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ADAMO EDUARDO DA SILVA FERREIRA

Simulation and techno-economic analysis of production processes of novel generation L-Asparaginases using recombinant *Escherichia coli* and *Pichia pastoris*

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Simulação e avaliação técnico-econômica de processos de produção de L-Asparaginases de nova geração utilizando *Escherichia coli* e *Pichia pastoris* recombinantes

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“Do or do not. There is no try.”

Master Yoda,

The Empire Strikes Back

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ABSTRACT

L-Asparaginase (L-asparagine amino hydrolase, E.C.3.5.1.1) is an important enzyme that presents antitumor properties being clinically used in the treatment of Acute Lymphoblastic Leukemia. The distinct microbial formulations available on the market differ in pharmacokinetics, technology, dosage, treatment protocol, and market value. Despite being a biopharmaceutical widely used as a first or second line of treatment, its production is restricted to a few laboratories around the world. Besides, techno-economic analyzes of the L-Asparaginase production process are scarce in the literature. The main objective of this work is to carry out a complete techno-economic analysis of the production of novel L-Asparaginases featuring two distinct technologies: PEGylation and glycosylation, produced by recombinant *Escherichia coli* and *Pichia pastoris*, respectively. Through laboratory studies described in the literature, the scale-up and simulation of different scenarios were carried out using SuperPro Designer software aiming at the production of 1 kg of enzyme/year. For the production of PEG-Asparaginase by *Escherichia coli*, the best scenario presented a unit production cost of 7.57 USD/mg, and the following economic indexes: Return on Investment of 26.8%; Internal Rate of Return of 19.9%; Net Present Value of 16.06 million USD; and Payback Time of 3.7 years. Regarding the production of glycosylated Crisantaspase by *Pichia pastoris*, the unit cost of production was estimated at 3.42 USD/mg, with the following economic indexes: Return on Investment of 44.2%; Internal Rate of Return of 33.5%; Net Present Value of 34.75 million USD; and Payback Time of 2.3 years. Currently, the values of the possible alternatives for the treatment of Acute Lymphoid Leukemia available on the market, which feature similar technology, vary between 15.66 USD/mg (Spectrila) and 36.70 USD/mg (Oncaspar). The attractive economic parameters corroborate with the potential for the development of improved and competitive biopharmaceuticals, capable not only of supplying the annual Brazilian demand but also of driving technological and industrial development.

Keywords: Techno-Economic Analysis. Bioprocess Simulation. L-Asparaginase. *Pichia pastoris*. *Escherichia coli*.

RESUMO

L-Asparaginase (L-asparagina amino hidrolase, E.C.3.5.1.1) é uma importante enzima com propriedades antitumorais utilizada no tratamento da Leucemia Linfoide Aguda. As formulações microbianas disponíveis no mercado diferem em farmacocinética, tecnologia, dosagem, protocolo de tratamento, e valor de mercado. Apesar de ser um biofármaco amplamente utilizado como primeira ou segunda linha de tratamento, sua produção é restrita a poucos laboratórios ao redor do mundo. Além disso, análises técnico-econômicas de processos de produção de L-Asparaginase são escassos na literatura. Dentro desse contexto, o principal objetivo deste trabalho é apresentar uma completa análise técnico-econômica para a produção de novas L-Asparaginases com duas tecnologias distintas: PEGuilação e glicosilação, produzidas respectivamente por *Escherichia coli* e *Pichia pastoris* recombinantes. Através de ensaios laboratoriais descritos na literatura, foi realizado o escalonamento e simulação de diferentes cenários através da utilização do software SuperPro Designer visando a produção de 1 kg de enzima/ano. Para a produção de PEG-Asparaginase por *Escherichia coli*, o melhor cenário apresentou um custo unitário de produção de 7,57 dólares americanos/mg, e os seguintes índices econômicos: Return on Investment de 26,8%; Internal Rate of Return de 19,9%; Net Present Value de 16,06 milhões de dólares americanos; e Payback Time de 3,7 anos. Com relação a produção de Crisantaspase glicosilada por *Pichia pastoris*, o custo unitário de produção foi estimado em 3,42 dólares americanos/mg, com os seguintes índices econômicos: Return on Investment de 44,2%; Internal Rate of Return de 33,5%; Net Present Value de 34,75 milhões de dólares americanos; e Payback Time de 2,3 anos. Até o presente momento, os valores das possíveis alternativas para tratamento da Leucemia Linfoide Aguda disponíveis no mercado as quais apresentam tecnologia similar, variam entre 15,66 dólares americanos/mg (Spectrila) e 36,70 dólares americanos/mg (Oncaspar). Os bons parâmetros econômicos corroboram para o potencial desenvolvimento de biofármacos melhorados e competitivos, capazes não apenas de suprir a demanda anual brasileira, mas também de impulsionar o desenvolvimento tecnológico e industrial.

Palavras-chave: Análise Técnico-Econômica. Simulação de Processos. L-Asparaginase. *Pichia pastoris*. *Escherichia coli*.

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LIST OF ACRONYMS AND ABBREVIATIONS

ALL	Acute Lymphoid Leukemia
CAPEX	Capital Expenditures
IRR	Internal Rate of Return
mPEG-NHS	methoxy-polyethylene glycolcarboxymethyl N-hydroxysuccinimidyl ester
NPV	Net Present Value
OPEX	Operational Expenditures
PBS	Phosphate Buffer Solution
PEG-ASN	PEG-Asparaginase
ROI	Return on Investment
USD	United States Dollar

SUMMARY

1 Introduction

Leukemia is a cancer that affects blood cells whose main characteristic is the presence of lymphocytes in the bone marrow and bloodstream, supplanting normal blood cells. Its main occurrence is in children under 15 years and adults over 55 years (BRUMANO et al. 2019; BRAZILIAN NATIONAL INSTITUTE OF CANCER, 2020). The incidence of new cases of leukemia expected for Brazil for each year of the triennium 2020–2022, will be 5,920 cases in men and 4,890 in women, corresponding to an estimated risk of 5.67 new cases per 100,000 men and 4.56 for every 100,000 women (BRAZILIAN NATIONAL INSTITUTE OF CANCER, 2020). In this context, L-Asparaginase is an enzyme clinically used as the main first-line therapeutic agent to treat Acute Lymphoid Leukemia – ALL (BRUMANO et al., 2019). This pharmaceutical has been used since 1970 which allowed an increase in the remission rate from 20% to 80–90% (MÜ; BOOS, 1998; SUTOW et al., 1971; TALLAL et al., 1969).

According to COSTA-SILVA et al. (2020), prokaryotic cells are reported as the main sources of commercial L-Asparaginases, with *Escherichia coli* reported as the first microorganism used to produce the enzyme commercially. *Dickeya dadantii* (formally named *Erwinia chrysanthemi*) can produce an alternative enzyme chosen as a second-line treatment for patients who developed hypersensitivity reactions. Nevertheless, due to collateral effects frequently caused by bacterial enzymes, new microorganisms have been studied as possible sources, such as eukaryotic cells. The search for new purification strategies and the concept of Quality by Design have also been applied to increase product quality and reduce the risks of undesired reactions (BRUMANO et al., 2019). TORRES-OBREQUE et al. (2019) developed a complete process production of a recombinant PEGylated L-Asparaginase (PEG-Asparaginase) produced by *Escherichia coli* BL21(DE3). More recently, DE ALMEIDA PARIZOTTO et al., (2021) described the production of a recombinant Crisantaspase of *Erwinia chrysanthemi* with N-glycosylation pattern produced by *Pichia pastoris* Glycoswitch®.

Even though both PEGylated and glycosylated enzymes are possible candidates for the first-line treatment of ALL, a comparison between the economics behind these two products is difficult without a systematic techno-economic study comparing the production processes. Moreover, studies regarding the production of L-Asparaginase and techno-economic analyses of

industrial production processes are very scarce in the literature, making difficult any comparison between novel or modified enzymes, expression systems, production process strategies, or even scientific/technological bottlenecks for large-scale production. In the context of process development for the production of bioproducts, there are some simulation software available in the market, some of them focused on pharmaceutical and biopharmaceutical processes (PETRIDES et al., 2011). SuperPro Designer (Intelligen, Inc. USA) is a tool capable of simulating discontinuous and semi-continuous bioprocesses. Different process scenarios can be created and modified dynamically, solving complete mass and energy balances. Through the generation of economic reports, it is possible to perform economic evaluations. Bottlenecks, scheduling inconsistencies, and critical operations can be identified, guiding the users toward debottlenecking efforts (FERREIRA; AZZONI; FREITAS, 2018a; PETRIDES; KOULOURIS; LAGONIKOS, 2002).

In this work, the design and simulation of different production processes of L-Asparaginases on a scale capable to supply the Brazilian annual demand for therapeutic applications were performed. Baseline process scenarios simulated in SuperPro Designer were created using literature references for *Escherichia coli* (TORRES-OBREQUE et al., 2019) and *Pichia pastoris* (DE ALMEIDA PARIZOTTO et al., 2021) with the intrinsic adaptations to industrial production. Finally, a complete techno-economic analysis was performed focused on the process scheduling, economic profitability, and feasibility of the process in different scenarios, determining the best operational parameters.

1.1 Objectives

General

This work aims to perform a complete techno-economic analysis and comparison of two processes of production of different L-Asparaginases that present two different technologies, PEGylation and glycosylation.

Specifics

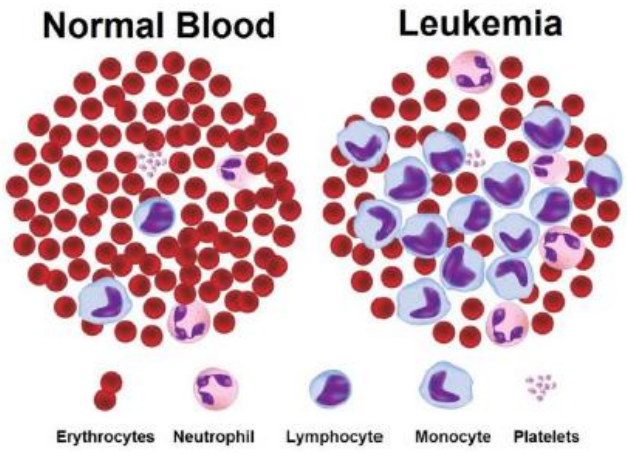
- Scale-up and simulate the production of the enzyme by procaryotic (*Escherichia coli*) and eukaryotic (*Pichia pastoris*) microorganisms, and then to perform a sensitivity analysis in some unit operations in upstream and downstream, identifying critical operation units and parameters for the generation of optimized scenarios.
- Estimate the production cost of the PEGylated and glycosylated L-Asparaginases and accomplish a techno-economic evaluation in all generated scenarios based on the economic indexes.
- Establish the best parameters to produce the enzyme based on economic profitability.

2 Literature Review

2.1 Acute Lymphoblastic Leukemia

Leukemia is a disease that affects blood cells whose main characteristic is the accumulation of cancer cells in the bone marrow that replace normal blood cells quickly or slowly, as shown in Figure 1. The type of leukemia depends on the blood cell and the velocity of growth, which characterizes the disease as acute or chronic. Among the 12 different leukemias, the four most common are Acute Myeloid Leukemia, Chronic Myeloid Leukemia, Acute Lymphoblastic Leukemia, and Chronic Lymphocytic Leukemia (BRAZILIAN NATIONAL INSTITUTE OF CANCER, 2020).

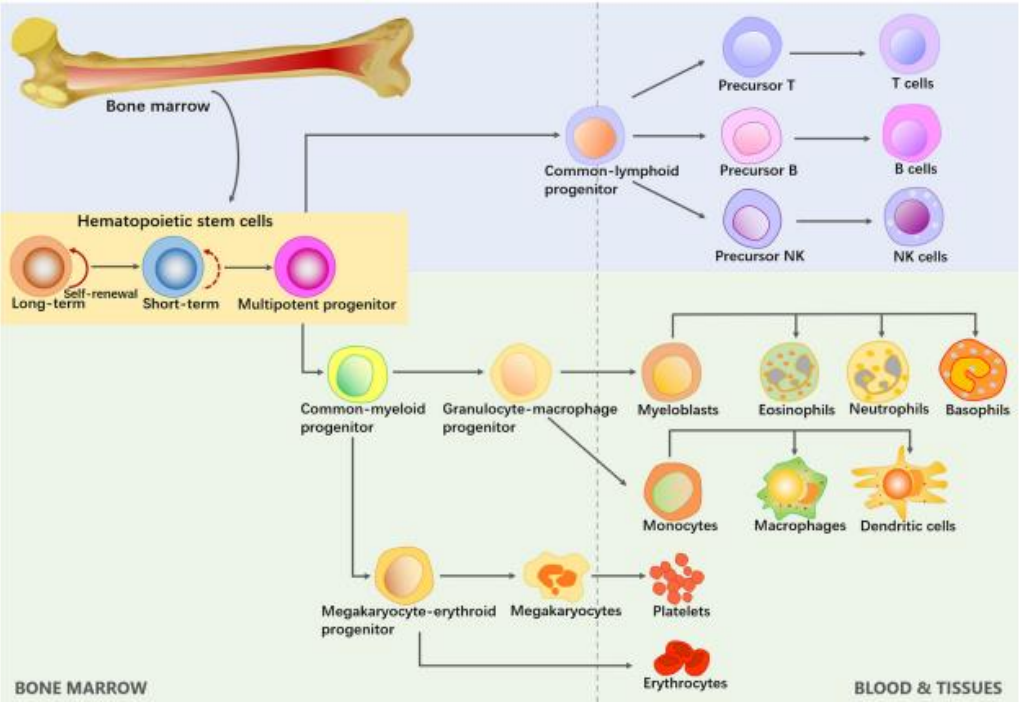
Figure 1 – Sample of blood cells in healthy individuals and leukemia patients.



Source: DESHPANDE; ALUVALU; GITE (2020)

In Acute Lymphoblastic Leukemia (ALL) cells resemble morphologically and immunophenotypically B-lineage and T-lineage precursor cells during the hematopoiesis (Figure 2). These cells have extensive involvement in the bone marrow and peripheral blood, but also may be limited to tissue infiltration and involvement of bone marrow up to 25% (ONCIU, 2009).

Figure 2 – Schematic model of human hematopoiesis.



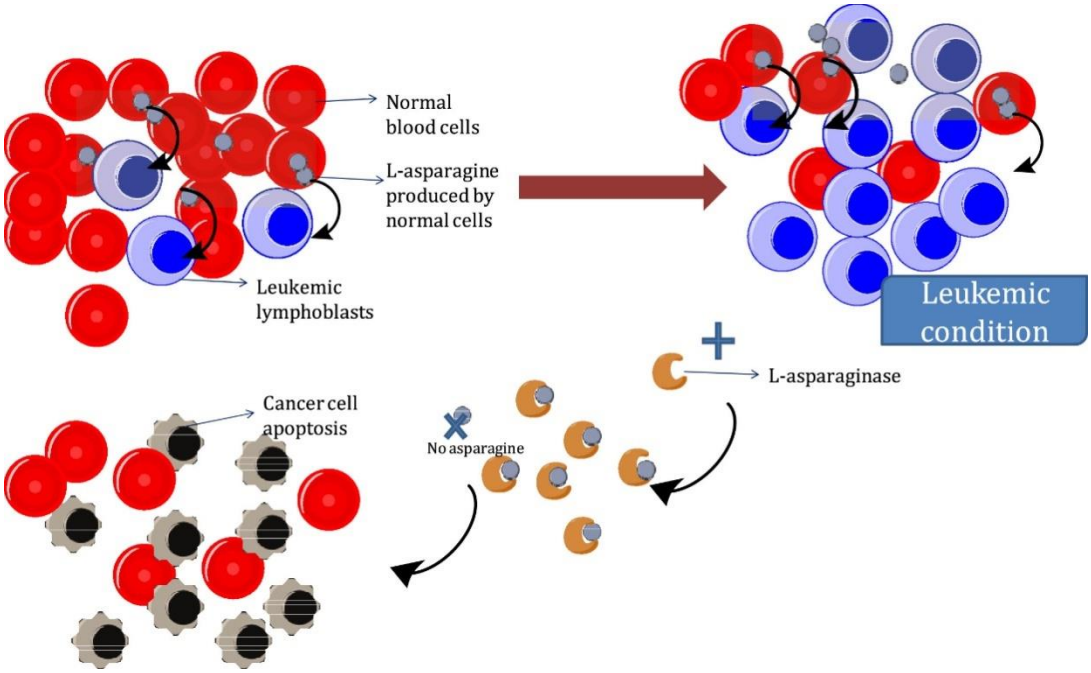
Source: ZHANG et al., (2019)

Most cases affect children up to 14 years old, with an incidence rate of about 3 to 4 per 100,000 patients, corresponding to 75% of all acute leukemias and 34% of all cancers in this age group. Even though Acute Myeloid Leukemia and Chronic Lymphocytic Leukemia are the ones that most affect adults, this group still represents about 4 out of every 10 cases of Acute Lymphoid Leukemia (ONCIU, 2009; BRUMANO et al., 2019).

2.2 L-Asparaginase

L-Asparaginase (E.C.3.5.1.1) is a biopharmaceutical enzyme used as the first line in the treatment of Acute Lymphoblastic Leukemia (LOPES et al., 2017). It acts through the inhibition of tumor growth by the hydrolysis of L-Asparagine necessary for the maintenance of tumor cells. The anti-leukemic effect occurs once tumor cells present a low level of expression of the enzyme L-asparagine synthetase, forcing them to depend on extracellular asparagine to perform protein synthesis, as presented in Figure 3. The remission rate has increased to almost 90% since it has been started to be used in the treatment protocol for leukemia (LOPES et al., 2017).

Figure 3 – Antileukemic action of L-Asparaginase in the bloodstream.



Source: SHAKAMBARI; ASHOKKUMAR; VARALAKSHMI (2019)

KIDD (1953) was the first that isolated the enzyme from guinea pig serum, although in insufficient amounts for therapeutic application: Karnofsky estimated a daily amount of between 2 and 6 liters for each patient (BAYARD CLARKS et al., 1970). The discovery that the prokaryotic microorganism *Escherichia coli* is able to produce L-Asparaginase in a biotechnological process made it possible to begin clinical trials, starting with Mashburn and Wriston in 1964 (COSTA-SILVA et al., 2020; MÜ; BOOS, 1998). Tests on large population groups began in the '70s, as well as the search for industrial-level production methods (SUTOW et al., 1971; TALLAL et al., 1969).

Several species of different microorganisms have been reported to produce L-Asparaginase. The main focus of the upstream step in L-Asparaginase production is the choice of the microorganism and the fermentation parameters. More than 123 species of bacteria are able to produce L-Asparaginase. *Escherichia coli* and *Erwinia chrysanthemi* are the main ones used for large-scale production of biopharmaceuticals due to their similarities in the mechanism of action, anticancer activity, and toxicity, although they present different pharmacokinetics profiles (LOPES et al., 2017; SHAKAMBARI; ASHOKKUMAR; VARALAKSHMI, 2019).

Studies determined that carbon and nitrogen sources are the key components of culture medium. The fermentation method (solid or submerged) for production by bacteria depends on the ability of the microorganism to metabolize the substrate in the culture medium in the form in which it is found (SHAKAMBARI; ASHOKKUMAR; VARALAKSHMI, 2019). In the seek to reduce the anaphylactic effects, hypersensitivity, and enzymatic inactivation, the search for therapeutic L-Asparaginase preparations by fungi has started, with the endophytic genus presenting a huge potential production. However, it is necessary to optimize growth conditions, enzyme yield, activity, stability against proteases, increase in half-life, and reduction of immunogenicity (LOPES et al., 2017).

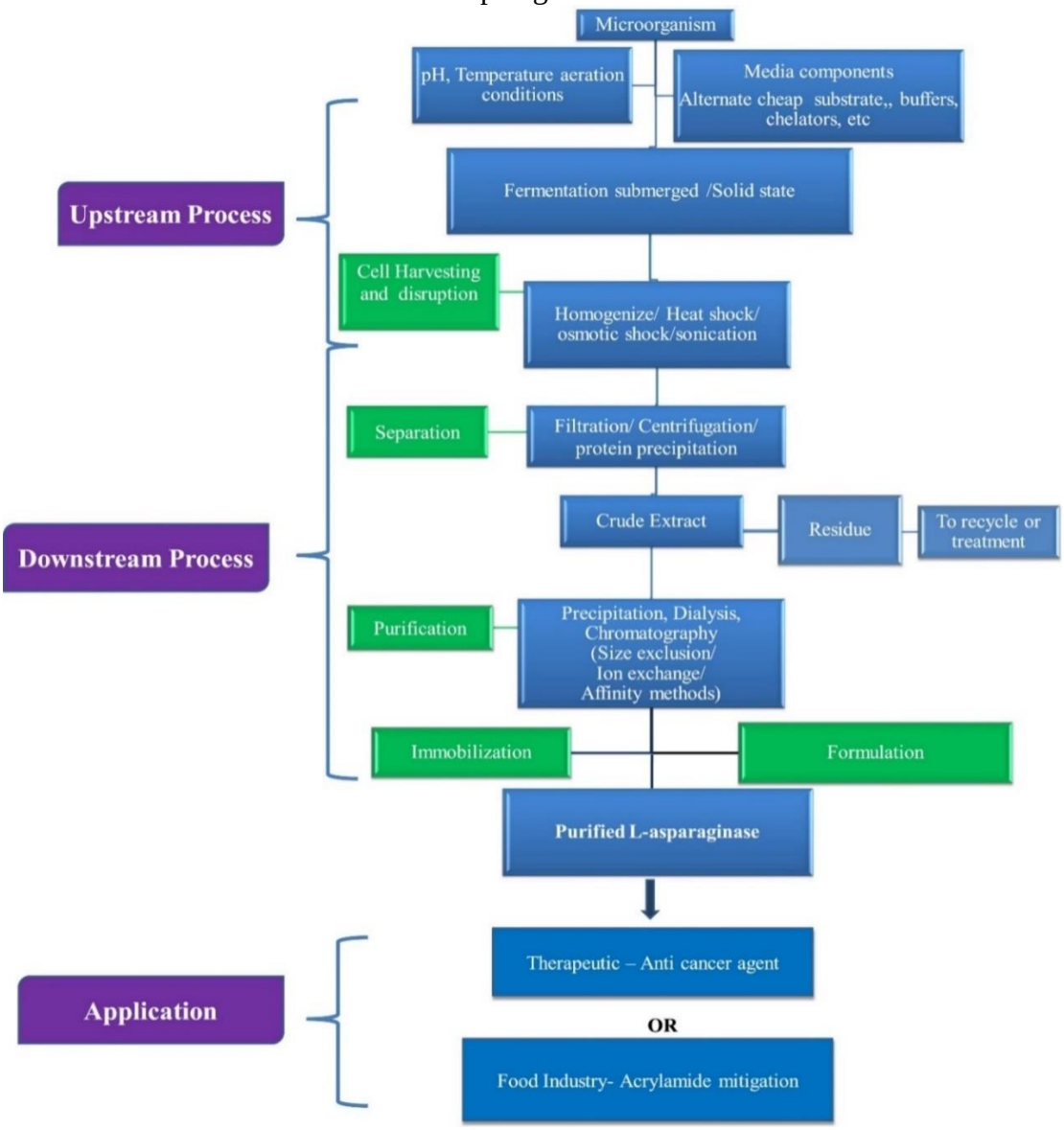
The interest in upstream and downstream costs has been a focus of the pharmaceutical industry, with downstream accounting for up to 80% of production costs. Therefore, the search for better schedulings and combinations of unit operations is important in terms of reducing the total cost (LOPES et al., 2017). Purification strategies depend on the microorganism involved and, consequently, whether the enzyme is produced intracellularly or extracellularly (SHAKAMBARI; ASHOKKUMAR; VARALAKSHMI, 2019).

Because classic laboratory scale purification strategies result in low yields, high process times, and high costs, it is necessary to search for new methodologies, such as the use of membranes (aqueous two-phase system) integrated with precipitation (ammonium sulfate) and/or fractionation. Industrial processes must have strategies that are fast, cheap, with high yields, and easily adaptable to large scale. Purification generally encompasses four stages: removal of insoluble (whole cells and/or debris), concentration, fractionation, and purification (TUNDISI et al., 2017).

Centrifugation is the first purification step in most of bioprocesses to separate solids from liquids, which means removing cells and/or debris. This operation features high efficiency, removing cells with the size of 0.5 μm and ease to scale-up (TUNDISI et al., 2017). Downstream techniques in L-Asparaginase production usually involve filtrations and more than one chromatography method in sequence (PRAKASH et al., 2021; SHAKAMBARI; ASHOKKUMAR; VARALAKSHMI, 2019). Filtration techniques use barriers and molecular size to separate the stream into retentate and filtrate, which brings up the possibility of directing the molecule of interest (TUNDISI et al., 2017).

Injectable biopharmaceuticals such as L-Asparaginase must present high purity. Therefore, a sequence of purification techniques is necessary. Although it is difficult to determine a single protocol for purification, it has been found that ion exchange and size exclusion are the most commonly used chromatography (LOPES et al., 2017). PRAKASH et al., (2021) determined 99.2% purity of an L-Asparaginase glutaminase free using ultrafiltration and gel filtration chromatography techniques. Figure 4 presents the main operations involved in the upstream and downstream production of the enzyme.

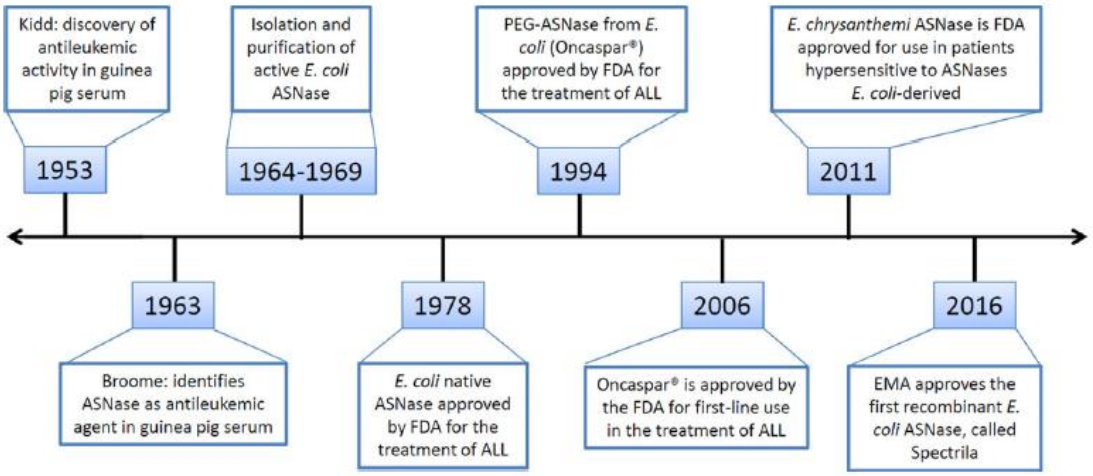
Figure 4 – General steps that are present in upstream and downstream production of L-Asparaginase.



Source: SHAKAMBARI; ASHOKKUMAR; VARALAKSHMI (2019)

Food and Drug Administration (FDA) approved the use of L-Asparaginase in 1978, and in 1994 approved a PEGylated version, with a novel technology capable to diminish side effects in patients. In 2011, Erwinase (L-Asparaginase from *Erwinia chrysanthemi*) was approved to be used as a second-line treatment in patients who developed hypersensitivity against *Escherichia coli* formulations (COSTA-SILVA et al., 2020). A summary of the main discoveries related to L-Asparaginase is presented in Figure 5. Table 1 presents an overview and the results obtained in recent research in the last years about L-Asparaginase.

Figure 5 – Timeline of discoveries and advances related to L-Asparaginase.



Source: TORRES-OBREQUE et al. (2019)

Table 1 – Studies about L-Asparaginase production by bacteria and fungus.

Microorganism	L-Asparaginase activity	Reference
Bacillus licheniformis	697.10 U mg^{-1}	(MAHAJAN et al., 2014)
Bacillus aryabhattai	680.50 U mg^{-1}	(SINGH et al., 2013)
Bacillus licheniformis	597.70 U mg^{-1}	(SUDHIR et al., 2016)
Rhizomucor miehei	1985.00 U mg^{-1}	(HUANG et al., 2014)
Penicillium sp.	13.97 U mg^{-1}	(PATRO, 2012)
Penicillium brevicompactum	574.24 U mg^{-1}	(ELSHAFEI et al., 2012)
Talaromyces pinophilus	145.00 U mg^{-1}	(KRISHNAPURA; BELUR, 2016)

The main L-Asparaginase formulations available on the market are Leuginase (Beijing, China), Spectrila (Medac, Germany), Elspar (Merck Sharp & Dohme, USA), and Oncaspar (Sigma-Tau, Italy). The prices of these pharmaceuticals vary significantly from 38.00 USD/dose to 1,619.10 USD/dose, according to the BRAZILIAN MINISTRY OF HEALTH (2017). Although all these formulations have high anti-leukemic activity, they differ in half-life time and toxicology, resulting in different pharmacokinetics, dosage, and treatment protocols (LOPES et al., 2017). The first and second-line treatment protocols depend on age and country, as presented in Table 2:

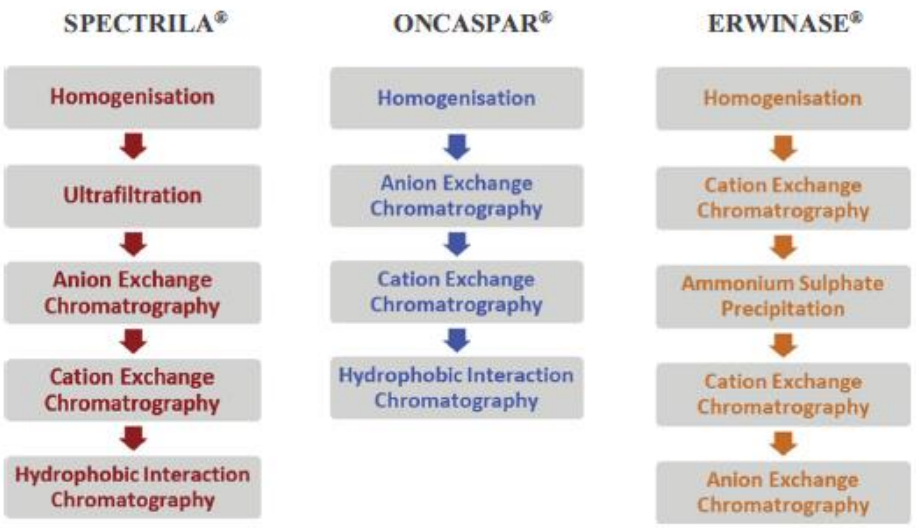
Table 2 – Treatment protocol for Acute Lymphoblastic Leukemia.

Age group	Treatment	North America, UK, Australia, New Zealand	Europe	Other Countries
Children	First-line	PEG-asparaginase	*E. coli* asparaginase	*E. coli* asparaginase
			Erwinia asparaginase	Erwinia asparaginase
	Second-line	Erwinia asparaginase	or PEG-asparaginase	or PEG-asparaginase
		E. coli asparaginase or	*E. coli* asparaginase or	
Adults	First-line	PEG-asparaginase	PEG-asparaginase	*E. coli* asparaginase
		Erwinia asparaginase		Erwinia asparaginase
	Second-line	or PEG-asparaginase	Erwinia asparaginase	or PEG-asparaginase

Adapted from: PIETERS; HUNGER STEPHEN; BOOS JOACHIM (2010)

According to BARROS et al., (2021), the process production of L-Asparaginase by *Escherichia coli* BL21 (DE3) proved to be viable regarding scaling up to industrial production, reaching about 70 g/L of dry weight and 43,954.79 U/L of volumetric activity. However, in the case of the most expensive L-Asparaginase (Oncaspar), the modification of the protein molecule is carried out during the production process in a reaction step known as PEGylation. Purification steps involved in the production of three commercial L-Asparaginases are presented in Figure 6.

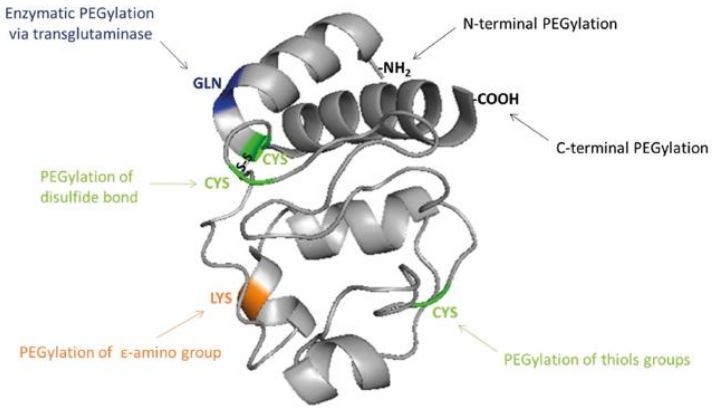
Figure 6 – Schematic of purification steps utilized during the production of different L-Asparaginases.



Source: COSTA-SILVA et al. (2020)

PEGylation is a chemical reaction that consists of the covalent attachment of at least one polyethylene glycol (PEG) chain to specific regions of the protein, usually amino acid residues. Some target groups are thiol groups of cysteines, lysine amines, and N-terminal, as shown in Figure 7, which have good reactivity when compared to other natural amino acid residues (ZHANG et al., 2012). The PEGylated protein market exceeds 8 billion USD per year, and there are currently 12 biopharmaceuticals approved for use in different diseases (GINN et al., 2014; TURECEK et al., 2016).

Figure 7 – Main potential targets in proteins where PEGylation reaction may take place.



Source: SANTOS et al. (2018)

Some advantages of PEGylated molecules include decreased immunogenicity, hiding of antigenic sites present on the protein surface, prevention against degradation by proteolytic enzymes, increasing of in vivo half-life due to increased molecular weight and hydrodynamic volume of the protein, which retards glomerular filtration rate, increased solubility in water and some organic solvents, and thermal/mechanical stability (JEVŠEVAR; KUNSTELJ; POREKAR, 2010; PASUT; VERONESE, 2009; PFISTER; MORBIDELLI, 2014; TORRES-OBREQUE et al., 2019).

N-terminal PEGylation is a site-directed reaction (GINN et al., 2014) that is based on the difference in pKa values between ε-amino groups of lysine residues (9.3-10.5) and the N-terminal α-amino group of proteins (7.6 to 8.0). For pKa values lower than 9.3, lysine residues will be protonated, preventing them from reacting with the active PEG (PASUT; VERONESE, 2009). After PEGylation, however, it is necessary to remove non-reacted products, such as non-reacted PEG, or even random PEGylated proteins. This separation is usually a challenge and leads to the inclusion of additional downstream processing steps, significantly raising the production cost of the PEGylated protein. Purification is usually carried out by chromatography, more specifically by size exclusion chromatography and ionic exchange chromatography (SHANG; YU; GHOSH, 2011; YU; GHOSH, 2010).

In order to improve immunogenicity issues arising from native formulation, the first PEGylated form of L-Asparaginase was developed in 1979 through random PEGylation with a 5 kDa agent, protecting the antigenic sites on the surface of the protein, a strategy known as a steric

hindrance. Clinical trials of PEG-Asparaginase began in 1984 (PASUT; VERONESE, 2009). The PEGylated molecule proved to be a possible substitute for the native one in the treatment of children and teenagers, mainly due to its similar profile to the native form (MEDAWAR et al., 2020). Studies proved that the use of PEG-Asparaginase as a chemotherapy drug led to fewer hypersensitivity reactions and hepatitis. The bigger interval between doses reduced hospital time, resulting in lower treatment costs (DAI; HUANG; LU, 2021).

Pichia pastoris is a methylotrophic yeast that features several advantages, such as fast growth (which impacts high biomass and protein concentrations), fermentation easy to scale up, and the possibility of genetic and post-translational modifications. It is a widely employed system for the production of recombinant proteins, being present in the production of more than 5000, and biopharmaceuticals and industrial enzymes (BAGHBAN et al., 2018). The capability to assimilate methanol as a carbon source and energy arose the interest in exploiting it, leading to studies that developed media and parameters that made it possible to reach high cell densities of over 130 g/L (CREGG et al., 2000).

A remarkable example of metabolic engineering in this yeast is the humanization of protein by glycosylation (PEÑA et al., 2018). In an attempt to overcome the challenges of the PEGylation process in the production of PEGylated L-Asparaginase, and aiming to substitute PEG for a more organic molecule, it has been studied the production of a recombinant Crisantaspase in *Pichia pastoris*, with the post-translational modification of glycosylation, binding molecules of glycans that are naturally present in the human organism to the protein surface (EFFER et al., 2020). An outstanding approach has been developed in a fully controlled environment that presents induction strategies by methanol and oxygen control in fed-batch mode (DE ALMEIDA PARIZOTTO et al., 2021).

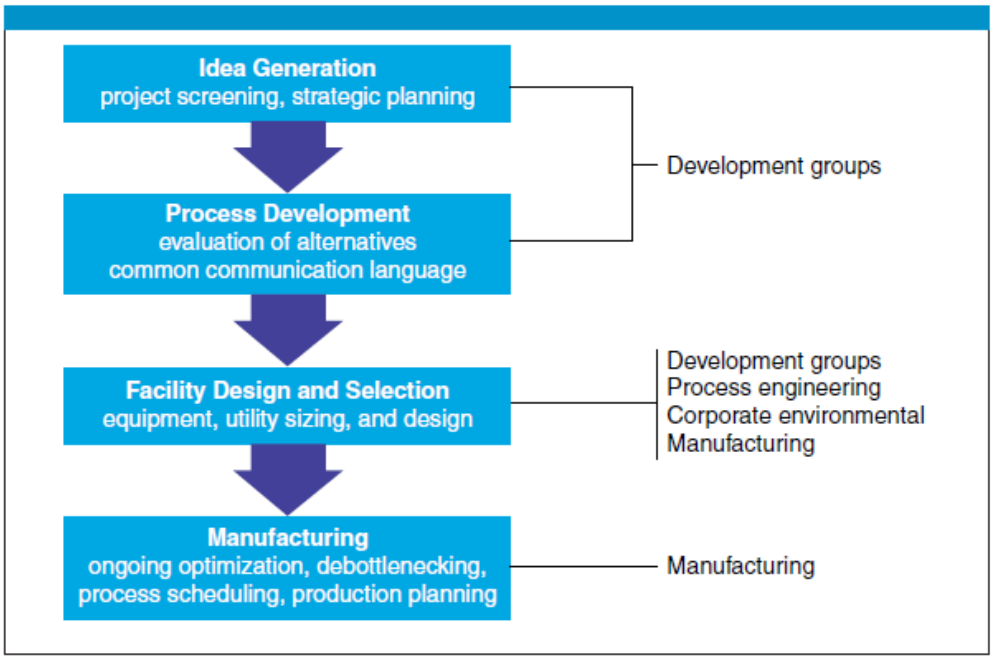
2.3 Modeling and Simulation of Processes

As important tools in technic-economic studies, the use of computer-aided process design and simulation software have been started in the 1960s, in the chemical and petrochemical industries to reproduce continuous processes and their transient behavior (TOUMI et al., 2010).

However, the batch and semi-continuous processes, widely found in pharmaceutical production are best modeled with simulators that account for time-dependency and sequence of events. This type of software was first released in the 1980s (PETRIDES et al., 2011).

The gathering of information about the process must be the first step to build a simulation model, by the creation of flow diagrams containing information about material inputs, operational parameters, and estimates about missing data, even though these assumptions are updated as the process progress (TOUMI et al., 2010). In this context, simulation tools are used by scientists and engineers to investigate several process scenarios described in detail, that respond to a set of "what if?" analyses, quickly evaluating not only parameters processes like synthesis, characterization, and formulation of the product but also cost-of-goods analysis, such as key steps, capital, and operational costs, and production throughput (PETRIDES et al., 2011; PETRIDES; KOULOURIS; LAGONIKOS, 2002). Usually, the main analysis tasks and outputs of batch simulators are the visual representation of the process; performing material and energy balances; sizing of equipment and utilities; estimation of capital and operational costs; process scheduling and cycle time analysis; throughput analysis; and environmental impact (PAPAVASILEIOU et al., 2007; TOUMI et al., 2010). All the benefits of the use of bioprocess simulation software are presented in Figure 8.

Figure 8 – Benefits of using simulation tools in general processes.



Source: PETRIDES; KOULOURIS; LAGONIKOS, (2002)

Simulation tools can be helpful to evaluate the impact of critical parameters on the key performance indicators (KPIs), and this information is used to guide Research and Development jobs judiciously (PAPAVASILEIOU et al., 2007). As the pharmaceutical industry faces constant pressure to develop new drugs, and make them available for treatment as soon as possible, it may be possible to keep the remaining parameters related to the scale-up process, leading to uncertainty in plant throughput, capital and operational costs, and environmental impact (ACHILLEOS; CALANDRANIS; PETRIDES, 2006).

Process simulation tools used for batch process design usually employ deterministic models, which do not account for random variation in their calculations, leading to scenarios that present the “average” or “expect” behavior. However, Monte Carlo simulation constitutes an easy and trustful approach to quantifying the risk associated with uncertainty, by combining the simulation software with a tool that englobes probabilistic and stochastic modeling (ACHILLEOS; CALANDRANIS; PETRIDES, 2006).

The debottlenecking theory can be applied to increase plant throughput by changes that increase the batch size or reduce the plant cycle time with a set of equations. Graphical information such as Gantt charts may be useful to identify equipment that presents a long cycle

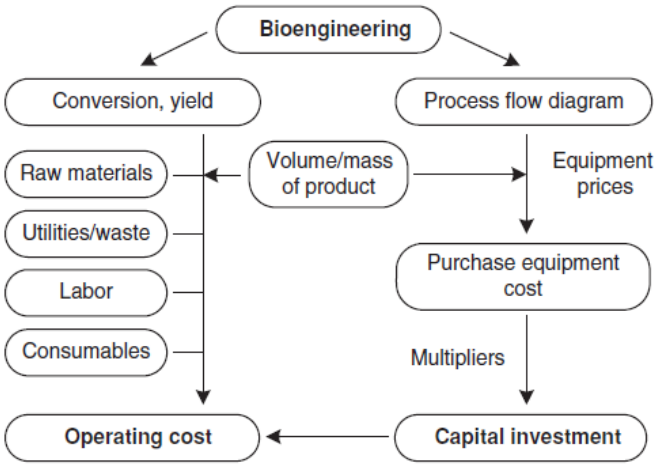
time and how auxiliary equipment impact the plant cycle time (PETRIDES; KOULOURIS; LAGONIKOS, 2002).

2.4 Economic Analysis of Bioprocesses

The development and commercialization of a new biopharmaceutical product usually takes 7 to 12 years, requiring investments that can reach up to 500 million USD. The construction of a new industrial plant, in addition to being a time-consuming process is one of the largest capital expenditures, influencing directly the development of new products (PETRIDES; KOULOURIS; LAGONIKOS, 2002).

The first step in an economic analysis consists of estimating the money necessary for capital investment, which is generally based on the value of all the equipment needed to operate the facility. From that, the operational costs related to the process can be determined from different costs, such as raw material, energy, labor, effluent treatment, etc (HEINZLE; BIWER; COONEY, 2006). Figure 9 shows the main variables related to obtaining capital investment costs and operating costs in a bioprocess.

Figure 9 – Steps involved in the determination of investment capital and operating costs.



Source: HEINZLE; BIWER; COONEY (2006)

In the economic analysis of bioprocesses, production costs are divided into two major categories: Capital Cost (also known as capital investment or capital expenditures – often called

CAPEX) and Operating Cost (also known as operating expenditures, often called OPEX). CAPEX is the addition of the amount of money needed to supply the plant (the fixed capital investment) with the working capital needed for operation (HEINZLE; BIWER; COONEY, 2006).

Computer models are a reliable tool that can be used to estimate all the equipment necessary, their uses, and batch working. Software that presents deep levels of detailing may serve as a starting point for negotiations. From there, it is possible to perform a cost analysis involving investment capital and operating costs (PAPAVASILEIOU et al., 2007; PETRIDES; KOULOURIS; LAGONIKOS, 2002). One of the methods for determining CAPEX is the use of multipliers for equipment acquisition prices, which can be obtained through vendor quotations, literature, or the amount paid for the same or similar equipment in another project. Usually, either the size of the fermenter or the annual production is the key element and from that, the dimensions of the other equipment are determined (HEINZLE; BIWER; COONEY, 2006; PETRIDES et al., 2014).

The purchase price of the equipment is the basis for a reliable estimate of the total capital investment. From the percentage values of the cost of the acquired equipment, the fixed capital cost items are estimated, such as process piping, insulation, electrical systems, etc. From these, the indirect cost of the plant, and after that, the fixed capital investment is calculated (HEINZLE; BIWER; COONEY, 2006). Table 3 details averages and ranges of multipliers for the fixed capital cost estimate, which for small to medium size biotechnology facilities are usually in the range of 50 to 200 million USD (HARRISON et al., 2015).

Table 3 – Main parameters to estimate the fixed cost in a process.

Cost Item	Average Multiplier	Range of Multiplier Values
Total Plant Direct Cost (TPDC)		
Equipment Purchase Cost (PC)		
Installation	0.50 × PC	0.2–1.5
Process piping	0.40 × PC	0.3–0.6
Instrumentation	0.35 × PC	0.2–0.6
Insulation	0.03 × PC	0.01–0.05
Electrical	0.15 × PC	0.1–0.2
Buildings	0.45 × PC	0.1–3.0
Yard improvement	0.15 × PC	0.05–0.2
Auxiliary facilities	0.50 × PC	0.2–1.0
Total Plant Indirect Cost (TPIC)		
Engineering	0.25 × TPDC	0.2–0.3
Construction	0.35 × TPDC	0.3–0.4
Total plant Cost (TPC)	TPDC + TPIC	
Contractor's fee	0.05 × TPC	0.03–0.08
Contingency	0.10 × TPC	0.07–0.15
Direct Fixed Capital (DFC)	TPC + Contractor's fee and contingency	

Source: HARRISON et al. (2015)

OPEX is defined as the sum of costs related to plant operation and recovery of the invested capital, which is the money needed to produce the desired product and recover the capital invested. The operating cost can be divided into variable, fixed, and plant overhead costs (HEINZLE; BIWER; COONEY, 2006).

Variable costs depend mainly on the amount of product produced. This category comprises the following costs: raw material, which depends on the amount needed per year multiplied by the price; consumables (filtration membranes, chromatography resins, and activated carbon), which are completely related to the downstream step; labor, which is determined by the working hours times the hourly wage, a value that is extremely dependent on the location in

which the facility is located; Operating supplies, which include items needed for the day-to-day of the plant and protective devices for the workers such as clothing, tools; laboratory (offline analysis), quality control (QC), and quality assurance (QA), which are derived from labor costs and for bioprocess can be taken up to 60% of the total labor cost; Utilities that correspond to the energy consumed for heating, cooling, evaporation/distillation, aeration, agitation, and centrifugation; treatment of wastewater, emissions, and solid wastes; and royalty expenses (HEINZLE; BIWER; COONEY, 2006).

The fixed costs, also known as facility-dependent, are independent of the amount produced and encompass costs of depreciation, plant maintenance and repair, insurance and local taxes, and overhead costs (FERREIRA; AZZONI; FREITAS, 2018a). Plant overhead costs, which are part of the operating cost are intrinsically linked to the operation of facilities that are not directly related to the process; for example, medical service, safety and protection, storage facilities, plant superintendence, packaging, cafeteria, and others. General expenses consist of the costs of running the company (administration), selling the product (distribution and marketing), and developing novel processes and products (R&D) (HEINZLE; BIWER; COONEY, 2006).

The right input of data in the software results in the Unit Production Cost (UPC), which is calculated as the total product cost allocated to the annual amount of product (HEINZLE; BIWER; COONEY, 2006). The results of an economic analysis help in the decision-making of industries concerning processes, and in this sense, a wide range of economic indicators allow evaluating the attractiveness of a process through a profitability analysis. A common approach to verify the cost-effectiveness of a bioprocess can be carried out through the analysis of the following indexes:

- Net Present Value (NPV): The most popular and sophisticated evaluation index, determines whether the process will be economically successful throughout the lifetime of the plant based on the analysis of discounting future cash flows at a predetermined rate by the present value. Positive values indicate that the process is profitable, and when comparing different scenarios of the same process, the one with the highest NPV will be more attractive (CZINKÓCZKY; NÉMETH, 2020; WANG et al., 2020; ŽIŽLAVSKÝ, 2014).

- Payback Time: the required amount of time for the total investment cost to be balanced by the annual net profit. A shorter payback time points that the project is more attractive and profitable because this means that the initial investment will be paid off in a short time (CHAROENSIDDHI et al., 2018; VILLEGAS-MÉNDEZ et al., 2022).
- Return on Investment (ROI): quantifies the net benefit of an investment per year divided by the investment cost. Widely used in the comparison of projects, those that present an ROI between 5 and 10% are disregarded (MORENO-SADER et al., 2019; VILLEGAS-MÉNDEZ et al., 2022).
- Internal Rate of Return (IRR): an indicator that reflects the efficiency of the investment, obtained through the discount rate that makes the NPV zero. An economic process feasible must present an IRR higher than the interest rate chosen (CZINKÓCZKY; NÉMETH, 2020; MUSSATTO et al., 2015).
- Gross Profit: calculated by subtracting the annual operating cost from the annual revenue, including depreciation (CZINKÓCZKY; NÉMETH, 2020; HEINZLE; BIWER; COONEY, 2006).
- Gross Margin: the measure of the company's efficiency in transforming raw materials into products, calculated as the ratio of gross profit to revenues (HEINZLE; BIWER; COONEY, 2006).

In short, in the comparison between different scenarios of a bioprocess, the most attractive will be the one that presents the highest values of NPV, ROI, and Gross Margin and the lowest Payback Time.

3 Material and Methods

3.1 Design Basis and Process Scenarios

All scenarios for the production of L-Asparaginase were designed, simulated, and economically evaluated using SuperPro Designer v12, a tool marketed by Intelligen, Inc. (Scotch Plains, NJ, USA), which performs process simulation and economic evaluation. Two different microorganisms were chosen: a prokaryote (*Escherichia coli*) and a yeast (*Pichia pastoris*). For all generated scenarios, it was chosen a plant lifetime of 15 years. All charts and tables were generated using Microsoft Excel from Microsoft Office to analyze the reports created by SuperPro.

3.1.1 *Escherichia coli*

The proposed process is based on the fermentation of a recombinant *E. coli* BL21 (DE3) with pET15b vector which hosts the gene for the production of ASNase from *Dickeya dadantii*. The baseline flow diagram, created in the software, is provided in Figure 10. Aiming for a better analysis of the process, it was divided into the following sections, which are going to be described in detail later:

- Upstream
- Recovery
- IEC (Ionic Exchange Chromatography)
- PEGylation
- SEC (Size Exclusion Chromatography)
- Formulation

A total of six scenarios were created: Baseline, S1, S2, S3, S4, and S5. In all scenarios, the fermentation is conducted initially in batch mode until it reaches a titer of 3.92 g/L, according to the process proposed by TORRES-OBREQUE et al. (2019) and first simulated by FUGANHOLI (2021). In scenarios S1 to S5, it was assumed that, after the batch fermentation, a

fed-batch mode is then conducted, feeding the fermenter with a stream that contains a glucose concentration equal to 670 g/L, allowing the fermenter to reach a biomass concentration of 10 g/L, 20 g/L, 30 g/L, 40 g/L, and 65 g/L respectively for each scenario. The fed-batch parameters to reach each of these final concentrations are given in Table 5.

The plant was assumed to be located in Brazil and constructed in 2022. The annual operating time was assumed to be 330 days. For all scenarios, the process was designed to have an annual production of 1 kg/PEG-Asparaginase, which corresponds to approximately the Brazilian annual demand (BRAZILIAN MINISTRY OF HEALTH, 2017). A list of the key assumptions to develop the baseline process is given in Table 4.

Table 4 – The design basis for the production of PEGylated L-Asparaginase.

Parameter	**Assumption**
Production Scale	1.00 kg of PEG-ASN/yr
Annual Operating Time	7920 h (330 days)
Process Cycle Time	34 h
Fermentation Cycle Duration	6 h
Concentration of Biomass in Broth	3.92 g/L
Nominal Volume of Seed Fermenter	41 L
Nominal Volume of Main Fermenter	452 L
Temperature	37.0 °C
Pos-Induction Temperature	39.6 °C
Aeration	1 VVM
μ	0.78 h^{-1}
Glucose Concentration	10.0 g/L
pO_2	20%

Source: this work.

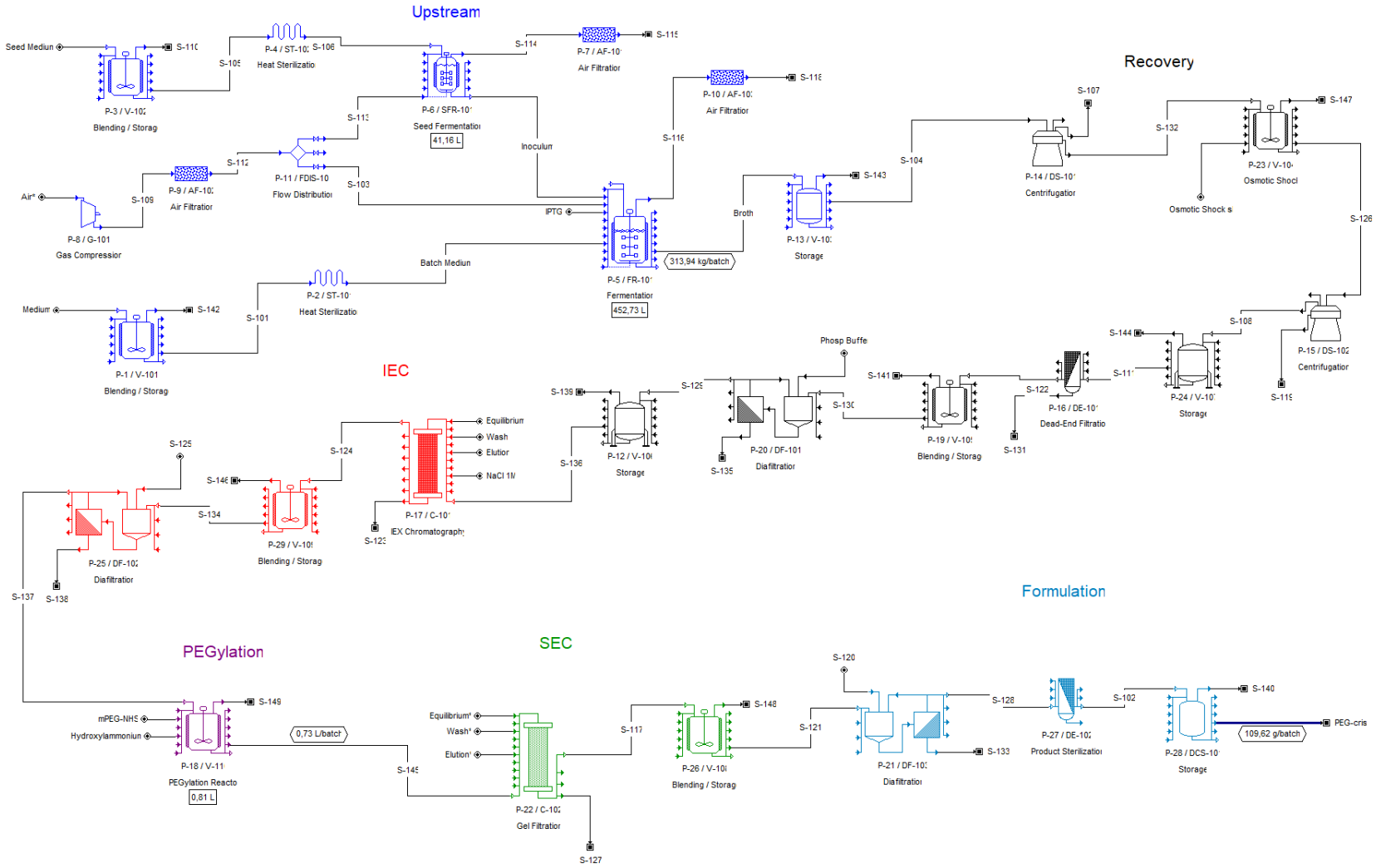
Table 5 – Parameters for fed-batch fermentation in all scenarios considering $\mu = 0.30\ h^{-1}$.

Scenario*	**Fermentation Time (h)**
S1 (10 g/L)	3.12
S2 (20 g/L)	5.43
S3 (30 g/L)	6.78
S4 (40 g/L)	7.74
S5 (65 g/L)	9.36

* Final biomass concentration.

Source: this work.

Figure 10 – Baseline process flow diagram for the process production of PEG-ASN by recombinant *E. coli* BL21 (DE3). This is an improvement of the process proposed by TORRES-OBREQUE et al. (2019) and first simulated by FUGANHOLI (2021).



Source: this work.

The development of scenarios with the corresponding data to each process generates a Unit Production Cost (UPC), which is by definition “calculated by the division of the Annual Operating Cost by the annual mass flow rate basis (which can be the total flow or component flow)”, previously specified in the software as the “Unit Reference”. Based on the UPC, it is possible to define the Unit Production Revenue (UPR) according to the profit factor desired according to the market in which the product is going to be sold and its competitiveness regarding the options already available. In this work, it was set a profit of 30% in the UPC of the baseline scenario, and this value was reapplied in all other scenarios. Hence, reports containing economic data can be generated, allowing the analysis of parameters such as feasibility and attractiveness of the bioprocess.

3.1.2 *Pichia pastoris*

The proposed process is based on the production of a glycosylated Crisantaspase by *Pichia pastoris* as described in data previously published by DE ALMEIDA PARIZOTTO et al., (2021). The baseline flow diagram generated is presented in Figure 11. In the same way as *Escherichia coli* scenarios, it was divided into sections, which are:

- Upstream
- Recovery
- IEC (Ionic Exchange Chromatography)
- SEC (Size Exclusion Chromatography)
- Formulation

Two process scenarios were created: Baseline and P1. Starting from data presented in the literature, in the scenario Baseline were assumed scale-up factors to the streams corresponding to seed, main, and fed-batch mediums in the upstream section, which led to an annual production of 8 kg Crisantaspase/year. Based on this scenario, it was possible to create P1 by using the tool of Scale-Up/Down on the software, where it was set an annual production of 1 kg/year granting a better comparison with *Escherichia coli* scenarios. The main parameters of this scenario are

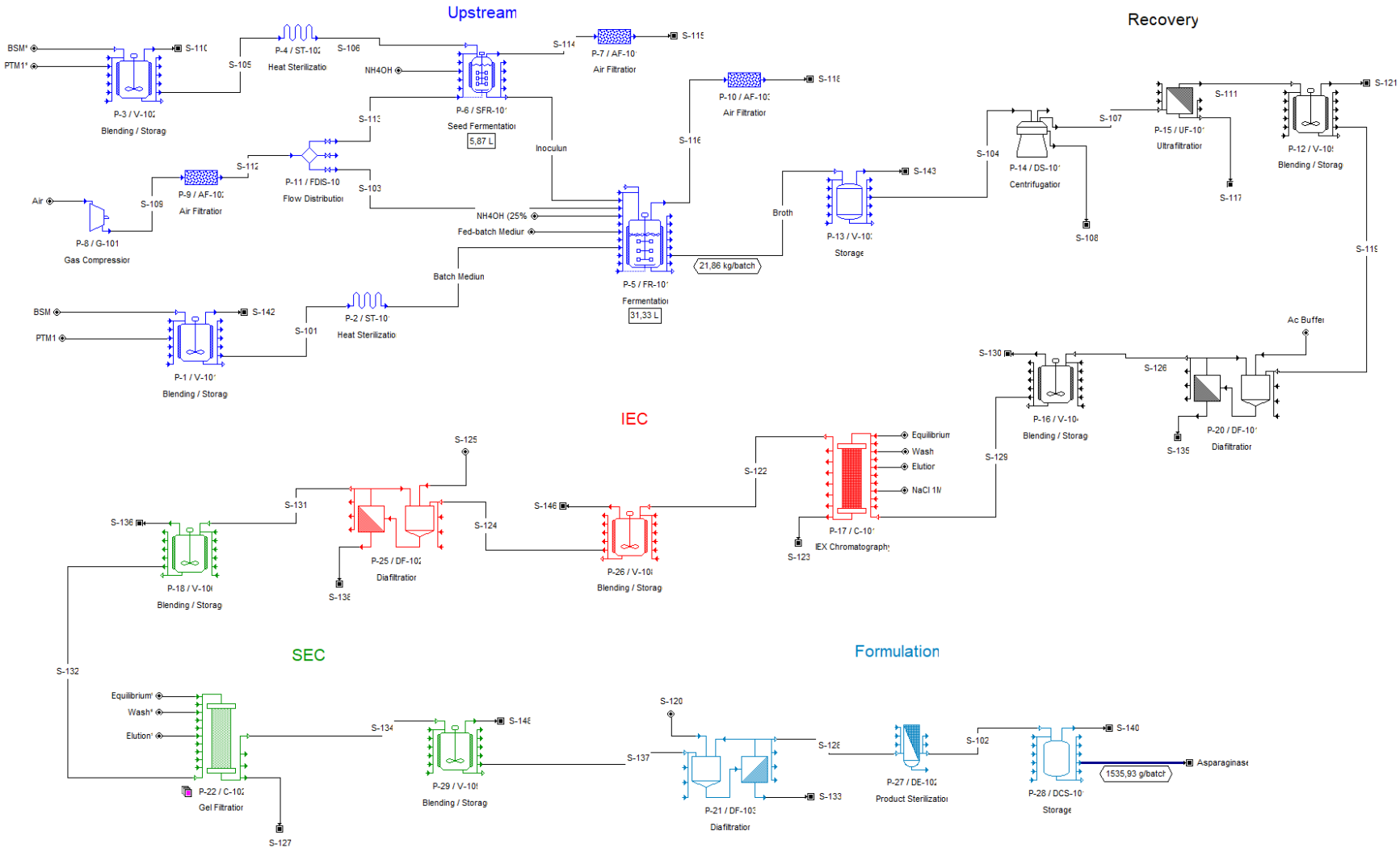
presented in Table 6. Parameters such as plant location, year of construction, and operation time remained unchanged.

Table 6 – Key parameters for the production of glycosylated Crisantaspase.

Parameter	**Assumption**
Production Scale	1.00 kg of Crisantaspase/yr
Annual Operating Time	7920 h (330 days)
Process Cycle Time	155 h
Fermentation Cycle Duration	120 h
Concentration of Biomass in Broth	65.0 g/L
Concentration of Crisantaspase in Broth	4.5 g/L
Nominal Volume of Seed Fermenter	6 L
Nominal Volume of Main Fermenter	31 L
Temperature	30.0 °C
Pos-Induction Temperature	35.0 °C
Aeration	1 VVM
μ	0.26 h^{-1}
Glycerol Concentration	95.0 g/L
pO_2	20%

Source: this work.

Figure 11 – Baseline process flow diagram for the production of glycosylated Crisantaspase by *Pichia pastoris*.



Source: this work.

3.2 Flowsheet Sections

3.2.1 *Escherichia coli*

3.2.1.1 Upstream

This section describes the fermentation step to produce inoculum and biomass. The key parameters such as seed train/main fermentation data and the final concentration of biomass in the fermentation broth were based on data previously published (TORRES-OBREQUE et al., 2019). This process was first simulated by FUGANHOLI (2021) designed for a pilot plant scenario, using Luria-Bertani (LB) medium and simplified process considerations. Here, in order to make possible industrial-scale production and a more precise simulation, the culture medium was replaced according to RIESENBERG et al., (1991), which employs glucose as the main carbon source and diammonium phosphate as a nitrogen source. This also includes different stoichiometric equations to represent fermentation. This section is composed of a seed train to generate inoculum, the main fermenter, and all supporting equipment that are essential to large-scale production, such as compressors, filters, sterilizers, and blending/storage tanks for medium preparation.

The seed train is composed of one seed fermenter having an expansion factor of 10x, producing biomass in a concentration of 4.0 g/L, which is inoculated in the main fermenter. The medium is composed of 10.0 g/L of glucose, 4.0 g/L of diammonium phosphate, and salt components in different concentrations, whereas the medium in fed-batch mode is composed of 670.0 g/L of glucose, 227.0 g/L of diammonium phosphate, and the same salt components present in batch fermentation (HORN et al., 1996). Each of these components is dissolved in water, sterilized, and then distributed to the respective fermenter. All fermentation steps are aerated with 1.0 VVM (volumes of air per volume of liquid per minute) of sterile air. The temperature in both fermentation is kept constant at 37.0 °C with chilled water. In the main fermentation, an induction step with IPTG 1.0 mM (final concentration) was performed, and the temperature was raised to 39.6 °C. The pH is maintained at 6.8 in all fermentation steps. The fermentation time is assumed to be 6 h in the seed train, and 6 h in the main fermentation, unlike the process simulated by FUGANHOLI (2021), in which the fermentation times for seed train and main were respectively 16 h and 5 h. Although FUGANHOLI (2021) generated

different scenarios varying the final cell concentration, the potential of fed-batch fermentation was not explored, with all cultures operating in batch mode, as opposed to most large-scale processes (HEINZLE; BIWER; COONEY, 2006).

In the fed-batch scenarios, the time of fermentation was increased according to the desired final concentration of biomass assuming $\mu = 0.30\ h^{-1}$. A conversion factor of 0.4 g biomass/g glucose was assumed for both fermentations. The fermentation step was modeled with the mass stoichiometric equation adapted from (FERREIRA; AZZONI; FREITAS, 2018b), which is based on atomic balances of carbon, hydrogen, nitrogen, and oxygen, resulting in the following equation:

180 Glucose + 10 Di-ammonium Phosphate + 93 Oxygen → 72 Biomass + 135 Carbon Dioxide + 76 Water

Considering that the elemental formula of cell biomass is $CH_{1.88}O_{0.49}N_{0.24}$. The reaction was set to achieve the target concentration of 3.9 g/L of biomass, and the heat released by fermentation was assumed to be 3746.4 kcal/kg of oxygen. Given the assumptions of this section, the final fermentation broth is expected to contain biomass and residual medium.

3.2.1.1 Recovery

The Recovery section was designed to represent an osmotic shock protocol, once produced L-Asparaginase is kept in the periplasmic space of *Escherichia coli*. The main equipment related to this section are centrifuges, storage tanks, dead-end filters, and a diafiltration system. The fermentation broth is sent to a disc-stack centrifuge where centrifugation takes place for 1 h to separate the bacterial cells from the L-Asparaginase present in the supernatant with a 98% of yield in the cell components removal, and the heavy stream presents 14.94% of biomass with a concentration of 150 g/L. The liquid is transferred to a blending tank and an osmotic shock protocol is carried out at 4 ºC, according to TORRES-OBREQUE et al., (2019): a hypotonic solution composed of Tris-HCl 33.0 mM, EDTA 1.0 mM, 20% of sucrose, is added with the purpose to release the pure enzyme in the supernatant. To represent the osmotic shock process, it was modeled the mass stoichiometric reaction given below:

1.00 Biomass → 0.50 L-Asparaginase + 0.30 Contaminating Proteins + 0.15 Debris + 0.05 Endotoxins

The supernatant is sent to a disc-stack centrifuge to separate the L-Asparaginase present in the liquid from cell debris and biomass. It is assumed that 98% of the biomass is removed. For the filtration steps, Cytiva's product portfolio was consulted to collect values and specifications of cartridges for dead-end filtration and diafiltration, in order to update the simulation performed by FUGANHOLI (2021), which used the SuperPro Designer database.

Next, the solution goes through a CFP-4-E-55 Hollow Fiber Cartridge (Cytiva) to remove any residual cells and debris. The last step of this section is a tangential flow filtration (TFF) with a UFP-10-E-55 Hollow Fiber Cartridge (Cytiva), where the solution is first concentrated with a concentration factor of 20x, and then diafiltered with 6 volumes of PBS Buffer 20 mM pH 5.5 to prepare the solution to the ion exchange chromatography. The rejection coefficient of the UF membrane for L-Asparaginase is assumed to be 0.99.

3.2.1.3 Ion Exchange Chromatography (IEC)

The first purification step is a cation-exchange chromatography to remove endotoxins and contaminating proteins. Despite the process first simulated by FUGANHOLI (2021) scaling the ion exchange chromatography performed by TORRES-OBREQUE et al., (2019), economic data related to the acquisition of columns suitable for chromatography on an industrial scale were not collected. Here, not only a new dimensioning of the column was carried out, but also the consultation of resins available for acquisition via bioprocess companies.

The chromatography column and resin were updated according to Cytiva products. An AxiChrom 300 Column (Cytiva) is used in this step due to its dimensions fitting the scale-up and the possibility to use SP Sepharose Fast Flow resin, which is well established in the industrial process, because of the highly cross-linked agarose resin enabling higher flow rates and productivity.

The column was equilibrated with a Phosphate Buffer Solution (PBS) 20 mM pH 5.5 at a linear velocity of 596 cm/h and 5 BV (Bed Volumes). Then, it was loaded with the

retentate solution at the same velocity, which was kept in all other steps. It was assumed that the biding capacity is 50 mg of L-Asparaginase/mL and a 69% yield. Next, the column was washed with the same buffer and 3 BV. The bounded L-Asparaginase was eluted by isocratic elution with a Phosphate Buffer Solution 20 mM pH 7.5, and 3 BV. The column regeneration step occurred with NaCl 1M, and 5 BV. The Cleaning-in-Place (CIP) protocol consisted of three steps, with NaOH (0.1 M), Water, and PBS 20 mM pH 5.5. The eluted solution was collected in a blending tank and stored until it was sent to diafiltration for 3 hr to remove any chromatography buffers, contaminating proteins and endotoxins. This step uses 6 volumes of PBS buffer 20 mM, pH 7.5, to prepare the eluate to site-specific PEGylation. The rejection coefficient of the membrane for all molecules was set as 1.

3.2.1.4 PEGylation

This section includes a stirred tank reactor, where the N-terminal site-specific PEGylation takes place to produce the final product PEG-Asparaginase. A mPEG-NHS 10 kDa solution is added to the diafiltered solution from the previous step in a ratio of 1:50 (enzyme: PEG). The reaction was kept under stirring at 22 °C for 30 min. Then, hydroxylamine 2 M (10% v/v) was added to cleave any unstable and random PEGylation sites. To represent the reaction, the following molar stoichiometric reaction was modeled:

$$1.00\ \text{L-Asparaginase} + 50.00\ \text{mPEG-NHS} \rightarrow 48.00\ \text{mPEG NR} + 0.70\ \text{PEGylation BP} + 0.35\ \text{PEG-ASN}$$

where BP means the PEGylation byproducts randomly generated, and NR is the mPEG-NHS not reacted. It is assumed a yield equals 50% and there is no enthalpy related to this reaction.

3.2.1.5 Size Exclusion Chromatography (SEC)

The final purification step is size exclusion (gel filtration) chromatography. FUGANHOLI (2021) determined that this step was the main bottleneck of the whole process with an operation time equal to 20 hr. Therefore, the search for new purification strategies was carried out, respecting the steps performed by TORRES-OBREQUE et al., (2019). The

column and resin were also updated in the same way that ionic exchange chromatography. The PEGylated solution is sent to a BPG 100/750 column (Cytiva) packed with Superdex 200 resin (a composite matrix of dextran and agarose). It was chosen a linear velocity of 137 cm/h and it was kept in all chromatography steps.

The column is first equilibrated with PBS buffer 20 mM, pH 7.5, and 2 Bed Volumes. The loading is carried out with a sample volume corresponding to 4% BV. It was assumed a PEG-Asparaginase recovery yield of 77% and a 100% resin capacity utilization. Next, an isocratic elution takes place with 1 BV of the same buffer, and the eluant volume in the product stream of 0.473 BV. Finally, the column is washed with the same parameters as the equilibration step. The eluted solution was then stored in a receiver tank before the sterilization.

3.2.1.6 Formulation

This section was not carried out by TORRES-OBREQUE et al., (2019). According to the plasmid DNA production (HEINZLE; BIWER; COONEY, 2006), a formulation section was defined, which is composed of: a diafiltration (to concentrate the enzyme to the desired concentration of 10 mg/mL of PEG-Asparaginase, and remove any traces of previous downstream steps), a representative dead-end filtration (to remove endotoxins and to ensure product sterilization), and a disposable storage tank, which is the final section of the whole process, storing the final product.

3.2.2 *Pichia pastoris*

3.2.2.1 Upstream

The upstream section includes the seed train to generate inoculum volume (yeast biomass), and the main fermentation to produce extracellular Crisantaspase according to DE ALMEIDA PARIZOTTO et al., (2021). An expansion factor of 30x for Basal Salt Medium (BSM) and Pichia Trace Metal Solution (PTM1) in the seed train was defined, whereas for the

main fermentation this value was set as 100x. The production fermenter is inoculated by the broth from the seed, which presents a yeast concentration equal to 30 g/L. The medium for the fermentation is described by GURRAMKONDA et al., (2009); 95.0 g/L of glycerol, 15.7 g/L of ammonium sulfate, and a PTM1 solution. Fermentation is aerated with 1 VVM of sterile air. Temperature is kept constant at 30 °C, and the pH 5.0 is controlled with NH_4OH 25% (v/v). The fermentation time is assumed to be 20 h in the seed fermentation and 24 h in the main fermentation. The mass stoichiometric equation, which describes the biomass growth in seed and batch fermentation steps is given below:

1.00 Glycerol + 0.05 Ammonium Sulfate + 0.59 Oxygen → 0.45 Yeast + 0.61 Carbon Dioxide + 0.58 Water

The elemental formula of yeast was set to be $CH_{1.67}O_{0.50}N_{0.17}$. This reaction took place until it reaches a yeast concentration of 30 g/L. After glycerol exhaustion in batch fermentation and aiming to produce more biomass, and consequently Crisantaspase, an induction fed-batch phase started with a solution containing pure methanol and PTM1 (DE ALMEIDA PARIZOTTO et al., 2021). Here an expansion factor of 100x was applied to guarantee the correct scale-up. The fed-batch time is assumed to be 96 h, resulting in a total fermentation time of 120 h. The mass stoichiometric equations of the fed-batch step are given below:

1.00 Methanol + 0.05 Ammonium Sulfate + 0.59 Oxygen → 0.45 Yeast + 0.61 Carbon Dioxide + 0.58 Water

1.00 Methanol + 0.04 Ammonium Sulfate + 1.08 Oxygen → 0.05 Crisantaspase + 0.82 Carbon Dioxide + 0.25 Yeast + 1.00 Water

Given these assumptions, the fermentation broth is expected to contain: 65.0 g/L of yeast; 4.5 g/L of glycosylated Crisantaspase; and a residual medium.

3.2.2.2 Recovery

The objective of this step is to separate the yeast biomass of the Crisantaspase present in the fermentation broth. This step is composed of centrifuge, ultrafiltration/tangential flow filtration systems, and blending/storage tanks. The broth is sent to a disc-stack centrifuge

where centrifugation takes place for 30 minutes. It is assumed that 98% of the yeast is removed. The supernatant goes through an 0.45 um Cartridge CFP-4-E-55 (Cytiva) for 30 minutes to remove any vestige of cell debris. Finally, the liquid is sent to a diafiltration system having an Ultrafiltration Cartridge UFP-10-E-55 (Cytiva) with a MWCO of 10 kDa, where it is first concentrated to 10 mg/mL of Crisantaspase, and then diafiltered with 6 volumes of Sodium Acetate Buffer 50 mM pH 5.2. The rejection coefficient of the membrane for Crisantaspase is assumed to be 0.99. The retentate is stored in a tank before going to the ion exchange chromatography.

3.2.2.3 Ionic Exchange Chromatography (IEC)

This step is similar to the one performed in the PEGylated scenarios, with changes in buffers and operational parameters. The ion exchange chromatography uses the same column and resin to remove residual molecules. The column was equilibrated with Sodium Acetate Buffer Solution 50 mM pH 5.2 at a linear velocity of 596 cm/h and 3 BV (Bed Volumes). The retentate solution was loaded at the same velocity, which was also kept in the other steps. It was assumed that the biding capacity is 50 mg of Crisantaspase/mL and a 56.63% yield. Next, the column was washed with the same buffer and 1.5 BV. The bounded Crisantaspase was eluted by isocratic elution with Sodium Acetate Buffer 50 mM pH 5.2 + NaCl 1 M + glycine 100 mM, and 3 BV. The column regeneration occurred with NaCl 1M, and 3 BV. The Cleaning-in-Place (CIP) protocol consisted of three steps, with NaOH (0.1 M), Water, and Ethanol 20%. The eluted solution was stored in a blending tank and later sent to a tangential flow filtration system to remove any chromatography buffers for 3 hr. This step uses 6 volumes of Sodium Acetate Buffer. The rejection coefficient of the membrane for Crisantaspase was defined as 0.99. The retentate was received and stored in a blending tank.

3.2.2.4 Size Exclusion Chromatography (SEC)

The final chromatography step is performed in a BPG 100/750 column (Cytiva) packed with Superdex 200 resin (Cytiva). The linear velocity of 137 cm/h was unchanged in all chromatography steps. The column is equilibrated with Sodium Acetate Buffer 50 mM pH 5.2, and 2 Bed Volumes. The loading is carried out with a sample volume corresponding to

4% BV, a Crisantaspase recovery yield of 54.93%, and a 100% resin capacity utilization. Finally, an isocratic elution takes place with 1.5 BV of the same buffer, and the eluant volume in the product stream of 0.083 BV. After, the column is washed with Water For Injection (WFI), and 1.5 BV. A Cleaning-in-Place protocol with NaOH (0.5 M), Water, and Ethanol 20% was performed. The eluted solution was stored in a receiver tank before going to the formulation step.

3.2.2.5 Formulation

The only change in this step compared to the *Escherichia coli* scenarios was the buffer in the diafiltration, which was changed to the Sodium Acetate Buffer previously used in size exclusion chromatography. The tangential flow filtration uses an UF Membrane (Biotech) present in the SuperPro database, concentrating the liquid solution to a Crisantaspase final concentration of 10 mg/mL. After a dead-end polishing filtration, a disposable storage tank stores the final product.

3.3 Estimation of Operating and Capital Costs

The following operating costs were considered in this work: raw materials; utilities; labor; waste treatment; and facility-dependent costs (which encompass plant maintenance, depreciation, and overhead expenses). The prices of utilities were the default values provided by SuperPro. In the downstream section, the values of chromatography columns and consumables (which include resins and cartridges) were updated by having the Cytiva products portfolio according to the required specifications in each simulated scenario. The hourly cost of labor remained unchanged with the values of SuperPro databank. The prices of raw materials were obtained either from the SuperPro databank or the website molbase.com. The selling price for both PEG-Asparaginase and Crisantaspase was set as 12.80 USD/mg, which corresponds to the Unit Production Cost in the *Escherichia coli* Baseline scenario plus a profit equal to 30%. An annual production rate of 1 kg per year was estimated, quite enough to supply Brazilian annual demand taking into account the average number of annual cases,

doses needed per treatment, price of Leuginase per dose, and the total amount spent by the Brazilian Ministry of Health (2017).

The following items were considered in the calculation of capital costs: Direct Fixed Capital (DFC), and Working Capital. The DFC is composed mainly of the equipment purchase and installation costs, but also the costs of piping, instrumentation, buildings, and so on, which were estimated based on the default factors of SuperPro.

The purchase cost of equipment was estimated based on the databank for cost functions of SuperPro Designer, whereas the Working Capital was estimated as the amount necessary to cover 30 days of labor, raw materials, utilities, and waste treatment expenses.

4 Results and Discussion

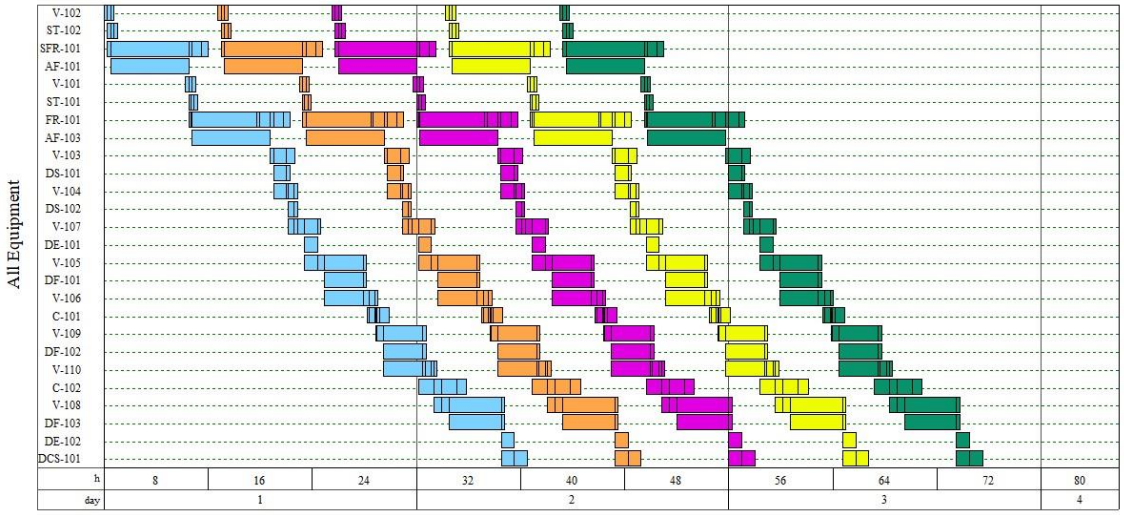
4.1 Process Scheduling

The Baseline scenarios for the production of L-Aparaginases using each microorganism were modeled according to the assumptions described in the Methodology section. A very useful output of modeling and simulation of processes is the possibility to visualize the complete scheduling of the process through the equipment occupancy chart, which shows the occupation of the pieces of equipment as a function of time. All processes equipment are listed on the y-axis and the elapsed time on the x-axis, with the horizontal bar representing the occupation of each equipment during its respective time (TOUMI et al., 2010). This type of representation enables the identification of bottlenecks that limit the maximum number of batches (or plant cycle time), described in the software as “the unit procedure with the longest duration”, also known as scheduling bottleneck (PETRIDES; KOULOURIS; LAGONIKOS, 2002).

For the *Escherichia coli* Baseline scenario, the occupancy time of each equipment is presented in Figure 12. The 32.5 hours cycle time is required to operate the entire process for a single batch. A cycle time slack (the amount of time between the beginning of a new batch according to the longest operation – in this case, the fermenter) was defined as equal to 1 hr. The volume of the P-6 seed fermenter and the P-5 fermenter are respectively 41 L and 452 L, and it is expected to contain in the broth fermentation 1.2 kg/batch of biomass with a

concentration of 3.92 g/L. Some amounts of residual elements from the culture medium are present as well. This batch time allows the processing of 902 batches per year. At the end of the process, 1.10 g/batch of PEG-Asparaginase is produced with a concentration of 10 mg/mL, which results in the stipulated annual production of 1 kg. The overall process parameters for all simulated scenarios are presented in Appendix B.

Figure 12 – Equipment occupancy chart for five consecutive batches in the *Escherichia coli* Baseline scenario.

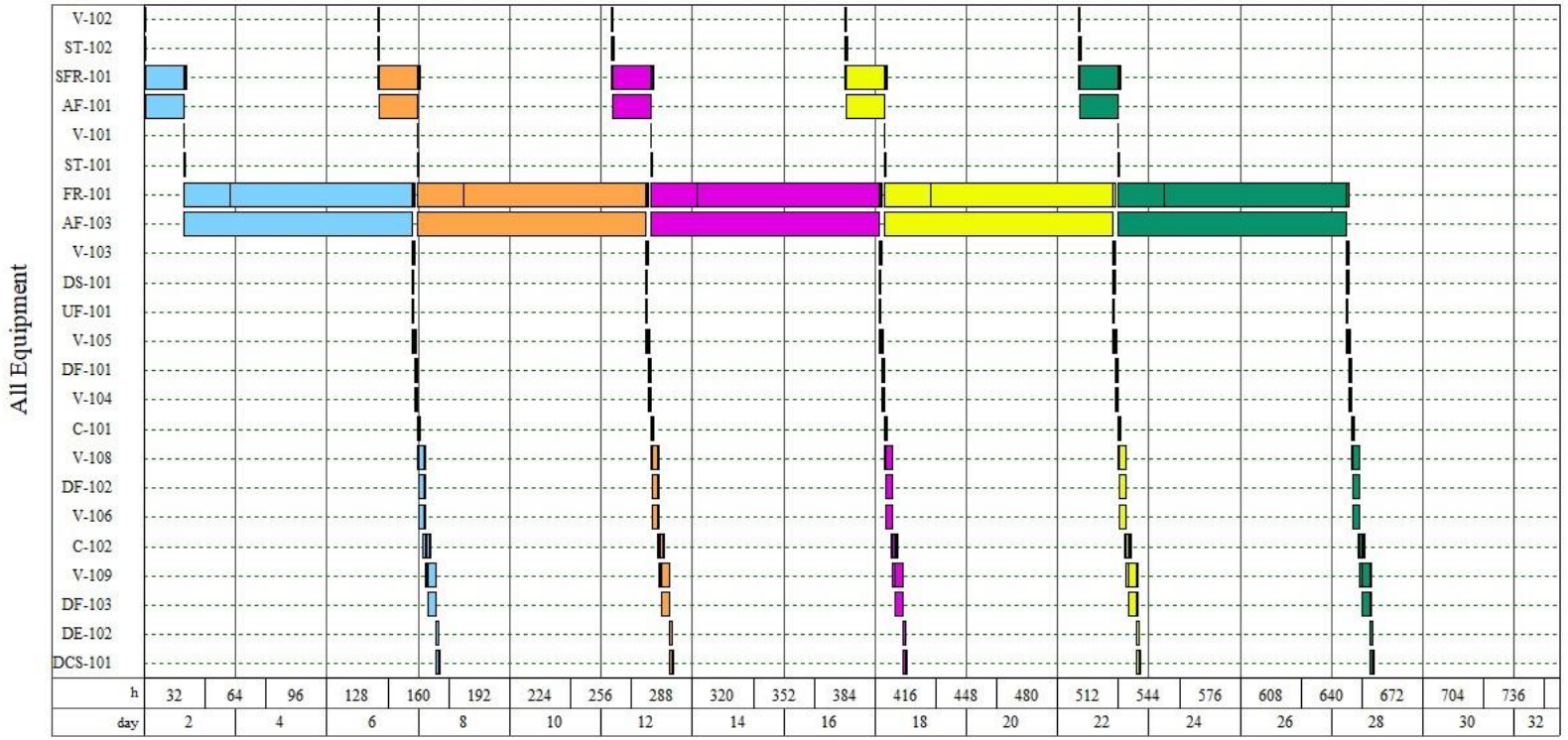


Source: this work.

The equipment occupancy chart for the same annual production of 1 kg of the production of Crisantaspase glycosylated by *Pichia pastoris* (in other words, scenario P1) is presented in Figure 13. Main fermentation is the longest procedure. This behavior was already expected since the following techniques were used: fed-batch cultivation; constant feeding rate and constant methanol concentration (generally above 4 g/L) aiming to achieve high cell concentration (DE ALMEIDA PARIZOTTO et al., 2021; GURRAMKONDA et al., 2009).

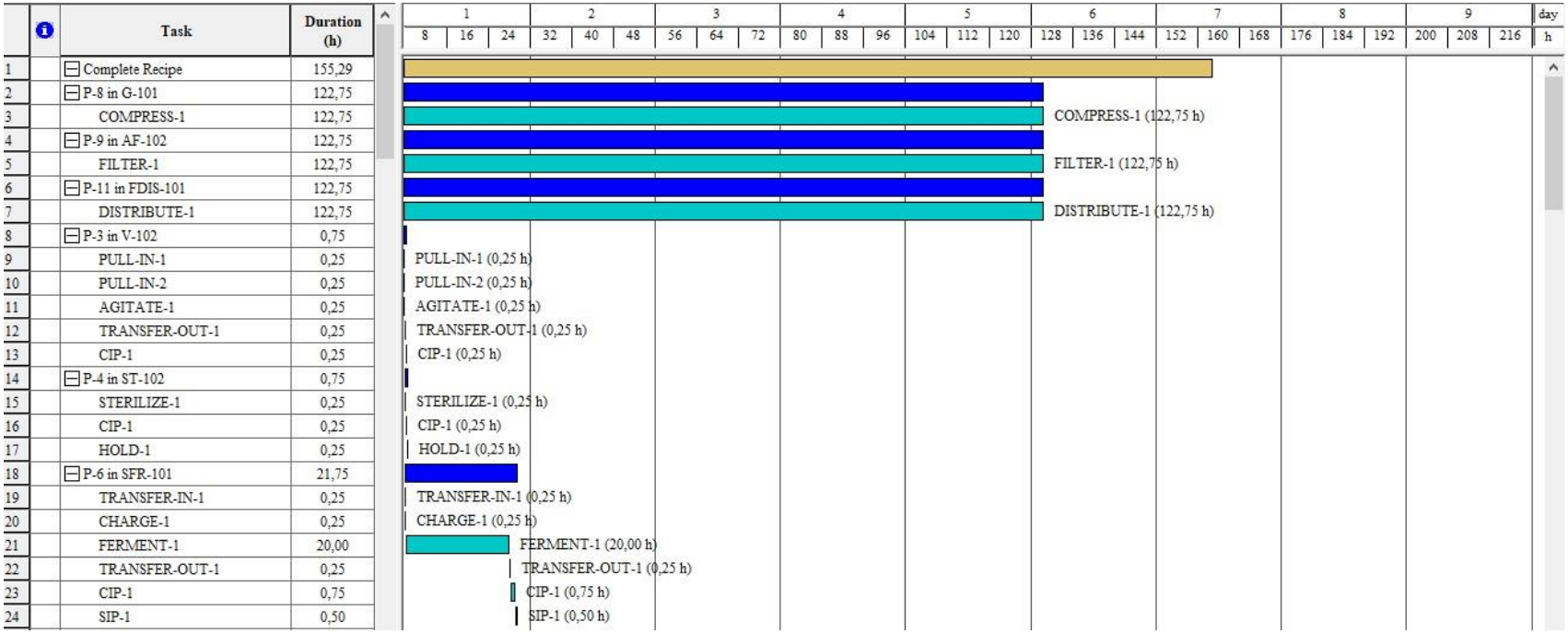
The analysis of the Gantt chart, which consists of a chart of horizontal bars representing each equipment present in the entire process, as well as their respective procedures involved and the duration of each operation on the longitudinal axis, shows that the set of operations that occur in the main fermenter (FR-101) constitute the scheduling bottleneck of the process, as presented in Figure 14.

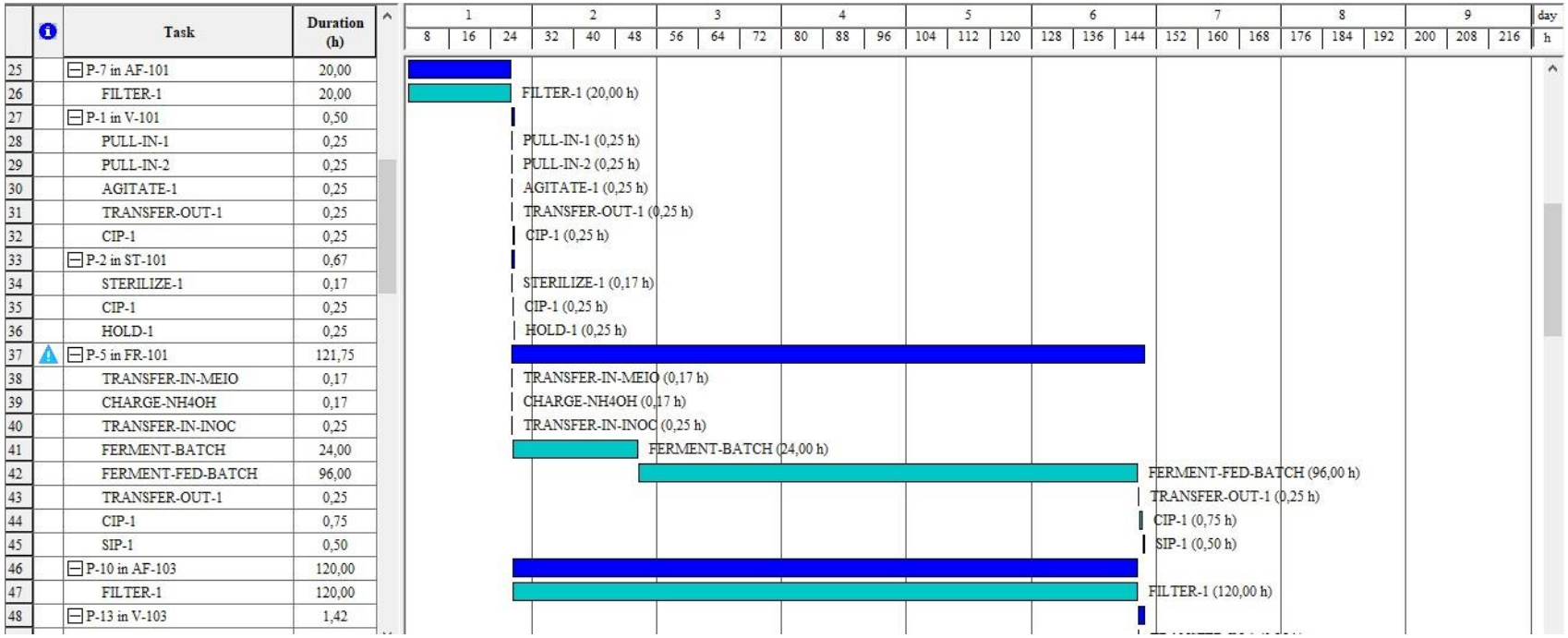
Figure 13 – Equipment occupancy chart for five consecutive batches in the P1 scenario.

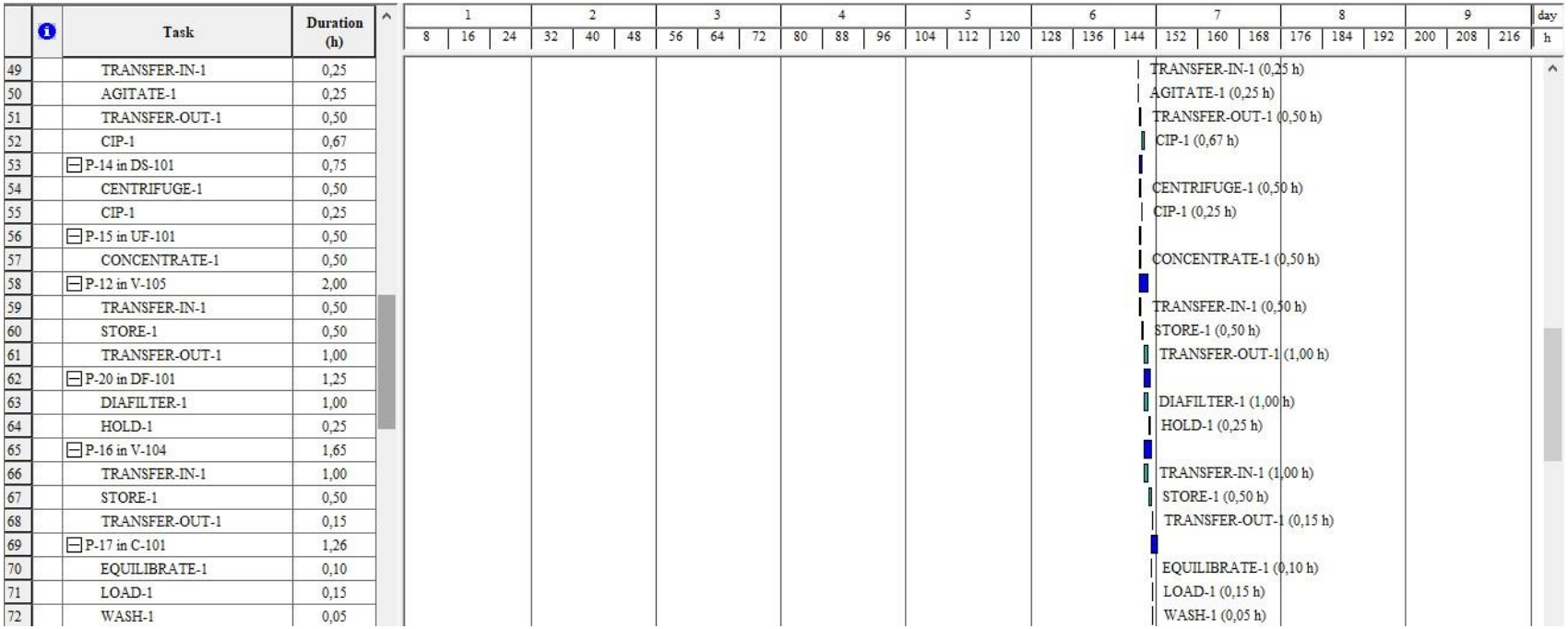


Source: this work

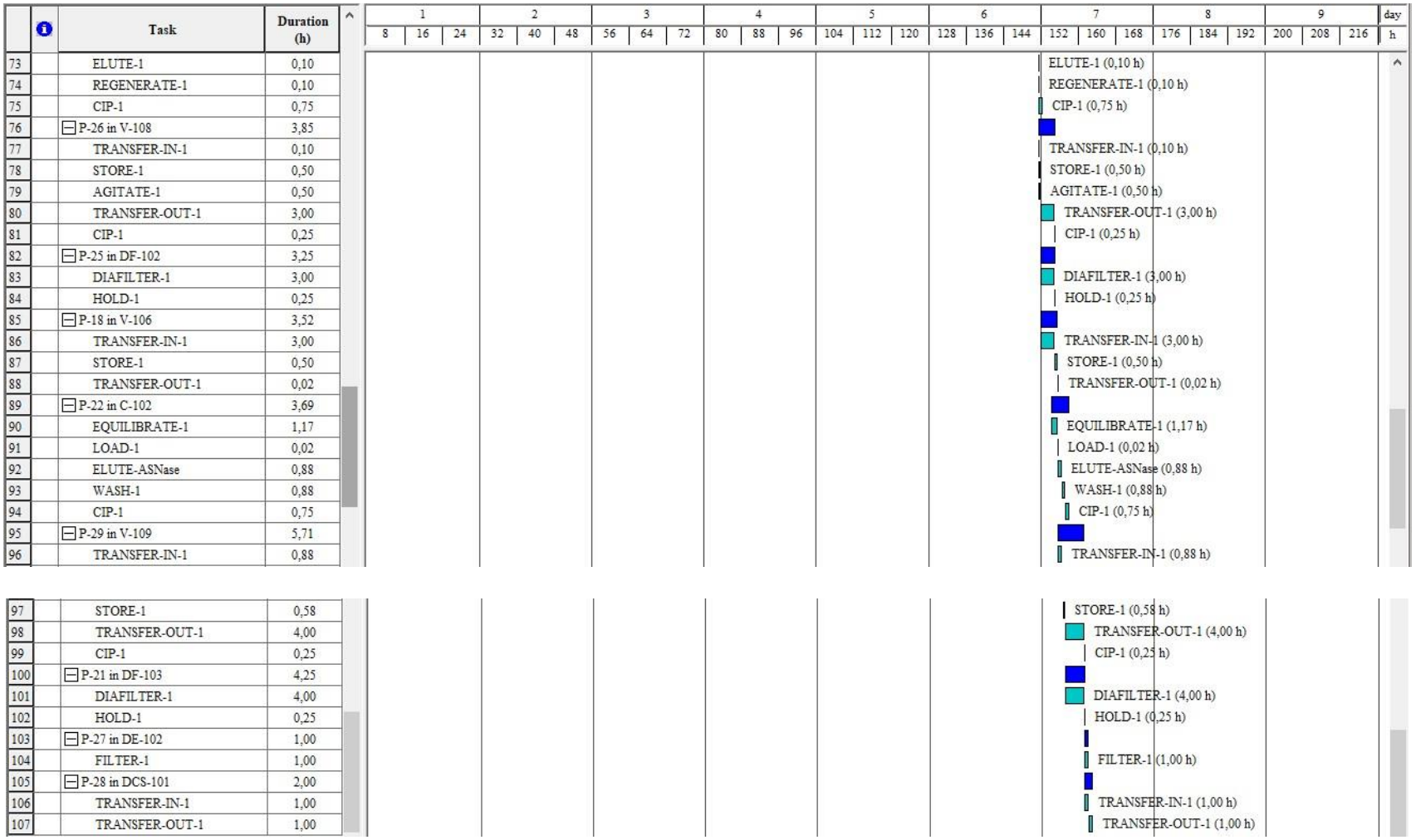
Figure 14 – Gantt Chart of a single batch of the glycosylated Crisantaspase baseline process production.




Task
Duration (h)
25 P-7 in AF-101 20,00
26 FILTER-1 20,00
27 P-1 in V-101 0,50
28 PULL-IN-1 0,25
29 PULL-IN-2 0,25
30 AGITATE-1 0,25
31 TRANSFER-OUT-1 0,25
32 CIP-1 0,25
33 P-2 in ST-101 0,67
34 STERILIZE-1 0,17
35 CIP-1 0,25
36 HOLD-1 0,25
37 P-5 in FR-101 121,75
38 TRANSFER-IN-MEIO 0,17
39 CHARGE-NH4OH 0,17
40 TRANSFER-IN-INOC 0,25
41 FERMENT-BATCH 24,00
42 FERMENT-FED-BATCH 96,00
43 TRANSFER-OUT-1 0,25
44 CIP-1 0,75
45 SIP-1 0,50
46 P-10 in AF-103 120,00
47 FILTER-1 120,00
48 P-13 in V-103 1,42
1 2 3 4 5 6 7 8 9 day
8 16 24 32 40 48 56 64 72 80 88 96 104 112 120 128 136 144 152 160 168 176 184 192 200 208 216 h
FILTER-1 (20,00 h)
PULL-IN-1 (0,25 h)
PULL-IN-2 (0,25 h)
AGITATE-1 (0,25 h)
TRANSFER-OUT-1 (0,25 h)
CIP-1 (0,25 h)
STERILIZE-1 (0,17 h)
CIP-1 (0,25 h)
HOLD-1 (0,25 h)
TRANSFER-IN-MEIO (0,17 h)
CHARGE-NH4OH (0,17 h)
TRANSFER-IN-INOC (0,25 h)
FERMENT-BATCH (24,00 h)
FERMENT-FED-BATCH (96,00 h)
TRANSFER-OUT-1 (0,25 h)
CIP-1 (0,75 h)
SIP-1 (0,50 h)
FILTER-1 (120,00 h)



	Task	Duration (h)
49	TRANSFER-IN-1	0,25
50	AGITATE-1	0,25
51	TRANSFER-OUT-1	0,50
52	CIP-1	0,67
53	P-14 in DS-101	0,75
54	CENTRIFUGE-1	0,50
55	CIP-1	0,25
56	P-15 in UF-101	0,50
57	CONCENTRATE-1	0,50
58	P-12 in V-105	2,00
59	TRANSFER-IN-1	0,50
60	STORE-1	0,50
61	TRANSFER-OUT-1	1,00
62	P-20 in DF-101	1,25
63	DIAFILTER-1	1,00
64	HOLD-1	0,25
65	P-16 in V-104	1,65
66	TRANSFER-IN-1	1,00
67	STORE-1	0,50
68	TRANSFER-OUT-1	0,15
69	P-17 in C-101	1,26
70	EQUILIBRATE-1	0,10
71	LOAD-1	0,15
72	WASH-1	0,05



Source: this work.

The volumes of each fermenter are respectively 5 L and 31 L for the seed and main fermenter. The total cultivation time is 120 hr with the first 24 hr operating in batch, followed by a fed-batch with methanol induction for 96 hr. The batch time of 155 hours and cycle time slack of 1 hour assured the realization of 64 batches per year. A mass of 21.86 kg/batch is produced, and the fermentation broth is expected to contain 65 g/L of yeast, 4.5 g/L of Crisantaspase, and residual components of the medium in low concentration. At the end of the process, 15.62 g of Crisantaspase are produced per batch, which guarantees an annual production of 1 kg. The simulation output parameters are presented in Appendix B.

4.2 Economic Evaluation

4.2.1 Comparison of Process Scenarios

4.2.1.1 Escherichia coli

Table 7 presents the main results of the economic analysis for each scenario including all the profitability metrics described in the Methodology section. Different production scenarios were simulated evaluating the impact of the increase of the final biomass concentration of the main bioreactor on the economic metrics of the processes, always for the production of 1.0 kg of enzyme per year. According to the parameters defined in the Literature Review section, all scenarios were found economically viable. An important point to consider is that the fed-batch processes (processes S1 – S5) led to a gradual decrease in the Total Capital Investment (TCI) and Unit Production Cost (UPC) from 19.89 million USD and 9.83 USD/mg to 17.47 million USD and 7.57 USD/mg. This is mainly because the process optimization leads to an increase in cell concentration, and consequently smaller equipment (mainly tanks and fermenters) are necessary to produce the same amount of product. Once the equipment purchase cost is straightly related to the estimation of Total Capital Investment through the multiplier factors (HEINZLE; BIWER; COONEY, 2006), smaller equipment have an impact on the reduction of capital-cost.

Processes that require small equipment also have the operating-costs reduced due to the decrease in facility-dependent costs, such as depreciation, maintenance, and repair

(HEINZLE; BIWER; COONEY, 2006). Comparing all the modeled scenarios, S5 is the one that presents the best economic indexes, such as the highest Return on Investment, Internal Rate on Return, Net Present Value, and the lowest Payback time. Furthermore, in industrial biotechnological processes, the fed-batch mode is indispensable in order to achieve high cell concentrations (FERREIRA et al., 2021a). The equipment occupancy chart and the Gantt Chart for the S5 scenario are presented in Figures 15 and 16 respectively. For this cell concentration, the batch time is 41.96 hr and it is possible to process 436 batches per year.

Table 7 – Total Capital Investment, Unit Production Cost, Annual Production, and profitability metrics found for the production of PEG-ASN according to the final biomass concentration obtained in the main bioreactor.

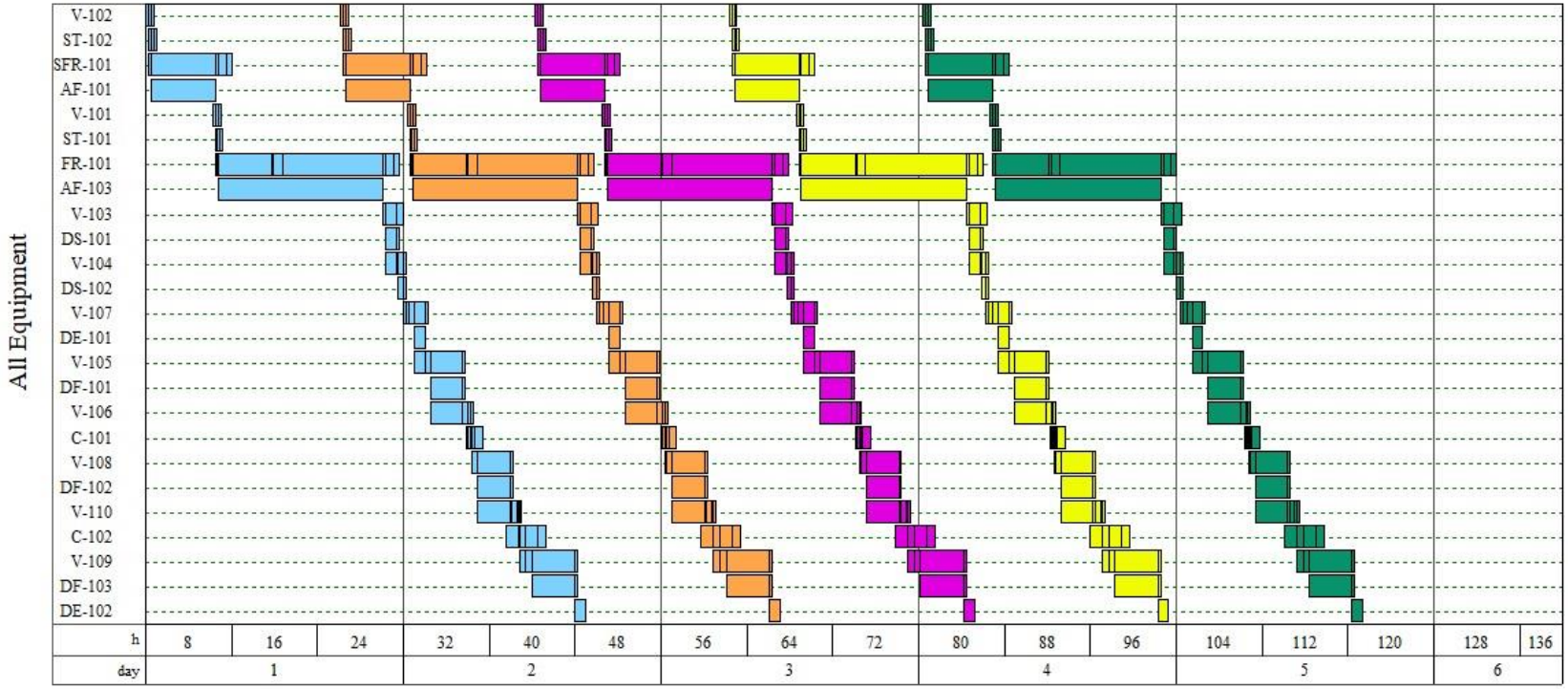
	***E. coli* Baseline (3.92 g/L)**	***E. coli* S1 (10.00 g/L)**	***E. coli* S2 (20.00 g/L)**	***E. coli* S3 (30.00 g/L)**	***E. coli* S4 (40.00 g/L)**	***E. coli* S5 (65.00 g/L)**
Total Capital Investment (million USD)	19.89	18.67	17.92	17.73	17.65	17.47
Unit Production Cost (USD/mg)	9.83	8.59	8.06	7.81	7.69	7.57
Return on Investment (ROI)[2]	17.82%	22.40%	24.74%	25.76%	26.24%	26.81%
Payback Time (years)[2]	5.61	4.47	4.04	3.88	3.81	3.73
Internal Rate of Return (IRR)[2]	11.17%	15.81%	18.01%	18.98%	19.41%	19.93%
Net Present Value [1] (NPV) (million USD)	5.24	11.14	13.75	14.92	15.47	16.06
Production (kg/year)	1.00	1.00	1.00	1.00	1.00	1.00

[1] Considering an annual interest rate equal to 7%.;

[2] Revenue Value: 12.80 USD/mg

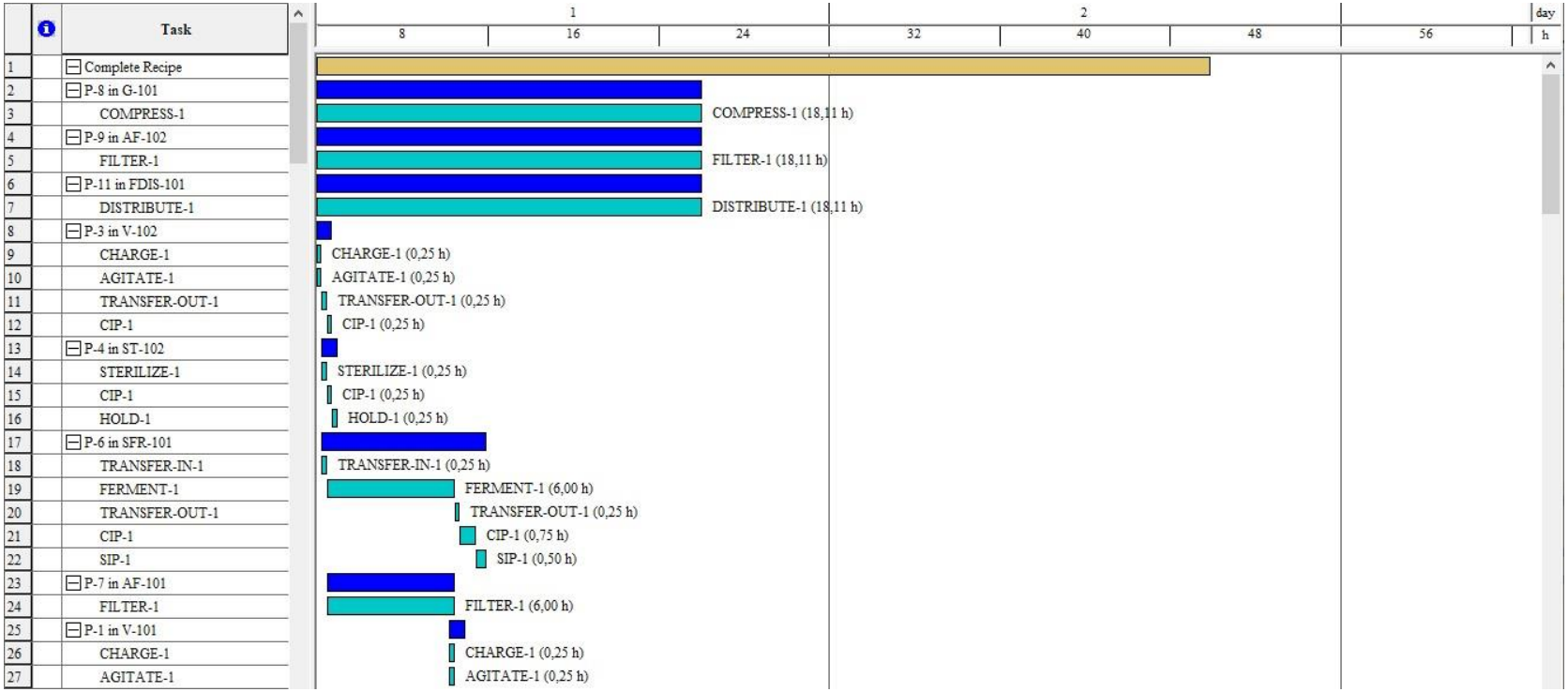
Source: this work.

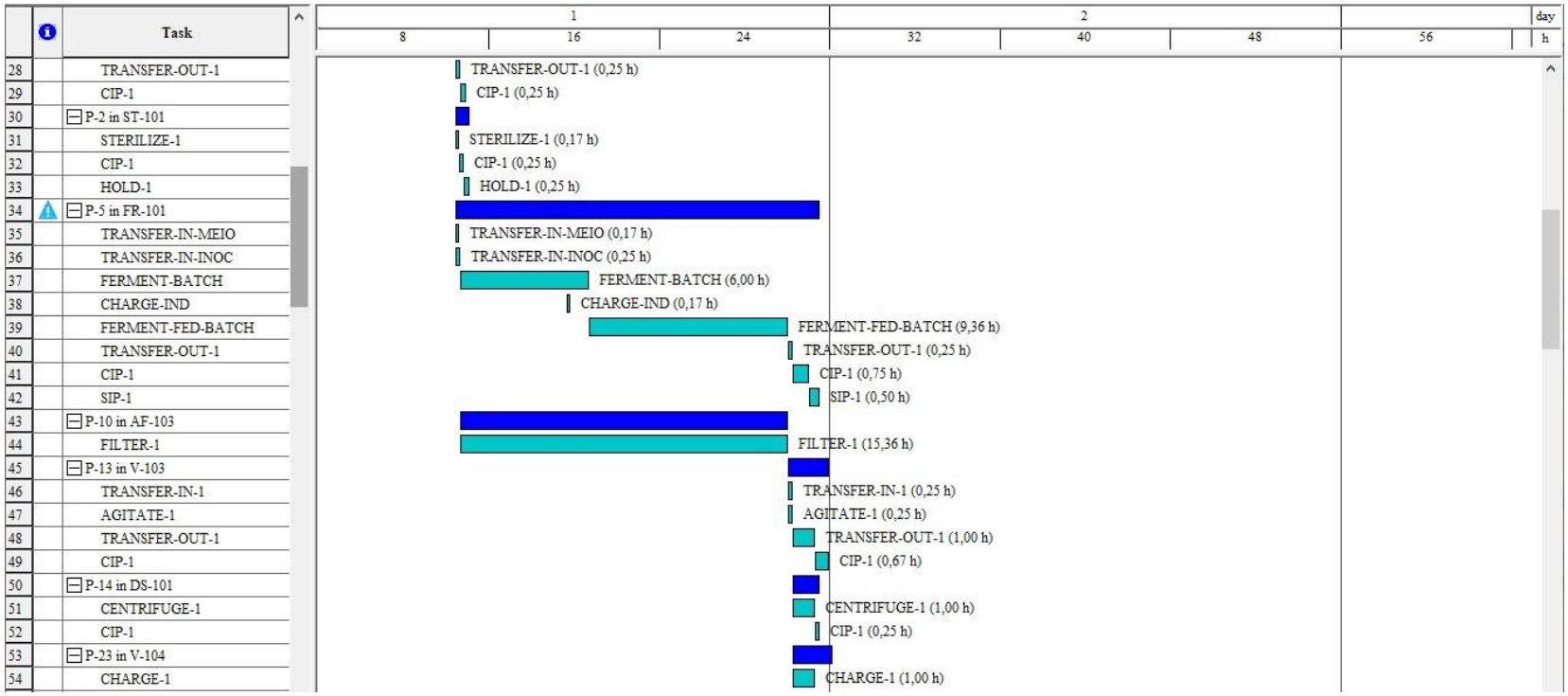
Figure 15 – Equipment occupancy chart for five consecutive batches in the S5 scenario.

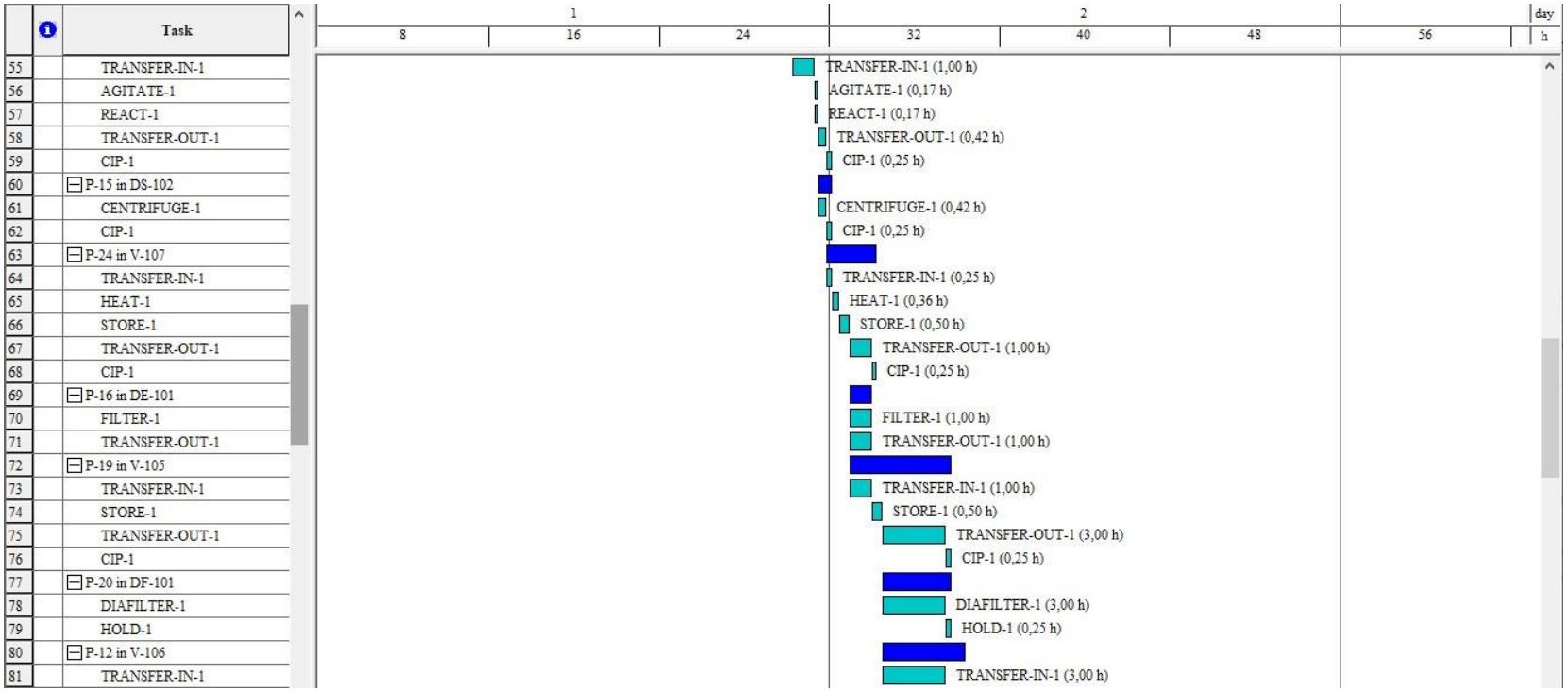


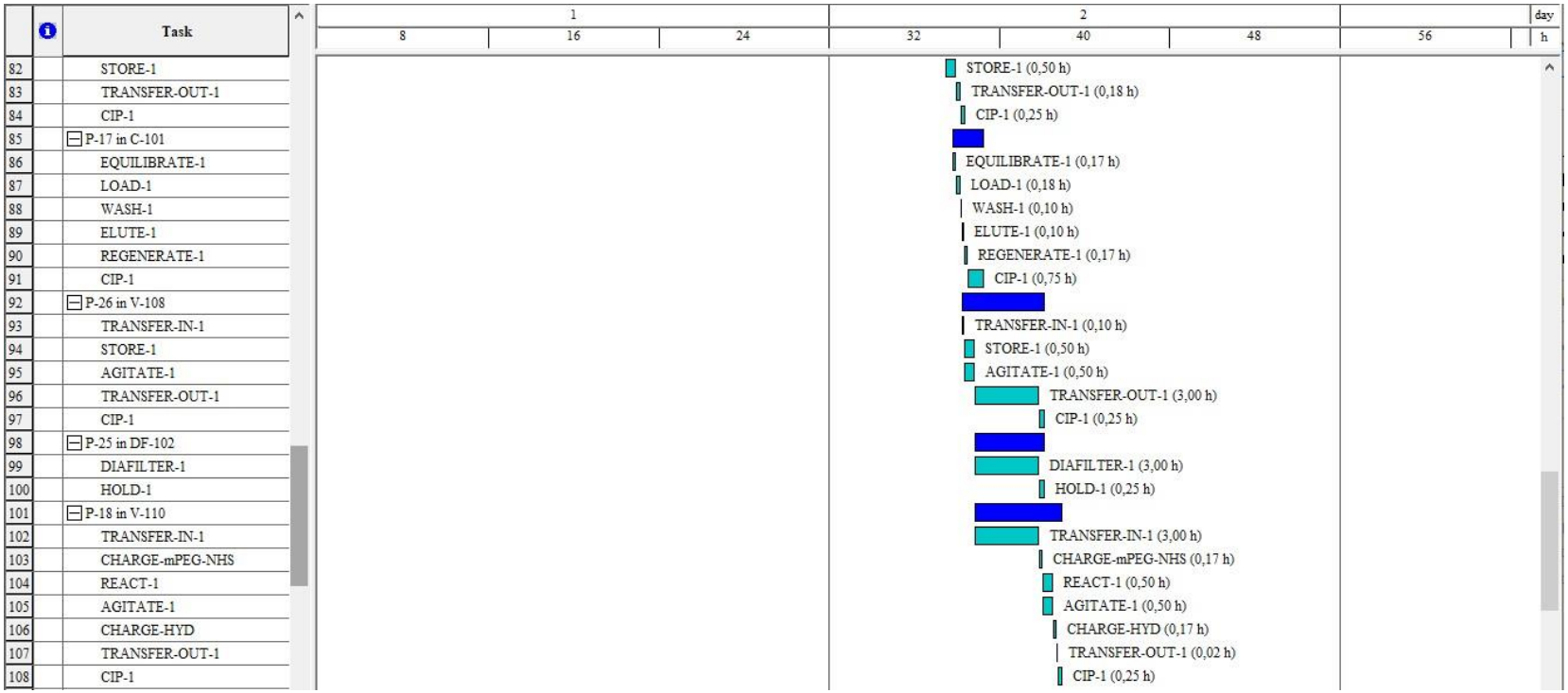
Source: this work.

Figure 16 – Gantt Chart of a single batch of the PEGylated L-Asparaginase process production.

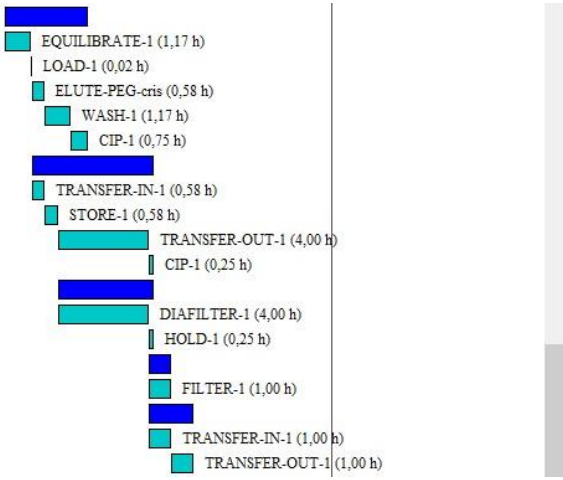



Task
1
2
day
8
16
24
32
40
48
56
h
28 TRANSFER-OUT-1
29 CIP-1
30 P-2 in ST-101
31 STERILIZE-1
32 CIP-1
33 HOLD-1
34 P-5 in FR-101
35 TRANSFER-IN-MEIO
36 TRANSFER-IN-INOC
37 FERMENT-BATCH
38 CHARGE-IND
39 FERMENT-FED-BATCH
40 TRANSFER-OUT-1
41 CIP-1
42 SIP-1
43 P-10 in AF-103
44 FILTER-1
45 P-13 in V-103
46 TRANSFER-IN-1
47 AGITATE-1
48 TRANSFER-OUT-1
49 CIP-1
50 P-14 in DS-101
51 CENTRIFUGE-1
52 CIP-1
53 P-23 in V-104
54 CHARGE-1
TRANSFER-OUT-1 (0,25 h)
CIP-1 (0,25 h)
STERILIZE-1 (0,17 h)
CIP-1 (0,25 h)
HOLD-1 (0,25 h)
TRANSFER-IN-MEIO (0,17 h)
TRANSFER-IN-INOC (0,25 h)
FERMENT-BATCH (6,00 h)
CHARGE-IND (0,17 h)
FERMENT-FED-BATCH (9,36 h)
TRANSFER-OUT-1 (0,25 h)
CIP-1 (0,75 h)
SIP-1 (0,50 h)
FILTER-1 (15,36 h)
TRANSFER-IN-1 (0,25 h)
AGITATE-1 (0,25 h)
TRANSFER-OUT-1 (1,00 h)
CIP-1 (0,67 h)
CENTRIFUGE-1 (1,00 h)
CIP-1 (0,25 h)
CHARGE-1 (1,00 h)


Task
1
2
day
8
16
24
32
40
48
56
h
55 TRANSFER-IN-1
56 AGITATE-1
57 REACT-1
58 TRANSFER-OUT-1
59 CIP-1
60 P-15 in DS-102
61 CENTRIFUGE-1
62 CIP-1
63 P-24 in V-107
64 TRANSFER-IN-1
65 HEAT-1
66 STORE-1
67 TRANSFER-OUT-1
68 CIP-1
69 P-16 in DE-101
70 FILTER-1
71 TRANSFER-OUT-1
72 P-19 in V-105
73 TRANSFER-IN-1
74 STORE-1
75 TRANSFER-OUT-1
76 CIP-1
77 P-20 in DF-101
78 DIAFILTER-1
79 HOLD-1
80 P-12 in V-106
81 TRANSFER-IN-1
TRANSFER-IN-1 (1,00 h)
AGITATE-1 (0,17 h)
REACT-1 (0,17 h)
TRANSFER-OUT-1 (0,42 h)
CIP-1 (0,25 h)
CENTRIFUGE-1 (0,42 h)
CIP-1 (0,25 h)
TRANSFER-IN-1 (0,25 h)
HEAT-1 (0,36 h)
STORE-1 (0,50 h)
TRANSFER-OUT-1 (1,00 h)
CIP-1 (0,25 h)
FILTER-1 (1,00 h)
TRANSFER-OUT-1 (1,00 h)
TRANSFER-IN-1 (1,00 h)
STORE-1 (0,50 h)
TRANSFER-OUT-1 (3,00 h)
CIP-1 (0,25 h)
DIAFILTER-1 (3,00 h)
HOLD-1 (0,25 h)
TRANSFER-IN-1 (3,00 h)


Task
1
2
day
8
16
24
32
40
48
56
h
82 STORE-1
83 TRANSFER-OUT-1
84 CIP-1
85 P-17 in C-101
86 EQUILIBRATE-1
87 LOAD-1
88 WASH-1
89 ELUTE-1
90 REGENERATE-1
91 CIP-1
92 P-26 in V-108
93 TRANSFER-IN-1
94 STORE-1
95 AGITATE-1
96 TRANSFER-OUT-1
97 CIP-1
98 P-25 in DF-102
99 DIAFILTER-1
100 HOLD-1
101 P-18 in V-110
102 TRANSFER-IN-1
103 CHARGE-mPEG-NHS
104 REACT-1
105 AGITATE-1
106 CHARGE-HYD
107 TRANSFER-OUT-1
108 CIP-1
STORE-1 (0,50 h)
TRANSFER-OUT-1 (0,18 h)
CIP-1 (0,25 h)
EQUILIBRATE-1 (0,17 h)
LOAD-1 (0,18 h)
WASH-1 (0,10 h)
ELUTE-1 (0,10 h)
REGENERATE-1 (0,17 h)
CIP-1 (0,75 h)
TRANSFER-IN-1 (0,10 h)
STORE-1 (0,50 h)
AGITATE-1 (0,50 h)
TRANSFER-OUT-1 (3,00 h)
CIP-1 (0,25 h)
DIAFILTER-1 (3,00 h)
HOLD-1 (0,25 h)
TRANSFER-IN-1 (3,00 h)
CHARGE-mPEG-NHS (0,17 h)
REACT-1 (0,50 h)
AGITATE-1 (0,50 h)
CHARGE-HYD (0,17 h)
TRANSFER-OUT-1 (0,02 h)
CIP-1 (0,25 h)

109	P-22 in C-102
110	EQUILIBRATE-1
111	LOAD-1
112	ELUTE-PEG-cris
113	WASH-1
114	CIP-1
115	P-29 in V-109
116	TRANSFER-IN-1
117	STORE-1
118	TRANSFER-OUT-1
119	CIP-1
120	P-21 in DF-103
121	DIAFILTER-1
122	HOLD-1
123	P-27 in DE-102
124	FILTER-1
125	P-28 in DCS-101
126	TRANSFER-IN-1
127	TRANSFER-OUT-1



Source: this work.

4.2.1.2 Pichia pastoris

Table 8 presents the main results of the economic analysis based on the same profitability metrics defined for *Escherichia coli* scenarios. The use of scale-up factors for seed, batch fermentation, and fed-batch with methanol induction strategy previously described in the Methodology section were applied to the process described by DE ALMEIDA PARIZOTTO et al., (2021) resulting in an annual production of 8 kg, requiring a Total Capital Investment estimated in 23.57 million USD.

With the purpose to accomplish a sharp comparison between the different microorganisms, the P1 scenario was created, setting the same annual production amount of 1 kg of glycosylated Crisantaspase. The Total Capital Investment required was lower compared to the best scenario for the production of PEGylated L-Asparaginase (S5). In processes that use *Escherichia coli* as microorganism complex steps are required, such as osmotic shock (CACHUMBA et al., 2016; COSTA-SILVA et al., 2020), or additional steps, which in this process is represented by PEGylation (BARROS et al., 2021; MEDAWAR et al., 2020). As a consequence, the costs related to equipment (acquisition and facility-cost) and raw materials are significantly increased. The Unit Production Cost of 3.42 USD/mg found for P1 represents almost half when compared to S5 scenario.

According to the values of specific activity and dose of Leuginase, Erwinase, and Oncaspar, and the budget for the acquisition of each one in the last bid of the Brazilian Ministry of Health (2017) it is possible to construct Table 9, which displays the values in USD/mg for each pharmaceutical. The results of the economic analysis suggest that even in the most conservative scenario, which means the closest to simply scaling up the process developed by TORRES-OBREQUE et al., (2019) without any operational changes (corresponding to the *Escherichia coli* baseline scenario), the Unit Production Cost is almost 4 times lower when compared to Oncaspar, the only biopharmaceutical that features PEGylation technology currently available in the market and used as first or second-line treatment (LOPES et al., 2017; PIETERS; HUNGER STEPHEN; BOOS JOACHIM, 2010; SANTOS et al., 2018). For the scenario with the best economic parameters (S5), the Unit Production Cost of PEG-Asparaginase is approximately 5 times lower.

The rise in cell concentration and as a consequence the decrease in equipment volumes and raw material required may justify the diminish in the Unit Production Cost. This new

biopharmaceutical can be classified in the class of biobetters (SANTOS et al., 2018) due to the N-terminal site-directed PEGylation technology, absent in Oncaspar, which features random PEGylation (MENEGUETTI et al., 2019). Among all the possible drugs listed by the Ministry of Health, Spectrila also presents recombinant DNA technology, which makes possible to establish a pattern comparison with glycosylated Crisantaspase produced by *Pichia pastoris*. The Unit Product Cost to produce this form of enzyme resulted in a value 4.5 times lower than Spectrila.

Table 8– Total Capital Investment, Unit Production Cost, Annual Production, and profitability metrics for the production of glycosylated Crisantaspase.

	***P. pastoris* Baseline (65.00 g/L)**	***P. pastoris* P1 (65.00 g/L)**
Total Capital Investment (million USD)	23.57	15.98
Unit Production Cost (USD/mg)	0.80	3.42
Return on Investment (ROI)[2]	253.37%	44.24%
Payback Time (years)[2]	0.39	2.26
Internal Rate of Return (IRR)[2]	120.36%	33.45%
Net Present Value [1] (NPV) (million USD)	401.08	34.75
Production (kg/year)	8.00	1.00

[1] Considering an annual interest rate of 7%.;

[2] Revenue Value: USD = 12.80

Source: this work.

Table 9 – Estimated cost of different L-Asparaginase formulations.

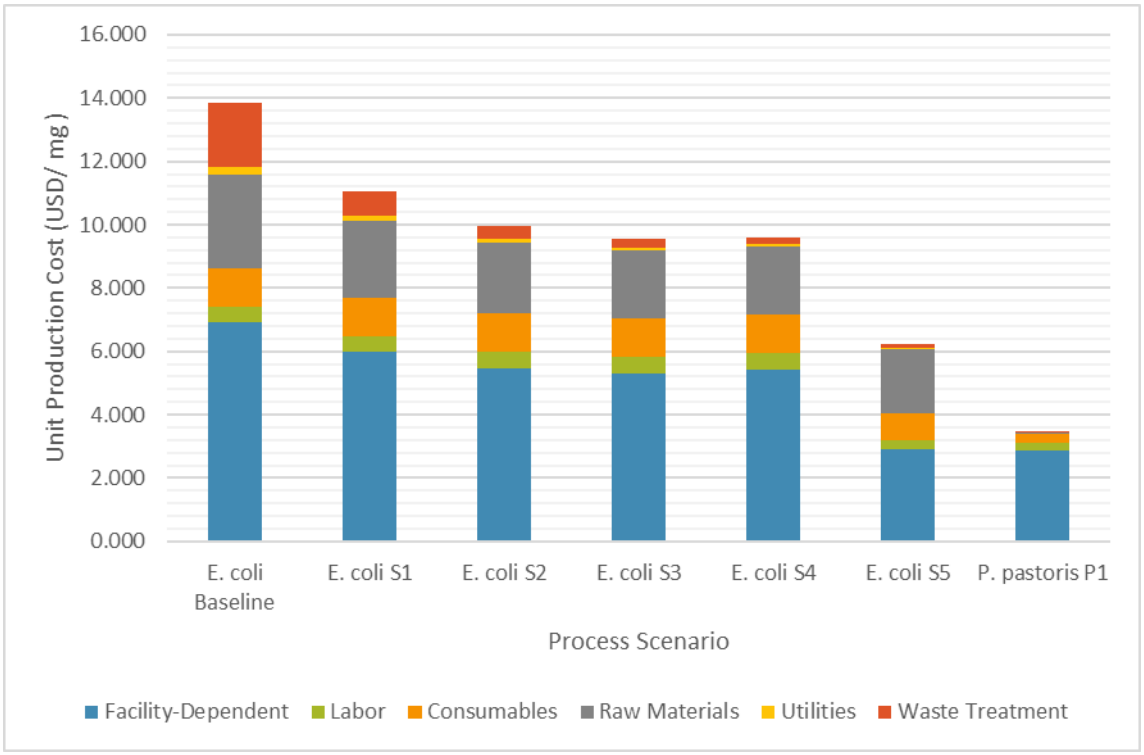
	Specific Activity (U/mg)	**Dose (UI)**	**Value (USD)**	**Price (USD/mg)**
Leuginase	269	10000	38.00	1.02
Spectrila	269	10000	582.17	15.66
Oncaspar	85	3750	1619.1	36.70

Source: this work.

Figure 17 complements the analysis of the tables displaying the Unit Production Cost for all simulated scenarios, broken down by cost categories such as raw materials, labor, consumables, utilities, and waste treatment. In all scenarios, the facility-dependent cost is the largest component, accounting for between 35% and 84% depending on the scenario. Facility costs, which include plant maintenance costs, plant depreciation, and overhead costs are

closely related to the equipment purchase costs and presents the same behavior as the Total Capital Investment. Batch scenarios usually require higher capital costs (FERREIRA et al., 2021a). The cost of raw material in the *Escherichia coli* scenarios varies according to the increase of cell concentration in fermentation, requiring lower volumes of culture medium. In some processes, the impact of the culture medium on the cost of raw materials may represent up to 84% (FERREIRA et al., 2021a). PEGylation of biopharmaceuticals generally involves the use of different mPEG formulations. One of the most common is mPEG-NHS, an amino reactive (SANTOS et al., 2018). The production of more L-Asparaginase per batch requires a bigger amount of mPEG to produce PEG-Asparaginase through the N-terminal site-specific reaction (MENEGUETTI et al., 2019). This is a component of high added value, consequently increasing raw material cost. Due to the absence of PEGylation reaction and the simplicity of the culture medium, the raw material cost for *Pichia pastoris* is considerably smaller, representing the fourth. The cost of consumables is high in both scenarios, which is composed of the filtration membranes and resins used in the recovery and chromatography sections. Diafiltration systems remove many impurities present in the extracellular broth, such as cellular debris, and salts, performing a separation of the biomolecule of interest from these impurities (FERREIRA et al., 2021a). Waste treatment cost decreases according to the rising in cell concentration in each scenario because of the decrease in the volumes of raw materials and volumes filtered in the purification steps, which implies the generation of less liquid and solid waste.

Figure 17 – Breakdown of the Unit Production Cost for all evaluated scenarios.



Source: this work.

4.2.2 Cost Composition

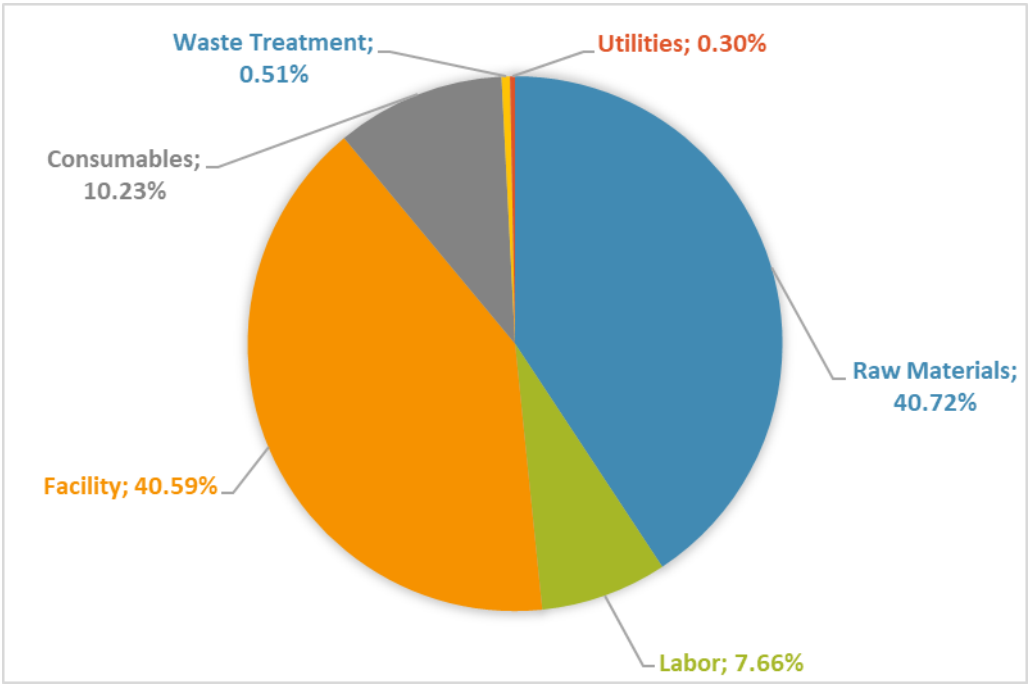
4.2.2.1 Escherichia coli

Figure 18 displays the cost composition for the production of PEG-Asparaginase in the S5 scenario. Raw material costs account for 40.72% of the unit production cost of biopharmaceutical. The use of cheaper carbon sources and induction strategies are a possible approach to reduce raw material costs and consequently the overall production costs (FERREIRA; AZZONI; FREITAS, 2018a). Among raw materials, mPEG-NHS and sucrose make up approximately 63% and 36% respectively. Sucrose is one of the components present in the hypotonic solution used in the osmotic shock aiming to release the enzyme from the periplasmic space (TORRES-OBREQUE et al., 2019). The rising in cell productivity in fermentation leads to the need for a bigger volume of hypotonic solution to perform the osmotic shock since more cells are produced. The use of strategies that involve cell disruption, such as homogenization, may be an alternative to diminish the impact of raw material in the Recovery section. It is important to point out that mPEG-NHS is intrinsically a

high-value-added component, so it might be difficult to diminish its impact on the costs. The optimization of the upstream section impacts directly the production of PEG-Asparaginase in downstream since more reagent is required for the PEGylation reaction to happen. The use of different enzyme:PEG molar ratios or a recycling strategy of the non-reacted L-Asparaginase after gel filtration chromatography may be interesting approaches.

Facility-dependent, which includes the costs related to plant maintenance, depreciation, insurance, local costs, and costs not directly associated with the process (FERREIRA; AZZONI; FREITAS, 2018a) accounts for 40.59%. Consumables costs make up 10.23% of total unit production cost. This category includes the procedures that take place in the downstream section and their respective consumables: dead-end filtration cartridges, ultrafiltration membranes used in tangential flow filtration, diafiltration, and resins used in ionic exchange and gel filtration chromatography. The characteristics and values of all consumables updated according to Cytiva's product portfolio are presented in Appendix A. Labor costs represent almost 8% of the total cost. Despite the possibility of using values from the literature, this cost varies drastically according to the location and labor laws of the country in which the plant is installed (HEINZLE; BIWER; COONEY, 2006).

Figure 18 – Cost composition of the PEG-Asparaginase production in the S5 scenario.



Source: this work.

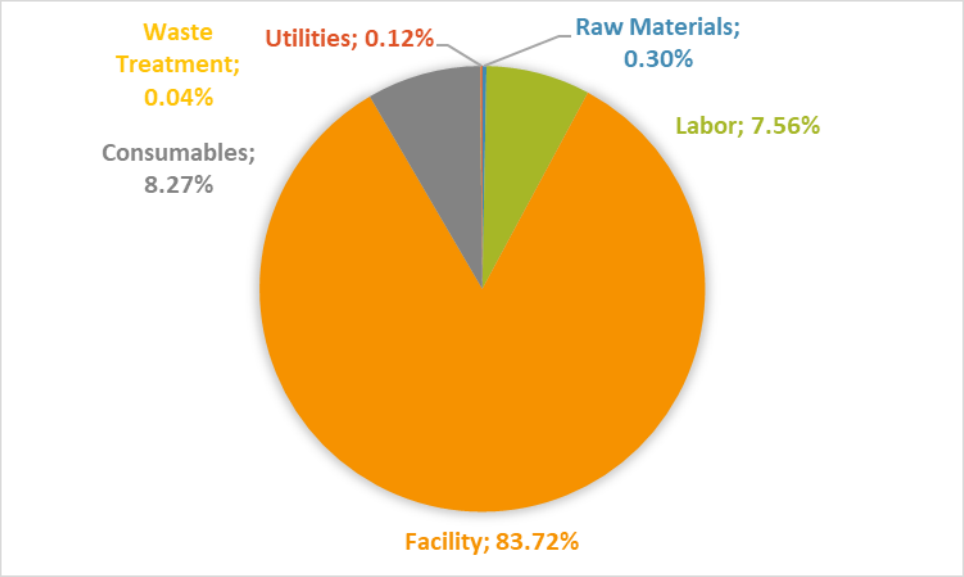
4.2.2.2 Pichia pastoris

Figure 19 shows the composition of the unit production cost for the production of glycosylated Crisantaspase. Facility-dependent costs make up almost 84%. It is important to point out that this value may be overestimated since it is straightly proportional to the equipment acquisition cost, which is given by SuperPro models based on the USA market. The use of factors to adjust the required total capital investment according to the country, number of equipment that the company already owns, amount of equipment that would be imported, exchange rate fluctuations, and possible government incentives may drastically modify facility costs.

Consumables cost corresponds to 8.27%. In the same way as the *Escherichia coli* scenarios, this category is represented by cartridges and membranes used in ultrafiltration, diafiltration, and resins for ion exchange and gel filtration in chromatography steps. The values were also updated according to the particularities of each unit procedure and can be seen in Appendix A. Labor costs are significant, corresponding to third place with approximately 8%. The number of workers is defined by batch, so estimates are usually made based on the use of each piece of equipment, and the information provided by a good model for simulation (PAPAVASILEIOU et al., 2007).

Raw material costs come in a distant fourth place, unlike the PEGylated L-Asparaginase production process, in which is the main driver of the total production costs. The choice of a culture medium that presents simple components, the fact that the enzyme is secreted into the extracellular medium, simplifying recovery, and the absence of the PEGylation contribute to the reduction of costs related to this step. NaOH (0.5 M), WFI, and Acetate buffer + 1M NaCl have an impact of 43%, 16%, and 13% respectively on raw material costs. NaOH (0.5 M) is used in a standard procedure throughout the plant in the clean-in-place steps of equipment such as fermenters and tanks. WFI is related to the composition of buffers while Acetate buffer + NaCl 1 M is the elution solution in ion exchange chromatography.

Figure 19– Cost composition of the glycosylated Crisantaspase in the P1 scenario.



Source: this work.

4.2.3 Breakdown of Production Costs by Process Section

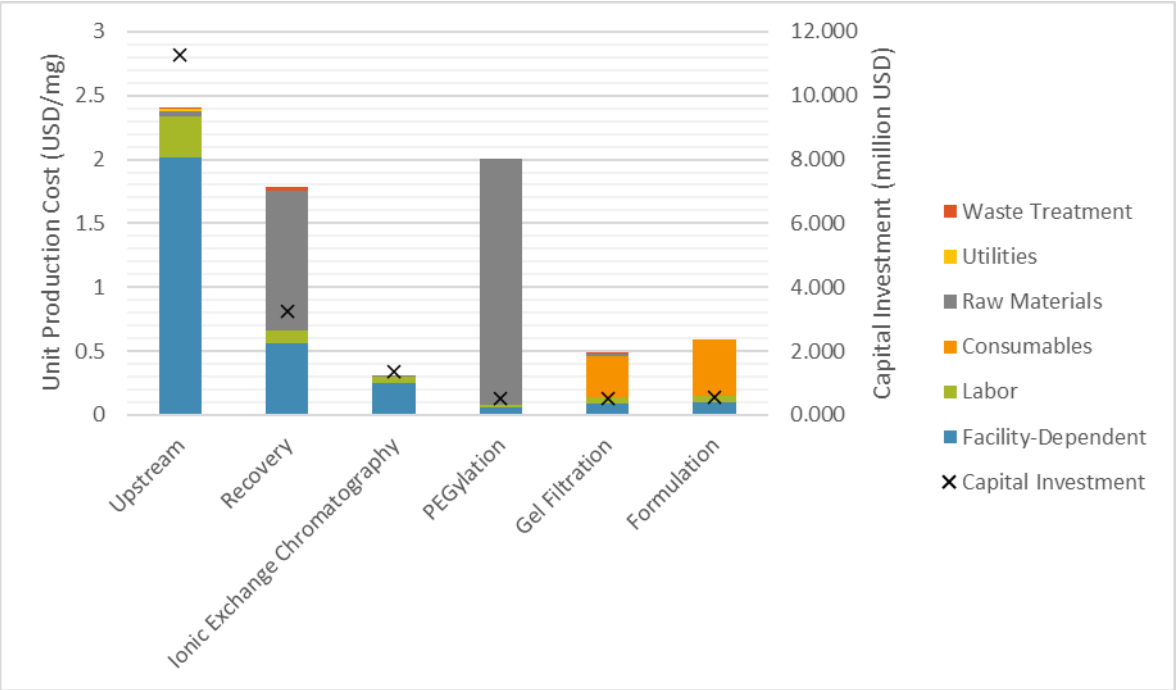
4.2.3.1 Escherichia coli

Among all the developed scenarios, the S5 was the most attractive concerning economic parameters. Therefore, it is useful to analyze the capital and operating costs to better understand the main cost drivers of PEG-Asparaginase production. The relationship between the operating costs, which are represented in detail by each process section, and the capital investment is shown in Figure 20. The largest operating costs correspond to the Upstream section, followed by PEGylation. The recovery section comes close in third place. However, concerning capital costs this behavior is the opposite since facility-dependent costs have an impact that rises Recovery costs. Facility costs decreased throughout the process since unlike the upstream, which demands a bigger number of equipment (such as fermenters, blending tanks for culture medium, sterilizers, and air filters), the other sections require less. As a result, capital investment decreases considerably given that facility costs and capital costs are straightly related (FERREIRA et al., 2021a).

Consumables costs are mainly concentrated in two sections: Gel Filtration and Formulation within downstream processes (due to Superdex 200 resin and UFP-10-E-55

Hollow Fiber Cartridge), being also present as a small fraction in Recovery through microfiltration and ultrafiltration membranes for separation of cells and protein concentration respectively. Most of the raw material costs are associated with the Recovery section (due to the volume of hypotonic solution to perform the osmotic shock) and PEGylation (due to mPEG-NHS), which corresponds to more than 90% of the costs.

Figure 20 – Breakdown of the production cost and capital investment by process section (S5).



Source: this work.

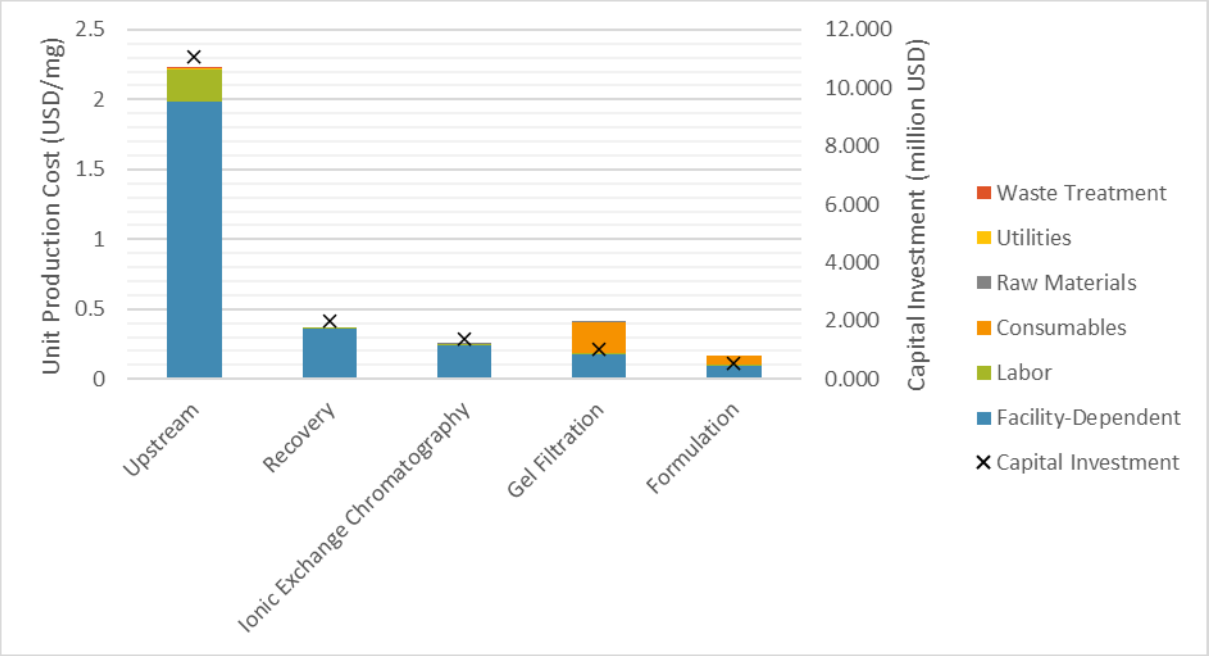
4.2.3.2 Pichia pastoris

The same scenario analysis was performed to understand the main cost drivers in the production of glycosylated Crisantaspase, as presented in Figure 21. In a similar behavior to the PEG-Asparaginase production process, the largest capital and operating costs are associated with the Upstream section, followed by Gel Filtration and Recovery, in distant second and third places respectively. In all evaluated sections, facility-dependent costs are predominant: in the Upstream it corresponds to 90% while in Recovery and Ion Exchange Chromatography it corresponds to almost the entire costs. The high impact of the facility cost on the Upstream was expected since the same equipment was used for the fermentation of both microorganisms, with the new volumes of blending tanks, fermenters, and other

equipment being determined by the SuperPro Designer. The decrease in facility costs followed the same trend as capital costs, with a six-fold reduction in value in the other sections.

Consumables costs are significant in the same two sections as verified in the S5 scenario, both in the Downstream section: Gel Filtration (due to Superdex 200 resin) and Formulation (due to UFP-10-E-55 Hollow Fiber Cartridge and sterilization cartridge). Labor costs are significant in the Upstream section due to the greater number of equipment, which implies the biggest number of employees, meanwhile, in the other sections, it represents only a small fraction of the total cost.

Figure 21 – Breakdown of the unit production cost and capital investment by process section (Scenario P1).



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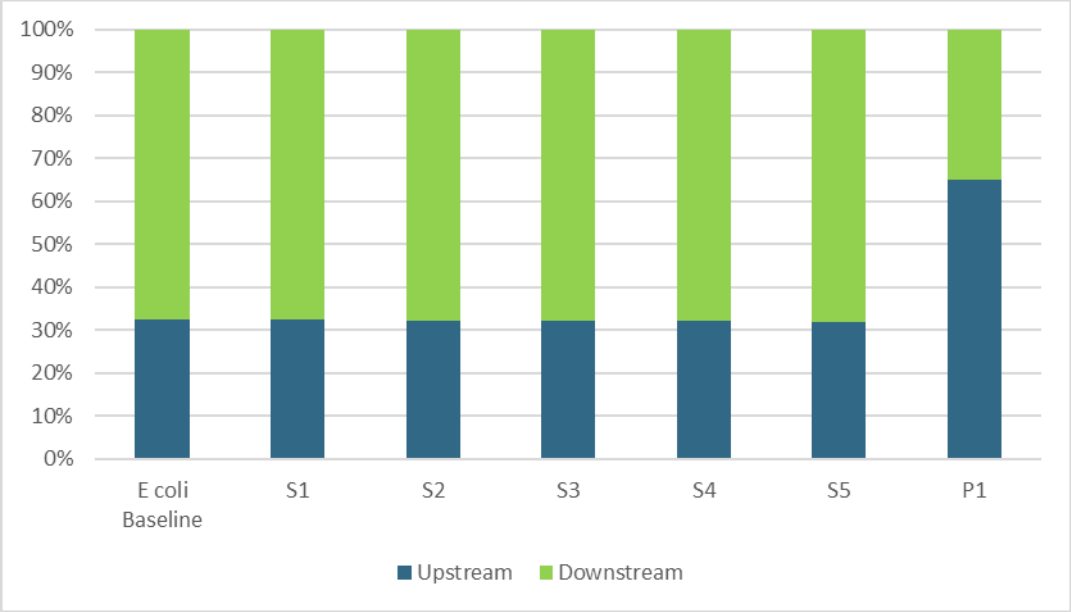
4.2.4 Upstream and Downstream

Another important point in the economic analysis of a process is to identify if the unit production cost is concentrated either in upstream or downstream processes, allowing the R&D staff a more effective focus, which is presented in Figure 22 for all evaluated scenarios. For *Escherichia coli* scenarios, upstream is composed only of fermentation, while downstream encompasses Recovery, Ion Exchange Chromatography, PEGylation, Gel

Filtration, and Formulation. The division for the *Pichia pastoris* scenario was performed as follows: upstream consists exclusively of fermentation while downstream consists of Recovery, Ion Exchange Chromatography, Gel Filtration, and Formulation.

Figure 22 – Upstream and Downstream distribution cost in all evaluated scenarios.



Source: this work.

According to LOPES et al., (2017), downstream might reach up to 80% of the total production cost of proteins in bioprocesses. The results show that downstream has greater participation in the costs for the production of PEGylated L-Asparaginase, corresponding to approximately 70% of the total production costs, mainly due to the strong impact of the costs involved in the site-specific PEGylation. The greater simplicity of the process involving *Pichia pastoris*, especially concerning purification techniques, ends up reducing downstream costs and, consequently raising the upstream to almost 65%.

4.3 Comparison between *Escherichia coli* and *Pichia pastoris*

The following tables summarize the main scheduling data and economic parameters obtained by the simulation of the two best scenarios analyzed for each microorganism.

Table 10 – Summary of the main scheduling information in the best simulated scenarios.

	***Escherichia coli* (S5)**	***Pichia pastoris* (P1)**
Annual Operating Time (h)	7920	7889
Batches per Year	436	64
Maximum Number of Batches per Year	461	64
Batch Time (h)	42	155
Minimum Cycle Time (h)	17	122

Source: this work.

Although both processes have similar annual operating times, the unit operations involved in upstream and downstream that are intrinsic to each microorganism and the desired final product influence directly key parameters, such as the maximum number of batches per year. Considering the same cell concentration in the analyzed scenarios, the minimum cycle time of the PEGylated L-Asparaginase production process proved to be approximately 10 times lower than the scenario using *Pichia pastoris*. The choice of the appropriate culture medium, induction strategies in the fed-batch mode, and the fact that prokaryotic organisms naturally have a higher maximum growth rate than eukaryotic organisms impact a longer fermentation time. The relationship between the high batch time of almost four times that of the process using *Escherichia coli*, and the maximum number of batches per year shows that the process using *Pichia pastoris* is more effective once fewer batches are required to produce the same final product mass.

The high value of fermentation time (which means, the minimum cycle time) is following the equipment occupancy chart, which shows that the main fermentation is the bottleneck of the process. Studies aimed at the optimization of fermentation, especially the time and increase in cell concentration, rising the process productivity, may rise the maximum number of batches per year.

It is important to point out that after the maximum number of batches is reached, some unit operations in multitasking industrial plants can be directed to the production of other biopharmaceuticals, for example, avoiding the idleness of equipment, allowing greater economic return through the sale of different products.

Table 11 – Summary of the main economic data for the best simulated scenarios.

	***Escherichia coli* S5 (65.00 g/L)**	***P. pastoris* P1 (65.00 g/L)**
Total Capital Investment (million USD)	17.47	15.98
Unit Production Cost (USD/mg)	7.57	3.42
Return on Investment (ROI)[2]	26.8%	44.2%
Payback Time (years)[2]	3.73	2.26
Internal Rate of Return (IRR)[2]	19.9%	33.5%
Net Present Value [1] (NPV) (million USD)	16.06	34.75
Production (kg/year)	1.00	1.00

[1] Considering an annual interest rate of 7%.;
[2] Revenue Value: 12.80 USD/mg
Source: this work.

While in the process simulated by FUGANHOLI (2021) an unit production cost of 12.37 USD/mg was determined, in this work the baseline scenario for the production of PEGylated L-Asparaginase presented a value of 9.83 USD/mg. The application of different approaches in fermentation, such as changes in the culture medium, fermentation times for inoculum and main fermentor, and fed-batch mode, as well as changes in some unit operations in downstream (in particular the replacement of filter cartridges, resins, and chromatographic columns with updated values), were all responsible for considerably decreasing the production costs to 7.57 USD/mg in the S5 scenario. It is important to point out that the huge potential of *Escherichia coli* to reach high cell densities can further decrease this value, rising the attractiveness of the process and its competitiveness, given a large number of benefits of the PEGylated biopharmaceutical compared to those currently available in the market (BARROS et al., 2021; SANTOS et al., 2018).

Considering the same cell concentration and annual production, the humanized Crisantaspase process presented the best economic parameters, with an unit production cost corresponding to half of the value found in the best scenario of PEGylated L-Asparaginase. The use of less equipment in the process (absence of the PEGylation reaction), simpler steps to scale up and operate industrially (such as the replacement of osmotic shock by filtration in the cell clarification section), cheaper raw materials (culture medium, absence of osmotic

shock solution in recovery, and high impact of mPEG-NHS on operating costs) reflect a nearly 2 million USD decrease in Total Capital Investment.

5 Conclusion

This work presented for the first time the techno-economic analysis of different production processes of the enzyme L-Asparaginase: PEGylated and glycosylated. Despite the challenges and uncertainties involved in the simulation and scheduling of bioprocesses for large-scale production, all developed scenarios showed potential for the production of new biopharmaceuticals to be used in the treatment of Acute Lymphoblastic Leukemia.

The techno-economic analysis of the production of PEG-Asparaginase using *Escherichia coli* in the scenario that presents the best economic parameters (S5 - 65 g/L) resulted in an enzyme cost of 7.57 USD/mg, which is lower than the cost of Oncaspar, the only drug available in the market featuring similar properties. Facility independent costs, raw material, and consumables costs are the three more significant. However, it is important to point out that PEG-Asparaginase features a novel PEGylation strategy (site-specific), thus allowing greater reproducibility and batch-to-batch control of industrial production. The investigation of better fermentation parameters, such as reaching higher cell concentrations, the replacement of osmotic shock by a cell recovery process more suitable for large scale, and PEGylation optimization may further reduce the total costs.

The techno-economic analysis of the production of glycosylated Crisantaspase using *Pichia pastoris* results in a baseline scenario able to produce 8 kg Crisantaspase/year, which is enough amount to supply the Latin America market and probably the global market. With respect to Brazilian demand, scenario P1 resulted in an enzyme cost of 3.42 USD/mg. This value is approximately five times lower than Spectrila, an L-Asparaginase which presents recombinant DNA technology. Facility-dependent costs, which are associated with the equipment, correspond to almost 84%, while consumables costs (resins and filtration cartridges) are also considerable. The increase in cell concentration in fermentation to values around 130 g/L may also further reduce facility and raw material costs.

The main fermentation proved to be the main bottleneck scheduling in the production process of both enzymes. The use of more than one fermenter in parallel (stagged mode) is a possible approach to reduce the fermentation time and consequently increase the maximum number of batches per year. However, it is necessary to verify the impact of costs related to the acquisition of new equipment on the total costs involved in the process. In addition, future

studies may consider accounting Research and Development, Quality Control, Quality Assurance, and updated indexes such as different annual rates, wages, local taxes, equipment acquisition, and currency to comprehend the impact on the total cost.

Finally, future clinical trials are still necessary in order to determine the dosage and treatment protocol of each drug. It is expected that PEG-Asparaginase has a smaller dose interval than glycosylated Crisantaspase, thus requiring lower annual production. The comparison between these two enzymes (products) under the view of pharmaco-economic fundamentals is also an interesting study to be developed in the future.

REFERENCES

ACHILLEOS, Evdokia C.; CALANDRANIS, John C.; PETRIDES, Demetri P. **Risk Assessment Quantifying the Impact of Uncertain Parameters in the Batch Manufacturing of Active Pharmaceutical Ingredients**. [s.l: s.n.].

BAGHBAN, Roghayyeh; FARAJNIA, Safar; GHASEMI, Younes; MORTAZAVI, Mojtaba; ZARGHAMI, Nosratollah; SAMADI, Naser. New Developments in Pichia pastoris Expression System, Review and Update. **Current Pharmaceutical Biotechnology**, *[S. l.]*, v. 19, n. 6, p. 451–467, 2018. DOI: 10.2174/1389201019666180718093037.

BARROS, Thaís; BRUMANO, Larissa; FREITAS, Marcela; PESSOA JUNIOR, Adalberto; PARACHIN, Nádia; MAGALHÃES, Pérola O. Development of processes for recombinant L-asparaginase II production by Escherichia coli Bl21 (De3): From shaker to bioreactors. **Pharmaceutics**, *[S. l.]*, v. 13, n. 1, p. 1–15, 2021. DOI: 10.3390/pharmaceutics13010014.

BAYARD CLARKS; IRWIN KRAKO; JOSEPH BURCHEN; DAVID KARNOFY; ROBERT GOLB; MONROE DOWLI; HERBERT OE-ITGE; ALLAN LIPTO. CLINICAL RESULTS OF TREATMENT WITH E. COLI L-ASPARAGINASE IN ADULTS WITH LEUKEMIA, LYMPHOMA, AND SOLID TUMORS. *[S. l.]*, 1970.

BRAZILIAN MINISTRY OF HEALTH. Explanations L-asparaginase. Available at: <https://www.gov.br/saude/pt-br/acesso-a-informacao/gestao-do-sus/articulacao-interfederativa/cit/pautas-de-reunioes-e-resumos-cit/2017/junho/2-b-l-asparaginase_cit_22_06_2017.pdf>. Accessed 23 Aug. 2022.

BRAZILIAN NATIONAL INSTITUTE OF CANCER. Estimative 2020: Cancer incidence in Brazil. Available at: <https://www.inca.gov.br/sites/ufu.sti.inca.local/files//media/document//estimativa-2020-incidencia-de-cancer-no-brasil.pdf>. Accessed 23 Aug. 2022.

BRUMANO, Larissa Pereira et al. **Development of L-asparaginase biobetters: Current research status and review of the desirable quality profiles**. **Frontiers in Bioengineering and Biotechnology** Frontiers Media S.A., , 2019. DOI: 10.3389/fbioe.2018.00212.

CACHUMBA, Jorge Javier Muso; ANTUNES, Felipe Antonio Fernandes; PERES, Guilherme Fernando Dias; BRUMANO, Larissa Pereira; SANTOS, Júlio César Dos; DA SILVA, Silvio Silvério. **Current applications and different approaches for microbial L-asparaginase production**. **Brazilian Journal of Microbiology** Elsevier Editora Ltda, , 2016. DOI: 10.1016/j.bjm.2016.10.004.

CHAROENSIDDHI, Suvimol; LORBEER, Andrew J.; FRANCO, Christopher M. M.; SU, Peng; CONLON, Michael A.; ZHANG, Wei. Process and economic feasibility for the production of functional food from the brown alga Ecklonia radiata. **Algal Research**, *[S. l.]*, v. 29, p. 80–91, 2018. DOI: 10.1016/j.algal.2017.11.022.

COSTA-SILVA, T. A.; COSTA, I. M.; BIASOTO, H. P.; LIMA, G. M.; SILVA, C.; PESSOA, A.; MONTEIRO, G. **Critical overview of the main features and techniques used for the evaluation of the clinical applicability of L-asparaginase as a biopharmaceutical to treat blood cancer**. **Blood Reviews** Churchill Livingstone, , 2020. DOI: 10.1016/j.blre.2020.100651.

CREGG, James M.; CEREGHINO, Joan Lin; SHI, Jianying; HIGGINS, David R. **Recombinant Protein Expression 23 MOLECULAR BIOTECHNOLOGY REVIEW 23 Recombinant Protein Expression in Pichia pastoris**. [s.l: s.n.]. Disponível em: www.kgi.edu/html/.

CZINKÓCZKY, Réka; NÉMETH, Áron. Techno-economic assessment of Bacillus fermentation to produce surfactin and lichenysin. **Biochemical Engineering Journal**, *[S. l.]*, v. 163, 2020. DOI: 10.1016/j.bej.2020.107719.

DAI, Zhan Jing; HUANG, Yan Qin; LU, Yun. Efficacy and safety of PEG-asparaginase versus E. coli L-asparaginase in Chinese children with acute lymphoblastic leukemia: A meta-analysis. **Translational Pediatrics**, *[S. l.]*, v. 10, n. 2, p. 244–255, 2021. DOI: 10.21037/TP-20-178.

DE ALMEIDA PARIZOTTO, Letícia et al. Increased glycosylated L-asparaginase production through selection of Pichia pastoris platform and oxygen-methanol control in fed-batches. **Biochemical Engineering Journal**, *[S. l.]*, v. 173, 2021. DOI: 10.1016/j.bej.2021.108083.

DESHPANDE, Nilkanth; ALUVALU, Rajanikanth; GITE, Shilpa. **A Brief Bibliometric Survey of Leukemia Detection by Machine Learning and Deep Learning Approaches**. [s.l: s.n.]. Disponível em: https://digitalcommons.unl.edu/libphilprac.

EFFER, Brian et al. Glycosylation of Erwinase results in active protein less recognized by antibodies. **Biochemical Engineering Journal**, *[S. l.]*, v. 163, 2020. DOI: 10.1016/j.bej.2020.107750.

ELSHAFEI, Ali Mohamed; HASSAN, Mohamed Mohamed; ABD-ELMONTASR ABOUZEID, Mohamed; MAHMOUD, Dalia Ali; ELGHONEMY, Dina Helmy. **Purification, Characterization and Antitumor Activity of L-asparaginase from Penicillium brevicompactum NRC 829British Microbiology Research Journal**. [s.l: s.n.]. Disponível em: www.sciencedomain.org.

FERREIRA, Rafael Da Gama; AZZONI, Adriano Rodrigues; FREITAS, Sindelia. Techno-economic analysis of the industrial production of a low-cost enzyme using E. coli: The case of recombinant β-glucosidase. **Biotechnology for Biofuels**, *[S. l.]*, v. 11, n. 1, 2018. a. DOI: 10.1186/s13068-018-1077-0.

FERREIRA, Rafael G.; AZZONI, Adriano R.; SANTANA, Maria Helena Andrade; PETRIDES, Demetri. Techno-economic analysis of a hyaluronic acid production process utilizing streptococcal fermentation. **Processes**, *[S. l.]*, v. 9, n. 2, p. 1–16, 2021. a. DOI: 10.3390/pr9020241.

FERREIRA, Rafael G.; GORDON, Neal F.; STOCK, Rick; PETRIDES, Demetri. Adenoviral vector covid-19 vaccines: Process and cost analysis. **Processes**, *[S. l.]*, v. 9, n. 8, 2021. b. DOI: 10.3390/pr9081430.

FUGANHOLI, NICOLA SGRIGNOLI. Análise Técnico-Econômica para a produção de biofármacos de L-asparaginase, Dissertação de Mestrado. Universidade de São Paulo, 2021. DOI.org/10.11606/D.9.2021.tde-05082021-093409.

GINN, Claire; KHALILI, Hanieh; LEVER, Rebecca; BROCCHINI, Steve. **PEGylation and its impact on the design of new protein-based medicines**. **Future Medicinal Chemistry** Future Science Ltd, , 2014. DOI: 10.4155/fmc.14.125.

GURRAMKONDA, Chandrasekhar; ADNAN, Ahmad; GÄBEL, Thomas; LÜNSDORF, Heinrich; ROSS, Anton; NEMANI, Satish Kumar; SWAMINATHAN, Sathyamangalam; KHANNA, Navin; RINAS, Ursula. Simple high-cell density fed-batch technique for high-level recombinant protein production with Pichia pastoris: Application to intracellular production of Hepatitis B surface antigen. **Microbial Cell Factories**, *[S. l.]*, v. 8, 2009. DOI: 10.1186/1475-2859-8-13.

HARRISON, Roger G.; TODD, Paul; RUDGE, Scott R.; PETRIDES, Demetri P. **Bioseparations science and engineering**. 2nd Edition ed. [s.l: s.n.].

HEINZLE, Elmar; BIWER, Arno P.; COONEY, Charles L. **Development of Sustainable Bioprocesses : Modeling and Assessment**. [s.l: s.n.].

HORN, U. et al. **High volumetric yields of functional dimeric miniantibodies in Escherichia coli, using an optimized expression vector and high-cell-density fermentation under non-limited growth conditions**. [s.l: s.n.].

HUANG, Linhua; LIU, Yu; SUN, Yan; YAN, Qiaojuan; JIANG, Zhengqiang. Biochemical characterization of a novel L-asparaginase with low glutaminase activity from Rhizomucor miehei and its application in food safety and leukemia treatment. **Applied and Environmental Microbiology**, *[S. l.]*, v. 80, n. 5, p. 1561–1569, 2014. DOI: 10.1128/AEM.03523-13.

JEVŠEVAR, Simona; KUNSTELJ, Menči; POREKAR, Vladka Gaberc. PEGylation of therapeutic proteins. **Biotechnology Journal**, *[S. l.]*, v. 5, n. 1, p. 113–128, 2010. DOI: 10.1002/biot.200900218.

KIDD, John G. **REGRESSION OF TRANSPLANTED LYMPHOMAS INDUCED IN VIVO BY MEANS OF NORMAL GUINEA PIG SERUM II. STUDIES ON THE NATURE OF THE ACTIVE SERUM CONSTITUENT HISTOLOGICAL MECHANISM OF THE REGRESSION: TESTS FOR EFFECTS OF GUINEA PIG SERUM ON LYMPHOMA CELLS IN VITRO: DISCUSSION***. [s.l: s.n.]. Available at: http://rupress.org/jem/article-pdf/98/6/583/1185282/583.pdf.

KRISHNAPURA, Prajna Rao; BELUR, Prasanna D. Partial purification and characterization of L-asparaginase from an endophytic Talaromyces pinophilus isolated from the rhizomes of Curcuma amada. **Journal of Molecular Catalysis B: Enzymatic**, *[S. l.]*, v. 124, p. 83–91, 2016. DOI: 10.1016/j.molcatb.2015.12.007.

LOPES, André Moreni et al. **Therapeutic l-asparaginase: upstream, downstream and beyond**. **Critical Reviews in Biotechnology** Taylor and Francis Ltd, , 2017. DOI: 10.3109/07388551.2015.1120705.

MAHAJAN, Richi V.; KUMAR, Vinod; RAJENDRAN, Vinoth; SARAN, Saurabh; GHOSH, Prahlad C.; SAXENA, Rajendra Kumar. Purification and characterization of a novel and

robust L-asparaginase having low-glutaminase activity from bacillus licheniformis: In vitro evaluation of anti-cancerous properties. **PLoS ONE**, *[S. l.]*, v. 9, n. 6, 2014. DOI: 10.1371/journal.pone.0099037.

MEDAWAR, Camile Valle; MOSEGUI, Gabriela Bittencourt Gonzalez; VIANNA, Cid Manso de Mello; COSTA, Talita Martins Alves Da. PEG-asparaginase and native Escherichia coli L-asparaginase in acute lymphoblastic leukemia in children and adolescents: a systematic review. **Hematology, Transfusion and Cell Therapy**, *[S. l.]*, v. 42, n. 1, p. 54–61, 2020. DOI: 10.1016/j.htct.2019.01.013.

MENEGUETTI, Giovanna Pastore et al. Novel site-specific PEGylated L-asparaginase. **PLoS ONE**, *[S. l.]*, v. 14, n. 2, 2019. DOI: 10.1371/journal.pone.0211951.

MORENO-SADER, Kariana; JAIN, Prerna; TENORIO, Luis Carlos Ballestas; MANNAN, M. Sam; EL-HALWAGI, Mahmoud M. Integrated Approach of Safety, Sustainability, Reliability, and Resilience Analysis via a Return on Investment Metric. **ACS Sustainable Chemistry and Engineering**, *[S. l.]*, v. 7, n. 24, p. 19522–19536, 2019. DOI: 10.1021/acssuschemeng.9b04608.

MÜ, H. J.; BOOS, J. **Use of L-asparaginase in childhood ALL Critical Reviews in Oncology/Hematology**. [s.l: s.n.].

MUSSATTO, Solange I.; AGUIAR, Luís M.; MARINHA, Mariana I.; JORGE, Rita C.; FERREIRA, Eugénio C. Economic analysis and environmental impact assessment of three different fermentation processes for fructooligosaccharides production. **Bioresource Technology**, *[S. l.]*, v. 198, p. 673–681, 2015. DOI: 10.1016/j.biortech.2015.09.060.

ONCASPAR: SUMMARY OF DRUG CHARACTERISTICS. Available at: < https://www.ema.europa.eu/en/documents/product-information/oncaspar-epar-product-information_pt.pdf>. Accessed in: 23 Aug. 2022.

ONCIU, Mihaela. **Acute Lymphoblastic Leukemia**. **Hematology/Oncology Clinics of North America**, 2009. DOI: 10.1016/j.hoc.2009.04.009.

PAPAVASILEIOU, V.; KOULOURIS, A.; SILETTI, C.; PETRIDES, D. Optimize manufacturing of pharmaceutical products with process simulation and production scheduling tools. **Chemical Engineering Research and Design**, *[S. l.]*, v. 85, n. 7 A, p. 1086–1097, 2007. DOI: 10.1205/cherd06240.

PASUT, Gianfranco; VERONESE, Francesco M. **PEG conjugates in clinical development or use as anticancer agents: An overview**. **Advanced Drug Delivery Reviews**, 2009. DOI: 10.1016/j.addr.2009.02.010.

PATRO, Krishna Raju. Extraction, purification and characterization of L-asparaginase from Penicillium sp. by submerged fermentation. **International Journal for Biotechnology and Molecular Biology Research**, *[S. l.]*, v. 3, n. 3, 2012. DOI: 10.5897/ijbmbr11.066.

PEÑA, David A.; GASSER, Brigitte; ZANGHELLINI, Jürgen; STEIGER, Matthias G.; MATTANOVICH, Diethard. **Metabolic engineering of Pichia pastoris**. **Metabolic Engineering** Academic Press Inc., , 2018. DOI: 10.1016/j.ymben.2018.04.017.

PETRIDES, Demetri; CARMICHAEL, Doug; SILETTI, Charles; KOULOURIS, Alexandros. Biopharmaceutical process optimization with simulation and scheduling tools. **Bioengineering**, *[S. l.]*, v. 1, n. 4, p. 154–187, 2014. DOI: 10.3390/bioengineering1040154.

PETRIDES, Demetri P.; KOULOURIS, Alexandros; LAGONIKOS, Pericles T. **The Role of Process Simulation in Pharmaceutical Process Development and Product Commercialization**. [s.l: s.n.]. Available at: www.intelligen.com.

PETRIDES, Demetri; SILETTI, Charles; JIMÉNEZ, José; PSATHAS, Petros; MANNION, Yvonne. **Facility Optimization Optimizing the Design and Operation of Fill-Finish Facilities using Process Simulation and Scheduling Tools**. [s.l: s.n.]. Available at: www.ISPE.org.

PFISTER, David; MORBIDELLI, Massimo. **Process for protein PEGylation**. **Journal of Controlled Release** Elsevier, , 2014. DOI: 10.1016/j.jconrel.2014.02.002.

PIETERS, ROB; HUNGER STEPHEN; BOOS JOACHIM. L-asparaginase treatment in acute lymphoblastic leukemia _ Enhanced Reader. *[S. l.]*, 2010.

PRAKASH, Pragya; CHANDRAYAN, Sanjeev; TIWARI, Purnima; SINGH, Hare Ram; JHA, Santosh Kumar. Development of a downstream process for purification and purity analysis of glutaminase free L-asparaginase using UPLC, DLS-ZP and DSC-TGA. **Journal of Taibah University for Science**, *[S. l.]*, v. 15, n. 1, p. 458–467, 2021. DOI: 10.1080/16583655.2021.1984694.

RIESENBERG ’, D.; SCHULZ ’, V.; KNORRE, W. A.; POHL ’, H. D.; KORZ, D.; SANDERS, E. A.; ROR, A.; DECKWER, W. D.; JEW, Theropre. **High cell density cultivation of Escherichia coli at controlled specific growth rate**. [s.l: s.n.].

SANTOS, João Henrique Picado Madalena; TORRES-OBREQUE, Karin Mariana; MENEGUETTI, Giovanna Pastore; AMARO, Beatriz Panichi; RANGEL-YAGUI, Carlota Oliveira. **Protein PEGylation for the design of biobetters: From reaction to purification processes**. **Brazilian Journal of Pharmaceutical Sciences** Faculdade de Ciencias Farmaceuticas (Biblioteca), , 2018. DOI: 10.1590/s2175-97902018000001009.

SHAKAMBARI, Ganeshan; ASHOKKUMAR, Balasubramaniem; VARALAKSHMI, Perumal. **L-asparaginase – A promising biocatalyst for industrial and clinical applications**. **Biocatalysis and Agricultural Biotechnology** Elsevier Ltd, , 2019. DOI: 10.1016/j.bcab.2018.11.018.

SHANG, Xiaojiao; YU, Deqiang; GHOSH, Raja. Integrated solid-phase synthesis and purification of PEGylated protein. **Biomacromolecules**, *[S. l.]*, v. 12, n. 7, p. 2772–2779, 2011. DOI: 10.1021/bm200541r.

SINGH, Yogendra; GUNDAMPATI, Ravi Kumar; JAGANNADHAM, Medicherla V.; SRIVASTAVA, S. K. Extracellular l-asparaginase from a protease-deficient bacillus aryabhattai ITBHU02: Purification, biochemical characterization, and evaluation of antineoplastic activity in vitro. **Applied Biochemistry and Biotechnology**, *[S. l.]*, v. 171, n. 7, p. 1759–1774, 2013. DOI: 10.1007/s12010-013-0455-0.

SPECTRILA: SUMMARY OF DRUG CHARACTERISTICS. Available at: < https://www.ema.europa.eu/en/documents/product-information/spectrila-epar-product-information_pt.pdf>. Accesed in: 23 Aug. 2022.

SUDHIR, Ankit P.; AGARWAAL, Viplove V.; DAVE, Bhaumik R.; PATEL, Darshan H.; SUBRAMANIAN, R. B. Enhanced catalysis of l-asparaginase from Bacillus licheniformis by a rational redesign. **Enzyme and Microbial Technology**, *[S. l.]*, v. 86, p. 1–6, 2016. DOI: 10.1016/j.enzmictec.2015.11.010.

SUTOW, Wataru W.; GARCIA, Florentina; STARLING, Kenneth A.; WILLIAMS, Thomas E.; LANE, Daniel M.; GEHAN, Edmund A.; ANDERSON HOSPITAL, M. D.; INSTITDE, - Ticinor; LOUIS, St. **L-ASPARAGINASE THERAPY I N CHILDREN W I T H**

ADVANCED LEUKEMIA T h e Southwest Cancel-Chemotherapy Study Group. [s.l: s.n.].

TALLAL, Lisa; TAN, Charlotte; OETTGEN, Herbert; WOLLNER, Norma; MCCARTHY, Michael; HELSON, Lawrence; BURCHENAL, Joseph; KARNOFSKY, David; MURPHY, M. Lois. **E. COLI L-ASPARAGINASE IN T H E TREATMENT OF LEUKEMIA AND SOLID TUMORS IN 131 CHILDREN**. [s.l: s.n.].

TORRES-OBREQUE, Karin; MENEGUETTI, Giovanna Pastore; CUSTÓDIO, Débora; MONTEIRO, Gisele; PESSOA-JUNIOR, Adalberto; DE OLIVEIRA RANGEL-YAGUI, Carlota. Production of a novel N-terminal PEGylated crisantaspase. **Biotechnology and Applied Biochemistry**, *[S. l.]*, v. 66, n. 3, p. 281–289, 2019. DOI: 10.1002/bab.1723.

TOUMI, Abdelaziz; JÜRGENS, Christian; JUNGO, Carmen; MAIER, Bernd A.; PAPAVASILEIOU, Victor; PETRIDES, Demetri P. **Design and Optimization of a Large Scale Biopharmaceutical Facility Using Process Simulation and Scheduling Tools**. [s.l: s.n.]. Available at: www.ISPE.org.

TUNDISI, Louise L.; COÊLHO, Diego F.; ZANCHETTA, Beatriz; MORIEL, Patricia; PESSOA, Adalberto; TAMBOURGI, Elias B.; SILVEIRA, Edgar; MAZZOLA, Priscila G. **L-Asparaginase Purification**. **Separation and Purification Reviews** Taylor and Francis Inc., , 2017. DOI: 10.1080/15422119.2016.1184167.

TURECEK, Peter L.; BOSSARD, Mary J.; SCHOETENS, Freddy; IVENS, Inge A. **PEGylation of Biopharmaceuticals: A Review of Chemistry and Nonclinical Safety Information of Approved Drugs**. **Journal of Pharmaceutical Sciences** Elsevier B.V., , 2016. DOI: 10.1016/j.xphs.2015.11.015.

VILLEGAS-MÉNDEZ, Miguel Ángel; MONTAÑEZ, Julio; CONTRERAS-ESQUIVEL, Juan Carlos; SALMERÓN, Iván; KOUTINAS, Apostolis; MORALES-OYERVIDES, Lourdes. Coproduction of Microbial Oil and Carotenoids within the Circular Bioeconomy Concept: A Sequential Solid-State and Submerged Fermentation Approach. **Fermentation**, *[S. l.]*, v. 8, n. 6, p. 258, 2022. DOI: 10.3390/fermentation8060258.

WANG, Junqi; CUI, Zhifang; LI, Yangyang; CAO, Leipeng; LU, Zhoumin. Techno-economic analysis and environmental impact assessment of citric acid production through different recovery methods. **Journal of Cleaner Production**, *[S. l.]*, v. 249, 2020. DOI: 10.1016/j.jclepro.2019.119315.

YU, Deqiang; GHOSH, Raja. Purification of PEGylated protein using membrane chromatography. **Journal of Pharmaceutical Sciences**, *[S. l.]*, v. 99, n. 8, p. 3326–3333, 2010. DOI: 10.1002/jps.22103.

ZHANG, Chun; YANG, Xiao-Lan; YUAN, Yong-Hua; PU, Jun; LIAO, Fei. **Site-Specific PEGylation of Therapeutic Proteins via Optimization of Both Accessible Reactive Amino Acid Residues and PEG Derivatives**. [s.l: s.n.].

ZHANG, Pan; ZHANG, Chen; LI, Jing; HAN, Jiyang; LIU, Xiru; YANG, Hui. **The physical microenvironment of hematopoietic stem cells and its emerging roles in engineering applications**. **Stem Cell Research and Therapy** BioMed Central Ltd., , 2019. DOI: 10.1186/s13287-019-1422-7.

ŽIŽLAVSKÝ, Ondřej. Net Present Value Approach: Method for Economic Assessment of Innovation Projects. **Procedia - Social and Behavioral Sciences**, *[S. l.]*, v. 156, p. 506–512, 2014. DOI: 10.1016/j.sbspro.2014.11.230.

APPENDIX A – SUPPLEMENTARY INPUT DATA FOR THE PROCESS SIMULATION

In this section, it is provided supplementary data required to carry out the techno-economic analysis, such as the cost of membranes, resins, and chromatography columns according to Cytiva's portfolio (Table 10), and the cost of raw material of PEG-Asparaginase (Table 11) and glycosylated Crisantaspase (Table 12).

Table 12 – Cost of consumables and equipment in the Downstream section.

Description	Price (USD)
Superdex 200 Prep Grade, 1L	3,972.14
SP Sepharose Fast Flow, 300 mL	582.00
CFP-4-E-55 Hollow Fiber Cartridge	4,385.00
UFP-10-E-55 Hollow Fiber Cartridge	3,721.00
BPG 100/750 Column	22,069.68
AxiChrom 300/300/PMMA/20PE/PED incl. FAT in Umeå	114,580.00

Source: this work.

Table 13 – Cost of raw materials to produce PEG-Asparaginase.

Bulk Material	Unit Cost (USD)	Annual Amount	Annual Cost (USD)	%
Air	0.000	20,338 kg	0	0.00
Boric Acid	0.080	1 kg	0	0.00
Carbenicillin	9.600	767 g	7,359	0.24
Citric Acid	0.100	26,063 g	2,606	0.08
Cobalt(II) Chlo	0.060	1,092 g	65	0.00
Copper(II) Chlo	2.400	1 kg	2	0.00
Di-NH4 Phosphat	0.048	1,184 kg	56	0.00
EDTA Disodium	2.740	21 kg	57	0.00
Ferric Citrate	0.170	26 kg	4	0.00
Glucose	0.400	3,466 kg	1,386	0.04
Hydroxylammonium	1.000	4 kg	4	0.00
IPTG	750.000	2 kg	1,432	0.05
KH2PO4	0.068	204 kg	14	0.00
Manganese Cl	0.033	972 g	32	0.00
Mg Sulfate	0.400	116 kg	47	0.00
Monoammonium ph	0.048	838 kg	40	0.00
mPEG-NHS	88.000	21,906 g	1,927,711	62.51
NaCI (1 M)	5.670	1,020 kg	5,781	0.19
NaOH (0.1M)	0.001	1,307 kg	1	0.00
NaOH (0.5 M)	0.199	161,434 kg	32,049	1.04
Sodium Molybdat	0.100	1,092 g	109	0.00
Sucrose	0.100	10,886,412 g	1,088,641	35.30
TF 20mM pH 5.5	0.200	11,538 kg	2,303	0.07
TF 20mM pH 7,5	0.200	69,593 kg	13,888	0.45
Thiamine.HCl	1.000	0 kg	0	0.00
TRIS HCl	0.107	264 kg	28	0.00
Water	0.180	1,800 MT	324	0.01
Zinc Acetate	1.000	3 kg	3	0.00
TOTAL			3,083,944	100.00

Source: this work.

Table 14 – Cost of raw materials to produce glycosylated Crisantaspase.

Bulk Material	Unit Cost (USD)	Annual Amount	Annual Cost (USD)	%
Air	0.000	11,690 kg	0	0.00
Amm. Sulfate	0.080	16 kg	1	0.01
Ammonia	0.200	26 kg	5	0.05
Biotin	1.000	0 kg	0	0.00
Boric Acid	0.080	0 kg	0	0.00
CaChloride	0.330	0 kg	0	0.00
Ethanol 20%	0.150	7,685 kg	1,154	11.37
Ferric Chloride	1.000	0 kg	0	0.00
Glycerol	1.800	95 kg	171	1.68
KH2PO4	0.028	9 kg	0	0.00
KI	0.040	0 kg	0	0.00
KOH	4.000	4 kg	16	0.16
Methanol	0.580	268 kg	155	1.53
Mg Sulfate	0.400	5 kg	2	0.02
Mn Sulfate	1.000	0 kg	0	0.00
NaAc + NaCl 1M	5.850	222 kg	1,296	12.77
NaAc Buffer	0.003	22,645 kg	76	0.74
NaCI (1 M)	5.670	216 kg	1,227	12.09
NaOH (0.1M)	0.001	192 kg	0	0.00
NaOH (0.5 M)	0.199	22,099 kg	4,387	43.22
PTM1	0.186	1 kg	0	0.00
Sodium Molybdat	0.100	2 g	0	0.00
Sulfuric Acid	0.070	0 kg	0	0.00
Water	0.180	175 MT	32	0.31
WFI	0.200	8,137 kg	1,627	16.03
Zinc Sulfate	0.800	0 kg	0	0.00
TOTAL			10,150	100.00

Source: this work.

APPENDIX B – SUPPLEMENTARY OUTPUT DATA FROM THE SIMULATION

In this section, it is provided additional data generated by the simulation, such as the overall process parameters for all evaluated scenarios (Table 13), the annual operating costs (Tables 14 and 15), and the related capital costs incurred to build the plant (Tables 16 and 17), and equipment specifications and purchase costs estimated by SuperPro Designer (Tables 18 and 19).

Table 15 – Overall process parameters.

Scenario	Annual Operating Time (hr/year)	Batch Size (mg)	Recipe Batch Time (h)	Recipe Cycle Time (h)	Number of Batches Per Year
Escherichia coli Baseline	7,916.25	1,108.65	32.5	8.75	902
S1	7,917.22	1,503.76	35.54	11.87	665
S2	7,907.71	1,798.56	37.81	14.18	556
S3	7,914.28	1,968.50	40.57	15.53	508
S4	7,906.08	2,092.05	40.35	16.49	478
S5	7,919.81	2,293.58	41.96	18.11	436
P1	7,888.54	15,625.00	155.29	122.75	64

Source: this work.

Table 16 – Annual operating cost of PEG-Asparaginase.

Cost Item	USD	%
Raw Materials	3,084,000	40.72
Labor-Dependent	580,000	7.66
Facility-Dependent	3,074,000	40.59
Consumables	775,000	10.23
Waste Treatment/Disposal	39,000	0.51
Utilities	22,000	0.30
Transportation	0	0
Miscellaneous	0	0
Advertising/Selling	0	0
Running Royalties	0	0
Failed Product Disposal	0	0
TOTAL	7,574,000	100

Source: this work.

Table 17 – Annual operating cost of glycosylated Crisantaspase.

Cost Item	USD	%
Raw Materials	10,000	0.30
Labor-Dependent	259,000	7.56
Facility-Dependent	2,864,000	83.72
Consumables	283,000	8.27
Waste Treatment/Disposal	1,000	0.04
Utilities	4,000	0.12
Transportation	0	0
Miscellaneous	0	0
Advertising/Selling	0	0
Running Royalties	0	0
Failed Product Disposal	0	0
TOTAL	3,421,000	100

Source: this work.

Table 18 – Fixed capital estimate summary to produce PEG-Asparaginase.

3A. Total Plant Direct Cost (TPDC)	USD
1. Equipment Purchase Cost	2,721,000
2. Installation	1,036,000
3. Process Piping	952,000
4. Instrumentation	1,088,000
5. Insulation	82,000
6. Electrical	272,000
7. Buildings	1,224,000
8. Yard Improvement	408,000
9. Auxiliary Facilities	1,088,000
TPDC	8,871,000

3B. Total Plant Indirect Cost (TPIC)	
10. Engineering	2,218,000
11. Construction	3,105,000
TPIC	5,323,000

3C. Total Plant Cost (TPC = TPDC+TPIC)	
TPC	14,194,000

3D. Contractor's Fee & Contingency (CFC)	
12. Contractor's Fee	710,000
13. Contingency	1,419,000
CFC = 12+13	2,129,000

3E. Direct Fixed Capital Cost (DFC = TPC+CFC)	
DFC	16,323,000

Source: this work.

Table 19 – Fixed capital estimate to produce glycosylated Crisantaspase.

3A. Total Plant Direct Cost (TPDC)	USD
1. Equipment Purchase Cost	2,542,000
2. Installation	941,000
3. Process Piping	890,000
4. Instrumentation	1,017,000
5. Insulation	76,000
6. Electrical	254,000
7. Buildings	1,144,000
8. Yard Improvement	381,000
9. Auxiliary Facilities	1,017,000
TPDC	8,261,000

3B. Total Plant Indirect Cost (TPIC)	
10. Engineering	2,065,000
11. Construction	2,892,000
TPIC	4,957,000

3C. Total Plant Cost (TPC = TPDC+TPIC)	
TPC	13,218,000

3D. Contractor's Fee & Contingency (CFC)	
12. Contractor's Fee	661,000
13. Contingency	1,322,000
CFC = 12+13	1,983,000

3E. Direct Fixed Capital Cost (DFC = TPC+CFC)	
DFC	15,201,000

Source: this work.

Table 20 – Major equipment specifications and purchase costs to produce PEG-Asparaginase.

Quantity/Standby/ Staggered	Name	Description	Unit Cost (USD)	Cost (USD)
1 / 0 / 0	SFR-101	Seed Fermenter Vessel Volume = 4,58 L	475,000	475,000
1 / 0 / 0	FR-101	Fermenter Vessel Volume = 65,35 L	475,000	475,000
1 / 0 / 0	ST-101	Heat Sterilizer Rated Throughput = 191,80 L/h	139,000	139,000
1 / 0 / 0	ST-102	Heat Sterilizer Rated Throughput = 12,79 L/h	114,000	114,000
1 / 0 / 0	C-101	PBA Column Column Volume = 0,45 L	108,000	108,000
1 / 0 / 0	DS-101	Disk-Stack Centrifuge Throughput = 45,74 L/h	106,000	106,000
1 / 0 / 0	DS-102	Disk-Stack Centrifuge Throughput = 296,80 L/h	106,000	106,000
1 / 0 / 0	G-101	Centrifugal Compressor Compressor Power = 0,20 kW	70,000	70,000
1 / 0 / 0	V-103	Receiver Tank Vessel Volume = 50,82 L	48,000	48,000
1 / 0 / 0	V-108	Blending Tank Vessel Volume = 1,53 L	45,000	45,000
1 / 0 / 0	V-102	Blending Tank Vessel Volume = 3,55 L	45,000	45,000
1 / 0 / 0	V-110	Blending Tank Vessel Volume = 0,84 L	45,000	45,000
1 / 0 / 0	V-101	Blending Tank Vessel Volume = 35,52 L	45,000	45,000
1 / 0 / 0	V-105	Blending Tank	45,000	45,000

		Vessel Volume = 117,57 L		
1 / 0 / 0	V-109	Blending Tank	45,000	45,000
		Vessel Volume = 9,89 L		
1 / 0 / 0	V-104	Blending Tank	45,000	45,000
		Vessel Volume = 138,46 L		
1 / 0 / 0	DE-102	Dead-End Filter	39,000	39,000
		Filter Area = 10,00 m2		
1 / 0 / 0	DF-101	Diafilter	27,000	27,000
		Membrane Area = 2,10 m2		
1 / 0 / 0	DF-103	Diafilter	27,000	27,000
		Membrane Area = 2,10 m2		
1 / 0 / 0	DF-102	Diafilter	27,000	27,000
		Membrane Area = 2,10 m2		
1 / 0 / 0	DE-101	Dead-End Filter	24,000	24,000
		Filter Area = 2,10 m2		
1 / 0 / 0	C-102	Gel Filtration Column	21,000	21,000
		Column Volume = 18,80 L		
1 / 0 / 0	V-107	Vertical-On-Legs Tank	18,000	18,000
		Vessel Volume = 117,69 L		
1 / 0 / 0	V-106	Vertical-On-Legs Tank	18,000	18,000
		Vessel Volume = 2,74 L		
1 / 0 / 0	AF-101	Air Filter		
		Rated Throughput = 209,39		
		L/h	7,000	7,000
1 / 0 / 0	AF-102	Air Filter		
		Rated Throughput = 386,57		
		L/h	7,000	7,000
1 / 0 / 0	AF-103	Air Filter		
		Rated Throughput = 2486,62		
		L/h	7,000	7,000
		Unlisted Equipment		544,000
			TOTAL	2,721,000

Source: this work.

Table 21 – Major equipment specifications and purchase costs to produce glycosylated Crisantaspase.

Quantity/Standby/ Staggered	Name	Description	Unit Cost (USD)	Cost (USD)
1 / 0 / 0	FR-101	Fermenter Vessel Volume = 31,33 L	475,000	475,000
1 / 0 / 0	SFR-101	Seed Fermenter Vessel Volume = 5,87 L	475,000	475,000
1 / 0 / 0	ST-102	Heat Sterilizer Rated Throughput = 14,29 L/h	114,000	114,000
1 / 0 / 0	ST-101	Heat Sterilizer Rated Throughput = 72,11 L/h	114,000	114,000
1 / 0 / 0	C-101	PBA Column Column Volume = 1,09 L	108,000	108,000
1 / 0 / 0	DS-101	Disk-Stack Centrifuge Throughput = 43,86 L/h	106,000	106,000
1 / 0 / 0	G-101	Centrifugal Compressor Compressor Power = 0,12 kW	70,000	70,000
1 / 0 / 0	V-103	Receiver Tank Vessel Volume = 24,37 L	48,000	48,000
1 / 0 / 0	V-109	Blending Tank Vessel Volume = 7,88 L	45,000	45,000
1 / 0 / 0	V-104	Blending Tank Vessel Volume = 5,57 L	45,000	45,000
1 / 0 / 0	V-102	Blending Tank Vessel Volume = 4,01 L	45,000	45,000
1 / 0 / 0	V-108	Blending Tank Vessel Volume = 3,78 L	45,000	45,000
1 / 0 / 0	V-106	Blending Tank	45,000	45,000

		Vessel Volume = 3,79 L		
1 / 0 / 0	V-101	Blending Tank	45,000	45,000
		Vessel Volume = 13,35 L		
1 / 0 / 0	V-105	Blending Tank	45,000	45,000
		Vessel Volume = 13,90 L		
1 / 0 / 0	DE-102	Dead-End Filter	39,000	39,000
		Filter Area = 10,00 m2		
1 / 0 / 0	DF-103	Diafilter	27,000	27,000
		Membrane Area = 2,10 m2		
1 / 0 / 0	DF-102	Diafilter	27,000	27,000
		Membrane Area = 2,10 m2		
1 / 0 / 0	UF-101	Ultrafilter	27,000	27,000
		Membrane Area = 2,10 m2		
1 / 0 / 0	DF-101	Diafilter	27,000	27,000
		Membrane Area = 2,10 m2		
2 / 0 / 0	C-102	Gel Filtration Column	21,000	42,000
		Column Volume = 42,60 L		
1 / 0 / 0	AF-101	Air Filter	7,000	7,000
		Rated Throughput = 265,41 L/h		
1 / 0 / 0	AF-102	Air Filter	7,000	7,000
		Rated Throughput = 223,32 L/h		
1 / 0 / 0	AF-103	Air Filter	7,000	7,000
		Rated Throughput = 1223,47 L/h		
		Unlisted Equipment		508,000
			TOTAL	2,542,000

Source: this work.