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Degree project in Biotechnology KBTM01

Development of a Water Activity Control and Reaction Monitoring System for Acidolysis and Transesterification Reactions using Immobilized Lipases in a Rotating Bed Reactor

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Popular summary

Development of a Water Activity Control and Reaction Monitoring System for Acidolysis and Transesterification Reactions using Immobilized Lipases in a Rotating Bed Reactor

Enzymes are biomolecules found in all organisms on Earth. In the human body, these molecules perform different kinds of reactions, for example enzymes can degrade starch from food to glucose or to degrade alcohol, which is toxic, to less toxic compounds. As soon as the working principle of enzymes was understood, they were employed in various industries. The most common industry, where enzymes are used, is the food industry. In the food industry enzymes are used to produce among other things, margarine and cocoa butter substitutes from cheap oils. In other words, cheap substrates are used to produce more valuable products with the help of enzymes.

In the industrial applications enzymes are stored in big tanks, called reactors. These reactors are big vessels, usually made from metal. A human body can be understood as the reactor, where millions of enzymes are performing various conversions.

Enzymes, naturally, are working in aqueous media, meaning that water is very important for enzymes to operate optimally. In order to employ enzymes in the industry the amount of water in the reactor should be considered. The amount of water in the industrial reactor is very important, the same way as it is important in the human body, and it must be controlled in order to keep enzymes active. The same goes for temperature, pH and other factors, that could influence how good the enzyme will perform.

Every organism has its own optimum environment in order to feel and operate well. The same applies for enzymes. If the amount of water, substrate and product is controlled and kept at the optimum level, the enzyme will operate at its highest rate, meaning that more product will be produced in shorter period. That idea was the main for this project, to find optimum conditions for enzymes to operate in reactors. In order to find and maintain optimum conditions for enzymatic reactions a system which controls water amount in the reactor was made. This system was able to keep the optimum conditions for the enzyme in the reactor, thus increasing the amount of final product.

Preface

The degree project was carried out at Lund University, Faculty of Engineering, Department of Chemistry, Division of Biotechnology within the group of Bioorganic Chemistry. The project started in 2019-01-28 ended in 2019-06-17.

Hypothesis of the project: The hypothesis is that by controlling the water activity lipase catalysed conversion could be performed at higher rate and reach higher yields.

Aim of the project: To construct a water activity control system capable of controlling the water activity to test the main hypothesis.

The main objectives were: 1. Design and construction of a water activity control system. 2. Optimise the system and use it for investigating the effect of water on the catalytical performance of immobilized lipase preparations in solvent free system. 3. Design, optimise and control the acidolysis reaction in a SpinChem rotating bed reactor.

Acknowledgement

I would like to say a special thanks to Carl Grey for guiding me through this project. It was an amazing experience having you as my supervisor.

I would also like to thank Patrick Adlercreutz, Ariana Causevic, Cecilia Tullberg and all the department for having and helping me.

From the bottom of my heart, I want to say thank you for my family. Thank you for your support, for believing in me, for everything you have done for me. If not for you, I would not be who I am right now, thank you so much.

Mama, tėti ir broliai, ačiū Jums už viską, dalis šito projekto ir visų mano pasiekimų priklauso Jums, ačiū Jums iš visos širdies už šią nuostabią galimybę mokytis ir tobulėti.

I had such a great time here in Lund, I have met so many wonderful people. I am grateful to you, my close friend Emmanuel Roosevelt D'Costa. Thank you for help and for being such a great friend to me. I am happy for the opportunity to meet all my other friends here; I had a great time with you guys!

Abstract

Controlled water activity in an enzymatic reaction can influence enzyme activity and productivity. A small layer of water around an enzyme gives it a required flexibility to catalyze reactions at a higher rate in contrast with the one that is dry (Adlercreutz, Dicko, Larsson, & Månsson, 2017). The hypothesis of this research project is that the product yield and productivity of acidolysis and transesterification reactions will be increased with the controlled water activity.

The acidolysis reaction is performed in two steps, where the first one is hydrolysis of the ester followed by reversed hydrolysis creating the new ester with the desired acid. Higher water activity promotes hydrolysis and vice versa for the reversed hydrolysis.

In this degree project a modified SpinChem rotating bed reactor was developed for the acidolysis and transesterification reactions. The reactor was equipped with an automatic water activity control system which was designed in the laboratory. The automatic water activity control system was made from two sub-systems. A nitrogen sparging and a relative humidity sensing system. The latter was kept in the headspace of the reactor while the nitrogen sparger was installed in the bottom of the reactor and was connected to dry and wet nitrogen. An Arduino UNO R3 micro-controller was used for communication between mentioned systems. The automatic water activity control system was designed in a way where a 3-way valve was switched between the dry and wet nitrogen source based on the setpoint of the humidity ant the actual value in the reactor, and where the flow of nitrogen gas was regulated by the Aalborg thermal mass flow controller based on the difference between the setpoint and the actual relative humidity.

A software for the communication between the sub-systems was written using C++ programming language using Notepad++ free source code editor. The code was later imported in the Arduino integrated development environment (IDE) and transferred to the micro-controller. The code was written in the way were the micro-controller was able change the parameters of the gas flow and switch the 3-way valve between the dry or wet nitrogen based on the data received from the relative humidity sensor.

The automatically controlled relative humidity system was able to maintain water activity in the reactor with the standard deviation ranging from 0.015 to 0.058 a_w units when going from 0.1 to 1.00 a_w respectively.

Automatically controlled water activity system was later used in a transesterification and acidolysis reactions. Different water activities were maintained in the reactor while the conversion of a

substrates was tracked with the gas chromatography. Results suggested that the water activity has a significant influence on the enzyme activity during transesterification reaction. In some cases, the activity of an enzyme in the reactor was increased several times by increasing the water activity. During acidolysis reaction the water activity had of a great importance on final substrate conversion and reaction rate. At the water activity of 0.9, 2.5 times more product was converted after 5 hours, compared to the water activity of 0.25. This shows that there is a great importance of controlling the water activity of enzymatic processes.

List of abbreviations

a_w – water activity.

Water activity – partial vapor pressure of water in a solution divided by the vapor pressure of pure water at the same temperature.

TL IM - 1,3 – position specific lipase obtained from *Thermomyces lanuginosus*, immobilized on a silica gel carrier.

RM IM - 1,3 – position specific lipase obtained from *Rhizomucor mehei*, immobilized on a resin carrier.

CalB - non – position specific lipase obtained from *Candida antarctica* B, immobilized on an acrylic resin carrier.

Benchmark 1 and 2 – immobilized preparations obtained from the company AAK.

Water activity control system – a system used to control the water activity in the reactor.

RH – relative humidity.

Oo – octyl octanoate.

1-oct – 1-octanol.

Oa – octanoic acid.

Da – decanoic acid.

Stdevp – standard deviation.

MFC – mass flow controller.

RHS – relative humidity sensor.

LCD – liquid crystal display.

Microcontroller/microcomputer – Arduino UNO R3.

U – enzyme activity, the amount of substrate converted per amount of time, μmol/min.

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1. Theory

Lipases are hydrolytic enzymes, known as carboxylesterases (EC 3.1.1.3), that hydrolyse ester bonds in di-, tri- and monoacylglycerols (Jaeger & Eggert, 2002). These enzymes also show a possibility to hydrolyze other compounds containing ester linkages (Derewenda, 1994). Furthermore, lipases were found to be very interesting enzymes for food, biofuel, pharmaceutical, and other industries, since they were found to be active in non-conventional organic media when performing esterification, interesterification and transesterification (Adlercreutz, 2013; Dossat, Combes, & Marty, 2002; Pyo, Nuszkiewicz, Persson, Lundmark, & Hatti-Kaul, 2011; Stergiou et al., 2013; X. Xu, Balchen, Høy, & Adler-Nissen, 1998).

Lipases are known to utilize a ping-pong bi-bi reaction mechanism. The latter mechanism can be seen in Fig. 1.

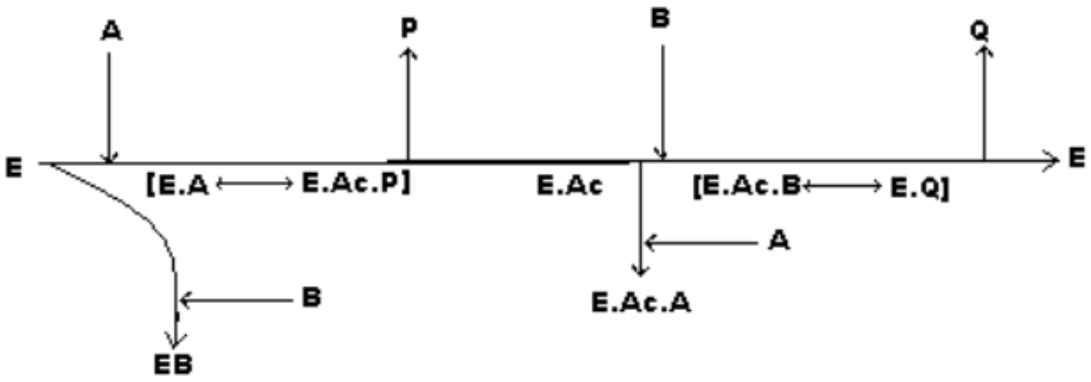

Fig. 1. Ping-pong bi-bi mechanism with the inhibition of alcohol and acid. and acyl group respectively. E, A, B, P, Q, Ac – enzyme, acid, alcohol, water, ester, acyl group respectively. E-B and E.Ac.A are the dead-end inhibition complexes of enzyme with alcohol and acid respectively (Chowdary & Prapulla, 2005).

Ping-pong bi-bi mechanism in Fig. 1 above explains the dead-end inhibition by acid or alcohol. From the figure can be seen that an enzyme E can form an acyl-enzyme complex (E.A), after binding with the acyl donor - alcohol (A). After the latter formation a water is released as a product (P). When water is released enzyme with acyl group (E.Ac) can bind the acid (B) and form acylated enzyme alcohol complex (E.Ac.B) which is later converted to the ester (Q), free enzyme (E). Irreversible inhibition might occur when acid (A) is bind to a acylated enzyme (E.Ac), or when alcohol (B) is bind to the free enzyme (E) forming a dead-end complexes (Chowdary & Prapulla, 2005).

1.1 Industrial application of lipases

Enzymes in the food industry are usually used to process food. For example, phospholipases are used to produce mayonnaise and to refine vegetable oil. Another example is esters produced by using lipases are used as a flavoring agent (Aravindan, Anbumathi, & Viruthagiri, 2007).

1.1.1. Lipases in food industry

One of the biggest food industries is margarine production industry utilizing immobilized lipases. Lypozyme TL IM is used to produce margarine from palm stearin and coconut oil (Zhang et al., 2001). Another industry, where lipases are used, is the dairy industry to hydrolyze the milk fat. Lipases from different microorganisms, *Rhizomucor miehei, Aspergillus niger, Aspergillus oryzae,* in the latter industry are used to produce enzyme modified cheese, to enhance the flavor and to reduce cheese ripening time i.e. (Aravindan et al., 2007; Kilcawley, Wilkinson, & Fox, 1998). Lipases are also used in cocoa butter substitutes production industry. 1,3-position specific lipases are used to convert low-cost vegetable fats and oils to cocoa butter substitutes, the structure of molecules of cocoa butter substitutes, in this case, is similar as one retrieved from cocoa beans (Undurraga, Markovits, & Erazo, 2001).

1.1.2. Lipases in cosmetics industry

Lipases are also used in cosmetics industry showing their versatility in different industries. In cosmetics, lipases can be used as an active/functional compound. The latter application includes face cleaners, additives in face masks, nose cleaners. Functional lipases work directly on the skin, creating certain compounds and releasing them constantly over time. Active lipases are used in the industry to produce cosmetic preparations and they are not used further in the process. Lipases also have the interest to be used in perfumery industry in order to release perfume evenly over time when applied to the skin, this is one of the functional lipase example (Ansorge-Schumacher & Thum, 2013).

1.1.3. Lipases in biofuel industry

In biofuel industry lipases are used to produce biodiesel by performing transesterification and esterification on vegetable oils with short chain alcohols. The usage of lipases became more interesting in the latter industry due to the convenient usage and much easier final product recovery, compared to biofuel production by chemical means. Compared to chemical transesterification of biodiesel, the process for biodiesel production by lipases is less energy intense, it does not require acidic or alkaline catalysts thus it does not create alkaline wastewater after the process, which requires special treatment (Fukuda, Kondo, & Noda, 2001; Nelson, Foglia, & Marmer, 1996).

1.2 Factors influencing enzyme activity and performance

There are many factors that can influence the activity of an enzyme. It is important to keep in mind that uncontrolled factors like water amount, solvent (if used), differences between polarities of a substrate and immobilized preparation, temperature and pH can have a negative influence on the

enzyme activity (Adlercreutz et al., 2017). In order to maximize the enzyme activity and optimize the process all mentioned factors should be considered and well designed. In the following section several factors that could influence the activity of an enzyme will be discussed.

1.2.1. Partitioning effect

Partitioning effect is a phenomenon where molecules are having stronger interactions with one phase, thus they are moving closer to that phase. The partitioning effect is one of the factors that could influence enzyme activity and equilibrium of final products in the reactor. It is important to stress that in order to achieve high reaction rates a substrate must always be available for an enzyme to catalyze, meaning that a substrate must be partitioned close to an enzyme or in an enzymes microenvironment. The partitioning effect might take place when a substrate and a carrier of an immobilized enzyme have different polarities, in this case, a carrier might attract products or solvents leaving less substrate available to convert in the microenvironment of an enzyme (Adlercreutz et al., 2017). The partitioning effect might also occur due to unfavorable solvation energy of substrates in the solvent, meaning that the solvent would keep substrate thus leaving a small fraction of it to be reached by the enzyme (Schmitke, Wescott, & Klibanov, 1996). In order to achieve high reaction rates and to avoid the latter negative effects, a substrate and a carrier must be matched (Bloomer, Adlercreutz, & Mattiasson, 1990).

Another example of decreased enzymatic activity could be seen due to hydrophilic hindrance formation around immobilized particles. In one study, where immobilized Lipozyme was used in the packed bed reactor, a negative effect of glycerol, which was formed during transesterification of high oleic sunflower oil, was spotted. It was concluded that glycerol adsorbed on immobilized particles resulting in significant enzyme activity loss. This happened due the latter phenomenon, resulting in diffusion limitations between hydrophilic layer around the enzyme and hydrophobic substrate (Dossat, Combes, & Marty, 1999).

1.2.2. Water influence

The enzyme activity might also be influenced by the amount of water in the reaction medium. It is known that a certain amount of water in the surroundings of an enzyme might increase the reaction rate considerably (Adlercreutz et al., 2017; Arroyo, Sánchez-Montero, & Sinisterra, 1999).

The amount of water in a reactor must be strictly controlled because fluctuations in the amount of water might result in a decreased activity of an enzyme thus reducing the productivity. The most convenient way of controlling the water activity in a reactor is by monitoring the thermodynamic

activity or the relative humidity of water which can be measured by sensors in a headspace of a reactor. The water activity can be calculated when the relative humidity is known. The relative humidity of 50% would give the water activity of 0.5, 70% - 0.7 and so on (Adlercreutz et al., 2017). Thus, it is easier to monitor the relative humidity and use the water activity, instead of measuring the water concentration in the medium, to describe the amount of water bind to the enzyme, since at the thermodynamic equilibrium, the water activity in all phases (liquid, gas, the microenvironment of enzyme) is the same (Adlercreutz et al., 2017).

A study was conducted on the catalytic rate of lipases in different reactions at various water activities. Results from the latter research suggested that lipases have similar activity profiles in different reactions, meaning that the water is more important as a lubricant for an enzyme than a reactant, hence showing the usefulness of the water activity as a concept over concentration/ amount when comparing different reaction systems (Wehtje & Adlercreutz, 1997). These results might be important to keep in mind when two-step reactions are performed, where hydrolysis and reversed hydrolysis are happening at the same time i.e. In the latter case an important thing would be to keep the water activity at the optimum level at which an enzyme would operate in both reactions at the fastest rate. However, if the hydrolysis is the reaction that is preferred one could increase the water activity to the point where the water properties as a substrate would be more dominant compared to the effect of the water as a lubricant. In the latter case, reversed hydrolysis should be suppressed while hydrolysis induced.

1.3 The relative humidity control possibilities

The relative humidity is one of the ways to measure water amount that is bound on the enzyme. It is important to note, that the water activity, at equilibrium, is the same in all phases of the reactor, meaning that sensors could be used in the headspace of the reactor in order to predict the amount of water that is bound to the enzyme phase (Adlercreutz, 2008).

There are several ways of controlling the water activity in the reaction medium, including using saturated salt solution, using pairs of salt hydrates or by using sensors (Adlercreutz, 2008). The latter method will be described further since it was used in the degree project.

Controlling the water activity by using sensors is a convenient and relatively simple method. There are several types of sensors, that work under different principles, that could be used to measure the relative humidity, including; Capacitance, coulometric, dew point, gravimetric, hygroscopic, infrared, microwave, piezoelectric psychrometric, radio-frequency sensor, resistance dependent sensor, saturated salt or thermal conductivity sensor (Kulwicki, 1991). A sensor that was used in this research

project was a resistance dependent sensor, meaning that it was measuring conductivity dependence on different amounts of water absorbed (Kulwicki, 1991). The working principle of the latter sensor was a measurement a conductivity difference between two electrodes in between which there was a polymeric substance capable of absorbing water. At increasing water amount in the reactor, the relative humidity in the headspace of the reactor was increasing, meaning that the polymeric compound in the sensor was absorbing more water thus resulting in decreased conductivity. It is important to note, that polymeric compounds used for measurement of the relative humidity, are less sensitive to the contaminants that could be present in the atmosphere compared to other types of sensors, meaning that the degradation of polymer is not observed, compared with other types of materials, making the latter sensor reliable over long term usage (Kulwicki, 1991).

1.4 Variations of reactors

There are a lot of important factors to consider before starting to use biocatalysis on industrial scale. One of the most important aspects in chemical or biochemical engineering is the type of the reactor. The most common reactor that is used in the industry is a packed bed reactor due to its simplicity, low cost of operation and performance compared to other type of reactors (Nemec & Levec, 2005). For biotechnological use, another type of reactors are gaining more interest due to better mixing properties and cheaper operational costs. In this section different types of reactors will be compared.

1.4.1. A stirred tank reactor

A stirred tank reactor (STR) is a reactor where stirrer is used to mix the reaction medium, schematics explaining STR is shown in Fig. 2. It is widely used in an industry due to its flexibility. An STR have several important advantages compared with other reactors. The mass transfer efficiency in an STR is usually very efficient, meaning that substrate will always be available for immobilized preparation. An STR is commonly used reactor because of its well-studied and understood characteristics and most importantly an easier way of maintaining aseptic conditions in the medium. However, an STR has severe disadvantages when it comes to scaled up processes. The most important problem is the power input required to operate the reactor at optimal conditions, meaning that in order to achieve good mass transfer and heat dispersion a very high-power input is required. Another disadvantage is the mechanical damage of an immobilized preparation. Extensive agitation might lead to damaged particles and debris in the reactor, resulting in reduced activity and more complicated downstream process. Another problem that is common in larger scale STRs is heat transfer limitations. It is harder to keep the same temperature in the whole reaction medium, meaning that in certain regions enzymes will perform catalysis at reduced rate and the heat produced while the reactor is stirred cannot be

dissociated efficiently meaning the regions of low and high temperatures are created (Berovic et al., 1996).

1.4.2. An airlift and fluidized bed reactor

Another type of reactor that could be used for enzymatic synthesis is an airlift or fluidized bed reactor (ALR and FBR respectively), Fig. 2. An ALR and FBR uses jets of gas or liquid coming from the bottom of the reactor and raising the reaction medium to the top of the reactor, the medium then returns through a down-comer to the bottom of the reactor. A good mixing and oxygen transfer is achieved by circulation of liquid and by introducing small bubbles of gasses, if necessary (Berovic et al., 1996). Another advantage of an ALR and FBR is that there is no mechanical mixing, meaning that an immobilized preparation would not be damaged and debris would not be created, resulting in easier downstream processing and easier separation liquid from particles (Najafpour, 2007). A research was conducted where isomaltose was produced by enzyme dextransucrase, obtained from *Leuconostoc mesenteroides*. A fluidized bed reactor was designed in order to evaluate hydrodynamics, kinetics of enzymatic reaction and thermodynamics. Results shown that a FBD was behaving as an ideal stirred tank reactor (STR) in means of the hydraulic retention time and mixing efficiency (Ergezinger, Bohnet, Berensmeier, & Bucholz, 2006). An FBR also expressed advantages like lower shear stress, compared to a STR, lower operational costs, easier scale up process.

1.4.3. A rotating bed reactor

Rotating bed reactors (RBR), Fig. 2, is a reactor designed for immobilized preparations. The working principle is that the rotating bed is moving inside the reactor, creating a flow of liquid through the bed similar to a packed bed column, thus transferring substrate close to an enzyme to perform catalysis. Immobilized preparation can be placed in the rotating bed to isolate it from the reaction medium thus, making downstream process easier by removing a need to separate immobilized preparation from products. These reactors are being employed in transesterification reactions more frequently due to their advantages like high centrifugal force, which creates good mass transfer, thus reducing the hydraulic retention time and increasing the productivity (Chen, Huang, Lin, & Shang, 2010). A lot of studies have been performed to evaluate rotating bed reactors and most of them shown, that the latter reactors show increased mass transfer, better mixing microenvironment and reduced activity loss compared to conventional stirred tank or fixed bed reactors (Mallin, Muschiol, Byström, & Bornscheuer, 2013; J. Xu et al., 2017).

1.4.4. Packed bed reactor

Fluids in a packed bed reactor (PBR), Fig. 2, are always flowing in one direction thus creating only axial flow pattern. PBRs are usually used in industry due to its simplicity, low operational cost and relatively simple scale up process due to its simple design (Blanch & Clark, 1996).

A PBR is commonly used in glucose isomerization to fructose in order to produce high fructose corn syrup (Blanch & Clark, 1996). A successful use of PBR in the production of biodiesel was also reported. However, several disadvantages were noticed during the latter process. First of all, a higher flow rates of oils had a damaging effect on enzymes immobilized on particles and inactivation of an enzyme by the methanol, which is present during process (Hama et al., 2007). Mass transfer limitations were also observed in the process were oils were used to produce structured lipids (X. Xu et al., 1998). A successful production of cocoa butter substitutes using Novozyme lipase Lipozyme™ in PBR was also reported (Undurraga et al., 2001). In the latter research palm oil was used as a substrate to create structure lipids for cocoa butter substitutes, final result shown that a PBR was an efficient reactor for the production and that the final composition of product was similar to cocoa butter (Undurraga et al., 2001).

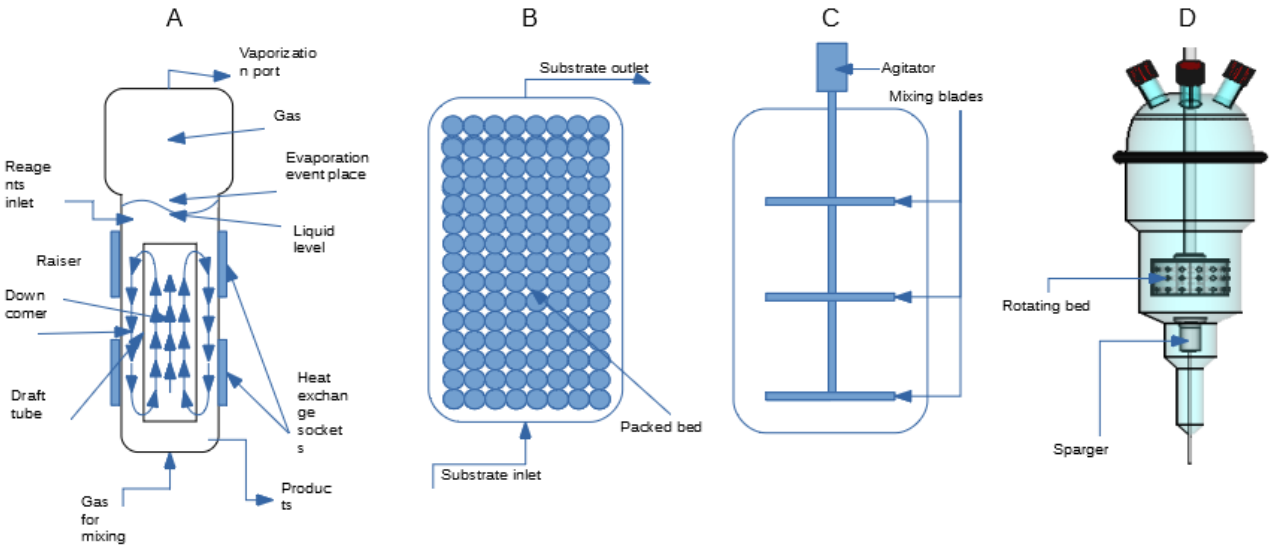

Fig. 2. Variations of reactors. A – airlift reactor (if gas is replaced with liquid – liquidized bed reactor), B – packed bed reactor, C – stirred tank reactor, D – rotating bed reactor.

2. Materials and methods

2.1 Materials

2.1.1 Transesterification in SpinChem and in vials

TL IM (1,3 – position specific lipase obtained from *Thermomyces lanuginosus*, immobilized on a silica gel carrier), RM IM (1,3 – position specific lipase obtained from *Rhizomucor mehei*, immobilized on a resin carrier), CalB (non – position specific lipase obtained from *Candida antarctica* B, immobilized on an acrylic resin carrier), the enzyme benchmark 1 and 2 were obtained from the company AAK. Methyl laurate (Sigma Aldrich, ≥98% purity), cyclohexane (Sigma Aldrich, 99.5% purity, anhydrous), 1-octanol (Sigma Aldrich, ≥99% purity), Mili-Q water, immobilized enzyme (Novozym, Lypozyme TL IM), RM IM, Benchmark 1 (AAK), CalB (Novozym, 435), Benchmark 2 (AAK)).

2.1.2 Acidolysis in SpinChem

Octyl octanoate (Sigma Aldrich, ≥98% purity), decanoic acid (Merck, >98% purity), TL IM (Novozymes, Lypozyme TL IM), RM IM (Novozym, 40086, RM IM), cyclohexane (Sigma Aldrich, 99.5% purity, anhydrous), Mili-Q water.

2.1.3 Water activity control system

Three-way valve (Dynamco D1L3202, 24VDC), mass flow controller (Aalborg, GFC17), Arduino UNO R3, jumper wires, relative humidity sensor AM2320, alcohol sensor MQ-3, nitrogen gas source, water bottles with sparger to saturate nitrogen gas.

2.2 Methods

2.2.1. Design of hardware for automatic a_w control system

The nitrogen gas line had a mass flow controller (MFC) and a three-way valve installed. MFC was used to monitor and regulate the flow of the nitrogen while the three-way valve was used to switch nitrogen gas between wet or dry by turning the 3-way valve resulting in either dry or wet gas The reactor was also equipped with a relative humidity sensor (RHS). The RHS was used to monitor relative humidity (RH) in the headspace of the reactor. Arduino UNO R3 micro-controller was used for communication and control of MFC, the three-way valve and the RHS.

In Fig. 3 below a logical diagram is shown, of how the automatic water activity control system is working.

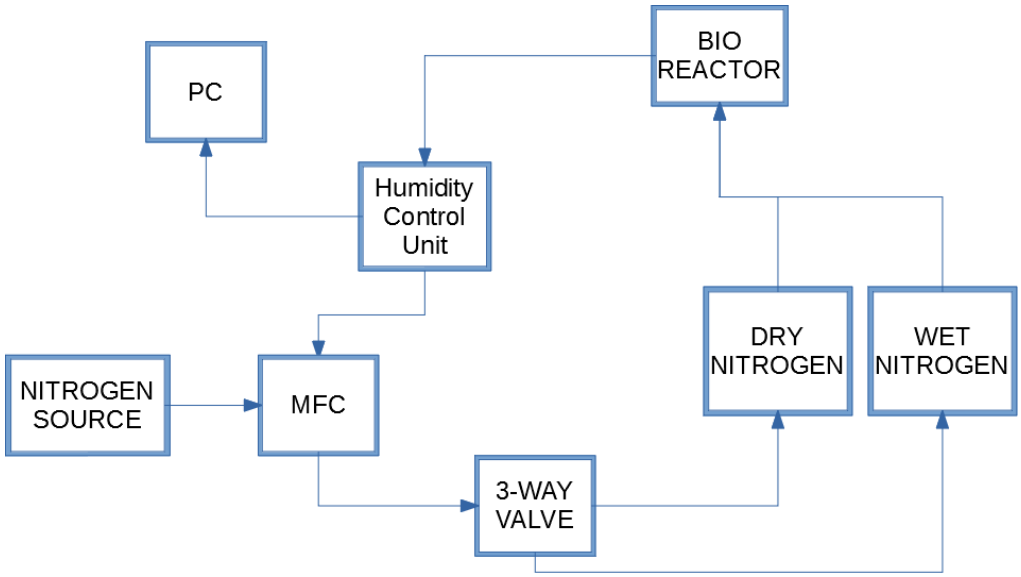

Fig. 3. Logical diagram of information flows in the relative humidity control system. This scheme shows a simple loop where the relative humidity is being compared to the setpoint (the desired relative humidity in the reactor) and being controlled by adjusting the nitrogen flow through the dry or wet nitrogen source.

The nitrogen gas flow will be controlled by the MFC, the 3-way valve will be controlled by humidity control unit (or Arduino Uno R3 microcontroller) and switched between dry or wet nitrogen based on the actual water activity in the reactor by switching the 3-way valve on or off and setting the nitrogen flow through water filled bottle or supplying nitrogen straight from the bottle (in case when dry nitrogen is needed). When nitrogen was flowing through water filled bottle it was saturated with water thus delivering that water to the reactor to increase the humidity.

2.2.1.1. Wiring of water activity control system

The mass flow controller and the 3-way valve were supplied by a 24 VDc external supply. A 24 VDc external electricity circuit was isolated with relays from the Arduino UNO, the LCD, and the RHS 5 VDc electrical circuit in order to prevent damage to latter components. The mass flow controller was controlled directly by the microcomputer. The flow of the nitrogen gas was regulated remotely by changing the output voltage from the microcontroller. A pulse width modulator (PWM) pin on the micro-computer was used in order to change the voltage signal. 5 VDc represented maximum flow, or 2 L/min, while 0 VDc – 0 L/min. Any other voltage received from the microcontroller was proportional to the gas flow rate. The mass flow controller was giving a flow signal output to the

microcontroller which was recalculated to the actual flow of the gas and displayed on the LCD of the a_w system. The signal that was received from the MFC was a number from 0 to 1024, which represented a flow of 0 to 2 L/min respectively. All pins (common grounds, reference signal, etc.) of the MFC was connected to the microcomputer according to the Aalborg mass flow controller operation manual. The RHS and the LCD were connected directly to micro-controller. The operational voltage of latter components is 5 VDc, hence it does not require any auxiliary power supply to operate. Wiring scheme and picture of the first prototype, can be seen in Fig. 4.

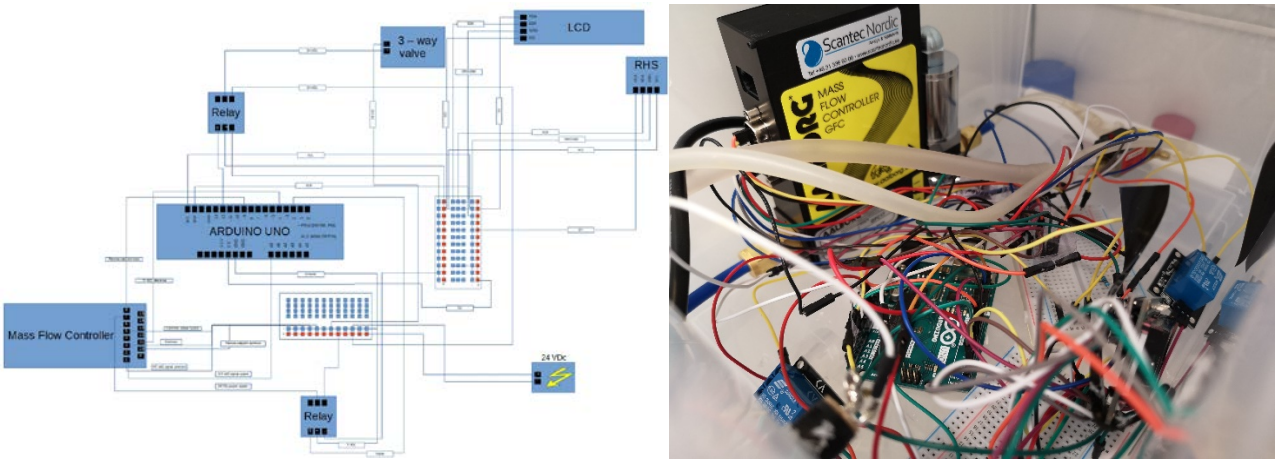

Fig. 4. Electrical scheme of the water activity control system. was used in the laboratory. All main components can be seen here, including: 3-way valve, mass flow controller, Arduino UNO microcontroller, LCD. Picture on the left is the wiring scheme, one on the right is the water activity control system.

2.2.1.2. Design of software for automatic water activity control system

The code for the automatic water activity control system was written using the C++ programming language. Code editor Notepad++ was used in order to develop the firmware, which was later transferred into the Arduino integrated development environment (IDE), the code can be seen in the Appendix G. The firmware was designed in the way that the data from RHS would be continuously monitored and compared with the desired water activity in the reactor (the setpoint) and so that the hardware would give a reasonable response in a case where the actual water activity and the setpoint do not match.

2.2.2. Reactor set up

A Spin Chem rotating bed reactor was equipped with a sparger to supply dry or wet nitrogen gas. The schematic view of the reactor is shown in Fig. 5

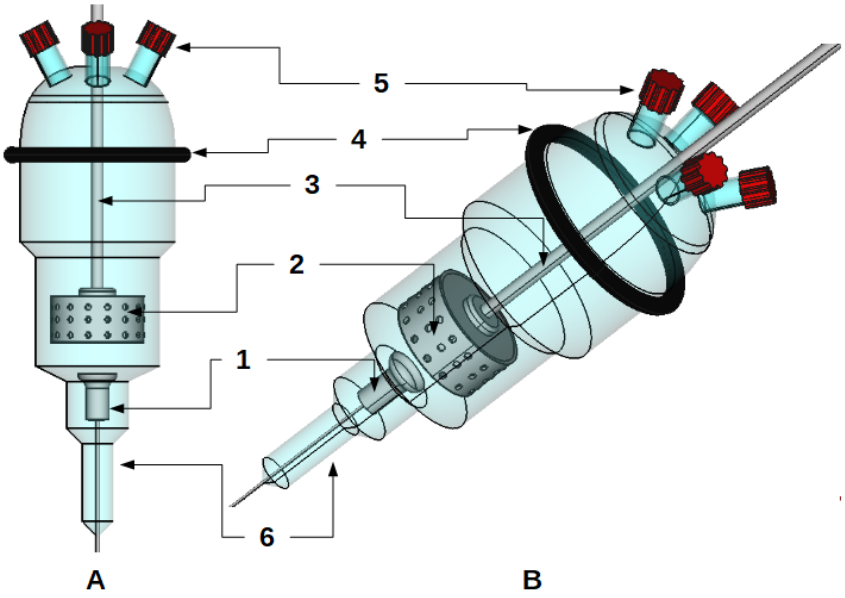

Figure 5. Drawing of SpinChem rotating bed bioreactor. A – side view. B – pictorial (isometric) view. 1 – aerator. 2 – Cartridge. 3 – shaft for cartridge support. 4 – connector for upper and lower part of the reactor (reactor consists of two parts made from glass; O-ring is placed in between to ensure good isolation). 5 – upper ports (used to install humidity and temperature sensor). 6 – bottom port (normally used for removing media from the reactor, in this case clog of the port was replaced with aerator which is marked with number 1 in the figure).

The reactor operating volume is 125-500 mL, however, after installing the sparger, a minimal amount of substrate used was ~175 mL. This was done due to the elevation of rotating bed, it could not have been placed lower due to installed sparger and in order to immense the bed completely in the liquid, a higher amount of substrate had to be used.

The relative humidity sensor was installed in the head space of the reactor through one of the ports marked with number 5 in Fig. 5. The temperature of the reactor was controlled with the water bath. Hot water was circulated through the reactor socket, surrounding the lower part of the reactor (Fig. 5, number 4). The sketch was designed with FreeCAD 0.17, open-source computer aided drawing software.

The reactor is originally equipped with the rotating bed with four sections used to pack with an immobilized enzyme. The sketch that was designed with the FreeCAD 0.17, open-source computer aided drawing software can be seen in Fig 6. The cartridge could store around 8-16 g of an immobilized enzyme, depending on the weight of the carrier used. A net with very fine pores was originally installed in the rotating bed to prevent escaping of the enzyme to the reaction medium.

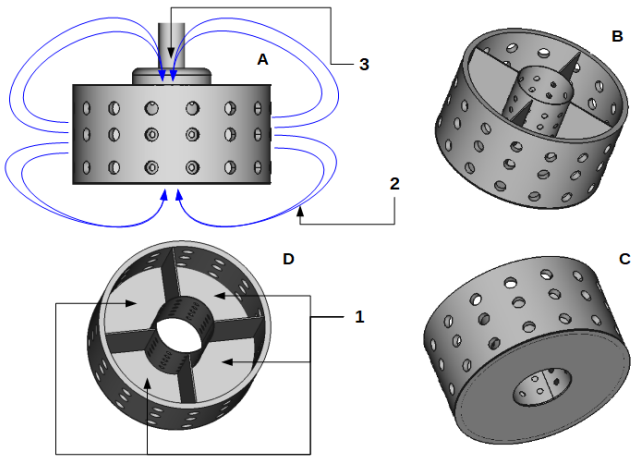

Fig. 6. The rotating bed of the Spin Chem rotating bed reactor. Picture A shows side a view while pictures B, C, D – an isometric view of the rotating bed. 1 – compartments that were used to pack the immobilized enzyme. 2 – blue lines represent simplified hydrodynamics of liquids in the reactor. 3 – shaft which is connected to the top the rotating bed, the other end is connected to the motor.

2.2.3. Transesterification reaction

Reaction was run at 60ºC, 900 RPM. Substrates was used at 1:1.2 molar ratio (100 mL of methyl laurate and 75 mL of 1-octanol). Enzyme (Lypozyme TL IM, Benchmark 1, CalB IM, RM IM, Benchmark 2) was added to one or more cartridge compartments, 2, 4 or 13 grams of immobilized enzyme preparation was used in different reactions. Samples were taken every 10 minutes for the first hour, then every 30 minutes for the second hour and every 1 hour for 2 hours. Nitrogen sparging was used for removal of the side product methanol in order to push the reaction equilibrium towards product formation. Samples were taken with 10 mL pipette through open port in the upper part of the reactor and transferred to 10 mL glass container. From glass container samples were taken with 100 µL syringe and transferred to 1.5 mL septum capped vials. Septum capped vials contained 900 µL cyclohexane. After sampling reaction mixture was poured back to the reactor to keep reaction volume as constant as possible. Transesterification reaction can also be called alcoholysis. The transesterification reaction is explained in Eq. 1.

$$RCOOR^1 + R^2OH \overset{Ez}{\leftrightarrow} RCOOR^2 + R^1OH \quad \text{(Eq. 1)}$$

Where: R^1 - organic group of an ester;
R^2 – organic group of alcohol;
Ez – enzyme.

2.2.4. Acidolysis

Reaction was run at 60°C, 900 RPM. Substrate was used at 1:2 molar ratio (octyl octanoate: decanoic acid, 97 mL and 75 mL respectively). Amount of RM used was 4 g. Samples were taken every 30 min with a 10 mL pipette through open port in the upper part of the reactor and transferred to 10 mL glass container. From the glass container samples were taken with a 100 µL syringe and transferred to 1.5 mL septum capped vials. Septum capped vials contained 900 µL cyclohexane. After sampling excess reaction mixture was poured back to the reactor to keep reaction volume as constant as possible.

An acidolysis reaction was performed in the Spin Chem rotating bed reactor. The relative humidity (or the water activity) was monitored during the reaction. The acidolysis is a twostep reaction, in the first part (1-2) of the reaction, relative humidity was kept high to induce hydrolysis of the ester substrate (octyl octanoate). In the second part (3-4) of the reaction, where the enzyme will be acylated by acid (decanoic acid), the relative humidity will be kept as low as possible in order to remove the water that is formed, in order to suppress hydrolysis and increase main product yield. The acidolysis reaction is done in several steps described below in Eq. 2.

$$1.\ Octyl\ octanoate + E \cdot OH \rightarrow E \cdot octanoate + Octanol \quad \text{(Eq.2)}$$
$$2.\ E \cdot octanoate + H_2O \rightarrow E \cdot OH + Octanoic\ acid$$
$$3.\ E \cdot OH + Decanoic\ acid \rightarrow E \cdot decanoate + H_2O$$
$$4.\ E \cdot decanoate + Octanol \rightarrow Octyl\ decanoate + E \cdot OH$$

Where: $E \cdot OH$ = Free enzyme

$E \cdot octanoate/decanoate$ = acylated enzyme

In the first step of the reaction mechanism, the acyl-enzyme complex will be formed, and 1-octanol released as a product. In the second step, the enzyme will be regenerated and octanoic acid will be released. In the third step, the enzyme will form an acyl-enzyme complex with decanoic acid thus release water. The last step will result in the formation of octyl decanoate and free enzyme.

2.2.5. Hydrolysis reaction in vials

The reaction catalysed was hydrolysis of octyl octanoate. Products formed were octanoic acid and 1-octanol. A total volume in the vials at the beginning of the reaction was set to 2 mL with the included amount of water added, meaning the substrate amount was decreasing with increasing water content (1800 µL of substrate + 200 µL water, 1900 µL of substrate + 100 µL of water i.e.). The reaction

temperature was 60 ºC, the amount of immobilized enzyme preparation was 100 mg in every vial. Reaction time was 10 minutes, samples were taken every 2 minutes. 100 µL of the sample was diluted in 900 µL cyclohexane. GC analysis was performed afterward to evaluate and compare the water effect on the reaction rate.

2.2.6. Analytical measurements

Transesterification and acidolysis reaction samples were analyzed using gas chromatography method. Varian 430-GC instrument was used together with NukolTM column, 15 m x 0.25 mm x 0.25 um film thickness. A gas flow of 1.5 mL/min, 1 µL injection volume, a split ratio of 50, injector and detector temperatures of 250 and 300 ºC respectively, were used. The temperature gradient program was set to increase 40 ºC /min. Starting temperature of the column was set to 80 ºC for 1 minute and the final temperature of 220 ºC was kept for 2 minutes. Total time of run was 6.5 min.

2.2.7. Saturated salt solutions

Saturated salt solutions were prepared to find out the amount of water, that the mixture of methyl laurate and 1-octanol at the molar ratio of 1:1.2, holds at different water activities. Saturated salt solutions can keep constant water activity in the substrate by equilibrating the water that is present in the salt solution. There is some extra water in the salt solution so that water could diffuse to the substrate or vise versa. This keeps the water activity steady in all phases. The salts that were used were: KOH (a_w = 0.093), KCH_3CO_2 (a_w = 0.231), $Mg(NO_3)_2$ (a_w = 0.544), HCl (a_w = 0.843), $MgCl_2$ (a_w = 0.331), NaCl (a_w = 0.755). The mixture was transferred to 5 mL glass containers and placed into the enclosed saturated salt solutions for equilibration.

2.2.8. Karl Fischer titrator

Karl Fischer titrator was used to measure water content in the reactor and in the samples placed in saturated salt solutions. 100 µL of sample was transferred to the Kar Fischer titrator equipment through septum, with a glass syringe. Triplicates of each sample were done.

3. Results and discussion

3.1 Design and optimization of the water activity control system

The water activity control system was designed in order to control and to maintain a desired relative humidity in a rotating bed reactor over long periods of time.

Several problems were faced in the process including condensation of the water in gas transfer tubes, condensation of water in the head space of the reactor due to temperature differences, little amount of water transferred to the reactor. In order to avoid condensation of the water in the tubes and the headspace of the reactor, a heat exchanger was placed in the hot water bath in order to increase the temperature of nitrogen gas, tubes and the headspace of the reactor were insulated with aluminium foil in order to reduce heat loss and to reduce the condensation problem. When the condensation problem was solved the amount of water transferred to the reactor increased, resulting in a possibility to have higher relative humidity in the reactor.

Another optimization process was done in the firmware, where the system was programmed to reduce the nitrogen flow while approaching the desired relative humidity. The further the actual relative humidity, in the reactor, goes from the setpoint, the higher the nitrogen flow is. This could be used in the industry in order to reduce cost of the process.

3.2 Optimization of the automatic water activity control system

The automatic water activity control system was used to keep the relative humidity in the reactor stable during an enzymatic transesterification and acidolysis reactions. In order to check if the humidity sensor corresponded to changes in a water amount in a reasonable manner, a saturated salt solution experiment was conducted. In this experiment, the substrate that was used in the transesterification reaction was placed in the saturated salt solutions with different water activities and kept for several days until the equilibrium of water activity was reached. After several days the substrate with different water activities was checked using Karl Fischer titrator equipment. The water activity in the rotating bed reactor was changed by the automatic water activity control system, samples of the reaction medium were taken and analysed with Karl Fisher titrator. Then both saturated salt solution and samples from the reactor results were compared in Fig. 7.

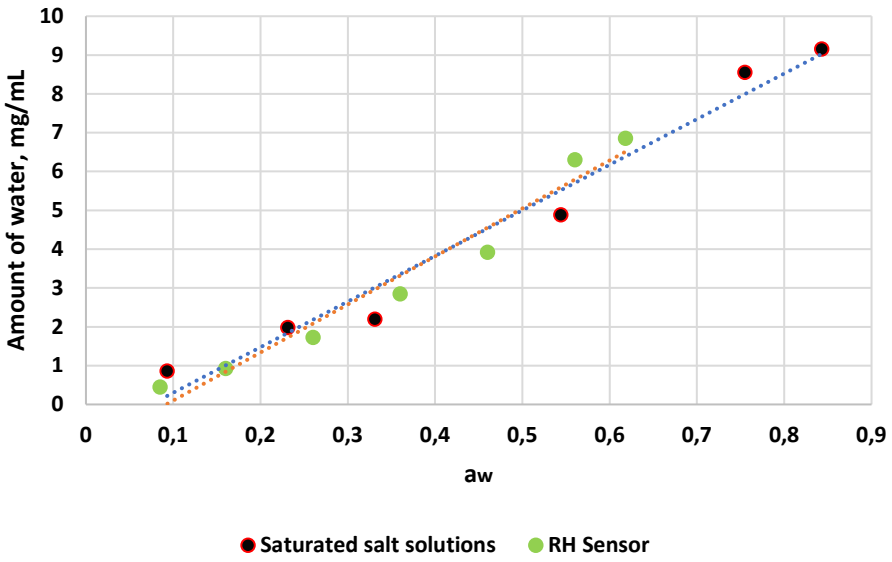

Fig 7. Standard curve of the water amount at certain water activity in the mixture of Methyl laurate and 1-Octanol (1:1.2 molar ratio) equilibrated in the saturated salt solutions (black dots) and the amount of water in the reactor during the transesterification reaction (where methyl laurate and 1-octanol was used as a substrate) at different water activities (measured with the relative humidity sensor), (green dots).

From the results in Fig. 7 the relative humidity sensor corresponds to changes in water content in a reasonable manner. Correlation factor between two data sets, one from saturated salt solutions and another from the reactor, was calculated to be 0.98, meaning that the relative humidity that is shown by the sensor is correct and can be trusted.

The stability of the automatic water activity control system was also tested under different water activities for a prolonged amount of time to check if the system is stable. Results are shown in Fig. 8 below.

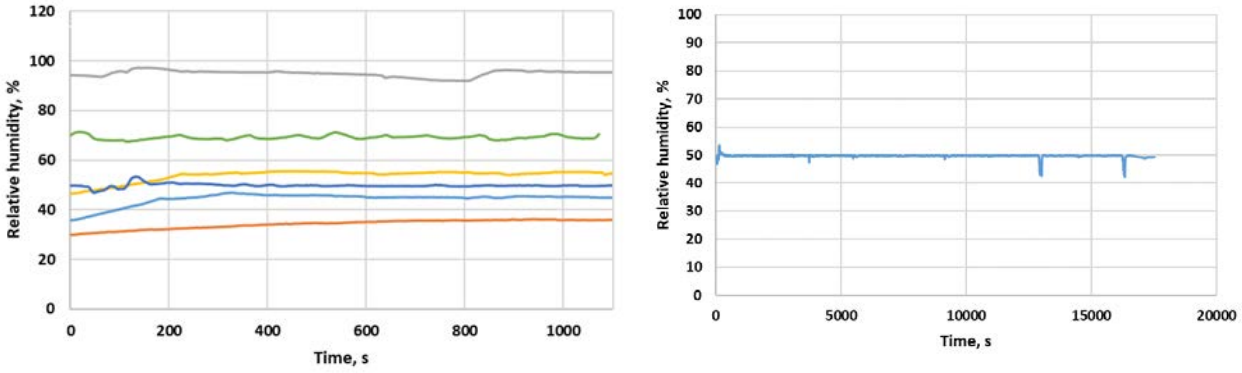

Fig 8. Data collected from the automatic water activity control system under operation. Graph on the left side shows several different water activities and how the system was able to maintain selected water activity. On the right-side graph there is a data collected from 5 hours long reaction.

This step was performed in order to check if the automatic water activity control system can maintain the set water activity in the reactor. The results show that in both short time and long-time experiments

the system was stable without any major deviations meaning that the system can be used further for the research purposes.

3.3 Real time reaction tracking system

During testing of the water activity control system, it was noticed that the relative humidity sensor is very sensitive to the changes in the methanol concentration in the reactor. This observation raised a concern that the system might be difficult to use in processes where methanol or any other volatile alcohols are produced or consumed. It was therefore decided to investigate a sensitivity of the sensor to methanol and the calibration curve shown in Fig. 9 was made.

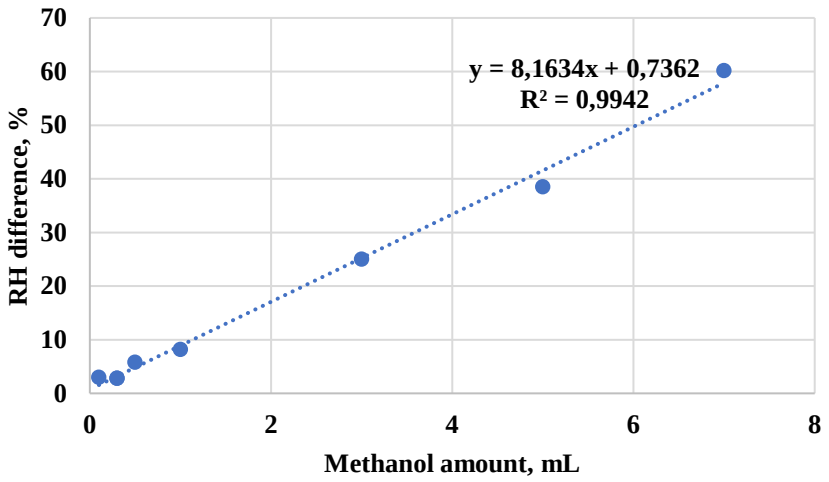

Fig 9. Response of the AM2320 relative humidity sensor measured in the relative humidity plotted against the amount of methanol added to the reactor.

From the figure above it can be seen that the relative humidity sensor is sensitive to the changes in methanol concentration in the reactor medium in a linear manner. Once this phenomenon was tested a new idea emerged of using the sensor to monitor a transesterification reaction online, since the methanol is released during the reaction and as it was known by now that it can be detected by the sensor.

Another experiment was conducted where methanol evaporation kinetics were tested. The reactor was filled with the methyl laurate and 1-octanol and the empty rotating bed (without the immobilized enzyme preparation) in order to mimic the methanol evaporation conditions during the transesterification reaction.

The data of the evaporation kinetics were collected every 1 second, sent and saved directly in the control computer which was connected to the automatic water activity control system. The response of the detector was then plotted against time, the results are shown in Fig. 10 below in the text.

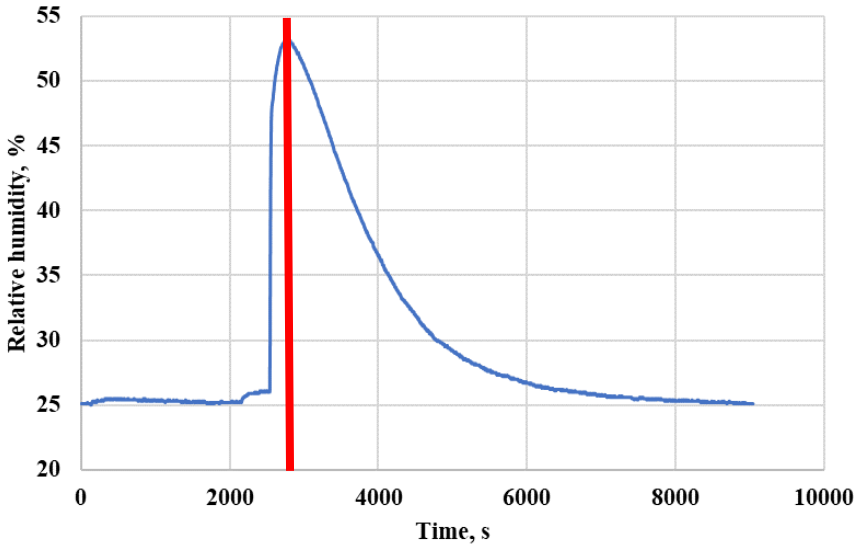

Fig 10. Methanol evaporation kinetics monitored with the relative humidity sensor AM2320. 3 mL of methanol was added to the reactor at t=2500 s, and the response of the sensor was monitored over time. The data was collected for 2,5 hours every 1s. Reactor set up conditions: 60°C, rotating bed speed 900 RPM, nitrogen gas flow – 2 L/min, working volume - 175 mL (100 mL Methyl Laurate + 75 mL 1-Octanol). The red line is the starting point from which the data was taken to calculate methanol evaporation model.

After conducting the methanol evaporation kinetics experiment, it was clear that the system can track the transesterification reaction in real time. The methanol evaporation kinetics curve was further used to calculate evaporation rate dependence on the actual methanol concentration in the reactor. The evaporation kinetics curve, from Fig. 10 (starting from red line in the plot), was divided into small parts (200 seconds each). Each 200 second part was used as a model to determine of how fast the methanol is evaporated, based on the starting concentration of the methanol at t=0 (of a small 200 seconds part of the curve). When several small parts were joined together a methanol evaporation model was made.

The model of the evaporation rate dependence on the methanol amount in the reactor, expressed as the relative humidity, was created and it is shown in Fig. 11 below.

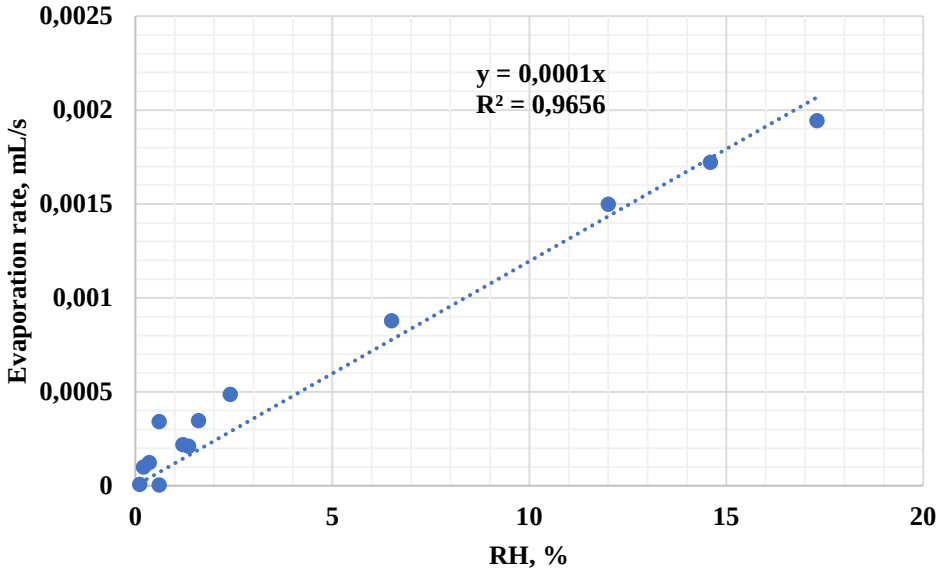

Fig 11. Response of the AM2320 sensor in relative humidity plotted against methanol evaporation rate. It can be seen from the graph that the higher the concentration of methanol (or the RH) the higher is the evaporation rate. The evaporation rate was calculated from Fig 9, 10. where slopes of different parts of the curve from Fig. 10 were associated with the amount of methanol, Fig. 9, in the reactor.

The model of methanol evaporation dependence on the actual methanol concentration in the reactor was used to track the transesterification reaction in real time. In Fig. 11 above, X axis marked with RH shows the detector response to the methanol amount in the reactor by the change in relative humidity. The relative humidity can be recalculated to the amount of methanol by using calibration curve from Fig. 9. In the transesterification reaction methanol was produced and was measured by both the GC and the system described using the RH-sensor. The results of the experiment are shown in Fig. 12.

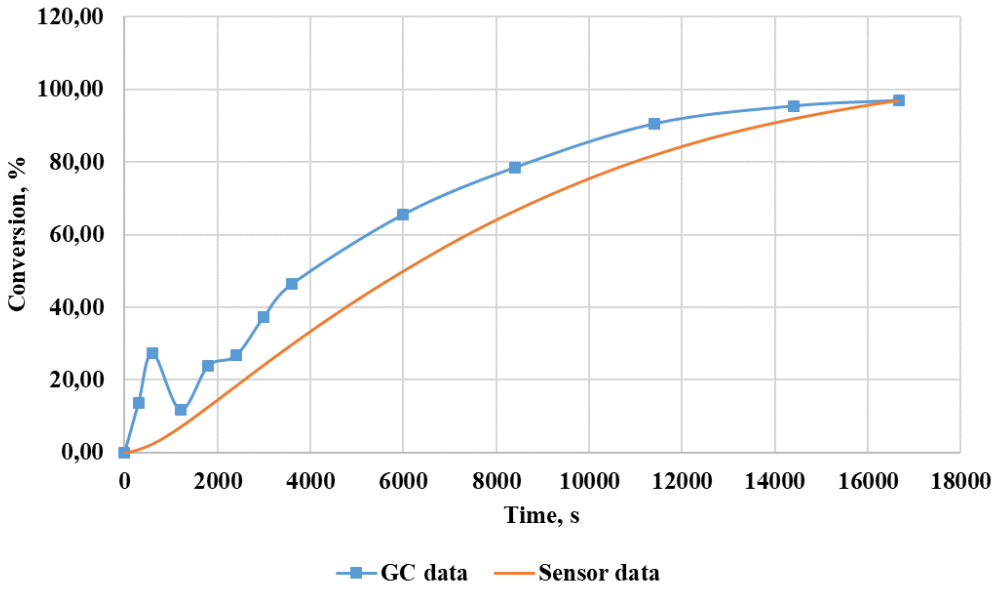

Fig 12. Substrate conversion in the transesterification reaction. TL IM amount – 13,11 g. The blue line (upper) represents the conversion measured using GC (gas chromatography) system while red line represents conversion calculated based on the detector response to the methanol produced in the reactor.

It can be seen from the results above, that the system has a great potential to be used in the reactions where methanol is consumed or created. 96,99% and 96,87 % of methyl laurate was converted according to the GC, where consumption of substrate was measured, and the relative humidity sensor in the head space of the reactor, respectively. However, the actual conversion profile did not exactly overlap the curve that was obtained from the GC since it seem to have a certain off set, but at the end of the reaction the conversion rates obtained by both the GC and the system were almost the same. The conversion profile obtained by the system might be different due to the inaccuracy of the methanol evaporation rate model and due to the time, that is required for the methanol to go from liquid phase to the gas phase where the relative humidity sensor is placed.

Other important data can be seen during the reaction, including enzyme activity, *i.e.* the enzyme activity can be calculated when the amount of methanol produced is known at any given time. Enzyme activity and substrate conversion profile from the transesterification reaction can be seen in Fig. 13.

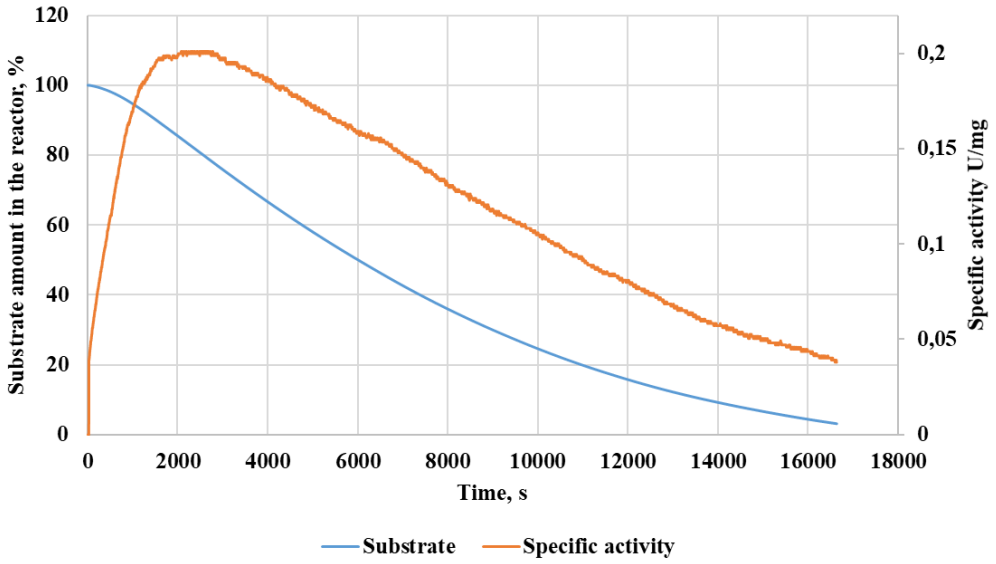

Fig 13. The conversion profile of a substrate (methyl laurate) and the specific enzyme activity during the transesterification reaction. TL IM amount – 13,11 g.

The conversion of the substrate and the specific enzyme activity was calculated based on the amount of methanol that has been produced in the reactor. It can be observed from the data that enzyme activity decreases with decreasing substrate amount. The peak activity of the enzyme was found to be around 0,18 U/mg. The specific activity of the enzyme drops as the amount of the substrate decreases. This is expected due to the decreased availability of the substrate for the enzyme.

To conclude this section of the degree project work it is possible to state that the automatic water activity control system is reliable to use for controlling the water activity in the reactor. The system can maintain the relative humidity in the reactor constant over long periods of time. The system also has a potential of tracking the biochemical/chemical reactions were volatile alcohols are produced or consumed. In this degree project, the methanol evaporation model was calculated to be true and reliable only for certain reaction conditions. If one would change temperature, stirrer speed, sparging or the reactor itself, the evaporation model should be designed and evaluated for new conditions.

The capability of tracking the reaction based on a change of volatile compounds in the reactor medium also has several severe disadvantages. The possibility of controlling the water activity becomes problematic in cases were volatile alcohols are present in the reactor because the sensor gives the same signal for both the water and the volatile compounds. This disadvantage can be overcome by decreasing the amount of enzyme used in the reaction, thus reducing the reaction rate, amount of methanol produced and allowing the automatic water activity control system to control the relative humidity, without the interference of volatile compounds. The latter solution is only applicable for research purposes, where the activity of an enzyme is being monitored at different water activities i.e.

However, if the system would be used for the purposes of production with high enzyme loadings this issue should be evaluated, and a more advanced solution might be necessary, for example a new sensor which would not be sensitive to methanol.

A possible solution to the problem mentioned above is to use an alcohol sensor, which would be non-specific for water. This solution was tried in the laboratory, however reliable results could not be obtained. Due to the lack of time, the issues with the sensor could not be identified. If an alcohol sensor could be implemented, then it would be possible to evaluate actual relative humidity of the system at high enzyme loadings by subtracting the influence of alcohol from the relative humidity value that is given by the relative humidity sensor. The latter process would require reliable equipment and further investigation and optimization of the system.

Another important aspect of the automatic water activity control system is the tubing that goes from the wet nitrogen source to the reactor. Due to temperature differences in the wet nitrogen source and the environment where tubes are placed the water can start condensing on the walls of the tubings. This problem could lead to a phenomenon when the automatic water activity control system would not be capable of maintaining the relative humidity stable due to loss of the water or due to the burst of liquid from the tubings reaching the reactor. The easiest solution for this problem is to isolate the tubings or make them as short as possible in order to reduce the contact time of the wet nitrogen with the cooler tubing walls. Another solution might be to maintain the temperature around the tubings the same as in the wet nitrogen supply source however, this might be a problem when the process is operated at high temperatures. In this case the temperature of the nitrogen should be high enough not to cool down the reaction medium and warm enough not to lose the water while traveling through the piping system, meaning that extensive heating of the tubings might be necessary and costly.

It is also important to stress, that the relative humidity is sensitive to the temperature. In order to read a correct value of the water activity in the all phases, temperature must be the same in all phases.

3.4 Water activity influence on the enzymatic transesterification and hydrolysis rate

In this part of the project the dependence of enzymatic activity on the water activity in the reactor was determined. The main hypothesis of the project was that water activity influence the activity of enzymatic process in the reactor. Results that were obtained during several hydrolysis and transesterification reactions are presented in Fig. 14., 15. The model reaction for this research part was transesterification where methyl laurate and 1-octanol were converted to octyl laurate and methanol.

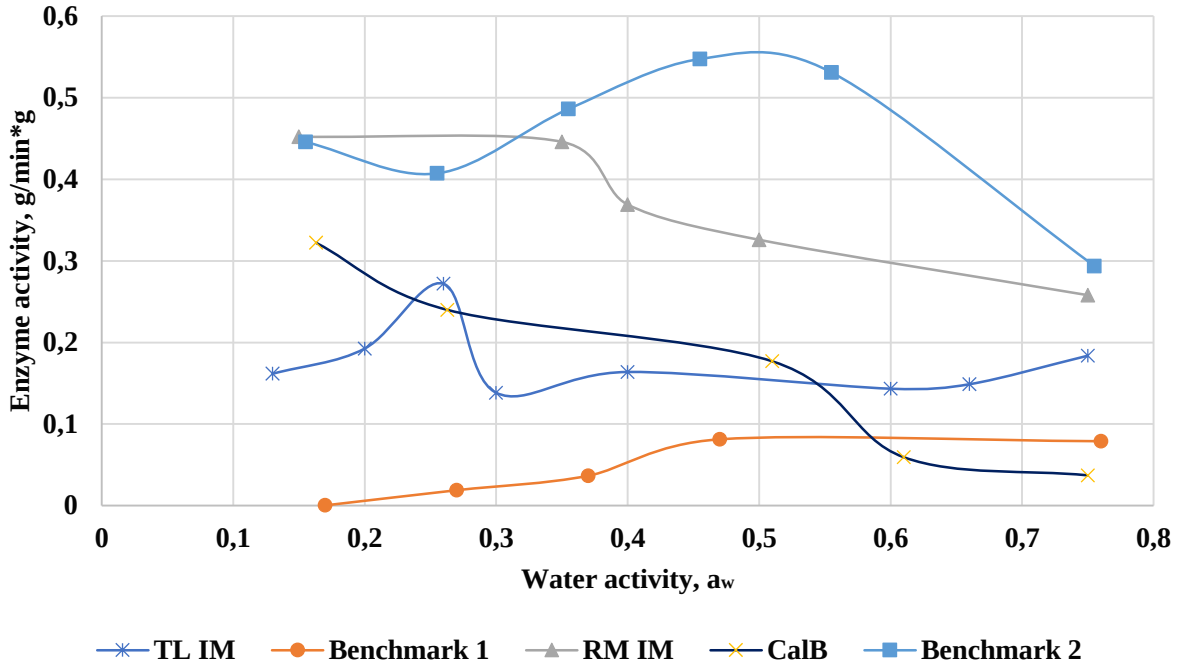

Fig. 14. Enzyme activity profiles during transesterification reaction. The amount of octyl laurate produced per minute per gram of enzyme in SpinChem reactor at different water activities.

Results from Fig. 14 above show how the enzyme preparations have different activities at different water activities. TL IM shows higher activity, when performing a transesterification reaction, at lower water activities. As the water activity increases, the activity decreases until it reaches a point where it does not increase nor decrease anymore. Results suggest that at certain water activities the activity of an enzyme could be increased almost twice, showing the importance of controlling the water activity in the industrial scale.

Other enzyme, RM IM has similar properties as the TL IM described in a paragraph above. It expresses the highest activity at lower water activities. A more significant decrease in the enzyme activity can be seen when water activity is increased from 0.35 to 0.4. Further rise in the water activity results in a decrease of the enzyme activity in more linear manner.

CalB shows the highest activity at the lowest water activity. When transesterification reactions were performed at higher water activities the latter enzyme showed quite dramatic decrease in the relative activity resulting in a very little activity at the water activity of 0.75.

The last two enzymes that were analysed were Benchmark 1 and 2. These enzymes were received from the company AAK. The enzyme benchmark 1 showed no activity whatsoever, at low water activities. The activity of the enzyme increased with increasing water activity and stabilised at the water activity of 0.45. These results can be quite important keeping in mind that the relative enzyme

activity can be increased four times when increasing the water activity from 0.25 to 0.45. This means that an industrial process can be improved quite a lot, if the water activity could be increased and kept at the latter levels. Another enzyme, benchmark 2, was also received from the same company. The latter enzyme was completely different from the first one. It expressed way higher relative activity at low water activities, compared to benchmark 1, reaching the maximum activity at the water activity of around 0.45 - 0.5 and further drastically decreasing at higher water activities.

In conclusion for this section it could be stated that the different enzymes tested expressed different properties at different water activities. This was expected because the immobilized preparations were different. Furthermore, the carriers that were used in all preparations were different, meaning that they could have had an influence on partitioning effect of substrates and/or products hence making it harder or easier for the substrate to enter the carrier particle and to reach the enzyme.

The water activity showed an important role in the transesterification reaction. Different enzymes catalyse the reaction better at certain water activities, meaning that certain aspects, *e.g.* productivity, yield could be increased in bigger scale production if controlling the water activity.

Simultaneously with transesterification reactions, which are shown in Fig. 14 above, the hydrolysis was also monitored. It was known from previous experiments that methyl laurate is also hydrolysed to lauric acid during the transesterification reaction so the formation rate of lauric acid was monitored and are shown in the Fig. 15 below in the text.

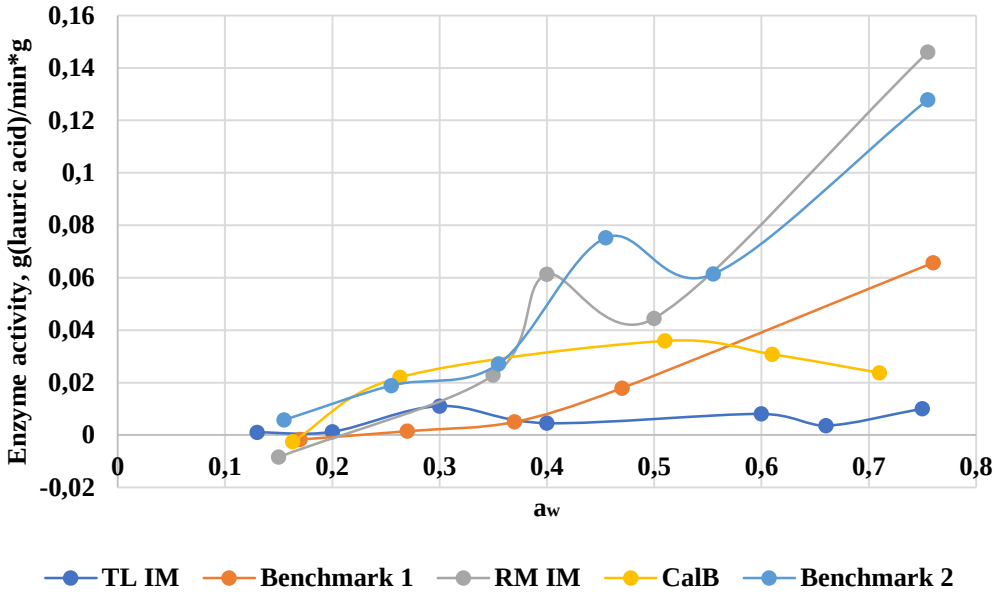

Fig. 15. Activity of hydrolysis (where methyl laurate was hydrolysed to lauric acid) reaction catalysed by different enzymes at different water activities. Amount of lauric acid per minute per gram of enzyme produced at different water activities are presented.

It was expected that with increasing water activity the relative hydrolysis rate would increase but this was not the case for all enzymes tested.

Unexpected results were observed with TL IM and CalB. TL IM showed very small hydrolytic activity at low water activities but almost no increase at higher water activities. CalB has shown an increase in hydrolytic activity when higher water activities were picked however, at the water activity of around 0.6 the enzyme shown a decrease in the hydrolytic rate.

The highest relative hydrolysis rate was expressed by RM IM and benchmark 2 at the water activity of 0.75, the highest water activity tested. These two enzymes show an interesting increase in the hydrolysis rate at the water activity 0.4 and 0.45 respectively. A small activity peak at the latter water activities was observed showing a little decrease and a tendency to increase afterwards. In order to explain the latter phenomenon transesterification and hydrolysis of the enzyme Benchmark 2 will be explained further in text.

Hydrolysis was measured in the same experiment as in the Fig. 14.

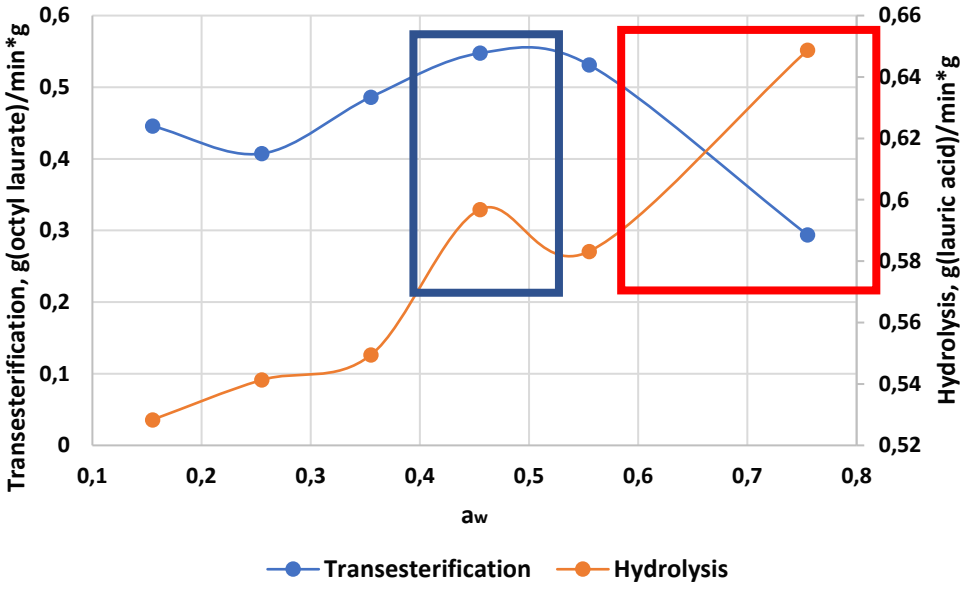

Fig. 16. Results of the enzyme Benchmark 2 activity at different water activity levels during transesterification and hydrolysis reactions. The blue line represents production of octyl laurate (transesterification), the red line – production of lauric acid (hydrolysis).

In the Fig. 16, enzyme expresses a local maximum at around 0.45 a_w in both transesterification and hydrolysis reactions. One would expect that hydrolysis would further increase with increasing water activity because water is consumed in this reaction. However, we can see a little drop in activity when

the water activity was increased further (blue region in the graph). This happened because each enzyme has their own optimum water activity at which they operate at the highest rate (Fig. 15) so the blue region in the graph shows the optimum activity of the enzyme where water does not influence the reaction as a substrate. However, once the water activity is increased to even higher levels, water starts behaving not only like a lubricant, but it also increases the hydrolysis rate by acting as a substrate in the medium (red region in the graph). This phenomenon explains the decrease in the transesterification reaction while at the same time enzyme performs hydrolysis at a higher rate.

3.5 Water influence on the carrier, hydrolysis in small scale

In order to understand how water inhibits the TL IM in the hydrolysis reaction several small-scale experiments were conducted. In these experiments octyl octanoate was hydrolysed to octanoic acid and 1-octanol. The formation of latter products was analysed and are shown in Fig. 17 below in the text.

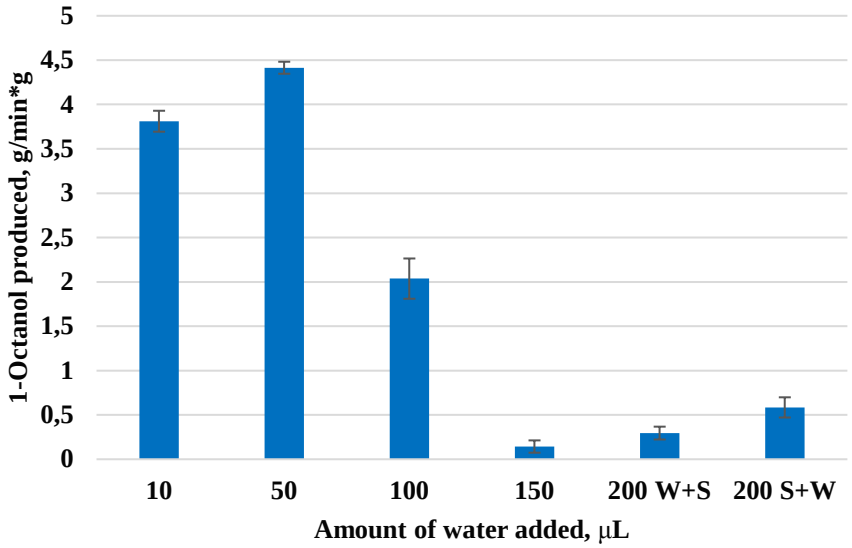

Fig 17. Activity of TL IM lipase at different amounts of water present in the reaction medium. The reaction catalysed was hydrolysis in vials. 200 W+S – water was added before substrate, 200 S+W – substrate added before water.

Results from Fig. 17 shows how different amounts of water inhibits the hydrolysis reaction. As it was already said higher water activities should increase hydrolytic rate of the enzymes which is their natural biological function. The latter experiment showed that small addition of water could help to increase the hydrolysis rate hence this can be associated with the hydration level of the enzyme. Small amount of water is explained to work as a lubricant for the enzyme; it helps to maintain the flexibility of the enzyme thus increasing the catalytic activity (Adlercreutz et al., 2017). However, higher amount of water added has resulted in very drastic decrease in the relative hydrolytic activity. The

carrier that is used to immobilize an enzyme is made from silica gel beads and it is known that silica can absorb water. Thus, the decrease in hydrolytic activity can be explained by the water layer formation around the carrier particles. In this case the substrate, that is less polar compared to water, cannot enter the immobilized preparation and be hydrolysed by the enzyme.

It was also interesting to evaluate if a way of how water is added makes any difference to the hydrolytic activity of the enzyme. In every small experiment which are shown in Fig. 17, 18 the water was added first on the immobilized preparation to wet it but, in the last experiment (histogram named S+W 200) that is shown in the latter figure, the substrate was added first. The latter experiment has shown a little increase in the relative hydrolytic activity however, the increase is not significant and was seen due to the saturation of enzyme with the substrate before the water was added.

In order to check if higher amounts of the water will inhibit another type of carrier, which is more hydrophobic, other set of small-scale experiments were conducted. In these experiments the same hydrolysis reaction of octyl octanoate was tested however, the enzyme used was RM IM, 1,3 – position specific lipase obtained from *Rhizomucor mehei*, immobilized on a hydrophobic resin carrier. Different amount of the water was added in each reaction and initial rates of product formation was determined by GC. Results are shown in Fig. 18 below.

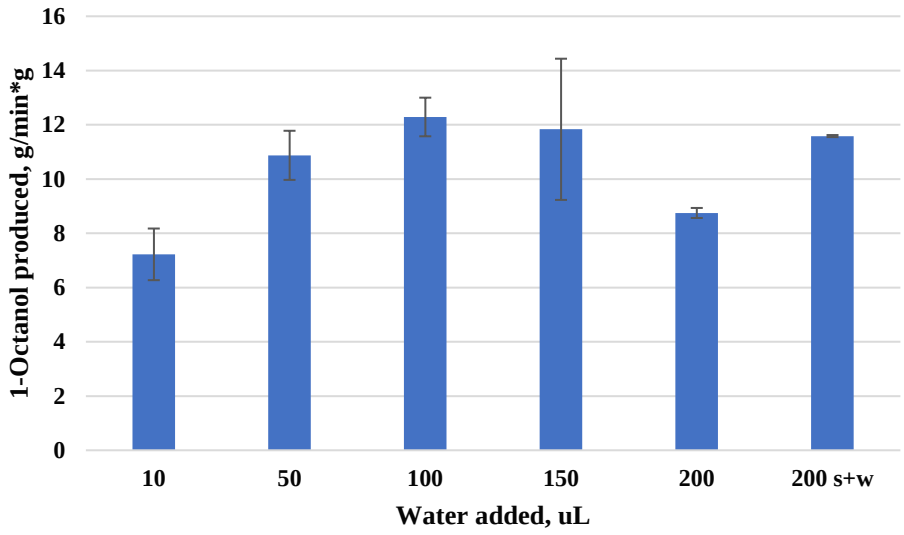

Fig 18. Activity of RM IM lipase at different amounts of water present in the reaction medium during hydrolysis reaction in vials. 200 W+S – water was added before substrate, 200 S+W – substrate added before water.

The results in Fig. 18 show that RM IM was not inhibited so much, compared to TL IM, by increasing water amount in the reaction medium. The carrier that the enzyme RM is immobilized on is more

hydrophobic compared to one that TL is immobilized on. This might be the reason why RM still shows high activity at high water amounts added, the water is not attracted to the carrier as much as to one made from silica, resulting in smaller water barrier and making it easier for the substrate to reach the enzyme. However, it is hard to compare these two experiments together since both the enzyme and the carrier are different. In order to confirm effects of the carrier, an experiment, where the TL would be immobilized on more hydrophobic carrier (compared to the original one), must be performed. This could be done in order to understand better effects of both the enzyme retrieved from TL and the carrier support material.

In the next graph, Fig. 19, relative initial activities of both RM and TL immobilized preparations are plotted.

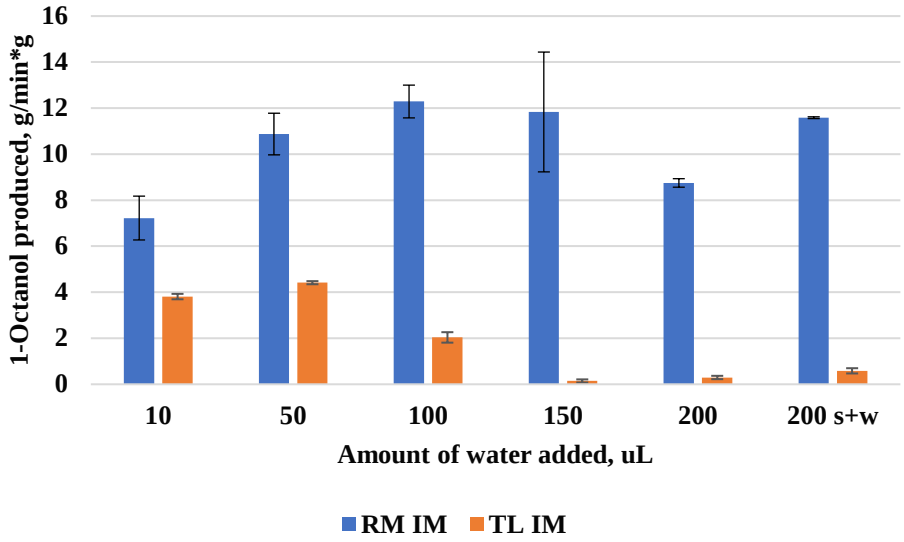

Fig. 19. Activity of RM IM and TL IM lipase at different amounts of water present in the reaction medium during hydrolysis reaction in vials. 200 S+W – substrate added before water, in all other cases water was added before substrate.

In Fig. 19 RM IM is much faster at hydrolysing the target ester at all tested water activities and that the activity of it was not inhibited by increasing amounts of water, compared to TL IM. For these reasons the RM IM was picked for further acidolysis experiments.

3.6 Water activity influence on acidolysis

The main idea of the project was to control the water activity during the acidolysis reaction in order to hydrolyse the ester faster in the beginning of the reaction at high a_w and to promote reversed hydrolysis after the first step by decreasing the a_w. It was thought that by keeping the water activity high in the beginning of the reaction, hydrolysis would be the most dominant reaction and by drying

out the reaction medium, after the hydrolysis step, reversed hydrolysis would be the dominant reaction.

The acidolysis was performed several times at different water activities with both the ester and the acid in the reactor and one experiment was designed as a two-stage reaction, where the ester was hydrolysed first, and the acid added later. Results are shown in Fig. 20.

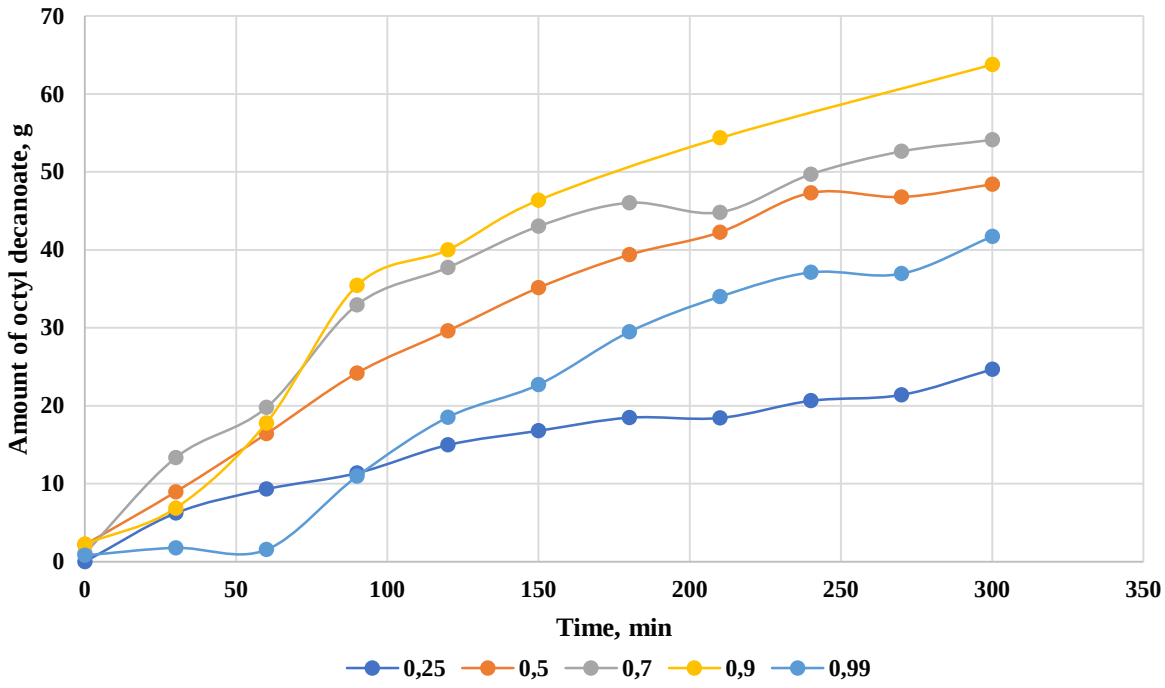

Fig. 20. Amount of octyl decanoate produced by RM IM at different water activities during acidolysis reaction. At the water activity of 0.99, only hydrolysis was performed for the first hour, after one hour second substrate (decanoic acid) was added.

From the results of acidolysis it can be seen the water activity has a significant impact on the final amount of product formed after 5 hours of reaction. When the water activity was increased to 0.5, the final amount of product formed was 2.2 times higher compared to the water activity of 0.25. The final amount of product was 2.6 times higher at the water activity of 0.9 compared to the 0.25 one, however, the increase of final product amount is less drastic at higher water activities than 0.5. At different water activities an influence on the total rate of the hydrolysis is expected. In order to understand how the acidolysis reaction works, the amount of 1-octanol formed during the first step of the reaction was also monitored. At higher water activity the amount of alcohol in the reactor should increase, since the hydrolysis would be induced and vice versa for lower water activities where hydrolysis should not be dominant. The results of the total amount of 1-octanol present in the reactor over time is shown in Fig. 21.

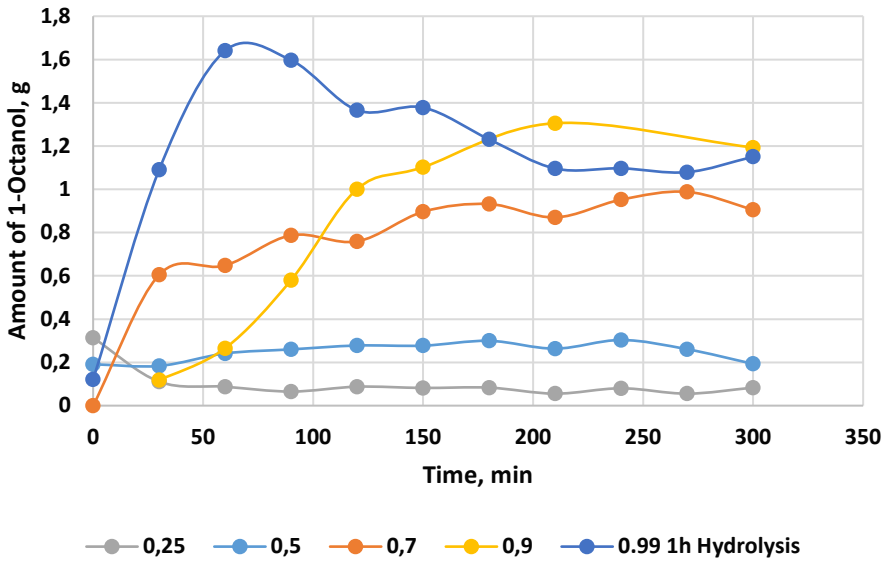

Fig. 21. Amount of 1-octanol in the reactor during acidolysis reaction performed by RM IM. At the water activity of 0.99, only hydrolysis was performed for the first hour, after 1 hour decanoic acid was added.

The amount of free alcohol in the reactor shows how fast the reversed hydrolysis is compared to the hydrolysis. An increase of the amount of alcohol would show that the ester is hydrolysed faster than the alcohol and acid are used in the reversed hydrolysis. However, the amount of alcohol in the reactor was increasing most dominantly when the hydrolysis of the ester was performed without decanoic acid present in the reactor. After the acid was added, the amount of the alcohol was decreasing to the same point as the other experiments until it levelled off. At the same time, the formation rate of the product was observed to be very similar to the experiments without higher concentration of 1-octanol at the beginning of the acidolysis (Fig. 20), meaning that the reaction limiting step might be the third step of acidolysis (Eq. 2), the binding of the acid substrate.

The acidolysis reaction was expected to work in a different way, it was expected that higher water activity would increase hydrolysis and low water activity – reversed hydrolysis. However, the results that were obtained during the experiments showed an interesting behaviour. It can be observed, that the reaction is limited on the third step (Eq. 2), since the amount of product does not increase with an increasing amount of 1-octanol in the reactor. However, in the later part of the reaction the rates of hydrolysis and reversed hydrolysis seem to match, since the level of 1-octanol does not change much. This would indicate that the total reaction rate is limited on both steps, at least in the final stages of acidolysis. The results showing amount of 1-Octanol and production of octyl decanoate in the same reaction, is shown in Fig. 22.

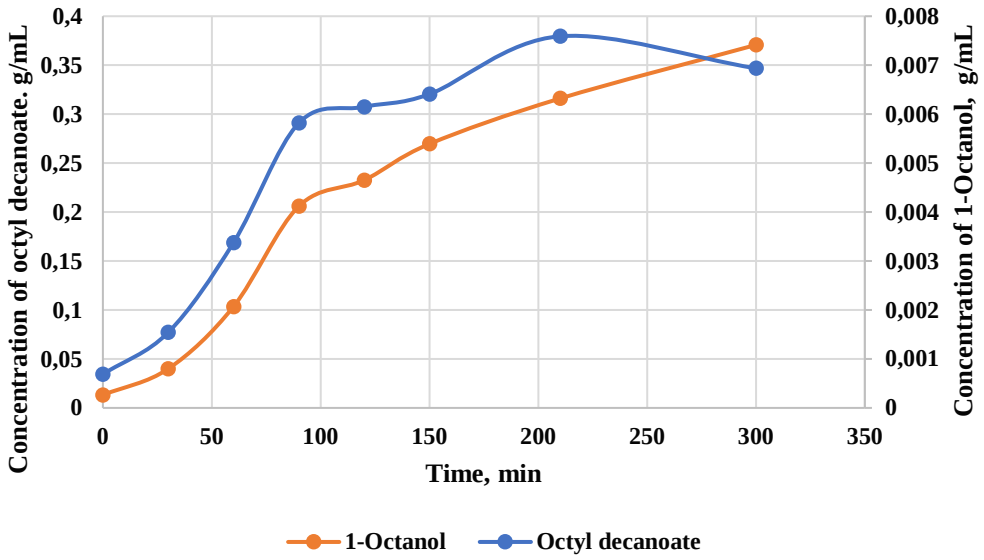

Fig. 22. Concentration of 1-Octanol in the reactor in comparison with the product – octyl decanoate, at a_w of 0.9, during acidolysis reaction. Acidolysis was performed by RM IM.

Results in Fig. 22 shows that the amount of 1-octanol in the reactor is very low at all times. This shows that the alcohol that is produced during the acidolysis is consumed very efficiently and this can suggest, together with the results from Fig. 20 and 21, that both, hydrolysis and the biding of the acid are slow.

4. Conclusion

1. The automatic water activity control system was designed and optimized for use in scientific research. The equipment expressed good reliability, safety and repeatability in the context of keeping the desired water activity in the reactor. The equipment had several shortcomings, including poor quality of jumper wires, which sometimes led to the malfunction of the humidity sensor, due to the unstable feeding of power; The wet nitrogen was showing a tendency to condensate in tubings, before entering the reactor, resulting in too low amount and slow water delivery to the reactor; The alcohol sensor that was used did not show a dependable response to the alcohol in the reactor. The relative humidity sensor was very sensitive to volatile alcohols, meaning that the water activity cannot be controlled in reactions where volatile alcohols are created or consumed.
2. The enzyme activity at different water activities was monitored and evaluated. The automatic water activity control system was used to maintain the water activity in the reactor; the enzyme activity was measured by means of sampling the medium and evaluating substrates consumed and products formed by the gas chromatography. Different immobilized preparations expressed different activity profiles in the range of water activities.
3. Miniaturised vial experiments were conducted in order to evaluate how different amounts of water can affect lipase activity in hydrolysis reaction. Two immobilized preparations were tested, RM IM and TL IM. Both preparations expressed different hydrolytic activities in vials. It was understood, that silica gel, that is used to immobilize TL, was keeping water around, creating water barrier and preventing the entrance of hydrophobic substrate to the active site of enzymes, resulting in diminished activity.
4. The acidolysis reaction was evaluated at different water activities with the RM IM. It was noticed, that higher water activities resulted in more product formed after 5 hours of reaction. The acidolysis reaction was happening in two steps, in the first step 1-octanol was released and later consumed in the second step. It was noticed that the amount of 1-octanol in the reactor is always very low, meaning that the hydrolysis of ester was slow, regardless of the water activity. It was thought that in order to increase the first step speed, which is hydrolysis, the water activity must be increased, however, at increased water activities both esterification and hydrolysis was performed equally well, meaning that the hydrolysis could be slower in general, compared with reversed hydrolysis. However, a little increase in the octanol was observed at different water activities, but the acidolysis rate in the beginning of the reaction,

was very similar at every water activity, meaning that the third step in reaction mechanism, where acid is biding to the enzyme, could be rate limiting.

5. Future work and recommendations

The automatic water activity control system demonstrated a good and repeatable operation however, further improvements might be necessary in order to use the equipment for longer time. Firstly, jumper wires that are being used right now, should probably be replaces with more reliable wires, in order to reduce the probability of energy supply loss in the sensor. Pipes that go from wet nitrogen source to the reactor must be isolated and kept as short as possible, in order to avoid condensation.

In the real time reaction tracking system, a better mathematical model, that would fit the GC data better, could be made by performing more experiments and by understanding how methanol evaporates from the reactor. The methanol evaporation rate is also very sensitive to the temperature and nitrogen flow speed. If any of these parameters would change a new methanol evaporation kinetics would be present so the system would need to be optimized for it from the beginning.

In the "water influence on the carrier, hydrolysis in small scale" part a TL immobilized on another carrier could be used in order to compare the influence of water on the carrier only, because in the latter experiment two different enzymes were used on different carriers.

6. Warnings and risks

A very important suggestion is that before filling the reactor with substrate, the nitrogen should be supplied in order to avoid the leakage of substrate into the nitrogen pipes. Without the pressure inside the pipes, a substrate will leak through the pipes into the wet nitrogen tank or into the 3-way valve. It is very important to make sure, that the sparger is installed correctly and that the substrate will not leak from the reactor. The sparger can be installed with the parafilm in order to seal the gap between plastic sparger and the glass structure of the reactor. It is also very important not to touch any wires inside the prototype box when the power supply is ON, in order to avoid serious injuries or death. One should know that the current of 1 ampere, that is flowing through the system, is enough to cause a fatal damage to a human being, please always think before doing something with the equipment. One should also keep in mind, that the equipment is just a prototype and it is not tested with safety regulations and it can only be used by personnel who completely understands risks and how to handle the equipment. It might also break down, do not try to fix it with the power supply on, do not try to open the lid when power supply is on. It is very important that the user understands completely how the system works and what kind of injuries, and how, it might cause, before using it. Please do contact me if you are not sure what to do and if you will try to fix something without being too sure of how things work. Make sure that the electronics in the fume hood are dry, the box of the equipment should not have any bottles with liquid on top, please be very careful when operating the system.

7. Sources of errors

- Human error (Errors in data entry / experimental techniques): sampling, preparing substrates, weighing, pipetting, preparing GC samples.

- Systematic error (Errors in the experiment itself): the water activity control system - the relative humidity sensor values could drift, the temperature in the head space greatly influence the relative humidity (temperatures in the medium and head space should be similar).

- Random error (caused by environmental factors, unpredictable factors): amount of enzyme on immobilized preparations.

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9. Appendix

Raw data exported from a GC and summarized using Excel. The enzyme that was used was TL IM. The data is plotted in the figure 19 in the text.

200ul (substrate + water)			200 ul water (water + substrate)			150ul water		
slope	108,50mg	102,50mg	slope	108,80mg	102,50mg	slope	104,90mg	103,90mg
1-oct	36,84	51,64	1-oct	28,85	16,47	1-oct	16,10	5,54
octyl octanoate	-795,5	-613,68	octyl octanoate	-574,24	-206,77	octyl octanoate	-134,0	-419,84
octanoic acid	42,31	74,36	octanoic acid	36,63	36,36	octanoic acid	12,00	66,54
slope/mg			slope/mg			slope/mg		
1-oct	0,34	0,50	1-oct	0,27	0,16	1-oct	0,15	0,05
oo	-7,33	-5,99	oo	-5,28	-2,02	oo	-1,28	-4,04
oa	0,39	0,73	oa	0,34	0,35	oa	0,11	0,64
Standard deviation		avg slope/mg	Standard deviation		avg	Standard deviation		avg
1-oct	0,08	0,42	1-oct	0,05	0,21	1-oct	0,05	0,10
oo	0,67	-6,66	oo	1,63	-3,65	oo	1,38	-2,66
oa	0,17	0,56	oa	0,01	0,35	oa	0,26	0,38
100ul w			50ul w			10ul w		
slope	100,60mg	104,70mg	slope	103,20mg	100,90mg	slope	105,40mg	100,40mg
1-oct	131,30	170,96	1-oct	333,63	316,34	1-oct	298,72	267,44
octyl octanoate	-499,14	-1081,54	octyl octanoate	-506,02	-663,78	octyl octanoate	-983,1	-1056,5
octanoic acid	201,74	226,65	octanoic acid	383,60	363,40	octanoic acid	342,43	210,03
slope/mg			slope/mg			slope/mg		
1-oct	1,31	1,63	1-oct	3,23	3,14	1-oct	2,83	2,66
oo	-4,96	-10,33	oo	-4,90	-6,58	oo	-9,33	-10,52
oa	2,01	2,16	oa	3,72	3,60	oa	3,25	2,09
Standard deviation		avg	Standard deviation		avg	Standard deviation		avg
1-oct	0,16	1,47	1-oct	0,05	3,18	1-oct	0,09	2,75
oo	2,68	-7,65	oo	0,84	-5,74	oo	0,60	-9,93
oa	0,08	2,09	oa	0,06	3,66	oa	0,58	2,67

Appendix B

Raw data exported from a GC and summarized using Excel. The enzyme that was used was RM IM. The data is plotted in the figure 19 in the text.

10 ul water			50 ul water			100 ul water		
slope	50 mg	50	slope	51,8 mg	53,2 mg	slope	50 mg	50,7 mg
1-oct	226,2	294,9	1-oct	440,1	382,5	1-oct	468,8	423,3
octyl octanoate	-350,0	-364,8	octyl octanoate	-575,8	-986,9	octyl octanoate	-744,9	-958,9
octanoic acid	211,1	305,7	octanoic acid	444,5	330,9	octanoic acid	481,8	412,0
slope/mg			slope/mg			slope/mg		
1-oct	4,5	5,9	1-oct	8,5	7,2	1-oct	9,4	8,3
oo	-7,0	-7,3	oo	-11,1	-18,5	oo	-14,9	-18,9
oa	4,2	6,1	oa	8,6	6,2	oa	9,6	8,1
stdevp slope/mg		avg	stdevp slope/mg		avg	stdevp slope/mg		avg
1-oct	0,7	5,2	1-oct	0,7	7,8	1-oct	0,5	8,9
oo	0,1	-7,1	oo	3,7	-14,8	oo	2,0	-16,9
oa	0,9	5,2	oa	1,2	7,4	oa	0,8	8,9
150 ul water			200 ul (water+substrate)			200 ul (Substrate+Water)		
slope	50,9 mg	50,3 mg	slope	51,3 mg	51,4 mg	slope	54,7 mg	52 mg
1-oct	530,0	334,9	1-oct	316,9	331,2	1-oct	458,5	433,1
octyl octanoate	-1388,4	-1528,1	octyl octanoate	-1216,0	-1754,5	octyl octanoate	-1130,8	-1164,5
octanoic acid	442,8	331,3	octanoic acid	324,2	380,3	octanoic acid	456,1	428,8
slope/mg			slope/mg			slope/mg		
1-oct	10,4	6,7	1-oct	6,2	6,4	1-oct	8,4	8,3
oo	-27,3	-30,4	oo	-23,7	-34,1	oo	-20,7	-22,4
oa	8,7	6,6	oa	6,3	7,4	oa	8,3	8,2
stdevp slope/mg		avg	stdevp slope/mg		avg	stdevp slope/mg		avg
1-oct	1,9	8,5	1-oct	0,1	6,3	1-oct	0,0	8,4
oo	1,6	-28,8	oo	5,2	-28,9	oo	0,9	-21,5
oa	1,1	7,6	oa	0,5	6,9	oa	0,0	8,3

Appendix C

Concentrations of water in the SpinChem bioreactor at different water activities. These results are plotted in the figure 7 in the text.

a_w	0,085	0,16	0,26	0,36	0,46	0,56	0,618
mg/mL 1	0,47	0,91	1,77	2,87	3,96	6,39	6,89
mg/mL 2	0,42	0,95	1,68	2,82	3,87	6,22	6,81
Avg mg/mL	**0,45**	**0,93**	**1,72**	**2,84**	**3,92**	**6,30**	**6,85**

Appendix D

Concentrations of water in the saturated salt solutions at different water activities. These results are plotted in the figure 7 in the text.

aw	0,093	0,231	0,331	0,544	0,755	0,843
mg/mL 1	0,742	1,841	2,237	5,035	8,673	9,161
mg/mL 2	0,737	1,826	2,27	5,006	8,767	9,348
Avg mg/mL	**0,7395**	**1,8335**	**2,2535**	**5,0205**	**8,72**	**9,2545**

Appendix E

Transesterification rate - octyl laurate production of different enzymes at different water activities. Results are plotted in the figure 14 in the text.

TL IM		RM IM		CalB		Benchmark 1		Benchmark 2	
Area of peak/min/ mg	aw	Area of peak/min/ mg	aw	Area of peak/min/ mg	aw	Area of peak/min/ mg	aw	Area of peak/min/ mg	aw
208,15	0,13	332,18	0,75	414,80	0,16	0,44	0,17	573,89	0,16
247,93	0,20	419,58	0,50	308,87	0,26	24,36	0,27	524,33	0,26
350,10	0,26	475,14	0,40	227,80	0,51	47,17	0,37	625,97	0,36
178,16	0,30	574,15	0,35	76,43	0,61	104,70	0,47	704,86	0,46
210,99	0,40	582,40	0,15	47,80	0,75	101,67	0,76	683,74	0,56
184,54	0,60							378,25	0,76
191,65	0,66								
236,66	0,75								

Appendix F

Raw data extracted from chromatograms of acidolysis reaction, data is plotted in figure 20, 21, 22 in the text.

25 RH					
Time, min	1-oct	decanoic acid	Area/octanoic acid	Area/Octyl decanoate	octyl octanoate
300	60,0	57818,4	6111,5	13300,9	40258,2
270	40,0	53346,1	5229,5	11534,7	38188,4
240	57,8	56591,3	4790,7	11128,2	39858,9
210	40,0	55116,6	4577,3	9939,9	40076,1
180	60,0	60566,9	4467,1	9961,3	43870,2
150	59,0	60501,5	23301,6	9051,5	42802,3
120	63,2	63239,3	3796,4	8067,8	46848,6
90	47,0	55884,4	2613,9	6127,1	42317,9
60	63,0	58747,9	2267,5	5017,9	44883,2
30	80,0	57683,4	1574,2	3355,4	45188,7
0	226,0	66699,0	411,7	0,0	51520,5

50 RH					
Time, min	Area/1-Octanol	Area/decanoic acid	Area/octanoic acid	Area/Octyl decanoate	Area/octyl octanoate
300	139,3	29579,7	8865,7	26097,0	15476,8
270	188,1	36726,9	10464,3	25218,2	19835,4
240	218,8	44013,0	11484,3	25505,9	24759,1
210	190,1	41424,8	10190,3	22784,6	23968,5
180	216,1	43471,1	9628,6	21225,2	26188,4
150	200,0	28744,4	5656,2	18942,2	18999,9
120	200,5	45166,9	7221,5	15960,8	30138,8
90	187,6	45717,2	5981,8	13047,7	31783,4
60	174,0	44237,5	4101,1	8842,8	33340,7
30	132,5	47060,4	2255,0	4827,5	37352,0
0	137,9	54839,0	801,0	1174,9	45088,3

90				
Time, min	Area/1-Octanol	Area/decanoic acid	Area/octanoic acid	Area/Octyl decanoate
300	860,5	36765,8	15802,4	34372,5
270	170,9	8360,2	3639,8	8272,2
240	473,9	21320,9	8591,4	18607,9
210	941,7	36050,0	13522,3	29299,8
180	428,4	15584,0	5464,9	11702,5
150	795,1	38532,5	11679,8	24996,2
120	762,9	38664,1	9699,6	21563,1
90	721,9	41686,8	9064,9	19094,0

60	418,7	30541,8	4457,2	9591,9
30	191,3	21776,0	1766,3	3701,4
0	85,4	29696,4	564,2	1223,8

70					
Time, min	Area/1-Octanol	Area/decanoic acid	Area/octanoic acid	Area/Octyl decanoate	Area/octyl octanoate
300	653,4	40730,9	13550,3	29170,7	19399,1
270	712,8	41436,0	13274,7	28369,8	20163,9
240	686,9	40600,4	12194,2	26786,5	20475,4
210	627,5	39759,2	11480,3	24159,6	20684,9
180	672,2	43992,1	11706,7	24819,5	23977,1
150	646,4	45755,4	10927,2	23192,6	26287,3
120	547,5	45361,1	9558,6	20341,0	27351,6
90	568,2	49270,1	8384,2	17762,7	31761,2
60	467,2	43003,2	5229,2	10660,8	30070,6
30	436,0	50452,7	3838,7	7186,6	36649,8
0	0,0	58772,9	514,8	575,4	45923,3

99,99					
Time, min	Area/1-Octanol	Area/decanoic acid	Area/octanoic acid	Area/Octyl decanoate	Area/octyl octanoate
300	829,8	47541,0	10667,0	22484,3	21725,0
270	778,4	45339,7	9689,3	19912,6	21287,2
240	791,2	49183,2	9673,0	20008,0	24046,8
210	790,5	50266,1	8865,6	18329,5	25766,3
180	888,0	50620,5	7894,2	15892,2	27076,3
150	994,0	48706,6	6368,5	12242,6	27270,4
120	984,8	51418,4	5403,3	9988,0	30579,1
90	1151,6	53137,5	3771,1	5904,9	34382,9
60	1183,3	59988,9	1585,8	829,9	41004,2
30	786,2	144,8	2019,5	959,2	46656,7
0	87,6	750,3	431,4	439,4	52521,2

Appendix G

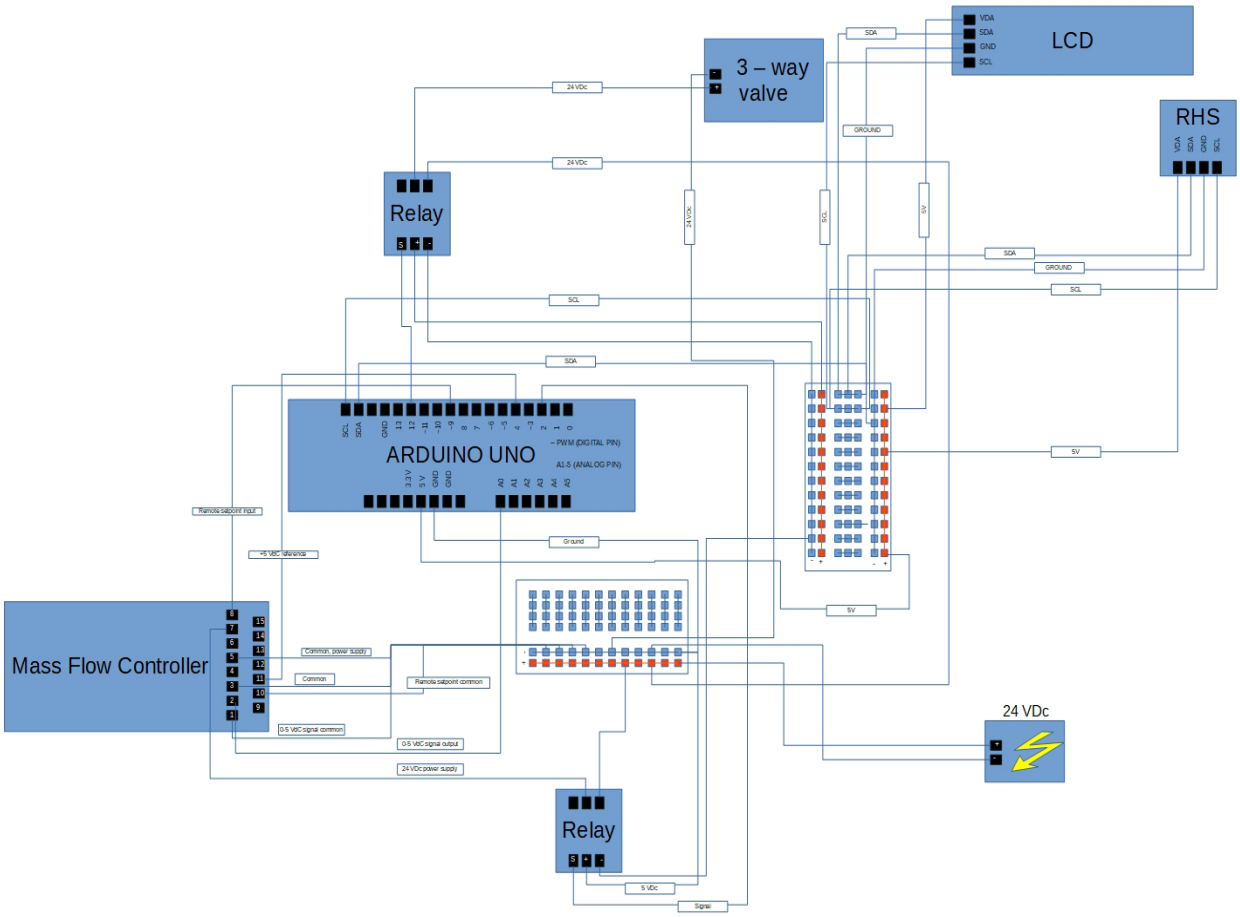
Code that was written for the water activity control system.

```
const int AOUTpin=2;
int value;
int pwmPin = A0;
int val = 0;
int setpoint = 10;
int pwmPin2 = 9;
int refPin = 4;
int relay5v = 2;
int relaysignal = 3;
const int ledPin =  12;
unsigned long previousMillis = 0;
int period = 880;
unsigned long time_now = 0;
unsigned long ttt = 0;
unsigned long currentMillis = 0;
unsigned long previousLedMillis = 0;
#include <Wire.h>
#include <LiquidCrystal_I2C.h>
LiquidCrystal_I2C lcd(0x27, 2, 1, 0, 4 ,5, 6, 7, 3, POSITIVE);
#include "Adafruit_Sensor.h"
#include "Adafruit_AM2320.h"
Adafruit_AM2320 am2320 = Adafruit_AM2320();
void setup() {
 pinMode(relay5v, OUTPUT);
 pinMode(relaysignal, OUTPUT);
 pinMode(pwmPin2, OUTPUT);
 pinMode(pwmPin, INPUT);
 pinMode(6, OUTPUT);
 pinMode(ledPin, OUTPUT);

 Serial.begin(9600);
  am2320.begin();
 digitalWrite(refPin, HIGH);
 digitalWrite(relay5v, HIGH);
 digitalWrite(relaysignal, HIGH);
 digitalWrite(6, HIGH);
   lcd.begin(16,2);
   lcd.setBacklight(HIGH);
}
void loop() {
if (setpoint > 0){
 Serial.print(" Time: ");
 time_now = millis();
 ttt = time_now / 1000;
 Serial.print(ttt);
 while(millis() < time_now + period){}
}
if (setpoint > 0){
 Serial.print(" ");
  Serial.print("Hum: ");
  Serial.print(am2320.readHumidity());
  Serial.print(" ");
  while(millis() < time_now + period){}
}
if (setpoint > 0){
```

```
    Serial.print("Temp: ");
    Serial.println(am2320.readTemperature());
    while(millis() < time_now + period){}
}

int val = analogRead(pwmPin);
float voltage = val * (5/1023.00);
float flow = ((voltage*2)/5);
value = analogRead(AOUTpin);
float mc = (am2320.readHumidity())- 19.50;
float mcd = ((mc-0.4587)/8.6957);
float mcc = (-1/(-97.04))*log(mcd/0.1879);
if (setpoint > 0){
    Serial.print("AlcVal: ");
    Serial.print(value);
    while(millis() < time_now + period){}
}
float difference = setpoint - (am2320.readHumidity());
if (setpoint > 0){
    Serial.print(" Flow: ");
    Serial.print(flow);
}
  lcd.clear();
  lcd.setCursor(0,0);
  lcd.print("Flow: ");
  lcd.print(flow);
  lcd.print(" L/min");
  lcd.setCursor(0,1);
  lcd.print("RH:");
  lcd.print(am2320.readHumidity());
  lcd.print(" T:");
  lcd.print(am2320.readTemperature());

if (difference>0.2){
  digitalWrite(ledPin, HIGH);
  }
  else {
    digitalWrite(ledPin, LOW);
    }

if (difference>3){
  analogWrite(pwmPin2, 255);
    }
else if ((difference>-1) && (difference <1)){
  analogWrite(pwmPin2, 105);
    }
else if ((difference >=-3) && (difference <-1)){
  analogWrite(pwmPin2, 255);
  }
else {
  analogWrite(pwmPin2, 255);
}
  }
```

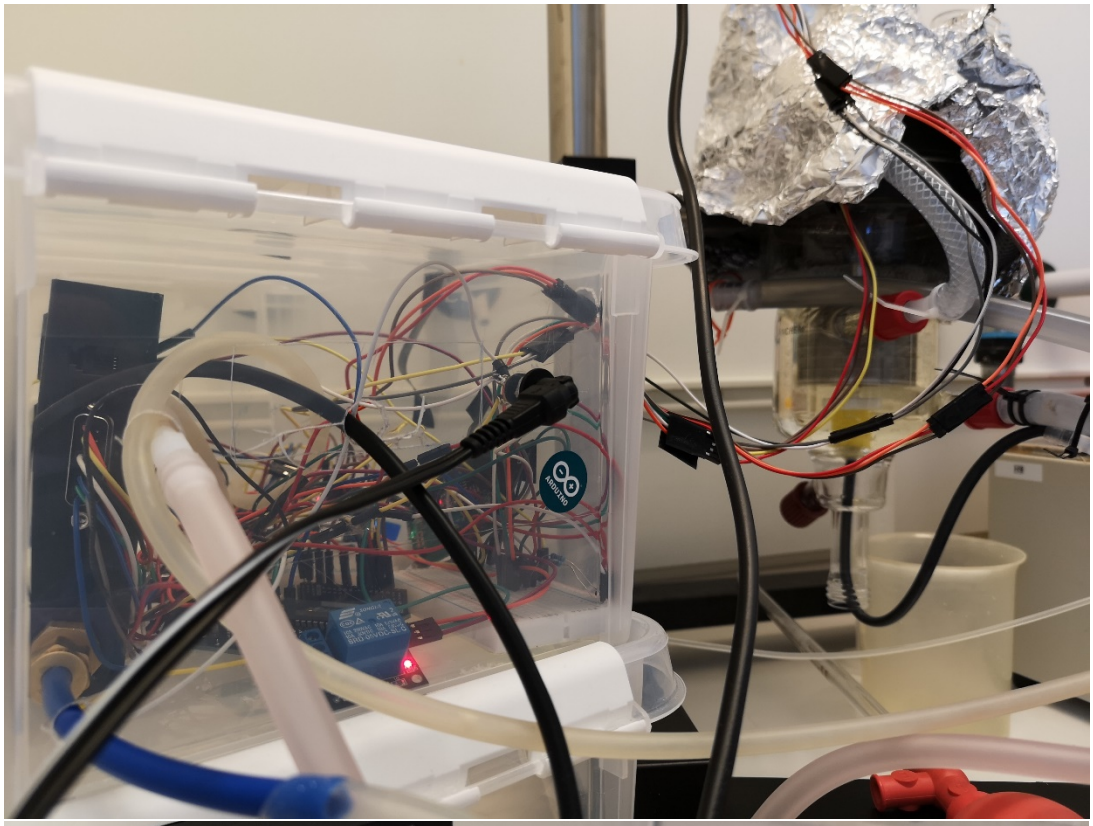
Appendix H

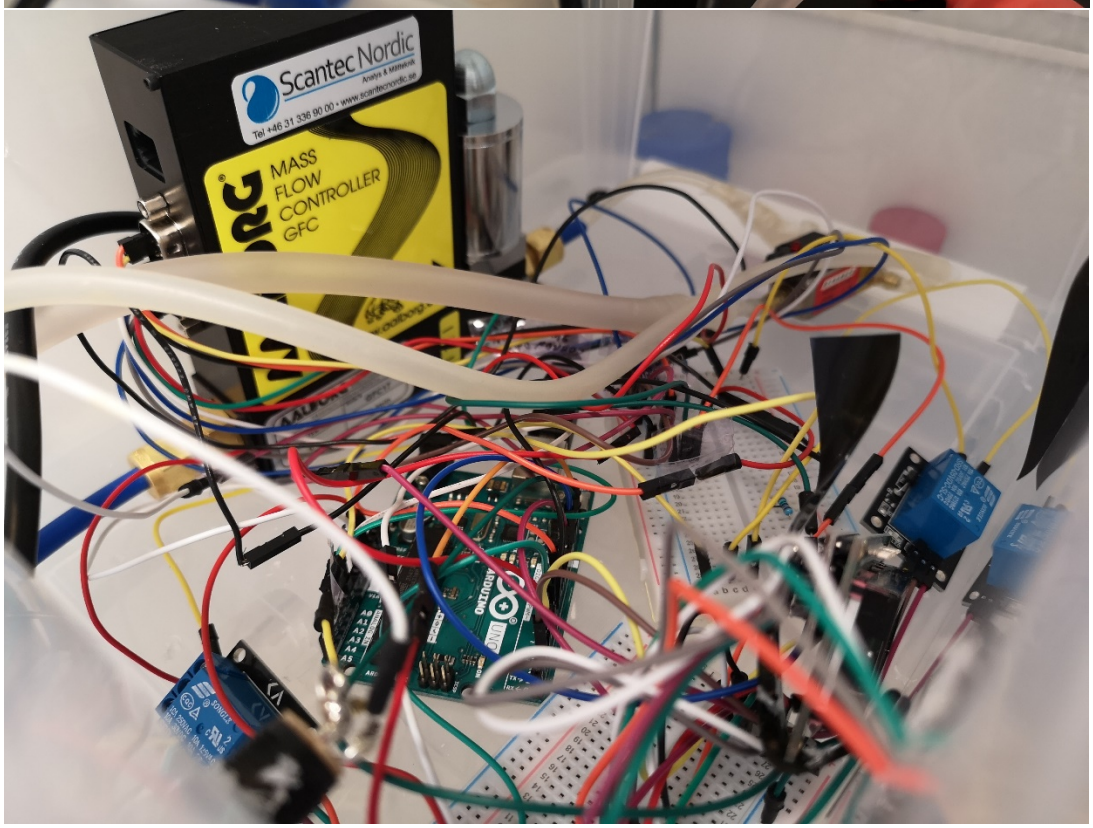
Electrical wiring diagram of the water activity control system.

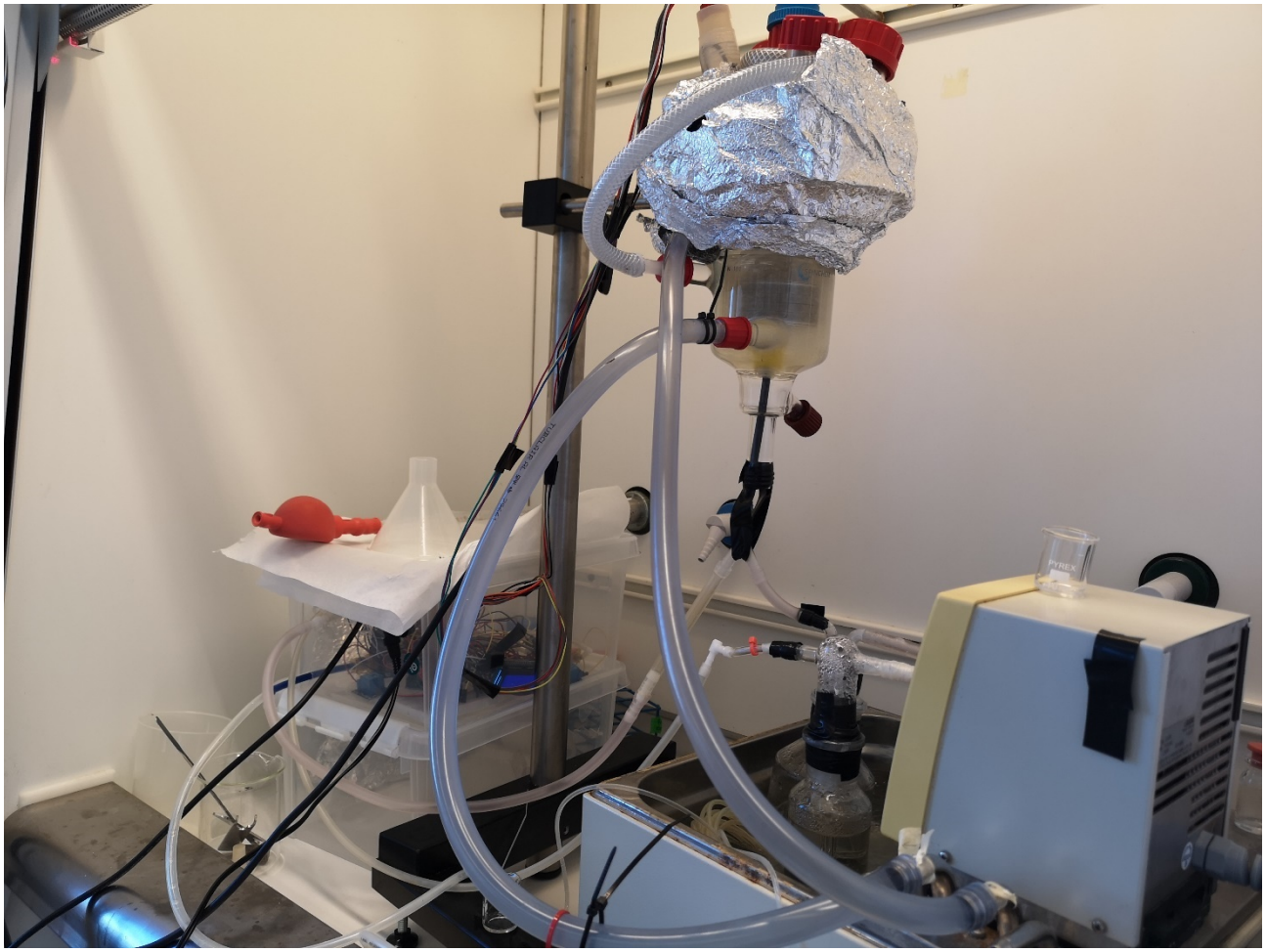


Appendix I

The prototype of the water activity control system that was constructed and used in the laboratory for research purposes.






PYREX