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Shaken flasks by resonant acoustic mixing versus orbital mixing: Mass transfer coefficient k_La characterization and *Escherichia coli* cultures comparison

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ABSTRACT

Shaken flasks are widely applied in bioprocesses due to their flexibility and ease of operation. Resonant acoustic mixing (RAM) enables non-contact mixing by the application of low frequency acoustic energy, and is proposed as an alternative to solve oxygen limitations in orbital mixing (OM). The aim of this study is to experimentally determine empirical k_La correlations for RAM and compare it with OM by its measurement at different shaking frequencies, nominal flask volumes, and filling volumes. The maximum k_La here obtained were $131.3 \pm 5.1\ h^{-1}$ for OM and $435.4 \pm 11.7\ h^{-1}$ for RAM. Empirical correlations were validated for k_La as a function of shaking frequency and superficial area/filling volume ratio and rendered adequate values for the adjusted R^2 with an accuracy of ±30%. Further, we compared the *Escherichia coli* kinetics of growth, glucose uptake, dissolved oxygen tension (DOT), and organic acids production in RAM and OM at two equivalent initial k_La. Similar *E. coli* kinetics were observed at an initial k_La of $46\ h^{-1}$, but not at $92\ h^{-1}$ where differences in DOT and culture parameters were found, mainly in growth rate and biomass yield on glucose, which is the result of different oxygen transfer rates due to the increased gas pressures in RAM.

1. Introduction

Orbital shaken flasks are well established bioreactors mainly due to its flexibility, low cost and ease of operation; hence, they are widely applied for screening experiments during the early stages of bioprocesses development, such as clone selection and determination of culture optimal conditions [1–3]. The insufficient supply of oxygen through the gas–liquid interface or/and the flask closure is a major deficiency in using orbital shaken flasks as culture bioreactors [1]. Since drastic effects on aerobic cultures (such as decreased metabolism, development of anaerobic metabolism or low production of desired metabolites) are often generated by differences in the availability of oxygen, changes in the operational conditions are needed in order to avoid oxygen limitation [1,4–8]. During microbial cultures, the available oxygen is given by Eq. (1), where the oxygen transfer rate (OTR) is defined as the rate of oxygen transferred through the gas–liquid interphase (liquid surface in shaken flasks) into the bulk liquid as described in Eq. (2), as follows:

$$\frac{dC_L}{dt} = \mathrm{OTR} - OUR \quad (1)$$

$$\mathrm{OTR} = k_La(C_L^* - C_L) \quad (2)$$

where $(C_L^*–C_L)$ corresponds to the oxygen concentration gradient between the interfacial saturation and the liquid bulk, and the k_La is the volumetric mass transfer coefficient which is used to compare

Abbreviations: A, superficial area of the liquid at the inner diameter of the motionless flask, cm^2; *a, b,c*, coefficients for the empirical models specific for each agitation system; adj R^2, adjusted *R*-square; A/V, superficial area/filling volume ratio, cm^{-1}; C_L^*, oxygen concentration at the interfacial saturation; C_L, oxygen concentration in the liquid bulk; d, maximum inner flask diameter, m; d_0, shaking diameter, cm; DOT, dissolved oxygen tension, % air saturation; DOF, degrees of freedom; Fr, Froude number; a, acceleration of gravity, $m.s^{-2}$; k_La, volumetric oxygen transfer coefficient, h^{-1}; n, shaking frequency, s^{-1}; Ph, phase number; RAM, resonant acoustic mixing; RMSE, root mean squared error; V_N, nominal volume, mL; V_F, filling Volume, % liquid volume/nominal flask volume; V, villing volume, mL.

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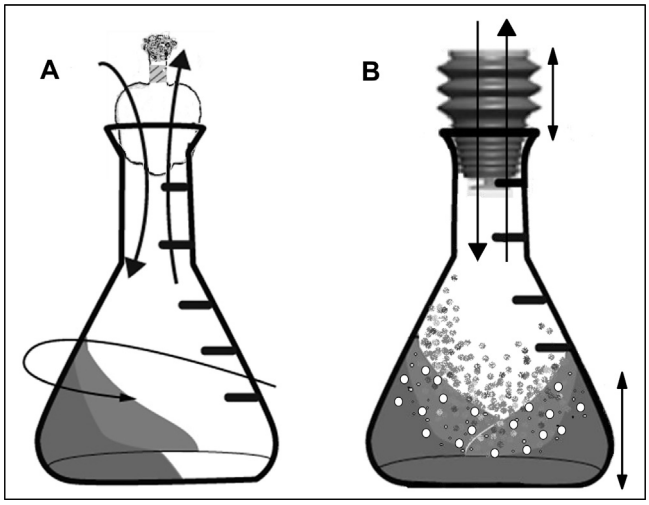

Fig. 1. Aeration of flasks shaken by two different systems. (A) Orbital movement in two dimensions, where the aeration of unbaffled flasks is accomplished only by diffusion [1,4]. The first resistance is through the plug, where the gas transfer occurs only by diffusion [13,32]; and (B) the RAMbio system oscillates in one dimension through low frequency acoustic resonance generating axial flow patterns; then, aeration is accomplished by diffusion, the entrapment of droplets that separate from the liquid bulk, and the formation of small bubbles [24]. The closure has an active participation, so the headspace is actually oscillated while mixing, creating a substantial driving force pumping gas molecules into and out of the flask [19].

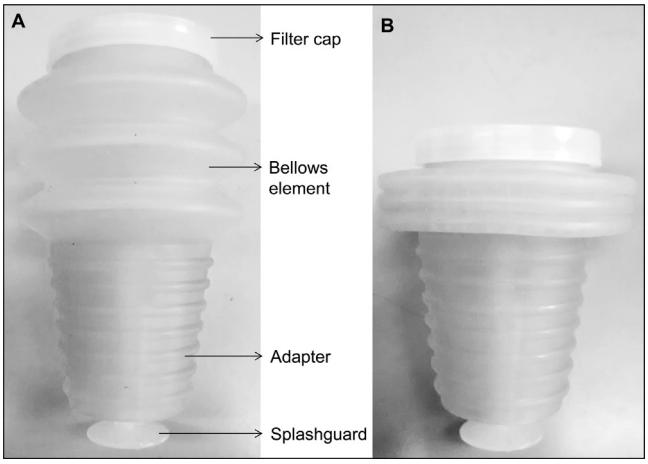

Fig. 2. Oxypump stoppers. (A) These silicone plugs, are comprised of four main parts with specific functions. The special design of the plug intends to enhance gas exchange within the flask, while preventing airborne particles from contaminating the culture and droplets from escaping from the flask. (B) Modified Oxypump stoppers with the bellows element fixed, consequently the pumping movement is cancelled.

the efficiency of bioreactors and their mixing devices, and also as an important bioprocess scaling-up criteria [8–11]. The empirical correlations for the prediction of the k_La in orbital shaken flasks are based on considering the operational parameters, for example the shaking frequency, the internal diameter of the flask, the filling volume, and the shaking diameter, along with the physicochemical properties of the liquid [8,10,12,13].

Oxygen limitation occurs when the oxygen uptake rate (OUR), which depends on the specific oxygen uptake rate and the biomass concentration, exceeds the oxygen transfer rate (OTR) (Eq. (1)). This situation was previously difficult to avoid in shaken flasks, since monitoring in small-scale bioreactors required the development of suitable technologies [3,8]. Nowadays, this limitation has been overcame, and technologies like the RAMOS device for OTR online recording [14,15] or the optical oxygen probes for DOT in-situ measurements [6,9,16], have proven its worth for evaluating the role of oxygen availability during screening experiments, along with a deeper study of the mass transfer phenomena and successful scale-up strategies.

New mixing technologies should be evaluated as an alternative to solve oxygen transfer limitations in orbital shaken flasks. The resonant acoustic mixing (RAM) is a non-contact mixing technology, that induces a low-frequency acoustic field to facilitate mixing through mechanical resonance [17,18]. This newly introduced technology has potential for mixing multiphase systems, as was recently evaluated for powders homogenization using low concentration of active pharmaceutical ingredient (acetaminophen, 3% w/w) and lubricant (magnesium stearate, 1% w/w). A highly efficient mixing performance was achieved by taking less mixing time to reach better blend homogeneity when compared to other batch blenders [18].

Introducing RAM to biotechnology begins with the RAMbio system (RAMbio®, Applikon® Biotechnology, Foster City CA, USA) which was designed to mix microbial cultures grown in flasks (Fig. 1), and provide them with plenty of oxygen when used along with the standard flexible silicone plugs (Oxy-Pump® stoppers by Applikon Biotechnology®). Those stoppers are capable of keeping the media inside the flask, with a membrane at the top through which air and gases are filtered (Fig. 2A). Hence, the mixing and aeration mechanisms differ with those from the orbital mixing (Fig. 1). Scientific information regarding the performance of the RAMbio system is scarce, and just a few technical notes have been published [17]. Performance of *Escherichia coli* K12 expressing Green Fluorescent Protein (GFP) was compared in shaken flasks (at both 20% and 40% medium volume/nominal volume) using an orbital shaker mixer (and Whatman Bugstopper closures with a shaking diameter of 1.9 cm) against a RAMbio acoustic mixer (and Oxypump® stoppers) for batch growth and protein production in rich media. Moreover, this technical report claimed that the RAM mixer at 20 g and the orbital mixer at 400 rpm are equivalent, lacking any engineered criteria [17]. At the end of cultures, the use of the RAM mixer resulted in an improved growth and biomass production (measured by optical density at 600 nm), as well as in an increased protein production (monitored by flow cytometry), versus traditional orbital shaken flask cultures.

The aim of this paper is to quantitatively compare the mass transfer phenomena in shaken flasks, particularly the oxygen transfer coefficient (k_La), in both mixers, the resonant acoustic mixer and the orbital mixer, as a function of the main operational conditions, including the shaking frequency, the flask geometry and the filling volume. Moreover, to compare the two mixing approaches based on the same initial k_La, *E. coli* cultures were carried out to evaluate growth kinetics, glucose consumption, dissolved oxygen tension (DOT), and acetate production profiles. In the present work, we introduce the first approximation to the understanding of the oxygen transfer phenomena in resonant acoustic shaken flasks.

2. Materials and methods

The experiments conducted during this work were performed in conventional Erlenmeyer flasks (unbaffled, Duran®, Erlenmeyer flask, narrow neck, Borosilicate Glass, USA) shaken by two different agitation systems; the resonant acoustic mixing in comparison with an orbital mixer.

2.1. Resonant acoustic mixing

The resonant acoustic mixer: RAMbio (Applikon® Biotechnology, Foster City CA, USA) operates by applying low frequency (58–66 Hz), high intensity acoustic field to induce oscillation at res-

Table 1
Representative dimensions of the Erlenmeyer glass flasks (Duran®) used through the experimentation.

Nominal volume (mL)	Maximum flask diameter[a]	Maximum neck diameter[a]	Neck height[a]	Total height[a]	Glass thickness[a]
250	85	40	54	165	1.5
500	105	34	36	180	
1000	131	42	46	223	

[a] Dimensions are given in mm.

onance. Working at the resonant frequency allows potential energy to be stored in springs and efficiently transferred to two balanced plates where the flask clamps are located. The acoustic wave generates micro-mixing zones throughout the flask while facilitating the bulk movement of the culture. The device acceleration can be adjusted from 3 to 20 g (29.4–196 m.s^{-2}).

The RAMbio was operated using the Oxypump® stoppers (Fig. 2A). These silicone plugs, are comprised of four main parts. First, the splashguard maintains liquid contents under vigorous agitation inside the flask without wetting the filter cap. The adaptor keeps the closure attached to the flask neck in a secure fashion. The bellows element works by pumping gases while oscillating and repeatedly changing the flask headspace content. Finally, the filter cap on the top, which is a hydrophobic gas-permeable barrier (0.2 μm) is intended to exclude small particles and/or microorganisms [19].

2.2. Orbital mixing

For comparison, a traditional orbital shaker (New Brunswick Scientific C251, Eppendorf, Inc. CT, USA) was operated with a shaking diameter of 25 mm, from 50 to 350 rpm. Cotton plugs were used as closures for the flasks, which are extensively used in the literature [1,4–7,12–14], and were made manually with equal amounts of gauzes and cotton. Plug weighs were: 6.16 ± 0.16 g (250 and 500 mL flasks) and 8.21 ± 0.15 g (1000 mL flasks). The respective density of the cotton plugs was around 0.1–0.2 g/cm^3.

2.3. Volumetric mass transfer coefficient (k_La) measurements by the gassing-out method

Three different nominal volumes of conventional Erlenmeyer glass flasks were used (250-, 500- and 1000-mL, Duran®). The necks of the flasks were modified to fit the Oxypump® stoppers, dimensions are shown in Table 1.

We choose the use of water–air and the gassing-out method as a universal approach to describe the mass transfer capabilities of the RAM system, compared to orbital mixing.

For this, each flask was filled to working volume (10, 15, 20, 25 or 40% deionized water volume/nominal flask volume). Then, oxygen was removed from water by adding Na_2SO_3 with $CoCl_2$ as catalyst to achieve a final concentration under 6×10^{-3} M and 5×10^{-7} M, respectively. The sulphite oxidation reaction was used for displacing dissolved oxygen in the liquid phase, and the k_La measurement took place only when the oxidation reaction was over, hence the impact of the reaction kinetics was not considered [20]. The mixer was started once assuring oxygen-free water, and dissolved oxygen tension (DOT) was recorded online, as described above in Section 2.4. All experiments were conducted at 30 °C and 37 °C.

The mass transfer coefficient (k_La) was obtained as the resulting linear slope by plotting the logarithmic expression against time, as shown in Eq. (3); this expression results after integration of Eq. (1) (between two different times), considering OTR as described in Eq. (2) and being OUR = 0 [11,21].

Since Eq. (3) is valid for a linear response, only data measured between 10 and 60% DOT were used for k_La estimation. It is worth noticing that the optical sensor (Section 2.4) renders higher sensitivity at DO levels below 60% [6].

$$\ln\left(\frac{C_L^* - C_{L2}}{C_L^* - C_{L1}}\right) = -k_La \times (t_2 - t_1) \quad (3)$$

For flasks with orbital shaking, all conditions tested met the criteria of an in-phase operating system, as we evaluated the Ph and Fr number under all operational conditions in order to avoid out-of-phase operation, which is accompanied by strong decreases in power input, mixing performance, and mass transfer [22].

2.4. Dissolved oxygen tension (DOT) measurements

DOT measurement for k_La estimation and during *E. coli* cultures was recorded online with the oxygen optical meter Fibox 3 using a PSt3 sensor (PreSens, Regensburg, Germany). The sensor was attached to the bottom of each Erlenmeyer flask, which was placed over a coaster [9,16]. The contact of the optical sensor with the liquid phase depends on its position inside the flask. For our experiments, the patch was placed at the bottom near the wall flask, and the respective distances from the patch to the flask bottom center were: 19 mm (250 mL flask), 28 mm (500 mL flask), and 40 mm (1000 mL flask). We were careful not to lose contact of the sensor with the liquid phase. A special clamp arrangement was designed under each flask, so the coaster was located around 2 mm distance below the sensor patch in one of each orbital or acoustic shaker. The optical sensor was calibrated to read 0% DOT with a solution of Na_2SO_3 (0.3 M) and $CoCl_2$ ($\leq 5 \times 10^{-7}$ M); and 100% DOT with water aerated in the respective shaker (following the supplier's methodology of calibration).

The response time of the sensor (t_r) t_{60} (0–60% air sat.) is ~3 s. Assuming a first order dynamic response of the electrode, characterized by a constant time, a simple criterion for the suitable selection of the electrode usually is: $t_r < 1/k_La$ [20]. The truncation of the first part of the electrode response curve helps for applying the first-order approximation [21]. Therefore, we discarded data below 10% air sat. in order to ensure we met the aforementioned criteria for all k_La measurements. Furthermore, changes in DOT profiles of microbial cultures took several minutes, hence, the response time of the electrode was fast enough and no considerations on these measurements were needed.

2.5. Use of an empirical correlation for k_La estimation in orbital shaken flasks

The empirical correlation reported by Klöckner and Büchs [8] (Eq. (4)) was used for the evaluation of k_La. This correlation was made by measuring OTR using a 1 M sodium sulphite system as a chemical oxygen consumer:

$$k_La_{Sulf} = 3.212.10^{-4} \times d^{1.92} \times n^{1.16} \times d_0^{0.38} \times V^{-0.83} \quad (4)$$

where the operating parameters are considered in SI units; maximum inner shake flask diameter (d) [m], shaking frequency (n) [s^{-1}], shaking diameter (d_0) [cm], and filling volume (V) [m^3].

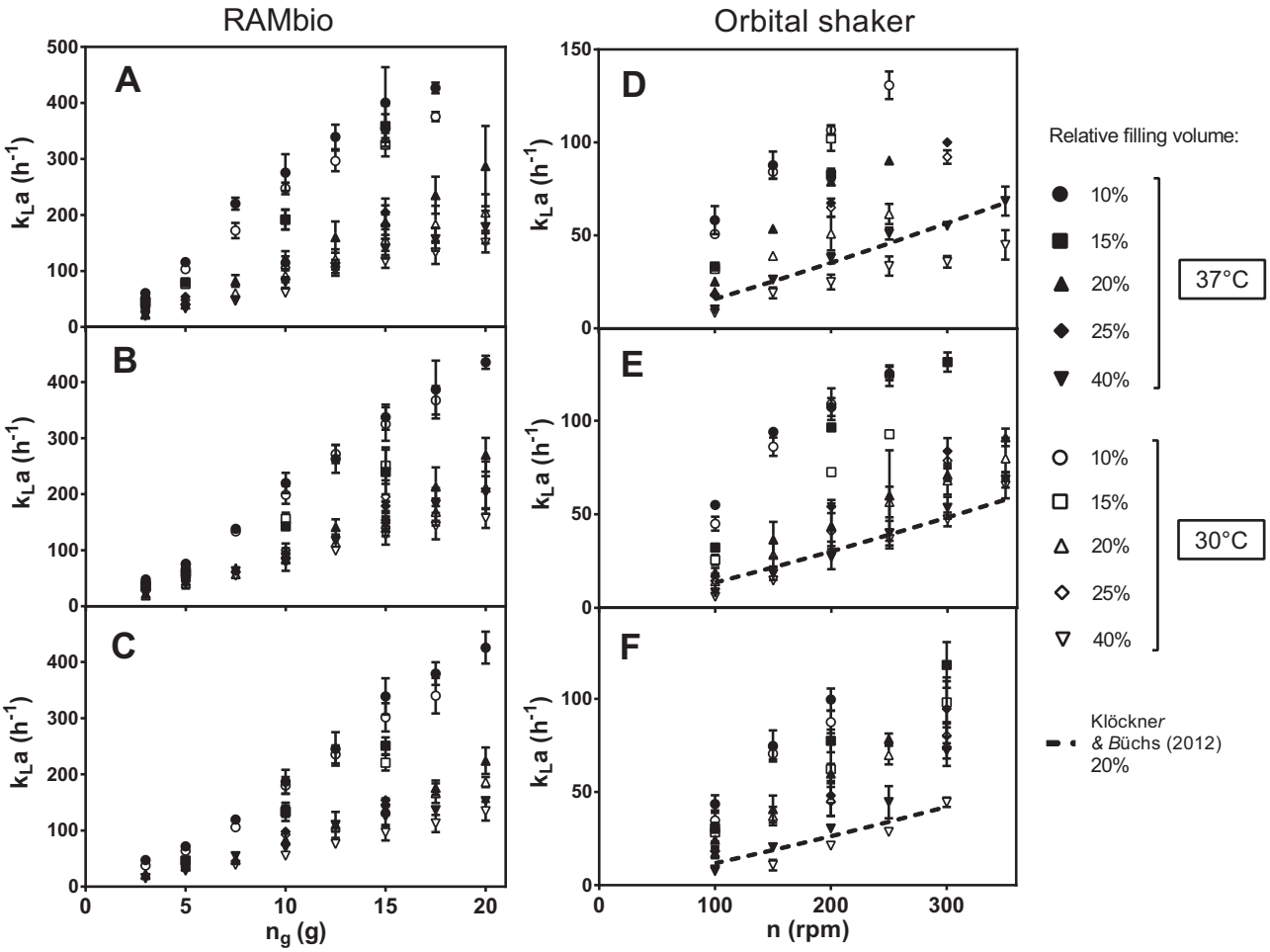

Fig. 3. k_La values of flasks shaken by two different agitation systems, obtained with different flasks sizes and varying the relative filling volume, at 30 and 37 °C. Nominal volumes of the flasks shaken by resonant acoustic mixing (A) 250-mL, (B) 500-mL and (C) 1000-mL; and by orbital mixing (D) 250-mL, (E) 500-mL and (F) 1000-mL. Mean and standard deviation of at least three independent experiments are shown.

Table 2
A/V ratios of the flasks used with different filling volumes. Where A refers to the superficial area (cm^2) of the liquid at the maximum inner flask diameter in motionless condition; and V refers to the filling volume (cm^3).

V_N (mL)	250					500					1000				
V_F (% v/v)	10	15	20	25	40	10	15	20	25	40	10	15	20	25	40
V (mL)	25	37.5	50	62.5	100	50	75	100	125	200	100	150	200	250	400
A/V (cm^2/cm^3)	2.11	1.41	1.06	0.84	0.53	1.63	1.09	0.82	0.65	0.41	1.29	0.86	0.64	0.51	0.32

V_N: nominal volume.
V_F: filling volume (deionized water volume/nominal flask volume).

2.6. Empirical correlations for k_La estimation in shake flasks

The empirical correlations of k_La based on the operational parameters were obtained using MATLAB R2012b software as well as the goodness of fit comprising: the adjusted *R*-square (adj R^2), the Root Mean Squared Error (RMSE), and the degrees of freedom (DOF). The operational parameters considered were the shaking frequencies, and the A/V ratio, where A refers to the superficial area of the liquid at the inner diameter of the motionless flask (cm^2); and *V* refers to the filling volume (cm^3).

2.7. Culture conditions of E. coli BL21-Gold (DE3) rSMD and culture media

The biological model used was a recombinant strain of *E. coli* BL21-Gold (DE3)-rSMD [23] maintained at −70 °C in Luria-Bertani (LB) (grams per liter: tryptone, 10.0; yeast extract, 5.0 and NaCl 5.0), supplemented with 50% v/v glycerol.

All cultures were performed in 500-mL flasks with 100 mL of semi-defined medium (SM) at 37 °C, unless otherwise mentioned. The culture media was prepared as previously described somewhere else [24,25] with some modifications. SM composition in grams per liter of distilled water was: glucose, 10.0; Na_2SO_4, 2.0; $(NH_4)_2SO_4$, 2.7; NH_4Cl, 0.5; K_2HPO_4, 19.0; $NaH_2PO_4{\cdot}H_2O$, 5.2; Citric acid, 1.0; $MgSO_4$, 0.24; thiamine, 0.01; and casamino acids, 2.0 and mineral salts. The pH of the culture medium was set to 7.0 ± 0.1 with HCl 2 N and sterilized at 121 °C for 20 min. Separately sterilized stock solutions of glucose, $MgSO_4$ and mineral salts were added to the medium once it was cold. Ampicillin was supplemented to a final concentration of 50 mg/L. Thiamine, casamino-acids and ampicillin stock solutions were filter sterilized (0.22 μm), and added to the medium just before the inoculum. The

Table 3
Coefficients of the empirical models (Eq. (4)), and goodness of fit (DF, adj R^2 and RMSE) for k_La estimation in shaken flasks, as function of the shaking frequency (g, rps) and the A/V ratio, with RAM and orbital shaking at 30 and 37 °C.

Agitation system	T (°C)	a	b	c	DF[a]	adj R^2[b]	RMSE[c]
RAM	30	10.570	1.089	0.771	544	0.908	29.0
Orbital		16.610	1.087	0.872	317	0.832	12.4
RAM	37	9.881	1.161	0.745	510	0.926	30.4
Orbital		18.350	1.140	0.788	313	0.863	12.0

[a] Degrees of freedom.
[b] Adjusted R-square.
[c] Root mean squared error.

final composition of the mineral salts in the medium was (in milligrams per liter): $CaCl_2{\cdot}H_2O$, 0.74; $ZnSO_4{\cdot}7H_2O$, 0.18; $MnSO_4{\cdot}H_2O$, 0.10; Na_2-EDTA$\cdot 2H_2O$, 22.25; $FeCl_3{\cdot}6H_2O$, 16.7; $CuSO_4$, 0.10; and $CoCl_2{\cdot}6H_2O$, 0.18.

Inoculum culture was grown in SM with 4% v/v of LB, instead of casamino-acids, and ampicillin concentration of 100 mg/L. The flasks were incubated at 30 °C with orbital shaking (200 rpm), and after 16–18 h the culture broth was centrifuged (12,100 × g, 15 min), suspending the cells in SM fresh medium. Enough volume of this suspension was taken using a sterile pipette to start all cultures with an optical density (OD) of 0.25–0.3 A.U. at 600 nm.

For evaluation of the Oxypump stoppers role on the microbial culture performance, an *E. coli* culture was developed as described above. The flask shaken by the RAMbio system had a modified stopper where the pumping movement of the bellows element was cancelled as shown in Fig. 2B.

2.8. Cell concentration, quantification of glucose and extracellular metabolites

Growth of the transformed and uninduced *E. coli* BL21-Gold (DE3) rSMD was followed by optical density (OD) at 600 nm (Spectronic Genesys 20, Thermo USA), samples were diluted when OD values were higher than 0.7, where 1 OD was equivalent to 0.50 g dry cell weight per liter [23].

Organic acids and glucose were detected from 1 mL centrifuged (17,900 × g, 15 min) sample of the culture broth. Samples for pH kinetic measurements were also taken (Corning, pH meter 430, USA).

Concentration of organic acids (acetate, oxalate, citrate, malate, succinate, and formate) was determined by high-pressure liquid chromatography (HPLC) analysis; HPLC (Shimadzu, Japan) using an AminexHPX-87H column (Bio-Rad Hercules, CA) equipped with an UV detector. Quantification was made running a commercial standard (Bio-Rad Hercules, CA) as established by the supplier. A mobile phase of 4 mM H_2SO_4 was used at 0.6 mL/min, and run at 30 °C; wavelength UV detector set at 215 nm. Glucose and lactate concentrations were measured using the Biochemistry Analyzer YSI2900 (YSI Life Sciences, USA).

2.9. TEM analysis

Morphology of *E. coli* was analyzed under transmission electron microscopy. Cell samples were taken at exponential phase of growth culture, washed three times with 0.16 M sodium cacodylate buffer at pH 7.2 at 4 °C, fixed with 4% v/v paraformaldehyde and 2.5% v/v glutaraldehyde in sodium cacodylate buffer pH 7.4 during 2 h at 4 °C. Post-fixed samples with 1% v/v osmium tetraoxide during 90 min at 4 °C were rinsed twice in chilled buffer and six times in cold distilled water. Then, samples were dehydrated in ethanol series and embedded in Epon/Araldita [26]. Thin sections were stained with uranyl acetate and lead in citrate, and observed with a ZEISS Libra 120 plus electron microscope.

2.10. Statistical analysis

The *E. coli* cultures were made at least in duplicate. Two-way ANOVA for independent samples and multiple comparisons tests using Tukey (Test for Post-ANOVA) with a threshold significance level of 0.05 were carried out to estimate the statistical differences between the cultures parameters, considering as independent variables the agitation system and the initial k_La. The analyses were done using GraphPad Prims 6.01 (2012).

3. Results and discussion

3.1. k_La in flasks shaken by RAMbio and orbital mixer

The k_La values were determined in flasks shaken by the RAMbio system against the orbital mixer under different operational conditions, in order to get a better understanding of the oxygen transfer phenomena with resonant acoustic mixing. Measurements were performed by varying the shaking frequencies (3–20 g and 100–350 rpm with RAM and orbital, respectively), the nominal flask volumes (250-, 500- and 1000-mL), and the filling volumes (10, 15, 20, 25 and 40% deionized water volume/nominal flask volume), at 30 and 37 °C.

The measured k_La values in function of the shaking frequency are presented in Fig. 3. Under all tested conditions, the RAM system achieved higher k_La values than those obtained with the orbital shaker. For example; in the 500-mL flask with 20% of filling volume at 37 °C, the acoustic mixer achieved a k_La value of $270.3 \pm 30.2\ h^{-1}$ (Fig. 3B), while the orbital shaker rendered $91.1 \pm 4.7\ h^{-1}$ (Fig. 3E). This could be explained by the extra mechanisms of aeration under the resonant mixing condition with acoustic wave comprising axial flow patterns, allowing the formation of drops in the headspace of the flask, and micro sized bubbles trapped in the fluid, which easily generate an increased gas–liquid interfacial area [27]. Nevertheless, a wider comparison of the RAM and the orbital mixing systems would require taking into account the volumetric power input, and the fluids hydrodynamics. In the literature, higher values of k_La for orbital shaken flask (unbaffled) can be found, but these values are obtained considering exceptional operational conditions, such as higher shaking diameters and lower filling volumes. For example, a k_La value of $565.2\ h^{-1}$ has been reported for a 50-mL flask with a relative filling volume of 4% (sulphite solution), with a shaking frequency of 450 rpm, and a shaking diameter of 7 cm [28]. The maximum k_La values here obtained for the orbital shaker were $131.3 \pm 5.1\ h^{-1}$ in the 500-mL flask with a relative filling volume of 15%, and a shaking frequency of 300 rpm at 37 °C (Fig. 3E); whereas for the resonant acoustic mixer a value of $435.4 \pm 11.7\ h^{-1}$ was obtained in the 500-mL flask with a relative filling volume of 10% at 20 g and 37 °C (Fig. 3B). Higher k_La values were not further measured due to the loss of contact between the patch (a PSt3 sensor attached to the flask bottom: Fibox 3 by PreSens, Regensburg, Germany) and the liquid phase while mixing. This depends on the patch position inside the flask [29], and occurred while working in specific operational conditions, including orbital mixing: the

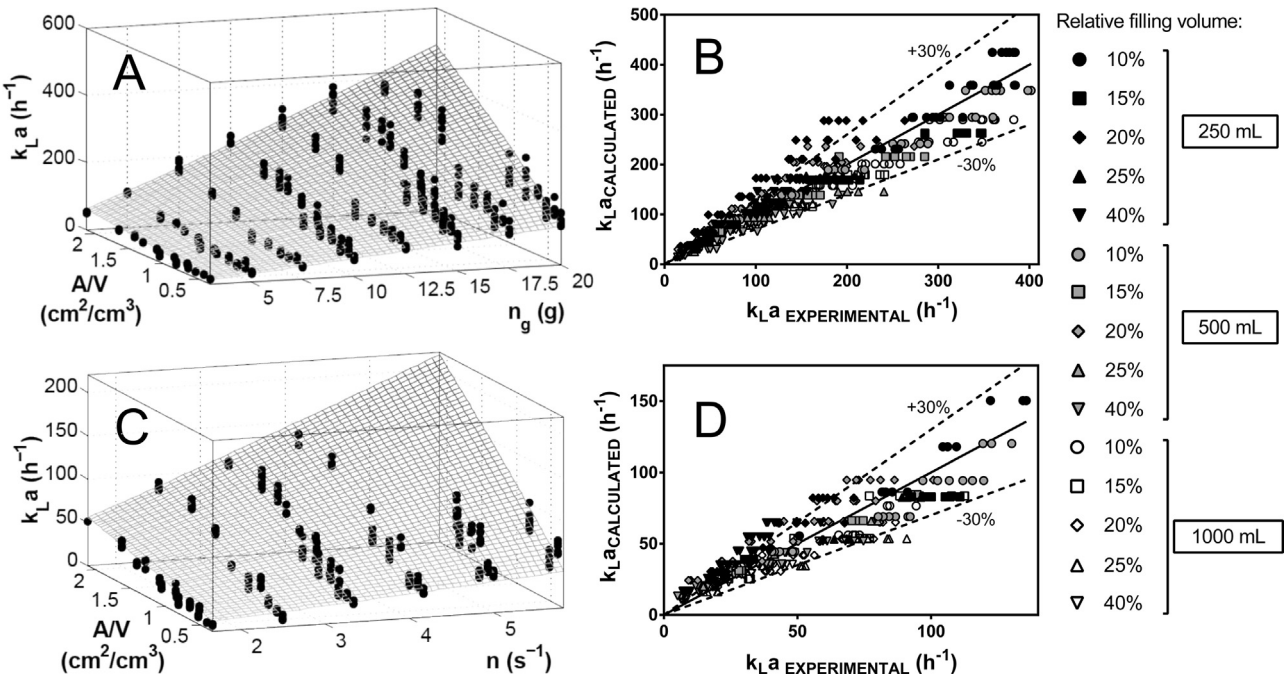

Fig. 4. Validation of the empirical model proposed for k_La estimation in shaken flasks at 30 °C. In the 3D graphics, the k_La is a function of the main operational parameters (*n* and A/V) where the proposed model is represented by the surface, and its comparison with the experimental data, the dark dots; is presented for flasks shaken by (A) a RAM (an interactive version of this plot can be found on the online version) and (C) an orbital mixer. Calculated k_La against measuring k_La values are also shown for shaken flasks with (B) RAMbio and (D) orbital mixing; for different flask sizes: 250-, 500- and 1000-mL, 10–40% relative filling volumes, 3–20 g and 50–350 rpm shaking frequencies, at 30 °C; k_La was determined with the gassing-out method as described in Materials and methods.

250 mL flask (V_F = 10% higher than 250 rpm, V_F = 15–20% higher than 300 rpm), the 500 mL flask (V_F = 10% higher than 300 rpm) and the 1000 mL flask (V_F = 10% higher than 250 rpm). Whereas, at 20 g in the 250 mL flasks (V_F = 10%) the loss of contact with the patch occurred in the RAM. This limitation in measuring the DOT in shaken flasks by the optical sensor probes was previously reported at high shaking frequencies and low filling volumes [29]. In *E. coli* BL21-pET28a cultures high values of DOT were observed using the optical probe, while a full development of oxygen limitation was detected by OTR measurement by the RAMOS device, which implied an interaction between the oxygen probe and the flask headspace [29].

The k_La values measured in orbital shaken flasks are comparable to those predicted by a previously reported model [8] for each flask at 20% of relative filling volume (Fig. 3B and E–F). The experimental values of k_La obtained were around 1.6 and 2.0 times higher, at 30 and 37 °C, respectively, than those predicted; with the values being greater at the highest shaking frequencies. Those differences rely firstly on the methodology used to determine the k_La: the reported model was validated using the RAMOS device which establishes a balance of gas pressure in the headspace of the flasks [4,5]; while in this work we measured the DOT. Furthermore, other variables have to be taken into account, such as the geometry of the flask neck, the gas diffusivity through the cotton plugs, and the physicochemical features of the liquid (the model was obtained for a 1 M sodium sulphite system at 22.5 °C).

3.2. Influence of the operational conditions on the k_La

From Fig. 3 it is clear that the k_La increases with the shaking frequency for both agitation systems. As previously reported, the gas–liquid transfer area, for orbital shaken flasks, includes the surface exposed to the surrounding air and the film on the flask wall. At high shaking frequencies, the centrifugal force increases and higher the water crawls higher to flask wall [28]. Upon increasing agitation intensity the liquid-side boundary layer thicknes and therefore, the mass-transfer resistance decreases and the gas–liquid mass-transfer rate reaches higher values. This seems to be true also for RAM shaken flasks, including the aforementioned development of droplets and small bubbles. The influence of the flask geometry was evaluated by varying the flask nominal volume; when this was incremented, the k_La increased as well. Conversely, increasing the filling volume causes the reduction of this coefficient. The aforementioned effects have been widely discussed before for orbital shaken flasks [4,12,13,30,31], and herein were observed for the RAM system.

We looked for a practical model that allowed comparing the aeration capacity of the RAM system to the traditional orbital mixing. This first approximation would help in establishing the RAM operating conditions in order to achieve non-limiting oxygen cultures when the needs for oxygen of the biological model are known.

We used the A/V ratio as the variable that groups together both effects the flask geometry and the filling volume (Table 2). Hence, the effects of A/V and n (given in g or rps) over the k_La were quantified using empirical models. We kept the model simple with the best fitting of all data, represented by Eq. (5), where *a*, *b* and *c* are specific coefficients for each agitation system, and their values are listed in Table 3.

$$k_La = a \times n^b \times \left(\frac{A}{V}\right)^c \quad (5)$$

The values for the *b* coefficient are near 1.1, which is consistent with previous reports for k_La in orbital shaken flasks, where this coefficient is between 1.0 and 1.4 [8,12,13,30,31]. Besides, Eq. (5) is in good agreement with existing models; a narrow comparison is available in Fig. S1. As already discussed, differences are expected due to the specific operating and physicochemical conditions of each system. Interestingly, the values for the *b* and the *c* coefficients were very close between the RAM and the orbital system, suggesting a similar impact of each of the evaluated operational

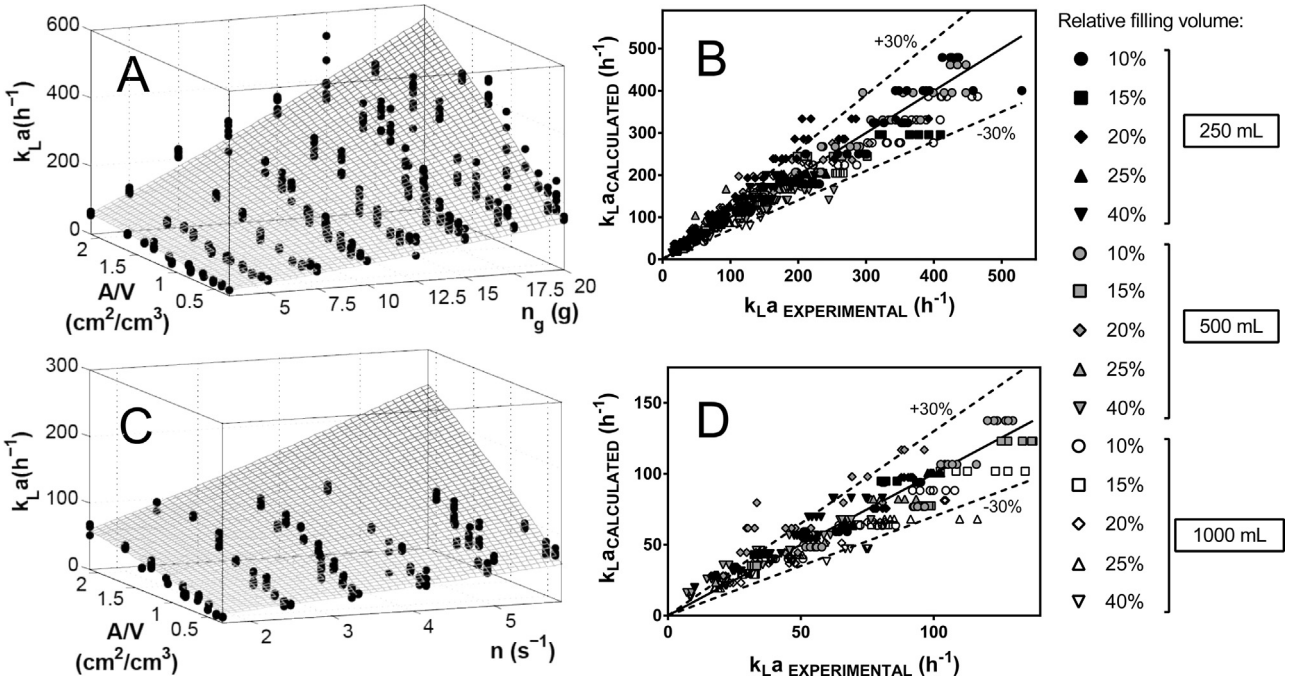

Fig. 5. Validation of the proposed empirical model for k_La estimation in shaken flasks at 37 °C. In the 3D graphics, the k_La is a function of the main operational parameters (n and A/V) where the proposed model is represented by the surface, and its comparison with the experimental data, the dark dots; is presented for flasks shaken by (A) a RAMbio and (C) an orbital mixer. Calculated k_La against measuring k_La values are also shown for shaken flasks with (B) RAM and (D) orbital mixing; for different flask sizes: 250-, 500- and 1000-mL, 10–40% relative filling volumes, 3–20 g and 50–350 rpm shaking frequencies, at 37 °C; k_La was determined with the gassing-out method as described in Materials and methods.

parameters over the k_La, even when the aeration mechanism in each system is completely different.

The models proposed in function of the operational parameters are represented in 3D plots in addition to their validation as a comparison of the measured against calculated k_La values. They are shown for each system at 30 °C in Fig. 4 (4A-Bfor the RAM system, 4C-D for the orbital system), and at 37 °C in Fig. 5 (5A-B for the RAM system, 5C-D for the orbital system). An interactive plot of Fig. 4A is available in the online version of this manuscript (Fig. S2). The models describe a positive interaction between the shaking frequency and the A/V ratio, and are fairly consistent with the experimental data for k_La estimation in shaken flasks as it is observed in the plots of Figs. 4 and 5, in addition to the goodness of fit (Table 3), covering high and adequate values for the adjusted R^2 along with the Root Mean Squared Error (RMSE) that was equal or less than 30% for each agitation system. On the other hand, data at 30 and 37 °C had to be fitted separately based on the statistical analysis that established significant differences between both temperatures. The evaluation of the k_La under two temperatures is not enough to include the temperature effect in the empirical model, but it does help to visualize the effect of this parameter under common laboratory conditions for microbial growth.

On the other hand, several outliers at low k_La values (>30%) are observed (Figs. 4 and 5). When the filling volumes are low (≤15%) and the A/V is high, the k_La increased faster with the shaking frequency than when higher filling volumes are used (Fig. 3). Using the A/V ratio helped in diminishing those differences, but it seems the model could underestimate the k_La values when lower A/V are used, mainly in the RAM system. These differences could be caused by the flow patterns formed that might be similar at higher accelerations, but different at lower accelerations [18].

Improving RAM performance for oxygen transfer would imply designing other geometries able to enhance the contact between gas–liquid phases, where hydrodynamics of the RAM system need to be taken into account.

3.3. E. coli *BL21-Gold (DE3) rSMD cultures in flasks shaken by RAM and orbital systems*

We studied the influence of the aeration mechanisms in shaken flasks over transformed (uninduced) *E. coli* BL21-Gold (DE3) rSMD growth, glucose consumption, DOT, acetate production and pH profiles. Our first interest was to determine if increasing the oxygen transfer rate into the RAM could prevent the accumulation of organic acids, particularly acetate. First, we chose common conditions to grow bacteria in shaken flask: 500-mL flasks with handmade cotton plugs, 20% of relative filling volume and a shaking frequency of 200 rpm. This approximately corresponded to a k_La value of 46 h^{-1}, as previously measured (Fig. 3E). A higher shaking frequency was used: 350 rpm, as high as the orbital shaker used could operate without unbalanced movement, which corresponded to a k_La value of 92 h^{-1}, previously measured (Fig. 3B). The comparison between the RAM and the orbital shakers was made by adjusting the shaking frequencies in each system in order to set two similar initial k_La (Table 4).

We discarded any effect over the k_La due to the broth composition by working with equivalent initial k_La values measured in water–air system, due to the water-like characteristics of the culture media. However, when working with complex media, non-Newtonian fluids or high-density cultures, corrections due to changes in the physicochemical properties of the culture (mainly due to changes in the oxygen diffusivity and the apparent viscosity) need to be further considered.

The *E.coli* culture performance at equivalent initial k_La values of 46 h^{-1} is presented in Fig. 6A-D, and main kinetic parameters are summarized in Table 4. Similar final biomass, measured as optical densities were found in the cultures after 23 h, complete glucose consumptions, and the same final acetate concentrations. In general, no significant differences in the global performance of the cultures were found (Table 4). Therefore, establishing the same aeration capabilities for each agitation system in terms of the initial

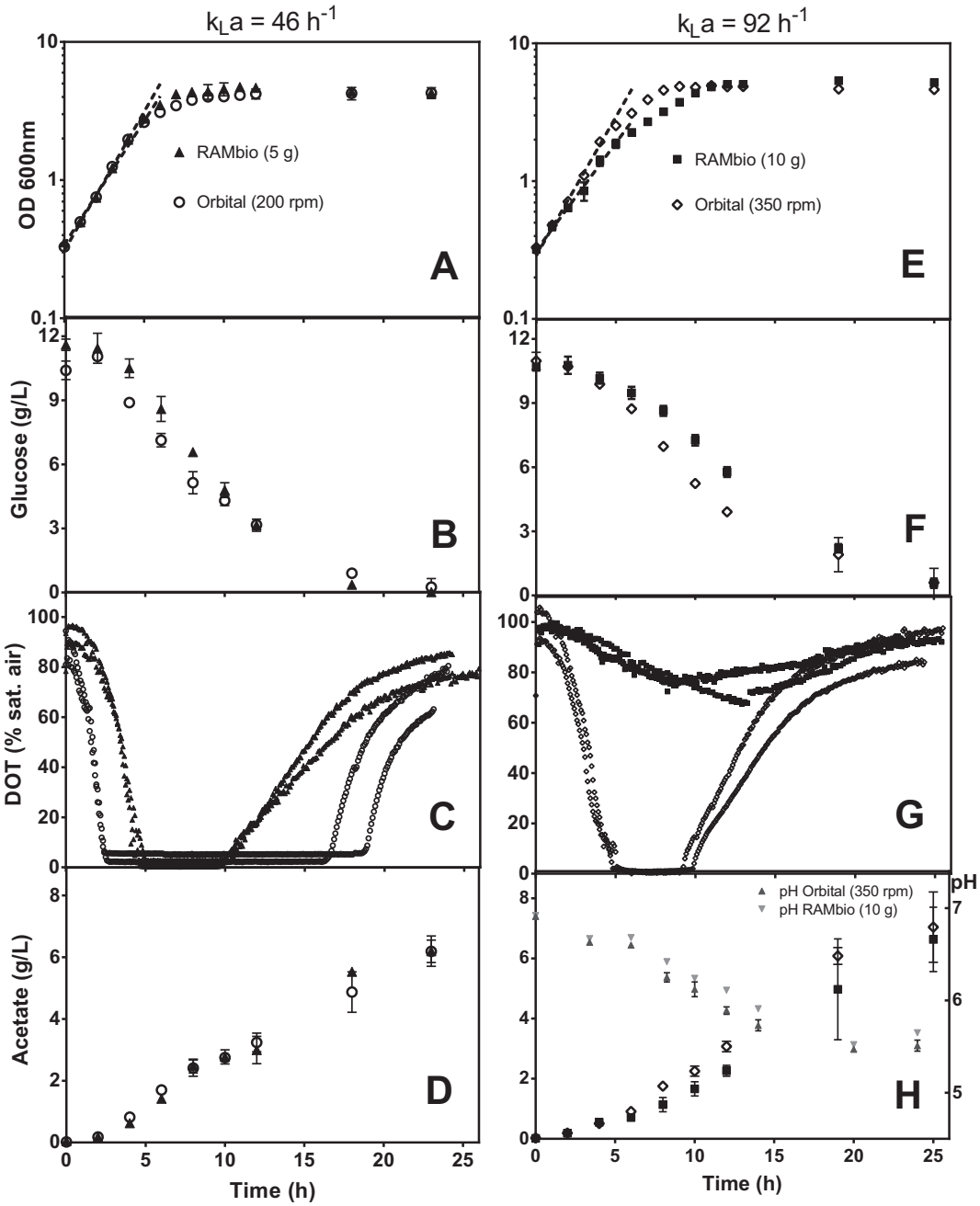

Fig. 6. Culture development of *E. coli* BL21 (DE3 gold) rSMD grown in flasks shaken by RAMbio and orbital systems at two equivalents values of initial k_La. (A), (E) Biomass growth, where the specific growth rate is represented as dotted lines; (B), (F) glucose uptake; (C), (G) DOT profiles, (D) acetate accumulation, and (H) acetate accumulation and pH drop; throughout cultures grown in shaken flasks (nominal size of 500-mL with 20% of filling volume) with RAM and orbital mixing at two equivalents values of initial k_La (46 and 92 h^{-1}), with SM medium at 37 °C. Mean and standard deviation of at least two independent experiments are shown for biomass, glucose, acetate concentrations and pH measurements. For DOT profiles, two typical trends are shown.

Table 4
Operational conditions and main kinetic parameters of the *E. coli* cultures. Mean and standard deviation for at least two independent cultures are shown.

n_g, n	RAM 5 g	Orbital 200 rpm	RAM 10 g	Orbital 350 rpm
k_La^* (h^{-1})	45.7 ± 0.8	46.2 ± 1.9	93.8 ± 4.6	91.1 ± 4.7
μ (h^{-1})	0.46 ± 0.01	0.46 ± 0.00	0.36 ± 0.02	0.46 ± 0.01
OD600 nm final	4.3 ± 0.2	4.3 ± 0.4	5.2 ± 0.2	4.6 ± 0.3
Yx/gluc (g DWC/g gluc)**	0.31 ± 0.01	0.32 ± 0.00	0.59 ± 0.05	0.38 ± 0.00
q_s (g gluc/g DWC h)**	1.53 ± 0.09	1.47 ± 0.02	0.60 ± 0.03	1.23 ± 0.02
$Y_{ac/x}$ (g ac/g DWC)**	1.31 ± 0.01	1.48 ± 0.06	0.82 ± 0.15	0.99 ± 0.05
q_p (g ac/g DWC.h)**	0.61 ± 0.01	0.69 ± 0.03	0.29 ± 0.04	0.46 ± 0.01
T (h), DOT → 0%	5.0 ± 0.2	15.0 ± 1.7	0	4.4 ± 0.5
pH final	5.30 ± 0.01	5.34 ± 0.01	5.46 ± 0.03	5.39 ± 0.03

* Mean and standard deviation for at least three independent experiments with deionized water, for 500-mL nominal volume flasks with 20% of filling volume, at 37 °C.
** Calculated at 10 h time point.

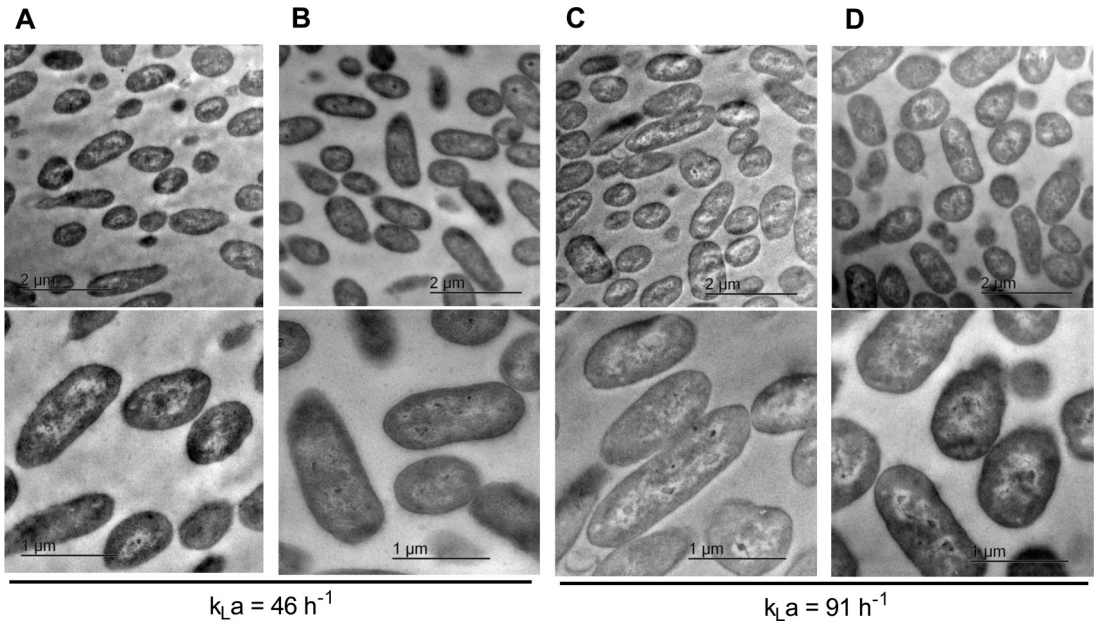

Fig. 7. Cross-sections of *E. coli* BL21 (DE3 gold) viewed under the transmission electron microscope (TEM). Examination of *E. coli* BL21-Gold (DE3) cells cultured in shaken flasks with (A), (C) RAMbio and (B), (D) orbital mixing at two equivalents values of initial k_La (46 and 92 h^{-1}), with SM medium at 37 °C. Cells harvested at 8 h of growth culture (Fig. 5). (Scale bars of 2.0 and 1.0 µm).

k_La (46 h^{-1}), allowed us to perform reproducible *E. coli* BL21-Gold (DE3) cultures.

The DOT profiles showed oxygen-limiting development during the culture. Interestingly, we observed a shorter time period of oxygen depletion (DOT = 0% air sat.) in the cultures with RAM than with orbital mixing (Fig. 6C). This faster drop on DOT could be an indication of different lag phases of growth, but this was not the case as shown in Fig. 6A. Instead, the DOT differences seem to be related with an enhanced capacity of the RAMbio system and the Oxypump stoppers to replenish the gas phase with fresh air, therefore maintaining during the culture a maximum value of the oxygen partial pressure within the headspace of the flask, hence ensuring a higher OTR value.

Previously, cultures of *E. coli* K12 expressing the green fluorescent protein (GFP) in the RAMbio exhibited a greater specific growth rate (20% faster), as well as a greater amount of overall biomass (reaching 300% more biomass concentration with 40% of filling volume), and recombinant protein production (specific GFP expression) than cultures from the orbital platform, although there was no approach that enabled the establishment of the same aeration performance in each agitation system [17]. At present, we are able to predict the k_La values at which the comparison was made; thus, for the RAM, using the model proposed in the present work, the k_La value was 333.4 h^{-1} (20% filling volume, 50 mL water/250 mL flask; and a shaking frequency of 20 g at 37 °C, assuming a nominal flask volume of 250-mL with a maximum inner flask diameter of 82 mm), whereas the k_La value for the orbital mixing is 70.7 h^{-1} (considering Klöckner and Büchs [8] model; at a shaking frequency of 400 rpm, same flasks and a shaking diameter of 19.05 mm). Based on this, it is evident the observed improved performance of the OTR with the RAM, where the DOT only briefly dropped below 20%, while the DOT dropped precipitously at the beginning of the orbital shaker cultures and did not recover until growth ceased nearly 24 h after inoculation.

Likewise, in Fig. 6E–H, biomass growth, glucose uptake, DOT profiles, acetate production and pH are presented during the cultures. Shaking frequencies were proposed to set equivalent initial k_La values of 92 h^{-1} in shaken flasks by each mixer (Table 4). Similar final biomass, glucose consumption, and final acetate concentrations were found after 25 h of growth. The pH profiles are the consequence of organic acids production (mainly acetate), as in Fig. 6H both parameters render symmetrical responses.

In comparison to Fig. 6C, significant differences in the DOT profiles were observed; for RAMbio DOT remains above 65% throughout the whole culture, whereas the cultures grown in the orbital mixer exhibited an oxygen depletion period of 4–5 h (from 5 to 10 h of culture, Fig. 6G). As we proposed above for Fig. 6C, the differences could be due to the Oxypump stoppers active performance in gas transfer; in contrast to the passive diffusion through the cotton plug. Previously, Mrotzek et al. [32] found a decreased gas exchange relative to the surrounding atmosphere between 25 and 38% when using cotton plugs with a bulk density between 0.06 and 0.25 g/cm^3. The diffusion limiting condition of the plugs becomes greater when working at higher OTR, like increasing the shakingfrequencies (Figs. 7 and 8).

Even when the oxygen transfer rate was enhanced, the performance of the bacteria culture did not improve. Substantial differences in the culture parameters were found (Table 4, Fig. 9A–E) for cultures grown in the RAMbio than the orbital shaker, including lower specific growth rate, lower specific glucose uptake, lower specific acetate productivity, and higher biomass-glucose yield. No significant differences were observed in acetate-biomass yield (Fig. 9E).

The increase in acetate concentration, and the consequent pH reduction, are not related to oxygen limitation. Great acetate production starts and remains even when enough dissolved oxygen is available. This is evidenced by comparing the DOT and concentration of acetate profiles at all conditions evaluated (Fig. 6C and D and Fig. 6G and H). Acetate production in aerobic *E. coli* cultures can occur at high growth rates and/or high glucose uptake rates [33,34]. Such response is induced by an imbalance of the glucose uptake, energy production and biosynthesis fluxes, mostly if high glucose concentration media are used [15,35]. Finally, no concentration of lactate, oxalate, malate, or formate was found; furthermore, despite the amount of succinate detected, the method used had not a proper resolution for its quantification. Citrate concentration remained close to 1 g/L through the whole cultures.

There are no previous reports of the impact over microbial development when mixed at mechanical resonance. Samples of the cells were taken during exponential growth phase and observed under transmission electron microscopy (TEM). No visible differences were found for microscopy morphology of *E. coli* cells grown on each mixing condition evaluated, as shown in Fig. 7.

The diminished performance of *E. coli* cultures at the high shaking frequency could be related to the hydrodynamic patterns, since working with shaking flaks mass and momentum transfer phenomena are hard to isolate. An *E. coli* BL21 (DE3 gold) rSMD culture was grown in the RAMbio system at a higher shaking frequency (12.5 g), but similar k_La value ($94.3 \pm 3.4\ h^{-1}$), in order to assess the hydrodynamic impact over the cultures, when separated from the mass transfer phenomena. This was achieved by changing the A/V ratio (1000 mL flask with 45% filling volume). Table 5 shows that the kinetic parameters of growth, and glucose consumption were no different from the culture grown in the orbital system. The kinetic performance of this culture was increased in comparison to the culture at 10 g (Fig. S3A–D) in terms of biomass production and glucose consumption. No significant differences were found in the pH measurements. Otherwise, the DOT profiles show lower values of dissolved oxygen, surely due to the diminished headspace achieve in relation to the selected filling volume.

Previous reports concluded that any changes in biological performance with yeast and bacteria due to variations in agitation or aeration intensity cannot be attributed to hydrodynamic stresses associated with the turbulence generated by impellers or with bursting bubbles, since estimation of the Kolmogoroff microscale suggest that those organisms are smaller enough [36]. For the RAM technology, those microscale eddies are assumed to be near 50 μm [37]. Moreover, *E. coli* cells are highly resistant to mechanical stress, as evaluated in continuous cultures of *E. coli* W3110, with controlled DOT (40% and 10% air sat.) in lab stirred tank reactors [38]. While the gas flow rate was maintained at 1 vvm, the impeller speed was increased from 400 rpm to 1200 rpm ($1\ kWm^{-3}$ to $30\ kWm^{-3}$) for 7 h. The only effect observed was over the outer polysaccharide layer of the cell, as shown by SEM, and supported with flow cytometry and TEM analysis [38]. Hence, we conclude that the hydrodynamics of the RAMbio has not any impact on the *E. coli* cultures performance or the morphology.

3.4. *The role of the Oxypump stoppers over* E. coli *BL21-Gold (DE3) rSMD cultures grown in flasks shaken by the RAMbio*

The role of the active gas transfer generated by the Oxypump stoppers was further analyzed. For this, we used a modified Oxypump stopper, where the pumping movement of the bellows element was cancelled (Fig. 2B). By using this special closure, the k_La values were not affected (Fig. S4A and B).

Except for the closures used, *E. coli* was grown under the same conditions as the previous cultures (Section 2.7–2.8). It was incubated in the RAMbio system at 10 g, having a k_La value of $96.1 \pm 3.4\ h^{-1}$ (Table 5). The results are in Fig. 6E–H showing biomass growth, glucose uptake, DOT profiles and pH measurements in comparison to the cultures evaluated at the same operational conditions and the normal Oxypump stoppers. Main parameters are summarized in Table 5.

Astonishingly, cultures grown with the modified Oxypump stopper recovered their performance. Higher values of specific growth rate and specific glucose uptake were achieved in comparison with the cultures using normal Oxypump stoppers (Fig. 9A,B and D). The DOT profiles were significantly different, with the modified Oxypump, dissolve oxygen reached lower values, it drop near 35% air sat. (With normal Oxypump the DOT was always above 65%). This result evidences the active gas transfer through the closure, which allows a faster replenishment of the flask headspace.

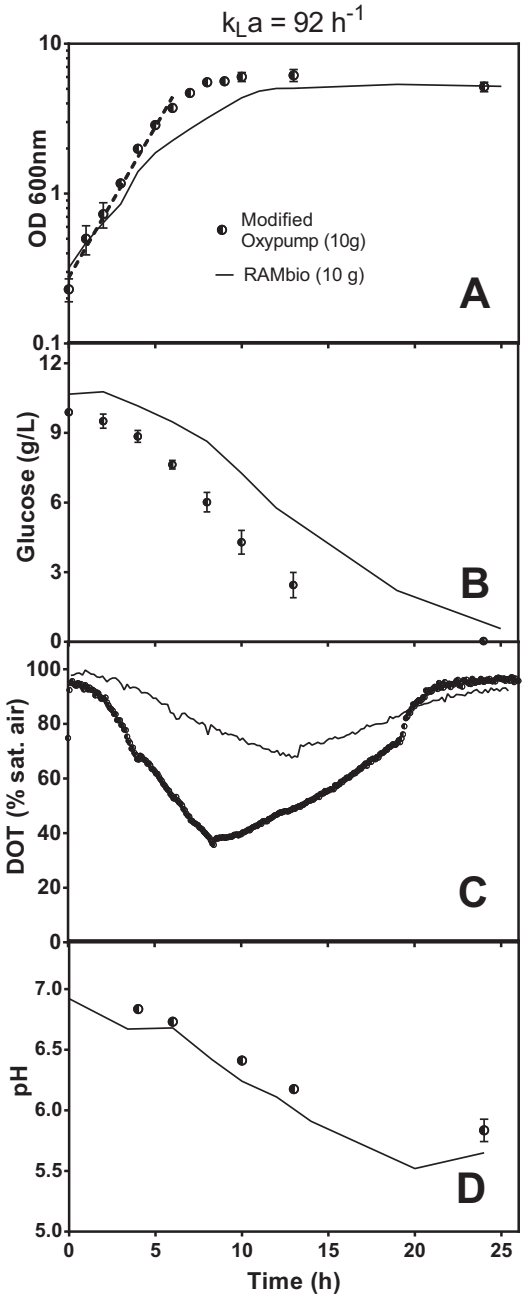

Fig. 8. Culture development of *E. coli* BL21 (DE3 gold)-rSMD grown in flasks shaken by RAMbio coupled with the modified Oxypump stoppers. (A) Biomass growth, where the specific growth rate is represented as dotted lines; (B) glucose uptake; (C) DOT profiles and (D) pH drop, throughout cultures grown in shaken flasks (nominal size of 500-mL with 20% of filling volume) with RAMbio coupled to the modified Oxypump stoppers, at an initial k_La value equal to 92 h^{-1}, with SM medium at 37 °C. Mean and standard deviation of at least two independent experiments are shown for biomass, glucose and pH measurements. Solid lines represent data of the same culture conditions when the normal Oxypump stoppers were used (Fig. 6E–H).

It also reinforces the discussion about the limitation of the passive transfer closures. Since diminishing the DOT renders better *E. coli* culture performance, it seems that the oxygen available plays a main role over cells metabolism. Previous reports had shown that maintaining high DOT values during *E. coli* cultures, could have unpredictable effects over growth rate, plasmid stability, plasmid content and in the recombinant protein production [39].

Even when the *E. coli* cultures where uninduced, the stress associated with producing the recombinant rSMD and maintaining the

Table 5
Operational conditions and main kinetic parameters of additional *E. coli* cultures. Mean and standard deviation for at least two independent cultures are shown.

n_g, n	RAM		Orbital	
	10 g Modified Oxypump	12.5 g	*E. coli* BL21 DE3 gold (10 g)	*E. coli* BL21 DE3 gold (350 rpm)
k_La^* (h^{-1})	96.1 ± 3.4	94.3 ± 3.4	93.8 ± 4.6	91.1 ± 4.7
μ (h^{-1})	0.49 ± 0.01	0.51 ± 0.05	0.73 ± 0.06	0.69 ± 0.02
OD600 nm final	5.2 ± 0.4	4.9 ± 0.5	13.77 ± 0.7	9.8 ± 0.3
Yx/gluc (g DWC/g gluc)**	0.52 ± 0.00	0.40 ± 0.02	0.61 ± 0.02	0.48 ± 0.01
q_s (g gluc/g DWC h)**	0.95 ± 0.02	1.29 ± 0.19	1.19 ± 0.13	1.43 ± 0.00
T (h), DOT → 0%	0	0	6.7 ± 0.6	7.2
pH final	5.84 ± 0.09	5.71 ± 0.16	6.79 ± 0.01	6.84 ± 0.04

* Mean and standard deviation for at least three independent experiments with deionized water, for 500-mL nominal volume flasks with 20% of filling volume, at 37 °C.
** Calculated at 10 h time point.

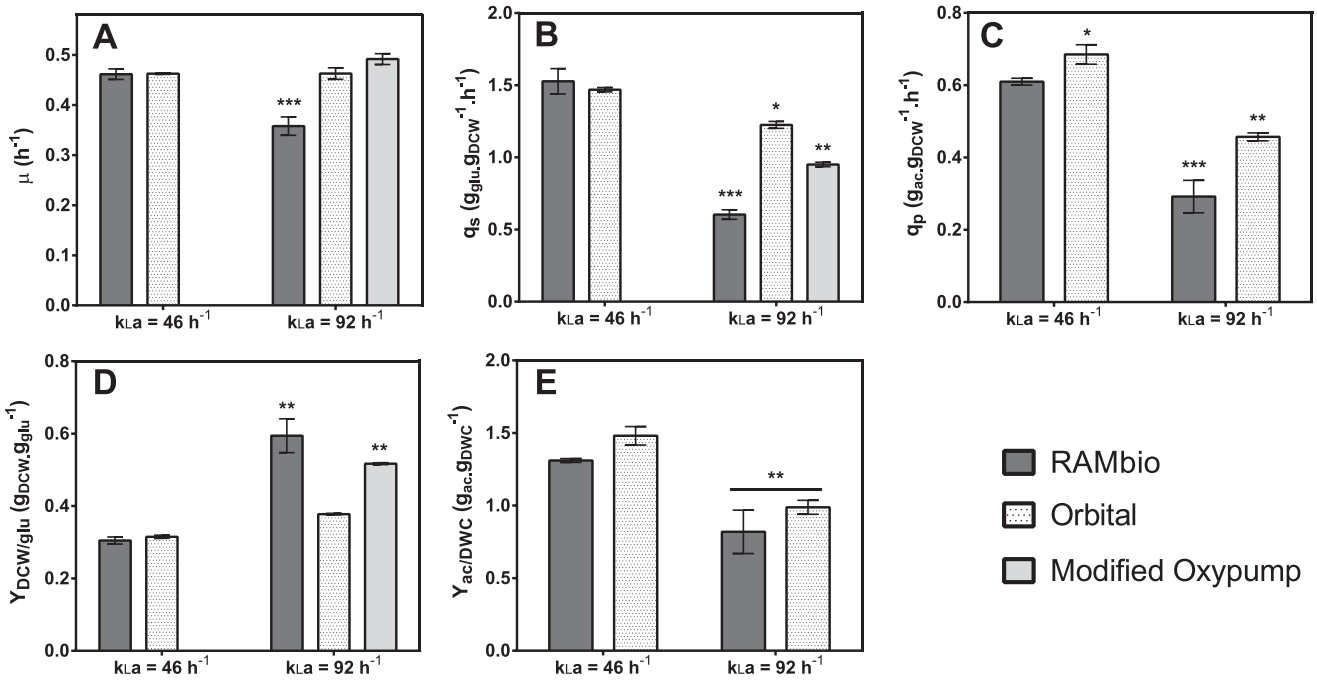

Fig. 9. Culture parameters comparison of *E. coli* BL21 (DE3 gold) rSMD grown in flasks shaken by the RAMbio, the orbital system and by RAMbio coupled with modified Oxypump stoppers; at two equivalents values of initial k_La. (A) Specific growth rate (obtained during exponential growth); and (B) specific glucose uptake, (C) specific acetate production, (D) biomass-glucose yield and (E) acetate-biomass yield calculated at 10 h of cultures (Fig. 5) grown in shaken flasks (nominal size of 500-mL with 20% of filling volume) with RAM and orbital mixing at two equivalents values of initial k_La (46 and 92 h^{-1}), with SM medium at 37 °C. Mean and standard deviation of at least two independent experiments are shown. Statistical differences with a threshold significance level of 0.05 are considered: * significant ($0.01 < P < 0.05$), ** very significant ($0.001 < P < 0.01$) and *** extremely significant ($P < 0.001$).

plasmid led to less biomass being produced than with the wild type [36]. *E. coli* BL21 DE3 gold (wild type) was grown with the same conditions as previous experiments (Fig. 6E–H), in absence of ampicillin. Table 5 summarizes the main parameters of those cultures, while the biomass growth, glucose uptake and DOT profiles can be analyzed from Fig. S5A–C. The performance of those cultures is clearly superior, as they reached higher specific growth rate and specific glucose uptake. In addition, they were enhanced when growing in the RAMbio system, due to the increase oxygen supply. The higher final pH values indicate that the acetate production may be diminished or even avoided, therefore using more glucose to biomass production.

Setting k_La equivalent initial values allowed performing similar cultures of *E. coli* while growing in flasks shaken by the RAMbio and the orbital systems. However, irreconcilable differences were reached, since the mechanisms of mass transfer in each system operate under different principles. A better approach would include the comparison of the OTR profiles during bacterial cultures. The use of this criterion gives worth to the information among several cultures development.

We are interested in the evaluation of the RAM performance over recombinant protein production for induced cultures under higher k_La operating conditions, as well as different biological host performance grown under the complete range of action of RAM technology (0–20 g), including non-Newtonian cultures.

4. Conclusions

Orbital mixers are widely used as the main agitation systems for aerobic cultures in shaken flasks. Recently, resonant acoustic mixing (RAM) has been introduced as an alternative for aeration and mixing supply in shaken flasks. In the present work, a characterization of the RAM system is presented in terms of the k_La coefficient, and an empirical model for k_La estimation in shaken flasks by RAM and orbital mixing is presented. The resulting equations can be applied to calculate the initial oxygen transfer coefficient for low viscosity cultures, using relevant cultivation parameters, such as the shaking frequency, the flask diameter, and the filling volume, within an accuracy range of ±30%. To our knowledge, this is the first k_La correlation that has been defined and validated for

RAM shakers. Understanding RAM performance for oxygen transfer would imply designing other geometries able to enhance the contact between gas–liquid phases, where hydrodynamics of the RAM system need to be taken into account.

Furthermore, *E. coli* cultures were grown in shaken flasks by RAM and orbital mixers, and had a similar performance at the same initial k_La of 46 h^{-1}. Conversely, at 92 h^{-1} the cultures of *E. coli* exhibited great differences in the DOT profiles and the culture parameters, which could be due to differences in the oxygen transfer rate caused by the increased gas pressures generated in the RAM system at a higher shaking frequency.

Authors' contributions

GIRC conducted experiments, performed the data analysis, and wrote the manuscript. RIGC and NAVC helped to conduct *E. coli* experiments and reviewed the manuscript. MATR coordinated the experiments, wrote and reviewed the manuscript. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bej.2015.10.015.

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