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COMPARATIVE ANALYSIS OF THE EFFECTS OF HYDRODYNAMIC CONDITIONS IN SUBMERGED CULTURING OF RECOMBINANT BACTERIA

Use of recombinant microorganisms in biotechnology is one of the leading branches of its development. Structure peculiarities of recombinant bacteria require the use of more sparing culturing conditions, first of all, stirring conditions. This requires more detailed study of the effects of hydrodynamic processes in fermenter, as well as mechanisms of this effect. Provided literature review contains analysis of studies of scientists worldwide in this area, devoted to the effects on biomass accumulation, synthesis of metabolites, and morphological structure of recombinant bacteria cells. As a result of the evaluation performed, we have arrived to the conclusion that, besides the stirring device rotation rate, the following factors exert essential effects: stirrer impeller structure, nutrient medium composition and pH, and aeration intensity, as well as the ratio between these parameters. In majority of cases, maximum metabolite synthesis conforms to maximum biomass increment, but this does not happen always; at high stirring rates, the change of external cellular structure and biosynthesis productivity decrease are observed. All these phenomena are related with development of shear stress in recombinant bacteria cells, but the mechanisms of this effect are not investigated in them. Thus, optimization of submerged culturing processes requires continued more detailed of the effects of hydrodynamic processes on recombinant bacteria growth and development.

Key words: recombinant bacteria, shear stress, stirring, stirring rate, submerged culturing.

Introduction. Biotechnology development is closely related with the use of recombinant microorganisms carrying foreign DNA responsible for synthesis of a certain metabolite inserted via gene engineering manipulations. Recombinant DNA technology has enabled to create producers for large-scale synthesis of many bioactive substances, as well as to obtain products with improved properties or in higher quantities. There are several requirements to microorganisms used in creation of recombinant strains, which include the absence of pathogenicity and toxicity, as well as possibility of culturing on simple nutrient media. Most frequently, various bacteria of *Bacillus*, *Erwinia*, *Pseudomonas*, *Rhizobium*, and *Escherichia coli* genera, as well as microscopic fungi *Saccharomyces cerevisiae* are used for creation of recombinant microorganisms.

Presence of foreign DNA in recombinant microbial strains increases their sensitivity to culturing conditions. Thus, the necessary ratio between all process conditions has to be found. One of the limiting factors in submerged culturing of recombinant microorganisms, irrespective of their genus, for assurance of maximum end product yield, is stirring regimen, which is related with sensitivity of the latter to various mechanical effects, which might cause damage or rupture of bacterial cells and loss of foreign DNA.

In view of the fact that stirring processes affect recombinant microorganisms, a need appears in comparative analysis of the effects of various stirring regimens and establishment of the optimal rate values to be used in industrial and laboratory conditions during the process of submerged culturing of recombinant bacteria. Besides, the regularities between culturing regimens have to be identified, and peculiarities of the mechanisms of stirring effects in submerged culturing conditions on morphological structure of recombinant bacteria cells, their viability and productivity have to be determined.

Main part. Experiments in the effects of hydrodynamic process regimens on productivity and viability of recombinant bacteria were conducted by scientists of many countries of the world. Having analyzed our results, the main of which are stated in the table, we have arrived to the conclusion that more sparing process regimens need to be applied for recombinant microorganisms versus the ordinary strains. Let's take a closer look at culturing conditions and experiments conducted by scientists in different recombinant bacteria species, and compare the effect of stirring regimens on biomass increment, proteins accumulation and external structure of microbial cells, and try to establish the causes of such effect.

Comparison of stirring regimens in submerged culturing of recombinant microorganisms [1–12]

No.	Name of producer	Stirring rate, rpm	Stirring rate enabling accumulation of maximum biomass quantity, rpm	Stirring rate enabling accumulation of maximum target product quantity, rpm
1	<i>Sphingomonas paucimobilis</i> SP 304 [1]	200–800	200	400
2	<i>Thermus thermophilus</i> HB 27 [2]	200–500	500	500
3	<i>Bacillus licheniformis</i> NCIM-2042 [3]	200–400	300	300
4	<i>Escherichia coli</i> LipR1 [4]	200–350	300	300
5	<i>Escherichia coli</i> BL 21 [5]	200–550	400	400
6	<i>Bacillus subtilis</i> DB 104 [6]	130–150	150	150
7	<i>Corynebacterium glutamicum</i> MCNB 10025 [7]	200–1200	410	–
8	<i>Escherichia coli</i> strain B [8]	600–1700	–	–
9	<i>Escherichia coli</i> NCIB 10000 [8]	500–1500	–	–
10	<i>Bacillus cereus</i> [8]	600–1700	–	–
11	<i>Staphylococcus epidermidis</i> [8]	600–1400	–	–
12	<i>Escherichia coli</i> SGK25/pIF TREN [9, 10]	50–400	200	400
13	<i>Escherichia coli</i> B/pTG201 [11]	50–200	50	–
14	<i>Escherichia coli</i> TERI BD 18 [12]	150–200	150	–

During investigation of stirring and aeration effects on $\alpha 2$ -interferon synthesis by recombinant strain *Escherichia coli* SGK25/pIF TREN, the experiments were carried out for three cases [9, 10]: in variable aeration intensity and stirring duration, in variable aeration and constant stirring rate, in constant aeration and variable stirring rate. During the experiment, the stirring rate was varied

within the range of 50 to 400 rpm. The highest biomass quantity was accumulated with constant aeration and stirring intensity 200 rpm (Fig. 1, a), and the maximum metabolite yield was seen at constant aeration intensity and stirring rate 400 rpm (Fig. 1, b) [9, 10].

In studies of the effect of stirring and aeration on growth kinetics of bacteria *Thermus thermophilus* HB 27, it was established that the culture growth rate increases with increase of stirring and aeration intensity. Nevertheless, the increase of stirring rate has more weighty contribution than the increase of aeration intensity. Increase of stirring device rotation rate from 200 to 300 rpm resulted in increase of bacterial growth rate by a factor of 2, and the highest biosynthesis productivity (biomass accumulation and product yield) were seen at 500 rpm [2].

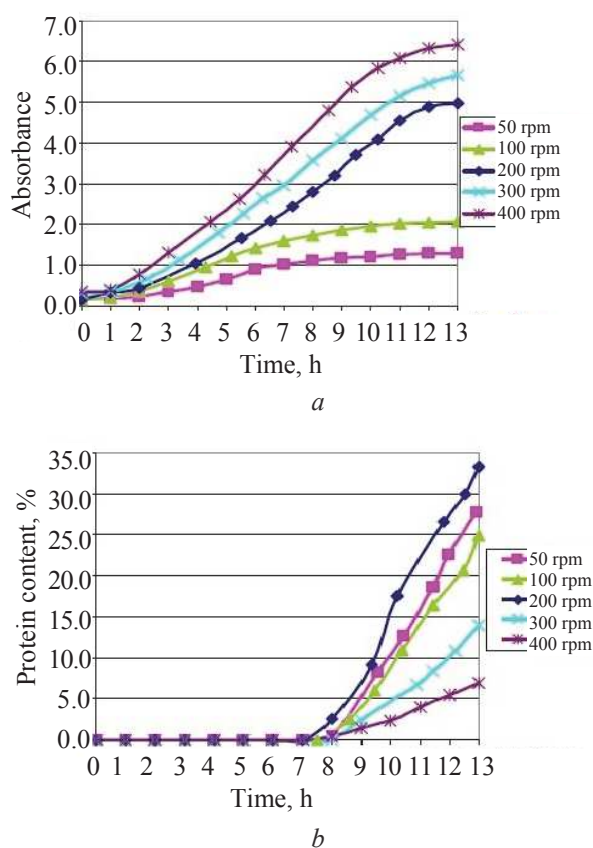


Fig. 1. The relation between stirring device rotation rate and protein synthesis by *Escherichia coli* SGK25/pIF TREN cells at fixed air supply rate [9]:
 a – accumulated biomass quantity;
 b – quantity of protein $\alpha 2$ -interferon synthesized by the cells

In submerged cultivation of recombinant *Bacillus licheniformis* NCIM-2042, the experiments were carried out for stirring rates 200, 300 and 400 rpm, as well as for three different values of aeration intensity. The maximum yield of enzyme

protease was observed at stirring rate 300 rpm. Deviation from this value either downward (to 200 rpm) or upward (to 400 rpm) has negative effect in the enzyme synthesis and biomass accumulation. Nevertheless, with increased aeration intensity, the maximum protease yield was observed at 200 rpm [2].

To determine the effect of stirring on the synthesis of enzyme lipase by recombinant *Escherichia coli* LipR1, the experiments were carried out for the stirring rate within the range of 200 to 350 rpm. The maximum biomass accumulation and enzyme synthesis level was observed at 300 rpm. Lower stirring rates had negative effects on bacteria development process and lipase biosynthesis. Stirring rate increase to 350 rpm led to the decrease of the culture growth and the enzyme synthesis [3].

When the stirring effect on synthesis of amino acid L-phenylalanine with another recombinant *Escherichia coli* BL 21 strain was considered, the maximum product yield is observed at 400 rpm (Fig. 2). The increase of accumulated biomass quantity and synthesized amino acid would be observed at stirring rate within the range of 200 to 400 rpm. Further increase of stirrer rotation rate to 500 rpm would result in decrease of the target product yield parameters [5].

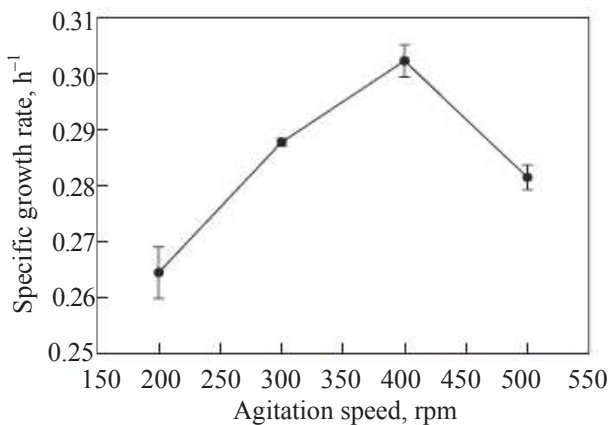


Fig. 2. Relation between amino acid L-phenylalanine synthesis by bacteria *Escherichia coli* BL 21 and stirring rate [5]

The effect of stirring rate on stability of plasmids during culturing of recombinant strain *Escherichia coli* B/pTG201, immobilized by the way of formation of carrageenan gel beads, was studied in two types of nutrient medium LB and M9 and in stirring rotation range of 50 to 200 rpm. The increase of stirring device rotation rate in both nutrient media resulted in decrease in number of cells in gel beads (accumulated biomass quantity for LB medium was decreased from 3.68 cells/ml at 50 rpm to 1.61 cells/ml at 200 rpm, and the same

for M9 medium was decreased from 2.06 cells/ml at 50 rpm to 1.39 cells/ml at 200 rpm), which was caused by potent shear stress in them [11].

The study of complex effects of pH and stirring duration on recombinant *Escherichia coli* strain TERI BD 18 has shown that lower biomass quantity is accumulated at pH 7.5 and stirrer rotation rate 200 rpm compared to pH 6.5 and 150 rpm. In the first case, the accumulated biomass quantity was 1.35 g/(l · h), and in the second case it was 1.73 g/(l · h) [12].

The study of stirring effect on the synthesis of enzyme endoxylanase by recombinant strain *Bacillus subtilis* DB 104 using submerged culturing [6] was carried out for 130 and 150 rpm. The obtained results have shown that the enzyme synthesis at stirring rate 150 rpm was twice as high as at the rate 130 rpm.

The experiments on stirring effect on biotin synthesis during submerged culturing of recombinant *Sphingomonas paucimobilis* SP 304 were carried out at stirring device rotation rate within the range of 200 to 800 rpm, using various stirrer structures. No signs of cellular growth and the vitamin synthesis were observed at 200 and 800 rpm (Fig. 3). The highest biomass concentration was formed at 600 rpm, and maximum biotin yield was observed at 400 rpm [1]. Based on the obtained results, the authors have arrived to the conclusion that this fact is related with shear stress appearing in microbial cells.

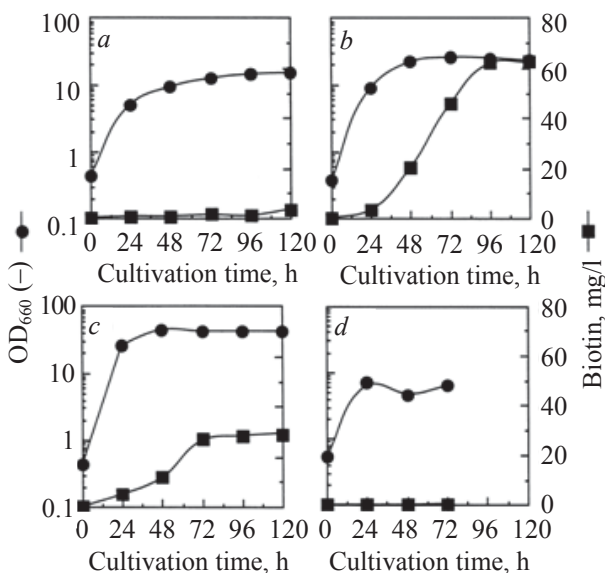


Fig. 3. Biomass and biotin accumulation level during culturing of *Sphingomonas paucimobilis* SP 304 depending on the culturing time [1]: a – 200 rpm; b – 400 rpm; c – 600 rpm; d – 800 rpm

Similar results were obtained for the use of various stirrer types. The maximum biomass increment was observed at 600 rpm irrespective of impeller

type. Biotin synthesis was the maximum at 200 rpm when the anchor stirrer was used, and at 400 rpm when turbine stirrer and Maxblend® stirrer (with structure shown in Fig. 4) was used [1].

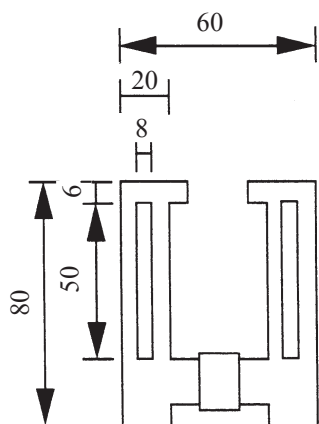


Fig. 4. Schematic illustration of Maxblend® stirrer structure [1]

Besides, the authors [1] have established that external structure of cells depends on the culturing conditions. Microscopic analysis of cultural fluid after 120 h of culturing (Fig. 5 – photographs of the culture are shown for experiments with turbine stirrer only) has revealed that the cell dimensions were larger at 200 rpm compared to the rest of the cases. Bacterial cells had oval shape at low stirrer rotation rates (200–600 rpm) and elongated shape at high rates (800 rpm), provided the turbine stirrer was used. Elongation of cells was also seen for the use of propeller stirrer at 400 rpm and more. In both cases, when the cells had elongated shape, level of biotin synthesized by recombinant bacterial cells was decreased [1].

In submerged culturing of recombinant strain *Corynebacterium glutamicum* MCNB 10025 in normal (410 rpm) and stress (1200 rpm) conditions, it has been found out that intensive stirring rate results in decrease in dimensions of microbial cells and formation of cells with irregular shape (Fig. 6) [7]. Microscopic analysis has also revealed that bacterial cellular wall had become thicker. Most likely, this is related with effect of intensive external mechanical stress. According to the authors' hypothesis [7], in this way the cells try to protect their internal structure from external effects and damages.

The study [8] investigated the effect of stirring on cell dimensions and shapes. Two recombinant *E. coli* strains were tested: *Escherichia coli* strain B and *Escherichia coli* NCIB 10000, as well as one *Bacillus cereus* strain and one *Staphylococcus epidermidis* strain. Linear relation between cell volume and its growth rate vs. stirring intensity (varying from 500 to 1500 rpm) was observed in

all cases – increase of stirrer rotation rate lead to the increase in cell volume for each of the tested microorganisms. Comparison of end biomass yield at different stirring rates shows that the enlargement is related with increase of water content in cells.

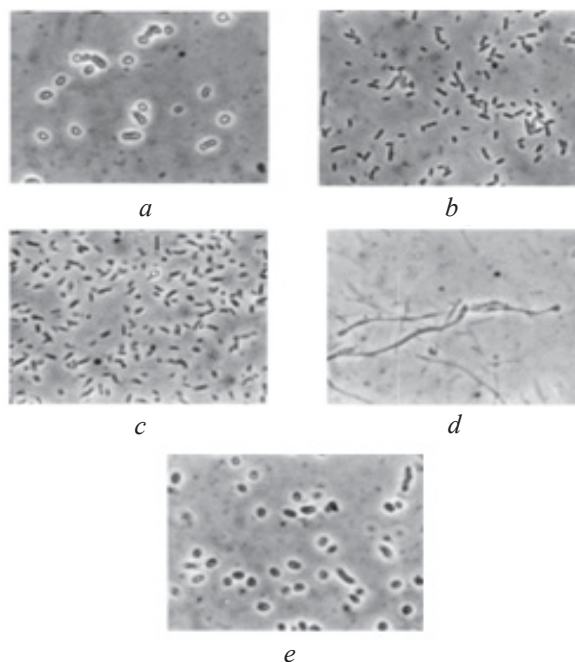


Fig. 5. Morphologic structure of *Sphingomonas paucimobilis* SP 304 cells in submerged culturing using turbine stirrer after 120 h of culturing depending on stirring device rotation rate [1]: a – 200 rpm; b – 400 rpm; c – 600 rpm; d – 800 rpm; e – test tube

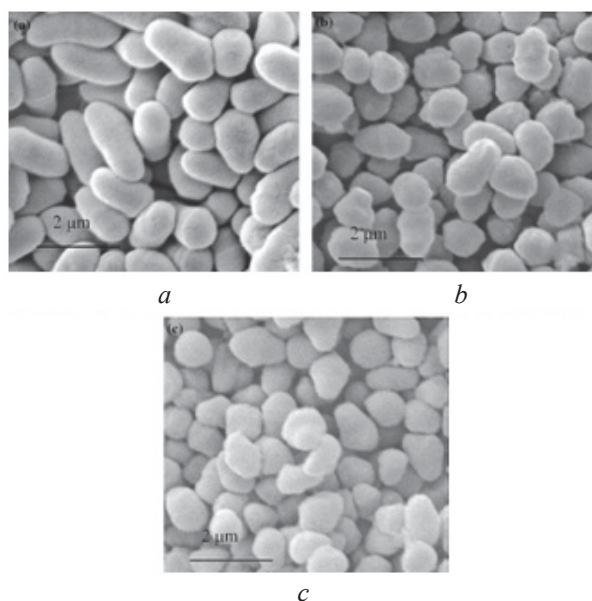


Fig. 6. Image of *Corynebacterium glutamicum* MCNB 10025 cells under microscope at various stirring conditions [7]: a – 400 rpm; b, c – 1200 rpm

The authors of [8] made an assumption that the enlargement of cells at increase of stirrer rotation rate is related with shear stress appearing during stirring; their negative effect on the viability of cells was not proven. Nevertheless, they did not understand how shear stress could promote the stable development of population of larger cells. In view of the above, they have come to the conclusion that the enlargement of cells is not associated with effect of shear stress occurring at stirring.

Conclusions. We can see from the above information that stirring rate, as was assumed, exerts essential influence in submerged culturing of recombinant bacteria and represents one of the limiting factors of this process. Besides, the ratio between stirring duration, aeration intensity, and nutrient medium composition is of great importance. According to literature data, optimal stirring duration for recombinant bacterial culturing that can be used in industrial conditions is 150–400 rpm. Nevertheless, maximum quantity of accumulated biomass does not always conform to the maximum level of metabolites synthesis, and biomass accumulation and metabolite synthesis processes are affected not only by stirring device rotation rate, but also by the structure of stirrer used in the process.

It is worth mentioning that, in some cases, no critical value of stirring device rotation rate was established, which makes the pattern incomplete. In majority of cases, the cells do not receive sufficient quantities of oxygen supplied to them at low stirrer rotation rate, and mechanical damage of cells occurs at high stirrer rotation rate. Thus, optimal stirring duration first of all depends on the culture used as a producer. Negative effect of shear stress appearing in cells at high stirring rates is observed for recombinant bacteria. This results in decreased metabolites synthesis level and decreased biomass accumulation quantity.

Still, unfortunately, not all reviewed studies investigated the stirring effect on the intensity of metabolites synthesis by examined recombinant bacterial strains, which is a more essential parameter than the accumulated biomass quantity and cellular volume.

That is why, in further studies, the parameters worth attention are not limited to the end result caused by the change of stirring rate, but also include the nature of this effect. This can be achieved by studying mechanisms of mechanical stirring effect on microbial cells based on experimental and theoretical studies of shear stress appearing in cells by the way of creation of mathematical model of hydrodynamic and heat-and-mass-exchange processes in a fermenter.

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