Commissioning and equipment assessment of a semi- industrial bioreactor

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Abstract

Tasks regarding the commission and equipment assessment on the fermenter situated in PILOT PLANT Research Center were carried out. The bioreactor consists in a pilot-scale fermenter with a capacity of 150L. Piping and Instrumentation Diagrams were elaborated, in which a posterior Sterilization Standard Operating Procedure was designed. In such SOP, Steam In Place method was implemented. The sterilization was designed as a batch process, in which the reaction medium is sterilized simultaneously to the fermentation vessel. This way, the use of additional equipment was avoided and the risk of contamination between sterilization and fermentation start was minimized. Optimal saturated vapor temperature was taken as 121ºC. The required holding time for equipment sterilization (spare parts) was determined to be 10 minutes, whereas a holding time of 20 minutes was considered for medium sterilization. Other key factors taken into account for the design of the procedure were condensate removal, air evacuation and post-sterilization integrity, The SOP is still in need of validation. A sealing test revealed the inoperability of the safety device (burst disk). This device was then replaced and tested with success.

Gas-liquid mass transfer capacity of the fermenter was assessed, applying the hydrogen peroxide method. A K_La of 25±3.3 $h^{-1}$was determined when the stirrer run at 450 rpm. However, due to supersaturation effect, this value could be an overestimation of the actual value. Comparison with other typical K_La values for pilot-scale bioreactors led to the conclusion that the aeration capacity of the fermenter is low and might be insufficient for some biochemical processes. A change in the impeller type is proposed in order to address this problem.

List of symbols

a	Specific interfacial surface (m^2interfacial durface/ liquid-m^{-3})
A	Arrhenius constant (s^{-1})
c	Concentration (mole/L or mol/m^3)
c_{H2O2}	Concentration of the hydrogen peroxide solution
c_{H2O2}	Concentration of the hydrogen peroxide solution
DO	Dissolved oxygen, measured in 10% basis
DO'	Dissolved oxygen, in 100% basis
E	Activation energy (kcal/mole)
$J_{A,l}$	Molar superficial flow of gas A in the liquid phase ($mol/s/m^2$)
$J_{A,g}$	Molar superficial flow of gas A in the gas phase ($mol/s/m^2$)
J_A	Molar superficial overall flow of gas A ($mol/s/m^2$)
k	Death rate constant (s^{-1})
k_l	Mass transfer coefficient in the liquid phase ($m \cdot s^{-1}$)
k_g	Mass transfer coefficient in the gas phase ($mol \cdot s^{-1} \cdot atm^{-1} \cdot m^{-2}$)
K_La	Volumetric gas-liquid mass transfer coefficient referred to the liquid phase (h^{-1}, s^{-1})
MW	Molar weight
m_{sol}	Wight of hydrogen peroxide solution sample (g)
$\dot{m}_{H2O2}$	Hydrogen peroxide mass flow (given by peristaltic pump)
N	Number of viable organisms
P	Pressure
q_A	Volumetric overall flow of gas A ($mol/s/liquid\text{-}m^3$)
Qair	Air flow
R	Gas constant (1.987 cal/mole/K)
r	Molar rate (mole/L/s)

t Time (s)

T Temperature

U Absolute uncertainty

V_L Liquid volume (L)

V_{MnO4-} Used volume of titration solution in the determination of hydrogen peroxide solution concentration

π Partial pressure (atm)

ε Relative uncertainty

Superscripts

* Saturation conditions

‘ Referred to 100% basis

Subscripts

0 Initial value

t Value at a time ‘t’

s Steady state value

l Liquid phase

g Gas phase

A referred to gas solute A

i Gas-liquid interface

sat Saturation conditions

tr Referred to mass-transfer process

gen Generated

H2O2 Referred to hydrogen peroxide solution

MnO4- Referred to permanganate solution (titration solution)

Acronyms

SAL	Safety Level Assurance
SIP	Sterilization In Place
rpm	Revolutions per minute
vvm	Vessel volumes per minute

Table of Contents

CHAPTER 1:
Introduction and Theory

1 Introduction

Fermentation has a long history. Microorganisms have been used from early times by humans for food and medical applications. They are key agents for obtaining very important products, such as bioethanol, antibiotics, insulin, etc., and its industrial application has a series of big advantages: they are highly selective, its production processes normally occur in mild conditions, they can convert a wide range of raw materials and they have, as a general rule, less impact on the environment. All of these qualities make biochemical processes a very attractive answer to the sustainability challenges our society faces today.

This project aims to contribute to the commission a pilot-scale fermenter for educational purposes. Diverse engineering skills as redaction of operational procedures, equipment qualification, and elaboration of technical representations of process units have been applied.

2 Theory

In this section, the theoretical aspects related to fermentation processes and equipment as well as the other subjects covered in the present project are introduced. The main scientific works related to each of the points are also here reviewed.

2.1 Fermentation processes overview

The first question to be answered is what do we understand by a fermentation process? In the general use of the word, fermentation means the production of alcohol by the action of microorganisms. The more scientific biochemical term means strictly “an energy-generation process in which organic compounds act as both electron donors and terminal electron acceptors”[1]. However, in an industrial microbiology context, the term is used in a broader sense and

"fermentation" is used to describe "any process for the production of product by the mass culture of a microorganism" [1], and can involve or not aeration.

The first two major processes involving microorganisms in an industrial context were alcohol and vinegar production. According to Stanbury et al. [1], the first big-scale breweries were built in the early 1700s, and since then thousands of different industrial applications have been discovered for numerous microorganisms strains and cultures, from the production of enzymes to the cultivation of monoclonal antibodies.

The growth and development of microorganisms have been broadly studied, and a typical life-curve in batch cultivation can be seen in Figure 1. In this plot, the natural logarithm of substrate and microbial concentrations, represented by "S" and "X" respectively, is represented versus time.

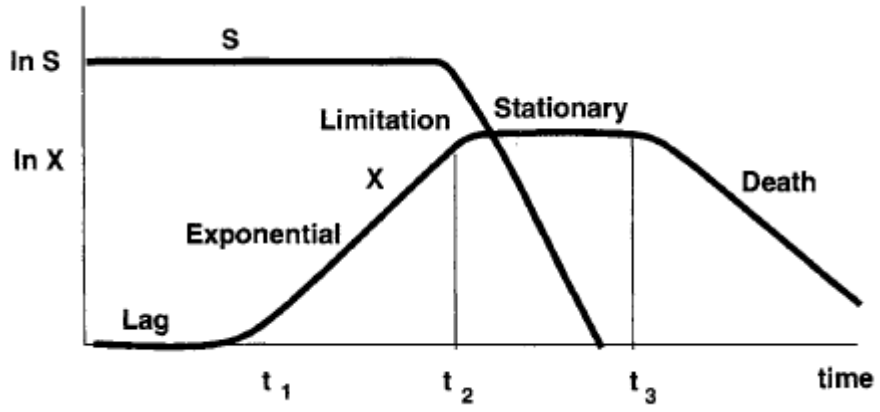


Figure 1. Substrate evolution and typical microbial growth in a batch culture (S= Substrate concentration, X= Biomass concentration), extracted from [2].

Looking at this representation, there are 4 recognizable different phases:

1) Lag phase: during this phase, biomass doesn't increase and no substrate is consumed. Microorganisms are adapting to the media, generating the necessary enzymes and preparing to degrade the

substrate. Commercially, this phase is required to be as short as possible.

2) Exponential growth phase: after the adaptation phase, they start to grow and, as there is an excess of substrate, they grow exponentially at a constant, maximum rate. During this phase, microorganisms produce substances known as primary metabolites, which are substances related to basic functions during the development, and reproduction of the microorganisms. Examples of primary metabolites are ethanol or lactic acid.

3) Stationary phase: due to the substrate depletion and the aging of the bioburden, the biomass stops growing in number, but they remain metabolically active. During this phase microorganisms generate secondary metabolites, defined by Hogg [3] as "substances […] usually not required for essential metabolic or cell maintenance purposes. Examples include toxins pigments and most antibiotics".

4) Death phase: the number of viable cells starts decreasing.

Once the life-cycle has been introduced, one can understand the different uses of the microbial activity.

In the book "Principles of Fermentation Technology" [1] written by Stanbury et al., already mentioned in this section, they divide commercial fermentation processes in five groups:

1) Microbial biomass production (f.i., yeas production for the bakery industry).

2) Intracellular substances production, such as enzymes (amylases, cellulases, …)

3) Microbial metabolites production, primary or secondary (examples of this kind of processes are production of ethanol, citric acid, vitamins, …).

4) Recombinant products production, making use of recombinant DNA technology. Some examples of products produced by genetically modified organisms include insulin (essential for palliating the effects of the diabetes disease) and interferon (used in treatments against cancer, multiple sclerosis, hepatitis C…) among other important substances.

5) Transformation processes, where microorganisms work as chiral catalysts making possible the addition or the removal of certain functional groups in highly complex molecules. Acetic acid production belongs to this type of fermentation, as well as some steroids and antibiotics.

Depending on what are the desired products out of the industrial process, the operating conditions will vary. However, a typical industrial fermentation process can be divided into 4 main steps, pictured in Figure 2.

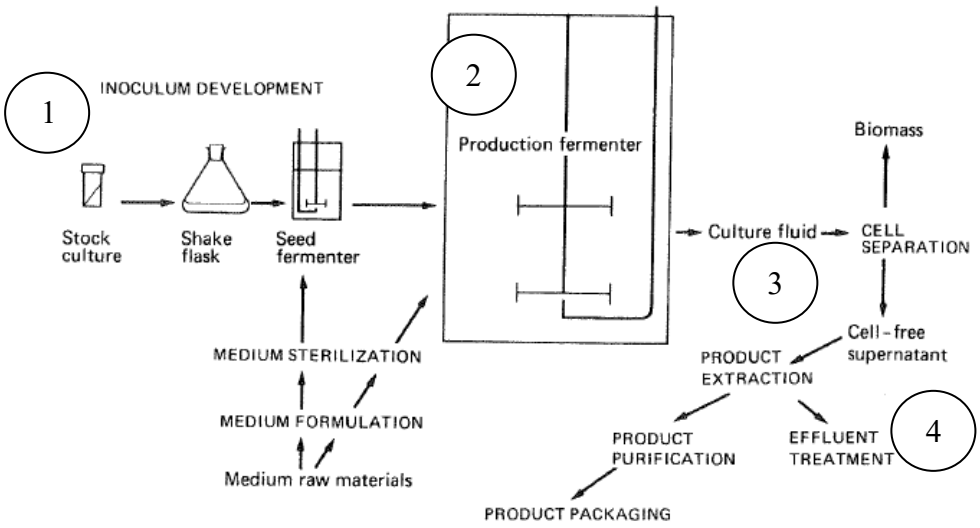


Figure 2. Generic representation the steps involved in an industrial fermentation process, extracted and adapted from [1].

1) Incubation: First, the culture is inoculated in a small flask where germination occurs. When it starts growing, it keeps being moved to gradually larger recipients. The last recipient to where they are transferred in this phase is called seed fermenter, which consists in a small replica of the proper fermentation vessel, with the medium and the

conditions that cells will find later in the production phase, making the adaptation of the culture quicker and more controlled.

The fermentation medium usually consists in a water based solution containing the appropriate nutrients (with a carbon and nitrogen source), salts and other elements essential for the growth of the culture. As it will be discussed later (see subsection 2.3 in this chapter) the absence of foreign organisms in this liquid is crucial for the success of the process.

2) Production: following seed fermentation, the culture is transferred to main fermenter, where the parameters such as temperature, pressure, pH,… are controlled to optimize production of the desired product.

3) Product recovery: the desired product, which can vary from the same biomass to segregated metabolites, has to be separated and purified from the media and the rest of elements involved in the process.

4) Effluent treatment: as last step, but not less important, generated waste streams, such as gases or purged microorganisms, need to be treated.

Now that an overview through the main aspects related to the process has been exposed, let's go deeper into the practicalities of the necessary equipment and processing units to carry out such a process in an industrial or semi-industrial scale.

2.2 Bioreactor types and operation

As exposed, different objectives can be pursued out of a fermentation process: biomass production, primary metabolites, secondary metabolites …Besides that, the nature of each particular culture is different: different kinetics, different optimal conditions… Hence, each process needs different operational conditions and equipment configuration.

The main types of bioreactors can be classified as stirred tanks, tubular and column reactors, although more complex configurations are also common, as airlift fermenters or fluidized bed bioreactors.

Another classification can be done attending to the different ways that they can be operated: batch operation, semi-continuous or fed-batch operation, continuous operation.

In batch operation, reactor is filled with medium, then the cell culture is inoculated and the production phase starts. When the desired concentrations are reached, the reactor must be emptied and cleaned. In this type of processes, biomass, substrate and reaction products concentrations continuously vary with time, therefore, the reaction conditions also change at each moment (see Figure 3).

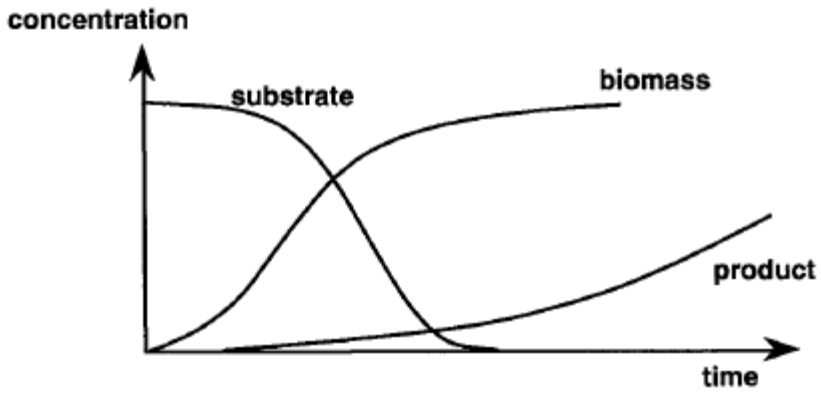


Figure 3. Biomass, product and substrate concentration variation with time for batch operation, extracted from [2].

The reactor is not productive during the filling, emptying and cleaning, but only during the production phase. Thus, a low productivity, linked to the higher final price of the product constitutes the main drawback of this kind of operation mode. Other important disadvantage is that the reproducibility of the quality and characteristics of each batch is difficult to achieve. Main advantages for bath production are a lower capital investment, a simpler control of the process and lower risk of contamination.

Semi continuous operation (also called fed batch operation) shares the first phases with batch operation: vessel is filled with medium and then inoculated with the culture. The difference comes with the addition of a substrate flow

during the production phase. This flow as well as the substrate concentration can be constant or be adjusted in order to follow different feeding strategies, such as slow constant feeding (to obtain a linear growth of biomass), exponential feeding (to maintain constant substrate concentration), or feeding connected to a feedback control loop (in order to monitor some important component for the process) [2].

Continuous operated reactors are fed with a flow rate equal to the one that is continuously leaving the unit. Therefore, under certain conditions, a steady state is reached, in which the production rate is constant and conditions and concentrations do not vary (ideally) for a certain position in the reactor. This operation mode has the highest productivity of the three, due to the fact that no profitable stages, as cleaning, filling or emptying, are needed. Also the quality control is better. However, the required equipment investment is higher as well as the complexity of the process.

With independency of the operating method, a certain number of parameters that affect the reaction progress need to be controlled. Temperature, pressure, stirring rate and pH have a high influence in the metabolic pathways of microbiological growth, as well as aeration in aerobic processes. Then, depending on the selected procedure, some other parameters must be additionally controlled. Table 1, extracted from [2], compiles the main variables to be controlled in bioreactors.

Table 1. Variables to control for the three operation methods, from [2].

Batch	Continuous	Semicontinuous
Initial medium composition and inoculum	Inlet medium composition	Feed and initial substrate composition
Temperature, pressure	Temperature, pressure	Temperature, pressure
pH if controlled	pH if controlled	pH if controlled
Reaction time	Liquid flow rate (residence time)	Liquid flow rate (residence time)
Aeration rate	Aeration rate	Feeding rate and control program
Stirring rate	Stirring rate	Aeration rate
		Stirring rate

Finally, Dunn et al. [2] also show a comparative analysis for the three different exposed bioreactor modes (see Table 2).

Table 2. Comparison between the different modes of operation, [2].

Mode of operation	Advantages	Disadvantages
Batch	Equipment simple. Suitable for small production.	Downtime for loading and cleaning. Reaction conditions change with time.
Continuous	Provides high production. Better product quality due to constant conditions. Good for kinetic studies.	Requires flow control. Culture may be unstable over long periods.
Fed batch	Control of environmental conditions, e.g. substrate concentration.	Requires feeding strategy to obtain desired concentrations.

2.3 Piping and Instrumentation Diagrams

Piping and Instrumentation Diagrams (P&ID) are schematic and standardized representations of the equipment, piping and instrumentation of a process [13]. They complement the information that other relevant diagrams include, as the Process Flow Diagram (PFD) and the Block Diagram, providing a more detailed overview of the process.

As stated by Toghraei [14], P&ID "is often considered to be the gold standard for the proper design, operation and maintenance of plants" in the chemical and biochemical process industries.

P&IDs are important in the design and installation of a plant because they collect in one place all the relevant information needed for that purposes. They are also a key tool in the operating phase: for starting the plant up, in controlling and maintenance tasks and during technical stops. Other areas for which these kind of diagrams are very important are qualification, optimization, HAZOP analysis and risk assessment studies [14]. Therefore, it is essential to dispose the updated P&IDs prior to the design or the enforcement of any operational procedure.

2.4 Sterilization

Fermentations, as previously presented, are carried out by microorganism cultures growing in a certain medium and under certain conditions (pressure, pH, temperature …). Most often in industrial biotechnology processes, a pure culture is used, the so called production organism. As already explained, this organism is inoculated and grows in the medium in the initial phases of the fermentation process.

However, some other microorganisms different from the production organism can get to interfere in the process if the correct measures are not taken. They can come from different sources:

1) The reaction medium, rich in nutrients, can harbor non desired microorganisms.

2) Equipment, where bioburden of previous fermentations can remain active or latent (spores) attached to the components surfaces. This is especially relevant when, as in this case, the equipment is used to run different processes with different cultures.
3) Ambience: air, people… Microbes and bacteria are present everywhere in the environment, and the surroundings of a laboratory are not an exception.

As explained by Stanbury et al. [1], severe consequences may be derived from the appearance of unwanted microorganisms in the process:

- Growth of both production and foreign organisms would occur, with the consequent increase in substrate consumption and loss in productivity.
- The growth of the foreign organism can be quicker than the one of the production organism, displacing the second in the process.
- The presence of the foreign microorganism in the final product may constitute a health or application issue.
- The undesired organisms can carry out parallel fermentations that would difficult the separation and purification of the final product.
- The desired product may be degraded by the foreign microorganisms.
- If the foreign microorganisms contain bacteriophages, this could destroy the desired culture.

Therefore, it is crucial for the success of the process to assure that only the desired microorganisms are growing in the medium. This can be achieved by first, assuring sterile conditions previous to the inoculums, that is to say, putting the medium and the equipment through a sterilization cycle; and secondly, keeping aseptic conditions along the fermentation process.

Medium and equipment can be sterilized separately or simultaneously, and several sterilization techniques can be applied. As one of the objectives of the project consists in the development of a sterilization procedure, a more deep discussion about these and other relevant aspects will be addressed in the following sections.

With sterilization, foreign organisms that were present in the medium and equipment are destroyed, and the only source of biological contamination left is

the ambience. In aerated processes, the feeding of air can be a critical point, since it can drag any kind of bioburden present in the environment of the installation. Hence, appropriate filters, able to retain particles of the size of a bacterium (usually 0.2 μm) need to be installed.

Typically, fermenters are sampled 5 times per day in an industrial process [4]. If the equipment is used for educational or research purposes, this frequency can be even higher. Taking these samples involves a risk of contamination, so an appropriate sampling system (typically using steam) must be also designed. These elements, together with keeping the installation always with a positive pressure and assuring that the vessel is correctly tightened, are the necessary measures to maintain aseptic conditions after the sterilization and throughout the process.

Once that biological contamination effects and main preventive lines of action have been exposed, let's dig a little bit deeper in the sterilization process itself.

2.4.1 Sterilization methods and techniques

Rubbo et. al [5] defined sterilization as "the process whereby microorganisms of all kinds are inactivated, killed, or removed from materials".

Microbiological organisms can be destroyed by the action of numerous agents, and this has led to the development, through the years, of different sterilization methods, including the usage of chemical reagents or physical pathways as dry-heat, steam, filtration, radiation or ultrasonic treatments [6] (see Figure 4). However, steam sterilization has a series of advantages that make this procedure the most extended one in big scale industrial fermentation processes for both medium and equipment sterilization: steam is non-toxic, readily obtainable and relatively easy to control [7].

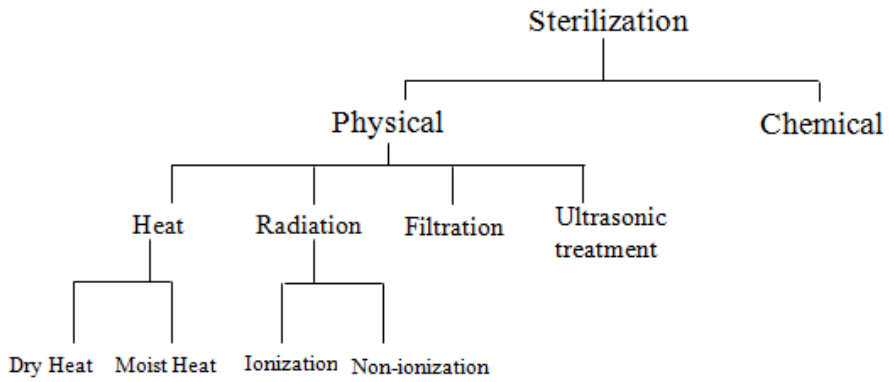


Figure 4. Types of sterilization methods, adapted from [8].

Destruction of microorganisms by moist heat generates a death rate proportional to the total number of organisms present in the to-be-sterilized system, as a first order chemical reaction. Typical steam sterilization cycles consist in maintaining contact between the steam and the contaminated elements at a certain temperature for the appropriate amount of time. Nevertheless, the death kinetics and the calculation of the relevant sterilization parameters will be further considered later on this chapter. Let's now see how steam can be applied for the sterilization to equipment and medium.

2.4.2 Equipment sterilization

There are two main ways to put in contact the steam and the different parts of the fermentation set up, depending on the size of the reactor and the different spare parts: autoclave sterilization and steam in place. These methods are described below.

For small-scale fermentations, the size of the equipment makes preferable the usage of the autoclave for sterilization, as it is the most effective, simplest and less time consuming technique to achieve a proper steam-surface contact within the elements to be sterilized. It is also less expensive to have dismountable small equipment and 1 autoclave for sterilization than have all small reactors made to be in-situ sterilizable.

The autoclave was invented by Charles Chamberland in 1879 [8]. It consists essentially of a large steel vessel through which the steam is circulated, a vacuum pump (optional) and a steam injection system and a drain collector. A scheme of an autoclave (without vacuum system) is pictured in Figure 5.

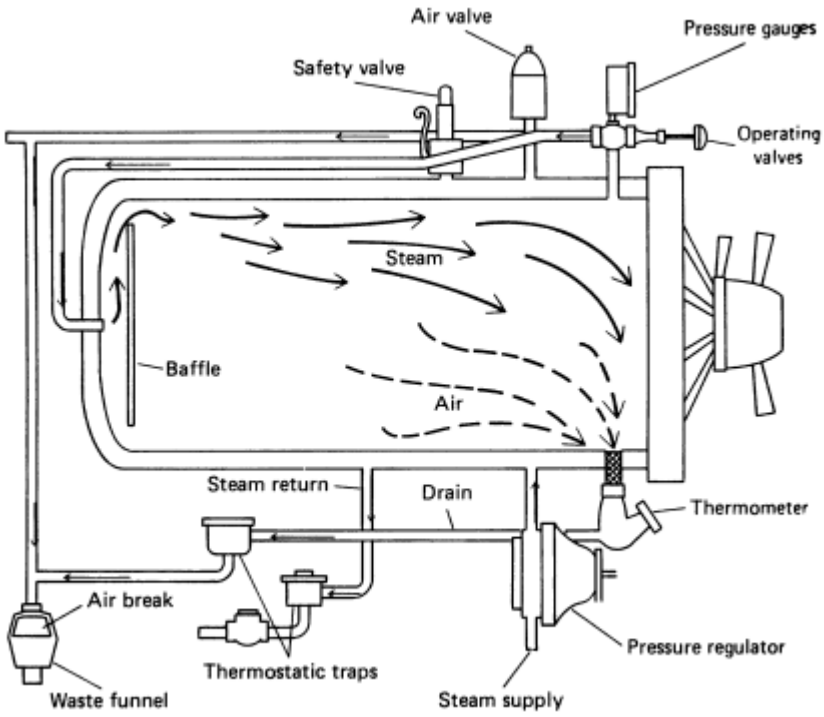


Figure 5. Autoclave scheme, extracted from [9].

The different elements to sterilize are introduced in the metallic vessel or chamber. Then, the chamber closes and the retained air is evacuated, normally using a vacuum pump (also removed by displacement with steam). Next, the chamber is pressurized again by filling it with steam till the desired temperature is achieved. This high temperature is hold for the needed amount of time, while the steam is continuously circulating through the autoclave chamber. Once the required time of sterilization has elapsed, the chamber opens and its content is cooled and dried.

However, as the size of the equipment and the production scale increases, it results completely unpractical to dismantle and autoclave all the processing elements that need to be sterilized. Thus, the preferred method for sterilizing non dismountable, big scale equipment is Steam-in-place

(SIP) ([4], [10]). This technique aims to reproduce the same sterilization process that occur in an autoclave but the processing equipment remains disposed as normally used during the fermentation. The saturated steam is injected into the equipment (directly in pipes, valves, filters, fittings… and either directly or indirectly in the fermentation vessel), contacting the surface of the different elements, heating them to the needed temperature for a determined amount of time. As the steam heats the installation, condensate is generated and, therefore, needs to be removed.

Therefore, when big scale fermentation equipments are designed, the SIP system must be designed in parallel, adding the required steam connections as well as valves, drains and steam traps in the right points of the system [11].

2.4.3 Medium sterilization

Destruction of the undesired living matter of the medium where the production culture is meant to grow can be done as a batch or a as a continuous process.

Batch sterilization consists in gathering the medium in a vessel, heating it up to a certain temperature (above the boiling point of the reaction liquid) and holding this temperature for a determined period. The temperature is achieved usually as the consequence of injecting hot steam in a jacket embracing the media recipient. This process can be carried out inside the proper process vessel, where the fermentation will take place later, or in a separate unit.

The advantages of using a separate cooker include saving time between fermentations, more efficient heating or less fermenter corrosion. On the contrary, additional equipment is needed and some problems in the transportation of the sterile medium to the fermentation equipment may occur.

Continuous sterilization systems are weather indirect or direct heat exchangers (the latter with steam injection), see Figure 6. The substrate mixture goes through one of these sterilization units previously to be fed into the process. A preheating and a cooling phase after the sterilization are usually required.

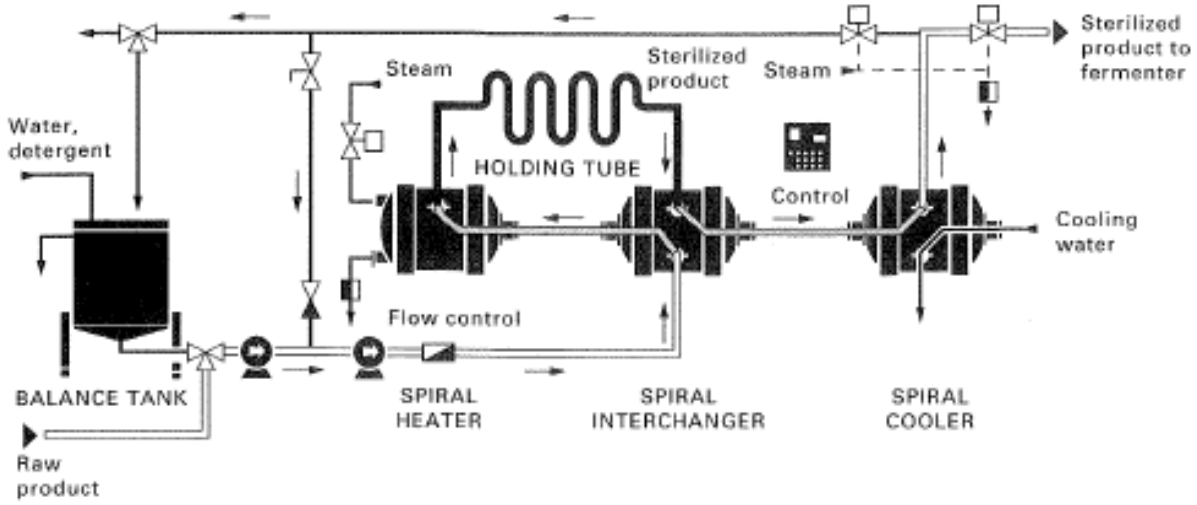


Figure 6. Flow diagram for a continuous sterilization system, extracted from [1].

The main advantage of this system is the lower nutrient degradation of the medium, but, by contrast, it requires a higher capital investment (additional equipment is needed) and due to the higher degree of complexity, the risk of contamination is also higher. It is usually applied when having continuous-fed fermentation processes.

2.4.4 Death kinetics for steam sterilization

The microorganisms' death rate by moist heat, i.e., saturated steam, can be described as shown in Eq. 1. By integration, it leads to Eq. 2.

This relation has been proved to describe the death mechanism with an appropriate degree of accuracy ([6], [11]).

$$\frac{-dN}{dt} = kN$$

Eq. 1

$$\frac{N_o}{N_t} = e^{-k\,t}$$

Eq. 2

Where N is the number of viable organisms present, t is the time of the sterilization treatment, N_o is the nº of viable org. present at t=0, N_t is the nº of viable org. present after a treatment period t and k is the death rate constant.

The value of k depends on the species as well as on the temperature, following an Arrhenius relation, as shown in Eq. 3.

$$\ln k = \ln A - E/RT$$

Eq. 3

Where A is the Arrhenius constant, E is the activation energy, R the gas constant and T is the absolute temperature.

From Eq. 2 and Eq. 3, one can calculate the exposure time to a certain temperature needed to reduce the bioburden to an acceptable number. However, to carry out these calculations, some values must be determined: k (and therefore A, E and T), No and Nt.

The span of different microorganisms that can be source of biological contamination of the process is huge, so usually an indicator organism is used in calculations and validations of sterilization procedures. The most common serilization indicator are the spores of *Bacillus stearothermophilus* (B. st.), the most resistant known organisms to moist heat effect. This way, by calculating the conditions under which this specie is destroyed, one can assure the destruction of all other potentially harmful microorganisms.

The characteristic parameters in Eq. 3 for *B. stearothermophilus* spores was determined in 1959 by Deindoerfer and Humphrey [12].

$$E \text{ (Activation energy)} = 67.7 \text{ kcal/mole}$$

$$A \text{ (Arrhenius constant)} = 1E36.2 \ s^{-1}$$

Regarding the temperature, as the Arrhenius relation shows, high temperatures have a positive effect in death rate. However, temperature is limited by three factors:

- As SIP needs saturated steam to be effective (see [7], [11]), high temperatures imply an increase in the working pressure during the sterilization. For safety and economic reasons, rather low pressures are preferred.
- Fermentations are normally carried out at ambience temperature. Thus, heating all the equipment to the desired sterilization temperature requires time. The higher sterilization temperature is, the more time that will be necessary, loosing productivity in the overall process.
- A more specific drawback appears regarding the sterilization of the fermentation medium and vessel. Here, together with the death of the microorganisms by moist heat, destruction of nutrients takes place, what results in a lower fermentation yield. Thus, the temperature shouldn't be unnecessarily increased, but kept in a reasonable number that still allows adequate sterilization.

Taking into consideration all of this points, 121 ºC appears to be the commonly agreed temperature in industrial SIP sterilization ([1]), since it leads to reasonable exposure times and does not compromise the feasibility of the posterior fermentation process.

Another important concept for the determining the conditions for the sterilization need to be subjected is the Sterility Assurance Level (SAL) [7]. SAL represents the probability of a single viable *B. stereotermophilus* spore surviving the sterilization process, thus, a SAL of 10^{-3} means that there is one chance in a thousand to have a viable spore of B.st. after the sterilization. The designer of the sterilization procedure has to decide with SAL is the most appropriate for the particular process: the higher the SAL, the less contamination probability, but the more time (and subsequent cost) needed to achieve it.

For the calculations here considered, the assumptions made by Dion and Parker ([7]) are here considered: a starting population of one million organisms and a Sterility Assurance Level of 10^{-4}.

With this data and substituting in Eq. 2 and Eq. 3:

$$\ln k = \ln 10^{36.2}\ \mathrm{s}^{-1} - \frac{67.7E3\ cal/mole}{1.987 \frac{cal}{mole \cdot K}\ (121 + 273)\ K} = -3.122$$

Eq. 4

$$k = 0.044\ s^{-1}$$

Eq. 5

$$t = \frac{\ln N_o - \ln N_t}{k} = \frac{\ln 10^6 - \ln 10^{-4}}{0.044\ s^{-1}} = 523.3\ s\ \approx 9\ min$$

Eq. 6

Other researches and estimations will lead to different results of this estimated time. However, an exposure time of 10-20 min is commonly agreed in the sterilization with 121ºC saturated steam ([6], [7], [4]).

Once than the kinetics theory of moist heat sterilization has been exposed, let's focus in the more practical aspects of this technique.

2.4.5 Operational considerations about SIP

Regarding the design of the sterilization procedure, Agalloco [11] as well as other authors ([7], [10]), highlight the importance of four key aspects that must be considered:

First of all, it must be assured that the steam fed for the SIP sterilization is saturated steam. Neither superheated vapor nor super cooled water, even at the same temperature, will be as effective as saturated steam [11]

Secondly, it is important to take into consideration that the air inside the equipment must be displaced correctly out of it in the initial phases of the sterilization cycle. If the air fails to be evacuated, this can lead to a bad steam-equipment contact and a slower and poorer heating.

As commented previously, when the steam contacts the metallic surfaces of the equipment and transfers heat to it, the vapor condensates. A big amount of condensate will be formed and therefore, will need to be drained out properly. Insufficient condensate removal would result in a decrease of the sterilization efficiency [11].

Finally, when the sterilization cycle has been completed, it is extremely important to assure the post-sterilization integrity, that is to say, “maintain sterile conditions in the vessel from the start of cool-down until the system is ready to use”[11]. As already commented in previous sections, this can be achieved by pressurizing the equipment to a pressure slightly higher than the atmospheric, and, of course, avoiding vacuum when steam flow is stopped inside the set up, which could also damage the mechanical properties of the equipment.

All of these aspects will be then considered and further discussed in the elaboration of the SIP operating procedure in the equipment under study.

2.5 Equipment qualification

Qualification can be defined as the "assessment of installation and operational characteristics and on parameters, which may influence the product quality" [15]. It is a requirement of Good Manufacturing Practices (GMP), and it pursues the avoidance of mistakes.

Qualification has to be done in a very carefully way, documenting all the checking process and it is normally divided in three stages, that have to be completed in thee shown order:

- Installation Qualification, which consists in checking that the available equipment is the adequate one, which its documentation coincides with the characteristics of the installed lay out…
- Operating Qualification, that includes tests such as pump function, calibration of instruments or emergency stops.
- Performance Qualification, where the whole plant is tested in some specific characteristics as production capacity or cleaning procedures.

As part of the Installation Qualification, usually a checkup of the Piping and Instrumentation Diagrams is done, documenting if the components that appear in the plans as well as their characteristics are correct. This part of the Installation Qualification has been applied for the review of the elaborated P&IDs.

2.6 Mass transfer characterization

In most of the fermentation processes, a gas-liquid mass transfer process takes place: most large-scale fermentation processes are aerobic (acetic acid, Single Cell Protein or enzyme production among others), and thus, require oxygen supply; CO_2 and methane are common gaseous products in fermentations, and its generation also implies gas-liquid interaction. Therefore, characterizing the mass-transfer performance of the studied fermentation layout is important for the assessment of the equipment.

In this project, the purpose of the mass transfer study is to determine if the equipment is suitable to satisfy the oxygen demand of certain microbiological processes of interest. In order to do so, two important points have to be studied: on the one hand, one must study how oxygen dissolves into the reaction medium; on the other hand, oxygen requirements of the organisms involved in the process must be also understood.

The process of oxygen solution is commonly agreed to follow the two film model proposed by Whitman in the early 20's [16]. Figure 7 illustrates the transfer steps that this theory involves, including:

1. Diffusion of solute gas from the bulk gas phase to gas film.
2. Diffusion of solute gas from gas film to the gas–liquid interface.
3. Transport across the gas–liquid interface.
4. Diffusion of solute gas through the liquid film to the bulk liquid.

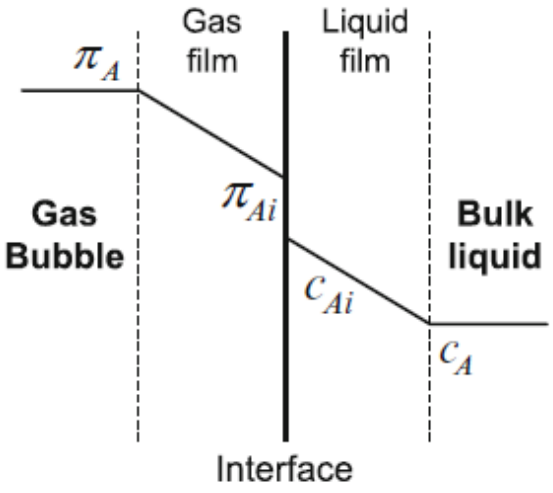


Figure 7. Concentration profiles of a gaseous compound A for the two film model for gas-liquid mass transfer process, extracted from [17].

Note that concentrations in the bulk of the gas phase and in the bulk of the liquid phase are assumed to be constant.

In each of the films, a driving force (partial pressure gradient for gas phase or concentration gradient for liquid phase) generates the mass flow from one side to

the other, and a mathematical expression for this flux (J_A, in mol/s/m^2) can be written as in Eq. 7 and Eq. 8, where k_g and k_l are mass transfer coefficients for the gas and the liquid side respectively.

$$J_{A,g} = k_g(\pi_A - \pi_{A,i})$$

Eq. 7

$$J_{\mathrm{A,l}} = k_{\mathrm{l}}(c_{A,i} - c_A)$$

Eq. 8

As explained by Villadsen et al. [17], since the interfacial concentrations are not directly measurable, it is more practical to calculate the overall flux of the considered gas solute from the gas phase to the bulk liquid as the product of an overall mass transfer coefficient and the driving force in the liquid phase (see Eq. 9).

$$J_{\mathrm{A}} = \mathrm{K_L}(c_A{}^* - c_A)$$

Eq. 9

Where $c_A{}^*$ is the saturation concentration in the bulk liquid that corresponds with the bulk gas partial pressure of the solute.

Equilibrium data (saturation concentrations) between gas and liquid phase for oxygen at different temperatures are collected in Table 3.

Table 3. Solubility of O_2 for various temperatures in pure water [17].

Temperature, ºC	O_2 solubility, mmoles/liter
0	2.18
10	1.70
15	1.54
20	1.38
25	1.26
30	1.16
40	1.03

According to this theory, therefore, there are two resistances to mass transport (one for each of the films). Since the diffusivity in the gas phase is high and the viscosity of gas is much lower than that of liquid (physical properties that affect mass diffusion and convection processes) the mass transfer resistance in the gas phase can be considered negligible. That is to say, the gas absorption process within a liquid is controlled by the liquid-phase side [18, 19].

Volumetric overall flow, q_A (mol/s/l-m^3) can be obtained from Eq. 9 multiplying by a (m^2 interfacial surface / m^3 of liquid), see Eq. 10.

$$q_A = J_A a = K_L a(c_A^* - c_A)$$

Eq. 10

Stanbury et al. point out in their book "Principles of Industrial Fermentation" [1] the difficulty of determining in an independent way K_L and a. However, there are several methods to determine the two terms combined, $K_L a$. This product is also known as volumetric mass-transfer coefficient, and its units are usually h^{-1} or s^{-1}.

Citing the authors [1], “the volumetric mass-transfer coefficient is used as a measure of the aeration capacity of a fermenter. The larger the $K_L a$, the higher the aeration capacity of the system.”

K_L is mainly dependent on the properties and hydrodynamics of the liquid. However, the interfacial area a is dependent on the amount of gas hold up and bubble sizes in the agitated vessel, which are determined by the flow field, power consumption and gas dispersing characteristic of the impeller, sparger, etc.

The Hydrogen Peroxide Method for $K_L a$ determination

Having in consideration the materials and sensors available in the laboratory and in the installation itself, it has been considered that the most appropriate method for measuring the reactor $K_L a$ is the Hydrogen Peroxide Method, a chemical, indirect way to determine the mass transfer coefficient.

The main idea consists in adding a constant rate of H_2O_2 in the interior of the reactor filled with water to a certain liquid volume (V_L), and in the presence of an enzyme named *catalase*, which decomposes the peroxide into water and oxygen as shown in Eq. 11.

$$2H_2O_2 \xrightarrow{catalase} 2H_2O + O_2$$

Eq. 11

The kinetic rate for this reaction is first order with respect to both H_2O_2 and the enzyme. The O_2 is formed within the liquid phase, and, since the dissolved oxygen raises concentrations above the saturation point, O_2 is transferred to the gas phase with a rate proportional to the value of $K_L a$, in the inverse pathway of the usual process.

If the addition of H_2O_2 is continuous, a steady state is reached where the oxygen generation rate (half of the fed peroxide rate, see Eq. 12) equals the amount of oxygen transferred to the gas phase (see Eq. 13). With known peroxide concentration and flow rate, $K_L a$ can be determined if the oxygen concentration

in the liquid is measured in the already mentioned steady state, c_s (see Eq. 14 and Eq. 15).

$$r_{O2,gen} = \frac{\dot{m}_{H2O2} \cdot c_{H2O2}}{2 \cdot V_L}$$

Eq. 12

$$r_{O2,tr} = K_L a \cdot (c_s - c_0)$$

Eq. 13

$$r_{O2,tr} = r_{O2,gen}$$

Eq. 14

$$K_L a = \frac{\dot{m}_{H2O2} \cdot c_{H2O2}}{2 \cdot V_L \cdot (c_s - c_0)}$$

Eq. 15

Mass transfer requirements in fermentation processes

Now that the $K_L a$ definition and measurement has been addressed, let's focus on the interpretation of its possible values.

A lot of data on *Saccharomices cerevisae* cultivation are available on literature ([20], [21]). Hence, this organism has been selected in this study to compare its oxygen requirements with the current aeration available in the studied fermenter.

From examples shown by Villadsen et al. [17] , some characteristic parameters for *Saccharomices c*. can be obtained. In one of the calculations they state determined that the oxygen demand for that cultivation with a dilution rate of 0.2 h^{-1} (with which the metabolism is purely respiratory), a substrate concentration of 28 g/L and an oxygen yield coefficient of 0.425 mol O_2/ C-mol glucose was 79.3 mmol O_2 /L/h. It is also stated that the oxygen concentration must be maintained above 4% of the saturation concentration. Optimal temperature at this conditions is 34.8 ºC [19].

From the equilibrium data for oxygen and water (see Table 3), c_{sat} can be calculated by interpolation, considering a partial pressure of 21% for oxygen in air:

$$c^* = c_{sat} = 0.21 \cdot 1.1 \frac{mmol\ O_2}{L} = 0.23\ mmol\ O_2\ /L$$

Eq. 16

Substituting in Eq. 10, the minimum value for the volumetric mass-transfer coefficient can be calculated (see Eq. 17).

$$K_L a = \frac{q_{o_2}}{{c_{o_2}}^* - c_{o_2}} = \frac{79.3 \frac{mmol\ O_2}{L\ h}}{(1 - 0.04) \cdot 0.23\ mmol\ O_2/L} = 360\ h^{-1}$$

Eq. 17

This value will be taken as a magnitude reference. Villadsen et al. [17] also state that common K_La values for pilot-scale and industrial fermenters are not higher than 500 h^{-1}.

Other K_La values obtained for a different culture can be seen in Table 4.

Table 4. Volumetric mass-transfer coefficient values for a 300 dm^3 fermenter containing a culture of S. aureofaciens, extracted from [1].

Method of K_La determination	Measured oxygen uptake rate (mmoles $dm^{-3}h^{-1}$)	K_La (h^{-1})
Static gassing out	—	58.2
Dynamic gassing out	6.6	58.2
Oxygen balance	20.1	108.0

3 Set up description and objectives

The fermenter that PILOT PLANT Research Center owns is pilot-scale fermenter with a capacity of 150L. A picture of the installation, developed by the German multi-national company BioEngineering, can be seen in Figure 8.

Figure 8. Pilot-scale 150L fermenter set up in PILOT PLANT laboratory.

The set up consists in a stirred jacketed tank. The tank is connected to a gas feeding system provided with gas in-house filters and connections and flowmeters for air, oxygen, CO_2 and N_2. The tank also has connections for 4 different metering pump inlets that can be working at the same time. The original purpose for these 4 inlets is the feeding of acid, base, antifoam and substrate mixtures. It also has gas filters for the gas exhaust pipeline.

The jacket of the vessel is connected to a heat exchanger in which steam and cooling water can be fed in order to control the operating temperature. The fermenter has been designed for *in situ* sterilization, what can be deduced by drain valves and steam piping disposed all over the equipment.

The equipment is also provided with several sensors, a control panel (including all the available separate control modules for the different variables) and software for the controlling and data logging tasks (BioScada). All of these elements are distributed provided by BioEngineering as well.

Regarding the aeration and mixing characteristics of the reactor, it is featured with a propeller-type stirrer (see Figure 10), four baffles disposed axially equidistant, and a sparger, under the already mentioned propeller. A front scheme is pictured in Figure 9.

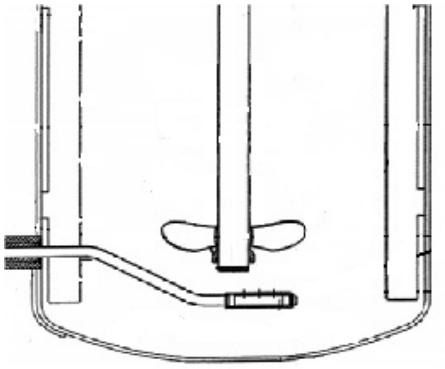


Figure 9. Front sheme of the interior of bioreactor's vessel.

This equipment was previously utilized by a Danish company for mammalian cell cultivation tests, and now it is meant to serve as an educational and research tool within the Chemical and Biochemical Engineering Department of DTU.

In order to be able to run a biological process in it, some tasks needed to be addressed first, and those tasks constituted the main objectives of this project. While preparing the equipment for its utilization, the student has been able to learn and apply important engineering skills for the commissioning of a big-scale reaction lay out.

Some work in the assessment and commissioning of the reactor has already been done by other student [22]. In this work, the focus was in checking the sensors, controlling modules and developing calibration procedures. However, additional aspects needed still to be covered.

First of all, the completion of the piping and instrumentation diagrams needed to be done. As already explained, these diagrams are the heart of the documentation of every processing set up, and are basic for controlling tasks or the development of Standard Operating Procedures (SOPs).

Some P&ID representations were already drafted prior to starting the present project. They were elaborated for the above mentioned previous work in the installation [22]. However, some connections for certain valves and pipes were unknown (they were not identifiable to the naked eye). Also some identification numbers for valves and other fittings were missing or needed checking. Thus, a plan for updating and upgrading the diagrams was set. It is further explained in CHAPTER 2:, subsection 2.

It has also been exposed earlier in this chapter that a validated sterilization procedure is crucial prior to running a biochemical process in any kind of equipment. Hence, the elaboration of a Sterilization Standard Operational Procedure is one of the key objectives of the project.

The validation of the SOP, once redacted, was also outlined as an objective under the scope of this project. As part of this task, a sealing test was programmed.

Finally, the assessment of the aeration-agitation capacity of the reactor and the experimental determination of mass-transfer coefficients is also covered.

CHAPTER 2:
Materials and Methods

1 Piping and Instrumentation Diagrams

The following method was used to elaborate the needed diagrams for the fermenter: as already explained, some connections were not easy to understand to the naked eye, so in order to avoid the disassembling of all the unknown connections and valves to elaborate the piping diagrams, pressurized air has been used.

The air was fed into the pipes, either through the steam inlet (see connection point 1B in P&ID nº 2,3,4,5 and 6 in Appendix I) or the exhaust (through valves V-323 and V-324, P&ID nº 4). The pressure of the air was maintained between 1 and 2 bar-g approximately.

When the air was fed into the installation, the different valves were closed and opened and the air path was followed by identifying through which points it was coming out of the equipment.

The drain pipe (see connection point 2A in P&ID nº 2 and 6, Appendix I) was submerged in a water recipient, in order to make easier the detection of air coming out through this point. Other methods used to detect air releases in other points were, for instance, auditory recognition or pipetting soap to see if air bubbles were formed.

The Piping and Instrumentation Diagrams have been developed using Microsoft Office Visio Professional 2013; a software tool specialized in diagrams. More specifically, mostly the “Process Engineering” shapes menu has been used. Table 5 and 0 compile a list compiling the different symbols used.

Table 5. Symbols and their description used for the fermenter Piping and Instrumentation Diagrams, part I.

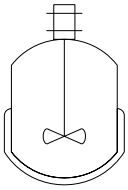
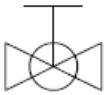
Symbol	Description
	Process pipe
	Auxiliary pipe (steam, condensate, instrumentation)
	Electrical signal
	Jacketed, stirred vessel
	Manual membrane valve*
	Check valve
	Gas filter
	Pressure reducing valve
	Powered automatic valve (solenoid actuator)
	Pump

Table 6. Symbols and their description used for the fermenter Piping and Instrumentation Diagrams, part II.

	Manual globe valve
	Ball manual valve
	Y strain
	Instrumentation bubbles (on field)
	Steam trap
	Heat exchanger

* This symbol, normally used as a generic representation of manual valves, has been used throughout the report representing a membrane valve

2 Sterilization Standard Operational Procedure

The Standard Operating Procedure (SOP) for sterilizing the pilot-scale fermenter (described in section 1, CHAPTER 3:) was elaborated based on the following documents:

- Piping and Instrumentation Diagrams of the fermenter (Appendix I).

- Standard Operating Procedures already developed for the installation in a previous project ([22]).

- IFM module manual, "Operating instructions: IFM Temperature control LP and P". BioEngineering ([23]).

All the cited literature about sterilization in section2, CHAPTER 2: was also considered while the elaboration of the SOP.

3 Sealing test

In order to test the watertightness and the absence of leakages in the installation, a 24h sealing test was tried with pressurized air. The outlined procedure was:

1. Tightening the top of the vessel

2. Closing valves V-320 (P&ID nº 4, Appendix I), V-213 (P&ID nº5, Appendix I),V-211, PRV-3.3, V-313, V-212, V-312, V-520, V-556, V557 (P&ID nº 2, Appendix I)

3. Opening valves V-214, PRV-3.1, V-311, PRV-3.4, V-314, V-316, V-317, V-315, V-310 and V-301 (P&ID nº 4, Appendix I).

4. Start feeding pressurized air (at 2 bar-g) into the equipment through V-214 (see P&ID nº4, Appendix I).

5. Fill the reactor with the pressurized air until 1.5 bar-g are reached.

6. Close V-316 and V-317 (P&ID nº 4, Appendix I) and stop the feeding of pressurized air.

7. Let the equipment pressurized for 24h, keeping track of the pressure (barometer PI-13, P&ID nº 1, Appendix I).

4 Equipment qualification

A test sheet for each piping and instrumentation diagram was elaborated. Sheets include the symbol of each present element, a short description and the identification tag of the element, as well as space for stating constancy if the correct element, its position and its tag correspond to the ones that one can find in the actual lay out. Also some space for writing down pertinent observations of the elements was added.

Then, with these test sheets and the P&IDs, all the items were checked in the installation in the correlative order.

The test sheets already filled after the qualification of the representations can be found in Appendix III.

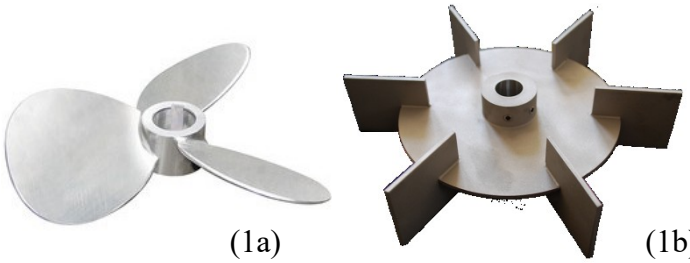
5 Mass transfer characterization

5.1 K_La determination: PVC vessel

The first approach to K_La measurement was done by using a PVC vessel with approximately $1m^3$ of capacity, as training for the student. This installation serves as a practical learning tool for Chemical Engineers students in DTU practical learning about agitation and aeration in stirred tanks. This tank is equipped with different agitation appliances and with the necessary devices to measure K_La under different conditions. A manual elaborated by the professors in charge of the practical courses presents a detailed description of the installation and the procedure (based on the Hydrogen Peroxide method – see 2.6 in CHAPTER 1: -) needed for the K_La determination, it can be seen in ref. [24].

Six experiments were carried out in this vessel using two different impellers (Propeller and Rushton turbine, see Figure 10) accommodated in the stirring

shaft and with different agitation speeds with each one (15%, 20% and 25% of the motor).



(1a) (1b)

Figure 10. Image of different agitation impellers used:
(1a) Propeller; (1b) Rushton turbine.

5.2 K_La determination: fermenter

Once the 'training' experiments were done, the method was applied by the student to the fermenter under study with the available equipment, tools and devices.

The list of the used materials comprises the following items:

- Level measurement: level control system provided by Bioengineering AG consisting in 2 sterilizable pressure sensors (Bio-Weight probes), IFM measurement and control unit and a connecting cable. The display shows the measured level in liters with a precision of one decimal digit (e.g. 80.0 L).

- Dissolved Oxygen measurement: Clark polarographic sensor, IFM measurement and control unit and a connecting cable, all of them provided by Bioengineering AG. The display shows the result as a percentage, normally referring the saturation dissolved oxygen concentration, with a decimal digit (e.g. 76.5%).

- Temperature measurement: Bioengineering AG sterilizable Pt-100 probe, IFM measurement and control unit and connecting cables. The result is displayed at the module in Celsius degrees (e.g. 22.2ºC).

- Air flow measurement: Bioengineering AG mass flow controller, IFM measurement and control unit and connecting cable. The display shows the result in Nl/min with one decimal digit (e.g. 48.4 Nl/min).
- Metering pump: Millipore peristaltic pump XX 80 002 30 working at 435 rpm
- Chronometer (hundredths of second precision).

The list of the used checmicals includes:

- Hydrogen peroxide solution, approximately 4%-5% in weight basis (the exact concentration is determined by titration for every prepared solution).
- *Catazyme®,* high activity catalase commertial preparation provided by Novozymes.
- $KMnO_4$ solution, 0.1M.
- H_2SO_4 solution, 20% w/w.

Experimental procedure:

In first place, the level piezo-resistive pressure transmitters of the fermenter were calibrated. Calibration was carried out from the IFM display, using the function menu 'CALIB MEAS'. The total capacity of the fermenter is 150L, thus, the two references used for calibration were 10% and 60% of the total volume, i.e., 15L and 90L respectively.

These volumes were measured by weighting buckets of 5 kg of water and assuming a density of 1 kg/L. The weighting was done with a high capacity balance. Then, the fermenter was filled up to 80.0L with tap water.

Previously to the experiments, the Dissolved Oxygen Probe (DO-probe) was also calibrated. The calibration was a 'Single point calibration' carried out from the IFM display, using the function menu 'CALIB MEAS'. The point chosen as reference was the DO in saturated conditions for the room temperature. These

conditions were achieved by feeding an air flow of 48NL/min through the sparger, agitating the vessel content with a high stirring speed (400 rpm).

Normally, 100% would be assigned to the DO value for saturation point: in a typical biochemical process no higher DO values would be reached and the DO electronic module does not show values above 120 %.

However, as the utilization of the hydrogen peroxide method requires measuring DO concentrations above the saturation point. Then, in order to be able to collect the DO data in the experiments, the saturation point was set to 10%. Later, these results were corrected assuming linear behavior of the DO-probes (a calculation example can be seen in CHAPTER 3:, section 5).

The metering pump was used to feed the hydrogen peroxide inside the fermenter. It was calibrated measuring the volume of tap water displaced (using a graduated cylinder) in a minute for various positions. The desired flow rate was achieved when the metering pump was set to 3.5 points in the graded scale.

For each set of experiments (4 as maximum) 10mL of the catalase preparation were added to the vessel.

Five different stirrer speeds were selected as study cases. Each experiment was initiated by feeding air through the vessel; when a stable initial DO value was reached, the addition of the hydrogen peroxide was initiated and the data for DO, temperature and air flow were collected minute by minute until the steady state was reached. Then, the addition of H_2O_2 was stopped and the oxygen concentration was dropped to its original value.

Finally, the H_2O_2 solution used in each of the experiments was titrated to determine the exact concentration. In order to do so, 1.5g of solution was accurately weighted (m_{sol}), and then diluted with demineralized water up to 20 mL and 20 mL of 20% H_2SO_4 solution was added. The solution was titrated with the $KMnO_4$ solution until a color change to red. The reaction taking place is presented in Eq. 18.

$$2\ MnO_4^- + 5\ H_2O_2 + \ 6H^+ \rightarrow 2Mn^{++} + 5\ O_2 + \ 8\ H_2O$$

Eq. 18

The volume of $KMnO_4$ (V_{MnO4-}) utilized in the titration was used to calculate H_2O_2 as shown in Eq. 19. Each titration was repeated 3 times.

$$c_{H_2O_2}(\%\, w/w) = \frac{\frac{5\ mol_{H_2O_2}}{2\ mol_{MnO_4^-}} \cdot\ c_{MnO_4^-} \cdot V_{MnO_4^-} \cdot MW_{H_2O_2}}{m_{sol}} \cdot 100$$

Eq. 19

CHAPTER 3:
Results and Discussion

1 Piping and Instrumentation Diagram

The P&IDs corresponding to the fermenter under study are pictured in Appendix I. A general P&ID has been plotted, and then, 6 additional detail diagrams have been elaborated, in order to present all the relevant data in a clear way.

- Overview, P&ID nº 1
- Inlet filters, P&ID nº 2
- Metering pumps, P&ID nº 3
- Exhaust filters, P&ID nº 4
- Product outlet, P&ID nº 5
- Cooling system, P&ID nº 6
- Steam supply, P&ID nº 7

The pilot-scale fomenter has been already designed, manufactured and installed. Therefore, the purpose of these diagrams was to serve as a tool for the operation, controlling and understanding of the fermentation equipment. Having this in mind, it was considered appropriate to omit certain mechanical or design information, as length, schedule, or the material for the pipes and fittings or data concerning the structure or the foundations of the plant. This way, the plans can remain simpler and clearer, but still containing the most useful information.

The information that was considered relevant to include in these diagrams was the following:

- Equipment: tags and connections.
- Valves and fittings: type, connections and tag numbers.
- Pipes: type (process, steam, condensate) and tags where necessary.
- Instrumentation: type, location.
- Reagents and process flow: inlets, outlets and drains.

After elaborating the P&IDs some parts of the lay out whose function was previously unclear were then understood:

1) The exhaust section (see Appendix I, P&ID nº4) consists of two identical filters. They can be isolated from each other, so the exhaust gas can be released through either FO3 or FO4. This allows changing or cleaning a filter when it is fooled without stopping the fermentation process.

2) In the metering pumps section (see Appendix I, P&ID nº3) the purpose of having two valves in the same part of the pipe (for example, V110 and V111) is related to the sterilization cycle.

   When the vessel is being sterilized, V110 remains open, while V111 is closed, draining the air and then the condensate through V-521. On the contrary, when the rest of the metering pumps section pipes are being sterilized, V110 remains closed (in order to avoid the unclean steam to enter the vessel), and V111, open, to allow condensate draining.

It needs also be commented that during the elaboration of the P&IDs, it was found that some valves and equipment units were tagged whereas others did not. Therefore, some suggestions on tag numbers were proposed.

Tag numbers on steam supply are clearly differentiated by having 4 digits (starting in 1000) instead of 3. The rest of the suggested tags were given tryng to match the already existing ones.

2 Sterilization operation procedure

The Standard Operating Procedure (SOP) for sterilizing the pilot-scale fermenter was elaborated. The followed sterilization method was Steam in Place.

The Sterilization SOP can be read in Appendix II.

Here we discuss some aspects that were taken in consideration when elaborating this procedure:

- Batch sterilization of the medium within the fermentation vessel was selected as the optimum method for the studied set up. Continuous and separated sterilization of the medium were discarded in order to avoid the utilization of additional equipment units and the problems of transferring of sterile media.

- Steam pressure
  As justified above (see 1.2), the optimal temperature for steam sterilization is 121ºC. This corresponds, in saturated conditions, with a pressure of 1.03 bar-g (using Eq. 20).

$$P\ (bar-g) = \frac{\exp\left(A - \frac{B}{T+C}\right)}{750\ mmHg/bar} - Patm$$

Eq. 20

Where A, B, C (parameters for Antoine's equation) for water are 18.3036, 3816.44, -46.13 respectively [25], and T refers to absolute temperature in Kelvin.

However, barometers, both the one installed in the equipment and the one in the steam supply have a 0.1 bar-g. precision. Considering this fact, together with the pressure drop that will take place in the equipment, it has been decided to set the pressure to 1.1 bar-g, With this pressure, the saturated steam is expected to reach 127ºC, what will speed up the pre-heating process. This steam will be used for both, direct injection in the equipment and into the vessel jacket.

- Sterilization time

  Eq. 6 shows that holding 121ºC for a period of 9 min is enough to assure a SAL of 10^{-4} (see 2.4.4,CHAPTER 1:) if the assumptions made are correct. Therefore, a 10 min period (rounded figure) is established as needed time for the sterilization of the equipment (valves, pipes, filters, etc). However, regarding the joint sterilization of the reaction vessel and medium, a period of 20 min is specified, being this a recommendation from the manufacturer.

- Sterilization sequence

  Throughout the SOP, the layout has been considered as an integration of 5 different sections:

  - Inlet filters
  - Exhaust filters
  - Metering pumps
  - Product outlet
  - Vessel

  As it can be seen in the document, the four first enumerated sections are sterilized in first place and then the vessel. This is the order recommended by Chisti [4] and, by having sterilized the inlet filters previous to the vessel, it assures that the latter can be kept under positive pressure after its sterilization with clean air flowing from the inlet to the exhaust in a continuous way until the fermentation process starts.

  Hence, as it results essential to sterilize the air inlet section prior to the vessel, and exhaust filters, metering pump and product outlet sections can be sterilized simultaneously, the proposed order optimizes the required time for completing the sterilization under the selected conditions.

- Vessel Sterilization

  As justified above the medium and the vessel were decided to be sterilized simultaneously, by indirect heating: steam will be injected into the jacket surrounding the vessel until 121ºC are reached in the medium, and then the temperature will be hold for the required 20 minutes. This procedure will be

controlled by the temperature controller in the sterilization. See Appendix II for a more detailed explanation.

- Valve opening and closing order

  During the elaboration of the SOP it has been evaluated the adequate order of opening the valves. In order to let the air exit the pipes and units before the steam enters the installation, it has been decided to open always in first place the drain valves, and then the steam feeding ones.

  If this is not done this way, air could accumulate inside the equipment, leading to the subcooling of the steam and having a less efficient sterilization.

  Also in order to avoid vacuum in the installation when the sterilization is finished and the steam feeding is stopped, the valves have to be closed in the opposite direction of the flow, this way the pipes keep pressurized.

  Considerations regarding air removal and vacuum avoidance when the vessel is sterilized have also been taken into account: the pre-programmed sterilization procedure (based on the temperature controller) requires the operator to open the gas exhaust valve – see SOP for specific tags – in the warming up phase (for air removal), and once the sterilization ends and the medium condensation temperature, pressurization is required.

- Assuring aseptic conditions after the sterilization

  As soon as the sterilization is done, the vessel and the rest of the equipment has to be filled with pressurize air, keeping a slightly higher pressure over the atmospheric one, in order to prevent the air in the ambiance to enter the already sterilized installation.

3 Sealing test

For this test, if the pressure would have been maintained for the 24h, the installation would have been proved no to have any leakage, and therefore, the inlet of hot steam inside the equipment would not have supposed any risk.

However, the pressure of 1.5 bar-g was not possible to achieve inside the equipment. That was due to the fact that the rupture disk of the vessel was found to be broken, as shown in Figure 11.

Figure 11. Dismantled rupture disk.

A new rupture disk was ordered, replaced and tested, maintaining the vessel pressurized with 1.5 bar-g during 24h. However, after this succesful trial and due to time constrictions, the sterilization procedure could not be validated.

4 Equipment qualification

The developed test sheets provided an important tool for validating the elaborated Piping and Instrumentation Diagrams. Position and type of all the elements plotted were checked to be right. The existing tag numbers were also confirmed, whereas the elements lacking such a tag were identified (observations field). See the result from this P&ID validation in Appendix III.

5 Mass transfer characterization

The results of the experiments in the installation of the PVC vessel, done as a first contact with the procedure of K_La determination, are shown in Figure 12.

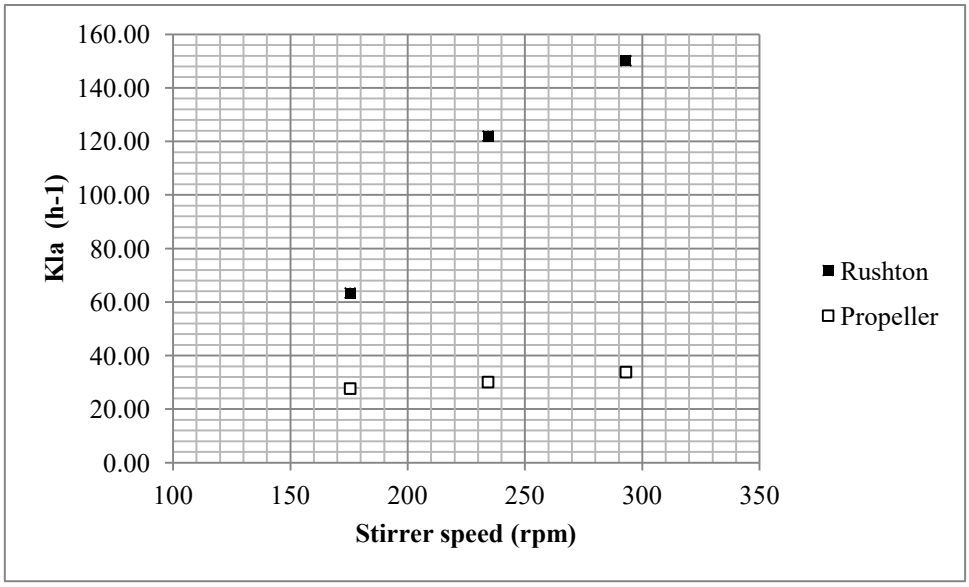


Figure 12. K_La values measured for a stirred tank with different impeller types and at various stirrer speeds.

From these results one can easily deduct that the aeration capacity is higher for the Rushton turbine than for the propeller. As expected, increasing stirrer speed has a positive effect for gas-liquid mass transfer [26]. It is also noticeable from the results that the design of the Rushton turbine has a better perfonce regarding mass-transfer efficiency. The uncertainties for these values were not calculated, as they were just carried out as a tool to understand the methodology.

After applying the methodology in the fermenter object of this project, the obtained results are pictured in Figure 13. A compilation of the obtained experimental curves can be seen in Appendix IV.

The experiments were carried out K_La in the fermenter were measured for different stirrer speeds, reproducing the conditions in the experiments in the PVC vessel (same Q_{air}/ V_L ratio = 0.6 vvm, same m_{H2O2}/V_L ratio).

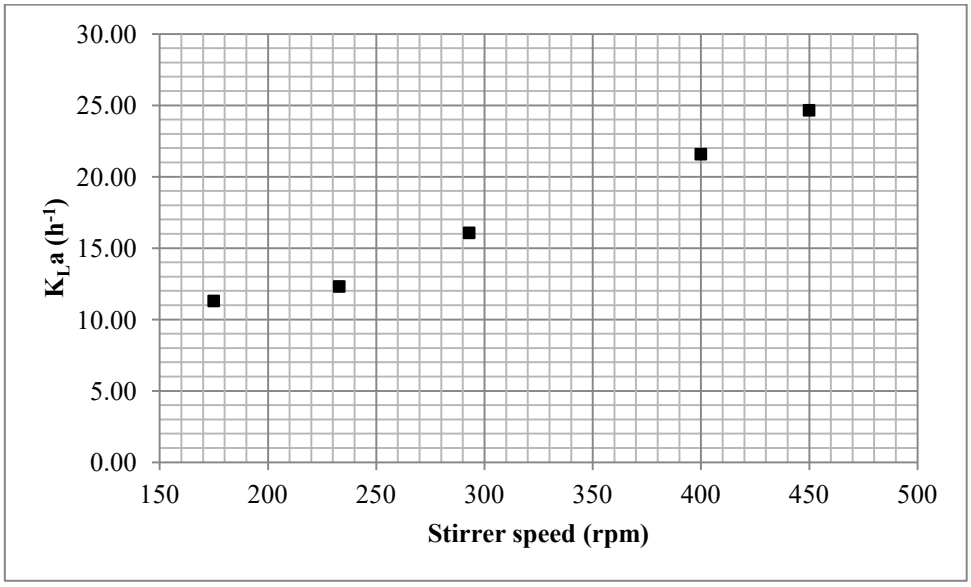


Figure 13. K_La values measured in the fermenter at various stirrer speeds.

In the following section 5.1, the calculation of K_La from experimental data is illustrated. Then, in 5.2, it is explained how the uncertainty for these results has been obtained. Finally, in section 0, all the obtained results are assessed and discussed.

5.1 Fermenter K_La determination from experimental data

In order to explain how the presented values have been obtained from the experimental set up, here are presented the obtained curves for one of the cases (293 rpm).

For this experiment, the reactor volume was 82.3L. The experimental curve for dissolved oxygen is shown in Figure 14, whereas the measurements of T and Q_{air} are pictured in Figure 15.

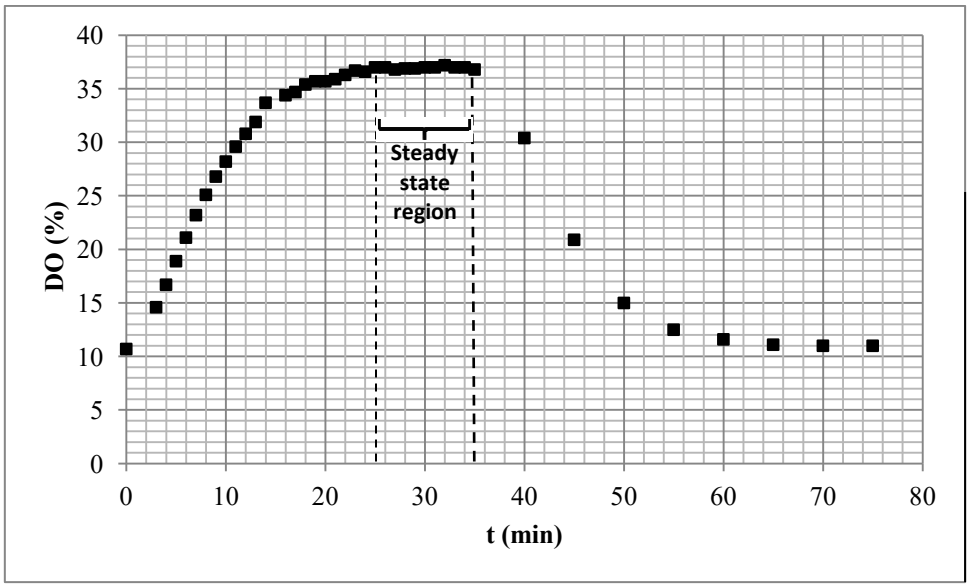


Figure 14. Dissolved oxygen percentage versus time with the stirrer at 293 rpm.

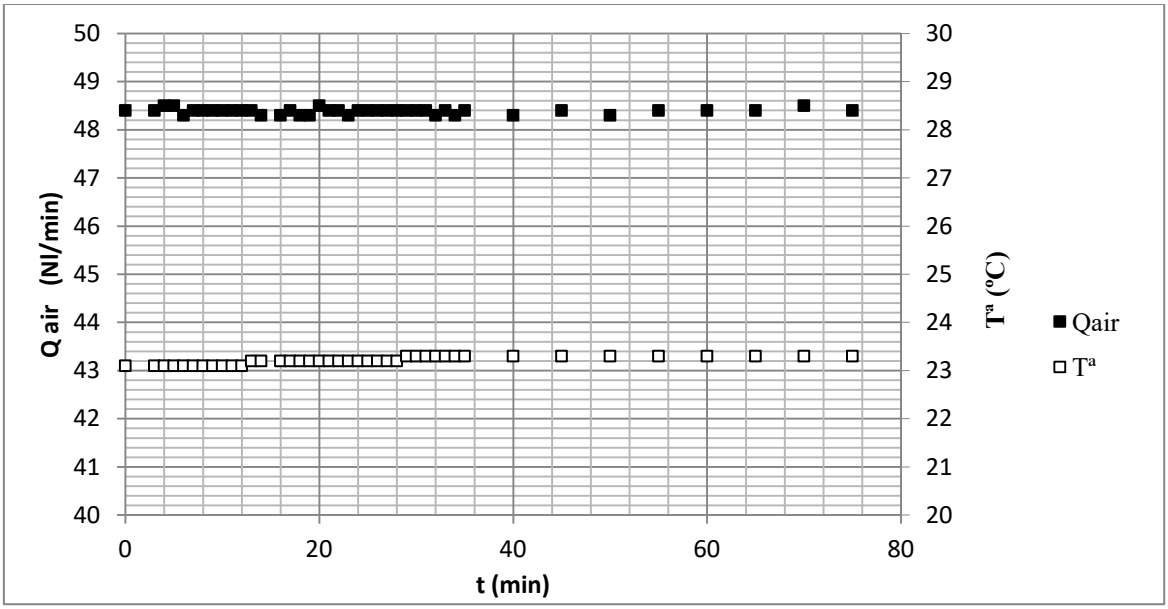


Figure 15. Temperature and air flow measured versus time during Kla determination experiment with the agitator stirring at 293 rpm.

Other known data were:

- $\dot{m}_{H2O2}$, feeding mass flow of the hydrogen peroxide solution, 0.027kg/min (constant for all the experiments).
- c_{H2O2}, concentration of the hydrogen peroxide solution, 4% w/w (this concentration is determined by titration as explained above – see Materials and Methods -).
- MW_{H2O2}, hydrogen peroxide molecular weight, 34.016 g/mole ([27]).

From the experimental curve given by the DO-probe (see Figure 14), two different levels of dissolved oxygen are determined: DO_s, representing the percentage of oxygen saturation achieved in the steady state with addition of hydrogen peroxide; and DO_0, representing the initial percentage of oxygen saturation, ideally equal to 10% (for these experimental data, 10% corresponds to saturated conditions, see 5.2), however, due to the uncertainty in the DO-probe used for the measurements, values for DO_0 slightly different from 10% are taken as acceptable.

In this case, as it can be seen from Figure 14, the values read in the DO-probe for this experiment for DO_0 and DO_s are 10.7% and 37% respectively. The value of DO_0 is calculated by calculating the average value within the steady state region, indicated in Figure 14 for this case.

As already mentioned, these values (DO_0 and DO_s) refer to a scale where 10% represents saturation conditions and therefore, these values must be converted to a 100% basis (DO'_0 and DO'_s, respectively). For this conversion, it is assumed that the polarographic DO-probes have a linear response in the region under study (as justified in [28]). Hence, the conversion is also linear (see Eq. 21 and Eq. 22)

$$DO'_0 = DO_0 \cdot \frac{100\%}{10\%} = 107.0\%$$

Eq. 21

$$DO'_s = DO_s \cdot \frac{100\%}{10\%} = 369.6\%$$

Eq. 22

Then, the actual difference in dissolved oxygen concentrations, $(c_s - c_0)$ in Eq. 15 can be written as follows (Eq. 23).

$$(c_s - c_0) = (\mathrm{DO'_s} \cdot \mathrm{c_{sat}} - \mathrm{DO'_0} \cdot \mathrm{c_{sat}}) = \mathrm{c_{sat}} \cdot (\mathrm{DO'_s} - \mathrm{DO'_0})$$

Eq. 23

Having this in consideration, the previous expression for K_La, Eq. 15 can be determined by using the expression in Eq. 24.

$$K_L a = \frac{\dot{m}_{H2O2} \cdot c_{H2O2}}{2V_L \cdot (c_s - c_0) \cdot MW_{H2O2}} = \frac{\dot{m}_{H2O2} \cdot c_{H2O2}}{2V_L \cdot \mathrm{c_{sat}} \cdot (\mathrm{DO'_s} - \mathrm{DO'_0}) \cdot MW_{H2O2}}$$

Eq. 24

With the results shown in Figure 15, an average temperature ($\bar{T}$) and air flow (Q_{air}) were calculated, and they were assumed to be constant at this value during the whole experiment. In this case, $\bar{T}$ = 23.2 ºC, Q_{air} = 48.4 Nl/min.

Having the average temperature, the solubility of pure oxygen in water at the calculated average temperature was determined by interpolation using the data in Table 3. This data are a list of solubilities for pure oxygen at 1 atm, extracted from [17]. In this case, interpolating for T = 23.2 ºC a solubility of 1.30 mmol O_2/Lwas obtained.

Since what it is fed is no pure oxygen but air, the actual oxygen saturation concentration (c_{sat}) is the 20.8% (volume percentage of oxygen in air within average temperature and humidity levels [29]) of the previous value (see Eq. 25).

$$c_{sat} = 0.208 \cdot 1.30 \frac{mmol\ O_2}{L} \cdot 10^{-3} \frac{mol}{mmol} = 2.71 \cdot 10^{-4}\ mol\ O_2 / L$$

Eq. 25

Substituting in Eq. 24. the previously discussed values for each of the variables, K_La value is obtained (Eq. 26).

$$K_L a = \frac{0.027\,\frac{kg\ sol}{min} \cdot \frac{1000\ g}{1\ kg} \cdot \frac{60\ \text{min}}{1\ \text{h}}\, 0.0397\,\frac{g\ H_2O_2}{g\ sol}}{2\,\frac{mol\ H_2O_2}{mol\ O_2}\ \ 82.3L \cdot 2.71 \cdot 10^{-4}\,\frac{mol\ O2}{L}\,\left(\frac{369.6-107}{100}\right) 34.016\,\frac{g\ H_2O_2}{mol\ H_2O_2}}$$

$$= \ 16.06\ h^{-1}$$

Eq. 26

5.2 Uncertainty of the experimental results

Every result obtained by experimentation has, inevitably, an associated uncertainty. The uncertainty derived from this experimental procedure is here analyzed and discussed. In order to calculate it, the equation of error propagation was applied to Eq. 24, resulting in Eq. 27.

$$U_{Kla} = \left[\left(\frac{\delta K_L a}{\delta m_{H2O2}}\right)^2 {U_{m_{H2O_2}}}^2 + \left(\frac{\delta K_L a}{\delta c_{H2O2}}\right)^2 {U_{c_{H2O_2}}}^2 + \left(\frac{\delta K_L a}{\delta V_L}\right)^2 {U_{V_L}}^2 + \left(\frac{\delta K_L a}{\delta c_{sat}}\right)^2 {U_{c_{sat}}}^2 + \left(\frac{\delta K_L a}{\delta DO'_s}\right)^2 {U_{DO'_S}}^2 + \left(\frac{\delta K_L a}{\delta DO'_0}\right)^2 {U_{DO'_0}}^2\right]^{1/2}$$

Eq. 27

It was considered that the value of the molecular weight for the peroxide used in the calculation was accurate enough to discard its uncertainty as non-significant, and therefore, the term for this variable was not consider in Eq. 27.

Each of expressions for the partial derivatives is shown, from Eq. 28 to Eq. 33.

$$\frac{\delta K_L a}{\delta m_{H2O2}} = \frac{c_{H2O2}}{2V_L \cdot \mathrm{c_{sat}} \cdot (\mathrm{DO'_s} - \mathrm{DO'_0}) \cdot MW_{H2O2}}$$

Eq. 28

$$\frac{\delta K_L a}{\delta c_{H2O2}} = \frac{\dot{m}_{H2O2}}{2V_L \cdot \mathrm{c_{sat}} \cdot (\mathrm{DO'_s} - \mathrm{DO'_0}) \cdot MW_{H2O2}}$$

Eq. 29

$$\frac{\delta K_L a}{\delta V_L} = -\frac{\dot{m}_{H2O2} \cdot c_{H2O2}}{4{V_L}^2 \cdot \mathrm{c_{sat}} \cdot (\mathrm{DO'_s} - \mathrm{DO'_0}) \cdot MW_{H2O2}}$$

Eq. 30

$$\frac{\delta K_L a}{\delta c_{sat}} = -\frac{\dot{m}_{H2O2} \cdot c_{H2O2}}{4V_L \cdot \mathrm{c_{sat}}^2 \cdot (\mathrm{DO'_s} - \mathrm{DO'_0}) \cdot MW_{H2O2}}$$

Eq. 31

$$\frac{\delta K_L a}{\delta DO'_s} = -\frac{\dot{m}_{H2O2} \cdot c_{H2O2}}{4V_L \cdot \mathrm{c_{sat}} \cdot \mathrm{DO'_s}^2 \cdot MW_{H2O2}}$$

Eq. 32

$$\frac{\delta K_L a}{\delta DO'_0} = \frac{\dot{m}_{H2O2} \cdot c_{H2O2}}{4V_L \cdot \mathrm{c_{sat}} \cdot \mathrm{DO'_0}^2 \cdot MW_{H2O2}}$$

Eq. 33

The following comprises a discussion for how each absolute uncertainty for the relevant variables was calculated or estimated.

Hydrogen peroxide mass flow, $\dot{m}_{H2O2}$

The mass flow, provided by a displacement metering pump operating at a certain speed, was determined by collecting the displaced volume in 1 minute in a test tube, repeating this measure 3 times (Table 7).

Table 7. Values for experimental determination of peroxide oxygen mass flow rate.

V_1	26.5 mL
V_2	27 mL
V_3	27 mL
$\bar{V}$	26.83 mL
SD	0.3 mL

With this data, then the mass flow rate is calculated:

$$\dot{m}_{H2O2} = \frac{V}{t} \cdot \rho = 1614\ g/h$$

Eq. 34

From this formula, applying the principle of error propagation, one can obtain the uncertainty related to the mass flow. The uncertainty for the volume has been estimated as the standard deviation of the three values taken (U_V = 0.3mL), whereas the uncertainties for time and density have been considered not relevant.

$$U_{m_{H2O2}} = \frac{\delta m_{H2O2}}{\delta V} U_V = \frac{\rho}{t} \cdot U_v = 17\ g/h$$

Eq. 35

Hydrogen peroxide solution concentration, c_{H2O2}

As already presented (see CHAPTER 2:, 5.2) the concentration of the peroxide solution was determined through titration. The used volume of permanganate is collected in Table 8 for the three measurements.

Table 8. Values for experimental determination of the peroxide oxygen solution concentration.

$V_{MnO4,\ 1}$	7 mL
$V_{MnO4,\ 1}$	7.1 mL
$V_{MnO4,\ 1}$	6.9 mL
SD	0.1 mL

$$c_{H_2O_2} = 0.03967 \frac{g\ H_2O_2}{g\ sol.}$$

Eq. 36

From these results and applying the error propagation formula to Eq. 19, the uncertainty for this variable can be calculated (Eq. 37). The resultant expressions for the derivatives can be seen from Eq. 38 to Eq. 40.

$$U_{c_{H2O2}} = \left[\left(\frac{\delta c_{H2O2}}{\delta V_{Mno4}}\right)^2 \cdot {U_{V_{MnO_4}}}^2 + \left(\frac{\delta c_{H2O2}}{\delta c_{Mno4}}\right)^2 \cdot {U_{c_{MnO_4}}}^2 + \left(\frac{\delta c_{H2O2}}{\delta m_{sol}}\right)^2 \cdot {U_{sol}}^2\right]^{1/2}$$

Eq. 37

$$\frac{\delta c_{H2O2}}{\delta V_{Mno4}} = \frac{5}{2} \cdot \frac{c_{Mno4} \cdot MW_{H2O2}}{m_{sol}}$$

Eq. 38

$$\frac{\delta c_{H2O2}}{\delta c_{Mno4}} = \frac{5}{2} \cdot \frac{V_{Mno4} \cdot MW_{H2O2}}{m_{sol}}$$

Eq. 39

$$\frac{\delta c_{H2O2}}{\delta m_{sol}} = -\frac{5}{4} \cdot \frac{c_{Mno4} \cdot V_{Mno4} \cdot MW_{H2O2}}{{m_{sol}}^2}$$

Eq. 40

Absolute uncertainty of the titration volume,$U_{V_{MnO_4}}$, is again estimated as the standard deviation of the three measured volumes, 0.1 mL. Uncertainty for the titration solution of permanganate, $U_{c_{MnO_4}}$, is known and its value is 1E-3 M. The uncertainty for the weight of the solution sample $U_{m_{dis}}$ is estimated as 0.05 g (half of the last digit of the balance used for weighting). Again, the uncertainty for the molecular weight is despised.

Substituting these numbers and the ones in Table 9, the overall uncertainty for the concentration of the peroxide solution is obtained (Eq. 41).

Table 9. Some values needed for calculation of $U_{c_{H2O2}}$.

c_{Mno4}	0.1 M
V_{Mno4}	$7 \cdot 10^{-3} L$
m_{sol}	1.5 g
MW_{H2O2}	34.016 g/mole

$$U_{c_{H2O2}} = 9.6 \cdot 10^{-4} \frac{gH_2O_2}{g\ sol.}$$

Eq. 41

Liquid volume in the bioreactor, V_L

Volume is measured by differential pressure probes (PT 01 and PT02 in P&ID nº1, Appendix I). The uncertainty for this measurement, U_V, was estimated in previous works as 0.4L.

Saturation concentration of oxygen in water, c_{sat}

C_{sat} was calculated as expressed in Eq. 42.

$$c_{sat} = 0.208 \cdot {c_{sat}}^*$$

Eq. 42

20.8% refers to the partial pressure of oxygen in humid, average temperature air. This value is precise enough to consider its uncertainty irrelevant. This percentage is then multiplied by the pure oxygen solubility in water. This value was determined by interpolation using data from [17], collected in Table 3. Villadsen et al. do not specify the uncertainty for these values, therefore it is estimated as 0.005 mmol O_2/L (half of the last significant figure of the data). In linear interpolations between two close values, it can be assumed that the uncertainty in the extrems is the same as in each intermiediate value. Therefore, $U_{c^*_{sat}} = 0.005\ mmol\ O_2/L$. Hence, through the error propagation principle $U_{c_{sat}}$was calculated as shown in Eq. 43.

$$U_{c_{sat}} = 0.208 * U_{c^*_{sat}} = 1.04 \cdot 10^{-6} M$$

Eq. 43

Dissolved oxygen percentages, DO'_0 and DO'_s

It has already been said that the DO-probes installed in the fermenter give a measurement of the dissolved oxygen in the liquid phase as a percentage related to the saturation concentration. For the determination of the K_La, these probes were calibrated in a way that a lecture of 10% equals saturation conditions for air at 1atm.

The uncertainty of these lectures has two contributions:

- The uncertainty of the device itself, estimated as the last digit given in the measurement, that is to say, 0.1%.
- The uncertainty associated to the variability of the measured values.

It can be seen in the section above (5.1) that the value for DO_s in each experiment was calculated as an average value of the several measurements taken in the stationary state. Thus, each of these values has an associated standard deviation, calculated in Table 10.

Table 10. Standard deviation of the DO_s measurements.

Case	450 rpm	400 rpm	293 rpm	234 rpm	176 rpm
SD (%)	0.073	0.15	0.11	0.43	2.0

A Fisher test was carried out to compare the variances of each experiment. The result of this test revealed that each group of data does not have comparable variances, thus, the group standard deviation was not used, but individual deviations were used instead for the uncertainty calculations instead.

Both sources of uncertainty were, hence, considered for estimation of U_{DO_s}. It was also assumed that $U_{DO_0} = U_{DO_s}$, see Table 11.

Table 11. Estimated uncertainty of the DO measurements

Case	450 rpm	400 rpm	293 rpm	234 rpm	176 rpm
U_{DO_0}, U_{DO_s}	0.17%	0.25%	0.21%	0.53%	2.1%

Then, DO_0 and DO_s have to be converted to a 100% basis, DO'_0 and DO'_s multiplying by 10 (see Eq. 21and Eq. 22), and so do their relative uncertainties

(applying one more time the principle of error propagation), see .Eq. 44, Eq. 45 and Table 12.

$$U_{DO'_0} = 10 \cdot U_{DO_0}$$

Eq. 44

$$U_{DO'_s} = 10 \cdot U_{DO_0}$$

Eq. 45

Table 12. Estimated uncertainty of the DO' data.

Case	450 rpm	400 rpm	293 rpm	234 rpm	176 rpm
$U_{DO'_s}$, $U_{DO'_s}$	1.7%	2.5%	2.1%	5.3%	21%

With all the estimated uncertainties, the measured and calculated values, and having Eq. 27, K_La final uncertainties are calculated (Table 13).

Table 13. Absolute uncertainty for K_La results.

Stirrer speed	450 rpm	400 rpm	293 rpm	233 rpm	175 rpm
U_{Kla} (h-1)	3.32	4.95	3.94	11.29	38.17

5.3 Discussion on mass-transfer results

After having gone through the values and uncertainties calculations, the final values are presented in Table 14 and pictured in

Table 14. Determined K_La values and their absolute and relative uncertainties

Stirrer speed	175 rpm	233 rpm	293 rpm	400 rpm	450 rpm
K_La (h^{-1})	11	12	16	22	25
U_{kla} (h^{-1})	38	11	4.0	5.0	3.3
ε_{kla}	338%	92%	25%	23%	13%

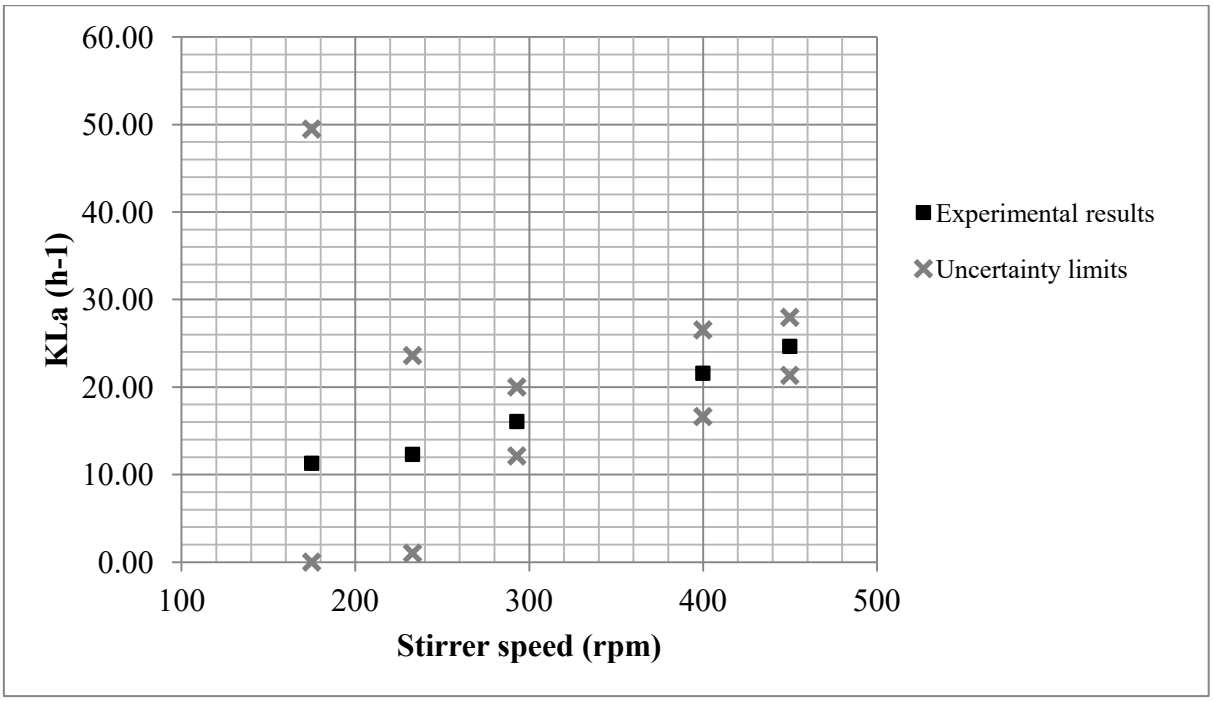


Figure 16. Experimental results and uncertainty limits for the volumetric mass-transfer coefficient in the fermenter.

Uncertainties are extremely high for low stirring speeds (175, 233 rpm) and are drastically reduced for medium-high mixing velocities (above 293 rpm).

The contributions of each variable to the final uncertainty (see Eq. 27) have been disaggregated in Table 15.

Table 15. Individual contribution to Ukla of the different variables

Stirring speed	450 rpm	400 rpm	293 rpm	233 rpm	175 rpm
m_{H2O2} (%)	0.64	0.22	0.19	0.01	0.00
c_{H2O2} (%)	3.20	1.10	0.97	0.07	0.01
V_L (%)	0.03	0.01	0.01	0.00	0.00
c_{sat} (%)	0.02	0.01	0.01	0.00	0.00
$DO'_0 + DO'_s$ (%)	96.11	98.66	98.82	99.92	99.99
Total (%)	100.0	100.0	100.0	100.0	100.0

The main source of error in the measurements, with great difference, is the uncertainty in DO measurements. Therefore, probes would give more accurate results in conditions n which the content of the reactor is homogeneous (high stirring speeds) than when the mixture is not efficient (low stirrer speeds).

In order to reduce this important source of uncertainty in the measurements, the following suggestions can be applied.

1. Extending the time that the stationary state is hold. This way, more lectures of the dissolved oxygen level could be recorded and this will lower the associated standard deviation.

2. Proceed to condition check of the DO-sensors, following the instructions in DO-control manual [30]. (p. 13), and carry out the appropriate maintenance tasks there specified.

Nevertheless, it has to be pointed that due to operational issues the probes were calibrated at 10%. Therefore, even when the minimum uncertainty for these probes can be as low as 0.1%, it will become instantly 10 times higher, due to the needed correction. In the light of this fact, working on a new way to record DO data in 100% basis would be highly recommended.

Keeping the uncertainty problem aside for a moment, another important issue must be addressed in order to evaluate the obtained results properly. In 1996, V. Linek et al. [31] stated that applying the hydrogen peroxide method to determine mass transfer coefficients can lead to overestimated values, due to a phenomena

known as “supersaturation effect”. This effect consists in the fact that when the liquid in the tank is under high supersaturation conditions, the importance of oxygen bubble nucleation increases. The generation of bubbles due to supersaturated conditions increases de K_La measured value.

They as well as other authors ([32], [33]) consider a supersaturation of 200% the maximum acceptable limit to avoid the appearance of the discussed effect. Moreover, a research group focused in mass-transfer within the Chemical and Biochemical department of DTU pointed as 150% the limit of oxygen saturation to obtain reliable results.

As the reader can check (Appendix IV) the experiments that were carried out for the fermenter K_La determination lie out of this upper limit (20% for DO_s experimental data), and therefore, suppose an overestimation of the real mass-transfer capacity of the equipment.

In the light of this fact, new experiments were designed and carried out in order to obtain measurements within the reliability limits. As the objective was to lower the driving force $(c_s - c_0)$- see Eq. 15- and the real Kla is a characteristic value of the fermenter, the new experiments were designed with a higher liquid volume (V_L) and a lower hydrogen peroxide concentration (c_{sat}). Finally, some trials were done with higher air flow-rate (Q_{air}) in order to operate at better mass-transfer conditions. To sum up, the new tested conditions were:

- V_L = 95L, c_{H2O2} =1% w/w, 0.6 vvm (Q_{air} = 52NL/h), 200 rpm
- V_L = 95L, c_{H2O2} =1% w/w, 0.6 vvm (Q_{air} = 52NL/h), 350 rpm
- V_L = 95L, c_{H2O2} =1% w/w, 1 vvm (Q_{air} = 88NL/h), 200 rpm
- V_L = 95L, c_{H2O2} =1% w/w, 1 vvm (Q_{air} = 88NL/h), 300 rpm

None of these conditions led to obtain DO’s lower than 150% either, and due to time constrictions, no more trials could be tested. However, in the light of the results, it can be concluded that in order to obtain more consistent results for the mass transfer coefficient in this fermenter:

- The hydrogen peroxide method might be applicable for studying mass-transfer performance for high stirring speeds (400-500 rpm), with a high liquid volume (95L), low hydrogen peroxide concentration (1% w/w) and 1.0 vvm.

- When the conditions are more unfavorable for the mass-transfer processes, the mass transfer efficiency of the fermenter is so low that the hydrogen peroxide method cannot give reliable results, and other methods have to be considered.

Besides the fact that no results within the reliability limits were achieved, some conclusions can be extracted from this study: K_La results are very likely to be overestimated, and still all the obtained values are much lower than the one calculated for the Saccharomices cerevisae culture (360 h^{-1}), the reference value given by Villadsen et al. [17] (500 h^{-1}), and the values collected in Table 4.

Therefore, it can be concluded that aeration capacity of the fermenter object of study is low, and, depending on the specific process, is very likely to be insufficient. A possible enhancement to address this problem could be to substitute the propeller with a Rushton turbine (see Figure 10), as it has been proved to provide a higher mass-transfer rate.

CHAPTER 4:
Conclusions

1) P&IDs of the bioreactor set up were elaborated, using pressurized air as an aid in determining connections between certain valves and fittings. By developing the P&IDs, the possibility of alternate the use of exhaust filters (FO3 and FO4, see P&ID nº4 , Appendix I) for a continuous operation, or the duplication of valves in the metering pumps section (F.i., V110 and V111, P&ID nº3, Appendix I). Some tag numbers needed for the operating procedures were suggested. (See Appendix I).

2) A Standard Operating Procedure for batch sterilization of the fermentation equipment and medium by following a Steam In Place method was designed (see Appendix II). First, a sterilization of air inlet filters, exhaust filters, metering pump section and product outlet parts was proposed, followed by a in-vessel sterilization of the fermentation medium.

   Critical parameters for each part of the SOP (sterilization temperature, pressure and holding time) were calculated and/or justified.The main 4 factors in SIP were analyzed and considered, including vapor saturation, air evacuation, condensate removal and post-sterilization integrity.

   A thermocouple, type J, has been proposed to be installed in the drain collector (before connection **2A** in P&ID nº 3 and/or 6) in order to control temperature during sterilization.

   This procedure is still in need to be validated.

3) Sealing test revealed an inoperable burst disk. It was then replaced and tested successfully.

4) Test sheets for equipment qualification were elaborated, permitting the audition of the previously developed diagrams (see Appendix III).

5) The gas-liquid mass transfer efficiency of the fermenter was evaluated, resulting to be low. Experimental data, obtained through the hydrogen peroxide method is compiled in Appendix IV. The selected experimental conditions and set up led to supersaturation effect and high uncertainties, which made experimental results unreliable. New experimental conditions to avoid supersaturation effect were tried, without any success. Hence, it was concluded that the hydrogen peroxide method was not the most adequate for this specific set up. In spite of the reliability of the results, magnitude comparison with K_La values from other authors, led to the conclusion that aeration capacity of the fermenter under study is low. Changing the impeller type for a Rushton turbine would help to increase the mass-transfer rate, even though it is not clear in which extent and further analysis should be done regarding this point.

References

1. Stanbury, P.F., A. Whitaker, and S.J. Hall, *Principles of fermentation technology*2013: Elsevier.

2. Dunn, I.J., et al., *Biological reaction engineering*1995: VCH, Weinheim New York Basel Cambridge.

3. Hogg, S., *Essential microbiology*2013: John Wiley & Sons.

4. Chisti, Y., *Assure bioreactor sterility.* Chemical engineering progress, 1992. **88**: p. 80-80.

5. Rubbo, S.D. and J.F. Gardner, *A Review of Sterilization and Disinfection as applied to Medical, Industrial and Laboratory Practice.* A review of sterilization and disinfection as applied to medical, industrial and laboratory practice., 1965.

6. Augurt, T.A. and J. van Asten, *Sterilization techniques.* Kirk-Othmer Encyclopedia of Chemical Technology, 2000.

7. Dion, M. and W. Parker, *Steam Sterilization Principles.* Pharmaceutical Engineering, 2013. **33**: p. 60-69.

8. Oyawale, F. and A. Olaoye, *Design and Construction of an Autoclave.* Pac J Sci Technol, 2007. **8**: p. 224-230.

9. Judelson, H., *Operation of the autoclaves.* 2004.

10. Cappia, J.-M., *Principles of steam-in-place.* Pharmaceutical Technology, 2004. **28** (10; SUPP): p. s40-s46.

11. James P. Agalloco, F.J.C., *Steam Sterilization-in-Place Technology and Validation*, in *Validation of Pharmaceutical Processes*2007, CRC Press. p. 760.

12. Deindoerfer, F. and A. Humphrey, *Analytical method for calculating heat sterilization times.* Applied microbiology, 1959. **7**(4): p. 256.

13. Turton, R., et al., *Analysis, synthesis and design of chemical processes*2008: Pearson Education.

14. Toghraei, M., *Principles of P&ID Development.* Chemical Engineering, 2014. **121**(4): p. 62.

15. Kioerboe, L., *GMP and quality in pharmaceutical, biotech and food industry. Lecture notes. Course 28855.* Departmen of Chemical and Biochemical Engineering, DTU, 2016.

16. Whitman, W.G., *The two film theory of gas absorption.* International Journal of Heat and Mass Transfer, 1962. **5**(5): p. 429-433.

17. Villadsen, J., J. Nielsen, and G. Lidén, *Bioreaction engineering principles.* NY: Springer, 2011.

18. Carpani, R. and J. Roxburgh, *Studies on fermentation aeration: I. The oxygen transfer coefficient.* The Canadian Journal of Chemical Engineering, 1958. **36**(2): p. 73-77.

19. Finn, R., *Agitation-aeration in the laboratory and in industry.* Bacteriological reviews, 1954. **18**(4): p. 254.

20. von Meyenburg, K., *Katabolit-Repression und der Sprossungszyklus von Saccharomyces cerevisiae*1969: ETH.

21. Bijkerk, A.H.E. and R.J. Hall, *A mechanistic model of the aerobic growth ofSaccharomyces cerevisiae.* Biotechnology and Bioengineering, 1977. **19**(2): p. 267-296.

22. Rasmussen, J., *Qualification of a Bioreactor*, 2016: DTU, PILOT PLANT Research Center.

23. *Operating instructions: IFM Temperature control LP and P* in *IFM manual*, BioEngineering.

24. *Aeration, Exercise 1D.* Chemical and Biochemical unit operations, 2016(Department of Chemical and Biochemical Engineering, Technical University of Denmark).

25. Sinnott, R.K., *Chemical engineering design: SI Edition*2009: Elsevier.

26. Banerjee, U.C., *Effect of stirrer speed, aeration rate and cell mass concentration on volumetric oxygen transfer coefficients (KLa) in the cultivation of Curvularia lunata in a batch reactor.* Biotechnology Techniques, 1993. **7**(10): p. 733-738.

27. Constantine, M. and E. Cain, *Hydrogen peroxide handbook*, 1967, DTIC Document.

28. Kanwisher, J., *Polarographic oxygen electrode.* Woods Hole Oceanographic Institution, 1959.

29. *Portable Gas Detection. MSA Gas Detectors Display 20.8% Standard Oxygen Reading.* Technical Bulletin, 2003. **ID 0800-84-MC**.

30. *Operating instructions: IFM DO-control*, in *IFM manual* BioEngineering.

31. Linek, V., P. Havelka, and J. Sinkule, *Supersaturation effect in steady-state and dynamic methods for measuring kLa in gas-liquid dispersions.* Chemical Engineering Science, 1996. **51**(23): p. 5223-5226.

32. Moucha, T., V. Linek, and E. Prokopová, *Gas hold-up, mixing time and gas–liquid volumetric mass transfer coefficient of various multiple-impeller configurations: Rushton turbine, pitched blade and techmix impeller and their combinations.* Chemical Engineering Science, 2003. **58**(9): p. 1839-1846.

33. Vasconcelos, J., et al., *Alternative ways of applying the hydrogen peroxide steady state method of kLa measurement.* Chemical Engineering Research and Design, 1997. **75**(5): p. 467-472.

List of symbols

a	Specific interfacial surface (m^2interfacial durface/ liquid-m^{-3})
A	Arrhenius constant (s^{-1})
c	Concentration (mole/L or mol/m^3)
c_{H2O2}	Concentration of the hydrogen peroxide solution
c_{H2O2}	Concentration of the hydrogen peroxide solution
DO	Dissolved oxygen, measured in 10% basis
DO'	Dissolved oxygen, in 100% basis
E	Activation energy (kcal/mole)
$J_{A,l}$	Molar superficial flow of gas A in the liquid phase ($mol/s/m^2$)
$J_{A,g}$	Molar superficial flow of gas A in the gas phase ($mol/s/m^2$)
J_A	Molar superficial overall flow of gas A ($mol/s/m^2$)
k	Death rate constant (s^{-1})
k_l	Mass transfer coefficient in the liquid phase ($m \cdot s^{-1}$)
k_g	Mass transfer coefficient in the gas phase ($mol \cdot s^{-1} \cdot atm^{-1} \cdot m^{-2}$)
K_La	Volumetric gas-liquid mass transfer coefficient referred to the liquid phase (h^{-1}, s^{-1})
MW	Molar weight
m_{sol}	Wight of hydrogen peroxide solution sample (g)
$\dot{m}_{H2O2}$	Hydrogen peroxide mass flow (given by peristaltic pump)
N	Number of viable organisms
P	Pressure
q_A	Volumetric overall flow of gas A ($mol/s/liquid\text{-}m^3$)
Qair	Air flow
R	Gas constant (1.987 cal/mole/K)
r	Molar rate (mole/L/s)

t Time (s)

T Temperature

U Absolute uncertainty

V_L Liquid volume (L)

V_{MnO4-} Used volume of titration solution in the determination of hydrogen peroxide solution concentration

π Partial pressure (atm)

ε Relative uncertainty

Superscripts

* Saturation conditions

‘ Referred to 100% basis

Subscripts

0 Initial value

t Value at a time ‘t’

s Steady state value

l Liquid phase

g Gas phase

A referred to gas solute A

i Gas-liquid interface

sat Saturation conditions

tr Referred to mass-transfer process

gen Generated

H2O2 Referred to hydrogen peroxide solution

MnO4- Referred to permanganate solution (titration solution)

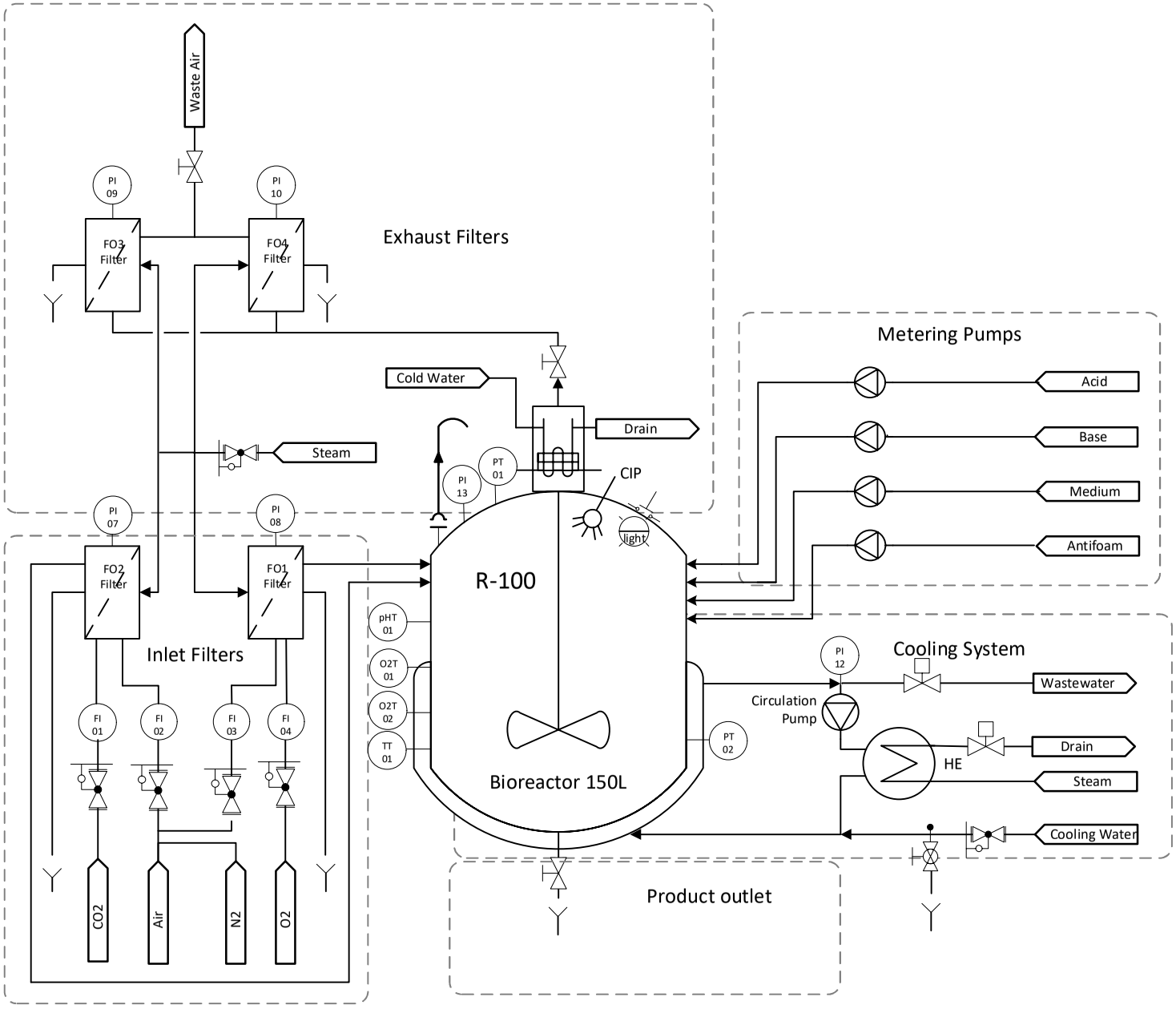
Acronyms

SAL	Safety Level Assurance
SIP	Sterilization In Place
rpm	Revolutions per minute
vvm	Vessel volumes per minute

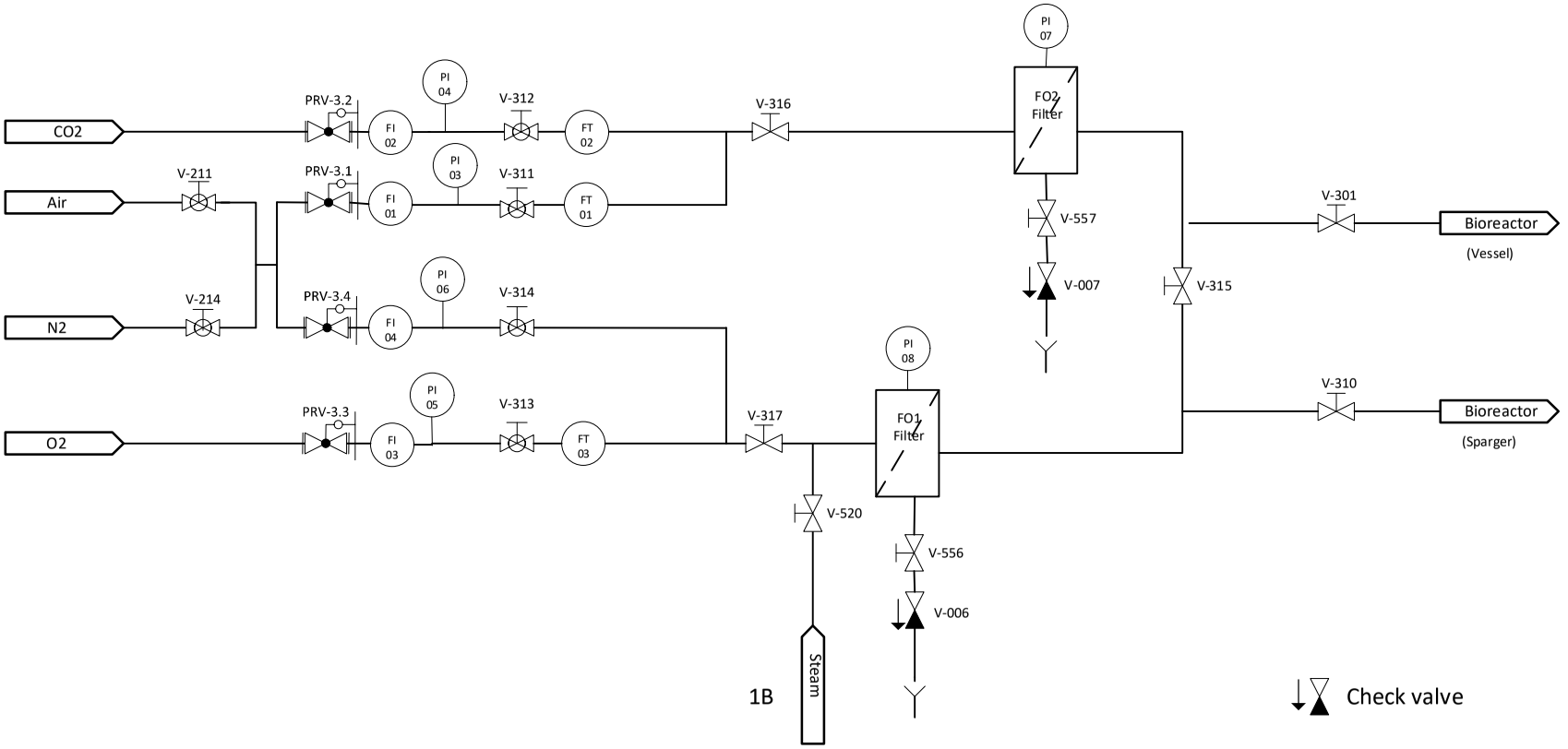
Appendix I

Piping and Instrumentation Diagrams

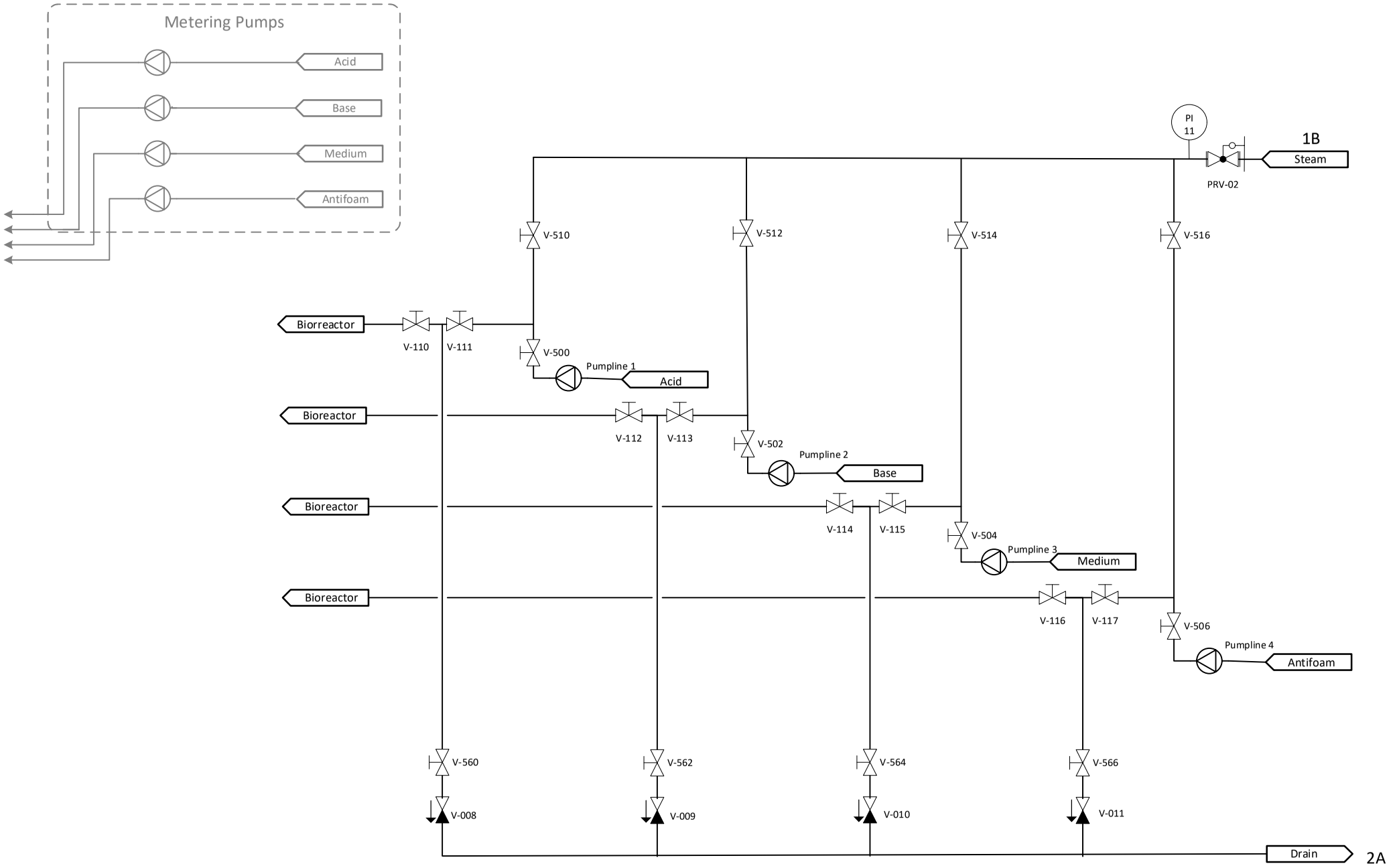
P&ID nº 1 - Overview



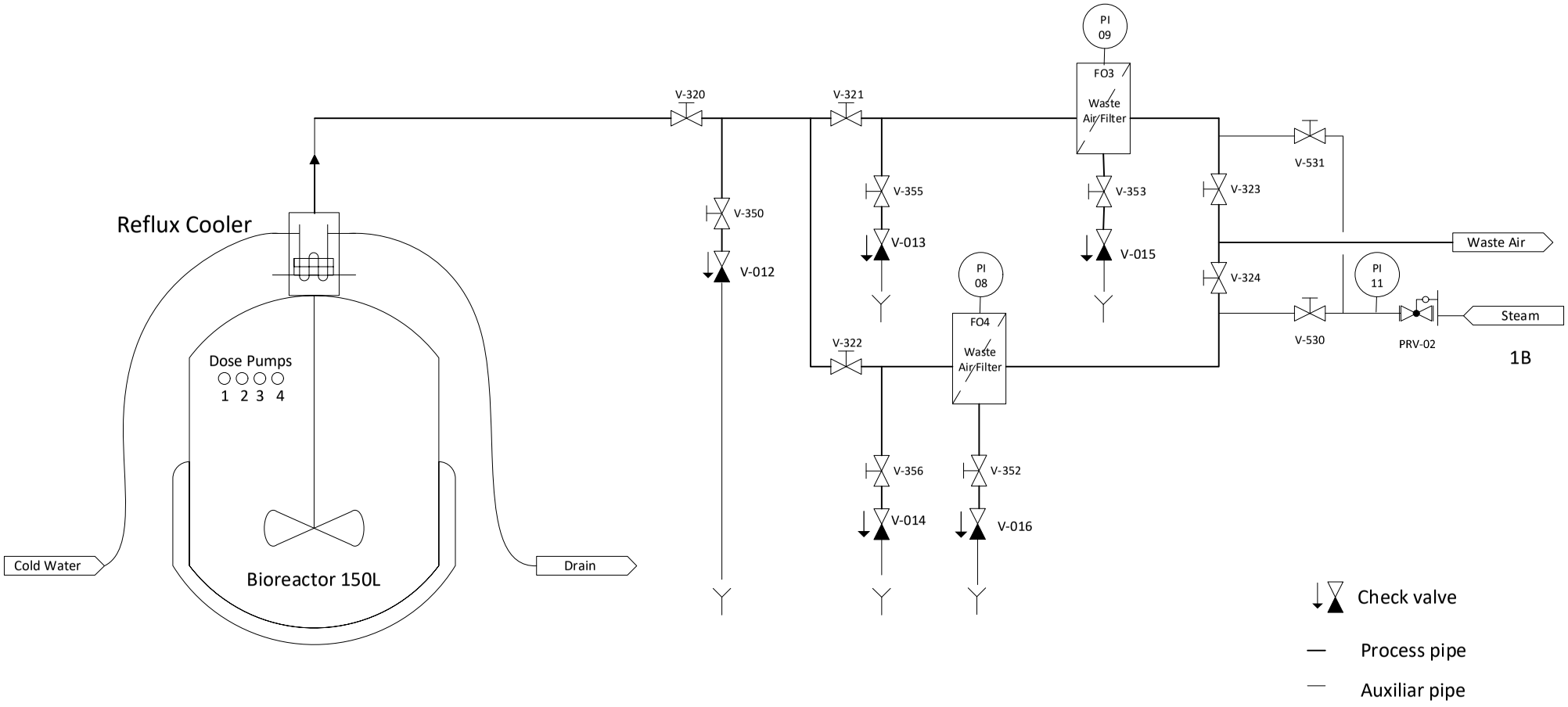
P&ID nº 2 - Inlet Filters



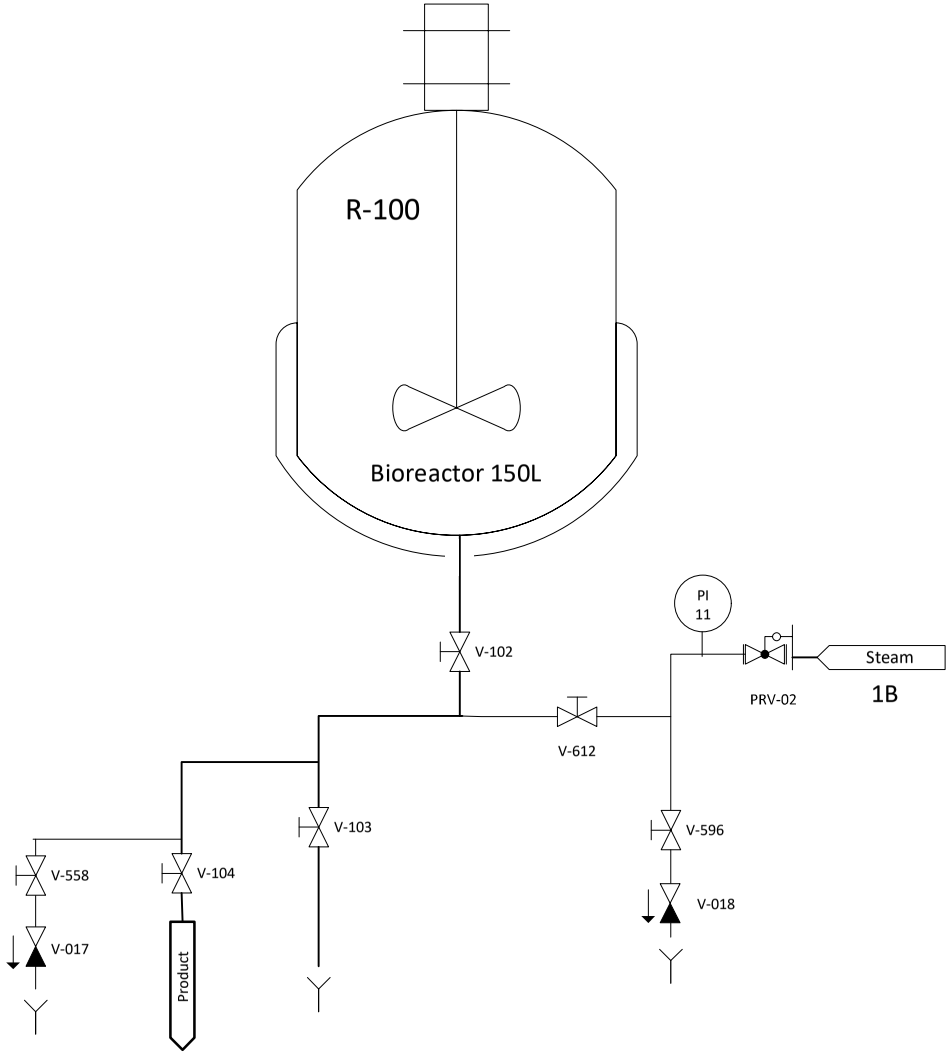
P&ID nº 3 - Metering Pumps



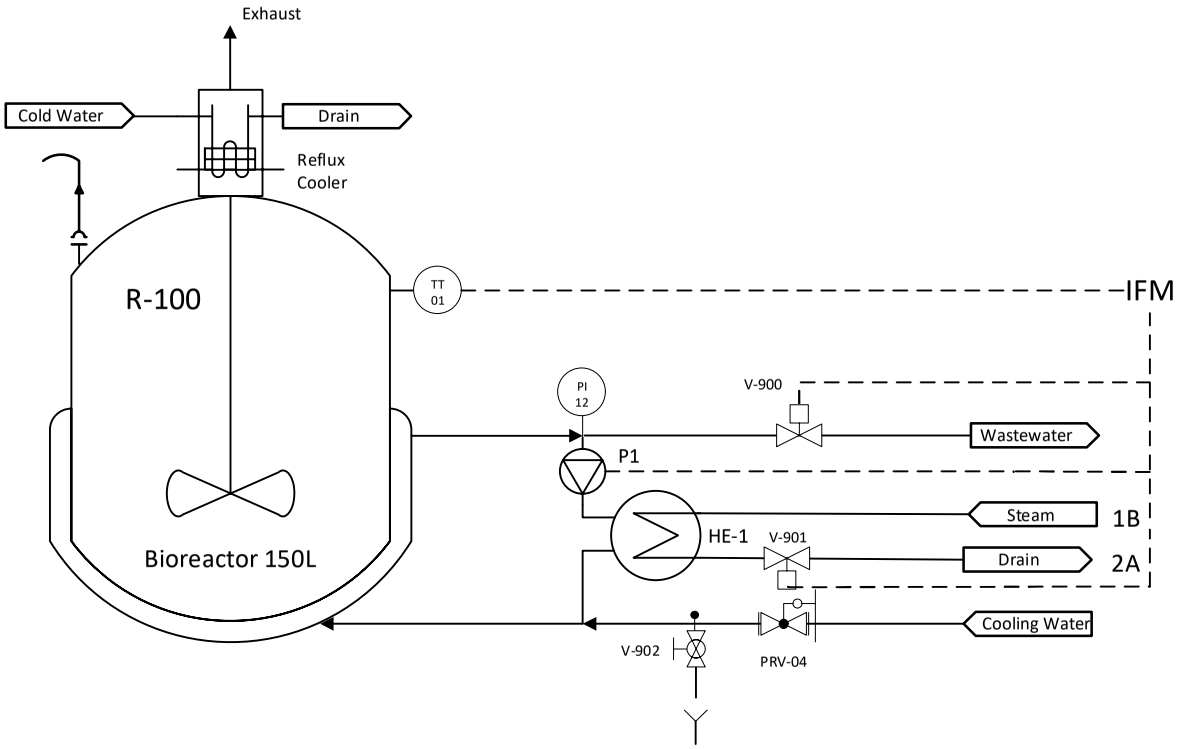
P&ID nº 4 - Exhaust Filters



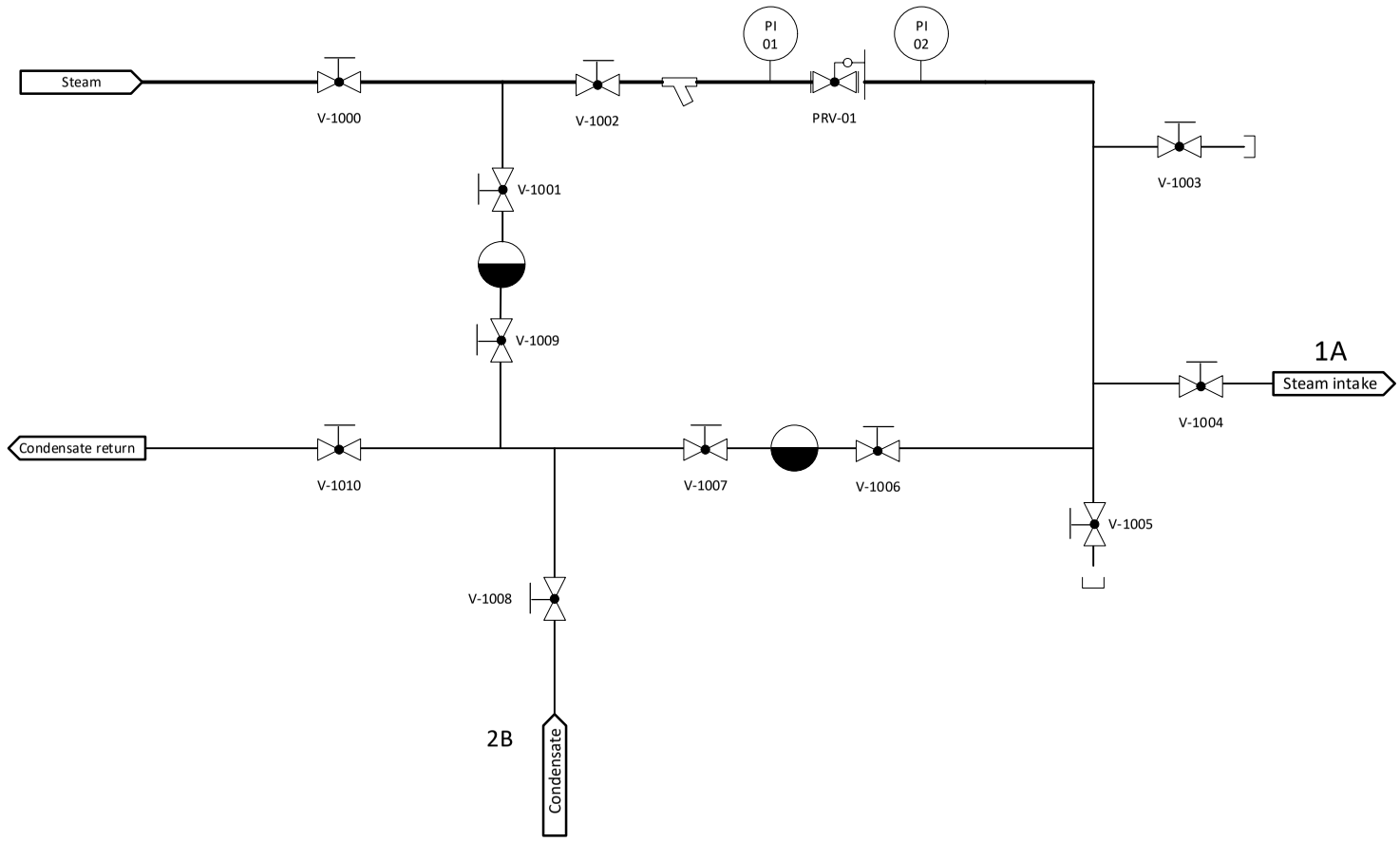
P&ID nº5 - Product outlet



P&ID nº6- Cooling System



P&ID nº7 - Steam supply



Appendix II

Sterilization Standard Operating Procedure

SOP 5 - Sterilization

Author: *Melania Artigas Ortega*

Approved by: _____

Revision register

Version	Revision date	Reviewed by	Significant changes
5.01	*09/01/17*	*MAO*	*New Sterilization SOP*

1. Purpose

The purpose of this SOP is to specify the procedures for sterilize the BioEngineering 150L fermenter in PILOT PLANT Department, DTU.

2. Related documents

The identifying numbers of the valves, pipes and connections in this procedure refer to the P&ID of the installation, shown in the Appendix A of this document.

For further information regarding the temperature controller, consult "Operating instructions: IFM Temperature control LP and P" [1].

3. Description

Figure 1shows schematically the main parts that configure the pilot-scale fermenter layout. A Steam-In-Place method is used to sanitize the equipment. The inlet filters, exhaust filters, metering pumps and product outlet sections are sterilized in first place, with direct steam injection. Next, the vessel is sterilized, achieving the needed temperature in the vessel filled with the reaction medium by indirect heating (steam is injected in the jacket of the reactor).

4. Material list

The following items are needed for the correct execution of the procedure (explained in point 5):

- Clean steam, obtained from the general steam supply that provides it for all the processing equipments present in the laboratory.

- Infrared temperature measuring device, testo 845. This non contact laser thermometer is used for measuring the temperature in some critical points of the layout as the filters' housing and the vessel.

- Thermocouple installed in the drain collector will be used in order to control the sterilization of the air inlet, exhaust, metering pumps and product outlet sections. (Thermocouple type J, measure range: 0-350ºC).

- Temperature control system of the fermenter and the programmed sterilization cycle included. The control loop consists in a Pt100 sensor (TT-01, P&ID nº1, Appendix A) placed into the vessel that transmits the measurement to a PID module for temperature control (called Intelligent Front Module or IFM). This control module regulates two solenoid valves in charge of the steam and cooling water flow respectively into the vessel jacket. A scheme of the set up is pictured in Figure 2.

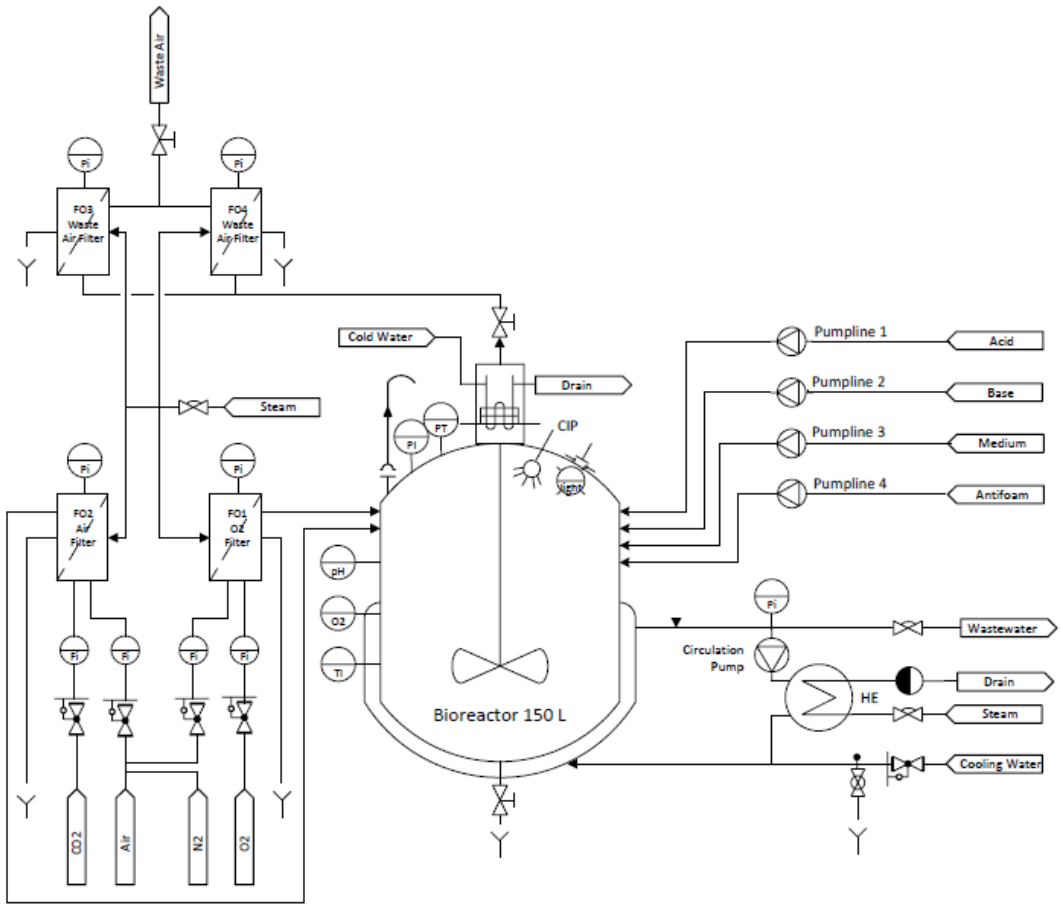


Figure 1. Schematic overview of the installation

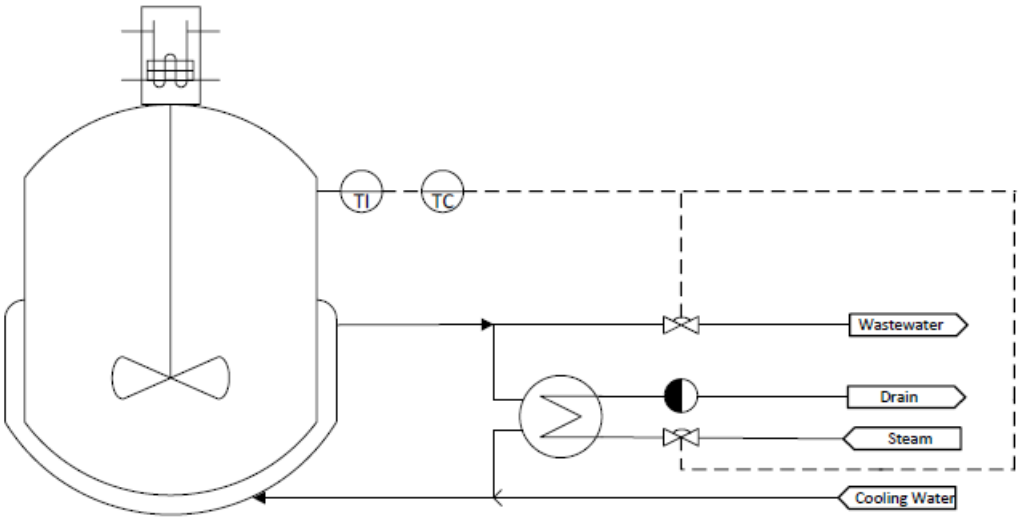


Figure 2. Representative scheme of the temperature control loop.

5. Procedure

A. Preparation

1. Remove the hose connections in the metering pumps section for acid (connected to V-500), base (V-502), substrate (V-504) and antifoam (V-506) - see P&ID nº 3, Appendix A - and autoclave the ones corresponding to the antifoam and substrate metering separately.

2. Before manipulating the steam supply, the operator must ascertain that valves V-1001, V-1002, V-1003, V-1004, V-1005, V-1006, V-1007, V-1008, V-1009, V-1010 are closed and PRV-1 is loosened (closed). See P&ID nº 7, Appendix A.

3. Check that the following valves are closed, in order to assure safe conditions when steam supply is connected:

   a. V317, V316, V301, V310, V520, V557,V556 (Inlet filters, P&ID nº 2, Appendix A)

   b. V510, V512,V514, V516 V111, V113, V115, V117 V500, V502, V504, V506, V110, V112, V114, V116, V560, V562, V564, V566 (Metering pumps, P&ID nº3, Appendix A)

   c. V530, V531, V324, V323, V321, V322, V320, V350, V355, V356, V352, V353 (Exhaust filters, P&ID nº4, Appendix A)

   d. V102, V104, V558, V103, V612 (Product outlet, P&ID nº5, Appendix A)

   e. PRV-02 (See either P&ID nº3 or nº4, Appendix A)

4. Connect the steam inlet and assure that it is correctly adjusted and tightened (this connection is indicated in the P&ID in Appendix A with number 1: **1A** – P&ID nº 7-, and **1B-** P&ID nº 2,3,4,5,6[1]).

[1] There is just one connection point, but it is pictured in all of the different subsections.

5. Connect the drain collector to the main condensate pipe and tighten (see connection details in P&ID in Appendix A with number 2: **2A** – P&ID nº 3,6[2] **-** and **2B**, P&ID nº 7).

6. If not already opened, open V-1000 (P&ID nº7, Appendix A)

7. Open valve V-1002 (P&ID nº7, Appendix A)

8. Make sure that the valves of the pressure indicators (PI-1 and PI-2)[3] are opened (P&ID nº7, Appendix A)

9. Open V-1008 and V-1010 (P&ID nº7, Appendix A).

10. Open PRV-01 to 1.10 bar-g (P&ID nº7, Appendix A)

11. Open V-1004 (P&ID nº7, Appendix A)

B. Inlet filters, exhaust filters, metering pumps and product outlet sterilization

1. Set PRV-02 to 1.10 bar-g. (See either P&ID nº3 or nº4, Appendix A). For that purpose, adjust PRV-01 if necessary (P&ID nº7, Appendix A).

2. Open the following valves in the indicated order:
   a. V557, V556, V520, V315 (Inlet filters, P&ID nº 2, Appendix A)

   b. V510, V111, V560
      V512, V113, V562
      V514, V115, V564
      V516, V117, V566 (Metering pumps, P&ID nº3, Appendix A)

   c. V355, V356, V352, V353
      V530, V531, V350, V321, V322 (Exhaust filters, P&ID nº4, Appendix A)

   d. V558, V612 (Product outlet, P&ID nº5, Appendix A)

[2] Again the connection is unique, but schemed in both representations.
[3] These valves are not pictured in the P&I diagrams, they are attached to the instrumentation.

3. Monitorize the temperature in FO1, FO2 (P&ID nº2), FO3, FO4 (P&ID nº4), and every minute with the IR laser thermometer pointing to the black labels disposed in the equipment. Monitorize the condensate temperature in the drain. When the temperature in the drain and filters reaches 121ºC, the sterilization conditions are optimum and have to stay stable for 10 min.

   If one of the measurements with the IR device gives a temperature lower than 115ºC, repeat after 30s. If the low temperature persists, start counting the 10 min when 121ºC are reached again.

4. Once the equipment has stayed at the sterilization temperature during the required time, close the following valves in the indicated order, so vacuum is avoided inside the pipes [2]:

   a. V-556, V-557, V-520 (Inlet filters, P&ID nº 2, Appendix A)

   b. V-566, V-564, V-562, V-560
      V-111, V-113, V-115, V-117,
      V-510, V-512, V-514, V-516 (Metering pumps, P&ID nº3, Appendix A)

   c. V350, V-355, V-356, V-353,
      V-352, V-531, V-530 (Exhaust filters, P&ID nº4, Appendix A)

   d. V-558, V612 (Product outlet, P&ID nº5, Appendix A)

5. Loosen (close) valve PRV-02 (see either P&ID nº3 or nº4, Appendix A)

C. Vessel sterilization:

Preparation

1. Check that the following valves are closed:

   V-310, V-301 (P&ID nº2)

   V-111, V-113, V-115, V-117 (P&ID nº3)

   V-102, V-612 (P&ID nº5)

2. The following valves need to be opened:

   V-110, V-112, V-114, V-116 (P&ID nº3)

   V-320, V-321, V323 (P&ID nº4)

3. Fill the reactor with medium
4. Set agitator to moderate speed
5. Check the sterilization parameters in the controller (select PARA, then STERIL PAR) :

   STER. TEMP: 121ºC

   STER. TIME: 20min

   RESTART < : 120ºC

   BOIL TEMP: 95ºC

   PRESSURIZE: 100 ºC

   For instructions on how to operate de controlling module, consult the controller manual, “Operating instructions: IFM Temperature control LP and P” chapter, [1].

Sterilization:

Description of the process

The temperature control allows the automation of the different required steps to sterilize the vessel of the fermenter, having specified the parameters above. The process has 4 phases:

- Preheating: the steam starts heating the jacket, and therefore, the content of the vessel. During this phase, the exhaust[4] is open (manually) to avoid excessive pressure in the vessel and to allow the release of the air in the vessel. This way saturation conditions can be hold into the equipment.
- Heating: once the medium starts boiling (BOIL TEMP is reached), the exhaust must be closed manually (the drain- V-350 - is opened partially). The temperature inside the fermenter keeps rising.
- Sterilization: when STER.TEMP (121ºC) is reached in the vessel (measured by TT-01, see P&ID nº1, Appendix A), a timer starts counting 20 min. If the temperature drops below the value specified as RESTART, the timer stops and starts again when STER.TEMP is reached.
- Cooling: once the sterilization period has been completed, steam flow is stopped and the cooling water starts lowering the temperature. To avoid vacuum in the vessel, a message appears when the temperature specified in PRESSURIZE is reached, requesting the operator to open the exhaust valves again.

Together with the sterilization of the vessel and the medium, the sterilization of the fragment of pipes used to connect antifoam and substrate hoses to valves V-506 and V-504, respectively (P&ID nº3, Appendix A) will be carried out. These pipe parts are highlighted in red in Figure 3.

[4] Specific valves are not mentioned here, but in the "Operation" section, where the particular instructions to be carried out are stated.

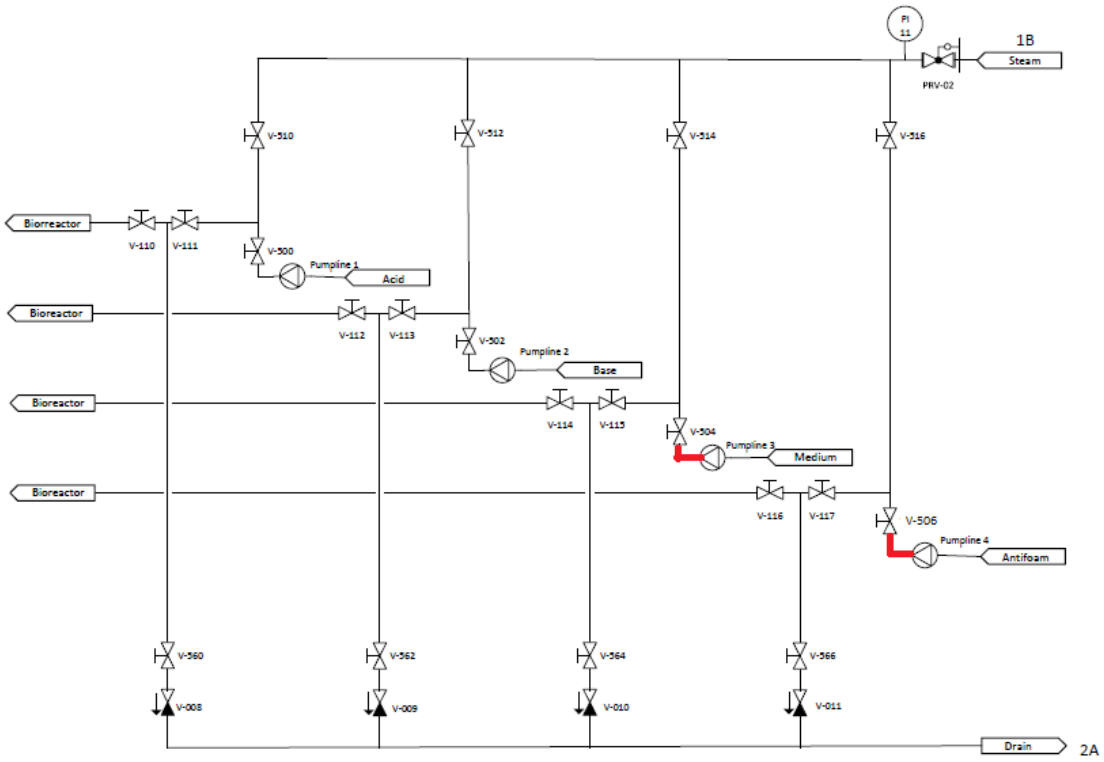


Figure 3. P&ID nº 3, metering pumps section, with highlighted pipes that are sterilized while vessel sterilization takes place.

Operation

The required actions are:

1. Connect an autoclaved hose to V-504 and other one to V-506. Fill a 5L bucket with tap water (ambiance temperature), and submerge the free extreme of both hoses in the bucket.

2. Open PRV-01 to 2 bar-g (P&ID nº7, Appendix A)

3. Select and start STERILIZE in the controller [1].

4. When this message appears:

   “CLOSE THE VALVE. WHEN DONE PRESS OK”

   - Close valve V-321 (P&ID nº4)

- Open V-350 partially, adjusting to condensate flow (P&ID nº4)
- Make sure V-320 is still opened (P&ID nº4)

Then press “OK”

5. Open valves V-504 and V-506 for 2 min, letting the steam generated in the vessel go through the hoses and be cooled within the water in the bucket. Repeat this action 5 times while the vessel is being sterilized.

6. When this message appears:
   “OPEN THE VALVE. WHEN DONE PRESS OK”
   Open valve V-321 and V-323 (P&ID nº4). Then press “OK”.

The sterilization is finished when the vessel is cooled to the operating temperature and the controller changes to CONTROLLD mode.

E. Maintenance of the sterile conditions:

Once the cycle has been completed, immediately pressurize with sterile air: open V-214, PRV-3.1, PRV-3.4, V-311, V-314, V-316, V-317, V-310, V-301 (P&ID nº2) to allow the air flow through the inlet filters and close V-350 (P&ID nº4).

6. References

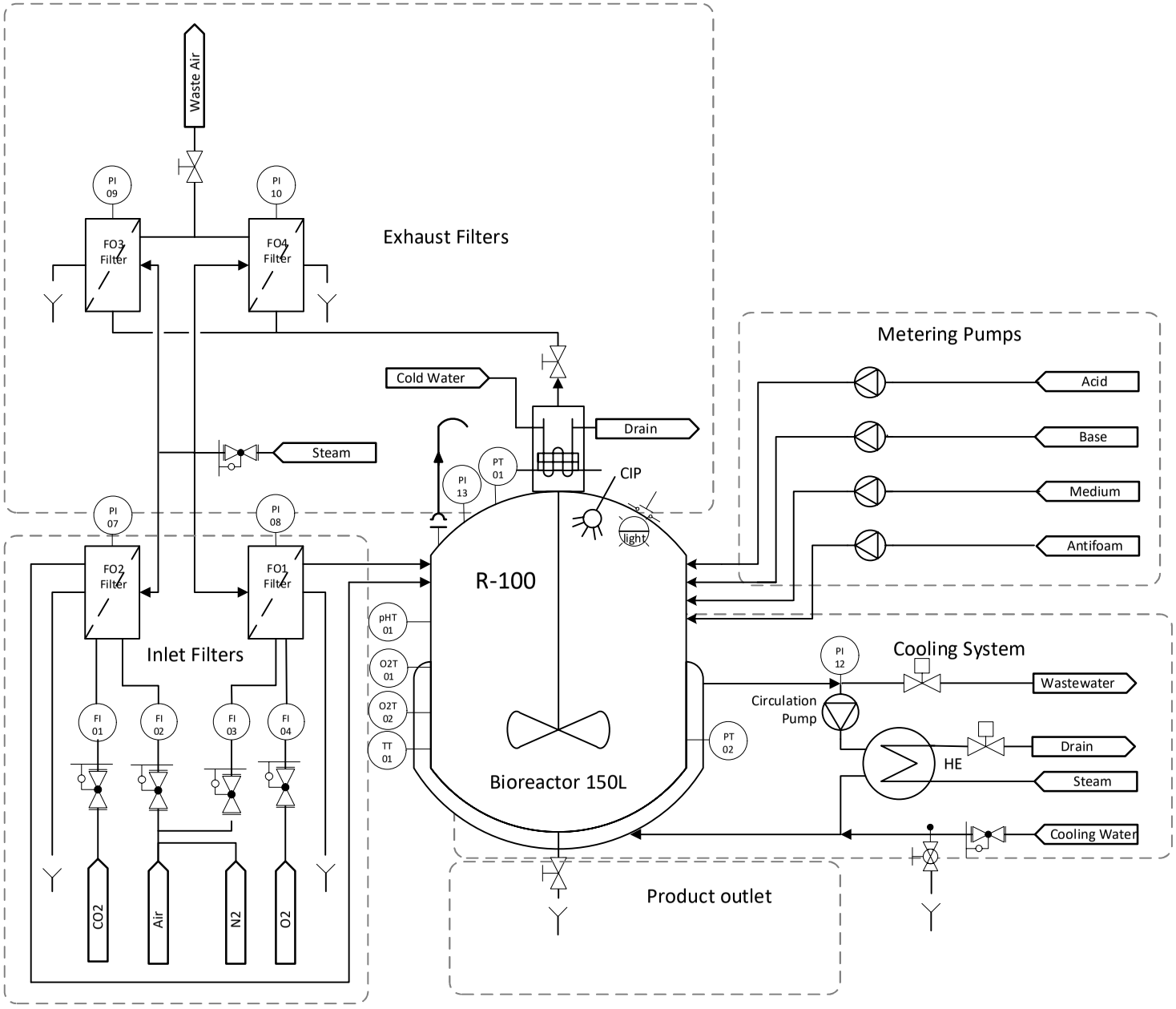
[1] IFM module manual, “Operating instructions: IFM Temperature control LP and P”. BioEngineering.

[2] Chisti, Y. (1992). Assure bioreactor sterility. *Chemical engineering progress*, 88, 80-80.

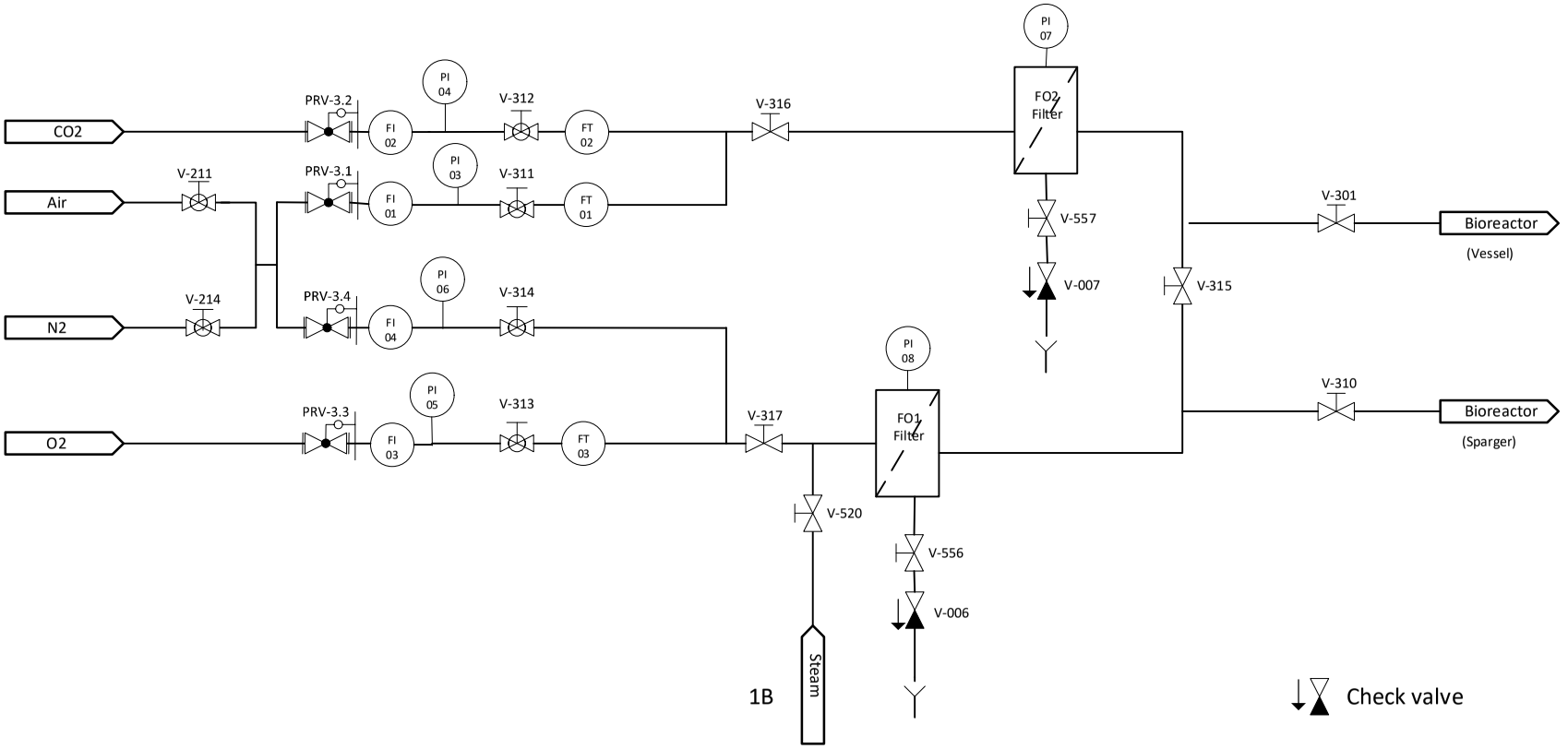
Appendix A

Piping and Instrumentation Diagrams

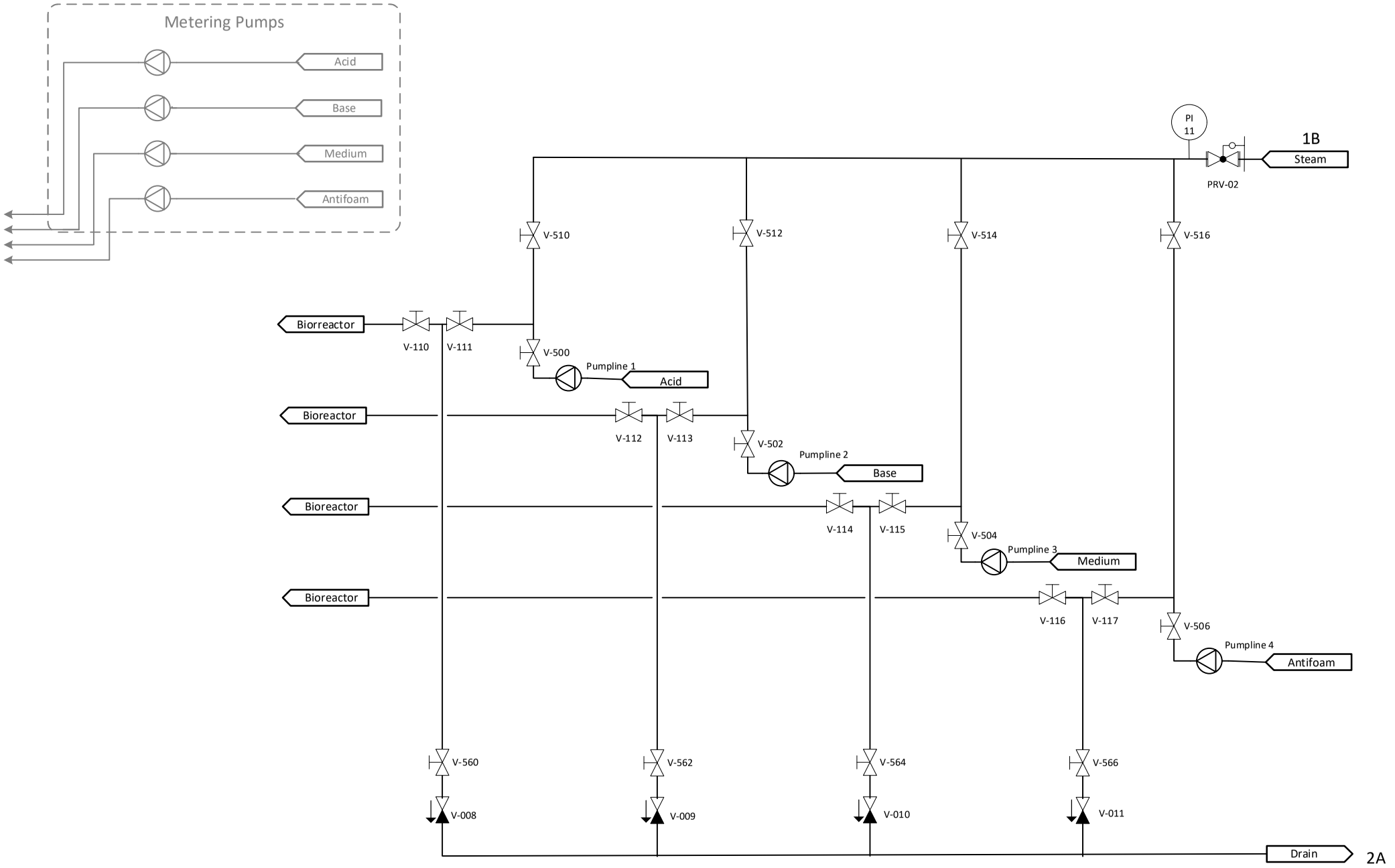
P&ID nº 1 - Overview



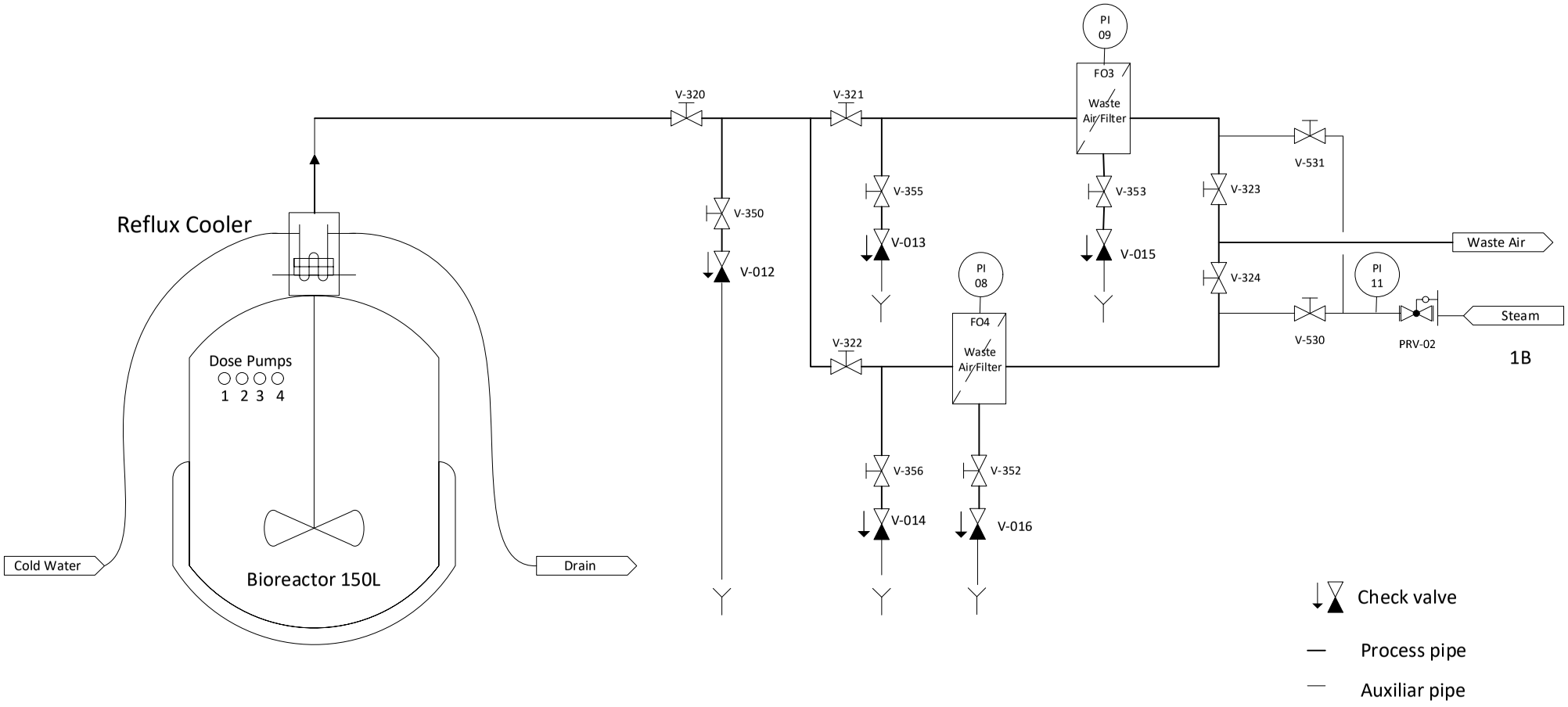
P&ID nº 2 - Inlet Filters



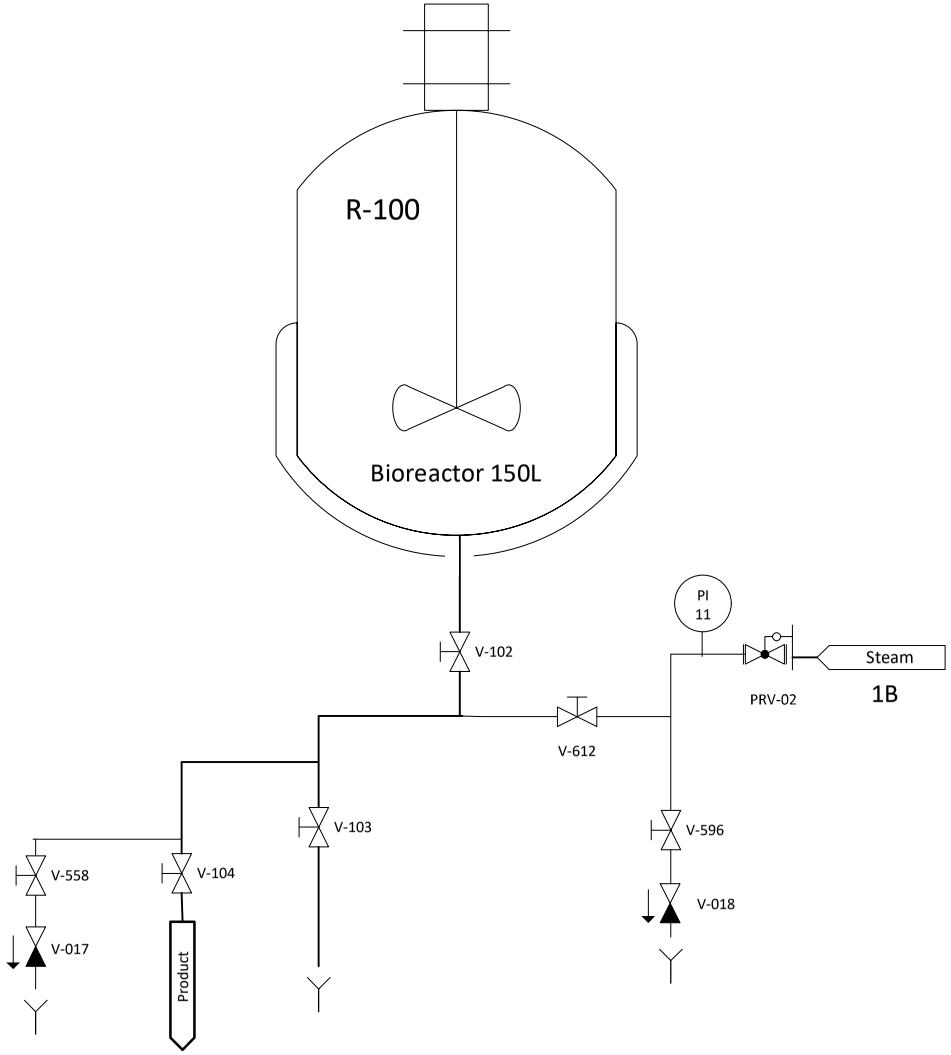
P&ID nº 3 - Metering Pumps



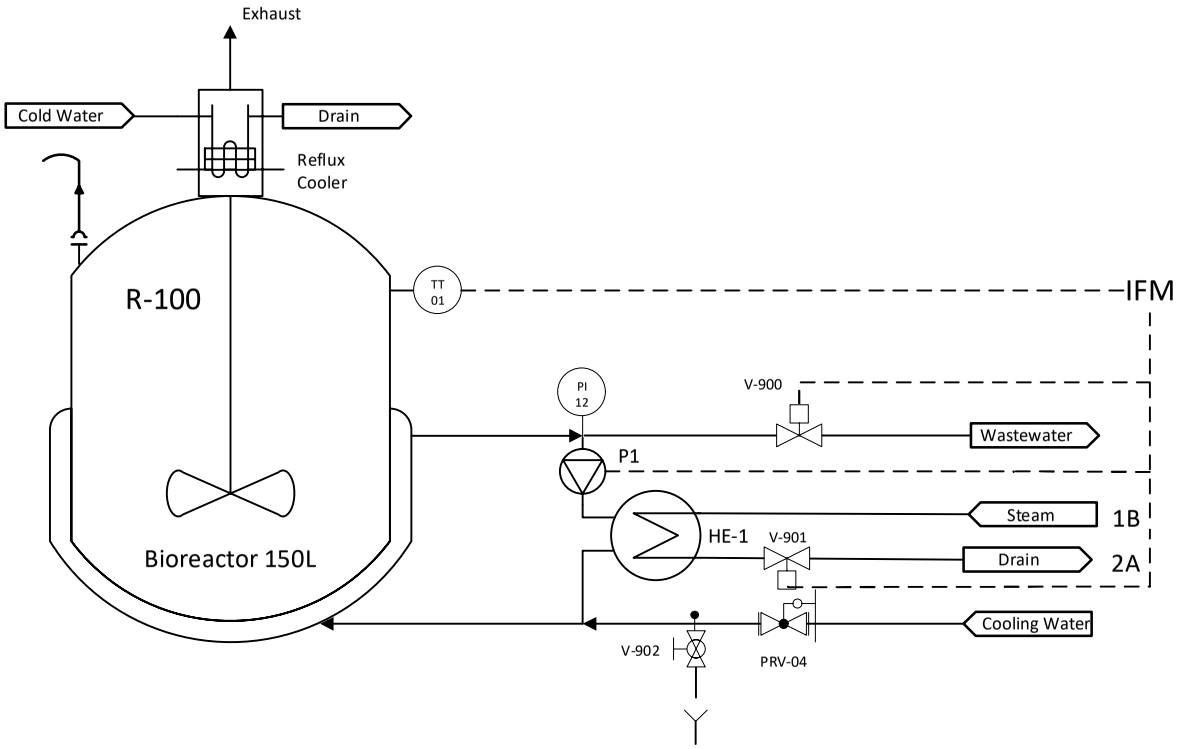
P&ID nº 4 - Exhaust Filters



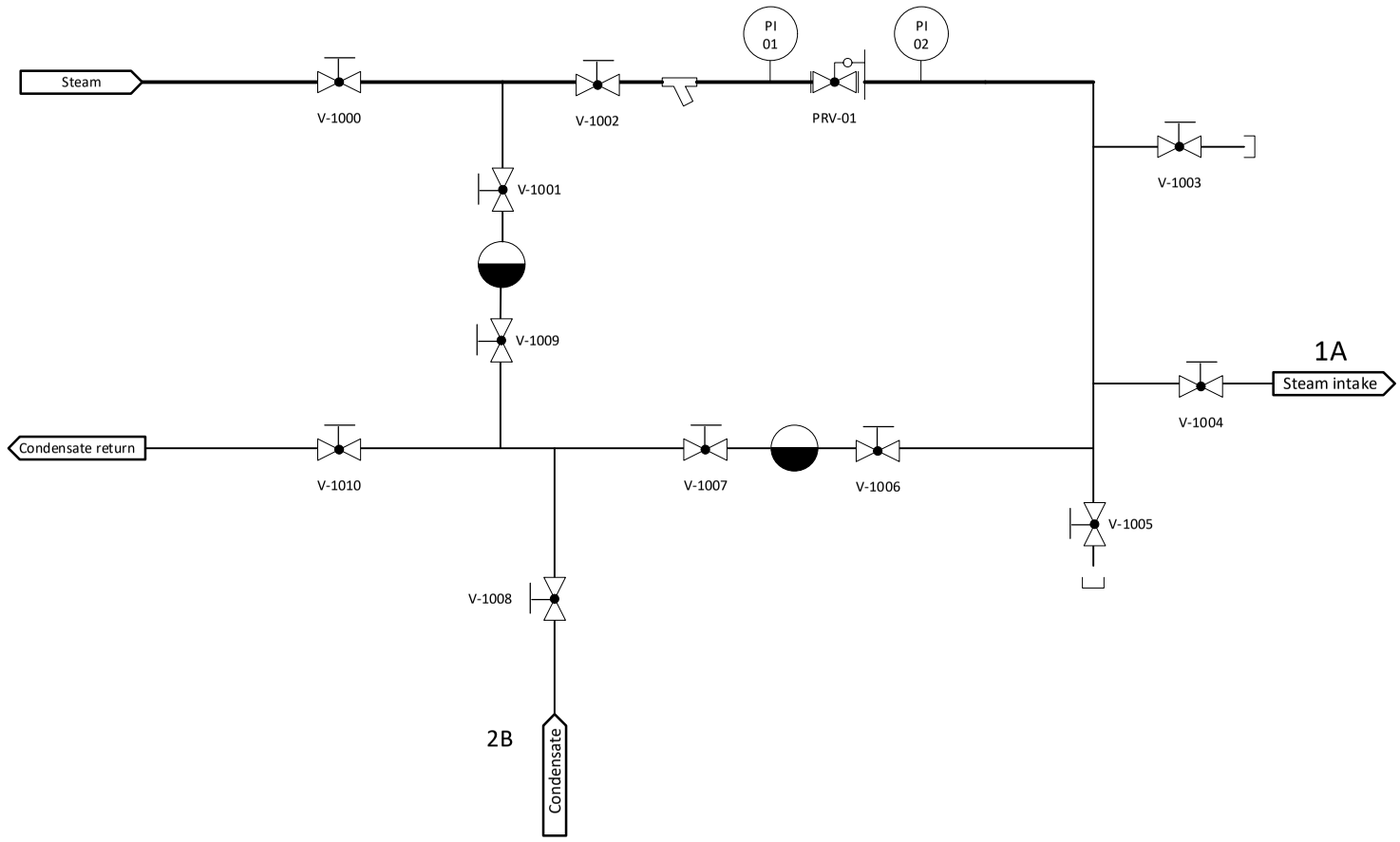
P&ID nº5 - Product outlet



P&ID nº6- Cooling System



P&ID nº7 - Steam supply



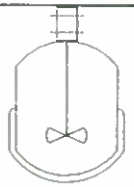
Appendix III

Test sheets for equipment qualification

TEST SHEET
P&ID component check list

P&ID nº: 1 Lay out section: *Reaction vessel*

Inspection responsible: Melania Artigas Start date: 17/01/17 End date: 17/01/17

TEST POINT	SUBJECT	IDENTIFICATION CODE	SYMBOL	TYPE OF ELEMENT CHECKED ON FIELD	ID -CODE CHECKED ON FIELD	UBICATION CHECKED ON FIELD	OBSERVATIONS
1	Jacketed Stirred Vessel	R-100		☒ Yes ☐ No	X	☒ Yes ☐ No	
2	Pressure trans-mitter	PT-01		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Proposed tag number*
3	Barometer	PI-12		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Proposed tag number*
4	pH trans-mitter	pHT-01		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Proposed tag number*

TEST POINT	SUBJECT	IDENTIFICATION CODE	SYMBOL	TYPE OF ELEMENT CHECKED ON FIELD	ID -CODE CHECKED ON FIELD	UBICATION CHECKED ON FIELD	OBSERVATIONS
5	O_2 trans-mitter	O_2T - 01	O2T	☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Proposed tag number*
6	O_2 trans-mitter	O_2T - 02	O2T	☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Proposed tag number*
7	Tem-perature transmitter	TT - 01	TT	☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*TYPE: Pt 100* *Proposed tag number*
8	Pressure transmitter	PT-02	PT	☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Proposed tag number*

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TEST SHEET
P&ID component check list

P&ID nº: 2 Lay out section: *Inlet filters*

Inspection responsible: Melania Artigas Start date: 17/01/17 End date: 17/01/17

TEST POINT	SUBJECT	IDENTIFICATION CODE	SYMBOL	TYPE OF ELEMENT CHECKED ON FIELD	ID -CODE CHECKED ON FIELD	UBICATION CHECKED ON FIELD	OBSERVATIONS
1	Pressure reducing valve, manual	PRV-3.2		☑ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
2	Ball valve, manual	V-211		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
3	Ball valve, manual	V-214		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
4	Pressure reducing valve, manual	PRV-3.1		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
5	Pressure reducing valve, manual	PRV-3.4		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
6	Pressure reducing valve, manual	PRV-3.3		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
7	Flowmeter, air/N_2	FI-01		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*

TEST POINT	SUBJECT	IDENTIFICATION CODE	SYMBOL	TYPE OF ELEMENT CHECKED ON FIELD	ID -CODE CHECKED ON FIELD	UBICATION CHECKED ON FIELD	OBSERVATIONS
8	Flowmeter, CO_2	FI-02	FI	☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
9	Flowmeter, O_2	FI-03	FI	☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
10	Flowmeter, air/N_2	FI-04	FI	☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
11	Barometer	PI-03	PI	☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
12	Barometer	PI-04	PI	☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
13	Barometer	PI-05	PI	☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
14	Barometer	PI-06	PI	☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
15	Ball valve, manual	V-312		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
16	Ball valve, manual	V-311		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
17	Ball valve, manual	V-314		☒ Yes ☐ No	☒ Yes ■ No	☒ Yes ☐ No	

TEST POINT	SUBJECT	IDENTIFICATION CODE	SYMBOL	TYPE OF ELEMENT CHECKED ON FIELD	ID -CODE CHECKED ON FIELD	UBICATION CHECKED ON FIELD	OBSERVATIONS
18	Ball valve, manual	V-313		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	
19	Flow transmitter, air/N_2	FT-01		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	Tag number suggestion
20	Flow transmitter, CO_2	FT-02		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	Tag number suggestion
21	Flow transmitter, O_2	FT-03		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	Tag number suggestion
22	Membrane valve, manual	V-316		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	
23	Membrane valve, manual	V-317		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	
24	Membrane valve, manual	V-520		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
25	Filter	F02		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	
26	Barometer	PI-07		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
27	Membrane valve, manual	V-557		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	

TEST POINT	SUBJECT	IDENTIFICATION CODE	SYMBOL	TYPE OF ELEMENT CHECKED ON FIELD	ID -CODE CHECKED ON FIELD	UBICATION CHECKED ON FIELD	OBSERVATIONS
28	Check valve	V-007		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
29	Filter	F01		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	
30	Barometer	PI-08		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
31	Membrane valve, manual	V-556		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	
32	Check valve	V-006		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
33	Membrane valve, manual	V-315		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	
34	Membrane valve, manual	V-310		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	
35	Membrane valve, manual	V-301		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	

Date and signature: 17/01/17

TEST SHEET
P&ID component check list

P&ID nº: 3 Lay out section: *Metering pumps*

Inspection responsible: Melania Artigas Start date: 17/01/17 End date: 17/01/17

TEST POINT	SUBJECT	IDENTIFICATION CODE	SYMBOL	TYPE OF ELEMENT CHECKED ON FIELD	ID -CODE CHECKED ON FIELD	UBICATION CHECKED ON FIELD	OBSERVATIONS
1	Membrane valve, manual	V-516		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	
2	Membrane valve, manual	V-514		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	
3	Membrane valve, manual	V-512		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	
4	Membrane valve, manual	V-510		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	
5	Membrane valve, manual	V-506		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
6	Membrane valve, manual	V-504		☒ Yes ☐ No	☒ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
7	Membrane valve, manual	V-502		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	

TEST POINT	SUBJECT	IDENTIFICATION CODE	SYMBOL	TYPE OF ELEMENT CHECKED ON FIELD	ID -CODE CHECKED ON FIELD	UBICATION CHECKED ON FIELD	OBSERVATIONS
8	Membrane valve, manual	V-500		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
9	Barometer	PI-11		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	Tag number suggestion
10	Pressure reducing valve, manual	PRV-02		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
11	Membrane valve, manual	V-117		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	
12	Membrane valve, manual	V-116		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	
13	Membrane valve, manual	V-115		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	
14	Membrane valve, manual	V-114		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	
15	Membrane valve, manual	V-113		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	
16	Membrane valve, manual	V-112		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	

TEST POINT	SUBJECT	IDENTIFICATION CODE	SYMBOL	TYPE OF ELEMENT CHECKED ON FIELD	ID -CODE CHECKED ON FIELD	UBICATION CHECKED ON FIELD	OBSERVATIONS
17	Membrane valve, manual	V-111		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	
18	Membrane valve, manual	V-110		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	
19	Membrane valve, manual	V-566		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	
20	Membrane valve, manual	V-564		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	
21	Membrane valve, manual	V-562		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	
22	Membrane valve, manual	V-560		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	
23	Check valve	V-011		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
24	Check valve	V-010		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
25	Check valve	V-009		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*

TEST POINT	SUBJECT	IDENTIFICATION CODE	SYMBOL	TYPE OF ELEMENT CHECKED ON FIELD	ID -CODE CHECKED ON FIELD	UBICATION CHECKED ON FIELD	OBSERVATIONS
26	Check valve	V-008		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*

Date and signature: 4 10/17

TEST SHEET
P&ID component check list

P&ID nº: 4 Lay out section: *Exhaust filters*

Inspection responsible: Melania Artigas Start date: 17/01/17 End date: 17/01/17

TEST POINT	SUBJECT	IDENTIFICATION CODE	SYMBOL	TYPE OF ELEMENT CHECKED ON FIELD	ID -CODE CHECKED ON FIELD	UBICATION CHECKED ON FIELD	OBSERVATIONS
1	Membrane valve, manual	V-320		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	
2	Membrane valve, manual	V-350		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	
3	Check valve	V-12		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
4	Membrane valve, manual	V-321		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	Missing metallic tag
5	Membrane valve, manual	V-355		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	
6	Check valve	V-013		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
7	Membrane valve, manual	V-322		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	

TEST POINT	SUBJECT	IDENTIFICATION CODE	SYMBOL	TYPE OF ELEMENT CHECKED ON FIELD	ID -CODE CHECKED ON FIELD	UBICATION CHECKED ON FIELD	OBSERVATIONS
8	Membrane valve, manual	V-356		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	
9	Check valve	V-014		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
10	Filter	F03		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	
11	Barometer	PI-09		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
12	Membrane valve, manual	V-353		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	
13	Check valve	V-015		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
14	Filter	Filter		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	
15	Barometer	PI-10		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
16	Membrane valve, manual	V-352		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	

TEST POINT	SUBJECT	IDENTIFICATION CODE	SYMBOL	TYPE OF ELEMENT CHECKED ON FIELD	ID -CODE CHECKED ON FIELD	UBICATION CHECKED ON FIELD	OBSERVATIONS
17	Check valve	V-016		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
18	Membrane valve, manual	V-323		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	
19	Membrane valve, manual	V-324		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	
20	Membrane valve, manual	V-531		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	
21	Membrane valve, manual	V-530		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	

Date and signature: 17/01/17

TEST SHEET
P&ID component check list

P&ID nº: 5 Lay out section: *Product outlet*

Inspection responsible: Melania Artigas Start date: 17/01/17 End date: 17/01/17

TEST POINT	SUBJECT	IDENTIFICATION CODE	SYMBOL	TYPE OF ELEMENT CHECKED ON FIELD	ID -CODE CHECKED ON FIELD	UBICATION CHECKED ON FIELD	OBSERVATIONS
1	Membrane valve, manual	V-102		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	
2	Membrane valve, manual	V-558		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
3	Membrane valve, manual	V-104		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	
4	Membrane valve, manual	V-103		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	
5	Membrane valve, manual	V-612		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	
6	Check valve	V-008		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*

TEST POINT	SUBJECT	IDENTIFICATION CODE	SYMBOL	TYPE OF ELEMENT CHECKED ON FIELD	ID -CODE CHECKED ON FIELD	UBICATION CHECKED ON FIELD	OBSERVATIONS
7	Check valve	V-008		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
8	Membrane valve, manual	V-597		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	

Date and signature: 17/01/17

TEST SHEET
P&ID component check list

P&ID nº: *6* Lay out section: *Cooling system*

Inspection responsible: Melania Artigas Start date: 17/01/17 End date: 17/01/17

TEST POINT	SUBJECT	IDENTIFICATION CODE	SYMBOL	TYPE OF ELEMENT CHECKED ON FIELD	ID -CODE CHECKED ON FIELD	UBICATION CHECKED ON FIELD	OBSERVATIONS
1	Barometer	PI-12		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
2	Circulation pump	P1		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
3	Heat exchanger	HE-1		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
4	Automatic valve	V-900		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
5	Automatic valve	V-901		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
6	Ball valve, manual	V-902		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*

TEST POINT	SUBJECT	IDENTIFICATION CODE	SYMBOL	TYPE OF ELEMENT CHECKED ON FIELD	ID -CODE CHECKED ON FIELD	UBICATION CHECKED ON FIELD	OBSERVATIONS
7	Pressure reducing valve	PRV-04		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*

Date and signature: 17/01/17

TEST SHEET
P&ID component check list

P&ID nº: 7 Lay out section: *Steam supply*

Inspection responsible: Melania Artigas Start date: 17/01/17 End date: 17/01/17

TEST POINT	SUBJECT	IDENTIFICATION CODE	SYMBOL	TYPE OF ELEMENT CHECKED ON FIELD	ID -CODE CHECKED ON FIELD	UBICATION CHECKED ON FIELD	OBSERVATIONS
1	Globe valve, manual	V-1000		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
2	Globe valve, manual	V-1001		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
3	Steam trap	ST-02		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
4	Globe valve, manual	V-1002		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
5	Y strain	Y-01		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
6	Barometer	PI-01		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
7	Pressure reducing valve, manual	PRV-01		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*

TEST POINT	SUBJECT	IDENTIFICATION CODE	SYMBOL	TYPE OF ELEMENT CHECKED ON FIELD	ID -CODE CHECKED ON FIELD	UBICATION CHECKED ON FIELD	OBSERVATIONS
8	Barometer	PI-02		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
9	Globe valve, manual	V-1003		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
10	Globe valve, manual	V-1004		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
11	Globe valve, manual	V-1005		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
12	Globe valve, manual	V-1006		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
13	Steam trap	ST-03		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
14	Globe valve, manual	V-1007		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*

TEST POINT	SUBJECT	IDENTIFICATION CODE	SYMBOL	TYPE OF ELEMENT CHECKED ON FIELD	ID -CODE CHECKED ON FIELD	UBICATION CHECKED ON FIELD	OBSERVATIONS
15	Globe valve, manual	V-1008		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
16	Globe valve, manual	V-1009		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*
17	Globe valve, manual	V-1010		☒ Yes ☐ No	☐ Yes ☒ No	☒ Yes ☐ No	*Tag number suggestion*

Date and signature: A/01/17

Appendix IV

Experimental data for $K_L a$ determination

Case 1: Stirring speed = 450 rpm, $V_{L=}$ 80.0L

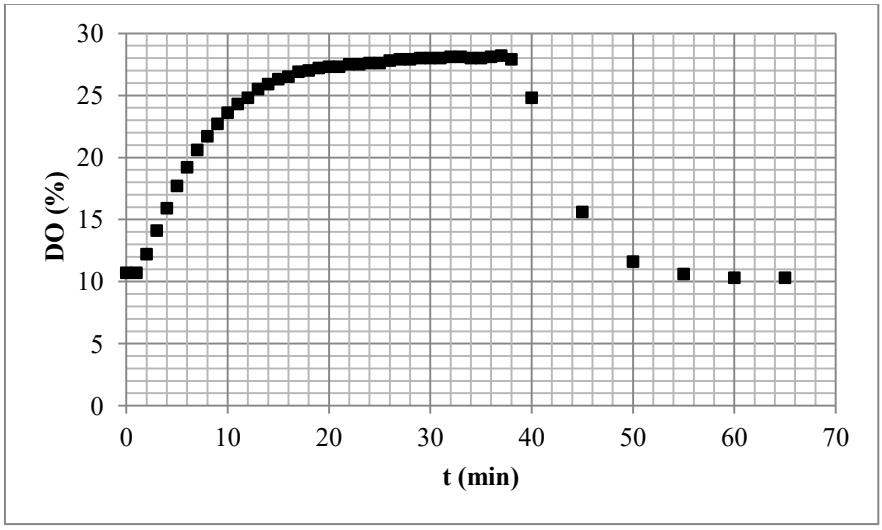

Figure 1 - DO (%) experimental curve for case 1.

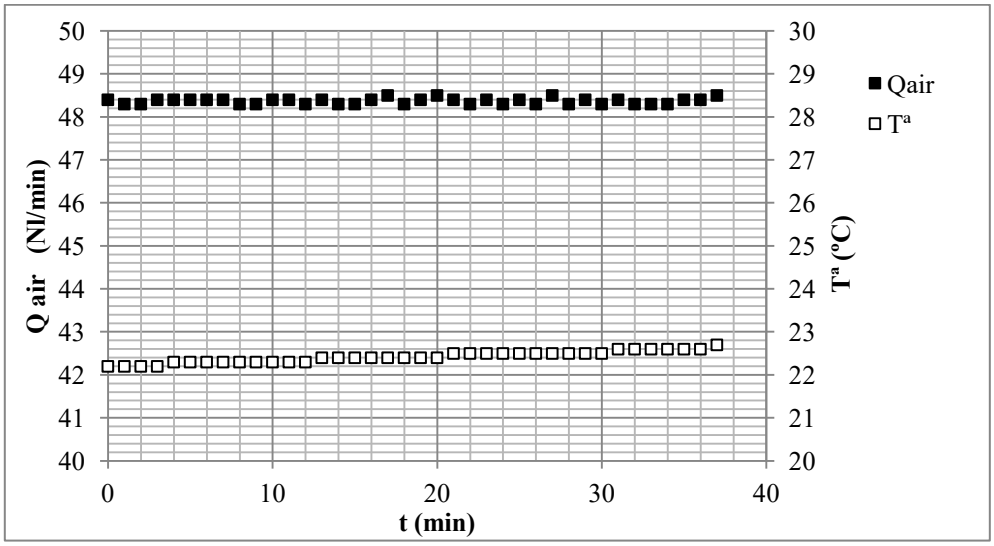

Figure 2 - Tª and Q air experimental curves for case 1.

Case 2: Stirring speed = 400 rpm, $V_{L=}$ 81.6L

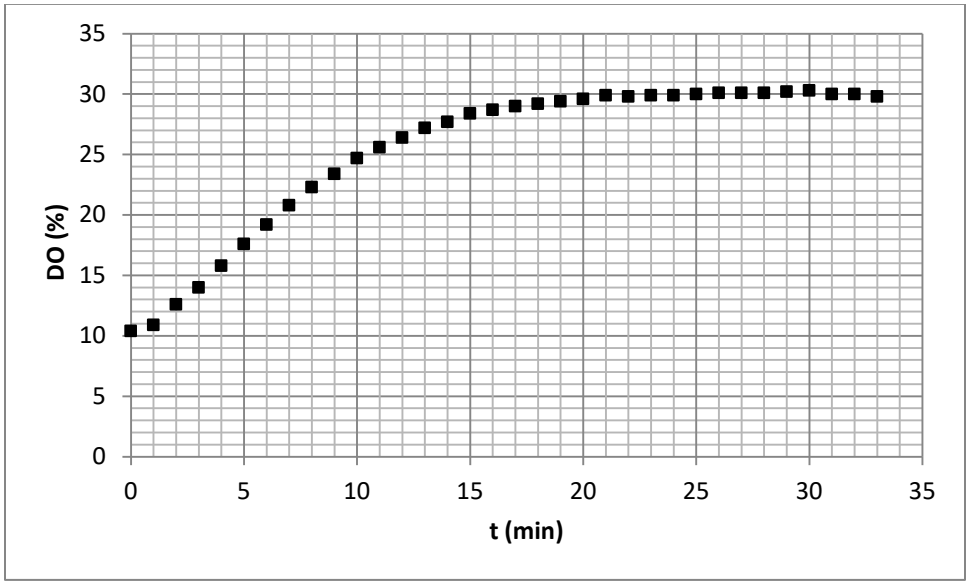

Figure 3 - DO (%) experimental curve for case 2.

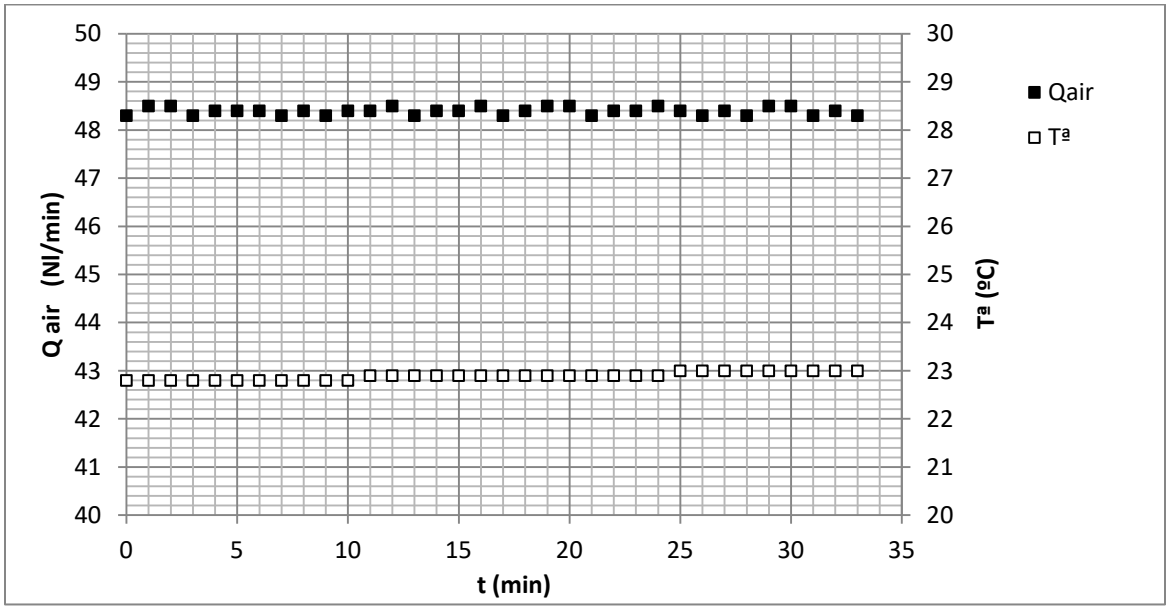

Figure 4 - Tª and Q air experimental curves for case 2.

Case 3: Stirring speed = 293 rpm, $V_{L=}$ 82.3L

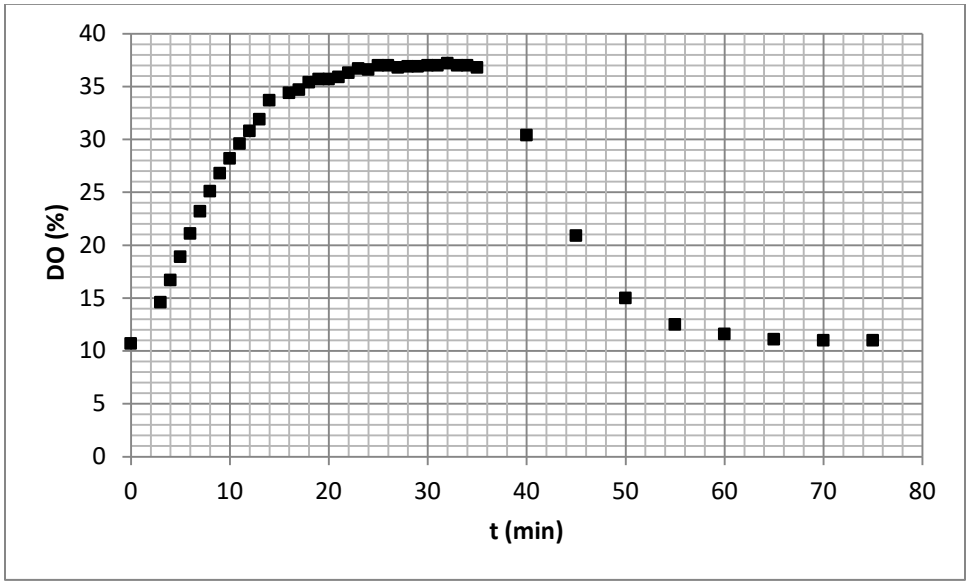

Figure 5 - DO (%) experimental curve for case 3.

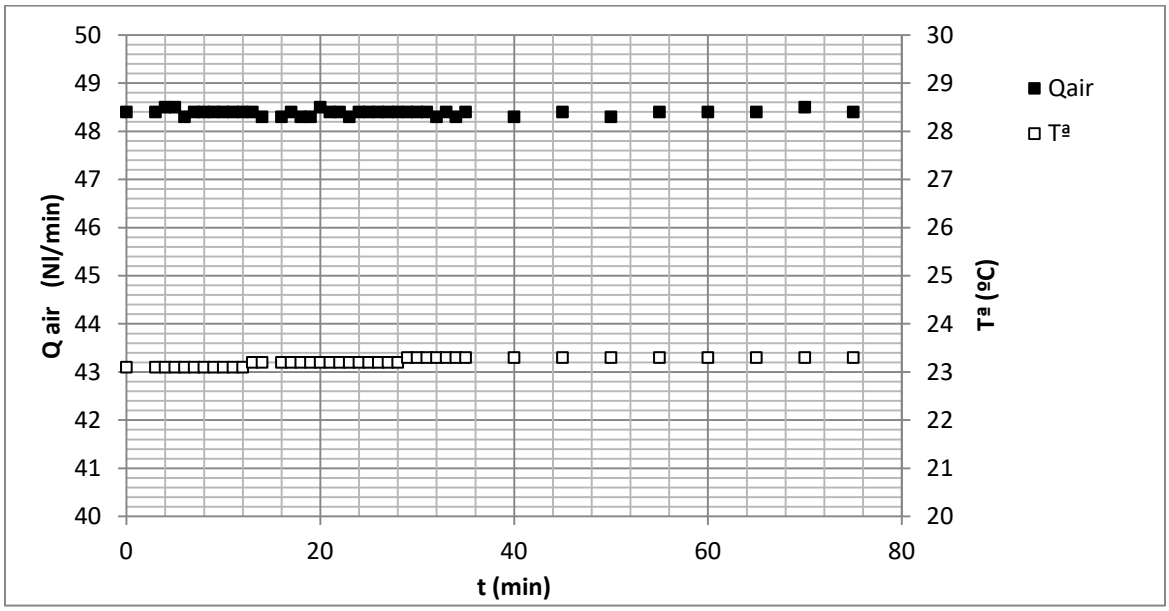

Figure 6 - Tª and Q air experimental curves for case 3.

Case 4: Stirring speed = 234 rpm, $V_{L=}$ 80.0L

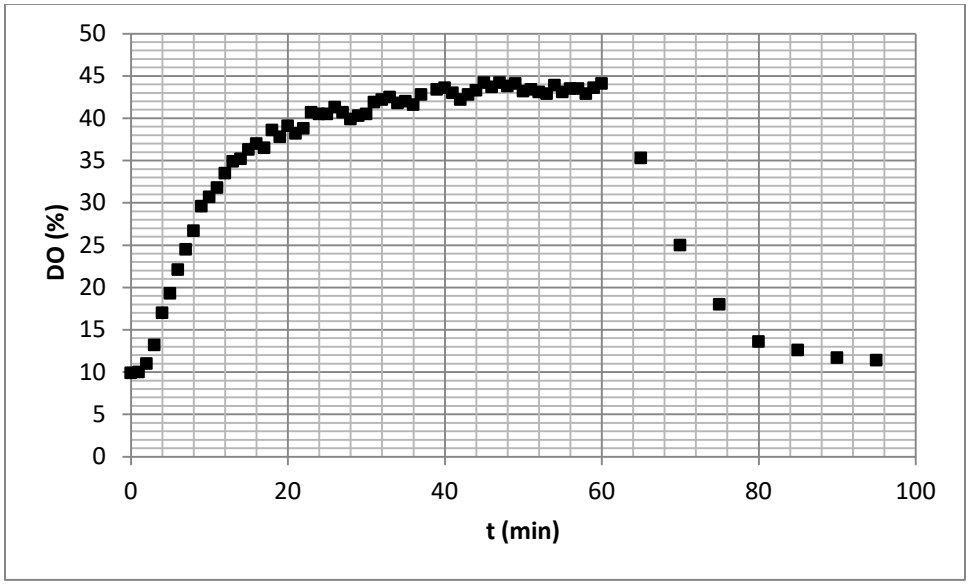

Figure 7 - DO (%) experimental curve for case 4.

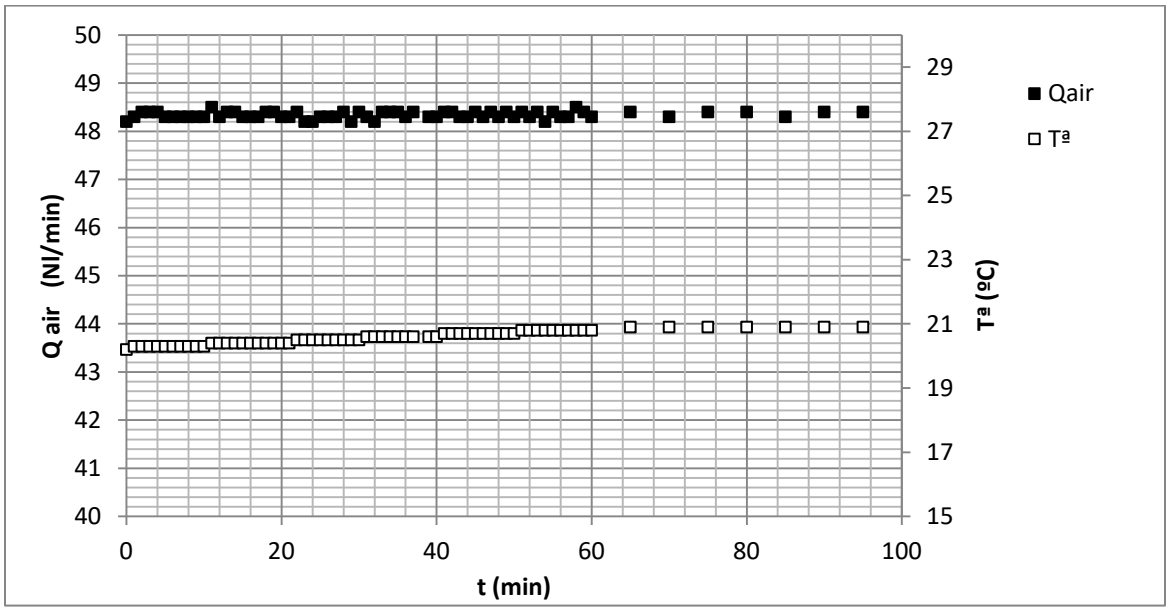

Figure 8 - Tª and Q air experimental curves for case 4.

Case 5: Stirring speed = 176 rpm, $V_{L=}$79.9L

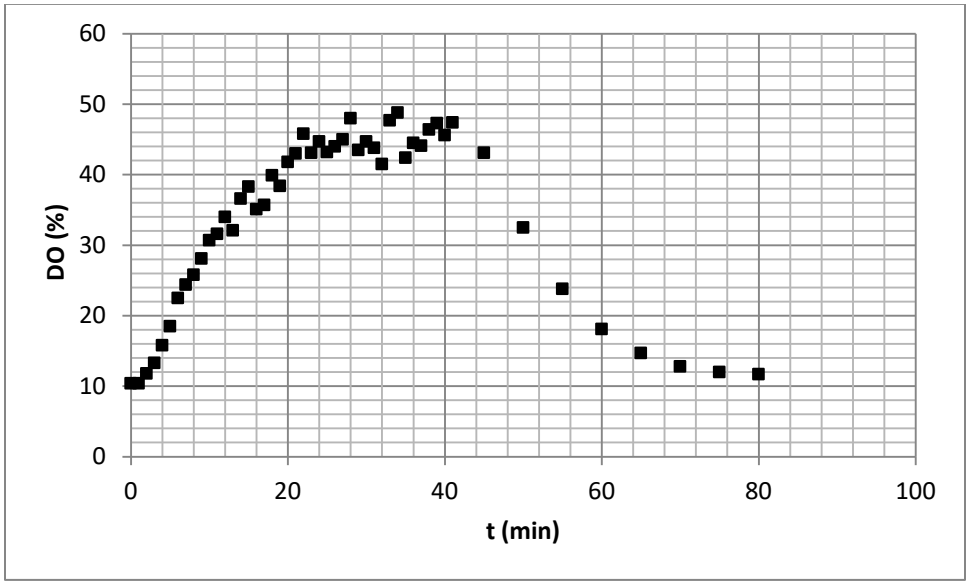

Figure 9 - DO (%) experimental curve for case 5.

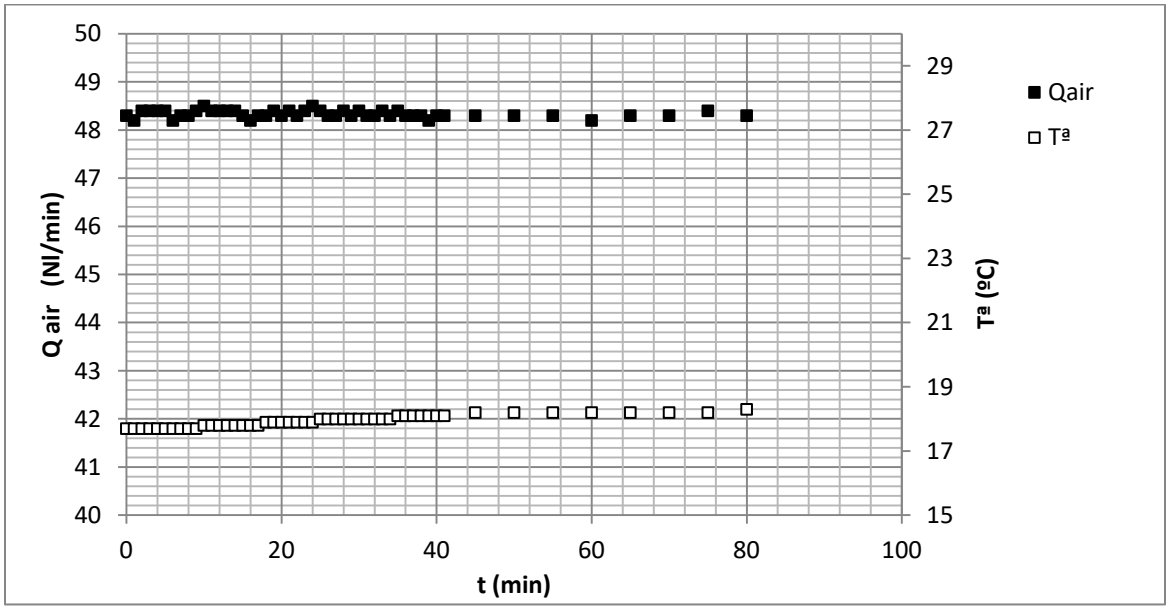

Figure 10 - Tª and Q air experimental curves for case 5.