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RESEARCH ARTICLE

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Alginate production by *Azotobacter vinelandii* as a virtual sensor to estimate effective shear rate on stirred tank bioreactors

ABSTRACT:

A virtual sensor was designed based on the alginate production by *Azotobacter vinelandii* with the objective to estimate the effective shear rate generated by Rushton turbines in a lab stirred fermenter. This virtual sensor can simulate the growth rate of bacterium. One of the components of this sensor is the volumetric oxygen transfer coefficient (k_La) which was determined experimentally by the method of gas elimination with nitrogen (only at the beginning of the culture). The experimental value of k_La matched with a correlation which is used for non-Newtonian fluids. The main components of such correlation are the superficial velocity (Vs), power consumption (P/V)-determined experimentally by a dynamometer bearing- and effective viscosity (μ_{eff}). The values of the effective viscosity of the broth cultures of *A. vinelandii* depends on the correlation used to estimate the shear rate (γ), flow index behaviour (n) and consistency index (K). In this context, the main component of our virtual sensor is the Monod equation; the sensor could stimulate the growth of *A. vinelandii* for cultures performed at 140, 180, 200, and 340 rpm and the kinetics were simulated at by means of the software SSBP.

KEY WORDS:

Azotobacter vinelandii, Consistency index, flow behaviour index, oxygen transfer rate, volumetric oxygen transfer coefficient.

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INTRODUCTION:

There are relatively few works in the literature that deal with the shear rate (γ) generated by the impellers in stirred tank fermenters. The shear rate is caused through the agitation speed of the impellers and causing velocity gradients on the fluid at eddy levels. The characterization of this parameter in practice is highly important, since it determines the apparent viscosity of the broth cultures, power consumption, mixing characteristics, hydrodynamic stress and mass transfer phenomena (Coulson and Richardson, 1990).

The shear rate is one of the most important parameters to characterize in stirred tank fermenters, but unfortunately this operational parameter is not easy to determine. This parameter is particularly important in cell cultures where the mass transfer, mixing characteristics and hydrodynamic stress determine the metabolite yields of such cultures. The broth cultures might behave like Newtonian or non-Newtonian fluids. In the last, the shear rate generated is dependant of the power consumption, flow index behavior (n), consistency index (K) and therefore on the agitation speed and the type of impeller used; including the airflow rate conditions (Campesi *et al*., 2009).

The main purpose of this current investigation is to design a virtual sensor, is to say a soft sensor that allows us to estimate the effective shear rate generated by the impellers through the alginate production by the Gram-negative bacterium *Azotobacter vinelandii*. This is because there are some discrepancies between the values obtained through empirical correlations reported in the literature generated by the movement of the impellers, for example, Campesi *et al*. (2009) reported that during *Streptomyces clavuligerus* cultivations, the shear rate caused by the Rushton turbines was three times order of magnitude higher than the common correlations normally used. The same values were reported by Witchterle *et al*. (1984). Therefore, in this current research, we propose the design of a virtual sensor to corroborate such discrepancies; the final objective is to simulate the growth of *A. vinelandii* during the alginate production by means of software. This kind of soft-sensors are widely used in bioprocess ingineering research and for industrial applications in order to measure, monitor, model and control common process (Luttman *et al*., 2012). The virtual sensor was designed based on an empirical correlation which is commonly used to estimate the volumetric oxygen transfer coefficient (k_La) in stirred tank fermenters and this equation was reported previously by García-Ochoa and Gómez (1998). The main components of this correlation are: superficial velocity (Vs), power consumption (P/V) and effective viscosity (μ_{eff}). The last parameter is determined by the Ostwald-de Waele model and this is dependand on the shear rate, flow index behavior (n) and consistency index (K). Here, the shear rate generated by the impellers determines the apparent viscosity of the broth culture and therefore have a great implication in the efficiency of oxygen transport on the bulk of the liquid. Our virtual sensor also includes the next correlations proposed by some authors in order to estimate the shear rate: Metzner and Otto (1957), Bowen (1986), Calderbank and Moo-Young (1959), Witchterle *et al*. (1984), Kelly and Gigas (2003), Vogel and Kroner (1999), Sánchez-Pérez *et al*., (2006). The values of the shear rate by using different correlations affects the k_La and therefore influence the oxygen transfer rate (OTR).

The main component of our virtual sensor is the Monod equation, where the limiting substrate is the oxygen. The whole oxygen concentration supplied to the culture during the alginate production by *A. vinelandii* is dependant on the k_La values obtained with the shear rate correlations previously mentioned. Then, at the end of the cultures, the total oxygen concentration could be estimated by the OTR and its values are determined by the type of shear rate correlation used. The mathematical solution of this virtual sensor component (Monod equations) is solved using a software denominated Software Simulator Bioprocess (Reyes *et al*., 2016). Because of *A. vinelandii* is obligate aerobic bacteria that exhibits high respiratory activity (1983) and under these conditions can synthesize alginate; a copolymer made of mannuronic and guluronic acid. Therefore, the oxygen was considered a limiting substrate, this was due to that at low agitation speeds the alginate yields are relatively low when compared with cultures performed at high agitation levels. Finally, because the values of k_La obtained are dependent of the shear rate correlations used and therefore the total oxygen concentration supplied to the culture changes; then the modelling better trials of growth of *A. vinelandii* with the virtual sensor during the alginate production means that are sensing the effective shear rate caused by the Rushton turbines.

MATERIAL AND METHODS:

Microorganism:

The strain used was *Azotobacter vinelandii* ATCC-9046

Culture media and inoculums development:

The formulation of the culture Burk medium was (g/L): sucrose (20); yeast extract (3.0); K_2HPO_4 (0.66); KH_2PO_4 (0.16);

$CaSO_4$ (0.05); NaCl (0.2); $MgSO_4.7H_2O$ (0.2); $Na_2MoO_4.2H_2O$ (0.0029); $FeSO_4$ (0.027). The initial pH was adjusted to 7.0, using NaOH 2 N. The inoculum was developed in 500 mL Erlenmeyer flasks during 24 h, containing 100 mL of culture medium and mixed at 200 rpm (29°C) in an orbital incubator shaker (New Brunswick Scientific Co., Model G25).

Fermentation experiments:

The cultures were carried out in a 1.5-L stirred fermenter equipped with two Rushton turbines with an initial working volume of 1.0 L and aerated at an airflow rate of 0.8 L min^{-1}. Dissolved oxygen was not controlled and was monitored with a polarographic oxygen sensor (Ingold), and its signal was amplified and acquired by a computer via an interface board (Peña *et al*., 2000). Samples of 20 mL were withdrawn for analytical measurements. The pH left varies freely and monitored by a sensor probe (Ingold, USA). The temperature incubation was controlled at 29°C with pump water into the loop serpentine.

Analytical methods:

The biomass and alginate concentration were determined gravimetrically as described previously by Peña *et al*. (1997). Specific growth rate was calculated trough the logistic model previously reported by Klimek and Ollis (1980). The molecular mass of the alginate was estimated by gel permeation chromatography a serial set of ultrahydrogel columns (UG 500 and linear waters), using a HPLC system with a differential refractometer detector (Waters, 410).

Experimental determination of volumetric transfer rate (k_La):

The technique consisted of the oxygen elimination of the medium of nitrogen gas into the vessel (Quintero, 1987). Once the oxygen concentration in the broth bulk was zero, the medium was aerated at 0.8 L min^{-1} with air and the oxygen concentration reached the saturation (Cg*). k_La was determined by the equation 1.

Experimental determination of the power input:

To determine the specific power consumption, we used the method previously reported by Reséndiz *et al*. (1991); where the dynamometer allows the torque reaction trough the speed of the impellers. A cell detects the reaction forces with the lever arm. The torque has a relationship with the lever arm and inlet force, according to the next equation 2: Where B is the lever arm (m), M refers to torque and F is the administrated force (Newtons). The power consumption by the impellers was estimated as follows eq 3: ω refers to the angular velocity of the impellers (2 πN).

Correlations used for determining volumetric transfer rate (k_La) at high viscosities:

For determining k_La at high viscosities we used the correlations reported by Garcia-Ochoa and Gomez-Castro (1998) eq 4: Where C_2 is a constant, μ_{eff} is the effective viscosity (Pa*s) according to the Ostwald-de Waele model eq 5: K refers to the consistency index (Pa*s), γ is the shear rate generated by the impellers (s^{-1}), and n is the flux index behavior (-).

Estimation of the effective shear rate (γ):

We probed several correlations to estimate the effective shear rate (γ) in broth cultures of *A. vinelandii* for alginate production: (Bowen 1986, Eq. 6); (Vogel and Kroner, 1999, Eq.7); (Metzner and Otto, 1957, Eq. 8); (Bowen, 1986, Eq. 9); (Witchterle *et al*., 1984, Eq. 10); (Sánchez-Pérez *et al*., 2006, Eq. 11); (Calderbank and Moo-Young, 1959, Eq. 12). The estimation of volumetric oxygen transfer coefficient (k_La) was performed in the bases of index flow behaviour (n) and index coefficient (K) through whole fermentations. The cultures shown a complex dynamic rheology when the alginate begins to produce, therefore the k_La diminished since the beginning of the cultures. The OTR was determined by the equation 13. For ensuring the maximum transfer rate, C_L was equal to zero. The whole oxygen transfer by each correlation into the cultures was estimated in the growth phase of *A. vinelandii* and therefore allows estimate $Y_{X/O2}$ (g/g) yields as follows eq. 14.

Rheology determination of the reconstituted alginates:

The alginate was recovered from broth (see section 2.2). Alginate was recovered by precipitation with 3 volumes of propan-2-ol. The cake was dried in an oven at 60°C during 24 h and later milled in a mortar. This powder was reconstituted in Burk`s medium to yield bacterial model fermentation broths as a normal occurring (Peña *et al*., 2007). The consistency index (K) and flow index behavior (n) was determined using a rheometer (Contraves, Reomat 120) with DIN125 device.

Modelling biomass accumulation as a virtual sensor:

To estimate the overall oxygen supply to the broth alginates cultures at high viscosities we used the biomass accumulation as a sensor. This was used because the effective viscosity affects the oxygen transfer rate and the last depend on the shear caused by the impellers in the Ostwald-de Waele model. Then, the model used to estimate the biomass accumulation of *A. vinelandii* was eq. 15. The death of microorganisms is not considered. Where r_X is the specific growth rate, and it was determinate by Monod equation as follows eq. 16. The growth of *A. vinelandii* at high viscosities the limiting substrate is oxygen. Therefore, the virtual sensor explains the oxygen transfer using the correlations previously explained. The value of Ks is dependent on the relative oxygen diffusion coefficients in aqueous alginate concentration; we used the correlation proposed by Fujita *et al*. (1961). Eq. 17, Where eq. 18. B_d is a measure of the minimum size of the hole required to accommodate diffusing molecules; f_B (0, T) is the free volume of pure oxygen at temperature T. We used the oxygen as a limiting substrate. Therefore, the oxygen rate consumption in the culture follows the next equation eq. 19, Where rO_2 is the specific oxygen consumption rate and this was determinate as follows eq. 20:

$$(\ln(1-C(t)/Cg^*) = k_L a \quad \text{Eq. 1}$$

$$M = F * B \quad \text{Eq. 2}$$

$$P = \varpi * M \quad \text{Eq. 3}$$

$$k_L a = C_2 * Vs^{2/3}\left(\frac{P}{V}\right)^{0.6} * \mu_{eff}^{-2/3} \quad \text{Eq. 4}$$

$$\mu_{eff} = K.\gamma^{n-1} \quad \text{Eq. 5}$$

$$\gamma_{av} = 4.2N\left(\frac{D_i}{D_t}\right)^{0.3}\frac{D_i}{W} \quad \text{Eq. 6}$$

$$\gamma_{av} = \left[0.038\frac{P}{VK}\left(\frac{D_t}{D_i}\right)^{3}\frac{D_i}{h_b}\right]^{1/1+n} \quad \text{Eq. 7}$$

$$\gamma = k_i N \quad \text{Eq. 8}$$

$$\gamma_{max} = 9.7N\left(\frac{D_i}{D_t}\right)^{0.3}\frac{D_i}{W} \quad \text{Eq. 9}$$

$$\gamma_{max} = N(1+5.3)^{1/n}\left(\frac{N^{2-n}D_i^2\rho}{K}\right)^{1/(n+1)} \quad \text{Eq. 10}$$

$$\gamma = \left(\frac{1}{K}\frac{P}{V}\right)^{1/(n+1)} \quad \text{Eq. 11}$$

$$\gamma_{av} = k_i\left(\frac{4n}{3n+1}\right)^{1/(n-1)} N \quad \text{Eq. 12}$$

$$OTR = k_L a(Cg^* - C_L) \quad \text{Eq. 13}$$

$$\sum_{t_i}^{t_f} OTR = O_2 \quad \text{Eq. 14}$$

$$V\frac{dX}{dt} = rXV \quad \text{Eq. 15}$$

$$r_X = \mu = \frac{\mu_{max} * O_2}{Ks + O_2} \quad \text{Eq. 16}$$

$$\ln\frac{D_l}{D_o} = -\frac{B_d}{f_B(0,T)}\frac{\phi}{1-\phi} \quad \text{Eq. 17}$$

$$\phi = \rho w_p / \rho_p^0 \quad \text{Eq. 18}$$

$$V\frac{dO_2}{dt} = rO_2 V \quad \text{Eq. 19}$$

$$rO_2 = \frac{r_X}{Y_{x/O_2}} \quad \text{Eq. 20}$$

All real-time simulations were performed by the software SSBP previously reported by us (Reyes *et al*., 2016).

RESULTS AND DISCUSSION:

Alginate production and Kinetics of *A. vinelandii*:

The alginate production and growth kinetics of *A. vinelandii* are shown in figure 1. The maximum specific growth rate (μ_{max}) was dependent of the agitation speed on the impellers, for example, at 140 rpm μ_{max} was around 0.093 h^{-1} while at 340 rpm μ_{max} reached values of 0.16 h^{-1} (Fig. 1) for biomass.

For all culture conditions, the final biomass concentration was around 5.6 g/L. This trend can be explained by the fact of the accumulation of an intracellular polymer known as poly-β-hydroxybutyrate (PHB) that is synthesized under anaerobic conditions (Galindo *et al*., 2007). The PHB production has been reported for *A. vinelandii* for batch cultures no matter if they are wild strains (Page and Knosp, 1989; Peña *et al*., 2011) or mutant strains (Page and Knosp, 1989). In cultures performed at low agitation rates, the PHB accumulation can be up to 63% (w/w) of the dry weight of the biomass (OTR_{max}; 3 mmol $L^{-1}h^{-1}$) but when the cultures were performed at 9.0 mmol $L^{-1}h^{-1}$, the PHB accumulation was only 17% (w/w) (Díaz-Barrera *et al*., 2007). The alginate production by *A. vinelandii* in all culture conditions tested were growth-associated. The yield reduction of the alginate in low agitation speeds may be due to the oxygen limitations, for example the cultures performed at 140 rpm the alginate production was only 3.2 g/L, but when the agitation speed was increased at 340 rpm the alginate production reached values of 8.0 g/L (Fig. 1). This could be as a result of better conditions of mixing and aeration, where these parameters are critical to the optimization of the production of microbial polysaccharides (Galindo *et al*., 2007). However, several studies reported the efficient conversion of sucrose to alginates is achieved when the oxygen is controlled between 1 to 10% of oxygen saturation (Parente *et al*., 2000; Peña *et al*., 2000; Sabra *et al*., 2000).

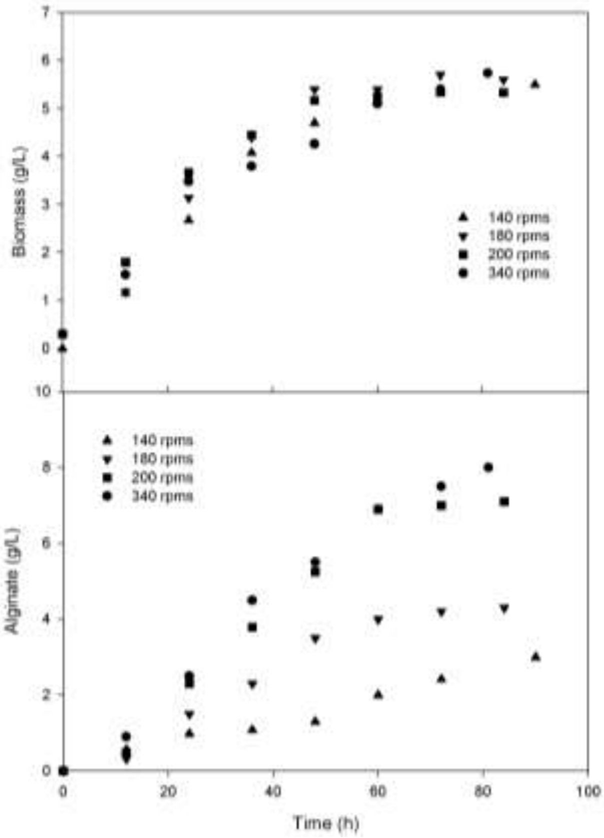

Fig. 1. Kinetics of the alginate production and biomass accumulation by A. vinelandii in cultures carried out in stirred tank fermenter.

Components of the virtual sensor:

Shear rate at high viscosities:

One of the major components of the virtual sensor proposed in the current research is the shear rate (γ). This parameter can be estimated through multiple correlations proposed in the literature, however, the reported values at specific conditions may vary among them, even in several orders of magnitude. Therefore, with the objective of validating our virtual sensor proposed in this work, first, we compare the values of the shear rate in the culture of *A. vinelandii* during the alginate production at 340 rpm (Fig. 2a) (from 0 to 5.0 g/L of alginate). The objective is to discern which of these correlations would generate greater values of shear rate and which of them could be in accordance with the batch modelling of *A. vinelandii* growth. Thus, demonstrating that the empirical correlations proposed by Vogel and Kroner (Vogel and Kroner, 1999), as well as Witchterle *et al*. (1984), the values are found plus about two or three orders of magnitude up with respect to the others (Fig. 2). This result has also been reported by Campesi *et al*. (2009), but their correlation proposed was not included in our virtual sensor because they also considered the airflow rate (only reported for 0.5 and 1.0 1 min^{-1}). The correlations that generate greater shear rate values is indicative of less apparent viscosity of the broth culture, therefore the k_La values could be increased. Our virtual sensor also includes empirical correlations for Newtonian and non-Newtonian fluids. In the case of alginate production by *A. vinelandii*, for the cultures performed at 340 rpm, the shear rate values keeping almost constants through all the culture when are used the next correlations: Bowen (1986), Metzner and Otto (1957), Calderbank and Moo-Young (1959). On the other hand, the shear values estimated with the correlations proposed by Witchterle *et al*. (1984), Vogel and Kroner (1999), and Sánchez-Pérez *et al*. (2006), the values vary significantly since the beginning of the culture. Figure 2 shows the shear rate values evaluated to 5.0 g/L of alginate, but this trend remains similar up to 8.0 g/L (Data not shown). The other cultures carried out at 140, 180, and 200 rpm exhibit the same behavior, respect to the shear rate, showed in figure 2. Shear rate generated by the impellers determines the apparent viscosity and its values influence the oxygen transfer rate (OTR) into the fermenter. Then, the amount of oxygen supplied into the stirred tank depends basically on the apparent viscosity of broth cultures; in this case, *A. vinelandii* has high values of oxygen uptake rate (OUR) and bacterium only growth under aerobic conditions, therefore, high shear rate values are desirable in cultures with polysaccharide production such as alginate because of their high viscosity (Fig. 2).

In a non-Newtonian fluid, apparent viscosity is dependent on the shear rate values. In the case of alginate solutions, its behaviour is pseudoplastic (Chen *et al*., 1985), therefore a linear relationship is not expected (Fig. 2b), besides the values of apparent viscosity also is dependent of n and K values, alginate concentration and by the mean molecular weight of the polymer.

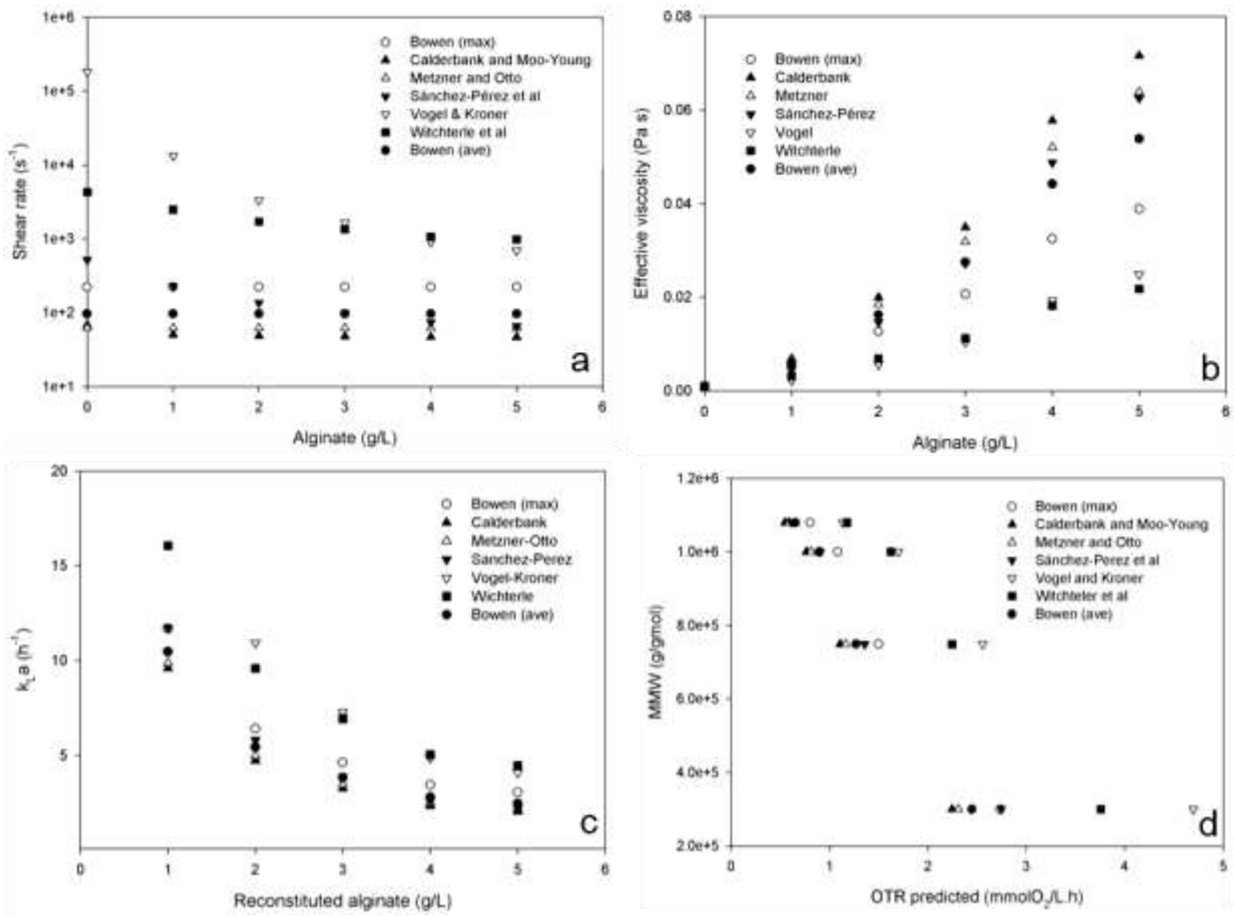

Fig. 2. a) Profiles of the shear rate generated in the cultures at 340 rpm with A. vinelandii at different alginate concentrations.

b) Effective viscosity of the cultures of A. vinelandii at 5 g/L of alginate in cultures carried out at 340 rpm.

c) Theoretical volumetric oxygen transfer coefficient at 340 rpm: simulations obtained with the shear rate correlations used in this work.

d) Quality of the alginates produced by A. vinelandii at 340 rpm at different theoretical values of OTR.

Effective viscosity in cultures of *A. vinelandii*:

The pseudoplastic non-Newtonian rheological behaviour of the alginates produced by *A. vinelandii* has been described by Peña *et al*. (2007). This behaviour exerts a profound effect on the bioreactor performance affecting the mixing pattern, the power requirement, heat and mass transfer (Gavrilescu *et al*., 1993). These phenomena are attributed to the effective viscosity sensing on the tip of the impellers of the fermenter, therefore the apparent viscosity of the broth is determinate by the shear rate generated by the agitation speed of these impellers. The apparent viscosity of the liquid-phase influences negatively the k_La because it offers resistance to the gas-liquid oxygen transfer. In this work, the effective viscosity was estimated by direct determination of n and K by the Ostwald-de Waele model. Figure 2c shows the apparent viscosity of the cultures carried out at 340 rpm and it corresponds to the shear rate obtained in figure 2a. With the correlations proposed by Calderbank and Moo-Young (1959), Metzner and Otto (1957), and Bowen (1986) the apparent viscosity is higher, while the lower value of apparent viscosity was obtained with correlations of Witchterle *et al*. (1984) and Vogel and Kroner (1999), therefore, high k_La values could be expected when compared with the other correlations.

Volumetric oxygen transfer coefficient (k_La) and oxygen transfer rate (OTR):

The basis of our virtual sensor is the work reported by García-Ochoa and Gómez (1998), who reported an empirical correlation to estimate k_La in non-Newtonians fluids. This equation involves the next parameters: superficial gas velocity (Vs), effective viscosity (μ_{eff}) and power consumption (P/V). These authors experimentally determined the volumetric oxygen transfer coefficient by dynamic method in xhantam gum solutions for wide operation intervals in stirred tank fermenters. In our case, the k_La and power consumption only were determined before the fermentations begin. The experimental data obtained are shown in table 1. The power law parameters (n and K) are shown in table 2 for several reconstituted alginates at several concentrations.

Table 1. Experimental determination of k_La and power input drawn in Virtis fermenter (1.5 L with 1.0 L of working volume).

Parameters	140 rpm	180 rpm	200 rpm	340 rpm
k_La (h^{-1})	8.69	12.67	14.5	28.6
P/V (W/L)	0.027	0.056	0.075	0.27

Table 2. Power low parameters (n and K) of bacterial alginate solutions

Alginate concentration (g/L)	Index flow behaviour, m (-)	Consistency index, K (Pa sm)
1.0	0.80	0.015
2.0	0.70	0.064
3.0	0.66	0.13
4.0	0.63	0.24
5.0	0.61	0.064

For example, the cultures carried out at 340 rpm during the alginate production are showed k_La values estimated with different correlations of shear rate (γ), it means that the k_La is dependent of the apparent viscosity on the broth culture. When the alginate concentration is equal to zero, the k_La value estimated by using all shear rate correlations is around 28 h^{-1}, it means, a similar value to the experimental data obtained. Figure 2c shows the hipothetical k_La evolution through the fermentation at several alginate concentrations. For example, at 5.0 g/L of alginate, the major values were obtained with Witchterle *et al*. (1984) and Vogel and Kroner (1999) correlations (4.4 y 4.0 h^{-1}, respectively), whereas the correlation proposed by Metzner and Otto (1957) was only about 2.6 h^{-1}. The same profiles of k_La can be observed in the cultures performed at 140, 180, and 200 rpm. In order to quantify the total amount of oxygen supplied to the broth cultures during the alginate production by *A. vinelandii*, we used the oxygen transfer rate (OTR), which was estimated by using different values of shear rate obtained with the correlations proposed. The data for OTR for all cultures performed were: at 140 rpm; OTR_{max} was around 2 mmolO_2 $L^{-1}h^{-1}$, at 180 rpm was 3.1 mmolO_2 $L^{-1}h^{-1}$, finally at 200 and 300 rpm, the OTR_{max} were 3.7 and 8.0 mmolO_2 $L^{-1}h^{-1}$, respectively. When the alginate begins to synthesize and accumulate in the broth culture, the OTR diminished drastically and their value depends on the shear rate correlation used in the virtual sensor. For example, at 140 rpm the OTR value diminished from 3.1 to 0.66 mmolO_2 $L^{-1}h^{-1}$ at alginate concentrations for 3.0 g/L and these values were achieved with Witchterle *et al*. (1984) correlation; but these values are less when were used the other shear rate correlations.

For the case of the cultures performed at 340 rpm, OTR_{max} diminished from 8.0 to 1.0 mmolO_2 $L^{-1}h^{-1}$ (from 0 to 5.0 g/L, respectively), but OTR drops near to zero when the alginate concentration is increased. Under these conditions, the relative respiration rate (RRR) of *A. vinelandii* diminished drastically; this parameter has been defined as the current respiration rate divided by the maximum respiration rate (Charoenrat *et al*., 2005). In this context, Díaz-Barrera *et al*. (2007) reported high values of RRR for *A. vinelandii* when the cultures are performed at low agitation rates as compared with cultures carried out at high agitation speeds. This observation is according to the results of the correlations used to estimate the shear rate, for example, at 140 rpm after about 40 hours of cultivation, the yield ($Y_{X/O2}$) varies from 3.2 g/g (attained with Vogel and Kroner correlation) to 4.17 g/g (obtained with $Bowen_{max}$ correlation). In contrast, at high agitation speeds (340 rpm), $Y_{X/O2}$ varies from 1.15 g/g (attained with Vogel and Kroner correlation) to 1.8 g/g (obtained with Metzner and Otto correlation). In this sense, Boiardi (1994) reported yields ($Y_{X/O2}$) for *A. vinelandii* of 4.0 gmolx/gmolO_2.

Quality of alginates produced in the effective shear rate:

The oxygen transfer rate affects considerably the mean molecular weight (MMW) of the alginate produced by *A. vinelandii* (Díaz-Barrera *et al*., 2007; Díaz-Barrera *et al*., 2009). Díaz-Barrera *et al*. (2007) found that the MMW of the alginate increased as OTR_{max} decreased in cultures performed without pH and oxygen control, conditions used in this work. In figure 2d, depicted the MMW of the alginate synthesized by *A. vinelandii* at 340 rpm. The main difference with previous report is the likely profiles of the OTR in the cultures of *A. vinelandii* at high viscosities. The MMW of the alginate produced by the bacteria increased as OTR_{max} (at the beginning of the culture) decreased. For the cultures carried out at 340 rpm, the OTR_{max} was 8.0 mmol$O_2h^{-1}L^{-1}$, but when the alginate concentration was increased the OTR profiles likely followed the Witchterle *et al*. (1984) correlation or the proposition made by Vogel and Kroner (1999). In this case, the maximum MMW of the alginate was obtained at 1.0 mmol$O_2h^{-1}L^{-1}$ and the MMW obtained was 1.08 x 10^6 g/gmol. The OTR obtained from the other correlations for estimate the shear rate works under low oxygen dissolved in the broth culture due to the higher apparent viscosities perceived by the impellers of the stirred tank. The cultures performed at 140, 180, and 200 rpm (data not shown) followed the same profile depicted in figure 2d. Other authors reported that the MMW of the polymer remains almost constant when the dissolved oxygen tension was kept at constant values (0.5%) when the agitation speed is varying (Lozano *et al*., 2011).

Virtual soft-sensor for effective shear rate:

Once quantified the total oxygen supplied to the cultures of *A. vinelandii* during alginate production, these data were used in our virtual sensor based in the Monod equation, where the oxygen was considered as a limiting substrate. The virtual sensor allows us to determine the growth modelling of *A. vinelandii* and this was based mainly on the values of $Y_{x/O2}$, which were attained using the different correlations of shear rate proposed. Is well known that *A. vinelandii* exhibits a high respiratory activity (Post *et al*., 1983), this bacterium can achieve oxygen consumption rates from 100 to 200 $mmolO_2h^{-1}gprotein^{-1}$, it means 6-14 times more than *E. coli*. In this manner, under oxygen-limited growth conditions, the OTR could be similar to the oxygen uptake rate (OUR). Therefore, the different values of OTR obtained using shear rate correlations could sense through our virtual sensor which of these equations explained better the growth of *A. vinelandii* when produced alginates and indirectly could estimate the effective shear rate caused by the agitation of the Rushton turbines. Figure 3a shows the results obtained with the virtual sensor when several data of shear rate are introduced in the Monod equation at 140 rpm during the alginate production by *A. vinelandii*. All simulations were conducted through the software denominated “Software Simulator Bioprocess” (Reyes *et al*., 2016), which was designed by us. This virtual sensor of shear rate has a purpose of discern which correlations for shear rate proposed in the literature simulates better the growth of *A. vinelandii* through the indirect method of k_La. This parameter has been used recently by Campesi *et al*. (2009) to determine the average shear rate caused by Rushton impellers during *S. clavuligerus* cultivations. At 140 rpm, the total oxygen supplied to the culture through 80 hours of cultivations varies according to the shear rate correlation used, for example, with Calderbank and Moo-Young proposal (Calderbank and Moo-Young, 1959), 1.3 gO_2 are provided to the cultures, while the correlation proposed by Witchterle *et al*. (1984) are supplied 3.63 gO_2. Figure 3a shows all the trial simulations of the virtual sensor and indicate that the Witchterle *et al*. (1984) and Vogel and Kroner (1999) correlations could model better the growth of the bacteria.

Figure 3b shows the results achieved with the virtual sensor in the cultures performed at 180 rpm after about 60 hours of cultivation. The amount of oxygen supplied varies from 1.68 gO_2 (Calderbank and Moo-Young correlation) from 2.9 gO_2 (Witchterle correlation). According to our virtual sensor, the correlations that model best the growth of *A. vinelandii* were those proposed by Withchterle *et al*. (1984) and Vogel and Kroner (1999). This same tendency was observed in the cultures carried out at 200 rpm (Fig. 3c) and 340 rpm (Fig. 3d). In this way, the virtual sensor proposed in this work indicates that Witchterle *et al*. (1984) and Vogel and Kroner (1959) correlations are the most suitable for explaining the growth of *A. vinelandii*. The above confirms a previous report made by Campesi *et al*. (2009) who demonstrated in cultures of *S. clavuligerus* that the shear rate generated by Rushton turbines is higher that reported by classical correlations commonly found in the literature.

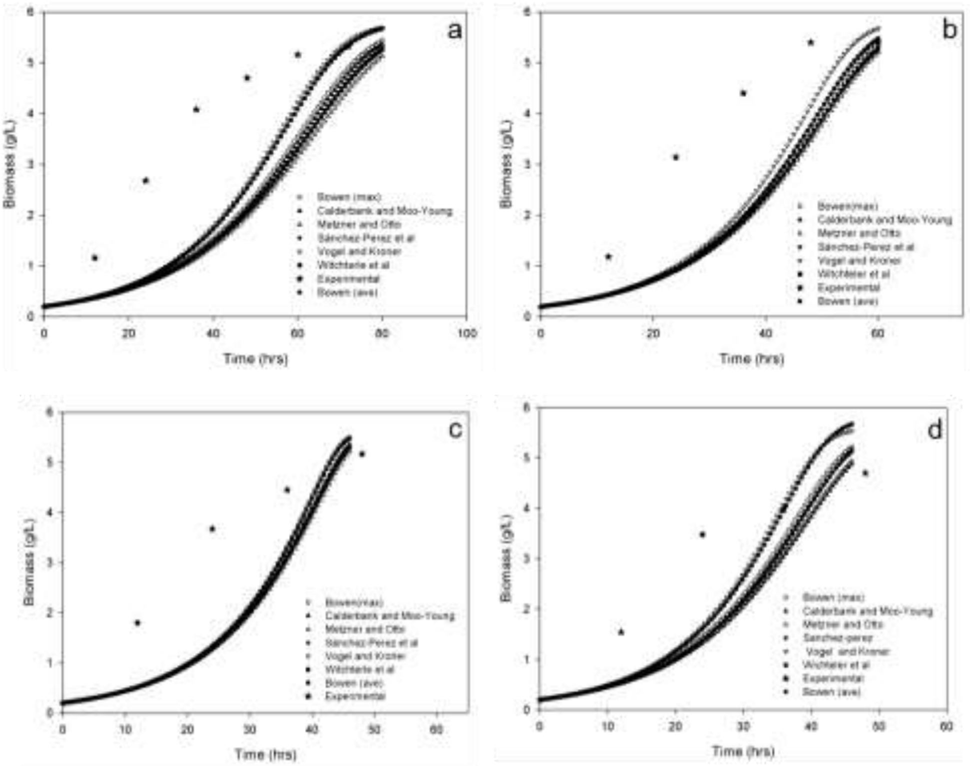

Fig. 3. a) Biomass simulation using virtual sensor and different correlation of shear rate at 140 rpm.
b) Biomass simulation using virtual sensor and different correlations of shear rate at 180 rpm.
c) Biomass simulation using virtual sensor and different correlations of shear rate at 200 rpm.
d) Biomass simulation using virtual sensor and different correlation of shear rate at 340 rpm.

CONCLUSIONS:

The design of a virtual sensor was developed based in the Monod equation to simulate the growth of *A. vinelandii*, which can produce alginate in aerobic conditions. Therefore, the Monod equation considered oxygen as a limiting substrate which are affected by the k_La values, this in turn depend on the shear rate values. The last parameter was estimated through correlations normally found in the literature. The choice of k_La as a characteristic parameter is because is affected by the apparent viscosity of the broth culture, besides was reported as an appropriate parameter in order to estimate the average shear rate during the cultivation of *S. clavuligeris*. The modelling performed by the virtual sensor demonstrated that the correlations reported by Witchterle *et al.* (1984) and Kroner were the most suitable to model the growth of *A. vinelandii*, respect to the other correlations tested. However, the model of the kinetics was not perfect due to the synthesis of the intracellular metabolite poly-□-hydroxybutyrate, the cell aggregate formation and by the presence of an alginate capsule around the cells. This shows that the shear rate generated by the Rushton turbines could be between two or three orders of magnitude higher than the common correlations normally used, such as the Metzner and Otto correlation. Finally, for further investigations is highly recommended the use of aerobic microorganisms that are capable of synthesize polysaccharides, as well as the use of gas-off analyzers (CO_2 and O_2) in order to perform a gas balance in order to confirm our models based in the virtual software.

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LIST OF SYMBOLS:

B = Lever arm (m)

B_d = Minimum size of hole required to accommodate difussing oxygen molecules (cm^2)

Cg* = Oxygen solubility ($mmolL^{-1}$)

C_L = Oxygen concentration in bulk medium ($mmolL^{-1}$)

C_2 = Constant in eq. 2 (-)

D_i = Impeller diameter (m)

D_t = Tank diameter (m)

D_l = Oxygen diffusion in liquid coefficient (cm^2/s)

D_O = Oxygen diffusion coefficient (cm^2/s)

F = Force (Newtons)

f_B (O, T) = Free volume of pure oxygen at temperature T.

K = Consistent index (Pa s^n)

K_i = Proporcionality constant eq.7; eq. 11 (-)

k_La = Volumetric oxygen transfer coefficient (s^{-1})

Ks = Saturation constant (gL^{-1})

h_B = Height of the impeller blade (m)

M = Torque (Newton.m)

MMW = Mean Molecular weight of the alginate ($gg^{-1}mol^{-1}$)

N = Rotational impeller speed (s^{-1})

n = Flow index (-)

OTR = Oxygen transfer rate ($mmolO_2L^{-1}h^{-1}$)

OUR = Oxygen uptake rate ($mmolO_2L^{-1}h^{-1}$)

O_2 = Oxygen concentration (g/L)

P = Power input drawn (W)

rO_2 = Specific oxygen consumption (gO_2L^{-1} h^{-1})

r_X = Specific growth rate (h^{-1})

t = Time (h)

V = Working volume (m^3)

Vs = Superficial gas velocity (ms^{-1})
X = Biomass (g/L)
$Y_{x/O2}$ = Yield biomass/oxygen (gg^{-1})
W = Width of the impeller blade (m)

GREEK LETTERS:

γ = Shear rate (s^{-1})
γ_{av} = Average shear rate (s^{-1})
γ_{max} = Maximum shear rate (s^{-1})
ρ = Density of fluid (kgm^{-3})
ρw_p = Alginate density in the broth (kgm^{-3})
ρ_p^o = Pure alginate density (kgm^{-3})
ω = angular velocity of the impeller ($2\pi N$)
μ = Specific growth rate (h^{-1})
μ_{max} = Maximum specific growth rate (h^{-1})
μ_{eff} = Effective viscosity (Pa s)
ϕ = Volume fraction of alginate in solution (-)