Modeling of an aerated bioreactor under non ideal mixing conditions

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Abstract

The purpose of this work is to improve a kinetic model in order to be able to predict the behaviour of *Trichoderma reesei* cultures to produce cellulases in non-ideal mixing conditions in order to explain scale-up problems. The modelling calculations were implemented in VBA language from Excel and the model was validated with experimental results performed in the laboratory within the scope of a PhD theses.

The model was improved by adapting its kinetics and including new features like, the storage of oxidative power inside the cells and the recirculation of bubbles of air. The improved model was proven to be able to predict the behaviour of the cultures performed in the laboratory and supplied new ideas to guide further investigations in the laboratory about *Trichoderma reesei* cultures.

The assumptions that were taken into account were confirmed to be in agreement with the reality for the conditions that were used to operate the system.
Keywords: *Trichoderma reesei*, Kinetic Model, Scale-up, non-ideal mixing conditions

1. Introduction

The climate changes, due to the use of fossil fuels to produce energy, that we have witnessed for the past years have been generating a lot of concerns between the scientific community and has become imperative to find a solution to replace this type of fuels (Dahman et al. 2019).

The use of biomass has gained a lot of support as a feedstock for the production of biofuels as it can help in the mitigation of the greenhouse effect by reducing the emissions of CO2 (Sigoillot e Faulds 2011). One of these biofuels is bioethanol that can be blended with gasoline reducing its amount in the final fuel that is sold to the consumers (Shelley Minteer 2016). The production process of this biofuel starts with the pre-treatment of the lignocellulose in order to separate its major components: cellulose, hemicellulose and lignin (Arora et al. 2019). Cellulose it's the most abundant one and it's a polymer of glucose molecules. These sugar molecules are obtained by enzymatic hydrolysis and are then used as substrate to a fermentation operation in each the bioethanol is obtained. The hydrolysis step represents 20% of the total costs of the hole process and it's manly affected by the enzyme activity (Sun e Cheng 2002). To reduce the costs associated with this step, the onsite production of the required enzymes, cellulases, have been considered and studied. The IFPEN (Institut français du pétrole - énergies nouvelles) have been working on the development of a process to produce such enzymes.

The process in question consists on the use of *Trichoderma reesei* cultures, a filamentous fungus with a big power of secretion of cellulases. This process requires the employment of bioreactors with a capacity of hundreds of cubic meters capacity and consequently it's necessary to do a scale-up of the process. Thus it's important to perform a study of the impact of the scale-up conditions on the performance of the culture. A PhD student at IFPEN, Tamiris Roque Gonçalves, used a scale down approach to study the impact of the heterogeneities, induced by the scale up, on the production of the enzymes by the fungus *Trichoderma reesei*.

The scope of this work is to improve an existing bioreactor model in order to adapt it to the heterogeneous conditions by the addiction of new bio-kinetics mechanisms to help to understand the behaviour of the cultures in such conditions. To validate the model, the experimental results from the PhD already mentioned, were used.

2. Materials and Methods

2.1. Experimental Results

To validate the performance of the model experimental results obtained by the PhD student were used. The experimental procedures carried out in the laboratory to obtain this data are explained bellow.

2.2. The Strain

The strain TR30060 was used in both sets of results. These strain is a descendant of CL847 strain that was genetically improved by the Biotechnology department of IFPEN in collaboration with the company PCAS Proteus (Longjumeau, France).

2.3. Experimental procedure

the effect of physicochemical heterogeneities induced by scale-up on the behaviour of the fungus Trichoderma reesei has been studied using a scale down approach that consist on a "Bi-zone" system that has two bioreactors connected, each characterized by different concentrations of dissolved oxygen saturation, since one of the reactors is operated as aerobic (DO=40%) and the other as anaerobic (DO = 0%). Although, initially in the growth phase the experience starts with only one reactor of 3,5 L of total volume with an initial volume of medium of 2,5 L. During this phase the temperature is kept constant at 27 ℃ and the pH controlled at 4,8 thanks to the addition of an ammonia solution (5,5 N) and the agitation speed is fixed at 800 rpm. The initial glucose concentration in the bioreactor is 30 g/L. It is considered that the growth phase is finished when the concentration of residual glucose in the medium is less than 5 g/L, which means that the fungus has consumed almost all of the sugar initially introduced. Then the production phase begins. This phase is carried out at 25 ℃ for approximately 230 hours, with a pH regulated at 4,0 by injecting an ammonia solution at 5,5 N, and a stirring speed fixed at 1000 rpm. The fed-batch is ensured by injecting lactose at 210 g/L with a flow rate of 4 mL/h in order to maintain a residual sugar concentration close to zero. After the fed batch begins the dissolved oxygen is controlled at 40% through a PID system and 96 hours after the start of the fed-batch, this aerobic bioreactor is connected to another bioreactor of 2,0 L of total volume, containing 0,5 L of medium that was withdraw from the aerobic reactor. The fermentation medium then circulates between the two bioreactors with a flow rate of 150 mL/min. The feeding with lactose is carried out only in the aerobic bioreactor. Two peristaltic pumps ensure the circulation of the broth between the two bioreactors throughout the production phase. Also a PID system controls the weight of the anaerobic reactor in order for the liquid volume to be kept at 0,5 L. Figure 1 demonstrates the experimental setup of the configuration that was just described. Beyond the bizone experience, other experience was carried out using this system that is the inverted bizone in which the reactor that is unaerated is the bigger one and the small one the aerobic reactor. This case is the

contrary of the bizone experience and so it is called inverted bizone.

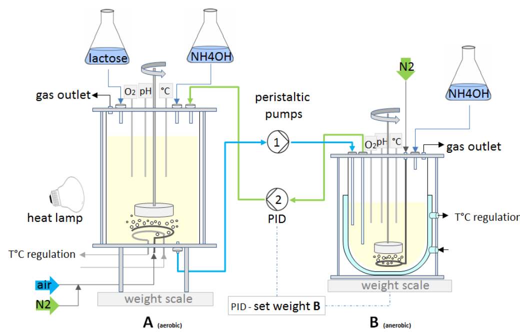

Figure 1: Experimental set up that was used in the laboratory to perform cultures of *Trichoderma reesei* to produce cellulases in conditions to study the scale up problems like bad mixing that can lead to gradients of substrate and zones with low dissolved oxygen levels.

2.4. IFPEN process for cellulase production by *Trichoderma reesei*

IFP Energies nouvelles (IFPEN) has developed an optimized process for the production of cellulases, in liquid phase in a stirred tank reactor, which includes two phases: growth phase and a production phase (Figure 2). The growth phase is carried out in batch mode using excess of glucose as a carbon source. From an industrial point of a view, this phase can be carried out in several stages in fermenters of increasing capacity, aiming to gradually increase the volume until the production fermenter can be inoculated. The second phase is dedicated to the production of cellulases. Its operating mode is fed-batch, allowing the reactor to be fed with lactose at an optimal limiting flow rate to induce the secretion of cellulases by the fungus, and prevent the its repression by the increase of glucose due to the hydrolysis of lactose.

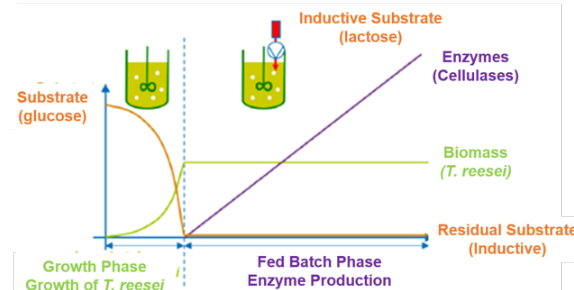

Figure 2: Schematic representation of the IFPEN protocol for cellulase production. Source: (Jourdier 2012).

2.5. IFPEN growth and production model

IFPEN has also been developing a simple model to describe the behaviour of the cultures of *Trichoderma reesei* that are carried out following the previous explained strategy. The model considers that the cells are all equal, growing and producing at the same rate. It is also assuming that the composition of the cellulase cocktail produced to be the same every time and so, there are no discrimination of the production of different cellulases. Additionally, the model doesn't discriminate the different sugars involved in the process (glucose, galactose and lactose) considering that it's all substrate. This model contemplates the specific growth rate, production rate and consumption rate of oxygen and substrate.

Regarding the growth, this phenomenon is described by a Monod type equation and a Michelis Menten term related with the oxygen available in the medium as presented in the Equation 1.

$$\mu = \frac{\mu_{\text{max}} \times [S]}{K_{\text{S}\mu} + [S]} \times \frac{[O_2]}{K_{O_{2\mu}} + [O_2]} \tag{1}$$

The μ_{max} represents the maximum growth rate at which the cells can grow and the constants $K_{s\mu}$ and $K_{O_{2\mu}}$ are the growth half saturation constants related with sugar and oxygen respectively. The [S] and the [O_2] represent the substrate and oxygen concentrations, respectively.

The specific production rate takes into account the inhibition by the biomass and substrate due to the increase of the first during the growth phase and the great amount of sugar present. It also varies according to Michaelis-Menten terms for oxygen and substrate as presented in the Equation 2:

$$q_{\text{p}} = \left(\frac{q_{\text{pmax}} - q_{\text{pmin}}}{1 + \frac{[X]}{K_{\text{iXP}}}} + q_{\text{pmin}}\right) \times \frac{[S]}{K_{\text{SP}} + [S]} \times \times \frac{[O_2]}{K_{O_2\text{P}} + [O_2]} \times \frac{K_{\text{iSP}}}{K_{\text{iSP}} + [S]} \tag{2}$$

The q_{pmax} represents the specific maximum theoretical production rate. The q_{pmin} is the specific theoretical minimum production rate. The constants K_{iXP} and K_{iSP} represent the inhibition of production by the biomass and substrate, respectively. The constants K_{SP} and K_{O_2P} are the production half saturation constants related with sugar and oxygen, respectively. The [X] represents the biomass concentration (the cells amount).

Both the consumption of oxygen and substrate contemplate the amount that it's spent for the growth, the production and the maintenance of the cells as described in the Equations 3 and 4 below:

$$q_{\text{s}} = \frac{\mu}{Y_{\text{x/s}}} + \frac{q_{\text{p}}}{Y_{\text{p/s}}} + m_{\text{s}} \tag{3}$$

$$q_{O_2} = \frac{\mu}{Y_{\text{x/O}_2}} + \frac{q_{\text{p}}}{Y_{\text{p/O}_2}} + Y_{O_2/\text{s}}{}^{\text{m}} \times m_{\text{s}} \tag{4}$$

The $Y_{x/s}$ and $Y_{p/s}$ represent the yield of biomass and enzyme on substrate, respectively. The m_s represents the specific rate of consumption of substrate for the cells maintenance. The Y_{x/O_2} and Y_{p/O_2} represent the yield of biomass and enzyme on oxygen, respectively. Finally the $Y_{O_2/s}{}^m$ represent the oxygen yield on substrate in maintenance reaction which is different depending on the sugar that is fed to the culture, meaning that there are two values for this variable: one for glucose and other for lactose.

The values for each parameter presented were obtained from the report “Modèle de production d'enzymes 2019” from Romain Rousset and are presented in the Table 1 below. Due to confidentiality agreements the values of q_p presented throughout this work are normalized as well the values of protein concentrations.

Table 1: Values of the Kinetic Parameters of the existing model.

Parameter	Value	Units
μ_{max}	0,07 – 0,1	h^{-1}
$K_{s\mu}$	1	gS/L
$K_{O_{2\mu}}$	0,0001	gO_2/L
q_{pmax}	80 – 100	%
q_{pmin}	40	%
K_{iXP}	30	gX/L
K_{iSP}	10	gS/L
K_{SP}	0,001	gS/L
K_{O_2P}	0,0001	gO_2/L
$Y_{x/s}$	0,5	gX/gS
$Y_{p/s}$	0,5	gP/gS
m_s	0,01	gS/gX/h
Y_{x/O_2}	1,04	gX/gO_2
Y_{p/O_2}	1,22	gP/gO_2
$Y_{O_2/gluc}{}^m$	1,07	gO_2/gS
$Y_{O_2/lac}{}^m$	1,12	gO_2/gS

2.6. Mass Balance Equations

To develop the mass balances, the two operation modes were considered, the batch and fed-batch. So a general equation for all the components can be written to modulate the liquid phase, as presented below:

$$\frac{dm_{\mathsf{i}}}{dt} = r_{\mathsf{i}} V_{\mathsf{L}} + (Q_{\mathsf{i}_{\mathsf{in}}} - Q_{\mathsf{i}_{\mathsf{out}}}) \quad (5)$$

In which dm_i/dt represents the variation of mass of i during time (g/hr), r_i is the rate of i (g/L/h), the V_L is the volume of liquid (L), $Q_{i_{in}}$ is the inlet flow rate of i (g/h) and $Q_{i_{out}}$ is the outlet flow rate of i (g/h).

Taking into account that there is no biomass or enzymes entering and leaving the reactor, and there is only inlet of sugar during the fed-batch and the oxygen that is transfer from the gas phase, the air, to the liquid phase is all used by the cells, the equations for the concentration of each component are presented below.

$$\frac{dm_{\mathsf{i}}}{dt} = \frac{d[i]V_{\mathsf{L}}}{dt} = [i]\frac{dV_{\mathsf{L}}}{dt} + V_{\mathsf{L}}\frac{d[i]}{dt} \rightleftharpoons \quad (6)$$

$$\rightleftharpoons \frac{d[i]}{dt} = \frac{1}{V_{\mathsf{L}}}\frac{dm_{\mathsf{i}}}{dt} - \frac{[i]}{V_{\mathsf{L}}}\frac{dV_{\mathsf{L}}}{dt}$$

$$\frac{d[X]}{dt} = \mu[X] - \frac{[X]}{V_{\mathsf{L}}}\Delta Q_{\mathsf{v}} \quad (7)$$

$$\frac{d[P]}{dt} = q_{\mathsf{p}}[P] - \frac{[P]}{V_{\mathsf{L}}}\Delta Q_{\mathsf{v}} \quad (8)$$

$$\frac{d[S]}{dt} = -q_{\mathsf{s}}[X] + \frac{Q_{\mathsf{fb}}[S_{\mathsf{fb}}] - [S]\Delta Q_{\mathsf{v}}}{V_{\mathsf{L}}} \quad (9)$$

$$\frac{d[O_2]}{dt} = -q_{\mathsf{O_2}}[O_2] + k_{\mathsf{L}}a([O_2]^* - [O_2]) - \frac{[O_2]}{V_{\mathsf{L}}}\Delta Q_{\mathsf{v}} \quad (10)$$

$$\Delta Q_{\mathsf{v}} = \frac{V_{\mathsf{L}}}{dt} = Q_{\mathsf{base}} + Q_{\mathsf{fb}} - Q_{\mathsf{evp}} \quad (11)$$

Notice that the $q_i = r_i/[i]$ (gi/gX/h). The i accounts for the S, P and O_2.

The [P] represents the concentration of proteins or enzymes. The $[O_2]^*$ represents the concentration of oxygen in the equilibrium. The $[S_{fb}]$ represents the lactose concentration of the solution that is fed to the reactor during the production phase. The ΔQ_v represents the variation of the volume of liquid inside the reactor, the Q_{base} represents the ammonia flow rate that was fed to the reactor, the Q_{fb} represents the substrate inducer (lactose) solution that is fed to the reactor during the production phase and the Q_{evp} is the amount of water that is lost through evaporation by the outlet of air. The first two flow rates are known from the laboratory data, but the last one was calculated by doing a mass balance to the water in the gas phase in the reactor, as demonstrated below in Equation 12:

$$Q_{\mathsf{air,\,in}} H_{\mathsf{a,\,in}} + Q_{\mathsf{evp}} = Q_{\mathsf{air,\,out}} H_{\mathsf{a,\,out}} \quad (12)$$

The $Q_{air,\,in}$ and the $Q_{air,\,out}$ represent the air flow rate at the inlet of the reactor and outlet, respectively. The inlet air flow rate is known and the outlet is assumed to be equal to it. The $H_{a,\,in}$ and the $H_{a,\,out}$ are the absolute humidity at the inlet and outlet of the reactor, respectively. To simplify the calculations, it was assumed that the air at the inlet is dry which means that the inlet humidity of the air is zero. The outlet humidity of the air was calculated by the Equation 13:

$$H_{\mathsf{a,\,out}} = \frac{Pi}{P - Pi} \quad (13)$$

The Pi represents the partial pressure of water and the P the total pressure inside the reactor that is 1 atm. It was also assumed that the air comes out saturated, meaning that the relative humidity is 100% and that the Pi it's equal to the vapour pressure of water (P_V). Therefor the Pi is replaced by Pv, vapour pressure, in the equation 13. The vapour pressure was obtained from the NIST website for a temperature of 25 ºC. Note that this humidity is define in moles so it was necessary to change to mass units using the molar masses of the air and water that are 28,96 g/mol and 18,02 g/mol, respectively. Also the air flow rate was passed from volumetric units to mass units considering the air as an ideal gas. In the Table 2 the values of all parameters included in this calculation are presented for the conditions used in the first part of the results (1 reactor system) and for the conditions used in the second part of the results (2 reactors system with two different configurations: bizone and inverted bizone. Regarding the setup employed in the second part of the results, the evaporation of the anaerobic reactor was calculated assuming that the nitrogen has the same psychometric properties of the air.

Table 2: Values of the Kinetic Parameters of the existing model.

Parameter	Value	Units
$Q_{air,\,in}$	0.3	L/min
$Q_{air,\,in}$ bizone aerobic	0.08	L/min
$Q_{air,\,in}$ bizone anaerobic	0.25	L/min
$Q_{air,\,in}$ inv. bizone aerobic	0.25	L/min
$Q_{air,\,in}$ inv. bizone anaerobic	0.25	L/min
P_v	23.75	mmHg
$H_{a,\,out}$	0.032	mol H_2O/mol Air
Q_{evp}	1.53×10^{-7}	L/s
Q_{evp} bizone aerobic	3.17×10^{-8}	L/s
Q_{evp} bizone anaerobic	9.57×10^{-8}	L/s
Q_{evp} inv. bizone aerobic	5.96×10^{-8}	L/s
Q_{evp} inv. bizone anaerobic	9.57×10^{-8}	L/s

As the model had to be adapted for two reactors it was necessary to include a term in each mass balance for each component to account with the recirculation lines between the two reactors. This term has the following construction: $Q_{r\,1\text{-}2}[i]_1$-$Q_{r\,2\text{-}1}[i]_2$. The $Q_{r\,1\text{-}2}$ is the flow rate that comes from reactor 1 to reactor 2 and the $[i]_1$ is the concentration of the component in the reactor 1.The other variables follow the same logic. This term is an example for the reactor 2 mass balances. For the reactor 1 the term is written on the other way around.

It was also necessary to modulate the gas phase in order to know the variation of the concentration of oxygen in the liquid phase. To do that it was necessary to do a mass balance to the oxygen in the gas phase to then calculate the molar fraction of oxygen in the gas phase that will affect the oxygen concentration in the equilibrium according to the Henry's Law and consequently the OTR (oxygen transfer rate) varies during the fermentation. This calculations were done by knowing the inlet conditions of the air its flow rate which was used to calculate the oxygen and nitrogen inlet flow rates assuming the air as an ideal gas and that its composition is 21% molar of O_2 and 79% molar of N_2. The equations that represent the explanation given above are presented below:

$$\frac{d[O_2]_{\mathsf{gas}}}{dt} = \frac{Q_{\mathsf{O_{2_{in}}}} - Q_{\mathsf{O_{2_{out}}}} - OTR \times V_{\mathsf{gas}}}{V_{\mathsf{L}}} \quad (14)$$

$$f_{\mathsf{O_2}} = \frac{[O_2]_{\mathsf{gas}}}{32} \times \frac{RT}{P} \times 1000 \quad (15)$$

$$K_{\mathsf{H}} = \frac{[O_2]^*}{pO_2} \quad (16)$$

$$OTR = k_{\mathsf{L}}a(K_{\mathsf{H}} \times f_{\mathsf{O_2}} \times P \times 32 - [O_2]) \quad (17)$$

$$Q_{\mathsf{O_{2_{out}}}} = Q_{\mathsf{N_2}}\frac{1 - f_{\mathsf{O_2}}}{f_{\mathsf{O_2}}} \quad (18)$$

The R, represents the ideal gas constant, the T represents the temperature of operation and the K_H represents the Henry's law constant for oxygen that has the value of 0,0013 mol/L.atm at 25 ºC obtained from the NEBIST website. The $[O_2]_{gas}$ represents the concentration of oxygen in the gas phase. The $Q_{O_{2_{in}}}$ and the $Q_{O_{2_{out}}}$ represent the inlet and outlet flow rate of oxygen

in the reactor. The V_{gas} is the volume of gas inside the reactor. The f_{O_2} is the molar fraction of oxygen. The Q_{N_2} is the inlet flow rate of nitrogen in the reactor.

To solve the differential equations, the Euler method was used, by giving initial values to the variables. This method, is an explicit one, which means that the values of the variables are calculated from the values from the time step before.

2.7. k_La and Hold-up Calculations

The values of the partial mass transfer coefficient of oxygen and the hold-up of gas inside the reactors were calculated for the experiences with the setup of two reactors in order to modulate the gas phase and the transfer of oxygen from the air to the liquid. These constants were calculated for the conditions of the bizone configuration and the inverted bizone configuration (these experiences are explained below in the experimental procedure chapter). For the modelling work with the data that was obtained with one reactor, the value of the k_La was considered to be 0,1 s^{-1} and the hold-up parameter wasn't calculated, because the gas phase wasn't taken into account for the calculations in that part and so, the oxygen concentration in the liquid phase was set constant at 40% of the saturation as it was done in the laboratory.

The calculations of these variables were done according to the explanation given by (Cappello et al. 2020), and are presented below in the following equations.

It begins with the calculation of the shear rate, for bioreactors smaller than 5 m^3, through the Metzner & Otto correlation, as presented in the Equation 19.

$$\dot{\gamma} = 11.5N \quad (19)$$

The N represents the stirring rate of the impeller.

Then the apparent viscosity was calculated by the Equation 20.

$$\mu_{\text{app}} = K\dot{\gamma}^{\text{n-1}} \quad (20)$$

The K and the n are parameters that define the rheology of the medium and it varies during the fermentation. As these calculations were only done for the second part of the results and in this part only the production phase was taken into account (further explanation about this is given in the presentation of the second part of the results), these parameters were calculated for that step of the process. Therefor the n is equal to 0,2 and the K is calculated according with the Equation 21:

$$K = 0.003[X] \quad (21)$$

Then it was required to calculate the value of Pg/V that is the input of power per cubic meter in the reactor. Pg is defined by the Equation 22:

$$Pg = RPD \times Pu + Pa \quad (22)$$

In which RPD is the relative power demand and it's obtained by the Equation 23. The Pu is the power required if there is no aeration. The Pa is the power linked to the injection of gas on the bottom of the reactor.

$$RPD = max[RPD_{\text{lim}}, exp(-15.36{N_{\text{p}}}^{0.16} {Q_{\text{G}}}^{0.62}T^{\text{-1.7}}(D/T)^{0.51})] \quad (23)$$

According to (Gabelle 2012a) the value of RPD_{lim} is 0,33. The N_p is the power number of the impeller, D is the impeller diameter, T is the reactor diameter and the Q_G is the gas flow rate.

Then the Pa and the Pu were calculated by the Equations 24 and 25, respectively:

$$Pa = \rho L.g.V_{\text{sg}} \quad (24)$$

$$Pu = \rho L.Np.N^3.D^5 \quad (25)$$

The V_{sg} represents the gas superficial velocity, the ρL represents the liquid density that we considered to be mostly water and so the value given was 1000 Kg/m^3 and the g represents the gravity acceleration that has the value of 9,8 m/s^2.

Finally, with the following correlation, represented by the Equation 26, the k_La and the gas hold up (α_G) were obtained.

$$Z(\equiv \alpha_{\text{G}}, k_{\text{L}}a) = c_1(Pg/V)^{c_2}{V_{\text{sg}}}^{c_3}{\mu_{\text{app}}}^{c_4} \quad (26)$$

In the Table 3 the values of the constants c_1, c_2, c_3 and c_4 are presented for both the k_La and the hold-up.

Table 3: Values of the constants that were used to calculate the k_La and the hold-up.

Constant	k_La (s^{-1})	Hold up
c_1	0.000586	0.042
c_2	0.45	0.32
c_3	0.18	0.44
c_4	-0.48	-0.07

In the Table 4 the values of the parameters needed to calculate the k_La and the hold-up are presented.

Table 4: Values of the Parameters used in the k_La and hold-up calculations.

Parameter	Big Reactor	Small Reactor
N (rpm)	1000	1000
Np	2	2
D (m)	0.08	0.055
T (m)	0.16	0.11

In the Table 5 below the values of k_La and hold-up obtained for both reactors in both configurations (bizone and inverted bizone) are presented.

Table 5: Values of K_La and hold up that were calculated for both reactors for the bizone and inverted bizone configurations.

Parameter	Big Reactor	Small Reactor
k_La bizone (s^{-1})	0.11	0.18
k_La inv. bizone (s^{-1})	0.11	0.14
Hold-up biozone	0.018	0.046
Hold-up inv. biozone	0.029	0.045

3. Considerations taken into account

During the entire work it was considered that the systems were perfectly mixed which means that it was considered that all the elements of volume have the same residence time. In the end of the results it was verified if this assumption is very far from a case where the reactor has a behaviour according to a residence time distribution that is represented by the Equation 27:

$$E(t) = \frac{1}{\theta}e^{\text{-t/}\theta} \quad (27)$$

The E(t) accounts for the residence time distribution, the θ represents the average residence time and the t represents all the other residence times

4. Results and Discussion

4.1. Modelling of the Scale up Problems

The scope of this work is the modelling of cultures under conditions that represent scale-up problems, their effect on the production of proteins and see if the model can explain the results or do we need to improve it. As presented in the methods, the experiences that were performed to study this were the bizone cultures, that consist on a system with two reactors with different sizes, connected between each other. One of the reactors is operated under aerobic conditions and the other under anaerobic conditions. This represents the problem of poor mixing in big bioreactors, with hundreds of cubic meters, that can lead to bad dispersion of the bubbles of air and therefor to the creation of zones with low levels of oxygen in the liquid. Also the

lactose it's only fed in the aerobic reactor which represents the problem of having gradients of nutrients due to its feeding on the top of big bioreactors. Two type of experiences, as mentioned above, will be approached: the bizone experience and the inverted bizone experience. In the first one the bigger reactor is the aerobic one and the small is the anaerobic. This case represents the case where the bad mixing induces the formation off small zones that are unaerated. The inverted bizone, as the name suggests, it's the other way around which that the bigger reactor is the anaerobic one representing the possibility of the mixing being so bad that the zones that have low levels of dissolved oxygen are big. To compare the production results from these experiences Tamiris also did a control experience that consists on a one reactor system operated under aerobic conditions with the total volume of liquid of the 2 reactors system and with the same dissolved oxygen concentration (40%).

The production is the phase that matters and to simplify the computations and the calculations the simulations were started around 160 hours. Also because the growth was carried out in a single reactor, only 4 days after the beginning of the fed batch the system was operated with two reactors as explained in the methods. So it was considered during the modelling work that the growth phase is the same for all the considered cultures and doesn't affect the subsequent phase, the production.

4.2. Bizone Experience Modelling

First in the Figure 4 a comparison between the experimental results of the bizone culture K1439 and the control experience K1452 is presented.

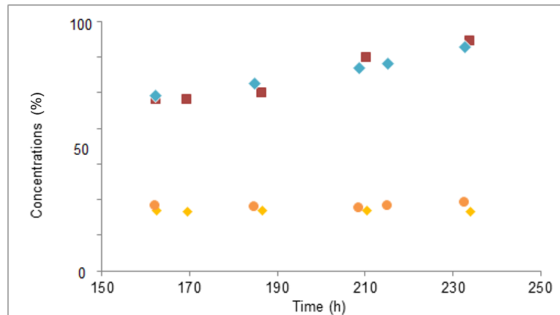

Figure 3: Comparison between the experimental results of the experiences bizone K1439 and control K1452. The ◆ represents the biomass concentration from the bizone experience K1439 and the ● represents the biomass concentration from the control experience K1452. The ◆ represents the proteins concentration from the control experience K1452 and the ■ represents the proteins concentration from the bizone experience.

As it can be seen in the Figure 4 there is no loss of production between the control experience and the bizone experience, which means that the q_p that was measured in the laboratory is the same for both experiences. The values of the experimental q_p are presented below in the Table 6. What this results says is that the anaerobic reactor isn't big enough to affect the global production of the system since in this reactor there is no biological activity due to the fact that the dissolved oxygen concentration is close to zero.

Table 6: Experimental values of q_p from the experiences K1439 (bizone) and K1452 (control).

Parameter	Value	Units
q_p exp. control	72	%
q_p exp. bizone	72	%

Now let's see if the initial model can predict the same results for both experiences. In the Figure 4 the modelling results for both experiences, bizone and control, are presented. The parameters used in this modulation are the ones presented in Table 1 and in the Table 7 below.

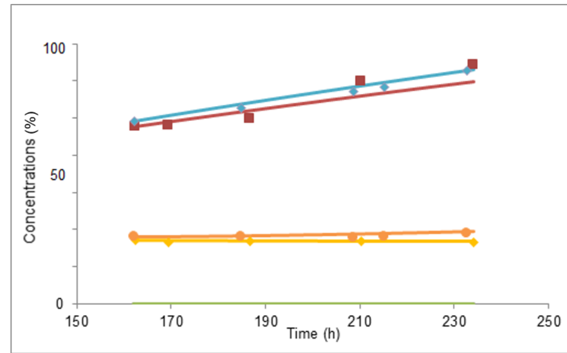

Figure 4: Modelling results of the cultures K1439 (bizone) and control (K1452). The markers have the same legend as Figure 4. The — represents the model predictions of biomass concentrations from the bizone culture. The — represents the model predictions of biomass concentrations from the control culture. The — represents the model predictions of Protein concentrations from the bizone culture. The — represents the model predictions of Proteins concentrations from the control culture. The — represents the model predictions of Substrate concentrations from the bizone culture. The — represents the model predictions of Substrate concentrations from the control culture.

Table 7: Values of the Kinetic Parameters that were used in the modelling results in this part of the thesis.

Parameter	Value	Units
μ_{max} (K1439)	0.08	h^{-1}
μ_{max} (K1452)	0.08	h^{-1}
q_{pmax} (K1439)	88	%
q_{pmax} (K1452)	88	%

From the analysis of the results showed in the Figure 4 it can be seen that the model has a good explanation of the control experience giving the same value for the q_p that was found experimentally. For the bizone experience the model gives a good prediction for the biomass concentrations and for the proteins even though, the proteins curve isn't perfectly overlapped with the experimental points giving a q_p value that is slightly smaller compared with the experimental data (64 %). This is acceptable once we are dealing with living beings and its behaviour is not always predicted by a straight line as this model makes it. Although, the difference of q_p from the model of the bizone compared with the experimental isn't significant since it is explained by the experimental error of the measures that were carried out in the laboratory.

Besides the experience K1439, Tamiris did also other bizone experiences as repetitions of the K1439. The model was also applied to these cultures using the same values of the kinetic parameters already presented as a way to confirm that these values are the optimal ones and give a good prediction of all the cultures in question.

Although, it was necessary to make a modification in the model regarding the maintenance coefficients. In the first attempts of modelling the bizone experience the values of dissolved oxygen concentration of the anaerobic reactor turned to negative after a certain time. This happened because the values of q_{O_2} were to high due to the maintenance term since there is almost no oxygen the terms of growth and production were small. To solve this problem, it was decided to put the maintenance coefficient of substrate changing according to a Monod type equation for the substrate and a Michaelis-Menten term for the oxygen as presented below in the Equation 28. This to say that as the oxygen available decreases so does the amount of substrate that can be metabolized for the maintenance and so the values of q_{O_2} decrease allowing the dissolved oxygen concentration to remain positive.

$$m_{\mathrm{s}} = m_{\mathrm{s\,max}} \frac{[S]}{K_{\mathrm{s\,M}} + [S]} \times \frac{[O_2]}{K_{\mathrm{O_2\,M}} + [O_2]} \tag{28}$$

The $m_{\mathrm{s\,max}}$ represents the maximum value that the maintenance can reach, the $K_{\mathrm{s\,M}}$ is the Monod constant for the

substrate for the maintenance and the $K_{O_2\,M}$ is the Michaelis-Menten term for the oxygen for the maintenance. The values of these new kinetic constants that best fit the experimental points are presented below in the Table 8.

Table 8: Values of the parameters that define the new maintenance mechanism.

Parameter	Value	Units
$m_{s\,max}$	0.01	gS/gX/h
$K_{s\,M}$	0.001	gS/L
$K_{O_2\,M}$	0.0001	gO_2/L

4.3. Inverted Bizone Experience Modelling

After checking that the model has a good prediction of the bizone experiences and after finding the correct kinetic values we moved forward to test the model on the inverted bizone experience. To start, in the Figure 5 a comparison between the experimental results of the control experience K1452 and the inverted bizone K1455 is presented.

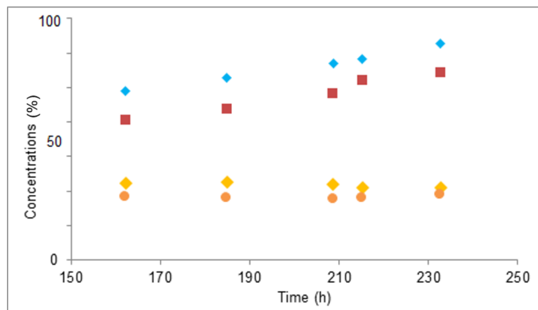

Figure 5: Comparison between the experimental results of the experiences inverted bizone K1455 and control K1452. The ◆ represents the biomass concentration from the bizone experience K1439 and the ● represents the biomass concentration from the control experience K1452. The ◆ represents the proteins concentration from the control experience K1452 and the ■ represents the proteins concentration from the bizone experience.

As it can be seen in the Figure 5 there is a loss of q_p from the inverted bizone compared with the control that was verified experimentally. This means that the loss of biological activity in the system, due to the fact that the bigger reactor is anaerobic, isn't negligible. Although there is still some production even though smaller than in the control. In the Table 11 the value of q_p obtained experimentally for the inverted bizone experience is presented.

Table 9: Value of the experimental q_p for the inverted bizone experience.

Parameter	Value	Units
q_p exp. inverted bizone	44	%

So, now the aim of this work is to see once again if the model can explain this loss of q_p that was verified in the laboratory or is it necessary to improve the model in order to have a good prediction of the results. In the Figure 6 the first attempt of modelling the inverted bizone is presented. All the modelling attempts of this experience were done using the same kinetic parameters that were used in the bizone modelling.

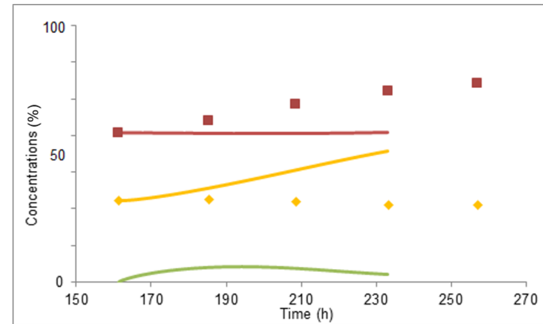

Figure 6: Modelling results for the culture K1455 (inverted bizone) in which the marker ◆ represents the experimental points of Biomass concentration and the ■ represents the experimental points of Protein concentration. The — represents the model prediction for the biomass concentration, the — represents the model prediction for the proteins concentrations and the — represents the model predictions for the substrate concentration.

By analysing the Figure 6 it can be clearly understood that the model doesn't predict at all the behaviour of the inverted bizone culture. The results of the model are quite logical taking into account its features. As there is a big part of the volume of the system that is unaerated the sugar starts to accumulate because as there is no oxygen there is no production, therefore the substrate isn't consumed. After a certain value of substrate concentration, the fungi start to grow in the aerobic reactor instead of producing. However, like explained before, this isn't what happened experimentally, so it was required to think what can be implemented in the model in order for it to predict the behaviour of this culture. Like it was said before in the laboratory it was verified that even that the production suffered a loss there is still some biological activity that as it was seen the model doesn't predict. This can only mean that experimentally, there is some activity/production in the anaerobic reactor and, in order for it to occur there must exist some kind of transport of oxygen from the aerobic reactor to the anaerobic reactor that it is not being taken into account. Notice that the dissolved oxygen in already being taken into consideration in the recirculation between the reactors but as it can be seen in the Figure 6 isn't enough to explain the experimental results.

At this point we started to think about phenomena that could explain the additional transport of oxygen to the anaerobic reactor and we came up with to possible theories:

1. Recirculation of bubbles of air inside together with the broth;

2. The cells can store oxygen inside of them.

During the course of the experience it was verified that there were some bubbles of gas that were transported between the two reactors but its flow rate wasn't known. To include this phenomenon in the model it was necessary to include some terms in the equations that define the gas phase of each reactor. The new equations for the gas phase for one of the reactors as an example, considering the recirculation of gas are presented below:

$$\frac{d[O_2]_{\text{gas R1}}}{dt} = \frac{(Q_{O_2\,\text{in R1}} - Q_{O_2\,\text{out R1}} - OTR_{\text{R1}} \times V_{\text{L R1}})}{V_{\text{gas R1}}} + \\ + \frac{(Qrg_{2\text{-}1} \times f_{O_2\,\text{R2}} - Qrg_{1\text{-}2} \times f_{O_2\,\text{R1}}) \times 32}{V_{\text{gas R1}}} \quad (29)$$

$$f_{O_2\,\text{R1}} = \frac{[O_2]_{\text{gas}}}{32} \times \frac{RT}{P} \times 1000 \quad (30)$$

$$OTR_{\text{R1}} = k_l a_{\text{R1}} (K_{\text{H}} \times f_{O_2\,\text{R1}} \times P \times 32 - [O_2]_{\text{R1}}) \quad (31)$$

$$Q_{N_2\text{ out R1}} = Q_{N_2\text{ in R1}} + (Qrg_{2\text{-}1} \times (1 - f_{O_2\text{ R2}}) \tag{32}$$

$$-Qrg_{1\text{-}2} \times (1 - f_{O_2\text{ R1}})) \times 28$$

$$Q_{O_2\text{ out R1}} = \frac{Q_{N_2\text{ out R1}}}{28} \frac{1 - f_{O_2}}{f_{O_2}} \times 32 \tag{33}$$

As the gas flow rate of the recirculation wasn't known, some tests were carried out with arbitrary values in an attempt to find the value of gas hold-up inside the recirculation lines that could explain the results. The value for the gas hold-up in the recirculation that was found to give a good explanation of the results was 40%. In the Figure 7 it's presented the modelling results of the inverted bizone experience with 40% of hold-up in the recirculation lines.

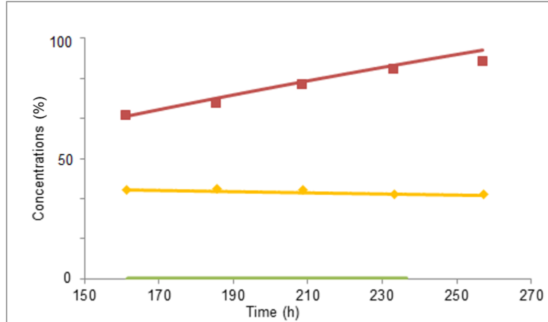

Figure 7: Modelling results for the culture K1455 (inverted bizone), considering the gas recirculation with 40% of hold-up, in which the marker ◆ represents the experimental points of Biomass concentration and the ■ represents the experimental points of Protein concentration. The — represents the model prediction for the biomass concentration, the — represents the model prediction for the proteins concentrations and the — represents the model predictions for the substrate concentration.

With this results presented in the Figure 7 it can be said that the recirculation of gas can be one explanation for the experimental results of the inverted bizone culture since the model has a good to the experimental points. Although, in order to have this results it was necessary to consider a gas hold-up of 40% inside the recirculation lines which probably isn't real because this value is too big.

So to confirm our ideas Tamiris repeated the experience in the lab during which she measured the gas flow rate in the recirculation lines between the reactors and found that the real gas hold-up is something around 17%. With this new value the modelling of the inverted bizone was repeated and the result can be found below in the Figure 8.

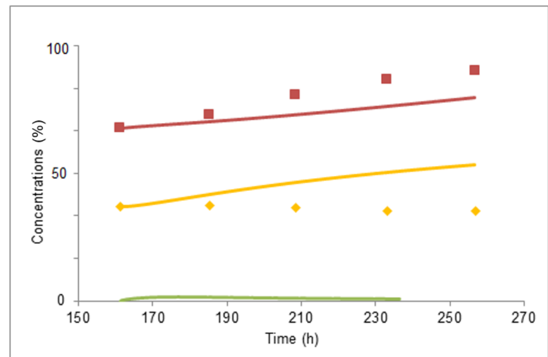

Figure 8: Modelling results for the culture K1455 (inverted bizone), considering the gas recirculation with 17% of hold-up, in which the marker ◆ represents the experimental points of Biomass concentration and the ■ represents the experimental points of Protein concentration. The — represents the model prediction for the biomass concentration, the — represents the model prediction for the proteins concentrations and the — represents the model predictions for the substrate concentration.

By analysing the Figure 8 it can be understood that the using the model with recirculation of gas with 17% of hold-up isn't enough to explain the experimental results of the inverted bizone. However, by comparing these result with the one presented in the Figure 5 we can see that the using 17% of hold-up the model can explain a part of the results giving some production and consequently less substrate accumulation.

Nonetheless, the recirculation of gas by itself isn't enough to improve the quality of the model, therefore we started to think about the second option that consists in considering that the fungi can store oxygen inside the cells. This theory consists on saying that when the fungi are in the aerobic reactor they take the oxygen from the liquid which is transformed in an intermediary molecule that functions as an oxidative power transporter. This intermediary can be accumulated inside the cells until a maximum value that defines the storage capacity of this molecule and to be used in the metabolism of the cells instated of the oxygen directly. The reaction of transforming the oxygen into W was modulated as a first order on the oxygen concentration.

To implement this theory, we had to change the kinetics of the model by creating a new component, the intermediary molecule that we called W, and define the maximum amount of W that the cells can store that we called W_{max}. First we defined the W_{max} as proportional to the biomass concentration by the Equation 34:

$$[W_{max}] = A[X] \tag{34}$$

Then a mass balance to the W was required. This balance is presented below by the Equation 35:

$$\frac{d[W]}{dt} = K_W[O_2]([W_{max}] - [W]) - q_W[X] \tag{35}$$

The first term defines the amount of W that can be formed and stored inside the cells and the second term is the amount of W that is consumed by the biomass to grow, produce and for maintenance. The KW represents the quickness of the transport and transformation of oxygen to W. The q_W represents the specific consumption of W that now replaces the q_{O_2} in the model.

The idea with this theory is to say that when the fungi are in the aerobic reactor they store the W close to the value of W_{max} and as they consume it they store again close to the maximum value. When the fungi arrive to the anaerobic reactor they start to consume the W that is stored inside the cells but now as the oxygen levels are very low the W isn't replenished as fast as it is consumed and the W concentration drops to low values (the W_{max} value is around 0,05 gW/L). As the fungi come back to the aerobic reactor they refill the storage of W again.

The other equations that define the model were also modified by replacing the oxygen concentration and the Michaelis-Menten constants and the yields related with oxygen by the W concentration and the constants related with the W. The new equations of the model are presented below:

$$\mu = \frac{\mu_{max} \times [S]}{K_{s\mu} + [S]} \times \frac{[W]}{K_{W\mu} + [W]} \tag{36}$$

$$q_p = \left(\frac{q_{pmax} - q_{pmin}}{1 + [X]/K_{iXP}} + q_{pmin}\right) \times \frac{[S]}{K_{SP} + [S]} \times \tag{37}$$

$$\times \frac{[W]}{K_{WP} + [W]} \times \frac{K_{iXP}}{K_{iXP} + [S]}$$

$$q_W = \frac{\mu}{Y_{x/W}} + \frac{q_p}{Y_{p/W}} + Y_{W/s}{}^{m} \times m_s \tag{38}$$

$$m_s = m_{s\,max} \frac{[S]}{K_{s\,M} + [S]} \times \frac{[W]}{[W] + K_{W\,M}} \tag{39}$$

The values of the Michaelis-Menten constants and the yields related with the W were kept the same as the ones that were presented in the Table 1 since it was considered that 1 gram of oxygen is equal to 1 gram of W.

The mass balance to the dissolved oxygen was also changed because now the decrease of oxygen in the medium isn't due to its direct consumption but due to the amount of oxygen that is transferred to inside the cells. The new mass balance to the oxygen in the liquid phase is presented below by the Equation 45:

$$\frac{d[O_2]}{dt} = \frac{-K_{\mathrm{W}}[O_2]([W_{\mathrm{max}}] - [W])}{Y_{\mathrm{W/O_2}}} + \qquad (40)$$
$$+k_{\mathrm{l}}a([O_2]^{*} - [O_2]) - \frac{[O_2]}{V_{\mathrm{L}}}\Delta Q_{\mathrm{v}}$$

The Y_{W/O_2} is the yield of the transformation of oxygen to W that it was assumed to be 1.

After making these implementations, some attempts of modelling the inverted bizone were made in order to try to find the correct values for the new variables that define this new mechanism of storage of oxygen. The optimal values that were found for the new parameters are presented in the Table 10.

Table 10: Values of the kinetic parameters of the Oxygen Storage theory.

Parameter	Value	Units
K_W	195	gW/gO_2
A	0.0528	gW/gX

After finding the right values for the new kinetic parameters we were able to modulate the inverted bizone experience. In the Figure 9 it's presented the modelling of the inverted bizone experience considering the storage of oxygen inside the cells.

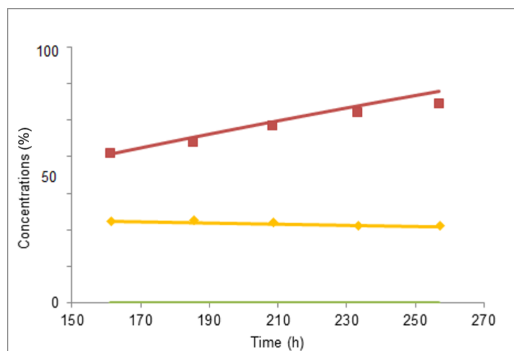

Figure 9: Modelling results for the culture K1455 (inverted bizone), considering the storage of oxygen inside the cells, in which the marker ◆ represents the experimental points of Biomass concentration and the ■ represents the experimental points of Protein concentration. The — represents the model prediction for the biomass concentration, the — represents the model prediction for the proteins concentrations and the — represents the model predictions for the substrate concentration.

In the Figure 9 it can be seen that this theory works very well since the model gives a good explanation of the experimental results. In the Table 17 the value of q_p obtained by the model with the W kinetics is presented.

Table 11: Value of q_p predicted by the model with the W kinetics.

Parameter	Value	Units
q_p model inverted bizone	48	%

The q_p predicted by the model is practically the same that was obtained experimentally (error of 9%) which means that the model gives a good prediction of the experimental results. Even thought, this idea is just a theory that we don't know if this is what really happens. Furthermore, we couldn't find any bibliographic information about the possibility of storage oxygen by any filamentous fungus. This is just an apparent kinetics to try to give an explanation of the experimental results. Experimentally this process of storage of oxidative power is probably much more complex than the way we choose to model it but as it was explained before in the first part of the results of this theses, the objective is to keep the model as simple as possible.

To validate this new kinetics, the bizone and the control experiences were also modulated with it using the same values of the parameters that were employed in the modelling of the inverted bizone. The results obtained with the new kinetics were exactly the same that were reached with the oxygen kinetics, which validates the new kinetics.

However, it was already shown that there was recirculation of bubble of gas between the two reactors with 17% hold-up that is responsible for a part of the productivity of the culture as it was seen in the Figure 8. This way, we thought that probably the more realistic approach is to consider the two phenomena at the same time: the bubble recirculation and the storage of oxygen. So a modelling experience was done considering the 17% of hold-up and the W kinetics. However, the value of A was changed (0,002 gW/gX) in order to give a lower value of W_{max}, something around 0,02 gW/L, since now there is no need to use a value so big of W because there is also the oxygen that is transported through the bubbles of air in the recirculation. In the Figure 10 the result of the modelling of the inverted bizone considering the two phenomena is presented.

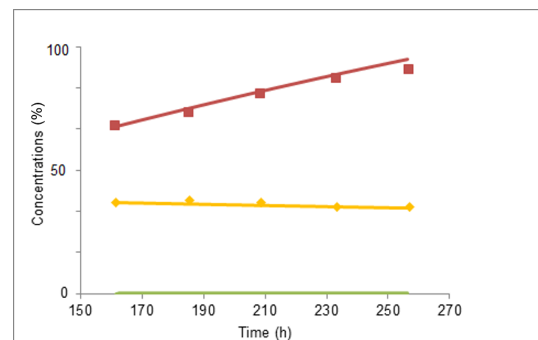

Figure 10: Modelling results for the culture K1455 (inverted bizone), considering the 17% of hold-up in the recirculation and the W kinetics, in which the marker ◆ represents the experimental points of Biomass concentration and the ■ represents the experimental points of Protein concentration. The — represents the model prediction for the biomass concentration, the — represents the model prediction for the proteins concentrations and the — represents the model predictions for the substrate concentration.

From the Figure 10 it can be seen that the combination of the two phenomena gives a good explanation of the experimental results with the same q_p that was obtained by the model with the W kinetics alone.

5. Conclusions

By going through the analyses of the results presented it can be said that, the initial model is enough to explain the results of the bizone experience but, overestimates the effect of large heterogeneities which are behind the experimental results of the inverted bizone experience. To improve the model two different phenomena were considered: the bubble recirculation and the storage of oxygen inside the cells. The first one only explains a part of the differences between the initial model and the experimental results. The storage of oxygen was proven to be a possible explanation for all the experiences. A combination of both phenomena is the most realistic approach that was used giving a good prediction of the experimental results of the inverted bizone experience.

The assumption of a perfectly mixed system in the conditions that were used in the laboratory was proven not to be introduce a significant error in the results given by the model. However, it was found that if other conditions are used the RTD has to be taken into account in the calculations in order not to have a big deviation between the model predictions and the reality.

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