Influence of the Operating Parameters on the *E. Coli* BL21 (DE3) Growth in Fedbatch Bioreactor

GABRIELA ISOPENCU[1*], ALINA TANASE[1], ANAMARIA JOSCEANU[2], VASILE LAVRIC[1]

[1]Politehnica University of Bucharest, Faculty of Applied Chemistry and Materials Science, Department of Chemical and Biochemical Engineering, 1-7 Polizu Str, 011061, Bucharest, Romania

[2]Politehnica University of Bucharest, Faculty of Applied Chemistry and Materials Science, Department of Analytical Chemistry and Environmental Engineering, 1-7 Polizu Str, 011061, Bucharest, Romania

The interdependences between environment changes and cellular responses to external stimuli are still not fully understood. Accordingly, in many cases the influence of the individual cellular heterogeneity on the productivity of some metabolites at industrial scale cannot be justified. This study intends to highlight the response of a bacterial population to the modification of certain external stimuli (operating parameters) during the fed-batch cultivation of an E. coli strain. The purpose is to understand how the population of E. coli responds, thus paving the way to a better production of secondary metabolites, such as recombinant proteins. The envisaged operating parameters were temperature and rotational speed of the stirrer, while the response parameters considered were the pH, pO_2 and the optical density (OD) of the growing medium. At the same time, the cellular apoptosis, responsible for the reduction of living cells' concentration, was determined measuring the cells viability by flow cytometry on samples taken at given intervals. The population tends to synchronize in all periods of growth, but more in the stationary phase.

Keywords: fed - batch, external stimuli, viability, optical density, flow cytometry, E. coli

Assessment of physiological stages of individual bacterial cells (grown in small environments – laboratory scale) may be useful in predicting and monitoring bacterial population behaviour at the industrial scale. Bacterial cells in different stages of the cell cycle respond individually at the action of external stimuli from the environment leading to heterogeneous populations [1].

Each bacterium has its own metabolic system, so even in the case of an isogenic population of bacteria that grow in apparently homogeneous conditions, latter it can be induced changes at the single cell level.

Experimental studies have shown that, after a perturbation applied to a culture medium through an external stimulus, only a fraction of the population responds to the change immediately, the rest providing a secondary response, out of phase; the former bacteria were in the appropriate cellular cycle phase, while the latter were, probably, in the lag phase when the perturbation occurred [2, 3]. However, depending on variables that are specific to the bacterial species growth, the correct mechanism would be the inherent response of the bacterial cells, such as: slow growing or rapid adjustment to the concentration of the substrate. These types of variables are likely to influence experimental interpretations, especially when species genetically modified are used [4].

Escherichia coli is a microorganism used as a host for the production of recombinant proteins and in metabolic engineering. For applications in which the product of interest is a recombinant protein, it is recommended to use *E. coli*, since recombinant proteins are produced by excretion, process which allows easier detection and purification. In these conditions the product can be separated into an optimal conformation, unaffected by the conditions in the culture medium and cell associations resulting from proteolytic degradation [5].

Production of recombinant protein with high yields is an area intensively studied in industrial biotechnologies. Various configurations of bioreactors are considered for obtaining recombinant proteins using *E. coli* as a vector [6, 7]. Different feeding scheme for batch bioreactors are reported with the purpose to relief the physiological changes of the cells population [8].

In the fed-batch bioreactors the processes for obtaining bioproducts imply repeatedly feeding the substrate (the carbon/energy source specially) in high concentrations to prevent the medium to reach the limit of the inhibition. Moreover, these concentrations should be correlated with the oxygen transfer capacity of the bioreactor, to ensure the oxygen flow needed for the growth of microorganisms. For maintaining the substrate concentration at this level limit, cellular productivity should remain constant. Since the concentration of biomass increases, specific rate of growth will progressively decrease. In literature are specified the mechanisms of interaction between biomass concentration and specific growth rate, in particular in industrial processes, where conceptually is established that specific growth rate would decline over time [8-10].

Fed batch operating favors the production of the secondary metabolites, as are the recombinant proteins. Since the mechanisms of interaction between the parameters specific growth rate versus production yield of secondary metabolites is not well-known, the process of obtaining recombinant protein is not fully optimized [11].

In discontinuous cultures the amount of carbon source available for cells is decreasing and induces, after reaching the minimum concentration limit of the carbon source, a state of starvation of individuals, which determine the production of approximately 30 proteins. [9, 10].

At this point in the process, it is indicate the measurement of the respiratory active and inactive cells, through the oxygen consumption. The amount of the oxygen consumed should be linked to the content of RNA and stability of plasmids, which are affected by the starvation state, since the cells are trying to maintain their

* *email: g_isopencu@chim.upb.ro*

viability. Production of RNA polymerase is directly dependent on the specific rate of cellular growth, the latter adversely affecting the production of secondary metabolites, in particular recombinant proteins; therefore, the feeding schemes with the carbon/energy source could result in a large variability of secondary metabolites production [9].

Other factors that influence the specific cellular growth rate in close correlation with the concentration of carbon / energy source are: temperature, *p*H and dissolved oxygen concentration in the medium. Therefore it is necessary to adjust these parameters at the levels necessary to maintain a higher yield of the useful bioproduct (such as recombinant proteins).

The flow-cytometry (FC) technique, using fluorescent colored samples to identify physiological state of individual cells, is a rather new analytical method used to estimate the heterogeneity of bacterial population [12].

FC is a powerful technique for the rapid analysis of single cells in a mixture, by means of light-scattering and fluorescence measurements. In biotechnology, the power of this method lies both on the possibility of determining a wide range of cell parameters at single cell level, and in the ability to obtain information about their distribution within cell populations, providing valuable information for bioprocess design and control [12, 13].

The main objective of this paper is the study of the intrinsic dynamics of bacterial populations of *Escherichia coli* BL21 (A3) (untransformed) growing in a fed batch bioreactor, monitoring the operating parameters and using spectrophotometric methods and FC to record the response of the bacterial population to the external stimuli.

Experimental part

Materials and methods

Cultivation conditions chosen to study the behaviour of this new strain of *E. coli* are the same as those used for the strain K12-MG1655 (see the experiments reported by [2, 3, 14]) but at different operating parameters.

Strain

The bacterial strain used in the experiments was E. coli BL21(DE3)[15], with genotype fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdSλ DE3=λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5.

Selection of the strain was motivated by its further use for gene expression of S-layer protein from achaean hyperthermophile *Pyrococcus abyssi*. BL21 (A3) represents the classical strain used for bacterial expression of the genes cloned in expression vectors of T7 to obtain recombinant proteins. The doubling time of *E. coli* BL21 (A3) strains at 37°C is 30 min.

Culture medium

The culture medium consists of a fraction sterilized by autoclaving (fraction 1) and a fraction sterilized by filtration (fraction 2), to which trace elements are added in a 1.5.L working volume.

Composition of fraction 1: 6 g Na_2HPO_4; 3 g KH_2PO_4; 0.5 g NaCl; 1 g of NH_4Cl (g/L).

Composition of fraction 2: 5 mL glucose 20 %; 2 mL $MgSO_4$ 1M; stock solution of mineral salts in traces (*p*H 7, conservation on 4°C).

Trace elements (100x stock solution): 5 g EDTA; 0.8 g $FeCl_3$; 0.05 g $ZnCl_2$; 0.0001 g $CuCl_2$; 0.0001 g $CoCl_2$; 0.0001 g H_3BO_3; 0.016 g $MnCl_2$.

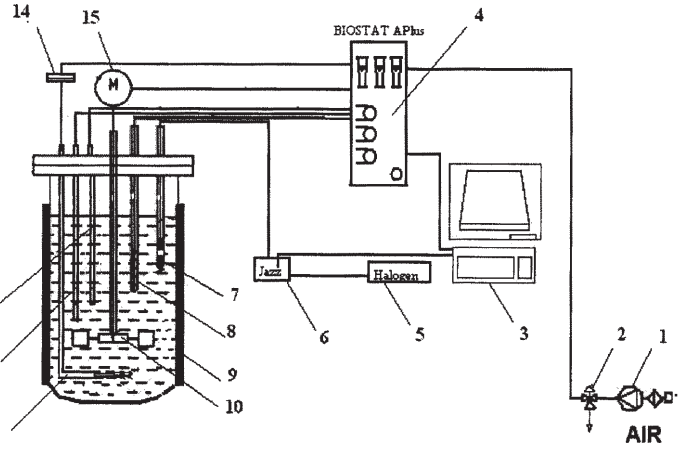

Fig. 1. Experimental setup for Escherichia coli BL21 (DE3) cultivation. 1 - Compressor; 2 -Tap air flow regulation with purge; 3 –Laptop with dedicated software for bioreactor and spectrophotometer; 4 - Central control unit of the bioreactor; 5 –Spectrophotometer source light; 6 – Spectrophotometer portable unit; 7 -Spectrophotometer sensor; 8 - pH-meter; 9 -Bioreactor glass tank with heating jacket; 10 -Mechanical stirrer; 11 -Gas distributor - metal sintered glass; 12 -Temperature sensor; 13 - Oxygen sensor; 14 -Air filter; 15 - Drive motor stirrer.

Cultivation

The bioreactor, previously sterilized, was inoculated with 1% *E. coli* in the exponential growth phase (the inoculum concentration of bacterial cells is determined as OD = 1.987 at 600 nm).

The experiments were carried out in a 2.5 L bioreactor with a working volume of 1.5 L (Sartorius BIOSTAT® A Plus). The bioreactor was equipped with air sparger having set the air flow at 0.33 l/min. The stirrer speed was varied in the range of 100-170 rpm during the bacterial constant growth rate. A polarographic oxygen electrode was used to register the DO level in the medium. The bioreactor was also equipped with *p*H sensor for measurement and *p*H adjustment. Temperature was controlled between 27 and 37°C by the bioreactor electrical jacket. The bacterial growth was monitored on-line with an optical spectrophotometer Ocean Optics Inc. Jaz (UV-VIS) with immersion sensor. Both the bioreactor and spectrophotometer were controlled online by the corresponding software. A schematic representation of the experimental setup is presented in figure 1.

Flow cytometry

The cell viability after cultivation was measured using Flow System Apogee. The ratio between live and dead cells was established using specific florescent markers.

Sample preparation took into consideration the following aspects: a) reduction of the amount of particulates in the sample by dilutions to approximately 10^4 - 10^6 CFU/mL; b) the requirement of minimum of four samples: one unstained control, two single-color compensation controls and one test sample; the correlation of the contacting time of the dye with the sample function of type of cells and dye.

The cytograms were obtained after the calibration and proper settings of the Flow System Apogee.

Results and discussions

Experiment 1

First set of determinations was carried out under the following operating conditions:

-in the lag period, representing the adjustment of the bacterial culture to the conditions of bioreactor, temperature

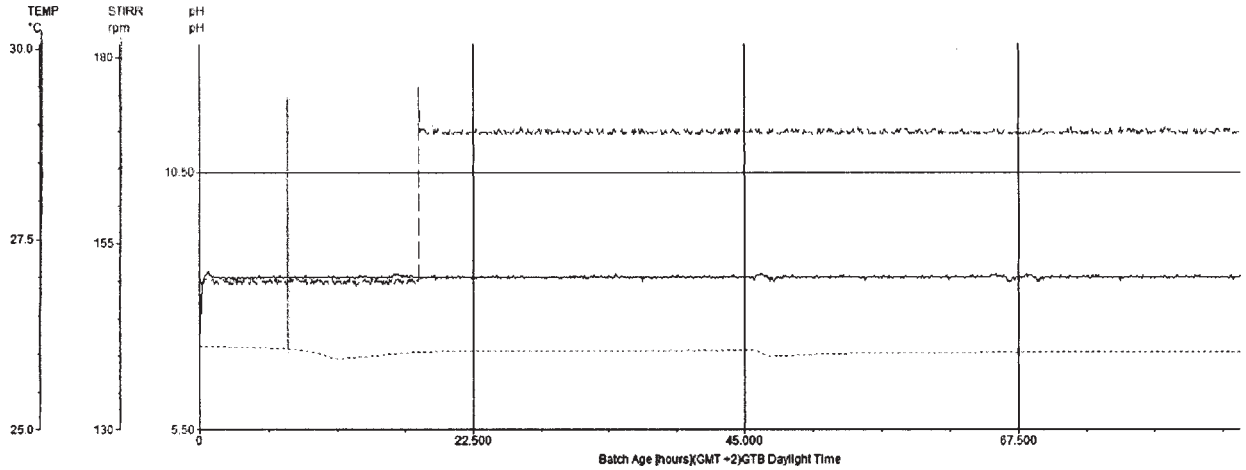

Fig. 2. The parameters measured in the bioreactor for an Escherichia coli BL21 (DE3) cultivation – Experiment 1

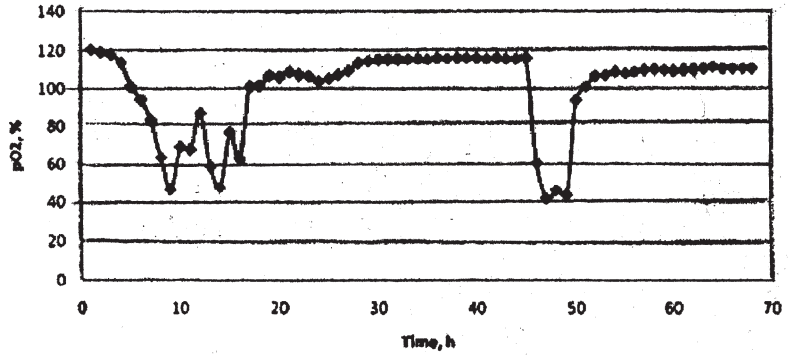

Fig. 3. Oxygen partial pressure in bioreactor during E. coli strain cultivation - Experiment 1

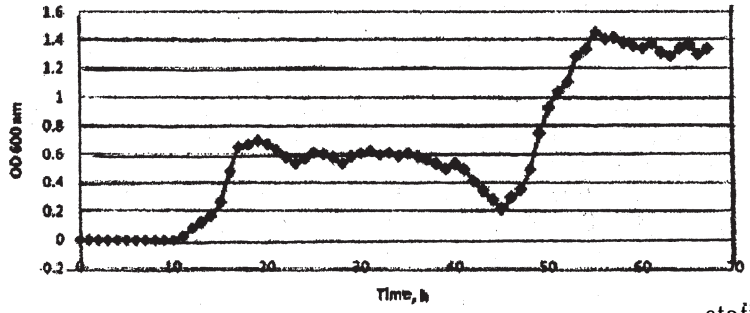

Fig. 4. OD dynamics in Experiment 1

has been set to 27°C, stirring rate at 150 rpm, *p*H of the medium being of 7.3;

-after approximately 20 h of operation, due to the decrease of oxygen consumption, the culture has been fed with fresh medium (fraction 2) and stirring has been raised to 170 rpm.

In figure 2 are presented all the growth parameters as recorded by the bioreactor's sensors for the entire experiment.

It is observed that during the start-up phase of the culture, up in the adjustment of a bacterial communities in the exponential growth phase, the oxygen concentration varies in agreement with bacterial population dynamics: decreases as the bacterial population increases, in the first 10 h of cultivation, after which periodic fluctuations appear, relatively wide and thick, for approximately another ten hours (fig. 3).

This behavior imposed the increase of the stirring up to 170 rpm. That ensures a bacterial growth for a period of 20 h with relatively constant rate of growth in the stationary phase, with small variation of the cell concentration in the bacterial population.

Recorded data of OD are in close correlation with the oxygen consumption data. In the first 10 h of bioreactor operation, the adjustment of bacterial strain to environmental conditions took place. After 10 h , the culture entered in the exponential growth phase and the OD reached higher values during the next 10 h. After this, the stationary growing phase follows, corresponding to secondary metabolites production, during which OD had fluctuations around 0.6, with local minima and maxima. After 40 h of cultivation, the population of *E. coli* strain started to decline, which has led to the need of a feeding with fraction 2 of the culture medium, in the same concentration and with the same amount as at the beginning (at t = 45h). After re-feeding, an immediate exponential increase in cellular mass occurred, reaching the maximum after another 10 hours of cultivation, when populations enters again into the stationary phase of growth (fig. 4).

The OD variation is similar with the concentration of the dissolved oxygen and indicates that the OD variations are generated especially by the changes in the fraction of living cells in the culture [2, 3].

Therefore, OD can be used for the characterization of bacterial culture and the study of intrinsic dynamics of the community. This hypothesis should be substantiated by the analysis of cytograms obtained on samples taken at the end of cultivation.

Cytograms present the concentration of cells, in a predetermined volume of a sample, resulted from a cycle of counting the individual microorganisms. Cytograms are built using the coordinates LALS-SALS (Large Angle Light Scatter - Small Angle Light Scatter) - for samples unstained and stained, giving the total amount of counted microorganisms. For stained samples, cytograms are built in coordinates Org - Grn (Orange- Green) - to highlight the

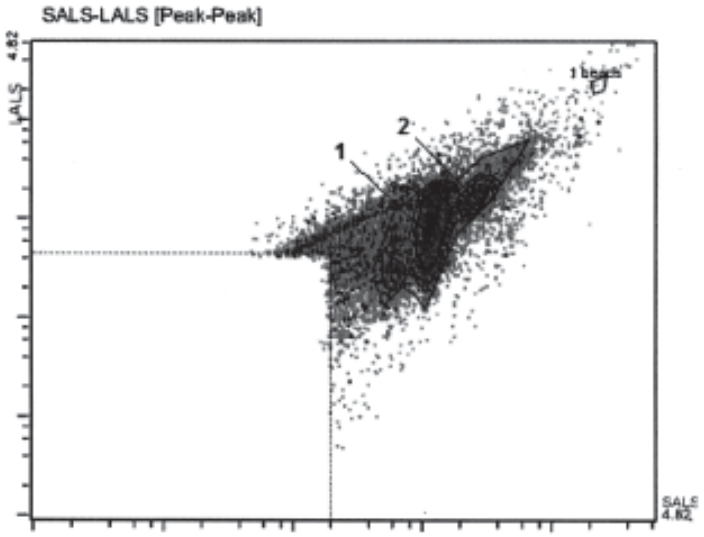

Fig. 5. Cytogram of estimated population of E. coli– unstained sample. Experiment 1

living/dead cells amount (according to the specific marker used).

Flow cytometry technique applied on a sample taken at the end of the batch process, after 70 h of cultivation, reports two clusters in the bacterial population: one group of 1890 cells/μL (1) and another group having 220 cells/μL (2). These two distinct groups differ through the cells dimension, affected by the different stages they are in the cellular life cycle (fig. 5).

To refine the results, the sample was stained with florescent marker Syber Green II - SyG (to evidence the live cells) and with Propionium iodine - PI (to mark the dead cells).

After staining, the two populations were more clearly differentiated as marked gates: 2e1 and 3 in LALS – SALS diagram (with estimated population number of 1894 cells/μL).

The Org – Grn diagram indicates the emission of the cells after staining with different florescent markers: the dead cells are stained by PI, because their membrane became permeable to this florescent marker and the population is near the Org axis (gate 6 with 760 cells/μL, resulted by gating the 3 gate), while the live cells are stained with SyG and their position is marked by gates 4 and 5 (with approximately 600 cells/μL resulted by gating the 2e1 gate).

The cells which are not included in gates 6 or 4 and 5 are in an intermediate life cycle and that determine an incomplete staining with one or the other florescent

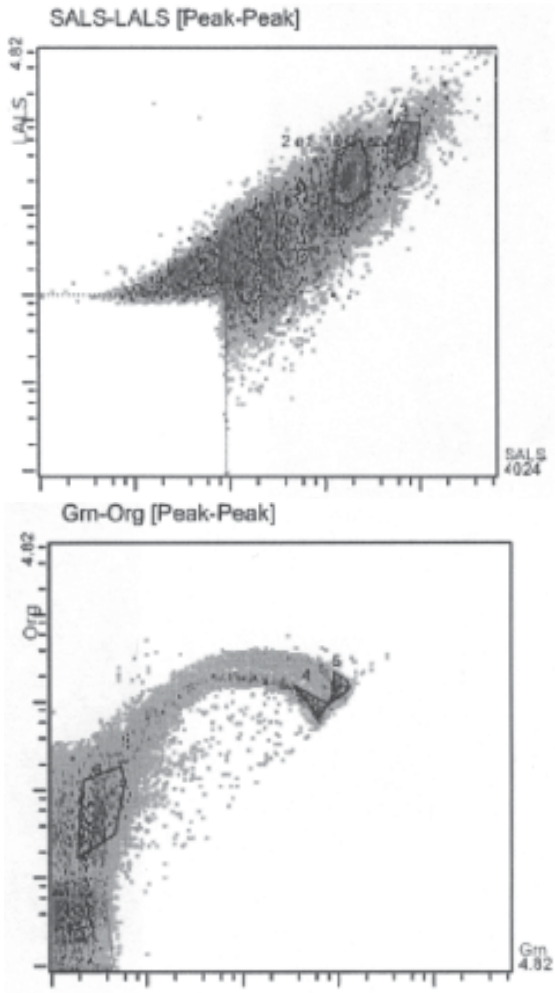

Fig. 6. Cytogram of estimated population of E. coli – stained sample with Syber Green II and Propionium iodine. Experiment 1.

markers. This is evidenced by the "*comet tail*" present in the Org-Grn diagram (fig. 6, right hand side diagram).

Experiment 2

The second experiment was carried out to study the stability of the culture characteristics function of the operating parameters. The experimental conditions, recorded by the bioreactor dedicated software (fig. 7), were:

-in the lag period the temperature has been set to 27°C , stirring at 130 rpm, *p*H of the medium being of 7.3;

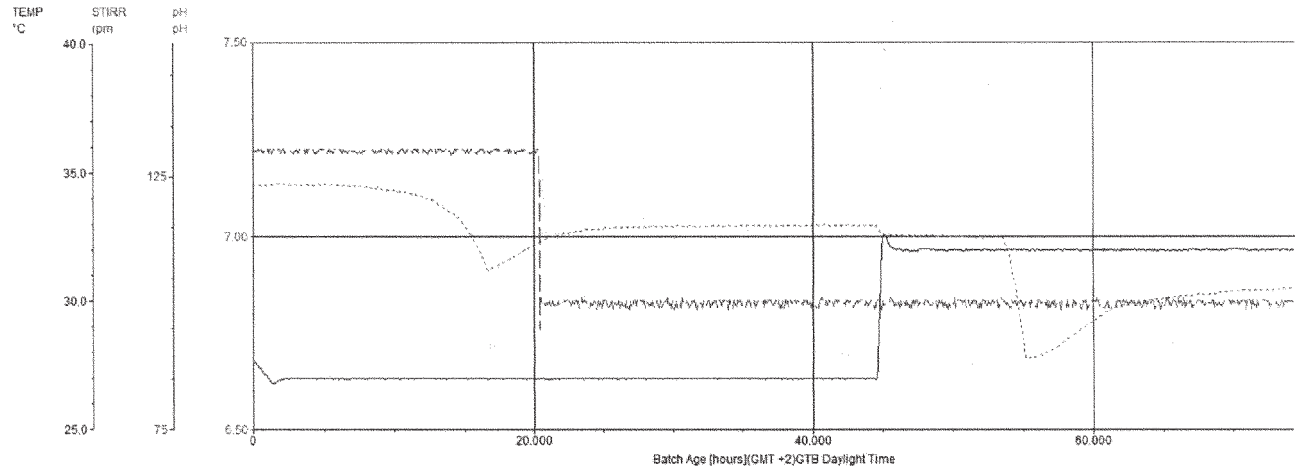

Fig. 7. The parameters measured in the bioreactor for E. coli BL21 (DE3) cultivation – Experiment 2

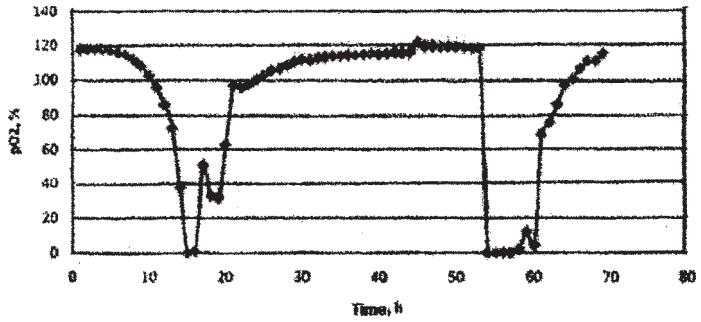

Fig. 8. Oxygen partial pressure in bioreactor during E. coli strain cultivation. Experiment 2

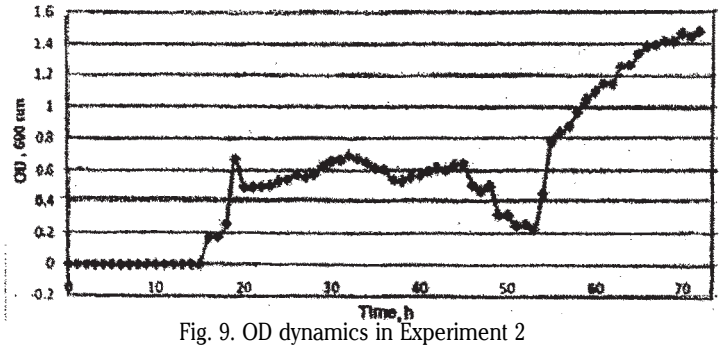

Fig. 9. OD dynamics in Experiment 2

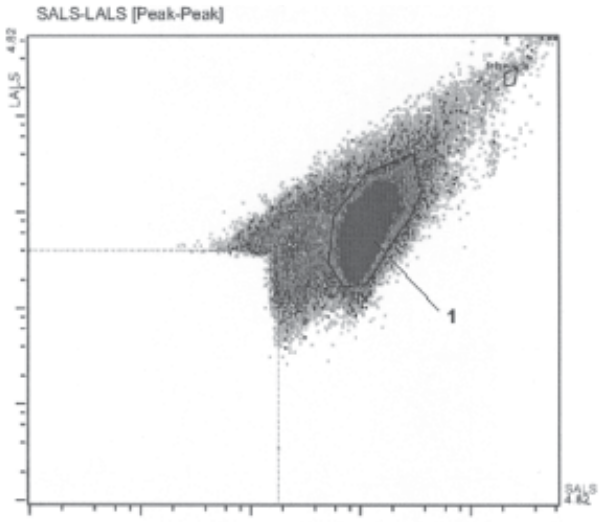

Fig. 10. Cytogram of estimated population of E. coli – unstained sample. Experiment 2

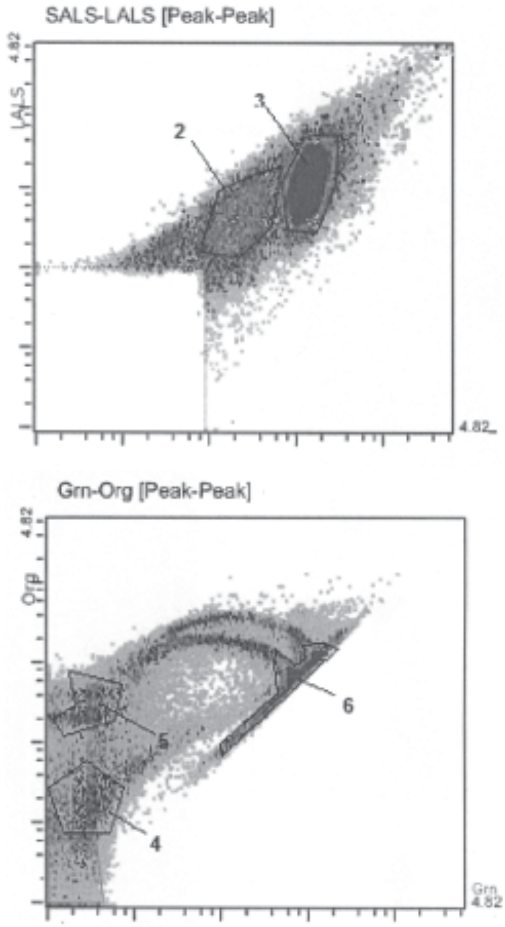

Fig. 11. Cytogram of estimated population of E. coli – stained sample with Syber Green II and Propionium iodine. Experiment 2

-when *p*H of medium was stabilized, the rotational speed was reduced to 100 rpm;

-after approximately 42 h of operation in these conditions, when the *p*H was stabilized at value of 7 and the temperature has been raised to 32°C;

-when the operating parameters are stabilized and due to lower consumption of oxygen, re- feeding has been carried out after approximately 53 h of cultivation.

The behaviour of the bacterial culture was recorded and the characteristic parameters are shown in figure 8, regarding the oxygen concentration, and in figure 9, which presents the cells' concentration as given by the optical density of the culture media.

The same dynamics of the bacterial culture growth corresponding to the external stimuli was emphasized in the Experiment 2, as in the Experiment 1. Still, some exceptions should be noted: the lag period is with approximately 5 h longer, due to the *p*H variation in the range 6.8-7.3; the exponential growth period is shorter that in Experiment 1; there is more manifested the cells concentration variation, prolonged in the stationary phase of growth and evidenced by the local minimum and maximum with larger amplitude than in Experiment 1.

Changes of operating parameters have been carried out under the same critical points of experiment (when the oxygen consumption decrease, concomitantly with the cell concentration decreasing), trying to extend the stationary growth phase, optimal for the production of secondary metabolites.

Refueling the culture medium by supplementing with the fraction 2 (sterilized by filtration) corresponds to a strong decrease in partial pressure of oxygen (fig. 8).

Measuring the absorbance and consumption of the oxygen highlight periods of maximum cellular development, with intense metabolism (high oxygen consumption) respectively, between hours 20 and 45, periods having the same trend as indicated in [3].

At the end of the experiment, the population composition was estimated by FC analysis. For an unstained sample the result indicates 5112.6 cells/μL (gate 1– fig. 10).

Special particularities of cellular growth at the end of the Experiment 2 have been also highlighted by FC analysis of the samples colored with specific florescent marker which can differentiate living / dead cells. The differences resulting from metabolic pathways of growth and development of cells cycle are shown in Figure 11.

The analysis evidences also two clusters in the bacterial populations (gate 2 and 3 with estimated cells concentration of 43193 cells/μL and 4885.4 cells/μL) in SALS-LALS diagram.

The Grn-Org diagram splits these populations in live cells (gate 6 – stained by SyG, estimated at 27576 cells/μL

resulted from gating 2) and dead cells (gate 4 and 5 – stained by PI, estimated at 1821 cells/μL resulted by gating 2). The great difference between SALS-LALS diagram and Grn-Org diagram is given by the cells dissipated on the cytogram field out of the market gates. The gates represent the zones with large amount of bacterial cells in the same physiological stage and the cells around those gates are in intermediate live cells cycle. The physiological state determines the amount of florescent markers which can attach to the RNA contained in the bacterial cells.

Conclusions

The complexity of the batch culture due to the variation of substrate concentration from a maximum value at the beginning of the experiment, to a value close to zero at the end of the experiment, changes the intrinsic synchronism behaviour in a bacterial culture. However, by analyzing the OD and the dynamics of the dissolved oxygen, it is possible to identify periods in which at least a part of the population gets into synchronism.

Initial low concentration of substrate, as well as its continuous decrease, due to the consumption by bacteria, determine the appearance of a transitional stationary phase, in which the microorganisms have less and less substrate, that determine an accentuate dynamics of internal metabolic pathways.

Even under these circumstances, the activities of bacterial population as a whole were emphasized, as evidenced by local increases and reductions of OD (fig. 4, 9). During the period of accelerated decline of cellular mass, too, because of starvation (consumption almost total of substrate), signs of synchronism still exists: figure 10, between 45 h and 53 h – at low stirring, and also the periodic variations of OD both before and after the addition of fresh medium.

A more thorough analysis was performed using FC, which quantified not only the living vs. cells concentrations, but also the stage of cellular cycle in which the living cells are - the existence of the intrinsic synchronism should be proven if a significant number of living cells are located at the same stage of cell development.

Acknowledgement: The work has been funded by the Human Resources Program - Research projects to stimulate formation of young independent research teams of the Romanian Ministry of Labour, Family and Social Protection, through the Financial Agreement PN-II-RU-TE-2012-3-0136 Dynamics of Escherichia coli population transformed to express archeal heterologous protein S –layer.

References

1. REZAEINEJAD, S., IVANOV, V., Microbiol. Res., **166**, 2011, p. 129
2. CHI FRU, E., OFIŢERU, I. D., LAVRIC, V., GRAHAM, D. W., Appl. Microbiol. Biotechnol., **89**, nr. 3, 2011, p. 791
3. OFIŢERU, I. D., CHI FRU, E., FERDEŞ, M., GRAHAM, D. W., LAVRIC, V., Appl. Microbiol. Biotechnol., **94**, nr. 1, 2012, p. 185
4.. LIDSTROM, M.E., KONOPKA, M.C., Nature Chem. Biol., **6**, 2010, p. 705
5.. YE NI, R. CHEN, Biotechnol Lett., **31**, 2009, p. 1661
6.. HORTSCH, R., WEUSTER-BOTZ, D., Appl. Microbiol. Biotechnol., **90**, 2011, p. 69
7.. HORTSCH R, WEUSTER-BOTZ D., Adv. Appl. Microbiol., **73,** 2010, p**.** 61
8.. SANDEN, A.M., PRYTZ, I. TUBULEKAS, I FORBERG, C, HA LE, HEKTOR, A., NEUBAUER, P., PRAGAI,Z., HARWOOD, C., WARD, A., PICON, A., TEIXEIRA DE MATTOS,J., POSTMA, P., FAREWELL, A., NYSTROM, T., REEH, S., PEDERSEN, S., LARSSON, G., Biotechnol. Bioeng., **81**, nr. 2, 2003, p.158
9.. HOLLENDER, J., VAN DER KROL, D., KORNBERGER, L., GIERDEN, E., DOTT, W., World J Microb Biot, **18**, 2002, p. 355
10.. ZENGIN, G.E., ARTAN, N., ORHON, D., CHUA, A.S.M., SATOH, H., MINO, T., Bioresource Technol., **101**, 2010, p. 4000
11.. GOGULANCEA , V., ISOPENCU, G., FERDEŞ, M., OFIŢERU, I.D., LAVRIC, V., U.P.B. Sci. Bull., Series B, *acceptat spre publicare*
12.. DIAZ, M., HERRERO, M., GARCIA, L.A., QUIROS C., Biochemical Eng. J., **48**, 2010, p. 385
13.. DE ROY, K., CLEMENT, L., THAS, O., WANG, Y., BOON, N., Water Res., **46**, 2012, p. 907
14.. LAVRIC, V., GRAHAM, D., J. Theor. Biol., **264**, 2010, p. 45
15. STUDIER, F.W., MOFFATT, B. A., J. Mol. Biol., **189**, nr. 1, 1986, p. 113

Manuscript received: 5.06.2014