Development of wine fermentation using immobilized cells

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December, 2015

Abstract: The wine industry is a great interest area in Europe due to the appearance of emerging markets. The recent appearance of immobilized yeast cell lines, allowed the passage of a wine making process traditionally done in batch to a continuous process, increasing the profitability of this sector and preserving wine quality. This study aims to adapt a kinetic model, originally designed for a system of free cells, to operate with immobilized cells facilitating the continuous operation design. Once validated the model, data related with sugar, ethanol and cell concentration evolution obtained from previous fermentations with immobilized cells (beads concentration of 152.6 $g.L^{-1}$ and 39.6 $g.L^{-1}$) was compared to the behaviour proposed by the model. Once the model did not followed the data during the fermentation, it was performed a parametric estimation. New data was obtained (beads concentrations of 39.6 $g.L^{-1}$ and 9.1 $g.L^{-1}$) from 6 fermentations (batch), screening the product and reducing sugars concentration evolution, as well as cell number, in different time points of fermentation. After comparing the experimental results with the behaviour proposed by the original model, it was possible to observe considerable differences. Due to this, the model was manipulated in order to achieve a better correlation. With the changes applied to the model some improvements were obtained, mainly for the concentration of 39.6 g L^{-1}. In brief, it was possible to adapt the model to follow the experimental data, which can be considered a promising result to facilitate the development of a continuous fermentation.

Keywords: Winemaking; Alcoholic fermentation; Immobilized cells; Kinetic equations; Modelling; Batch/Continuous operation

Nomenclature: t is the fermentation time (h);P(t) and S(t) are respectively the concentrations of product and substrate in $g.L^{-1}$; N(t) is the concentration of nitrogen assimilated by yeast ($g.L^{-1}$); X(t) is the total number of cells in the medium ($cells.L^{-1}$);N_{max} corresponds to the maximum concentration of assimilable nitrogen ($g.L^{-1}$);T(t) is the fermentation temperature (ºC); T_{ucd} (t) is the temperature corresponding to the end of the cell growth phase, which is equal to the temperature of the stationary phase (°C) (which remains constant); $K_1(T)$ is the biomass growth rate in the exponential phase; X_{max} is the maximum biomass concentration versus the initial nitrogen concentration; K_S is the affinity constant of sugar transporters ($g.L^{-1}$); K_{SI} is the constant for the inhibition of sugar transporters by ethanol ($L.g^{-1}$);α_S is other constant; $K_2(T)$ is a function depending on temperature. Overall this equation describes the active transport of glucose per transporter ($g.h^{-1}$); K_N is the affinity constant of yeast cells for nitrogen transport ($g.L^{-1}$);K_N is the constant for the inhibition by ethanol of nitrogen transport in yeast cells ($L.g^{-1}$); α_N is other constant;K_3 (T) is a function depending on temperature. Overall, this function describes the active transport of nitrogen per cell ($g.h^{-1}$).

Functions: ν_{ST} is the specific rate of glucose transport (h^{-1}); N_{ST} corresponds to the synthesis of glucose transporters from a fraction of absorbed nitrogen (g); ν_N is the specific rate of nitrogen transport ($g.h^{-1}$).

1. Introduction

The process by which the wine is produced is called alcoholic fermentation. Fermentation processes may be classified according: how the substrate is added and the production withdrawn. Thus, in a batch fermentation, the substrate is added initially, and when it reaches the end of the fermentation, the product is discharged out of the tank. The continuous operation involves successive addition of substrate to the fermentation medium at the same rate at which the product is withdrawn[1]. In the last few years, the French winery industry as the European one, has suffered competition from new emerging economies[2]. To struggle this, the investigation of new oenological techniques has risen in France and in the rest of European Union. In winemaking, the continuous fermentation system should be capable of respond to a major problem that is the inhibitory effect of the products formed during the growth of the microorganisms. However, when immobilized cells are used, this problem is softened as immobilized cells are more tolerant to inhibitory end products such as ethanol. Alcoholic fermentation is the biological transformation of grape sugars (primarily glucose and fructose) in ethanol and carbon dioxide, according to the Equation (1). This

production of alcohol is accompanied by the formation of by products such as glycerol, acetic acid, lactic acid, alcohols and esters.

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 \quad (1)$$

During alcoholic fermentation, the sugars contained in the grape are converted into alcohol by a series of complex reactions. Glycolysis, process common to fermentation and respiration, is the starting point, in which glucose is converted into pyruvic acid[3]. In fermentation conditions, pyruvic acid is decarboxylase into acetaldehyde, which is itself reduced to ethanol. The fermentation is generally conducted by yeasts of the specie Saccharomyces Cerevisiae. Although these are slightly present on the grapes (2-3%)[4], during fermentation they become the majority and thus constitute the principal motor of the fermentation.

Nowadays the wines are produced by batch fermentation, which means that the juice is placed in a vessel and the entire batch is kept there until fermentation is completed, normally after 5–10 days. The passage of the production process typically in batch for a continuous process will be important in this industry as a continuous operation permits production (with strict control), large volumes of the same wine with the guarantee of a standard quality [5]. In a batch process, the fermentation takes place in a tank where it is placed grape juice, keeping this space until fermentation end. In modern wineries are used cylindrical-conical vessel made of stainless steel. For many years, the systematic ability to control these tanks (temperature, aeration and agitation), made possible the use of this technology easily and successfully[6].However, batch fermentations have inherent inefficiencies, such as the low speed of the process and the dependency on the growth of cell populations [7].

Recently, they were developed several bioreactors developed in continuous operation. For example, the membrane reactor, where the reactions are continuously occurring with greater efficiency, because high cell densities are reached. Its application, among other models of continuous reactors are referenced in Strehaiano et al. (2006) [8] .Experiments at laboratory level have shown that the enormous potential of bioreactors technologies can bring in the winemaking. However, the scale-up to pilot or industrial scale, usually presents a number of challenges that require further study and knowledge, such as : the possible reaction instability of the yeast cells in the reactor due to the huge physical, chemical and biological stress [5;9].

The major step for the implementation of wine continuous fermentation was given with the appearance of immobilized cells on the market, which allows to obtain a standard product with greater safety [10]. Currently, it has been developed several immobilization techniques. Mostly are grouped into four categories, as described Verbelen et al.2006[26]: attachment to a surface; entrapment within a porous matrix; containment behind barriers and cell aggregation or flocculation. Nowadays the entrapment of cells in a porous matrix gel is a common technique in the food industry[8] .In the entrapment within a porous matrix there are two forms of entrapment for this technique. In one hand, the cells may be introduced into the porous material and after their growth, their mobility is reduced through the matrix and due to the presence of other cells. In the other hand, the matrix is synthesized in situ around the cells, or the matrix is not originally made[3]. The calcium alginate gel, used for stabilization and film formation, is the most common material used in this technique[11].

To describe the fermentations were developed mathematical models. Over the past fifteen years, numerous research models describing the kinetics behaviour of alcoholic fermentation have been published. The majority of the models are deterministic models which take into consideration main phenomena that affect the kinetics of alcoholic fermentation. There are other models resulting from the macroscopic analysis of the alcoholic fermentation process that require the estimation of fewer parameters, although their physiological significance is difficult to interpret[13]. The model presented herein was proposed by Malherbe et al. (2004)[13] is based on physiological considerations and takes into account the main phenomena that directly affect the kinetics in these specific conditions, such as: nitrogen in yeast synthesis (growth phase); the transport of sugar into yeast cells; ethanol inhibition; and temperature, including anisothermal conditions, which is known to be very common in wine-making[13]. The model is represented by the following equations:

$$\frac{dS}{dt} = -X(t).V_{ST}\big(S(t),P(t),T(t)\big).N_{ST}\big(N_{max}(t) - N(t),X(t),T_{ucd}(T)\big) \quad (2)$$

$$\frac{dN}{dt} = -X(t).V_N(N(t), P(t), T(t)) \quad (3)$$

$$\frac{dX}{dt} = K_1(T(t)).X(t).(1 - \frac{X(t)}{X_{max}(N_{init})}) \quad (4)$$

Where:

$$X_{max}(N_{init}) = \gamma_3(N_{init}^2) + \gamma_4 N_{init} + \gamma_5 \quad (5)$$

$$v_{ST}(S, E, T) = \frac{K_2(T)S}{S + K_S + K_{SI}SE^{\alpha_S}} \quad (6)$$

$$N_{ST}(N_{max}(t) - N(t), X(T), T)k_2(T) = \lambda_a \frac{N_i(t)}{X(t)} + \lambda_b T(t) + \lambda_c \frac{N_i(t)}{X(t)} T(t) + \lambda_d \quad (7)$$

$$v_N(N, E, T) = \frac{K_3(T)N}{N + K_N + K_{NI}NE^{\alpha_N}} \quad (8)$$

The concentration of ethanol, product of interest, is calculated from the amount of CO_2 which is released during the reaction. Assuming Gay-Lussac-like relationships[14], the concentrations of glucose S(t) and ethanol E(t) (or P(t)), together with *d*S/*d*t and *d*E/*d*t, are calculated applying the following Equations (9)(10) :

$$S(t) = S_0 - 2.17CO_2(t) \quad (9)$$

$$E(t) = 0.464[S(0) - S(t)] \quad (10)$$

Throughout the description made it is removed that the great novelty of this model is to take into account the physiological phenomenon of nitrogen assimilation, studying its effect.

Finishing the presentation of the model, there are some assumptions that should be taken into account. This model has a limiting step in the fermentation activity related to the number of existing hexose transporters in the plasma membrane, and not in the intracellular environment, because it is proved that the measured intracellular glucose concentration is very low. High levels of ethanol exerts a mechanical effect on glucose assimilation ability of both cells. The nitrogen, as already demonstrated, is implicitly linked to the synthesis of glucose transporters, present in small quantities, and a limiting factor on cell growth and activity of yeast.

2. Materials and Methods

2.1 Preparation of fermentation

2.1.1 Fermentation medium

The laboratory tests were conducted with a fermentation medium derived from a commercial grape juice ("100% grape juice Carrefour Discount, France"), in order to simulate the real conditions existing in a winery. The acquired commercial juice, contains 150 g.L^{-1} of reducing sugars, 2.3 g.L^{-1} of malic acid, 161 mg.L^{-1} of assimilable nitrogen and a pH of 3.45. Therefore, and in order to respect the industrial grape juice requisites, were added 60 g.L^{-1} of reducing sugars, which 50% are glucose and 50% fructose; 225 mg.L^{-1} of ammonium sulphate and 5 g.hL^{-1} of sulphur dioxide It was adjusted also the concentration of malic acid to 5 g.L^{-1}. These mixtures were homogenized by using the ultrasonic homogenizing device. The prepared medium was placed into Erlenmeyer flasks previously and it was sterilized, for approximately 30 min. (T = 121 ° C and P = 2 bar), in autoclave.

2.1.2 Immobilized yeasts

The yeast used is from the *strain S.cerevisiae var. bayanyus*. This immobilized yeast is provided by the Portuguese society Proenol Lda. designating this as ProRestart®, because is often used in slow fermentations. It is a yeast encapsulated in alginate (natural polysaccharide extracted from algae) acclimatized to alcohol and other difficult conditions. Since it is an encapsulated yeast, resists more easily to external aggressors such as alcohol.

2.1.3 Yeast rehydration

Before adding the beads to the fermentation medium, different sequential steps, provided by the fabricator, were followed in order to provide the appropriate operational conditions.

Initially, it was necessary to remove the beads, leaving them at room temperature. Then it was prepared a rehydration solution with a glucose concentration of 40 g.L^{-1}, at 37 ° C. For each gram of beads were added 5 grams of rehydration solution, leaving the suspension on rehydration for 5 hours before medium inoculation.

2.1.4 Batch fermentation

Batch fermentation tests were carried out in a 500 mL Erlenmeyer flasks, previously sterilized. The beads present in the rehydration medium, were added to 250 mL of fermentation medium. To avoid problems related to cell growth, the temperature of fermentation medium and rehydration solution was controlled in order to ensure a difference smaller than 10ºC between them.

The agitation and temperature were set in 100 rpm and 20°C respectively, in order to enable a good homogenization of the medium and good mass transfer between beads and medium.

2.2 Analytic methods

The manipulation of the Erlenmeyer flasks for samples (3 mL) and beads (4) removal at each time, was always done inside the fume hood (Faster® KBN), in order to avoid contaminations.

After separating the precipitate from the supernatant (free cells released from immobilized cells) by centrifugation, samples for later analysis were stored in the freezer (-20ºC) to prevent cell growth, keeping the sample characteristics.

2.2.1 CO_2 determination

During the fermentation, CO_2 released was controlled by weight loss measurement. This was calculated by weighing the Erlenmeyer flasks. Thus, in this study, measurements were taken every 30 minutes, at the various concentrations tested. The measurement was also made before and after the sample collection (for further analysis) in order to not introduce error. This sample quantity (3 mL) was the same throughout all time, and the same for all concentrations.

The validity of this technique as a monitoring method of a fermentation has been shown to be precise and accurate [15].

2.2.2 Biomass analysis

The concentration of cells in the medium and beads was determined using a Thoma haemocytometer[16]. The percentage of viable cells was estimated by counting after methylene blue staining. The reagent consists in a mixture of 0.1 g of methylene blue in 1L of sodium citrate 2% (w / v) , which is kept in a freezer, away from light and air[40]. During analysis, each sample was mixed, by agitation (vortex), with the same volume of methylene blue (100 µL each). This technique allow the differentiation between dead cells from live cells, since dead cells appear with blue colour while the others remain colourless.

Since the final goal is to have comparable data, and knowing that depending on the counting, the sample was further diluted or were counted more large squares, an equation was used in order to calculate the cell concentration per ml of sample:

$$X\ (\ cells\ concentration\ .mL^{-1}\ ) = Dilution \times \frac{Number\ of\ Cells\ Counted}{Number\ of\ Large\ Squares\ Counted} \times \frac{1}{4.10^{-6}} \quad (11)$$

Throughout the experiments both immobilized cells (which grow within the beads) and free cells (which are being released into the environment) were counted. To count the immobilized cells collected in each time point (4 beads), it was necessary to dissolve the carrier material (alginate gel) in a solution (1 mL) of sodium citrate 2 % (w / v), using an agitation by vortex (10min.).

2.2.3 HPLC

The HPLC was used to measure sugars (total) and ethanol presents in the sample. HPLC measurements were performed on columns Rezex ROA-H+, 250 mm long x 4.6 mm wide, with a mobile phase of H_2SO_4 1.87 mmol.L^{-1} circulating 170 µL.min^{-1} at 30ºC, in a Thermo Scientific Acella™ HPLC system (Thermo Fischer Corporation) mounted with an external detection device. Before starting the measurements related to ethanol and sugars (glucose, sucrose and fructose), standard solutions were prepared. It was weighed 500 mg (of each standard compound: ethanol, sucrose, fructose and glucose) in Sartorius CP214 Analytical Balance (more precision) and then were added 50 mL of water, performing a concentration of 10 g.L^{-1} (for each compound). Successive dilutions were done to prepare solutions of 8,6,4,2 g.L^{-1} (concentration of each standard compound). To minimize errors in the detection device, water samples were also prepared.

2.2.3 DNS method

This method was used to dose and monitor the consumption of reducing sugars. Thus, this method was as proof of the data collected from HPLC. The method principle says that the reducing sugars react with DNS (dinitrosalicylic acid reagent: dinitrosalicylic acid; rochelle salt; phenol; sodium bisulphite and sodium hydroxide) by reducing it to 3-amino-5-nitrosalicylic acid [17]. This reaction is accompanied by a colour change, from a yellow colour (corresponding to the DNS reagent) into an orange / red colour.

2.2.3 Assimilable nitrogen determination

The major nitrogen source for yeast are cations (NH_4^+), present in the medium. In addition, there are nitrogen groups present in amino acids (NH_3^+).

Initially, the sample pH is very low (about pH 3 or lower) .The pH of the solution is adjusted to a pH of 8. The amino groups after this adjustment, will form free carboxyl groups that react with the ammonia present in the medium. After addition of the formaldehyde, free protons are formed. That will be neutralized by NaOH (sodium hydroxide). Thus, knowing how much base is added, it is estimate the nitrogen present in the medium and accessible to yeasts.

2.3 Modelling

The computational methods used in this work are very important in order to interpret and use the data from the experiment. Since it works with kinetic models, integration and parameter estimation are the engine of this work. This was made on the Matlab® software[19] and the Microsoft Excel® program.

The Excel numerical integration was performed essentially using the Euler method:

$$y_{n+1} = y_n + hf(t_n, y_n) \quad (12)$$

where $f(t_n, y_n) = y'(t)$ and $t_n = t_0 + nh$.

To identify the value of a parameter, the most commonly used formula is the method of least squares, represented by the following formula [43]:

$$(\theta) = min \sum_{t=1}^{N} (\delta_{exp}(t) - \delta_{mod}(t, \theta))^2 \quad (13)$$

where J is the objective function; δ_{exp} represents the measurements collected from the experiments; δ_{mod} represents the prediction result by the model corresponding to the point of measurement in experimentation; θ is the parameter to be determined; N is the number of measurements. The Solver function of Excel is used to minimize the Equation (13).

It was used a program developed in modelling applied to the dynamics of microbial populations, developed at LGC (Laboratoire de Genie Chimique) where this work was done. This aid program, termed as Modelight[18] is a workspace that contains four types of files: Data, Functions, Models and Results.

3. Results and Discussion

3.1 Model validation with article results

Before the analysis of the data obtained in the experimental part, it was first required the validation of the kinetic model presented by Malherbe et al. (2004)[13]. According to the results obtained *(*Figure 1) and as expected, the model of Malherbe et al. (2004)[13](his differential equations and their parameters), allows for a very good correlation with their own results outlined in its scientific publication.

The simulated curve for *d*CO_2/*d*t fits very well the points removed from the article, and this curve is also representative of what happened with the biomass and product during the experience. While the biomass grown and the respective product (ethanol) was produced, the release of CO_2 was increasing exponentially. When the biomass entered in a stationary phase and the substrate was disappearing, CO_2 production was decreasing until a certain point (t = 120 hours) where the derived *d*CO_2/*d*t approached zero, indicating the end of the fermentation. The other simulated curves, *d*N/*d*t and *d*X/*d*t, also prove that the model follows the results described in the article.

As can be seen, from the moment (t~25 hours) in which the nitrogen present in the fermentation medium is exhausted (in this case nitrogen was not added over time), the cell population stabilizes and enters in a steady state.

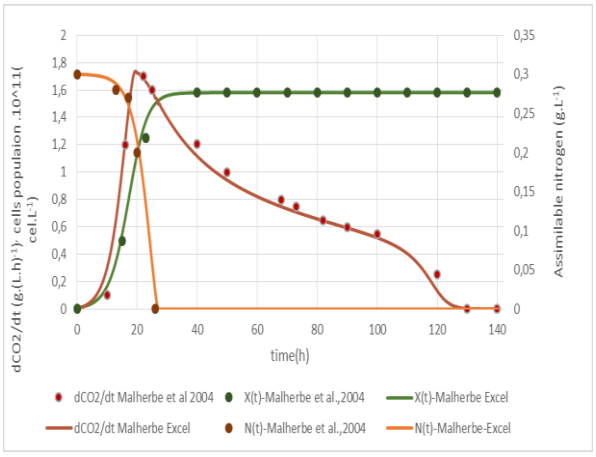

Figure 1 - Simulation of a fermentation with S_{init}= 202 $g.L^{-1}$, N_{init}=0, 28 $g.L^{-1}$ and a constant Temperature T=24ºC (isothermal conditions). Comparison with the results obtained by article (Malherbe et al., 2004)[13]. In this fermentation were used free yeast cells.

3.2 Initial Simulations with existing Data

Initially, simulations were made with data on previous laboratory fermentations using the same immobilized yeast. Concentrations (beads in the fermentation medium) used, were 152.5 $g.L^{-1}$ (high concentration cell) and 39.6 $g.L^{-1}$ (intermediate concentration) [22].

3.2.1 Experimental Data simulation

Before being made the simulations, were made some integrations with the model purpose by Malherbe et al.(2004)[13] , using the respective parameters, which proved to be far from monitoring variables evolutions (over time),measured in the study of Kassim[22] . Once the parameters found in Malherbe et al. (2004)[13] proved incapable of describing these results, it proceeded to parameter estimation using Matlab and Excel. As the model with its original parameters proved insufficient to describe these experiments, parameter estimatation were made in order to get reliable simulations able to correspond the experimental data.

The estimation of parameters was difficult due to non-linearity and interdependence between the measured variables (sugars, biomass, nitrogen and ethanol). The parameters were estimated, taking into account the physical meaning of each, and the estimation was not performed simultaneously for all but limiting each estimation to a number of parameters to minimize convergence difficulties in the objective function, which is a minimum function (Equation (13)). The strategy used for both software was to start by "blocking" all the parameters involved in the differential equations of the model, except those related to the growth of the biomass, ie d*X*/d*t* (Equation 4). The functions, linked to cell growth, are K_1 (T) and X_{max} (N_{init}), which relate to a linear function and a polynomial function, respectively. The first function involved the parameters Y_1 and Y_2 (K_1 (t) = Y_1T- Y_2), while the second, as demonstrated in Equation (5), involved the parameters Y_3, Y_4 and Y_5. These parameters (as will be discussed subsequently) were determined in Malherbe et al. (2004)[13] by parameter estimation also using a similar methodology to the proposal (simultaneous estimation of a limited number of parameters).However, it was performed at 24 °C (T) with a certain initial concentration of biomass (free yeast),X_0, and from a certain initial concentration of assimilable nitrogen (N_{init}).

Estimations were made solely directed to the parameters of the above functions (K_1(T) and X_{max} (N_{init})), in order to correct the correlation between biomass measured experimentally and the calculated by the model. The data relating to the estimation, both using the Matlab software and Microsoft Excel, are represented in Table 1.

Table 1 - Calculated parameters in order to adjust the model to the experimental data. The original parameters are presented (the article[13]) and the calculated parameters depending on the used programs.

Parameters	Originally calculated (Article)	Estimation by Excel Beads Concentration 152.6 $g.L^{-1}$	Estimation by Matlab Beads Concentration 152.6 $g.L^{-1}$	Estimation by Excel Beads Concentration 39.6 $g.L^{-1}$	Estimation by Matlab Beads Concentration 39.6 $g.L^{-1}$
k_2	$1.05x10^{-11}$	$9.58x10^{-9}$	$9.58x10^{-9}$	$3.05x10^{-11}$	$3.05x10^{-11}$
K_s	$1.50x10^{1}$	$5.93x10^{1}$	$5.93x10^{1}$	$3.11x10^{1}$	$1.50x10^{1}$
K_{si}	$1.20x10^{-2}$	$2.26x10^{-2}$	$2.20x10^{-2}$	$9.42x10^{-3}$	$1.20x10^{-2}$
α_s	1.25	1.26	1.26	1.25	1.25
k_3	$1.00x10^{-12}$	$5.84x10^{-13}$	$5.83x10^{-13}$	$1.00x10^{-12}$	$1.00x10^{-12}$
K_N	$3.00x10^{-2}$	$3.22x10^{-2}$	$3.21x10^{-2}$	$3.52x10^{-5}$	$3.50x10^{-5}$
K_{NI}	$3.50x10^{-2}$	$3.46x10^{-2}$	$3.40x10^{-2}$	$3.50x10^{-2}$	$3.11x10^{-2}$
α_n	1.50	$6.53x10^{-1}$	$6.50x10^{-1}$	-1.04	1.50
λ_a	$3.35x10^{11}$	$1.93x10^{11}$	$1.93x10^{11}$	$3.35x10^{11}$	$3.35x10^{11}$
λ_b	$6.10x10^{-2}$	$1.11x10^{-1}$	$1.11x10^{-1}$	$3.56x10^{1}$	$3.58x10^{1}$
λ_c	$3.00x10^{9}$	$2.80x10^{+9}$	$2.80x10^{9}$	$3.00x10^{9}$	$3.00x10^{9}$
λ_d	-1.00	$-3.25x10^{-1}$	$-3.20x10^{-1}$	$4.79x10^{-1}$	$4.90x10^{-1}$
Y_1	$2.87x10^{-2}$	$2.18x10^{-2}$	$2.10x10^{-2}$	$1.81x10^{-2}$	$1.60x10^{-1}$
Y_2	$-3.76x10^{-1}$	$-4.36x10^{-1}$	$-4.36x10^{-1}$	$4.52x10^{-2}$	$5.10x10^{-2}$
Y_3	$-6.49x10^{11}$	-7.43	-7.43	-7.43	-7.43
Y_4	$6.98x10^{11}$	$1.69x10^{10}$	$1.69x10^{10}$	1.00	1.00
Y_5	$7.00x10^{+9}$	$-1.04x10^{9}$	$-1.04x10^{9}$	$4.41x10^{8}$	$4.41x10^{8}$

Referring to Figure 2 (higher concentration) is perceptible to note that the parameters calculated by the two tools are similar (in most cases equal), since the curves are similar and overlapping. The parameters change from the original model was evident, because the model assumes a cell growth (as it was designed to lower cell concentrations) that did not happen in the case of the estimation using a concentration of 152.6 $g.L^{-1}$ of beads. Once corrected biomass, X (t), there were changes also in other system equations, since this biomass are interdependent. Changes in other parameters (not all) were also performed.

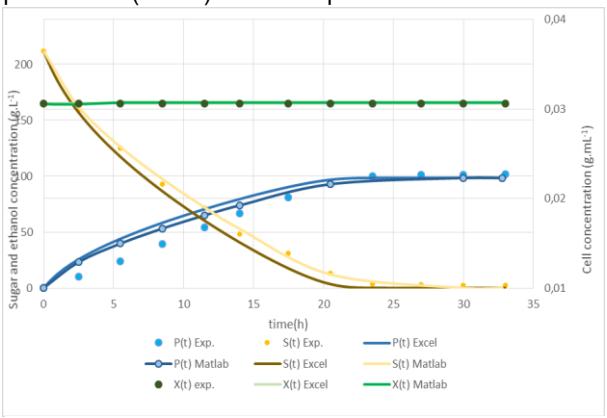

Figure 2- Simulations with estimated parameters (obtained by software Matlab and Excel) from existing experimental data (Kassim, 2012)[10,] using the model developed by Malherbe et al. (2004)[13]. .Constant Temperature (T) = 20 °C; S_0 = 212 $g.L^{-1}$; X_0 (theory) = $3.81x10^8$ Cellules.mL^{-1} (0.035 $g.mL^{-1}$); N_0=0,161 $g.L^{-1}$; Beads Concentration = 152.5 g (beads).L $(medium)^{-1}$.

Observing the behaviour of the substrate (S(t)), where they enter N_{ST} and V_{ST} functions (Equation2)), it can see a better fit in relation to the simulation made with parameters estimated by Matlab *(Figure* 2). Function N_{ST} is represented by the Equation (7), discriminating the number of hexose transporters. It is important to note that is considered λ_0 equal to one unit in the Equation (7), since nitrogen is not added during fermentation in Kassim (2012)[20]. Thus the λ parameters were estimated, as shown in Table 1. Simultaneously, it estimated the parameters for V_{ST} function (Equation (6)), which is a nonlinear regression model related do the hexose transport, where were estimated the parameters: K_2; K_S; K_{SI} and α_s . For some of these parameters can be seen that these are physically measurable parameters, K_S is a constant affinity for sugar transporters (expressed in $g.L^{-1}$) and K_{SI} is the constant for inhibition for the sugar transporters in the presence of ethanol (expressed in $L.g^{-1}$), which led to the research and care in the preparation of the ranges of these parameters in your estimation.

At the same that it made the estimation of parameters involved in the differential equation relating to substrate consumption S(t), it was also made the estimation of v_N function parameters, which describes the active transport of nitrogen (Equation (8)) belonging to the differential equation relating to nitrogen assimilable existing in the medium .The function v_N is written in the same way as the function V_{ST}, however the parameters relate to nitrogen (it was made the same preparation of the ranges). The reason for done this estimation, simultaneously for S(t) and N(t) parameters,was related to the fact that the value of N(t) enters into the fucntion N_{ST} (respect to the substrate), and thus a simulation made a posteriori to the nitrogen (N(t)) would have a direct impact on the equation of the substrate, affecting its fit. In turn, the biomass was initially set, as already mentioned it was determined a priori.

It is assumed that once was adjusted the substrate consumption between the model and experimental data, the product (P(t)) calculated by simulation for each of the software will also adjusted on the same extent.There was,again, a better fit for the product estimated by Matlab.

An overview to the Table 1, it can be seen that most parametric changes were in the parameters relating to cell growth (Y), which is explained by the use of higher concentrations than those related to article Malherbe et al. (2004)[13], which used free cells inoculate 106 cells.ml-1 (2 and 3 times smaller than the cells inoculated beads in concentrations of 39.6 $g.L^{-1}$ and 152.6 $g.L^{-1}$, respectively).

3.3 Simulation for the new experiments

In addition to the parametric changes that were justified in the previous chapter, relating to cell growth, it was detected some failure in the previous experimental data. Thus, they were done new tests in batch to obtain data for the concentration (of beads) of 39.6 $g.L^{-1}$ and also to obtain new data for a concentration of 9.1 $g.L^{-1}$, in order to validate the model proposed by Malherbe et al.(2004)[13]. The choice of these concentrations was purposeful, since the idea was to validate the model to do the reactor modelling proposed by Kassim[20], which proved the successful use of two reactors in series with these concentrations. The batch tests were prepared in triplicate for each of these concentrations. Thus, at 20 °C as indicated in the *Material and Methods* were prepared six Erlenmeyer flasks (from the same initial medium) and the variables were monitored: biomass, substrate and ethanol. In addition it was performed some experiments related to the measurement of nitrogen. It was done a constant measurement of beads diameter, in order to be understood what was happening with cell growth within them.

3.3.1 Simulation with new Data (original parameters)

After the treatment and analysis of new data, new simulations were performed in order to study the correlation between the Model[13] with the initial conditions used in the new assays, and experimental data (new assays). These simulations were made to two concentrations, and its analysis was also made independently in order to take conclusions about the Malherbe et al. (2004)[13] model. Firstly, it was studied the model with experimental results for a concentration of 39.6 $g.L^{-1}$. It is recalled that this study had been done in Section *3.2.1*, but with data from Kassim (2012)[20], which appeared to have some inaccuracies particularly in terms of biomass (biomass has not reached stable values in its steady state). Observing Figure 3, it can be view a good fit between the substrate measured experimentally and

predicted by the model. In consequence, the adjustment for the product is also good.

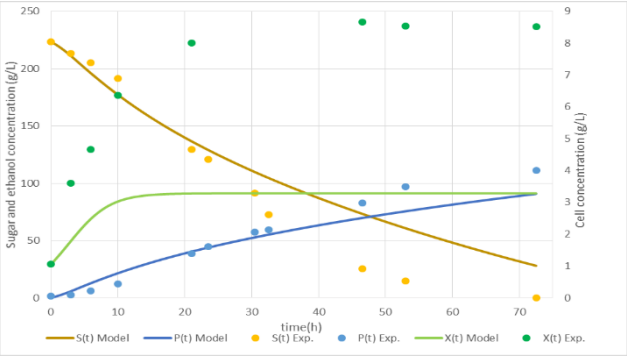

Figure 3- New experiments and simulation results made with the original model (original parameters) for a bead concentration of 39.6 $g.L^{-1}$ in the fermentation medium. Constant Temperature (T) = 20 °C; S_0 = 223.5 $g.L^{-1}$ (average); X_0 (real) = $5.76x10^7$ $Cellules.mL^{-1}$ (1.05 $g.L^{-1}$); N_0=0.385 $g.L^{-1}$.

However in relation to biomass, as can be seen in Figure 4, this correlation was not verified: modeled biomass would reach a maximum around 3 $g.L^{-1}$, while experimentally there is an increase to about 9 $g.L^{-1}$, remaining constant after that.

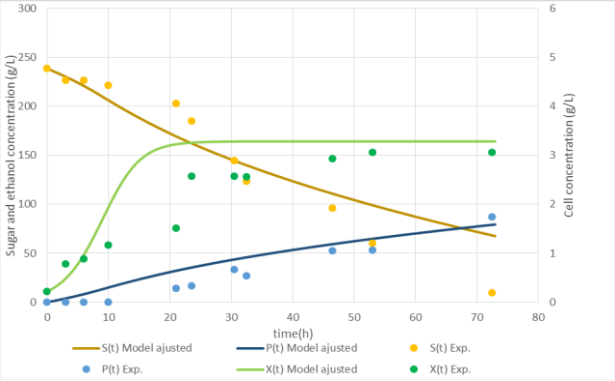

Figure 4 - New experiments and simulation results made with the original model (original parameters) for a bead concentration of 9.1 $g.L^{-1}$ in the fermentation medium. Constant Temperature (T) = 20 °C; S_0 = 238.52 $g.L^{-1}$; X_0 (real)= $1.84x10^7$ $Cellules.mL^{-1}$ (0.22 $g.L^{-1}$); N_0=0.385 $g.L^{-1}$.

As in the previously referenced Section *3.2.1*, it was not obtained a total fit between the data from the laboratory and the original model proposed by Malherbe et al. (2004)[13]. Even using the parameters calculated by the parameter estimation in the previous studies, Table 2, the fit would not be achievable. Thus, it opted for another strategy and directly analyze the model and its structure, as it will be seen later.

It was also studied the fit between the model originally proposed and prepared for further tests on a concentration of beads 9.1 $g.L^{-1}$. It can be seen in Figure 4 that the differences between the results proposed by the model and the experimental data were softened. It is verified experimentally that after about 72 hours (duration of the test fermentation), the substrate reaches values close to 0-1 $g.L^{-1}$,while the model predicts that is only reduced to approximately half of the initial substrate, i.e. around 60 $g.L^{-1}$. Consequently the adjustment related to the product is also inaccurate. At the level of biomass it was also found inaccuracies, especially at the stage where there is the passage of cell growth to a steady state, verifying that experimentally occurs at the beginning of the second day of fermentation, while in the model occurs after 15 hours in relation to beginning of the fermentation.

Comparing Figure 3 and Figure 4, it was conclude that in Figure 4 was obtained a better fit, as the model followed better the experimental points. This was an expected result, since it is working at a lower concentration bead, which side the model proposed by Malherbe et al. (2004)[13] developed for lower cell concentrations. However, they remained errors in modelling. Looking at Figure 3 and Figure 4, it came across something in common in the two Figures: the fact that the model predict values of maximum biomass concentration (Xmax) equal, $1.79x10^{11}$ $cells.mL^{-1}$ (or 3.28 g.L-1), which would hardly be explained as they presented different biomass initial concentrations (X_0), 0.22 and 1.05 $g.L^{-1}$ for the lower and higher concentration, respectively. Reading the mathematical model, the expression is easily associated with the Xmax, Equation (5), which influences the dX/dt, influencing all the fermentation kinetics, since are dependent variables (X, S and N). The model assumed that the nitrogen is the main limiting nutrient (present in low quantities) for cell growth, suggesting a nonlinear function to calculate X_{max} according with the available assimilable nitrogen and when it depletes, cell growth ends. This function, Equation (5), was built for the concentration of biomass (free) tested in the article. This function took into account only the N_{init}, i.e., if it is applied the same values of γ_3, γ_4 and γ_5 to different concentrations with the same N_{init}, similar values would be obtained, as those obtained in Figure 3 and Figure 4. Nevertheless, as can be concluded, that value depends not only on the initial nitrogen, but also on X_0. In this way, and once a model is built to be applied in different conditions, this function had lost its effect in the model. It can be recalculated these parameters (γ_3, γ_4 and γ_5) for these new tests, however it was chosen for future simulations eliminate these parameters of the model, entering directly X_{max} value measured experimentally. Another model "controversy" was the nitrogen, referring to the cells role. In the mode, it was pointed just as an important factor on

cell growth ,i.e., its restricted to affect only the exponential part of the growth. Then, it was also proposed that the nitrogen required for cellular maintenance was also a limiting factor, and thus could not be negligible [21]. Knowing, *a priori* from Kassim´s data[20], that the prepared medium would contain around 0.385 mg.L^{-1} of assimilable nitrogen, it was studied the veracity and evolution of it. First, it was assayed an experimental method presented in Section *Material and Methods*, where it is used a ratio between the assimilable nitrogen and the amount of base added in the titration. However this study proved inconclusive, since the degree of accuracy did not allow the measurement and monitoring of small amounts nitrogen present in the samples. It was only demonstrated that nitrogen is rapidly consumed, as expected. It was also used a correlation presented by Colombiè et al.(2005)22. it was obtained the values for initial concentrations of assimilable nitrogen, 263.42 and 421.09 g.L^{-1}. This difference between estimated values for the initial nitrogen was not in agreement with the expected, since both tests were prepared from the same fermentation medium, then the values have to be similar. Once the value obtained from the tests with the lowest concentration was below the expectations, it was decided to simulate the amount corresponding to 421.09 g.L^{-1}.

3.3.2 Simulation with new Data (new approach)

With the conclusions drawn from previous section, it was performed new simulations with the changes highlighted in the model and in respect to the amount of initial assimilable nitrogen. It was also made a parametric estimation on the level of parameter $k_2(T)$ for the lower concentration, due to the oscillation previously observed in the values compared with the originally proposed .The results can be seen in Figure 5 and Figure 6.

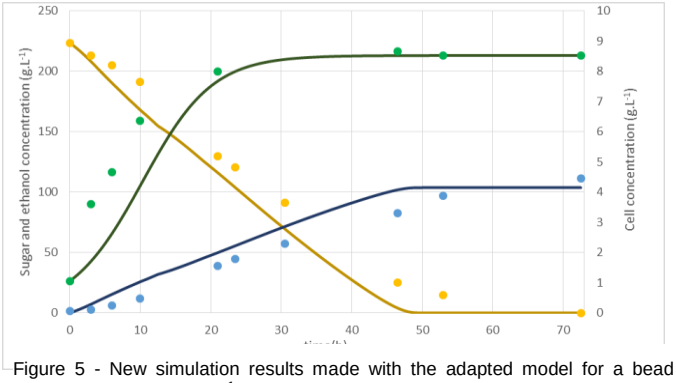

Figure 5 - New simulation results made with the adapted model for a bead concentration of 39.6 g.L^{-1} in the fermentation medium. Constant Temperature (T) = 20 °C; S_0 = 223.5 g.L^{-1} (average); X_0 (real) = 5.76x10^7 Cells.mL^{-1} (1.05 g.L^{-1}); N_0=0.421 g.L^{-1};

Comparing Figure 5 with Figure 3 (application of the original model), it can be seen that the modified model fits better to the experimental results. It was reached the

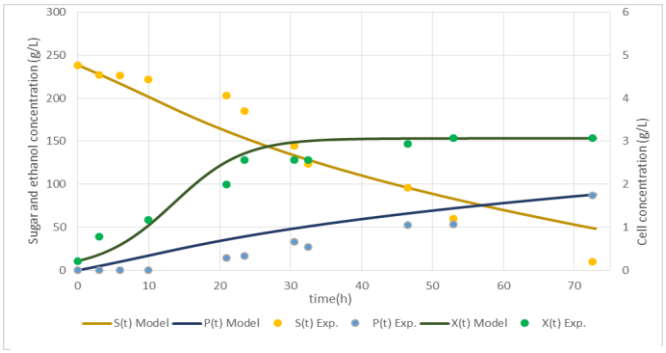

Figure 6 - New simulation results made with the adapted model for a bead concentration of 9.1 g.L^{-1} in the fermentation medium. Constant Temperature (T) = 20 °C; S_0 = 238.52 g.L^{-1}(average); X_0 (real)= 1.84x10^7 Cells.mL^{-1} (0.22 g.L^{-1}); N_0=0.421 g.L^{-1} ;

same value of X_{max} (between experiments and model) due to the elimination of certain parameters in dX/dt. Thus it was proved that the expression presented by the article to calculate the X_{max} became incoherent. In relation to the reaction rate, it was observed that the substrate is consumed faster in the prediction of the model. Consequently, the concentration of ethanol for any time (t) is higher than the predicted by the experiments. However these differences are low and may be due to the fact that the nitrogen (by model description) is directed to cell growth and glucose transporters synthesis while may also play an important role in the cellular maintenance. Thus, the model estimated more glucose transporters than those existing in reality and thus explain the differences between substrate concentrations. These differences may also be due to the model has been constructed for free cells, and fermentations were done with immobilized cells on beads, preventing cells to the same access to substrate, i.e., there was inaccessible biomass. Referring to Figure 6 in general, it can be seen that the modified model fits the experimental results.

3.4 Reactor's modelling

As already mentioned, the main objective of this study was apply the model to the immobilized cells. In this part, it is presented the results obtained by simulation based on some data obtained from the Kassim[20] study, that instead of applying a kinetic model, introduced experimental results, i.e. the experimental rates were taken directly from the experiments.Two CSTR in series were simulated by Kassim[20], with concentration of

residual sugar equivalent to 30-40 $g.L^{-1}$ (output of first reactor) and equal to 10 $g.L^{-1}$ (output of second reactor). Taking into account the conclusions reported in this study, it was chosen a new operating mode: a two-stage reactor[20]. The best combination (for a two-stage reactor) reported in this study was related to 39.6 / 9.1 ($g.L^{-1}$). For the first stage it was chosen a concentration of 39.6 $g.L^{-1}$, in order to convert a large proportion of sugar (210 $g.L^{-1}$ to 40 $g.L^{-1}$).Once in the first stage the majority conversion of sugar will be converted, it was chosen a low concentration of beads to operate in the second stage (9.1 $g.L^{-1}$).As the process in the CSTR better followed the experimental data, this type of operation was selected to simulate this operation. Some operating parameters were found in Kassim[20] study and set *a priori* of simulation, such as Q, C_0 and C_f.

It was calculated a global volume (in this case for 2 columns) of 6479 x 10^3 L, above expectations from the results of Kassim (2012)[20], 4781 x 10^3 L. It was obtained a residence time of 69 hours. These differences between values were lied to the substrate consumption rate on the second stage (referring to the concentration of 9.1 $g.L^{-1}$), which was observable in Figure 6 (it can be seen a slowly rate when the substrates crossed 10 $g.L^{-1}$ of available). Thus, despite the second floor had been a residual concentration, the utilisation of this low concentration increased the residence time of the fermentation.

5. Conclusion and Future perspectives

This project aimed to apply a kinetic model, originally developed for a system with free yeast cells, in order to follow alcoholic fermentation with immobilized cells. The strategy adopted was inherently study the mathematical model and it was found a few fails. In terms of biomass, came across X_{max} calculation was not valid for high concentrations of initial biomass, being eliminated the parameters of γ_3, γ_4 and γ_5. Moreover, the experimental results showed that the contribution of nitrogen for cellular maintenance was not negligible, as the model assumed[50]. In addition to these variables that were corrected through the experiences, the parameter estimation and optimization returned a new value of k_2. In the end, with changes made to *Malherbe*[13] model, it was obtained a good correlation between the predicted mathematically and the results of fermentation. It is concluded that the model was feasible to design a continuous system. In the future, some changes to the model can also be applied, especially regarding nitrogen (dN/dt), which can be added an expression which takes into account the nitrogen used to cellular maintenance (Monod kinetics for example).

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