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Simulation of a bioreactor to produce recombinant insulin

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Agraïr primerament al Dr. Bringué, per ajudar-me i introduir-me al món dels processos biotecnològics.

També vull donar-li les gràcies als meus pares pel suport que em donen dia a dia, per animar-me quan jo no em veia capaç de seguir i per fer-me saber sempre que estan molt orgullosos de mi.

I finalment a les meves amigues, per estar en els bons i en els no tan bons moments. Per fer que tot sigui més fàcil sempre, gràcies.

CONTENTS

SUMMARY

This project has focused on the study, modelling and simulation of the fermentation process to produce recombinant proinsulin in Escherichia coli, as a key step in the process of manufacturing human insulin from the proinsulin method. Given the current market situation for this important protein, the project is based on the need to explore accessible, efficient and replicable production strategies on an industrial scale.

To this end, a mathematical model based on differential matter balances was developed, which describes the evolution of the main variables of the process (biomass, glucose, volume and proinsulin) in a fed-batch culture. From this model, two operational configurations were implemented: one based on a single tank where both the growth and induction phases take place, and another in which these phases are carried out separately in a cyclic system of two connected tanks.

Both strategies were simulated using Euler's method, applying a pH-stat type feed and with realistic operating conditions. The results obtained allowed the analysis of the dynamics of the system in each configuration, evaluating the evolution of the key variables and the productivity of the process.

Finally, both configurations were compared under a scenario of continuous operation for 365 days, which allowed estimating the annual production of proinsulin in each case. Based on these results, the advantages and limitations of each strategy were analyzed, in order to reflect on which of the models is more viable depending on the specific operating conditions. This comparison seeks to serve as a guide for future implementations of the process on a larger scale, providing solid information for decision-making in industrial contexts.

Keywords: Proinsulin, bioreactor, fermentation, simulation, Escherichia coli, fed-batch, productivity, insulin.

RESUM

El present projecte s'ha centrat en l'estudi, modelat i simulació del procés fermentatiu per produir proinsulina recombinant en *Escherichia coli*, com a etapa clau dins del procés de fabricació de la insulina humana a partir del mètode de la proinsulina. A causa de la situació actual del mercat d'aquesta proteïna tan important, el projecte parteix de la necessitat d'explorar estratègies de producció accessibles, eficients i reproduïbles en l'àmbit industrial.

Per aconseguir-ho, s'ha desenvolupat un model matemàtic basat en balanços de matèria diferencials, que descriuen l'evolució de les variables principals del procés (biomassa, glucosa, volum i proinsulina) en un cultiu fed-batch. A partir d'aquest model, es van implementar dues configuracions operatives: una basada en un únic tanc on es produïen tant la fase de creixement com la d'inducció, i una altra on aquestes fases es realitzen per separat en un sistema cíclic de dos tancs.

Ambdues estratègies van ser simulades utilitzant el mètode d'Euler, aplicant una alimentació de tipus pH-stat i amb condicions d'operació realistes. Els resultats obtinguts van permetre analitzar la dinàmica del sistema en cada configuració, avaluant l'evolució de les variables Claus i la productivitat del procés.

Finalment, es van comparar les dues configuracions sota un escenari d'operació continu durant 365 dies. Gràcies a això es va poder estimar la producció anual de proinsulina en cada cas. Arran dels resultats obtinguts, es van analitzar els avantatges i limitacions de cada estratègia amb el fi de reflexionar sobre quin dels dos models resulta més viable en funció de les condicions operatives específiques. Aquesta comparació vol ser una font d'informació per orientar futures implantacions industrials.

Paraules clau: Proinsulina, biorreactor, fermentació, simulació, Escherichia coli, fed-batch, productivitat, insulina.

SUSTAINABLE DEVELOPMENT GOALS

This project is part of a global context marked by a growing need to promote qualitative access to health, in particular to essential medicines such as insulin. The constant increase in cases of diabetes around the world means that the demand for this drug is increasingly high. However, its production is concentrated in a small number of large pharmaceutical companies, which generates a critical dependence on them. In this context, the present work, which focuses on the study and simulation of the fermentation process for the production of proinsulin, aims to provide a technical analysis that can serve as a basis for developing efficient and scalable production systems.

This contribution can be analyzed through the framework of the five "P's" of sustainable development: People, Planet, Prosperity, Peace and Partnerships.

First, from a **people'**s perspective, the work has a clear potential impact, as improving efficiency in proinsulin production can translate into more affordable and accessible insulin for millions of people. In terms of prosperity, the development of replicable systems can benefit regions with emerging technological capacity, creating new industrial opportunities. Finally, in the field of **Partnerships**, the project promotes transferable and adaptable knowledge in different environments, favouring future collaborations in the technological field.

Moreover, the project is directly aligned with several Sustainable Development Goals (SDG's) established by the Uited Nations. The most relevant is **SDG 3: Good Health and Well-being**, particularly **target 3.8**, wich seeks to ensure universal access to affordable essential medicines. It is also linked to **SDG 9: Industry, Innovation and Infrastructure**, as it promotes applied research, technological development, and the optimization of production process with potential for industrial implementation.

In addition, to offering a technical tool for analyzing insulin producton processes, this project aims to contribute meaningfully to social and economic impact, aligning with the values and goals of sustainable development.

NOMENCLATURE

C_X: Biomass concentration [g/L]

C_S: Glucose concentration [g/L]

C_P: Proinsulin concentration [g/L]

V: Volume [L]

F: Feed flow [L/h]

μ: Specific growth rate [h^{-1}]

t: time [h]

q_p: Specific product production rate [g proinsulin/ g biomass·h]

$Y_{X/S}$: Biomass yield on substrate [g biomass/ g glucose]

1. INTRODUCTION

Insulin is a peptide hormone essential for regulating the metabolism of carbohydrates, fatty acids and proteins. This is secreted by the beta cells of the islets of Langerhans in the liver and its main function is to facilitate the uptake of glucose by peripheral tissues, especially skeletal muscle and adipose tissue, and its reserve in the form of glycogen in the liver. This action is very important to maintain glycemic homeostasis and prevent hypoglycemia, a characteristic condition of diabetes mellitus.

1.1. History

The discovery of insulin represents one of the most transcendental milestones in the history of medicine [7]. Until the beginning of the twentieth century, type 1 diabetes was a lethal disease, especially in children, since there was no effective treatment beyond extremely restrictive diets in carbohydrates that managed to slightly prolong the patient's life [7]. In 1921, Frederick Banting and Charles Best, working in John Macleod's laboratory at the University of Toronto, managed to isolate a pancreatic extract that, when administered to diabetic dogs, significantly reduced blood glucose levels. The subsequent purification of the extract by biochemist James Collip allowed insulin to be administered for the first time in January 1922 to a human patient, Leonard Thompson, a terminally ill teenager. The clinical results were immediate and successful. This discovery was awarded the Nobel Prize in Medicine in 1923, marking the beginning of a new era in the treatment of diabetes.

1.2. Characteristics and functions

From a biochemical point of view, insulin is composed of 51 amino acids organized in two chains (A and B) joined by disulfide bridges [6]. Insulin was the first human protein whose

complete structure was determined in 1955 by biochemist Frederick Sanger and, decades later, thanks to David Goeddel and his team, in 1979, it was the first hormone produced using recombinant DNA technology, achieving its synthesis on a large scale from genetically modified bacteria [6]. This fact revolutionized the availability and use of insulin for clinical use.

The mechanism of action of insulin involves the binding of the hormone to its specific receptor on the membrane of the target cells, which activates an intracellular signaling cascade [5-8]. This signalling promotes, among other effects, the translocation of the glucose transporter GLUT4 to the cell surface, thus allowing glucose from the extracellular environment to enter the cell [5-8]. This regulatory capacity is altered by different types of *diabetes mellitus*, a chronic metabolic disease whose main characteristic is hypoglycemia, that is, containing low levels of glucose in the blood.

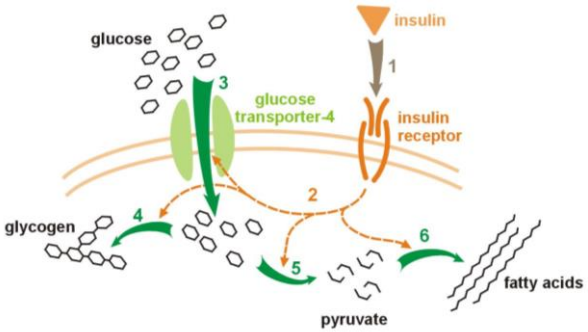

Figure 1. *Diagram of insulin-stimulated GLUT4 translocation and glucose metabolism.*
[Jonathan Zander, 11/12/13 via Wikimedia Commons, Creative Commons Attribution]

1.2.1. Classification of diabetes

As mentioned above, there are different types of diabetes that differ mainly in their relationship with insulin. In type 1 diabetes, there is no insulin production due to an autoimmune process that selectively destroys beta cells in the liver [1]. This absolute deficiency of insulin causes glucose to be not captured by the tissues, which leads to severe hyperglycemia, weight loss and, if not properly treated, death. This type manifests itself more frequently in childhood or adolescence

[1]. Treatment requires the exogenous administration of insulin chronically to replace the absent hormonal function and ensure the patient's survival.

On the other hand, type 2 diabetes is a multifactorial pathology that accounts for approximately 90% of diabetes cases in adults [3]. In this type, insulin secretion is present, but there is a progressive resistance of its action in peripheral tissues. Over time, the secretory function of the liver deteriorates, resulting in a relative insulin deficit. Factors such as obesity, sedentary lifestyle, genetic predisposition and old age contribute to the development of this condition [3]. During the early stages of type 2 diabetes, it can be treated with oral drugs, but many patients end up needing insulin treatments (as in type 1) to achieve appropriate glycemic control [3].

1.3. Industrial production

The production of insulin on a global scale has been one of the most important advances in modern biotechnology [9]. This hormone is essential for the treatment of diabetes and thanks to the development and improvement of obtaining techniques, it was possible to improve the quality, quantity, safety of clinical use and accessibility around the world. Initially, it was extracted from the liver of cattle and, with the discovery and advancement of genetic engineering, insulin is now manufactured through the synthesis of microorganisms such as bacteria and genetically modified yeasts [9].

The current process of obtaining it is based on introducing the human insulin gene into organisms such as Escherichia coli or Saccharomyces cerevisiae. These systems allow the hormone to be expressed in a controlled manner and on a large scale. In bacteria, insulin is produced as inclusion bodies that require different stages of solubilization, folding, and purification. In contrast, in the case of yeast, the ability to secrete proinsulin directly into the culture medium has been developed, simplifying the hormone recovery steps.

There are two main methods of recombinant insulin production today: the proinsulin method and the two-chain method [22]. The first consists of expressing a single proinsulin chain that is enzymatically processed to obtain a functional hormone. The second is based on the synthesis

of the two chains that make up insulin separately, which will subsequently be joined by disulfide bonds. Both methods are effective, but the proinsulin method is considered more efficient from an industrial point of view.

Currently, the annual production of insulin worldwide exceeds 60 tons, with constant growth due to the increase in the diabetic population. The production of this hormone is highly concentrated in three large pharmaceutical companies: Novo Nordisk, Sanofi and Eli Lilly [21]. These companies control 96% of the global market [21].

2. OBJECTIVES

Given the growing global demand for insulin and the concentration of its production in a few pharmaceutical companies, it is key to open this competitive market to other companies and thus be able to diversify the production of this essential hormone. In this context, the main objective of this project is to simulate and analyze the fermentation process for the production of recombinant proinsulin in Escherichia coli, using a comparative approach between two fed-batch operation strategies.

The study focuses exclusively on the bioreactor stage, a critical stage where the insulin precursor is generated. To do this, the simulation of two different configurations is proposed: one based on a single tank and the other cyclical with two tanks. Thanks to the simulation, the dynamic behavior, productivity and applicability in an industrial environment of both strategies will be evaluated.

This analysis seeks to make known what each of these two strategies offers in terms of production, production continuity and industrial viability so that companies can choose which type of configuration suits them best.

3. PROINSULIN METHOD

The production of recombinant insulin represents one of the greatest achievements of modern biotechnology, allowing the safe, efficient and scalable supply of this hormone essential for the treatment of diabetes mellitus.

In this context, the most commonly used strategy in the pharmaceutical industry involves the expression of a precursor form of insulin, human proinsulin, in bacteria such as Escherichia coli or Saccharomyces cerevisiae. The production process encompasses multiple interdependent phases, from genetic design and construction of the expression system, to large-scale fermentation, isolation of the recombinant protein and its final purification.

The proper implementation of these stages makes it possible to obtain an insulin with the pharmaceutical quality standards demanded internationally. The following sections describe in detail the typical industrial protocol for obtaining recombinant proinsulin, addressing both the technical and biotechnological aspects involved in each step of the process.

3.1. Desing and construction of the expression system

The first step of this method consists of taking the gene that codes for human proinsulin and introducing it into a plasmid, a small circular fragment of DNA that serves as an expression vector. This plasmid is **pET-9a**, which contains **T7** as a promoter [10]. This promoter ensures that the transcription process can be done, but it needs to be activated, since under normal conditions, T7 does not activate itself. **Isopropyl β-D-1-thiogalactipyranoside (IPTG)** is used, a molecule analogous to lactose, which activates a specific enzyme of our promoter (RNA polymerase T7) and which causes the transcription process to begin. This plasmid, apart from containing the proinsulin gene, also finds different essential sequences:

- An Escherichia coli (E.Coli)-compatible replication source; which allows the plasmid to be copied inside the bacterium.
- A gene for resistance to antibiotics, such as kanamycin or ampicillin; which helps detect the bacteria that contain the plasmid.
- A multi-clone site; which facilitates the insertion of the gene of interest.

Once the expression system has been built, it is introduced into bacteria of the chosen strain, in this case **E.Coli BL21(DE3)** [10]. This strain is genetically modified to contain on its chromosome a gene encoding RNA polymerase T7, but as mentioned above, this gene can only be activated in the presence of IPTG [10]. Therefore, by introducing the plasmid and then adding IPTG, the cell begins to produce large amounts of RNA from the inserted gene. The insertion of the plasmid into the bacterium can be done by different mechanisms; by electroporation, where electric powders are used to open pores in the bacterial membrane, or by treatment with sodium chloride followed by thermal shock. Once the plasmid is introduced and transcription has been given, the bacteria are seeded in media containing antibiotics with the aim that only the bacteria that have the plasmid incorporated with the gene of interest survive. Finally, these colonies are considered transformed and can now be used to continue with the process.

3.2. Fermentation and biomass production

Once the expression system has been designed and built, the next step is to scale up production through controlled fermentation. To achieve high proinsulin production, bacteria are grown in highly nutrient-rich culture media containing carbon sources such as glucose or glycerol. This process is carried out in bioreactors, which are industrial scale, can have volumes of up to 10,000 L. In industry, a staggered feeding strategy, also known as feed-batch [10-11], is used, where the substrate (glucose) is added in a controlled and progressive manner. This strategy prevents bacteria from quickly consuming sugar, which could cause catabolic repression and slow down production. During fermentation, some conditions must be carefully monitored:

- The pH of the crop must be kept in an optimal range of between 6.8 and 7.2 [10]

- The temperature is set at 37 °C during the bacterial growth phase [10-12]. When sufficient cell density is achieved (measured by optical absorbance at 600 nm, OD600 > 50), the temperature is reduced to 30 °C [12]. This decrease in temperature favors a folding of the protein, preventing the production of insoluble aggregates that later hinder purification [10].
- Oxygenation is a very important variable to control, since E.Coli BL21(DE3) needs oxygen to grow efficiently. The oxygen dissolved in the medium must be at least 40% [10-12]. In order to control this level, cascade control systems are used that automatically adjust the agitation in the bioreactor and the air flow.

Once the cells have grown sufficiently, IPTG is added to activate the expression of the proinsulin gene [10-12-13]. IPTG induces RNA polymerase T7 (present in the E.Coli genome), which, in turn, transcribes the cloned gene into the plasmid, thus initiating the massive production of proinsulin in cells [10].

3.3. Training and recovery of inclusion bodies

Induced the expression of proinsulin in E.Coli BL21 (DE3) bacteria, the production system enters a key stage: the formation of inclusion bodies. Due to an overexpression of the proinsulin gene induced by IPTG, the recombinant protein accumulates in large quantities in the bacterial cytoplasm in the form of inclusion bodies, which are insoluble and biologically inactive aggregates, mostly composed of proinsulin. These inclusion bodies favor subsequent purification, as they are physically separated from the rest of the cellular content. To recover them, the biomass must first be cultivated, that is, the cultured bacteria must be collected, and subjected to cellular disruption. This step is performed using a high-pressure homogenizer (800-1200 bars) [10], which breaks the cell walls by mechanical force, releasing all the intracellular content. During this process, protease inhibitors are added to prevent the bacteria's own enzymes from degrading the protein. Once the cells are lysed, the inclusion bodies are separated from the rest of the lysate by centrifugation [10], since the aggregates are much denser than the other soluble fractions. The resulting pellet contains the inclusion bodies, which are carefully washed to remove traces of unwanted bacterial membranes and proteins. This washing is carried out in different stages [10]:

1. Washed with Triton X-100 [10], a non-ionic detergent that solubilizes and removes lipids from cell membranes.
2. Washed with diluted urea or SDS [10], which makes it easier to detach unwanted proteins from the bodies.
3. Washed with ionic buffers [10], which allow the added proinsulin to be recovered in a purer form, although in an insoluble state

3.4. Solubilization and Folding

The next step is to transform the recovered proinsulin, as it is still insoluble and inactive. To achieve this, solubilization is carried out, where the purified inclusion bodies are incubated with reducing agents, such as urea or guanidine-HCl [10]. In addition, reducing agents are added, such as β-mercaptoethanol or DTT [10], which have the function of breaking the disulfide bridges incorrectly formed during bacterial expression. This solubilization step transforms the aggregated proinsulin into a linear, fully deployed protein. But it is still inactive, to make it biologically active it needs to fold properly. The next step is folding, a delicate process where the denatured protein is diluted in a controlled manner in a buffer containing a mixture of reduced glutathione (GSH) and oxidized glutathione (GSSG) [10]. This combination produces a redox environment that favors the formation of the three disulfide bonds specific to human insulin; two between the two chains A and B, and an additional one within the chain A. Folding is a critical step, as incorrect folding can result in new non-functional aggregates or the protein not being properly folded. For the process to take place correctly, different parameters such as pH, temperature, protein concentration and dilution rate are optimized. At the end of this stage, proinsulin is obtained correctly folded and soluble, but still inactive.

3.5. Enzymatic processing and final purification

To obtain functional insulin, enzymatic processing is required, where the protein is incubated with trypsin and carboxypeptidase B [10]. These two enzymes remove peptide C, a sequence that connects chains A and B, and allows the chains to bind correctly using the previously formed disulfide bridges. With this stage, active and structurally correct insulin is achieved, but it still requires purification to comply with pharmaceutical standards. This purification process applies several chromatography techniques [10], starting with ion exchange (separating molecules according to their electrical charge), followed by gel filtration (exclusion by size) and, finally, high-resolution liquid chromatography in reverse phase (RP-HPLC), which guarantees high purity. Once purified, insulin is formulated pharmaceutically. Preservatives, zinc ions and other excipients such as glycerol or protamine are added [10], depending on the insulin to be obtained (fast-acting, intermediate, or long-acting). Finally, the product is carefully packaged in vials, cartridges or pre-filled pens, respecting quality and safety regulations for clinical use.

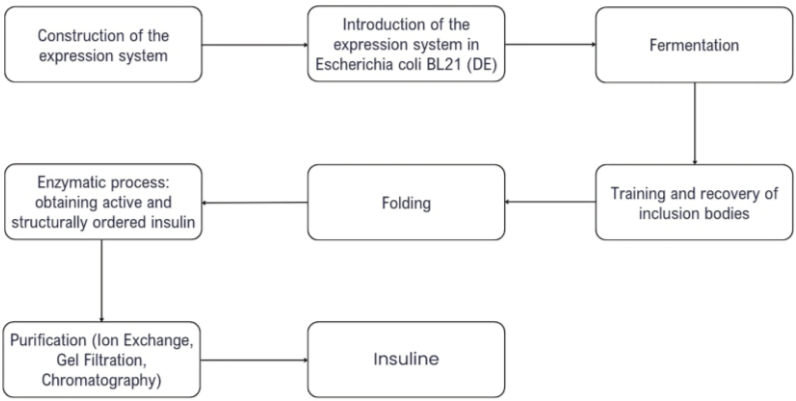

Figure 2. *Schematic representation of process involved in insulin production*

4. FERMENTATION PROCESS

Once the general process of recombinant insulin production has been described, the next step is to focus on the design of the stage targeted in this project: the biotechnological system where the process takes place, namely, the bioreactor.

As previously discussed, fed-batch fermenters are commonly used in this type of process, as they allow precise control over cell growth and the expression of the protein of interest [10]. This mode of operation prevents the accumulation of toxic by-products and optimizes both cell yield and productivity. The fermenters used in these processes are typically stirred and aerated reactors, due to their efficient mixing capabilities and good oxygen transfer performance [11].

For the development of this study, the 5-liter BioFlo III bioreactor manufactured by New Brunswick Scientific Co. has been selected as the technological basis. This bioreactor is widely used in laboratory-scale production [12–13] and has several features that make it especially suitable for the present project.

In the following section, focused on process simulation, a pH-based feeding strategy (pH-stat) will be introduced. This approach uses the pH value as an indicator of substrate depletion—glucose in this case—so that nutrient addition is automatically triggered when a pH variation is detected. The BioFlo III is capable of controlling pH within a range of 2.00 to 12.00 with an accuracy of ±0.01, and when a change is detected, it automatically activates the medium feed pumps [14]. This allows sustained cell growth without substrate accumulation and prevents the formation of toxic by-products.

In addition, this fermenter features precise temperature control via an RTD sensor, a wide agitation range (50–1000 rpm)—although high agitation is not needed in this case—and an aeration system capable of achieving high oxygen transfer rates [14].

5. BIOREACTOR SIMULATION

In order to analyze the proinsulin production process, a mathematical simulation of the fermentation process in fed-batch mode has been developed.

This simulation makes it possible to predict the evolution of variables such as biomass, glucose, crop volume and product concentration, under different operational strategies. Two different process configurations have been modeled and, as will be seen in the next section, compared:

1. **Single-stage fermentation:** In this strategy, both the cell growth phase and the induction phase are carried out within the same bioreactor, sequentially. During the first stage, the culture is fed to achieve high cell density [12]. The expression of the product is then induced by the addition of IPTG, without transfer of the medium or change of tank.
2. **Fermentation in two stages, cyclical process:** In this case, the process is divided into two differentiated stages. In the first tank, only the cell growth phase takes place until a predetermined biomass is reached. Once achieved, a fraction of the cultured volume is transferred to the second tank, where the induction phase begins [13]. Being a cyclical process, the mode of operation is more similar to continuous than to fed-batch, due to the fact that at the end of one phase the other begins and so on, but the feeding in the two tanks is still as described above, therefore, everything and it seems to be a continuous process, is still fed-batch.

Both models have been constructed by resolving differential mass balances [10] for the main variables of the system: biomass (C_x), glucose (C_s), proinsulin (C_p) and volume (V). The equations were solved numerically using Euler's method at constant time steps, implemented in an Excel spreadsheet.

The procedure followed to carry out the simulation of the process is detailed below, but first it is necessary to introduce a series of key concepts that allow a better understanding of its approach. Likewise, the main previous considerations that have been taken into account during the development of the model are presented.

5.1. FUNDAMENTAL PRIOR TO SIMULATION

5.1.1. Operations modes in microbial cultures

The mode of operation plays a decisive role in the behaviour of the culture, affecting cell growth kinetics, metabolite synthesis, and the accumulation of the desired product [11]. The following are the main operation modes commonly used in bioreactors:

1. Batch Culture:

   This is the most basic and traditional operation mode. The microorganism is inoculated into a medium having all the necessary nutrients from the start, with no further addition of substrates or removal of product until the end of the process. The culture goes through several characteristic phases: lag, exponential, stationery and death.

   The advantages of this method include operational simplicity and a minimal risk of contamination. However, its main limitation is its relatively low productivity compared to more advanced systems [11].

2. Continuous Culture:

   In continuous culture, fresh medium is constantly fed into the reactor while part of the culture is simultaneously withdrawn, keeping a constant total volume. This mode allows the system to reach a steady state, in which the cell growth rate equals the dilution rate. Advantages of this approach include higher productivity and the ability to operate under stable conditions for extended periods. Nevertheless, it entails greater operational complexity and a higher risk of contamination, which may compromise process stability [11].

3. Fed-Batch Culture:

The fed-batch system combines the simplicity of batch culture with enhanced control over cell growth. Instead of adding the entire substrate at the beginning, it is gradually fed into the bioreactor according to a predefined strategy. This prevents both nutrient limitation and the accumulation of inhibitory or toxic by-products [11].

Due to this regulatory capability, fed-batch culture is widely used to produce recombinant proteins, such as proinsulin, as it allows for the optimization of conditions for product expression.

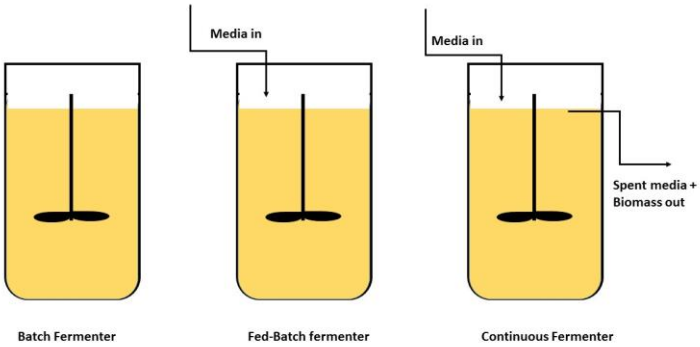

***Figure 3**. Diagram of bioreactor operation modes: batch, fed-batch, and continuous. [Dhanus Micro Notes: Batch, Fed Batch and Continuous Fermentation], author not identified.*

Various feeding strategies can be implemented depending on the specific requirements of the process [11]:

- **Constant flow feed:** his consists of adding the substrate at a fixed speed throughout the operation.
- **Exponential feeding:** It is based on supplying the substrate following an exponential flow profile to maintain a constant growth rate.
- **pH-stat:** Substrate addition is automatically triggered when a pH change is detected in the medium, which can indicate substrate depletion.

- **DO-stat:** The system detects changes in dissolved oxygen as an indirect signal of the metabolic state of the crop.

5.1.2. Global kinetics of the process and inhibition considerations

The cellular metabolism of *E. coli* is based on the use of glucose as its main carbon and energy source. Under aerobic conditions, glucose is metabolized through glycolysis and the tricarboxylic acid (TCA) cycle, producing ATP, biosynthetic precursors, and CO_2 as a byproduct [11]. Part of the carbon is incorporated into cellular biomass, while another fraction is directed toward proinsulin synthesis [11].

The fermentative process can be divided into three main reactions:

1. Conversion of glucose into biomass (cell growth):

   During the growth phase, glucose is primarily degraded via glycolysis and the Krebs cycle. This generates ATP (energy), reducing power (NADH/NADPH), and metabolic precursors necessary for synthesizing new cellular components [11].

   $C_6H_{12}O_6 + NH_4^+ + O_2 \rightarrow$ E. coli (biomass) + $CO_2 + H_2O$

2. Production of recombinant proinsulin:

   The E.Coli BL21 (DE3) strain is genetically modified to carry a plasmid containing the proinsulin gender under the control of an inducive promoter, T7 [10]. Upon induction (with IPTG) [10], the cellular machinery redirects part of its biosynthetic toward the synthesis of the recombinant protein [17].

   $C_6H_{12}O_6 + NH_4^+ + O_2 \rightarrow$ E. coli (pET-proinsulin) + Proinsulin + $CO_2 + H_2O$

3. Acetate formation:

   When glucose supply is excess or oxygen becomes limiting, E.Coli cannot fully process all the pyruvate via the Krebs cycle. Under these conditions, enters a state known as overflow metabolism, redirecting pyruvate to fermentative pathways that lead to acetate production [15].

   $C_6H_{12}O_6 \rightarrow$ Acetate + CO_2 + ATP

The main kinetic challenge in this system is the accumulation of acetate, a byproduct of overflow metabolism. The associated inhibitory effects include:

- **Inhibition of cell growth:** Acetate lowers intracellular pH, causes osmotic imbalances and can interfere with cellular functions, reducing the specific growth rate [16].
- **Inhibition of recombinant protein expression:** The metabolic stress caused by acetate can reduce the efficiency of the expression system and promote the formation of inclusion bodies, which are non/functional aggregates of proinsulin [18].
- **Diversion of carbon flux:** Carbon directed toward acetate formation is lost for biomass or protein production, lowering the overall process yield in terms of product per substrate consumed [15].
- **Oxidative stress and oxygen limitation:** In high-density fermentations, low oxygen availability exacerbates the formation of undesired byproducts and reduces respiratory efficiency, which may trigger the accumulation of reactive oxygen spices (ROS) that are harmful to the cells [18].

5.1.3. Stages of the process

As explained in section 3.2, during the fermentation process it is possible to differentiate two fundamental stages: the cell growth phase and the proinsulin gene expression induction phase. In the first stage, the main objective is to increase the biomass of Escherichia coli BL21 (DE3), feeding the crop glucose in a controlled manner to avoid the formation of unwanted by-products, such as acetate, which could negatively affect growth and future recombinant protein production.

Once the desired cell density is reached, the second stage is initiated by the addition of IPTG, an inducer that activates the T7 promoter present in the plasmid containing the proinsulin gene. This event marks the beginning of the expression of the product of interest, and from that moment on, the cell redirects part of its metabolism towards the synthesis of recombinant proinsulin, which also entails a greater demand for energy and cellular resources.

Therefore, it is important to note that proinsulin is not produced during the growth phase, as the aim is only to increase biomass. It is in the induction phase that the expression of the recombinant gene is activated and proinsulin production effectively begins.

5.2. Model assumptions

To simplify the mathematical treatment of the process and focus on the most relevant aspects of crop dynamics, several reasonable assumptions have been adopted:

1. The bioreactor is considered to work as a **perfectly agitated system** [10-12-13], where all variables (biomass, glucose, product) are evenly distributed.
2. It is further assumed that there are **no limitations for oxygen or nutrients other than glucose and yeast extract** [12]. This assumption is justified based on the characteristics of the bioreactor used in the study: a 5-liter BioFlo III [12-13], which has automatic control oxygenation, temperature and agitation [14]. These variables are regulated
3. During each of the phases, the corresponding **specific growth rate is kept constant** [12-13]. This assumption is based on the fact that by applying a pH/stat feeding strategy and by keeping oxygenation, temperature and pH constant using the bioreactor's automatic control system, the conditions of the medium are stabilized, allowing the specific growth rate to approach a constant value during each stage [12-13].
4. The system's feeding follows a **pH-stat strategy**, activating when glucose concentration drops below a predefined threshold [12-13].
5. The glucose concentration of the fed medium has been **adjusted to maintain the glucose/yeast ratio** [12].
6. During operation, the temperature remains **constant at 37 ºC during the growth phase and reduces to 32ºC during the induction phase** [12].
7. The pH of the crop is automatically controlled to a constant **value of 7.0** [12]
8. The formation of by-products, like acetic acid, **is not modeled**, but the simulation is designed on the control of the previous parameters mentioned above so as not have problems with the formulation of acetate.

5.3. Simulation. Calculations and procedures performed

5.3.1. Initial parameters and culture medium

In order to correctly simulate the fermentation process, it is essential to establish both the initial parameters of the system and the composition of the culture medium used. These elements define the fermentation conditions and allow the model to correctly represent the behavior of Escherichia coli crop under controlled conditions.

First, the initial parameters have been defined, such as biomass concentration, initial volume, glucose concentration, as well as specific growth rates and feed flow [12]. The following table shows the initial parameters chosen and to which model they belong, where "I" refers to the single-step process and "II" to the two-step process:

Table 1. Initial parameters

Model	Variable	Value
I, II	V_0	2,415 L
I, II	$C_{X,0}$	2,54 g/L
I, II	$C_{S,0}$	10 g/L
I, II	$C_{Y,0}$	8,16 g/L
I	μ_{creix}	0,4 h^{-1}
I	μ_{ind}	0,15 h^{-1}
II	μ_{creix}	0,32 h^{-1}
II	μ_{ind}	0,0625 h^{-1}
I	F	0,1 L/h
II	F_{creix}	0,3 L/h
II	F_{ind}	0,1 L/h
I, II	$Y_{X/S}$	0,941 g X/ g S
I, II	q_p	0,00405 g P/ g X ·h

It should be noted that some parameters, such as specific growth rates (μcreix , μind) and feed flows (F), have different values depending on the model considered. In the case of the two-tank model, these speeds have been reduced compared to the single-tank model in order to ensure a balanced development of the cycle, maintaining the condition that the growth and induction phases have the same temporal duration [13]. A lower growth rate allows better control of the increase in biomass and volume, avoiding exceeding the operational limits of the system[12-13]. Likewise, the feed flow has also been adjusted according to the needs of each phase and the operating volume of each tank.

Secondly, the composition of the culture medium and the feeding medium is presented, designed to promote efficient growth without generating inhibitory effects [12]:

Table 2. Components of medium culture and feeding medium [12]

Components	Initial medium (g/L)	Feeding medium (g/L)
KH_2PO_4	5,00	0
K_2HPO_4	3,00	0
$(NH_4)_2SO4$	1,67	1,50
$FeSO_4 \cdot 7H_2O$	0,10	0
$CaCl_2 \cdot 2H_2O$	0,02	0
$MgSO_4 \cdot 7H_2O$	2,00	1,50
Trace metak	80 mL/L	0
Thiamine-HCl	0,10	0
Ampicillin	0,20	1
Glucose	10,00	250
Yeast Extract	8,16	204

As can be seen, the base medium includes a series of inorganic salts, trace metals, vitamins and antibiotics, as well as glucose and yeast extract as the main sources of carbon and nitrogen,

respectively. In the case of the feeding medium, the formulation is simplified in glucose, yeast, some inorganic salts and ampicillin. However, in the mathematical simulation of the process, it has been decided to model only the contribution of glucose (250 g/L) and yeast extract (204 g/L), since these two components are the majority in terms of mass and the main responsible for the dynamics of cell growth [12]. The rest of the components present in the feeding medium are not explicitly included in the matter balances, since their variation has a much smaller impact on the simulated variables. This simplification allows the model to focus on the limiting factors and facilitates the resolution of the associated differential equations.

5.3.2. Differential material balance

This section presents the differential matter balances used to simulate the dynamic behaviour of the fed-batch fermentation process. These balances allow us to describe the evolution of the main variables of the system. Each equation has been proposed according to the physical and biological processes involved [11], considering that it is a bioreactor operating in a fed-batch regime with no volume output, and that the feed is carried out with concentrated liquid medium:

1. Volume balance:

   The volume of the culture increases because of feeding, since there is no withdrawal of the liquid. The balance sheet is [11]:

   $$\frac{dV}{dt} = F \quad (1)$$

   Where **F** represents the average flow rate entering the reactor (in L/h), and may vary during the process depending on the feeding strategy.

2. Biomass balance:

   Biomass is generated by cell growth and can be diluted as a result of increased volume due to feeding. There is no outflow, therefore, the balance is [11]:

   $$\frac{dC_X}{dt} = \mu C_X - \frac{F}{V} \cdot C_X \quad (2)$$

   The difference between biomass generation by growth and dilution of biomass concentration due to the input of cellless medium is plotted. This balance applies in both

the growth and induction phases, although with different values of the specific growth rate [12].

3. Substrate balance:

   Glucose is consumed by cells as the main source of energy and carbon, and is replenished through food. Its balance sheet includes a consumption term for growth and an input term [11]:

$$\frac{dC_S}{dt} = -\frac{\mu \cdot C_X}{Y_{\frac{X}{S}}} + \frac{F}{V}(C_{S,f} - C_S) \quad (3)$$

   The first term represents glucose consumption per unit of biomass formed and the second is the net glucose intake per feed [10]. This balance reflects the activation dynamics of the pH-stat feed. As we will see in the next section, when the glucose concentration drops below 0,5 g/L, the flow of food is activated [12-13].

4. Product balance:

   Proinsulin is produced only during the induction phase [10], and accumulates in the system. There is considered to be no output of the product [11]:

$$\frac{dC_P}{dt} = q_p \cdot C_X - \frac{F}{V} \cdot C_P \quad (4)$$

   This balance is only activated after the induction of IPTG [11-12-13].

5.3.3. Methodology

Once the matter balances and initial parameters were defined, the simulation of the fermentation process was implemented by means of a numerical resolution in Excel, using Euler's method[19]. This explicit method was applied with a constant time step of 0,1 h, which allowed a detailed monitoring of the evolution of the system variables: biomass concentration (C_X), glucose (C_S), product (C_P) and volume (V). At each time interval, these variables were updated based on the previously defined differential balances. The feeding strategy implemented in both models was pH-stat [12-13]. The feeding was automatically activated when the glucose concentration in

the reactor dropped below a predefined threshold of 0,5 g/L. This condition was evaluated at each step of time and, when it was met, glucose-fed medium and yeast extract were introduced.

5.3.3.1. Simulation of the single-tank model

In this case, the entire growth and induction process was carried out in a single reactor. The simulation started with an initial biomass of 2,54 g/L and a volume of 2,415 L [12]. The growth phase operated under pH-stat control with a constant feed flow rate of 0,1 L/h, activated whenever glucose dropped below the threshold. The specific growth rate during this phase was $\mu = 0.4\ h^{-1}$. Once the biomass reached 28,4 g/L [12], which occurred at 6,3 hours, the induction phase was considered to begin [12], simulating the addition of IPTG. From that point on, the specific growth rate was reduced to and proinsulin production was activated [12]. The feed was maintained at 0,1 L/h until the end of the process, where the biomass reached a value of approximately 42,5 g/L [12] which was completed at 9,6 total hours. This model allowed the evolution of the crop to be evaluated under a sequential strategy in a single operating environment.

5.3.3.2. Cyclic model simulation

In this model, the process was divided into two independent tanks: the first phase is grown and the second is induced [13]. The growth phase began with the same initial conditions (X = 2,54 g/L, V = 2,415 L), but with an initial feed flow rate of 0,3 L/h, and a specific rate of $\mu = 0,32h$. Once the target biomass (28,4 g/L [12]) was achieved, 60% of the volume was transferred to the next tank [13]. The total duration of this first stage was set at 8 hours [12-13]. Volume transfer marked the beginning of the induction phase, in which a specific reduced growth rate was set ($\mu = 0,0625\ h^{-1}$), and proinsulin production was activated [12]. The feed flow rate was reduced to 0,1 L/h and remained constant throughout this stage. The induction phase also lasted 8 hours [12-13], so that the condition that the growth time was equal to the induction time, a fundamental characteristic of the cyclic design considered, was met [13].

It should be noted that the values used for growth rate and feed flow are different from those of the single-tank model because the staged design requires tighter process control. Lower values were used to ensure that crop conditions did not get out of control throughout the cycle and that

both phases could be completed correctly in the expected time. In addition, by dividing the process into two separate phases, each can better adapt to the needs of the cell at that time, both to grow and to produce the desired product [13].

6. RESULTS AND DISCUSSION

This section presents the results obtained from the simulation carried out with each of the configurations studied: the single-tank model and the two-tank cyclic model. The results are shown and analyzed independently for each case, with the aim of examining in detail the evolution of the main variables of the system (biomass, glucose, volume and proinsulin) and the effect of the operating parameters of each strategy.

Each set of results includes a series of graphs that illustrate the dynamic behavior of the simulated variables over time, which allows a clearer interpretation of the information they offer.

Finally, a comparative section between both models is included, in which the differences in terms of productivity, system control and total duration of the process are discussed, in order to evaluate advantages and limitations of each strategy.

6.1. Single-tank model

The results obtained from the simulation of the fermentation process conducted in a single tank are presented below.

In this configuration, the biomass is grown to a target value and then proinsulin production is induced in the same environment without the need for transfers.

The following charts show the dynamic behavior of this configuration:

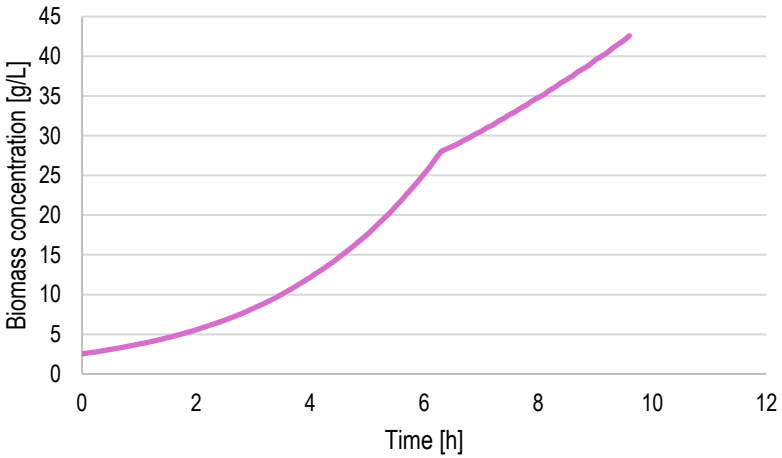

Figure 4. *Evolution of biomass concentration over time.*

An exponential growth has been observed since the beginning of the process, starting from a first value of 2,54 g/L [12]. Throughout the first 6.3 hours, the specific growth rate is μ= 0,4 h^{-1} [12], which is reflected in the slope of the curve.

When the target biomass of 28,4 g/L [12] is reached, the induction phase [12] begins, which is shown by the change in slope from that point on. During this second stage (from 6.3 hours to 9,6 hours), the specific growth rate is reduced to μ= 0,15 h^{-1} [12], and although the biomass continues to increase, it does so at a slower rate. This transition reflects the change of stage within the same tank, from one of active growth to one of product expression.

The process ends when a concentration value of approximately 43 g/L is obtained [12], this occurs after 9,6 hours.

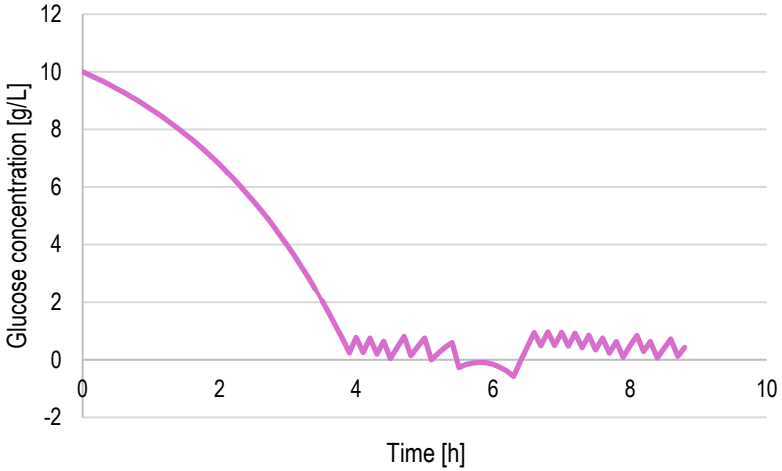

Figure 5. *Evolution of glucose concentration over time during fermentation in a single tank*

Initially, a progressive decrease in glucose is observed due to its consumption by the crop. After approximately 4 hours, the concentration falls below the established threshold of 0,5 g/L, which activates the pH-stat feeding strategy.

This behavior is reflected in the oscillations that appear on the graph from that point on. Intermittent feeding keeps glucose within a range close to the threshold, without allowing it to build up excessively. However, at some points of the curve it is observed that the glucose concentration takes negative values, which is not physically possible. This behavior is due to the nature of the mathematical method used to develop the simulation, the Euler method with discrete time steps. Sometimes, the consumption calculated in one step can exceed the actual value of available glucose. This produces temporary negative values that actually correspond to a situation of total glucose depletion. This situation is corrected by bringing in food for more than one step of time in a row, until the concentration of glucose in the medium is stabilized again.

Finally, throughout the induction phase (from 6.3 hours onwards), this pattern of oscillations is maintained, which indicates that the system continues to actively consume glucose, as can be seen in Figure 3.

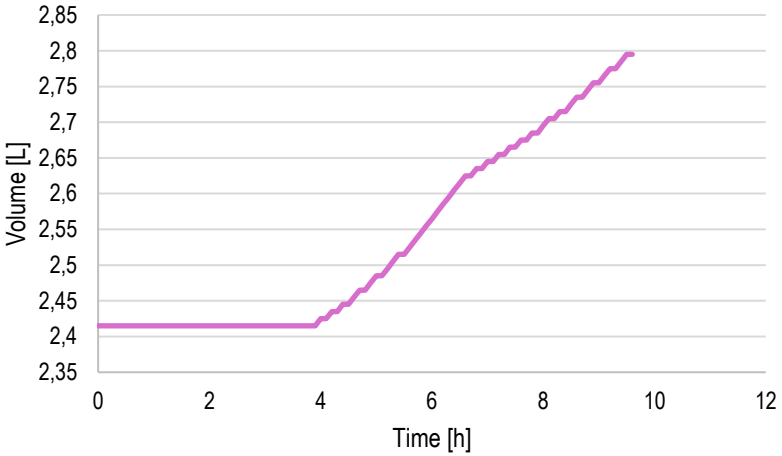

Figure 6. *Progressive growth of crop volume over time as a result of the fed-batch feeding strategy*

The initial volume is 2.415 L [12], and as the system activates the pH/stat feed, this increases as medium is added with a constant flow rate of 0,1 L/h from approximately 4 hours, when glucose falls below the established threshold. This increase can be seen in the small peaks that appear in the graph that refer to the increase in volume, that is, the entry of food.

At the end of the process, the total accumulated volume reaches a value of 2,795 L, which represents a moderate and controlled thanks to the constant feed input when required (see Appendix 1).

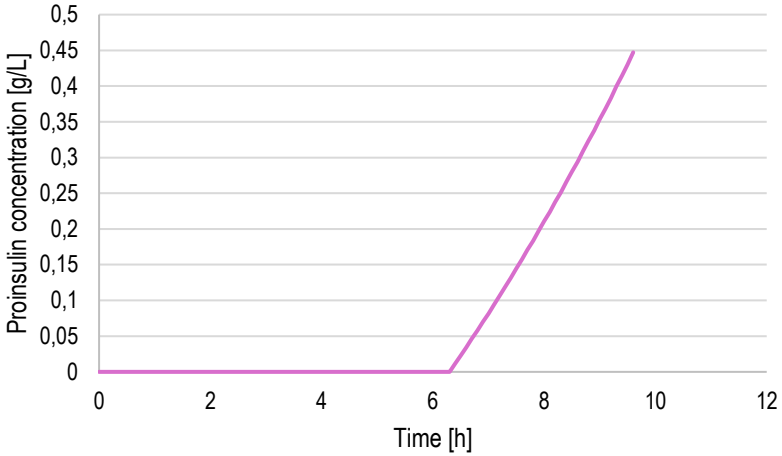

Figure 7. *Evolution of proinsulin concentration over time*

As expected, the curve remains at zero throughout the growth phase, and it is not until 6,3 hours when production begins, that is, when IPTG is added to start induction.

From that moment on, an increasing linear curve is observed, which reflects a sustained production of proinsulin proportional to the biomass present. The final value reached, approximately 0,45 g/L, represents the total accumulation of the product at the end of the crop. This concentration represents a final production of 1,25 grams of proinsulin.

6.2. Cyclic model

As we saw in section 5.3.3, the cyclical process is divided into two tanks [12-13]: one dedicated to growth and the other to the induction phase. This strategy allows differentiated conditions to be applied at each stage [13], adapted to the specific requirements of the crop and proinsulin production.

The first tank operates for 8 hours at a pH-stat feed rate, initially using a flow rate of 0,3 L/h until the target value (28,4 g/L) is reached [12]. Once achieved, 60% of the volume is transferred to the second tank [13], from there the induction phase begins, also lasting 8 hours, with a reduced specific growth rate (μ = 0,0625 h^{-1}) and constant feeding at 0,1 L/h.

Below are the graphs illustrating the evolution of the main variables of the system in each tank:

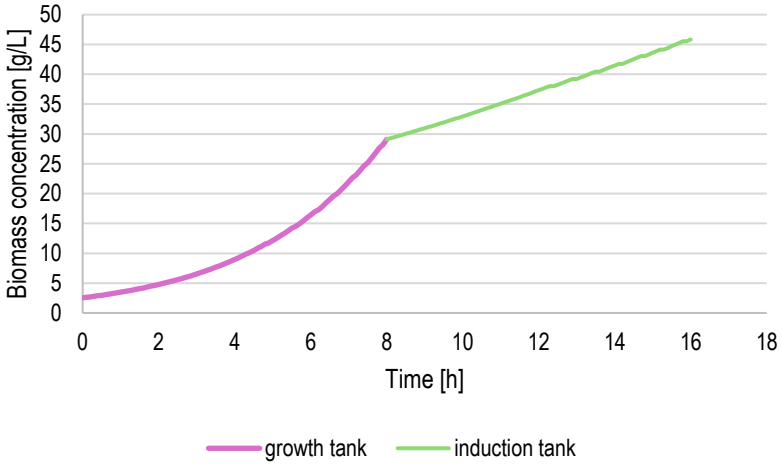

Figure 8. *Evolution of biomass concentration over time*

The pink curve corresponds to the growth tank (0–8 h) and the green curve to the induction tank (8–16 h). Progressive growth is observed in the first tank, starting with an initial biomass of 2,54 g/L [12] and reaching the target value of 28,4 g/L [12] at the end of the phase (8 h). This growth is driven by a specific rate of $\mu = 0{,}32\ h^{-1}$ and a pH-stat feeding strategy, with a flow rate of 0,3 L/h.

Once the desired biomass is reached, 60% of the volume is transferred to the second tank [13], where the induction phase begins. At this stage, the biomass continues to increase more moderately, due to the reduction of the specific growth rate to $\mu = 0{,}0625\ h^{-1}$. The slope of the green curve is lower, as is to be expected under conditions of induced production. At the end of the process, at 16 hours a final biomass of approximately 46 g/L is reached.

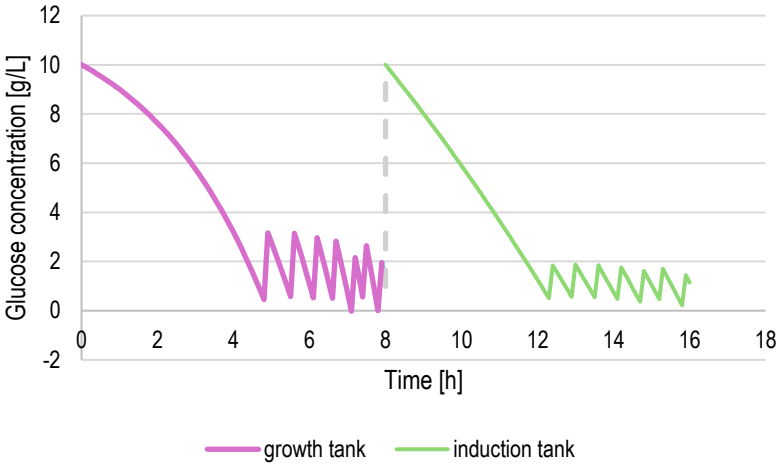

***Figure 9**. Glucose variation as a function of time in each tank*

The two stages of the process are clearly distinguished: the growth phase (pink curve, from 0 to 8 h) and the induction phase (green curve, from 8 to 16 h), each with its own consumption and feeding profile. In the first stage, corresponding to the growth tank, a progressive decrease in glucose is observed due to consumption by the cells. When the threshold of 0,5 g/L is reached, the pH-stat type feeding system is activated, which introduces medium with glucose through a controlled flow rate. This mechanism causes an oscillatory pattern, characterized by brief increases and repeated decreases in concentration, which is maintained until the end of this stage.

At the end of the growth phase (at 8 hours), 60% of the volume is transferred to the induction tank [13]. The gray area refers to the jump between one tank and the other, that is, it represents the transfer of volume. During induction, although the cell growth rate is lower, glucose consumption continues and the pH-stat feed is reactivated when the threshold is reached again. The resulting pattern is similar to that of the previous stage, but less pronounced, reflecting more moderate consumption.

This glucose behavior is consistent with that observed in the single-tank model, where oscillations caused by the pH-stat feeding strategy are also recorded. In both models, glucose is maintained in adequate ranges to avoid both limitation and excessive accumulation [11-12]

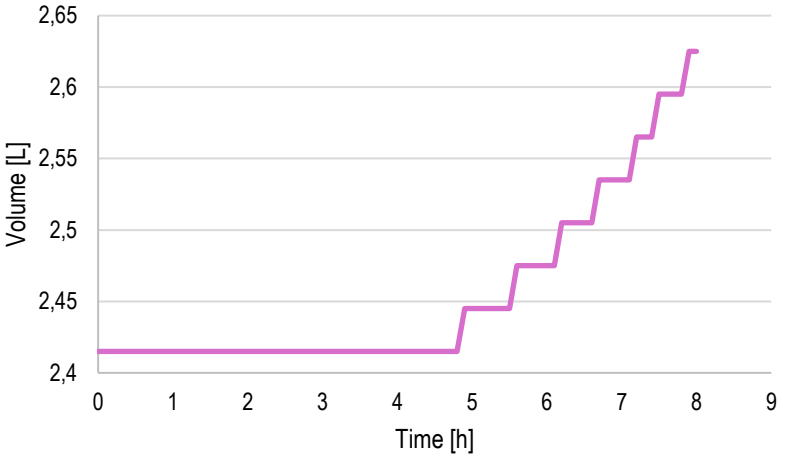

Figure 10. *Volume variation as a function of time*

It starts with an initial volume of 2,415 L [12], which is progressively increased due to the pH-stat-controlled feeding. In this case, the feed flow rate is 0,3 L/h, therefore, the steps representing the volume variation are larger than in Figure 5 or in the induction tank of this model. At the end of the growth phase, the total volume reaches 2,625 L.

Although not shown here (see Appendix 2), a similar volumetric behaviour is observed in the induction tank. After the transfer of 60% of the initial volume, the feed is continued at a constant flow rate of 0,1 L/h for the next 8 hours until a final volume of 1,645 L is reached.

As expected, the volume increases more in the growth phase than in the induction phase since in one the feed flow is higher than in the other, also because in the growth phase more glucose is consumed, so more impulses will be sent than in the induction phase.

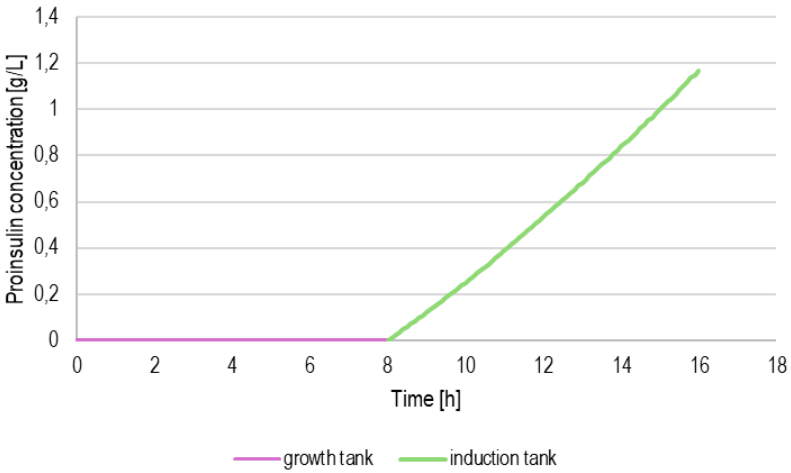

Figure 11. *Evolution of proinsulin concentration over time*

As a visual support for the operation of the feeding system, a graph is included in Appendix 1 with the evolution of the feed flow during the process, where it is possible to observe at what times the media input is activated and how it is related to the evolution of the volume.

As expected, the curve remains at zero throughout the growth phase (0–8 h), as proinsulin production is not activated until the target biomass is reached and induction is initiated [12]. From the beginning of the second stage, in the induction tank (green curve), a progressive increase in the concentration of proinsulin is observed.

The curve has an increasing and sustained slope, indicating a constant production rate throughout the 8 hours of induction. This behavior is consistent with the maintenance of stable conditions (constant feeding, increasing volume, and biomass in activity), which favor the synthesis of the product. The final concentration achieved is 1,16 g/L, which represents a considerable increase compared to the single-tank model. At the end of each cycle, 1,92 grams of proinsulin are obtained, which is also greater than what is obtained at the end of the single-tank model.

6.3. Comparative of the two models

Once both simulation models have been analyzed separately, it is interesting to compare them to see what advantages and disadvantages each one presents, this comparison is focused on the main variables of interest: final biomass concentration, proinsulin production, volumetric productivity and total process time.

Both models were designed with practically the same conditions, initial parameters and requirements, such as reaching 28,4 g/L of biomass before induction or that in both cases a pH-stat feeding strategy was used. It was done in this way so that the comparison between the two would be as realistic as possible.

The following table shows the results obtained at the end of each process:

Table 3. Parameter values at the end of each process

Parameter	Single-tank model	Cyclic model
Biomass concentration (g/L)	42,59	45,84
Proinsulin concentration (g/L)	0,45	1,16
Proinsulin (g)	1,25	1,92
Volume (L)	2,795	1,645 (second tank)
Time (h)	9,6	16
Volumetric productivity (g/L·h)	0,0468	0,0725

From a time point of view, the single-tank model is shorter, which can be an operational advantage if you are looking for speed in the cycles. However, as it is a single system, the conditions of growth and production must coexist in the same environment, which can limit the efficiency in the expression of the product. In addition, this model requires stopping the process

since the tank must be emptied, cleaned and filled between one cycle and another, which increases preparation time and reduces operational continuity.

On the other hand, the two-tank model, although longer, allows a clear separation between phases. This offers the advantage of being able to adapt the conditions of each stage (growth rate, flow rate, volume and fresh medium), which translates into a higher production of proinsulin (1,16 g/L vs. 0,45 g/L). Additionally, it operates in a similar way to a continuous system in successive cycles, since while a batch is in the induction phase in the second tank, the first tank can be started again with a new crop. This reduces the need for frequent cleanings and increases the operational efficiency of the assembly.

To better visualize the difference in frequency of operation, Gantt charts were constructed for each process, showing how many complete cycles can be performed in a 24-hour period. In the case of the single-tank model, also considering the cleaning, emptying and filling times, where it has been decided that it takes 2 hours to clean [20], two processes can be completed per day.

On the other hand, the two-tank model, by operating in a cyclical and chained way, allows a new growth cycle to be started while the induction of the previous one is completed, which allows two and a half processes interspersed per day, thus optimizing the use of the equipment.

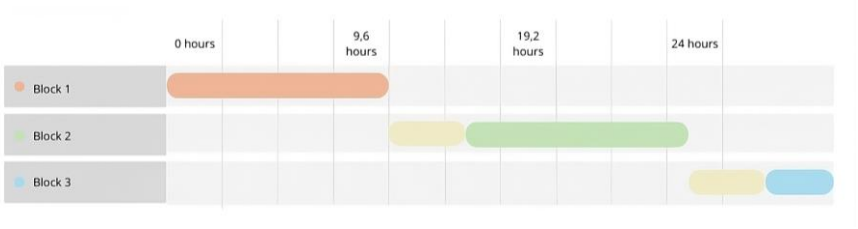

Figure 12. *Gannt chart of single-tank model*

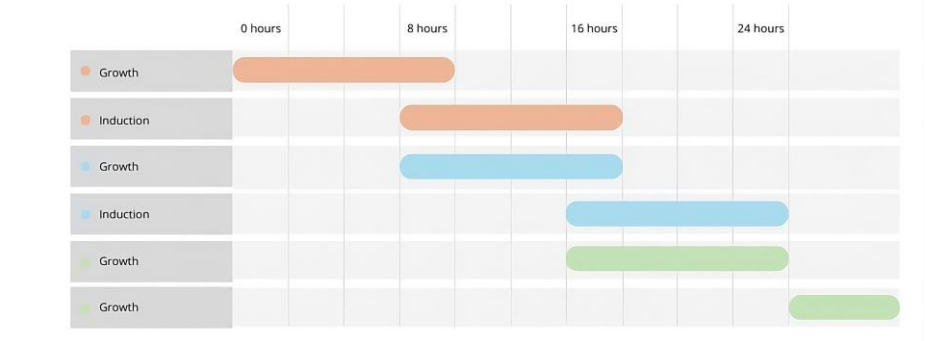

Figure 13. *Gannt chart of cyclic model*

In addition to the batch analysis, it is relevant to estimate the total production of proinsulin that can be achieved under real operating conditions over time. To this end, the daily and annual production has been calculated in both models, considering continuous operation for 365 days a year 24 hours.

In the single-tank model, each complete process lasts 11,6 hours, allowing for two full cycles per day. In each of these cycles, 1,25 grams of proinsulin are produced, the result of a final concentration of 0,45 g/L in a total volume of 2,795 liters. This means a daily production of 2,5 grams. If this operation is maintained constant throughout the year, the estimated annual production amounts to 912,5 grams of proinsulin.

On the other hand, the two-tank model allows for a more efficient cyclical organization, with a duration of 16 hours per cycle (8 hours of growth and 8 hours of induction). Thanks to this structure, up to two and a half cycles can be carried out interleaved per day. In each of them, 1,92 grams of proinsulin are produced per cycle, reaching a final concentration of 1,16 g/L in a volume of 1,645 liters. This translates into a daily production of 3,84 grams (up to the induction phase no proinsulin is produced, so there are only two complete cycles) and an annual production of approximately 1401,6 grams of proinsulin.

Finally, although the two-tank model has proven to be more efficient in terms of yield and productivity, especially by allowing a better adaptation of the cultivation conditions in each phase, in industrial practice it is still more common to use a single tank to carry out both growth and induction. This preference is mainly due to economic criteria and operational simplicity, since operating with a single bioreactor significantly reduces the costs associated with the installation.

In addition, the use of a single tank simplifies the overall management of the process. By not requiring intermediate transfers or duplication of equipment, operational logistics are facilitated and possible operational errors are minimized. Although the separation of phases in separate tanks allows for a more precise optimization of the conditions for each stage, it is also possible to achieve product quality with a single fermenter.

Added to this advantage is a key aspect from an industrial point of view: maintenance. A single-tank system means a simpler infrastructure, with fewer sensors, pumps, valves, and power lines.

On the other hand, the two-tank model, by requiring two units to be operational simultaneously, involves duplicating certain components of the system.

7. CONCLUSIONS

Throughout this work, the simulation and comparative analysis of two fed-batch culture strategies for the production of recombinant proinsulin in Escherichia coli BL21 (DE3) has been carried out: a conventional single-tank model and a cyclic model based on two differentiated tanks for the growth and induction phases.

The production of recombinant insulin represents a critical area within pharmaceutical biotechnology, both because of its high global demand and because of its health importance. The treatment of diabetes mellitus, an ever-growing chronic disease, depends heavily on sustained access to this hormone.

In a competitive and highly regulated market, optimizing production efficiency is essential not only for industrial profitability, but also to ensure treatment availability globally. The simulation developed in this work has been based on the operating conditions of a 5-liter laboratory bioreactor (BioFlo III) [12-13], which has allowed working with realistic parameters. It should be noted that, for the productivity comparison between the two models, a continuous operation scenario on an industrial scale has been considered, estimating the amount of proinsulin that could be produced in 24 hours for 365 days a year.

The results show that the two-tank model allows for significantly higher production of proinsulin, both on a batch and cumulative basis. On an industrial scale, this model could reach an annual output of approximately 1401.6 g, compared to the estimated 912.5 g in the single-tank model. This difference is mainly due to the higher final concentration of product and the possibility of chaining cycles without the need for stops, which maximizes the use of time and useful volume.

Despite these advantages, the single-tank model remains the most common in industrial environments, due to its operational simplicity, reduced need for equipment, and lower installation and maintenance costs. In addition, this more compact design allows acceptable product quality

to be achieved with a lower technical and economic investment. In short, both models have specific strengths depending on the objectives and resources available.

While the two-tank model is more competitive in terms of performance and process continuity, the single-tank model represents a robust and cost-effective solution, especially in facilities with scale constraints. This comparison provides a useful basis for decision-making in the design of biotechnological processes for the production of insulin and other products of high therapeutic value.

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ACRONYMS

1. IPTG: Isopropyl β-D-1-thiogalactipyranoside
2. E.Coli : Escherichia coli BL21 (DE3)
3. RNA: Ribonucleic acid
4. DNA: Deoxyribonucleic acid
5. ATP: Adenosine triphosphate
6. GLUT4: Glucose Transporter type 4
7. DTT: Dithiothreitol
8. RTD (sensor): Resistance Temperature Detector

Appendices

APPENDIX 1: FOOD INPUT DURING THE PROCESS

1. Single-tank model:

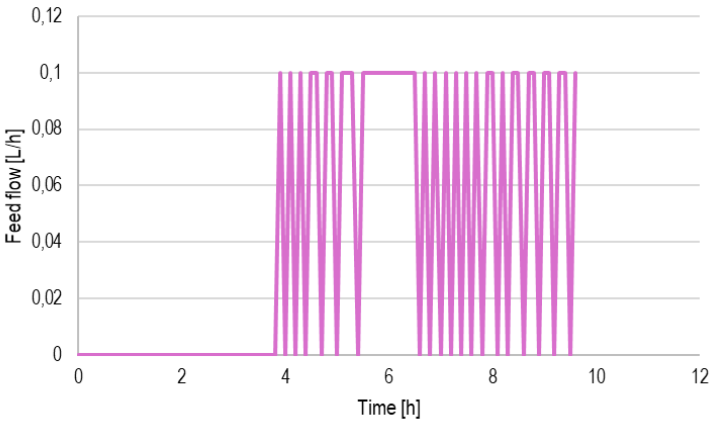

Figure 14. *Food input related to volume increase during the process*

2. Cyclic model:

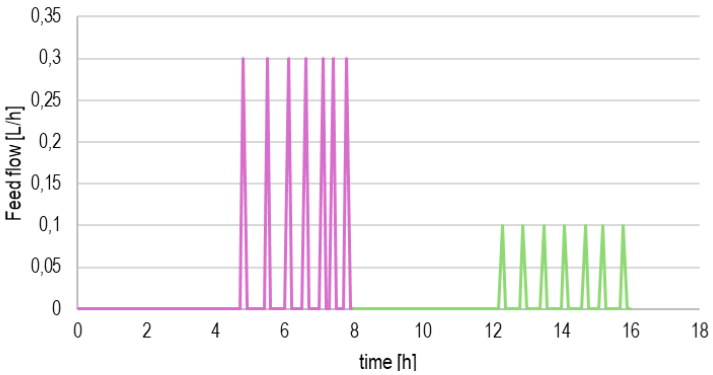

Figure 15. *Food inputs during the process. As explained above, the feed flow rate of the grow tank is higher than in the induction tank*

APPENDIX 2: VOLUM VARIATION

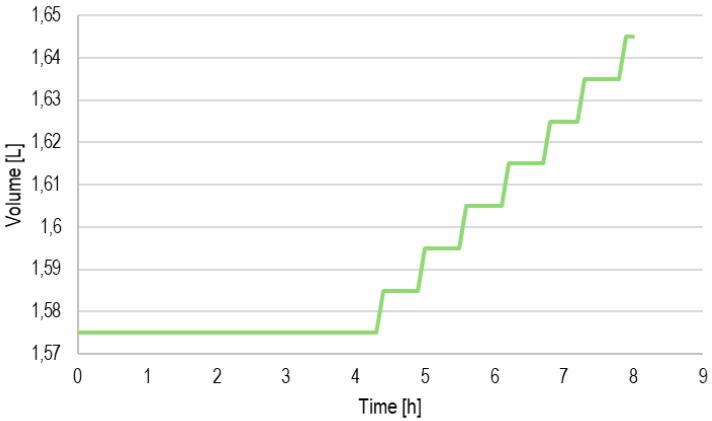

Figure 16. *Variation of the volume in the induction tank of the cyclic configuration*