EFFECT of FLUID DYNAMIC CONDITIONS on 2,3-BUTANEDIOL PRODUCTION by *Raoultella terrigena* in SBTR: OXYGEN TRANSFER and UPTAKE RATES

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ABSTRACT

BACKGROUND: The fluid dynamic conditions play a key role in the development and scaling-up of bioprocesses. In aerobic cultures, oxygen is an essential substrate for microbial growth, production and culture maintenance; an effective gas-liquid transfer must be achieved. Changes in fluid dynamics due to stirrer speed can affect the culture negatively, causing hydrodynamic stress (increasing shear stress) or oxidative stress (by an increase of available oxygen in the liquid phase).

RESULTS: Under oxygen-limiting conditions, specific growth rate increases with stirrer speed, and several fermentation products were specifically released to the culture medium. BD production increased with stirrer speed, reaching a maximum at 400 rpm. When the agitation was increased over 550 rpm, the metabolic flux was mainly routed to increase the cell growth. Negative effects of fluid dynamic conditions on biomass production were observed at 1900 and 2000 rpm. Cellular response to shear stress conditions was also shown in the large increase with time of the broth viscosity.

CONCLUSIONS: *R. terrigena* is able to adapt the carbon metabolic flux to the availability of oxygen, producing fermentation products, alcohols or directing microbial growth. Moreover, cells can withstand aggressive agitation conditions (until 1600 rpm).

KEYWORDS: 2,3-Butanediol; *Raoultella terrigena*; Fluid dynamics; Hydrodynamic and oxidative stress; Oxygen mass transfer coefficient; Stirred tank bioreactor.

NOMENCLATURE

a:	Specific interfacial area (m)
BD:	2,3-butanediol
C_j:	Concentration of compound j ($g \cdot L^{-1}$)
C_{O2}:	Concentration of oxygen in the medium ($mol \cdot L^{-1}$)
C_{O2}*:	Oxygen concentration in equilibrium with gas concentration ($mol \cdot L^{-1}$)
C_X:	Biomass concentration ($g \cdot L^{-1}$)
CFU:	Colony Forming Units
CMD:	Cell dry mass ($g \cdot L^{-1}$)
CO_2:	Carbon dioxide
Da:	Damköhler number (-)
d_b:	Bubble diameter (m)
D_L:	Mass diffusion coefficient ($m^2.s^{-1}$)
DO:	Dissolved oxygen
F_{O2}:	Oxygen molar flow rate ($mol \cdot s^{-1}$)
K:	Consistency index in a power-law model ($Pa.s^n$)
k_L:	Mass transfer coefficient ($m \cdot s^{-1}$)
k_La:	Volumetric oxygen mass transfer coefficient (s^{-1})
N:	Stirrer speed (rpm)
n	Flow behaviour index (-)
NADH:	Nicotinamide adenine dinucleotide phosphate
OD_{600}:	Optical density at 600 nm
OTR:	Oxygen transfer rate (mmol $O_2 \cdot L^{-1} \cdot s^{-1}$)
OUR:	Oxygen uptake rate (mmol $O_2 \cdot L^{-1} \cdot s^{-1}$)
PDO:	1,3-propanediol
t:	Time (s or h)
V:	Volume of the liquid in the vessel (L)
Yi :	Molar yields of compound j (-)

Greek Letters

ε:	Energy dissipation rate per unit mass ($W \cdot kg^{-1}$)
η:	Effectiveness factor for oxygen uptake rate (-)
η_{MT}:	Effectiveness factor for oxygen transfer rate (-)
Φ:	Gas hold-up (-)
μ_a	Apparent liquid viscosity (Pa·s)
μ_{max}:	Specific maximum growth rate (h^{-1})
ρ_L:	Liquid density ($kg \cdot m^{-3}$)

Subscripts

AA:	referred to acetate
Ac:	referred to acetoin
BD:	referred to 2,3-butanediol
CO_2:	referred to carbon dioxide
DO:	referred to dissolved oxygen
Et:	referred to ethanol
G:	referred to glycerol
L:	relative to liquid phase
La:	referred to lactate
max:	referred to maximum value
Su:	referred to succinate
X:	referred to biomass
0:	referred to initial value

Superindexes

IN:	referred to inlet.
max:	referred to maximum value
OUT:	referred to outlet

INTRODUCTION

In aerobic bioprocesses, oxygen is a key substrate employed for cell growth and maintenance, and also for other metabolic routes, including product and by-product synthesis. Bioprocess development and performance are dependent not only on an efficient biocatalyst, but also on operating conditions and physiochemical properties of gas and liquid phases, affecting the relative rates of different phenomena taking place.

One of the most important factors affecting aerobic bioprocesses is the gas-liquid oxygen transfer rate, because the rate of oxygen consumption (OUR) by cells is usually high, compared to the rate of oxygen transfer (OTR) [1-5]. A balance between both OTR and OUR determines the dissolved oxygen (DO) concentration, which may significantly affect the process performance [6-10].

In stirred tank bioreactors (STBR), when stirrer speed is increased, thus increasing the mass transfer coefficient, the fluid dynamics can affect the culture, provoking hydrodynamic stress (by increasing the shear stress) or oxidative stress (by increasing *DO* concentration). This may affect the growth rate [11-14], but also the production rate of the different metabolites [15], or both [16, 17].

Raw glycerol, the main by-product in biodiesel manufacture (10% w/w), has been considered as a feedstock for other platform chemicals in order to reduce its negative impact over the economic viability of biodiesel production and its accumulation as industrial waste [18-20]. Bioconversion of glycerol presents advantages over the chemical way, such as more selectivity, moderate operating conditions and less energy demand, and allows to obtain useful chemical platforms like 1,3-propanediol, ethanol, D-lactic acid, propionic acid or 2,3-butanediol (BD) [21, 22].

BD has a large number of applications in the synthesis of many raw materials [23, 24] and also presents potential uses in perfumes, fumigants, moistening agents, pharmaceuticals,

plasticizers, food and explosives [25, 26]. Several bacteria, belonging to genera *Bacillus, Enterobacter* and *Klebsiella*, are able to produce BD using glucose and other sugars (mannose, lactose or fructose) as substrates [23, 25, 27]. There are several bacterial strains able to produce BD from glycerol, by a metabolic pathway where glycerol is degraded by oxidative and reductive routes, depending on the DO concentration [28, 29]. Recently, *Raoultella terrigena* has been proposed as biocatalyst to produce BD from glycerol [30]. The *DO* concentration is considered one of the most important variables affecting bioprocess performance, due to its influence on the key enzymes of the pathway and, hence, over the cell metabolic flux [23, 25]. At low DO concentrations, glycerol is converted into 3-hydroxypropionaldehyde by glycerol dehydratase, and subsequently reduced to 1,3-propanediol by a dehydrogenase; both enzymes are regulated by coenzyme B12 and NADH, respectively [31, 32]. At higher DO concentrations, glycerol is firstly converted to pyruvate and then channeled into a mixture of short chain organic acids (succinic, lactic, acetic and formic acids), ethanol, acetoin and BD [23]. In this case, a higher DO level leads to a biomass increase and, hence, to a higher BD production. Nevertheless, the BD production could be reduced by the deviation of metabolic flux to tricarboxylic acid cycle [28, 33]. Considering these published results, at least, if the oxygen supplied is lower than demanded, fermentation products, such as ethanol and acetic acid, would be synthetized; while if the OTR is increased, metabolic flux could move to BD and acetoin production. However, if the oxygen provided exceeds microbial demand, some authors have detected that only biomass and carbon dioxide (CO_2) are produced [23].

Accordingly to the methodology proposed in previous works [9, 13, 16], OTR and OUR must be related with potential hydrodynamic and/or oxidative cellular stress, and OUR could be used as stress indicator [34]. The effects of changing fluid dynamic conditions by increasing stirrer speed can induce cell stress, either hydrodynamic or oxidative. Hydrodynamic stress is caused in agitated cultures by collisions between cells and between cells and the components

of the reaction system, which involves shear injuries and mechanical damages [35]. Oxidative stress is produced when the ability of the biological system to readily detoxify the medium, due to the reactive intermediates and the reactive oxygen species is unbalanced [36]; this disequilibrium of the normal redox state of the cells can cause toxic effects through the production of peroxides and free radicals able to cause cellular damage or changes in the cell metabolism.

The aim of this work is to study the influence of fluid dynamics on biomass growth rate, by-products and BD production by *R. terrigena* CECT 4519 cultures in a STBR, changing stirrer speed from 100 to 2000 rpm. The results will be explained as the coupling of OTR and OUR and, therefore, by the OD concentration level reached with time in each run. Also viable cells have been measured and several parameters have been defined taking into account the relative rates of the different phenomena taking place.

EXPERIMENTAL

Microorganism and medium

Raoultella terrigena CECT 4519 has been the biocatalyst employed in this work. Cells were stored at -80ºC in 50% w/w glycerol-saline serum solution before inoculation. Medium M92x was used for all cultures, with the following composition (per liter of deionized water): 2 g of NH_4Cl, 6 g of KH_2PO_4, 12 g of Na_2HPO_4, 1 g of NaCl, 0.246 g of $MgSO_4 \cdot 7H_2O$, 0.011 g of $CaCl_2$ and 1.5 g of yeast extract. The initial glycerol concentration was 30 g/L in all runs, including the inoculum build-up. The two steps inoculum build-up were carried out in shaken flasks with 50 mL of working volume, at 30 ºC ~~of temperature~~ and 210 rpm in an orbital shaker, for 14 and 4 h, respectively. These conditions were established in a previous work [37].

STBR batch culture procedure

Experiments were carried out in a 3 L BIOSTAT® B-Plus (Sartorius AG Germany), with a working volume of 2000 mL. The bioreactor consisted on a un-baffled cylindrical vessel of

inner diameter 13.5 cm and height 20 cm, with agitation by means of a dual six blade Rushton turbine system (5.3 cm diameter) coupled to an electrical motor (Kollmorgen 3ΦPM, Danaher Motion, Czech Republic). The reaction equipment was ~~previously~~ sterilized by autoclaving at 121 ºC for 20 min. The experiments were carried out employing 12.5 % (v/v) of an inoculum at adequate operating conditions determined in previous works [30, 37]: 30 ºC temperature, 0.25 g/L as initial biomass concentration, pH monitored at 5.5 after free evolution from 6.9 using 2 M NaOH and 2 M HCl solutions and airflow of 1.5 vvm. Stirrer speed was modified from 100 to 2000 rpm and the outlet gas bioreactor stream was coupled to a combined oxygen-carbon dioxide sensor for determining outlet gas composition. Culture samples were withdrawn during each run to determine biomass concentration and to quantify broth metabolite concentrations by HPLC, previously having removed the biomass by centrifugation (14,000 x *g* during 10 min).

Analytical methods

Biomass concentration was determined by measuring the culture optical density at 600 nm (Shimadzu UV-visible spectrophotometer UV-1603). The cell dry mass (CDM), obtained after drying biomass at 105 ºC until constant weight, was related with optical density (OD_{600}), according to the following equation:

$$CDM\ (g/L) = 1.616\ x\ OD_{600} \quad (1)$$

Colony viable counting was carried out in several runs in order to be compared with the OD measurements described above. Agar plates with LB medium [38] were seeded (3 for each sample) from saline serum serially diluted samples taken at different times from the STBR. Microbial colonies appearing in the dishes after incubation at 30 ºC within 24 h were counted and the biomass concentration was determined as Colony Forming Units (CFU) per mL of culture applying the corresponding dilution factor.

Glycerol and other metabolites (succinate, lactate, acetate, ethanol, acetoin and BD) were quantified by HPLC (Agilent Technologies, 1100 Series), using a Rezex RHM-Monosaccharide H+ (8 %) column (300 x 7.8 mm, Phenomenex), coupled to both refractive index and diode array detectors. The sample volume was 5 μL and 0.005 M H_2SO_4 was employed as mobile phase at 0.6 mL/min of flow rate. Column temperature was monitored at 65 ºC, while refractive index detector operated at 55 ºC.

Bioreactor outlet gas stream composition was determined using a combined oxygen-carbon dioxide sensor BioPAT® Xgas (Sartorius AG, Germany), connected to the STBR outlet gas stream. The sensor was calibrated before every run by circulating 3 L/min of compressed air during 30 min.

Broth apparent viscosity *(μ_a)* was determined by measuring the shear stress *(τ)* at different shear rates *(γ)* in a Brookfield® Synchro-Electric Viscosimeter (Brookfield, USA), according to Ostwald de Waele rheological model:

$$\tau = K \cdot \gamma^n = \mu_a \cdot \gamma \tag{2}$$

THEORETICAL BACKGROUND

Growth rate modeling

Microbial growth rate has been described according to equation (3), employed in previous works for different bioprocesses [10, 17, 39]:

$$\frac{dC_X}{dt} = \mu_{max} C_X \left(1 - \frac{C_X}{C_X^{max}}\right) \tag{3}$$

If this equation is integrated with the boundary condition *($t = 0;\ C_X = C_{X0}$)*, the logistic growth equation is obtained:

$$C_X(t) = \frac{C_{X_0} exp(\mu_{max} t)}{1 - \frac{C_{X_0}}{C_X^{max}}[1 - exp(\mu_{max} t)]} \tag{4}$$

OTR and OUR determination

The oxygen mass balance in the liquid phase, assuming well-mixed culture, can be established by [5]:

$$\frac{dC_{O_2}}{dt} = OTR - OUR \tag{5}$$

OTR depends on the mass coefficient transfer, k_L, the specific interfacial area, a, and the driving force of the oxygen transfer, which is the difference between the equilibrium dissolved oxygen concentration in the liquid phase at working pressure and temperature, $C^*_{O_2}$, and the DO concentration in the broth, C_{O_2}, according to:

$$OTR = k_L a \cdot \left(C^*_{O_2} - C_{O_2}\right) \tag{6}$$

OTR can be determined by an oxygen mass balance in the gas phase by the difference between the inlet and outlet oxygen molar flow rates ($F_{O2}^{IN}$and F_{O2}^{OUT}, respectively), as follows:

$$\frac{F^{IN}_{O_2} - F^{OUT}_{O_2}}{V} = OTR \tag{7}$$

Once OTR is determined and DO concentration is measured in the liquid phase, k_La can be calculated directly from equations (6) and (7), yielding:

$$k_L a = \frac{F^{IN}_{O_2} - F^{OUT}_{O_2}}{V \cdot \left(C^*_{O_2} - C_{O_2}\right)} \tag{8}$$

Then OUR can be calculated according to equation (5), once OTR and DO oxygen profile are known, calculating dC_{O2}/dt by numerical methods, such as those described elsewhere [17].

Volumetric mass transfer coefficient (k_La) prediction

In order to evaluate the validity of the experimental measurements of OTR and k_La, calculated as indicated above, these values were compared to those given by semi-empirical

prediction methods widely used in the literature for k_L and a [40, 41]. If the rheological behavior of the broth is described by the Ostwald de Waele model (see equation 2), the following equation is obtained to predict the mass transfer coefficient:

$$k_L = \frac{2}{\sqrt{\pi}} \cdot \sqrt{D_L} \left(\frac{\varepsilon \cdot \rho_L}{K}\right)^{\frac{1}{2(1+n)}} \quad (9)$$

The interfacial area *(a)* can be calculated from the values of the average bubble size *(d_b)* and the gas hold-up *(Φ)*, assuming spherical bubbles [42].

$$a = \frac{6\Phi}{d_b} \quad (10)$$

Calculation of the average energy dissipation rate per mass unit, ε, the gas hold-up, *Φ*, and the average bubble size, d_b, are detailed in previous works [40, 41].

OTR and OUR analysis

Oxygen transfer and uptake rate limitations have been analyzed employing the methodology previously described [5], defining two dimensionless parameters, the observed effectiveness factor *(η)* and the *Damköhler* number *(Da)*, in order to evaluate whether the overall process rate is limited by oxygen transport or by the biochemical reactions forming the cell metabolism [7, 9].

The effectiveness factor for the oxygen uptake rate indicates the degree of utilization of oxygen by the cells during the culture process, and is defined as the ratio of the observed oxygen uptake and the uptake rate without mass transfer limitations, according to:

$$\eta = \frac{OUR}{OUR_{max}} \quad (11)$$

If $\eta < 1$, the transport is the rate-limiting step of the bioprocess; if η is close to 1, the cellular activity is not limited by oxygen transfer.

The *Damköhler* number is useful for determining the rate-limiting step of the bioprocess, comparing the potential OUR and OTR maximum values as follows:

$$Da = \frac{OUR_{max}}{OTR_{max}} \tag{12}$$

This dimensionless parameter indicates the ratio between the potential rates of oxygen consumption and transport rates, also indicating if the bioprocess is transport-limited, biochemical reaction-limited, or whether it occurs under intermediate operation conditions. If $Da > 1$, the biochemical reaction rate is faster than the oxygen supply rate and, therefore mass transfer resistance is the controlling step of the overall process rate. However, if $Da \leq 1$, the oxygen consumption rate is slower than OTR and the overall process rate is then limited by the rate of chemical reactions [5].

In this work, another dimensionless parameter is defined, η_{MT}, or effectiveness factor for oxygen transfer rate, as the ratio between the experimental OTR and the potential OTR maximum values, in a similar way to that done for the chemical phenomenon, according to:

$$\eta_{MT} = \frac{OTR}{OTR_{max}} \tag{13}$$

The value of η_{MT} provides an idea of the limitations in oxygen transfer throughout the biochemical process. If $\eta_{MT} < 1$, the bioprocess is oxygen transfer-limited; if η_{MT} is close to 1, the cellular activity is not limited by oxygen transfer. It must be taken into account that k_La values can decrease with the time course of a bioprocess due to physical changes in the liquid phase, such as an increase in viscosity or a decrease in surface tension, and also the driving force, in equation (6), can be smaller if DO concentrations increase.

The value of OTR_{max} is easily calculated from equation (6) with C_{O2} equal to zero.

$$OTR_{max} = k_La \cdot C^*_{O_2} \tag{14}$$

RESULTS and DISCUSSION

Experiments at eleven different stirrer speeds were performed (100, 250, 400, 550, 700, 850, 1000, 1300, 1600, 1900 and 2000 rpm). Experimental results of *R. terrigena* growth at different stirrer speeds are shown in Figure 1 (A-B), together with the DO concentration profiles (C-D) and the glycerol concentration profiles (E-F). Maximum biomass and metabolite concentrations achieved in the different runs are given in Table 1. Experimental growth data were used to determine the kinetic parameters of equation (4) by nonlinear regression, minimizing the sum of squared residuals as convergence criterion. The parameter values obtained in all the runs are given in Figure 2. In this figure are also shown the ratio between molar yield, Y_j, and the maximum theoretical yield, Y_j^{max}, for each product; these latter values were calculated as described by Doran [43]. Finally, in Figure 2 are also given the OTR_{max} values calculated as previously indicated. All these results suggest that microbial metabolism is clearly affected by fluid dynamic conditions.

Influence of oxygen transfer rate on biomass growth and substrate consumption rates

The reproduction of experimental biomass concentration by equation (4), using the kinetic parameter values given in Figure 2, is also presented in Figures 1A and 1B; as can be seen the model of equation (4) is able to fit all the experimental results; all the fittings passed F-test at 95% of confidence interval. Moreover, Figures 1 and 2 show that biomass growth rate increased with stirrer speed between 100 and 550 rpm; then, the growth rate remained constant between 550 and 1600 rpm; but at higher stirrer speeds, in the runs carried out at 1900 and 2000 rpm, a slight decrease in growth rate can be observed.

These results are in agreement with the *DO* concentration profiles (Figure 1C and 1D) and glycerol concentration evolution (Figure 1E and 1F). Changes in fluid dynamic conditions involve three different behaviors regarding microbial growth and substrate consumption. When the culture evolves under limiting oxygen conditions, that is, when DO concentration

becomes zero, the biomass growth rate and the glycerol uptake rate increase with agitation (see Figure 1A, 1C and 1E). In these runs, OTR is equal to OUR according to equation (5), because DO concentration remains equal to zero. If stirrer speed is higher than 400 rpm, the oxygen supplied to the liquid phase is not consumed completely, which means that OTR is higher than OUR, and therefore DO concentration is greater than zero. If the batch is conducted between 550 and 1600 rpm of stirrer speed, the biomass growth rate reaches its highest value and the biomass concentration profiles overlap, together with the evolution of glycerol concentration (see Figure 1B, 1D and 1F). Under this range of agitations, the DO concentration virtually does not change during batch time. That is, OTR is slightly greater than OUR, but the value of k_La is so high that, to maintain a coherent value of OTR, the driving force, the difference between the oxygen saturation concentration and DO concentration, decreases to a value close to zero, and then DO concentration profile is practically constant (Figure 1D).

A negative effect of very high agitation conditions over microbial growth can be observed at 1900 and 2000 rpm, although DO and glycerol concentration profiles remain close to those values obtained for stirrer speeds between 550 and 1600 rpm.

These effects of fluid dynamic conditions, through stirrer speed, can be clearly seen in Figure 2A, where the values of the two kinetic parameters of equation (4) are shown. First, both μ_{max} and C_X^{max} increase in parallel as the stirrer speed increases; then, these parameters remain constant between 550 and 1600 rpm; and finally, these values decrease, when 1900 and 2000 rpm of stirrer speed are employed.

Influence of oxygen transfer rate on cell metabolism

In Figure 2 the evolution of several variables with respect to the stirrer speed employed in each run can be observed. Thus, the metabolite yields reached respect to their maximum

theoretical values and the total CO_2 produced can be seen in Figure 2B; in Figure 2C the maximum values of the oxygen transfer rate (OTR_{max}) are given.

The three different bacterial behaviors, described above, are more evident when the metabolic response to the oxygen supplied is analyzed. When OTR is low, related fermentation products, such as ethanol and lactate, are the main final metabolites in the culture medium. BD yield reaches its maximum value at 400 rpm, when OTR_{max} is close to $5.5 \cdot 10^{-3}$ mmol $O_2 \cdot L^{-1} \cdot s^{-1}$. If stirrer speed is increased from that value, the cellular metabolism changes, being clearly directed to biomass production; thus, the maximum values of μ_{max} and C_X^{max} are reached, several pathways of mixed acid route are inactivated and CO_2 production clearly increases (see Figure 2A, 2B and 2C). These observations agree with previous works, where the influence of the dissolved oxygen level was studied in different bioprocesses. For example, Park et al. [44] observed that if the stirrer speed was higher than 350 rpm, the production of 2,3-BD decreased dramatically while the biomass growth increased in cultures with *Klebsiella oxytoca* using glucose as sole carbon source. In this work[44], the biomass profiles overlap from 550 to 750 rpm. Ji et al [45] have proposed a two-stage agitation speed strategy for enhancing 2,3-BD final concentration with engineered strains of *K. oxytoca*, because the production declined from 300 rpm, in favor of biomass growth [45].

The OTR_{max} values are higher than 0.017 mmol $O_2 \cdot L^{-1} \cdot s^{-1}$ in the runs from 700 rpm to 1300 rpm of stirrer speed, when the biomass concentration profiles overlap. At 1600 rpm, a significant decrease in CO_2 production and OTR_{max} are observed and this fact is more evident at 1900 and 2000 rpm. Thus, it can be assessed that mixed acid route remains blocked to produce alcohols, diols and organic acids for stirrer speeds of 850 rpm and higher, being CO_2 and biomass the main metabolic products until 2000 rpm (Figure 2B).

These observations allow to define four culture behaviors with respect to the fluid dynamic conditions: an initial fermentation zone (100-400 rpm), where an increase in OTR favors BD

an acetoin production; an intermediate region, whose border is highly sensitive to OTR (since at 550 rpm the metabolic change is evident in terms of mixed acid route products distribution); a third zone (700-1300 rpm), where the culture maximizes microbial growth and carbon dioxide is the main by-product; and a last agitation range (1600-2000 rpm), where the increase in stirring speed carries on a decrease in microbial growth rate, in OTR_{max} and in CO_2 production. According to these observations it can be suggested that *R. terrigena* is really sensitive to changes in dissolved oxygen availability, as occurs with other bacterial strains able to produce 2,3-BD [44-46].

Theoretical values of k_La have been calculated (equations 9 and 10) at several times in various runs carried out under different stirrer speeds. It was necessary to measure the broth viscosity, describing its evolution with time using the Ostwald de Waele rheological model (equation 2). With the k_La values the theoretical maximum values of transport rate, OTR_{max}, were calculated. Then values of η, Da and η_{MT} were determined using the experimental measured values of OTR and OUR. The evolution with time of these three dimensionless numbers, together with the viscosity and the k_La profiles are presented in Figure 3. The broth viscosity increases with time (see Figure 3A), especially when the culture is conducted under non-limiting oxygen conditions and the metabolic change previously described is observed (from 550 rpm). This change in broth viscosity with time is dramatic when the biomass growth reaches the stationary growth stage (see also Figure 1B). This may be due to the secretion of cell polysaccharides at later stages of growth as a cellular response to the aggressive agitation conditions, as quoted by other authors [47]. The production of these polysaccharides is reinforced by the fact that when the broth viscosity reaches its highest values (from 1000 rpm), the total CO_2 produced at the end of the fermentation decreases when stirrer speed is increased (see also Figure 2C). Broth viscosity profiles influence the evolution of k_La predictions, involving a sharp decline of this coefficient with time, according

to Figure 3 (B). These observations presume that theoretical OTR_{max} is getting smaller with time, as given by equation (14).

When the run is conducted under limiting oxygen conditions (e.g. 400 rpm), *Da* is always close to 1 and the rate-limiting step is the OTR. If stirring speed is higher, *Da* decreases with time, as a result of OUR_{max} being lower from run to run (see Figure 3C). The latter increase of this parameter in the experiments conducted at 550 and 700 rpm is due to the critical decrease in k_La (see also Figure 3A), which affects directly the theoretical value of OTR_{max}, according to equation (14).

The time ~~of~~ course of η_{MT} at different agitation conditions is shown in Figure 3D. When the oxygen transport is the rate-limiting step, the levels of experimental OTR are close to the theoretical OTR_{max} and $\eta_{MT} = 1$ throughout the run. However, for runs conducted from 700 rpm, the overall process rate is limited by the biochemical reaction rate (see also *DO* profiles in Figure 1C and 1D), and η_{MT} decreases with time after reaching its maximum value. For the runs carried out with N > 700 rpm, cellular stress provokes low values of OUR, and consequently also low values of OTR, which causes the driving force of equation (6) to be reduced. Moreover, the increase in broth viscosity affects k_La negatively and hence, the measured OTR is significantly lower than the predicted value of OTR_{max}.

The evolution with time of the effectiveness factor *(η)* is presented in Figure 3E. If the rate controlling step is the oxygen transfer rate (e.g. 400 rpm), η remains close to 1, which means that OUR takes its maximum possible value [5]. Conversely, if the biochemical reaction rate is the slowest process step (from 550 rpm), η takes the value 1 in the early stages of the run, till the steady state growth phase is reached, and then decreases with time. This drop is more pronounced the higher the stirrer speed is, indicating that the oxygen consumption by the culture is negatively influenced by the stress conditions.

Evaluation of cellular stress under high agitation conditions

Experimental data on biomass growth, described above and shown in Figures 1A and 1B, permits to establish different growth rate ranges when stirrer speed is changed. Biomass quantification by OD is based on the culture sample absorbance, which is compared with a water pattern at a certain wave length. The absorbance is related with broth turbidity, but does not inform about cell integrity or damage due to hydrodynamic stress or oxidative stress. Figure 4 presents bacterial growth profiles under several agitations, measured by viable colony counting in LB agar plates. The results confirm OD measurements, but also reflect that *R. terrigena* cells are affected by shear stress at high agitation conditions, especially after 18 h of time growth, when the number of colonies forming units decreases significantly. This behavior can explain the decrease in total CO_2 released and OTR_{max} in the runs carried out from 1300 rpm. If culture cells are damaged, and OUR is lower, OTR is accordingly reduced, which affects the driving force value in equation (6). The growth rate decline observed at 1900 rpm by OD measurements (see Figure 1B) is confirmed by viable colony counting, reaching a final number of colonies similar to that achieved at 400 rpm (see Figure 4). This analysis reinforces the previous~~ly~~ description of four different regions that can be observed in the culture behavior when different stirrer speeds are employed. The first one corresponds to runs conducted at low agitation(from 100 to 400 rpm of stirrer speed) when the process is carried out under oxygen transport limiting conditions; the biomass growth rate increases with increasing ~~of~~ agitation and mixed acid route metabolites are produced. When the culture is conducted under non-limiting transport conditions (stirrer speed higher that 550 rpm), all the oxygen supplied is directed to bacterial growth and no by-products are obtained, except CO_2; this behavior is maintained in all the runs carried out with stirrer speeds between 550 and 1300 rpm. If the stirrer speed is even higher (1600-2000 rpm), cells are affected by stress, which is reflected in the decrease in total CO_2 released and the decrease of the value of

OTR_{max}, which happens from 1300 rpm. Cellular stress involves lower values of OUR, reducing consequently OTR according to equation (6), because the DO concentration remains close to C_{O2}* throughout the run (see also Figure 1D). If the runs are conducted at the maximum stirrer speeds studied, 1900 and 2000 rpm, cells are affected by hydrodynamic stress, and the values of CO_2 produced and OTR_{max} are significantly lower; thus, bacterial growth rate is slower and the biomass concentration when the stationary growth stage is reached is lower too (see Figures 2A and 2C).

CONCLUSIONS

The BD production bioprocess with *R. terrigena* CECT 4519 has been studied under different fluid dynamic conditions in a STBR. Stirrer speed has been changed from 100 to 2000 rpm. Cellular metabolism is clearly affected by fluid dynamic conditions and *DO* concentration level. At low stirrer speed, under oxygen-limiting conditions, growth rate increases with the agitation, as clearly shown for the kinetic parameter values of equation (4). Under these conditions, fermentation products are synthetized, such as ethanol and lactate. Also BD production increases with the stirrer speed, reaching a maximum at 400 rpm. For stirrer speed higher than 550 rpm, the metabolic flux changes to biomass production, although substrate uptake rate remains constant from 550 to 1600 rpm. In experiments carried out employing very high stirrer speed, 1900 and 2000 rpm, biomass production is negatively affected most probably by hydrodynamic stress. In the runs conducted under no-limiting oxygen conditions, the broth viscosity increases dramatically during the steady state growth phase, reducing the values of volumetric mass transfer coefficient *(k_La)*, and the maximum theoretical value of *OTR*. Cellular stress due to fluid dynamic conditions is observed by the changes in broth viscosity and the decrease of the number of colonies forming units after 18 h of culture.

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Table 2. Experimental results of the conversion of glycerol by *R. terrigena* through mixed acid route at different agitations: biomass and metabolite concentrations achieved at the end of the experiments. Confidence interval is also given.

N (rpm)	C_X ($g \cdot L^{-1}$)	C_{Su} ($g \cdot L^{-1}$)	C_{La} ($g \cdot L^{-1}$)	C_{AA} ($g \cdot L^{-1}$)	C_{Ac} ($g \cdot L^{-1}$)	C_{Et} ($g \cdot L^{-1}$)	C_{BD} ($g \cdot L^{-1}$)	C_{CO2} ($g \cdot L^{-1}$)
100	6.91 ± 0.58	0.74 ± 0.09	2.49 ± 0.37	0.76 ± 0.11	0.48 ± 0.07	5.20 ± 0.62	4.28 ± 0.61	9.1 ± 1.01
250	10.1 ± 0.62	0.89 ± 0.11	1.46 ± 0.24	0.79 ± 0.14	1.02 ± 0.16	4.27 ± 0.54	5.74 ± 0.82	9.4 ± 1.21
400	16.5 ± 0.71	0.88 ± 0.15	0.78 ± 0.12	0.76 ± 0.17	1.56 ± 0.24	3.33 ± 0.47	5.85 ± 0.86	9.8 ± 1.23
550	22.5 ± 0.74	0.18 ± 0.02	0.03 ± 0.01	0.09 ± 0.02	0.95 ± 0.12	0.08 ± 0.01	1.18 ± 0.20	11.1 ± 1.55
700	22.1 ± 0.77	0.07 ± 0.01	0.03 ± 0.01	0.14 ± 0.01	0.13 ± 0.02	0.15 ± 0.03	1.14 ± 0.17	15.6 ± 1.46
850	22.3 ± 0.78	0 ± 0.01	0 ± 0.01	0.10 ± 0.01	0 ± 0.01	0 ± 0.01	0 ± 0.01	16.8 ± 1.82
1000	22.5 ± 0.78	0 ± 0.01	0 ± 0.01	0 ± 0.01	0 ± 0.01	0 ± 0.01	0 ± 0.01	13.8 ± 1.94
1300	22.9 ± 0.73	0 ± 0.01	0 ± 0.01	0 ± 0.01	0 ± 0.01	0 ± 0.01	0 ± 0.01	12.6 ± 1.33
1600	23.5 ± 0.79	0 ± 0.01	0 ± 0.01	0 ± 0.01	0 ± 0.01	0 ± 0.01	0 ± 0.01	7.3 ± 1.58
1900	21.1 ± 0.72	0 ± 0.01	0 ± 0.01	0 ± 0.01	0 ± 0.01	0 ± 0.01	0 ± 0.01	6.9 ± 1.07
2000	18.9 ± 0.71	0 ± 0.01	0 ± 0.01	0 ± 0.01	0 ± 0.01	0 ± 0.01	0 ± 0.01	6.5 ± 0.96

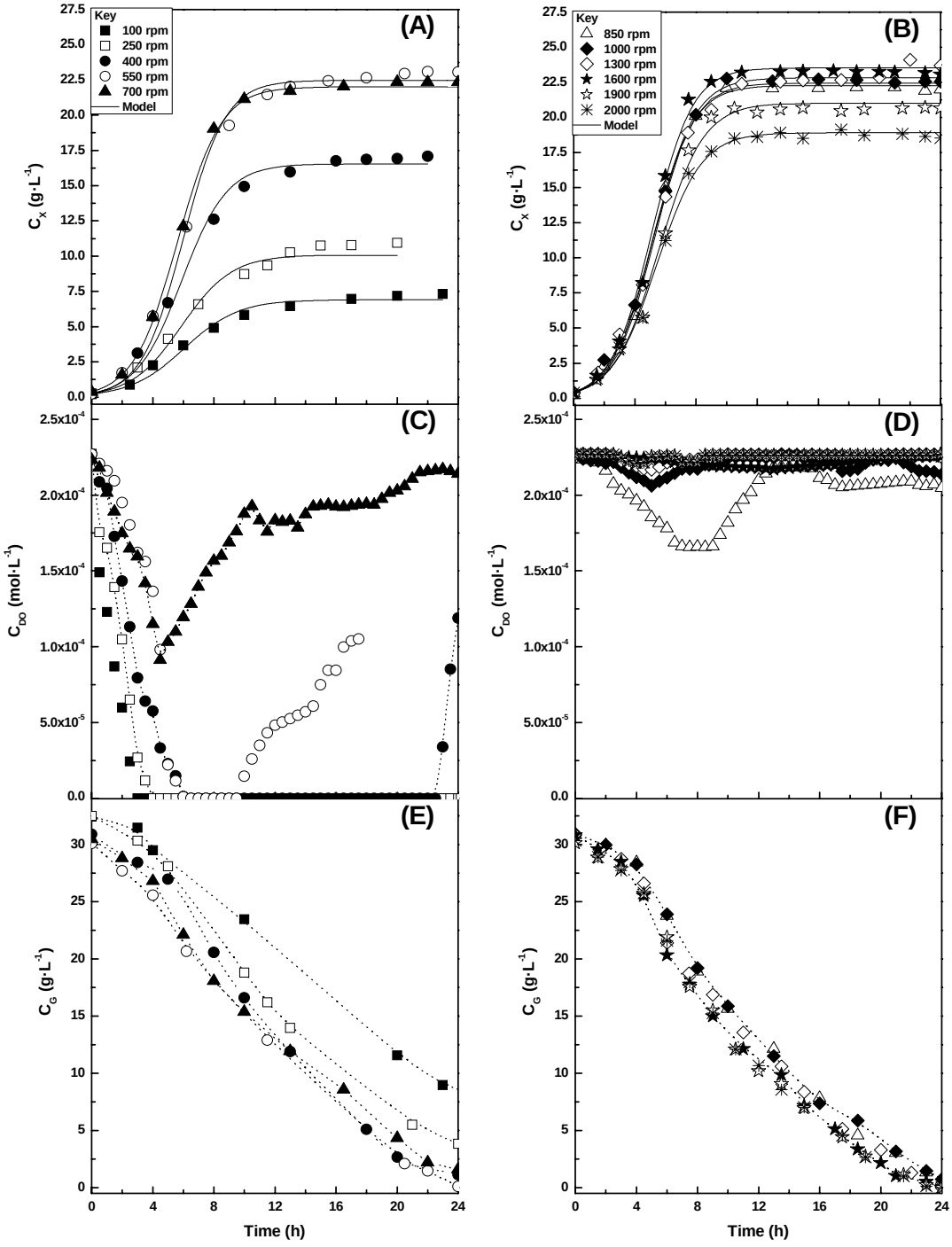

Figure 3. Biomass (A-B), dissolved oxygen (C-D) and glycerol (E.F) concentrations time course in *R. terrigena* batch cultures under different agitation conditions.

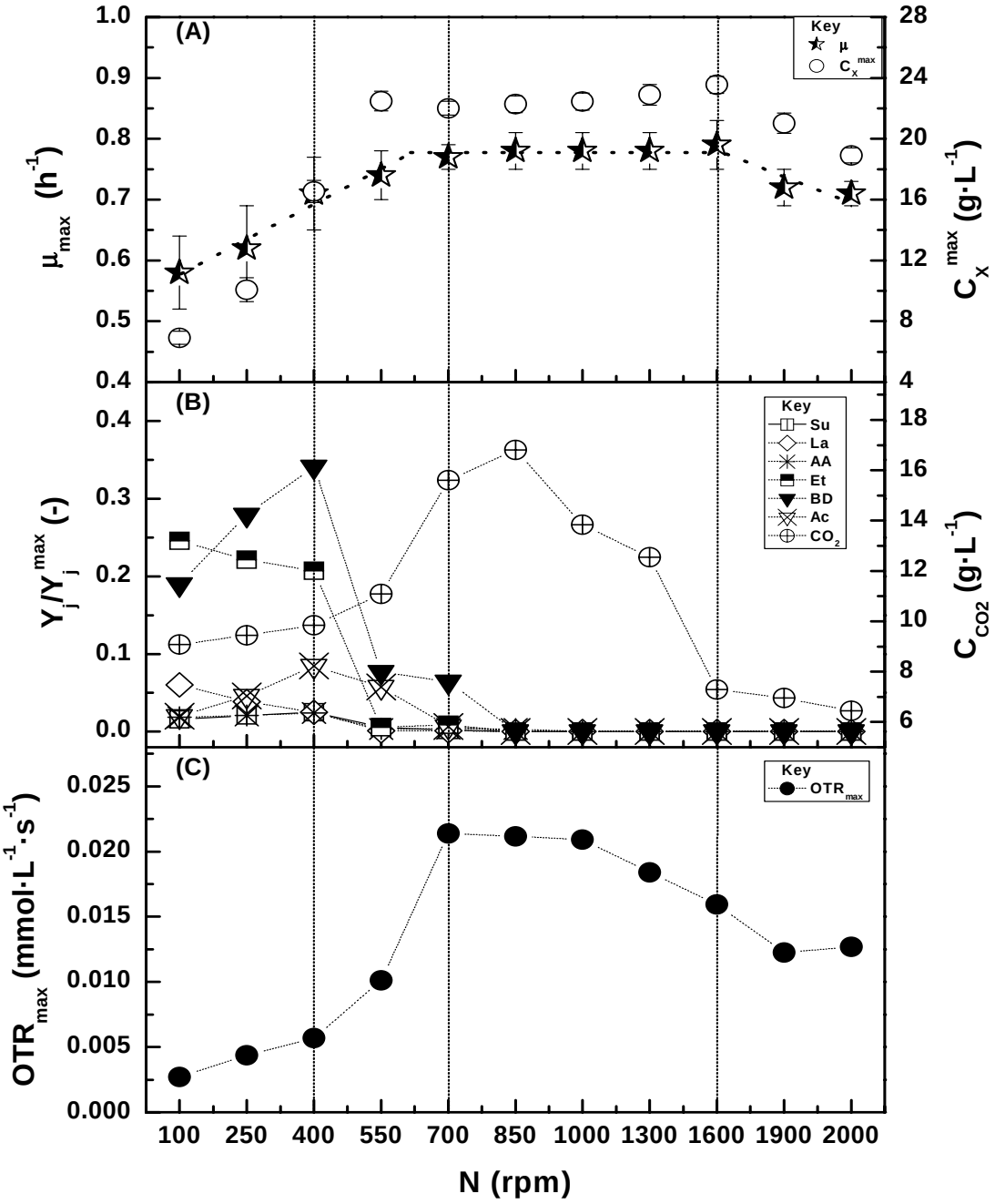

Figure 2. Kinetic growth parameters (A), relative metabolite yields respect to the maximum theoretical value and CO_2 produced (B) and maximum oxygen transfer rate (C) for *R. terrigena* cultures in runs conducted at several agitation speeds.

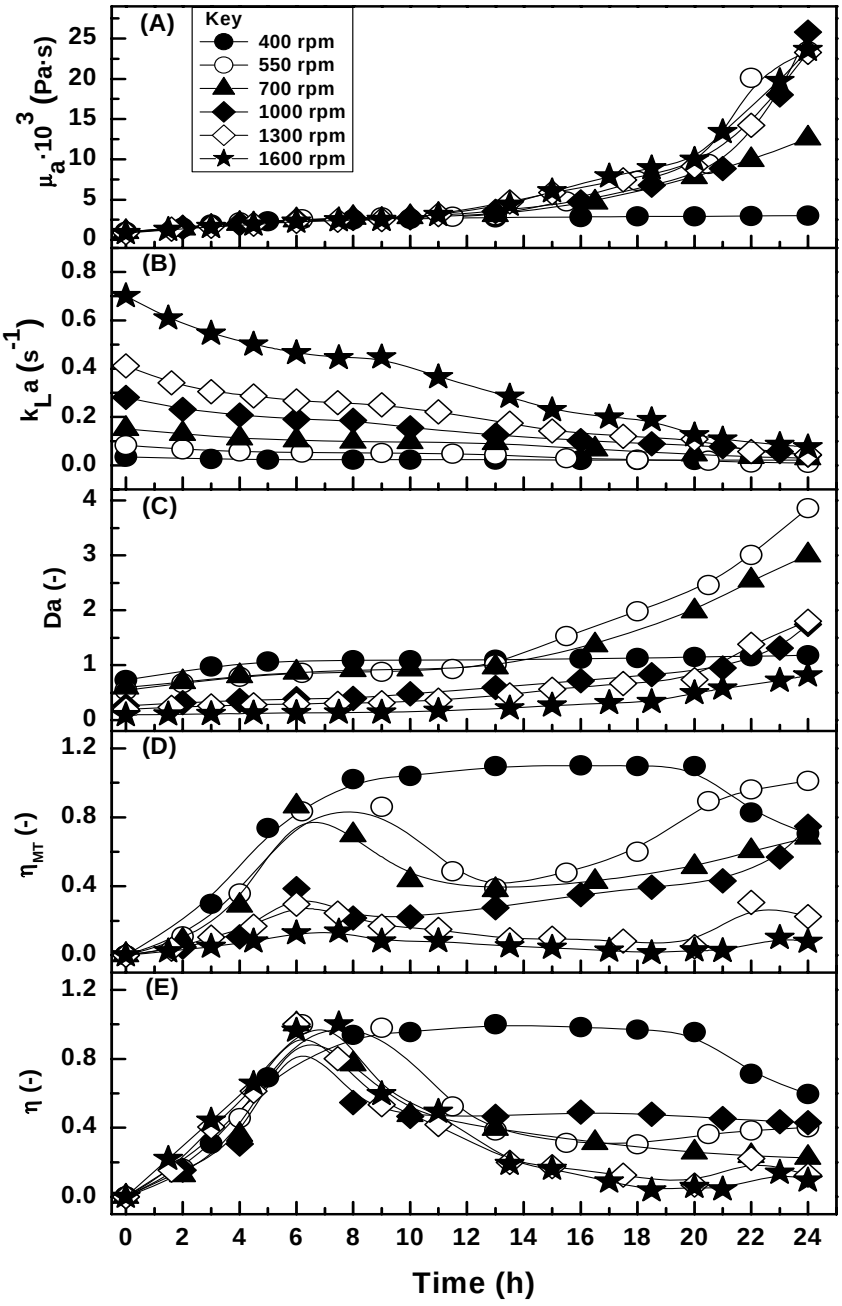

Figure 4. Evolution with time of broth viscosity (A); volumetric mass transfer coefficient (B); *Damköhler* number (C); effectiveness factor for oxygen transfer rate (D); effectiveness factor for oxygen uptake rate (E) for *R. terrigena* cultures carried out at different agitation conditions.

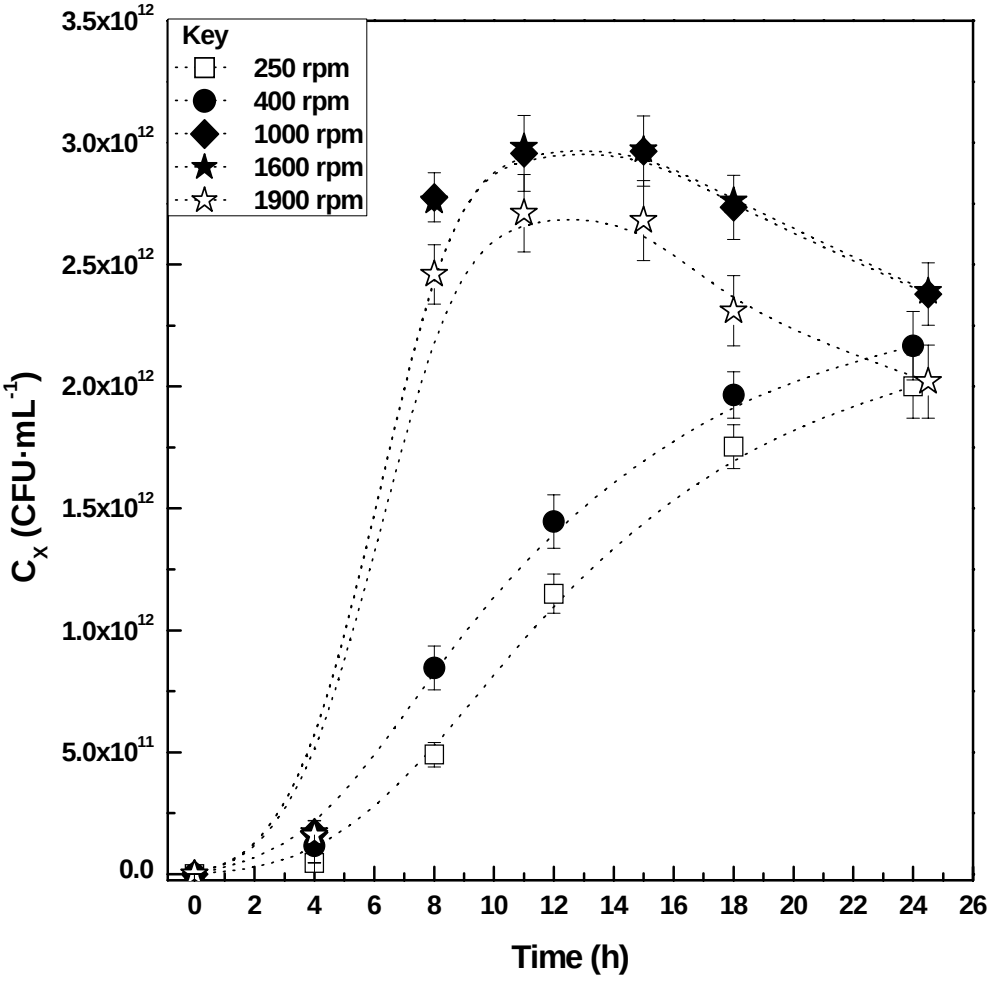

Figure 4. Time of course of *R. terrigena* colonies forming units (CFU) in LB medium agar plates in runs carried out with different stirrer speeds to evaluate culture hydrodynamic stress.