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EXPERIMENTAL ARTICLES

Relationship between Astaxanthin Production and the Intensity of Anabolic Processes in the Yeast *Phaffia rhodozyma*

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Abstract—The astaxanthin synthesis in the yeast *Phaffia rhodozyma* was shown to depend on the rate of growth occurring in the first two days of cultivation. The growth rate of the yeast culture studied was preset by the cultivation conditions, among which the C : N ratio was decisive. The intense anabolic processes coupled with active culture growth during the first 24 h significantly inhibited the synthesis of the key enzymes involved in astaxanthin synthesis, which led to a marked decrease in the carotenoid production. It was demonstrated that, for the maximum yield of astaxanthin to be obtained from 1 l of nutrient medium, it is necessary to carry out cultivation, beginning with the first day, at a growth rate significantly lower than μ_{max}. The optimum budding rate of the mutant strain *Ph. rhodozyma* VKPM Y-2409 consistent with the maximum astaxanthin synthesis was determined. The specific astaxanthin productivity of the strain studied was about 7.0 mg/g of dry biomass at a budding rate of <0.5.

Key words: astaxanthin, *Phaffia rhodozyma*, carotenoid synthesis, anabolism intensity.

Astaxanthin (AX) is a carotenoid pigment rather widespread in nature. It can be isolated from the flowers of certain plants, marine microorganisms and animals, algae, and lichens, as well as from the yeast fungus *Phaffia rhodozyma* [1]. The ability of a number of animals, such as birds, fish, and crustaceans, to accumulate astaxanthin in their tissues was the reason for the active study of the yeast *Phaffia rhodozyma* as a natural source of this pigment. Astaxanthin is actively used in agriculture as an additive in mixed feed for hens [2, 3] and artificially bred trout (*Salmo irideus*) [4]. The addition of astaxanthin results in deep red staining of the muscular tissues of trout and hen egg yolk, which significantly increases the competitiveness of these products in the market.

The synthesis of astaxanthin, as that of other carotenoids, is strongly dependent on environmental factors. The remarkable antioxidant properties of AX, determined by its chemical structure, aid in the survival of AX-synthesizing microorganisms under unfavorable conditions. Such microorganisms are able to bind free radicals generated in the process of their vital activity. AX possesses a large number of active double bonds and can therefore effectively act as a trap for these radicals, performing the function of their quenching.

The influence of different cultivation factors on the astaxanthin synthesis in *Ph. rhodozyma* has been highlighted in the literature rather widely [5–8]; however, many questions concerning the mechanisms of regulation of the synthesis of carotenoids, including AX, remain unclear. It was noted that the conditions for the optimum biomass growth and the maximum astaxanthin synthesis differ appreciably [9]. The source of carbon, as well as its concentration, plays an important role in the AX synthesis in the yeast *Ph. rhodozyma.* The AX synthesis was established to be liable to glucose repression; an initial glucose concentration exceeding 5% markedly inhibits the AX synthesis [8]. The AX level increases significantly as compared to the control if cultivation is carried out under the conditions of carbon limitation [9]. Accordingly, high levels of the carbon source in the medium, especially in the lag phase and in the early exponential phase, cause the AX synthesis to be decreased and the synthesis of by-products, such as ethyl alcohol and acetaldehyde, to be increased. The yeast cultivation under conditions of stringent nitrogen limitation at the stage of exponential growth dramatically decreased the AX content in the biomass of *Ph. rhodozyma* [9]. At high C : N ratios, a significant increase in the synthesis of both lipids and carotenoids was observed in the cells of this yeast. The authors noted that, in AX superproducers, nitrogen utilization is much slower than in the wild strains, which may indicate the possibility of nitrogen involvement in the regulation of AX biosynthesis. Experiments in continuous cultures showed that mild carbon limitation throughout the cultivation process should be considered to be the optimal conditions for AX synthesis [10, 11].

In this work, we attempted to reveal the relations between the rate of anabolic processes in a log-phase

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culture of *Ph. rhodozyma* and the synthesis of its main carotenoids. The influence of such important factors for AX synthesis as light and aeration was not considered, and all the experiments were carried out under conditions optimal in terms of these parameters. The main attention was given to the conditions of the synthesis of the enzyme systems that in the stationary phase determine the synthesis of the predominant carotenoids, of which the most important is AX.

MATERIALS AND METHODS

This work used the mutant strain *Phaffia rhodozyma* VKPM Y-2409 with abolished catabolite repression, capable of accumulating up to 10 mg of carotenoids (the total of pigments) per gram of dry biomass. The choice of the strain for this work was not fortuitous since the high level of AX synthesis in this mutant enabled us to study the influence of the main cultivation factors on AX synthesis in greater detail. On the other hand, choosing this strain, we understood that not all the regularities revealed would be valid for all *Ph. rhodozyma* yeasts; this may especially true for the ability of the mutant strain to grow well and synthesize carotenoids at sufficiently high glucose concentrations in the medium (>10%). The yeast was cultivated in medium containing glucose, peptone, salts (g/l, KH_2PO_4, 1.0; $MgSO_4$, 0.5; NaCl, 0.1; $CaCl_2$, 0.1); vitamins (μg/l, biotin, 50; thiamine, 500; Ca pantothenate, 2000; folic acid, 2; inositol, 1000; nicotinic acid, 400; *p*-aminobenzoic acid, 200; pyridoxine hydrochloride, 400; riboflavin, 200); and trace elements (μg/l, boric acid, 500; $CuSO_4$, 40; KI, 100; $FeCl_3$, 200; $MnSO_4$, 400; Na_2MoO_4, 200; $ZnSO_4$, 400). The initial glucose concentration in the medium was the same in all of the experiments, namely, 20%, which is optimal for astaxanthin synthesis by the strain studied when cultivated in flasks. The amount of nitrogen (peptone) in the medium was varied. The amine nitrogen content in a 1% solution of the peptone used was 0.18 mg/ml. The amounts of glucose and the nitrogen source were converted to the carbon and nitrogen contents, respectively, and the C : N ratio was calculated. The yeast was grown on a circular shaker (250 rpm) in 750-ml flasks and in 3-l Anglicon fermentors at 17°C under constant illumination (60 lx) for seven days; pH 5.0 in the fermentors was maintained by a KOH solution. A 50% glucose solution was used as additional feeding if required. Cycloheximide (CHI), a protein synthesis inhibitor, was added to the medium at a concentration of 0.05% wt/vol, which virtually completely inhibited protein synthesis and, accordingly, the growth of this yeast culture [12]. Complete cessation of protein translation in the strain studied was controlled using [^{14}C]-labeled leucine.

CHI at the above-mentioned concentration was added to the culture of a certain growth phase. [^{14}C]-labeled leucine at a concentration of 0.5 μCi was added to CHI-containing and CHI-free cultures in parallel. Sampling was carried out every 20 min; the biomass was collected from the Millipore filter, washed three times with distilled water, and dried at 60°C. Its radioactivity was measured on a Delta 300 apparatus. The mass of dry cells (g/l) in the samples analyzed was determined after their lyophilic drying. The titer of the cells was determined by counting them in a Goryaev chamber, the cells not as yet separated from the maternal bud cell being counted as independent cells. The specific budding rate was determined as the 1-h cell titer difference. The growth intensity of the yeast culture studied was defined as the capacity of accumulating dry biomass per unit time and expressed in g/h. The carotenoids were extracted from the biomass by acetone with simultaneous cell destruction with Sigma glass beads (425–600 μm) in a shaker [13]. The sum of the carotenoids was determined according to the conventional technique [8] using a Shimadzu UV-120 spectrophotometer. The measurements were carried out in quartz cells at $\lambda = 474$ nm, which corresponded to the maximum astaxanthin absorption in petroleum ether. The maximum AX absorption in acetone was in the same range (471–477 nm); therefore, the specific strain productivity was calculated using a formula in which the data on the coefficient of astaxanthin extinction in petroleum ether were used:

$$C = \frac{VA_{474} \times 100}{21m},$$

where C is the sum of carotenoids in 1 g of dry biomass; V is the acetone volume; A_{474} is the spectrophotometer values; m is the weighed portion of dry yeast biomass; and 2.100 is the extinction coefficient of 1% astaxanthin. The carotenoid percentage was determined using high-performance liquid chromatography (C-18 column). Pure crystalline astaxanthin and β-carotene preparations (Sigma) were used as the standards. All the experiments were staged in five replicates with no less than three experimental cycles. Statistical analysis was carried out using standard methods (Student's *t*-test) with the Microsoft Excel 2000 software package (the probability test $P < 0.05$ was taken to be sufficient for a significant difference between groups of data).

RESULTS AND DISCUSSION

Earlier we showed [14] that the growth intensity of *Phaffia rhodozyma* can be changed to a significant degree by varying the amount of available nitrogen in the medium at an optimal content of the other growth factors, including the carbohydrate component (glucose). In other words, the growth intensity of the yeast studied depends on the carbon to nitrogen ratio (C : N) in the medium. In this work, we demonstrated that a low initial C : N ratio (<5) influences negatively the synthesis of carotenoids in *Ph. rhodozyma*. A more detailed study of strain Y-2409 showed a C : N ratio decrease from 40 to 1.5 to result in a substantial reduction in the sum of carotenoids (Σ_{car}) from 10.1 to

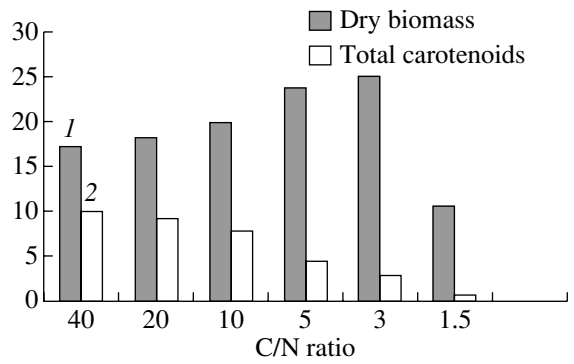

Fig. 1. Influence of the C/N ratio on (*1*) biomass growth (g/l) and (*2*) accumulation of carotenoids (mg/g).

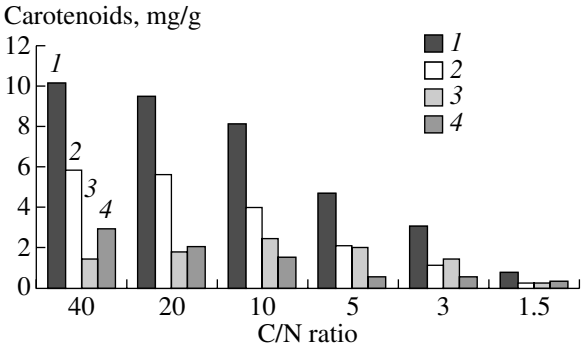

Fig. 2. Dependence of the content of the main carotenoids on the C/N ratio: (*1*) total carotenoids; (*2*) astaxanthin; (*3*) β-carotene; (*4*) foenicoxanthin.

Acetyl CoA → Acetoacetyl CoA → Hydroxymethylglutaryl CoA →
Mevalonate → Mevalonate-PP → Isopentyl-PP → Geranyl-PP →
Farnesyl-PP → Geranylgeranyl-PP → Phytoin → Phytofluin →
ς-Carotene → Neurosporene → Lycopene → γ-Carotene → β-Carotene →
Echinenone → 3-Hydroxyechinenone → Foenicoxanthin → *trans*-Astaxanthin

Fig. 3. Pathways of carotenoid synthesis in the yeast *Ph. rhodozyma*.

0.8 mg per gram of dry biomass. Simultaneously, we observed an active growth of the biomass, attaining the greatest value at the C : N ratio = 3.0–5.0 (Fig. 1). At the lowest C : N ratio, 1.5, a certain biomass decrease was observed, which was likely to be linked to the classical effect of inhibition of culture growth by an excess of nitrogen in the medium [15]. On the other hand, a high C : N ratio accompanied in this case by a decreased amount of available nitrogen in the medium, led to a marked decrease in the growth of the biomass of the *Ph. rhodozyma* culture studied and simultaneously contributed to the absolute increment in the sum of carotenoids (Fig. 1). The study of the content of the main pigmented carotenoids showed that the C/N dependence of AX, whose share in *Ph. rhodozyma* may constitute more than 80% among the other carotenoids, is the strongest [13]. The amount of AX synthesized decreased dramatically with the C : N ratio (Fig. 2). A similar picture was observed for foenicoxanthin (FX), another main carotenoid of this yeast. Absolutely different is the character of the synthesis of β-carotene (Fig. 2), which is the precursor of the carotenoids enumerated above (Fig. 3). The amount of β-carotene does not actually depend on the initial C : N ratio in the medium (Fig. 2) and slightly changes after three days of cultivation (table). On the basis of the results obtained, we suggested that large amounts of nitrogen in the medium, stimulating the active biomass growth, are likely to inhibit directly or indirectly the synthesis of the enzymes mediating the conversion of β-carotene to AX via a series of sequential reactions in the yeast *Ph. rhodozyma* (Fig. 3). In this case, the inhibition of the enzyme synthesis by certain metabolites synthesized in the log phase of culture growth probably occurs. The synthesis of most secondary metabolites is known to be the most intense in the stationary phase after cessation of the growth of the microorganisms producing them [16]. The mechanisms of the control of the synthesis of secondary metabolites are an interesting, but not studied in depth, aspect of the biochemical regulation. The synthesis of carotenoids also assigned to the group of secondary metabolites has its specific features. The synthesis of carotenoids begins not after complete cessation of the producer culture growth but much earlier. Thus, Johnson and Lewis [7] demonstrated that, in a natural *Ph. rhodozyma* strain in the process of cultivation in a fermentor, the active synthesis of carotenoids began at the early stages of the stationary phase (30–40 h) and continued until the end of cultivation (128 h). On the other hand, the most active synthesis of the enzymes involved in the synthesis of secondary metabolites in certain microorganisms begins in the first hours of cultivation [17], which might have occurred in *Ph. rhodozyma*.

We staged a series of experiments with the addition to the nutrient medium of cycloheximide (CHI), which is a powerful inhibitor of the translation occurring in 80S ribosomes when protein is synthesized in eukaryotic cells [12]. Adding CHI at different stages of yeast cultivation, beginning from the first day, we artificially

Dynamics of the carotenoid biosynthesis by *Ph. rhodozyma* Y-2409 and its dependence on the presence of cycloheximide*

Days	Biomass, g/l		Total carotenoids, mg/g		Astaxanthin, mg/g		β-Carotene, mg/g		Foenicoxanthin, mg/g	
1	6.9		0.8		0.45		0.11		0.24	
2	7.3		1.5		0.83		0.19		0.48	
3	11.9		3.1		1.78		0.63		0.69	
+ CHI	C	+ CHI	C	+ CHI	C	+ CHI	C	+ CHI	C	+ CHI
4	15.7	15.0	4.51	4.40	2.74	1.87	0.75	2.13	1.02	0.4
5	17.0	15.3	6.43	6.00	3.96	3.58	0.93	2.38	1.54	0.04
6	17.6	15.5	8.12	7.31	4.92	4.36	0.98	2.93	2.22	0.02
7	17.9	15.7	9.21	8.40	5.54	5.13	1.15	3.25	2.52	0.02

* CHI was added only once after three days of cultivation.

blocked the synthesis of virtually all novel proteins, including carotenogenesis enzymes (Fig. 4). With the experiments staged in such a fashion, the carotenoids were formed by means of enzymes already synthesized, before the addition of CHI. Analyzing the amount of the carotenoids formed, it is possible to assess with rather high probability the contribution of one or another enzyme involved in carotenogenesis to the sequential conversion of these pigments (Fig. 3). It was shown that most of the carotenoids (>80%) are formed in *Ph. rhodozyma* with the involvement of the enzymes synthesized in the cell during the first three days of cultivation. Thus, the addition of CHI to the one- and two-day culture resulted in the complete cessation of its growth with an actual cessation of the synthesis of all carotenoids. The addition of CHI after three days of growth affected but slightly the change in the sum of the accumulated carotenoids compared to the control (table). It follows from the data shown that the enzymes involved in carotenogenesis are synthesized in the greatest amount at the interval between the second and fourth days, which corresponds to the transition from the logarithmic growth phase to the early stationary phase when the yeast *Ph. rhodozyma* is cultivated in both the flasks and the fermentor (Figs. 5, 6). The picture of the synthesis of the main individual pigmented carotenoids such as astaxanthin, foenicoxanthin, and β-carotene revealed on addition of CHI appears to be rather complicated (Fig. 7). All the enzymes involved in the carotenoid conversion at the last stages of synthesis are probably liable to inhibition by the metabolites synthesized in the log phase of the growth of the yeast culture studied. To study the work of the specific enzymes involved in carotenogenesis is a formidable task from the technical point of view since many enzymes are highly labile. It is only possible to interpret with a certain degree of probability what happens to the enzymes if the proportion of sequentially converted carotenoids changes on addition of CHI. Thus, FX is converted to AX with the use of the enzyme β-carotene hydroxylase (Fig. 3), most of which is likely to be produced during the first three days of cultivation (Fig. 7), which corre-

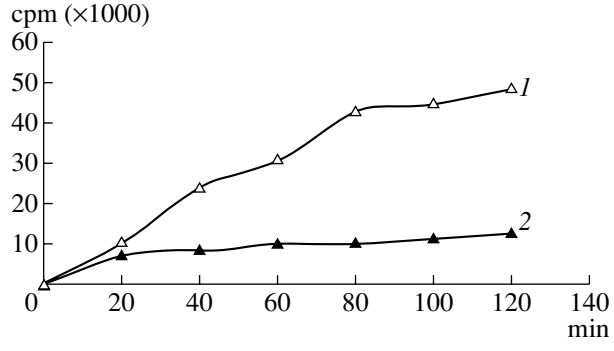

Fig. 4. Incorporation of carbon-labeled leucine into the protein of the yeast *Ph. rhodozyma* Y-2409 (*1*) in the control and (*2*) upon addition of CHI.

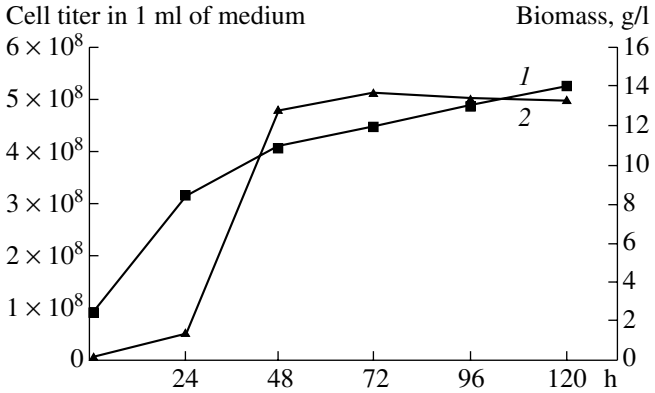

Fig. 5. Dynamics of (*1*) biomass and (*2*) cell titer during the growth of *Ph. rhodozyma* in a flask.

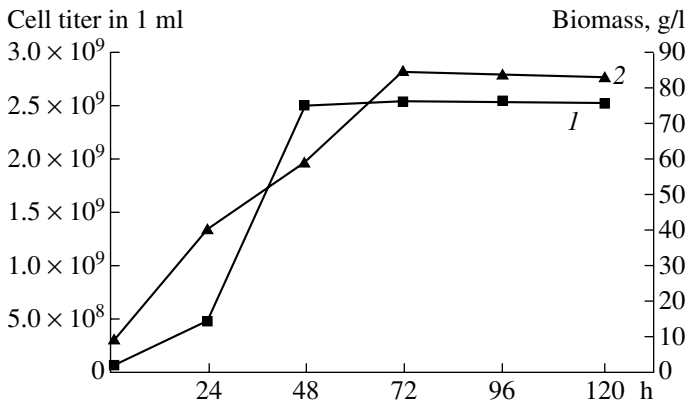

Fig. 6. Dynamics of (*1*) biomass and (*2*) cell titer during the growth of *Ph. rhodozyma* in a fermentor.

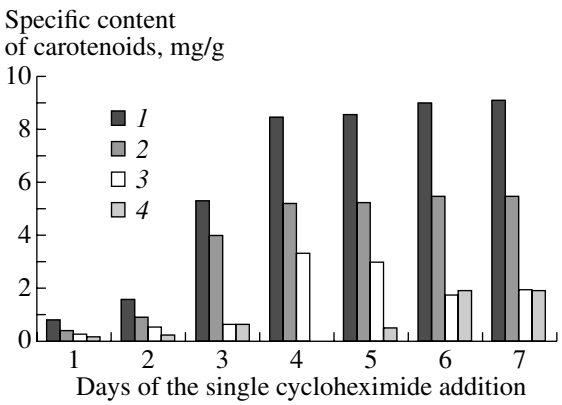

Fig. 7. Effect of cycloheximide on the biosynthesis of the main carotenoids: (*1*) total carotenoids; (*2*) astaxanthin; (*3*) β-carotene; (*4*) foenicoxanthin.

sponds to the biosynthesis of 96% of astaxanthin as compared to the control (table). As for foenicoxanthin, the enzyme (β-carotene ketalase) synthesis at the stage of conversion of β-carotene to FX occurs with high probability at the early stationary stage, which corresponds to three or four days of cultivation (table; Fig. 7). Analyzing the specific features of metabolism in *Ph. rhodozyma*, it may be suggested that acetyl-CoA concentration in the cells is a weak spot. On the one hand, it this compound is the precursor of reserve substances such as lipids, and, on the other hand, it is a compound that is actively used by the cell for synthesizing primary metabolites such as the Krebs cycle organic acids, fatty acids, and amino acids involved in protein synthesis [16]. This metabolism stage proceeds with active energy consumption. During the first day from the beginning of cultivation under conditions conducive to active biomass growth, no excess acetyl-CoA is actually formed in the cell, because it is actively utilized by the cell in anabolic processes. Under conditions that are not optimal for growth, as well as when glucose is in excess, the synthesis of all the enzymes involved in the synthesis of secondary metabolites, including carotenoids, is activated. In [18], a stoichiometric analysis showed that the astaxanthin production increased with a decrease in the NADP level. NAD actively collects reductive equivalents from many NAD-dependent substrates, including NADP. In the period of active growth of the culture in the log phase, most of the energy supplied to the cell is used up for anabolic processes, primarily, for protein synthesis. It is at this stage that the inhibition of the synthesis of the enzymes involved in the transformation of early carotenoids into later carotenoids (one of which is AX) occurs (Fig. 3). A similar suggestion was put forward by Flores-Cotera *et al.* [19]. They concluded that the limitation of the rate of protein synthesis plays a decisive role in the carotenoid synthesis in *Ph. rhodozyma.* The protein synthesis in the growing cells always correlates with biomass growth [20]. Approximately after two days, the intensity of budding in the yeast studied decreased dramatically; a biomass increase was observed for more two days owing to the young buds growing to an adult state. By the example of the yeast strain studied, we showed that a change in the cell culture titer made a decisive contribution to biomass growth (Figs. 5, 6). Analysis of the data obtained led us to the conclusion that the synthesis of the enzymes involved in carotenogenesis and the budding rate reflecting the intensity of anabolic processes are inversely related to each other. When measuring the budding rate more meticulously, we succeeded in showing that the carotenoid synthesis is a function of the intensity of budding (Fig. 8). The budding rate of the producer strain strongly depended on the preset C : N ratio and differed substantially during the first 48 h, virtually ceasing at 70 h after the beginning of cultivation. As seen from the data obtained, an excess of the average budding rate >0.5 during the first 48 h of cultivation is essential for the AX biosynthesis decrease. In this case, the maximum culture growth, accompanied by inhibition of the synthesis of secondary metabolism enzymes, including the synthesis of late carotenoids such as AX, is observed. The maintenance of the budding rate <0.5 during the first two days in the producer strains leads to a considerable increase (twofold) in the synthesis of carotenoids, especially AX (Fig. 8). However, it should be noted that a substantial decrease in the budding rate (<0.2) associated with a high specific synthesis of carotenoids may result in a significant decrease in the biomass level. The high biomass increment in the fermentor during the first two days of growth substantially decreases the synthesis of carotenoids (including AX) and, on the contrary, a low rate of culture growth in the flasks ensures the maximum carotenoid yield (Figs. 5, 6, 9) in *Ph. rhodozyma.* The conclusion that cultivation should be carried out under conditions optimal for the budding rate for each specific strain, which will allow both a high biomass and a high level of carotenoids to be obtained, is evident.

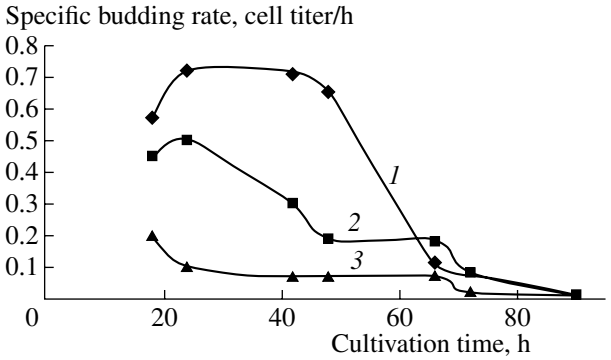

Fig. 8. Relationship between astaxanthin synthesis and the budding rate of *Ph. rhodozyma* developing at different C/N ratios: (*1*) C/N = 5 (AX = 3.4, dry biomass = 24.5); (*2*) C/N = 20 (AX = 8.1, dry biomass = 14.5); (*3*) C/N = 40 (AX = 7.9, dry biomass = 9.4).

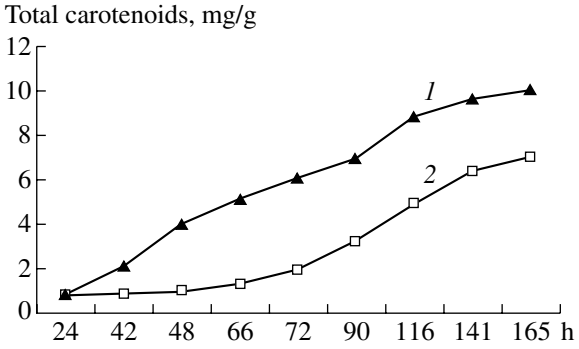

Fig. 9. Dynamics of the accumulation of carotenoids by the yeast *Ph. rhodozyma* Y-2409 during growth in a (*1*) flask and (2) fermentor.

With such a mode of cultivation, conditions favorable for synthesizing the enzymes mediating the conversion of β-carotene to foenicoxanthin and then to astaxanthin are created (Fig. 3). Thus, we succeeded in demonstrating that the synthesis of the enzymes involved in the sequential conversion of β-carotene to AX is subject to regulation by the main compounds involved in the anabolic processes. Acetyl-CoA, as the main building and energy-producing intermediate of primary and secondary metabolites, seems to play a role that is far from being the least important. Apparently, this regulation is effected by the principle of competitive inhibition and begins at the early stages of culture growth during the first 48 h of cultivation. For this reason, if the task of obtaining the maximum yield of AX per fermentor unit volume is set, the process of cultivation during the first two days should be carried out under the $\mu < \mu_{max}$ conditions. The growth parameters to be maintained for the maximum yield of carotenoids to be obtained may be different for each producer strain, but the general approaches based on the specific features of carotenoid synthesis revealed in the yeast *Ph. rhodozyma* will be the same.

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