Faculty of Chemistry and Chemical Engineering

Babeş-Bolyai University

Cluj-Napoca

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PhD Thesis Abstract

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Keywords: biocatalysis, enzymatic esterification; 2,5-Bis(hydroxymethyl)furan fatty acid esters; solvent-free reaction; sustainable synthesis; pyruvate decarboxylase; unconventional yeasts; chiral acyloins; factorial design optimization; substrate spectrum

1. General introduction

The worldwide high demand of chemical products, from commonly used household substances to pharmaceuticals and fuels, has led to a long-term negative impact on both human society and natural environment. In a quest to reduce the downsides of traditional chemical processes, researchers, scientists and chemical industries started to develop new "greener" processes and technologies with a lower environmental impact, while also putting emphasis on their long-term sustainability.

In order to achieve more sustainable and economical processes, catalysis has always been the chemist's closest aide and has led to the development of the modern-day chemical industry. However, due to certain disadvantages of classical catalysis, in present times both biocatalysis and biotransformations have played an important role as a greener substitute that enabled new processes with high productivity and much lower environmental impact. Biocatalysis is currently recognized a truly green and sustainable technology since the constant advances in biotechnology have made it cost-effective, environmental-friendly and industrially applicable.

The present thesis highlights the utility of known commercially available biocatalysts in the development of new strategies by incorporating renewable resources into value added products in greener technologies, while also focusing on investigation and development of new biocatalysts for the synthesis of various chiral compounds of interest for the pharmaceutical and fine chemical industry by employing whole-cell biocatalysis.

The first part of the thesis, ***Lipase-catalyzed synthesis of 2,5-Bis(hydroxymethyl)furan fatty acid diesters***, describes the investigation of lipase-catalyzed esterifications of 2,5-bis(hydroxymethyl)furan with various free fatty acid resources to obtain diesters with applications as bio-additives for increasing the cetane number of biofuels, among others. Based on their renewable nature, a green and sustainable procedure was developed, by using either a green solvent as reaction medium or a solvent-free approach, which offered high yields in mild reaction conditions.

The second part of the thesis, ***New whole-cell biocatalysts for the synthesis of optically active acyloins***, describes the identification and optimization of new strains of yeasts for the selective synthesis of (*R*)-phenylacetylcarbinol through their innate pyruvate decarboxylase activity, while also systematically exploring the intrinsic substrate domain of these microorganisms for the synthesis of optically actives analogues of (*R*)-phenylacetylcarbinol. Finally, the possibility to use sol-gel immobilization of the whole-cell microorganisms in order to obtain a recyclable biocatalyst for the production of (*R*)-phenylacetylcarbinol was investigated in both batch and continuous flow mode.

2. Chapter I. Lipase-catalyzed synthesis of 2,5-bis(hydroxymethyl)furan fatty acid diesters

2.1. Introduction. Literature overview

Biomass-based platform chemicals have become an increasing interest for researchers and industry, starting from their production and purification from renewable resources, to downstream processing towards relevant chemical building blocks or finished end-of-the-pipe products. In general, biomass is considered the ideal renewable resource as an alternative to fossil-based resources and has been proposed as a sustainable source of organic compounds for the production of fuels and chemicals. Among the many renewable building blocks, 5-hydroxymethylfurfural (HMF) and its derivatives are considered important versatile intermediates due to their rich chemistry and availability from sustainable carbohydrates feedstocks,[1] with an impressively wide variety of added value derivatives and application-oriented compounds such as bio-based green solvents, polymers, resins, etc.[2]

As a HMF-based chemical building block, 2,5-bis-(hydroxymethyl)furan (BHMF) represents a very potent resource for the chemical industry, with several applications already being reported in the literature such as foams,[3] polymers,[4–6] resins[7] and as an intermediate in pharmaceuticals.[3] Due to its symmetrical structure and the bearing of two sterically unhindered hydroxyl functional groups, BHMF is especially interesting as a bio-based monomer building block for bio-polymers production through esterification reactions; however, the research in the area of BHMF esters is still scarce.[8] Triacylglycerols and fatty acids also represent an attractive resource due to their availability from cheap and sustainable sources such as oils, but also from high free fatty acid feedstocks (HFFA). By integrating such resources into BHMF-based

products, fatty acid esters of BHMF were previously reported in the literature, with two patented procedures claiming the production of BHMF diesters as bio-based plasticizers through chemocatalytic diesterification with free fatty acids; and the biocatalytic synthesis of BHMF monoesters with free fatty acids with the aim to develop novel biodegradable non-ionic surfactants.[9,10] However, the biocatalytic synthesis of BHMF diesters from renewable resources, as potent biofuel additives, has not been investigated thoroughly and represents an appealing research area that was investigated in the current chapter of the present thesis.

2.2. Aim of the study

The aim of the study focuses on developing biocatalytic strategies for a green and sustainable production of BHMF fatty acid diesters as potent biofuel additives, solely based on renewable resources. The first approach was based on the lipase mediated double esterification of BHMF with commercially available saturated fatty acids in green organic solvents. For this purpose, the screening of several lipases and solvents was investigated. Immobilized CaL-B and 2-MeTHF proved to be an excellent combination for synthetizing BHMF fatty acid diesters and further optimization of reaction conditions was carried out in order to develop a cost-efficient high-yielding process. After testing the applicability of the product diesters as potent additives for increasing the cetane number of commercial biodiesel, in the second approach the synthesis of BHMF fatty acid diesters was investigated by using cheaper and readily available sunflower oil and fatty acid mixtures derived from sunflower oil as fatty acid sources. Switching between the solid saturated fatty acids to fatty acids mixtures (FAM) with a high content in liquid unsaturated fatty acids resulted in a completely new approach towards BHMF diesters production. Further, the use of FAM as fatty acid source was investigated with the purpose of developing a solvent-free heterogeneous enzymatic procedure for the double esterification of BHMF. Finally, the "greenness" of both methods was assessed by quantitative sustainability metrics.

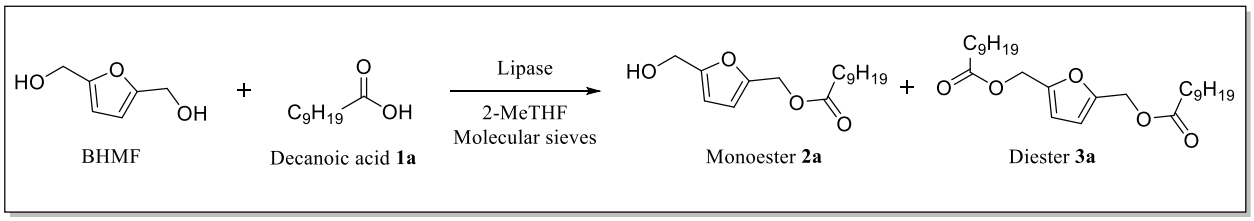
2.3. Results and discussions

2.3.1 Enzymatic synthesis of BHMF esters with saturated fatty acids in 2-MeTHF

2.3.1.1. Biocatalyst screening

In order to develop a cost-effective procedure for the synthesis of BHMF fatty acid diesters, the esterification of BHMF with decanoic acid was selected as a model reaction. By

using the afford mention as substrates, 2-MeTHF was selected as a green solvent based on its excellent applicability in several organic[11] and enzymatic reactions.[12–16] In these conditions, the screening of several lipases with previously reported high activity in biodiesel[17] production was performed in the presence of molecular sieves at room temperature (**Scheme 2.1**).



Scheme 2.1. Lipase-catalyzed esterification of BHMF with decanoic acid in 2-MeTHF.

In spite of using 4 equivalents of decanoic acid (two-fold excess), only 3 of the tested enzymes were capable of transforming BHMF into esters with a significant overall conversion (OC) as shown in **Table 2.1**. However, only Novozym 435 (immobilized lipase B from *Candida antarctica)* provided a complete transformation of the substrate (**Table 2.1**, OC > 99%, entry 1) and an excellent conversion into the corresponding diester (C_{DE} = 92.1%) as the major product of the reaction. Based on these results, further experiments were performed with Novozym 435 as biocatalyst.

Table 2.1. Biocatalyst screening for the esterification of BHMF with decanoic acid in 2-MeTHF*.

Entry	Enzyme	C_{ME} (%)[a]	C_{DE} (%)[b]	OC (%)[c]
1	Novozym 435	7.3	92.1	99.4
2	SWCNT-CaL-B	53.9	29.5	83.4
3	Lipozyme MM IM	38.5	17.6	56.1
4	Lipozyme TL IM	3.7	0.8	4.5
5	PS Amano IM	12.4	0.3	12.7
6	PS-C Amano II	10.5	0.2	10.7

*Results after 24 h reaction time. [a]Conversion into monoester C_{ME}; [b]Conversion into diester C_{DE}; [c]Overall conversion of BHMF into esters OC.

2.3.1.2. Screening of reaction solvent

Although excellent results were obtained with Novozym 435 as biocatalyst in the esterification of BHMF with decanoic acid in 2-MeTHF, other solvents were also assessed as reaction media using the same model reaction. Surprisingly, Novozym 435 gave good to excellent results in all tested solvents similar to 2-MeTHF (**Table 2.2**), however, 2-MeTHF was considered the optimum solvent based on its performance but also taking into account the availability from renewable resources and its environmentally benign nature.

Table 2.2. Comparative performance of different solvents on the enzymatic esterification of BHMF with decanoic acid.

Entry	Solvent	C_{ME} (%)	C_{DE} (%)	OC (%)
1	2-MeTHF	7.3	92.1	99.4
2	THF	37.7	55.1	92.8
3	DIPE	5.9	92.5	98.4
4	MTBE	5.6	92.4	98.0
5	Acetone	7.1	91.9	98.9
6	ACN	31.1	62.8	93.9
7	1,4-Dioxane	12.0	86.8	98.8

Results after 24 h reaction time.

2.3.1.3. Optimization of fatty acid : BHMF molar ratio

After the selection of a proper biocatalyst and solvent, the step by step optimization of reaction conditions was further performed. The first parameter investigated was the fatty acid : BHMF molar *ratio*, since the previous screening was performed directly with a double stoichiometric excess of fatty acid (decanoic acid : BHMF, 4:1 molar *ratio*). From this perspective, experiments were carried out by using different decanoic acid : BHMF molar *ratios* between 2:1 and 6:1. As shown in **Table 2.3**, a stoichiometric amount of fatty acid resulted in a high overall conversion into esters (OC = 93.2%) but the distribution of the products was unsatisfactory after 24 h, with C_{DE} only reaching 64.4%. Increasing the molar *ratio* provided an optimum at a molar *ratio* of 4:1, with a high C_{DE} of 93.2 %, while a further increase of the *ratio* did not result in a higher conversion into the diester product.

Table 2.3. The effect of fatty acid : BHMF molar *ratio* on the enzymatic esterification of BHMF with decanoic acid.

Entry	Decanoic acid/BHMF molar *ratio*	C_{ME} (%)	C_{DE} (%)	OC (%)
1	2 : 1	28.8	64.4	93.2
2	2,5 : 1	24.8	74.0	97.5
3	3 : 1	14.3	84.8	99.1
4	3,5 : 1	12.9	86.1	99.1
5	4 : 1	6.3	93.2	99.5
6	5 : 1	6.9	92.9	99.9
7	6 : 1	5.9	93.8	99.7

Results after 24 h reaction time.

2.3.1.4. Influence of molecular sieves as water adsorbent

In order to further shift the equilibrium of the enzymatic reaction, the optimization of molecular sieves was investigated. Accordingly, the enzymatic esterification reactions were performed with varying amounts of molecular sieves at room temperature. As expected, the reaction performed in the absence of molecular sieves provided an unsatisfactory overall

conversion of 60% and a three times higher content of monoester product than the diesteric product after 24 h (**Table 2.4**). The addition of even small amounts of molecular sieves (25 mg) to the reaction resulted in a substantial increase of the overall conversion (OC = 96.2%) as well as producing the diester as the major product of the reaction (C_{DE} = 63.0%). Further increase of the adsorbent content gradually improved the reaction output and, although a complete conversion of BHMF was achieved with only 50 mg molecular sieves, the nearly quantitative conversion of BHMF into the decanoic diesters was reached only at a higher content of 200 mg of molecular sieves (C_{DE} = 98.4%).

Table 2.4. The effect of molecular sieves on the enzymatic esterification of BHMF with decanoic acid.

Entry	Molecular sieves (mg)	C_{ME} (%)	C_{DE} (%)	OC (%)
1	0	46.3	13.5	59.8
2	25	33.2	63.0	96.2
3	50	7.8	91.9	99.8
4	100	5.9	94.1	99.9
5	150	2.5	97.4	99.9
6	200	1.5	98.4	99.9

Results after 24 h reaction time.

2.3.1.5. Optimization of reaction temperature and biocatalyst recyclability

Further, the influence of temperature on the enzymatic reaction rate was studied at various temperatures in the 20-60 ºC range. As can be seen in **Table 2.5**, a complete conversion into esters was achieved at all temperatures testes within 24 h of reaction, with a continuously increased conversion into diester product with increasing temperature, with the exception of a local minimum at around 40 ºC. Nonetheless, only slight differences were detected for the conversion into decanoic diester product between reactions performed at temperatures below and above 40 ºC.

Table 2.5. The effect of temperature on the enzymatic esterification of BHMF with decanoic acid.

Entry	Temperature (ºC)	C_{ME} (%)	C_{DE} (%)	OC (%)
1	20	7.9	91.9	91.9
2	30	2.3	97.3	97.3
3	35	2.8	96.7	96.7
4	40	11.1	88.6	88.6
5	45	3.1	95.7	95.7
6	50	2.6	97.1	97.1
7	60	2.2	97.6	97.6

Results after 24 h reaction time.

Since the enzymatic esterifications gave similar results at different reaction temperatures after 24 h, the optimum reaction time was further investigated by analyzing the time course

profile of the reactions at 30 ºC and 50 ºC. According to the time course profile obtained at 30 ºC (**Figure 2.1 A**), a complete transformation of BHMF was observed after 6 h (OC > 99%), while the conversion into diester reached a favorable conversion of more than 96% in the desired product only after 12 h. In comparison, the time course profile obtained at 50 ºC (**Figure 2.1 B**) demonstrated that complete conversion of BHMF was achieved in just 2 h, moreover with a similar selectivity of more than 96% towards diester formation being reached in less shorter, 6 h reaction time.

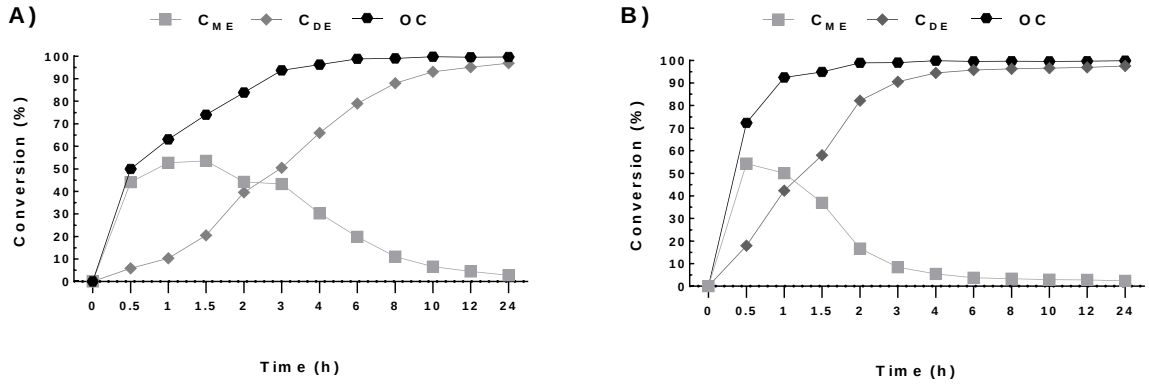


Figure 2.1. The time course of the esterification reaction performed at **A**) 30 ºC and at **B**) 50 ºC.

Although a higher reaction rate was clearly achieved at 50 ºC than 30 ºC, the optimum temperature should be selected also based on its influence on the stability and recyclability of the biocatalyst. From this perspective, the reusability of the enzyme was tested in repeated batch experiments at 30 ºC and 50 ºC. As depicted in **Figure 2.2 B**, the activity of Novozym 435 gradually decreased at 50 ºC in the course of 10 repeated batches, resulting in a severe decrease of both the overall conversion (decreased by ~35%) and conversion into diester (decreased by ~75%). In comparison, a much lower impact was observed on the recyclability of the enzyme at 30 ºC (**Figure 2.2 A**) with a maximum decrease of only ~8% of the overall conversion after 10 consecutive uses and with a drop of only ~25% of the conversion into diester. Thus, taking into account both the recyclability of the biocatalysts and conversion of the substrate, performing the reaction at 30 ºC with a 12 h reaction is preferable in order to maintain a good stability of the catalyst over several reaction cycles.

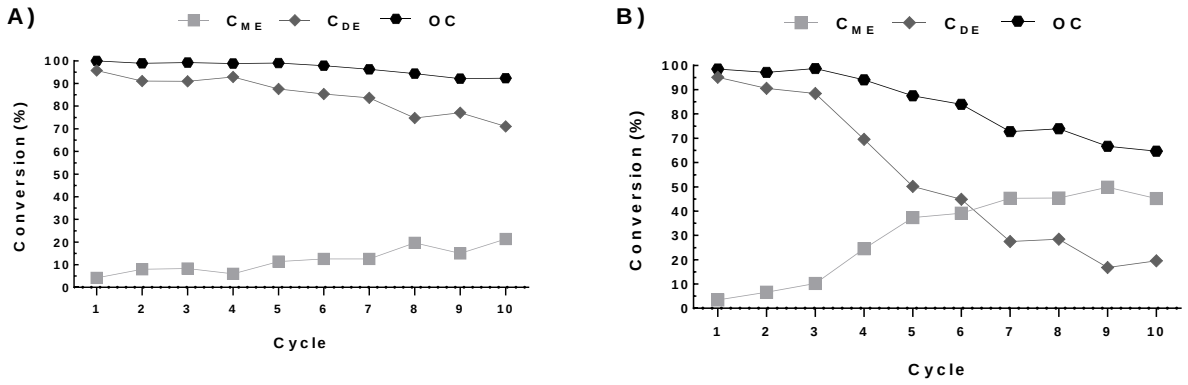


Figure 2.2. The reusability of Novozym 435 at **A**) 30 ºC and at **B**) 50 ºC using 12 h batch runs.

2.3.1.6. The influence of fatty acid

In the determined optimum conditions, the possibility to employ other saturated fatty acids and also the influence of the alkyl chain length of these acids was further investigated. As presented in **Table 2.6**, the esterification of BHMF with the other fatty acids resulted in quantitative conversion of the substrate into esters, with overall conversions higher than >99% in all cases and only some moderate differences detected. Since no correlation can be established between the fatty acid length and selectivity, it was concluded that the enzymatic diesterification of BHMF with the fatty acids tested can be indiscriminately achieved using Novozym 435 with a similar efficiency.

Table 2.6. Enzymatic esterification of BHMF with fatty acids **1a-e**.

Entry	Fatty acid	C_{ME} (%)	C_{DE} (%)	OC (%)
1	Decanoic acid (**1a**)	3.1	96.9	>99.9
2	Dodecanoic acid (**1b**)	4.5	95.5	>99.9
3	Tetradecanoic acid (**1c**)	10.7	89.3	>99.9
4	Hexadecanoic acid (**1d**)	1.0	99.0	>99.9
5	Octadecanoic acid (**1e**)	2.6	97.4	>99.9

Results after 12 h reaction time.

2.3.1.7. Optimization of BHMF and fatty acid concentration.

In order to increase the productivity of the batch esterification process, optimization of the concentration of reactants in 2-MeTHF was performed. The reactions were carried out in optimum conditions with the same biocatalyst load and amount of molecular sieves, while keeping constant the molar ratio of reactants. As presented in **Table 2.7**, increasing the concentration of the reactants up to saturation still resulted in excellent overall conversions

(> 99%) after 12 h of reaction, with more than > 93% conversion into the decanoic diester even at a 0.5 M concentration of BHMF. Moreover, evaluation of the time-course of reactions indicated an increased reaction rate with increasing concentration up to 0.4 M BHMF, followed by a slight decrease at 0.5 M concentration. Based on the results obtained, further reactions were performed at the saturation limit of reactants.

Table 2.7. The effect of BHMF concentration on the enzymatic esterification reaction at a decanoic acid: BHMF molar *ratio* of 4:1.

Entry	BHMF (M)	Decanoic Acid (M)	C_{ME}(%)	C_{DE}(%)	OC(%)
1	0.05	0.2	4.5	95.1	99.6
2	0.1	0.4	4.9	94.1	99.0
3	0.2	0.8	4.7	94.8	99.4
4	0.3	1.2	4.3	95.2	99.6
5	0.4	1.6	4.9	94.8	99.7
6	0.5	2	6.2	93.4	99.6

Results after 12 h reaction time.

2.3.1.8. Optimization of biocatalyst dosage

Finally, the biocatalyst dosage was investigated in order to ensure the minimum enzyme load for a cost-effective biocatalytic strategy. Surprisingly, more than >99% overall conversion was achieved after 12 h even at the lowest biocatalyst loading of 5 mg/mL. Examining the time course of product distribution for each reaction revealed that the rate at which the diester product is formed is clearly slower as the amount of biocatalyst is reduced; however the conversion into diesters reached a suitable conversion only after 12 h or reaction time regardless of the enzyme load used as depicted in **Figure 2.3**.

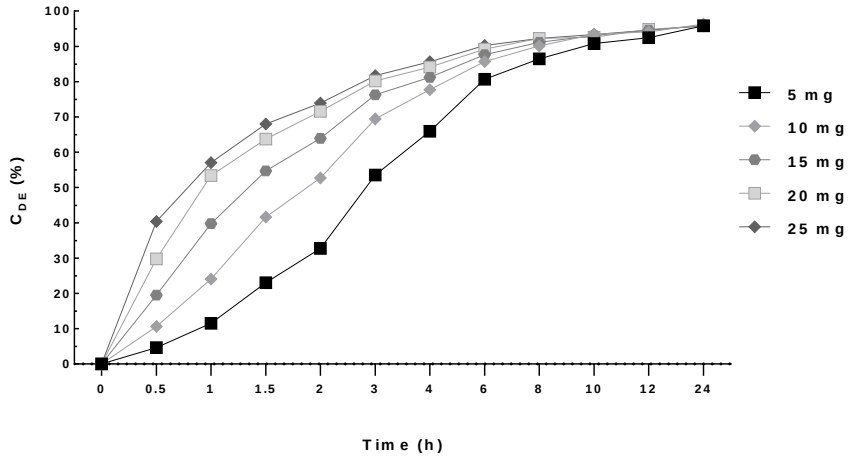


Figure 2.3. Time-course of the conversion of BHMF into diester **3a** (C_{DE}) for different biocatalyst dosages.

Prolonging the reaction time to 24 h, a further slow increase was detected for the conversions into diester in all cases. Accordingly, a 0.5% enzyme load was considered optimum and was used in further investigations.

2.3.1.9. Preparative scale enzymatic esterification of BHMF with fatty acids 1a-e in optimum conditions

Based on the results obtained at analytical scale with the optimized procedure, preparative scale experiments with fatty acid **1a-e** were performed. However, because the solubility of the fatty acids in 2-MeTHF decreased as the molecular mass of the fatty acid increased, the volume of the reaction mixture also increased accordingly, in order to completely dissolve the fatty acids. The final volumes and concentrations used, as well as conversions into diesters and final yields obtained are presented in **Table 2.8**.

Table 2.8. Preparative scale conversions and isolated yields obtained for the enzymatic esterification of BHMF with fatty acids **1a-e**[*].

Entry	Fatty acid	Volume of reaction mixture (mL)	Concentration of BHMF(M)	Concentration of fatty acid(M)	C_{DE} (%)	Yield (%)
1	**1a**	100	0.5	2	96.7	82.5
2	**1b**	100	0.5	2	96.8	84.4
3	**1c**	110	0.45	1.8	97.4	89.7
4	**1d**	166	0.3	1.2	99.1	91.3
5	**1e**	250	0.2	0.8	99.7	92.2

[*]Results after 24 h reaction time.

2.3.1.10. Large-scale synthesis of BHMF decanoic diester and performance evaluation as biodiesel additive

In order to assess the utility of BHMF fatty acid diesters as biodiesel additives, a large scale synthesis was performed starting from 64 g BHMF (0.5 mol) and decanoic acid in identical conditions as the preparative procedure. After successfully obtaining the chemically pure decanoic diester (188.2 g, η=86.2%), 3 L of additivated biodiesel (5 wt.%) was prepared and tested in an accredited biofuel analysis facility. The results revealed that the cetane number of the additivated biodiesel increased with ~4.5 % as shown in **Table 2.9**, while the other tested physico-chemical properties were maintained similar to that of standard biodiesel.

Table 2.9. Performance test results of diester **3a**, standard biodiesel and additivated biodiesel with **3a**.

Entry	Sample	Gross calorific value [J/g]	Density at 15 °C [kg/m^3]	Flash point [°C]	Cetane number
1	Diester **3a** (pure compound)	34340	n.d.	n.d.	n.d.
2	Biodiesel (standard)	39645	888.4	188	54.9
3	Additivated biodiesel (5 wt% of diester **3a** added)	39440	891.0	220	57.4

2.3.2. Enzymatic synthesis of BHMF esters with unsaturated fatty acids, sunflower oil and FAM in 2-MeTHF

Further, sunflower oil and fatty acids mixture (FAM) obtained by complete hydrolysis of sunflower oil were investigated as renewable fatty acid feedstocks. Oleic and linoleic acid were first tested for BHMF esterification with Novozym 435 in 2-MeTHF, checking whether these unsaturated fatty acids are converted to their corresponding BHMF diesters with similar efficiency and selectivity. Using a 4:1 molar ratio (fatty acid : BHMF), quantitative conversions were observed (OC > 99%), similar to the previously tested saturated fatty acid after 24 h of reaction time as presented in **Table 2.10** (entry 1 and 2). As expected, when FAM obtained by hydrolysis of sunflower oil was used, excellent results were obtained with a complete conversion of BHMF and a high C_{DE} = 93.5% (**Table 2.10**, entry 4). As another approach, the transesterification of BHMF with sunflower oil was also tested (**Table 2.10**, Entry 3), however, a low conversion was observed, mainly into the corresponding fatty acid monoesters.

Table 2.10. Testing of oleic acid, linoleic acid, sunflower oil and FAM as fatty acid source for the synthesis of BHMF fatty acid diesters.

Entry	Fatty acid source	C_{ME} (%)	C_{DE} (%)	OC (%)
1	Oleic acid	8.2	91.7	99.9
2	Linoleic acid	14.9	85.0	99.9
3	Sunflower oil	22.2	1.1	23.3
4	FAM	6.5	93.5	>99.9

Results after 24 h reaction time.

Considering the results obtained with FAM as fatty acid source, the mass ratio of 2-MeTHF/FAM (wt./wt.) was also investigated with decreasing the amount of solvent in the reaction mixture (1 mL to 0 mL), corresponding to a 2-MeTHF: FAM mass *ratio* between 1.06-0.00 (wt./wt.). While preparing the reaction mixture, it was observed that lowering the 2-MeTHF content below ~0.5 mL (0.53 wt./wt.), the reaction mixtures becomes heterogeneous due to oversaturation in BHMF. Analyzing the reaction mixtures after 24 h, the overall conversion of the substrate was higher than >99% regardless of the amount of solvent used, while the

conversion into diesters slightly decreased by ~ 2% as presented in **Table 2.11**. In the absence of solvent, the esterification proceeded with continuous solubilization of BHMF in the reaction mixture and provided a high conversion of 68.6% into the desired diesters with an excellent 96.3% overall conversion of the substrate (**Table 2.11**, Entry 1).

Table 2.11. Influence of 2-MeTHF content on the enzymatic esterification of BHMF with FAM.

Entry	Mass ratio of 2-MeTHF/FAM (wt./wt.)	C_{ME} (%)	C_{DE} (%)	OC (%)
1	0	27.7	68.6	96.3
2	0.26	8.5	91.3	99.9
3	0.53	9.4	90.3	99.7
4	0.79	9.1	90.6	99.7
5	1.06	6.4	93.6	>99.9

Results after 24 h reaction time.

2.3.3. Solvent-free enzymatic synthesis of BHMF esters with FAM

In order to further explore a solvent-free procedure as an alternative for the synthesis of BHMF diesters, several factors were reevaluated and optimized in order to develop a reliable and cost-effective process.

2.3.3.1 The influence of temperature on the enzymatic esterification in solvent-free conditions

With the aim to increase the efficiency of the enzymatic esterification reaction, the influence of temperature was evaluated for reactions performed in solvent free conditions. Since no solvent was added to the reaction mixture, water removal was achieved using a 15 mbar vacuum as an alternative to molecular sieves. Reactions were investigated at different temperatures in the range of 30-60 ºC and the results are presented in **Table 2.12**. Based on results, it was observed that a temperature of >40 ºC facilitated the efficient removal of water from the reaction and provided high conversions into diesters between 96.9-98.7% (**Table 2.12**, Entries 2-4). The time-courses of reactions performed in the 40-60 ºC range are presented in **Figures 2.4 A-C** and showed that the reaction rate increases with temperature with an exponential dependence of the conversion in time at 50 ºC and 60 ºC, while at 40 ºC the conversion showed an unexpected linear dependence as depicted in **Figure 2.4 A**. Comparing these results, it was concluded that at 50 ºC and 60 ºC, the determining step of the process is represented by the enzyme catalyzed esterification, while at 40 ºC and below, the rate limiting

step of the process is controlled by either diffusion or by the gradual solubilization of BHMF into the reaction mixture.

Table 2.12. The influence of temperature on the enzymatic esterification of BHMF with FAM in solvent-free conditions and vacuum.

Entry	Temperature (ºC)	C_{ME} (%)	C_{DE} (%)	OC (%)
1	30	42.9	45.9	88.8
2	40	3.4	96.6	>99.9
3	50	1.8	98.2	>99.9
4	60	1.3	98.7	>99.9

Results after 24 h reaction time.

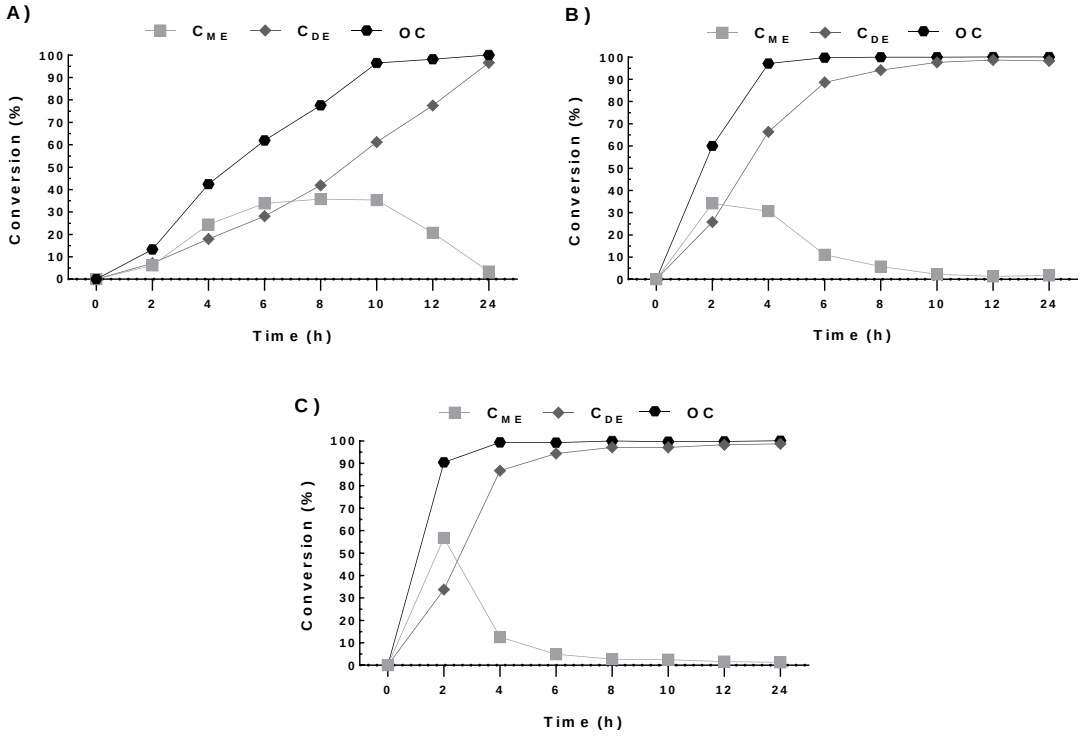


Figure 2.4. Time-course of solvent-free esterifications of BHMF with FAM at **A**) 40 ºC, **B**) 50 ºC and **C**) 60 ºC

2.3.3.2. The influence of BHMF : FAM *ratio*

Next, the FAM : BHMF molar *ratio* was investigated for the solvent-free procedure. Reactions were carried out with decreasing equivalents of FAM (at 60 ºC, under vacuum), lowering the amount of fatty acid source down to an approximately stoichiometric *ratio* of FAM to BHMF. In these conditions, the overall conversion reached more than 99% after 12 h of reaction time even at a *ratio* of 2:1, while the conversion into diesters showed a gradual slight

decrease as the amount of fatty acids in the mixture was reduced (**Table 2.13**). A conversion into diesters of ~95% for an approximately stoichiometric molar *ratio* of 2:1 offered a higher overall efficiency and was considered to be optimum for the solvent-free procedure.

Table 2.13. The influence of FAM equivalents on the enzymatic esterification of BHMF in solvent-free conditions and vacuum.

Entry	Acid equivalents of FAM	C_{ME} (%)	C_{DE} (%)	OC (%)
1	2	5.1	94.6	99.7
2	2.25	3.7	96	99.7
3	2.5	2	97.6	99.5
4	3	1.8	97.9	99.7
5	4	1.6	98.2	99.7

Results after 12 h reaction time.

2.3.3.3. The influence of biocatalyst dosage

Further, the optimum amount of biocatalyst necessary for the efficient esterification of BHMF into diesters in solvent-free conditions was investigated. Semi-preparative scale experiments were performed at 60 ºC using 5 mmol of BHMF (640 mg) and a stoichiometric amount of FAM, with enzyme loadings between 0.5-2.5% (amount of enzyme reported to amount of BHMF). The results obtained after 12 h indicated that, as expected, an enzyme load of 0.5% proved to be insufficient for a attaining a desirable conversion into diesters (**Table 2.14**, Entry 1), however, an enzyme load equal or higher than 1% provided overall conversions greater than 99% and excellent conversions into diesters (**Table 2.14**, Entries 2-5). When the reaction time was extended to 24 h, nearly quantitative conversion into diesters was achieved even at a 1% enzyme loading and was considered optimum in this study.

Table 2.14. The effect of biocatalyst dosage on the enzymatic esterification of BHMF in solvent-free conditions and vacuum.

			After 12 h			After 24 h		
Entry	Biocatalyst dosage (mg)	Enzyme load (%)	C_{ME} (%)	C_{DE} (%)	OC (%)	C_{ME} (%)	C_{DE} (%)	OC (%)
1	50	0.5 %	44.3	52.3	96.7	11.3	88.1	99.4
2	100	1 %	8.4	91.3	99.7	2.8	97.1	>99.9
3	150	1.5 %	6.9	92.9	99.8	3.1	96.9	>99.9
4	200	2 %	7	92.8	99.8	2.8	97.1	>99.9
5	250	2.5 %	5.1	94.6	99.7	3	96.9	>99.9

2.3.3.4. Preparative solvent-free enzymatic synthesis of BHMF diesters with FAM

Lastly, the preparative scale production of BHMF diesters with FAM in solvent-free conditions was performed using the previously determined optimal conditions (15 mbar vacuum,

60 ºC, 2:1 molar *ratio* of FAM : BHMF, 1% enzyme load, 24 h reaction time). The reaction mass was worked-up and the fatty acid diesters products were isolated with an 89.6% yield.

2.3.4. Assessment of sustainability metrics

The quantitative assessment of sustainability and "greenness" of both developed procedures in this study was performed in accordance with recent literature.[18,19] In order to compare the two procedures, the preparative scale synthesis of BHMF diesters with stearic acid in 2-MeTHF (Method A) and with FAM in solvent-free conditions (Method B) were taken into consideration based on the fact that stearic acid (284.48 g/mol) has a molecular weight similar to the mean molecular weight calculated for FAM (≈ 280.8 g/mol). The calculated metrics, presented in **Table 2.15**, show that both methods display very good sustainability parameters, with the solvent-free procedure having superior indicators.

Table 2.15. Compared sustainability metrics for the enzymatic production of BHMF fatty acid esters.

		Sustainability metric				
Entry	**Method**	**E-Factor**	**Atom Economy**	**Atom Efficiency**	**Mass Intensity**	**Reaction Mass Efficiency**
1	A*	1.05	94.8	87.5	10.6	48.2
2	B**	0.11	94.8	84.9	1.9	87.4

*Enzymatic esterification of BHMF with stearic acid in 2-MTFH in presence of molecular sieves.
** Enzymatic esterification of BHMF with FAM in solvent-free conditions under vacuum.

2.4. Conclusions

This study demonstrates the efficient biocatalytic valorification of 2,5-bis-(hydroxymethyl)furan as a renewable platforms chemical, by lipase-mediated diesterification with biogenic fatty acids for a sustainable production of BHMF fatty acid diesters, as potent renewable biofuel additives. Investigating of several commercial and home-made immobilized lipases for the diesterification of BHMF with saturated fatty acids in 2-MeTHF, as a biogenic green solvent, provided Novozym 435 as a suitable biocatalyst with an excellent compatibility, with both the substrate and solvent used. In optimum conditions (4:1 molar *ratio* of fatty acid : BHMF, 200 mg molecular sieves, 30 °C and a 0.5% enzyme loading,), excellent conversion of the substrate into diesters was obtained even at high concentrations of the reactants, while also providing a good recyclability of the biocatalyst. Furthermore, a similar selectivity and high yields were observed for the diesterification of BHMF at analytical, preparative and large scale reactions. The study also describes the first investigation of fatty acid diesters of BHMF as reliable additives for increasing the cetane number of commercial biodiesel.

As a promising alternative, the use of liquid fatty acids or liquid mixtures of fatty acids derived from sunflower oil enabled the development of a solvent-free strategy for the synthesis of BHMF diesters in heterogeneous conditions. Despite the low solubility of BHMF in FAM, it was observed that the esterification of substrate occurs by the gradual solubilization into the reaction mixture. Optimization of the solvent-free procedure revealed that the enzymatic esterification can be carried out in solvent-free conditions by substituting the molecular sieves with vacuum; however a minimum temperature of 40 °C is necessary for an efficient separation of water, while a 60 °C provided a maximum yield with a high reaction rate. Further improvement could be accomplished by decreasing the molar ratio of FAM : BHMF, achieving nearly quantitative conversion even at a stoichiometric ratio between the reactants with a 1% enzyme loading.

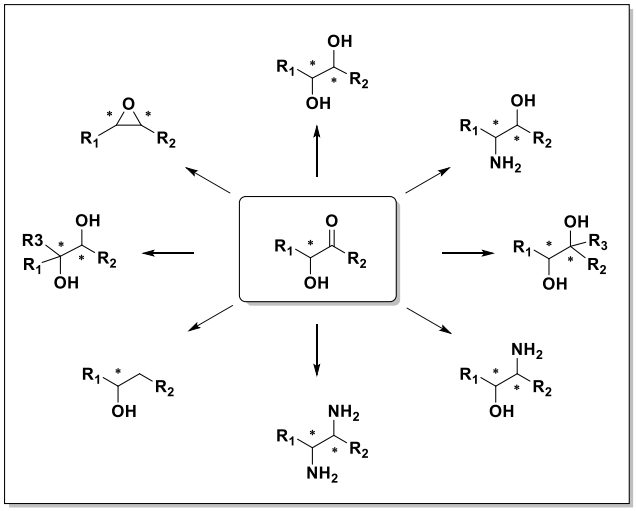
Finally, a quantitative evaluation of the sustainability of both procedures was made by performing esterification reactions at preparative scale with both methods described in this chapter. The result supported that both methods gave very good green metrics values, however in terms of sustainability, the solvent-free procedure provided superior metrics.

3. Chapter II. New whole-cell biocatalysts for the synthesis of optically active acyloins

3.1. Introduction. Literature overview

3.1.1. Optically active α-hydroxyketones (acyloins)

α-Hydroxyketones or acyloins, are chemical compounds bearing a hydroxyl group attached to an α carbon adjacent to a carbonyl group. From a structural point of view, acyloins possess a chiral carbon responsible for their optic activity (and in most cases, their biological activity) and a prochiral carbon that can be further exploited to obtain another stereocenter, thus greatly expanding their possible applications as chiral building blocks. The α-hydroxyketone motif, which can be regarded as a versatile functional group, has found important applications in the synthesis of many chiral precursors or chiral building blocks. As consequence, these valuable compounds can be upgraded to many other types of chiral compounds (**Figure 3.1**) such as 1,2-diols, epoxides, amines (or diamines), alcohols and amino alcohols.[20]



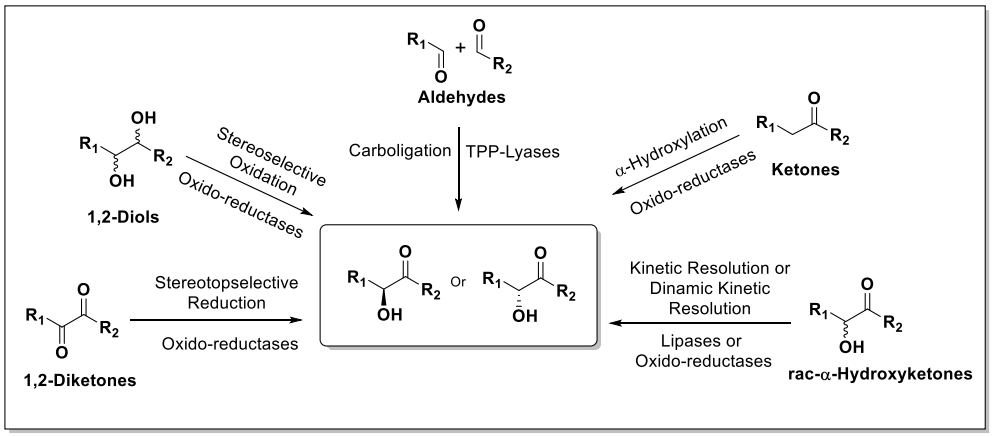
Scheme 3.1. Optically active compounds that can be obtain starting from α-hydroxyketones as chiral precursors.

Based on this specific versatility and chirality, α-hydroxyketones have found various applications especially in the pharmaceutical sector with many examples of biologically active acyloins or acyloin derivatives being described in the literature.[21,22,23,24,25] Taking into account their current and future importance, many chemical strategies for their asymmetric synthesis were reported and can be generalized as: asymmetric condensation of two different parent aldehydes; α-hydroxylation of ketones (by means of enolate oxidation); ketohydroxylation of olefins; selective and asymmetric oxidation of 1,2-diols or stereoselective reduction of 1,2-diketones;

kinetic resolution or dynamic kinetic resolution by racemic acyloins oxidation, etc.[21,26] Besides the chemical strategies mentioned, biocatalytic strategies can be successfully employed in the synthesis of asymmetric acyloins. By means of biocatalysis, these compounds can be obtained in economically relevant yields with high chemo-, regio- and enantioselectivities, while also endorsing the principles of a green and sustainable chemistry.[27]

3.1.2. Biocatalytic pathways towards optically active α-hydroxyketones

Due to their synthetic significance, the production by biocatalytic means of asymmetric α-hydroxyketones has received an abundance of attention and several enzymatic strategies were developed over time. From this perspective, many classes of enzymes were successfully exploited for the synthesis of these compounds such as hydrolases, oxido-reductases and lyases; as presented in **Scheme 3.2**.[21]



Scheme 3.2. General biocatalytic strategies used to obtain optically active α-hydroxyketones.

Although several methods were reported by using lipases and oxido-reductases, the most researched and economically viable method to obtain α-hydroxyketones remains the use of thiamine pyrophosphate (TPP) dependent lyases. TPP-dependent lyases are enzymes involved in a multitude of metabolic pathways and catalyze a large variety of reactions, being able to perform oxidative/non-oxidative decarboxylation and asymmetric bond formation (C-C, C-O, C-S and C-N bonds) and C-C bond cleavage.[28] Besides their biological role, the promiscuous applicability of TPP dependent enzymes has been already greatly demonstrated by numerous examples presented in literature over the last few decades.[29]

TPP-lyases catalyze the formation of enantiomerically enriched α-hydroxyketones by a stereoselective umpolung (polarity inversion) C-C bond formation between an aldehyde and a

suitable "donor".[20] Briefly, the catalytic cycle begins with a nucleophilic addition of the TPP cofactor (anion form) to a "donor" aldehyde. After deprotonation, an enamine carbanion is formed. The carbanion acts further as nucleophile and attacks the carbonyl carbon of the "acceptor" aldehyde, leading to the formation of the C-C bond. In the case of decarboxylase type lyases, the enamine carbanion is formed by decarboxylation of a "donor" 2-ketoacid, that is first attacked by the TPP anion to generate an intermediate 2-hydroxy adduct. The enamine carbanion formed by releasing the carbon dioxide reacts further with the carbonyl acceptor to form the desired α-hydroxyketone. The stereoselectivity of the C-C bond forming reaction is, as in most enzymatic reactions, controlled by the enzyme's structure and helps to discriminate between the two enantiotopic faces of the "acceptor" aldehyde.[30] Several TPP-lyases have been reported as efficient catalysts for the formation of a wide range of α-hydroxyketones, such as benzaldehyde lyase (BAL, EC 4.1.2.38), benzoylformate decarboxylase (BFD, EC 4.1.1.7), pyruvate decarboxylase (PDC, EC 4.1.1.1), phenylpyruvate decarboxylase (PhDC, EC 4.1.1.43) and indole-3-pyruvate decarboxylase (InPDC, EC 4.1.1.74).[20,31–35]

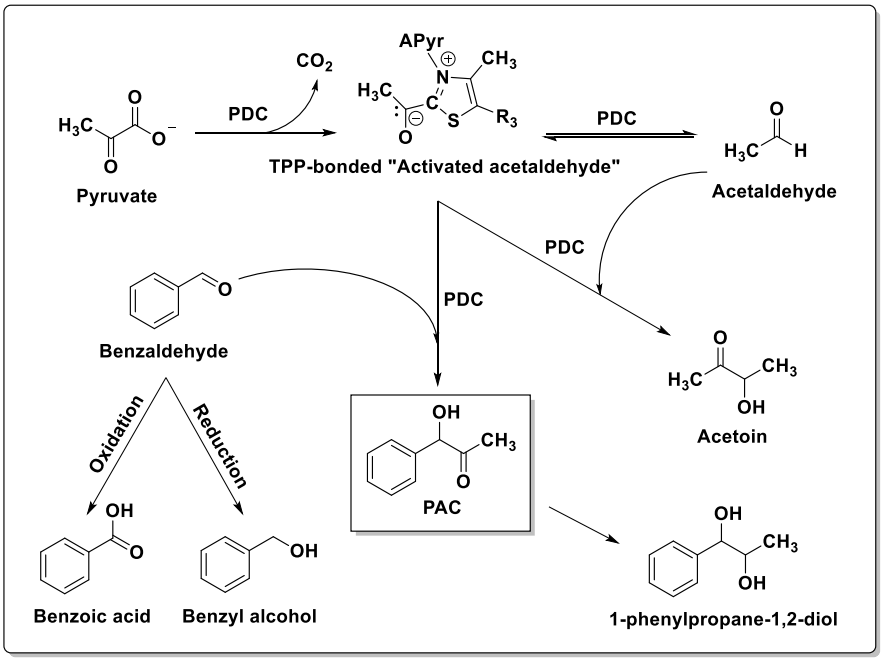
3.1.3. PDC mediated synthesis of optically active PAC and PAC analogues.

Pyruvate decarboxylase (E.C. 4.1.1.1) is the first exploited TPP and magnesium ions dependent enzyme in the synthesis of chiral α-hydroxyketones and also one of the oldest biocatalytic processes that was successfully implemented at an industrial scale. Genes that encode PDC enzymes have been identified and isolated from many organisms such as yeast or fungi (*Saccharomyces cerevisiae*, *Hanseniaspora uvarium*, Klyveromyces marxianus, Candida utilis, Rhizopus javanicus, etc.), bacteria (*Zymomonas mobilis* and *Acetobacter pasteurianus*) and plants (maize, rice, pea and tabaco).[20,36]

Besides its primary role in the cells of organisms in the glycolytic pathway, PDC also catalyzes the acyloin condensation as a side reaction if a suitable acceptor aldehyde is present. By means of this side reaction, the biocatalytic carboligation of benzaldehyde enabled the successful synthesis of enantioenriched (*R*)-phenylacetylcarbinol ((*R*)-PAC). The reaction was first reported by Neuberg nearly a century ago by using the wild-type PDC activity of whole cells of *Saccharomyces cerevisiae* and glucose as a source of pyruvate, which was further developed as a key biocatalytic step in the synthesis of ephedrine and pseudoephedrine.[21]

Pyruvate decarboxylases are one of the most studied enzymes for the synthesis of optically active acyloins and (*R*)-PAC. Research performed on PDCs and PDC mediated synthesis of acyloin compounds in the last decades was summarized in many reviews and related literature.[20,21,41,42,31,32,34,35,37–40] Although extensive research has been performed on PDC from *S. cerevisiae*, PDCs from *Candida utilis*, *Zymomonas mobilis* and *Acetobacter pasteurianus* were also recognized as powerful tools for the synthesis of acyloins.[41] Structurally, the active form of PDCs is a tetrameric holoenzyme made up of two identical dimers and requires 2-4 TPP molecules and magnesium ions that, besides providing the catalytic activity of the enzyme, also play an important role in stabilizing the tetramer quaternary structure.[43] Despite the fact that PDCs are found in many different species of organisms (prokaryotic and eukaryotic), the quaternary structures of these enzymes is relatively similar regardless of the enzyme's source.[44] The enzymatic mechanism of PDCs has been already well studied and described in literature.[29]

Regardless of the origin of PDC enzymes, there are two major strategies employed in the synthesis of PAC using PDC: whole microorganism cells (free or immobilized) or purified enzymes (free or immobilized). The major disadvantage of performing whole-cell synthesis of PAC from benzaldehyde is the formation of by-products due to side reactions.[45] **Scheme 3.3** shows the PDC-catalyzed formation of PAC using a whole-cell approach and usual side products.



Scheme 3.3. The general whole-cell PDC-catalyzed PAC synthesis and possible side-reactions and by-products.

In regards to the promiscuity and substrate scope of these enzymes, it has been shown that PDC can catalyze the decarboxylation of not only pyruvate, but also many 2-oxo acids (up to 2-oxohexanoate), hydroxy pyruvate and even *p*-substituted α-keto acids. Wild-type PDC from *S. cerevisiae* can also catalyze the formation of various PAC analogues starting from substituted aromatic aldehydes. It was also observed that *o*-substituted benzaldehydes are poor substrates for PDC in contrast to *m*- and *p*-substituents analogues.[46] The stereoselectivity of wild-type PDC enzymes is also an important factor for their synthetic application on industrial scale. Studies on C-C bond formation by PDCs and TPP-dependent enzymes revealed that they are mostly *R*-selective and the (*R*)-enantiomer is usually the major product of the biocatalytic reaction.[41]

3.2. Aim of the study

The aim of the study described in the present chapter of the thesis focused on the investigation of various unconventional new yeast strains as whole-cell biocatalysts for the enzymatic synthesis of (*R*)-phenylacetylcarbinol and (*R*)-PAC analogues. By screening of multiple lyophilized cells of various newly isolated strains of microorganisms, four strains showed a high activity in the acyloin condensation of benzaldehyde with sodium pyruvate. Next, the optimum conditions were determined by factorial design optimization of relevant factors such as temperature, pH and molar ratio of co-substrate.

In order to further explore the synthetic potentials of the selected yeast strains, the screening of various substituted aromatic aldehydes was performed and a preliminary substrate domain was established. Additionally, the possibility to immobilize the selected yeast strains by templated sol-gel entrapment and use them in both batch and continuous flow acyloin condensation reactions was investigated.

3.3. Results and discussions

3.3.1. Yeast strains

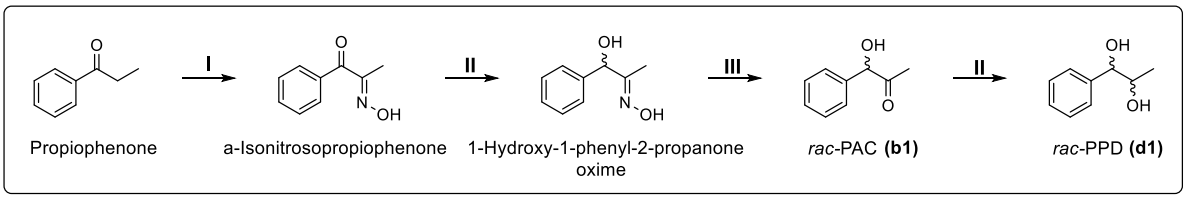
The yeast strains investigated in current study were received as lyophilized whole cells from Fermentia Microbiological Ltd (Budapest, Hungary) and were deposited in the strain collection of Witaria Ltd (Budapest, Hungary).Regarding the biocatalytic applications of the presented strains, these “unconventional” yeasts were scarcely reported in previous studies and some examples are provided in **Table 3.1**.

Table 3.1. Previously reported biocatalytic applications of the investigated yeast species.

Nr. Crt.	Yeast	Witaria Depository Code	Biocatalytic applications
1	*Pichia carsonii*	WY-1	Bioreduction of pro-chiral ketones[47]
2	*Lodderomyces elongisporus*	WY-2	Bioreduction of pro-chiral ketones[47–49]
3	*Wickerhamonyces subpelliculosus*	WY-3 WY-13 WY-21	Bioreduction of pro-chiral ketones[50]
4	*Candida norvegica*	WY-4	Bioreduction of pro-chiral ketones[47]
5	*Cryptococcus curvatus*	WY-5	Stereoselective reduction of oxoester clofibrate analogues[51,52]
6	*Debaryomyces hanseii*	WY-6	Bioreduction of coumarin[53]; Oxidation of alcohols, saccharides, amino acids, and organic acids[54]; Synthesis of (*R*)-PAC by acyloin condensation[45]
7	*Candida guilliermondii*	WY-7 WY-8	Esterification of oleic acid by extracellular lipases[55]; Production of long-chain α,ω-dicarboxylic acids[56]; Bioreduction of pro-chiral ketones[47]; Selective Hydrolysis of Nitriles[57]; Monoreduction of 1-phenylpropane-1,2-dione[58]; Reduction of D-xylose to xylitol[55]
9	*Debaryomyces fabryi*	WY-11	Monoreduction of 1-phenylpropane-1,2-dione[58]; Stereoselective reduction of ketopantoyl lactone[59]; Synthesis of (*R*)-PAC by acyloin condensation[45]
10	*Candida parapsilosis*	WY-12	Preparation of (*R*)-1,3-butanediol (BDO) by stereo-specific microbial oxido-reduction[47,60]; Stereoselective reduction of ketopantoyl lactone[59]

3.3.2. Chemical synthesis of the *rac*-Phenylacetylcarbinol and *rac*-1,2-Phenylpropandiol

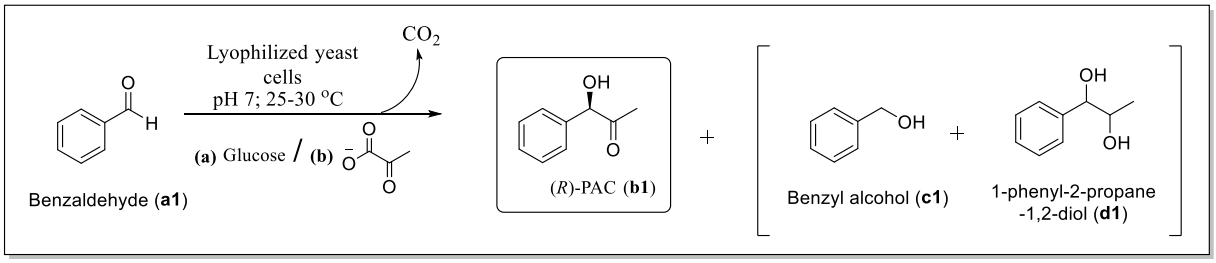
In order to set up the analytical methodology, allowing to monitor the PDC activity of the investigated strains, the chemical synthesis of racemic phenylacetylcarbinol (*rac*-PAC, **b1**) and racemic 1-phenyl-2-propane-1,2-diol (*rac*-PPD, **d1**) was carried out (**Scheme 3.4**). Benzyl alcohol (**c1)** is commercially available and was used as received. A chiral gas chromatographic procedure was set up in order to determine the conversion of the substrate, the distribution of the products and the enantiomeric excess of the desired PAC product.



Scheme 3.4. Chemical synthesis of *rac*-PAC (**b1**) and *rac*-PPD (**d1**): I. *n*-BuONO, CH_3OH, HCl, 0 ºC; II. $NaBH_4$, CH_3OH, r.t.; III. CH_2O sol., HCl, reflux.

3.3.3. Initial investigation of the pyruvate decarboxylase activity of yeast strains towards the synthesis of (*R*)-Phenylacetylcarbinol

With the aim to explore the potential of the native pyruvate decarboxylase activity of these yeast strains, a preliminary analytical scale screening was performed using benzaldehyde **a1** as a model substrate. Accordingly, whole cell biotransformations were set up using lyophilized cells in ambient reaction conditions with glucose or sodium pyruvate as co-substrate (**Scheme 3.5**). Another set of reactions were performed independently as control experiments in absence of the co-substrates. The biocatalytic activity was assessed in each case using three parameters: overall conversion of the substrate aldehyde (C), conversion of the substrate into the carboligation product PAC (**b1**) (C_{PAC}) and PAC enantiomeric excess (*ee*). Reactions were monitored by TLC while conversion of the substrate and enantiomeric excess of the product acyloin was monitored by chiral GC.



Scheme 3.5. PDC activity screening of selected yeast strains for the production of PAC and by-products formed.

As presented in **Table 3.2**, performing the reactions without the addition of external co-substrate did not produce significant amounts of PAC even after 24 h reaction time. However, a quick and high conversion (76.9-99.8%) of substrate into benzyl alcohol **c1** was observed for the yeasts *Pichia carsonii*, *Lodderomyces elongisporus*, *Debaryomyces fabryi*, *Candida parapsilosis and Wickerhamonyces subpelliculosus* ssp3 after only 4 h of reaction time. A moderate conversion of the substrate (~56%) was observed for the two *Candida guilliermondii* species and a low conversion into PAC of ~7.5% with moderate stereoselectivity (*ee* of 81.8% and 79.6%). Extending the reaction time to 24 h did not improve significantly the conversion of the substrate for any of the tested strains. The addition of glucose as co-substrate to the reaction media in order to generate *in situ* pyruvate molecules did not provide an improved the conversion of the substrate into the acyloin product. Moreover, a slight decrease in conversion of the substrate into benzyl alcohol was observed for most strains after 4 h of reaction time (**Table 3.2**).

Table 3.2. PDC activity screening of yeast strains.

Nr. Crt.	Yeast strain	No co-substrate[a]			Glucose Co-substrate[a,b]			Sodium Pyruvate Co-substrate[a,c]		
		C (%)	C_{PAC} (%)	*ee* (%)	C (%)	C_{PAC} (%)	ee (%)	C (%)	C_{PAC} (%)	ee (%)
1	***Pichia carsonii***	89.9	0.9	n.d.	>99	0.9	n.d.	**97.7**	**52.6**	**95.9**
2	***Lodderomyces elongisporus***	>99	1.0	n.d.	96.2	1.9	n.d.	**>99**	**58.9**	**96.1**
3	*Wickerhamonyces subpelliculosus* ssp1	2.2	1.3	n.d.	6.6	0.9	n.d.	10.8	4.8	59.4
4	*Candida norvegica*	14.0	1.0	n.d.	9.5	0.7	n.d.	17.5	16.7	89.2
5	*Cryptococcus curvatus*	15.5	1.3	n.d.	5.2	0.5	n.d.	14.6	10.6	78.2
6	*Debaryomyces hanseii*	1.6	1.1	n.d.	0.6	0.5	n.d.	25.1	24.5	87.8
7	***Candida guilliermondii***	56.7	7.5	81.8	42.8	4.5	88	**>99**	**83.3**	**98.0**
8	***Candida guilliermondii var***	55.3	7.6	79.6	57.7	4.8	90	**>99**	**83.2**	**97.2**
9	***Debaryomyces fabryi***	>99	0.8	n.d.	11.5	2.7	74	**>99**	**30.8**	**94.9**
10	***Candida parapsilosis***	>99	0.7	n.d.	>99	0.8	n.d.	**>99**	**33.4**	**95.5**
11	*Wickerhamonyces subpelliculosus* ssp2	9.2	1.5	n.d.	4.5	0.8	n.d.	28.7	23.6	92.6
12	*Wickerhamonyces subpelliculosus* ssp3	76.9	2.0	n.d.	47.4	0.6	n.d.	20.8	18.3	90.6

[a] Reaction conditions: 20 mg of aldehyde **a1** (in 100 µl of isopropanol), 150 mg lyophilized yeast, phosphate buffer pH 7 (5mL) containing 1 mM $MgCl_2$, 25°C-30°C, 4 h, 1000 rpm.[b] Reaction were performed with the addition of 68 mg of glucose (2 eq.). [c] Reaction were performed with the addition of 41,4 mg of sodium pyruvate (2 eq.).

Finally, in the presence of 2 equiv. of sodium pyruvate, it was observed that the addition of pyruvate favored the carboligase activity of all tested strains and showed an increased conversion into PAC, while a quantitative conversion of the substrate was achieved after 4 h of reaction time for six of the tested strains. Quantitative (>97%) overall conversions were achieved for the strains *Pichia carsonii*, *Lodderomyces elongisporus*, *Candida guilliermondii* and *Candida guilliermondii var* with a good to excellent selectivity towards the acyloin condensation product (C_{PAC} = 52.6-83.3%) an excellent stereoselectivity with (*ee* > 95%). In the case of the other six tested strains, the overall conversion of the substrate did not reach a satisfactory value (C < 30%), even after extending the reaction time to 24 h. The analysis of the product distribution also revealed that the only major side product of the whole cell mediated process was benzyl alcohol and only small amounts of 1,2-phenylpropandiol (< 1-2%) as minor side product, while oxidation by-products (benzoic acid and 1,2-phenylpropan diketone) could not be detected in the reaction mass. In addition to the presented results, the PDC activity of some of the tested strains (*Candida norvegica*, *Debaryomyces fabryi*, *Lodderomyces elongisporus* and *Pichia carsonii*) was also investigated with freshly harvested cells in the same conditions as the lyophilized form.[61] It was

found that, even though sodium pyruvate was used as a co-substrate, PAC was not detected in the reaction mixture and the substrate was converted by the present reductases only into the corresponding alcohol **b1**.

The stereoselectivity of the native pyruvate decarboxylase of these strains was also investigated. The stereoselectivity of the biocatalytic reactions and the absolute configuration of the product were easily established by measuring the optical rotation of PAC isolated and purified from the reaction mixtures. As final result, all tested strains produced (*R*)-PAC as major enantiomer. These results are also in accordance with reported literature that describes most PDCs of yeast origin as (*R*)-selective.[41,46]

3.3.4. Factorial design optimization of reaction conditions for selected yeast strains

After the successful screening of PDC activity of the yeast strains, the four selected strains (*Pichia carsonii, Lodderomyces elongisporus*, *Candida guilliermondii* and *Candida guilliermondii var*) were selected for further studies. Based on the results obtained, the reactions conditions for these biocatalysts were further investigated in order to improve the selectivity of these yeasts and increase the conversion of the substrate into (*R*)-PAC. In the case of the present study, the reaction conditions considered for optimization are the pH of the buffer used as reaction media, the temperature at which the reactions are performed and the molar *ratio* between the substrate (benzaldehyde) and pyruvate co-substrate used in the reaction (pyruvate equivalents). A factorial design optimization of the three factors was selected in order to evaluate the influence of each factor, as well as the possible interactions between them. In order to set up the optimization by means of Design of Experiments (DOE) method, the Minitab software was used to create the factorial design optimization and results analysis. Full factorial design experiments were chosen with triplicate measurement in the center points (*Ct Pt*) as the experimental design.[62] The results were analyzed using Minitab software[63] in order to obtain the model equations for the process' response to the factor variation with interaction terms up to the third order and Pareto charts of standardized effects for each factorial experiment.

Starting from the initial screening reaction conditions as center point (pH = 7, T = 30 ºC and 2 equiv. of pyruvate), a 2^3 full factorial design was proposed, using 2 levels and 3 factors. The upper and lower levels were defined by choosing the degree of variation from the base level: ΔpH=2; ΔT=10 ºC and ΔEquiv.Pyr. = 1 molar equivalent. Coded units for factors are x_1 (pH), x_2

(T), x_3 (Pyr. Equiv.) and response values are Y_1 (C) and Y2 (C_{PAC}). Interaction terms were defined as x_1x_2 (pH*T), x_1x_3(pH*Pyr.Equiv.), x_2x_3(T*Equiv.Pyr.) and $x_1x_2x_3$ (pH*T*Equiv.Pyr.). The enantiomeric excess of the obtained (*R*)-PAC is also reported but was not used as a response in the factorial experiment. A representative experimental design matrixes, as well as responses measured after each run, is presented in **Tables 3.3** for optimization of PAC production by the *Candida guilliermondii* strain. Representative model equations for the process' response to the factor variation with interaction terms up to the third order (Eq. 1 and 2) and Pareto chart of standardized effects (**Figure 3.1**) are provided below.

Table 3.3. The 2^3 full factorial design optimization of PAC production with *Candida guilliermondii* strain

	Uncoded values						Coded values				
Run	**T (ºC)**	**pH**	**Pyr. Equiv.**	**C (%)**	**C_{PAC} (%)**	***ee*$_{PAC}$ (%)**	**x_1**	**x_2**	**x_3**	**Y_1**	**Y_2**
1	5	20	1	80.8	70.7	98.2	-1	-1	-1	80.8	70.7
2	9	20	1	97.9	0.4	n.d.	+1	-1	-1	97.9	0.4
3	5	40	1	59.2	58.1	92.2	-1	+1	-1	59.2	58.1
4	9	40	1	74.9	0.0	n.d.	+1	+1	-1	74.9	0.0
5	5	20	3	99.6	93.9	94.4	-1	-1	+1	99.6	93.9
6	9	20	3	97.1	0.0	n.d.	+1	-1	+1	97.1	0.0
7	5	40	3	83.0	82.5	91.6	-1	+1	+1	83.0	82.5
8	9	40	3	80.0	0.1	n.d.	+1	+1	+1	80.0	0.1
9	30	7	2	99.9	87.2	96.7	0	0	0	99.9	87.2
10	30	7	2	98.9	82.3	96.3	0	0	0	98.9	82.3
11	30	7	2	99.4	85.1	96.2	0	0	0	99.4	85.1

$$\mathbf{C} = 84.060 + 3.413\,pH - 9.766\,T + 5.867\,Pyr.Equiv. - 0.253\,pH*T - 4.781\,pH*Pyr.Equiv. + 1.359\,T*Pyr.Equiv. + 0.105\,pH*T*Pyr.Equiv. + 15.324\,Ct\,Pt \quad (1)$$

$$\mathbf{C_{PAC}} = 38.212 - 38.080\,pH - 3.039\,T + 5.925\,Pyr.Equiv. + 2.976\,pH*T - 5.993\,pH*Pyr.Equiv. + 0.200\,T*Pyr.Equiv. - 0.109\,pH*T*Pyr.Equiv. + 46.64\,Ct\,Pt \quad (2)$$

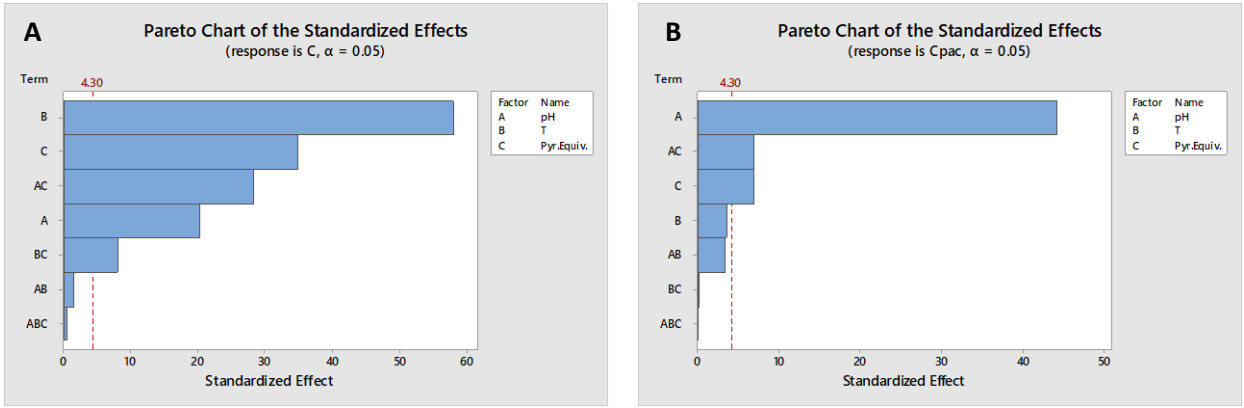


Figure 3.1. Representative Pareto charts of standardized effects for PDC activity of *Candida guilliermondii* strain when response is C (**A**) and C_{PAC} (**B**). The reference line (red line) is drawn at a significance level of 0.05 to indicate which effects are significant.

Analyzing the obtained results, it was observed that the influence of the investigated factors has a high similarity between strains with only low variations and a general overview of the influence of the investigated factors can be drawn. All three factors were shown to have a significant influence on the biocatalytic activity of the investigated cells and on the particular enzymatic activity of the PDC enzyme. A higher temperature (40 ºC) and an alkaline pH (9) is shown to have a negative effect on the PDC activity of the cells tested, leading to conversion into PAC of the substrate of under 1%. However, nearly quantitative conversions into benzyl alcohol are observed in this condition, which shows that the biocatalytic reductase activity of the cells is mostly unaffected. These results are also in agreement with previous data on the stability and activity of PDC enzymes, which report the complete dissociation of the PDC enzyme's tetramer structure in alkaline conditions and decreasing stability with higher temperatures.[44] As expected, increasing the amount of pyruvate available in the reaction media showed a clear increase in the amount of (*R*)-PAC formed during the biocatalytic process. By taking into consideration the overall conversion of the substrate, the amount of PAC produced and the *ee* obtained, the best results were obtained at a pH = 5, T = 20 ºC and 3 equivalents of pyruvate and are presented in **Table 3.4**.

Table 3.4. Results obtained for PDC activity of yeast strains after the first factorial optimization.

Nr. Crt.	Yeast Strain	Sodium Pyruvate Co-substrate [a]		
		C %	C_{PAC} %	ee_{PAC} %
1	*Pichia carsonii*	>99	79.5	95.3
2	*Lodderomyces elongisporus*	>99	77.3	95.5
3	*Candida guilliermondii*	>99	93.9	94.4
4	*Candida guilliermondii var*	97.2	94.7	95.9

[a] Reaction conditions: 20 mg of benzaldehyde **a1** (in 100 µl of isopropanol), 150 mg lyophilized yeast, phosphate buffer containing 64 mg of sodium pyruvate (3 eq.) and 1 mM $MgCl_2$, pH 5 (5 mL), 20 °C, 4 h, 1000 rpm.

Following the data obtained from the first set of optimization experiments, a second 2^2 factorial optimization was performed with a lower degree of freedom with 2 factors and 2 levels in order to further evaluate the effects of pH and pyruvate equivalents on the production of PAC. The temperature was maintained constant at T = 20 ºC since a lower level of temperature could not be investigated due to technical limitation and also to not greatly reduce the enzymatic reaction rate of the acyloin condensation reaction. The base levels (center point) for the two factors were pH = 5 and 3 equiv. of pyruvate. The upper and lower levels were defined by choosing the degree of variation from the base level established in the previous optimization

experiment: ΔpH = 1 and ΔEquiv.Pyr. = 1 equiv. Coded units for factors are x_1 (pH), x_2 (Pyr. Equiv.) and response values are Y_1 (C) and Y2 (C_{PAC}). Interaction term was defined as x_1x_2 (pH*Pyr.Equiv). A representative experimental design matrixes, as well as responses measured after each run, is presented in **Tables 3.5** for optimization of PAC production by the *Candida guilliermondii* strain. Representative model equations for the process' response to the factor variation with interaction terms up to the third order (Eq. 3 and 4) and Pareto chart of standardized effects (**Figure 3.2**) are provided below.

Table 3.5. The 2^2 full factorial design optimization of PAC production with *Candida guilliermondii* strain

	Uncoded values						Coded values			
Run	**T (ºC)**	**pH**	**Pyr. Equiv.**	**C (%)**	**C_{PAC} (%)**	***ee*$_{PAC}$ (%)**	**x_1**	**x_2**	**Y_1**	**Y_2**
1	20	4	2	5.8	5.7	98.2	-1	-1	5.8	5.7
2		6	2	98.2	95.5	98.2	+1	-1	98.2	95.5
3		4	4	3.8	3.8	97.8	-1	+1	3.8	3.8
4		6	4	99.4	97.5	96.3	+1	+1	99.4	97.5
5		5	3	97.2	96.3	94.4	0	0	97.2	96.3
6		5	3	95.8	94.4	94.8	0	0	95.8	94.4
7		5	3	96.1	94.6	94.7	0	0	96.1	94.6

$$\mathbf{C} = 51.804 + 46.967\,pH - 0.202\,Pyr.Equiv. + 0.801\,pH * Pyr.Equiv. + 44.558\,Ct\,Pt \quad (3)$$

$$\mathbf{C_{PAC}} = 50.620 + 45.868\,pH + 0.022\,Pyr.Equiv. + 1.015\,pH * Pyr.Equiv. + 44.481\,Ct\,Pt \quad (4)$$

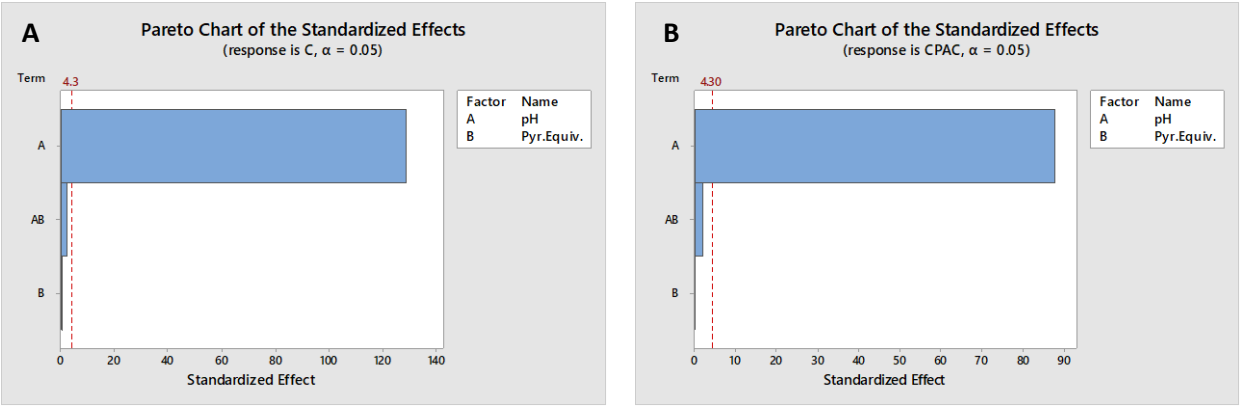


Figure 3.2. Representative Pareto charts of standardized effects for PDC activity of *Candida guilliermondii* strain when response is C (**A**) and C_{PAC} (**B**). The reference line (red line) is drawn at a significance level of 0.05 to indicate which effects are significant.

Based on the analysis of the responses C and C_{PAC}, the biocatalytic process showed a similar pattern for all four studied yeast strains. The pH of the reaction medium was the only significant factor and has a positive effect. Generally, increasing the pH (pH = 6) resulted in a slightly (~1-3%) higher conversion of the benzaldehyde into PAC while lowering the pH below

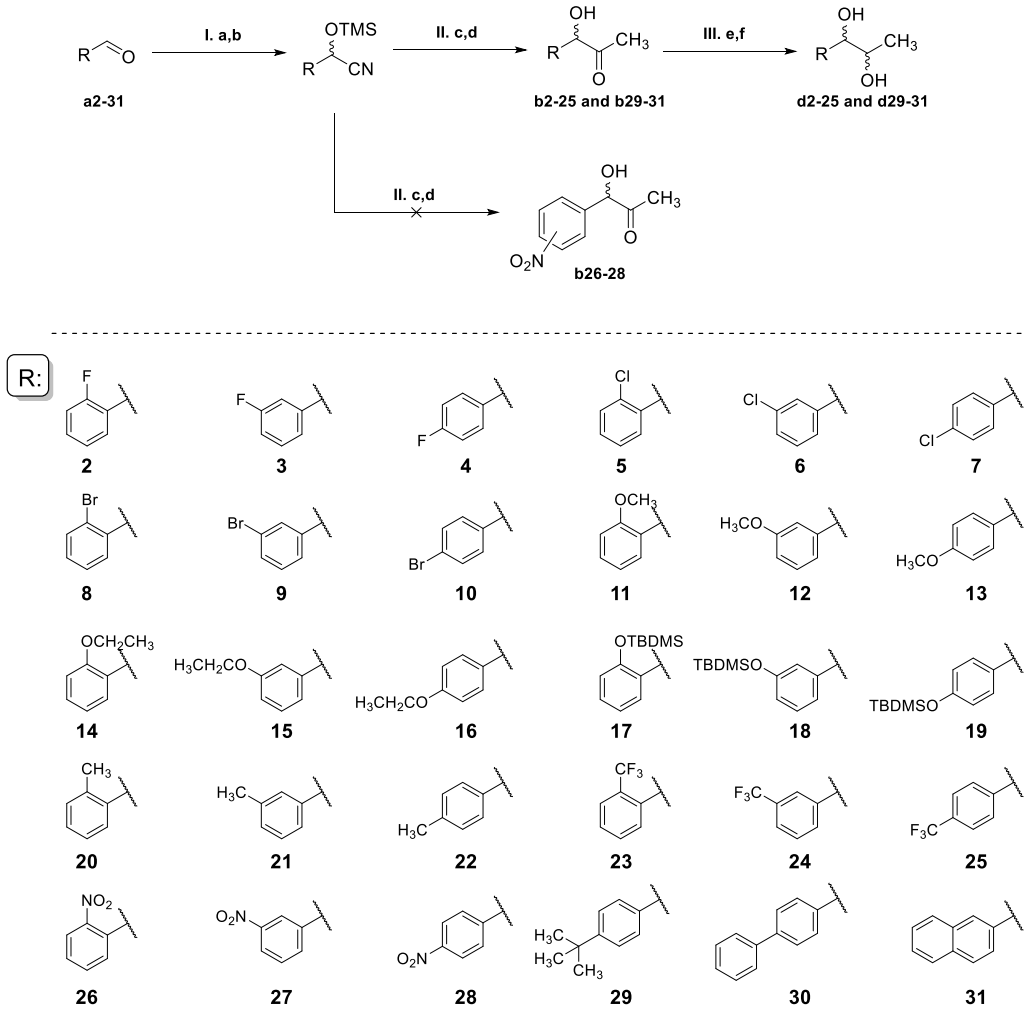
the center point (pH = 4) resulted in very low overall conversions and conversion into the acyloin product. Increasing the molar *ratio* between benzaldehyde and pyruvate did not result in significant improvement to justify the addition of supplementary pyruvate to the reaction. Finally, it was also observed that the *ee* of the (*R*)-PAC product was slightly higher at a pH of 6 and when only 2 equivalents of sodium pyruvate were added as co-substrate. The final optimum conditions (**Table 3.6**) were selected based on the experimental results obtained after the second set of experiments, taking into account that only minor differences where observed for the overall conversion of benzaldehyde and the conversion into condensation product C_{PAC}.

Table 3.6. Results obtained for PDC activity of yeast strains after the second factorial optimization in optimum conditions.

Nr. Crt.	Yeast Strain	Sodium Pyruvate Co-substrate[a]		
		C %	C_{PAC} %	ee_{PAC} %
1	*Pichia carsonii*	**>99**	**79.7**	**97.6**
2	*Lodderomyces elongisporus*	**>99**	**79.5**	**98.1**
3	*Candida guilliermondii*	**98.2**	**95.5**	**98.2**
4	*Candida guilliermondii var*	**98.1**	**93.5**	**98.3**

[a] Reaction conditions: 20 mg of aldehyde **a1** (in 100 µl of isopropanol), 150 mg lyophilized yeast, phosphate buffer containing 42 mg of sodium pyruvate (2 eq.) and 1 mM $MgCl_2$, pH 6 (5 mL), 20°C, 4 h, 1000 rpm.

3.3.5. Chemical synthesis of the racemic acyloins *rac*-b2-31 and their corresponding 1,2-diols *rac*-d2-31



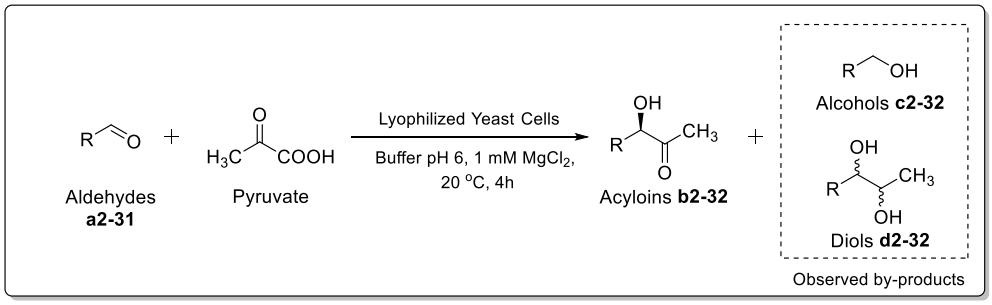
Scheme 3.6. Chemical synthesis of racemic acyloins *rac*-**b2–31** and 1,2-diols *rac*-**d2-31**. Reaction conditions: I. a) TMSCN, LiCl/THF, 25 °C, b) *n*-Hexane/1M Na_2CO_3 sol.; II. c) MeMgI/Et_2O, 25 °C, d) 5% HCl sol./ 0 °C; III. e) $NaBH_4$, methanol/0 °C; f) NaCl sat. sol.

In order to further investigate the substrate domain of the PDC enzyme present in the selected yeast strains, several *ortho*-, *meta*- and *para*-substituted benzaldehydes **a2-29** as well as biphenyl (**a30**) and naphthyl (**a31**) aldehydes were selected as substrates. Consequently, the chemical synthesis of the racemic acyloins and corresponding 1,2-diols was performed and the synthetic pathway adopted, which is depicted in **Scheme 3.6**. For nitro substituted aldehydes **a26-28** the proposed synthetic method was not applicable due to the electronic effects of the nitro group and therefore, the racemic acyloins **b26-28** and 1,2-diols **d26-28** could not be obtained but literature ^{1}H-NMR data were used to evaluate the conversion. The obtained racemic acyloins and

1,2-diols were further used in order to set up HPLC separations methods as well as a ^{1}H-NMR based method for monitoring conversion and enantiomeric excess of the product acyloins from biocatalytic reactions.

3.3.6. Substrate domain assessment of selected yeast strains

With all preliminary data on the PDC activity of the four lyophilized yeast strains in hand, the substrate scope of these enzymes was further investigated by testing their biocatalytic activity on *ortho*-, *meta*- and *para*- substituted benzaldehydes **a2-29,** as well as biphenyl-4-carboxaldehyde **a30** and 2-naphthaldehyde **a31** as bulky substrates. Reactions were carried out at analytical scale as previously done for benzaldehyde **a1** in the optimum conditions established through the factorial design optimization (**Scheme 3.7**). The reactions were stopped by biomass centrifugation and extraction in organic solvent prior to ^{1}H-NMR and HPLC analysis. As previously mentioned, the outcome of the biocatalytic reactions was monitored by a ^{1}H-NMR method as described in recent literature[45,64] and enantiomeric excess of the product acyloins were determined using the developed chiral HPLC methods. Results are expressed as overall conversion of the substrate aldehyde into products C (%), conversion of the substrate into acyloin condensation product C_B (%) and enantiomeric excess *ee* (%) of the obtained acyloin. The difference between the overall conversion and the conversion into acyloin represents the amount of substrate transformed into the corresponding alcohols (**c2-31**) or 1,2-diols (**d2-31**) due to the activity of the reductases also present in the cells. As a general observation, only very low amounts of 1,2-diols were detected (< 5%) while the major side-product was represented by the aromatic alcohols. Oxidation side-products could not be detected in the mixture after completion of the biocatalytic reaction.



Scheme 3.7. The PDC substrate scope investigation of lyophilized yeast cells in optimized conditions. Reaction conditions: 75 mg lyophilized cells, 0.1 mmole aldehyde **a2-32** in 50 µL isopropanol, 0.2 mmoles of sodium pyruvate (2 equiv.), 50mM phosphate buffer, 20 ºC, 4h, 1000 rpm.

The aldehydes tested were selected to have electron donating (substrates **a11-16** and **a20-22**) and electron withdrawing (**a2-10** and **a23-38**) substituents as well as some more sterically demanding bulky substrates (**a17-19** and **a29-31**). The results obtained are presented in **Figures 3.3-3.5**, and for comparison, the data is depicted simultaneously for all 4 tested strains per substrate.

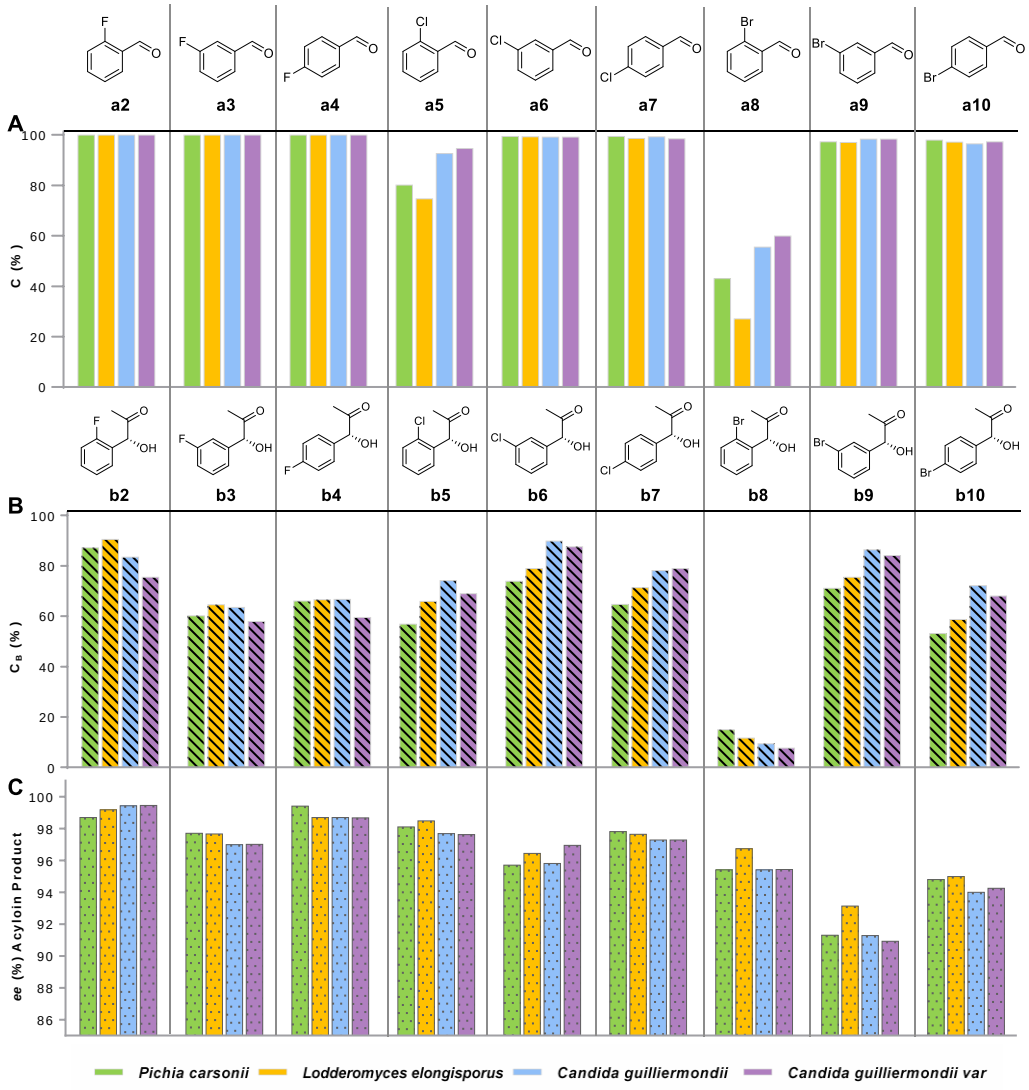


Figure 3.3. Compared results obtained for *Pichia carsonii*, *Lodderomyces elongisporus*, *Candida guilliermondii* and *Candida guilliermondii* var yeast strains in the acyloin condensation of substituted aldehydes **a2-10** in optimum conditions: **A)** Overall conversion of the substrate C (%); **B)** Conversion into acyloin product C_B (%); **C)** Enantiomeric excess of the acyloin product *ee* (%).

As depicted in **Figure 3.3 A**, all four yeast strains showed similar results on the halogen-substituted substrates **a2-10**. Excellent overall conversions of 96-99.9% were observed for all derivatives, similarly to the unsubstituted benzaldehyde, except for the *o*-Cl (**a5**, C = 74-94%)

and *o*-Br (**a6**, C = 26-60%) benzaldehydes. The PDC activity, however, generally showed a significant decrease when compared to the results obtained for benzaldehyde (C > 99). As can be seen in **Figure 3.3 B**, good to excellent conversions into the acyloin products (C_B = 53-91%) were observed for the halogen substituted substrates, except for *o*-Br benzaldehyde (C_B = 7-15%). The enantiomeric excess of the acyloins obtained (**Figure 3.3 C)** were also good to excellent (*ee* = 90-99.5%), with respect to the *ee* values obtained for benzaldehyde with the four strains.

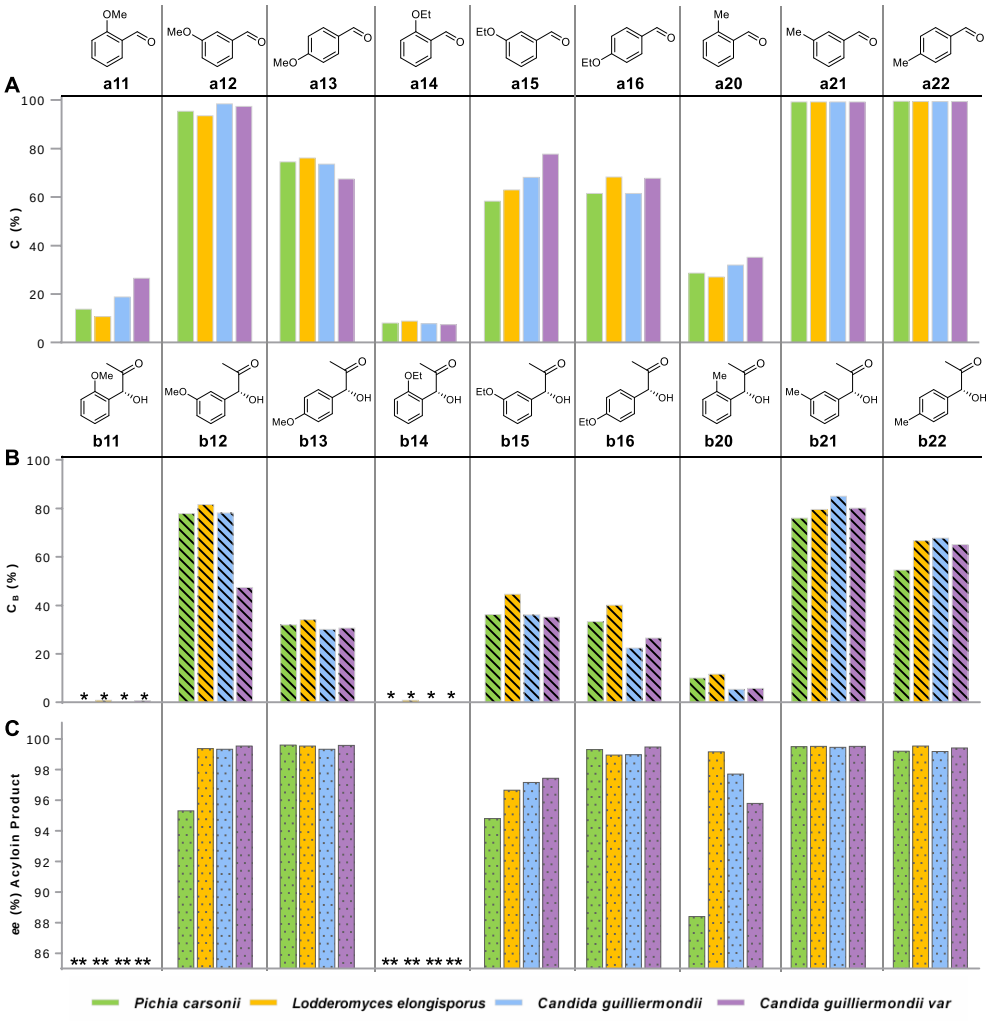


Figure 3.4. Compared results obtained for *Pichia carsonii*, *Lodderomyces elongisporus*, *Candida guilliermondii* and *Candida guilliermondii* var yeast strains in the acyloin condensation of substituted aldehydes **a11-16** and **a20-21** in optimum conditions: **A.** Overall conversion of the substrate C (%); **B.** Conversion into acyloin product C_B (%); **C.** Enantiomeric excess of the acyloin product *ee* (%).* No product formed (or <1%). ** Not determined.

For alkoxy and methyl substituted benzaldehydes, the tested strains provided good to moderate amounts of acyloins for benzaldehydes substituted in the *meta* and *para* position (C_B =

26-85%) as presented in **Figure 3.4**. The *o*-methoxy (**a11**) and *o*-ethoxy (**a14**) benzaldehydes were transformed with a low overall conversion into the corresponding benzyl alcohols and no acyloin product could be detected. The optical purities of the obtained acyloins were generally higher than those obtained for the halogen substituted substrates, as can be seen in **Figure 3.4 C**.

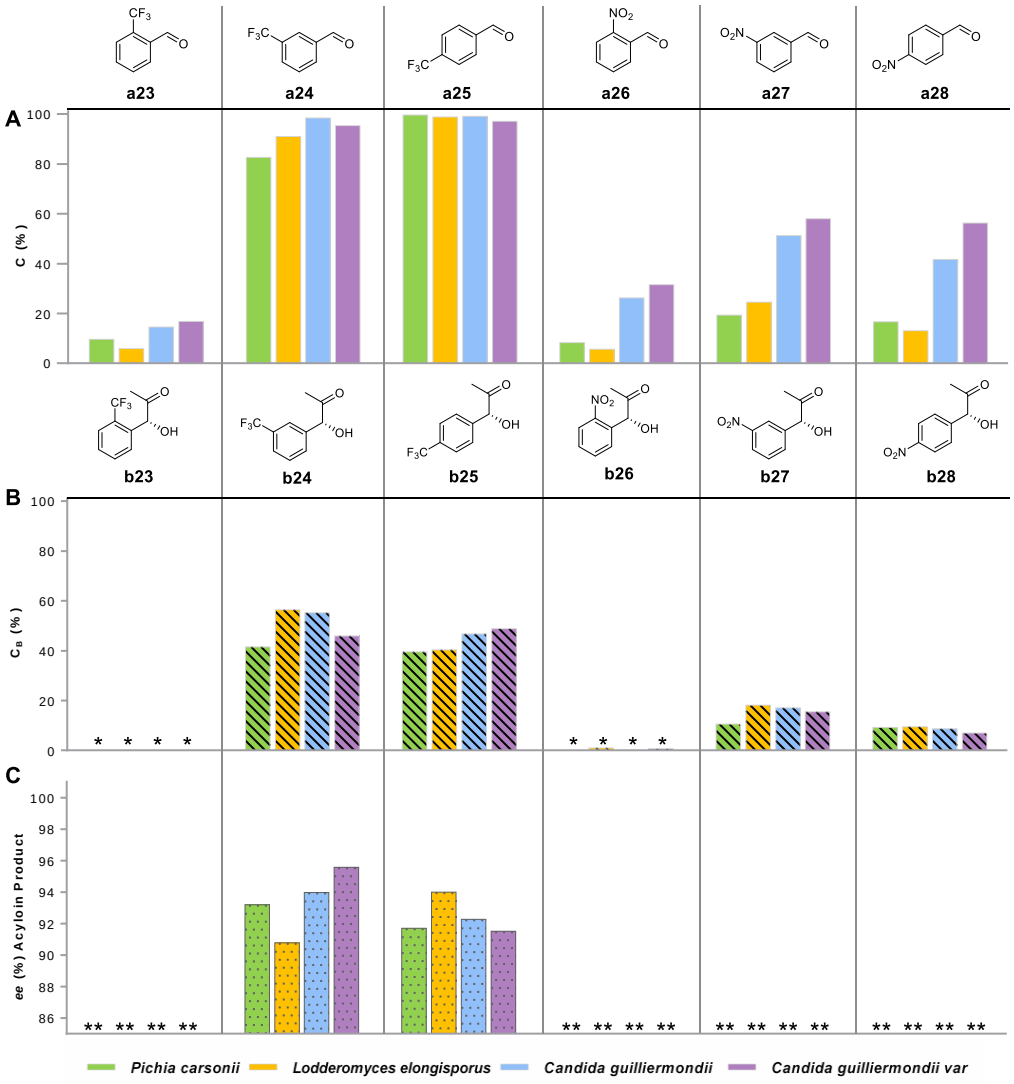


Figure 3.5. Compared results obtained for *Pichia carsonii*, *Lodderomyces elongisporus*, *Candida guilliermondii* and *Candida guilliermondii* var yeast strains in the acyloin condensation of substituted aldehydes **a23-28** in optimum conditions: **A.** Overall conversion of the substrate C (%); **B.** Conversion into acyloin product C_B (%); **C.** Enantiomeric excess of the acyloin product *ee* (%).* No product formed (or <1%). ** Not determined.

Following up the previous results, nitro- and trifluoromethyl-substituted benzaldehydes were also tested as substrates for acyloin condensation with the four yeast strains. As compared with the other tested *ortho*-substituted substrates, *o*-CF_3(**a23**) and *o*-NO_2 (**a26**) benzaldehydes provided low overall conversions (**Figure 3.5 A**) and no acyloin products. Excellent overall

conversions (C > 80%) where obtained for *m*-CF_3 (**a24**) and *p*-CF_3 (**a25**) benzaldehydes (39-57% conversions and optical purities above 90%). For the *m*-NO_2 (**a27**) and *p*-NO_2 (**a28**) substrates, the aldehydes overall conversion was lower than for the CF_3 analogues (C = 13-58%) and the conversion into the corresponding acyloins did not go above a 20% value, as can be seen in **Figure 3.5 B**. The optical purity for the nitro-substituted acyloins could not be determined.

Finally, bulkier substrates such as *tert*-butyldimethylsilyl (TBDMS) protected hydroxy benzaldehydes (**a17-a19**), *p-tert*-butyl benzaldehyde (**a29**) as well as biphenyl (**a30**) and naphthyl (**a31**) aldehydes were tested as substrates using the four selected yeast strains. As expected, the TBDMS protected hydroxy benzaldehydes were poor substrates for the PDC enzyme and not even the corresponding alcohols were formed during the biocatalytic reactions. The *p-tert*-butyl substituted benzaldehyde **a29**, however presented moderate overall conversions with all the tested strains (C = 48-63%) and the amount of acyloin formed was rather low (C_B = 5-8%, *ee* = 94-99.5%). Biphenyl-4-carboxaldehyde **a30** and 2-naphthaldehyde (**a31**) provided even lower overall conversions (C = 9-22%) than the *tert*-butyl analogue, with conversions into the acyloin product C_B < 10% and *ee* = 94-99%.

Analyzing the obtained data after testing the four yeast strains on the diverse substrate spectrum, several discussions can be elaborated regarding a preliminary substrate scope of the PDC present in these cells. Low variations between the strains with respects to the amount of produced acyloin and enantiomeric excess, except for some localized cases for certain substrates, were observed. All tested strains showed excellent biocatalytic PDC activity and selectivity for the halogen substituted substrates **a2**-**a10** with good to excellent conversions into the corresponding acyloins, except for the *o*-Br-benzaldehyde **a8**. In comparison, the *o*-alkoxy derivatives were not transformed by the yeast cells into the corresponding acyloins and only alcohols were observed as product, except for *o*-methyl benzaldehyde analogue **a20**. The *para*- and *meta*-substituted alkoxy and methyl benzaldehydes, on the other hand, were transformed with moderate to excellent conversions, depending on the position and type of substituent. Similar results were also seen for the trifluoromethyl (**a23-a25**) and nitro (**a26-29**) substituted benzaldehydes, where only the corresponding alcohols were detected in low amounts for the *ortho* derivatives, while the *meta* and *para* substrates were accepted as substrates by the decarboxylase enzyme and resulted in low to moderate amounts of acyloin detected in the reaction mixture. Lastly, "bulky" substrates were the poorest substrates among all tested

aldehydes. The TBDMS-protected hydroxy aldehydes were not transformed at all by the cells with only very low amount of alcohol and acyloin detected for the *para* substituted substrate **a19**. Although better results were obtained for the other sterically demanding substrates, the conversion into acyloin product was rather unsatisfactory.

As a general observation regarding the influence of the position, *ortho*-substituted benzaldehydes proved to be inappropriate substrates for the PDC enzyme of these strains as no acyloin (or < 1%) was formed in most cases with the exception of F, Cl, Br and methyl derivatives. As presented in **Figure 3.6 A** for the reactions mediated by *C. guilliermondii* (as representative strain) on *ortho*-substituted substrates, the amount of acyloin obtained drastically decreases as the volume or "bulkiness" of the substituent increases. The main reason for this low reactivity lies with the steric hindrance caused by these substituents that can obstruct the nucleophilic attack of the activated aldehyde bonded to the TPP, since in most cases the derivatives with the same functional group were accepted as substrates if the substituent was in *meta*- or *para*-positions. Also, this steric hindrance can also hamper the access of the substrate in the active site of the PDC enzyme. Nonetheless, the amount of acyloin obtained for the *meta*- and *para*-substituted substrates were comparable and that *meta*- derivatives generally gave higher conversions (C_B%) than their *para* analogues as depicted in **Figures 3.6 B** and **C**.

Taking into account the substituents electronic effects, it can be assumed that both, electron withdrawing substituents and the electron donating substituents also have a certain degree of influence on the activity of the PDC enzyme on the tested substrates. However, the conversion into acyloin product for the *para*- and *meta*-substituted substrates does not show a clear correlation between the electronic effect and the amount of the formed product.

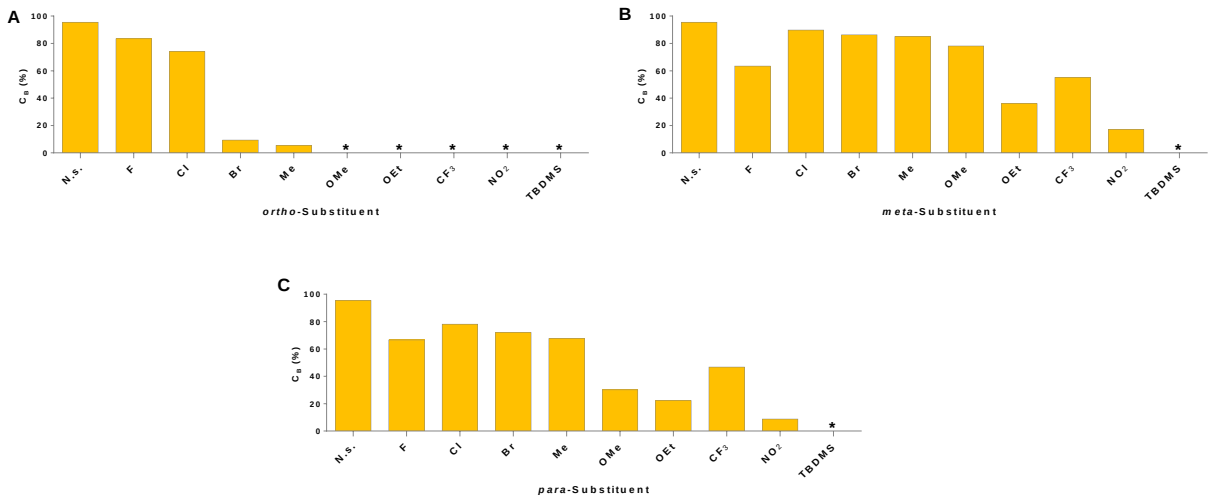


Figure 3.6. Influence of position and type of substituent on the acyloin condensation reactions mediated by lyophilized whole-cells of *C. guilliermondii*. **A.** Conversions into acyloin products (C_B) obtained for *ortho* substituted substrates; **B.** Conversions into acyloin products (C_B) obtained for *meta* substituted substrates; **C.** Conversions into acyloin products (C_B) obtained for *para* substituted substrates. * No product formed (or <1%). N.s.- no substituent.

In regards to the optical purity of the obtained acyloins using the four yeast strains as catalysts, the enantiomeric excess was high (90-98%) for all the acyloins obtained, however a direct correlation between enantioselectivities and substrate/substituent cannot be considered with the current data, as shown in **Figure 3.7**. Furthermore, it can be presumed that the enantiopreference of the PDC present in the investigated strains does not change when substituted aldehydes are employed as substrates instead of benzaldehyde. From this perspective, it can be stated that the *R*-enantiomer was the major product for all the substrates investigated. This conclusion is also supported by the literature published to present date.[35,65]

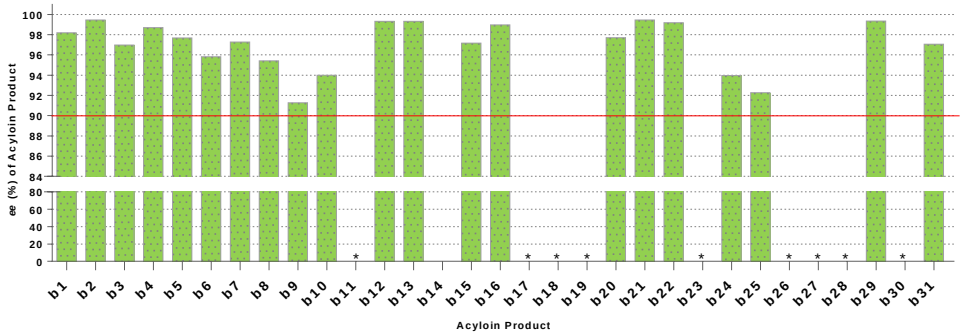


Figure 3.7. Optical purity (*ee*%) of acyloin products **b1-31** obtained by biocatalytic acyloin condensation reactions mediated by lyophilized whole-cells of *C. guilliermondii*. *No acyloin product formed or not determined.

3.3.7. (*R*)-Phenylacetylcarbinol production with immobilized cells of selected yeast strains in batch and continuous mode.

Besides the activity and stereoselectivity of the tested yeast strains, another important aspect of their potential use as biocatalysts resides in their recyclability and storage stability. Therefore, in addition to the previously described investigations, the possibility to employ two of the tested yeast strains, *Pichia carsonii* and *Lodderomyces elongisporus*, in immobilized form to produce (*R*)-PAC through acyloin condensation reactions was also investigated, in both batch and continuous flow mode. For the immobilization procedure, a so called "2nd generation" templated sol-gel entrapment method (**Figure 3.8**) was used as previously described, with hollow silica microspheres MAT540 as support for the immobilization.[66]

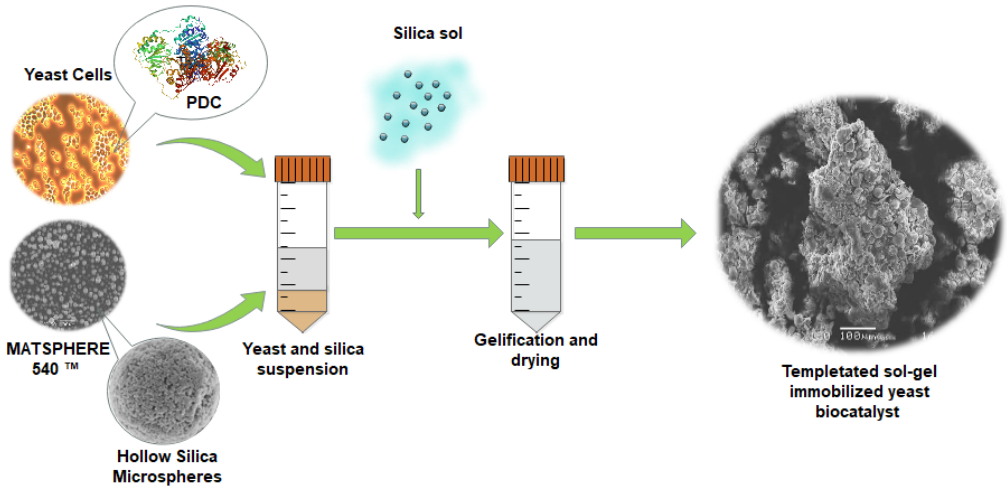


Figure 3.8. Immobilization of yeast cells by templated sol-gel entrapment.

Accordingly, the immobilized form of *Pichia carsonii* and *Lodderomyces elongisporus* was first tested in preliminary batch acyloin condensation using benzaldehyde as substrate. Biocatalytic reactions were set up using the immobilized yeast cells and the work-up and analysis were performed as previously described for reactions with lyophilized cells. As expected, the results presented in **Table 3.7** showed that the PDC activity of the immobilized yeast cells was lower than for the lyophilized form of the cells. It was also observed that while for the *L. elongisporus* immobilized biocatalyst the overall conversion and the amount of acyloin dropped only slightly (from 59 to 44%), the PDC activity of the *P. carsonii* strain decreased significantly and only minor conversion into the acyloin product was observed.

Table 3.7. Results obtained for lyophilized and immobilized *L. elongisporus* and *P. carsonii* biocatalysts mediated acyloin condensation of benzaldehyde in batch reactions.

Biocatalyst[a]	C (%)	C_{PAC} (%)	$ee_{(R)\text{-PAC}}$ (%)
Lyophilized cells of *L. elongisporus*	>99	59	96
Lyophilized cells of *P. carsonii*	98	53	96
Immobilized cells of *L. elongisporus*	92	44	93
Immobilized cells of *P. carsonii*	>99	5	92

[a] Reaction conditions: biocatalyst (150 mg), citrate buffer (2.5 mL, pH = 5, 100mM, containing 1 mM MgCl2) and sodium pyruvate (3 equiv.) as a co-substrate; benzaldehyde (40 mM in 100 µL isopropanol), 750 rpm, 25 ºC, 3 h.

Further, the recyclability of the immobilized biocatalyst was tested in batch reactions in the same conditions for the *L. elongisporus* biocatalyst comparison with the lyophilized form (**Table 3.8**). It was observed that the activity of the immobilized biocatalyst decreased by ~10% in the second run, while the lyophilized cells showed a more substantial loss of activity in comparison. From the results obtained, it was observed that the immobilized form the yeast cells helps to conserve the PDC activity of the cells tested, when compared to their lyophilized counterpart.

Table 3.8. Recyclability of immobilized and lyophilized *Lodderomyces elongisporus* whole cells in acyloin condensation of benzaldehyde.

	First Run			Second Run		
Biocatalyst	**C (%)**	**C_{PAC} (%)**	**$ee_{(R)\text{-PAC}}$ (%)**	**C (%)**	**C_{PAC} (%)**	**$ee_{(R)\text{-PAC}}$ (%)**
Lyophilized cells of *L. elongisporus*	>99	58	96	63	5	92
Immobilized cells of *L. elongisporus*	92	44	93	82	35	94

In spite of the modest activity obtained for the immobilized biocatalysts, further studies were performed in continuous-flow mode. Consequently, a continuous-flow system was devised and the acyloin condensation of benzaldehyde was performed using the same biocatalyst under two flow rates (**Figure 3.9**). For flow experiments, small amounts of TPP were added in the reaction mixture in order to compensate an observed gradual "washing-out" phenomenon of the TPP cofactor.

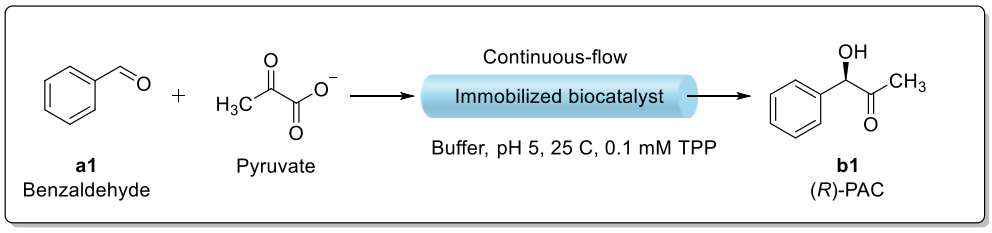


Figure 3.9. Biocatalytic acyloin condensation in continuous-flow with immobilized yeast cells.

In continuous-flow, a higher conversion into (*R*)-PAC was observed for both tested biocatalyst at a flow rate of 50 µL/min once the system was stabilized and reached the stationary

phase as described in **Table 3.9**. Increasing the flow rate to 100 µL/min resulted in a higher specific reaction rate in the flow system; however, this also resulted in a decrease of overall conversion of the substrate, as well as the amount of (*R*)-PAC produced.

Table 3.9. Acyloin condensation of benzaldehyde mediated by immobilized biocatalysts in continuous flow mode at different flow rates.

Biocatalyst	Flow Rate* (µL/min)	C (%)	C_{PAC} (%)	$ee_{(R)\text{-PAC}}$ (%)	r_{flow}
Immobilized cells of *L. elongisporus*	50	98	51	96	1.2
	100	81	37	94	1.8
Immobilized cells of *P. carsonii*	50	69	19	90	0.5
	100	59	15	91	0.7

Reaction conditions: benzaldehyde (20 mM), sodium pyruvate (60 mM), TPP (0.2 mM), $MgCl_2$ (1.0 mM) and 2-propanol (2 v/v %) in buffer (100 mM, pH 5.0), column filling weight: 410±13 mg, temperature: 25 °C, flow rate: 50 and 100 µl min^{-1}. *Specific reaction rate in the flow system (r_{flow}) according to following equation: $r_{flow} = ([P] \times f)/m_b$, where [*P*] – concentration of the product (µmol/mL), *f* – flow rate (mL/min), m_b – mass of the applied biocatalyst (g).

Although only modest conversions into (*R*)-PAC were obtained even in continuous-flow conditions, the operational stability of the biocatalyst however was excellent and could be maintained for 24 h without significant loss of enzymatic activity or optical purity (**Figure 3.10**).

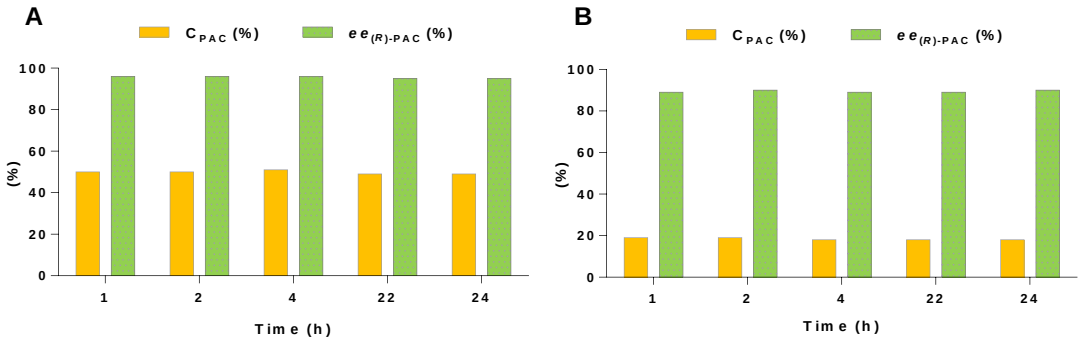


Figure 3.10. Productivity and operational stability of immobilized *L. elongisporus* (A) and *P. carsonii* (B) in acyloin condensation reactions performed in continuous-flow mode. Reaction conditions: benzaldehyde (20 mM), sodium pyruvate (60mM), TPP (0.2 mM), $MgCl_2$ (1.0 mM) and 2-propanol (2 v/v %) in buffer (100 mM, pH 5.0), column filling weight: 410 ± 13 mg, temperature: 25 °C, flow rate: 50 µl min^{-1}].

As a general overview of the described immobilized biocatalyst and its application, the results were modest in terms of the amount of (*R*)-PAC obtained during the biocatalytic reactions but further investigations can be carried out by immobilizing other yeast cells with higher PDC activity using the same process and by further optimizing the reactions conditions. Nonetheless, the obtained biocatalysts showed a good recyclability in bath reactions and remarkable operational stability when the biocatalytic reaction was performed in continuous-flow mode.

3.4. Conclusions

As concluding remarks of the current chapter, the proposed aims, to study the newly selected yeast strains, were successfully achieved. The screening of the twelve nonconventional yeasts revealed that four strains showed great results in terms of biocatalytic pyruvate decarboxylase activity in the carboligation reaction of benzaldehyde towards the synthesis of (*R*)-phenylacetylcarbinol (C_{PAC} >50%). Additionally, these unconventional yeast strains contain (*R*)-selective PDC enzymes and provide the corresponding (*R)*-PAC with high optical purity. Selected reaction conditions were successfully optimized through full factorial design experiments for the four selected strains and, in optimum conditions, quantitative conversions (>98%) of benzaldehyde and high conversions (>80%) into the desired (*R*)-PAC product were obtained, with excellent optical purities (*ee* > 97%). Further, the preliminary substrate scope of the PDC enzymes present in the yeasts was determined and it was found that the enzymes can accept a wide range of unnatural substrates. Except for local differences, the substrate scope of the innate PDC enzymes of the yeasts did not show consistent high variations for reactions involving the same substrate. Excellent biocatalytic PDC activity and selectivity was observed for a wide range of aldehydes and acyloin products with high optical purity were obtained for all substrates that were accepted by the PDC enzymes present in the cells.

Lastly, the biocatalytic applicability of *Pichia carsonii* and *Lodderomyces elongisporus* strains was also investigated in immobilized form by entrapping the yeast cells in a silica microparticle supported sol-gel matrixes. After immobilization, it was demonstrated that the investigated strains could be successfully used as recyclable biocatalysts for (*R*)-PAC synthesis in both batch and continuous-flow processes, however a decrease in activity was observed. Furthermore, it was shown that the developed biocatalysts have an excellent operational stability and can be operated continuously for more than 24 h without significant loss of PDC activity.

4. General conclusions

The current thesis deals with two relevant topics in biocatalysis, in accordance with the two chapters discussed.

The first aim of the thesis was to set-up a cost-effective biocatalytic procedure for the sustainable synthesis of 2,5-bis(hydroxymethyl)furan fatty acid diesters as value added products from renewable resources. The study demonstrated that BHMF fatty acid diesters could be successfully synthesized in high yield and selectivity by Novozym 435 mediated diesterification of 2,5-bis(hydroxymethyl)furan with fatty acids in 2-MeTHF as green solvent. Performing the reaction at a 100 g-scale enabled the synthesis of a sufficient amount of the targeted fatty acid diester, which was further tested and confirmed as a potent additive for increasing the cetane number of commercial biodiesel. As an alternative biocatalytic process, liquid fatty acids or liquid mixtures of fatty acids derived from sunflower oil enabled the biocatalytic synthesis of BHMF fatty acid diesters in solvent-free heterogeneous conditions. In terms of sustainability, both biocatalytic processes presented very good green metrics that support the overall sustainability of the procedures.

The second aim of the thesis was to investigate unconventional yeast strains as new biocatalysts for the synthesis of optically active acyloins. The study reported the screening of several unconventional newly isolated yeast strains in lyophilized form for the synthesis of (*R*)-phenylacetylcarbinol. After a preliminary selection based on the observed PDC activity, four strains of *Pichia carsonii, Lodderomyces elongisporus, Candida guilliermondii* and *Candida guilliermondii* var were identified as potent catalysts with a high PDC enzymatic activity. In optimum conditions, the substrate scope of the PDC enzymes present in the yeasts was further investigated. It was found that the yeast could perform the acyloin condensation of a wide range of unnatural substrates. Additionally, the *P. carsonii* and *L. elongisporus* strains were also investigated as biocatalyst for acyloin condensation reactions in their immobilized form. The obtained biocatalysts showed modest results in the synthesis of (*R*)-PAC in both batch and continuous flow reactions, however with excellent operational stability of the biocatalyst over a long operation time in continuous-flow.

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7. List of publications

The present thesis is based on the following publications. Unpublished data is also included.

Scientific publications:

1. Lăcătuş, M.A., Bencze, L.C., Toşa, M.I., Paizs, C. and Irimie, F.D., **2018**. Eco-friendly enzymatic production of 2, 5-bis (hydroxymethyl) furan fatty acid diesters, potential biodiesel additives. *ACS Sustainable Chemistry & Engineering*, *6*(9), pp.11353-11359.

2. Lăcătuş, M.A., Dudu, A.I., Bencze, L.C., Katona, G., Irimie, F.D., Paizs, C. and Toşa, M.I., **2020**. Solvent-free biocatalytic synthesis of 2, 5-bis-(hydroxymethyl) furan fatty acid diesters from renewable resources. *ACS Sustainable Chemistry & Engineering*, *8*(3), pp.1611-1617.

3. Nagy-Győr, L., Lăcătuş, M., Balogh-Weiser, D., Csuka, P., Bódai, V., Erdélyi, B., Molnár, Z., Hornyánszky, G., Paizs, C. and Poppe, L., **2019**. How to Turn Yeast Cells into a Sustainable and Switchable Biocatalyst? On-Demand Catalysis of Ketone Bioreduction or Acyloin Condensation. *ACS Sustainable Chemistry & Engineering*, *7*(24), pp.19375-19383.

4. Nagy-Győr, L., Farkas, E., Lăcătuş, M., Tóth, G., Incze, D., Hornyánszky, G., Bódai, V., Paizs, C., Poppe, L. and Balogh-Weiser, D., **2020**. Conservation of the Biocatalytic Activity of Whole Yeast Cells by Supported Sol–Gel Entrapment for Efficient Acyloin Condensation. *Periodica Polytechnica Chemical Engineering*, *64*(2), pp.153-161.

Other co-authored publications with complementary topics:

5. Dudu, A.I., Lăcătuş, M.A., Bencze, L.C., Paizs, C. and Toşa, M.I., **2021**. Green Process for the Enzymatic Synthesis of Aroma Compounds Mediated by Lipases Entrapped in Tailored Sol–Gel Matrices. *ACS Sustainable Chemistry & Engineering*, *9*(15), pp.5461-5469.

Conference presentations:

1. Lăcătuş M., Bódai V., Toşa M. I., Paizs C., Poppe L., Irimie F.D., Investigation of new biocatalysts with carboligase activity for the synthesis of chiral aromatic 2-hydroxyketones. National Conference of Doctoral Schools from the University Consortium - Chemistry Section, Iasi (Romania), **2018**

2. Lăcătuş M., Toşa M. I., Paizs C., Irimie F.D., An eco-friendly enzymatic approach for the production of 2,5-bis(hydroxymethyl)furan fatty acid esters, International Conference Young Researchers in Sciences, Cluj-Napoca (Romania), **2017**

3. Lăcătuş M., Bencze L.C., Paizs C., Irimie F.D., Potential of some yeast strains in the stereoselective biosynthesis of acyloins, 3rd Symposium Biotransformations, Warsaw (Poland), **2016**

4. Lăcătuş M., Nagy-Győr[2] L., Bencze L.C., Paizs C., Poppe L., Irimie F.D., Biocatalytic stereoselective acyloin condensations by new yeast strains, Young Researchers' International Conference on Chemistry and Chemical Engineering (YRICCCE I), Cluj-Napoca (Romania), **2016**

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