Investigation of the effect of aeration on growth dynamics, respiratory rate and pH changes of the aerobic bacterium *E. coli* BL21 cultivated in RTS-8 PLUS single-use bioreactor. Part 1

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INTRODUCTION

Disposable bioreactors with non-invasive sensors for measuring and monitoring cell concentration, O_2 and pH are in the biotechnology development trend. Biosan has developed a family of disposable RTS bioreactors, which, unlike the eccentric medium distribution mechanisms in Erlenmeyer flasks inherent to shakers, operate on the principle of a uniform centrifugal distribution of the medium arising from a rotating tube around its axis with a change of direction of rotation motion resulting in highly efficient mixing and oxygenation for aerobic cultivation.

RTS mixing principle has several advantages described earlier [1], which are automatic balancing, defoaming single-use, compact design, etc., but one of the more important points is that, due to the individual rotation principle and thermostating of each bioreactor tube, different conditions for supporting the temperature (from 4 to 70 °C) and aeration conditions from anaerobic to aerobic can be applied.

In RTS-1/RTS-1C bioreactors, the measurement is performed by photometric analysis of OD_{850} in a thin

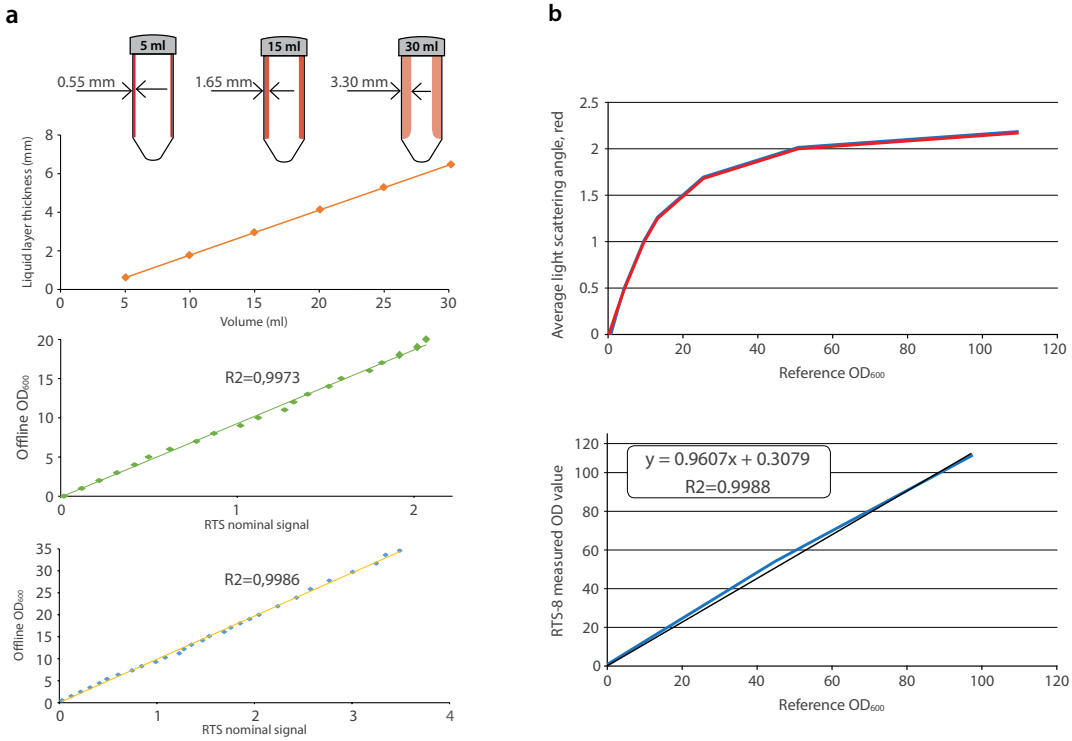

Figure 1a-b: *The principal differences in measuring the OD_{600} and light scattering in the RTS, RTS-U and RTS-8 devices.*

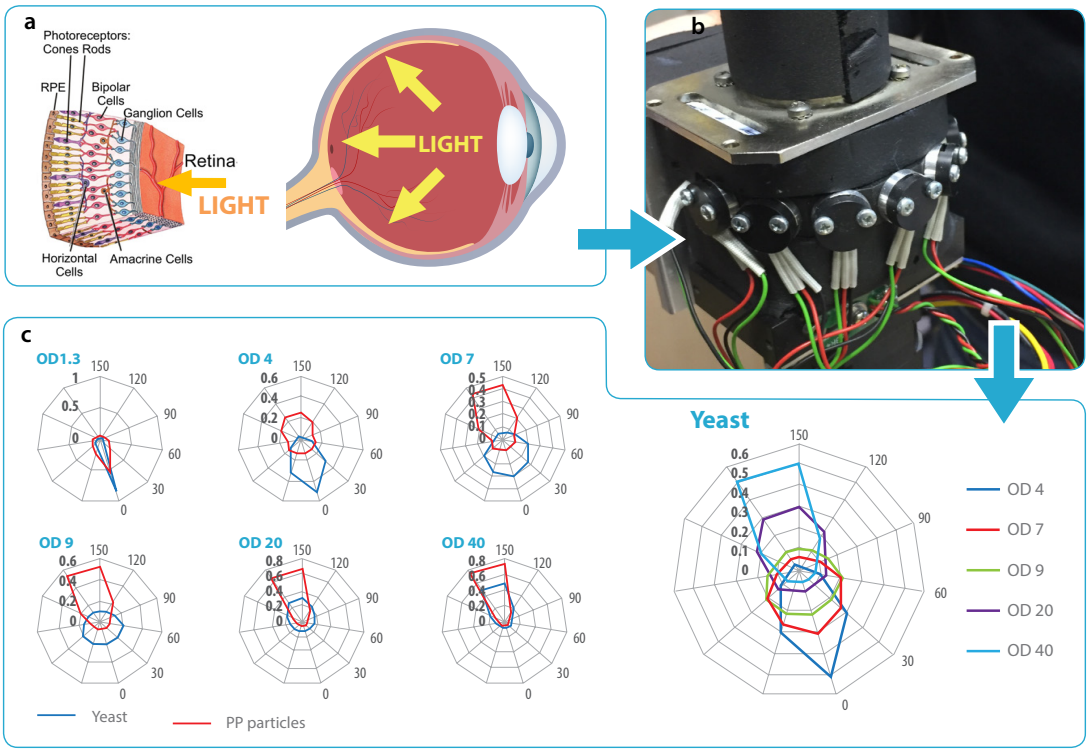

Figure 2 a-c: *a) Eye structure b) Photodiodes around the perimeter of the falcon tube c) Calculation of the average cosine of the light deflection collected by the sensors*

suspension layer formed by rotating the tube around its axis with a sufficiently linear portion of the OD_{850} calibration curve from the cell concentration (Fig. 1a). In RTS-8 and RTS-8 Plus instruments, the measurement is performed by photometric analysis of OD_{600} in a not rotating tube. To allow this to happen, a multi-angle analysis of the indicatrix is carried out, that is, the probability of the light scattering angle from the cell concentration, which is then being linearized by mathematical processing (Fig. 1b). This is an innovative solution for the noninvasive determination of cell concentration in a non-rotating single-use bioreactor falcon tube (optical path ~30 mm) by estimating the probability of the average angle of deflection of the incident light beam, which allows correctly measuring the OD_{600} in the range from 0 to 100. As can be seen from Fig. 2c, the behavior of the light beam depends on the concentration of microorganism cells, and the higher it is, the greater the angle of deviation of the incident light beam from zero (the deflection angle of the transparent liquid is usually 0°). If to arrange around the tube with a suspension of cells a perimeter of photodetectors (Fig. 2b), then the acceptable angle of light scattering can be fixed in the form of the largest signal on a specific photodetector. Since the deflection of the light beam tends to move from 0 to 180° as the concentration of cells increases, then by constructing a calibration curve of the dependence of the indicatrix on the cell concentration, we can control the growth of the bacterial cell culture in a non-rotating tube of 30 mm in diameter in a fairly wide range of cell concentrations allowing to measure anaerobic and microaerophilic cultures effectively in comparison to RTS-1 where the measurement is carried out during 2000 rpm rotation of the tube, which is what can adversely affect the viability of oxygen intolerant cultures.

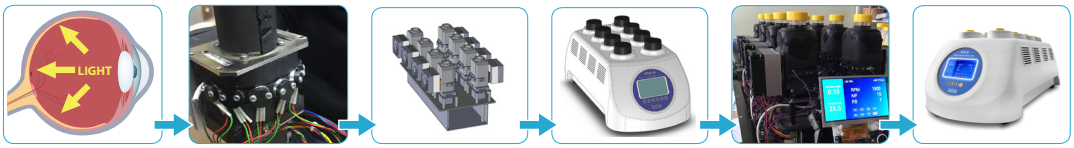

Figure 3: *Development cycle of the RTS-8 Plus*

This innovative measurement principle allowed to develop the possibilities of non-invasive measuring and monitoring of fermentation vital bioprocess parameters such as O_2 and pH of the medium by fixing additional disposable fluorescent pH and O_2 sensors (preSens, Germany) on the inner wall of the single-use bioreactor falcon tubes and integrating custom optics to RTS-8 Plus to be able to detect and measure these sensor spots. Thus, in combination with our OD_{600} measurement solution, it was possible to implement a parallel bioprocess on a single platform with independent conditions — 1) aeration, 2) temperature control, 3) OD_{600}, 4) pH of the medium and 5) O_2. Since 8 independent bioprocesses can be performed on a single platform in parallel and since on board each bioreactor, additionally to 8 multi-angle sensors for OD_{600}, there can be inserted pH and O_2 sensors, then this design was given an abbreviated name Reverse Tube Spinner Plus or RTS-8 Plus (Fig. 3). In this paper, we present the experimental data obtained for the RTS-8 Plus bioreactor for the first time.

FORMULATION OF THE TASK

In the parallel to the above mentioned optics, in bioreactor RTS-8 Plus, each tube is provided with an individual mechanism that performs a reverse-forward rotation (Reverse Spin) at the set speed. The resulting centrifugal forces instantaneously and evenly distribute the nutrient medium both horizontally along of the tube and vertically along the tube, thus providing a given area of the interphase between air and liquid proportional to the intensity of aeration. But in addition to these processes that determine the mass transfer, there is another barrier of oxygen diffusion into the tube — 1) the presence of cap openings, 2) their number, 3) their diameter, 4) the pattern of the openings and 5a) the oxygen transmissive material, 5b) carbon monoxide, 5c) other gases associated with the process. If we compile a balance equation for these components and then describe the general equation for the mass transfer of oxygen in air to the nutrient medium in a rotating single-use bioreactor falcon tube — a lot of thorough work on each process coefficient may be required. We went along the experimental path in the hope of describing these processes mathematically in the future. Earlier these dependencies (volume of medium and intensity of rotation) were carefully studied and analyzed from the point of view of the growth rate of the model microbes (*E. coli, P. Pastoris*) and on the basis of the known directly proportional dependence of the growth rate on aeration as well as the Fick's laws of diffusion and Henry's gas law, we reduced the understanding of the mass transfer process in our bioreactor relate to two obvious rules, which are — 1. overall oxygen transfer is proportional to the surface to volume ratio, thus by decreasing the working volume, gas–liquid mass transfer rate reaches higher values, 2. the higher the intensity of rotation, the faster and more efficiently the cell suspension spreads around the inner walls of the falcon tube, thus the higher the gas–liquid mass transfer rate. Earlier, we showed the dependence of the growth rate of *E. coli* and *P. pastoris* cells on the working volume of nutrient medium and the intensity of rotation of the single-use bioreactor falcon tubes in comparison to shake flasks [1]. It was found that the most intensive growth takes place at 5 ml working volume. However, how this affects the O_2 and the pH of the medium has not been investigated for RTS bioreactors. We took these patterns as the basis for our further research. In this report, we present no longer indirect experimental data on the effect of aeration both on the growth rate and OD_{600} yield, but on the parameters of the change in O_2 and pH of the nutrient medium during the process of aerobic fermentation. This data is obtained for four aeration modes: 1 and 2 modes correspond to intensive aeration and a high oxygen transfer rate (OTR). These modes are provided by rotating the tube at a speed of 2000 and 2700 rpm; 3–4 modes correspond to semi-anaerobic and practically microaerophilic aeration with low OTR values, provided by rotating the tube at a speed of 1000 and 1500 rpm. Although it is known from previous experiments [1] that 5 ml working volume is the most efficient in terms of aeration, the minimum working volume for the optical system to work correctly in RTS-8/RTS-8 plus is 7.5 ml, for using lower working volumes and to be able to measure OD it is recommended to use RTS-1/RTS-1C systems. In this experiment 10 ml working volume was chosen because in the past it was the recommended condition for aerobic cultivation and proved to be sufficient in previous experiments for non-rich broth medium's like LB.

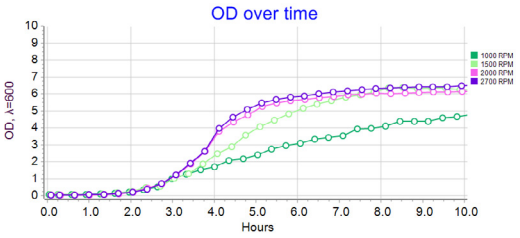

Figure 4: *Influence of the rotation intensity on the dynamics of cell growth using LB broth medium.*

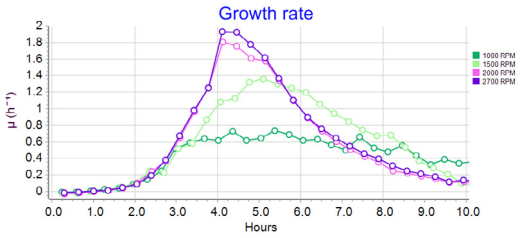

Figure 5: *The influence of rotation intensity on the growth rate of the* OD_{600}.

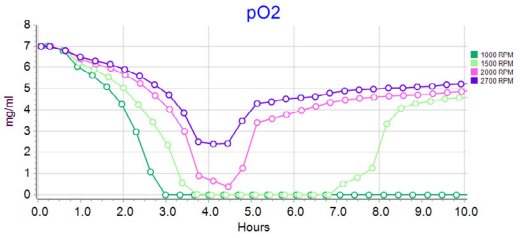

Figure 6: *Influence of the rotation intensity on the dynamics of the change in the concentration of oxygen in the cell suspension.*

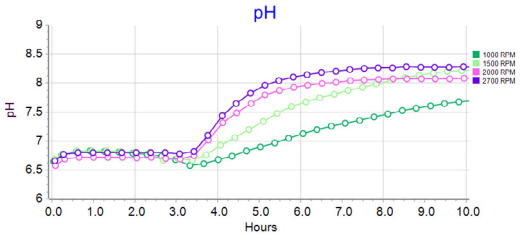

Figure 7: *Influence of the rotation intensity on the dynamics of pH change in the culture medium.*

MATERIALS AND METHODS

The single-use bioreactor falcon tubes were filled with nutrient medium and covered with screw caps provided with special breathing openings, which were closed with a membrane that was semi-permeable to oxygen. Then these 50 ml tubes were placed in RTS-8 Plus and the fermentation process was initiated synchronously.

Working volume was 10 ml, the cultivation temperature was 37 °C, the measurement intervals (MI) of the sensors were every 20 minutes, the Reverse Spin Time (RST) of changing the rotation of the tube 1 time per second, the intensity of rotation of the tubes was according to the signatures of the legends (Fig. 4). The aeration intensity was changed by changing the rotation speed or angular velocity (ω) of the bioreactor tube in discrete ranges of ω= 1000 rpm (green curve), ω = 1500 rpm (light green), ω = 2000 rpm (pink curve) and ω = 2700 rpm (purple curve).

RESULTS AND DISCUSSION

Let us consider the obtained data of the dependence of the change in growth rate, oxygen consumption and pH of the nutrient medium on the rotation intensity of the falcon tube. From the data obtained (Fig. 4), it is clear that an increase in the rotation intensity of the falcon tube leads to an acceleration of the growth rate of the optical density (OD_{600}) and therefore of cells whose concentration corresponds to the values of OD_{600}. The greatest difference in OD_{600} between all variants of rotation speed is at 4–5 hours of cultivation and then after 10 hours the OD_{600} of all variants is compared in the region of 6.0–6.5 OD_{600}, which is the maximum possible yield of biomass of *E. coli* BL21 cells using the LB medium.

And now let's observe the obtained data in the coordinates of the growth rate of OD_{600} depending on the time of fermentation. (Fig. 5)

From Fig. 5 it can be seen that an increase in ω from 1000 to 1500 and then 2000 rpm leads at every step to a 0.7-fold increase in the maximum growth rate of OD_{600} (the value is expressed in OD_{600}/h^{-1}). A further increase from ω 2000 rpm to ω 2700 rpm does not lead to a rise in growth rate. Therefore, in a single LB medium at 10 ml of the bioreactor working volume, the aeration conditions achieved at ω 2000–2700 rpm for this strain are not limiting. At the same time, the range below ω 2000 rpm leads to the oxygen limiting conditions. Moreover, the data presented in Fig. 6 confirm the above mentioned observations.

From the data presented in Fig. 6, it is seen that during the transition of the culture to the logarithmic growth phase, an increase in the intensity of oxygen consump-

Table 1: *Dependence of OD600 at which hypoxia is observed depending on the rotation intensity of the tube.*

ω (rpm)	μ_{max}	OD_{600}
2700	1.95	4
2000	1.8	3.75
1500	1.35	2.5
1000	0.7	1.75

tion from the medium consumed for aerobic generation of ATP is observed. If under intensive aeration conditions (corresponding to ω = 2000 rpm and ω = 2700 rpm) the cell culture does not fall into hypoxic shock, then for aeration intensity corresponding to ω from 1500 and below, hypoxia is observed - that is, a state in which the oxygen transfer rate (OTR) is lower than the intensity of oxygen uptake rate (OUR) by the culture. It is interesting to note that this transition is observed at OD_{600} in the medium corresponding to the values given in Table 1.

Table 1 is of practical interest and can serve as an orientation for scale-up of the bioprocess. In the future, we propose a more detailed study of the dependence of the mass transfer of oxygen on the intensity of rotation of the tube in order to obtain an algorithm for reducing the toxic effect of hypoxia during fermentation.

Now we will consider what happens with the pH of the nutrient medium during fermentation and how this parameter is affected by the intensity of aeration.

It is necessary to note two sections of the pH dependence on the fermentation time — 1. stable pH retention in the initial pH range of 6.8, 2. alkalization of the nutrient medium to pH 8.3 from the moment of oxygenation limitation. From the data obtained (Fig. 7), it follows that the change by microorganisms of the pH of the medium is not a response to oxygen limitation (hypoxia) but is the result of another process not associated with aerobic processes. Since the carbon source for the tricarboxylic acid cycle is, as a rule, the ketoacids that result from the deamination and deamidation of the amino acids present in the LB (tryptolytic hydrolysate of the milk protein of casein), then it becomes understandable regarding the alkalization of the nutrient medium to pH 8.3 — the point of equilibrium shift of the ammoniacal solution NH_4OH towards ammonia gas NH_3 after 4 to 5 hours of fermentation.

CONCLUSIONS

In this report, we presented experimental data reflecting the effect of aeration intensity on growth dynamics, growth rate, changes in oxygen consumption and pH change in the nutrient medium obtained using RTS-8 Plus. Non-invasive multi-angle measurement of OD_{600} based on the probability of deflection of the light scattering angle in combination with non-invasive measurement and monitoring of O_2 and pH realized in the innovative development of Biosan and preSens, Germany is effective in measuring and monitoring these parameters during parallel fermentation processes. On the basis of the data obtained, one can judge the prospects of using a disposable parallel bioreactor RTS-8 Plus with the ability to perform a parallel bioprocess with non-invasive measurement and monitoring of cell concentration, pH and O_2. The data obtained with this unique and innovative device can be used to further scale-up of the bioprocess on other types of bioreactors, since the results obtained give a complete idea of what happens to the dynamics of mass transfer of the most important limiting substrate — namely oxygen in the process of aerobic cultivation.

REFERENCES

[1] Bankovskis, V. et. al. (2017, May 01). Reverse-Spin® Technology — Innovative Principle of Microbial Cultivation, 2nd edition. Retrieved from biosan.lv/rts-tech