on the first category, industrial waste is commonly referred to as wood by-products, which are secondary materials generated during processing that are not the main product but can still be valued in various industrial applications.

These by-products are generated across three main processing stages: harvesting, primary processing, and secondary processing (Mehmood et al., 2010), as shown in **Figure 3.** During harvesting, residues such as bark, branches, leaves, stumps, and other forest debris are produced; however, these materials are often contaminated with soil and therefore unsuitable for bioproducts applications (Mehmood et al., 2010). Sawmilling and other primary processing operations generate by-products such as sawdust, wood chips, wood shavings, and cut-offs (Dissanayake et al., 2025). Secondary processing, including furniture and manufacturing, generates additional by-products such as cuttings, wood shavings, and panel trimmings, which can also be effectively reused (Mehmood et al., 2010).

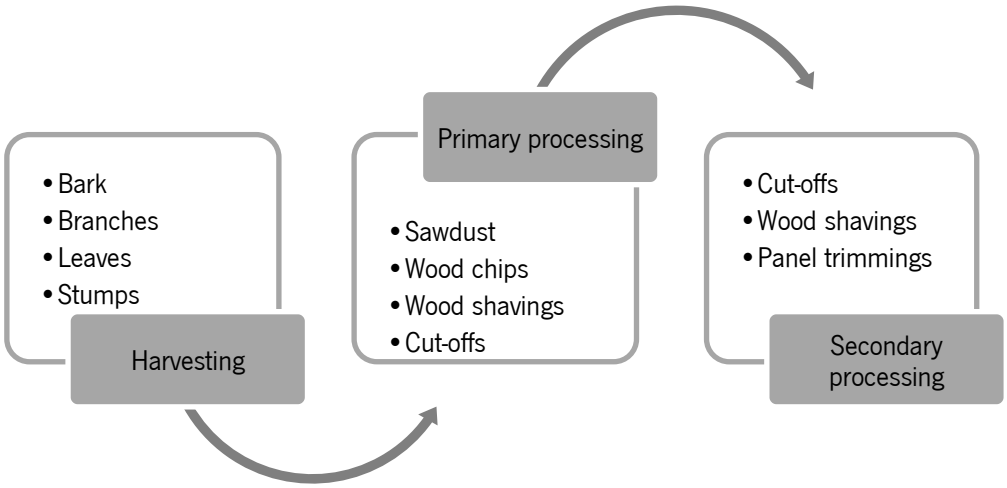


Figure 3: Wood by-products produced in the three stages of wood processing.

1.2.1. Wood shavings

Wood shavings are an inexpensive and widely available lignocellulosic by-product typically produced in large quantities during primary and secondary processing of wood products (Mallakpour et al., 2021). Traditionally, this by-product is used for low-value applications, including as a component in fertilizers, particle boards, animal bedding, particularly for chickens, or as a fuel source through direct combustion (Rominiyi et al., 2017). However, advancements in biorefinery technologies have significantly expanded their potential, positioning wood shavings as a valuable lignocellulosic feedstock, particularly for biofuel production (Adewumi, 2023; Rahmana et al., 2024).

Chemically, wood shavings are mainly composed of cellulose, hemicellulose, and lignin, which together constitute the structural framework of plant cell walls (**Figure 4**). Cellulose is a linear polysaccharide consisting of β-1,4-linked glucose units that form highly ordered microfibrils, contributing to the mechanical strength of the cell wall (Kumar et al., 2009; Marriott et al., 2016). It exists in both crystalline and amorphous regions, with the crystalline form being more resistant to enzymatic hydrolysis (Kleingesinds et al., 2018). Hemicellulose, by contrast, is an amorphous, branched polymer made up of diverse monomers like xylose, arabinose, mannose, and uronic acids, and is more readily hydrolyzed due to its lower structural order (Jönsson and Martín, 2016; Kumar et al., 2009). Lignin, the third major component, is a complex aromatic polymer composed of monolignols such as coniferyl, sinapyl, and coumaryl alcohol, interconnected mainly by β-aryl ether bonds (Renault et al., 2019; Zhu et al., 2022). It plays a crucial role in providing rigidity, hydrophobicity, and resistance to microbial degradation, while binding cellulose and hemicellulose into a cohesive three-dimensional matrix (Bimestre et al., 2022).

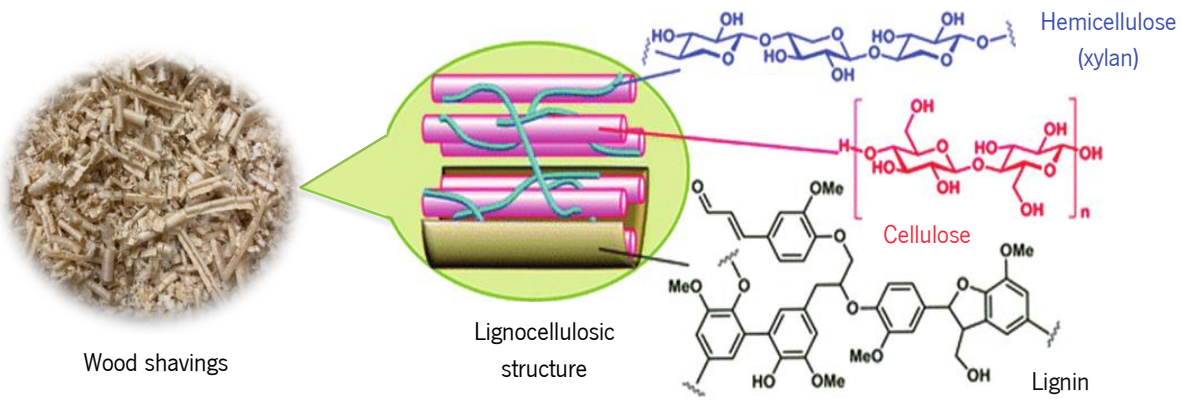


Figure 4: Structure and composition of lignocellulosic components (hemicellulose, lignin, and cellulose) of wood shavings.

In biorefineries, lignocellulosic biomass, which contains polysaccharide components such as cellulose and hemicellulose, undergoes a series of processing steps (**Figure 5**). The process begins with a pretreatment step, in which the biomass is subjected to physical, chemical, or physicochemical methods to reduce its recalcitrance and produce a solid fraction with enhanced accessibility to structural carbohydrates. This is followed by enzymatic hydrolysis, during which enzymes break down the pretreated biomass into a sugar-rich hydrolysate that serves as a key intermediate for microbial processes. Microorganisms then use the released sugars as a carbon source to produce biofuels, chemicals, and functional materials (Sheldon, 2014).

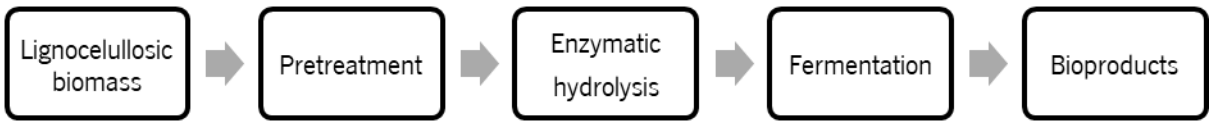


Figure 5: Principal steps in the conversion of lignocellulosic biomass into bioproducts.

1.2.2. Pretreatment

The recalcitrance of lignocellulosic biomass, primarily attributed to the extensive network of intramolecular and intermolecular hydrogen bonds, particularly between lignin and hemicellulose, presents a challenge to efficient biochemical conversion processes (Zoghlami and Paës, 2019). These interactions lead to the formation of lignin-carbohydrate complexes (LCCs), which generate a rigid and highly recalcitrant matrix that limits the effective separation and enzymatic hydrolysis of individual components (Brodeur et al., 2011).

Pretreatment is a critical process aimed at disrupting the structural rigidity of lignocellulosic material, thereby enhancing enzyme accessibility during subsequent enzymatic hydrolysis (Luo et al., 2021). An effective pretreatment should facilitate the delignification of biomass, modifying and/or removing hemicellulose, reducing cellulose crystallinity, and enhancing the surface area and porosity of the material, before an enzymatic hydrolysis (Zoghlami and Paës, 2019). Furthermore, it should minimize the formation of inhibitory compounds, lower production costs, and reduce overall energy consumption (Brodeur et al., 2011). Various pretreatment strategies have been applied and can generally be classified into four main categories: physical, chemical, physicochemical, and biological methods (Chen et al., 2017). Among them, hydrothermal pretreatment and dilute acid pretreatment are two widely studied approaches due to their effectiveness in breaking down the complex structure of lignocellulose.

Hydrothermal pretreatment (HP), a physicochemical method, uses water under pressure at elevated temperatures of 150 °C – 230 °C without the addition of external catalysts (Martín et al., 2022). This process is commonly performed using specialized equipment such as pressurized reactors and can operate at high solid loading conditions (Fan et al., 2024; Martín et al., 2022). During HP, the combined action of heat and pressure promotes the hydrolysis of hemicellulose, the solubilization of acetyl groups, and the redistribution or partial depolymerization of lignin, while preserving the cellulose fraction (Sun et al., 2022). The efficiency of HP is highly dependent on operational parameters, mainly temperature, residence time, and solid-to-liquid ratio (Ruiz et al., 2011). The harshness of pretreatment is often quantified using the severity factor, an empirical parameter that combines temperature and time into a single variable, enabling direct comparison of different pretreatment conditions (Chornet and Overend, 2017). In practice, these parameters are often optimized through experimental design approaches to enhance the solubilization of hemicellulose or cellulose retention. For instance, Dias et al. (2025) performed a 3^2 factorial experimental design to optimize the hydrothermal pretreatment of olive tree prunings, identifying 196 °C for 29 min as the optimal conditions for maximizing cellulose retention in the solid fraction. However, operating at higher severity levels characterized by high temperatures and prolonged reaction times can lead to the formation of degradation by-products such as furfural and 5-hydroxymethylfurfural (HMF) which can act as inhibitors in subsequent biological conversion steps (Martín et al., 2022). For example, hydrothermal pretreatment of eucalyptus biomass at 200 °C caused partial cellulose degradation and the formation of inhibitors compounds such as acetic acid, furfural, and HMF (Sun et al., 2014).

Hydrothermal dilute acid pretreatment (HDAP) uses dilute inorganic acids typically sulfuric acid (H_2SO_4), at concentrations between 0.2 % – 2.5 % (w/v), to intensify biomass deconstruction (Brodeur

et al., 2011). Under these acidic conditions, hemicellulose undergoes extensive chemical hydrolysis, resulting in increased release of fermentable sugars (Martín et al., 2022). Moreover, this approach enables effective operation at lower temperatures (100 °C – 200 °C) compared to HP (Carvalho et al., 2017). The main disadvantages of this pretreatment include the formation of inhibitory compounds from sugar degradation, such as furfural, HMF, and carboxylic acids (formic and levulinic acids) (Martín et al., 2022), and the need to neutralize the pretreated biomass before subsequent processing steps such as enzymatic hydrolysis or fermentation (Jönsson and Martín, 2016). In a study with beech wood, pretreatment with 0.5 % (w/v) sulphuric acid at 200 °C resulted in a higher glucose concentration but also led to increased formation of inhibitors such as furfural, HMF, and organic acids as the reaction time increased (Świątek et al., 2020). On the other hand, corn straw pretreated with 0.7 % (w/v) dilute sulfuric acid combined with 1.5 % (w/v) aluminum sulfate at 160 °C for 40 min removed 98 % of hemicellulose and improved enzymatic hydrolysis (Jiang et al., 2024).

1.2.3. Enzymatic hydrolysis

Following the pretreatment of lignocellulosic biomass to reduce recalcitrance, the next critical step is enzymatic hydrolysis. This process involves the breakdown of cellulose and any remaining hemicellulose into monomeric sugars through the catalytic action of specific enzymes (Martín et al., 2022).

Enzymatic hydrolysis is carried out using complex enzyme cocktails, typically composed of cellulose-active glycoside hydrolases and other enzymes derived from fungi or bacteria (Martín et al., 2022). Cellulose hydrolysis is facilitated by the synergistic action of three principal enzymes: endoglucanases, exoglucanases, and β-glucosidases (Van Dyk and Pletschke, 2012). Endoglucanases initiate the process by cleaving internal β-1,4-glycosidic bonds in long cellulose chains, producing shorter oligosaccharide fragments (Patel and Shah, 2021). Exoglucanases then act on the free chain ends, progressively releasing cellobiose units, which are subsequently hydrolyzed into glucose monomers by β-glucosidases (Van Dyk and Pletschke, 2012). In addition to cellulases, hemicellulases function as composite enzyme systems that act synergistically to cleave glycosidic linkages in hemicellulose, thereby deconstructing its complex, heterogeneous structure into oligosaccharides, disaccharides, and ultimately monosaccharides such as glucose, mannose, xylose, and arabinose (Tao et al., 2024).

Several factors strongly influence the efficiency of enzymatic hydrolysis. Among these, substrate characteristics, particularly cellulose crystallinity and the degree of polymerization, directly affect enzyme binding and activity (Sun and Cheng, 2002). Other important parameters include the solid porosity, lignin content, and residual hemicellulose content (Zoghlami and Paës, 2019).

Process conditions are also critical, with hydrolysis typically conducted under mild temperatures (45 °C – 55 °C) and pH values between 4.5 and 5.5 to maintain enzyme stability and activity (Amândio et al., 2023). Inhibitory compounds generated during pretreatment, such as soluble carbohydrates and aromatic substances, can significantly impair cellulase activity and reduce overall sugar yields (Jönsson and Martín, 2016). Another limitation of enzymatic hydrolysis is the low sugar concentration in the hydrolysate, which can compromise the process viability. To overcome this, hydrolysis is generally performed at a solid loading of at least 15 % (w/w) (Kristensen et al., 2009), which enhances sugar concentration while also reducing water use, energy consumption, and processing costs (Silva et al., 2020).

However, operating under high-solids conditions introduces technical challenges. Modenbach and Nokes (2013) observed that enzymatic hydrolysis of lignocellulosic biomass at high-solids loadings decreases glucan conversion efficiency due to factors such as high viscosity, mass transfer limitations, and enzyme inhibition. Similarly, Manzanares et al. (2011) and Cara et al. (2007) reported that solid loadings above 20 % led to significantly low glucose yields (approximately 50 %) in olive tree prunings. Studies on empty fruit bunches also demonstrated that increasing solid loading to 25 % – 30 % resulted in lower glucose yields, primarily attributed to inadequate mass transfer and mixing limitations (Kim et al., 2019).

Enzyme recycling has emerged as a promising strategy to reduce process costs. Cellulases, typically recovered from the liquid supernatant, can be recycled for multiple hydrolysis cycles. Ramos et al. (1993) reported that a commercial mixture of Celluclast and Novozym retained activity over five consecutive cycles, although hydrolysis efficiency gradually decreased with each reuse. Similarly, Weiss et al. (2013) demonstrated that significant cellulase activity can be recovered by recycling the insoluble biomass fraction, allowing a reduction in enzyme dosage by up to 30 % while maintaining comparable glucose yields.

1.2.4. Wood hydrolysates

Wood hydrolysates obtained after enzymatic hydrolysis of pretreated lignocellulosic biomass have emerged as substrates of significant relevance within the context of sustainable bioprocessing (Ünver et al., 2023). These hydrolysates are rich in fermentable sugars, offering a versatile feedstock for the efficient microbial production of valuable compounds, particularly by yeasts (Ezeorba et al., 2024) (**Table 2**). For instance, *Saccharomyces cerevisiae* has been widely applied to convert pine, spruce, and eucalyptus hydrolysates into bioethanol (Abdou-Alio et al., 2020; Amândio et al., 2023; Farías-Sánchez

et al., 2017; Mendieta et al., 2022), while oleaginous yeasts such as *Yarrowia lipolytica*, *Cyberlindnera jadinii*, *Wickerhamomyces anomalus*, *Rhodotorula toruloides* and *Blastobotrys adeninivorans* have been explored for lipids production under batch or fed-batch operations (Silva et al., 2025; Dias et al., 2024; Lapeña et al., 2020; Ünver et al., 2023).

Table 2: Examples of wood hydrolysates used as substrates for yeast-based processes to obtain bioproducts.

Hydrolysates	Yeast strain	Mode of operation	Bioproducts	References
Pine wood	*Yarrowia lipolytica* MUCL 28849	Batch	Lipids	Ünver et al. (2023)
	Saccharomyces cerevisiae IMR 1181	Batch	Bioethanol	Mendieta et al.(2022)
	Saccharomyces cerevisiae ITD-00185	Batch	Bioethanol	Farías-Sánchez et al. (2017)
Spruce wood	*Saccharomyces cerevisiae* ATCC 7754	Batch	Bioethanol	Abdou Alio et al. (2020)
	Blastobotrys adeninivorans LS3	Repeated Fed-batch	Biomass	Lapeña et al. (2020)
	Cyberlindnera jadinii LYCC 7549	Repeated Fed-batch	Biomass	Lapeña et al. (2020)
	Wickerhamomyces anomalus J21 (CBS100487)	Repeated Fed-batch	Biomass	Lapeña et al. (2020)
	Saccharomyces cerevisiae (wild type)	Fed-batch Batch	Lipids	Axelsson (2011)
Eucalyptus wood	*Rhodotorula toruloides* PYCC 5615	Batch	Lipids	Silva et al. (2025)
	Yarrowia lipolytica W29	Batch/ Two-stage repeated batch	Lipids	Dias et al. (2024)
	Saccharomyces cerevisiae PYCC 5246 (ATCC 24860)	Batch	Bioethanol	Amândio et al. (2021)

Lapeña et al. (2020) studied the cultivation of yeast species *Blastobotrys adeninivorans*, *Cyberlindnera jadinii*, *Wickerhamomyces anomalus* on enzymatically saccharified spruce hydrolysates obtained from sulfite-peptide pretreated biomass under repeated fed-batch conditions, focusing on the production of yeast biomass. Among the tested strains, *W. anomalus* exhibited the highest performance, achieving a biomass yield of 0.6 $g \cdot g^{-1}$.

In a study of Mendieta et al. (2022), *Saccharomyces cerevisiae* was used to convert pine sawdust pretreated with a soda–ethanol process through simultaneous enzymatic hydrolysis and fermentation, to produce bioethanol, achieving a maximum ethanol yield of 82 %.

Research conducted by Silva et al. (2025) *Rhodotorula toruloides* was cultivated on eucalyptus bark hydrolysate derived from steam explosion and enzymatic hydrolysis, achieving a lipid content of approximately 30 % (w/w), while *Yarrowia lipolytica* grown on a similar eucalyptus bark hydrolysate accumulated intracellular lipids up to 26 % (w/w) (Dias et al., 2024).

1.3. Glues

Glues, or adhesives, are materials designed to bond two or more surfaces together through mechanisms of adhesion and cohesion. Adhesion involves the attractive forces between different materials and determines the energy required to separate them (Desai et al., 2003). Cohesion refers to the intermolecular forces, such as hydrogen bonding, van der Waals interactions, and electrostatic forces that maintain the internal integrity of a material (Vamza et al., 2022).

The theoretical understanding of adhesion has undergone significant evolution. One of the earliest, the mechanical interlocking theory, introduced in 1925 by McBain and Hopkins, suggests that adhesion occurs when adhesives penetrate surface pores and cavities, with wettability playing a critical role (Packham, 2008). More recent models, including adsorption, diffusion, electrostatic, and thermodynamic theories, emphasize molecular-level interactions and the importance of surface energy, intermolecular compatibility, and polymer behavior (Ali et al., 2020).

Most commercially available glues are petroleum-based, derived from synthetic resins, polymers, and elastomers (Eisen et al., 2020). Nowadays, these adhesives are essential in various industries, including automotive, aerospace, medical, and construction, enabling the bonding of materials such as glass, metal, plastic, rubber, and wood (Ma et al., 2023; Tian et al., 2023). These materials offer several desirable properties, including high mechanical strength, excellent chemical and water resistance, thermal stability, and long-term durability (Ma et al., 2023).

Figure 6 illustrates the global adhesives market by type in 2022. Vinyl-based adhesives dominated the global market, accounting for 29 %, followed closely by acrylic adhesives at 23 %.

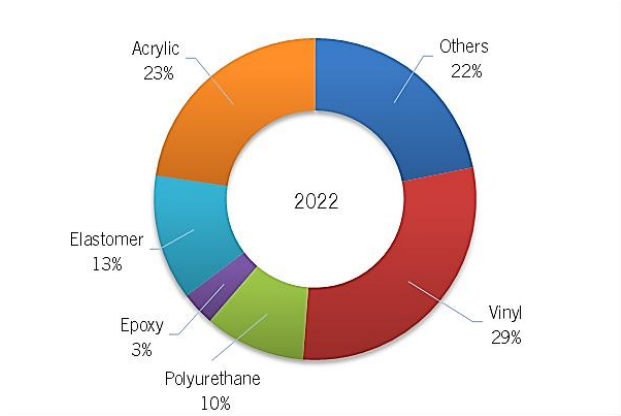


Figure 6: Global market of adhesives by type in 2022 (Ceresana, 2022).

The use of synthetic adhesives raises significant environmental and health concerns. Their production depends on non-renewable fossil resources, contributing to greenhouse gas emissions and long-term sustainability challenges (Ma et al., 2023). Moreover, many synthetic adhesives release volatile organic compounds (VOCs) during manufacturing and application, including hazardous substances such as formaldehyde, toluene, and isocyanates (David and Niculescu, 2021). These compounds are linked to respiratory irritation, neurotoxicity, and an increased risk of cancer (Salthammer et al., 2010). Considering these challenges, there is growing pressure on manufacturers to reformulate products in compliance with stricter safety and environmental standards. As a result, research is increasingly focused on developing alternative adhesives from renewable biological sources, commonly known as bio-based glues.

1.4. Bio-based glues

Increasing environmental awareness has fueled demand for eco-friendly adhesives with low or zero formaldehyde concentration. In response, bio-based glues, derived from renewable biomass, have gained significant attention in recent years as a promising and eco-friendly alternative to conventional petroleum-based adhesives. Their growing importance is driven by increasing demand for sustainable materials to meet the environmental and performance requirements of biomedical, pharmaceutical, and industrial applications (Duan et al., 2021; Raj et al., 2025; Muñoz et al., 2022).

In industrial applications, bio-based adhesives derived from natural polymers such as tannin, lignin, soy, and chitosan have been extensively developed for the wood and furniture industries (Gonçalves et al., 2021; Todorovic et al., 2024; Xi et al., 2022). In the biomedical field, protein and polysaccharide-based glues have shown promising potential. For instance, egg albumin-based hydrogels have been reported to support soft tissue regeneration and enhance wound healing (Wang et al., 2022). Similarly, Ferreira et al. (2017) developed a hybrid fibrin sealant combining buffalo-derived fibrinogen with a serine protease from snake venom that substitutes for thrombin, effectively mimicking the final step of coagulation and enabling tissue sealing and wound closure. Besides, polysaccharide-based hydrogels, such as those derived from starch, have also been engineered for controlled drug delivery, demonstrating the capability to encapsulate and release therapeutic agents effectively, showing potential for the pharmaceutical industry (Lee and Hwang, 2023) .

Bioglues offer several advantages, including abundance, biodegradability, low toxicity, and renewability, making them an attractive option for reducing the environmental impact of conventional synthetic glues, contributing to reduce the carbon footprint (Raydan et al., 2021). However, despite their advantages, many bioglues still face challenges related to performance, particularly poor water resistance and relatively low adhesion strength (Yu et al., 2024). To overcome these limitations, researchers have explored various strategies, including chemical and physical treatments to improve their performance (Dewantoro et al., 2024; Vallée et al., 2017).

1.4.1. Microbial-based bioglues

Microbial-based bioglues are an alternative to traditional plant-based bioglues. Microorganisms offer faster growth rates and can be produced under precisely controlled conditions in bioreactors (Palladino et al., 2024), making them more suitable for scalable production. Microbial production demands substantially less land and water compared to conventional agricultural sources, thereby enhancing their sustainability and overall resource efficiency (Fasolin et al., 2019). Finally, microbial systems are easily scalable and suitable for continuous industrial production, in contrast to plant sources, which have natural growth and harvesting limitations (Zhang et al., 2022). A comparative summary of these characteristics is presented in **Table 3**.

Table 3: Comparative overview of microbial and plant sources.

Parameter	Microbial source	Plant source
Growth rate	Fast	Slow/moderate
Production control	High	Limited
Land use	Minimal	Extensive
Industrial scalability	Excellent	Limited

Microorganisms naturally produce adhesive substances such as exopolysaccharides (EPS) and other biopolymers intracellularly, which play crucial roles in various biological processes and have significant industrial applications (Flemming and Wingender, 2010). Among these, EPS are the most extensively studied, high-molecular-weight polymers secreted under environmental stress (Mouro et al., 2024). They are primarily composed of polysaccharides, proteins, lipids, and nucleic acids (Costa et al., 2018), and are produced by bacteria, fungi, yeast, and microalgae (Osemwegie et al., 2020). Notable examples include homopolysaccharides, such as dextran and levan, and heteropolysaccharides, such as xanthan gum and alginate (Mouro et al., 2024).

In contrast to extracellular exopolysaccharides, intracellular biopolymers produced by microorganisms have been less studied. Nevertheless, they represent valuable resources for the development of bio-based glues, as their diverse chemical compositions can be harnessed and functionalized to impart strong and adjustable adhesive properties.

Proteins, for example, contain functional groups (e.g., amine, carboxyl, hydroxyl) which form hydrogen bonds and ionic interactions that stabilize folding and cohesion (Donald et al., 2011; Hubbard and Haider, 2010). Disulfide bonds between cysteine residues provide covalent crosslinks, imparting high mechanical strength and irreversible adhesion (Yammine et al., 2025; Yousefi and Abbasi, 2022). Polysaccharides, composed of long chains of monosaccharides connected by glycosidic bonds, form linear or branched structures (Dias et al., 2011). Their adhesive properties are attributed to two factors: their high density of polar functional groups, which promote strong adhesion to polar surfaces, and their high molecular weight, which supports cohesive secondary structures (e.g., helical, sheet, or spiral) (Lutz et al., 2022). Lipids contribute to hydrophobicity and barrier properties in adhesive formulations and are primarily composed of long-chain fatty acids, triglycerides, wax esters, and phospholipids (Mohamed et al., 2020). Although lipids are rarely used as the sole adhesive component due to their relatively weak cohesive strength, they are frequently blended with materials to enhance flexibility and water resistance (Debeaufort et al., 2000). Nucleic acids, primarily recognized as genetic materials, also

enhance adhesion by electrostatically interacting with other biopolymers, thereby stabilizing polymeric matrices (Meyer et al., 2024; Wang et al., 2022).

A key strategy for accessing these intracellular components is autolysis, a cost-effective process in which endogenous enzymes such as proteases, nucleases, and glucanases are activated to degrade the cell wall (Silva et al., 2025; Takalloo et al., 2020). Following autolysis, intracellular components can be harnessed and strategically modified to impart tailored adhesive properties through functionalization.

Functionalization, defined as the intentional modification of molecular structures to enhance specific properties, can be achieved via chemical and enzymatic treatments (Arcos Rosero et al., 2024). Chemical modifications, such as cross-linking, have been widely employed to improve cohesion, thermal stability, and water resistance (Luo et al., 2024). Cross-linking introduces covalent or strong intermolecular bonds between polymer chains, creating a three-dimensional network that improves cohesion, rigidity, and water resistance (Li et al., 2021). Several cross-linking agents have been employed for microbial proteins and polysaccharides. For example, glutaraldehyde reacts with amino groups in proteins to form Schiff bases, significantly increasing wet strength and durability (Yu et al., 2024). Similarly, glyoxal and other aldehydes interact with hydroxyl groups in polysaccharides, enhancing thermal and mechanical stability (Fathima et al., 2004; Monier et al., 2010). Epoxy-based reagents, such as ethylene glycol diglycidyl ether (EGDE), form covalent bonds through epoxide ring-opening with hydroxyl, carboxyl, or amino groups, generating dense polymer matrices with improved resistance to water and heat (Vasylieva et al., 2011). In parallel, enzymatic treatments offer an eco-friendly and more specific alternative to chemical cross-linkers, avoiding toxic residues. The enzyme transglutaminase has been widely studied for protein-based adhesives, where it catalyzes the formation of isopeptide bonds between glutamine and lysine residues (Singh and Kumar, 2019). This reaction produces a dense cross-linked protein network that significantly improves mechanical strength and water resistance, as demonstrated in yeast-derived adhesives (Kadimaliev et al., 2012). Additionally, hydrolytic enzymes such as amylases, cellulases, and proteases can be employed to partially depolymerize polysaccharides or proteins, exposing additional reactive groups that increase cross-linking efficiency in subsequent steps (Kumar et al., 2024).

Some studies have demonstrated that microbial adhesives, particularly those derived from yeasts, can be effectively functionalized to enhance their mechanical strength, water resistance, and thermal stability (**Table 4**). For example, yeast hydrolysates (≥ 55 % protein) cross-linked with EGDE (Bai et al., 2019), beer yeast waste treated with alkaline pretreatment followed by the addition of glutaric aldehyde and transglutaminase (Kadimaliev et al., 2012), and protein extracts from *Rhodotorula rubra* cross-

linked with glyoxal and resorcinol (Núñez-Decap et al., 2019), have all demonstrated enhanced adhesive performance in wood applications. Even unmodified protein extracts from yeasts such as *Rhodotorula rubra*, *Pichia pastoris*, and *Saccharomyces cerevisiae* have shown inherent adhesive properties (Núñez Decap et al., 2016).

Table 4: Summary of microbial adhesives, modification strategies, and their applications.

Source/Component	Type of Modification	Application	References
Yeast hydrolysate (≥ 55 % protein)	Crosslinking with EDGE	Wood adhesive	Bai et al. (2019)
Beer yeast waste	Alkaline pretreatment; crosslinking with glutaric aldehyde and transglutaminase	Wood adhesive	Kadimaliev et al., (2012)
Protein extract from yeasts *Rhodotorula rubra, Pichia pastoris, Sacharomyces cerevisiae*	No modification	Wood adhesive	Núñez-Decap et al., (2016)
Protein extract from yeast *Rhodotorula rubra*	Crosslinking with glyoxal and resorcinol	Wood adhesive	Núñez-Decap et al., (2019)

However, despite these promising results, the available literature on yeast-based glues remains limited, highlighting the opportunity for further research to explore their full potential and optimize their functionalization strategies.

2. OBJECTIVES

The main objective was to develop an integrated bioprocess from wood by-products to yeast biomass with potential for bioglue formulation. The proposed bioprocess involves the pretreatment and enzymatic hydrolysis of wood shavings, batch cultivation of yeast in wood shavings hydrolysate in Erlenmeyer flasks and in a bioreactor, lysis of yeast biomass, recovery of yeast lysates, and evaluation of their properties to develop adhesive formulations. The yeast *Yarrowia lipolytica* W29 was selected as the microbial platform owing to its ability to grow in lignocellulosic hydrolysates (Dias et al., 2024) and its unexplored potential for bioglues production.

Specific Objectives:

- Hydrolysis of low-cost by-products from wood processing (wood shavings) using two pretreatment methods: hydrothermal and hydrothermal dilute acid.
- Enzymatic hydrolysis of wood shavings and characterization of the resulting hydrolysates.
- Production of yeast biomass in wood shavings hydrolysate.
- Characterization of yeast biomass composition.
- Disruption of yeast cells by autolysis and characterization of the lysates.

3. MATERIALS AND METHODS

3.1. Yeast strain and maintenance

Yarrowia lipolytica W29 (ATCC 20460, isolated from soil) grew in YPD medium (20 g·L^{-1} glucose, 20 g·L^{-1} peptone, 10 g·L^{-1} yeast extract) for 2 days and was mixed with 20 % (v/v) of pure glycerol and stored at - 80 °C until the pre-inoculum preparation.

3.2. Wood shavings collection and characterization

Wood shavings (WS) of pine wood were collected at the carpentry workshop José Canossa, Unipessoal Lda in Arcos de Valdevez (Portugal). WS were stored at room temperature until utilization.

3.2.1. Quantitative acid hydrolysis

The carbohydrate composition of wood shavings, including cellulose, hemicellulose, and Klason lignin were assessed through a two-step quantitative acid hydrolysis in triplicate, based on NREL/TP-510-42618 protocol (Sluiter et al., 2008). In the first step, samples of WS (0.5 g) were mixed with 5 mL of 72 % (w/w) H_2SO_4 in test tubes. The tubes placed in a water bath at 30 °C for 1 h, with manual stirring every 10 min. After this period, distilled water was added to stop the reaction, and the content of the tubes was completely transferred into Schott bottles. In the second step, the acid concentration of the mixtures in the Schott bottles was adjusted to 4 % (w/w) H_2SO_4 with additional distilled water. The Schott bottles were sealed and placed in an autoclave at 121 °C for 1 h. Afterwards, the Schott bottles were cooled down, and the mass loss due to evaporation was quantified. The mixtures were filtered through a Gooch crucible (previously weighed and dried in the oven overnight at 105 °C). The Gooch crucibles containing the insoluble fraction were oven-dried at 105 °C for 24 h. After drying, the crucibles were cooled in a desiccator and weighed to determine the dry mass of the insoluble residue.

The concentration of glucose, xylose, arabinose, and acetic acid in the filtrate was quantified by high-performance liquid chromatography (HPLC) and the corresponding polymeric fractions (CP) – glucan (CGn), xylan (CXn), arabinan (CArn), and acetyl groups – were calculated in accordance with equation 1 (expressed as grams of polymeric fraction per 100 grams of dry substrate).

$$\mathrm{CP}\ (\%) = \mathrm{F} \times \mathrm{C_{est}} \times \frac{[\mathrm{S}]}{\rho} \times \frac{\mathrm{W+WHS \times H}}{\mathrm{WHS\ (1-H)}} \times 100 \qquad \textbf{(Eq. 1)}$$

Where F is a factor for sugar degradation (1.04 for CG_n, 1.088 for CX_n and CAr_n, and 1.00 for CG_A). C_{est} is a stoichiometric correction factor that accounts for the increase in molecular weight during hydrolysis, (162/180 for CG_n, 132/150 for CX_n/ CAr_n, 43/60 for CG_A). [S] is the monomer concentration ($g \cdot L^{-1}$) and ρ is the density of the analyzed dissolution (1024 $g \cdot L^{-1}$). W represents the weight of added water (g), corrected for losses during the autoclave. WHS is the sample weight (g), and H is the moisture content (expressed as grams of water per gram of wet sample).

Cellulose and hemicellulose content (%) were determined according to **equations 2** and **3**, respectively.

$$Cellulose\ (\%) = CG_n \quad \textbf{(Eq. 2)}$$

$$Hemicellulose\ (\%) = CX_n + CAr_n + CG_A \quad \textbf{(Eq. 3)}$$

The increase in weight of the Gooch crucible corresponds to the amount of Klason lignin in the sample. This content (%) was calculated according to **equation 4.**

$$Klason\ lignin\ (\%) = \frac{(W_{CDS} - W_C)}{W_{CHS} \times (1-H)} \times 100 \quad \textbf{(Eq. 4)}$$

Where W_{CDS} is the weight of Gooch crucible with the dry sample (g), W_C is the weight of the empty dry crucible (g) , W_{CHS} is the weight with the initial wet sample before hydrolysis (g) and H is the humidity content, expressed as grams of water per gram of wet sample. The content of ashes (%) was removed from lignin content.

3.3. Hydrothermal pretreatment (HP)

WS were subjected to a pretreatment in a 1.9-L stirred pressurized bench-top reactor (PARR 4520, Parr Instruments Company, Moline, Illinois, USA), equipped with a PID-controlled temperature system, agitation regulation, and a pressure gauge. A solid-to-liquid ratio of 1:5 (w/w) was maintained by mixing WS with water for a working volume of 500 mL, while agitation was consistently set at 120 rpm. Temperature (180 °C, 200 °C) and isothermal residence time (0 min, 15 min) were the variables tested to evaluate the severity of pretreatment. The severity factor [$\log R_0$] was used to quantify the harshness

of the pretreatment and was calculated for each condition using **equations 5** and **6**, which combine the influence of temperature and time.

Following the bioreactor cooling, the resulting slurry was filtered to separate the solid and liquid fractions. The solid fraction was washed with distilled water and stored at - 20 °C for enzymatic hydrolysis. For additional characterization, the solid fraction was dried at 60 °C before being subjected to quantitative acid hydrolysis.

$$[\log \mathrm{R}_0] = [R_0 Heating] + [R_0 Isothermal\ Process] + [R_0 Cooling] \quad \textbf{(Eq. 5)}$$

$$[\log \mathrm{R}_0] = \left[\int_0^{tmax} \frac{\mathrm{T}(t) - 100}{\omega}\, dt\right] + \left[\int_{ctrl}^{ctrf} exp\left[\frac{\mathrm{T}(t) - 100}{\omega}\right] dt\right] + \left[\int_0^{tmax} \frac{\mathrm{T}'(t) - 100}{\omega}\, dt\right] \quad \textbf{(Eq. 6)}$$

Where *tmax* (min) is the time required to reach the maximum pretreatment temperature, $ctrl$ and $ctrf$ (min) represent the start and end of the isothermal phase, respectively. T(t) and T′(t) (°C) correspond to the temperature profiles in the heating and cooling period , respectively, and ω is an empirical parameter related to the activation energy (with a value of 14.75 assuming pseudo-first-order kinetics). The severity factor [log R_0] was determined by integrating the area under the temperature-time curve.

3.4. Hydrothermal dilute acid pretreatment (HDAP)

A 3^3 factorial design was performed to evaluate the effect of time, solid loading and acid concentration on the hydrothermal dilute acid pretreatment of WS. The independent variables were time (A), solid loading (B) and acid concentration (C) (**Table 5**). The dependent variable chosen for the experimental design was the cellulose mass (in grams of the total dry weight). To ensure experimental robustness, the central point was assessed in triplicate, resulting in a total of 15 experimental runs. Statistical analysis, predictive modeling, and graphical representations were conducted using STATISTICA software (version 8.0). Differences with *p*-values < 0.05 were considered statistically significant.

WS were mixed with 100 mL of sulfuric acid solution prepared from concentrated sulfuric acid (95 %, w/w). The Schott bottles were placed in an autoclave operating at 121 °C. After the pretreatment, the material was cooled to room temperature. The resulting slurry was then filtered to separate the solid and liquid fractions. The solid fraction was thoroughly washed with distilled water to remove residual

acid and stored at - 20 °C until enzymatic hydrolysis. For additional characterization, part of the solid fraction was dried at 60 °C and later subjected to quantitative acid hydrolysis.

Table 5: Factors and levels used in the 3^3 factorial experimental design for the wood shavings in the hydrothermal dilute acid pretreatment process analysis. A: time (min); B: solid loading of WS (%, w/w) and C: acid concentration (%, w/w).

Variables levels	A	B	C
-1	30	10	1
0	45	15	2.5
1	60	20	5

3.5. Enzymatic hydrolysis

Enzymatic hydrolysis was conducted using pretreated solids obtained under selected pretreatment conditions: HP (200 °C, 0 min) and HDAP (45 min, 20 % solid loading, 5 % acid concentration).

Experiments were performed in 500 mL glass bottles with a working volume of 120 mL, using a solid loading of 10 % (w/w, dry basis). Hydrolysis was conducted in a shaking incubator at 50 °C and 180 rpm, in 50 mM citrate buffer (pH 4.8), for 72 h. All experiments were performed in triplicate.

The enzymatic hydrolysis was performed using the commercial enzyme cocktail Cellic CTec3, which contains cellulases, β-glucosidases, and hemicellulases, with an initial cellulase activity of 116 FPU per milliliter and an enzyme loading of 30 FPU per gram of dry solid. The wood shavings hydrolysates (WSH) obtained after enzymatic hydrolysis after multiple rounds of HP and HDAP were designated as WSH-HP and WSH-HDAP, respectively.

During the hydrolysis, samples were periodically withdrawn, centrifuged, and the supernatant was analyzed by HPLC. The enzymatic saccharification yield (%), defined as the percentage of cellulose converted into glucose was calculated using **equation 7**.

$$\text{Saccharification yield (\%)} = \frac{[\text{Glucose}]+1.053 \times [\text{Celobiose}]}{1.111 \times \text{cf} \times [\text{Biomass}]} \times 100 \qquad \textbf{(Eq. 7)}$$

Where [Glucose] is the concentration of glucose ($g \cdot L^{-1}$), [Cellobiose] is the cellobiose concentration ($g \cdot L^{-1}$), [Biomass] is the concentration of dry solid fraction at the beginning of enzymatic hydrolysis ($g \cdot L^{-1}$), cf corresponds to the cellulose fraction on a dry basis ($g \cdot g^{-1}$), 1.111 is the conversion factor from cellulose to equivalent glucose, and 1.053 is the conversion factor from cellobiose to equivalent glucose.

3.6. Erlenmeyer flask experiments

The ability of *Y. lipolytica* W29 to grow was evaluated in two different WSH media obtained after enzymatic hydrolysis: WSH-HP and WSH-HDAP. Each hydrolysate-based medium was tested separately in batch cultures in 250-mL Erlenmeyer flasks, and all experiments were performed in duplicate.

Yeast cells were pre-grown overnight in 50 mL of YPD medium, centrifuged, and resuspended to obtain an initial concentration of 0.5 $g \cdot L^{-1}$ in 55 mL of WSH-based medium supplemented with 5 $g \cdot L^{-1}$ of corn steep liquor and ammonium sulfate to obtain a C/N ratio of 10 (based on the total carbon and nitrogen content ($g \cdot L^{-1}$) of the hydrolysates. The experiments were conducted in an orbital incubator oven at 27 °C and 200 rpm for 96 h.

3.7. Bioreactor experiments

Y. lipolytica W29 batch experiments were carried out in a 2-L stirred tank bioreactor (BIOLAB, B. Braun, Germany) filled with 450 mL of WSH-HDAP (**Figure 7**). The culture medium was supplemented with 5 $g \cdot L^{-1}$ of corn steep liquor and ammonium sulfate to obtain a C/N ratio of 10 (based on the total carbon and nitrogen content ($g \cdot L^{-1}$) of the hydrolysate). The bioreactor with the medium was sterilized in an autoclave at 115 °C for 30 min before the inoculation. The bioreactor was inoculated with yeast cells cultivated for 16 h in 50 mL of YPD medium.

The medium pH was measured with a probe (405-DPAS-SC-K8S/325, Metler Toledo, Urdorf, Switzerland) and kept constant at 5.5 ± 0.5 by the automatic addition of HCl 2 M or NaOH 2 M, using peristaltic pumps. An optical oxygen probe (InPro6860i, Metler Toledo, USA and the respective meter (Solaris Biotechnology SRL, Italy) were used to measure the dissolved oxygen concentration in the medium. The experiment was conducted at 27 °C for 72 h at 300 rpm of agitation rate and 1 vvm of aeration rate.

At the end of the bioprocess, the culture was centrifuged at 8000 rpm for 10 min, and the biomass was collected and used for the autolysis process.

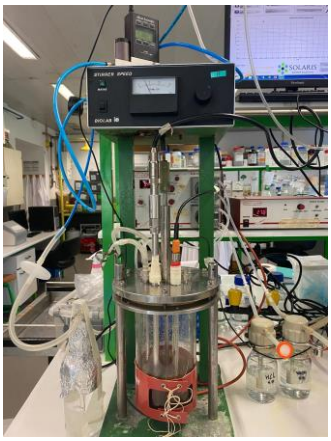


Figure 7: 2-L stirred tank bioreactor (BIOLAB, B. Braun, Germany) with WSH-HDAP medium.

3.8. Autolysis

Autolysis experiments were performed in Schott bottles with two different concentrations of yeast biomass (10 % and 20 % w/v, dry basis), dissolved in a solution containing 0.1 mM EDTA, 0.1 mM PMSF, and 1 % (w/v) boric acid.

Both cellular suspensions were incubated in a shaking incubator at 150 rpm and 55 °C for 96 h. During incubation, samples were periodically withdrawn to monitor cell viability and optical density (OD). At the end of the process (**Figure 8**), the suspensions were centrifuged at 8000 rpm for 10 min, and the supernatant (lysate) was lyophilized.

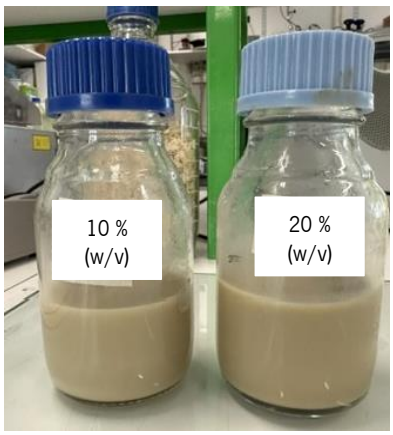


Figure 8: Autolysis assays performed with 10 % (w/v) and 20 % (w/v) cell suspension.

3.9. Analytical methods

3.9.1. Humidity

The moisture content of samples was measured by a humidity analyzer (ADAM PMB 53, United Kingdom).

3.9.2. Ash

A known quantity of sample (≈ 0.5 g) with known humidity was added to a porcelain crucible (previously placed in an oven at 105 °C for 24 h). The crucible was placed in the muffle furnace at 550 °C for 2 h. The crucibles were cooled in a desiccator with silica gel and then weighed. This procedure was executed in triplicate. The ash percentage (grams of ash per 100 grams of dry solid) was determined according to **equation 8**.

$$\text{Ash}\ (\%) = \frac{\text{WCA}-\text{WC}}{(\text{WCHS}-\text{WC})\times(1-\text{H})} \times 100 \qquad \textbf{(Eq. 8)}$$

Where WCA is the weight of the ceramic crucible with ashes (g), WC is the weight of the empty ceramic crucible (g), WCHS is the weight of the ceramic crucible containing the wet sample (g), and H is the humidity, expressed in grams of water per grams of sample.

3.9.3. Sugars concentration

The concentration of different sugars was determined by HPLC (LC 2060C, Shimadzu, Japan) with an Aminex HPX-87H column (300 mm × 7.8 mm, 8 μm particle size). The column temperature was maintained at 60 °C and was eluted with sulfuric acid 0.005 M at a flow rate of 0.5 mL·min^{-1}. Detection was performed using refractive index (RI) and UV (λ = 210 nm) detectors.

3.9.4. Biomass concentration

To evaluate yeast growth, biomass concentration was quantified by cell counting in a Neubauer counting chamber (Hirschmann, Eberstadt, Germany) using a binocular bright-field microscope (Leica DM 750, Wetzlar, Germany). In the case of autolysis, biomass concentration was determined

spectrophotometrically by measuring optical density at 600 nm. The absorbance and cell number were converted into cell dry weight ($g \cdot L^{-1}$) by a calibration curve: Abs (600 nm) = (0.56 ± 0.04) × Cell dry weight ($g \cdot L^{-1}$) + (- 0.0002 ± 0.0319) (confidence level 95 %) and a conversion factor: 3.69×10^{10} cells × g .

3.9.5. Total carbohydrates

The carbohydrates of yeast biomass were measured using the method described in Araújo et al. (2024). Briefly, 0.20 g of dry yeast biomass was incubated with 10 mL of 1.5 M H_2SO_4 at 100 °C for 20 min. After cooling at room temperature and neutralization with 12 mL of 10% (w/v) NaOH, the mixture was filtered and diluted to 100 mL with distilled water. Total carbohydrate content was estimated based on the concentration of reducing sugars from hydrolysis, quantified using the DNS method. The content of carbohydrates (%) was calculated using **equation 9**.

$$\text{Carbohydrates}\ (\%) = \frac{\text{C}}{\text{m}} \times 10 \qquad \textbf{(Eq. 9)}$$

Where c is the glucose concentration ($g \cdot L^{-1}$) and m is the mass of the sample (g).

3.9.6. Reducing sugars

Soluble reducing sugars were quantified using the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). For each assay, 0.1 mL of DNS reagent was mixed with 0.1 mL of the sample in test tubes, prepared in duplicate. For the blank, 0.1 mL of distilled water was used instead of the sample. Then, the test tubes were incubated in a bath at 100 °C for 5 minutes. After cooling at room temperature, 1 mL of distilled water was added to stop the reaction. The absorbance was measured at 540 nm. A calibration curve was prepared using glucose standard solutions ranging from 0 $g \cdot L^{-1}$ to 4 $g \cdot L^{-1}$. The absorbance was converted into glucose concentration using the calibration curve: Abs (540 nm) = (0.47 ± 0.05) × [glucose] ($g \cdot L^{-1}$) + (- 0.02 ± 0.05).

3.9.7. Total lipids

Lipids were extracted from lyophilized yeast biomass (10 mg) with a mixture (2 mL) of methanol and chloroform (1:1, v/v) and quantified by the phospho-vanillin colorimetric method (Lopes et al., 2018).

The extraction mixture was vortexed for 3 min to promote cell disruption, and a 250 µL aliquot was transferred to a test tube and heated at 100 °C until complete solvent evaporation. Then, 100 µL of pure sulfuric acid was added, vortex-mixed, and incubated at 100 °C for 15 min. After cooling at room temperature, 2.4 mL of phospho-vanillin reagent (1.2 $g \cdot L^{-1}$ vanillin dissolved in 85 % orthophosphoric acid) was added. The tubes were vortex-mixed, and absorbance at 490 nm was measured after 15 min. Lipid concentration ($g \cdot L^{-1}$) was calculated from a calibration curve prepared with olive oil solutions (0 $g \cdot L^{-1}$ – 2 $g \cdot L^{-1}$) dissolved in acetone. The absorbance was converted into lipid concentration using the calibration curve: Abs (490 nm) = (0.001 ± 5.9 × 10^{-5}) x [olive oil] ($mg \cdot L^{-1}$) + (-0.04 ± 0.05).

3.9.8. Total protein

The total protein content of yeast biomass and lysates obtained after autolysis was calculated by multiplying the total nitrogen content, measured by an elemental analyzer (FlashSmart 2000, Thermo Scientific, Massachusetts, USA), by a conversion factor of 6.25.

3.9.9. Colony-forming unit (CFU)

At the end of the autolysis process, cell viability was assessed by determining colony-forming unit (CFU). For this purpose, serial dilutions ranging from 10^{0} to 10^{-7} were prepared to obtain CFU counts between 30 and 300 in each dilution. An aliquot of 100 µL from each dilution was plated onto YPDA medium (20 $g \cdot L^{-1}$ peptone, $g \cdot L^{-1}$ glucose, 20 $g \cdot L^{-1}$ agar, and 10 $g \cdot L^{-1}$ yeast extract). Plates were incubated at 30 °C for 24 h – 48 h. The number of colonies per milliliter was calculated using **equation 10**.

$$\text{nº}\,\frac{\text{CFU}}{\text{mL}} = \frac{\text{nº of colonies per plate} \times \frac{1}{\text{DF}}}{\text{Volume added to the plate}} \qquad \textbf{(Eq. 10)}$$

Where DF is the dilution factor.

3.9.10. Total nitrogen (TN)

Total nitrogen (TN) in wood shaving hydrolysates was measured by a test kit (LCK 338 (Lange, Hach, Germany)).

3.9.11. Total organic carbon (TOC)

Total organic carbon (TOC) in wood shaving hydrolysates was measured by a test kit (LCK 387 (Lange, Hach, Germany)).

4. Results and Discussion

4.1 Characterization of wood shavings

The chemical composition of WS used in this study is presented in **Table 6**. The lignocellulosic material is primarily composed of structural polysaccharides, including cellulose and hemicellulose, along with a significant fraction of lignin. Literature values for pine wood by-products typically range between 38 % – 46 % cellulose, 22 % – 27 % hemicellulose, 24 % – 32 % lignin, and less than 1 % ash (Budiyantoro and Yudhanto, 2024; Nitsos et al., 2016; Normark et al., 2014; Rusanen et al., 2019) .

Table 6: Composition (dry basis) of wood shavings (WS). Data are the average ± standard deviation of three independent analyses.

Parameter (%, w/w)	**WS**
Ash	0.08 ± 0.03
Cellulose	49.0 ± 1.4
Hemicellulose	26.5 ± 0.5
Lignin	22.1 ± 1.7

In this study, the values obtained were generally consistent with those reported in the literature. However, the cellulose content of WS was slightly higher than the typical range. This difference may be attributed to natural variability associated with wood origin, age, and growth conditions (Han et al., 2022; Lachowicz et al., 2019). This composition indicates that WS is a suitable lignocellulosic feedstock for subsequent enzymatic hydrolysis to obtain a sugar-rich hydrolysate. The relatively high cellulose content provides a substantial source of fermentable sugars, enhancing the potential of WS as a raw material for yeast biomass production (Dias et al., 2024).

4.2. Hydrothermal pretreatment (HP)

In hydrothermal pretreatment (HP), water functions as both solvent and catalyst, while organic acids that may be released from the biomass enhance the disruption of the lignocellulosic matrix (Li et al., 2017). In this study, HP of WS was performed at two temperatures (180 °C and 200 °C) and two residence times (0 min and 15 min). The resulting solid fraction was analyzed to assess the effects of these treatment conditions on the content of cellulose, hemicellulose, and lignin, aiming to maximize

cellulose content for subsequent enzymatic hydrolysis. The mass of cellulose, calculated from the recovered dry solid mass and its cellulose fraction, was used as the primary indicator of pretreatment efficiency (**Table 7**).

Table 7: Composition (dry basis) of solid fraction obtained after hydrothermal pretreatment of wood shavings. Data are the average ± standard deviation of three independent analyses.

Temperature (°C)	180		200	
Residence time (min)	0	15	0	15
Parameter				
$[\log R_0]$	3.11	3.66	3.85	4.42
Heating rate (°C/min)	7.52	6.00	6.48	7.25
Solid composition				
Total solid (g)	100.9 ± 10.2	90.4 ± 5.4	96.6 ± 11.3	66.6 ± 4.2
Cellulose (%, w/w)	55.4 ± 5.1	68.9 ± 1.6	78.5 ± 2.0	80.1 ± 1.7
Cellulose mass (g)	11.7 ± 1.6	12.9 ± 0.8	15.2 ± 1.8	11.0 ± 0.7
Hemicellulose (%, w/w)	20.5 ± 0.8	12.4 ± 0.2	7.7 ± 0.1	5.5 ± 0.1
Lignin (%, w/w)	20.2 ± 0.9	24.3 ± 1.1	25.3 ± 0.7	27.1 ± 0.1

The severity factor, defined as a function of temperature and residence time (Ruiz et al., 2021), increased with higher temperatures and longer treatment times. As shown in **Table 7**, the severity factor ranged from 3.11 (180 °C, 0 min) to 4.42 (200 °C, 15 min). Increasing residence time from 0 min to 15 min raised the severity factor by approximately 0.55 – 0.56, while increasing temperature from 180 °C to 200 °C led to a greater increment of 0.77. These results indicate that temperature has a stronger influence on the severity factor than residence time, consistent with the previous report for pine wood (Nitsos et al. 2016) and other lignocellulosic biomasses (Batista et al., 2019; Dias et al., 2025).

The total solid recovery of WS decreased gradually with increasing pretreatment temperature, indicating the solubilization of hemicellulose and partial degradation of other biomass components. Specifically, a 66.6 % loss of solid mass was observed between 180 °C/0 min and 200 °C/15 min (**Table 7**). This reduction is primarily attributed to the removal of hemicellulose, resulting in the release of oligosaccharides, monosaccharides, and acetic acid into the liquid fraction (Ma et al., 2025). Similar behavior was reported by Zhou et al. (2010) for corn stover, where high temperatures led to low solid

recovery. On the other hand, hemicellulose was highly sensitive to HP, decreasing from 20.5 % at 180 °C/0 min to 5.5 % at 200 °C/15 min (**Table 7**). Nitsos et al., (2016) reported that HP of pine wood resulted in the removal of approximately 59 % – 85 % of the hemicellulose fraction under similar conditions. This reduction is mainly attributed to autohydrolysis reactions, where at elevated temperatures, water autoionization generates hydronium ions (H_3O^+) that catalyze hemicellulose hydrolysis and deacetylation. The released acetic acid amplifies this effect, generating additional H_3O^+ and promoting further depolymerization of hemicelluloses into low-molecular-weight oligomers (Sun et al., 2014). This removal benefits subsequent hydrolysis by increasing the cellulose content and improving the accessibility of cellulose to enzymes (Martín et al., 2022). Cellulose content increased with pretreatment severity from 55.4 % at 180 °C/0 min to 80.1 % at 200 °C/15 min. However, when comparing the condition 200 °C/0 min with 200 °C/15 min, only a slight increase in cellulose content was observed. The highest cellulose mass recovery (15.2 g) was achieved at 200 °C/0 min, while more severe conditions (200 °C/15 min) reduced the mass to 11.0 g, indicating that harsh conditions can lead to cellulose degradation. Similar effects were observed by Dias et al. (2025) and Sun et al. (2014), who reported cellulose loss in olive tree prunings and eucalyptus wood, respectively, when temperature reached 200 °C. Therefore, while higher severity pretreatment can improve enzyme accessibility through hemicellulose removal and cellulose concentration, it may also accelerate cellulose decomposition in certain biomasses, leading to reduced overall cellulose mass available for downstream enzymatic hydrolysis. Lignin content also showed a gradual increase with pretreatment severity. This behavior, with changes in polysaccharide composition, is typical of hydrothermal pretreatment, where selective hemicellulose removal leads to relative enrichment of both cellulose and lignin in the solid fraction (Margellou et al., 2023).

Overall, the hydrothermal pretreatment effectively fractionated WS by selectively removing hemicellulose while preserving most of the cellulose and concentrating lignin. Among the conditions tested, the pretreatment of WS at 200 °C for 0 min was selected because it yielded the highest cellulose mass (15.2 g), making this condition the most favorable for maximizing cellulose recovery and subsequent enzymatic hydrolysis efficiency.

4.3. Hydrothermal dilute acid pretreatment (HDAP)

Hydrothermal dilute acid pretreatment (HDAP), which involves the addition of dilute sulfuric acid to the reaction medium to intensify the deconstruction of the lignocellulosic structure (Brodeur et al., 2011) was also evaluated in this work.

In the HDAP of WS was investigated the effect of time (A), solid loading (B), and acid concentration (C), to maximize the mass of cellulose for subsequent enzymatic hydrolysis. To assess the effects of these factors, a 3^3 Box–Behnken factorial design based on response surface methodology (RSM) was applied. RSM is a statistical approach widely used for experimental design and process optimization, enabling the modeling of independent variable effects and the identification of optimal conditions for desired responses, thereby improving process efficiency and reducing the number of experimental trials required (Verma and Kumar, 2019). **Table 8** presents the experimental data of 15 experimental assays of the Box–Behnken matrix and the respective result of the mass of cellulose obtained. These results were used to identify the optimum pretreatment conditions with the specific goal of maximizing cellulose mass obtained. The adequacy of the fitted model was subsequently evaluated through ANOVA (**Table 9**).

Table 8: Experimental matrix showing the levels of independent variables A (time), B (solid loading), and C (acid concentration), and response (mass of cellulose, g) obtained after pretreatment of wood shavings.

Run	A	B	C	Cellulose (g)
1	0	1	-1	43.55
2	1	-1	0	22.76
3	0	1	1	52.36
4	0	-1	-1	24.04
5	-1	0	1	35.32
6	-1	1	0	42.29
7	-1	0	-1	31.93
8	1	0	1	34.13
9	0	-1	1	27.08
10	1	1	0	46.11
11	1	0	-1	37.10
12	-1	-1	0	21.37
13	0	0	0	38.21
14	0	0	0	36.18
15	0	0	0	35.64

In this study, the highest cellulose mass was obtained in run 3 (52.36 g), and notably, all runs with high cellulose recoveries (runs 1, 3, 6, and 10) shared a common solid loading of 20 %. In contrast, the lowest cellulose masses (runs 2, 4, 9, and 12) were obtained under the lowest solid loading condition of 10 %. These results indicate that a high solid loading favor the retention of cellulose in the solid fraction during HDAP. This can be explained by the lower liquid-to-solid ratio, which reduces cellulose exposure to the acid, limiting its solubilization.

Table 9: Analysis of variance (ANOVA) of the factors affecting cellulose mass in the solid fraction of pretreated wood shavings.

Source	Sum of squares	Degree of freedom	F value	*p*-value
A	10.56	1	2.61	0.1671
B	991.46	1	245.10	0.00001*
C	18.82	1	4.65	0.0835
AA	29.80	1	7.37	0.0421*
AB	1.48	1	0.36	0.5721
AC	10.11	1	2.50	0.1747
BB	1.83	1	0.45	0.5314
BC	8.32	1	2.06	0.2109
CC	2.27	1	0.56	0.4875
Error	20.23	5		
Total sum of squares	1095.66	14		
R^2	0.982			
Adjusted R^2	0.948			

* Statistically significant.

The ANOVA results (**Table 9**) confirmed that the proposed quadratic model was highly significant, with a coefficient of determination (R^2) of 98.2 % and an adjusted R^2 of 94.8 %, indicating an excellent fit to the experimental data. The fitted quadratic regression model (**Equation 11**) quantified the magnitude and the direction of the effects of each factor.

$$\text{Cellulose (g)} = 36.68 + 2.3A + 22.3B + 3.1C - 5.9A^2 + 1.2AB - 3.2AC - 1.4B^2 + 2.9BC + 1.6C^2 \quad \textbf{(Eq. 11)}$$

Figure 9 presents a Pareto chart derived from the Box–Behnken design, highlighting the relative contribution of main, quadratic, and interaction effects, as well as their significance level ($p < 0.05$). This analysis allows the identification of the most influential variables, thereby guiding process optimization by prioritizing the key factors that maximize cellulose mass (Pandey et al., 2024).

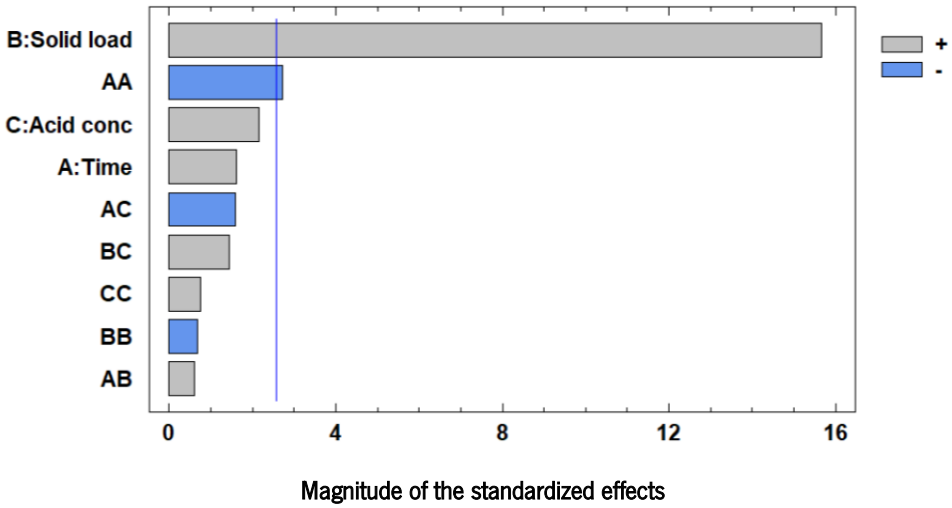


Figure 9: Pareto chart to evaluate the effects of factors A, B, and C on the mass of cellulose (g) after hydrothermal dilute acid pretreatment of wood shavings. Grey bars indicate positive effects; blue bars indicate negative effects. The vertical blue line represents the statistical significance threshold; bars that extend beyond this line have $p < 0.05$.

In mathematical model-fitting (**Eq.12**) , the regression coefficients indicated that solid loading had the most significant positive effect on cellulose mass ($p < 0.0001$), whereas the quadratic term of time was also significant ($p = 0.0421$) at the 95 % confidence level . In agreement, the Pareto chart (**Figure 9**) highlighted these two factors as the most influential in the Box–Behnken design. Solid loading was the most influential factor in the hydrothermal acid pretreatment of wood shavings, having a positive effect, indicating that increasing solid loading enhances the mass of cellulose . Conversely, the quadratic term of time had a negative effect, indicating that extreme reaction times negatively affect cellulose recovery. The effect of acid concentration was minor ($p = 0.0835$), approaching the 90 % confidence level, whereas all other main and interaction terms were less influential and not statistically significant at the 95 % confidence level. Overall, these results highlight solid loading and the squared term of reaction time as the key parameters for optimizing cellulose mass under HDAP conditions.

A two-dimensional response surface plot was constructed to visualize the optimal zone and the interactive effects of solid loading and acid concentration, which were found to be statistically significant on the cellulose mass of pretreated wood shavings (**Figure 10**).

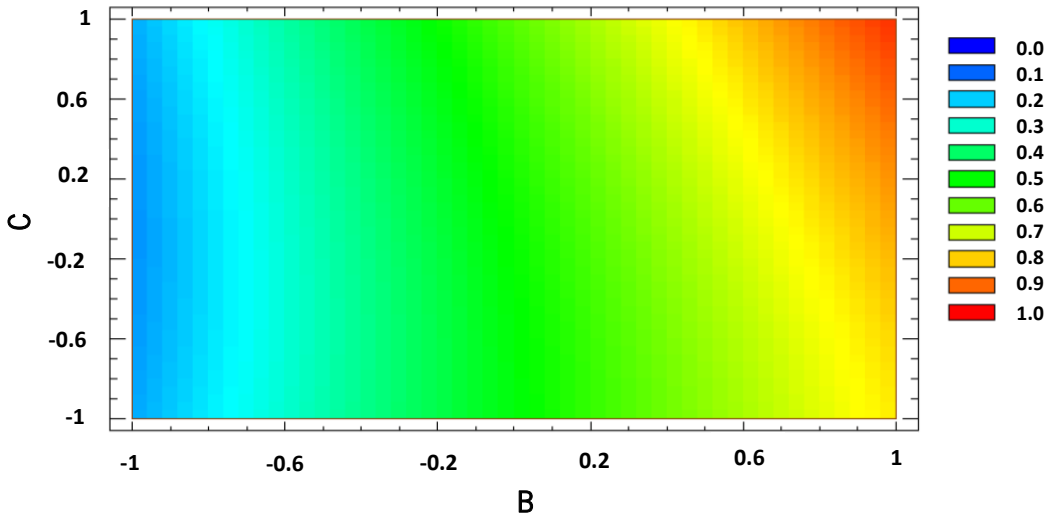


Figure 10: Response surface plot relating the effect of solid loading (**B**) and acid concentration (**C**) after hydrothermal dilute acid pretreatment of wood shavings.

The color gradient in the plot provides a clear visualization of the trend in cellulose mass, highlighting how it increases with solid loading (B) and acid concentration (C). The blue region corresponds to the lowest mass of cellulose, obtained at low solid loading and low acid concentration. As both parameters increase, the plot transitions through green and yellow regions, indicating intermediate cellulose levels, and ultimately reaches the red zone, which represents maximum cellulose mass at high solid loading combined with high acid concentration. This behavior reflects the dual influence of pretreatment conditions: increasing solid loading reduces cellulose solubilization by limiting the effective acid-to-biomass ratio, whereas high acid concentration breaks the glycosidic bonds in hemicellulose, promotes its solubilization, and partially disrupts the lignin structure, resulting in concentration of the cellulose fraction (Martín et al., 2022) .

The optimal conditions predicted to maximize the mass of cellulose in the solid fraction of WS were set at 20 % solid loading, 5 % acid concentration, and 46 min of residence time, yielding 50.9 g ± 4.4 g of cellulose. These conditions were nearly identical to those used in experimental run 3 (**Table 8**); therefore, for practical reasons, 20 % solid loading, 5 % acid concentration, and 45 min residence time were selected as the pretreatment conditions for WS, which were subsequently subjected to enzymatic hydrolysis.

Under these conditions, compositional analysis of solid fraction was also performed (**Table 10**). Hemicellulose removal reached approximately 80 % demonstrating that HDAP was highly effective in hemicellulose solubilization. In contrast, the cellulose and lignin content increased, reflecting their relative concentration due to the removal of hemicellulose. Comparable studies on hardwood sawdust and agricultural residues have reported hemicellulose reductions in the range of 80 % – 90 % under similar dilute-acid hydrothermal conditions (Gautam et al., 2025). Nevertheless, the relatively high residual lignin fraction could be a limiting factor for enzymatic saccharification, as lignin can act as a physical barrier and limit the access of enzymes (Siqueira et al., 2017).

Table 10: Composition (dry basis) of the solid fraction obtained at optimum parameter conditions (20 % solid loading, 5 % acid concentration, and 45 min residence time) in the hydrothermal dilute acid pretreatment of wood shavings.

Parameter	Solid fraction after HDAP
Cellulose mass (g)	50.9 ± 4.4
Cellulose (%, w/w)	62.9 ± 1.2
Hemicellulose (%, w/w)	5.3 ± 0.2
Lignin (%, w/w)	30.8 ± 0.6

4.4. Enzymatic hydrolysis and WSH characterization

Enzymatic hydrolysis involves the use of enzymes as catalysts to break down cellulose and remaining hemicellulose into monomeric sugars such as glucose and xylose, which can subsequently be metabolized by microorganisms during fermentation (Diamantopoulou et al., 2025). Several factors influence the enzymatic hydrolysis of cellulose in lignocellulosic biomass, including cellulose crystallinity, degree of polymerization, porosity, lignin content and hemicellulose content (Zoghlami and Paës, 2019).

In this study, the solid fractions of selected conditions of HP (200 °C, 0 min) and HDAP (45 min, 20 % solid loading, 5 % acid concentration) were subjected to enzymatic hydrolysis using the Cellic CTec3 cocktail. **Figure 11** represents the profile of sugars (glucose and xylose) and saccharification yield of pretreated solids obtained from HP (**Fig.11A**) and HDAP (**Fig.11B**).

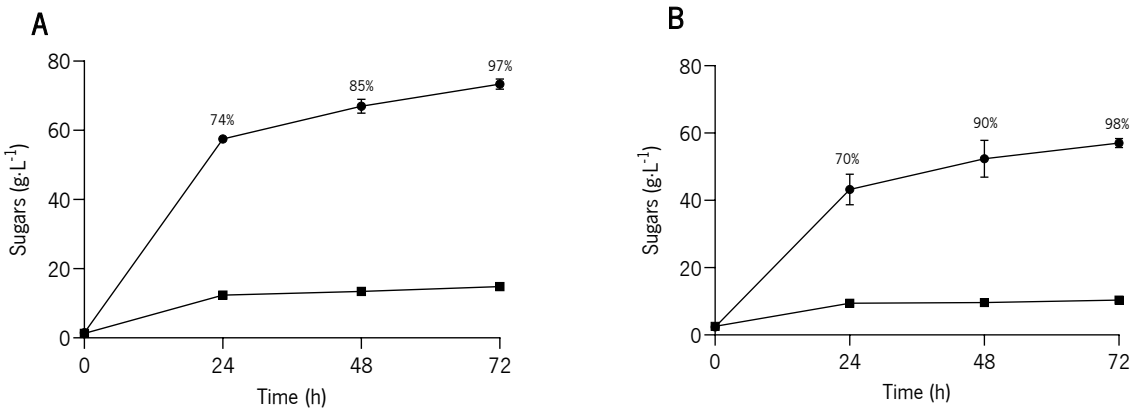


Figure 11: Profile of glucose (●) and xylose (■) concentration obtained in enzymatic hydrolysis of pretreated wood shavings carried out with two different pretreatments: hydrothermal pretreatment (**A**) and hydrothermal dilute acid pretreatment (**B**). The saccharification yield (cellulose to glucose conversion, %) is indicated above each glucose data point. The error bars represent the standard deviation of three independent replicates.

In general, the enzymatic hydrolysis revealed similar patterns of sugar release in pretreated solids of both pretreatment methods. In both cases, glucose concentration gradually increased throughout the 72 h of incubation, indicating that hemicellulose removal enhanced surface accessibility and exposed additional cellulose chain ends for efficient cellulase action. Notably, the hydrolysis of the HP fraction released substantially higher glucose concentration (73 $g \cdot L^{-1}$) than HDAP (57 $g \cdot L^{-1}$) although both achieved high saccharification yields, approaching almost 100 % after 72 h (**Figure 11**). These results indicate an almost complete conversion of cellulose in the pretreated solids to fermentable glucose. Variations in final glucose concentration are likely due to differences in hemicellulose and lignin content arising from the pretreatment methods applied. This observation is consistent with previous reports showing that partial removal of hemicellulose and lignin enhances enzymatic hydrolysis efficiency in various lignocellulosic feedstocks, including corn straw, sugarcane bagasse, and poplar wood (Hongdan et al., 2013; Jiang et al., 2024; Moniz et al., 2013; Shi et al., 2020). Other studies reported high saccharification yields under optimized enzymatic hydrolysis conditions. For example, Dias et al. (2025) achieved glucose yields of approximately 80 % during enzymatic hydrolysis of hydrothermal pretreated olive tree prunings (200 °C, 15 min) using 15 FPU/g of solid and 10 % solid loading, yielding a glucose concentration of 40 $g \cdot L^{-1}$. Similarly, dilute acid pretreatment of brewer's spent grains with 1.25 % sulfuric acid (120 °C,17 min) led to a 75 % glucose yield after enzymatic hydrolysis with 45 FPU/g enzyme loading (Mussatto et al., 2008).

Regardless of pretreatment conditions, xylose concentration showed little variation after 24 hours of hydrolysis. The prior solubilization of hemicellulosic sugars during pretreatment, including xylose and

xylo-oligosaccharides (Moniz et al., 2013), largely eliminated the substrate for xylanases, thereby restricting further release of xylose. On the other hand, cellobiose produced during cellulose hydrolysis is possibly rapidly converted into glucose by β-glucosidase (Brown et al., 2024), leading to a low concentration of cellobiose in both hydrolysates (< 1 $g \cdot L^{-1}$). Overall, the pretreatments effectively reduced pine wood biomass recalcitrance by decreasing cellulose crystallinity while increasing surface area and porosity, thereby facilitating efficient enzymatic conversion (Chen et al., 2024). Both hydrolysates obtained showed no detectable inhibitory compounds such as HMF, furfural, or formic acid, while cellobiose and acetic acid concentrations remained below 1 $g \cdot L^{-1}$. The resulting hydrolysates were characterized mainly by the presence of glucose and xylose as fermentable sugars. Specifically, the hydrolysate derived from hydrothermal pretreatment (WSH-HP) contained 73 $g \cdot L^{-1}$ of glucose and 15 $g \cdot L^{-1}$ of xylose. The hydrolysate obtained from hydrothermal dilute acid pretreatment (WSH-HDAP) was composed of 57 $g \cdot L^{-1}$ of glucose and 10 $g \cdot L^{-1}$ of xylose. Both hydrolysates were used as substrates for *Y. lipolytica* W29 biomass production in Erlenmeyer flask experiments.

4.5. Biomass production in Erlenmeyer flasks

Y. lipolytica exhibits a remarkable capacity to grow on a wide variety of carbon sources, including hydrolysates derived from lignocellulosic biomass such as olive tree prunings , sugarcane bagasse, wheat straw, corn stover, and eucalyptus bark (Caporusso et al., 2023; Dias et al., 2025; Dias et al.,2023; Tsigie et al., 2011; Zhong et al., 2025). However, to the best of our knowledge, only pine sawdust hydrolysate (Ünver et al., 2023) was tested as substrate for *Y. lipolytica* biomass.

In this study, Erlenmeyer flask experiments were performed to evaluate the growth of *Y. lipolytica* W29 in two WSH obtained after enzymatic hydrolysis of solid fractions resulting from distinct pretreatments: WSH-HP and WSH-HDAP (**Figure 12**).

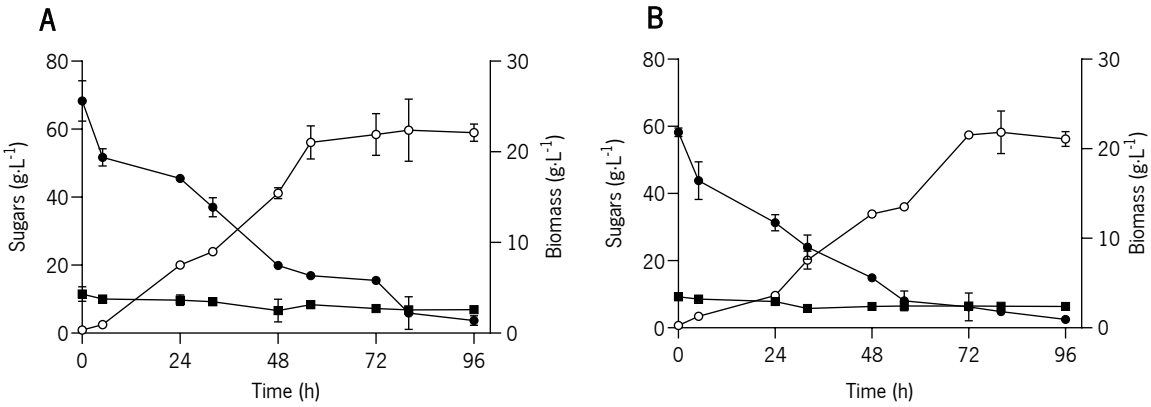


Figure 12: Profile of glucose (●), xylose (■) and biomass (○) concentration obtained in *Y. lipolytica* W29 batch cultures carried out in Erlenmeyer flasks with WSH-HP (**A**) and WSH-HDAP (**B**). The error bars represent the standard deviation of two independent biological replicates.

Regardless of the hydrolysate used, the *Y. lipolytica* W29 exhibited similar growth profiles, characterized by an exponential phase beginning at 8 h, followed by the onset of the stationary phase. However, the transition to the stationary phase occurred earlier in WSH-HP (56 h) than in WSH-HDAP (72 h). The maximum specific growth rates were similar in both hydrolysate-based media (**Table 11**), indicating that the intrinsic growth rate of the strain was not significantly affected by the composition of hydrolysate and the type of pretreatment applied to WS. Moreover, final biomass concentrations were comparable in both hydrolysates, reaching approximately 21 $g \cdot L^{-1}$ (**Figure 12**). Dias et al. (2024) reported maximum specific growth rates between 0.07 h^{-1} and 0.08 h^{-1} and final biomass concentration of 13 $g \cdot L^{-1}$ for same strain cultivated in eucalyptus bark hydrolysate under similar conditions. Similarly, Dias et al. (2023) observed maximum specific growth rates between 0.07 h^{-1} and 0.09 h^{-1} and final biomass concentrations of 8 $g \cdot L^{-1}$–12 $g \cdot L^{-1}$ when the strain was grown in a medium mimicking lignocellulosic biomass hydrolysate. Compared to literature, the results obtained in the present study showed slightly lower growth rates but substantially higher final biomass concentrations. This difference may be attributed to variations in the composition of the hydrolysates, primarily associated to sugar concentration and the presence of additional assimilable carbon sources, such as acetic acid, furfural, and 5-HMF, which can be metabolized by *Y. lipolytica* (Dias et al., 2023).

Table 11: Values of biomass yield ($Y_{X/S}$), maximum specific growth rate (μ) and glucose consumption rate (*GUR*), and obtained in *Y. lipolytica* W29 batch cultures carried out with WSH-HP and WSH-HDAP in Erlenmeyer flasks. Data are presented as the average ± standard deviation of two independent replicates. The absence of letters represents the absence of statistical differences.

Parameter	WSH-HP	WSH-HDAP
$Y_{X/S}$ ($g \cdot g^{-1}$)	0.36 ± 0.01	0.31 ± 0.03
μ (h^{-1})	0.057 ± 0.011	0.055 ± 0.009
GUR ($g \cdot L^{-1} \cdot h^{-1}$)	0.67 ± 0.05	0.58 ± 0.01

Biomass yields obtained in both hydrolysates were statistically equal (**Table 11**) and were similar with those reported by Dias et al. (2023) using a medium mimicking lignocellulosic biomass hydrolysate (0.3 $g \cdot g^{-1}$ – 0.4 $g \cdot g^{-1}$). In addition, minor differences were observed in glucose assimilation between the two hydrolysates. Although glucose was consumed at a slightly higher uptake rate in WSH-HP, this difference was not statistically significant (**Table 11**). At the end of cultivation, approximately 2 $g \cdot L^{-1}$ of glucose remained in the medium for both conditions. Xylose consumption under both conditions (**Figure 12**) exhibited a sequential sugar consumption: glucose was metabolized first, followed by delayed xylose assimilation. Dias et al. (2023) demonstrated that, in a medium mimicking lignocellulosic biomass hydrolysate containing a mixture of 50 $g \cdot L^{-1}$ glucose and 8 $g \cdot L^{-1}$ xylose, *Y. lipolytica* W29 and NCYC 2904 showed a preference for glucose. This pattern has been previously reported in other yeasts and is often attributed to glucose repression of xylose metabolism or competition at sugar transporters (Poontawee et al., 2017).

These results confirm that the production of *Y. lipolytica* biomass was not significantly affected by the compositional differences in sugar content among the WSH. For subsequent bioreactor experiments, hydrolysates obtained by enzymatic hydrolysis of HDAP were used , as this pretreatment provided easier operational practicality and compatibility with the available equipment compared to HP.

4.6. Biomass production in the bioreactor

Since *Y. lipolytica* W29 was able to grow in WSH, the process was scaled up from Erlenmeyer flasks to a 2-L lab-scale stirred-tank bioreactor, using WSH-HDAP as substrate. During the cultivation period,

pH, temperature, agitation, and aeration were controlled to maintain optimal growth conditions. The concentration of glucose, xylose and biomass was monitored throughout the time (**Figure 13**).

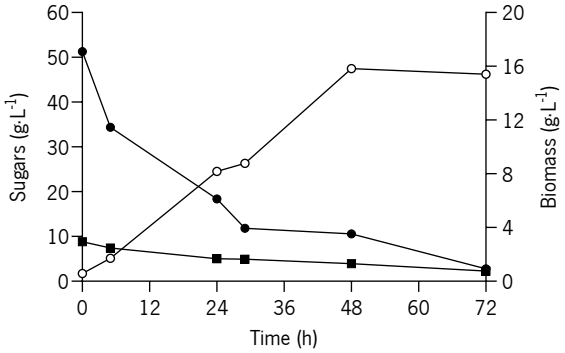


Figure 13: Profile of glucose (●), xylose (■) and biomass (○) concentration obtained in *Y. lipolytica* W29 batch culture in a lab-scale bioreactor.

In the bioreactor experiments, the growth profile of *Y. lipolytica* resembled that observed in Erlenmeyer flask cultures, displaying two distinct phases: an exponential phase followed by a stationary phase reached after 48 h of cultivation. No lag phase was observed, indicating rapid adaptation to the hydrolysate-based medium. This behavior in *Y. lipolytic* growth was also observed in lignocellulosic hydrolysates derived from eucalyptus bark and olive tree prunings (Dias et al., 2025; Dias et al., 2024). The final biomass concentration (15 $g \cdot L^{-1}$) and the biomass yield (0.27 $g \cdot g^{-1}$) of *Y. lipolytica* W29 cultures were similar to those obtained in Erlenmeyer flask experiments, whereas the maximum specific growth rate (0.092 $h^{-1} \pm 0.016\ h^{-1}$) was highest in the bioreactor, which can be attributed to the improved homogenous mixing and aeration provided by the controlled bioreactor conditions (Palladino et al., 2024). The value of specific growth rate is comparable to that reported for the same strain grown on medium mimicking lignocellulosic biomass hydrolysate (0.107 $h^{-1} \pm 0.003\ h^{-1}$) containing 40 $g \cdot L^{-1}$ of glucose, and in eucalyptus bark hydrolysate containing 56 $g \cdot L^{-1}$ of glucose, under batch conditions (Dias et al., 2023; Dias et al., 2024). The glucose consumption rate of 0.67 $g \cdot L^{-1} \cdot h^{-1}$ was 1.15-fold higher than in Erlenmeyer flasks (0.58 $g \cdot L^{-1} \cdot h^{-1}$). Glucose and xylose were consumed simultaneously during the exponential phase, a pattern that did not occur in the flak experiments. In bioreactor, residual glucose (3 $g \cdot L^{-1}$) and xylose (2 $g \cdot L^{-1}$) were detected after 72 h of cultivation.

In the bioreactor, dissolved oxygen was depleted within 3 h of the exponential phase and remained at nearly zero for the duration of the process. *Y. lipolytica* is a strictly aerobic yeast, meaning that cellular growth is strongly influenced by dissolved oxygen concentration (Kerssemakers et al., 2023). Dias et al. (2024) demonstrated that enhancing oxygen transfer increased *Y. lipolytica* W29 biomass production.

Oxygen depletion in *Y. lipolytica* W29 in batch cultures, can trigger citric acid accumulation and a yeast-to-mycelium transition (Timoumi et al., 2017). Jost et al. (2015) showed that *Y. lipolytica* redirected metabolism toward citric acid production under low oxygen conditions. In this experiment, citric acid accumulation could not be quantified due to interference from the citric acid buffer used during enzymatic hydrolysis, but such a shift remains plausible.

The morphology of *Y. lipolytica* cells was evaluated during cultivation by microscopic (**Figure 14**) , as this yeast is known to exhibit dimorphic growth, alternating between oval and filamentous forms depending on environmental conditions (Barth and Gaillardin, 1997). Morphological transitions in *Y. lipolytica* have been associated with factors such as nutrient limitation, pH, oxygen availability, and the presence of specific carbon sources (Barth and Gaillardin, 1997; Ruiz-Herrera and Sentandreu, 2002).

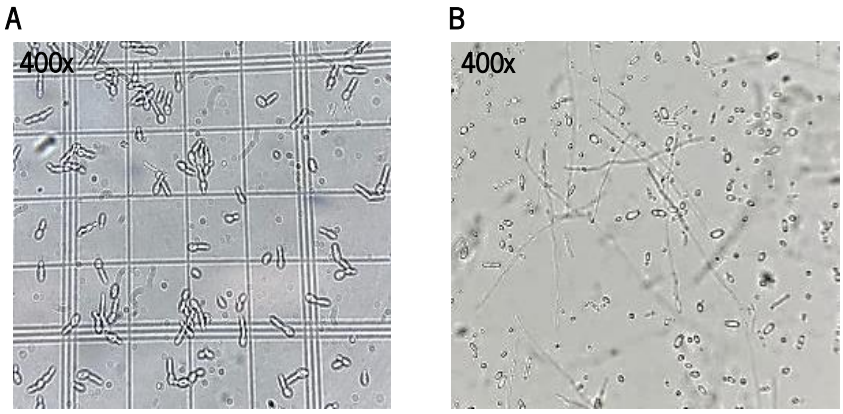


Figure 14: Microscopic observation of *Y. lipolytica* W29 cells at 0 h (**A**) and 72 h (**B**).

In this study, microscopic analysis revealed distinct morphological forms in *Y. lipolytica* W29 cells during bioreactor cultivation (**Figure 14**). At inoculation (**Figure 14A**), cells displayed the characteristic yeast-like morphology, appearing as oval cells (Jach and Malm, 2022). After 72 h of cultivation (**Figure 14B**), a distinct morphological shift was observed: alongside the presence of oval cells, elongated hyphal structures appeared, indicating a transition toward filamentous form. The beginning of hyphae forms coincided with the stationary phase and a long period of oxygen depletion (69 h), suggesting that oxygen limitation may have acted as a key driver of this morphological response. Similar findings have been reported in other *Y. lipolytica* strains, where restricted aeration induced hyphae formation (Timoumi et al., 2018). Bellou et al. (2014) demonstrated that the dissolved oxygen concentration is a critical factor affecting the morphological transition, independently of the carbon and nitrogen source. Overall, the bioreactor experiments demonstrated that *Y. lipolytica* W29 adapted rapidly to wood shavings hydrolysate media and is capable of simultaneously assimilating glucose and xylose without a lag phase.

4.7. Yeast biomass characterization

Yeast biomass is a renewable resource that offers significant potential to obtain valuable bioproducts. In particular, *Y. lipolytica* biomass is recognized as a rich source of proteins, carbohydrates, essential amino acids and B-vitamins and bioactive compounds such as mannoproteins (Silva et al., 2024). *Y. lipolytica* biomass has been used as an animal feed ingredient since 2010 and was approved for human consumption by EFSA in 2019, highlighting its safety and nutritional value (Drzymała et al., 2020; Jach and Malm, 2022). **Table 12** presents the characterization of *Y. lipolytica* W29 biomass obtained after bioreactor fermentation in WSH-HDAP.

Table 12: Composition (dry basis) of *Y. lipolytica* W29 biomass obtained in wood shaving hydrolysate-based medium in bioreactor experiments. Data are the average ± standard deviation of two independent replicates.

Parameter (%, w/w)	**Yeast biomass**
Protein	33.0 ± 0.2
Lipids	5.6 ± 0.6
Carbohydrates	11.1 ± 0.8
Ash	11.1 ± 0.4

The biomass of *Y. lipolytica* W29 obtained after cultivation in wood shaving hydrolysate-based medium was mainly composed of total proteins, followed by ash, carbohydrates, and a small fraction of lipids. Proteins were the major component, reflecting cellular metabolism and efficient assimilation of nitrogen sources during the bioprocess. The protein content of *Y. lipolytica* biomass is dependent on the cultivation conditions and the type of substrate used, according to literature. Protein content of *Y. lipolytica* growing in sawdust hydrolysate ranged between 30 % and 38 % (Ünver et al., 2023). *Y. lipolytica* grown on various industrial substrates such as glycerol, biofuel waste, and solid tallow, as well as on agricultural by-products including rye straw, rye and oat brans, soybean hydrolysates, and other residues, reported protein values typically range between 30.5 % and 56.4 % (Jach and Malm, 2022). In a study with glycerol, protein content in *Y. lipolytica* varied from 19 % to 48 % depending on substrate type, concentration, and nitrogen supply (Fabiszewska et al., 2021). The high protein content observed in this study is therefore consistent with values typically described in literature.

The ash content reflects the presence of inorganic minerals such as potassium, calcium, magnesium, copper, iron and zinc, which have been identified in previous studies with *Y. lipolytica* biomass (Czech et al., 2016; Silva et al., 2025). The lipid content reached is within the range usually reported for *Y. lipolytica* biomass produced under conditions aimed at maximizing cell growth (3 % – 8

%) (Silva et al., 2025; Torres-Añorve and Sandoval, 2025). This relatively low lipid content is consistent with the initial C/N ratio of 10, as excess nitrogen favors cell growth rather than lipid accumulation (Costa et al., 2024). In contrast, under high C/N ratio, *Y. lipolytica* can redirect metabolism toward lipid accumulation, reaching 20 % – 40 % of dry weight (Dias et al., 2023; Naveira-Pazos et al., 2023). According to the literature, the range of carbohydrate content in *Y. lipolytica* biomass varies depending on cultivation conditions and the type of carbohydrates measured. *Y. lipolytica* IMUFRJ 50682 biomass was composed of 39 % and 33 % of total carbohydrates when cultivated in glucose and glycerol, respectively (Silva et al., 2024). Two *Y. lipolytica* strains, growing in YPD medium, accumulated 29 % – 32 % of total carbohydrates (Torres-Añorve and Sandoval, 2025). The carbohydrate content of *Y. lipolytica* growing in sawdust hydrolysate ranged between 17 % and 22 % (Ünver et al., 2023). Sekova et al. (2015) reported that stress conditions, such as alkaline pH and elevated temperature, increased cytosolic carbohydrate content in yeast populations between 3.6 % and 11.8 %. The carbohydrate content of *Y. lipolytica* YB-423 ranged from 19 % to 36 %, depending on the composition of leftover corn leaves and stalks hydrolysates (Heppner and Livney, 2025). The content of carbohydrates obtained in this study is similar to others found in the literature. Yet, Papanikolaou and Aggelis (2011) concluded that in cases where a rapid cell proliferation occurs, the glycolytic flux is directed towards protein and nucleic acid synthesis rather than carbohydrate accumulation (Papanikolaou and Aggelis, 2011).

4.8. Autolysis of *Y. lipolytica* biomass

Autolysis is a biological process in which endogenous enzymes progressively degrade the cell wall, leading to the release of intracellular components such as proteins, nucleic acids, and other macromolecules (Takalloo et al., 2020). This mechanism provides an alternative to mechanical or chemical cell disruption, enabling the recovery of functional biomolecules under relatively simple operating conditions (Takalloo et al., 2020). In this study, the biomass obtained after fermentation was subjected to autolysis at two different solid loadings of 10 % and 20 % (w/v). The process was monitored by measuring biomass concentration (**Figure 15**).

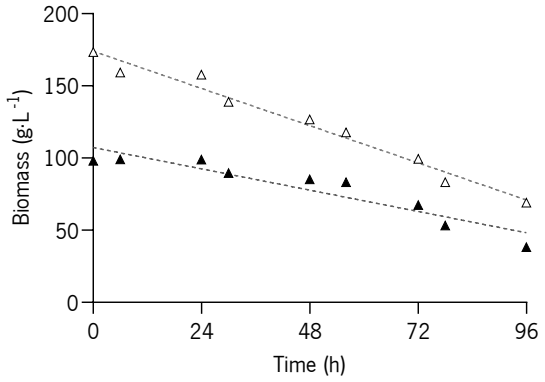


Figure 15: Profile of biomass concentration during autolysis of *Y. lipolytica* W29 biomass at solid loading of 10 % (▲) and 20 % (Δ) (w/v) .

During autolysis, biomass concentration (**Figure 15**) decreased regardless of solid loading studied, indicating the destructuring of cell wall and the solubilization of intracellular compounds, and the consequent decrease in yeast cell size. A similar trend has been reported in the work of Takalloo et al. (2020), where autolysis was linked to water diffusion from the extracellular medium into the cytoplasm, promoting cell lysis and resulting in a measurable reduction of optical density at 600 nm. This decline was adjusted to a linear regression model (**equations 12** and **13**) with high coefficients of determination (R^2 = 0.98 for 10 % and R^2 = 0.88 for 20 %) indicate a good correlation between biomass decrease and time.

$$\text{Biomass } (g{\cdot}L^{-1}) = (-0.62 \pm 0.09) \times [\text{Time}]\ (h) + (107.3 \pm 4.8) \quad \textbf{(Eq. 12)}$$

$$\text{Biomass } (g{\cdot}L^{-1}) = (-0.91 \pm 0.06) \times [\text{Time}]\ (h) + (159.1 \pm 7.2) \quad \textbf{(Eq. 13)}$$

Although, the rate of biomass decrease was higher in the 20 % suspension (0.91 $g{\cdot}L^{-1}{\cdot}h^{-1}$) compared to the 10 % suspension (0.62 $g{\cdot}L^{-1}{\cdot}h^{-1}$), both conditions exhibited a similar overall biomass reduction of approximately 40 % by the end of the process. This suggests that increasing the solid loading did not significantly influence the efficiency of the autolysis process. In contrast, a study with 15 % (w/v) suspension of *Saccharomyces cerevisiae*, cell lysis reached approximately 80 % (Takalloo et al., 2020), demonstrating that autolytic efficiency is highly dependent on the yeast strain.

At the end of the autolysis process , CFU concentration was measured to determine whether the ability of the cells to multiply was affected (**Table 13**).

Table 13: Variation of CFU concentration of 10 % and 20 % cell suspensions (dilution 10^0) at the beginning (t = 0) and end (t = 96) of autolysis. Data are the average ± standard deviation of two independent replicates.

Time (h)	10 % (CFU/mL)	20 % (CFU/mL)
0	3.93 x 10^8	6.94 x 10^8
96	1.45 x 10^2	3.99 x 10^2

In **Table 13**, a substantial decrease in cell concentration, evaluated by CFUs counting, is observed from the beginning (t = 0 h) to the end of the autolysis process (t = 96 h), showing a 6-log reduction of viable cells concentration. CFUs were only detected in the undiluted samples (10^0), and even in this dilution the number of colonies was below the standard quantification range of 30–300 colonies per plate (Pasrija and Kumari, 2025). These results indicate a drastic loss of cell viability and a decrease in metabolic activity, confirming the effectiveness of the autolysis process and suggesting that most yeast cells underwent extensive lysis, leaving only a minimal number of viable units.

Moreover, this reduction in CFU concentration aligns with the morphological and physiological changes associated with autolysis. During autolysis, yeast cells undergo structural alterations including membrane weakening, shrinkage and loss of cellular integrity (Moyo et al., 2025). These changes compromise the ability of cells to proliferate, which is reflected in their reduced capacity to form colonies on agar plates. As membrane permeability increases and cellular damage progresses, metabolic functions essential for growth and division are impaired, ultimately leading to a loss of viability (Gautério et al., 2023).

For evaluating the composition, the lysate obtained at the final of the autolysis was lyophilized (**Figure 16**) and characterized in protein, lipids, carbohydrates, and ash (**Table 14**).



Figure 16: Images of lyophilized lysates.

Table 14: Composition (dry basis) of lysates obtained after autolysis of 10 % and 20 % cell suspensions. Data are the average ± standard deviation of four independent analyses.

Parameter (%, w/w)	**Yeast lysates**	
	10 %	**20 %**
Protein	42.3 ± 0.3	45.3 ± 0.4
Lipids	1.9 ± 0.6	3.1 ± 1.5
Carbohydrates	8.2 ± 0.5	9.9 ± 0.3
Ash	33.5 ± 1.2	32.3 ± 1.4

The analysis of the yeast lysate composition at 10 % and 20 % concentration shows distinct trends in the major biochemical components. Raising the cell concentration in the autolysis process enhances the percentage of organic components, primarily proteins and carbohydrates, with only minor variation in inorganic components. Protein is the dominant macromolecule in the lysates and increases with cell concentration in the autolysis, while lipids and carbohydrates, although increasing, represent smaller fractions.

In comparison to initial yeast biomass (**Table 12**), the composition of lyophilized lysates (**Table 14**) indicates chemical alterations due to the degradation of cellular components and the relative concentration of the remaining constituents. During autolysis, extensive lysis of cell structures occurs, along with the loss of readily degradable fractions such as lipids and volatile organic compounds, which contributes to a reduction in overall organic matter (Oliveira, 2008). Consequently, after centrifugation and lyophilization, the lysates exhibit a higher relative proportion of more stable and less soluble components, including structural proteins, and inorganic minerals.

The significant increase in ash content from 11 % in the initial biomass to approximately 34 % in the lysates can be attributed to the concentration of inorganic minerals naturally present in the yeast (e.g. potassium, magnesium, calcium) as well as residual reagents such as EDTA, boric acid, and PMSF used in the autolysis medium, which remain after centrifugation and drying and contribute to the mineral fraction. The apparent increase in protein content (from 33 % to 45 %) is primarily related to changes in the relative composition of the biomass rather than to a higher absolute protein amount. According to Moyo et al. (2025), the autolysis process specifically enhances protein release by disrupting cellular structures and allowing cytoplasmic proteins to diffuse into the extracellular medium. The lipid content of the lysates decreased to 2 % – 3 %, compared to 6 % in the initial biomass. This reduction can be

attributed to the hydrophobic nature of lipids, which tend to remain associated with membrane fragments and cell debris, which are subsequently removed during centrifugation (Debeaufort et al., 2000).

Overall, the observed changes did not reflect an absolute increase in these components, but rather a concentration effect resulting from the selective removal of degradable organic fractions and the retention of less soluble and inorganic materials in the final lysate.

From an application perspective, the high protein content (45 %) of lysates is particularly advantageous for bioglue development. Studies on yeast-based adhesives primarily focus on protein content, as proteins can provide functional groups (e.g., amine, carboxyl, and hydroxyl) capable of participating in crosslinking reactions (Hubbard and Haider, 2010; Luo et al., 2024), thereby enhancing the mechanical strength of the bioglue.

5. CONCLUSIONS

The results of this study demonstrate the potential of using wood shavings (WS) as an alternative and low-cost substrate to produce *Y. lipolytica* biomass. The hydrothermal (HP) and hydrothermal diluted acid (HDAP) pretreatments applied to WS were effective in increasing the cellulose content in the solid fractions. Both solid fractions were subjected to an enzymatic hydrolysis, and the hydrolysates obtained were mainly composed of glucose (73 $g \cdot L^{-1}$ and 57 $g \cdot L^{-1}$) and a small amount of xylose (15 $g \cdot L^{-1}$ and 10 $g \cdot L^{-1}$). These wood shavings hydrolysates (WSH) were primarily used in Erlenmeyer flask experiments, and the results proved the ability of *Y. lipolytica* to grow in lignocellulosic hydrolysates-based medium without inhibition, regardless of sugar composition.

In the bioreactor experiments, the final biomass concentration and yield obtained were similar to those achieved in Erlenmeyer flasks. However, the bioreactor experiment exhibited higher specific growth and glucose consumption rates, demonstrating an efficient metabolic activity under controlled conditions.

The characterization of the resulting yeast biomass revealed that proteins and carbohydrates were the predominant components. Autolysis of the biomass using 10 % and 20 % (w/v) cell suspensions effectively promoted cell disruption and solubilization of intracellular material, resulting in a protein-rich lysate.

Overall, this study demonstrated that the proposed bioprocess aligns with the principles of a circular economy. WS, a low-value by-product of the wood industry, were biologically converted into *Yarrowia lipolytica* biomass and subsequently transformed through autolysis into protein-rich lysates. These lysates reveal potential application in bioglues production, offering an environmentally friendly alternative to petroleum-based adhesives.

6. FUTURE WORK PERSPECTIVES

Although this study demonstrated the feasibility of producing *Y. lipolytica* biomass from wood shavings hydrolysates and its potential valorization into protein-rich lysates for bioglue formulation, several aspects could be further explored.

Future work should focus on optimizing cultivation conditions to increase biomass yield. Since *Y. lipolytica* metabolism is strongly influenced by aeration, agitation, and nutrient availability, further studies should investigate different oxygen transfer rates, agitation rates, and C/N ratios in bioreactor cultures using wood shavings hydrolysate. In addition, implementing a fed-batch fermentation strategy could enable higher cell densities, leading to increased biomass concentration and productivity.

The autolysis process should also be further optimized by investigating the effects of cell concentration, pH, and incubation time on *Y. lipolytica* cells disruption efficiency and protein solubilization. Alternative or combined cell lysis methods, such as enzymatic or mechanical lysis, could be evaluated. Moreover, future work should include a detailed characterization of the lysates, including Fourier Transform Infrared Spectroscopy (FTIR) analysis before and after autolysis to assess structural alterations in functional groups and the determination of amino acid composition.

Finally, given the limited information available, future studies should explore possible functionalization strategies for the lysates through chemical treatments, for example using crosslinkers such as EDGE, glutaric aldehyde, glyoxal or enzymes like transglutaminase, which have been shown to be effective crosslinkers in yeast hydrolysates. It would also be important to evaluate key physicochemical and performance properties of the resulting bioglues, including density, viscosity, bonding strength, thermal stability, and shear strength under dry and wet conditions.

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