**Cláudia Filipa Duarte Bento**

Bachelor in Chemical and Biochemical Engineering

# Advanced Development of Sugar Nucleotide Regeneration Cascade

Dissertation submitted to fulfill Master's degree in

Chemical and Biochemical Engineering

Adviser: Dr. Thomas Rexer, PostDoc,
Max Planck Institute for Dynamics of Complex Technical Systems

Co-advisers: Reza Mahour, Phd,
Max Planck Institute for Dynamics of Complex Technical Systems

Rui Oliveira, Associated Professor,
Faculty of Science and Technology
NOVA University of Lisbon

Examination Committee:

| | |
|---|---|
| President: | Dr. Mário Eusébio |
| Main Examiner: | Dra. Ana Cecilia Roque |
| Supervisor: | Dr. Rui Oliveira |

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

**September, 2018**

**Advanced Development of Sugar Nucleotide Regeneration Cascade**

# Acknowledgements

First, I would like to thank Dr. Thomas Rexer and Prof. Udo Reichl for the opportunity to do my Master Thesis at Max Planck Institute Dynamics of Complex Technical Systems in Magdeburg. A special thanks to MSc. Reza Mahour for his guidance through all the internship and for teaching me everything I needed to succeed in this work. Thanks for showing me availability when it came to questions, doubts, dead ends and also for pushing me in a way that I learned how to work better under pressure.

Um agradecimento especial ao meu orientador na FCT, o Prof. Rui Oliveira pelo apoio durante esta tese especialmente na reta final.

A big thanks to my coworker Suzana Cvijetinovic for all the support in the working environment she gave me during this time. I will never forget all the good moments we had and all the life knowledge advices she transmitted to me. I am very happy that I had the pleasure to meet her.

I would also like to thank my friend Karina Pál for joining me in this adventure of living in Germany for 6 months. It was not an easy journey and she was a big support and helped me a lot with everything. We had a wonderful time and without her this experience would not had been the same.

Um agradecimento muito especial que tenho a fazer é aos meus pais que me possibilitaram toda esta experiência. Ao meu pai, por me incentivar a entrar nesta jornada tão fantástica e diferente e à minha mãe pelos telefonemas a dar-me motivação todos os dias para continuar. Sem o apoio deles nada disto seria possível o que contribuiu mais para os tornar pais tão excelentes.

Um obrigado à minha segunda mãe, Misa, que com 79 anos fazia videochamadas comigo todas as semanas e me dava sempre as palavras de apoio e orgulho que motivam sempre mais. Ao meu irmão, Guilherme, um grande obrigado por ter-me encorajado a fazer esta jornada, por me motivar a ser sempre melhor e por me ajudar sempre que eu preciso. A pessoa que me tornei hoje, os estudos que tenho assim como a decisão de fazer a tese na Alemanha foram resultado dos seus incentivos e apesar de ele ser um “chato”, agradeço bastante todos os bons conselhos que me deu.

Também devo agradecer a todos os meus amigos que me apoiaram não só nestes 6 meses, mas também ao longo destes 5 anos de faculdade. Um agradecimento especial a fazer é à Maria Pereira que foi um pilar durante todo o curso, assim como nestes 6 meses. Como sempre, foram os seus conselhos motivadores e perspicazes que me mantiveram sempre a cabeça focada no que é importante.

Last but not least, I also want to thank my office mates, José and Lars that made all work days much funnier and happier, to Weiwei Peng for all the help she provided me and for producing all the enzymes I needed to do my work and to Claire Telfer for all the good moments we shared in the lab. It was a very good experience to teach such a motivated person.

In general, a big thanks to all the master and internship students that were part of this institute that I had the pleasure to meet. All of them contributed to a very good environment and it was a very good experience to meet such different and wonderful people.

# Abstract

Sugar nucleotides are essential building blocks for the glycobiology of all organisms being intrinsically linked to cellular metabolism. However, the market price of sugar nucleotides is currently very high due to the inexistence of cost-effective manufacturing processes.

In this thesis, an in vitro enzyme process for the synthesis of sugar nucleotides UDP-Man, UDP-Glc and UDP-GlcNAc using low cost substrates was developed and studied in order to decrease the manufacturing costs of sugar nucleotides. The effects of pH and buffer composition on the cascades were investigated leading to an increase in the productivity of the target product. Another important discovery was the enzymatic production of the sugar nucleotide, UDP-Man, representing the first step towards the more economical production of this sugar.

Moreover, it was found that, by adjusting the concentration of the enzymes in the cascades, the productivity of the UDP-Glc and the UDP-GlcNAc cascade could be increased by 52% and 56%, respectively. Finally, it was observed that when high concentration of product if formed, *in situ* removal of sugar nucleotide might be necessary.

Given the results obtained, this thesis presents new data and ideas towards the establishment of a cost-effective in vitro enzymatic production process of UDP-Man, UDP-Glc and UDP-GlcNAc with very promising practical applications.

**Keywords**: sugar nucleotides, UDP-GlcNAc, UDP-Glc, UDP-Man, multi enzymatic system, *in vitro*, one pot synthesis

# Resumo

Os açúcares nucleotídeos são essenciais para a Glicobiologia de todos os organismos estando intrinsecamente ligados ao metabolismo celular. No entanto, pesquisas relacionadas com esta temática encontram-se limitadas devido a complicações na obtenção de açúcares nucleotídeos provocadas pelo seu elevado preço de fabricação. Nesta tese, é desenvolvido um processo multienzimático *in vitro* que se foca na síntese de açúcares nucleotídeos, UDP-Man, UDP-Glc e UDP-GlcNAc, através de uma eficiente cascata de enzimas usando substratos de baixo custo.

Os efeitos do pH e buffer nas cascatas foram investigados levando a um aumento da produtividade do produto em questão. Um ponto importante a frisar é que UDP-Man foi produzido, pela primeira vez, via enzimática dando assim o primeiro passo em direção á produção mais económica deste açúcar nucleotídeo.

Foi ainda provado que, ao ajustar as concentrações de enzimas nas cascatas, a produtividade de UDP-Glc e UDP-GlcNAc poderia ser aumentada em 52% e 56%, respetivamente. Para finalizar, observou-se que, quando se forma alta concentração de produto, pode vir a ser necessária a remoção *in situ* do produto.

Dados os resultados obtidos, esta tese apresenta novos dados e ideias para uma produção enzimática mais económica de UDP-GlcNAc, UDP-Glc e UDP-Man com aplicações práticas muito interessantes.

**Palavras-chave:** açúcares nucleotídeos, UDP-GlcNAc, UDP-Glc, UDP-Man, sistema multienzimático, *in vitro*

# Contents

# List of Figures

# List of Tables

# Acronyms

| | |
|---|---|
| **2D-PPK2** | 2-Domain-Polyphosphate Kinase |
| **ADP** | Adenosine Diphosphate |
| **ATP** | Adenosine Triphosphate |
| **GalU** | UTP-Glucose-1-Phosphate Uridylyltransferase |
| **Glk** | Glucokinase |
| **His-tag** | Hexahistidine-tag |
| **HPAEC** | High Performance Anion Exchange Chromatography |
| **Man B** | Phosphomannomutase |
| **Man C** | Mannose-1-Phosphate-Guanyltransferase |
| **Nahk** | N-Acetylhexosamine 1- Kinase |
| **PmPpA** | Inorganic Diphosphatase |
| **Poly$P_n$** | Polyphosphate |
| **PPK3** | Polyphosphate Kinase |
| **UDP** | Uridine Diphosphate |
| **UDP-Glc** | Uridine Diphosphate Glucose |
| **UDP-GlcNAc** | Uridine Diphosphate N-acetylglucosamine |
| **UDP- Man** | Uridine Diphosphate Mannose |
| **UMP** | Uridine Monophosphate |
| **URA6** | Uridine Monophosphate Kinase |
| **UTP** | Uridine Triphosphate |

# 1. Introduction

Sugar nucleotides are fundamental molecules in life. They are formed by the combination of a uridine or guanosine-containing nucleoside diphosphate (UDP or GDP) along with a sugar. The sugar can contain aldopentose (UDPXyl), aldohexose (UDP-Glc, UDP-Gal), aldohexosamine (UDP-GlcNAc, UDP-GalNAc) or uronic acid (UDP-GlcA) components [1]. In Figure 1.1 there is presented the example of the structure of the sugars nucleotides that are mainly approached in this project, UDP-N-acetylglucosamine (UDP-GlcNAc) and UDP-Glucose (UDP-Glc).

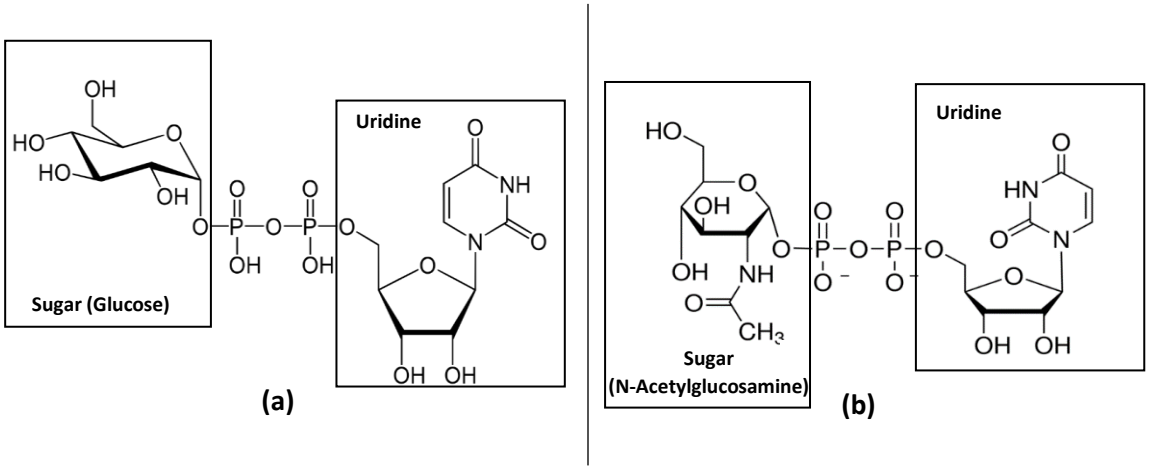



*Figure 1. 1: Sugar nucleotide structure. (a) Structure of UDP-Glucose* [53]*. (b) Structure of UDP-GlcNAc* [54].

Figure 1.1A illustrates UDP-Glucose, which is a sugar nucleotide composed by a uridine group and an aldohexose group (glucose). It is also presented, in Figure 1.1B, UDP-GlcNAc that has, also, a uridine group attached to an aldohexamine sugar (N-acetylglucosamine). These nucleotide sugars are essential building blocks for the glycobiology of all living organisms, therefore, the study and quantification of these compounds contributes to the understanding of their functions [1].

One of the most important cellular processes involving sugar nucleotides is glycosylation. Glycosylation comprehends important modifications that confer and controls a wide range of properties to a protein [2]. This process takes place mainly in the endoplasmic reticulum (ER) where various glycosyltransferases catalyze the transfer of sugar nucleotides forming a glycan. When the glycan is attached to the protein, it forms a glycoprotein with a specific purpose which can be a direct result of their structure [3].

Glycoproteins are included in the field of protein engineering technologies. This area has reached high advances regarding the achievement of desirable functional characteristics of proteins for the treatment of several diseases [4]. These glycoproteins, particularly therapeutic proteins, have a high demand and have stimulated the research for new, better, and faster sources for recombinant protein production leading to an increase of studies in this field [2] [5].

These glycoproteins are associated to many diseases related to glycans, and they have an important role on vaccination and host-pathogen interactions. For that reason, sugars nucleotides have a high impact on protein glycosylation, and thus on biological activity, stability and immunogenicity of glycoproteins [6]. With that in mind, it became essential to describe and produce sugar nucleotides in the biotechnological field, for instance, in biopharmaceutical production [7]. Nevertheless, this research has been limited by the high commercial price of sugar nucleotides [8].

These sugars can be produced chemically or via enzymatic synthesis. However, the chemical synthesis is somewhat problematic due to their complex molecular structure, low solubility of sugar nucleotides in organic solvents and the presence of several polar or charged functional groups [9] [10]. Chemical processes will always have the main use in the pharmaceutical industry, but, by combining or changing them to enzymatic processes, prices can decrease, and productivity can increase offering a wide broad of opportunities for this technology [11].

For that reason, enzymatic synthesis offers a promising highly efficient alternative allowing the regeneration of sugar nucleotides and promoting continued advances on understanding the biological roles played by glycans and their sugars contributing to the development of new therapeutic medicines [10]. In addition, with an industry that is continuously trying to improve efficiency the use of biocatalysts is becoming more cost-effective and additional sustainable solutions can be found, reducing the environmental footprint [11].

Although the enzymatic reactions to produce sugar nucleotides take place inside the cells, producing them *in vivo* can be problematic since the process is dictated by many factors such as the availability, activity, and correct subcellular localization of substrates and enzymes resulting in a lower yield of the reaction [9].

As a result, enzymatic reactions can be made outside of the cells, *in vitro*, which means that the process uses isolated enzymes to produce the final product, keeping the outstanding efficiency. This approach holds a very particular promise since individual enzyme expression contributes to promoting the transformation wanted to obtain only the desired product [12]. Also, since enzymes are usually derived from mesophilic organisms in moderate conditions, by using enzymes *in vitro*, optimal conditions can be determined resulting in the reduction of cost and time processing [13].

Usually, in cells are also present multi-enzymatic systems that can be recreated *in vitro*. These type of systems are based on the combination of several enzymatic transformations, offering

considerable advantages: the demand of time, costs and chemicals for product recovery may be reduced, reversible reactions can be driven to completion and the concentration of harmful or unstable compounds can be kept to a minimum [14].

The major advantage of *in vitro* multi-enzymatic systems compared to engineered cellular systems is their reduced complexity and therefore ease of reaction control. However, to find the optimal conditions, it is necessary to do an intensive study for all the reactions. All the reactions can be optimized by varying enzyme and substrate concentrations, testing different cofactors and buffers, variations of pH and temperature [15]. After optimization of the system, it is possible to obtain higher purity of the final product when compared to cellular systems where are side reactions occurring resulting in a complex mixture of compounds [16].

When dealing with multi-enzymatic systems, there is also a traditional “stop and go” approach to synthesis in which a sequential process takes place with many separation steps. However, regularly can exist problems regarding the insurance of the consecutive reactions and can be associated with a higher price. For that reason, a more straightforward and better approach for this type of systems was pursuit [17].

As a rival to this traditional approach to synthesis stands the one-pot strategy, in which multiple chemical transformations are performed in a single reaction vessel without intermediary purification steps [18], as it is shown in Figure 1.2.

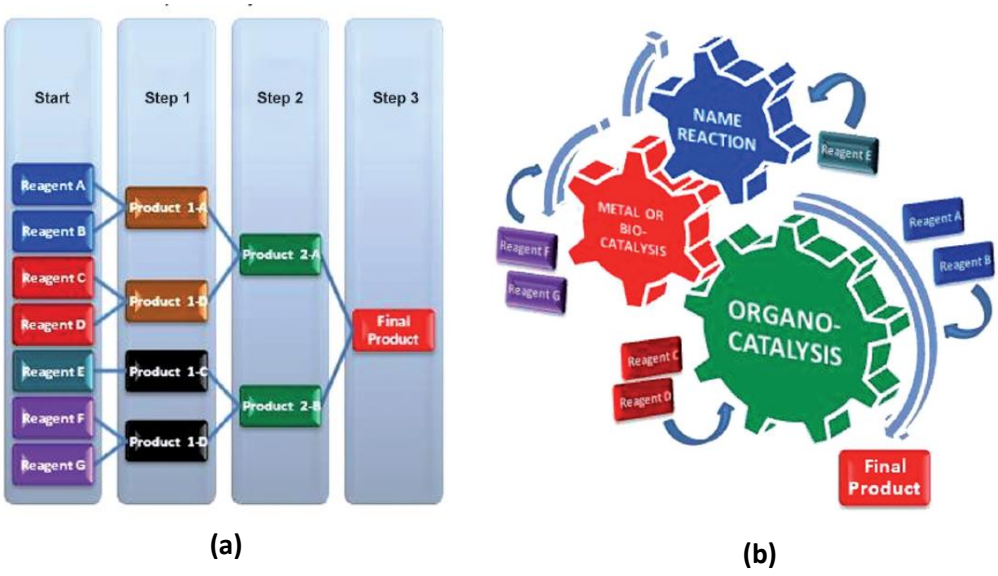



*Figure 1. 2: Comparison between stop and go and one-pot synthesis* [55]. *(a) Stop-and-go synthesis involving more separation steps and for that reason can increase the costs. (b) One- pot synthesis where no intermediary steps need to occur, decreasing the costs of production.*

In industrial processes, the one-pot approach has been adapted as an effective means of reducing time, costs and waste generation when compared to traditional synthesis where purification of intermediaries needs to be done at each step (Figure 1.2A). By reducing the number of isolation steps, an improvement on process efficiency and safety can be made leading to process research and development in the area of one-pot synthesis which is culminating in more efficient and practical processes [19].

Since sugar nucleotides production is already costly it is important to develop better and cheap ways to produce this product [20]. Therefore, high productivities can be reached when reactions are made using an *in vitro* multi-enzymatic system in one-pot based on a *de novo* cascade that regenerates the product in question, like is shown on Figure 1.2B [21].

Regarding the sugars produced in this thesis, a significant one is UDP-Mannose (uridine diphosphate mannose) whose presence was reported in mammalian tissues. It has been recently stated that in some areas of the brain there is a higher concentration of UDP-Man when compared to other organs. This leads to believe that if there is a better understanding of the role of UDP-Man in the process of glycosylation, then it can open new pathways for the treatment of neurological diseases [22].

Also, naturally in cells, sugar nucleotides such as UDP-GlcNAc and UDP-Glc act as activated sugars in the glycosylation process [23]. Besides this, a study was made that shows that both this sugar nucleotides concentrations are distinctly increased in human breast cancer, contributing to the tumor-promoting microenvironment [24]. Also, regarding UDP-Glc, it was proven that has effect in specific cell receptors that variate immune and inflammatory responses [21]. Furthermore, the research that can be made on these sugars may lead to a better understanding of their biological roles and importance [24].

Chemical synthesis of sugar nucleotides as UDP-Glc and UDP-GlcNAc was already made and reported in several papers [30-31]. Regarding enzymatic synthesis of these sugar nucleotides, it was also reported in articles [31]. For example, UDP-GlcNAc was already synthesized in vitro from low-cost substrates with a yield approaching 100 % [25] and UDP-Glc was synthetized catalyzed in one-pot with a yield of 70% and high purity [26]. However, in the particular case of UDP-Man there are no successful studies reported by *in vitro* enzymatic conversion.

In conclusion, sugar nucleotides have a crucial biological role, however, research related to this field is held down due to the high prices of fabrication. By resorting to a one-pot synthesis using an *in vitro* multi-enzymatic system, sugar nucleotides can be produced using low cost substrates, allowing the formation of this product with lower prices when compared to the traditional methods.

# 2. Theoretical Background

In this chapter, the relevant topics related to enzyme technology and sugar nucleotide production will be overviewed. In section 2.1. the process of glycosylation in which sugar nucleotides are involved will be explained followed by section 2.2. where it will be reviewed about what are enzymes and their structure. In section 2.3. an explanation is made about how each enzyme used in the cascade works.

## 2.1. Glycosylation

Glycosylation is a process that causes modifications on proteins trough the covalent attachment of an oligosaccharide on the protein. It comprises an assembly part and a processing part extending for three cellular compartments (Figure 2.1) [27]:

- the cytosol, where sugars are synthesized
- the endoplasmic reticulum (ER), where sugar nucleotides are catalyzed by glycosyltransferases that remove the sugars to form a glycan, which is a chain of oligosaccharides that is then attached to the protein
- the Golgi Complex, where more modifications take place

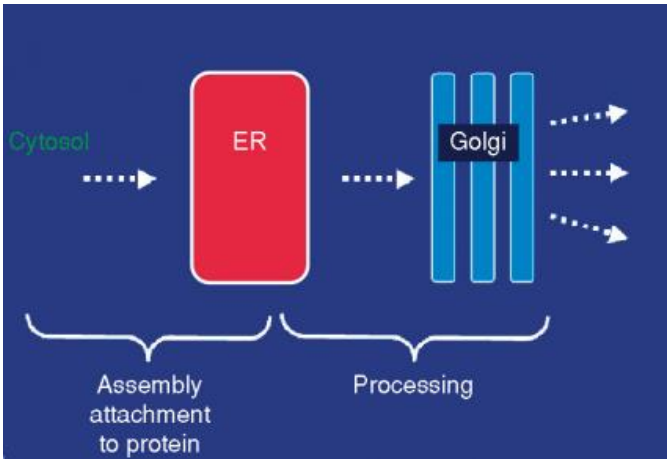



*Figure 2. 1: Glycosylation of a protein* [27]

It is possible to observe in Figure 2.1 that in the cytosol and ER occurs the assembly attachment to the protein, followed by the processing that takes place mainly in the Golgi Complex. When processing is over, all the post-translation modifications in proteins are over, defining their structure and functions.

Every living cell on the planet is covered with a dense and complex array of glycans and for that reason, it has many properties related to health [28]:

- Many diseases that affect humans involves glycans
- A significant majority of host-pathogen interactions involve glycans via recognition or degradation
- Most of therapeutic proteins must be glycosylated to be functionally effective
- Altered glycosylation is a universal feature of cancer and contributes to pathogenesis and progression
- Many vaccines are glycan based because virus has a protein capsule

Besides that, congenital disorders of glycosylation (CDG) are a fast-growing family of genetic diseases due to the defects in the synthesis of glycans [20].

## 2.2. Enzymatic synthesis of sugar nucleotides

Enzymes are biological proteins that catalyze different chemical reactions. All reactions that take place in living systems are catalyzed by enzymes. Enzyme processes replacing or supplementing chemical reaction have lowered the environmental footprint regarding energy consumption, water usage, and amount of synthetic chemicals ending up in the environment. These advantages led to more research and consequent improvement of enzyme technology [29].

### *2.2.1. Structure*

Enzymes are proteins composed by several amino acids. These amino acids are covalently bound through a peptide bond and their function is determined by their complex structure. The reaction takes place in a small part of the enzyme called the active site. The amino acids around the active site attach the substrate molecule and hold it in position while the reaction takes place. This makes the enzyme specific for one reaction only, as other molecules will not fit into the active site [29].

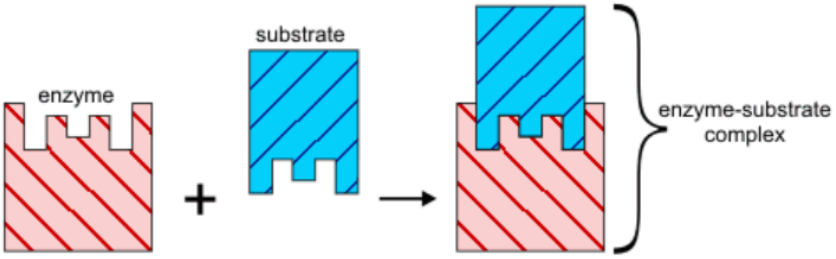



*Figure 2. 2: Model that represents how an enzyme works*

In Figure 2.2 it is possible to observe the simplest model to represent how an enzyme works. The substrate fits into the active site to form a reaction intermediate.

Many enzymes need cofactors or coenzymes to work correctly. These can be metal ions (such as $Fe^{2+}$, $Mg^{2+}$, $Cu^{2+}$) or organic molecules (such as FAD, NAD or coenzyme A). Many of these are derived from dietary vitamins, which is why they are so important. For this reason, it is vital to determine if the enzymes used are activated with co factor or with coenzymes [30].

### *2.2.2. Enzyme Assays*

When dealing with enzymes, it is imperative to determine the temperature and pH that allows them to achieve a higher yield of the final product. The best temperature needs to be determined since for high temperatures, enzymes can suffer denaturation and for very low temperatures the reaction can be prolonged, not allowing the achievement of a high yield. Regarding the pH, each enzyme has an optimal pH that maximizes the yield of production of the final product. For high or low values of pH, enzyme activity can be affected, and for that reason, the pH that promotes a higher production of the final product should be determined [31].

During the reactions, it is also necessary to add an appropriate buffer to preserve the stability of the enzymes. Buffers are used to adjust and stabilize the desired pH of the enzyme assay. They are strongly correlated with the yield of the reaction because if pH is drastically changed then enzyme activity can be decreased [32].

Regarding the buffers used in the reactions, literature usually recommends using Tris buffer (tris (hydroxymethyl) aminomethane). Biological buffers are considered non-toxic, biocompatible and environment-friendly which is the case of MOPS (3-(Nmorpholino) propane sulfonic acid) and HEPES (N-(2-hydroxyethyl) piperazine-N0-ethanesulfonic acid) [31].

Regarding the buffers available for enzyme assays, it is important to study each one of them, and their particularities, as it is possible to see in the following table 2.1 [33].

*Table 2. 1: Properties and pH range of different buffers used in literature*

| | MOPS | HEPES | BIS TRIS PROPANE | TRIS |
|---|---|---|---|---|
| **pH range** | 6.5 - 7.9 | 6.8 – 8.2 | 6.3 – 9.5 | 7 - 9 |
| **Properties** | It is recommended for use as a buffer in solutions with metal ions | It is relatively cheap, and it is biologically inert in most cases | High pH screening | The primary amine group of Tris reacts with metals ions |

Enzymes are stored in glycerol at -20°C [25], to keep protein stabilization. Storing proteins in glycerol is very common, mainly because it is shown that storing enzymes in this substance allows them to not loose stability [34]. However, it is debatable if this is the best substance to stabilize proteins. Recently was reported that sorbitol, which is also a polyhydric alcohol, is better than glycerol to preserve enzyme stability [35].

### *2.2.3. Application*

The use of biocatalysts is increasing very fast in the pharmaceutical industry. With an industry that is continuously striving to improve efficiency, a high number of studies had been conducted to increase the ability of biocatalysts to reach its full potential in pharmaceutical synthesis. In addition, such processes can deliver alternative ‘green’ solutions with reduced environmental footprints [11].

Besides pharmaceutical industry, enzymes are being used in numerous new applications in the food, agriculture, paper, leather, and textiles industries, resulting in significant cost reductions. This means that industries are embracing more and more enzyme technology, a trend strengthened by concerns regarding health, energy, raw materials, and the environment [36].

## 2.3. Enzymes used

The following subsections explain how each enzyme used in this project works and which are the substrates needed and final products obtained. It is also presented the EC number (Enzyme Commission Number) for each enzyme. This specific number consists in a classification scheme for enzymes, based on the chemical reactions they catalyze thus, allowing a properly distinction of one enzyme from another.

### *2.3.1. NahK*

Enzyme NahK, also known as N-acetylhexosamine 1-kinase (EC 2.7.1.162) uses ATP to phosphorylate substrate GlcNAc forming GlcNAc-1p and ADP. The reaction that is associated to this enzyme is shown in Equation 1.

$$ATP + GlcNAc \overset{NahK}{\longleftrightarrow} ADP + GlcNAc - 1p \qquad (1)$$

This enzyme is very important in the area of glycoscience because has a big affinity to N-acetylglucosamine which is a fundamental substrate of the process of glycosylation [37] [38].

### *2.3.2. Glk*

This enzyme is known as glucokinase (EC 2.7.1.2) and the main purpose is to use ATP to phosphorylate Glc, forming Glc-6p [39].

$$ATP + Glc \overset{Glk}{\leftrightarrow} ADP + Glc - 6p \qquad (2)$$

Also, this enzyme also phosphorylates mannose to form Man-6p, as shown in Equation 3.

$$ATP + Man \overset{Glk}{\leftrightarrow} ADP + Man - 6p \qquad (3)$$

### 2.3.3. Man B/C

In the case of glucose and mannose, enzyme Glk forms Glc – 6p and Man – 6p, respectively. However, to obtain the final product, it is necessary to acquire Glc and Man phosphorylated in the first carbon, in the form of Glc-1p and Man-1p. For that reason, enzyme Man B/C is used. This enzyme is co-expressed by phosphomannomutase (ManB) (EC 5.4.2.8), and mannose-1-phosphate-guanyltransferase (ManC) (EC 2.7.7.13) in *E.coli* using one plasmid [40].

The reactions this enzyme promotes are the following [41]:

$$Glc - 6p \overset{Man\ B/C}{\longleftrightarrow} Glc - 1p \quad (4)$$

$$Man - 6p \overset{Man\ B/C}{\longleftrightarrow} Man - 1p \quad (5)$$

### 2.3.4. URA6

Uridine monophosphate kinase, URA6 (EC 2.7.4.22) forms UDP through UMP. It removes a phosphor of ATP forming ADP. The phosphor removed is attached to UMP forming UDP, as shown in Equation 6 [42].

$$ATP + UMP \overset{URA6}{\longleftrightarrow} ADP + UDP \quad (6)$$

### 2.3.5. PPK3

Enzyme polyphosphate kinase (EC 2.7.4.1) phosphorylates ATP forming ADP consuming phosphate [43].

$$ATP + PolyP_n \overset{PPK3}{\longleftrightarrow} ADP + PolyP_{n+1} \quad (7)$$

### 2.3.6. GalU

UTP-glucose-1-phosphate uridylyltransferase (EC 2.7.7.9) is the most important enzyme of the system, since it produces the final product. Like the name already says, the enzyme uses as substrates UTP and Glc-1p to produce the final product. The reactions are shown in the following reactions [44]:

$$UTP + Glc - 1p \overset{GalU}{\longleftrightarrow} UDP - Glc + PP_i \quad (8)$$

$$UTP + GlcNAc - 1p \overset{GalU}{\longleftrightarrow} UDP - GlcNAc + PP_i \quad (9)$$

$$UTP + Man - 1p \overset{GalU}{\longleftrightarrow} UDP - Man + PP_i \quad (10)$$

### *2.3.7. PmPpA*

PmPpA, also known as inorganic diphosphatase (EC 3.6.1.1) and it is used to shift the equilibrium of the reaction of GalU to the direct way in order to increase the yield of the final product [45].

$$PP_i + H_2O \overset{PmPpA}{\longleftrightarrow} 2P_i \quad (11)$$

### *2.3.8. 2D-PPK2*

This enzyme, 2-domain-polyphosphate kinase (EC 2.7.4.1), is known to have affinity to AMP, GMP, CMP and UMP in this order. For this reason, the AMP that is formed in the reaction is consumed by this enzyme, forming ADP and ATP. The same happens with UMP, forming UDP and UTP but in lower affinity, since this enzyme has a higher affinity to AMP [43] [46].

The following reactions show how this enzyme processes AMP:

$$AMP + PolyP_n \overset{2D-PPK2}{\longleftrightarrow} ADP + PolyP_{n-1} \quad (12)$$

$$ADP + PolyP_n \overset{2D-PPK2}{\longleftrightarrow} ATP + PolyP_{n-1} \quad (13)$$

In a very similar way as AMP, also UMP is consumed by this enzyme being phosphorylated by polyphosphate forming UDP and UTP.

$$UMP + PolyP_n \overset{2D-PPK2}{\longleftrightarrow} UDP + PolyP_{n-1} \quad (14)$$

$$UDP + PolyP_n \overset{2D-PPK2}{\longleftrightarrow} UTP + PolyP_{n-1} \quad (15)$$

## 2.4. High Throughput Screening

As it was said before, there are many variables that need to be determined when an enzyme cascade is being established and optimized. High throughput screening (HTS) is a valuable tool to handle such complex systems involving many different variables with interactions. It involves recent scientific methods relevant to the field of chemistry and biology, in which hundreds of experimental samples are subjected to simultaneous testing under given conditions. This allows not only a faster sample reading but also an easier way to test different conditions instead of the regular way [47].

For that reason, a technique was implemented to analyze the amount of product in each sample, which was the Phosphate Assay test ($P_i$ Color Lock System). This test measures the amount of phosphate in a microplate through a colorimetric assay [48].

When the dye is added to the samples, forms a $P_i$-dye complex turning into a blue color, which is more intense when there is a high concentration of phosphate in the sample. Considering the quantity of phosphate measured in the samples, and the chemical reactions that take place, the obtained concentration of phosphate will be twice the value of the product obtained in the sample, based on the following chemical Equation 16:

$$UTP + Man - 1p \overset{GalU}{\longleftrightarrow} UDP - Man + PP_i \overset{PmPpA}{\longleftrightarrow} 2P_i \qquad (16)$$

Essentially, with this indirect method, it is possible to have a fast, quantitate value of the concentration of the final product in each sample that is taken from the system.

# 3. Materials and Methods

## 3.1. Choice of Enzymes

The enzymes used on this project were based on the article previously reported in literature for production of UDP-GlcNAc [25]. However, to produce sugar nucleotides UDP-Man and UDP-Glc data base website BRENDA [49] was used in order to find enzymes needed to create the functional enzymatic cascades. With research, the appropriated enzymes were found, and a series of experiments were conducted in order to evaluate if the cascade worked. In addition, the amount of enzymes and substrates that were necessary to make the cascade functional were also determined with a series of experiments of trial and error.

## 3.2. Protein Expression

This part of the project was made by laboratory technical assistant, whose work was to generate a specific DNA sequence in E. Coli that can be multiplied with the right medium and conditions, increasing the amount of protein generated. The DNA sequence generated specifies a string of six histidine residues that is used in the vectors for the production of recombinant proteins. The result is an expression of a recombinant protein with six histidine's that can be easily purified due to the string of histidine that binds to immobilized metal ions, as nickel.

## 3.3. Cell Lysis

Since the proteins of interest are inside the cells, it is necessary to do cell lysis using sonification which is a technique that uses mechanical forces in the form of ultrasonic sound waves to shear cell tissues. The sound waves provide cell rupture and release protein do the mixture. However, they can also release other proteins that can inhibit our protein of interest. For that reason, it is very important to purify them.

To perform cell lysis on cells, 5 mL of lysis buffer was added do the sample of biomass followed by vortex to lift the protein from the bottom. Lysis buffer was prepared with 50 mM HEPES at pH 7.5, 10 mM of Imidazole, 10 mM of MgCl2 and 300 mM of NaCl.

Then, the samples were placed in ice and using digital sonifier BRANSON it is started pulses of 0.5 seconds with pause every 2.5 seconds for 3 minutes. In the end, samples are centrifuged for 20 minutes at 16 000 g [40].

## 3.4. Enzyme Purification

For purposes of enzyme purification, it was used IMAC (immobilized metal ion affinity chromatography*),* connected to a computer with the software UNICORN. To use this technique, several buffers needed to be prepared:

- Elution buffer was made with 50 mM HEPES at pH 7.5, 500 mM of Imidazole, 10 mM of $MgCl_2$ and 300 mM of NaCl
- Equilibration buffer was made with 50 mM HEPES at pH 7.5, 10 mM of Imidazole, 10 mM of $MgCl_2$ and 300 mM of NaCl (same as lysis buffer previously used)

Firstly, all system needed to be washed with 20% ethanol followed by a wash with equilibration buffer with the flow of 5 mL/min. When it was finished, the flow rate was set to 0.5 mL/min.

His-Trap HP Column (1ml capacity) was prepared by adding droplets of water into the column until it is full to prevent bubbles. Then, water was injected through the column followed with equilibration buffer to wash out unbound proteins.

Samples were loaded, and the program started. The protein of interest is released when it is used elution with a buffer containing high concentration of imidazole that has strength enough to take the protein of interest out of the column. When separation is finished, the system was manually cleaned at 1 ml/min with water and then with cleaning agent and again with water. Finally, the whole system was cleaned with 20% ethanol [10].

By using this type of purification, the proteins generated with a six-histidine terminal bind to the nickel column while the other proteins are released from the column.

## 3.5. Enzyme Quantification

For enzyme quantification Bradford method was adopted. The main principle of this assay is that the binding of protein molecules to dye results in a color change from brown to blue.

To obtain the concentration of protein, calibration curves needed to be obtained. For that reason, a solution of 100 mg of BSA and 100 g of PBS was made in 250 mL. In the end, the solution was stirred for 5 minutes.

Then, in a 96 well plate, the solution prepared for the calibration curve was added in each well in triplicates (200 µL). In addition, it was also placed 200 µL of the samples containing the enzymes in triplicates. It is necessary to do a series of dilutions (always in triplicates) of the samples with enzymes to assure that obtained value falls within the calibration range.

After that, 50 µL of dye Bio-Rad was added to each well. In the end the plate was read in the plate reader at 595nm.

## 3.6. Reactions

For the reaction of the enzymes, it is necessary four essential things:

- Buffer (HEPES)

- Co-factor (usually $MgCl_2$)

- Reactants

- Enzymes

All reactions were carried out with a co-factor concentration of 30 mM MgCl2 at 30°C in 50 mM HEPES buffer at pH 7.5. The reaction was incubated at 1000 rpm in a thermomixer from Eppendorf AG. Sample aliquots of 25 µl were quenched in 400–1500 µl of MilliQ water, preheated in a closed Eppendorf tube to 90°C, followed by another 3 min of heating at 90°C to ensure enzyme inactivation. In the end, all samples are centrifuged at 12 000 g for 20 minutes to remove proteins.

## 3.7. Quantification of compounds

To quantify the compounds of the reaction High Performance Anion Exchange Chromatography (HPAEC) was used which separates compounds on an anion exchange column. The machine used was an IC5000 thermo and a column carbopac PA1. With an optimized gradient all the compounds appear as peaks and all measurements for quantification of analytes were based on UV detection. An example of the chromatogram is exposed in Figure 3.1.

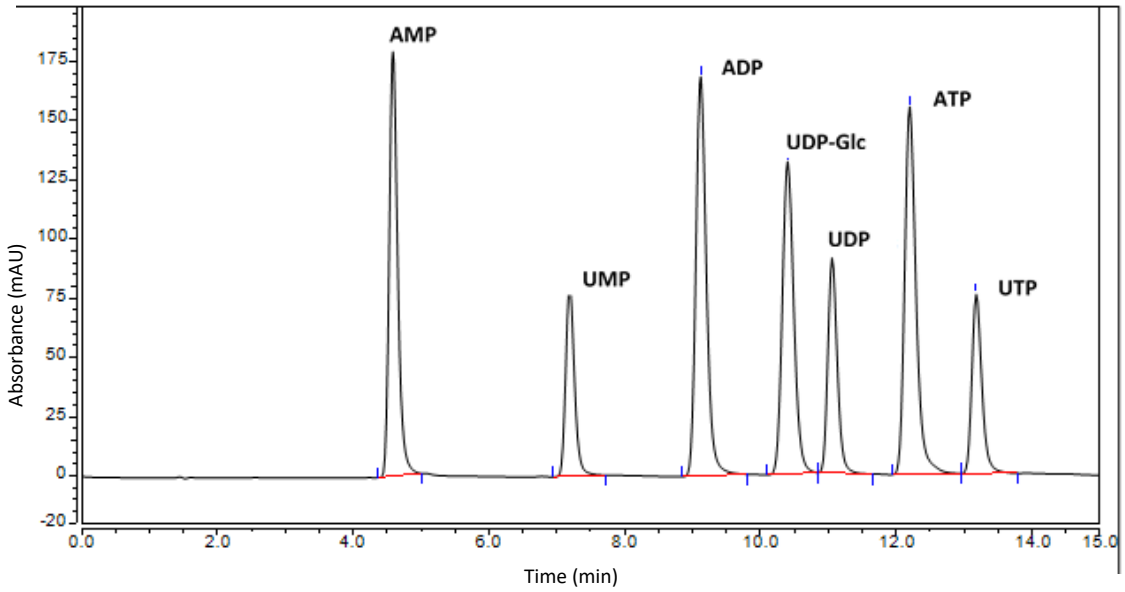



*Figure 3. 1: Example of a chromatogram using HPAEC*

To run the samples in chromatography, they were collocated in special vials appropriate for the machine and 4 eluents (A-water, B-1 mM NaOH and 1M NaOAc, C-100 mM NaOH and D-1M NaOAc).

Regarding Phosphate assay test, it includes a commercially available kit that contains a Golden mix to detect phosphate and a stabilizer to stabilize phosphate formation. In a 384 well plate, 50 µL of Golden mix are added to 70 µL of samples. After 5 minutes, 20 µL of stabilizer needs to be added and the solution is mixed with a pipette. Afterwards the plate is shaken at 1000 rpm for half an hour and is inserted in a spectrophotometer read at 635nm.

# 4. Results and Discussion

In this chapter, experimental work and obtained results will be discussed. In section 4.1. the establishment of the cascades to produce sugar nucleotides UDP-Man, UDP-Glc and UDP-GlcNAc is presented with an analysis of intermediary compounds. In section 4.2. it is presented a series of experiments and analysis that improved the cascade reactions to produce the sugar nucleotides. In order to improve the productivity of the cascade, a few issues were found when performing reactions. For that reason, section 4.3. focus on solving and analyzing the problems found with the purpose to develop and improve the reactions. In the end, because a high number of experiments still need to be performed to establish and improve the reactions to be prepared for the scale up, a high throughput screening test was studied to be implemented for these reactions (section 4.4). It is important to mention that mass balance was made for all reactions to reassure the reliability of the system. It has taken into consideration the conservation of the amounts of adenosine and uridine in the system.

## 4.1. Established Cascades

A significant step regarding the production of sugar nucleotides is the cascade establishment. It includes not only the choice of the enzymes used, but also the concentrations of enzymes and substrate that it is possible to be processed to create a functional cascade. In this thesis, three sugar nucleotides were successfully synthetized: UDP-Mannose, UDP-Glucose and UDP-GlcNAc.

All concentrations for the final product and intermediary compounds were calculated using a calibration curve for each compound (except UDP-Man which commercial standard is not available). For each analysis made in chromatography, the calibration curve for each substance was determined in the same run in order to avoid the possible biased error of the HPAEC.

### *4.1.1. UDP-Man Cascade*

UDP-Man Cascade has ATP, UMP, $PolyP_{14}$ and mannose as substrates. The enzymes in this cascade are shown in Figure 4.1

.

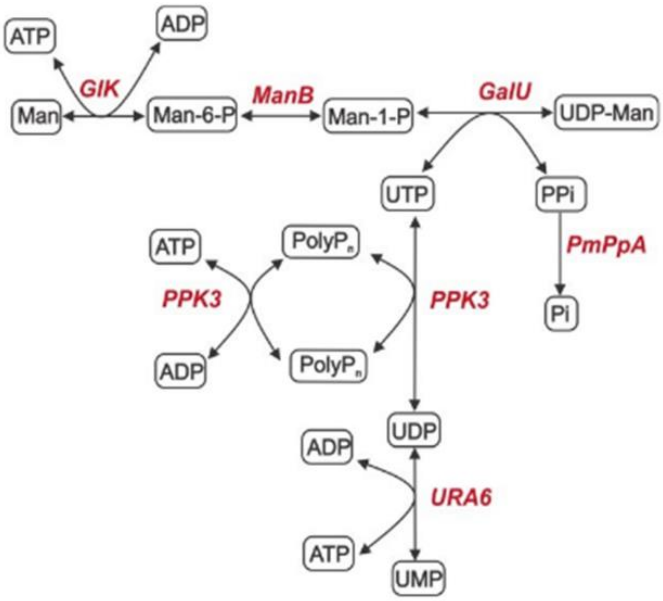



*Figure 4. 1: UDP-Man Cascade*

By the analysis of Figure 4.1 it is possible to observe that enzyme Glk converts Man into Man-6p. However, for the reaction to take place and form UDP-Man it is necessary to add enzyme Man B/C to convert Man-6p in Man-1p, which is the mandatory substrate for enzyme GalU to produce the final product.

Enzyme URA6 has the role of using ATP to form UDP. On the other hand, PPK3 converts ATP in ADP, as $PolyP_{14}$ into $PolyP_{13}$, phosphorylating UDP to produce UTP that in the end is used to produce the final product by enzyme GalU. For this reason, both enzymes, URA6 and PPK3, are crucial to transform UMP into UTP.

Experiments to establish the cascade of UDP-Man were made in the following conditions (Table 4.1).

*Table 4. 1: Conditions for UDP-Man cascade*

| | Glk | GalU | PPK3 | URA6 | PmPpA | Man B/C |
|---|---|---|---|---|---|---|
| **Enzymes concentrations (mg/mL)** | 0.5 | 0.1 | 0.1 | 0.1 | 0.05 | 0.2 |
| | **Mannose** | **UMP** | | **ATP** | | **PolyPn** |
| **Initial concentrations (mM)** | 2 | 2 | | 1.5 | | 4.5 |
| | **$MgCl_2$** | | | **HEPES** | | |
| **Other Concentrations (mM)** | 20 | | | 50 | | |
| | **Temperature (°C)** | | | **Shaking (rpm)** | | |
| **Conditions of the reaction** | 30 | | | 1000 | | |

The analysis of the concentration of substrates, product and intermediary compounds during the reaction for UDP-Man cascade is exposed in Figures 4.2 and 4.3.

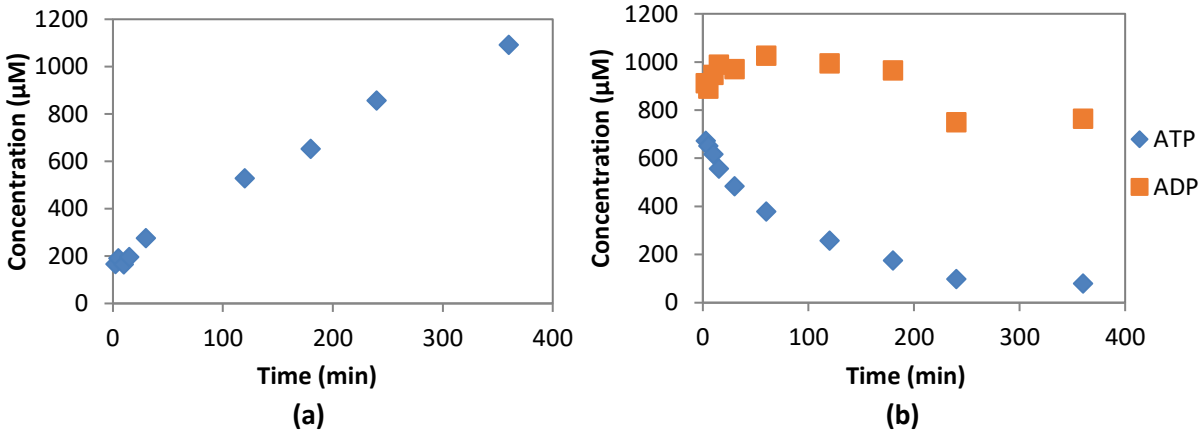



*Figure 4. 2: Results of AMP, ADP and ATP for UDP-Man cascade. (a) Concentration of AMP vs Time. (b) Concentration of ADP and ATP vs Time.*

It is possible to observe in Figure 4.2A that AMP concentration increases with time due to the fact that ADP and ATP can have their phosphate bond hydrolyzed forming AMP [50]. As shown in Figure 4.2B, ATP decreases along time because it is being consumed by enzymes PPK3 and Glk to produce the final product. ADP concentration increases in the beginning of the cascade reaction as ATP is consumed and turned into ADP by PPK3 but then it reaches equilibrium, which means that the rate of consumption of ADP is the same rate as formation.

In Figure 4.3 is presented the concentration of UDP over time.

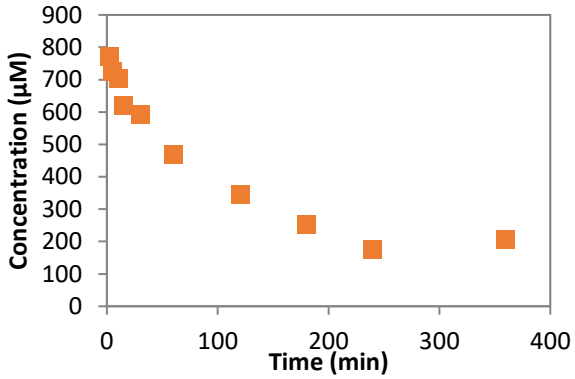



*Figure 4. 3: Concentration of UDP vs Time for UDP-Man Cascade*

It is possible to conclude from the analysis of Figure 4.3 that the intermediary product, UDP, is produced very fast when reaction starts. However, the concentration values start to decrease very quickly due to enzyme PPK3 that phosphorylates UDP by using ATP to form UTP.

The concentration of UDP-Man was not evaluated due to the fact that this substance is not commercially available.

### *4.1.2. UDP-Glc Cascade*

Regarding UDP-Glc cascade, it uses the same biocatalysts as in the UDP-Man cascade. Enzyme Glk converts glucose in Glc-6p and, once again, for the reaction to take place and form UDP-Glc it is necessary to add enzyme Man B/C to convert Glc-6p in Glc-1p, which is the mandatory substrate for enzyme GalU to produce the final product.

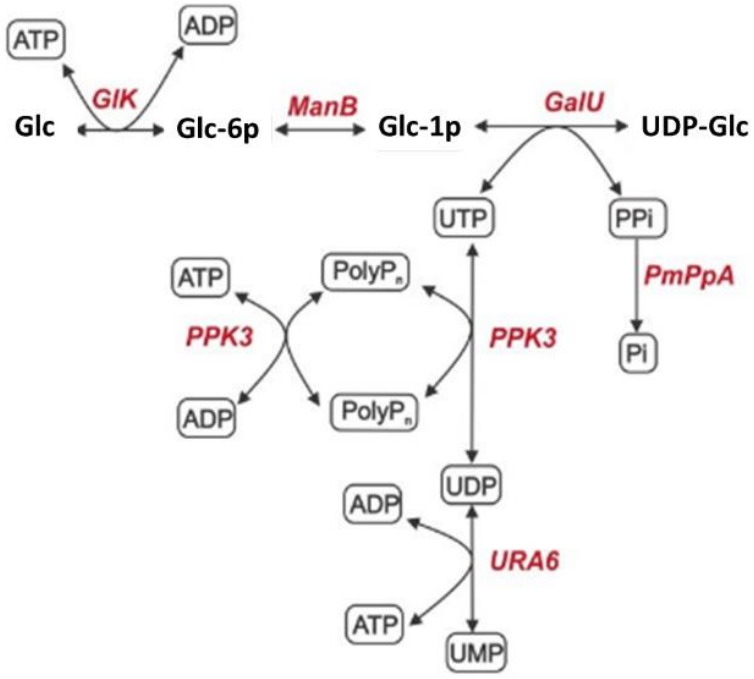



*Figure 4. 4: UDP-Glc Cascade*

The UDP-Glc cascade was established and the concentrations of enzymes and reactants that made the cascade functional were determined and are shown in Table 4.2.

*Table 4. 2:Conditions for UDP-Glc cascade*

| | Glk | GalU | PPK3 | URA6 | PmPpA | Man B/C |
|---|---|---|---|---|---|---|
| **Enzymes concentrations (mg/mL)** | 0.5 | 0.1 | 0.1 | 0.1 | 0.05 | 0.2 |
| | **Glc** | **UMP** | | **ATP** | | **PolyPn** |
| **Initial concentrations (mM)** | 1 | 1 | | 3 | | 3 |
| | **$MgCl_2$** | | | **HEPES** | | |
| **Other Concentrations (mM)** | 30 | | | 50 | | |
| | **Temperature (°C)** | | | **Shaking (rpm)** | | |
| **Conditions of the reaction** | 30 | | | 1000 | | |

The analysis of each reactant of this cascade is exposed in Figure 4.5.

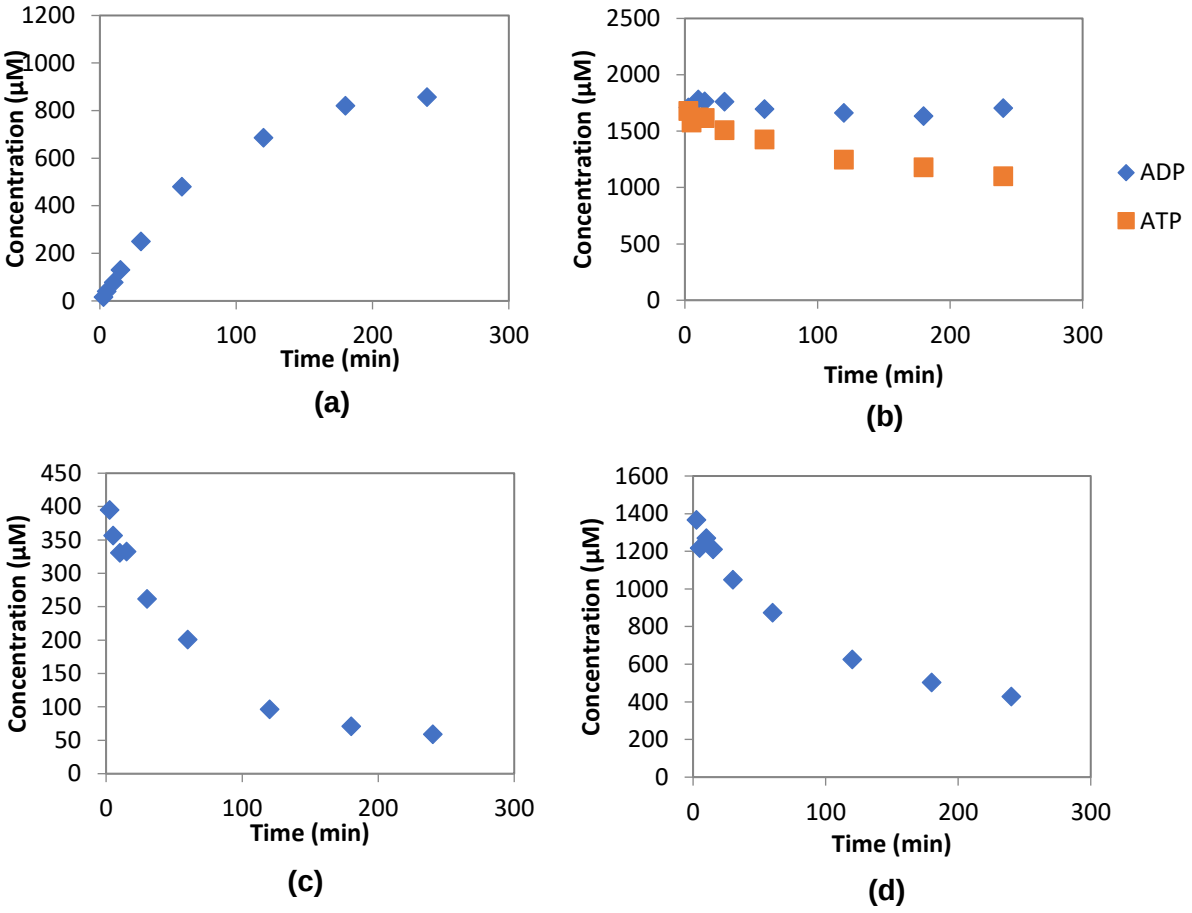



*Figure 4. 5: Results of AMP, ADP, ATP, UDP and Glc-6p for UDP-Glc cascade. (a) Concentration of AMP vs Time. (b) Concentration of ATP and ADP vs Time. (c) Concentration of UDP vs Time. (d) Concentration of Glc-6p vs Time*

Figures 4.5A and 4.5B show that, as in UDP-Man cascade, the concentration of AMP increases with time. Concentration of ADP increases in the beginning while concentration of ATP decreases. This happens because ATP is consumed in the beginning, forming ADP. But very fast both compounds reach equilibrium.

In Figure 4.5C UDP is consumed, meaning that enzyme PPK3 is efficiently consuming the substrate UDP, in order to form the main substrate for enzyme GalU, UTP.

Regarding Glc-6p concentration (Figure 4.5D) it decreases along time meaning that enzyme Man B/C is working very well, consuming Glc-6p and forming Glc-1p that will be used by enzyme GalU to produce the final sugar nucleotide, UDP-Glc.

In Figure 4.7 it is possible to observe the behavior of UDP-Glc and UMP during the reaction.

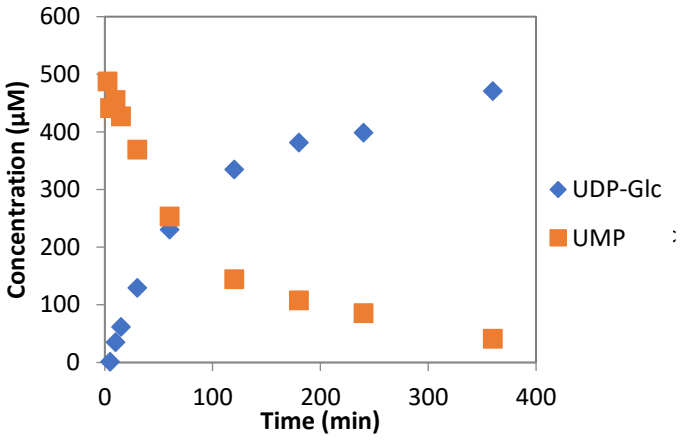



*Figure 4. 7: Concentration of UMP and UDP-Glc vs Time*

It is possible to observe in the previous figure that, while UMP is consumed, the product UDP-Glc is produced as expected. Since UMP is one of the main substrates for the cascade reaction to work it is expected that the concentration decreases which means that this substrate is consumed in high quantity, resulting in a formation of high concentrations of product.

For this cascade, the productivity obtained had the value of 4.24 $\frac{mg}{h \cdot g_{protein}}$.

### *4.1.3. UDP-GlcNAc Cascade*

About UDP-GlcNAc cascade (Figure 4.8), it is not necessary to have enzyme Man B/C because Nahk converts instantly GlcNAc into GlcNAc-1p.

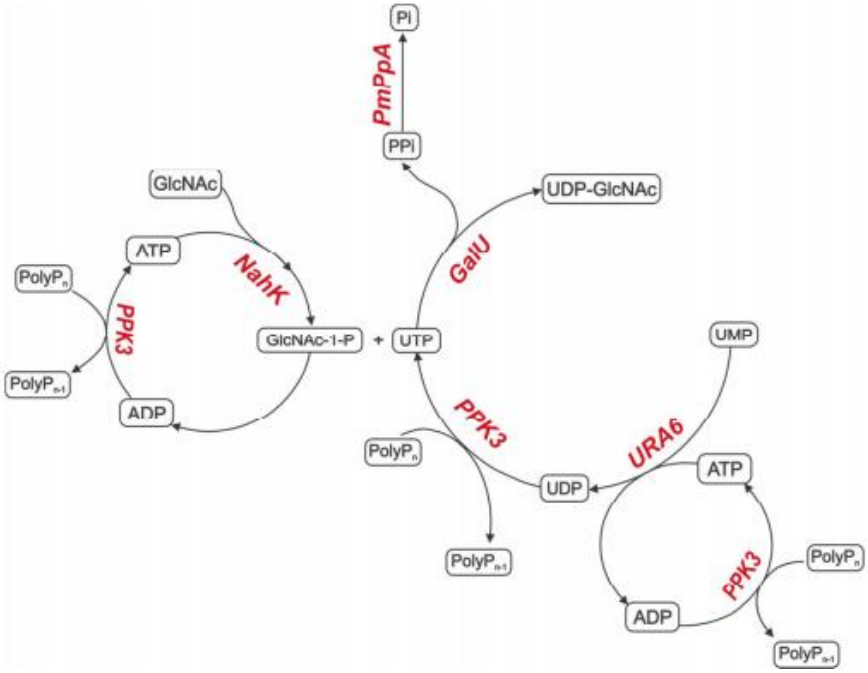



*Figure 4. 8: UDP-GlcNAc Cascade [25]*

The concentrations of enzymes and reactants that can make the cascade functional were determined and are shown in Table 4.3.

*Table 4. 3: Conditions for UDP-GlcNAc cascade*

| | **NahK** | **GalU** | **PPK3** | **URA6** | **PmPpA** |
|---|---|---|---|---|---|
| **Enzymes concentrations (mg/mL)** | 0.3 | 0.2 | 0.1 | 0.1 | 0.05 |
| | **GlcNAc** | **UMP** | | **ATP** | **PolyPn** |
| **Initial concentrations (mM)** | 2 | 2 | | 2 | 4 |
| | **$MgCl_2$** | | | **HEPES** | |
| **Other Concentrations (mM)** | 20 | | | 50 | |
| | **Temperature (°C)** | | | **Shaking (rpm)** | |
| **Conditions of the reaction** | 30 | | | 1000 | |

The results for the behavior of the different compounds are shown in Figure 4.9.

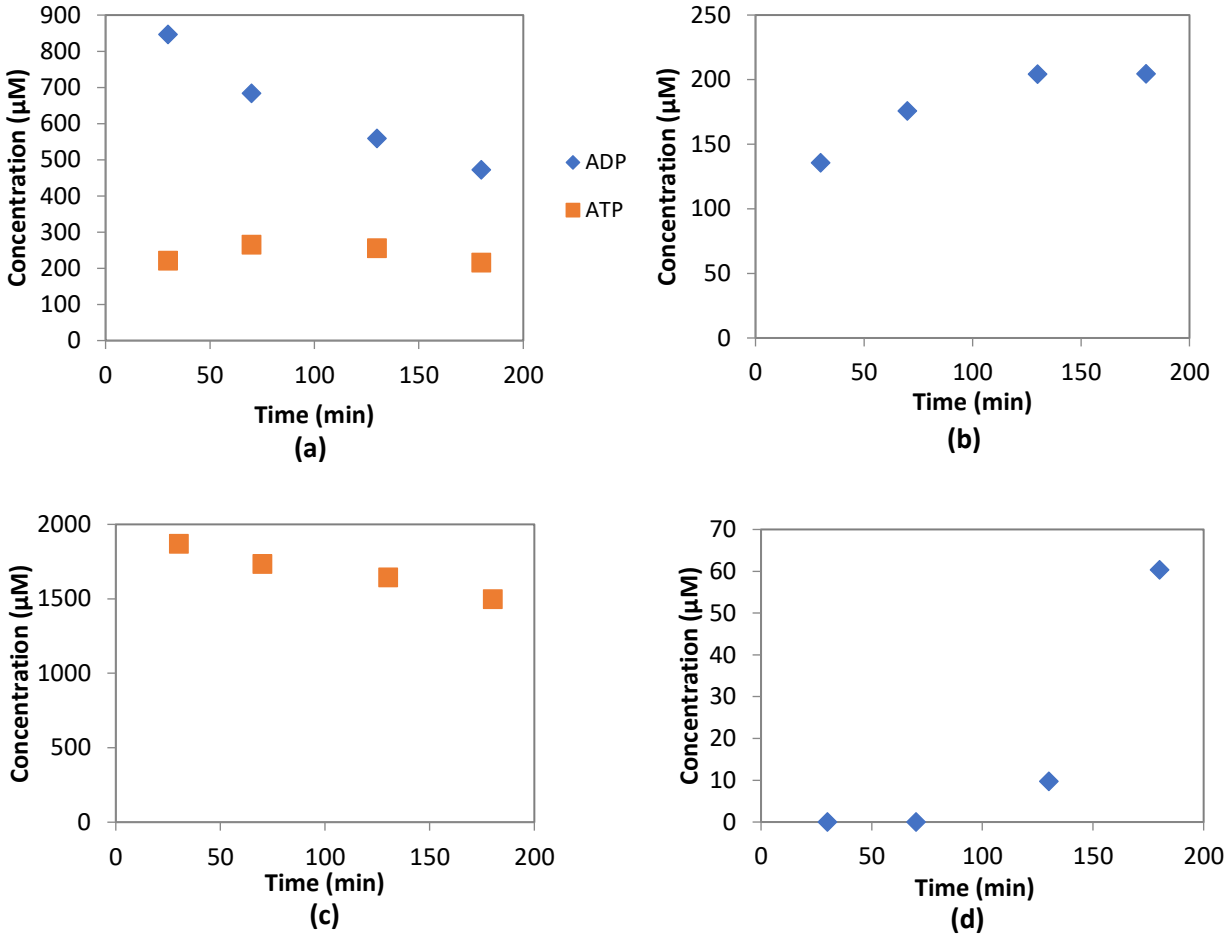



*Figure 4. 9: Results of ATP, ADP UMP, UDP-GlcNAc and UDP for UDP-GlcNAc cascade. (a) Concentration of ADP and ATP vs Time. (b) Concentration of UDP vs Time. (c) Concentration of UMP vs Time. (d)Concentration of UDP-GlcNAc vs Time.*

From Figure 4.9A, it is possible to observe that at the beginning of the reaction, while a significant amount of ATP is consumed, a high amount of ADP if formed, due to enzyme PPK3. As it happened in the previous cascades, in the end both compounds tend to reach equilibrium.

UMP, which is one of the main substrates for the cascade reaction, is being consumed very slowly, as it is possible to see in Figure 4.9C. For that reason, there is not a very high production of the main product, however, there is production of UDP-GlcNAc starting at 130 minutes, meaning that the cascade was established and works appropriately (Figure 4.9D).

Figure 4.9B shows that, opposite to the analysis made in the previous cascades, UDP increases over time. This means that in the first 130 minutes, the UMP consumed formed UDP, however, UDP did not form UTP for enzyme GalU, ending up being accumulated. After 130 minutes, UDP reaches

equilibrium which means that the amount of UDP consumed is the same as the UDP produced, having formation of UDP-GlcNAc.

The productivity obtained for this cascade was 1.48 $\frac{mg}{h \cdot g_{protein}}$.

## 4.2. Advanced Development of Sugar Nucleotide Cascade

After cascade reactions are established regarding enzymes and substrate concentrations, it is important to establish other conditions as best pH, temperature and buffer to be used in the reactions.

Rules for performing enzymes reactions, appropriate handling, preparation of assay, choice of the assay time and suggestions to avoid frequent and trivial errors need to be investigated. Only after the determination of this important aspects, it is possible to improve cascade reactions to produce sugar nucleotides.

In subsection 4.2.1. the best pH that can promote a higher formation of the final product was determined.

In subsection 4.2.2, the best buffer to use in enzymatic reactions is chosen. The choice of the best buffer is made based on the yield of the final product, showing that the buffer that reaches higher productivity can control pH shifts better not affecting enzyme activity.

All these factors together can contribute to higher productivity of the sugar nucleotide, maintaining enzyme activity and improving the cascade reactions.

### *4.2.1. Best pH for UDP-Glc Cascade*

Experiments were conducted for UDP-Glc cascade at different pH to determine the pH that allows a higher production of the sugar nucleotide UDP-Glc. The experimental design is shown in Table 4.4.

Table 4. 4: Conditions for different pH of UDP-Glc cascade

| | Glk | GalU | PPK3 | URA6 | PmPpA | Man B/C |
|---|---|---|---|---|---|---|
| **Enzymes concentrations (mg/mL)** | 0.5 | 0.1 | 0.1 | 0.1 | 0.05 | 0.2 |
| | **Glc** | **UMP** | | **ATP** | | **PolyPn** |
| **Initial concentrations (mM)** | 1 | 1 | | 3 | | 3 |
| | **$MgCl_2$** | | | **HEPES** | | |
| **Other Concentrations (mM)** | 30 | | | 50 | | |
| | **Temperature (°C)** | | | **Shaking (rpm)** | | |
| **Conditions of the reaction** | 30 | | | 1000 | | |

The results are shown in Figure 4.10.

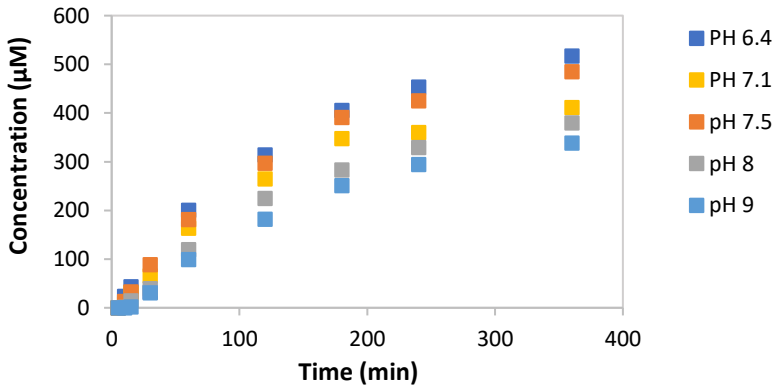



*Figure 4. 10: Concentration of UDP-Glc vs Time at different pH*

Analyzing Figure 4.10, it is possible to observe that for pH 6.4 and 7.5 there is higher productivity, meaning that this two pH's are adapted to do the reactions and enzymes are not affected at these pH values. However, because 7.5 is a neutral pH and it is this value that is mainly used in the literature [31], it was this pH that was chosen to do all the reactions for this cascade.

It is also possible to see that, at pH 9, enzymes do not have high productivity at alkaline environments, not producing that much final product as they could if the reaction was made at the advised pH in literature. This leads to believe that at pH's 7.1, 8 and 9 enzyme activity can be affected.

### *4.2.2. Different Buffers*

A series of experiments were made to evaluate different buffer effect in the reactions. The buffers were chosen based on the literature [51]. Since buffer MOPS, HEPES and tris/HCl are the buffers mainly used in enzymatic reactions they were the ones that were evaluated, together with bis-tris-propane buffer that has a large buffer capacity.

The results for the effect of different pH are exposed in the following Figure 4.11 based on the conditions of Table 4.3.

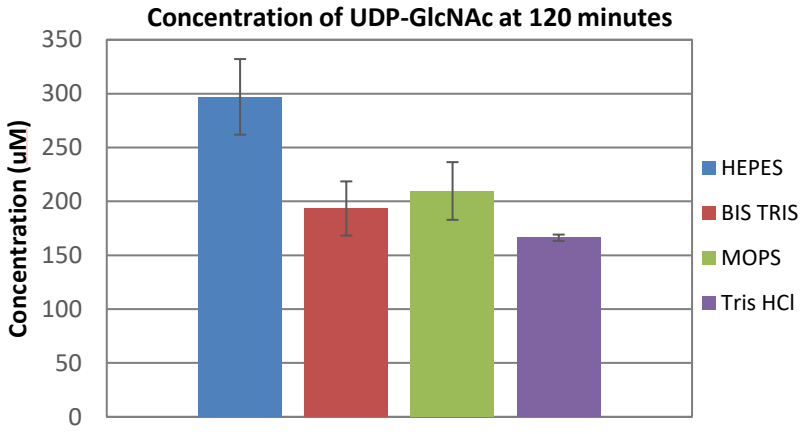



*Figure 4. 11: Concentration of UDP-GlcNAc at 120 minutes with different buffers*

The best buffer to perform the reactions is HEPES because it has higher productivity of final product than the other buffers improving cascade reactions. For that reason, all the experiments made after this were using HEPES buffer instead of the previous used Tris/HCl buffer.

## 4.3. Small Scale Experiments

One of the main goals to develop the process is to do the scale up to allow the industrial application of the final product. For that reason, it is important to do small scale experiments to investigate what type of problems can the process have and solve them. This way it is possible to get a better understanding of the process and better preparation for the scale up.

One of the problems that appeared during the reactions was the sub product production of AMP. This sub product is formed due to hydrolyzation of the phosphate bond of ADP and ATP which are present in the reactions. In Figure 4.12 it is possible to observe the high concentration of AMP reached during the reactions.

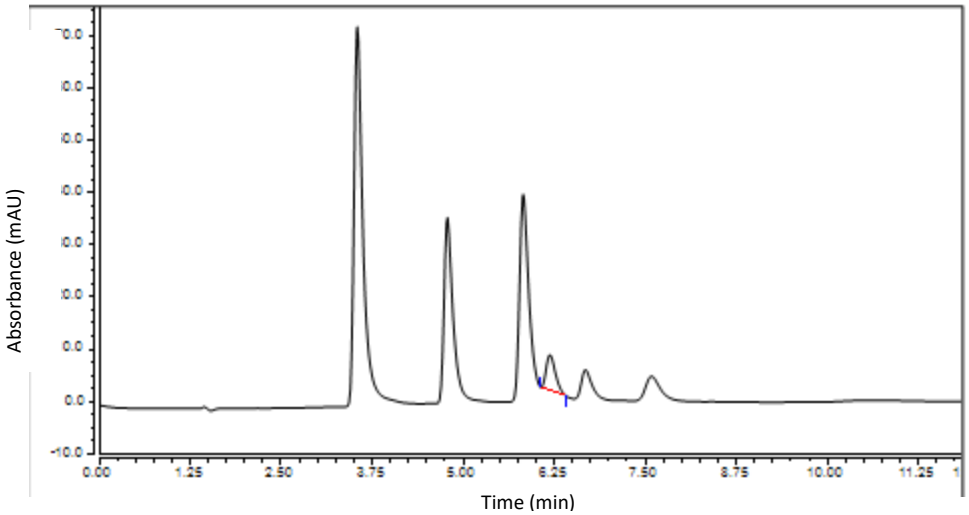



*Figure 4. 12: Chromatogram from a cascade reaction that shows the amount of AMP formed*

As it is possible to observe in the previous figure, high amounts of AMP were being formed during the reactions. This can lead to problems not only in the reaction, decreasing the productivity of the system but also in the purification process. Thus, an attempt to decrease the formation of this sub product was made.

In addition, the influence of high amounts of substrate on the reaction vial was analyzed. On the other hand, the influence of large quantities of product inside vials was also pursuit. Both these sets of experiments were made to observe how significant quantities of product or substrate can affect the reaction giving important information about the future design of the reactor.

In the end, enzyme stability was studied in the sense that when scale up is done it is very important to keep enzyme stability and productivity stable.

### *4.3.1. AMP formation*

After analyzing a high number of preliminary experiments, it was seen that high amounts of AMP were produced. This fact led to do a series of experiments to see how high amounts of substrates can lead to a more prominent formation of AMP.

The experiments were conducted with different concentrations of reactants and without any enzymes, to see how higher concentrations can affect the formation of this intermediary product. The experimental plan is shown in Table 4.5.

*Table 4. 5: Experimental plan to investigate the formation of AMP*

| | | UMP | UTP | UDP | ATP | ADP |
|---|---|---|---|---|---|---|
| **Initial concentrations (mM)** | **Experiment 1** | 1 | 1 | 1 | 1 | 1 |
| | **Experiment 2** | 2 | 2 | 2 | 2 | 2 |
| | **Experiment 3** | 3 | 3 | 3 | 3 | 3 |
| | **Temperature (°C)** | | | **Shaking (rpm)** | | |
| **Conditions of the reaction** | 30 | | | 900 | | |

The results for the analysis of AMP formation are shown in Figure 4.13.

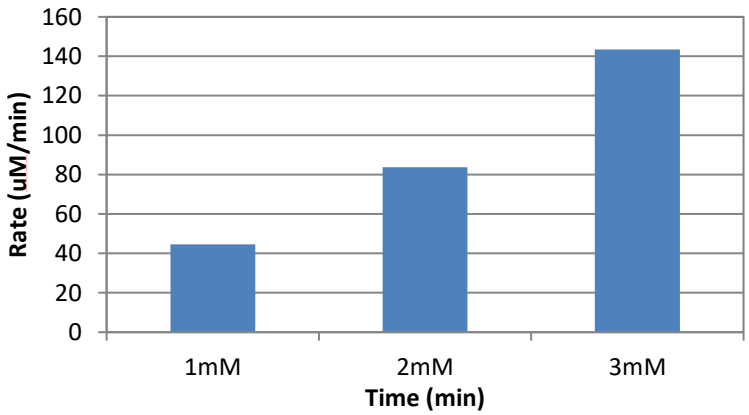



*Figure 4. 13: AMP rate of formation (µM/min)*

From the previous figure is possible to conclude that the higher concentrations of reactants, higher is the rate of formation of AMP. The possible explanation for this is the fact that when reactants concentration increases, the hydrolyzation of phosphate bounds in ADP and ATP also increases. This is of concern potentially leading to serious scale-up problems due to the fact that results show that ATP will hydrolyze very fast. For that reason, it is reinforced that measures should be taken to decrease the concentration of this compound.

### *4.3.2. Enzyme 2D-PPK2 experiments*

To reduce the accumulation of AMP, enzyme 2D-PPK2 was added to the cascade since this enzyme is known to catalyze AMP forming ATP and ADP [46].

The experimental plan is shown in Table 4.6.

*Table 4. 6: Experimental plan for UDP-GlcNAc cascade with and without 2D-PPK2*

| | **NahK** | **GalU** | **PPK3** | **URA6** | **PmPpA** | **2D-PPK2** |
|---|---|---|---|---|---|---|
| **Enzymes concentrations (mg/mL)** | 0.3 | 0.2 | 0.1 | 0.1 | 0.05 | 0.5 |
| | **GlcNAc** | **UMP** | | **ATP** | | **PolyPn** |
| **Initial concentrations (mM)** | 2 | 2 | | 2 | | 4 |
| | **$MgCl_2$** | | | **HEPES** | | |
| **Other Concentrations (mM)** | 20 | | | 50 | | |
| | **Temperature (°C)** | | | **Shaking (rpm)** | | |
| **Conditions of the reaction** | 30 | | | 1000 | | |

The results for the addition of enzyme 2D-PPK2 to the UDP-GlcNAc cascade are shown in Figures 4.14 and 4.15.

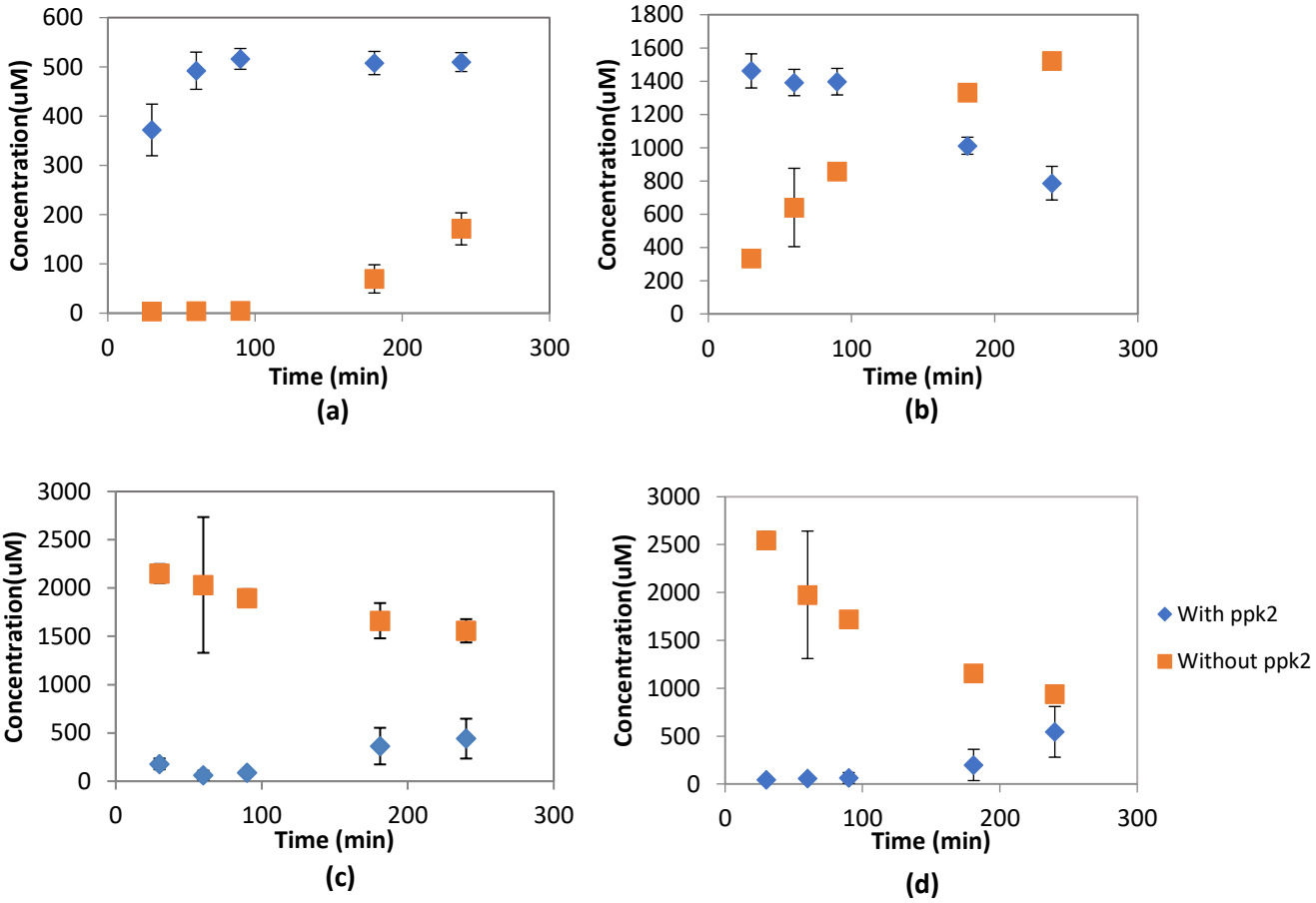



*Figure 4. 14: Experiments with and without enzyme 2D-PPK2 for UDP-GlcNAc Cascade. (a)Concentration of UDP-GlcNAc vs Time. (b) Concentration of AMP vs Time. (c) Concentration of UMP vs Time. (d)Concentration of ADP vs Time.*

By analyzing Figure 4.14A, it is possible to conclude that when enzyme 2D-PPK2 is added to the cascade there is a high increase of the product when compared to the cascade without 2D-PPK2 (increase of 56%). Faced to values showed in literature for the volumetric productivity obtained of UDP-GlcNAc (0.81 g $L^{-1}h^{-1}$) [25], the value obtained with enzyme 2D-PPK2 in this project was 18.74 g $L^{-1}h^{-1}$ which is a considerable higher productivity. However, it is important to mention that the values compared don't have the same concentration of reactants.

In Figure 4.14C it is possible to observe that UMP concentration has a much more severe decrease when enzyme 2D-PPK2 is added to the system. Because UMP is much faster consumed, there is much more production of the final product.

AMP concentration with this enzyme has a fast increase when the reaction starts reaching even higher values than when enzyme 2D-PPK2 is not in the system (Figure 4.14 B). This can be explained since when the reaction starts ATP is quickly consumed, forming ADP. However, when enzyme 2D-PPK2 is added to the system, ADP concentration reaches almost zero (Figure 4.14D), meaning that the following reaction is happening on the inverse direction, consuming all the ADP and forming high quantities of AMP.

$$AMP + PolyP_n \overset{2D-PPK2}{\longleftrightarrow} ADP + PolyP_{n-1}$$

Then, when AMP concentration is very significant, reaction starts sifting in the direct way, into forming ADP and ATP. For that reason, as it is possible to observe in Figure 4.14B, AMP concentration decreases, which was precisely the wanted objective.

The opposite happens with UDP concentration, as it is shown in Figure 4.15.

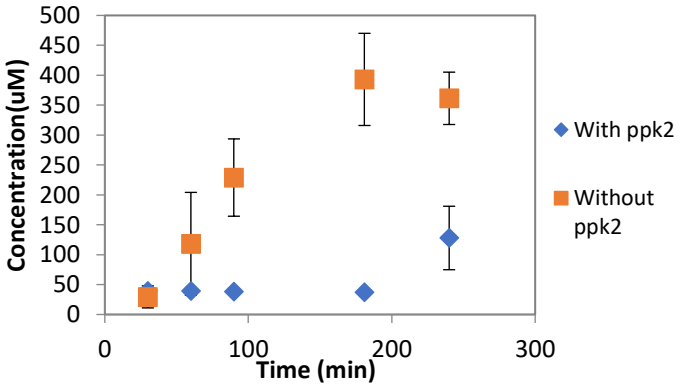



*Figure 4. 15: Concentration of UDP vs Time (Experiments with and without enzyme 2D-PPK2 for UDP-GlcNAc Cascade)*

UDP concentration with enzyme 2D-PPK2 increases much slower than the cascade without 2D-PPK2. This happens because UMP is consumed very quickly by 2D-PPK2, forming UDP that is also being consumed very fast to form UTP that is the main substrate to form the final product, UDP-GlcNAc.

Regarding UDP-Glc cascade, experimental conditions are shown in Table 4.7.

*Table 4. 7: Experimental plan for UDP-Glc cascade with and without 2D-PPK2*

| | **Glk** | **GalU** | **PPK3** | **URA6** | **2D-PPK2** | **PmPpA** | **Man B/C** |
|---|---|---|---|---|---|---|---|
| **Enzymes concentrations (mg/mL)** | 0.5 | 0.1 | 0.1 | 0.1 | 0.5 | 0.05 | 0.2 |
| | **Glc** | **UMP** | | **ATP** | | **PolyP$_n$** | |
| **Initial concentrations (mM)** | 3 | 3 | | 6 | | 6 | |
| | **$MgCl_2$** | | | **HEPES** | | | |
| **Other Concentrations (mM)** | 30 | | | 50 | | | |
| | **Temperature (°C)** | | | **Shaking (rpm)** | | | |
| **Conditions of the reaction** | 30 | | | 1000 | | | |

The results with concentrations of substrates and intermediary compounds for UDP-Glc cascade are presented in Figure 4.16.

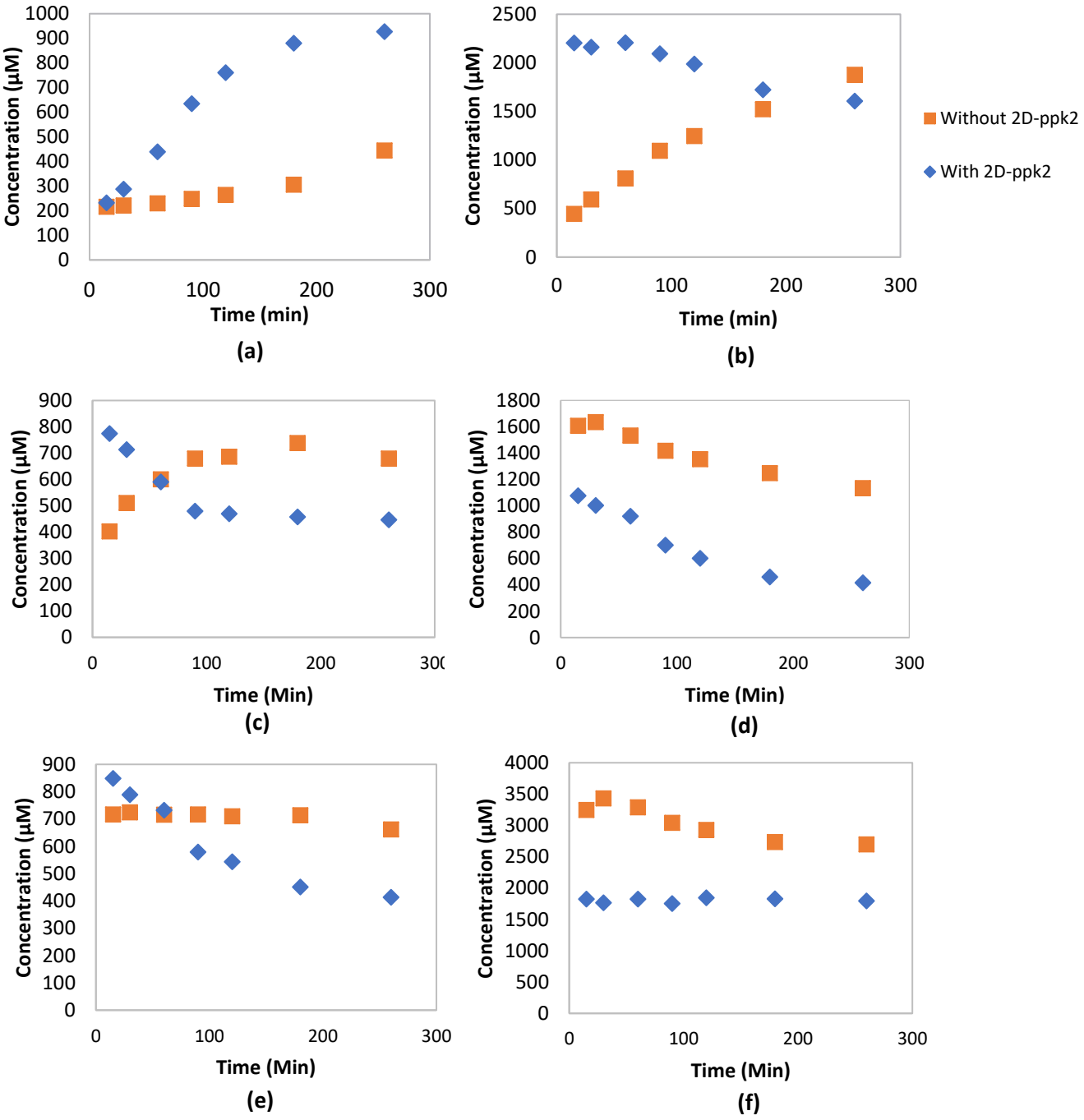



*Figure 4. 16: Experiments with and without enzyme 2D-PPK2 for UDP-Glc Cascade. (a) Concentration of UDP-Glc vs Time. (b) Concentration of AMP vs Time. (c) Concentration of UMP vs Time. (d)Concentration of UDP vs Time. (e) Concentration of UTP vs Time. (f) Concentration of ADP vs Time.*

In Figure 4.16A and Figure 4.16B it is possible to observe that AMP is being consumed, as expected, and formation of UDP-Glc increases when 2D-PPK2 is added to the system increasing the productivity by 52%. In literature, the volumetric productivity obtained for the production of this sugar nucleotide with different enzymes and substrates was 10 g $L^{-1}h^{-1}$[52]. In this project, the productivity obtained when enzyme 2D-PPK2 is added to the system is 31.51 g $L^{-1}h^{-1}$ having a considerable increase when compared to literature values.

In Figure 4.16D and 4.16E it is possible to see that when enzyme 2D-PPK2 is added to the system, concentration of UTP and UDP decreases due to the fact that enzyme 2D-PPK2 also has affinity to UMP, forming UDP and UTP that is more quickly consumed by enzyme GalU. On the other hand, when enzyme 2D-PPK2 is not present in the system, both compounds are less consumed.

Regarding UMP concentration (Figure 4.16C), it decreases faster with 2D-PPK2 then without the enzyme. This happens because when the enzyme is added to the system, UMP is also converted into more UDP and UTP increasing the concentration of substrate for enzymes PPK3 and GalU.

In Figure 4.16F it is possible to observe that in the first minutes, reactions take place on the reverse direction (ATP is forming ADP and AMP). When the reaction reaches 50 minutes, the reaction takes place on the forward directions, consuming AMP and ADP to form ATP. Afterwards, at 200 minutes, it reaches the equilibrium where the ADP consumed equals the ADP produced.

### *4.3.3. Enzyme Stability*

Enzymes are stored in glycerol to prevent them from losing activity when stored in the freezer. However, when the reactions are made, the dilutions to fulfill the final volume are made with water. Three experiments were conducted to analyze if enzymes loose activity in different environments: water, sorbitol and glycerol. The conditions for the experiments are shown in Table 4.3 and the results for the UDP-GlcNAc concentration in the final time point of the experiment is shown in Figure 4.17.

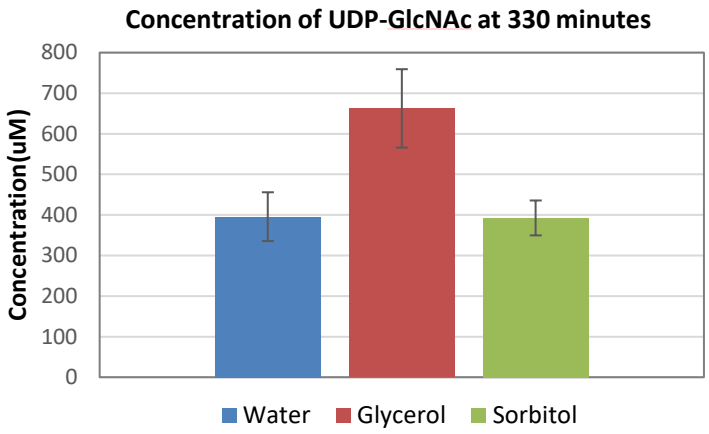



*Figure 4. 17: Experiments conducted in water, sorbitol and glycerol.*

In the previous figure, it is possible to observe that higher amounts of product in glycerol might imply higher enzyme stability. In fact, it is much more effective to use glycerol to perform the dilutions to the reaction to prevent the loss of activity of the enzymes, increasing the respective productivity.

In Figure 4.18 it is possible to observe the behavior of different compounds in the different environments.

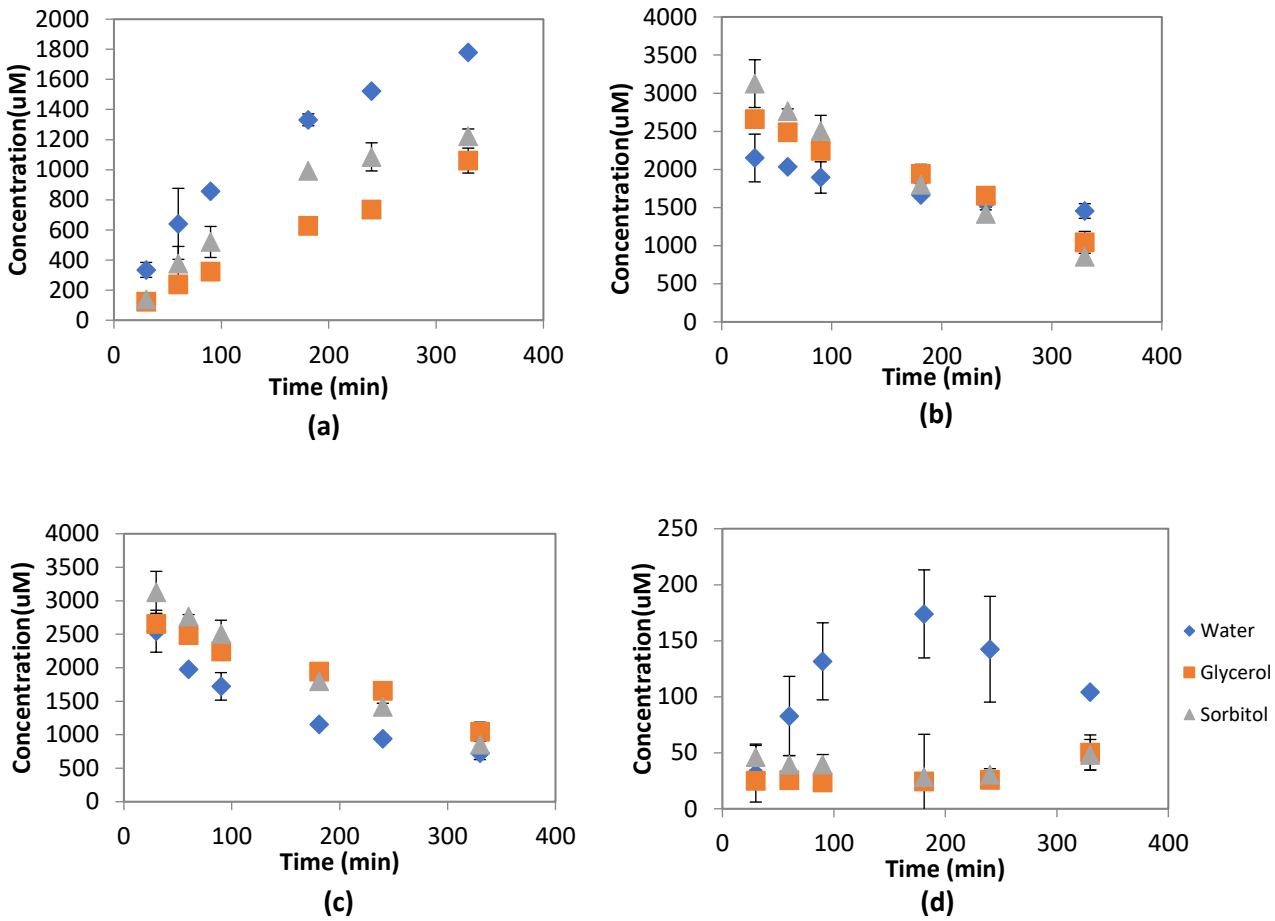



*Figure 4. 18: Results of AMP, ADP, UMP and ATP vs Time for the experiments conducted in water, sorbitol and glycerol. (a) Concentration of AMP vs Time. (b) Concentration of UMP vs Time. (c) Concentration of ADP vs Time. (d)Concentration of ATP vs Time*

In Figure 4.18A, the subproduct AMP is also formed in a higher amount when dilutions are performed in water. For that reason and to reinforce the choice of glycerol for the dilutions, lower amounts of AMP are formed.

Regarding the UMP concentration (Figure 4.18B), it seems to decrease with time. In the case of water, it decreases very fast but then starts decreasing slower when compared to the others. This means that when water is added the enzymes still work properly, but with time, they start loosing their stability, not consuming UMP that much. However, it should be necessary to run more reactions to confirm this fact due to standart deviation error that usually happens during experiments.

Regarding ADP concentration (Figure 4.18C) it is possible to see that when in water, it is more consumed when compared with glycerol and sorbitol leading to a higher concentration of ATP. This is confirmed when ATP concentration is analyzed (Figure 4.18D).

### *4.3.4. Product Inhibition with single enzyme*

Analyzing how the amount of product can affect the reaction can give a lot of important information to the process and how the scale up can be properly done. Experiments with enzymes PmPpA and GalU were conducted with different amounts of product inside the reaction vessel in order to have an appropriate study of the possible scale up to do in the future development of this process. The experimental plan for these experiments is shown on Table 4.8.

*Table 4. 8: Experimental plan for product inhibition experiments*

| | **GalU** | | **PmPpA** |
|---|---|---|---|
| **Enzymes concentrations (mg/mL)** | 0.2 | | 0.1 |
| | **UDP-Glc** | **UTP** | **Glc-1p** |
| **Initial concentrations (mM)** | 0 | 1 | 1 |
| | 5 | | |
| | 10 | | |
| | 15 | | |
| | 20 | | |
| | 25 | | |
| | 30 | | |
| | **$MgCl_2$** | | **HEPES** |
| **Other Concentrations (mM)** | 5 | | 30 |
| | **Temperature (°C)** | | **Shaking (rpm)** |
| **Conditions of the reaction** | 30 | | 900 |

The results of this experiment are shown in Figure 4.19.

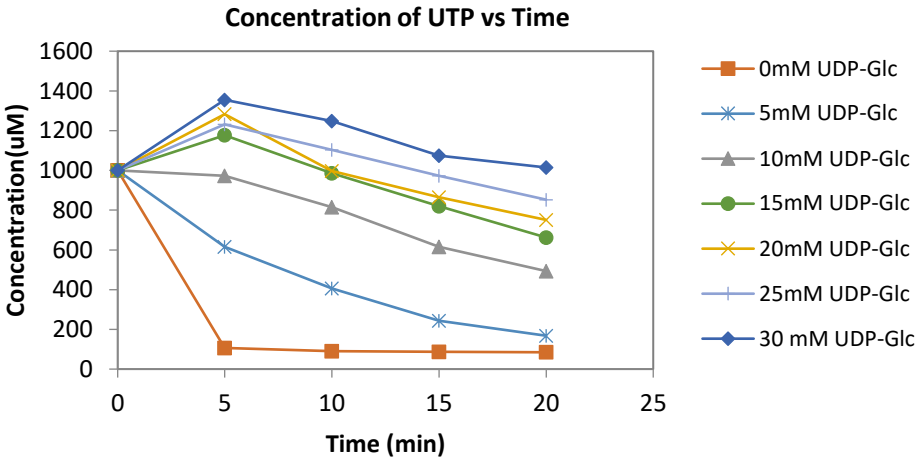



*Figure 4. 19:Concentration of UTP vs time (Product Inhibition)*

Regarding UTP concentration, it is possible to observe in the previous figure that the reaction is reversible, especially for high concentrations of UDP-Glc. Until 10 mM UDP-Glc that is already inside the vial, the reaction takes place in the forward direction. However, when the concentration of product increases above 10 mM of product inside the vial, in the first 5 minutes there is an increase of UTP concentration, meaning that the reactions are happening in a reversible direction, producing UTP and consuming UDP-Glc. This means that, for high concentrations of product inside the reactor, enzyme GalU starts consuming the product, producing more substrate, which is something that is not desirable at all, especially in a scale up process where the main goal is to produce the most significant amount of final product possible consuming the lowest amount of substrates allowing a high productivity. For that reason, in the future should be considered an *in situ* product removal when cascade reactions have present high concentration of product.

### *4.3.5. Substrate Loading with single enzyme*

The research of how different substrate loadings can affect the reaction is also very critical. For that reason, a series of experiments using different substrate concentrations were conducted (Table 4.9) to see how reactions are affected.

*Table 4. 9: Experimental conditions for substrate loading experiments*

| | **GalU** | **PmPpA** |
|---|---|---|
| **Enzymes concentrations (mg/mL)** | 0.2 | 0.1 |
| | **Glc-1p** | **UTP** |
| **Initial concentrations (mM)** | 1 | 1 |
| | 5 | 5 |
| | 10 | 10 |
| | 20 | 20 |
| | **$MgCl_2$** | **HEPES** |
| **Other Concentrations (mM)** | 5 | 30 |
| | **Temperature (*°C*)** | **Shaking (rpm)** |
| **Conditions of the reaction** | 30 | 900 |

The results for this experiment are shown in Figures 4.20 and 4.21.

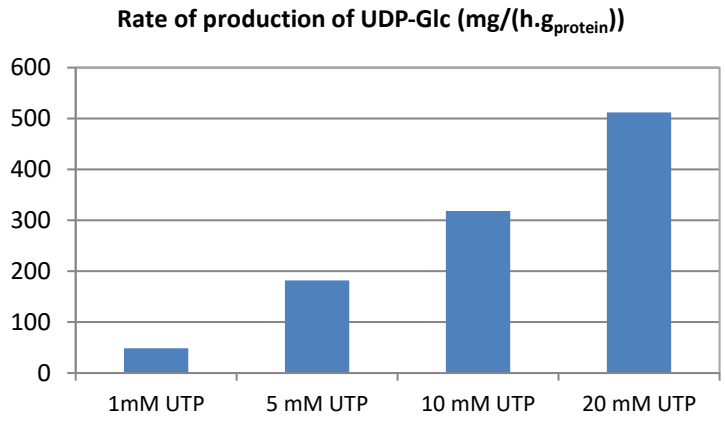



*Figure 4. 20: Rate of production of UDP-Glc (mg/(h.$g_{protein}$))*

As expected, the concentration of UDP-Glc increases when substrate concentrations are also increased.

Figure 4.20 shows in a more explicit way what was said before. The rate of production of sugar nucleotide UDP-Glc increases with the increase of reactants concentration. For that reason, substrate loading does not seem to cause any problems when scale up is done.

To confirm the thought mentioned before, rate of consumption of UTP was also calculated and it is presented on Figure 4.21.

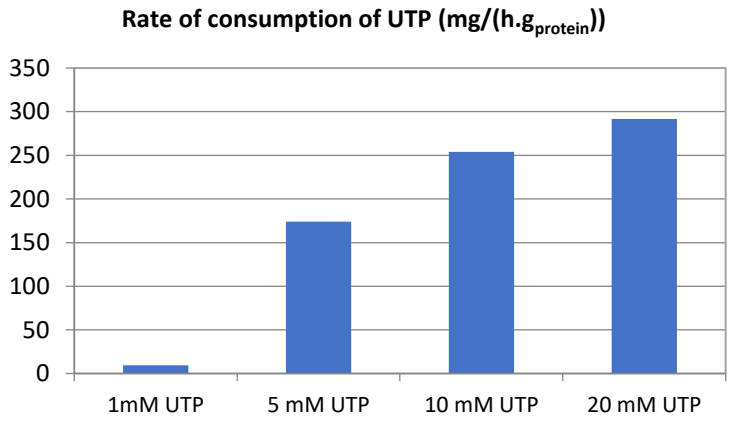



*Figure 4.21:Rate of consumption of UTP (mg/(h.g$_{protein}$))*

On the previous Figure 4.21, it is possible to observe that the rate of consumption of UTP increases when substrate concentration increases also. This means that enzymes PmPpA and GalU can process high concentrations of substrate and as the concentration of substrate increases, concentration of product also increases.

## 4.4. High Throughput Screening (Phosphate Assay)

As mentioned above, establishing an enzyme cascade is a very complex process that involves testing different environments with the purpose of finding the best conditions to maximize the production of the final product. For that reason, a phosphate assay was attempted to be established due to the fact that by using a 384 well plate, innumerous conditions could be tried in one experiment. However, to establish a method, several parameters should be defined, starting with the calibration curve (shown in Figure 4.22).

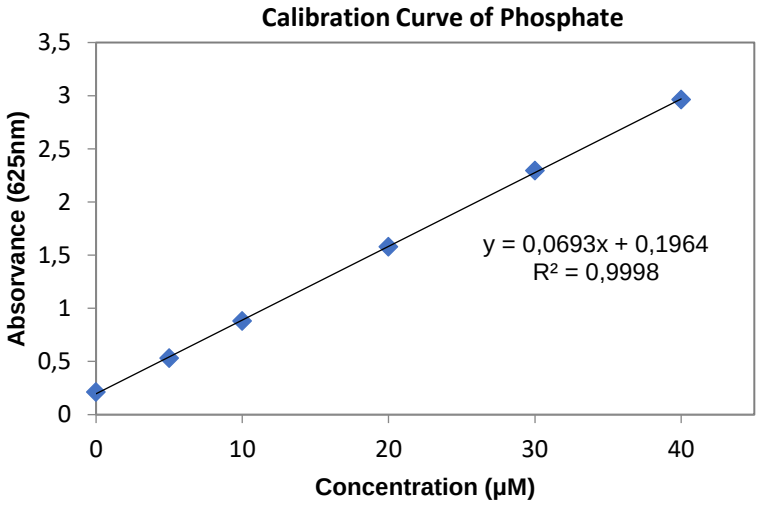



*Figure 4. 22: Calibration Curve of phosphate assay*

After obtaining the calibration curve that allows the calculation of phosphate in each sample that is going to be analyzed, it is possible to see that all the experiments that will be done in the future should fit in this curve (Figure 4.22), meaning that all dilutions made should fit between 0-40 µM.

Subsequently, new tests were made, particularly a sensibility test to the presence of PmPpA on the cascade. Experiments were conducted to analyze if this phosphate assay could detect free phosphate. For that reason, two experiments were made for the UDP-GlcNAc cascade: one with all the enzymes and another one without enzyme PmPpA.

*Table 4. 10: Experimental design for phosphate test with negative control*

| | NahK | GalU | PPK3 | URA6 | PmPpA |
|---|---|---|---|---|---|
| **Enzymes concentrations (mg/mL)** | 0.3 | 0.2 | 0.1 | 0.1 | 0.05 |
| | | | | | 0 |
| | **GlcNAc** | **UMP** | | **ATP** | **PolyPn** |
| **Initial concentrations (mM)** | 2 | 2 | | 2 | 4 |
| | **$MgCl_2$** | | | **HEPES** | |
| **Other Concentrations (mM)** | 20 | | | 50 | |
| | **Temperature (°C)** | | | **Shaking (rpm)** | |
| **Conditions of the reaction** | 30 | | | 1000 | |

The results for the phosphate concentration calculated are shown in Figure 4.23.

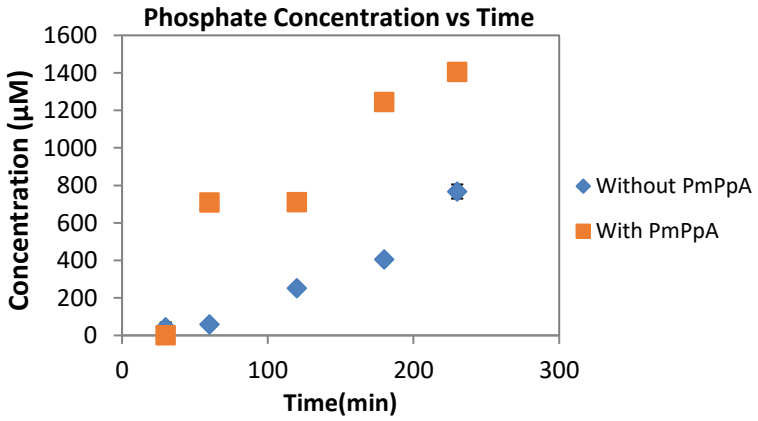



*Figure 4. 23: Phosphate concentration vs time with and without PmPpA using chromatography*

The experiments were made with and without enzyme PmPpA as negative control. It is also important to mention that, for effects of calculations, the contamination of phosphate due to reactants as ATP, UTP, ADP, UMP and AMP was considered by the subtraction of the concentration of phosphate of the reactants to the total phosphate concentration with the purpose to obtain a more accurate phosphate concentration.

$$Real\ phosphate\ Concentration = Total\ amount\ of\ phosphate - Amount\ of\ phosphate\ in\ reactants$$

As it is possible to observe in Figure 4.24, there is a big difference in the amount of phosphate present in the samples when PmPpA is added to the system. This happens because of the following reaction

$$UTP + GlcNAc - 1p \overset{GalU}{\longleftrightarrow} UDP - GlcNAc + PP_i \overset{PmPpA}{\longleftrightarrow} 2P_i \qquad (17)$$

When the enzyme PmPpA is not in the system, the phosphate formed is not very significant. For that reason, it can be said that in fact, enzyme PmPpA works appropriately and it forces the reaction to occur in the direct mode, producing more final product.

After phosphate concentration was obtained, UDP-GlcNAc concentration was calculated and is shown in Figure 4.24.

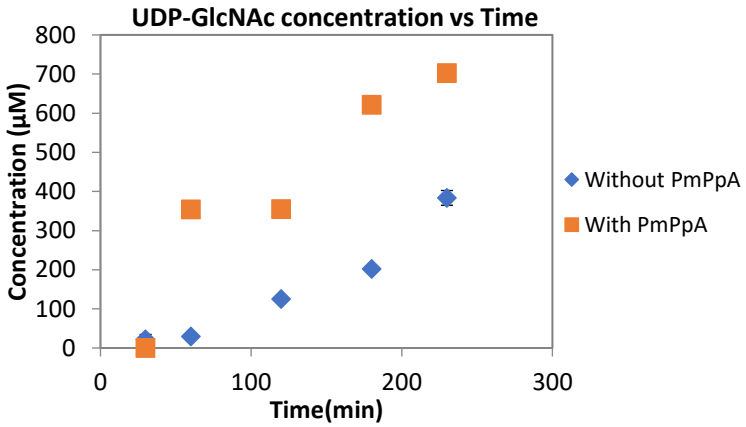



*Figure 4. 24: Estimated UDP-GlcNAc concentration vs time (phosphate assay)*

As it is possible to realize by the previous chemical reaction shown, the concentration of UDP-GlcNAc was considered as half of the phosphate concentration measured due to the reaction stoichiometry. It is also important to mention that for each sample, triplicates were made to analyze with this assay and standard deviation was calculated however, it was a very low deviation and for that reason, it is not very visible in Figure 4.24.

To realize if the results are similar with chromatography (HPAEC) which is already established and proved that it works properly all the samples were run in chromatography to compare with phosphate assay. The results are presented in Figure 4.25.

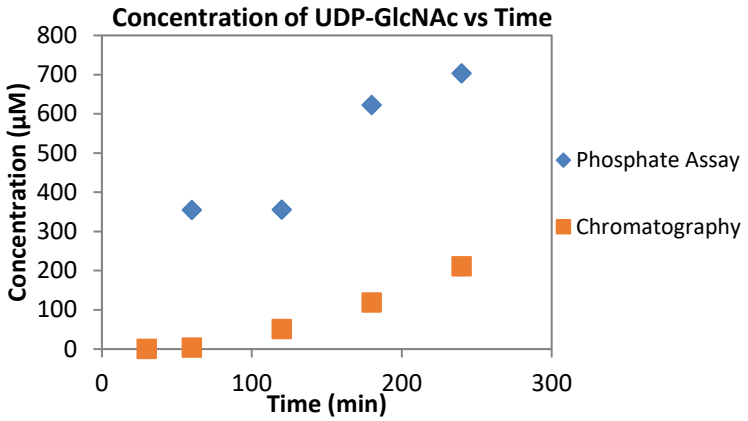



*Figure 4. 25:UDP-GlcNAc concentration vs time (phosphate assay and chromatography)*

Regarding the comparison between phosphate assay and chromatography results (Figure 4.25) it is possible to see that the outcomes are not close. This lead to believe that perhaps there is another font of contamination of phosphate in the reactants due to the phosphate assay having higher values of UDP-GlcNAc concentration than chromatography. Even though reactants contamination of phosphate was taking into account by putting them all together and measuring the absorbance of them, another technique should be tried.

In conclusion, an attempt to establish a high throughput measurement was made, however, it is a long and delicate subject that was proven that works as a qualitative method, however it requires much more testing due to the high phosphate contamination. With more testing, it can be possible to perform all the reactions in the spectrophotometer in a 384 well plate allowing to do innumerous experiments at once.

## 4.5. UDP-Glc Cascade Experiments

Finding the right model of a system can provide important information about it, thus allowing the optimization of the system and being able to predict behaviors. However, prior to the modelling it is important to collect the precise data without error associated.

For that reason, experiments for the cascade of UDP-Glc were conducted with the purpose of modelling. For this set of experiments, different initial concentrations were tested and are shown in Figure 4.26.

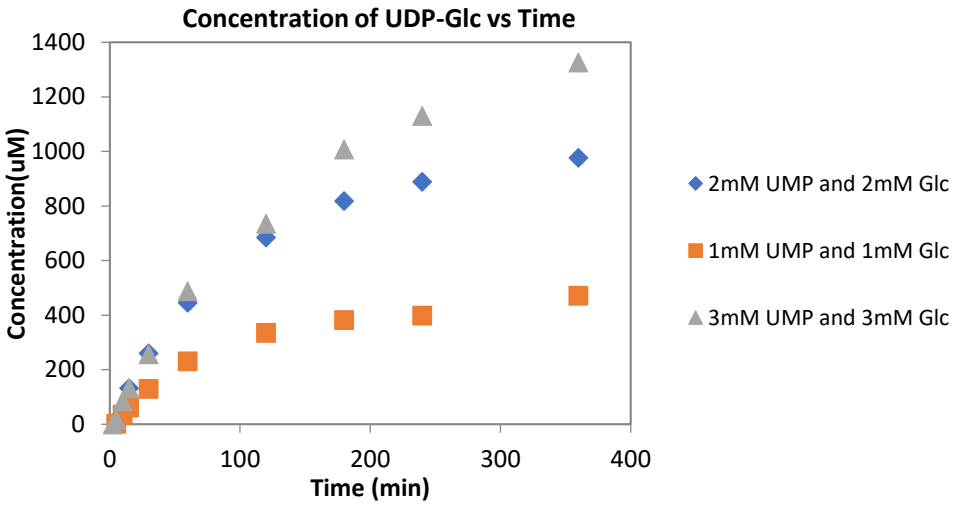



*Figure 4. 26: UDP-Glc modelation results*

It is possible to see that when substrate concentration increases, the concentration of product also increases as expected.

In the Figure 4.27 are the results for the experiments of 3mM UMP and 3mM Glc.

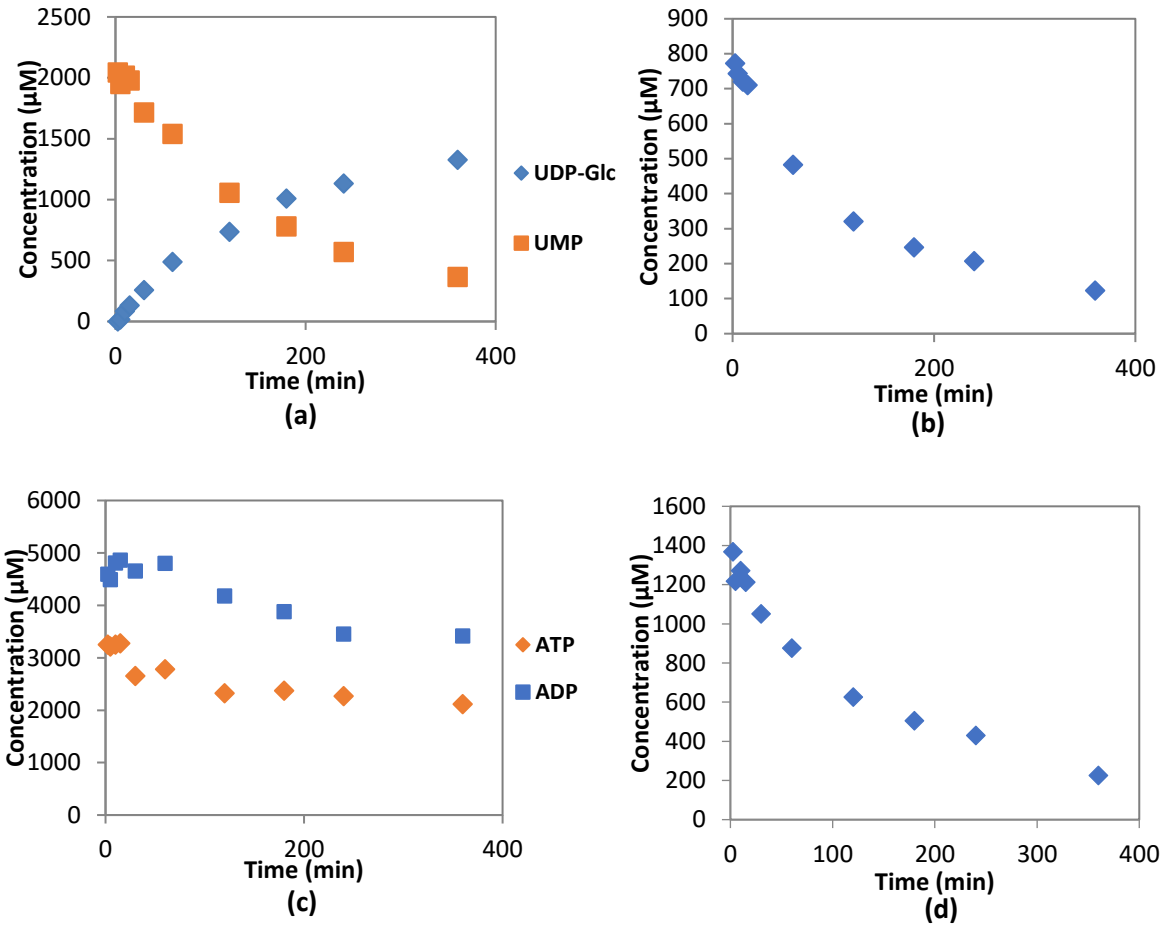



*Figure 4. 27: Results for UDP-Glc modelling experiments. (a) Concentration of UDP-Glc and UMP vs Time. (b) Concentration of UDP vs Time. (c) Concentration of ADP and ATP vs Time. (d) Concentration of Glc-6p vs Time.*
*Enzymes concentrations (mg/mL): Glk: 0.5; GalU: 0.1; PPK3: 0.1; URA6: 0.1; PmPpA: 0.05; Man B/C: 0.2.*
*Initial concentrations: Glc, UMP (3mM), PolyPn and ATP (6mM)*

As it is obvious in the previous figures, as UDP-Glc concentration increases, UMP and UDP concentrations decrease because these compounds are being consumed by URA6 and PPK3 to produce the final product.

In Figure 4.27C it is possible to realize that in the first minutes, concentration of ADP increases and ATP decreases because enzyme PPK3 is consuming ATP to form ADP. After 100 minutes both compounds reach equilibrium which means that the ADP and ATP that is consumed is also produced. Regarding Glc-6p concentration, it decreases through time. This shows that enzyme

Man B/C is working properly converting Glc-6p into Glc-1p which is the main substrate for the main enzyme, GalU, that produces the final product.

# Conclusions

The main achievement of this thesis was the successful establishment of in vitro enzymatic cascades to produce UDP-Glc and UDP-GlcNAc. In addition, for the first time, the enzymatic pathway to produce UDP-Man was found. More specifically, the concentration of enzymes and substrates were studied to make the cascade functional.

Subsequently, cascade conditions were improved to increase product yield, by using HEPES buffer at pH 7.5. Results showed an increased yield of sugar nucleotide production with HEPES buffer when compared to the buffer that was previously used to do the reactions.

When doing the experiments, problems were found, mainly the high production of sub product AMP. Enzyme 2D-PPK2 was added to the cascade to consume AMP resulting in a 52% and 56% productivity increase of the UDP-Glc and UDP-GlcNAc cascades, respectively.

Another problem found was that enzyme stability apparently decreases when the reaction takes place in water instead of glycerol. However more experiments should be performed to confirm this observation. In addition, it was also observed that when high concentration of product is formed, an *in situ* removal of the sugar nucleotide might be necessary.

Since there are many parameters that need to be tested to fully establish an enzyme cascade, an attempt to develop a high throughput screening test was made. In this thesis, the test was proven to work, however there is the need to do more experiments to have more precise results.

Another important aspect is that the efficient design of enzymatic cascades requires a mathematical model that embodies the many different parameters to study. As a first step to the establishment of such a model, dedicated experiments were performed with a very low error and precise time points.

# Future Work

As future work, the best pH for the cascade of UDP-GlcNAc and UDP-Man should also be studied. Also, the effect of different co factors should be studied to determine which one is the best to use on the reactions and correspondingly the effect of different temperatures for all the cascades.

Also, as upcoming work, all the cascades should be established with enzyme 2D-PPK2 to obtain higher yields of product. Moreover, more studies regarding enzyme stability should be done because if water affects enzymes, then all the reactions should start being done in glycerol.

Modelling should be done for all the cascades to optimize the system and get the optimal enzyme and substrate concentration. Only after that, product inhibition and substrate loading should be studied for all the cascade to conclude what type of reactor can be used in the future scale up.

Catalytic properties such as stability and reusability of enzymes should also be pursued, and the purification of the final product should be studied.

Regarding the phosphate assay test, it should be pursuit due to the great advantages that can bring to analyze different conditions of the reactions. The next step to improve this test should be doing the reactions directly in a 384 well plate. While doing a large number or experiments, contamination of reactants can also be considered with same shaking and temperature as the reaction, allowing a more effective counting of the phosphate contamination of substrate and hopefully giving a more accurate concentration of final product.

# Bibliography


[1] X. Qin, Xuan; Wang, "Quantification of nucleotides and their sugar conjugates in biological samples: Purposes, instruments and applications," *J. Pharm. Biomed. Anal.*, vol. 158, pp. 280–287, 2015.

[2] National Research Council, *Transforming Glycoscience: A Roadmap for the Future*. 2012.

[3] M. Helenius, Ari ; Aebi, "Roles of N-Linked Glycans in the Endoplasmic Reticulum," *Annu. Rev. Biochem*, vol. 73, pp. 1019–1049, 2004.

[4] H. A. D. Lagassé *et al.*, "Recent advances in (therapeutic protein) drug development," *F1000Research*, vol. 6, p. 113, 2017.

[5] M. D. Truppo, "Biocatalysis in the Pharmaceutical Industry : The Need for Speed," 2017.

[6] M. Rejzek, L. Hill, E. S. Hems, S. Kuhaudomlarp, B. A. Wagstaff, and R. A. Field, *Profiling of Sugar Nucleotides*, 1st ed., vol. 597. Elsevier Inc., 2017.

[7] Premier Biosoft, "Glycobiology." [Online]. Available: http://www.premierbiosoft.com/tech_notes/glycobiology.html. [Accessed: 25-Aug-2018].

[8] J. Rodríguez-Díaz, A. Rubio-del-Campo, and M. J. Yebra, "Metabolic engineering of Lactobacillus casei for production of UDP-N-acetylglucosamine," *Biotechnol. Bioeng.*, vol. 109, no. 7, pp. 1704–1712, 2012.

[9] L. Cai, "Recent progress in enzymatic synthesis of sugar nucleotides," *J. Carbohydr. Chem.*, vol. 31, no. 7, pp. 535–552, 2012.

[10] G. K. Wagner and R. A. Field, "www.rsc.org/npr | Natural Product Reports A survey of chemical methods for sugar-nucleotide synthesis," pp. 1172–1194, 2009.

[11] D. J. Pollard and J. M. Woodley, "Biocatalysis for pharmaceutical intermediates: the future is now," *Trends Biotechnol.*, vol. 25, no. 2, pp. 66–73, 2007.

[12] Sánchez-Moreno, "Chemistry, Molecular Sciences and Chemical Engineering," in *Comprehensive Chirality*, 2012, pp. 430–453.

[13] V. Tiwari, "In vitro Engineering of Novel Bioactivity in the Natural Enzymes," vol. 4, no. October, 2016.

[14] F. Lopez-gallego and C. Schmidt-dannert, "Multi-enzymatic synthesis," *Curr. Opin. Chem. Biol.*, vol. 14, no. 2, pp. 174–183, 2010.

[15] P. De Gracia and A. Santacoloma, *Multi-enzyme Process Modeling*. 2012.

[16] J. Ricca, Emanuele, Brucher, B. ; Schrittwieser, "Multi-Enzymatic Cascade Reactions: Overview and Perspectives," *Spec. Issue Biocatal.*, vol. 353, no. 13, pp. 2239–2262, 2011.

[17] L. M. Ambrosini and T. H. Lambert, "Multicatalysis : Advancing Synthetic Efficiency and Inspiring Discovery," vol. 10027, pp. 1373–1380, 2010.

[18] O. Reactions, Ł. Albrecht, H. Jiang, and K. A. Jørgensen, "Minireviews A Simple Recipe for Sophisticated Cocktails : Organocatalytic One-Pot Reactions — Concept , Nomenclature , and Future Perspectives," pp. 8492–8509, 2011.

[19] W. Zhao and F. Chen, "One-pot Synthesis and its Practical Application in Pharmaceutical Industry," no. 1, pp. 873–897, 2012.

[20] J. R. Cossy, "The Importance of Chirality in Drugs and Agrochemicals," vol. 1, 2012, pp. 430–453.

[21] L. LOVIGLIO, "De Novo," *WORD Watch.*, p. 1742.

[22] K. Nakajima, Y. Kizuka, Y. Yamaguchi, and Y. Hirabayashi, "Biochemical and Biophysical Research Communications Identi fi cation and characterization of UDP-mannose in human cell lines and mouse organs : Differential distribution across brain regions and organs," *Biochem. Biophys. Res. Commun.*, vol. 495, no. 1, pp. 401–407, 2018.

[23] J. A. Hanover, M. W. Krause, and D. C. Love, "Biochimica et Biophysica Acta The hexosamine signaling pathway : O-GlcNAc cycling in feast or famine," *BBA - Gen. Subj.*, vol. 1800, no. 2, pp. 80–95, 2010.

[24] A. Masarwah, "UDP-sugar accumulation drives hyaluronan synthesis in breast cancer," *Matrix Biol.*, no. 2018, 2017.

[25] A. R. Mahour, J. Klapproth, T. F. T. Rexer, A. Schildbach, S. Klamt, and M. Pietzsch, "Establishment of a five-enzyme cell-free cascade for the synthesis of uridine diphosphate N-acetylglucosamine," *J. Biotechnol.*, vol. 283, pp. 120–129, 2018.

[26] X. Ma and J. Sto, "High yielding one-pot enzyme-catalyzed synthesis of UDP-glucose in gram scales," vol. 333, pp. 159–163, 2001.

[27] J. Jaeken, "Glycosylation: General Overview," *Encycl. Neurosci.*, pp. 935–942, 2009.

[28] F. Schwarz and M. Aebi, "Mechanisms and principles of N-linked protein glycosylation," *Curr. Opin. Struct. Biol.*, vol. 21, no. 5, pp. 576–582.

[29] L. L., "Reference Module in Earth Systems and Environmental Sciences," in *Encyclopedia of Sustainable Technologies*, pp. 663–673.

[30] L. Lange, V. Parmar, and A. Meyer, "Biocatalysis," *Encycl. Sustain. Technol.*, vol. 3, pp. 663–673, 2017.

[31] H. Bisswanger, "Enzyme assays," *Perspect. Sci.*, vol. 1, no. 1–6, pp. 41–55, 2014.

[32] P. Pannuru, A. Rani, P. Venkatesu, and M. Lee, "The effects of biological buffers TRIS, TAPS, TES on the stability of lysozyme," *Biol. Macromol.*, 2018.

[33] G. Biotechnology, "User Guide for GoldBio Buffers," 2015. [Online]. Available: www.goldbio.com.

[34] K. Yasumatsu, M. Ohno, and C. Matsumura, "Stabilities of Enzymes in Polyhydric Alcohols," vol. 1369, 2014.

[35] N. Pazhanga, M.; Mehrnejadb, F.; Yaghub, P.; Hanieh, F.; Chaparzadeha, "Effect of sorbitol and glycerol on the stability of trypsin and difference between their stabilization effects in

the various solvents. Mohammad Pazhang," *Biotechnlogy Appl. Biochem.*, 2015.

[36] Z. Beilen, Jan; Li, "Enzyme technology : an overview," *Curr. Opin. Biotechnol.*, vol. 13, no. 4, pp. 338–344, 2002.

[37] T. Shi, P. Han, C. You, and Y. P. J. Zhang, "An in vitro synthetic biology platform for emerging industrial biomanufacturing : Bottom-up pathway design," *Synth. Syst. Biotechnol.*, 2018.

[38] K. Slámová and P. Bojarová, "Engineered N-acetylhexosamine-active enzymes in glycoscience," *Biochim. Biophys. Acta - Gen. Subj.*, vol. 1861, no. 8, pp. 2070–2087, 2017.

[39] BRENDA, "Information on EC 2.7.1.162 - N-acetylhexosamine 1-kinase." [Online]. Available: https://www.brenda-enzymes.org/enzyme.php?ecno=2.7.1.162. [Accessed: 16-Jul-2018].

[40] T. F. T. Rexer *et al.*, "One pot synthesis of GDP-mannose by a multi-enzyme cascade for enzymatic assembly of lipid-linked oligosaccharides," no. September 2017, pp. 192–205, 2018.

[41] BRENDA, "Information on EC 2.7.7.13 - mannose-1-phosphate guanylyltransferase." [Online]. Available: https://www.brenda-enzymes.org/enzyme.php?ecno=2.7.7.13. [Accessed: 17-Jul-2018].

[42] BRENDA, "Information on EC 2.7.4.22 - UMP kinase." [Online]. Available: https://www.brenda-enzymes.org/enzyme.php?ecno=2.7.4.22. [Accessed: 17-Jul-2018].

[43] BRENDA, "Information on EC 2.7.4.1 - polyphosphate kinase." [Online]. Available: https://www.brenda-enzymes.org/enzyme.php?ecno=2.7.4.1. [Accessed: 15-Jul-2018].

[44] BRENDA, "Information on EC 2.7.7.9 - UTP-glucose-1-phosphate uridylyltransferase." [Online]. Available: https://www.brenda-enzymes.org/enzyme.php?ecno=2.7.7.9. [Accessed: 15-Jul-2018].

[45] BRENDA, "Information on EC 3.6.1.1 - inorganic diphosphatase." [Online]. Available: https://www.brenda-enzymes.org/enzyme.php?ecno=3.6.1.1. [Accessed: 20-Jul-2018].

[46] B. Nocek, S. Kochinyan, M. Proudfoot, G. Brown, E. Evdokimova, and J. Osipiuk, "Polyphosphate-dependent synthesis of ATP and ADP by the family-2 polyphosphate kinases in bacteria," 2008.

[47] D. Cronk, *Chapter 8 - High-throughput screening*, Second Edi. Elsevier Ltd, 2012.

[48] I. Biosciences, "PiColorLock™ Gold Phosphate Detection System." [Online]. Available: https://www.innovabiosciences.com/images/stories/innova/PiColorLock Gold Phosphate Detection System protocol r6.pdf. [Accessed: 10-Aug-2018].

[49] "BRENDA - The Comprehensive Enzyme Information System." [Online]. Available: https://www.brenda-enzymes.org/.

[50] P. Biochemistry and M. Bethesda, "Adenosine Triphosphate-Adenosine Monophosphate transphosphorylase," 1957.

[51] E. Biocatalysis, "Enzymes as Catalysts. Structure–Functionality," in *Enzyme Biocatalysis: Principles and Applications*, 2008, pp. 4–10.

[52] B. Schmölzer, K.; Lemmerer, M.; Gutmann, A.; Nidetzky, "Integrated Process Design for

Biocatalytic Synthesis by a Leloir Glycosyltransferase: UDP-glucose Production with Sucrose Synthase," *Biotechnol. Bioeng.*, vol. 114, no. 4, pp. 924–928, 2016.

[53] M. Berbis, J. Sanchez-Puelles, F. Canada, and J. Jimenez-Barbero, "Structure and Function of Prokaryotic UDP-Glucose Pyrophosphorylase, A Drug Target Candidate," *Curr. Med. Chem.*, vol. 22, no. 14, pp. 1687–1697, 2015.

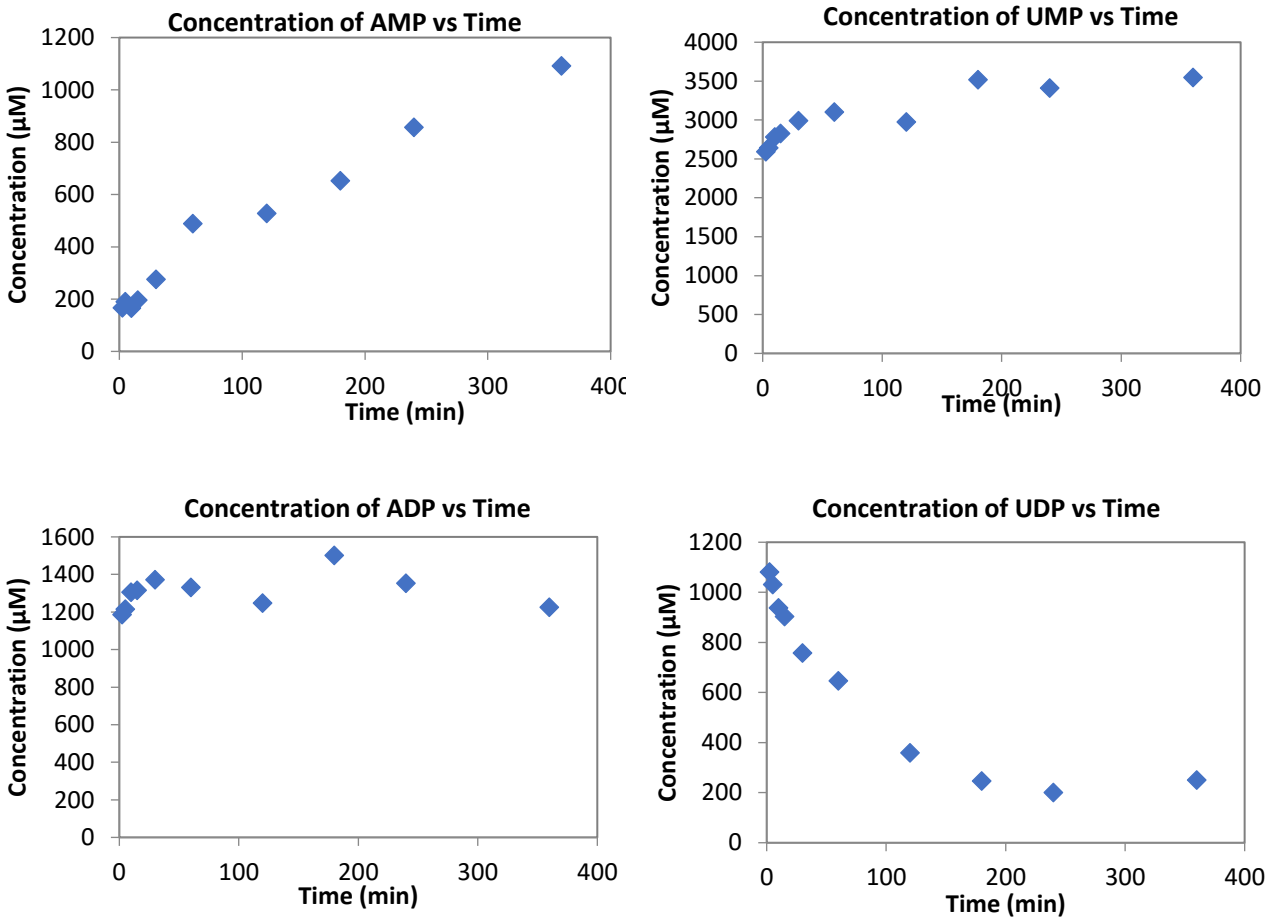
[54] Sigma-Aldrich, "Uridine 5'-diphospho-N-acetylglucosamine sodium salt." [Online]. Available: https://www.sigmaaldrich.com/catalog/product/sigma/u4375?lang=de&region=DE. [Accessed: 05-Jul-2018].

[55] Y. Hayashi, "Chemical Science," pp. 866–880, 2016.

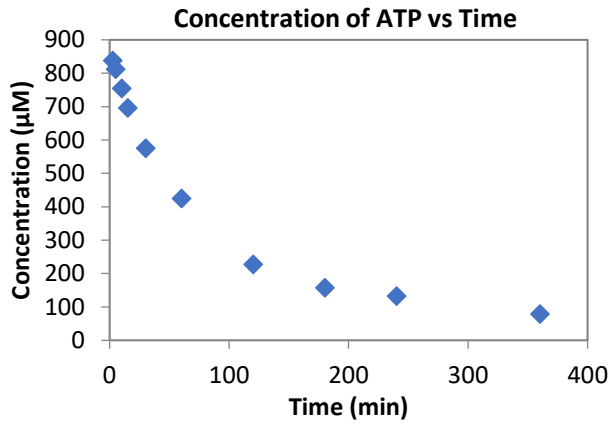
# Attachments

### Attachment 1

In this attachment are presented the results for UDP-Man cascade for 3mM Man and 3mM UMP



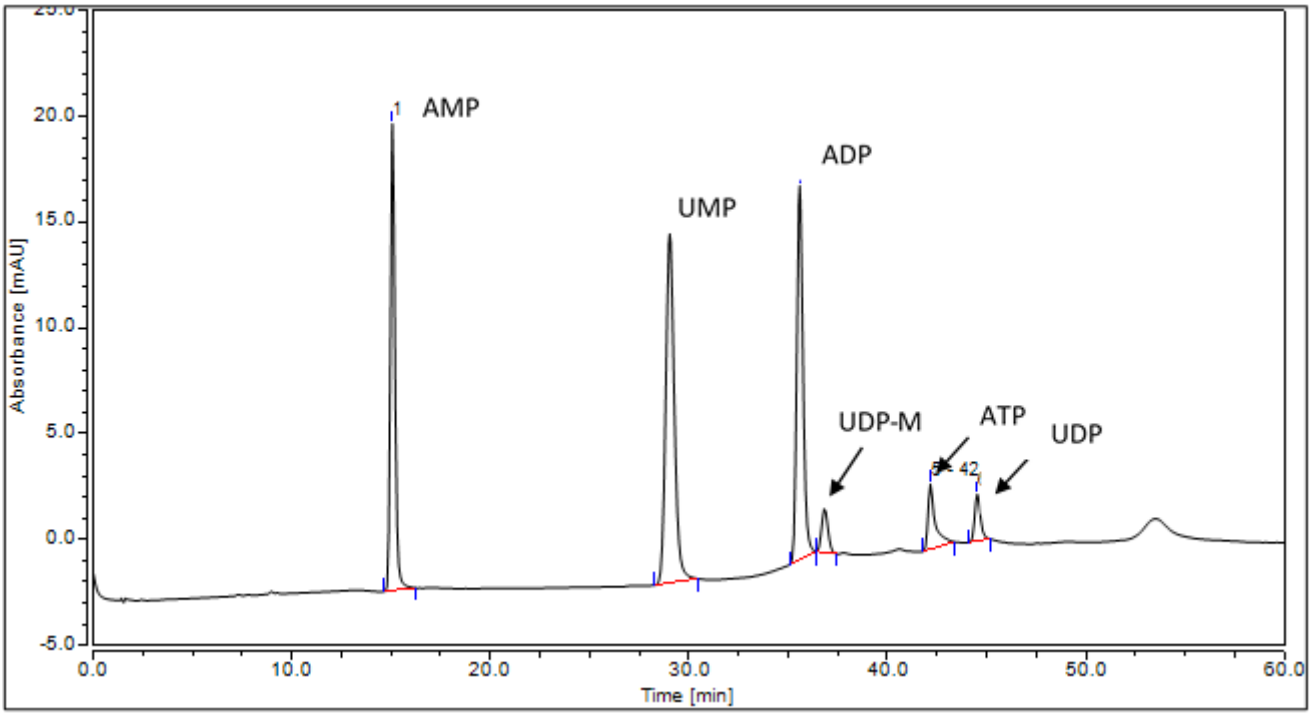

*Attachment 1: Experiments for UDP-Man cascade. (a) Concentration of AMP vs Time. (b) Concentration of UMP vs Time. (c) Concentration of ADP vs Time. (d) Concentration of UDP vs Time*




*Attachment 2: Experiments for UDP-Man cascade (concentration of ATP vs Time)*

## Attachment 2

In this attachment is presented the chromatogram for the UDP-Man cascade, where it is possible to observe that the sugar nucleotide in question is eluted at 38 minutes.




*Attachment 3: Chromatogram of UDP-Man Cascade*