TÉCNICO LISBOA

Potential and Challenges of Liquid-Phase Adsorption in Fermentative Processes

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I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

Due to the impact of the COVID-19 pandemic, this dissertation has a higher theoretical focus than experimental, with case studies complementing it.

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Abstract

As the world turns to decrease the environmental impact that it has perpetuated in the last decades, most industries are deciding to switch to bioprocessing to lower their carbon footprint. However, with most bioprocesses counting up to 80% of costs for only the downstream steps, this presents the biggest setback for the choosing of a more environmentally conscious process. Unlike many downstream methods, adsorption can reduce the operational costs of the fermentative processes, while having high efficiencies regarding product recovery. Unfortunately, since this technique has not been yet studied for these conditions, this thesis focus on the research regarding the adsorption of lysine, a fermentation-based product, alongside the single adsorption of medium components commonly found in fermentation media. The study with the highest removal of lysine was performed using activated carbon at a pH of 9.8 and 30 ℃, reaching an efficiency of 230 mg/g when in the presence of glucose, through polar interactions. The adsorption of most medium components is shown to be dependent of the ionic strength and pH of the environment and mostly independent of the temperature. As adsorption presents a more economically viable with lower operational and energy costs, there needs to be a decrease on the information gap regarding its use for the recovery of fermentation products.

Keywords

Bioprocess, Fermentation, Lysine, Adsorption, Medium Components

Resumo

À medida que o mundo começa a diminuir o impacto ambiental que causou nas últimas décadas, a maioria das indústrias está a dirigir-se para o bioprocessamento de forma a reduzir a sua pegada de carbono. Porém, 80% dos custos em bioprocessos reside nas etapas de purificação, representando assim o maior obstáculo para a escolha de um processo mais ambientalmente consciente. Ao contrário de muitos métodos de recuperação e purificação, a adsorção pode reduzir os custos operacionais dos processos fermentativos, devido à sua alta eficácia em relação à recuperação do produto. Consequentemente, uma vez que esta técnica ainda não foi estudada para tais condições, esta tese foca-se na pesquisa da adsorção de lisina, um produto fermentativo, juntamente com a adsorção individual de componentes encontrados em meios de fermentação. O estudo com a maior remoção de lisina foi realizado com carvão ativado a pH 9.8 e 30 ℃, alcançando uma eficiência de 230 mg/g na presença de glucose, por meio de interações polares. A adsorção da maioria dos restantes componentes fermentativos mostra-se dependente da força iónica e do pH e independente da temperatura. Como a adsorção apresenta-se economicamente mais viável com menores custos operacionais e energéticos, deve haver uma diminuição na falta de informação quanto ao seu uso para a recuperação de produtos fermentativos.

Palavras Chave

Bioprocesso, Fermentação, Lisina, Adsorção, Componentes do Meio Fermentativo

Contents

List of Figures

List of Tables

Acronyms

AC	Activated Carbon
AFAA	*L*-Aspartate Family of Amino Acids
AEC	S-(2-Aminoethyl)-*L*-Cysteine
AL	Activated Alumina
ATPS	Aqueous Two Phase System
ATR-FTIR	Attenuated Total Reflectance Fourier-Transform Infrared Spectroscopy
ATR-IR	Attenuated Total Reflectance Infrared
CS	Cocunut Shell
CTAB	Cetyltrimethylammonium Bromide
DVB	Divinylbenzene
EBA	Expanded Bed Adsorption
GRAS	Generally Regarded As Safe
h-BBN	Boron Nitride Surface
HCP	Hypercrosslinked Polymers
IA	Itaconic Acid
OAA	Oxaloacetate
PEP	Phosphoenolpyruvate
PEG	Polyethylene Glycol
pI	Isoeletric Point

POP Porous Organic Polymers

PPP Pentose Phosphate Pathway

PTS PEP-dependent Phosphotransferase System

PVC Polyvinyl Chloride

RQ Respiratory Quotient

SDS Sodium Dodecyl Sulphate

STIRS Surface Titration by Internal Reflection Spectroscopy

VGAC Vetiver Grass-Activated Carbon

VOC Volatile Organic Compound

XRD X-ray Diffraction

ZR Zirconium Chloride Octahydrate

1

Introduction and Background

Contents

1.1 Purpose of the research

In today's society, reducing the carbon footprint of industrial processes has become more important than ever. Most of them still rely on fossil resources that contribute to the quick deterioration of the global climate and environment, but are now looking for solutions to lower their emissions, energy usage and waste pollution. Some of these industries have been replacing their processes with biorefineries based procedures by, for example, using biomass as an initial resource [1].

Nonetheless, the use of biomass can bring risks to the process, as it contains a high percentage of water [2]. Due to this, most of the recovery downstream steps have to be altered. Methods such as distillation and spray-drying, are not suited for aqueous media, due to the high temperatures that they would have to work, which brings the setback of higher costs and energy usage. Aspects like these discourage companies to alter their fossil fuel based processes [3].

As a way to enforce a sustainable bio-economy, there has been an increase on the development of technologies to recover the produced components, with separation processes like chromatography and filtration being capable of processing aqueous medium. Unfortunately, the implementation and operational costs of units such as those previously mentioned still constitute the bigger part of the investment and not always make up for the change to a biorefinery process [4]. This need to improve the downstream steps can take a long time with more different and new processes being tested and implemented than ever before. Most are still looking for more cost saving methods to appeal industries to replace its process to more environmentally conscious ones.

One of the downstream processes that has yet to be applied at large scale is adsorption, who, at laboratory scale, has been considered more energy efficient and simpler compared to other methods that are currently being applied [5]. Unlike distillation and spray-drying, this method is not temperature dependent, meaning that there are no additional costs regarding energy usage towards increase or decrease of temperature, while still being able to process large amounts of aqueous media [3, 4]. However, up to today the application of adsorbents in fermentative media for the product recovery has not yet been studied in detail, neither its process efficiency and setbacks, like the release of contaminants and the removal of medium components that are not the intended product, affecting the efficiency of the technique [6].

As this thesis is inserted in a project regarding the removal of fermentation products through adsorption, the research will be focused on the application of this method for the recovery of lysine, an amino acid obtain through microbial fermentation. This research will describe studies concerning the production of lysine, its recovery through adsorption and the adsorption of components commonly found in the medium.

1.2 State of the art

Nowadays, different separation methods are being applied for the product recovery of many large scale processes. One of these processes is the fermentation of lysine, an amino acid that, like aspartic acid, glutamic acid, is essential for the industries of food and beverages, animal feed, pharmaceuticals and cosmetics [7].

The first time lysine was industrially produced dates back to the 1950s by a japanese pharmaceutical company called *Kyowa Hakko Kogyo* (currently *Kyowa Hakko Bio*) using a homoserine-auxotrophic mutant. In the 1960s, in Japan and in the USA, it was found that the various types of these microbial auxotroph organisms could be obtained by using UV-light or Co-60 irradiation and by penicillin methods employing glutamic acid producing bacteria [8]. Later in the 1970s and 80s, fermentations were carried out in mutants by adding additional amino acid auxotrophies and resistance to antimetabolites, resulting in a 40 to 50% yield growth in fed-bacth cultures for lysine production. In the late 1980s and 1990s genetic engineering started to be applied to various microorganisms capable of producing lysine and in the 2000s, *in silico* modelling and simulation approaches started to be applied for further strain improvement. As a result, the conventional style of selecting improved strains by their phenotypes, formerly the standard practice in the industry, is rapidly being replaced by a new method called genome breeding. Thanks to these new techniques, new organisms easier to cultivate, such as *Escherichia coli*, can play a role in the lysine fermentation process [9].

Currently, by the year of 2020, over 2 million tons of lysine are produced annually with several hundred thousands tones (approximately 800,000) being exclusively via bacterial fermentations [10]. In 2018 the lysine marked was valued at over 3000 million US dollars and is still growing every year at an estimate of rate 10%, thanks to the research of various companies and academic institutions [11]. Its fluctuation per metric ton is represented in the chart (figure 1.1 [11]).

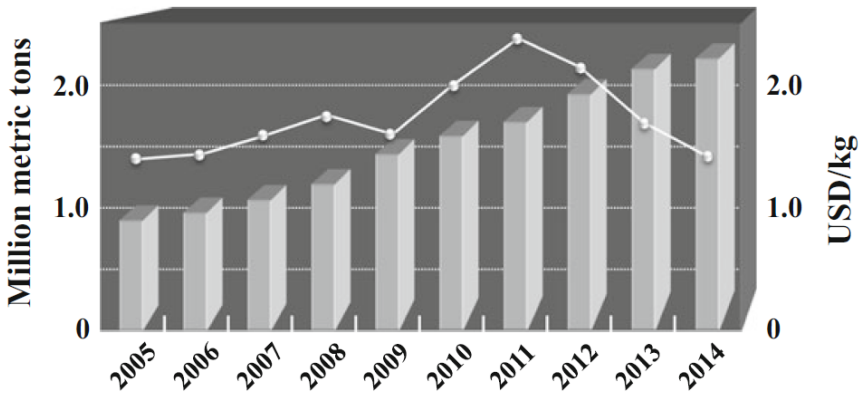

Figure 1.1: Annual evolution rate of global markets (bars) and prices (circles) for lysine in the years between 2005 and 2014.

Some of the main manufacturers of this amino acid are found in Asia (South Korea with *CJ Cheil-Jedang* and China with *Global Bio-Chem Technology Group*), North America (USA with *Archer Daniels Midland*) and Europe (*Evonik Industries*, Germany) with more than half of the global lysine production being controlled by China and the USA. [9, 11]. The substrates for the biotechnological lysine production are mainly produced in the fields of Brazil, Russia and Indonesia which provide a high quality carbon source for both the micro organism and end-product flow. The choice of this regions to produce the main carbon source translates in more economical and higher yield processes [11].

For the microbial fermentation of lysine, using strains of the gram positive *corynebacteria* (such as *Corynebacterium lilium*, *Brevibacterium dicvaricatum* and *Corynebacterium glutamicum*) showed adequate results, with the latter being the most adopted bacteria [10, 12]. Ever since the publication of the genome of the specific strain ATCC 13032 in 2003 for the production of lysine, researches using *C. glutamicum* in the fields of industrial microbiology and biotechonology have increased very quickly, as shown in figure 1.2 [13]. Nonetheless, transcriptomes and proteomes for this subspecies are still being interpreted to this day [11, 13].

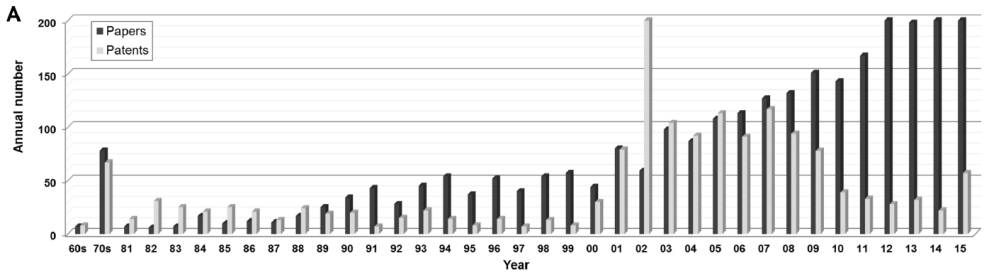

Figure 1.2: Reports of papers and patents regarding fermentation studies with *C. glutamicum* at an annual view.

Currently the industrial fermentation processes consist of two main parts, the lysine production and its downstream processing, going from purification/product recovery to wastewater treatment [10]. For most large scale bioprocesses, the downstream steps can constitute up to 80% of the overall cost, making it a major bottleneck for implementation, where in most refineries costs regarding downstream units round up to 45% of the total capital cost [14, 15]. Despite that, most used downstream techniques such as drying, distillation and evaporation tend to consume 90% more energy than membrane-based methods, according to a report from Nature Magazine in the United States [3]. Due to this, there is a constant necessity to improve the current separation methods while also developing new alternatives. Since fermentation broths are aqueous media very rich in many different components, the downstream must be thought with care, as to maximize the recovery of the product and exclude the unwanted components. These improvements must also consider the final use of the product since this aspect can dictate how strict and time consuming the downstream process is [16, 17].

The entire process is being constantly improved by considering numerous factors such as cost of

raw materials (most importantly the carbon source which varies from region to region), fermentation and purification yields, productivity and overall costs of the process [11, 18]. According to a projection study to implement a continuous lysine fermentation plant in 2012, it would be necessary to invest 140 M US$ in the project, with operational costs making up over 100 M US$. This simulation used chromatography as the main process for product recovery, with the obtaining of raw materials resulting in 46% of the annual costs [4]. This simulation took into account the inflation of the prices through the years of raw materials, product selling and operational costs. A more concrete example is the construction of a lysine fermentation plant in 1999 in Nebraska, USA, that costed about 100 M US$ and had the capacity to produce 75 kilotonnes annually [19]. By the year of 2018, feedstock prices for the fermentation of lysine are setted around 0.806 US$/kg, with lysine being sold at 2.632 US$/kg [20]. At the moment, fed-batch is the most common type of industrial process with fermentation tanks going up to a size of 500 kL, but recent improvements show that upgrading it to a continuous type fermentation increases the productivity of the overall process for more than 2.5-fold [9].

When recovering biomolecules such as proteins and amino acids, many separations methods are being applied in the downstream phase. Some of the techniques used in current processes include spray-drying and distillation and these are not considered suitable for the downstream of fermentation processes. This is due to the fact that fermentation media have a 90 to 95% of water content, making the energy costs necessary to recover the product notably high [2, 21]. Spray-drying consists on the fast evaporation of the water molecules by mixing with a dry high temperature gas and has seen its use in the recovery of probiotics in pharmaceutical processes [22, 23]. Similarly, simple distillation resides on the evaporation of the medium until only the product is left. Both of these demand high energy use to be able to extract the pre existing water.

One of the oldest methods is precipitation, mainly by the salting-out effect (which by the increase of the concentration of salts such as calcium hydroxide or calcium oxide, hydrophobic or hydrophilic molecules start to attract to each other in a modified formulation of emulsion) [24]. Most studies focusing on the precipitation of proteins are carried in a single protein and proceed to be spiked in fermentation broths [16]. Nonetheless, there must be an extra attention as to minimize irreversible denaturation of the product while separating it from the other components. Another disadvantage is the heavy consumption of additives that cannot be recovered, specially in large scale [17, 25].

Filtration is considered one of the most economical and easier processes to be scaled-up for the clarification of fluids, but finds difficulties with the many particles sizes and the flux rates that must be implemented to keep the normal run of the overall process [17]. One of the most used approach is large scale is chromatography, which can range from ion-exchange, affinity and HPLC chromatography. Despite its effectiveness, it is one of the most expensive methods. Having to use large amounts of resins that sometimes are not reusable and having low process throughput, it easily becomes the most

expensive step. Even with HPLC, which improves the speed and resolution, there must be an extra care due to denaturation or irreversible binding, making the choice of the mobile phase critical [16, 17]. The use of certain ion exchange resins can produce salts that are then released to the medium and can become wastes, that will need further proper treatment as to not be released in the environment. Most processes use buffers to avoid this situation, with some end up using ion suppressors, adding an additional cost to the process [26, 27].

A method that can be applied for the recovery of small molecules, like amino acids such as lysine, is an aqueous two phase system (ATPS). This system uses two immiscible solutions (an aqueous one and an aqueous polymer solution one, such as polyethylene glycol (PEG)) and, by playing with the electric charges and hydrophobicity, can separate the different molecules from one solution. There are studies that found this method to be successful reaching 100% yields of separation, mainly when separating fermentation based products such as carboxylic acids, while also being cost-effective and environmentally friendly by recycling some of the phase components and the used solutions [17, 28]. Unfortunately, for these kinds of systems there still is a gap regarding the scale-up, despite the variety of existing works dedicated to improve the method at large scale [25, 29].

One method that has been gaining attention in is adsorption. This technique is considered to be simple and relatively easy to apply in continuous mode [5]. It uses materials that are able to remove the desired component from the surrounding environment by adhesion to its surface. This occurs through chemical and physical interactions, such as covalent bond and electrostatic interactions. Some examples are activated carbons, zeolites with industrial and agricultural wastes being recently adopted. When assessing the different separation methods, it is revealed that adsorption is considered one of the favorites, due to its high level of capture of the desired product, keeping a high purity. Its process was heavily studied in the first half of the 20^{th} century and reaching its higher amount of researches in the late 40s. Today, it is one of the most economical, effective and versatile in many different industrial fields [30, 31]. Without counting the expense of the adsorbent materials, reports have shown a cost reduction of 50 to 70%, mainly to the replacement of three to four downstream steps to just one, reducing in half the overall process time and achieving the recovery and purity demanded [14].

In a study regarding the recovery of itaconic acid (IA) performed by Magalhães Jr *et al* [32], adsorption proved to be one of the most appropriate methods to extract this fermentation product from its broth (the overall comparison is shown in figure 1.3). Despite, not being the cheapest and neither the most modern, adsorption shows almost no waste production during the process, a higher selectivity and energy saving in the same conditions, concluding that it can be used in fermentations processes. This comparison also showed that it was the process that allowed a 100% yield of recovery alongside a high level of purity with overall adsorbed amounts of 0.42 g IA/g of adsorbent, with the acquired results supported by literature and other similar studies. When compared with other extraction techniques,

the recovery of components using adsorption presents more advantages, such as easier operations, lower resources costs, no requirement of chemical additives and it is able to reuse the carriers for later processes [33].

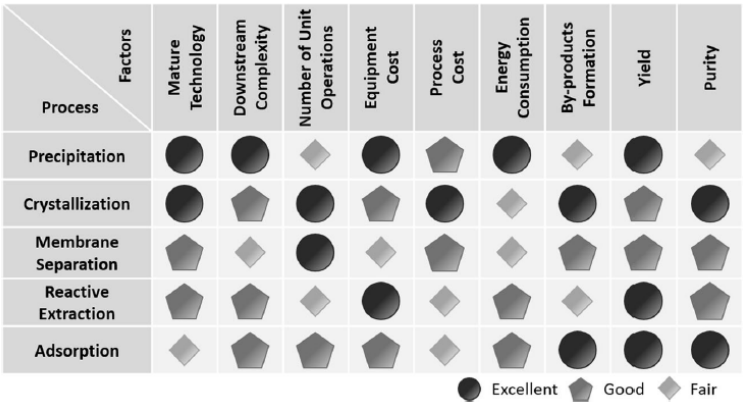

Figure 1.3: Comparison of advantages and disadvantages of separation methods for itaconic acid in the work by Magalhães Jr group.

Like the previous mentioned study, to apply the adsorption method in product recovery or component removal, it can by using adsorption bed columns, which that can be packed/fixed or fluidized/expanded.

In fixed bed columns, a typical addition to continuous processes, the adsorbates are constantly being flowed through a bed of the adsorbent material [34]. This presents an effective adsorption process that can be obtain by using higher flow rates for higher amounts of processed volume, being more adequate for large scale continuous processes. Nonetheless, there are disadvantages with this process - the interactions in these beds can only be optimized after being firstly applied, with theoretical and modelling calculations not begin enough to describe the process effectiveness [34, 35]. Fixed bed can be arranged in series or parallel modes. In series bed columns the bed is typically exhausted more fully than one large single fixed bed, where parallel beds are more efficient in meeting the substances concentrations requirements in the final discharge. Unfortunately, to regenerate the adsorbent, fixed beds require a total or partial shutdown [36]. In fluidized beds, the adsorbate has a better contact with the adsorbents with the help of the motion of the adsorbent material. They are mainly used when there are large volumes of product to be extracted, which is helpful for large scale processes. Unfortunately, the rapid mixing between the medium with the adsorbent might lead to smaller contact time, decreasing the adsorption efficiencies [34].

In fig. 1.4, there is a step-by-step representation of how an expanded bed works during the recovery of components. This system involves the integration of both solid-liquid separation and product recovery into one single unit, without creating excessive back-pressure and allowing higher flow rates compared to packed bed columns [37]. In the expanded bed column, the application of an upward flow allows the adsorbent material a better contact to the particles without blocking the bed. The direction of the flow is

the main difference between expanded and packed bed adsorption columns, with the last one typically being in a downward direction. The use of an upward flow is able to create a stable fluidization of the matrix and the increase of void volume due to the expansion of the bed, which lowers the fouling and column blockage observed with traditional packed bed [38, 39]. Large scale expanded bed columns can be found in the range of 50 to 300 L with even bigger volumes found nowadays [38]. The expanded bed adsorption (EBA) system has now been used in a lot of areas, such as protein purification and the pharmaceutical industry, with now developments of a new second-generation of EBA adsorbents, allowing the processing of higher flow rates ranging from 300 to 600 cm/h [14].

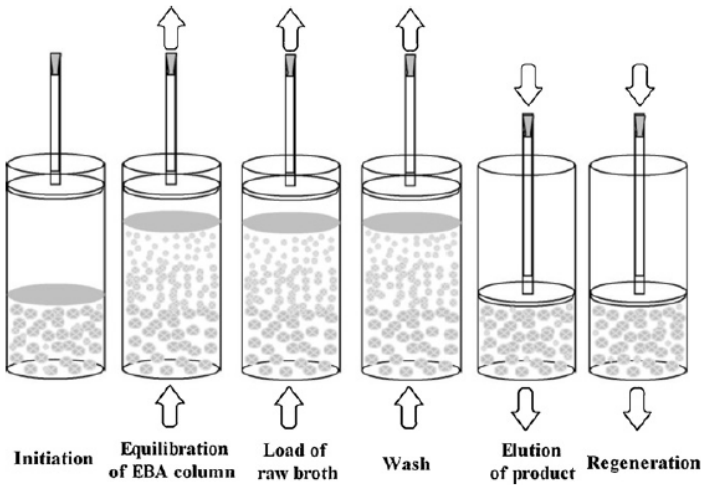

Figure 1.4: Overall working steps of an expanded bed adsorption.

1.3 Goals And Motivation

This master thesis aims to gather information regarding the application of adsorption in the fermentative processe of lysine by *Corynebacterium glutamicum* by summarizing various researches that apply different types of adsorbents for the recovery of lysine, while taking in account the presence of other medium components and how they can influence its recovery. With this information, this research work hopes to, understand how the the adsorption recovery process from the fermentation medium occurs in laboratory scale as to further predict how it will perform in larger scales.

Since one of the main topics is the production and capture of lysine, this amino acid must be supplied in the best conditions possible under the producion of the *C. glutamicum* bacteria. As explained in the previous topic, these processes are looking for ways to be less expensive while being more energy efficient. As to understand how to apply any product recovery technique, it is necessary to also understand how the fermentation works, including its possible improvements. One of the methods that can be

applied for the product recovery is adsorption, but this operation has not seen so much attention in large scale fermentations, due to still existing a gap of information regarding how it can be applied to the fermentation broths for the recovery of a desired product. If this approach is going to be practiced, we first need to have an understanding on its basics - how does the mass transfer works, what kind of material can be used and their characteristics for a well thought application and how in the end if it is efficient in the recovery of the desired product. Almost every study regarding the adsorption of said components is done isolated, meaning they're not performed in the more complex fermentation media but in much simpler solutions. Still, this data can bring us a step forward on how to indeed apply adsorption in real fermentation broths and eventually, applying it to large scale processes.

The production of lysine through fermentation was looked into with the variation of important parameters, such as operation temperature, pH, mode of operation and what type of organisms proved to be the most adequate for its goal. On the other hand, the study of adsorption was not only observed for lysine, but also for other components, such as sugars, phosphates, urea, etc, that are commonly found in such fermentation media, using different materials for the extraction of such species.

2

Literature Review

Contents

2.1 Lysine Fermentation

Lysine, or more specifically *L*-Lysine ($C_6H_{14}N_2O_2$), represented in figure 2.1, has a molecular weight of 146.19 Da and it is considered a basic amino acid due to having two amine groups, one on the alpha-position and other at epsilon-position with pk_a values at 2.2, 9.1 and 10.7. [12, 40]. It is considered a fundamental amino acid for humans and animals health due to its nutritional value [10, 41]. *L*-Lysine belongs to the *L*-aspartate family of amino acids (AFAAs), which means that, alongside other amino acids such as *L*-methionine and *L*-threonine, they can not be synthesized by humans or most farm animals. The only way to obtain it is by its consumption through meat (bovine, pork and poultry, which lysine is fed through feedstock), dairy (mainly cheese) and eggs. [42, 43].

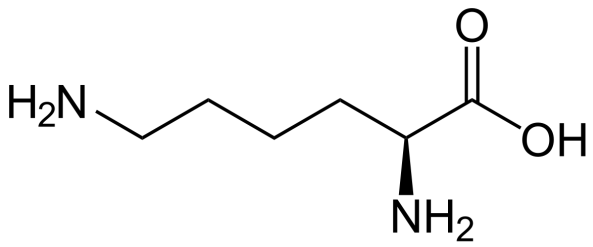

Figure 2.1: Stereochemical Sctruture of *L*-Lysine amino acid.

The production of lysine can happen by both chemical routes or by the biochemical method of microbial fermentation. It was observed that lysine produced by chemical synthesis methods resulted in racemic mixtures, having both the *L* and *D* monomers of the amino acid [10–12]. When using the biochemichal methods, it was noted a significantly larger production of *L*-lysine when compared to the synthetic processes, with the fermentative broths producing almost 100% of the *L* monomer [11, 12]. The explanation for this resides in the metabolic pathways, due to the most important intermediates are in a *L* assemble [43].

Having found a lot of different applications in many different fields, lysine became one of the most comercially important amino acid [44]. It is mainly used for supplement of feed stocks for the meat industry as a food additive, due to being a more economic option than threonine or methionine [12, 45]. It can also be used as a cross linking on collagen peptides (by interacting with arginine) and for the modification of histones. The use of *L*-Lysine in medicine goes from its helpful role to overcome diseases such as *angina pectoris*, unclogging of arteries and cancer prevention. It can also have its use in the cosmetic industry or as a chemical agent [10, 12].

2.1.1 Fermentation Parameters

The fermentation of lysine must follow established parameters so that the micro organisms in the medium can produce the amino acid at the best rate possible. Such parameters include temperature, osmotic

pressure of the medium and respiratory conditions, and its influence on the optimal stoichiometric metabolism will be described in this chapter in the following sections. This parameters will be heavily dependent on the organism used in the process, specially its specific strain. To obtain a successful fermentation, there must also be a special attention to its medium, mainly the carbon and nitrogen sources, vitamins (such as biotine) and salts [11, 43]. Most studies on this fermentation found in literature are focused on one parameter at the time, with a few researches giving attention to maximum two, usually temperature and pH.

2.1.1.A Appropriate Organisms

For the intent production of *L*-lysine, a primary metabolite, the used organisms must have two main properties namely, they must be homoserine auxotrophic (due to a defect of homoserine dehydrogenase) and S-(2-aminoethyl)-*L*-cysteine (AEC) resistent. Nowadays, most strains applied in the industrial processes are build to combine both traits described [9]. Experiments done in 70s when the lysine fermentation process was starting to grow, many bacteria were tested to check which one had the best production of the amino acid. Microorganisms such as the genera of *Brevibacterium*, *Corynebacterium*, *Microbacterium* and *Micrococcus* were put into the same fermentation conditions and mediums. All bacteria had positive results except *Microbacterium* which showed the lowest production overall in lysine concentration units. *Corynebacterium* showed the highest values at work conditions of 30 ℃ at neutral pH values of 7 and 7.5, achieving a final lysine concentration of 20.8 and 3.77 g/L, respectively [46].

Currently, the most used organism is *C. glutamicum* (fig. 2.2), with other species such as *Brevibacterium* (with its subspieces of *flavum* and *lactofermentum*) being occasionally used. *E.coli* strains have also been found to be effective in the same kind of fermentation when properly engineered [9, 47]. *C. glutamicum* was found as the better host, especially in large scale processes, due to its physiological properties, being generally regarded as safe (GRAS), having a fast growth rate, being genetically stable and capable of using a variety of different carbon sources (pentoses, hexoses, and alternative carbon sources), making it accessible to manipulation and cultivation in more harsh industrial conditions [13].

2.1.1.B Temperature

The temperature that the fermentation is settled can influence the overall production yield of lysine. Regarding this parameter, most processes regarding fermentation of lysine are done in mesophilic conditions, usually below 35 ℃. Being an exothermic process, studies show that an increase in the temperature of the medium lowers the viability of the entire process and can put it at risk since it affects the growth of the organisms and even the stability of the end product [11]. Due to this, a functional and operational cooling system is advisable to keep the temperature at its ideal for the growth of the bacteria and production of the amino acid, specially if the production plants are located in tropical regions.

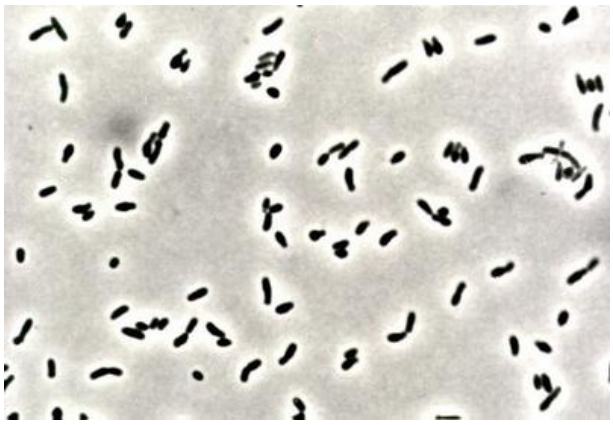

Figure 2.2: *Corynebacterium glutamicum.*

Many researches are still looking for economically and favourable processes where higher temperatures can be applied without bringing disadvantages to the process, considering the high costs of the cooling operations. Some studies involving thermo-tolerant bacteria such as *Bacillus licheniformis* and *Corynebacterium thermoaminogenes* have been performed, but no positive results have yet emerged from such works [11, 48]. Since *C. glutamicum* is the most used bacteria for *L*-lysine production, many studies have been using genomic breeding to check if it can bring advantages when talking about resistance to the temperature of the medium. Some genetic recombinants show production of lysine in temperatures only between 30 and 37℃, with most of the commercially sold strains are based in the repetition of random mutations and selections [49].

In the study performed by the group of Toshihiro Tateno, an increase of temperature in a recombinant *C. glutamicum* that can degrade starch with a higher efficiency by using an amilase from *Streptococcus bovis*, the overall yields of production lowered from 18.80% to 2.38% when temperatures increased from 30 to 40℃, while keeping the pH level constant at 7.0 [49]. This shows that, without a specific mutation in the temperature tolerance genes, *C. glutamicum* will not perform well in fermentation processes with higher temperatures.

The research group of J. Ohnishi [48] proved that a specific mutant of *C. glutamicum* that they called AHP-3 can operate functionally at 40℃ and provide an improvement of 20% in production yield for lysine (reaching a final titer of 85 g/L) when compared to the 30℃ culture, but with a decrease of 75% in the growth, as indicated in figure 2.3. They also concluded that when subjected to conditions of stress, adaptive responses are likely developed, but in this situation were not related to the rise of temperature. These findings opens new paths for future investigations and possible new applications in large scale processes, which can reduce its overall costs due to the lower of the cooling tariff.

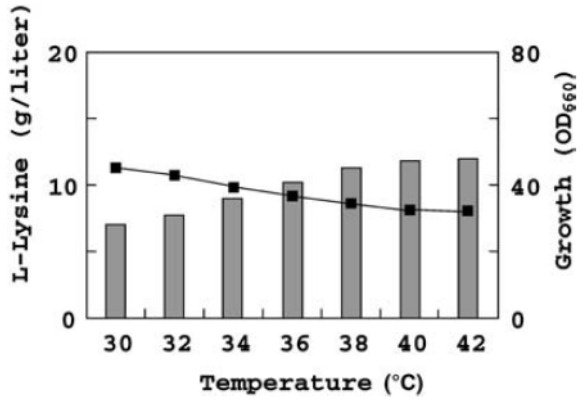

Figure 2.3: Growth levels (black squares) and *L*-lysine production (shaded column) of strain AHP-3 in jar-fermentor cultivation at temperatures from 30℃ to 42℃.

2.1.1.C Oxygen Supply

Being an aerobic process, the supply of oxygen to the lysine fermentation is considered critical for the productivity of this amino acid, since the level of oxygen in the medium is directly related to the metabolic activity, verified by a linear production of ATP. Nonetheless, the increase of the oxygen in the medium can later develop into oxidative stress and further decrease in the global productivity [11, 43]. To avoid events such as these, the process must be under the advisable oxygenation conditions, with studies showing the higest levels of production with an oxygen saturation of 50%, when happening at 35℃ at pH level of 7.5, according to Ahmed *et al* [50].

A study conducted by A Hadj Sassi *et al* [44], looked into how the oxygen and carbon dioxide could influence the overall production of lysine when using *C. glutamicum*. Performed in a bacth culture in 8L of working volume, 27℃, pH 7.2 and a carbon source of glucose (180 g/L inside the medium), the aeration rate was altered between 0.5 to 1 vvm, with the highest lysine production happening at 0.75 vvm with a 50% increase. This means a 30 to 35% saturation of oxygen in the medium, with a redox potential of -140 mV, and a 20% increase of final lysine concentration (reaching 48.5 g/L) when compared to cultures with a saturation below 20%. Unfortunately, the authors don't indicate how much the stirring rate was. This increase in oxygen availabilty also resulted in a smaller production of by-products, such as isobutyric acid.

When testing how the presence of CO_2 could influence the process, the authors maintained the ideal oxygen saturation and increased the concentration of CO_2 in the medium. This increase revealed a reduction in the production of lysine by 17%, with the carbon being redirected to the formation of lactic acid, due to the cells giving more importance to the glycolyic activity, enhacing the necessity of the removal of this component.

Thus, to maintain the level of oxygen in the fermentation constant, available and at the desired

value, strategies such as increase in oxygen partial pressure in the bioreactor, which increases the gas solubility and can be implemented to avoid shortage of oxygen in the medium, by changing the head-space oxygen content. This improves its presence in the medium during the process. Of course the choice is dependent of the characteristics of the medium such its rheology, properties of the end-product and overall cost of the procedure [51].

2.1.1.D Osmotic Pressure

Another important parameter to consider in the fermentation is the osmotic pressure that affect directly the *C. glutamicum* cells and can be a limiting factor in the production of lysine due to the osmostic stress effect in the medium. Since *C. glutamicum* is a gram-positive bacteria, the whole cells tend to contract in hyperosmotic stress instead of plasmolysis when osmotic pressure rises in the medium, mainly due to a higher intracellular pool of amino acids which gives them a higher turgor pressure [52].

The research done by the O. T. Skjerdal group [52] showed that, in their conditions of 30℃, pH 7,0 and an oxygen saturation of 30%, gram-positive baceria tolerate higher levels of stress than gram-negative bacteria like *E. coli*. For the gram positive bacteria tested, *C. glutamicum* tolerated higher levels of stress when compared to *B. lactofermentum*, such as increasing NaCl (2.1 osmol/kg), sucrose (1.6 osmol/kg) and lysine (1.7 osmol/kg) concentrations, by maintaining a bigger relative cell volume when compared to the same cells in an unstressed environment. Nonetheless, the growth rate had a decrease with the increase of the input stress.

2.1.1.E pH

Alongside osmotic pressure in the medium, the pH level must also be regulated as to facilitate the export rate of lysine to outside the cell, making it easier to collect. This excretion *via* an export system (that consist in a symport with two hydroxide ions) is highly dependent on the membrane electric potential which is consequently dependent on the pH and lysine gradient between the cell and the outside medium [53].

When tested in continuous, the best value of pH where the export rate of lysine reaches its maximum was founded to be at neutral values, with the export enzyme carrier activity reaching its higher capacity at a pH of 6.5 and an extracellular concentration of approximately 130 mM of lysine. It is near this neutral pH values that the carbon source reaches its highest levels of consumption and, eventually, we have the highest yields of production for lysine. When analysing different values of pH (below and above 6.5 and 7.5, respectively), it was noted that the biomass concentration stayed constant at 25 g/L, but the production of lysine decreased notably to below 60 mM in the medium [53].

Another study supported the idea that the best production of lysine happened at pH 7 (at 30℃), with the culture and its growth rate decreasing for lower pH levels, being observed by OD_{600}. This study was

also able to conclude that, for the *C. glutamicum* production of lysine, the best conditions were at pH 7 at 30℃, after testing different temperatures as well [49].

2.1.1.F Manipulation of Biochemical Routes

To improve the production of lysine in *C. glutamicum* the most common approach inside the metabolic engineering, has been to reduce the specific growth rate of the organisms by redirecting the carbon source to the synthesis of lysine [54]. As it has been studied, *C. glutamicum* displays two main pathways to synthesize lysine, both being diaminopimelate routes - aminotransferase pathway and succinylase pathway [55, 56]. The most important precursors are pyruvate and oxaloacetate (OAA), which means that an attempt of lowering their availability for other routes can improve the formation of this amino acid. Ideally, if we increase the replenishment and decreasing the consumption of precursors we improve its availability, thus increasing the lysine final concentration. This can be made possible by genetic manipulation of the expression of enzymes [55]. As seen in fig 2.4 ([55]), the biochemical production of lysine is heavily dependent on the presence and absence of a variety of enzymes. As pointed out by many studies, the overexpression and deregulation of certain enzymes is directly correlated to the improvement of the production of lysine. This manipulation is usually accomplished via plasmid.

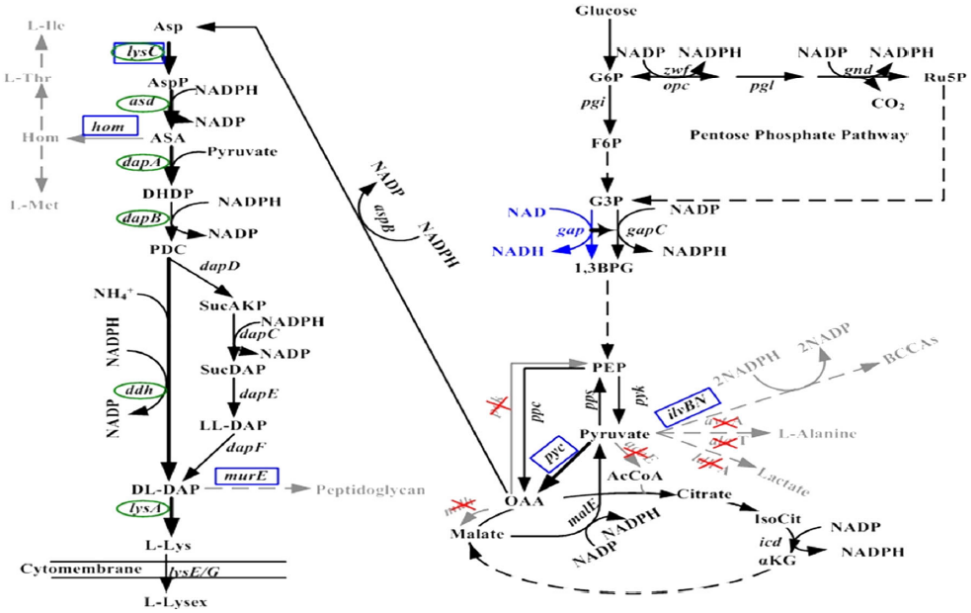

Figure 2.4: Schematic representation of metabolic engineering strategy for genetically modifying C. glutamicum strain for L-lysine production. Gray arrows represent the interrupted or attenuated pathways, red cross represents the gene knockout, green ring represent the gene over-expression, blue line and box represent the gene replacement, and the thick arrows represent the up-regulated pathway.

The research group of Jianzhong Xu decided to approach how the overexpression or deregulation of the enzyme genes could influence the production of lysine [55]. At 30℃, pH level of 7 and a carbon

source of mainly glucose, a fermentation of various recombinants of *C. glutamicum* was performed in a batch mode in shaking flasks.

The main objective of this study was to increase the quantity and availability of the precursor OAA. By knocking out the encoding phosphoenolpyruvate carborykinase gene (*pck*), and reducing the expression of the coding genes of *ilvBN* and *pyc*, the precursors were redirected to the lysine formation pathway, decreasing the production of by-products, such as lactate and *L-Alanine*. Further down the pathway, *hom* was also attenuated as to most of the AspP intermediate continued on the inteded route. The mutation of *murE*, who its believed to lower the cell growth and glucose consumption for it, showed a limitation in the synthesis of peptidoglycan (without provoking damage the cells) and a higher conversion rate of the carbon source by leading more diaminopimelate to the production of lysine rather than peptidoglycan. By adding this final mutation, the production of lysine achieved a final concentration of 130.82 g/L, with a glucose conversion efficiency of 47.06%. Unfortunately, most cells were left impaired due to the simultaneous over-expression of *lysA* and reduction of *murE*, leading to a dangerous lack of formation of cell-wall peptidoglycan. The authors conclude that, except, for the over expression of *lysA*, they obtained a viable strain capable of highly improving the production of lysine using *C. glutamicum*.

These results are supported by other studies regarding the influence of the encoding genes. The deletion of the *pck* is considered a common procedure for *C. glutamicum* to secure the production of OAA. The over expression of the enzymes coded by *lysC* and *lysA* that redirect the carbon intermediates to the formation of lysine, instead to the formation of peptidoglycan, are also described by other researches. The attenuation of *hom* gene is also advisable to lower the production of by-products as well [57–60].

The influence of the encoding gene *pyc* was studied more in depth by Peters-Wendisch *et al* [61]. The correspondent enzyme, PCx, can turn pyruvate to OAA in a simpler reaction that skips the Krebs Cycle (fig. 2.5). This gene is present in the wild type of *C. glutamicum*. Using non-mutant strains, the bacteria was able to produce 34$\pm$1 mM of lysine, but with its over expression it resulted in an increase up to 50$\pm$2 mM. On the other hand, its inactivation displayed a decrease of 60% (14$\pm$1 mM) for the same initial concentration of glucose of 40 g/L. By-products like threonine and homoserine registered final concentrations below 1 mM.

In the same study of Xu *et al* [55], it was looked into how the NADPH supply is important and critical for the formation of lysine, specifically for the precursors. As NADPH is a very much needed co-factor, its majority is produced through the pentose phosphate pathway (PPP). But, the enhance of PPP can lead to an increase of produced CO_2, decreasing the conversion of sugar to the lysine formation pathway. To avoid this situation, a gene from *E. coli* capable of producing GADPH was inserted to generate a higher concentration of NADPH during glycolysis. It is then concluded that increasing the NADPH with GADPH is a better decision than reinforcing the PPP as a way to decrease the production of by-products.

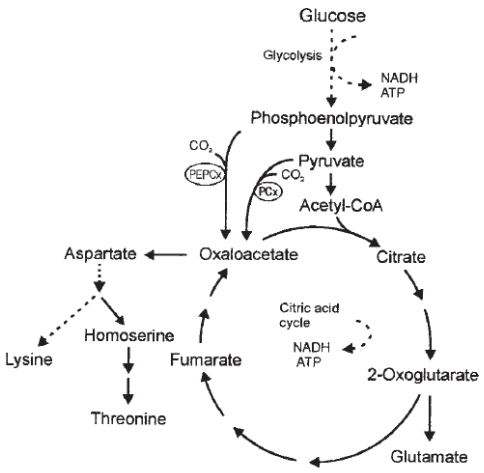

Figure 2.5: Diagramm of the central metabolism of *C. glutamicum* with a carbon source of glucose and the relationship between PCx and the formation of components such as lysine, homoserine and threonine.

Steffen N. Lindner *et al* [56] also studied how the lysine production pathway can be manipulated by focusing on the increase of phosphoenolpyruvate (PEP) as a way to increase the amount of OAA, by making the conversion of PEP to pyruvate irreversible through PEP-dependent phosphotransferase system (PTS, specific for the transport and phosphorylation of several sugars [11]) in a glucose intake by *C. glutamicum*. PEP is one of the most important precursors for the formation of lysine since its absence can limit its production. PEP is generated right after glycolysis so its directly dependent of the carbon source. For this study, the cultivation of the recombinant cells were done in 50 mL in shake flasks at 30℃, using glucose as carbon source.

A PTS-deficient strain with overexpression of inositol transporters (that are able to use alcohols as carbon source and also transport glucose) showed a slower growth of *C. glutamicum*, due to to the transporters having a low affinity for glucose at low concentrations such as 2.8 and 1.9 mM. On the other hand, at PTS-independent strains, the glucose uptake becomes relevant at higher concentrations than the ones previously tested. In these PTS-independent strains, there must a phosphorylation of glucose thats follows the uptake by the inosiol transporters. This route actually leads to an accumulation of more 10 to 20% of lysine than when using a PTS-dependent strain, with even lower production of by-products (such as lactate). Nonetheless, it was not proven to be a higher carbon flux for PEP and furthermore OAA. This works concludes its results with the idea that an efficent inhibition of PTS-dependent system, alongside an overexpression of the transporter of inositol, can increase the lysine production by 20%.

2.1.1.G Mode of Operation

With the necessary conditions provided for the growth of *C. glutamicum* and its production of lysine, the process can be performed in different ways, usually between batch, fed-batch or continuous.

In batch cultures, since the medium is not renewed, the bacteria has a tendency to suffer a catabolic repression and an additionally osmotic pressure that begins in the starting point due to the high concentrations of different components. Towards the end this stress increases, mainly due to the accumulation of the amino acid end product in the medium along other by-products, such as carboxilic acids, which can even lead to the deactivation of the overall fermentation. It is no longer considered a preferred method and it is only used in smaller fermentation plants. A fed-batch culture can operate as an alternative to batch operations since they can avoid loss of productivity due to product and by-product repression, thanks to the constant feed of low values of carbon and nitrogen components. A method called repeated fed-batch has seen a worldwide growth by operating in a semi continuous way - part of the culture broth is removed at the end of a specific time period, but leaves a fixed amount of 10-20%, serving as inoculum to next fermentation. However, this brings problems such as high risks of contamination and undesirable mutations due to the high number of generations [11, 43, 44].

A. Hadj Sassi [62] tested the differences in performing a batch and a fed-batch fermentation of *C. glutamicum* to produce lysine. Both processes occurred under the same conditions - glucose as carbon source, 27 ℃ at a pH of 7.2 and an oxygen saturation of 30% in 8L of working volume. For the case of fed-batch, there is a small but constant feed of carbon and nitrogen sources of glucose (from 0 to 20g/L by a feed pump) and phosphoric acid (at a constant concentration of 7.5 ml/L), which are proven to improve the kinetic parameters and physiological state of the culture. In both batch and fed-batch cultures, lysine production reached its peak during the lower growth rates (0.016 and 0.024 h^{-1}, respectively), explained by the higher consumption of carbon being towards the production of lysine, instead for the growth replication of cells. This study ends up showing the flaws of the fed-batch operation - there is a higher production of by-products, such as lactic and acetic acid, and therefore, a lower productivity for lysine. Nonetheless, in the overall process, it reaches a higher final lysine concentration of 110.6 g/L, with a respective yield of 0.70 g/g_{sugar}.

Osmotic stress still occurs due to the retention of lysine in the reactor. To overcome this problem, it has been suggested the use of osmoprotective compounds to the fermentation medium, such as glycine-betaine, glutamic acid and trehalose, as a way to reduce the stress in the medium [11, 62]. By reducing the stress in the cells and maintaining the respiratory quotient, they are able to maintain and even increase their growth rate and further production of lysine.

A continuous type fermentation ends up presenting the most advantages when compared to the previous modes of operation in large scale productions, since it averts problems like osmotic pressure and end-product repression thanks to its constant nutrient input and product and by-products removal [11]. Unfortunately, since continuous fermentations have longer working time periods, this means that despite reaching high end-product concentrations (60 to 100 g/L), their productivity and process yields are lower than with those of batch and fed-batch processes, with values of only 0.2-0.3 g/g of glucose

[63]. Other disadvantages are how the system has a higher vulnerability to contamination and the genetically destabilization of the used strain, making the work with mutant strains more complex, since it is imperative that these remain invariable throughout the process [11, 43].

In a research from Kiss and Stephanopoulos [63], it was tested how a fermentation of a strain of *C. glutamicum* in a continuous mode performed. Settled in a 3L of working volume, at 30℃ and a pH of 7 with an air flow of 1 vvm (volume of air per volume of liquid per minute) and a carbon source of glucose of 20 g/L and *L*-threonine of 100 mg/L in the feed, it reached steady state after the variables remained constant for 3 to 5 residence times, with only the first steady state having its behaviour studied and evaluated. At steady state, *L*-threonine was below the detection limit of 5 mg/L, with its specific uptake rate proportional to the rate of cell growth, leading to the conclusion that *L*-threonine was only used for biomass production.

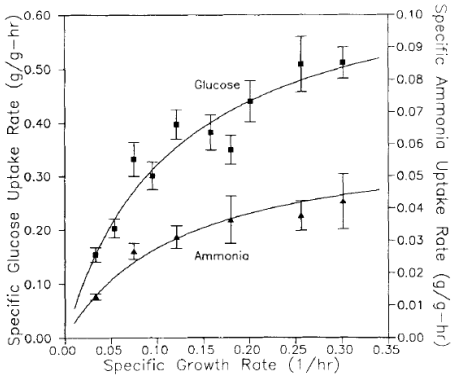

Figure 2.6: Specific glucose and ammonia uptake evolution regarding the specific growth rate during steady state. This shows how the nutrients uptake reach a steady value, meaning saturation.

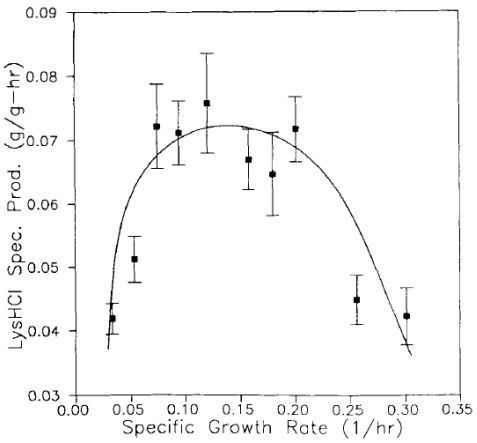

Figure 2.7: *L*-lysine specific productivity evolution regarding the specific growth rate.

The uptake of glucose and ammonia reached a saturation with the growing specific growth rate, as shown in fig. 2.6, while at the same time, the production of lysine dropped at the higher growth rates (fig. 2.7). The authors justify this event by saying that glucose and ammonia are now taking part in the biomass and lysine production at the same time. At specific growth rates between 0.1 and 0.2 h^{-1}, the specific production of lysine reaches its peak in this process. The decrease of the specific growth rate also increases the RQ due to the diversion of carbon to the synthesis of lysine, and therefore, increases the overall production yield, with its maximum above 0.25 g of lysine/ g of glucose.

From this research, and what was already talked in the previous topic of biochemical pathways, the main nutrients necessary for the production of lysine are also being used for the production of biomass and its cell growth, confirming the statement that lysine is a primary product. As many intermediate species participate in the cellular growth and multiplication, the formation of lysine ends up being linked

to it. With the constant feed of carbon and nitrogen to the medium, to improve the yield of the process, the nutrients must be redirect to the synthesis of lysine by adjusting the process conditions or by genetically engineering the organism that will be used [11, 63].

2.2 Principles of Adsorption

The concept of adsorption from a solution by a solid stems from the observation that the composition of a mixture after contact with a solid adsorbent is different from its original composition [64,65]. The material of the solid surface where the molecules will be retained will be characterized by its specific surface area and volume, since the efficiency of adsorption processes also depends on the solid surface. This solid phase is called adsorbent and the substances (adsorbate) accumulate in its surface due to different types of interactions [66, 67]. This phenomenon is a mass transfer process that leads to the removal of molecules from a fluid bulk phase to a solid surface. This mechanism represents one of the most important separation techniques nowadays [64, 65, 67, 68].

The adsorption on the solid surface happens when the molecules or atoms on the solid surface have residual surface energy. When some substances collide with the solid surface, they are attracted by stronger forces and adhere there. With the existence of different kinds of forces and interactions, this process can be divided into two categories namely physical adsorption and chemical adsorption [67,69].

Physical adsorption, or physisorption, occurs when the interaction between the adsorbent and the adsorbate is weak, translating in such as electrical attractive forces (weak Van der Waals forces, hydrogen bonds and polar iterations), without changing the chemical properties of both components. When it happens, it forms a single or multilayer of adsorbate on the material surface. It is also considered, energetically, a spontaneous process, by having low activation energy. The main features to expect from a physisorbent include high specific surface areas and high selectivity. Some of these materials include activated carbon and zeolites. On the other hand, for chemisorption to take place, there is the need for a chemical interaction to happen between the adsorbent and adsorbate, such as the establishment of covalent bonds due to a higher entalpy. With the formation of one single layer on the material surface, this type of adsorption is much stronger than physisorption. [67, 69–71].

As previous mentioned before, adsorption is considered a mass transfer phenomenon and it can be summed up in three steps: film diffusion, pore diffusion and surface reaction. The first step consist on the movement of the particle from the bulk phase to the external surface of the adsorbent, with the second step being the transport of the same particle from the external surface to the inside of the porous matrix. Both of these are represented in figure 2.8, as a schematic representation of the mass transfer. The third step of surface reactions consists in the attachment of the adsorbate inside the porous matrix of the adosorbent [72].

The overall adsorption process and rate are heavily dependent on the resistance of the two first steps, with the third and last step being the fastest, thus representing an insignificant resistance to the overall adhesion of particles to the adsorbent [72].

Usually, a suitable adsorbent has to allow for the component to desorb from its surface. The efficiency of adsorption is dependent of the conditions of the liquid environment, such as temperature,

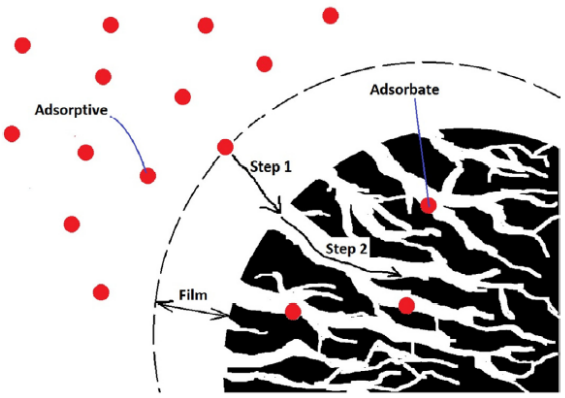

Figure 2.8: Schematic representaton of the mass transfer process in adsorption. Step 1 - film diffusion; Step 2 - pore diffusion. Surface reaction is not represented.

pH and concentration of components [65]. In theory, the adsorbent is supposed to be inert and has long been considered to be fundamental for the phenomenon, but throughout time this stance has been highly challenged, since the adsorbent's mechanical state has now seen its changes of the physical and chemical properties. If the adsorbent material suffers some sort of physical or chemical change during the process it cannot be considered absolutely inert, and it will most likely influence the efficiency of the adsorption process [73].

As it is described in image 2.9, which refers the removal of a dye from a wastewater flow [70], an adsorbing material must be able to remove, at least, the desired substance thanks to its surface characteristics. When it is time to recover the removed substance, the medium environment conditions must be changed (by altering the polarity, temperature, pH etc) so the component can be able to desorb from the adsorbent.

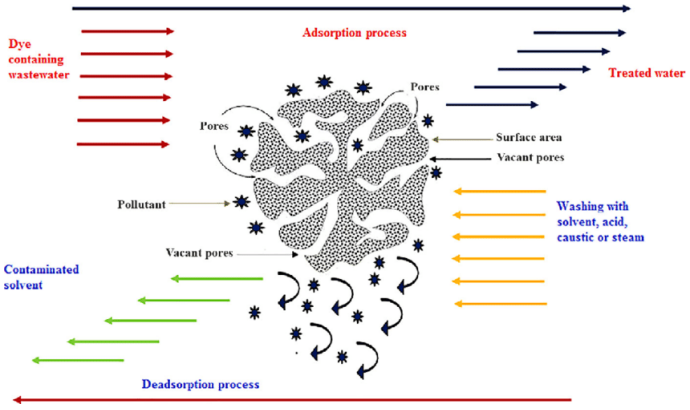

Figure 2.9: Processes of adsorption and dedsorption when applied in wastewater treatments.

Typically, adsorption is reversible as to further recover the desired substance. This is dependent of the adsorbent, which has a responsibility to remove the substances and later release them, with the

change of the environment conditions.

For most of the cases, adsorption is described at the equilibrium by means of equations called isotherms models [68, 74]. These models are called isotherms since the equilibrium they show between the adsorption and desorption rates are happening at constant temperature, and translate the adsorption capacity, mechanism and behaviour of the material [71]. Many models are being applied to the current researches where the most common ones are the Langmuir and Freundlich isotherm models.

Irving Langmuir developed its adsorption model in 1916 to describe the progress of the occupation in the distinct adsorption sites, and, to this day, has been widely used since its approach has described well various experimental data [70, 75]. It is based in three main assumptions, which are that the surface of the adsorbent material is energetically equivalent (homogenous), the adsorption sites only interact with one adsorbate molecule and these adsorption sites are considered well-defined, with the system reaching saturation when all of them become occupied [70, 76]. Since it can only be applied at equilibrium scenarios, the model translates in the equation 2.1:

$$q_e = \frac{q_{max} K_L C_e}{1 + K_L C_e} \tag{2.1}$$

where q_e is the concentration in the solid phase at equilibrium (mg/g), q_{max} is the Langmuir maximum adsorption capacity (mg/g), K_L is the Langmuir isotherm constant (L/mg) and C_e is the concentration of solute in solution at equilibrium (mg/L) [71].

In 1924, Freundlich established an alternate adsorption isotherm model. This new model described the adsorption of a single molecule in an aqueous solution at equilibrium [77]. The isotherm can be mathematically described by equation 2.2:

$$q_e = K_F {C_e}^{1/n} \tag{2.2}$$

where q_e is the concentration in the solid phase at equilibrium (mg/g), K_F is the Freundlich constant associated with adsorption capacity of the adsorbent [(mg/g).(L/mg)$^{1/n}$], C_e is the concentration of solute in solution at equilibrium (mg/L) and *n* is the Freundlich heterogeneity factor [71].

This model becomes a suitable substitute for the Langmuir isotherm, mainly in practical purposes, since the equation can describe a non-linear adsorption in a smaller range of adsorbate concentrations. Its mathematical simplicity makes it more accessible for usage while describing the adsorption process on adsorption sites which are estimated to be energetically heterogeneous, a condition more realistic of the adsorption systems [77].

The boundary layer of the gas or liquid phase is heavily dependent on the temperature criteria of the environment and the concentration of the components that are intended to be adsorbed. At low temperature and low concentrations in the bulk phase the density profile (or concentration profile) is

perpendicular to the surface generally exhibits a rather sharp step from the layer next to the surface to the bulk phase. In such a case, the concept of an adsorbed phase is well justified.

On the other hand, at high temperatures and high bulk concentrations, the density profiles exhibit a more gradual decay from the surface into the bulk solution, with the adsorbed phase becoming almost non existing [64]. The composition becomes more important rather than the concentration of the individual components present in the liquid phase [64]. When describing adsorption processes, the thermodynamic methods developed by Gibbs are the most used for the interpretation of experimental results as to check its spontaneity [73].

2.2.1 Types of Adsorbents

The type of adsorbent is crucial for the overall adsorption process. The material used must have a great affinity and select conditions for the components present in the solution that are intended to be removed at best efficiency [78–80].

The most important characteristics that make an adsorbent suitable are its large specific surface area, surface properties such as polarity, the availability of the adsorption sites and its overall degree of activation (which consists on the preparation of the material for its application as an adsorbent). The adsorption will end up following different kinds of forces, such as electrostatic attractions and repulsions, hydrogen bonds and polar interactions [81]. The adsorption capacity will be higher with a large available specific surface area, whereas selectivity depends on the characteristics of the surface of the adsorbent.

Nonetheless, having a higher quantity of adsorbent for the same amount of pollutant, will lower the ratio of adsorbed pollutant per unit of adsorbent mass. Regarding pH, this parameter affects the charge of the surface and of the adsorbate, and can determine how the interaction between the adsorbent and the adsorbate ends up happening [70]. When discussing temperature, its increase can lead to the slowdown of the adsorption, mainly due to the molecules in the medium gaining more energy and becoming least likely to adhere to the adsorbent surface, since adsorption is considered an exothermic process [82]. With temperatures ranging from 20 to 60 ℃, despite decreasing, the adsorption still was considered spontaneous, with small variations of temperature (less than 10 ℃) not altering considerably the molecular energies and neither the process for most materials [70, 83].

According to the nature of the material, adsorbents can be classified as organic and inorganic, while also being natural (such as wood, coal, lignite), synthetic (which are modified from natural adsorbents, such as zeolites and clay) and waste (such as ash, tea waste, peanut shells and fruit peels) [70, 79, 84]. Since most of the materials used as adsorbents have limitations regarding selectivity and difficulty in recycling, most of them have been facing improvements to strengthen their advantages and weaken its disadvantages. These modifications are usually about remodeling its structure while at the same time increasing the active adsorption sites, with one of the methods being the blend of two different

adsorption materials to form a composite [85].

The most commonly used material for adsorption is activated carbon (AC)/charcoal which can be produced by pyrolysis of carbonaceous organic materials such as coal, wood and husks, making them an amorphous carbon-based material that is able to capture other atoms or molecules from the environment that is inserted [67, 70]. It's mostly used as support for environmental applications like the removal of pollutants (such as heavy metals), catalysts support for chemical processes and even medical researches and procedures. The application of AC has been focused on the removal of organic pollutants from aqueous mediums but it can also extract inorganic compounds [86–88]. This adsorbent has an excellent adsorption capacity for most pollutants due to their porosity, high hydrophobicity and large specific surface area with a range than can go from 500 m^2 to 2000 m^2 per gram [70, 89]. Conventional activated carbons present an average porosity around 2 to 50 nm when mesoporous, above 50 nm when macroporous and below 2 nm if microporous [70]. Solid AC material is found to have a density range of 2000 to 2100 kg/m^3, but it lowers to the range of 400 to 500 kg/m^3 when considering the porosity and the air space present in between, in, for example, granular AC [90].

To turn the different source materials previously mentioned into adsorbents, they must be subjected to an activation which can happen *via* chemical or physical route. The type of activation chosen will dictate the diameter of the pore and the source material will highly influence its density and macro shape (whether powdered or granular), alongside its surface characteristics. For example, hard wood is a better choice as an adsorbent source material, since it produces a more stable and less crumbly AC [69]. Before performing the activation, they need to go through dehydration and carbonization steps by being heated in a range of temperatures between 600 and 800 ℃ in the absence of air [70]. Physical activation (being also called gasification) mainly uses hot gases, specially inert ones, such as CO_2 and steam, with sometimes using potassium phosphate as to produce mesoporous AC. This process allows the extraction of hydrocarbons to increase the porosity and the specific surface area. Chemical activation stands as an alternative to physical activation, where carbonization and activation tend to happen simultaneously, since the source material is mixed with a chemical agent right in the beginning. These chemical components can be phosphoric acid (H_3PO_4), potassium hydroxide (KOH), zinc chloride ($ZnCl_2$) and calcium compounds and they have the task to degrade or dehydrate the organic molecules that don't allow the deposition of hydrocarbons on the material surface, as well as to enlarge the pore size [69, 70, 91]. It's very common to see both methods being applied at the same time in a physicochemical activation, to easily achieve the wanted pore size at a higher production yield [91].

Another adsorbent material typically used are zeolites. Zeolites are hydrated crystalline microporous aluminosilicates, usually amorphous, with cavities of constant molecular dimensions (0.3–1.5 nm diameter), which makes them very selective about the components that can be adsorbed [70, 92]. This materials are employed as adsorbents in many areas and/or catalysts in wastewater treatments, petro-

chemical industries and storage of compounds [92–94].

Zeolites have different classifications mainly due to their diversity in chemical composition, crystal structure, effective pore diameter and natural occurrence [92]. When talking about natural zeolites (who are found in nature in zeolite-bearing rocks, namely zeolitic tuffs), it is quite easy to find them in nature in forms such as clinoptilolite, mordenite and phillipsite, with the even rarer offretite and paulingite. Structurally, three independent parameters must be considered regarding the classification of each zeolite: aluminosilicate framework (Si/Al ratio), exchangeable cations and zeolitic water [94, 95]. The Si/Al ratio is inversely proportional to the cation content, however it's directly proportional to the thermal stability of the zeolite [96]. Its composition also plays a part in the charge of the surface, which makes them, most of the times, negatively charged [97]. Nowadays, the majority of zeolites used are synthetic or altered versions of the natural material as to achieve higher levels of adsorption capacity for more components [93, 94].

Recently, a new alternative adsorbent has found its way in the separation and purification by adsorption. Nanoporous organic polymers are attracting attention thanks to their structural and functional versatility and applications [98, 99]. By using multi functional organic building blocks and putting them through a number of cross-linking reactions, it results in a variety of amorphous networks with a permanent porosity and high specific surface areas [99]. These porous and organic polymers are called POPs and are highly stable physically and chemically, even in extreme conditions such as high temperature, pressure and acidity. Their production displays almost no difficulties (fig. 2.10) [98, 100]. Inside this category, hypercrosslinked polymers (HCPs) are the ones who are seeing the fastest development in recent times. The synthesis of HCPs is based on Friedel–Crafts chemical reactions, providing fast kinetics to favor strong linkages which results in a highly crosslinked network with predominant porosity [100, 101]. This material has gaining more and more attention due to its requirements of low cost reagents and easy control and handle of the conditions necessary to produce a high yield functional material [100].

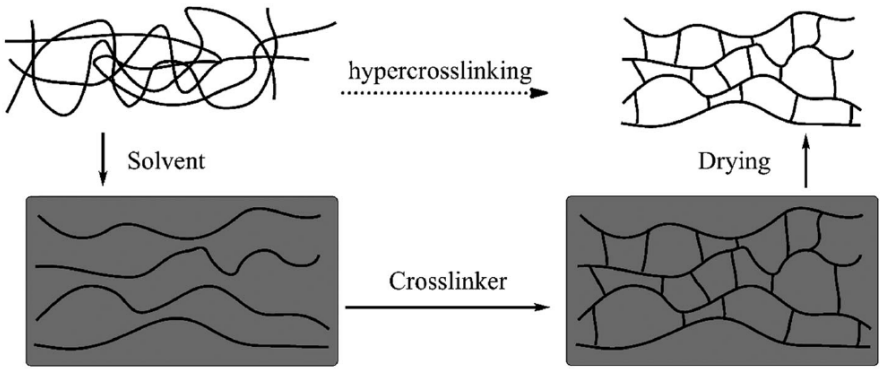

Figure 2.10: Simplified schematic representation of the hypercrosslinking process for the creation of polymer nets.

One material that can also be used as an adsorbent is silica gel (SiO_2), which is a porous, amor-

phous form of silica with an hydrophobic surface area [102, 103]. Having a completely different and unique internal structure than other SiO_2 based materials, it possess an ample network of connected microscopic pores. Its particles have a size of 2–20nm diameter wise and will aggregate to form the pores, usually in the range of 6–25nm, with large specific surface areas [104, 105]. With an isoeletric point of approximately 2, this adsorbent ends ups carrying a negative net charge in almost every working environment. This implicates that adsorption using this material would be mainly driven by electrostatic interactions [106, 107].

2.2.2 Biosorption Processing

When applied in bioprocesses, the adsorbent materials are commonly introduced in the downstream phases, since adsorption presents a separation technique. Unfortunately, adsorption as a method of product recovery has not been extensively researched, with its potential being applied in wastewater treatments and pollutant extractions. To first start to understand how adsorption can perform as a recovery method, we need to know how this process and its adsorbents perform in the different modes of operation, specially in their bed columns, which is what this section intends to do.

In a batch type operation, the adsorbent is added to the solution until the concentration of the pollutant has reached the intend level. After the adsorption, the adsorbent is later discarded or regenerated [70]. Most of the batch processes happen at a laboratory scale for the treatment of small amounts of effluent. This type of process is not feasible at an industrial point of view due to the large volumes of process streams that need treatment and ends up being limited to smaller amounts [108]. This mode of operations is useful to study intrinsic kinetics. For the many different adsorption systems, pseudo-second order kinetics provides a good description for the entire process time, describing accurately the mass transfer mechanisms [72].

For a continuous process, the adsorbent is present in a column adsorption system with a flow that could be either upflow or downflow, but almost always with a packed bed upflow carbon columns so to create a counter current and improve the removal of components. The liquid is fed through the top or the bottom of a stationary bed of adsorbent material with most designs consisting of a series of two and three packed beds, with parallel systems being also adopted. In figure 2.11, we can find a schematic representation of a continuous upflow adsorption process in a column [70, 109]. A portion of the used adsorbent is usually removed regularly from the bottom while a new portion of regenerated solid is added through the top [70]. As stated before, this requires a total or partial shutdown of the adsorption column [36]. Materials such as AC and zeolites have been proved to have a good performance in continuous processes for the removal and recovery of desired adsorbates [110, 111], unlike polymer nets like HCPs, where the high pressure drops in fixed beds make it impracticable to apply due to being a fine powder [112]. Researches of adsorption in a continuous type took place in fix bed columns. The

processes were mainly evaluated with consideration of the initial concentration of compound to extract, the height of the bed and the flow rate of the solution.

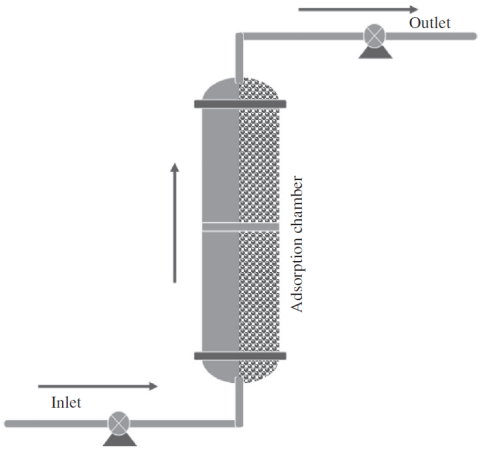

Figure 2.11: Representation of a continuous adsorption happening at an upflow stream.

In these columns, the rate and kinetic of the process can be followed and determined by the study of the breakthrough curve, which correlates the concentration of the desired component at the outlet with the passing time (as its represented in fig. 2.12). With the increasing occupation of the adsorption sites of the material present in the column, starting at breakthrough time (t_b), the concentration of the desired component will increase in the elluent. When this concentration becomes equal as the inlet, it means that the bed reached its saturation, at t_s. With this information, we can achieve the optimum operating conditions for the adsorption column, with the capacity of bed being calculated by mass transfer equations (in liquid phase studies, both heat effects and pressure drops are negligible). The higher the breakthrough time, the higher bed capacity [72].

Nonetheless, this scenario tends to assumes a zero axial dispersion which for many cases may not represent how the real adsorption system works.

At higher adsorbate initial concentrations, the driving forces in the column are typically the highest, resulting in a faster transport of the component to remove through the adsorption column. Unfortunately, this also leads to a quicker occupation of the binding sites due to smaller mass transfer zones. This conclusions were deducted by Mohammad Foroughi-dahr *et al* [108] and Bagher Hayati *et al* [109], where both researches tested the removal of congo red and heavy metals in fixed-bed systems, respectively. The extraction of congo red was performed using tea waste as an adsorbent, with the process occuring at 30℃, and achieving a removal efficiency of 3 mg/g at pH 6 and an inlet flow of 4.6 mL/min, the lowest flow applied. The removal of heavy metals showed a capacity of 432 mg/g for As(III) as arsenous acid, 494 mg/g for Co^{2+} and 470 mg/g for Zn^{2+} at bed height of 12 cm. All these metals had an initial concentration of 100 mg/L and entered the adsorption column at a flow rate of 0.5 mL/min.

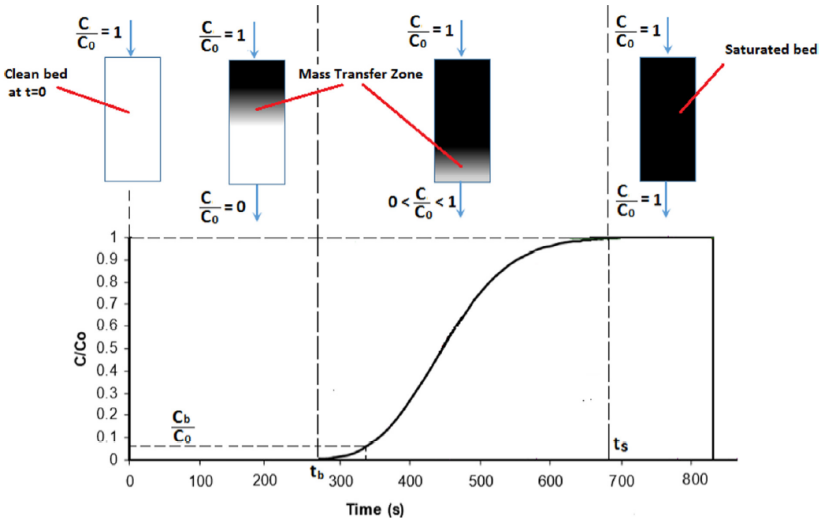

Figure 2.12: Represented is a typical S-shape breakthrough curve. The corresponding position of the mass transfer zone in the column is shown on top of the curve. C_b corresponds to the breakthrough concentration; C_0 means feed concentration.

The increase of adsorbent in the column translates to a change in the bed height. With more adsorbent, it's clear that a higher quantity can be removed from the solution due to having more binding sites available for the adsorption, thus increasing the breakthrough point, as concluded in the previous mentioned studies. With the increase of the bed height comes an increase of both time and breakthrough points, meaning a higher interaction and contact time between the available adsorbent and the components due to the increasing of the mass transfer area [109, 111]. In the study by Mohammad Amin Shavandi *et al* [111], the influence of the bed height of a zeolite packed bed adsorption column was observed in the removal of metals and residual oil. The highest removal efficiencies happened at the highest height of 15 cm, with a flowrate of 3 mL/min, of 1.455, 0.229, 0.0198 and 100 mg/g for Fe, Mn, Zn and residual oil, respectively. The research of Ki-Joong Kim [113], consisting in extraction of VOCs by modified AC in a fixed bed, showed that it is the increase of the bed height and not the diameter of the column that enhances the adsorption procedure due to the increase of contact probability.

Flow rate also has an important influence on the efficiency of the adsorption process. At higher rates there is a decrease in the contacting time between the surface of the adsorbent and the adsorbate. This means a reduction on the breakthrough point and that the rate of the adsorption lowers. On the other hand, at lower inlet rates, the breakthrough point increases, meaning a longer time in the column and a higher removal efficiency. This conclusions were drawn out from the previous mentioned researches in this topic [108, 109, 111]. In the heavy metal and residual oil extraction in a fixed-bed performed by Mohammad Amin Shavandi *et al*, the increase of the flow rate from 3 to 8 mL/min decrease the breakthrough point from 250 to 70, 250 to 50, 150 to 30 and 200 to 50 min for Fe, Mn, Zn and residual oil, respectively. The same type of conclusions were observed in the removal As(III), Co^{2+} and Zn^{2+} in the

work of Bagher Hayati *et al*, which used carbon nanotube (CNT) coated polyamidoamine dendrimer (PAMAM) as an adsorbent. The increase of the flow rate from 3 to 9 mL/min in the fixed-bed column (with a 12 cm height) proved the decrease of the adsorption capacity by the adsorbent, despite reaching equilibrium faster. It was also concluded that the adsorption process is controlled by intra-particle diffusion. Nonetheless, the increase of contact time proved that there isn't any increase in the removal of the compound [109].

It was concluded that a higher mass of the adsorbent, a lower flow rate and a lower influent concentration would be in favor of the adsorption process in a continuous type. Unfortunately, some aspects may find adversities when applied in larger scales. For example, the lower flow rates can translate into process speed problems, especially at an industrial scale with the large amount of medium solution that needs to be processed. The need for a higher quantity of adsorbent material can also lead to monetary complications due to increase of costs.

As it was previous mentioned, the fermentation of lysine also produces by-products, most of them being weak carboxilic acids, like acetic and lactic. These are usually found at the end of the microbial fermentations of lysine and can present a more vigorous competition for the adsorption sites when upon the product recovery from the broth due to being products as well. According to the following researches, the removal of acids is very dependent on the pH of the environment, despite showing some different behaviours according to the adsorbent used.

In the case of acetic acid, since it tends to interact with compounds who are more basic, the adsorption will be facilitate if the adsorbent applied has a basic nature - which is the case for some activated carbons. This conclusion is drawn from the study by Dina D.J.D *et al*, where, for the same concentration and temperature (20 ℃), pH is altered to test the adsorption in different AC adsorbents. Adsorbents with low values of impregnation ratios (who present macroporous and few microporous) displayed a higher capacity of adsorption for acetic acid. For this specific acid, it's also usual to see the formation of a multilayer in the surface with a growing thickness with the increase of adsorption time [114].

The same situation is observed when lactic acid is adsorbed by AC and a resin in similar conditions in a research performed by the group of Sahika Sena Bayazit [115]. For this case, lactic acid was adsorbed in a similar interaction as acetic acid, but adsorption reached a higher rate on the resin, due to being a weaker basic adsorbent.

A research by Kai Schute *et al* [112], shows us how the adsorption of itaconic acid (IA) proceeds in different adsorbents. All tests were performed under competition conditions with equimolar concentrations of glucose. At 20 ℃, the adsorbents who showed better efficency were HCP and A Supra EUR (an AC), and were the ones used in following procedures. According to the Langmuir isotherms, IA showed a ten time fold higher affinity towards the AC adsorbent than for HCP, specially for smaller concentrations. Despite that, both achieved equilibrium at 4 mmol/g adsorbed. When tested at temperatures similar to

fermentation processes (50 an 80℃) on HCP, neither studies displayed significant changes between themselves or when compared to the previous 20℃. The increase of pH on the medium reduced the adsorption of IA due to the continuous increase of electric repulsion between the adsorbate molecules and the hydrophobicity of HCP, and therefore, lowering the selectivity for IA. In a competitive adsorption with glucose, HCP showed a higher adsorption rate than AC. This is explained by the polarity difference between each adsorbate and the higher interactions with the hydrophobic surface of HCP. The group then applied both HCP and AC to real fermentation solutions at 20℃, and concluding similar behaviours and adsorption efficiencies for laboratory studies, but with a lower selectivity due to the presence of other components, such as ions, and increase polarity, similar to a salting-out effect.

2.2.3 Adsorption of Lysine

Since our desired product is an amino acid, we need to comprehend how these types of biomolecules act when subjected to this method of recovery. The interactions between proteins and interfaces are considered to be relevant in a large number of separation processes, with most being followed through techniques such as spectroscopy, fluorescence, electrophoresis, amongst others. The way proteins and amino acids are adsorbed can be led by different types of forces, but the most common found are by electrical attraction, hydrogen bonding and polar interactions, and its the chosen adsorbent and its characteristics that will dictate which interaction is going to dominate [116].

Since amino acids have chemical groups like hydroxides and amines alongside a diversity of side chain groups, these molecules are very sensitive to the acidity of the medium they are in, meaning that their charge is highly dependent on the pH level and that the adsorption for this molecules will mostly certain revolve around electric attraction and repulsion [97, 116, 117]. Many researches showed that the maximum removal takes place when the pH of the medium is near the isoeletric point (pI)/point of no charge of the amino acid [40, 116]. Acidic amino acids (such as glutamic acid) are expected to have a lower pI point while basic amino acids (such as lysine) will have a higher point of no charge, meaning that the highest adsorption rate is going to happen in acidic and basic environments, respectively [97]. A parameter that doesn't show any big influence in the adsorption process is temperature even when tested in different types of surface materials, like zeolites and activated carbons [97, 116, 117].

For larger peptides and proteins, adsorption in aqueous solutions to solid surfaces can be the result of various interactions such as redistribution of charged groups (mostly due to the existence of ions in the medium), changes in the state of hydration (the dehydration of hydrophobic parts of the protein and the adsorbent surface is driven by entropy gain and, therefore, promotes adsorption to occur spontaneously) and even rearrangements in protein structure. The later frequently happens when the intramolecular hydrophobic interaction is stronger than intramolecular electrostatic repulsion at a pH away from the isoelectric point. This can promote the break of the secondary structures and the loss of the structure of

the native protein [116, 117].

For the specific case of *L*-lysine, literature shows different behaviours under different adsorbent materials [107]. This amino acid has a pI 9.8 (figure 2.13 [118]) and has an amine group on its *R* side chain, translating in a third pk_a and a third electric state. At low pH values, lysine its considered a dication, where both amine groups and carboxilic group are protonated. With the increase of basicity in the medium, lysine starts to lose its protons, firstly by losing it on the carboxilic group (becoming a cation), turning into a zwitterion at around pH 10 by losing a proton on the α-NH_2 and then becoming an anion at pH 10.7 by deprotonation of the amine group in the *R* side chain [107, 119]. With lysine present in the aqueous medium, the increase of pH translates in a continuously deprotonation, with the molar fractions of each electric species varying during the process. Having four different overall net charges, the adsorption of lysine can well happen only using electrostatic interactions. An example of this is when silica based materials are used as an adsorbent, since it has a very low pI.

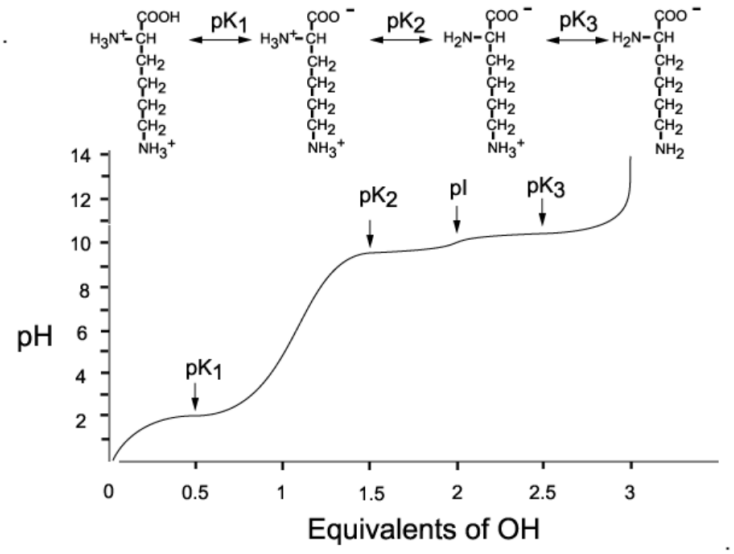

Figure 2.13: Titration curve of lysine amino acid - as you move from left to right accross the page, you are adding hydroxide to the solution and see the change of the overall charge of the amino acid. Lysine has three pk_a points, previously mentioned, with a pI at 9.8.

A study using Attenuated Total Reflectance Infrared (ATR-IR) spectroscopy followed through absorption bands and its correspondent spectra how the variation of pH influenced the adsorption on silica process [107]. When comparing the spectra of adsorbed and non-adsorbed lysine, the decrease of the pH level displayed a more intense peak in the non-adsorbed amino acid. This means an increase of non-adsorbed lysine at lower pH, and mainly a stronger presence of its cationic form. At pH levels lower than 7.1 the signal of adsorbed lysine was so low that the ATR-IR could not measure it,.The follwing tests were performed in the range of 7.1 to 9.8, with the spectra showing evidence that the carboxyl group is fully deprotonated. In the established pH range, adsorbed lysine showed unchanged dissociation states

of 81% cationic and 19% zwitterionic, both with a 5% error margins. With the increase of pH from 7.1, it does not come as a surprise that the adsorbed cationic lysine increases and the same non-adsorbed state decreases, since the silica surface becomes more negatively charged. But, in the case of lysine as a zwitterion, this species also shows an increase in its adsorption, and, at pH lower than 8.5, its molar fraction was superior in adsorbed lysine than non-adsorbed. The authors conclude that, when using silica as an adsorbent, it is the electrostatic interactions that rule the adsorption process but are probably complemented with hydrophobic relations between the zwitterioninc lysine and siloxane bridges of the silica surface.

Other studies support this statement, such as the research of Andrea J. O'Connor, *et al* [106]. By using mesoporous silica sieves, they saw how the the best adsorption rate happened at a pH of 6 at room temperature, by reaching a maximum capacity of 0.21 mmol/L, where the silica surface was negatively charged and lysine existed in a predominant state of a monovalent cation. They also explored how the ionic strength in the medium took a part in the adsorption, since its mechanism happened mainly to electrostatic interactions. The addition of sodium chloride led to a decrease in the adsorbed lysine since it boosted the electrostatic shielding and competition for the adsorption sites. The further analysis of the Langmuir isotherm also showed a possibility of another interaction playing a part in the process of the removal of the amino acid.

The research project by the group of L. Stievano *et al* [120] took in consideration the adsorption of lysine onto silica in aqueous medium, in the presence of another amino acid (glycine, with a pI of 6.02), by altering the pH. Both amino acids were tested using equimolar concentrations. It was observed that at pH 7, silica reached its maximum adsorption rate for lysine, since at that pH it is in its cationic state while glycine presents no electric charge. The increase of concentration of overall amino acids translated in an increase of lysine selectivity in the adsorption process, meaning that, it is the electrostatic interactions that rule the adsorption of different amino acids for the case of silica as an adsorbent.

The previous studies show us how lysine is best adsorbed when is in its cationic state, but other positive charged species are also found in the medium at the same pH ranges. Yanli Yang, *et al* [119], researches this topic by testing a competetive adsorption between lysine and Ca^{2+} in the surface of Na-Montmorillonite, a type of clay rich in silica, using X-ray diffraction (XRD) and attenuated total reflectance Fourier-transform infrared spectroscopy (ATR-FTIR). The study happened in three different pH levels (4, 7 an 10) at equimolar concentrations of lysine and $CaCl_2$, with an ionic strength of 0.01 M of NaCl. It was clear by the analysis of the spectrum that the presence of the calcium cation decreased the absorbance of interfacial lysine at $\sim$1413 cm^{-1}, meaning a decrease in adsorbed lysine and probably a substitution of the already adsorbed amino acid with Ca^{2+}, mainly at pH 4 and 7. The authors explain this event where the calcium cations had stronger interactions to the negatively charged adsorption sites due to their smaller size. They concluded that the presence of cations with higher positive charges could

dramatically reduce the adsorption of lysine. These cations however, can be applied on the desorption of the amino acid by replacing them in the adsorption sites.

The research group of Alisa D. Roddick-Lanzilotta *et al* [40] inspected how the adsorption of lysine would occur in another non-organic surface, this time, in a titanium one (TiO_2). The used titanium film had a pI of approximately 5 and the process was followed by the Surface Titration by Internal Reflection Spectroscopy (STIRS) technique at room temperature. With a variation of pH, it was expected that the electrostatic interactions dominated the adsorption process with the lysine only being adsorbed at pH levels above 5, where the TiO_2 had a negatively charged surface. Surprisingly, the highest amount of lysine was adsorbed near its pI of 9.8, in its zwitterionic and anionic state. The authors explain it by claiming that this is due to the hydrogen bonds interactions between the surface material and the still protonated amine in the *R* side chain. This seems to contradict the basis of adsorption in these kind of environments where there are obvious electrostatic interactions happening. The paper also mentions previous studies using the same type of material where lysine had the best adsorption rate at a pH of 7.3, drawing unclear conclusions to the efficiency of this adsorbent.

Not all adsorbents rely on electrostatic interactions for the adsorption of amino acids. Adsorption using hydrophobic materials, such as AC, can be a more suitable alternative in terms of energy, source and cost, but its potential has not been fully explored yet. In a research performed by Jeff Deischter *et al* [121], the adsorption of lysine onto different AC was tested in aqueous medium. Since AC is a hydrophobic and non-polar material, the adsorption of lysine shows its maximum at pH 10 with 294 mg/g, where lysine is a zwitterion. The lowest amount of lysine adsorbed happened at higher and lower pH values, where the cationic, dicationic and anionic states of lysine present a higher polarity.

Establishing a pH of 10, different types of AC were then tested at 30 ºC, using a concentration of 30 g/l of lysine. With adsorption capacities of 206 to 256 mg/g, *A SUPRA EUR* presented the higher amount of lysine adsorbed, meaning it had the best affinity to the zwitterionic form of lysine, due to the nonspecific enthalpy-driven hydrophobic effect and weak van der Waals forces. The same ACs were then put to test on a solution with equivalent masses of lysine and *D*-Glucose as to approach which adsorbent had the best selectivity. *KB-G* and *CW20* presented the higher separation factors (α) with 5.9 and 4.4, respectively, explained by having a high specific surface area and the most amount of oxygen groups. *A SUPRA EUR* had one of the worst selectivity with an α of 1.2. It was noted that in this competitive adsorption the better results happened using more hydrophilic carbons, probably due to glucose relying more in weaker van der Waals forces and physical adsorption based on hydrophobic interactions, with lysine relying in the interactions between its amine groups and the oxygen surface groups. Lastly, *CW20* was modified as to increase both selectivity and adsorption capacity. With the proper process, it was achieved a 13 fold increase in the separation (with an α now reaching 55.7), mainly due to enrichment in oxygen on the surface material. After the first recycling there was a decrease in the adsorption capacity,

due to the loss of some functional groups.

Curiously, not all AC show a good adsorption capacity for amino acids using hydrophobic interactions. In the study performed by Jinbei Yang [122] on a non-competitive adsorption of lysine, the use of an AC derived from coconut shell (CS) displayed the higher adsorption amount on pH 6, by removing 84% of the lysine present in the aqueous medium. At this pH, lysine is predominantly in dicationic and cationic states, which suggest that the surface of the CS AC is negatively charged, making the adsorption a process led by electrostatic interactions. The adsorption was also tested at 20, 30 and 40 ℃ at pH 6. The increase of temperature led to a decrease of the negative Gibbs energy, meaning that the adsorption process maintained its spontaneity and that it is an endothermic phenomenon when using this adsorbent.

2.2.4 Adsorption of Medium Components

The recovery of lysine by adsorption will happen when its still immersed in the fermentation medium. This presents some setbacks, since applying adsorbents in this environment will probably result in the parallel removal of other components that are not the wanted product, since many essential substances for the growth of the bacteria and production of lysine will still be in the broth at the end of the fermentation. To improve the recovery of lysine, we need to understand how adsorption performs for each medium component under similar conditions and adsorbents as to prevent it from occurring. The separation of each substance will be influenced by the type of adsorbent applied and the conditions of the environment that is inserted. Since the research of adsorption regarding recovery of products is scarce, many of the following researches focus on the recovery of one component at the time, with some performing competition studies.

2.2.4.A Microorganisms

When processing the fermentation broth for product recovery in the downstream, the presence of bacteria is usually low due to most adsorption processes implementing a microfiltration ahead [95, 112]. Nonetheless, some can flow through the membrane and end up affecting the recovery of our product by presenting competition to the adsorption sites. Like smaller biomolecules, the adhesion of cells is dictated by interfacial interactions, with surface charge and similar polarity being the most relevant, since cell surfaces present hydrophobic areas and hydrophobic molecules, which can even cause them to aggregate [123]. Regarding the cell surface net charge, most studied organisms present a pI in acidic values, which in most working conditions means a negatively net charge. Electrostatic interactions can then be responsible for the adsorption of cells, which are dependent on the working pH [124].

Different types of cells will have different behaviours when it comes to adsorption in surfaces. This is explored in the study by Jan Feuser *et al* [125], where gram positive and gram negative bacteria

(*Staphylococcus carnosus* and *E. coli*, respectively), yeasts and mammalian cells (mouse-mouse hybridoma cells) are tested to be adsorbed in a series of different ion exchange resins within an expanded bed adsorption, with the results obtained by a pulse response transmission process. At pH 7, none of the different cells adsorbed into the cation exchange matrices mainly due to electrical repulsion between the cell's negatively charged surface and matrices ligands. The decrease in the medium pH led to a rise in adsorption due to the increase of positive charges in the cells surface - at pH 5, only 38% of hybridoma cells were not adsorbed. In an anion exchange matrix, at pH 7, all cells except *E. coli* exhibit an adsorption behaviour, supporting the hypothesis that most cells are indeed negatively charged at neutral pH values and with adsorption being ruled by electrostatic interactions. In the same anion exchange matrix it was also tested the influence of ionic strengh by increasing the concentration of NaCl. As expected, this led to the suppression of the binding capacity of the cells, with over 50% being detected in the eluate. This study revealed the use of ion exchangers in expanded bed reactors to capture cells such as *S. cerevisiae* but not so much to bacteria, since the best result was a 40% adsorption of gram-positive *Staphylococcus carnosus* and 15% for *E. coli*.

For the specific case of *E. coli*, its adsorption was studied in three different types of AC (designated S, S-HCL and M) in a study by the group of J Rivera-Utrilla [126]. All of the used adsorbents presented points of zero charge at basic levels of pH, with 12.1 for S, 9.7 for S-HCL and 7.5 for M. *E. coli* presents a pI of approximately 3. All experiments using the adsorbent material were performed at a pH similar to their pI. S was able to adsorbed almost completely the bacteria present in the medium within 4 hours, where M was only able to reach 20% of its capacity. This is explained due to the material of M having smaller porous volume and for being richer in oxygen content, decreasing its hydrophobicity. S-HCL (demineralized S) showed a negligible adsorption capacity, due to the removal of mineral matter, increasing the oxygen content. But, when added electrolytes to the same medium, the adsorption rates became very high, reaching an adsorption of 87.8% when in the presence of Fe^{3+}, since its the one who creates the bigger ionic strength. The presence of this cations reduce the electrostatic repulsion interactions and give room for the van der Waals attractive interactions between the cells and the surface of the adsorbent. At the end, the best pH value for adsorption was found to be at 4.5 for S and S-HLC (both reaching similar rates), where the carbon samples and cell surface are electrical opposites, concluding that at a pH between the pI values of both the adsorbent and the organism, it would enhance the adsorption of these cells.

Regarding the cells of *C. glutamicum*, its adsorption was tested in different environments and materials, giving a special attention to the different interactions that occur during the process. As a gram-positive bacteria, the cell wall is rich in peptidoglycan with components such as teichoic and lipoteichoic acids on the external side which influence the overall cell surface net charge and polarity.

The study made by Jochen Büchs *et al* [127] followed how the consumption rate of phosphate af-

fected these components and the further cell adsorption in porous glass carriers. With cell growth happening above phosphate concentrations of 1.5 mM, at 30℃ and pH 7.2, phosphate saturated cells were compared with phosphate depleted ones. With isolectric points of around 3 and 2.5, respectively, it was found out the electrophoretic mobility of *C. glutamicum* was dependent on pH and the net charge of the cell surface. Only above pH 4 did the saturated cells become a little more mobile than the depleted ones, characteristic of gram positive bacteria, due to the higher presence of anionic groups such as teichoic acids. This means that the anionic nature of the cell wall is derived from the phosphodiester groups of this acids. A difference noted between saturated and depleted cells are the lipoteichoic acids, who are synthesised in the presence of phosphate, and reduce the polarity of the cell wall due to their hydrophobic nature. These molecules also help with the adhesion to surfaces. Regarding the adsorption of the cells to different carriers, it is expected that electrostatic interactions are the main reason for this process, making the adsorption heavily dependent on the pH. The presence of ions in the medium reduces the repulsive forces between cells and promote a better adsorption, with the highest rate being observed at the isoelectric point of the cells. For the case of glass carriers, the adhesion is favored by a higher hydrophobicity of the cells (displayed by the saturated ones).

Different materials have been tested for the adsorption of *C. glutamicum*. Tran Thi Minh Tam *et al* [128] researched the adsorption process on bacterial cellulose in a statistical form. For this case study, the main factors were the cell density and the time of the process, reaching an average of cells on the adsorbent of $47.7\,\% \pm 2$ billion clone forms per gram unit. This study occurred during a lysine fermentation at 30℃ at pH 7 and a shaking speed of 150 rpm.

A more specific study was performed by Xiufen Li *et al* [124] as to test the adsorption of *glutamicum* type cells in a polysulphone membrane (an anionic material), with the adsorption being expected to be dependent on the ionic strength and pH of the medium. Using a solution with a concentration of cells of 2 g/L, it was clear that the adsorption reached its peak at the pH of the pI of the cells (3.2), similar to previous studies and suggesting a binding regulated by electrostatic interactions and an affinity between the positive charges of the cell and a more negatively charged adsorbent. The process rate was lower on pH values below the pI but it decrease severely on values above it, due to repulsion forces. The effect of ionic strength was also observed, with results showing a decrease on adsorption on lower pH values but a rise on higher ones. This is due to what authors call a screening effect - the adsorption becomes less dependent on pH with the increase of salt concentration, since it reduces the repulsion forces and allows the positive charges to bind to the negative ones present in the surface, a similar conclusion from the study previously mentioned.

As to avoid the eventual adsorption of cells, specially *C. glutamicum*, an established pH different than the pI of the cells and lower ionic components concentrations will retard this removal and allow other components, such as lysine, to be recovered.

2.2.4.B Sugars

Sugars are commonly used as the carbon source for fermentations and productions of different components, such as lysine and itaconic acid. Being heavily present in the mediums, these molecules can affect the recovery of the product when using adsorption by presenting a competition to the adsorption sites which alters the products selectivity. The various types of saccharides are known to have different characteristics, such as polarity and hydrophobicity between themselves.

For the example of *D*-glucose, María Francisco *et al* [129] tested how its adsorption in different types of zeolites would be influenced by the presence of ionic liquids in the aqueous solution. Testing the process at the room temperature of 25 ℃, it was witnessed a higher removal of sugar on all adsorbents in the samples where the medium was purely ionic liquid (water-free). Reaching an approximately removal of 50% of glucose for every applied zeolite, this is explained by the weakened interactions between glucose and the water molecules, which favours the attraction between the OH groups and the active sites of the adsorbents. It was also found that ionic liquids richer in potassium cations favor the adsorption of glucose than smaller cations like sodium. The desorption process saw no problems, with it being performed at high temperatures such as 60 ℃ for a quicker final step. This study, which used zeolites, also concluded that the adsorbent with the lowest Si/Al ratio correlated to a smaller equilibrium adsorption for glucose.

The research group of Ziwei Liu [130] attempted a separation of a mix of eight different types of saccharides. These were in the range of 13 to 21 μL and the separation happened at neutral pH values in an AC column at 30 ℃. Three different types of AC were tested, varying their $ZnCl_2$ impregnation ratio (10, 40 and 70%). The material with the highest value performed a better separation, with trisaccharides and disaccharides being finely separated. For the monosaccharides group, *D*-ribose is separated from fructose and glucose successfully. The increase of carbons in the molecules (which increases its complexity) makes them more heterogeneous and therefore, more distinctive, explaining the better separation between trisaccharides, disaccharides and monosaccharides. This separation using AC is dominated by the electrostatic interactions between the hydroxyl groups of sugars, who bring the negative charges, and the zinc atoms at the adsorbent surfaces, who give a positively charge. Another force present in this adsorption are the hydrogen bonds between the hydroxyl groups and oxygen groups present in the surface material.

Another study using AC was performed by Michael R. Rosene and Milton Manes [131] by using the AC to separate glucose from an organic ternary solution with pnitrophenol and benzamide at room temperature. For this specific case of when sugar is in a multicomponent environment, specially with other organic compounds with benzamide, it's shown that it's one of the most weakly adsorbed, due to a higher competition from the other compounds, with the diagrams showing an almost non-existent no-competition region. An attempt on separating a mixture of glucose and fructose was studied by Guido

Schroer *et al* [132] using, as adsorbent, crosslinked boronic acid polymers. The chosen adsorbent was the one treated with phosphate electrolytes, due to a higher sorption rate, allowing the appearance and increase of porosity. The respective polymer applied had 20 mol% of DVB (divinylbenzene) since it was considered a good compromise between polymer durability while preserving the adsorption capacity, with fructose showing a higher association constant than glucose (4370 and 110, respectively). Indeed, at pH 10, the adsorbent shows a swelling of 212 and 178% in fructose, for 20 mol% of DVB and EDMA respectively, where in glucose the swelling is negligible when compared to the solution with no buffer. Nonetheless, the process takes a considerable amount of time, reaching equilibrium for fructose after 3 hours and for glucose, 90 minutes. The recovery of fructose must happen at acidic pH since that's where the sugar complexes are most unstable. For the adsorption competition test, a solution of 70:30 mol% of glucose:fructose was used. As the ratio of sugar to polymer increased, so did the selectivity toward the adsorption of fructose. More fructose means a quicker occupation of the sites in the surface, leaving almost none available for glucose. At the end, the adsorption of fructose using this kind of material reached a yield of 540 and 407 $mg_{Fru}/g_{polymer}$ for DVB and EDMA 20mol%, respectively.

Due to the chirality at many carbon atoms, the possibility of anomers, and several differences in three dimensional structures, the separation and purification of saccharides can become unpredictable [129]. Ahmed A. Darwish *et al* [133] performed a computational study (using *Quantum Espresso*) about how the orientation of glucose could influence the adsorption process in an aluminum and galium doped boron nitride surfaces (h-BBNs). In pristine h-BBNs, glucose is adsorbed in conditions that allow the lowest energy configurations and adsorption energy (0.06 eV). To improve the adsorption capacity, this material is then enriched with trivalent elements, namely, Al and Ga. These elements distort the hexagonal structure by creating a bipolar charge distortion in the surface net. In both Al and Ga-h-BBNs, the adsorption of glucose happens with the oxygen atom from the hydroxyl group (OH2) and reached higher energy levels, which was expected. With process energies of 1.27 and 0.79 eV, respectively, it was concluded that enriching the surface material with galium will provide a higher adsorption of glucose.

For saccharides, temperature is not seen as a parameter that adsorption might depend on, since its variation and further influence was not actively studied and documented. Instead, its the presence of other components that can determine how the adsorption process occurs, which is the case for the ionic strength of the medium. Kai Schute *et al* [112], in a research about adsorption competition between itaconic acid (IA) and glucose (both at equimolar concentrations) in HCP and AC (A Supra EUR) displayed a negligible influence of temperature on the adsorption of glucose, when studied at 20, 50 and 80 ℃. The increase of pH provided a small increase of adsorption for glucose, due to the decrease of it for IA and consequently, less competition for the adsorption sites on the HCP. According to the Langmuir isotherms, glucose showed a higher affinity for HCP than the AC, reaching adsorption values close to 1 mmol/g at 20 ℃ and pH 6.

The adsorption of sugars are then very dependent on the ionic strength of the medium, since they tend to strongly interact with water molecules, meaning that lower concentrations of ionic species can so hinder the adsorption onto the surfaces. Temperature and pH presented small variations to the overall removal of these molecules.

2.2.4.C Phosphates

Phosphates are present in the medium in the form of anions/salts and are essential for the growth and well functioning of the lysine producing bacteria. But since these species are electronic charged, they can present a competition for the adsorption sites when in certain environment conditions. In the removal of phosphates anions, most studies show that the cover of the surface of the adsorbent with metals components, like lanthanum, zirconium and/or iron, tend to attract, and remove, this types of components.

The following study tested how phosphate adsorption happens in zeolites who were modified with lanthanum in their surface. Yinhai He *et al* [134] performed it using an initial concentration of phosphate of 5 mg/L, and varying parameters such as temperature, pH and the ionic strength of the environment. By studying the pH evolution, it clearly shows how the adsorption of phosphate is dependent of this parameter, meaning, that its dependent of the electrostatic interactions with the adsorbent (that has a pI of 7.02). Since the phosphate species are anionic, below this value the adsorption efficiency reaches values above 80%. Above 7.02, the adsorbent increases its amount of negative charged sites, therefore increasing the electric repulsion and raising the competition with the hydroxides present in the solution, represented in 2.14. With the Langmuir isotherms correctly describing the process at pH 6, the variation of temperature show a rise on amount of adsorbed phosphate with the increase of temperature to 40 ℃. When discussing the removal efficiency in the presence of other anionic species, such as chloride, sulphate and nitrate, it shows a clear decrease due to higher competition for the adsorption sites, enhance of electric repulsion and formation of ionic complexes with the lanthanum.

The same conclusions were drawn out from a study using lanthanum doped AC by Jianyong Liu *et al* [135]. With a higher pI of 8.5, the highest rates of adsorption were observed at acidic pH as well, reaching approximately 5.5 mg/g. The increase of NaCl to 0.1 M in the medium showed a decrease in the adsorption efficiency for phosphate in 10% to 90.7%. The increase of temperature displayed a higher rate of adsorption for phosphate too, with over 9 mg/g begin adsorbed at 50 ℃ (fig 2.15).

Weiping Xiong *et al* [136] still applied AC nanofibers to test adsorption of phosphates, but this time modifying it with iron-zirconium. Like the previous researches, the highest adsorption rate was achieved at acidic pH of 4, reaching 26.3 mg/g. This was tested at room temperature. In the presence of other anions, such as fluoride, nitrate, chloride and sulfate, all compete with phosphate for the adsorption sites, with fluoride exercising a higher interaction with the adsorbent surface, reducing the rate in approximately

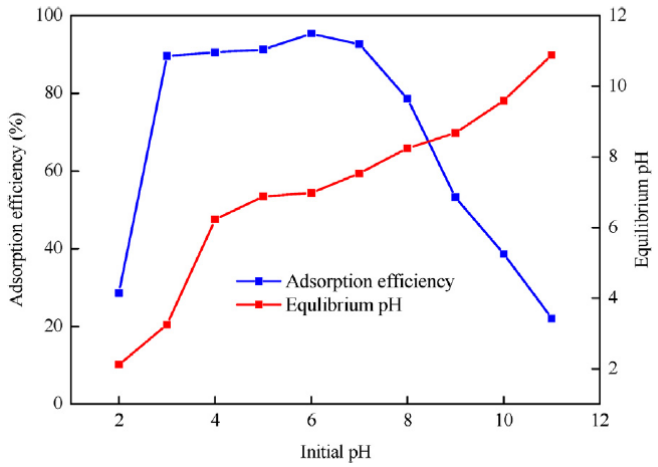

Figure 2.14: Removal of phosphate with evolution of pH in lanthanum modified zeolite.

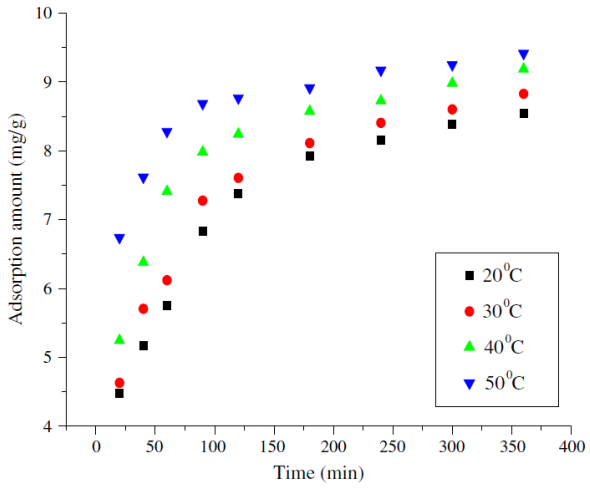

Figure 2.15: Effect of temperature on the phosphate uptake in AC.

50%. This reveals that the material interacts with phosphate also by ion exchange between its anions and the hydroxide ones.

Another study focused on the modification of AC was done by Jianmin Li *et al* [137]. This time, the adsorbent was prepared from vetiver grass-activated carbon (VGAC), using zirconium chloride octahydrate (ZR) and cetyltrimethylammonium bromide(CTAB) as modifiers. At 25℃ and an initial concentration of 15 mg/L, the increase of contact time and amount of adsorption lead to higher process yields, ending up by achieving equilibrium after 15 minutes and a removal efficiency of above 90% at amount higher than 0.3g. pH related points drew the same conclusions as the previous studies. Unlike in the temperature parameter, its increase to 45℃ show no noticeable change in the adsorption rates. At the end, the process efficiency was set at the range of 87.75–97.75% for removal of phosphate.

This addition of metals on the adsorbents surfaces improved the removal of phosphates from the solutions they were present, since the interactions between the components and the metal were much noticeable. As a large anionic and heavily charged component, it can present a considerable competition to the adsorption sites when present in fermentation broths. The use of adsorbents not modified with transitioning metals can so help to avoid the excessive adsorption of phosphates.

2.2.4.D Sulphates

Like phosphates, sulphates are essential for the growing and functioning of the bacteria since these species present a source for sulphur. Regarding sulphates anions, adsorption has been tested with mainly kinds of salts and ionic components. In a similar way, the adsorption of this anions can happen like the previously described phosphate anions.

In the research of Evgenia Iakovleva *et al* [138] there is a focus on removing sulphate and chloride from an aqueous medium. With working conditions at room temperature and alkaline environments, the adsorption was observed using different industrial wastes as adsorbents. The initial concentration for both adsorbates and adsorbent was 20 mg/L and 40 g/L. Three of the four tested alkaline adsorbents showed a preference for the smaller chloride anions, exhibiting a low competition. Nonetheless, the adsorption of sulphate anions had its lowest value at 54% and reached 99%. As expected, the main interactions that ruled this process were electrostatic ones.

The adsorption of sulphate ions was also studied in natural zeolites (flocculated and powder) by Cristiane da Rosa Oliveira *et al* [139]. With working conditions established at 25℃ and pH 6, the Langmuir isotherms show a higher adsorption in the powdered form of adsorbent with a capacity of 1.36 m_{eq}/g. Of course this process follows the electrostatic interactions between sulphates and the surface of the zeolite form.

A competition study between four anions, which included the sulphate anion, was done by the research group of Houssine Sehaqui [140], using cationic cellulose nanofibers. For this case, it is expected that electrostatic interactions will rule the process and it was observed that the adsorbent showed a preference for the species with highest negative charge, meaning sulphate and phosphate, despite their larger size. In all the tests performed, showed in 2.16, at different pH values, it was sulphate who reached the highest amount adsorbed, meaning a preference for multivalent anions, even when compared to the tri-negative phosphate anion, displaying more 6% of amount adsorbed. In a solution that included the four anionic species, the adsorption still favored the adhesion of multivalent ions but in this scenario, both sulphate and phosphate showed similar removal rates.

Sulphates present in complex molecules such as sodium dodecyl sulphate (SDS) were tested in an alumina rich adsorbent. B. Tamamusa *et al* [141] tested the variation of pH and salt concentration in this method at room temperature (20-25℃), with an initial concentration of adsorbate of 0.001M.

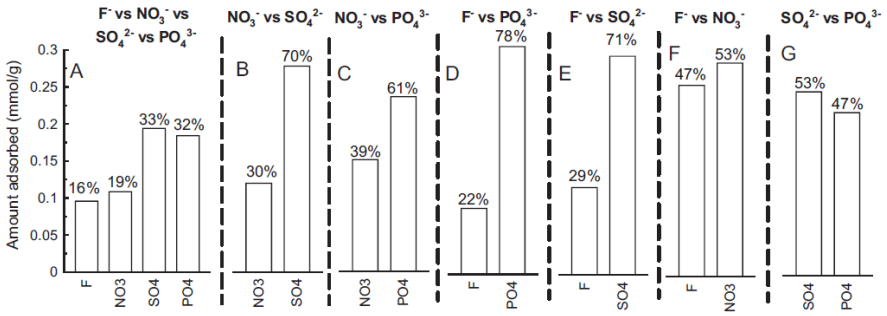

Figure 2.16: Selectivity of the cationic cellulose nanofiber, showed in molar %, regarding different anionic mixtures.

As expected, electrostatic interactions where the main strengths in the adhesion, showing the highest adsorption of SDS in acidic pH values, reaching above 0.6 mmol/g.

2.2.4.E Vitamins

As vitamins are commonly used for fermentations, such as biotin, these molecules can influence how the recovery of lysine from the medium happens, since they can still be present at the end of the process. Their removal must take in consideration their polar properties, since they are divided by their solubility in fat or water. Water soluble vitamins such as the ones of group B (B_1, B_2, B_3, B_6, B_7, etc) have a higher tendency to be adsorbed by polar materials, while fat soluble vitamins (A, D, E and K) get removed more often by non-polar adsorbents [142, 143].

Sergey N. Lanin *et al* [143] tested the adsorption of water soluble vitamins in different adsorbents (such as silica, HCP and AC) as a way to check the importance of polarity. Under the same working conditions, four different B vitamins were put to test in 1.1-1.2x10^{-4}M concentrations. The vitamins had weak interactions with silica based adsorbents, explained due to stronger interaction between the components and water molecules, while presenting stronger ones with HCPs and AC materials. In HCPs, adsorption occurred because of the contribution of the π - π interactions. Mainly, the increase in hydrophobicity of the adsorbent surfaces led to a lower rate of removal of vitamins. The polarity of the solution in the process was also studied, so different solvents were applied, concluding that the competition between highly polar water and the vitamin molecules for the adsorption sites decreases with addition of less polar organic solvents to the sample with protic solvents were considered practically inert.

Although no significant adsorption by zeolites was observed in the previous research, Zorica Basić *et al* [144] put to observation the variation of pH on the adsorption of three B vitamins (1, 2 and 6) on zeolites. Using concentrations of 1, 2 and 5 mg/L, respectively, and at 37℃, it was found that, at the acidic pH of 2, the adsorbent showed adsorption efficiencies of 21.87, 20.15 and 4.53%, respectively. This shows that zeolites have very small adsorption capacities for water soluble vitamins.

The adsorption of vitamin B_{12} on micro to nano AC was studied by the group of Rishabh Saraswat *et al* [145]. With work conditions established at neutral pH, at 30 ℃, while using initial concentrations ranging from 5 to 300 ppm, the AC beads showed a higher adsorption efficiency when compared to the polymeric beads when in batch, reaching differences of 35% and a maximum of 300 mg/g. The addition of Ni to the surface of the polymeric beads also displayed an increase on the adsorption of about approximately 10%, meaning an adsorption based on van der Waals forces.

The adsorption of water soluble vitamins is then under the control of the overall solvent and the ionic strength that it may present. In aqueous environments, these molecules show a higher tendency to interact with the water molecules rather than the surface of the adsorbents. The type of material is also important, specially a similar polarity may improve the removal of the vitamins.

The adsorption of fat soluble vitamins such as vitamin E, by Martin Hartmann *et al* [146], showed higher rates of extraction in mesoporous AC sieves when happening in non-polar solvents. With vitamin concentrations ranging from 0.25 to 60 g/L, the study was performed at 25 ℃, and it was observed the absence of solvent clusters, meaning a well mixture. The use of *n*-heptane as solvent lead to 82.8% of the total specific pore volume of the adsorbent, meaning an adsorption efficency above 80%, where *n*-butanol presented a lower value of 76.2%. P. Drott *et al* [147] also tested the adsorption of fat soluble vitamins, namely A and E, in IV medical bags made of poly vinyl chloride (PVC). Present in a mixture of lipids, carbohydrates, amino-acids and minerals, after 20h at room temperature, it was noted a decrease of 15 to 20% of vitamins. The authors also said that the decrease of the concentration on the medium was also derived due to photodegradation.

2.2.4.F Urea

Urea (CH_4N_2O) is added to the fermentation medium as it provides carbon dioxide to the cells, accelerating the initial growth of the culture. For a component like this, its removal by adsorption has been tested in many different materials. With a very low pk_a of 0.1 [148], urea is considering a cation for most common environments due to gaining a proton on the carbonyl oxygen [149], making the attachment on surfaces mainly dependent on electrostatic and polar interactions.

When studied in zeolites, V. Wernert *et al* [148] tested how the removal of urea would occur in a mixture of uremic toxins. The experiment was rolled in 37 ℃, with initial concentrations of 8.6 and 41 mM at 6 and 5.4 pH, respectively. Testing 15 different types of zeolites, the adsorption reached efficiencies above 30 mg/g and 140 mg/g for each initial concentration. Urea interacted with the adsorbent material by establishing hydrogen bonds between the amine groups and the existing surface oxygen groups, but at the end, it is the size of molecule that allowed adsorption into the zeolites. With urea being the smallest specie, the adhesion most likely takes place inside the pore system.

The research group of Tomohito Kameda [150] observed the adsorption of urea in AC. This study

occurred at 10, 30 and 60 ℃, but it's not specified what pH was established. With initial concentrations in the range of 50 to 2000 mg/L, the highest adsorbed amount was found to be at 10 ℃, with a value of 1.1 mg/g at a rate of 7.8 mg/(g.min), and decreased with the raising of temperature. This led to the conclusion that the process works in an exothermic route. The adsorption itself was explained by dipole-dipole interactions between the NH_2 group of the urea and the existing carbonyl and hydroxyl groups in the surface of the adsorbent. This managed to create a multilayer in the surface material, and concluding that it was a physical adsorption. Another study by the same group concluded that using spherical AC reached an adsorption maximum (at 10 ℃, no explicit value of pH) of 1.63 mg/g (meaning a removal of 65.2% of the initial urea). Despite that, this type of AC showed less adsorption efficiency for urea than AC fibers, mesoporous silica, zeolites, etc.

As to check the influence of pH, Safwat M. and Minerva E. Matta [149] decided to observe the adsorption of urea, both in granular activated alumina (AL) (pI of 8.8) and granular AC (pI of 7.1), in a batch study set at the constant temperature of 20 ℃. The adsorbents were put to test at three different pH values - 5, 7 and 9. The maximum removal efficiencies were both at the highest pH, reaching 24 and 31% for granular AL and granular AC, respectively, as seen in fig.2.17, at an initial concentration of urea of 1 g/L. At pH 9, both adsorbents are considered negatively charged, making the adsorption of urea ruled by electrostatic interactions. In lower pH values, the adsorption removal decreases due to the electric repulsion. In the end, AC showed a higher performance efficiency than AL.

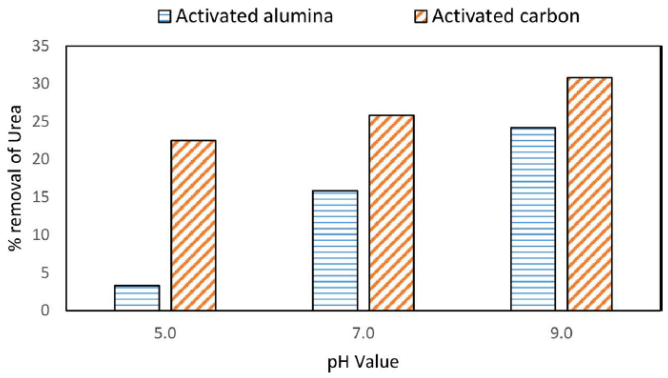

Figure 2.17: Removal efficiency of urea at different pH values using activated alumina and activated carbon.

This substance ends up showing a competition for the recovery of lysine due to its small size and higher adsorption at pH values near the pI of the amino acid. Nonetheless, the dipole interactions between urea and the surface of the adsorbents decrease with the increase of the temperature of the process.

3

Modelling Part

Contents

3.1 Adsorption of Itaconic Acid

In this first section, we have a hypothetical case of production and recovery of itaconic acid (IA) by microbial fermentation and adsorption, respectively. The parameters are established in the following table 3.1 with a simplified block diagram of the process represented in fig. 3.1.

Fermentation Reactor	Adsorption Step
Itaconic acid concentraction = 25 g/L	Adsorption capacity = 464 mg IA/g adsorbent
Glucose concentration = 5 g/L	Itaconic acid is completely adsorbed until capacity is reached
IA productivity = 5 g/(L.h)	Glucose is not adsorbed
Feeding rate is equal to glucose uptake	2 columns woking in paralel, switching after 1 hour cycles
Working Volume = 1.5 L	Working volume is constant

Table 3.1: On the left, we have the given conditions of operation for the fermentation reactor where the production of IA is happening in a fed-batch mode. On the right, the characteristics of the adsorption column alongside the parameters for the adsorption process itself.

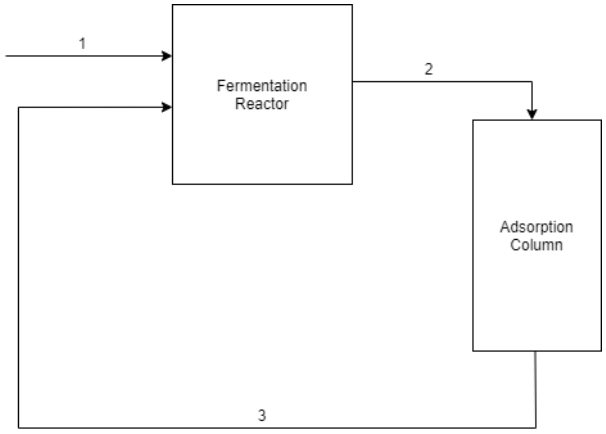

Figure 3.1: Simplified block diagram for the overall process of fermentation of IA and its recovery through adsorption in a column.

The first calculation intends to find out what volumetric flow rate there needs to be in the external loop as to maintain the product concentration constant in the fermentation reactor.

Knowing that the working volume of the reactor is 1.5 L and the process has a productivity of 5 g/(L.h), this means that, per hour, there is a production of 7.5 g of lysine. Assuming the fermentation is happening with an ideal mixing, this means that the concentration of IA (and glucose) is the same inside the reactor and on the outlet (flow number 2). With this information, we can also conclude that, since we don't have IA being fed to the reactor and not being consumed in any way, the produced mass is the same amount that exits the fermentor.

We can know calculate the volume rate that exits the reactor by dividing the produced mass with the concentration of IA that exits in the outlet, having a result of 0,3 L/h. Since we want to keep the working

volume of the adsorption column constant, the volume rate in flow 2 and 3 must be the same, meaning that the volumetric flow rate in the external loop must also be 0,3 L/h.

In the step of desorption it is used methanol, but every time it is applied, it reduces the adsorbents capacity by 20%. Since each column needs to be viable for at least 11 one-hour adsorption cycles (for a total of 22 cycles), we need to know how much adsorbent material there needs to be at the beginning of the process, so the adsorption is still 100% effective in the last of the cycles.

In each cycle, 7.5 g of IA are produced, meaning that for each cycle we need, at least, 16.16 g of adsorbent material (eq. 3.1).

$$\frac{7.5}{0.464}\frac{gIA}{\frac{gads}{gIA}} = 16.16gads \tag{3.1}$$

16.16 grams is the required amount of adsorbent material that needs to be present in the last adsorption cycle, meaning that after 10 cycles there must be 16.16 g of adsorbent in each column. The mathematical equation that represents this argument is established in equation 3.2.

$$16.16(1-0,20)^{10} = x \tag{3.2}$$

By solving the previous equation, we find out that to maintain the adsorption capacity to its maximum up to the 11th cycle, we need to implement 150.5 grams of adsorbent material in the column at the beginning of the process.

3.2 Dimensioning of Lysine Fermentation Reactors and Adsorption Columns

In a similar working process to the previous section, we now have a production of lysine in two scales, laboratory and industrial with each one having a daily production of 25 g and 420 kg, respectively. The following deductions are intend to set the dimensions for both the reactors for fermentation of lysine and its adsorption columns, in a fed batch operation.

For the first part of the sizing of the reactors, literature data was applied from an example of a fermentative preparation of lysine when using *C. glutamicum*, with example number three being the chosen one [151]. With this respective medium composition, it was found that there was a 32.3% (w/w) of sugar conversion into lysine, with 54.7% of it being located outside the biomass and able to retrieve. Assuming a total recovery of the intended 420 kg of lysine, there would need to be available approximately 2380 kg of sugar up to the end of the fermentation step. According to the recipe of the example in the patent, 26% of the sugar would be introduced within the sterile medium in the beginning of the process, where the remaining 74% being slowly introduced by the feed flow. The sugar in the

initial provided medium composes 8.6% of its total mass where in the fed batch feed represents roughly 46%.

The same example uses a 10 m^3 reactor for the fed-batch fermentation. This reactor is used as to hold the mass of the initial sterile medium of 3980 kg, alongside the total feed that will be receiving from the feed flow (2171 kg), totaling 6151 kg of broth. By applying the same mass compositions and conditions for the intended production of 420 kg of lysine, the initial sterile medium and final quantity of feed would now be 7181 and 3845 kg, respectively, adding to a total of 11027 kg. Assuming a linear expansion, the new reactor would need to have a volume of approximately 18 m^3. As for lab scale, the analogous calculations were made: an initial sterile medium of 427,8 g and a total feed of 227,6 are necessary to provide 141.5 g of sugar and therefore a production of 25 g of lysine. Since the reactor would need to be able to hold 655.4 g, its volume must be settled at roughly 1.07 L.

The downstream and recovery step using adsorption columns was deducted using equations and values found in literature. The choice on adsorbent material fell to the AC *CW 20*, as it reported a capacity of 230 mg/g [121]. This adsorbent can be provided by *Chemelco*, a chinese chemical industry, in the form of a macroporous powder with a specific surface is of approximately 1300 m^2/g [152]. To achieve a total removal of 420 kg of our product, the column would have to be filled with 1830 kg of adsorbent. With an average bulk density of 450 kg/m^3, the volume of the bed ends up being 4.06 m^3. With the recommendation made by the The Royal Society of Chemistry, the volume of the column must 1.5 time bigger than of the adsorbent bed to account the interstitial space, the total volume of the adsorption column is 6.06 m^3 [153]. With the established ratio for column design for height of the bed/column diameter being approximately 10 [154], to process the removal and further recovery, the designated column must have a diameter of 80 cm and an 8 m height. For the lab scale production of 25 g, the amount of adsorbent needed is around 108.7 g, making its bed and total column volume of the column $2.42x10^{-4}$ m^3 and $3.62x10^{-4}$ m^3, respectively. This translates in a column with a diameter of 3 cm and a height of 31 cm.

3.3 Flow Sheet Design for Lab Scale Process

For the last section, we intend to design a lab scale continuous process of lysine fermentation, including its recovery, in a flow sheet. The prepared medium for the fermentation is based upon the recipe used by the AVT laboratory for the fermentation of lysine using *C. glutamicum*, as expressed in table 3.2. The fermentation broth will be composed of a carbon source of glucose, sulphates and phosphate salts, urea, small concentrations of tracing metals (such as iron, magnesium, zinc, copper and niquel), calcium (added in the the form of $CaCl_2$), biotin vitamin, an acidic solution and a buffer called MOPS. The fermentation reactor has a designated working volume of 1.5 L, with the cultivation happening with

an agitation of at 350 rpm. The carbon source is being controlled by a valve, as is the aeration.

Substance	Medium Concentration (g/L)	Volumetric Flow Rate (mL/h)
$(NH_4)_2SO_4$	10.0	1050
KH_2PO_4	1.0	
K_2HPO_4	2.0	
$MgSO_4$*7 H_2O	0.25	
Glucose ($C_6H_{12}O_6$*H_2O)	10	150
Urea ($CO(NH_2)_2$)	2	150
$FeSO_4$*7H_2O	0.01	1.5
$MnSO_4$*H_2O	0.01	
$ZnSO_4$*7H_2O	0.001	
$CuSO_4$	0.0002	
$NiCl_2$*6H_2O	0.00002	
$CaCl_2$*2H_2O	0.01	1.5
Biotin	0.0002	1.5
Dihydroxybenzoic acid	0.03	1.5
MOPS Buffer	21	150

Table 3.2: Final concentrations for the necessary substances for the fermentation medium that must be established in the working volume of the reactor.

As previously mentioned, the best working condition for the production of lysine from *C. glutamicum* are at neutral pH values, with temperatures from 30 to 35℃. We so establish the conditions to a pH at 7.00 $\pm$ 0.02 (its adjustment can be made by the addition of NaOH or HCl) and a temperature of 30℃, since according to Kiss and Stephanopoulos [63], this conditions provide a lysine production of 0.8 g/L in glucose, with a productivity of about 0.075g/($g_{glucose}$.h), when at steady state. After the reaction, there will be a filtration step, as its considered typical for fermentation broths [112]. A microfiltration was chosen as a way to separate bigger components, like the *C. glutamicum* cells from the medium, since they have a length of 1–1.5 μm, and microfiltration is characterized for pores with a size ranging from 0.1 to 10 μm, meaning a membrane with a pore size smaller than 1 μm [95, 155]. For this step, there will be a re-circulation flow of the concentrate back to the reactor as to redirect the cells back to the reactor and to decrease the waste of the medium components. We will assume that all produced lysine was able to pass through the introduced membrane and into the adsorption column, due to the small size of the amino acid and assuming no aggregation occurred, with no amount being recirculated back to the reactor. The adsorption column will be a packed bed (as researches typically use this type of bed when operating in continuous mode) and it will use the modified AC *CW 20* as the adsorbent for this process, with a previous addition of NaOH to raise the solution pH to 10 and to achieve a removal of lysine up to 230 mg/g [121]. To represent the overall process in a schematic and simple approach, the software *SuperPro Designer* was used, and its representation is in fig. 3.2. With the established parameters, we can calculate that there needs to be at 15 grams of glucose in the medium for the fermentation to occur. Applying the productivity, we know now that in each hour it is produced 1.125g of lysine [99]. In the downstream, to recover the product with the same efficiency as the presented by the *CW 20* adsorbent,

4.89 g have to be present in the adsorption column. The recovery is a result due to the application of an acidic solution through the column.

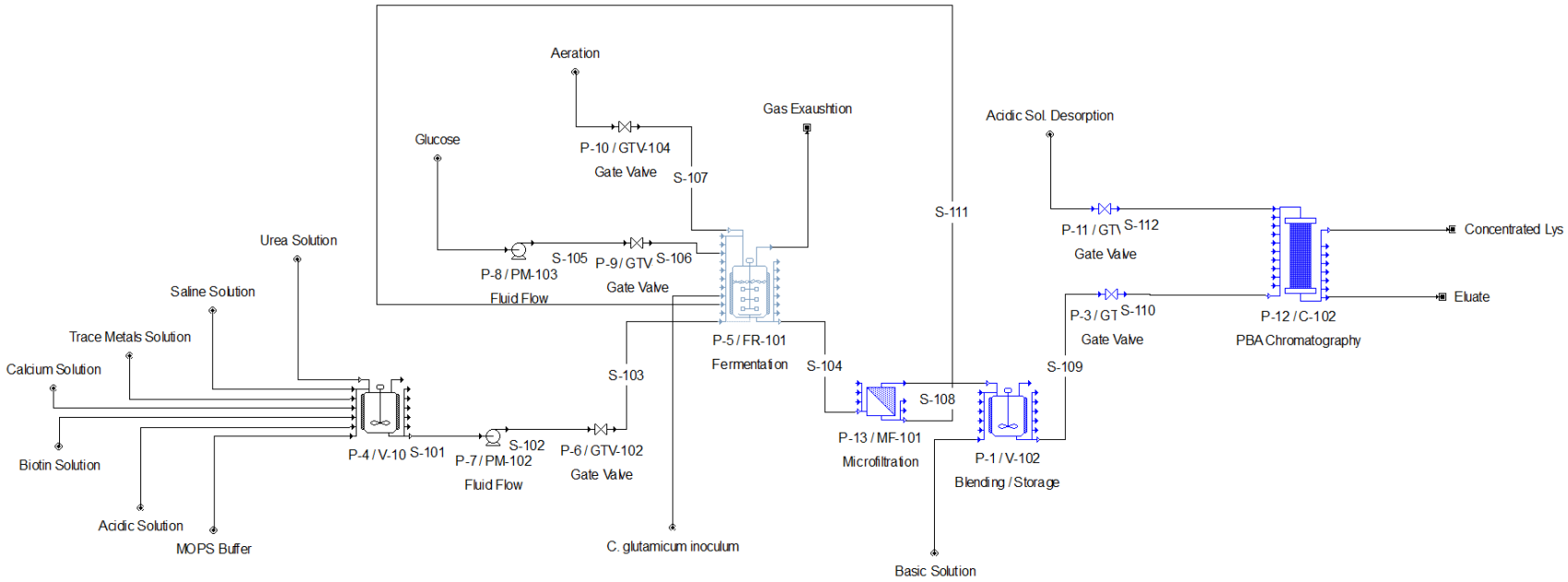

Figure 3.2: Schematic representation for a lysine production in continuous operation, including the medium preparation (in black), the fermentation reactor (in light grey) and the downstream steps (in dark blue) that include a microfiltration and a continuous adsorption. The valves and pumps in the flowing streams allow a better control of the process as to maintain its long-term viability. The used icons are a simple representation of the unit equipments.

4

Conclusions and Future Prospects

Contents

4.1 Conclusions

Despite most industrial processes are currently still working using fossil resources, there is already a change of scenery happening with biorefinery based processes starting to take place. The downstream phase still compromises the majority of the total costs of these type of processes, due to the number of necessary steps, cost of equipment and not satisfying yields, making it the major downside to turn to more environmentally conscious process designs. To this problem, adsorption presents itself as a solution, since it can be implemented in fewer steps, meaning lower operational costs and energy usage.

As stated before, this thesis focus on the specific case of the application of adsorbents to recover *L*-Lysine from its fermentation medium. As the global market for lysine is still on the rise with over 2 million tonnes being annually produced, bioprocesses such as microbial fermentations are still looked upon for improvements. The increasing necessity of substances like lysine for different industries is making a mark on the carbon footprint of today's society, mainly due to their current emissions and consumption of materials for processes with yields that could be higher. Parameters such as temperature, pH, oxygen supply and osmotic pressure have been well studied for the production of lysine, using *C. glutamicum* as host. Mesophilic temperatures, neutral pH values, oxygen saturation at a range of 30 to 35% for the gram-positive bacteria translate into the higher production yields for the amino acid.

Studies dedicated to advance the production of lysine are currently focusing on the genetic and metabolic manipulation of the bacteria capable of producing the amino acid, as a way to better employ the available carbon source towards the target product and create more resilient strains. Most of them are still incorporating over-expression, attenuation and deletion of enzymes that take part in the metabolic pathways. Researches have proven that these manipulations in bacteria, specially in *C. glutamicum*, show an increase in the production yields of lysine, alongside a reduction in the final concentration of by-products and cellular growth. This affects the operational costs, for example with the case of making the microorganism more thermo-tolerant by reducing the cooling expanses in the fermentation step, necessary to keep the viability. The increase of conversion efficiencies also translate in a reduction of costs regarding the source materials for the fermentation.

When it comes to the recovery step of lysine, adsorption has only been applied to laboratory prepared solutions that don't accurately resemble a fermentation broth. As different materials were tested for the product recovery in simpler environments, AC presented the higher adsorption capacities at the established conditions, which is the case for *A SUPRA EUR*, reaching a value of 256 mg/g in a non competitive environment. The conclusions that this adsorption happened due to electrostatic and polar interactions (being a physical adsorption), even when using other materials for adsorbents, such as silica, are also supported by the current literature.

Unsurprisingly, this types of studies are not loyal to a real fermentation broth, where there are many

more components competing for the same adsorption sites. To understand how this process would work when applied in real fermentation mediums, it was also researched how adsorption performs for the recovery of components typically found in these mediums. Most papers display the same conclusion that electrostatic interactions are the main reason for the adsorption on different materials. For these components, pH and ionic strength are the main parameters that end up controlling their adsorption efficiency, with temperature showing little influence in most of them. This presents a clear competition for the adsorption sites when working to recover lysine from the fermentation medium, since most components were adsorbed under the same conditions. The choice of the adsorbent material must then reside on the separation factors, with a preference for the highest α available for the removal of our product of interest. An approach that can retard the adsorption of unwanted substances is the changing of the conditions of the medium (such as pH, temperature, ionic concentration) ahead of the adsorption step, as is the case of filtration.

4.2 Future Prospects

As a simpler downstream method who is more economically viable due to lower operational and energy costs, adsorption has the potential to produce similar or better results for product recovery than most of the methods currently in usage. It has been widely used in bioprocessing through bed adsorption columns to extract contaminants and to purify wastewaters. Most researches regarding this method still focus on such topics, with very little information on how adsorption performs for product recovery.

This calls for extra research for the application of this technique for product recovery, mainly for industries still relying in petrochemical processes and intend to lower their carbon foot-print by switching to biorefinery ones (such as fermentative processes). The current available studies and information for recovery of fermentation products such as lysine are not loyal to the real-life situations, where the product has to be removed from mediums rich in a variety of components that present setbacks, such as the competition for the adsorption sites under the same conditions. The researches that present competition studies only focus on two components at the same time, such as lysine and glucose, which is still not enough for adsorption to reach its application in large scale processes.

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