UNIVERSITAT ROVIRA i VIRGILI

Biocatalytic Conversion of Xylan-based Furfural to 2-Furoic Acid via Organic-Peracid Mediated Oxidation Induced by Lipase Enzyme

Master Degree Thesis

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1. ABREVIATIONS

CAL-B: Candida antarctica lipase B

EtOAc: Ethyl acetate

FA: Furoic acid

FF: Furfural

GC: gas chromatography

HPLC: High-performance liquid chromatography

HMF: hidroxymetilfurfural

H_2O_2: hydrogen peroxide

PDB: Protein data bank

t-buOH: tert-Buthanol

p-NPP: Para–nitrophenyl palmitate

2. ABSTRACT

The sustainable conversion of renewable feedstocks into high-value-added products has gained more attention recently in the academic and industrial sectors. Herein, we have shown the conversion of furfural to 2-furoic acid from xylan via a facile, green, and efficient chemo-enzymatic approach. The production of furfural from xylan using a biphasic system of ethyl acetate and water (2:1 v/v) in a microwave reactor has been investigated. This is because the xylan easily undergoes hydrolysis upon microwave heating and forms furfural as a key intermediate in the organic phase. Short reaction time and energy efficiency, and green features are possible with the use of microwave technology. Subsequently, we thoroughly investigated the organic peracid-mediated oxidation of furfural to 2-furoic acids under mild conditions using immobilized lipase as a biocatalyst. When substrate loading was up to 40 mM, a good yield of 90% of 2-furoic acid was achieved at 40°C temperature. Moreover, the recyclability of the immobilized lipase has been evaluated, interestingly, it was found that lipase for this reaction can be recycled four times.

Keywords: Biocatalyst, Lipase enzyme, Oxidation, Recyclability, Biorefinery, Sustainability

3. INTRODUCTION

3.1 Environmental issue

Due to several factors, such as the global increase in population growth, the decrease in petroleum resources, the high oil prices, and the political and environmental concerns regarding CO_2 emissions and global warming effect, there is a pressing need in modern society to develop economical and energy-efficient processes for the sustainable production of fuels and chemicals.

Environmental pollution has become a very present political topic in our lives and numerous national and international policies are focused on reducing pollutant emissions. Nonetheless, the seriousness and urgency of some environmental problems push towards the adoption of new energy sources, capable of limiting the emissions of harmful and climate-altering gases. For this reason, we are always looking for new energy sources, possibly renewable, to try to achieve a circular economy.

In the first half of the last century, the use of nuclear energy began, which has many advantages, including the production of a large amount of energy with no greenhouse gas emissions in its process; the use of this energy source is not without disadvantages, including the disposal of radioactive waste and accidents that would cause catastrophes, as shown by those that occurred in Chernobyl in 1986 and Fukushima in 2011.

There are many sources of energy currently used, both renewable and exhaustible.

Solar energy is the main source of renewable energy on the planet, which is used to produce electricity through photovoltaic systems, consisting of solar panels, directly and instantaneously, without using fuel. The conversion from light to electricity takes place in the photovoltaic cell, made of silicon, a semiconductor which represents the fundamental component of the energy generator. Solar energy can also be used to obtain thermal energy, thus heating domestic hot water or rooms. This use of solar energy also does not involve the combustion of gas. Wind energy is the kinetic energy produced by the movement of air on the earth's surface, between areas of high and low pressure. This energy is exploited through wind turbines, which subtract part of its kinetic energy from the wind and transform it into mechanical energy. A wind farm is composed of a system of blades built with aerodynamic shapes, a rotor, a shaft and an electric generator. The wind makes the blades turn, which transmit the kinetic energy to the rotor to which they are connected, in turn it is connected to the shaft to which it transfers the mechanical rotation energy which then turn the generator which transforms, thanks to a dynamo, the mechanical energy into electrical energy.

Hydroelectric energy, on the other hand, exploits the gravitational potential energy of the large masses of water conveyed in dams, locks, canals and bridges. In a hydroelectric plant, a natural basin exists upstream or an artificial basin is built with a dam which forms a barrier and prevents the flow of water from flowing downstream. Through forced pipes the water is conveyed at great speed, until it reaches the valley where a plant containing the hydroelectric turbines and an alternator is located. Falling or moving water produces kinetic energy which, by moving the turbine, is transformed into electrical energy by the alternator.

According to 2016 World Energy Council statistics, by 2060, non-fossil fuels or renewable resources will be an important source of electricity because their usage will increase, as shown in Fig.1.

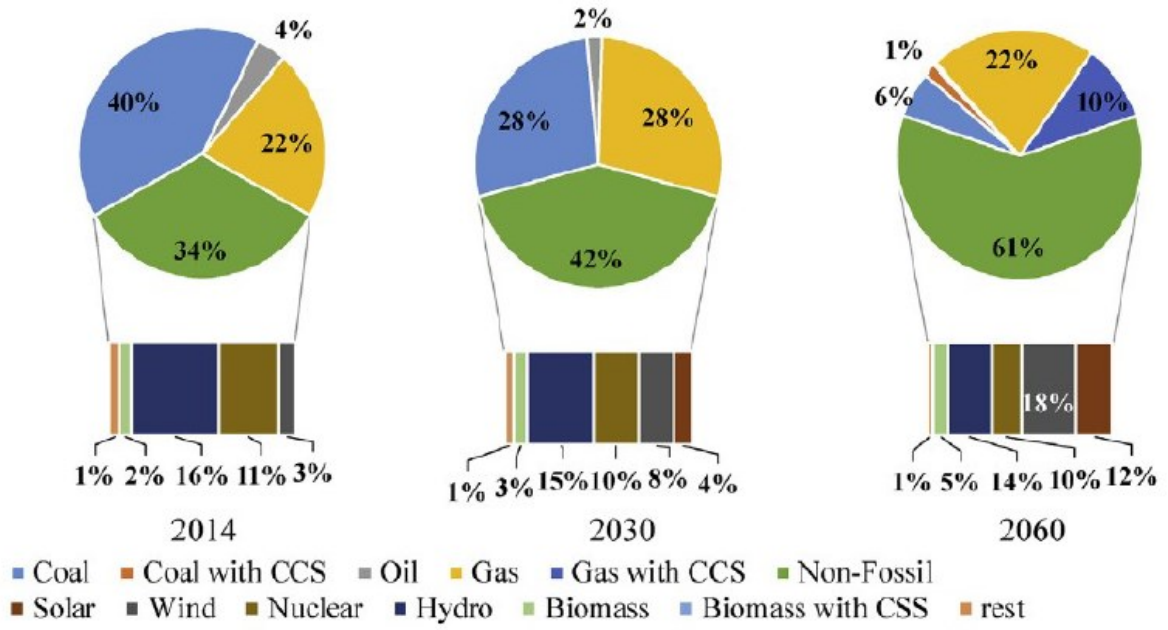


Fig.1 According to 2016 World Energy Council statistics, already from 2030 there will be a decrease in non-fossil fuels use for the increased use of renewable resources. From 2060, the situation will be even better. Adapted from 2016 World Energy Council.

Although these sources of energy are clean, they are still not very competitive and therefore could not satisfy the global energy demand on their own. For this reason, fossil fuels continue to dominate the energy market; in the situation in which we find ourselves, abruptly limiting the use of fossil fuels would mean not only slowing down human progress, but this would even lead to a regression as it would not be able to satisfy the energy demand. To counter pollution and limit the use of fossil

fuels, it is extremely important to continue the search for new energy sources and try to improve those that are already used.

According to April 2023 World Energy Pulse (fig 2.) , the main obstacles to progress are an escalating competition for sustainable technologies and high capital expenses (46%). Inadequate investments in power grid infrastructure contribute to the significant shortfall in comprehensive infrastructure planning.

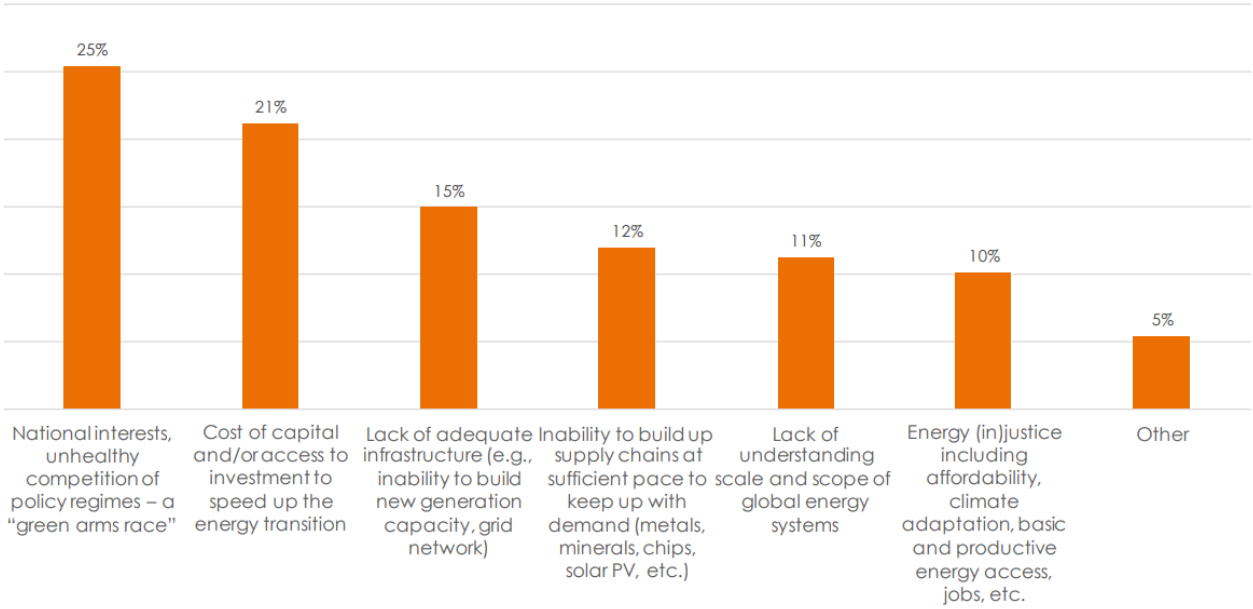


Fig. 2. Greatest obstacles in acceleration energy transitions according to April 2023 World Energy Pulse. Adapted with permission.

3.2 Biomass

In this scenario, biomass could be a good alternative as a renewable resource for the production of energy and chemicals. Biomass is a renewable resource that is carbon-neutral and has cleaner combustion compared to fossil fuels. It is estimated that biomass has the potential to fulfill approximately 25% of global energy needs. Moreover, biomass offers the opportunity to generate valuable chemicals, pharmaceuticals, and food additives. Several types of biomasses can be converted into fuel and chemicals, including wood, agricultural crops and waste, animal feedlot litter, food processing waste, and water treatment plant sludge. [1]

The typical compositions of some of them are indicated in **Tab.1**.:

Biomass	C	H	N	S	O	Ashes	Humidity	Organic fraction
Wood	50,8	6,06	42,7	0,07	42,7	1,84	19,8	78,4
Legume strow	43,3	5,62	0,61	0,12	50,35	9,8	1,62	73,74
Apricot kernel	44,39	5,74	0,37	0,05	49,45	8,52	0,17	75,14
hornbeam shell	41,78	5,36	0,60	0	52,26	9,52	2,28	78,83
hornbeam sawdust	45,18	6,59	0	0	48,23	0,45	8,78	78,14
rice husk	42,0	5,4	0,4	0	39,3	12,9	1,1	70,5
Straw	48,9	5,97	0,82	0,15	43,9	6,37	12,7	80,9
safflower seeds	60,46	9,08	3,10	0	27,36	2,2	5,7	80,8
Mud	50,2	7,09	5,63	1,77	34,9	25,7	32,5	41,8
Sawdust	52	6,07	0,28	0	41,55	0,1	-	-
Manure	50,2	6,50	6,50	0,85	34,6	17,2	43,6	39,2
Vegetable oils	75,4	11,7	0	12,9	0	0	0	100

Tab. 1. typical composition of some kind of biomasses

A promising kind of biomass is the lignocellulosic one. It consists predominantly of cellulose (34-54% by weight), hemicellulose (19-34% by weight), and lignin (11-30% by weight) [2]. Cellulose, which accounts for half of the organic carbon in the biosphere, is the primary constituent of biomass [2-4]. The conversion of lignocellulosic biomass into fuels and value-added chemical poses a significant challenge. The complex chemical composition of lignocellulosic feedstocks is the main hurdle in achieving high yields of desired chemicals and fuels.

Cellulose is structurally composed of anhydrous glucose units, while hemicellulose consists of various C5 sugar monomers. Conversely, lignin is a complex, three-dimensional, cross-linked biopolymer with hydrophobic and aromatic properties [5,6]. These variations in chemical composition and structure give rise to different chemical reactivities in cellulose, hemicellulose, and lignin. Furthermore, the intricate nature of biomass, combined with the inert chemical structure and

compositional ratios of carbon, hydrogen, and oxygen within biomass molecules, pose challenges in the chemo-catalytic conversion of biomass into fuels and chemicals.

Lignin is situated in the outer cell wall of biomass, while cellulose resides within a protective lignin shell. Hemicellulose, on the other hand, is found between the cellulose and lignin, as well as within the cellulose itself. The structure of the lignocellulosic matrix is remarkably dense, making it highly resistant to hydrolysis. As a result, a pre-treatment of lignocellulosic materials becomes necessary to unlock their structure, enabling enzyme penetration and eventual hydrolysis of cellulose and hemicellulose.

3.3 Biomass issue

Criticism has been directed towards the utilization of food crops such as sugar cane, corn, wheat, and sugar beet for renewable fuel production, referred to as first-generation biofuels. Concerns encompass the negative impacts on food prices and biodiversity as these crops are diverted from traditional use in human and animal nutrition. Additionally, first-generation biofuels often require subsidies to be cost-competitive and exhibit limited reductions in greenhouse gas emissions. Moreover, their production necessitates substantial water withdrawal for irrigation purposes. In contrast, non-edible lignocellulosic biomass emerges as a highly promising carbon source. The adoption of second-generation biofuels and biomass-derived chemicals derived from lignocellulosic materials has gained traction due to its substantial geopolitical and environmental advantages. Among the available alternatives, the utilization of lignocellulosic biomass stands out as one of the most attractive and promising approaches to attain sustainable and environmentally friendly production of fuels and chemicals.

3.4 Methods to convert biomass

Various processes exist for converting biomass into energy, mainly divided into biochemical and thermochemical.

Thermochemical methods are divided into two macro-categories: gasification and pyrolysis. Although thermochemical methods require more energy than biochemical ones, they are much faster and more efficient than biochemical ones [7]. Indeed, they are more suitable for industrial production; consequently, gasification and pyrolysis are currently among the most popular techniques for the valorization of biomass. However, it is important to underline that these

technologies have been studied and used for a longer time, so there has been more time to optimize them; unlike biochemical methods that have been less studied so far, which have a very high potential and need more studies to be optimized as well

3.4.1 Thermochemical processes: gasification and pyrolysis

The gasification process is a partial combustion of a fuel based on carbon and hydrogen (e.g. biomass) or of a hydrocarbon in the presence of a gasifying agent, in order to generate a fuel gas rich mainly in carbon monoxide and hydrogen, generally called syngas [7]. Synthesis gas is also made up of CO_2 , H_2O, CH_4 and, in the case of gasification with air, as well as unwanted products (H_2S, COS, NH_3, HCl, tar, char, powders, etc.).

Pyrolysis is a process of thermal conversion of organic substances/carbonaceous material such as solid biomass in the absence of oxygen or air to convert it into liquid fuels, solid carbon and gaseous compounds [8]. The condensable steam obtained is condensed into bio-oil, a fuel which, through the steam reforming reaction, can produce hydrogen.

3.4.2 Biochemical methods

The biochemical methods are digestion, fermentation and photosynthesis; fermentation can transform biomass into biogas, while biochemical processes can yield bio-alcohol. An emerging process is the valorization of oils into biodiesel, through a reaction called transesterification**.**

Presently, the most economically significant processes include wood combustion, bioethanol production from sugarcane or corn, and biodiesel production from oilseeds. However, further advancements are still required to enhance these processes [1].

3.4.3 Hydrolysis

A promising method to obtain chemicals in a sustainable way is the extraction of sugars from the lignocellulosic biomass and convert them then into chemicals. In this way, sugars are used as feedstock.

To release individual glucose units, acid and enzymatic hydrolysis are commonly employed. Hemicellulose, on the other hand, contains C5 sugars like xylose, galactose, mannose, and

arabinose. The dehydration of C5 sugars can lead to the formation of furfural, a versatile platform chemical with applications spanning solvents, resins, and fuel additives (fig.3). The large-scale production of organic and furanic chemicals from sugars presents a significant alternative to petroleum-based energy sources [1].

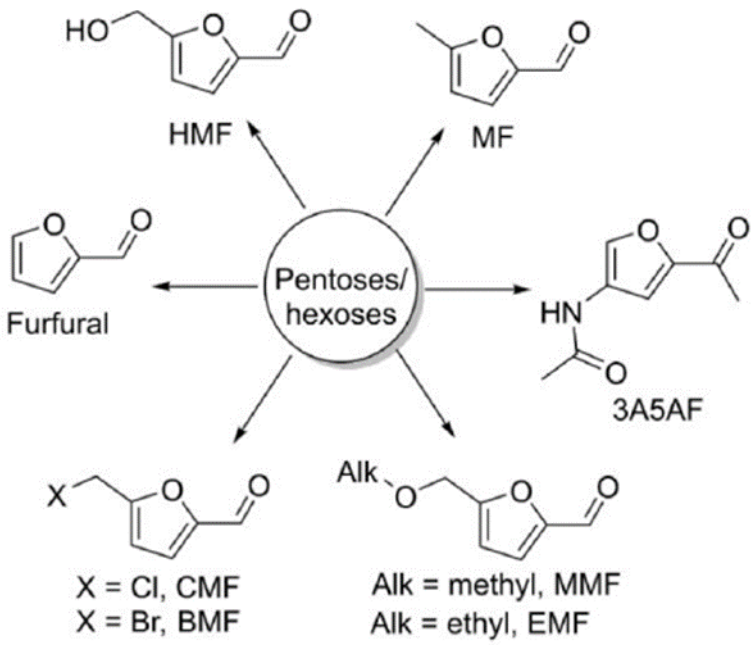


Fig. 3 From the hydrolysis of hemi-cellulose sugars, it is possible to obtain several compounds, like furfural, HMF, MF, 3A5AF, MMF, EMF, CMF, BMF. Adapted from [44].

3.4.4 The concept of biorefinery

As it can be seen, a huge amount of method for the valorization of the biomass are currently used and still studied; but to maximize the potential of the biomass, the concept of a biorefinery, which combines multiple primary conversion processes with advanced separation and downstream upgrading operations, must be considered: it is highly appealing as it maximizes feed utilization and enhances the value of products. Furthermore, a significant advantage of a biorefinery is its ability to produce valuable chemical co-products, thereby reducing vulnerability to fluctuations in energy prices [1] (fig.5).

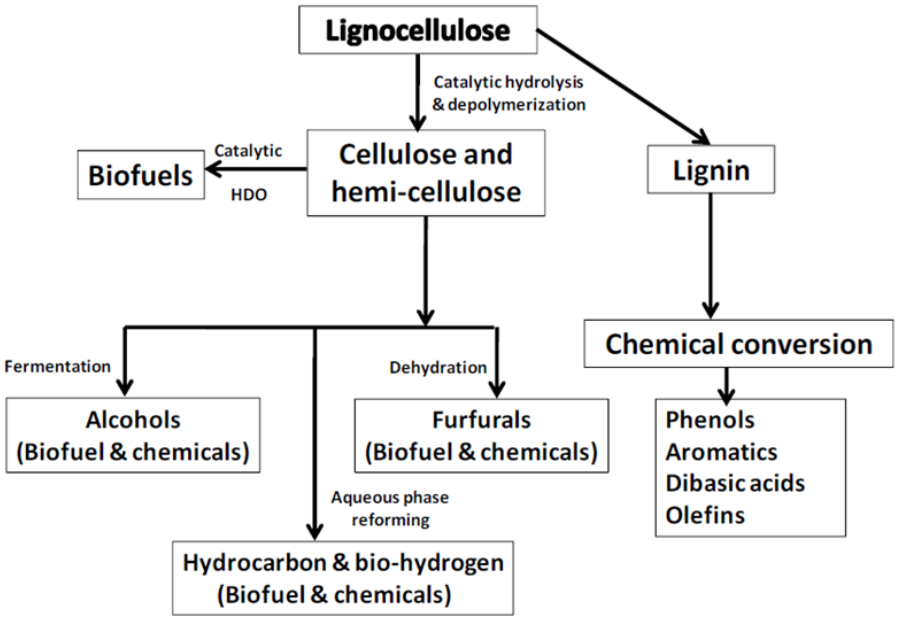


fig. 4. the possible scheme for a chemo-catalytic biorefinery. Adapted from [38].

4. FUFURAL

4.1 Definition of furfural and importance

Furfural, chemically known as furan-2-carbaldehyde or 2-furaldehyde ($C_5H_4O_2$), is a heteroaromatic furan ring containing an aldehyde functional group. Furfural serves as a renewable and versatile platform molecule for the synthesis of chemicals and fuels [9].

Recently, furfural has gained significant attention as one of the valuable chemicals derived from biomass and identified as a key product of lignocellulosic biorefineries [10]. In particular, urfural has been identified as one of the potential top 30 high-value bio-based molecules [11]. FF is produced from renewable agricultural sources such as food crop residues and wood wastes. China is the largest producer of FF, accounting for about 70% of the global production capacity, followed by the Dominican Republic and South Africa, which together contribute to approximately 90% of the global production. [12]

4.2 Furfural production

Furfural primarily finds application as a selective solvent, utilizing its aromatic nature and polarity for favorable interaction with aromatics and unsaturated compounds. Additionally, it possesses intermediate polarity, making it partially soluble in highly polar as well as non-polar substances [13]. The chemical reactivity of FF is largely attributed to its two functionalities: the aldehyde group and the aromatic ring.

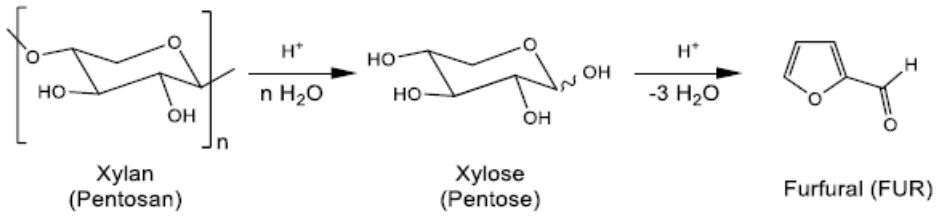


Fig. 5 simplified scheme of the conversion of xylan into furfural. Adapted from [13].

Furfural holds potential for the production of bio-based transportation fuels or sustainable chemicals through subsequent aldol condensation and hydrodeoxygenation reactions [14,15].

The industrial production of furfural began in 1921 with the Quaker Oats Company using oat hulls as raw material. However, this method faced limitations such as limited demand, high maintenance costs, low yields, and lack of significant improvements since 1980 [16,17].

Currently, it is produced commercially by acid-catalyzed transformation of pentosan sugars present in biomass. This involves hydrolysis of C5 polysaccharides to monosaccharides (mainly xylose) using H_2SO_4, followed by dehydration to FF. Finally, it is recovered through steam stripping to avoid further degradation and purified through double distillation [9].

The reaction kinetic mechanisms for furfural formation have been widely investigated and it is commonly described as a dehydration reaction via an unidentified intermediate compound. Studies have shown that simultaneous molecular breakdown and condensation reactions lead to the formation of a black residue referred to as humins [18]. However, the consensus on the specific molecular mechanism for the formation of these by-products has yet to be achieved.

The achievement of a high yield and selectivity in the production of furfural through the selective dissolution and conversion of hemicellulose relies on two critical factors: the choice of solvent and catalyst [19].

4.3 Catalyst

Catalysts can be categorized as homogeneous or heterogeneous. Homogeneous catalysts are dissolved within the reaction medium, facilitating their interaction with the solid biomass. However, the recovery of homogeneous catalysts from the reaction media poses challenges. On the other hand, heterogeneous catalysts offer the advantage of easy separation and recovery, aligning with the principles of green chemistry [20]. However, heterogeneous catalysts, such as solid acids, encounter issues related to their deactivation in aqueous solutions [21]. To effectively utilize hemicellulose in biomass and achieve high yield and selectivity in furfural production, it becomes essential to design effective catalysts suitable for use in the solvent system.

4.3.1 Homogenous acid catalyst

Over the past few decades, diverse acid catalysts, including mineral acids, Lewis acidic salts, and solid acids, have been developed for the hydrothermal decomposition of biomass to yield furfural [20]. Extensive research has been conducted on the reaction mechanism of hemicellulose and its model compounds using these catalysts.

Among homogenous catalyst, sulphuric acid is the most widely used catalyst in the production of furfural [22], but today also other acids are used and studied, as hydrochloric acid, tin chloride [23], aluminum chloride and iron (III) chloride hexahydrate [24].

4.3.2 Solid catalyst

A strategy to enhance FF yield, facilitate catalyst recovery, and enable reutilization involves the employment of solid catalysts. A wide range of materials, including zeolites, zeotypes, sulfonic ion-exchange resins, sulfonic-acid modified mesoporous silicas, sulfonated metal oxides, Keggin heteropolyacids, mesoporous niobium phosphate, and vanadyl pyrophosphates, have been extensively investigated in recent years [9].

Zhang et al. introduced a novel carbon solid acid catalyst based on carbonized sucrose in γ-valerolactone, achieving a furfural yield of up to 78.5% from xylose and demonstrating excellent catalyst reusability [25].

Zhu et al. obtained a similar furfural yield using a resin-based solid acid catalyst with an ordered mesoporous structure and confirmed its recyclability [26].

4.4 Reaction medium

In addition to the catalyst, the reaction medium also affects reaction rate, reaction pathway, and product distribution [27]. Kelly et al. discovered that protons exhibited higher activity in acetonitrile compared to water, leading to enhanced catalytic performance of acid catalysts [28]. Wang et al. analyzed the distribution of pentose conformers in solvent mixtures containing water and polar aprotic solvents like γ-valerolactone and isopropanol, finding that the proportion of active conformers significantly increased with the addition of organic solvent, thereby enhancing pentose dehydration [29,30].

Various solvents such as GVL, 2-MTHF and DMSO were used for the conversion of xylose [19].

A noteworthy approach to obtain FF from biomass is the utilization of H_2O-organic solvent biphasic systems [9]. This approach capitalizes on the partition coefficient of FF between H_2O and the organic solvent. FF demonstrates a higher affinity for the organic phase, which inhibits degradation reactions as catalysts are absent.

Furthermore, organic solvents demonstrate different abilities to extract furfural. In biphasic solvent systems, the utilization of the organic phase facilitates rapid extraction of furfural from the aqueous phase (reactive phase), minimizing side reactions and increasing furfural yield [31,32].

Ionic liquids have also been widely discussed as solvents and acidic catalysts for pentose dehydration, either in aqueous media or in the presence of organic solvents. They can enhance furfural yields when combined with xylose or xylan, organic solvents, and acidic catalysts. Ionic liquids can also serve as a reaction medium for furfural production from pentoses, higher saccharides containing pentoses, or pentosans [33].

4.5 Reactor

With the aim to optimize the extraction of sugars and furoic compound, reactors of ultimate generation and configurations have been employed.

4.5.1 Semi-continous reactor

The use of a semi-continous plant can improve the yields of the biomass extraction compounds, because water-soluble compounds can be quickly removed from the reaction zone; thank to this technique, the extracted compound are protected from secondary reactions [34]. In particular, Katarzyna et al. analyzed the hydrolysis of 3 different kind of biomasses (spruce wood, beech wood and miscanthus) using sulfuric acid as catalyst at 0,05 M, changing reaction time and temperature. It has been found out that the best biomass for the extraction of furfural is miscanthus at 200°C and 40 minutes, with a yield of 18,5%. [34]

4.5.2 Conventional vs Microwave Heating

In the last years, researchers have considered the microwave reactor as an appropriate alternative to the conventional reactors for the valorization of the biomass.

Microwave heating is different from conventional heating in many respects. Difference between these two heat sources have been discussed in a wealth of literatures are summarized below [35]:

Conventional Heating	Microwave Heating
By thermal or electrical sources, heating takes place.	By electromagnetic waves, heating takes place.
Heating of reaction mixture proceeds from a surface usually from the inside surface of reaction vessels.	Heating of reaction mixture proceeds directly inside the material avoiding the vessel.
The vessel surface is brought in physical contact with the source which is at a higher temperature (e.g., burner, mantle, oil bath, steam bath etc.).	No need of physical contact of reaction vessel with the higher temperature source. The reaction vessel is kept in the oven cavity and microwave source or the magnetron is kept little further.
Heating mechanism involve conduction of heat.	Heating mechanism involve dielectric polarization and ionic conduction.
In conventional heating, generally, the achievable highest temperature is limited by boiling point of a substrate.	In microwave, the temperature of a substrate can be raised higher than its boiling point, *i.e.*, superheating may take place.
In the conventional heating, all the components in a mixture are heated almost equally.	In microwave heating, specifically a particular component can be heated more depending on its dielectric characteristics.
Heating rate is less.	Heating rate is several (from 10 to 1000 in best cases) fold high.

Tab.2 Comparison between conventional heating and microwave heating. Adapted from [35].

In particular, for the production of furfural from hemicellulose, microwave reactor has been particularly appreciated, as it has been reported in the literature.

Yemis et al. conducted a study on the acid-catalyzed conversion of xylan to furfural using a microwave-assisted reaction. They achieved a furfural yield of 36.5 wt% by utilizing 0.1M HCl at 180 °C for 30 minutes [36]. The efficiency of various Brønsted acids in the conversion of xylan was investigated, revealing that HCl exhibited the highest catalytic effectiveness.

Yang et al. explored the conversion of xylan under microwave conditions in a GVL/H_2O solvent system, testing various metal salts as catalysts. They obtained 87.8% furfural yield at 130 °C using the $Al_2(SO_4)_3$ catalyst [37]. The same reaction system was also employed with other organic solvents such as MIBK, THF, and 2-MTHF to evaluate their effects on xylan conversion. It was observed that these three organic solvents yielded lower furfural yields compared to GVL [37].

4.6 Furfural compounds

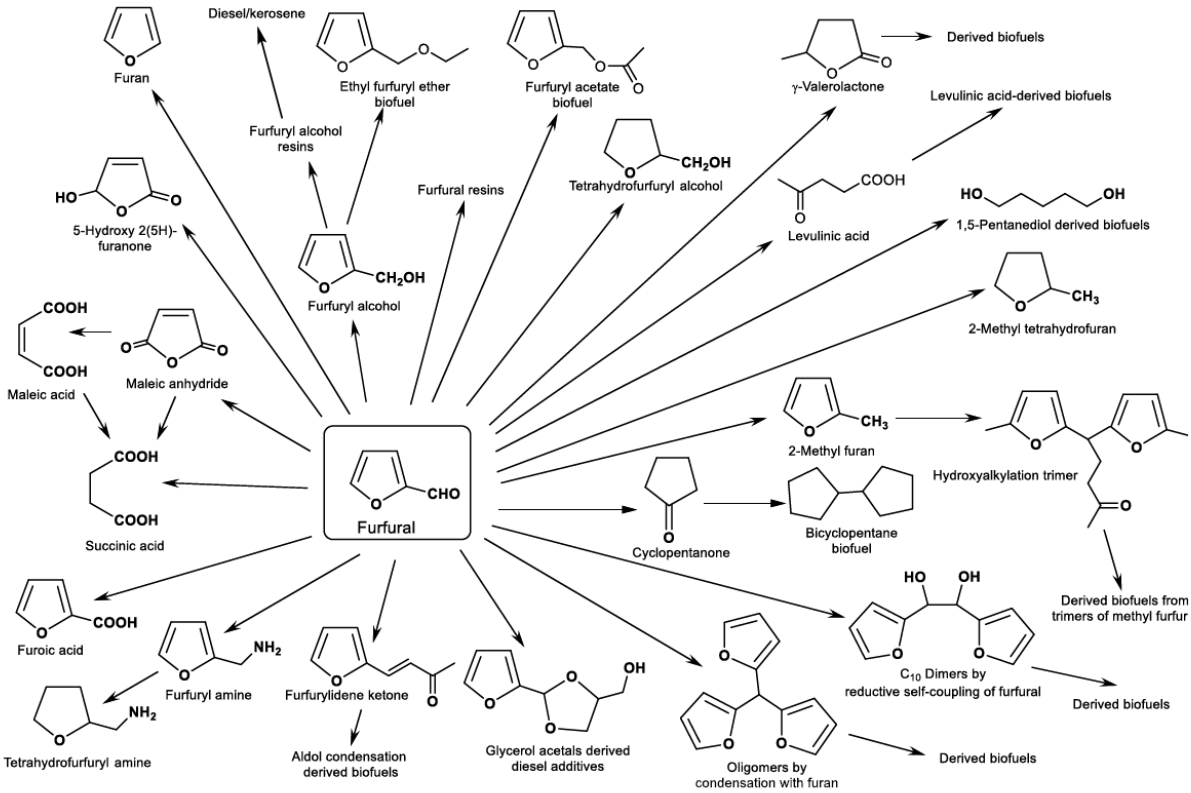


Figure 3. Summary of the furfural-derived chemicals and biofuels described in this review.

fig. 6 Summary of furfural-derived chemical and biofuels. Adapted from [45].

The primary derivative obtained from furfural is furfuryl alcohol, which accounts for approximately 65% of the total furfural produced. Industrial production of furfuryl alcohol involves hydrogenation of furfural using Cu-Cr catalysts, but the environmental concerns regarding the high toxicity of chromium in these catalysts have prompted research into more environmentally friendly alternatives that selectively hydrogenate the carbonyl group while preserving the C=C bonds [38]

Other industrial chemicals that can be synthesized from furfural include 2-methylfuran (2-MF) and tetrahydrofurfurylalcohol (THFA), both of which have applications in biofuels and as raw materials for resins and fuel additives. The synthesis of these chemicals involves the hydrogenolysis of furfural, and different catalysts have been explored for improved yields [38].

Cyclopentanone (CPO) is another C5 chemical that can be produced from furfural, mainly using Cu-based catalysts. [38].

Furfural can undergo decarboxylation to produce furan, which can then be hydrogenated to tetrahydrofuran (THF). Furan and THF are important industrial chemicals, and catalysts such as supported noble metals and mixed metal oxides have been studied for their production [38].

The oxidation of furfural can lead to the production of C4 chemicals like maleic anhydride (MAN), maleic acid (MA), and succinic acid (SA). Vanadium oxide-based catalysts have been investigated for the gas-phase oxidation of furfural to maleic anhydride, while other oxidants such as oxygen and hydrogen peroxide have been discussed as well. Copper nitrates combined with phosphomolybdic acids selectively convert furfural to maleic acid using oxygen as an oxidant, and aqueous oxidation with H_2O_2 and titanium silicate (TS-1) has also been explored [38].

5. FUROIC ACID

5.1 Utilities of furoic acid

Furoic acid, also known as furan-2-carboxylic acid, finds applications across various industries, including pharmaceuticals, agrochemicals, flavors, and fragrances [9]. Actually, it is used also as precursor of the nylon [10] fig. 7.

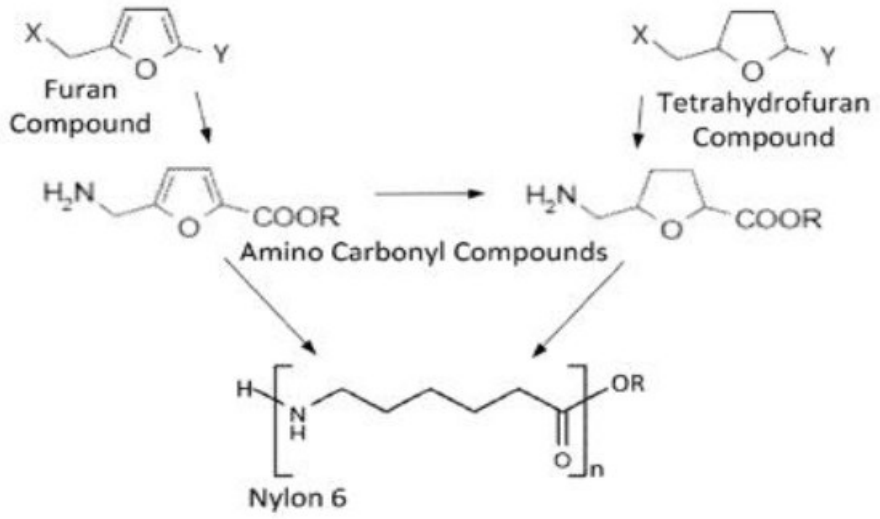


fig.7. scheme of the conversion of furfural and/or hydroxymetilfurfural used as precursor of Nylon 6. Adapted from [39].

Now, furoic acid from furfural can be produced through four routes:

- heterogenous catalyst
- homogenous catalyst
- (photo)electrochemical process
- biological process: enzyme and microbial (they will be shown better in the next chapter)

5.2 Heterogeneous catalyst

Industrially, furoic acid is produced through the Cannizaro disproportionation reaction of FF in an aqueous NaOH solution, resulting in the formation of furfuryl alcohol and sodium furoate [13] (Fig. 8):

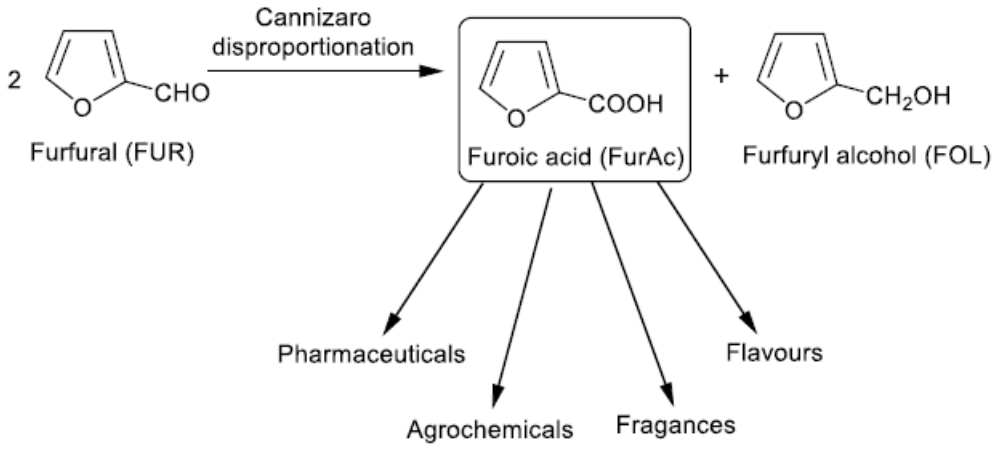


Fig. 8 synthesis of furoic acid from furfural as well as main industrial applications of furoic acid. Adapted from [9].

To yield furoic acid, the solution requires the addition of sulfuric acid, which also neutralizes it, leading to the formation of sodium bisulfate. Since the Cannizaro reaction is highly exothermic, temperature control is crucial. However, the previous synthesis methods involving strong oxidative reactants such as $KMnO_4$, MnO_2, or NaOCl cannot be considered environmentally friendly.

Efforts have been made to achieve selective oxidation to furoic acid using cheaper, less polluting, and less toxic oxidative reactants such as O_2. One approach involves catalytic selective oxidation of aqueous FF to furoic acid with O_2. Various catalysts based on noble metals (Ag, Au, Pt, or Pd) supported on different metal oxides (CuO, Fe_2O_3, Co_2O_3, NiO, TiO_2, CeO_2, ThO_2, Bi_2O_3, or Sb_2O_5) have been explored. Among them, a catalyst combining Ag and CuO-CeO_2 achieved the highest selectivity (96%) at temperatures ranging from 323 to 328 K [40]. This catalyst could be reused multiple times but required rejuvenation through transfer to an alkaline medium and the passage of O_2 to prevent deactivation. Deactivation of the catalyst was attributed to the formation of Cu_2O and Cu (reduction of CuO) and the deposition of organic molecules [41].

Additionally, the addition of Pb significantly enhanced the activity of C-supported Pt catalysts, allowing for complete conversion of FF to furoic acid [42,43].

However, these methods still had limitations, such as the need for a strong base co-feeding to maintain a high pH, which is necessary to prevent catalyst deactivation by furoate chemisorption. The addition of a base posed an environmental sustainability challenge for the process.

5.3 Homogeneous catalyst

Challenges arise in utilizing well-defined homogeneous complexes for furfural/HMF oxidation due to the easy breakdown of substrates under high temperatures in alkaline/aerobic conditions. This breakdown leads to the formation of polymeric products [44-45] Previous research by Goldberg et al. showcased active complexes for catalyzing the aqueous reforming of other aldehydes to acids but showed limited activity when furfural/HMF was used as a substrate [46-47].

A novel approach to catalytic homogeneous oxidation has been presented, wherein furfural is converted to furoic acid and HMF is transformed into FDCA [48]. The oxidation process employs alkaline water as the primary oxidant and is facilitated by well-defined ruthenium complexes featuring acridine-based PNP pincer ligands [49-50]. Furthermore, the reaction produces pure H2 gas as a byproduct. Through mechanistic investigations, it has been determined that the Ru complexes not only catalyze the oxidation of the substrate to acid but also facilitate rapid disproportionation of the substrate within the first hour. This disproportionation step plays a crucial role in preventing substrate decomposition.

5.4 Photochemical process

Exploration of alternative oxidation methods for furfural and HMF, electrochemical [51-53] approach has been undertaken. These methods aim to convert furfural and HMF into furoic acid and FDCA. In recent studies, a remarkable strategy has been devised to combine H2 production from water with biomass oxidation, enabling the (photo)electrochemical generation of H2, furoic acid, and FDCA from a mixture of water and furfural/HMF [54-59]. However, the implementation of these photoelectrochemical systems presents challenges due to the requirement for specialized materials and the difficulty of achieving rational improvements. Moreover, their practical application on a large scale is hindered by the need for sophisticated infrastructure and low working concentrations [60].

6. BIOCATALYTIC CONVERSION OF FURFURAL TO FUROIC-ACID

6.1 Biocatalyst for biobased furans valorization

Biocatalysts, such as enzymes and whole cells, has emerged as a promising technique in the chemical and pharmaceutical industries due to its high selectivity, mild reaction conditions, and environmental friendliness. These properties of biocatalysts align well with the inherent low stability of certain furanic chemicals, opening new possibilities for their conversion. [61]

The valorization of biobased furans, including furfural, HMF, and 2,5-furandicarboxylic acid (FDCA), has become a prominent focus in the biorefinery field. Catalysis, both chemo- and biocatalysts, provides a sustainable approach for the synthesis of furan-based products. However, the biocatalytic upgrade of furans has received relatively less attention compared to traditional chemical routes. [62]

According to Sheldon and colleagues, biocatalysts conform to 10 out of the 12 principles of green chemistry. Therefore, the application of biocatalysts, a green and sustainable technique, for the valorization of biobased furans can contribute to a shift in economic paradigms. For instance, the biocatalytic cascade oxidation of HMF can yield FDCA, a high-value biobased platform chemical. Furthermore, lipases enable the production of various furan-based polymers, such as polyesters and polyamides, at relatively low reaction temperatures.

By adhering to green chemistry principles, biocatalysts contribute to the realization of a circular bioeconomy and facilitates the production of valuable furan-based products. Tremendous advances in biocatalytic upgrading of biobased furans have been recently achieved, especially over the last five years.

6.2 Biocatalytic synthesis of Furoic Acid

The selective oxidation of the formyl group present in biobased furans resulted in the production of furan monocarboxylic acids such as FA and HMFCA [62].

HMFCA is a monomer for the manufacture of polyesters; also, it acts as a precursor for the synthesis of TPA and interleukin inhibitor. **Table 2** shows some of the biocatalysts, processes, and major results (conversion/yield) in the synthesis of furoic acids.

Catalyst	Furan/mM	Reaction conditions	Product	Yield (%)	Reference
CAL-B	HMF/50 Furfural/50	EtOAc/t-BuOH (1:1) 40°C, 24h	HMFCA FA	76 91	[63]
PAMO M446G, PTDH	HMF/5 Furfural/5	Tris-HCl buffer (50 mM, pH 7.5), 25°C, 12-16 h	HMFCA FA	85 60	[64]
BovALDH/AcALDH, NOX	HMF/20 Furfural/20	PB (50 mM, pH 8.5), 40°C, 4 h	HMFCA FA	90 >99	[65]
G. oxidans ATCC 621H	Furfural/100	Culture medium, pH 5.5-6, 30°C, 12 h	FA	98	[69]

Tab. 3 Examples of biocatalytic synthesis of FA. Adapted from [61]

6.2.1 Enzyme Catalysis

Dominguez de Maria and coworkers reported the chemoenzymatic synthesis of furan monocarboxylic acids from various biobased furans in ethyl acetate (EtOAc)/tert-butanol (t-BuOH), in which peracetic acid in situ formed from EtOAc and H_2O_2 by immobilized lipase B from Candida antarctica (CAL-B) oxidized the formyl group. The chemoenzymatic oxidation of furfural afforded FA with high yields, while HMFCA together with the byproduct, its acetate (23%), was produced from HMF. Notably, DFF could be oxidized to FDCA via this chemoenzymatic approach as well, in good yields [63].

Fraaije and Kumar reported the oxidation of furans by Baeyer−Villiger monooxygenases (BVMOs) using an enzyme called phosphite dehydrogenase (PTDH)/phosphite [64]. Furfural and HMF were converted to FA and HMFCA by a phenylacetone monooxygenase (PAMO) variant M446G, respectively, with 60−85% conversions; nonetheless, minor amounts of unknown byproducts were produced in both cases. Also, the variant PAMO M446G has been efficient, and good conversions (90−98%) were obtained at low substrate concentrations (<10 mM) within 24h. [64]

Using BovALDH and EcALDH coupled with NOX, both HMFCA and FA were obtained with >90% yields at 20 mM substrate concentration, but the substrate conversions sharply decreased when the substrate concentrations were more than 20 mM, possibly owing to the substrate inhibition. Furthermore, the addition of dithiothreitol was necessary to achieve high conversions in

the enzymatic oxidation; its possible role might be to protect the catalytic residue cysteine from oxidative damage [65].

6.2.2. Whole-Cell Catalysis

Several microorganisms were found to oxidize the formyl group present in biobased furans in the biological detoxification of inhibitors present in lignocellulosic hydrolysates [61].

From a synthetic viewpoint, whole-cell catalytic transformation of biobased furans remains challenging because these chemicals are well-known potent inhibitors to microorganisms, particularly at high concentrations.

In 2004, Nagasawa and co-workers reported whole-cell catalytic oxidation of furfural and HMF by Acetobacter rancens IFO3297 and Serratia liquefaciens LF14, respectively. A fedbatch strategy in which furfural and HMF were stepwise added with the concentrations of 50 and 10 mM, respectively, was applied to produce high titers of FA (977 mM, 110 g/L) and HMFCA (168 mM), owing to great substrate inhibition [66].

Nocardia corallina was used for the fed-batch synthesis of FA (approximately 86 mM) from furfural (adding 31 mM every time), with 92% yield [67].

Gluconobacter oxidans is a Gram-negative bacterium belonging to the family Acetobacteraceae. This microorganism is well-known for its incomplete oxidation ability. Incomplete oxidation leading to nearly quantitative product yields makes G. oxidans important in the industrial production of various valuable chemicals such as dihydroxyacetone and sugar acids [68].

Xu and co-workers reported the synthesis of FA by growing cells of G. oxidans ATCC 621H [69]. FA was obtained in a quantitative yield at the furfural concentration of 100 mM, but its yields markedly decreased at higher substrate concentrations. The production of FA was scaled up to the volume of 1 L in a compressed oxygen supply sealed and stirred tank reactor in a fed-batch manner, providing 1.6 g/L h productivity. Like HMFCA, FA is an acidic chemical; its accumulation would result in significant pH changes of the reaction media, particularly at high concentrations, thus reducing the catalytic performances of biocatalysts including activity and stability. Therefore, the addition of bases is generally required to neutralize the produced carboxylic acid.

The He group developed a one-pot chemoenzymatic route to directly synthesize FA from biomass by combining sulfonated tin-based solid acid catalysts and whole-cell biocatalysts (Fig. 9) [69]. The pretreated lignocellulosic biomass, such as rice straw and corncob, was concurrently hydrolyzed

and dehydrated to furfural in acidic aqueous solutions by solid acids, followed by base neutralization and dilution; subsequently, microbial cells were added to convert furfural to FA, with yields of 36−42% (based on xylan present in the biomass). In addition, both chemical and biological catalysts proved to be stable in the reuse test [69]. It is a promising strategy toward the cost-effective and sustainable production of high value furan-based products because of the following reasons: (1) the starting materials are inexpensive and readily available; (2) no isolation of any intermediate is required; (3) the catalysts can be facilely recovered and reused; (4) satisfactory FA yields are obtained. Also, this route suffered from such drawbacks as base addition and low catalytic activities of biocatalysts in the reaction mixtures tested.

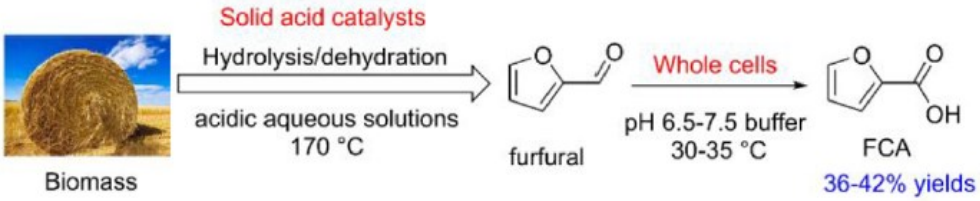


Fig. 9 one-pot chemoenzymatic synthesis of FA from biomass. Adapted from [52].

6.3 Lipase

Lipases, a type of enzyme, are able to catalyze a range of different reactions, including hydrolysis, esterification, transesterification, and interesterification (fig.10).

Lipases, like most globular proteins, can dissolve in water. However, they carry out their catalytic function on hydrophobic substances, specifically triglycerides and their derivatives. Consequently, lipases perform their catalytic activity at the interfaces between immiscible aqueous and lipid phases, commonly known as oil-water interfaces.

The interfacial behavior of lipases can be understood by examining their three-dimensional structure. The enzyme's active site is composed of hydrophobic functional groups. Additionally, located near the active site on the enzyme's outer surface, there exists a movable structure known as the lid. These lids found in lipases are amphiphilic in nature, meaning they consist of both hydrophobic and hydrophilic functional groups.

The lid of lipases plays a crucial role in regulating substrate access to the active site. It can adopt two distinct configurations:

1) CLOSED CONFIGURATION: When lipases are in an aqueous environment, the enzyme conceals its hydrophobic groups. In this state, the lid is closed, and the hydrophobic groups of the lid bind to the hydrophobic groups of the active site. Consequently, the active site becomes inaccessible to substrates, rendering lipases inactive.
2) OPEN CONFIGURATION: In the presence of a hydrophobic phase, the enzyme masks its hydrophilic groups. This causes the lid to open, and the hydrophobic groups of both the lid and the active site interact with the hydrophobic phase. As a result, lipases relocate to the interface between the aqueous and lipid phases. Substrates can now access the active site, allowing lipases to exhibit catalytic activity.

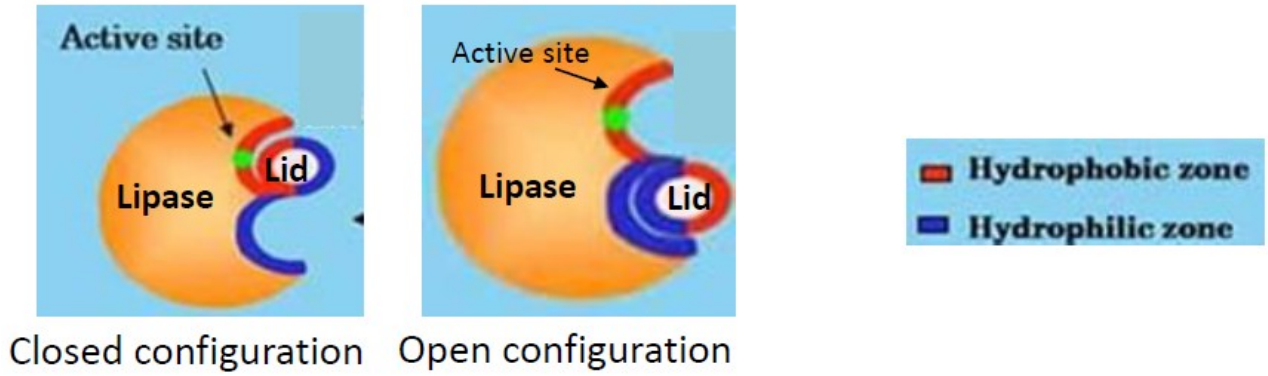


Fig. 10 representation of lipase closed configuration and open configuration.

Currently, microbial lipases have gained significant attention due to the rapid advancements in enzyme technology. They are considered the most important biocatalysts for a wide range of industrial applications. Lipase are considered for diverse industrial uses, including the fat and oleochemical industry, detergent industry, production of biodegradable polymers, food processing, flavor development, medical and pharmaceutical fields, pulp and paper industry, biosensors, waste treatment, cosmetics, and perfumery, as well as biofuel production (biodiesel) [70].

6.4 CAL-B

Lipases demonstrate remarkable stability, retaining their activity even in unfavorable conditions. The enzyme of interest in this study, CAL-B, originates from Candida antarctica, a yeast belonging to the α/B hydrolase family. CAL-B, widely utilized in various industries [71,72], possesses the enzyme code 3.1.1.3. Its highly selective nature enables it to engage in diverse chemical

transformations. CAL-B finds application in kinetic resolutions of racemic amines or alcohols, as well as in the desymmetrization of diacetates and diols.

Another application of CAL-B is the preparation of optically active compounds from meso reactants. The resulting optically pure compounds are not easily obtained through alternative routes and possess significant synthetic value. Figure 11 demonstrates the wide range of products that have been successfully synthesized utilizing CALB as a regio- or enantioselective catalyst [73].

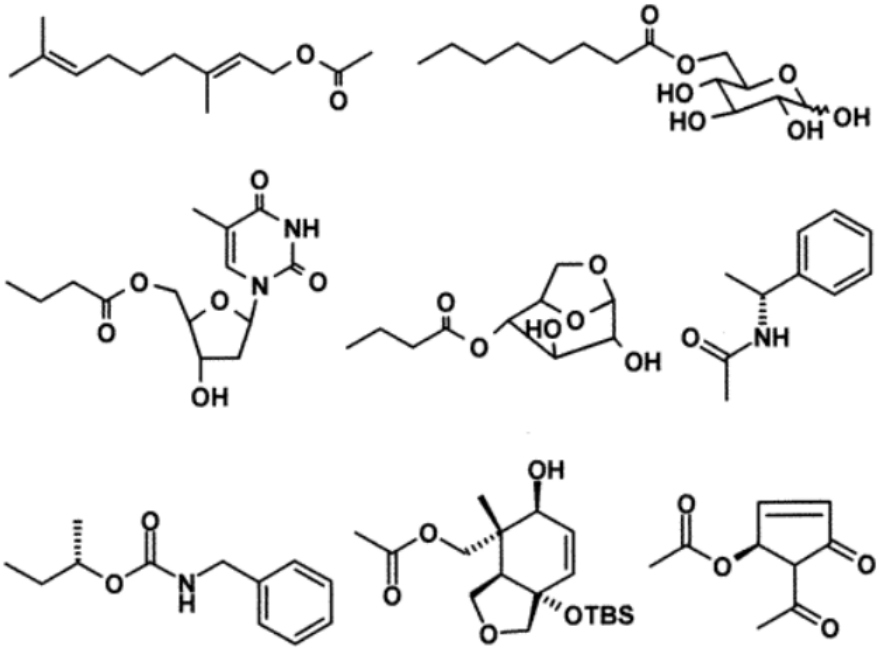


Fig. 11 Products that can be obtained from CAL-B. Adapted from [73].

While the study of this enzyme began relatively recently, until 2016, only eight crystallized structures had been deposited in the Protein Data Bank (PDB). Presently, there are over 200 deposited structures available.

Structurally, CAL-B is a macromolecule weighing approximately 33 kDa. Its unmodified enzyme comprises a polypeptide chain consisting of 317 amino acids. The enzyme contains seven beta chains that form a beta sheet at the molecule's core. Additionally, it possesses ten alpha helices, with helix 5, 6, and 10 contributing significantly to the active center's specificity and enzymatic activation. The active center, more precisely, comprises a catalytic triad: Ser105, His224, and Asp187. To maintain its conformation, CAL-B relies primarily on three disulfide bridges: Cys22-Cys64, Cys216-Cys258, and Cys293-Cys311 [71,74] (figure 12).

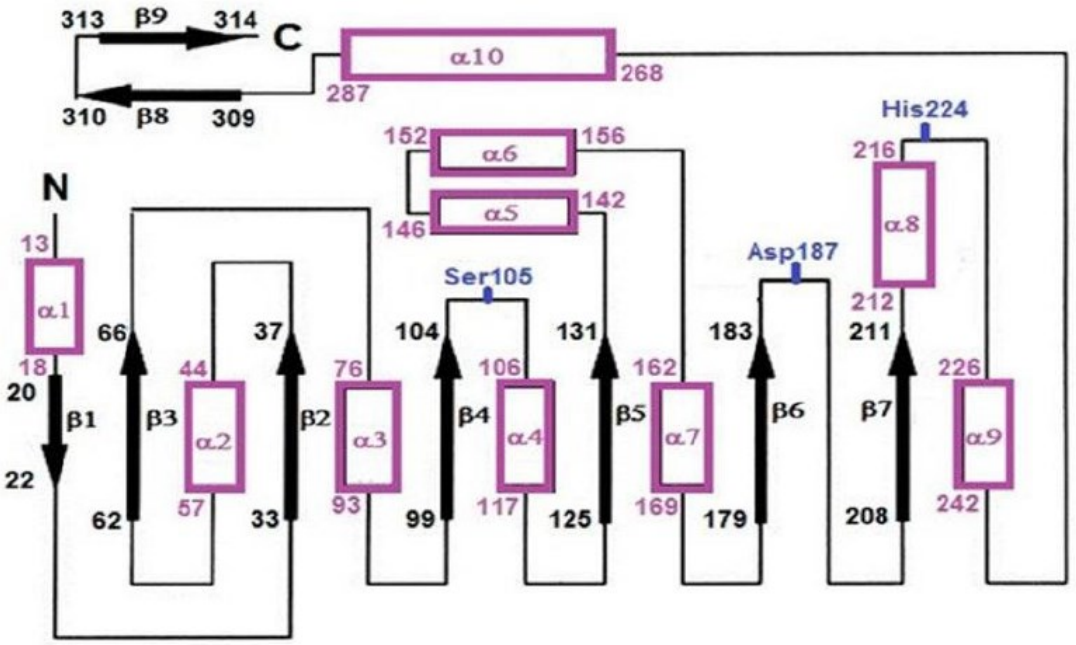


Fig. 12 structure diagram of the secondary structure of lipase Candida antarctica B. Adapted from [71].

6.5 Oxidative mechanism: Baeyer-Villiger

The mechanism for the oxidation of furfural to furoic acid is based on a Baeyer-Villiger reaction targeting a carbonyl group. The process is initiated by introducing ethyl acetate (EtOAc) as the acyl donor. The enzyme CAL-B then catalyzes the oxidation of EtOAc through the addition of hydrogen peroxide (H_2O_2). As a result, peracids are formed, which subsequently oxidize furfural. Ultimately, this sequence of reactions leads to the desired end product, furoic acid [63] (figure 13).

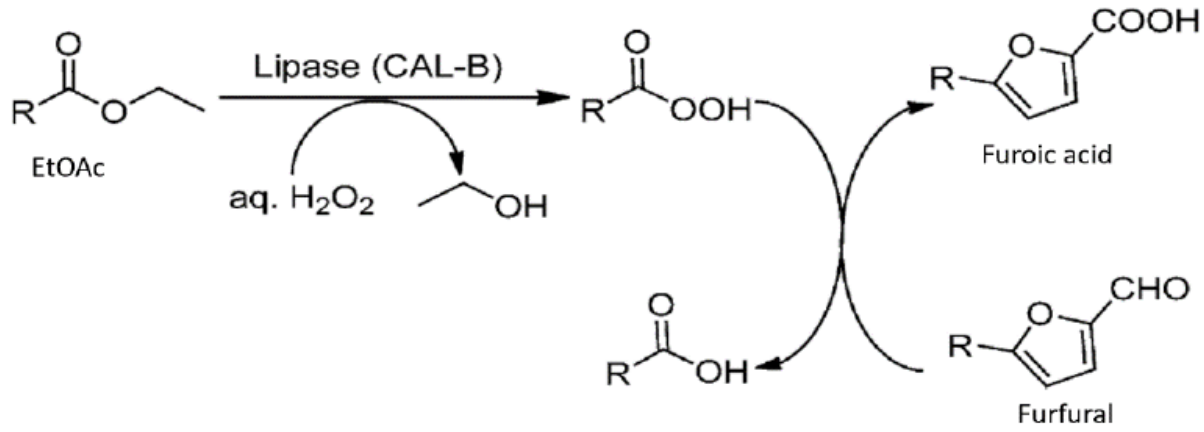


Fig. 13 Oxidative mechanism for the conversion of furfural into furoic acid. Adapted from [46].

The oxidative breakdown follows a two-step reaction mechanism (figure 14):

1) The peroxide group adds to the carbonyl, resulting in the formation of a tetrahedral intermediate.
2) A concerted migration occurs in which one of the carbon atoms adjacent to the oxygen undergoes a shift, leading to the elimination of either a carboxylic acid (when employing a peroxoacid) or an alcohol (when utilizing a hydroperoxide). Consequently, if the migrating carbon is chiral, the stereochemistry remains unchanged.

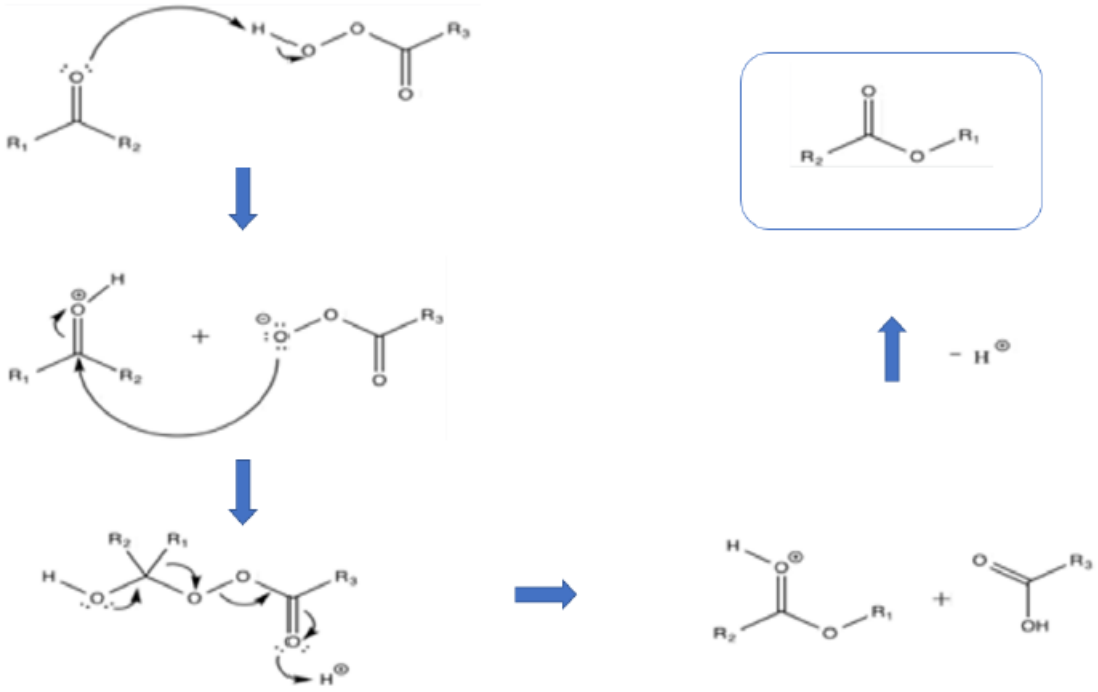


fig. 14 mechanism scheme of the Baeyer-Villiger reaction

The oxidation reaction described presents an opportunity for the targeted oxidation of furans. Specifically, sp2 aldehyde groups within the furan structure exhibit a higher susceptibility to oxidation by peracids, while sp3 alcohols remain unaffected. The enzyme CAL-B facilitates this process by utilizing hydrogen peroxide as a nucleophile in non-aqueous solutions, resulting in the generation of organic peracids [75]. As mentioned earlier, these peracids can subsequently initiate the oxidation of other compounds, such as furfural.

7. EXPERIMENTAL SECTION

The aim of this project is the biocatalytic conversion of bio-based furfural to 2-furoic acid, using CAL-B as biocatalyst. To achieve that, xylan has been used as model-feedstock to obtain furfural through a microwave reactor, testing the optimal hydrolysis conditions (xylan load and acid catalyst load). Optimal conditions of the enzymatic reaction have been previously tested with the use of commercial furfural, to find the optimal substrate load. Therefore, the optimal conditions of both the processes have been applied to the enzymatic reaction, using bio-based furfural, previously treated with activated carbon.

7.1 Study goal

Principal goals:

1. Conversion of xylan into furfural using microwave reactor
2. Demonstration of CAL-B use for the conversion of bio-based furfural into 2-furoic acid.

Specific goals:

1. Investigate the effect of the catalyst load (sulfuric acid) on the hydrolysis of the biomass to obtain furfural, using xylan as model compound.
2. Investigate the effect of the feedstock load on the hydrolysis of the biomass to obtain furfural, using xylan as model compound.
3. Investigate the effect of the furfural load on the furfural oxidation reaction using lipase (CAL-B).
4. Investigate the effect of hydrogen peroxide on the furfural oxidation reaction using lipase (CAL-B).
5. Investigate the recyclability of the lipase on the furfural oxidation reaction.
6. Characterize the kinetics of the lipase on the furfural oxidation reaction.
7. Investigate the effect of bio-based furfural on the furfural oxidation reaction using lipase (CAL-B).

7.2 Chemicals:

Chemical	Company	CAS number
Xylan	Biosynth carbosynth	9014-63-5
Furfural	Sigma-Aldrich	98-01-1
2-furoic acid	Sigma-Aldrich	88-14-2
Ethyl-acetate	Sigma-Aldrich	141-78-6
Tert-butanol	Sigma-Aldrich	75-65-0
D-Fructose	Fisher Scientific	57-48-7
L-Arabinose	Sigma-Aldrich	5328-37-0
D(+)-Galactose	MERCK	59-23-4
Potassium hydroxide	Sigma-Aldrich	1310-58-3
Sodium sulfate Anhydrous	J.T.Baker	7757-82-6
Sulphuric acid	Fisher Chemical	7664-93-9
p-NPP	Sigma Aldrich	1492-30-4
Hydrogen peroxide	Thermo scientific	7722-84-1
Ethanol	Scharlau	64-17-5
1-Propanol	Sigma-Aldrich	71-23-8
Formic acid	Sigla-Aldrich	64-18-6
Levulinic acid	Sigla-Aldrich	123-76-2
Acetic acid	J.T. BAKER	64-19-7

tab. 4 list of all the chemical used for the project

CAL–B enzyme was ceded by Purolite.

7.3 Calibration curve

7.3.1 Furfural

A calibration curve has been used to evaluate the concentration of furfural. For that, 6 solutions have been prepared, knowing the concentration of furfural:

- 0 mM
- 50 mM
- 100 mM
- 200 mM
- 400 mM
- 800 mM

The solution at 800 mM has been prepared using 3,31 ml of furfural for 50 ml of EtOAc. The other solutions have been prepared starting from the previous prepared. The table below shows the solutions preparations (table 5):

mM	Solutions
0	10 ml EtOAc
50	0,625 ml of 50 mM solution + 9,375 ml of EtOAc
100	1,25 ml of 50 mM solution + 8,75 of ml EtOAc
200	2,5 ml of 50 mM solution + 7,5 ml EtOAc
400	5 ml of 50 mM solution + 5 ml of EtOAc
800	3,31 mL of furfurale for 50 ml of EtOAc

tab. 5 resume of the solutions preparation for the furfural calibration curve

The concentrations of the calibration curve have been determined with the use of gas chromatography (Shimadzu GC-2010) and of the software GC-solution (figure 15).

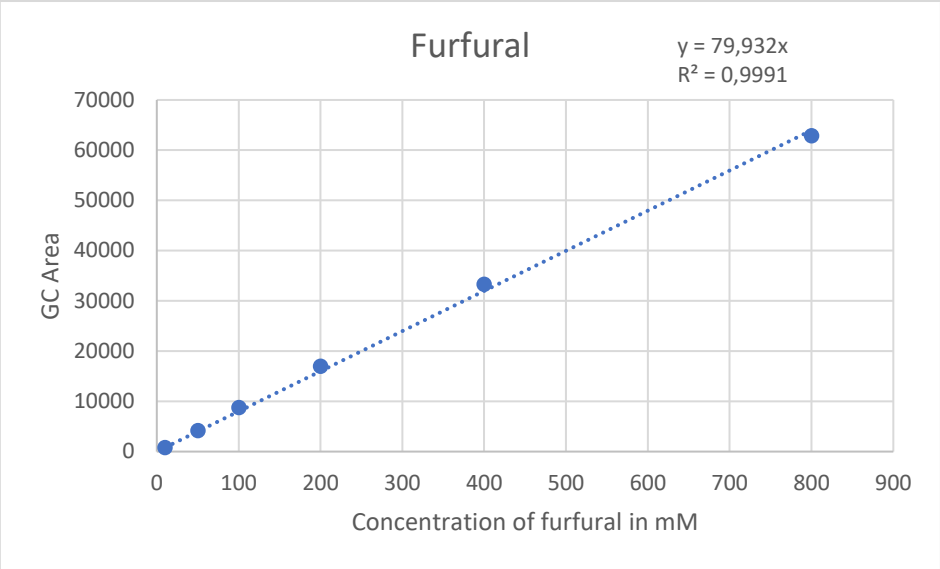


Fig. 15 Furfural calibration curve

7.3.2 Furoic acid

A calibration curve has been used to evaluate the concentration of furoic acid. For that, 8 solutions have been prepared, knowing the concentration of FA:

- 0 mM
- 5 mM
- 10 mM
- 20 mM
- 40 mM
- 80 mM
- 160 mM
- 320 mM

The solution at 80 mM has been prepared using 0,225 g of furoic acid for 25 ml of EtOAc. The other solutions have been prepared starting from the previous prepared. The table below shows the solutions preparations (table 6):

Concentration (mM)	Solutions
0	10 ml of EtOAc
5	0,15625 ml of 320 mM solution + 9,84375 ml of EtOAc
10	0,3125 ml of 320 mM solution + 9,6875 ml of EtOAc
20	0,625 ml of 320 mM solution + 9,375 ml of EtOAc
40	1,25 ml of 320 mM solution + 8,75 ml of EtOAc
80	2,5 ml of 320 mM solution + 7,5 ml of EtOAc
160	5 ml of 320 mM solution + 5 ml of EtOAc
320	0,9 g of FA into 25 ml of EtOAc

tab. 6 resume of the solutions preparation for the furoic acid calibration curve

The concentrations of the calibration curve have been determined using with the use of gas-chromatography (Shimadzu GC-2010) and of the software GC-solution (figure 16).

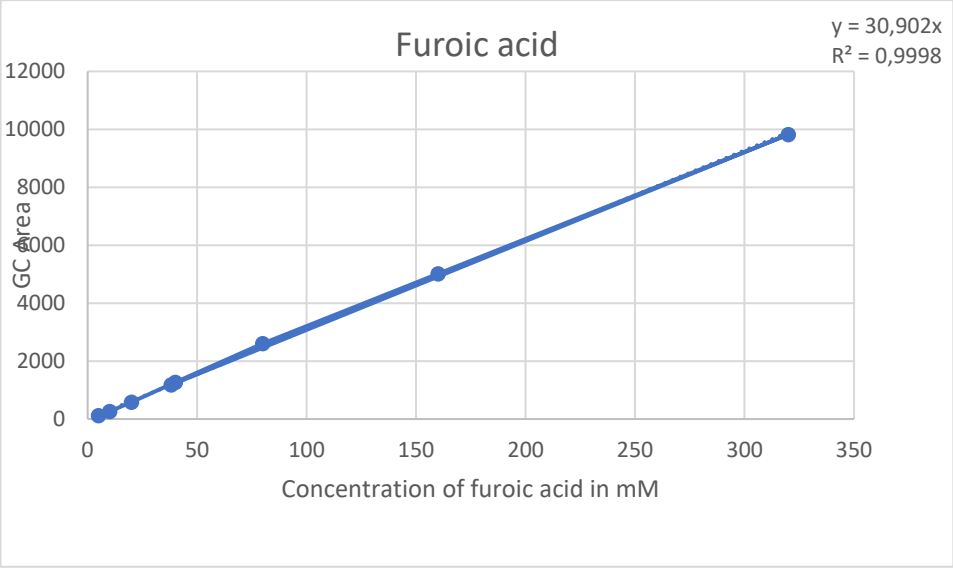


Fig. 16 Furoic acid calibration curve

7.3.3 Sugars

A calibration curve has been used to evaluate the concentration of the sugars that compose the biomass (xylan). For that, 6 solutions have been prepared, knowing the concentration of cellobiose, glucose, xylose, galactose, arabinose, mannose at:

- 0,25 g/L
- 0,5 g/L
- 2,5 g/L
- 5 g/L
- 10 g/L
- 20 g/L

The solution at 20 g/L has been prepared using 2 g of each sugar for 100 ml of pure water. The other solutions have been prepared starting from the previous prepared. The table below shows the solutions preparation (table 7):

Concentration (g/l)	Solutions
0,25	0,125 ml first solution + 9,875 ml pure water
0,5	0,25 ml first solution + 9,75 ml pure water
2,5	1,25 ml first solution + 8,75 ml pure water
5	2,5 ml first solution + 7,5 ml pure water
10	5 ml first solution + 5 ml pure water
20	100 ml = 2 g/l because we use 100 ml

tab. 7 resume of the solutions preparation for the sugars calibration curve

The concentrations of the calibration curve have been determined using with the use of HPLC (figure 17,18,19,20,21,22).

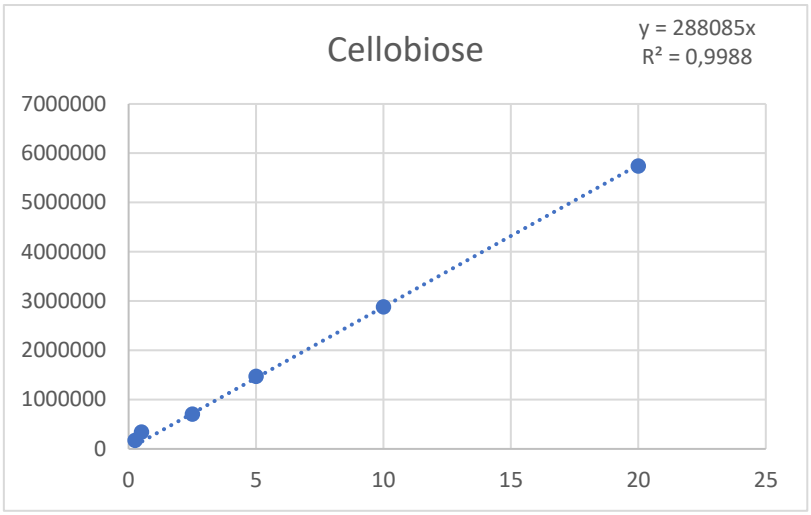


Fig. 17 cellobiose calibration curve

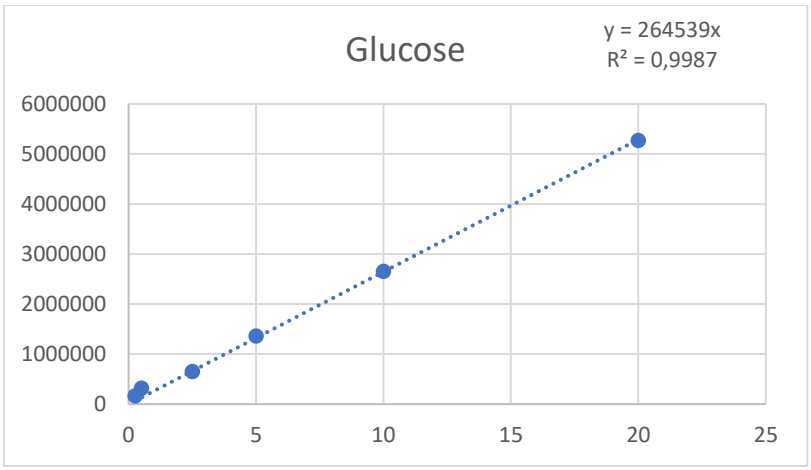


Fig. 18 glucose calibration curve

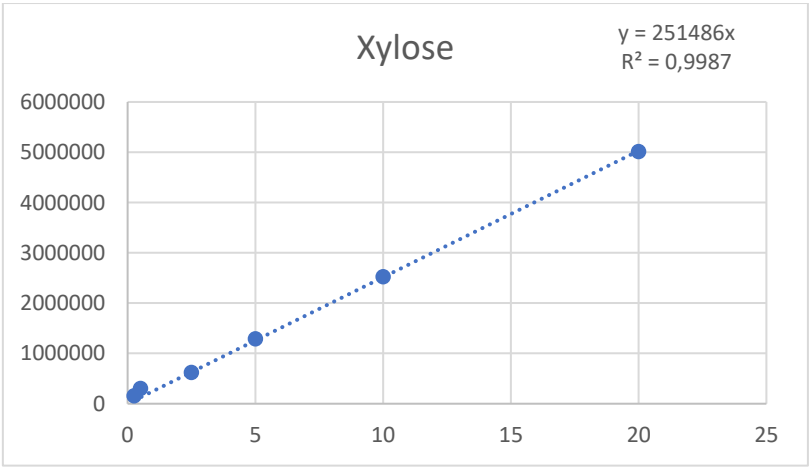


Fig. 19 xylose calibration curve

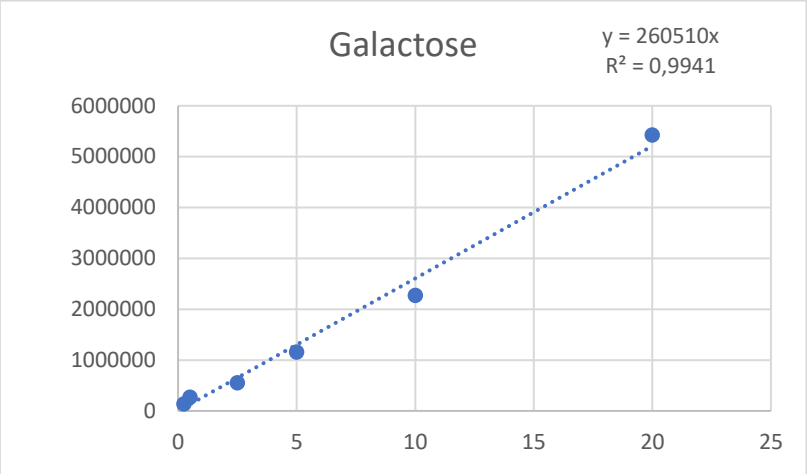


Fig. 20 galactose calibration curve

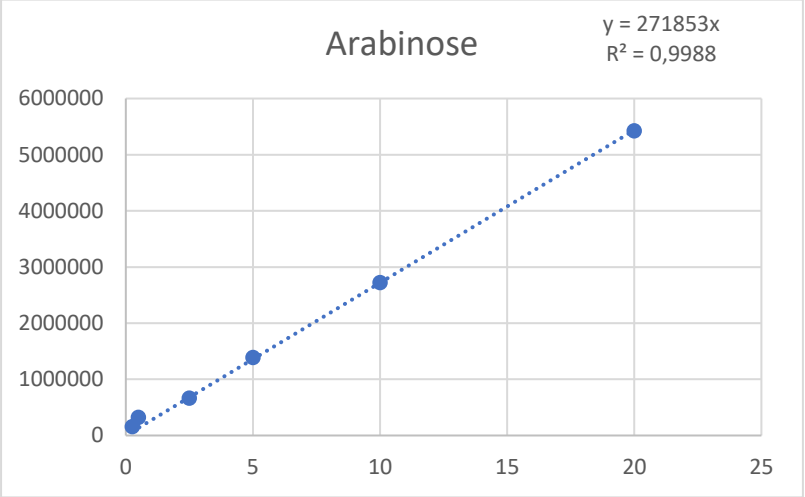


Fig. 21 arabinose calibration curve

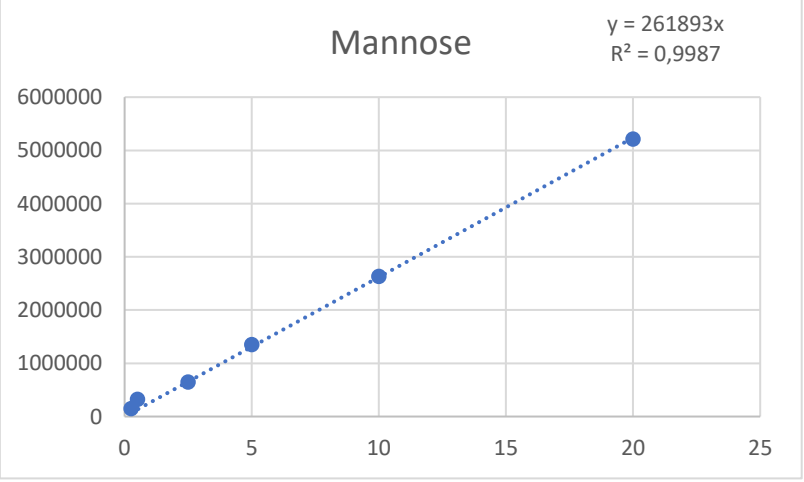


Fig. 22 mannose calibration curve

7.4 Compositional analysis of xylan:

In order to understand the composition of the xylan used, the analysis of it was done according to the National Renewable Energy Laboratory (NREL) method [76].

The following solution was added in a tube and then incubated at 30°C for 1 hour:

- 0.3 g of xylan
- 3 mL of 72% (w/w) H2SO4

In the while, the mixture was stirred every 10 minutes.

Following an hour, the acid concentration was reduced to 4% by incorporating 84 mL of deionized water. Subsequently, the samples were subjected to autoclaving at 121 ºC for one hour, and after cooling down, the caps were removed.

The samples obtained were collected into 2 ml vials and analyzed by HPLC analysis.

7.5 Obtention of furfural from xylan: microwave reaction

Furfural production from xylan was adapted from Mittal et al. [77].

The conditions used were:

- H_2SO_4 as catalyst
- 170 °C
- 20 min
- xylan as feedstock
- water:EtOAc → 1:2

In glass reactor tube were added, in this order:

- xylan
- 5 ml of diluted acid
- 10 ml of EtOAc

to create a biphasic system.

The microwave reactor used for the experiment was the synthWAVE Microwave Synthesis System, manufactured by Milestone in Sorisole, Italy. The reaction tube was placed inside this reactor. Various reaction conditions were examined, including different xylan loads (8w%, 4w%, and 2w%) and acid concentrations (0.05 M, 0.125 M and 0,250 M). The reaction took place under a pressure of 30 bar, with continuous stirring. After the completion of the reaction, the glass reactor was removed from the microwave and allowed to cool to room temperature.

Then, the solution has been filtrated using a syringe filter nylon 0,45 microl and eventually neutralized with a 2 M KOH solution. The organic phase and acquous phase have been separated with the use of a glass funnel. The acquous phase has been analyzed through HPLC analysis, the organic phase through GC analysis.

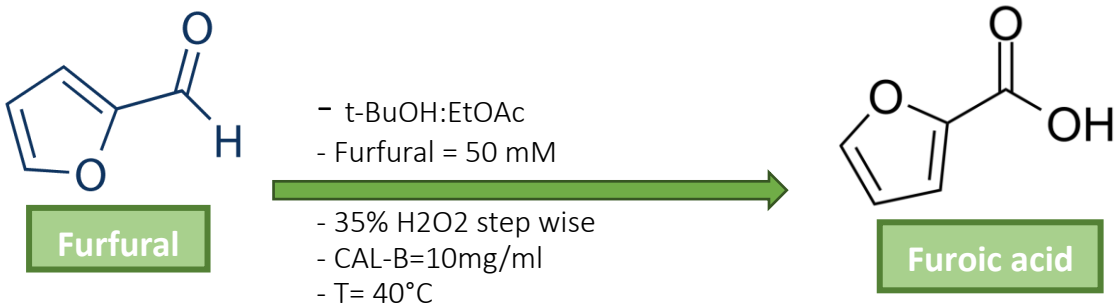
To use the resulting furfural for the enzymatic reaction, the organic phase has been properly diluted with EtOAc to arrive at the desired concentration.

7.6 Enzymatic reaction of furfural to 2-furoic acid: commercial furfural

7.6.1 Enzymatic reaction of furfural to 2-furoic acid

Furoic acid production from furfural, using CAL-B as biocatalyst, was adapted from Krystof et al. [63].

The operational conditions proposed are:



In a 50 ml round bottom flask on top of a cork support, 5 ml of tert-BuOH, 5 ml of EtOAc, 42 µl of furfural (using a micropipette) and 0,1 g of CAL-B (weighed on the analytical balance) were added.

A magnetic stirrer was also added into the round bottom flask and closed with a tight stopper to prevent evaporation. The bottom flask was submerged halfway into a container filled with liquid glycerin; in this way, the reaction temperature has been kept constant during all the reaction time.

The reaction has been carried out on top of a hot plate, with temperature of 40°C and 410 rpm stirring.

After one hour, 82,4 µl of 35% hydrogen peroxide was added, to start the reaction. Each hour that elapsed, the same amount of H_2O_2 was added during the first 6 hours. After 6 hours, the reaction was kept at temperature and constant stirring for 24 hours.

At the end of the reaction, a sample was extracted using a syringe and then filtering the sample using a syringe filter nylon 0,45 µl. The sample was collected into a vial of 2 ml for the GC analysis (Shimadzu GC-2010) using a TRB-5 column (Teknokroma, TR120232; length: 30 m, film thickness: 25 µm; inner diameter: 0.25 mm). The temperature program consisted of a first step at constant temperature (50 °C for 2 minutes), a second linear increase in temperature (from 50 ºC to 300 ºC in a gradient of 40 ºC/min) and a third step at constant temperature (300ºC for 7 minutes).

Furfural has a retention time of 3 minutes, furoic acid of 8 minutes.

7.6.2 Enzymatic reaction of furfural to 2-furoic acid: furfural load effect

Aiming to search for the optimal conditions, it has been of relevant importance to test the oxidation reaction changing the furfural load. The same reaction has been replicated, keeping constant all the other parameters (solvent ratio, H_2O_2 load, enzyme concentration, temperature, stirring, time) in the following furfural loads (table 8):

Conc of FF (mM)	Quantity (µl)
25	20,7
40	33,13
50	42
100	84
200	168
400	336
800	672

tab. 8 furfural load change to test the effect of the substrate concentration

After 24 hours, the samples have been collected using the same procedure and analyzed for a GC analysis, though the use of the same column and method.

7.6.3 Enzymatic reaction of furfural to 2-furoic acid: H_2O_2 load effect

With the intention to understand the H_2O_2 effect on the enzyme reaction, it holds considerable relevance to repeat the oxidation reaction changing the H_2O_2 load. The same reaction has been replicated, keeping constant all the other parameters (solvent ratio, furfural load, enzyme concentration, temperature, stirring) but choosing the optimal one as furfural load (40 mM), in the following H_2O_2 loads (table 9):

Conc of H_2O_2 (mM)	**Quantity (μl)**
0,41	41,2
0,82	82,4
1,64	164,8
3,28	329,6

tab. 9 H_2O_2 load change to test the effect of the oxidation effect.

After 24 hours, the samples have been collected using the same procedure and analyzed for a GC analysis, though the use of the same column and method.

7.6.4 Recyclability of the enzyme

In an effort to realize if the recyclability of the enzyme is possible, always the same reaction has been performed, keeping the conditions as before:

- Fufural: 50 mM
- Temperature: 40°C
- EtOAc: 5 ml
- T-buOH: 5 ml
- CAL-B: 0,1 g
- H2O2 35%: 6 x 82,4 microl
- Time: 24 h

When the first cycle finished after 24 hours, the solution has been separated from the enzyme using a syringe; a sample has been collected, filtrating it with the help of a nylon filter and analyzed by GC. Then, the enzyme has been washed using a buffer phosphate solution (pH 7); to have 200 ml of 50mM buffer phosphate solution, 1,74 g of potassium phosphate dibasic has been added to 200 ml of distillated water.

The instruments used to wash the enzyme are a glass funnel, an Erlenmeyer, and a paper filter 25μm . When the enzyme was dry, it has been collected into a viale and kept all night into a fridge at 4°C.

The next day, a new cycle was performed with the washed enzyme and using again the same conditions.

Until cycle number 4° the recycle has been repeated, using the same conditions, washing solution and all the samples have been analyzed by GC using the same column and the same method.

7.6.5 Enzymatic kinetics characterization

With the aim to characterize the lipase CAL-B, using the Michaelis Menten model, the oxidation reaction with the lipase has been carried out changing the reaction time at 40 mM of furfural load:

- 12 h
- 24 h
- 48h

The reaction time has been chosen considering the Michaelis Menten plot from the results of this analysis, it has been chosen a time of 17h to carry out the same enzymatic reaction but changing the furfural load as following (table 10):

Concentration of FF (mM)	**Quantity of FF (μl)**
15	12,42
20	16,56
40	33,13
60	49,68
80	66,26
100	82,8
150	126
200	164,8

tab. 10 Furfural load changing at 17h

All the other parameters have been the same.

After 17 hours, the samples have been collected using the same procedure and analyzed for a GC analysis, though the use of the same column and method.

7.7 Enzymatic reaction of furfural to 2-furoic acid: bio-based furfural

The experiment has been replicated using xylan-based furfural, applying all the optimal conditions obtained by the experiments performed with commercial furfural. In this case, the furfural was already present in the EtOAc solution.

It has been state that, the use of activated carbon are able to filtrate undesired products acid-pretreated lignocellulosic substrate [78]. For this reason, at the furfural solution in EtOAc from microwave reaction has been added 1w% of activated carbon, to filtrate the humins present in the solution that are formed during the hydrolysis of the xylan . The solution has been shacked for 1 minute and then filtered using a syringe filter nylon 0,45 microl.

Later, to obtain the proper concentration of furfural, the furfural solution in EtOAc has been diluted with the appropriate quantity of EtOAc.

Briefly, the conditions are:

- 5 ml of EtOAc with furfural
- 5 ml of t-BuOH
- 0,1 g of CAL-B
- H2O2 35%: 6 x 82,4 microl
- 40 °C
- stirr 410 rpm

After 24 hours, the samples have been collected using the same procedure and analyzed for a GC analysis, though the use of the same column and method.

7.8 Enzyme activity assay

The lipase activity was assessed according to the procedure described by Qin et al., with some modifications [79].

0,1 mL of 60 mM p–NPP dissolved in isopropanol was added to 0,6 mL of phosphate buffer (50 mM, pH 7.5); then, the lipase was added, and the mixtures were incubated at 45 °C for 15 min. The reaction was terminated by adding 5,3 mL of ethanol. Two controls, one with pure enzyme and another one without enzyme were run simultaneously. This was a qualitative assay, so the lipase activity was observed by eye. If no change of color was appreciated, the enzyme was considered inactive.

8. RESULTS AND DISCUSSION

8.1 Compositional analysis of biomass

With the aim to understand the conversions and the yields obtained by the different experiments performing the hydrolysis of xylan using a microwave reactor, before it is necessary to understand the composition of the feedstock: (Figure 23):

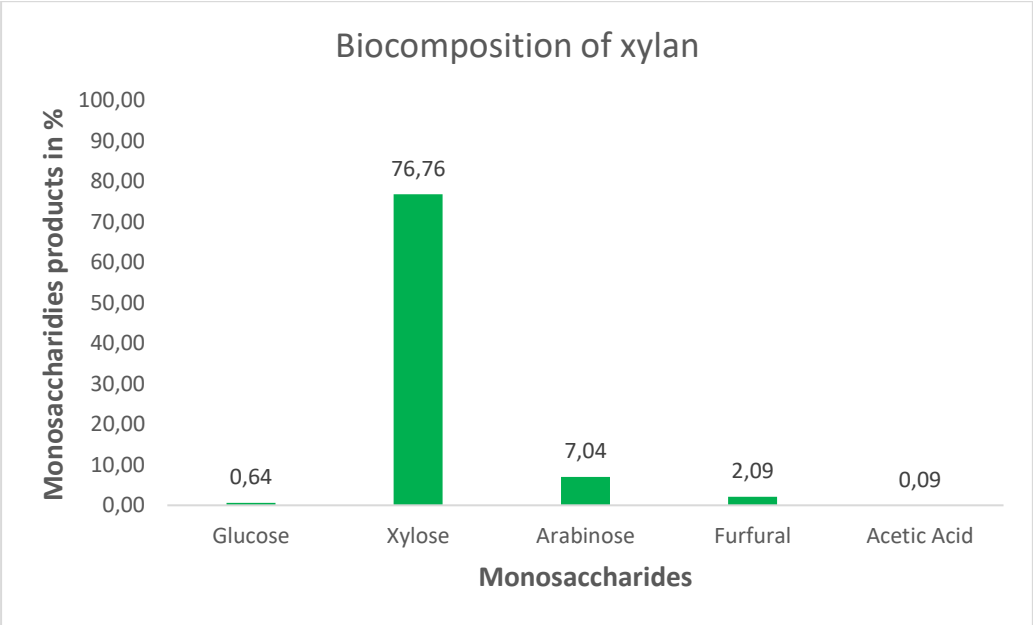
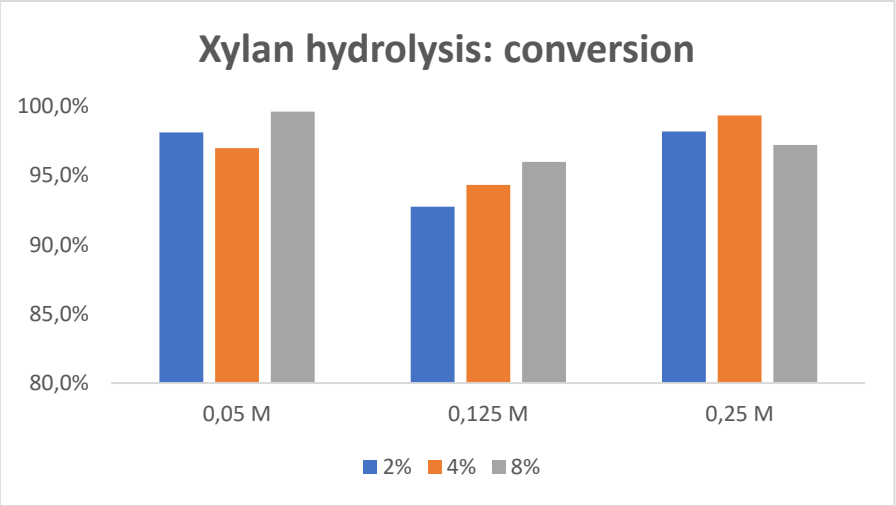


Fig. 23 biocomposition of xylan

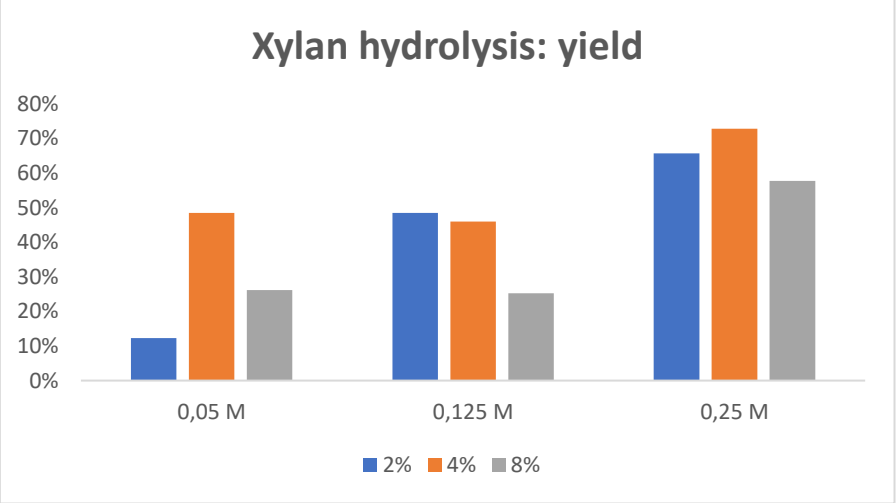
As it could be expected, xylose corresponds to the majority of the composition of the feedstock. For this reason, xylan can be considered as a good model for the extraction of furfural from the biomass.

8.2 Microwave reaction

A series of experiments for the hydrolysis of xylan, changing the xylan load and catalyst load, have been performed and then the samples obtained from the different experiments have been analyzed using a GC analysis (Figure 24):



(a)



(b)

Fig. 24 Resulting conversions and yields of microwave reaction of xylan, changing xylan load and acid catalyst load

Considering the conversions (figure 24.a) the results are always very high. The minimum conversion is obtained at 2% load of xylan and 0,125M of sulfuric acid (92,8%) and the higher is at 8% xylan load and 0,05M of sulfuric acid (99,6%).

About the yields (figure 24.b), considering the experiments with a sulfuric acid load of 0,05 M, the best yield is obtained at 4% load of xylan (48%). In the case of 0,125M of catalyst, the best yield is at 2% load of feedstock (48%), even if the difference with the 4% case is very low (46%). Again, with 0,25 M of sulfuric acid, the best condition is at 4% of xylan. Consequently, there is the same

trend at 0,05 M and 0,25 M; on the other hand, with 0,125 M, there is a decrease in the yield increasing the xylan load.

Between all the conditions, the highest yield (73%) is obtained with the conditions of 0,25 M of sulfuric acid and 4% load of xylan.

As it can be seen, using 8% of feedstock, the yield is always lower with respect to the case at 4%. It can be explained by the fact that the catalyst load is too low to properly convert the xylan into furfural. But increasing continuously the catalyst concentration is not a proper strategy, because increasing it there is also the increase of undesired reactions, like that producing humins. Considering this, it has been preferred to test the hydrolysis of xylan using a microwave reactor in that range of acid catalyst concentration.

Both production of furfural and humins are strongly influenced by the temperature; in particular, humis are highly produced exaggerating with the temperature. Therefore, a temperature of 170°C has been kept [60].

8.3 Enzymatic reaction of furfural to 2-furoic acid: furfural load effect

A series of experiments for the oxidation of furfural into furoic acid, changing the solvent, have been performed and then the samples obtained from the different experiments have been analyzed using a GC (Figure 25):

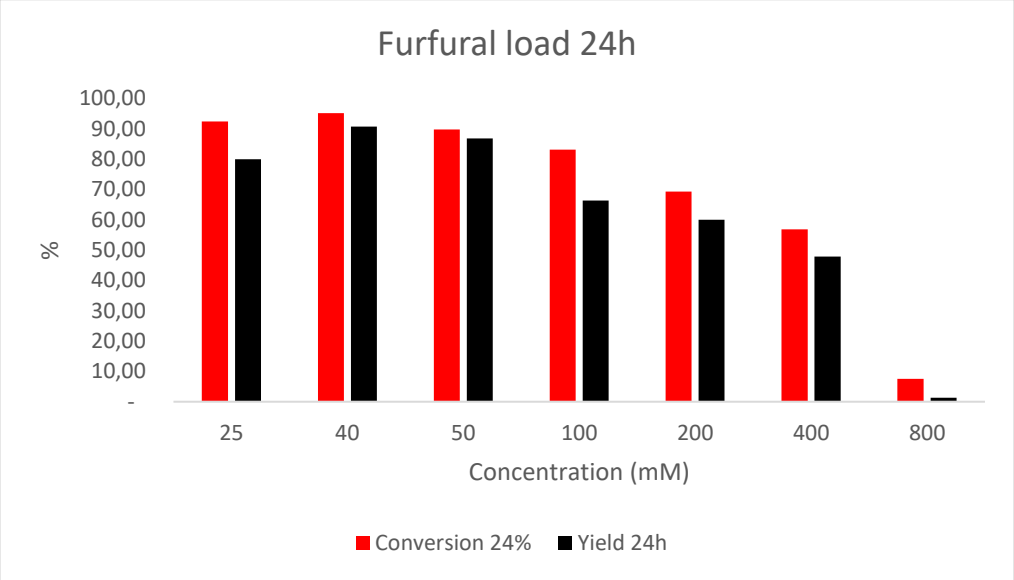


Fig. 25 Resulting conversions and yields changing the furfural load

As it can be seen from the graph, from 25 mM to 40 mM an increase in both conversion and yield is observed. Then, from 50 mM to the last condition at 800 mM, both conversion and yield continuously decrease; in particular, in the last case both are particularly low, respectively 7,54% and 1,26%. It indicates that there is a maximum of furoic acid production at 40 mM, with a conversion of 95,04% and a yield of 90%, keeping H_2O_2 and CAL-B concentration constants. For this reason, 40mM of furfural load can be considered as an optimal value for the production of furoic acid from furfural.

For this reason, a substrate inhibition could be present, due to the decrease in both conversion and yield, increasing the furfural load.

To better understand what is happening, it can be useful to see exactly how much furoic acid is produced from each condition (table 12):

FF load (mM)	yield of FA%	FA concentration (mM)
25	80%	19,97
40	91%	36,27
50	87%	43,39
100	69%	69,24
200	60%	120
400	48%	191,16
800	1,3%	10,08

Tab. 12 Resulting FA produced changing the furfural load

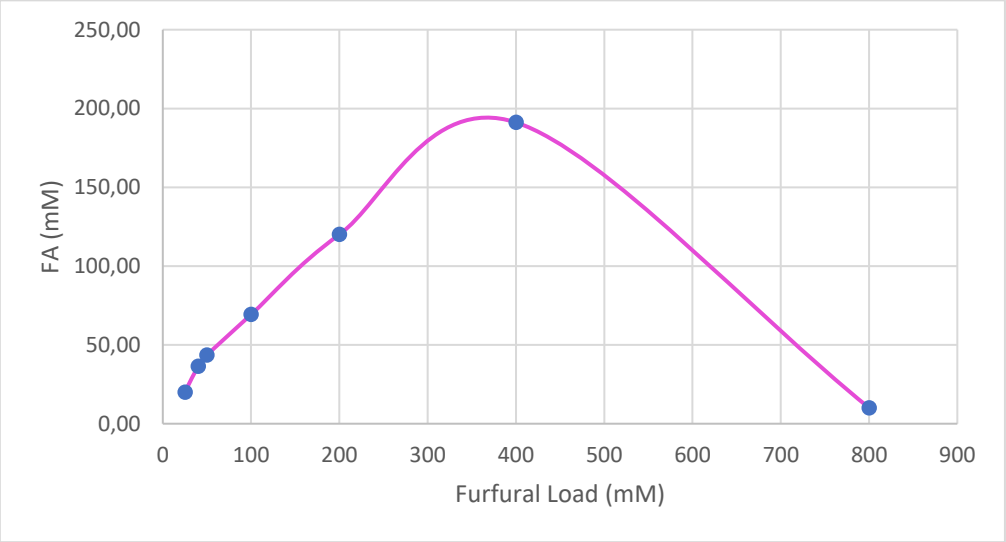


Fig. 26 Resulting FA produced changing the furfural load

It is noticeable that the enzyme is still working well at higher amount of furfural loads. In particular, at 400 mM there is a production of 191,16 mols of furoic acid. Again, 800 mM is the worst condition, with the production of 10,08 mol of furoic acid.

Then, it can be considered the amount of furfural that is converted in the different conditions:

FF load (mM)	FF converted (mM)	Conversion of FF
25	20,29	92,34%
40	33,44	95,09%
50	41,68	89,72%
100	80,44	83,07%
200	129,64	69,28%
400	208,22	56,81%
800	52,13	7,54%

Tab. 13 Resulting FF converted changing the furfural load

Taking into account the case at 25mM, it can be seen that 20,29 mM of furfural are converted; at 50mM, it can be seen that 41,68 mM of furfural are converted: more or less the double. In this case, doubling the initial concentration of substrate, it is double also the substrate that is converted. Again, passing from 50mM to 100mM, also in this case the furfural converted is almost the double. The situation is different arriving at 200mM of initial substrate concentration: the enzyme begins to not double it anymore, the same considering 400mM of furfural. A drastic decrease in furfural converted is present at 800mM. It could be expected that the concentration of converted furfural could not increase always, because the amount of enzyme is constant, therefore the enzymatic activity is limited to a certain conversion. But the furfural converted is not even constant at very high initial furfural concentrations. It could mean that there is a substrate inhibition starting from 200mM.

8.4 Enzymatic reaction of furfural to 2-furoic acid: H_2O_2 load effect

A series of experiments for the oxidation of furfural into furoic acid, changing the hydrogen peroxide load, have been performed and then the samples obtained from the different experiments have been analyzed using a GC (figure 27):

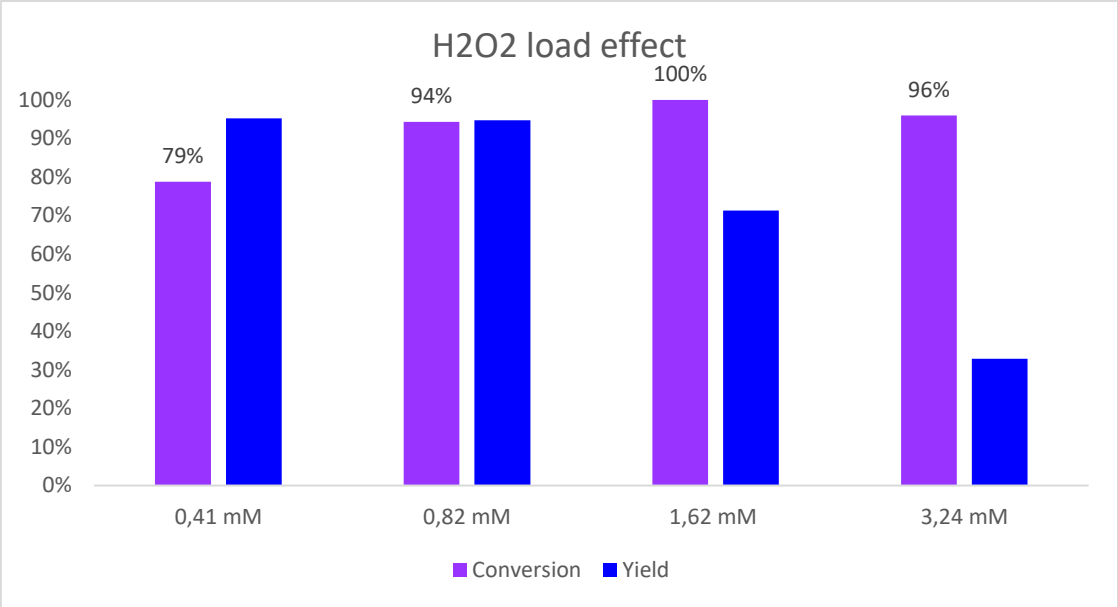


Fig. 27 H_2O_2 changing load effect

As it can be seen, from the minimum concentration of H_2O_2 and the last one, there is an increase in the conversion. In particular, from 0,82 mM and on, it is always very high (94%-100%) and more or less constant. But, even if the conversion is almost very high, from the 0,41 mM to the 3,24 mM there is a decrease of the yield in furoic acid: it is appropriate conclude that the enzyme is working, so no oxidant inhibition is present, but it is producing something different from furoic acid.

8.5 Enzyme recyclability

To better understand the possibility to scale up at industrial scale the production of furoic acid through the oxidation of furfural using CAL-B as biocatalyst, the recyclability has been tested, performed a series of reactions always using the same enzyme from the first to the last cycle. Then, the samples obtained from the different experiments have been analyzed using a GC analysis.

Two main conditions can be considered:

1) without washing the enzyme (Figure 32):

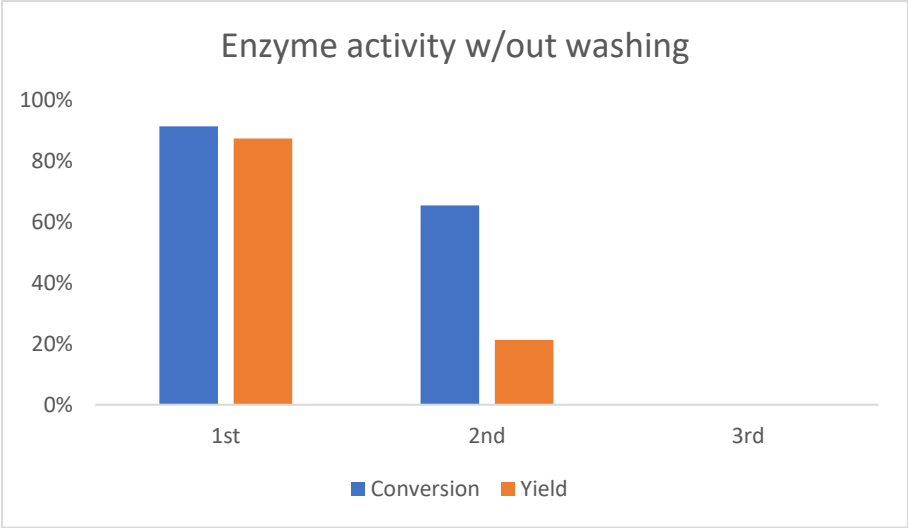


Fig. 32 Resulting conversion and yield from enzyme recyclability without washing step

2) using a buffer phosphate solution to wash the enzyme (figure 33):

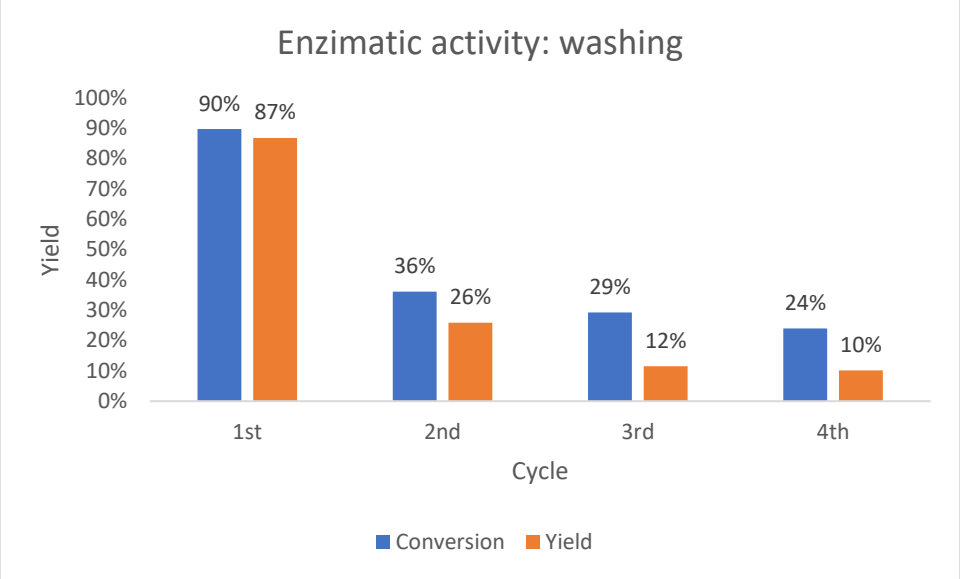


Fig. 33 Resulting conversion and yield from enzyme recyclability with washing step

About the first case, there is a decrease in both conversion and yield from the first to the second cycle, passing from 91% to 60% for the conversion and from 87% to 20% for the yield. At the third cycle, the enzyme was already completely disactivated.

About the second case, as it is possible to notice, passing from the first cycle to the second cycle, an impressive decrease in both conversion and yield are present. In particular, in the first cycle has

been obtained a yield of 87%, in the second cycle the yield already decreases at 26%. But differently from the previous case, the enzyme is still active at the end of the second cycle, demonstrating the necessity of a washing step between one cycle and another. From the second cycle to the last one, the decrease in both conversion and yield continues but with lower loss; the last cycle reaches a conversion value of 24% and a yield value of 10%.

After each cycle, the enzyme was firstly analyzed through the enzyme activity assay and then washed, to check the possibility to continue with the recyclability.

To explain the loss in conversion and yield , two points of view can be considered:

1) after each cycle, part of the enzyme is not immobilized anymore, due to the stirring that is needed to have the mechanocatalysis. The stirring is absolutely indispensable to obtain a better contact between the enzyme and the substrate, but at the same time there is a leaching process. This is appraisable due to the less presence of enzyme after the reaction (figure 34b) and for an increase of turbidity in the reaction medium (figure 34a).

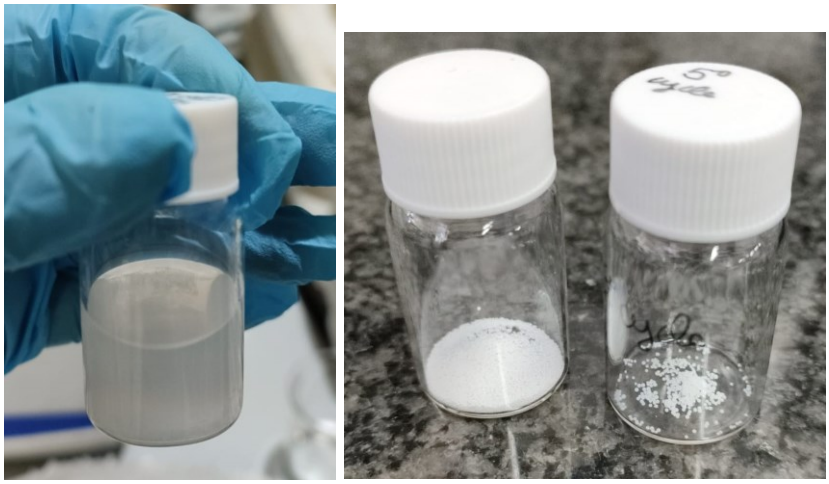


Fig. 34 Due to the enzyme leaching, there is the formation of turbidity in the reaction medium (a) and therefore reduction of the enzyme quantity (b)

2) enzymes, being protein, tends to denature with the time, due to the constant heat during the reaction and the increasing presence of the furoic acid during the reaction.

For all the explanations above, it can be stated that CAL-B cannot be properly recycled using the previous conditions. To perform a recycle process, the values in both conversion and yield should be higher.

8.6 Enzymatic kinetics characterization

With the aim to appropriately characterize the kinetics parameters of CAL-B in the oxidation of furfural into furoic acid, it has been performed a series of experiments at 40 mM of furfural changing the time: 12h, 24h and 48h (figure 28).

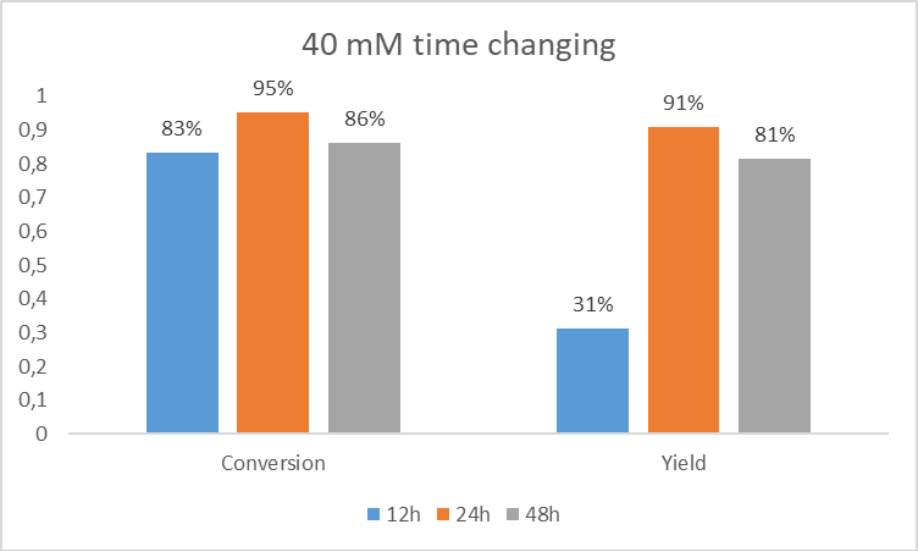


Fig. 28 Time changing effect at 40 mM of substrate

Both best yield and conversion are obtained at 24h. The decrease at 48h is probably due to the degradation of both furfural and furoic acid after too much time.

To thoroughly comprehend the kinetics parameters, it is necessary to plot the amount of furoic acid produced over the time:

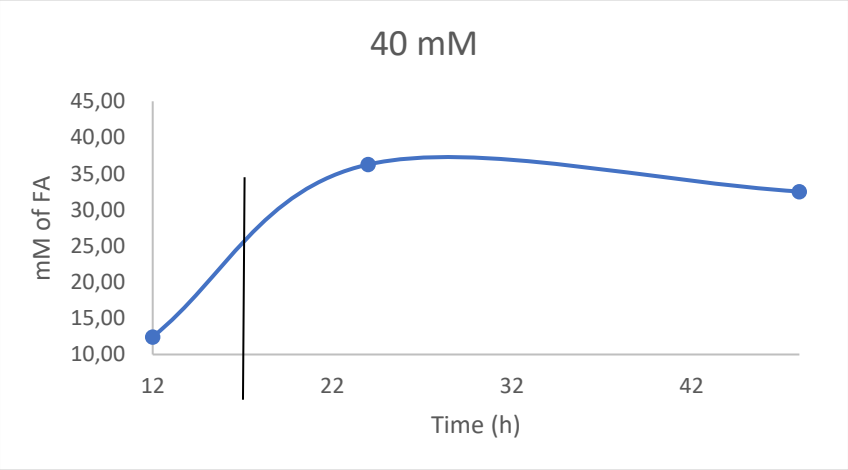


Fig. 29 Effect of the time changing with 40 mM of substrate

From this graph, it can be chosen a value on the tangent, in this case 17h. This has been the time taken for the next experiments, to understand the beginning velocity of the reaction, changing substrate load (Figure 30):

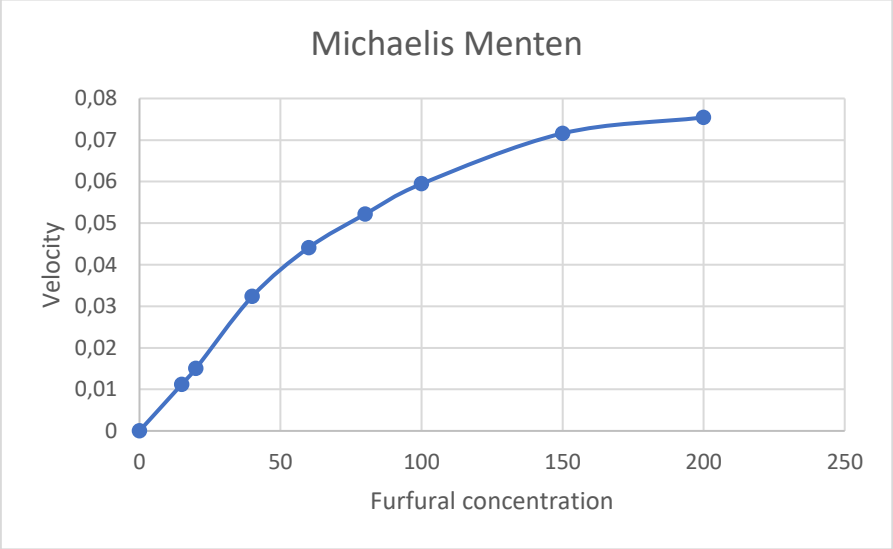


Fig. 30 Michaelis Menten graph changing the furfural load, at 17h reaction time

To evaluate the kinetics parameters (K_m and v_{max}) it is necessary to make the linearitation of the Michaelis Menten plot (figure 31):

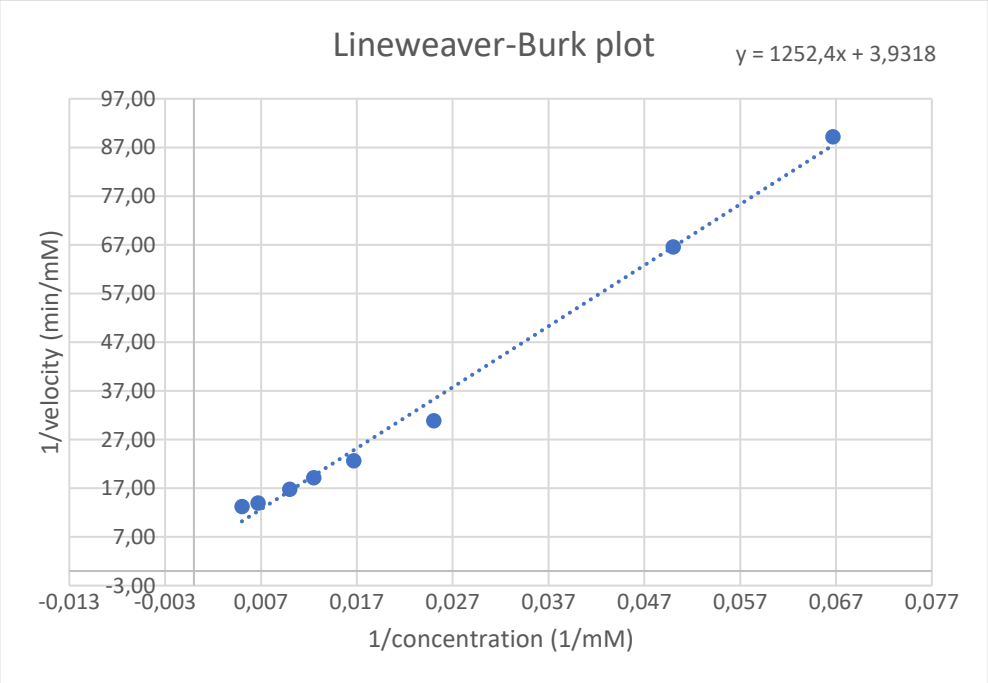


Fig. 31 Lineweaver-Burk plot

Considering the intersection with the x axis and y axis, the resulting values are:

v_{max}	0,25 mM/min
K_m	318,5 mM

Tab. 14 kinetcis parameters of the oxidation reaction of furfural to furoic acid

8.7 Enzymatic reaction of furfural to 2-furoic acid: bio-based furfural

A series of experiments for the oxidation of furfural into furoic acid, using bio-based furfural, have been performed and then the samples obtained from the different experiments have been analyzed using a GC analysis (Figure 35).

Stated that the best result in furfural yield from the hydrolysis of xylan is at:

- 0,250 M of sulfuric acid
- 4% of xylan

With the aim to analyze what happens changing the acid load but keeping the feedstock constant, also the hydrolase from these conditions has been used:

- 0,125 M of sulfuric acid
- 4% of xylan

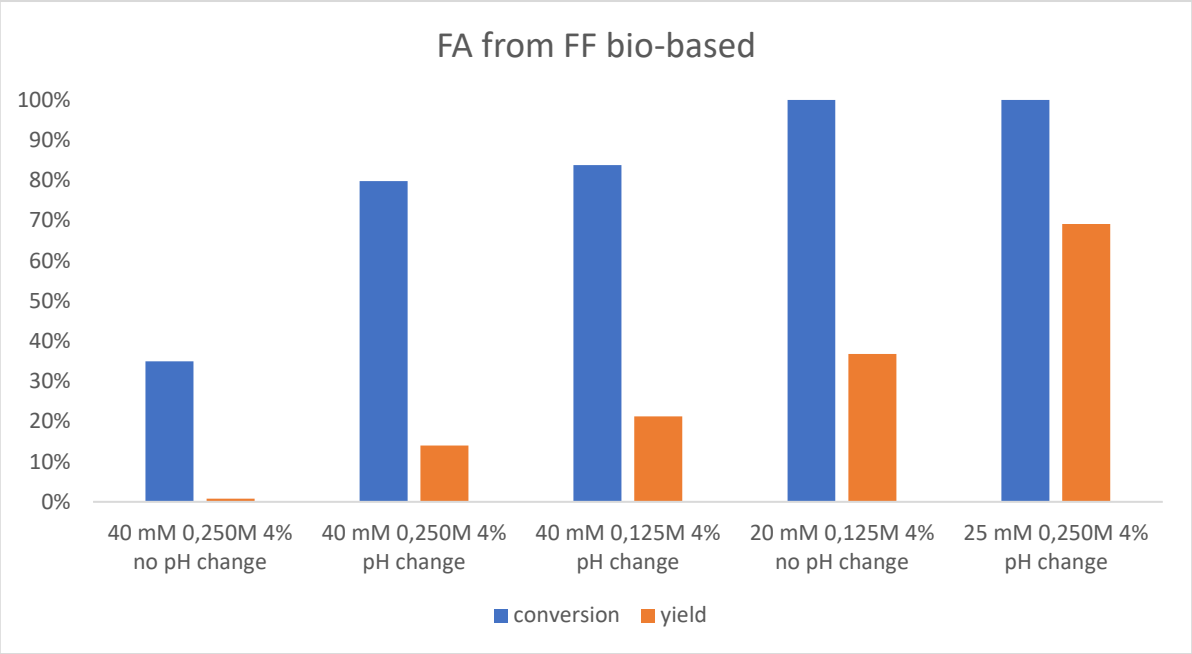


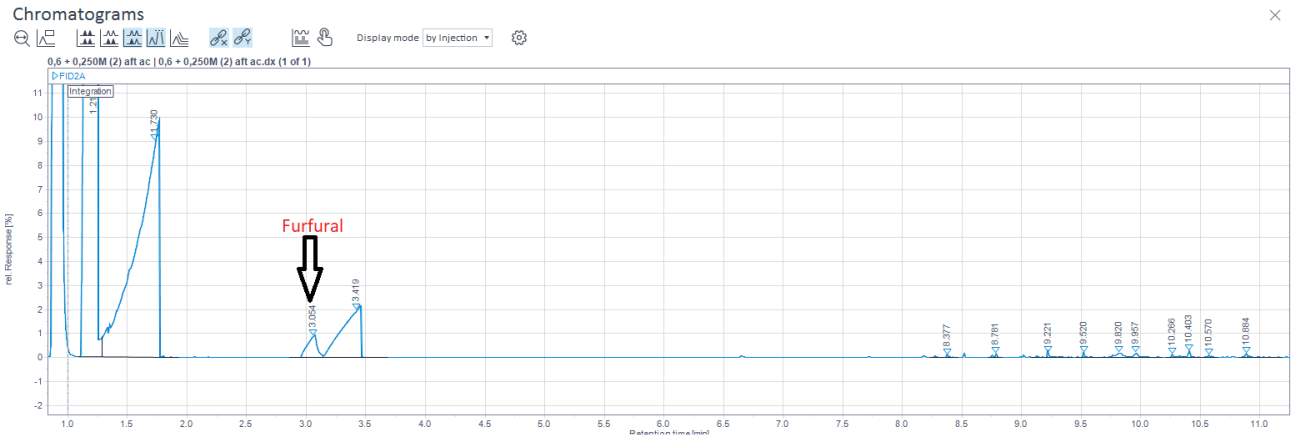
Fig. 35 Resulting conversion and yield from bio-based FF into FA, changing pH and FF load

For the treatment of the bio-based furfural, 1w% of activated carbon has been used and the solution has been shaked for 1 minute and then filtered; such low quantity of activated carbon and treatment time have been chosen because it can cause a reduction not only in humins concentration, but also in furfural concentration. Adopting such strategy results in furfural concentration loss of between 1-5% (table 15) and the enzyme results still active after each experiment performed.

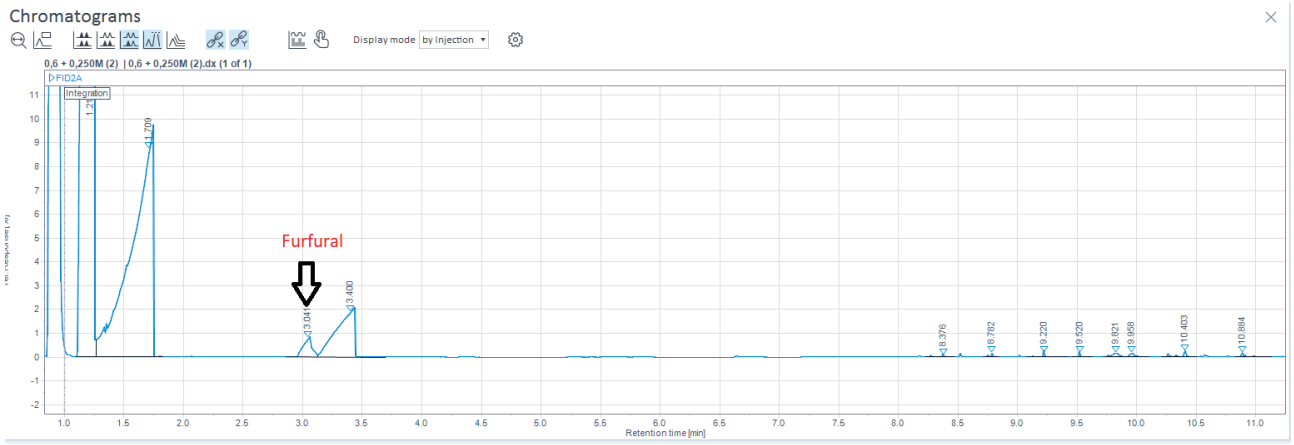
xylan %	H2so4 load	FF in mM before treatment	FF in mM after treatment	FF loss
4%	0,125	102,7179352	97,58	5%
4%	0,250	168,3958615	166,49	1%

Tab. 15 activated carbon treatment effect on the FF loss

For example, below are reported the gas chromatographs related to the case: 4% xylan load and 0,250M of sulfuric acid, before and after treatment:



(a)



(b)

Fig. 36 Gas cromatographs related to the case: 4% xylan load and 0,250M of sulfuric acid, before and after treatment. The area of FF before (a) is almost the same of that after the treatment (b).

Referring to Figure 35, the first two experiments have been performed basing on the optimal conditions found in the previous experiments:

- 0,250 M of sulfuric acid, 4% load of xylan
- 40 mM of commercial furfural

Firstly, the hydrolyzed obtained has not been neutralized. As a result, both conversion and yield are extremely low (35% and 0,8%). Then, neutralizing the solution, both conversion and yield have better results (79,79% and 14,03%).

A better result is obtained changing the furfural concentration at 25 mM, with a yield of 69,16% (Figure 37).

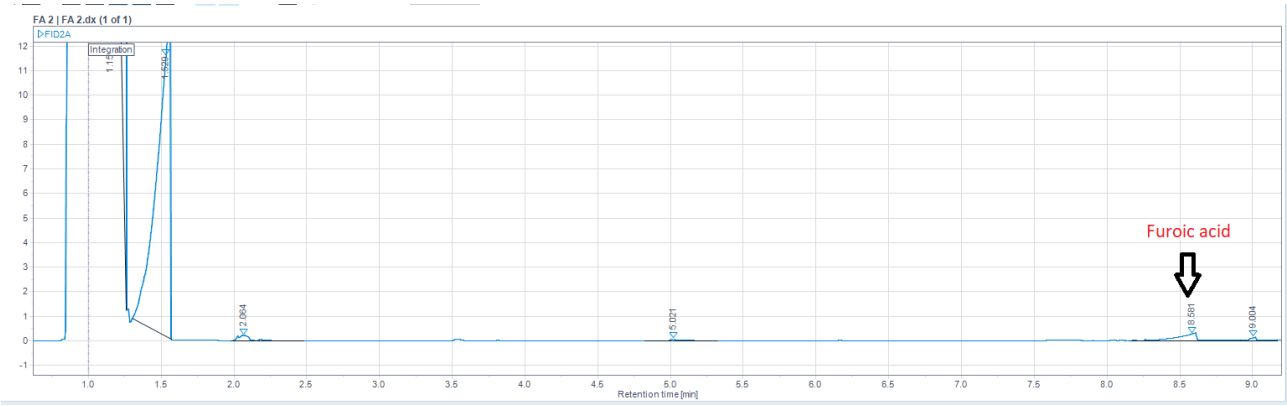


Fig. 36 Gas cromatographs related to the case: 4% xylan load, 0,250M of sulfuric acid, 25 mM FF, neutral pH.

Other experiments have been performed using the furfural bio-based from the hydrolysis with 0,125 M of sulfuric acid, keeping constant all the other parameters. It has been tested with 40 mM and 20 mM of furfural, reaching better results in the second case.

These results can be explained considering that a higher concentration of sulfuric acid produces more furfural but also humins. The resulting furfural of the hydrolysis reaction, to be properly used for the reaction with the enzyme, has been diluted with pure ethyl acetate, to reach the desired furfural concentration. Diluting more, to arrive at 20 or 25 mM of furfural concentration, results also in humins dilution. Probably, for this reason the enzyme performs better than the reactions with 40mM of furfural.

9. CONCLUSION

The demand for replacing fossil fuels with renewable alternatives that have a minimal environmental impact is growing. An attracting solution is utilizing products derived from lignocellulosic biomass. Among them, furanics compounds have being obtaining particular attention in the decades. The focus of this work is the biocatalytic conversion of furfural into 2-furoic acid using CAL-B as biocatalyst, combining in this way a renewable feedstock with a bioprocess.

It can be concluded that the main results of this work are:

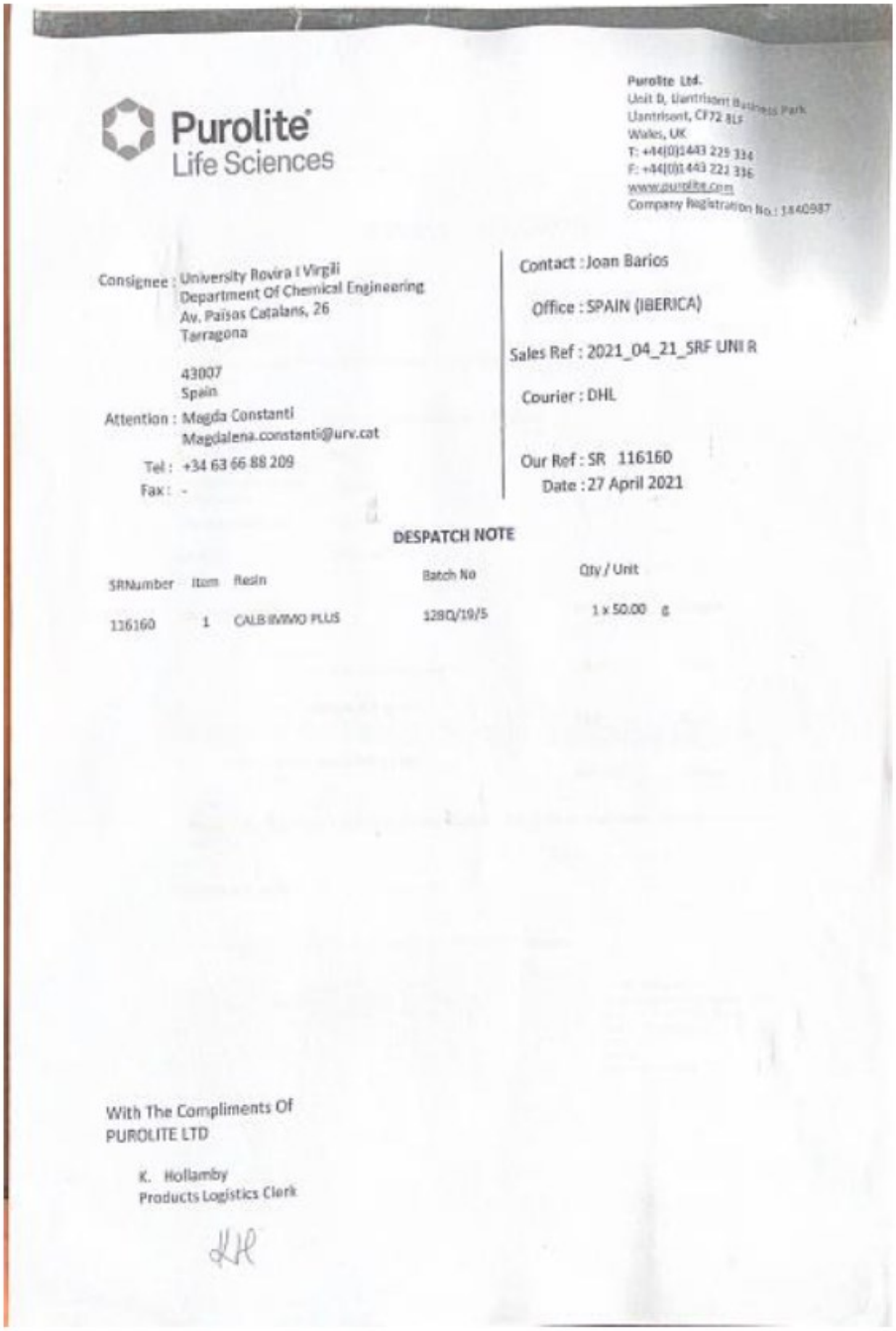
- Furfural from xylan can be obtained by using a biphasic system of water and ethyl acetate (1:2), with the help of a microwave reactor. The optimal conditions are: 170°C, 20 minutes, 0,25M of sulfuric acid and 4w% of xylan for a yield of 73%.
- The oxidation reaction can be carried out in a range of furfural load between 20-800 mM, with optimal condition at 40mM, with substrate inhibition starting around 200mM.
- The oxidation reaction can be carried out in a range of hydrogen peroxide load between 0,42-3,48 mM, with optimal condition at 0,84 mM.
- The recyclability of the enzyme is possible until the cycle number 4.
- The oxidation reaction can be carried out using xylan-based furfural and 1w% of activated carbon to filtrated humis, with optimal condition at 20mM of furfural as substrate and with a yield of 69%.

To continue this work, some suggestions are proposed to improve the research:

- Bio-based furfural oxidation reaction from biomass.
- To try other kind of washing solutions for the enzyme recyclability and also new recyclability configurations.
- To try different activated carbon loads

10. ANNEXES

10.1 ANNEXE 1. Certificate of analysis and first page of the safety data sheet for the CAL-B enzyme purchased by the Purolite company.



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Contact : Joan Barios
Office : SPAIN (IBERICA)
Sales Ref : 2021_04_21_SRF UNI R
Courier : DHL

Our Ref : SR 116160
Date : 27 April 2021

DESPATCH NOTE

SRNumber	Item	Resin	Batch No	Qty / Unit
116160	1	CALB IMMO PLUS	128Q/19/5	1 x 50.00 g

With The Compliments Of
PUROLITE LTD

K. Hollamby
Products Logistics Clerk

Purolite
Life Sciences

CERTIFICATE OF ANALYSIS

PRODUCT:

CalB immo Plus

Lipase from *Candida Antarctica B* immobilized by adsorption on ECR1030M

PRODUCT CODE:

LS02009

Appearance:	White to slightly yellow spherical beads
Supplied As:	Dry
Recommended Storage Temperature:	2 – 8 °C
Site of Manufacture:	Romania
Batch No:	1280/19/5

	Test/Characteristic:	Limits:	Results
1	**Activity:** 60°C; Synthesis of propyl laurate [1] PLU/g	> 9,000	9,800
2	**Total Moisture as Shipped:** %	< 5.0	3.3
3	**Particle size: 90% in range:** µm	300 – 710	Conform

[1] Unit definition PLU: One unit corresponds to the synthesis of 1 µmol per minute propyl laurate from lauric acid and 1-propanol at 60°C.

Best Before [Year- Month]: 2021 - 04

This document has been electronically produced and is valid without a signature.

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Purolite

SAFETY DATA SHEET

1. Section 1. Identification of the substance/mixture and of the company/undertaking

1.1. Product Identifier:

Trade name or designation of mixture: **CalB immo Plus™**

EC Number: Not applicable.

Registration Number: A registration number is not available for this substance as the substance or its uses are exempted from registration, the annual tonnage does not require a registration or the registration is envisaged for a later registration deadline.

1.2. Relevant identified uses of the substance or mixture and uses advised against

Identified uses: Immobilized Enzyme to be used as biocatalyst in biotransformations for the manufacture of Chemicals and substances.

Uses advised against: None known.

1.3. Details of the supplier of the safety data sheet

Supplier:
Purolite Ltd.
Llantrisant Business Park,
Llantrisant, Wales, UK CF72 8LF

Tel:+44 1443 229334
Fax:+44 1443 227073

Manufacturer:
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Tel:+40 2682 43004
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Purolite
150 Monument Road
Bala Cynwyd, PA 19004 USA

Tel:+1 610 668 9090
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Purolite (China) Co. Limited
Qiankong Economic Development Zone,
Qianyuan Town, Deqing County,
Huzhou City, Zhejiang, China 313216

Tel:+86 572 8422908
Fax:+86 572 8425345

Contact Person: Ken Shaner

Email: msds@purolite.com

1.4. Emergency Telephone Number:

+1 866 387 7344
+1 760 602 8703

10.2 ANNEXO 2. Summary protocol for the use of the Shimadzu GC-2010 gas chromatograph and TRB-5 column specifications

The Shimadzu GC-2010 Gas Chromatograph: an instrument employed for the separation and analysis of gaseous or volatile constituents within a sample. The process involves injecting the sample into the chromatography system, causing the components to separate within the chromatographic column. Once separated, a gas detector detects the components, and their records are graphed as a chromatogram.

Protocol:

Sample Preparation: The filtered sample is transferred into a container designed for sample injection into the chromatography system.

Equipment Calibration: Prior to analysis, it is crucial to calibrate the equipment to ensure result accuracy. This entails verifying gas flow and programming analysis parameters.

Sample Injection: The sample is injected into the chromatography system via a sample injection probe.

Separation of Sample Components: The chromatographic column's adsorbent material facilitates the separation of sample components. Carrier gas, typically nitrogen or helium, transports the components through the column.

Detection and Recording of Separated Components: The gas detector identifies the separated components, which are then recorded on the chromatogram.

Analysis of Results: Chromatogram analysis software is utilized to analyze the results, comparing them to reference standards for the identification and quantification of sample components.

The TRB-5 column, manufactured by Shimadzu, is a gas chromatography column with the following technical specifications:

- Column length: 30 m

- Silica pack diameter: 0.25 mm

- Composition of the silica packet: 95% silica, 5% cobalt

- Holding capacity: up to 5 mg

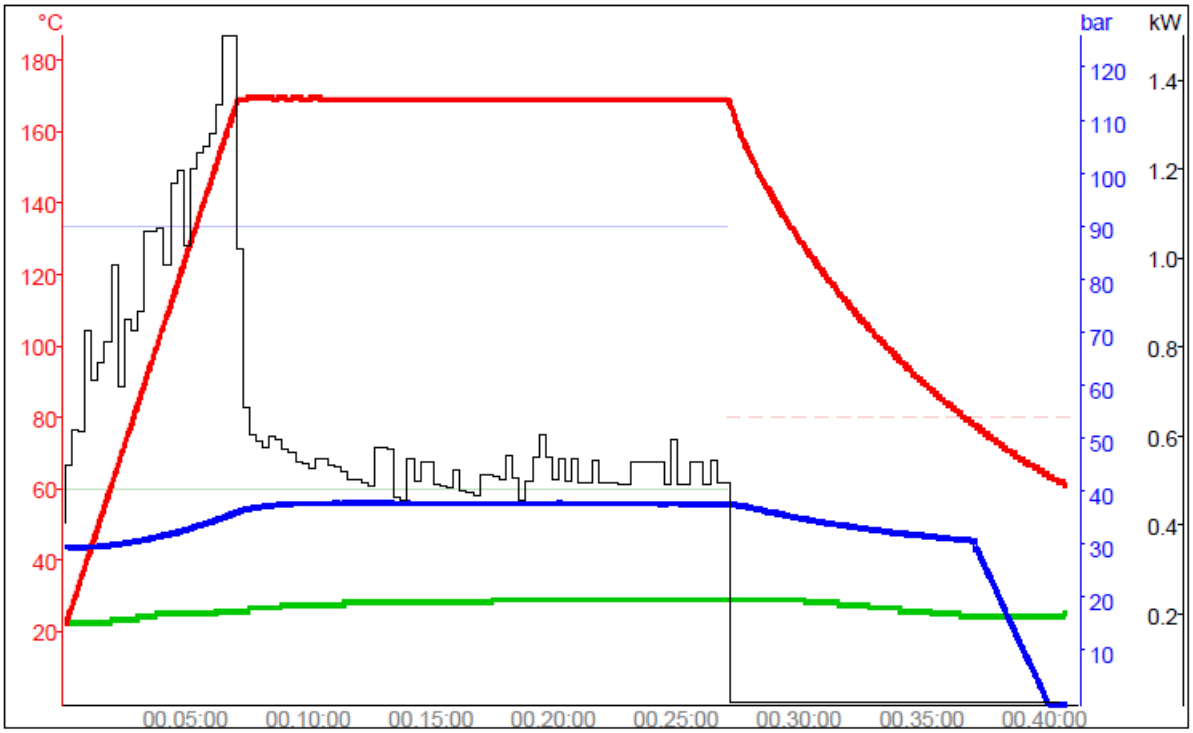
- Operating temperature range: -60 to 280°C

10.3 ANNEXO 3. Filtration and washing of the enzyme

Material: Stand, metal clamp, Büchner funnel, circular filter paper (of a size that covers the base of the funnel without exceeding it), Kitasato flask, glass rod.

Procedure: A filter paper circle, adequately sized to cover the Büchner funnel surface without exceeding it, will be prepared. Following that, the suspension is extracted with a syringe; the remaining solid (the enzyme) is washed with a buffer phosphate solution and then carefully poured onto the filter using a glass rod, ensuring that no liquid spills over. The solid residue retained in the filter can be rinsed using more sulfate buffer solution. Subsequently, the filter is placed on a piece of paper to facilitate the drying process, allowing the enzyme to dry as much as possible.

10.4 ANNEXO 4. Microwave reactor temperature program and results for obtention of furfural from xylan, determinated by GC



Parameter "barbara 170 20 min.mpr"

Stirrer	:	60 %
Start-Parameter	:	Current values
Load pressure	:	30.0bar
Vessel cooling	:	activated
beyond	:	25°C
Pressure after run	:	release
Release rate	:	10bar/min
Temperature below	:	80°C
Rack	:	
Type of vessels	:	
Number of vessels	:	1
Cooling system	:	

MW program "barbara 170 20 min.mpr"

Nr	t	T1	T2	P	E
1	00:07:00	170°C	60°C	90.0bar	1500W
2	00:20:00	170°C	60°C	90.0bar	1500W

xylan %	H2so4 load	FF in mM	Yield
2%	0,05	13,77	12%
2%	0,125	54,14	48%
2%	0,25	73,40	66%
4%	0,05	107,69	48%
4%	0,125	102,72	46%
4%	0,25	188,72	85%
8%	0,05	116,71	26%
8%	0,125	112,59	25%
8%	0,25	257,86	58%

Calculations:

To calculate the conversion, the following formula has been considered:

$$conversion = \frac{initial\ xylose\ moles - final\ xylose\ moles}{initial\ xylose\ moles}$$

where:

$$initial\ xylose\ moles = \frac{m_{xylan} * (x_{xylose})}{MW}$$

$$final\ xylose\ moles = mols\ of\ xylose\ aft\ microwave * V$$

m_{xylan}= 1,2 g or 0,6 g or 0,3 g

V= 5-15 ml

MW = 150,3 g/cm^3

x_{xylose}= 0,7676

To calculate the yield, the following formula has been considered:

$$yield = \frac{real\ mols\ of\ FF}{maximum\ mols\ of\ FF}$$

where:

$$real\ mols\ of\ FF = mols\ of\ FF * V$$

$$maximum\ mols\ of\ FF = \frac{m_{xylan} * (x_{xylose} + x_{arabinose})}{MW}$$

m_{xylan}= 1,2 g or 0,6 g or 0,3 g

V= 15 ml

MW = average of molecular weight of xylose and arabinose (150,3 g/cm^3 and 150,13 g/cm^3)

x_{xylose}= 0,7676

$x_{arabinose}$= 0,0704

10.5 ANNEXO 5. Results of the enzymatic reaction of furfural to 2-furoic acid: furfural load

Concentration (mM)	Conversion of FF	Yield of FA
25	92,3%	79,9%
40	95,1%	90,7%
50	89,7%	86,8%
100	83,1%	66,3%
200	69,3%	60,0%
400	56,8%	47,8%
800	7,5%	1,3%

10.6 ANNEXO 6. Results of the enzymatic reaction of furfural to 2-furoic acid: H2O2 load

Concentration	Conversion	Yield
0,41 mM	79%	95%
0,82 mM	94%	95%
1,62 mM	100%	71%
3,24 mM	96%	33%

10.7 ANNEXO 7. Results of the recyclability of the enzyme

Results without washing the enzyme:

Cycle	Concentration	Conversion	Yield
1st	50	91%	87%
2nd	50	65%	21%
3rd	50	0%	0%

Results washing the enzyme:

Cycle	Conversion	Yield
1st	90%	87%
2nd	36%	26%
3rd	29%	12%
4th	24%	10%

10.8 ANNEXO 8. Results and calculations of the enzymatic parameters characterization

40mM				
time	**mM of FA**	**Conversion**	**Yield**	**Product (mM/L)**
12	12,44	83%	31%	1.244,34
24	36,27	95%	91%	3.627,22
48	32,53	86%	81%	3.252,70

17h			
Concentration (mM)	**Conversion of FF**	**Yield of FA %**	**V_0**
0	-	0	0
15	100%	76%	0,0112
20	91%	77%	0,0150
40	88%	83%	0,0324
60	83%	75%	0,0441
80	79%	67%	0,0522
100	75%	61%	0,0595
150	70%	49%	0,0716
200	67%	38%	0,0754

where:

$$v_0 = \frac{mols\ FA}{L * min}$$

10.9 ANNEXO 9. Results of the enzymatic reaction of furfural to 2-furoic acid using bio-based furfural

Conditions	conversion	yield
- Furfural: 40 mM - H_2SO_4: 0,250M - xylan: 4w% - no pH change	35%	0,82%
- Furfural: 40 mM - H_2SO_4: 0,250M - Xylan: 4w% - pH change	79,79%	14,03%
- furfural: 40 mM - H_2SO_4: 0,125M - xylan: 4w% - pH change	83,83%	21,26%
- Furfural: 20 mM - H_2SO_4: 0,125M - Xylan: 4w% - no pH change	100%	36,82%
- Furfural: 25 mM - H_2SO_4: 0,250M - xylan: 4w% - pH change	100%	69,16%

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