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# Software Simulator Bioprocess (SSBP) to estimate hydrodynamic stress conditions in cell cultures performed in shaking bioreactors

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**Key words:** Energy dissipation rate, Hydrodynamic stress, Eddy length, Shake flasks, Shear rate

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**Abstract**

In order to estimate hydrodynamic stress conditions in cultures carried out in shake flasks we used a Software (SSBP). The systematic analysis was performed with mammal cells, plant cells, filamentous molds, protozoa and bacteria; all data were collected from the literature. The parameters more useful for the quantification of hydrodynamic stress was the energy dissipation threshold and critical length eddy. According to these parameters the mammal cell line AGE.1.HM®, *Rubia tinctorium*, *Penicillium purpurogenum*, *Streptomyces zaomyceticus*, bacteria on their capacity to colony forming units (FCU) and *Trichoderma harzianum* were the most tolerant to hydrodynamic stress in shake flask cultures. In counterpart, erythrocytes, CHO cells, *Penicillium citrinum*, *Ceratocorys horrida* and *Protoceratium reticulatum* were the most susceptible. The use of shear stress and shear rate to compare hydrodynamic stress, at least in shake flask cultures, cannot be useful when compares different biological systems. In this context, the Kolmogoroff theory, neither applied at all in shaking bioreactors. Finally, the highest exposition to hydrodynamic stress in terms of the maximum drop diameter in shake flask cultures corresponds to protozoan *Protoceratium reticulatum*, *Lactococcus lactis*, mammal cell line AGE.1.HM, mollusk larvae *Dreissena polymorpha* and *Rubia tinctorium* although with great exposition differences to energy dissipation thresholds.

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**Introduction**

The shake flasks are widely used in the development of new bioprocess in the academy, scientific and industrial fields. There are bioprocesses in which the hydrodynamic stress becomes a limiting step and this phenomenon includes cell cultures that use oil as a carbon source (Jones and Porter, 1998) an insoluble ingredient in the media culture formulation (Cull *et al*., 2002), where a second phase is formed. Other kinds of bioprocess include sensible cells to the hydrodynamic stress, such as animal cells (Chisti, 2001), plant cells (Kieran *et al*., 2000), filamentous fungi (Van Suidjam and Metz, 1981), or marine dinoflagellates (García-Camacho *et al*., 2007). The bottleneck in this kind of bioprocess is the necessity to scale up to stirred fermenters (Choi *et al*., 1996), thus the development of new industrial cell cultures is the main challenge, especially when hydrodynamic stress becomes in the limit parameter.

Unfortunately, there are relatively few works that report hydrodynamic stress in cultures carried out in shake flask cultures. The studies that address the quantification of the flow-related forces acting on suspended cells in a shake flaks were reported by Zhang *et al* (1995) analyzed the effect of the hydrodynamic environment and shear protectants in the viability of erythrocytes. García-Camacho *et al* (2007) analyzed the shear stress effects on the specific growth rate of the marine dinoflagellate *Protoceratium reticulatum*. Horvath and Crane (2010) investigated the effect of the hydrodynamic forces on the mortality of larval zebra mussel. Finally, Busto *et al* (2013) reported the effect of cell viability, biomass and anthraquinones production by the plant cell *Rubia tinctorium*. Those studies opened the possibility of quantification of the hydrodynamic stress in cell cultures carried out in shaking bioreactors. Actually, the parameters used by the quantification of the hydrodynamic stress in shake flask cultures are: energy dissipation rate (Büchs *et al*, 2000b), eddy length (Zhang, 2005), average shear rate and average shear stress (García-Camacho *et al*, 2007), and maximum drop diameter (Büchs and Zoels, 2001).

The motivation and aims of this study include the analysis of several cultures performed in shake flasks where the hydrodynamic stress is the main concern. This analysis incorporates a wide kind of cell cultures reported in the literature and covered animal cells, plant cells, marine dinoflagellates, mollusk larvae, filamentous molds and bacteria (Table 1, see materials and methods section). Still need to be investigated in these cultures is knowing the specific culture conditions for scale-up these bioprocess to fermenters.

Therefore, the necessity of this study is to understand the critical culture conditions for avoiding hydrodynamic stress and know the optimal operation in shake flasks environments for the development and scale-up specific bioprocess. In this investigation, we propose the analysis of the hydrodynamic stress conditions through the software SSBP, which was designed by us.

***Materials and methods***

We collect literature data for several cultures carried out in shake flasks, these bioprocess include animal cells, plant cells, marine dinoflagellates, larvae zebra mussel, filamentous molds and bacteria. The specific culture conditions were taken from the original author´s publications (Table 1).

*Software Simulator Bioprocess*

The Software Simulator Bioprocess (SSBP) was designed through the program Phyton (Reyes *et al*., 2015), one of the most used languages in the informatics world. For estimate hydrodynamic stress conditions of the biological systems proposed, we introduce some characteristics of the shake flaks cultures (Table 2). The parameters characterize shake flask cultures are described below.

*Power input drawn*

The SSBP estimate power input drawn by the use of the correlation proposed by Büchs *et al.,* 2000a. This equation is based on torque measurements in the drive on the shaking machine and appropriate compensation of the friction losses.

$$\frac{P}{V_L} = \frac{C\rho n^3 d^4 \mathrm{Re}^{-0.2}}{V_L^{2/3}} \quad (1)$$

Where P is the power input per mass unit for shaken flasks (W/L), *d* the maximum inner diameter of the flasks (m), $V_L$ the filling volume ($m^3$), n refers to agitation frequency ($s^{-1}$) and r is the density ($kg/m^3$) of the broth with a Büchs constant (C).

Reynolds (Re) was estimated as follows:

$$\mathrm{Re} = \frac{\rho n d^2}{\mu} \quad (2)$$

m refers to viscosity of the broth (Pa*s).

*Kolmogoroff microscale of turbulence*

Turbulent eddy length scale (l), is usually measured by the Kolmogoroff microscale of turbulence (Zhang *et al.,* 2005), and related to the rate of energy dissipation (e) (Büchs et al., 2000b) as follows:

$$\lambda = \left(\frac{\mu}{\rho}\right)^{3/4} \varepsilon^{-1/4} \quad (3)$$

The average rate of energy dissipation (e) ($Wkg^{-1}$) was estimated from the equation:

$$\varepsilon = \frac{P}{\rho_L V_L} = \frac{1.94 n^3 d^4}{V_L^{2/3} \mathrm{Re}^{0.2}} \quad (4)$$

*Average shear stress and average shear rate*

Finally, the SSBP estimated the average shear stress (Eq. 5) as follows (García-Camacho *et al*., 2007):

$$\tau_t = 0.0676 \left(\frac{d_p}{\lambda}\right)^2 (\rho u \varepsilon)^{0.5} \quad (5)$$

Where $d_p$ is average cell diameter (m). Shear rate was estimated by the equation 6:

$$\gamma = \frac{\tau_t}{\mu} \quad (6)$$

*Volumetric mass transfer coefficient ($k_La$)*

For estimate $k_La$, the SSBP works with the dimensional equation (Klöckner and Büchs, 2012):

$$k_L a = 0.5 . d^{\frac{73}{36}} . n . d_0^{\frac{1}{4}} . V_L^{-\frac{8}{9}} . D_{O_2}^{\frac{1}{2}} . \upsilon^{-\frac{13}{54}} . g^{-\frac{7}{54}} \quad (7)$$

Where d (m) is the maximum inner flask diameter, n is the shaking frequency ($s^{-1}$), do (m) is the shaking diameter, $V_L$ ($m^3$) is the working volume, D ($m^2/s$) correspond to the oxygen diffusion coefficient, u ($m^2/s$) is the kinematic viscosity and g ($m^2/s$) is the acceleration of gravity.

**Results and discussion**

The characterization of the engineering parameters in shake flask cultures considered viscosities and densities near to the water. Stress conditions in shake flask cultures were based on the energy dissipation rate threshold, it means before the cells show an apparent detrimental damage, reduction of viability, enzyme activity, specific growth rate, yields and productivities. Once an energy dissipation threshold was determined, the SSBP automatically estimated other parameters for asses hydrodynamic stress conditions.

*Average energy dissipation rate threshold*

According to energy dissipation threshold (Fig.1) the most susceptible cells to hydrodynamic stress were plant cell *S. acmella*, erythrocytes, *C. horrida*, *P. reticulatum*, *P. citrinum* and *B. licheniformis*. In contrast, the most tolerant cells to hydrodynamic stress were *R. tinctorium*, mammal cell line AGE.1.HM®, *T. harzianum*, *P. purpurogenum*, *S. zaomyceticus* and FCU´s. For a single comparison, the animal commercial line AGE1.HN® shows high tolerance to hydrodynamic stress in shake flasks environments, this contrasts when compares with CHO cells and erythrocytes.

This behavior may be as a result of a cell adaptation or selection of commercial cell lines. In this context, when CHO cells were grown under continuous operation mode in stirred fermenters and exposed at energy dissipation values from 0.32 to 2.5 W $kg^{-1}$, the cell suspensions begin with necrosis and apoptosis, respectively (Balandras *et al*., 2011).

**Table 1.** Analysis of hydrodynamic stress using SSBP for several biological systems in shake flask cultures.

| Cell system | Conditions | Biological indicator | Reference |
|---|---|---|---|
| Chinese Hamster Ovary (CHO) | 500 mL Erlenmeyer flask; 200 mL 60-200 rpm | CHO cell cultures for β galactosidase production | (Zainul Abidin and Anuar, 2011) |
| AGE.1.HM® cells | 500-100 mL nominal volume at 200-225 rpm | Specific growth rate (μ) | (Platas *et al*., 2013) |
| Erythrocytes | 500 mL shake flasks from 0 to 400 rpm. | Effect of the shear rate with the viability | (Zhang *et al*., 1995) |
| *Spilanthes acmella* Murr. | 250 mL shake flasks with 50 mL; 60, 90, 120, 150 and 120 rpm | Cell biomass growth and sphilantol synthesis | (Singh, R. Chaturvedi *et al*., 2012) |
| *Rubia tinctorium* | 100 mL Erlenmeyer flasks, 25 mL of culture; 100 and 360 rpm. | Down scale of shear effects | (Busto *et al*., 2013) |
| *Solanum aviculare* | 250 mL shake flasks with 50 mL 100-160 rpm | Cellular DNA distribution (flow cytometry) | (Yanpaisan *et al*., 1998) |
| *Ceratocorys horrida* Sterin | 125 mL containing 60 mL; 40 to 120 rpm | Effect of the flow on the morphology | (Zirbel *et al*., 2000) |
| *Protoceratium reticulatum* | 200 mL in a 1000 mL shake flasks; from 35 rpm. | Determination of shear stress thresholds | (Garcia-Camacho *et al*., 2007) |
| *Dreissena polymorpha* | 125 mL shake flasks with 100 mL; 100-400 rpm. | Hydrodynamic forces on the mortality of larval Zebra mussel | (Horvath and Crane, 2010) |
| *Aspergillus niger* | 250 mL with 100 mL of medium; 0, 100, 130 and 200 rpm | Effect of the agitation on morphological changes and tannase production | [Purwanto *et al*., 2009] |
| *Aspergillus niger* HFD5A-1 | 200 mL in 500 mL Erlenmeyer flasks | Effect of the agitation rates in the hyphae morphology and pectinase production | [Ibrahim *et al*., 2015] |
| *Penicillium citrinum* | 250 mL shake flasks with 50, 75, 100, 125, 150 mL: 100, 150 and 200 rpm | Examination of fungal morphology and stantin production | [Gomaa and Bialy, 2009] |
| *Penicillium purpurogenum* GH2 | 25 mL shake flasks in shake flask of a 25 mL | Effect of the oxygen in the pigment production | (Morales-Oyervides, 2015) |
| *Phanarochaete chrysosporium* | 35 mL medium in 125 mL Erlenmeyer flasks; 100, 150 and 200 rpm | Effect on the agitation on the ligninase activity | [Venkatadri, and Irvine, 1990] |
| *Trichoderma harzianum* | 500 mL with 80 mL of extractive medium | Effect of the power input drawn in the penthyl-pyrone production | [Rocha-Valadez *et al*., 2006] |
| *Streptomyces zaomyceticus* RC 2073 | 50 mL in a 250 mL Erlenmeyer flasks; 100, 125, 150, 175 and 225 rpm | Effect of the agitation rates on the production of antimicrobials | [Naik *et al*., 2015] |
| *Bacillus licheniformis* | 100 ml of liquid in 250 mL conical flasks | Effect of the agitation rate in the bacitracin biosynthesis | (Tahir *et al*., 2012) |
| *Pseudomonas aeruginosa* | 25, 50, 75, 100, and 125 mL in 250 mL Erlenmeyer flasks; 25, 50, 75, 100, 125, 150, and 75 rpm | Effect of bioprocess parameter in the production of rhamnolipids | (Vanavil *et al*., 2014) |
| *Bacillus amyloliquefacients* | 50 mL in 500 mL shake flasks; 50 to 250 rpm | Development a bioprocess for □-amylase production | (El Enshasy, 2007) |
| *Bacillus cereus* MS-6 | 50 mL of medium in 250 mL Erlenmeyer flasks; 30, 60, 90, 120, 150, 180 rpm. | Effect of agitation on L- asparaginase activity | (Madda, 2009) |
| *Bacillus sp*. | 50 mL of medium in 250 mL Erlenmeyer flasks; 110, 130, 150, 180 rpm | Effect of the agitation speed on the production of extracellular protease | (Akhavan-Sepahy and Jabalamel 2011) |
| *Lactococcus lactis* NHD1 | 200 mL in 500 mL Erlenmeyer flasks | Effect of the agitation speeds on the growth kinetics of recombinant *L. lactis* | (Ibrahim *et al*., 2010) |
| *Escherichia coli DH5A-1* | 100 mL in 250 mL Erlenmeyer flasks; 150, 250, 300 rpm. | Effect of the agitation on xilanase production in recombinant *E. coli* | (Mohd-Rusli *et al*., 2009) |
| *Escherichia coli, Pseudomonas sp., Bacillus sp.* | 30 mL in 250 mL shake flasks; 0 to 200 rpm | Effect of the agitation intensity in the capacity to form colony forming units (CFU´s) | (Sakil-Munna *et al*., 2014) |

Plant cell culture shows significant differences to hydrodynamic stress tolerance. For example, *S. aviculare* is less tolerant than *R. tinctorium* (Fig. 1). In stirred fermenters, the plant cells generally begin to suffer a mechanical damage at energy dissipation values of 2.3 $Wkg^{-1}$ (Thomas *et al*., 1994).

The most tolerant molds to hydrodynamic stress were *T. harzianum* and *P. purpurogenum*, and the less tolerant counterpart were *P. citrinum*, *A. niger* and *A. niger* HFD5A-1 (Fig. 1). For these biological systems when growth in stirred tank fermenters the cell damage begins between values from 0.025 $Wkg^{-1}$ to 25 W $kg^{-1}$ (Ayazi-Shamlou *et al*., 1994).

The protozoan cells (Fig. 1) supported relatively low values of energy dissipation thresholds. In order to avoid detrimental damage in *P. reticulatum* the protective polymer Pluronic F-68 was used for modified the interface tensile properties and diminished the hydrodynamic forces of the fluid motion (García-Camacho *et al*., 2007).

The dinoflagellate *C. horrida* exhibits rapid morphological changes for avoiding shear forces is the reduction of the cell size, development of shorter spines and reduced swimming would allow cells to sink away from turbulent conditions more rapidly (Zirbel *et al*., 2000).

**Table 2**. Platform of the Software Simulator Bioprocess (SSBP) for estimate engineering parameters of shake flasks cultures.

| *Initial considerations* | | *Assessment parameters* | |
|---|---|---|---|
| Density (kg/m$^3$) | _____ | P/V (W/L) | _____ |
| Shaking frequency (s$^{-1}$) | _____ | Re (-) | _____ |
| Maximum inner diameter (m) | _____ | l (m) | _____ |
| Viscosity (Pa*s) | _____ | $t_t$ ( mN/m$^2$) | _____ |
| Average cell diameter (m) | _____ | g (s$^{-1}$) | _____ |
| Nominal volume (m$^3$) | _____ | Mixing time (s) | _____ |
| Filling volume (m$^3$) | _____ | $k_La$ (h$^{-1}$) | _____ |
| | | e (Wkg$^{-1}$) | _____ |

The zebra mussel larvae (*D. polymorpha*) is able to bear with relatively low shear forces in shake flasks fluid motions (Fig. 1), but a longer exposition increase their mortality (Horvath and Crane, 2010).

Something amazing was the susceptibility of bacteria to hydrodynamic stress (Fig. 1) in shake flask cultures, because the general believed in these biological systems are supposed less sensitive to adverse hydrodynamic effects. This detrimental effect was focused on sublethal effects, such as: the enzyme activity, metabolite yields, specific growth rate and colony forming units (FCU´s). The hydrodynamic stress also has been observed in *Xanthomonas campestris* through morphological changes, therefore the yields and productivity of the xhantam gum diminished (Garcia-Ochoa *et al*., 2012).

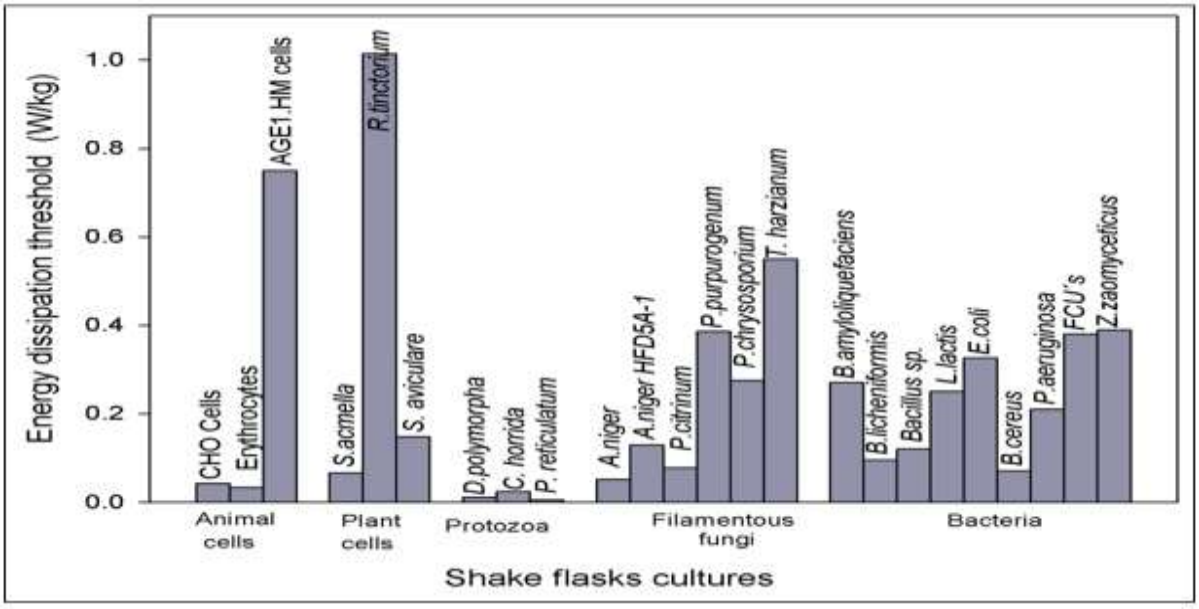


**Fig. 1.** Determination of energy dissipation threshold (ε) for the biological system analyzed in shake flasks cultures by the SSBP.

*Effect of the energy dissipation rate on the biological activity*

In order to analyze the sensibility degree of cells that grow in shake flask cultures, we used the method reported by Dunlop (Dunlop *et al*., 1996) (Fig. 2).

The reduction of the biological activity of the cells analyzed, for example, CHO cells the hydrodynamic stress begins at 0.042 W kg$^{-1}$ (99 % of viability), but at 0.0705 W kg$^{-1}$ the viability was only about 25 %, with the concomitant effect of the production of β-galactosidase (Zainul and Anuar, 2011).

The same behavior was observed for commercial cell line AGE1.HN, and erythrocytes slurries. For plant cell cultures, *S. acmella* the index aggregation was reduced about 1.86 % at 0.20 W $kg^{-1}$.

For a single comparison, the production of podophyllotoxin by *Podophyllum hexadrum* in shake flask cultures operated at 0.86 $Wkg^{-1}$ the cell viability falls around 30 % (Chattopadhyay *et al.*, 2001).

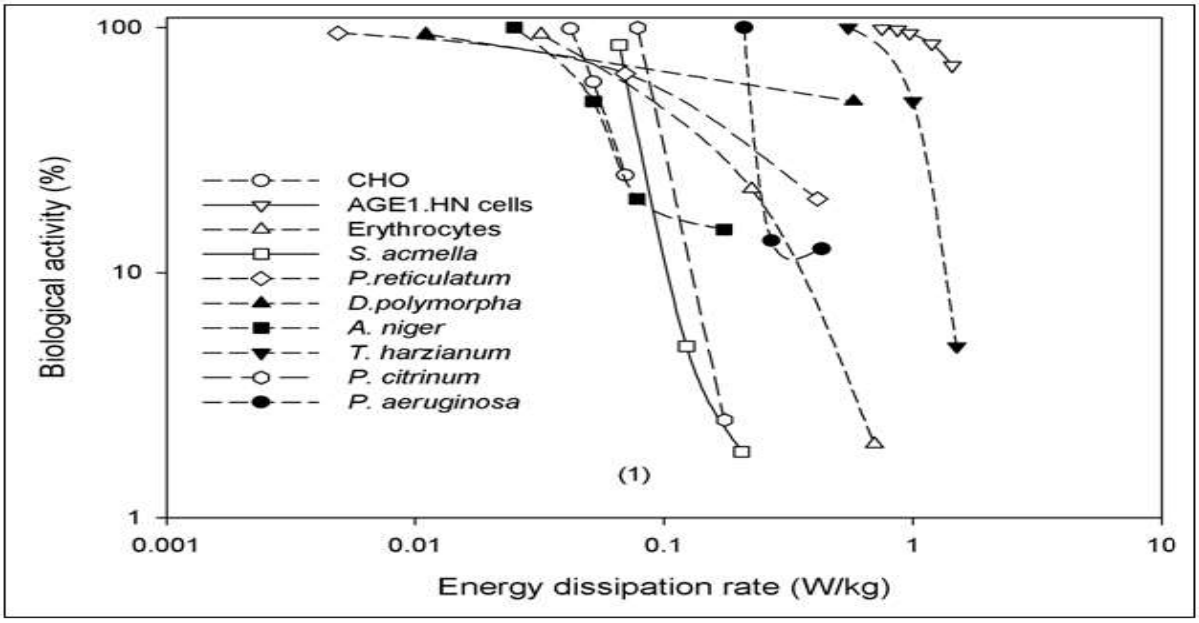


**Fig. 2.** Relationship between the energy dissipation rate and biological activity in cultures carried out in shake flasks.

Regarding filamentous molds (Fig. 2). *A. niger* in 0.025 $Wkg^{-1}$ the pellet size was maximized (10,000 μm), but the increase of the shear forces at 0.052 W $kg^{-1}$ the pellets size average was around 5,000 μm with the concomitant decreases of the tanasse activity, if the energy dissipation continued to increased (0.172 $Wkg^{-1}$) the pellet size is reduced at 1500 μm (Purwanto *et al.*, 2009).

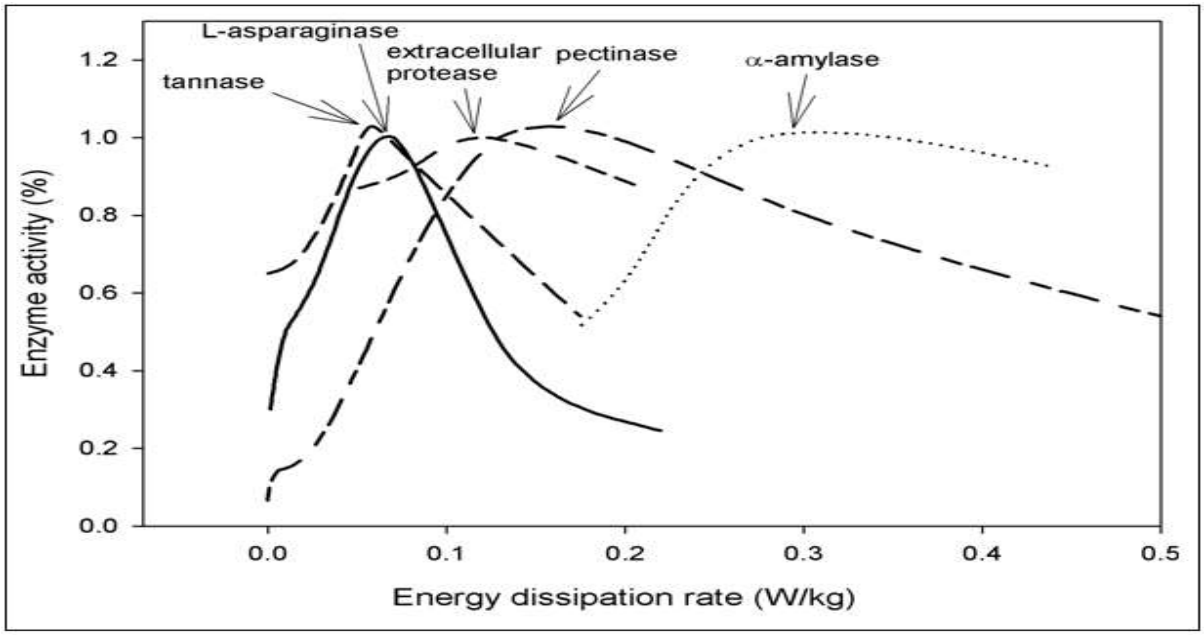


**Fig. 3.** Relationship between the energy dissipation rate and enzyme activity analyzed by the SSBP.

The same behavior was observed by *P. citrinum*, and *T. harzianum*. In the case of *D. polymorpha*, the viability diminished 50 % at 0.57 $Wkg^{-1}$ (Horvath and Crane, 2010). *P. reticulatum* was the most affected by the hydrodynamic stress (Fig. 2). In bacterial cultures the same phenomenon is observed in the yield index of rhamnolipids by *P. aeruginosa*, bacitracin biosynthesis by *B. licheniformis*, and in the capacity to form colony forming units (FCU´s) (Fig. 2). These results contrast with previous reports (Dunlop *et al*., 1996), where the insect cells, mammal and plant cells are highly sensitive to the hydrodynamic stress when compared with bacteria.

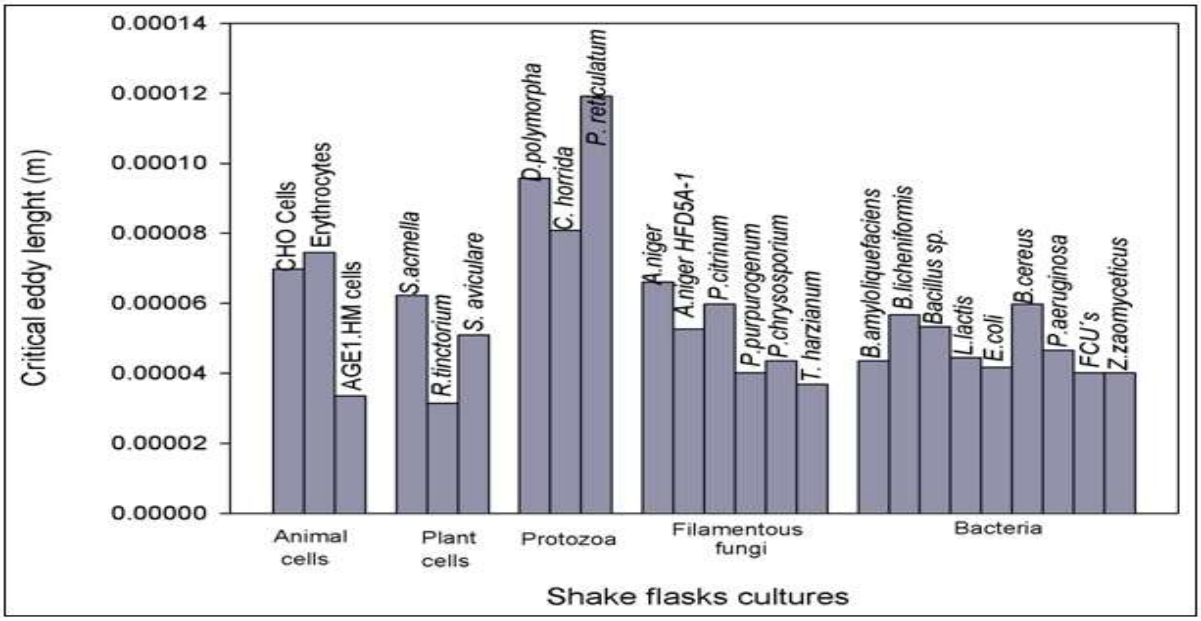


**Fig. 4.** Determination of critical eddy length scale (λ) for the biological system analyzed in shake flask cultures by the SSBP.

One of the main concerns in cultures carried out in shake flasks is the optimization of the production of enzymes of industrial interest. Fig. 3. show tannase and pectinase activity produced by *A. niger* and *A. niger* HFD5A-1, it seems to be optimal at 0.052 and 0.12 $Wkg^{-1}$, respectively. α-amylase activity got maximum values around 0.3 $Wkg^{-1}$. Finally, the L-asparaginase and protease activity reach the highest activity at 0.7 $Wkg^{-1}$ and 0.12 $Wkg^{-1}$, respectively. In the case of the filamentous molds of *A. niger*, the maximum enzyme activity was reached with a pellet morphology.

The adverse effects of the hydrodynamic stress also were observed in the production of extracellular protease expressed by *Bacillus* sp., production of xilanases by the recombinant *E. coli* DH5A-1, production of α-amylase by *B. amyloliquefacients* (Fig. 3).

*Critical eddy length* (λ)

The SSBP automatically estimated the values of critical eddy length (λ) when the energy dissipation threshold is given (Fig. 4). This criterion as indicative of the shear stress acting on the cells fits with the Fig.1. almost completely. The most susceptible biological systems to hydrodynamic stress were the protozoa *P. reticulatum, D. polymorpha, C. horrida*, erythrocytes, and CHO cells. Whereas the commercial mammal cell line AGE.1.HM®, *R. tinctorium*, FCU´s, *S. zaomyceticus* and *T. harzianum* were the most tolerant. Therefore, the eddy length could be used as a hydrodynamic stress parameter in shake flask cultures, because this parameter matches well with the energy dissipation threshold.

However, the isotropic turbulence theory of Kolmogoroff is defined as follows: if the eddies size of the fluids has a similar size or less as compared to the cells they could suffer adverse effects.

Eddies between 10 to 50 μm have been considered harmful to the cells are likely to become in a detrimental damage. Therefore, the cultures carried out in shake flasks with bacteria (1-10 μm) cannot be affected by these eddies, the hydrodynamic stress is not relevant if these cells are smaller than eddies sizes (Thomas, 1990).

In counterpart, eddies with a major sizes, drag the biological particles to a convective movement, but the smallest eddies have enough energy and these packed fluids have an effect over the cells, put down them into a hydrodynamic stress condition (Prokop and Bajpai, 1992).

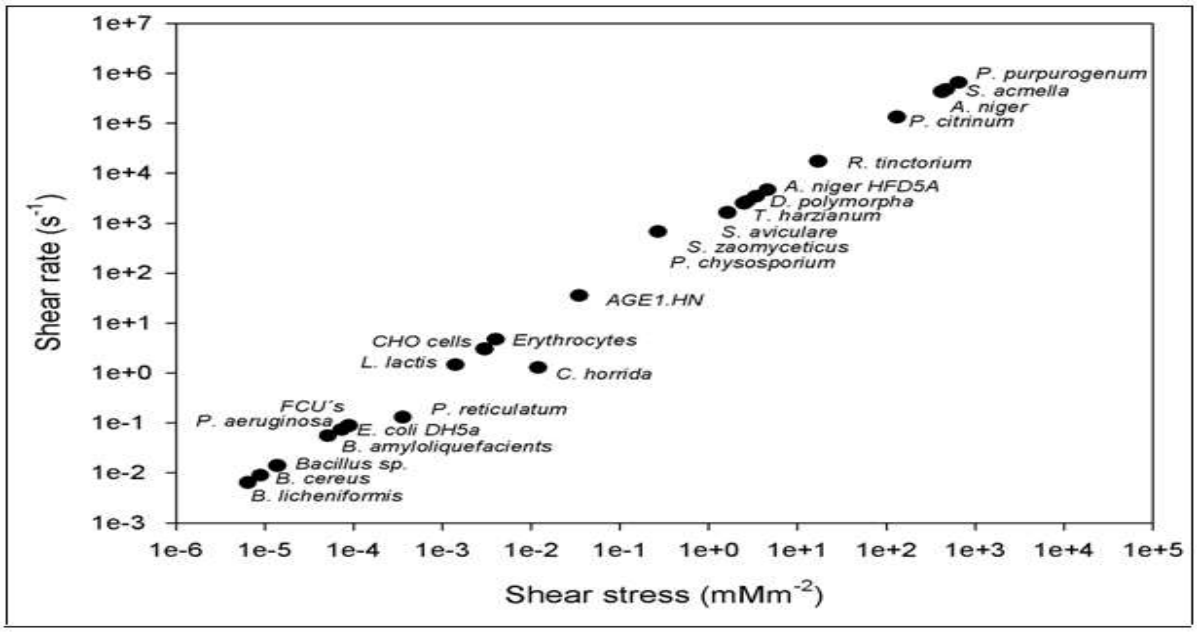


**Fig. 5.** Relationship between the shear stress threshold and shear rate in the biological systems analyzed by the SSBP.

One approach for evaluated the application of the Kolmogoroff theory on a suspended particle depends on the ratio between its size and λ. The analysis of the cultures when cell aggregates, the size/λ ratio increased considerably, in comparison with bacteria or animal cells. For example, *S. acmella*, *A. niger*, *A. niger* HFD5A-1, *P. purpurogenum*, *P. chrysosporium* and *T. harzianum* showed high/λratios (from 50 to 190). Therefore, these biological systems could be more exposed to the eddies energies, so the most fragile systems to hydrodynamic stress in shake flask cultures could be when the cells develop in cell aggregations, but this phenomena does not occur (Fig. 4). Then, the isotropic theory of Kolmogoroff cannot apply at all in shake flask cultures, because the great differences in diameter of the biological system analyzed. Furthermore, Büchs and Zoels (2001) reported that non-fully isotropic turbulent conditions will lead to even lower levels of hydrodynamic stress.

*Average shear stress and shear rate thresholds*

Hydrodynamic stress also can be quantified by the evaluation of the shear stress and shear rate in shake flask cultures. These parameters were successfully applied for determining the shear sensitivity of *P. reticulatum* (García-Camacho *et al.*, 2007). However, the values of shear stress applied to different biological systems vary considerably in shake flask cultures by the differences on the sizes and morphologies of the biological systems analyzed. According to the shear analysis, the filamentous mold, plant cells and vegetal cells are the most exposed to extensional and compression flow forces. For example, the shear stress thresholds for bacteria are about 6.0 x $10^{-6}$ $mNm^{-2}$ (Fig. 5). Filamentous molds are exposed to high shear stress thresholds (up to 1.2 $mNm^{-2}$) as the same phenomena occur when the cells suffer aggregation such as *D. polymorpha* (3.41 $mNm^{-2}$).

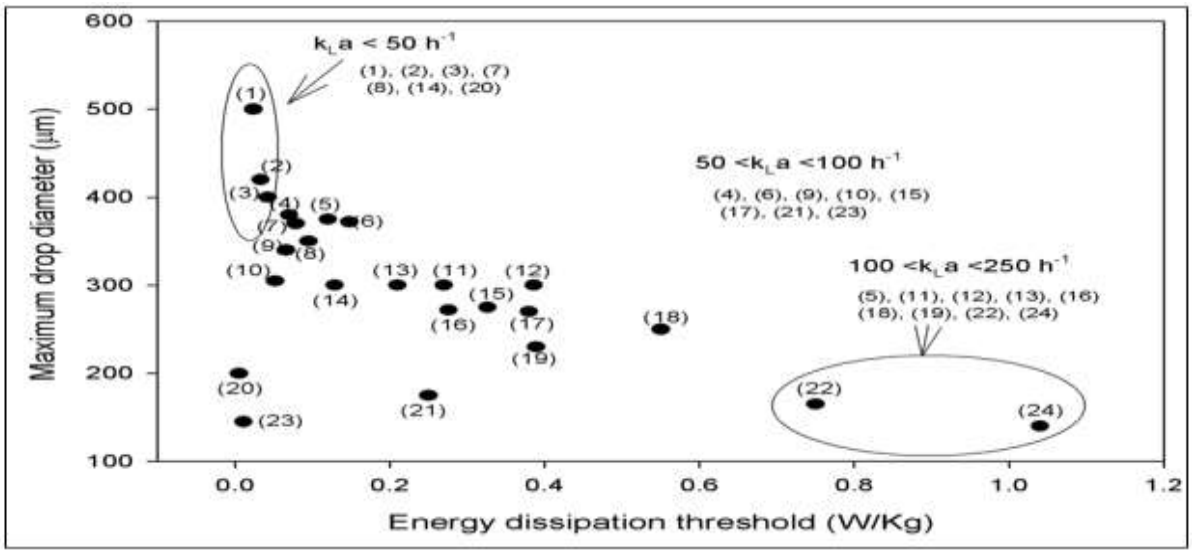


**Fig. 6.** Relationship between the maximum power tolerated and maximum drop diameters of different biological systems in cultures carried out in shake flasks. 1) *C. horrida*, 2) erythrocytes, 3) CHO cells, 4) *B. cereus*, 5) *B. subtillis*, 6) *S. aviculare*, 7) *P. citrinum*, 8) *B. licheniformis*, 9) *S. acmella*, 10) *A. niger*, 11) *B. amyloliquefacients*, 12) *P. purpurogenum*, 13) *P. aeruginosa*, 14) *A. niger* HFDA-1, 15) *E. coli* DH5a, 16) *P. chrysosporium*, 17) FCU´s, 18) *T. harzianum*, 19) *S. zaomyceticus*, 20) *P. reticulatum*, 21) *L. lactis*, 22) AGE.1.HM cells, 23*) D. polymorpha*, 24) *R. tinctorium.*

Therefore, the results depicted in Fig. 5. are not according to the susceptibility of hydrodynamic stress on the biological systems when the energy dissipation threshold and critical length eddy were used. However, the use of the shear stress as a parameter for quantifying shear sensitivity was successfully applied for *P. reticulatum* (Garcia-Camacho *et al*., 2007). But, only seems to apply when compares the hydrodynamic stress in biological systems with the same average cell diameters.

*Effect of the maximum drop diameter*

For assessment the maximum drop diameter in the cultures carried out in shake flasks (depicted in Table 2), we obtained these values on the basis of the experimental data previously reported (Büchs and Zoels, 2001) through extrapolation and interpolation. According to this parameter, the cells less exposed to hydrodynamic stress were *C. horrida*, erythrocytes, CHO cells; *B. cereus* and the most exposed cells to hydrodynamic stress were *R. tinctorium*, *D. polymorpha*, AGE.1.HM cells, and *L. lactis* (Fig. 6). These results match well with the energy dissipation threshold and critical eddy length. Therefore, these parameters could be used for quantifying the hydrodynamic stress in cultures carried out in shake flasks. Meanwhile, the shear stress and shear rate could be used for the comparison of the hydrodynamic stress between the same biological systems, where the average cell diameter is the same. And the isotropic theory of turbulence cannot be applied at all in shake flask cultures when the ratio of cell size and eddy size is too high (Büchs and Zoels, 2001).

The relationships between maximum drop size and energy dissipation rate for the cultures analyzed in shake flask show the magnitude of the hydrodynamic stress under the specific operational parameters correlated with the size of the flasks. As before reporting (Büchs and Zoels, 2001), the maximum drop diameter decreased when energy dissipation was increased and the drops diameter generated correspond with the breakup occurred in the boundary layer near the glass wall of the flasks.

The systematic analysis of the cultures analyzed allows avoid unfavorable conditions and unknown conditions where the optimization of the cell cultures didn`t have a chance.

In general terms, the analysis of the maximum drop diameter corresponds with the reports made by Büchs and Zoels (2001), where at the same agitation speed on the shaker means less drop diameter for the larger flasks and therefore there is a high probability to develop a bioprocesses at high hydrodynamic stress levels. Also, the increase of agitation speed generates the minor diameters of the maximum drops, and where the filling volume has not a significant influence in the drop dispersion. And the other hand, for the filamentous molds analyzed here: *A. niger*, *A. niger* AFDA-1, *P. purpurogenum*, *P. chrysosporium*, develop a larger pellets than those observed in stirred tank fermenters. Furthermore, the ratio between the maximum local energy dissipation rates is about 10 times lower in shake flasks than in stirred bioreactors (Peter *et al.*, 2006).

Finally, Reynolds number not fit at all with the maximum size diameter of the drops (data not shown). Furthermore, Peter *et al.*, (2006) proposed at a critical Reynolds number for turbulent flow of 60,000 for unbaffled shake flasks.

The cultures analyzed in this work never achieving these values when operated at energy dissipation thresholds and when these values got achieved, the hydrodynamic stress was evident with concomitant detrimental cell damages and low metabolite yields.

*Scale up challenges*

One of the main challenges in Biochemical Engineering is the scale up of the bioprocess from shake flask cultures to stirred fermenters and keep as the mainly bottleneck in the development of a new bioprocess. As a general rule, the scale up of a phototrophic culture of dinoflagellates is more difficult compared to the scale-up to the other kinds microbial and cell cultures.

The main criteria for scale up bioprocess from shake flasks to stirred fermenters are the power input drawn (Reyes *et al.*, 2003; Trujillo-Roldán *et al.*, 2013; Gamboa-Suasnavart *et al.*, 2013) and the volumetric oxygen mass transfer coefficient-$k_La$ (Shukla *et al.*, 2001).

But these reports considered only bacteria, yeast and filamentous molds, there a little known about the scale up from flasks to stirred fermenters to the others biological systems where the hydrodynamic stress is the main concern.

**Conclusion**

The use of the software SSBP showed a useful tool for the analysis of hydrodynamic stress in shake flask cultures. On the basis of the energy dissipation threshold the biological systems most tolerable to the hydrodynamic stress were AGE.HM cells, *R. tinctorium* and *T. harzianum*. On the other hand, the most susceptible cells to hydrodynamic stress were erythrocytes, CHO cells, *P. citrinum*, *C. horrida* and *P. reticulatum*.

These results fit almost completely with the analysis of critical eddy length and maximum drop diameter. Whereas the analysis of hydrodynamic stress carried out with shear stress, shear rate and size ratio/$\lambda$ were not a useful parameter, the main problem was the Kolmogoroff theory cannot be applied at all in shake flask cultures.

***Nomenclature***

P = Poer input drawn (W)
$V_L$ = Filling volume ($m^3$)
C = 1.94 (Büchs constant)
n = Agitation rate ($s^{-1}$)
g = Acceleration of gravity ($m^2/s$)
d = Maximum inner diameter of the flasks (m)
do = Shaking diameter (m)
Tm = Mixing time (s)
$d_p$ = Average cell diameter (m)
Re = Reynolds number (-)
$k_La$= Volumetric mass transfer ($h^{-1}$)
$DO_2$ = Diffusion coefficient ($m^2/s$)

Greek letters

$\rho$ = Density ($kg/m^3$)
$\mu$ = Viscosity (Pa*s)
$\varepsilon$ = Average rate of energy dissipation (W $kg^{-1}$)
$\gamma$ = Average shear rate ($s^{-1}$)
$\lambda$= Kolmogorov microscale (m)
$\tau_t$ = Average shear stress ($mN/m^{-2}$)
$\upsilon$ = Kinematic viscosity ($m^2/s$)

***Conflict of interest***

The authors have no conflict of interest to disclose.

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