Microbiology, Vol. 69, No. 2, 2000, pp. 152–156. Translated from Mikrobiologiya, Vol. 69, No. 2, 2000, pp. 191–196.
Original Russian Text Copyright © 2000 by Sharipova, Balaban, Nekhotyaeva, Leshchinskaya.

EXPERIMENTAL ARTICLES

The Influence of Carbon Sources and Mononucleotides on the Production of Extracellular Alkaline Phosphatase by *Bacillus intermedius*

M. R. Sharipova[1], N. P. Balaban, N. V. Nekhotyaeva, and I. B. Leshchinskaya

Kazan State University, ul. Kremlevskaya 18, Kazan, 420008 Tatarstan, Russia

Received January 21, 1999; in final form, April 26, 1999

Abstract—The biosynthesis of extracellular alkaline phosphatase in the streptomycin-resistant strains *Bacillus intermedius* S3-19 and S7 in the presence in the medium of 5'-nucleoside monophosphates and different sources of carbon—glucose, sodium pyruvate, sodium lactate, or glycerol—was studied. It was established that, in the presence of mononucleotides, the content of extracellular alkaline phosphatase in both strains increased; the maximal effect was caused by 5'-AMP at a concentration of 20 μg/ml. In medium with a low orthophosphate content, where active biosynthesis of alkaline phosphatase occurred, 1% glucose and 0.5% pyruvate stimulated this process 2.5–4 times, and 2% sodium lactate and sodium pyruvate, on the contrary, inhibited it by 20–40%. Analysis of the dynamics of growth and accumulation of extracellular phosphatase in the presence of different sources of carbon in the medium gives evidence of an interrelationship between the biosynthesis of alkaline phosphatase and carbon metabolism in *Bacillus intermedius*.

Key words: extracellular alkaline phosphatase of *Bacillus intermedius*, biosynthesis, mononucleotides, sources of carbon

Extracellular inorganic phosphate is known to be a specific regulator of the biosynthesis of alkaline phosphatase: the enzyme synthesis occurs only in its absence. There is evidence in the literature that alkaline phosphatase synthesis may also be depressed during growth in medium with a low orthophosphate content (where the synthesis of the enzyme normally proceeds after phosphate depletion). However, the synthesis of phosphatase under these conditions is regulated by factors other than the inorganic phosphate level. It was established that the synthesis of the alkaline phosphatases of *Bacillus subtilis* and *Bacillus licheniformis* 749/C is stimulated by the addition to the medium of glucose, glycerin, pyruvate, or lactate in low concentrations (0.1–0.3%) and is inhibited by 2% lactate [1, 2]. Lactate does not influence either the overall protein synthesis or the synthesis of other proteins secreted (penicillinases) [2]. Thus, the source of carbon specifically influenced the biosynthesis of alkaline phosphatase. When extracellular phosphatase is inhibited by lactate present in medium, *B. licheniformis* exhibits preferential (in comparison with glucose) incorporation of a lactate molecule into glycogen in the process of gluconeogenesis [2] (the levels of the biosynthesis of glycogen in the lactate- and glucose-grown cells are the same). It was established that the intracellular pool of inorganic and total phosphate (which changed during glucose catabolism) did not influence the biosynthesis of extracellular phosphatase. The authors postulate that gluconeogenic metabolism regulates the biosynthesis of secreted alkaline phosphatase in phosphate-deficient medium. Two membrane-bound alkaline phosphatases from *B. subtilis* SB15 cells grown on media with glucose and lactate were isolated and studied [3]. Both enzymes appeared to be dimers with a subunit molecular mass of 56 kDa and identical in amino acid composition. The protein molecules contained two Zn^{2+} atoms and were activated by Mg^{2+} ions. On the other hand, the enzymes differed substantially in their activity. The phosphatase from glucose-grown cells had a K_m for *p*-nitrophenyl phosphate 60% lower than the phosphatase from lactate-grown cells. On the contrary, the phosphatase synthesized on lactate-containing medium had a 25% higher K_i than the phosphatase synthesized on glucose medium. The transphosphorylase activity of the phosphatases differed by 30–40%. The enzymes differed in the rate of ATP hydrolysis. And, finally, alkaline phosphatase produced by cells grown on glucose was not inactivated when treated with antibodies against the alkaline phosphatase synthesized by cells grown on lactate. The authors believe that not only does lactate metabolism regulate the biosynthesis of alkaline phosphatase, but it can also cause its functional changes by subtly affecting the conformation of the protein molecule.

[1]Corresponding author (e-mail: margarita.sharipova@ksu.ru).

0026-2617/00/6902-0152$25.00 © 2000 MAIK "Nauka/Interperiodica"

The aim of the present investigation was to study the biosynthesis of extracellular alkaline phosphatase in two streptomycin-resistant strains of *B. intermedius*, S3-19 and S7, on media with various sources of carbon and in the presence of mononucleotides.

MATERIALS AND METHODS

The subjects of study were *B. intermedius* strains S7 (Str 500) and S3-19 (Str 500), which are streptomycin-resistant mutants of the wild strain *B. intermedius* 7P from the culture collection of the Department of Microbiology, Kazan State University.

The bacteria were grown on medium of the following composition (%): peptone, 2; $CaCl_2 \cdot 2H_2O$, 0.01, $MgSO_4 \cdot 7H_2O$, 0.03; NaCl, 0.3; $MnSO_4$, 0.01. A sterile solution of 0.1 mM $CoCl_2 \cdot 6H_2O$ was added to the medium before inoculation. When preparing the medium, we used phosphorus-depleted peptone (Semipalatinsk) containing 0.8 μg/g of inorganic phosphate [9]. Before sterilization, the pH of the medium was adjusted to 8.5 with a NaOH solution. The medium was sterilized at 1 atm. Cultivation was carried out in Erlenmeyer flasks, with a medium to flask volume ratio of 1 : 7, in a thermostated shaker (B. Braun, Germany) at 200 rpm and 30°C. A 24-h inoculum (1 vol %) grown on medium with streptomycin sulfate was used. The antibiotic was introduced to a concentration of 500 μg/ml before inoculation.

The mononucleotides 5'-AMP, 5'-GMP, 5'-UMP, and 5'-CMP (Reanal, Hungary), as well as glucose, sodium pyruvate, glycerin, and sodium lactate, were introduced into nutrient medium in the form of sterile solutions before inoculation.

Bacterial growth was monitored by measuring the culture optical density on a KFK-2 photoelectric colorimeter at 590 nm.

The culture productivity with respect to phosphatase was determined as the ratio of the phosphatase activity in the culture fluid to the amount of biomass and expressed in conventional units.

The specific growth rate(μ) and the specific enzyme accumulation rate (ε) in the culture fluid were calculated as described earlier [4].

The phosphomonoesterase activity of phosphatase was determined from its effect on the model substrate *p*-nitrophenyl phosphate (*p*-NPP) (Serva, Germany) according to the method described earlier [5].

The enzyme activity was expressed in micromoles of substrate hydrolyzed by 1 ml of enzyme solution in 1 min (μmol/min), using for conversion an A_{410}–*p*-NPP concentration curve.

The results were processed statistically according to conventional methods [6].

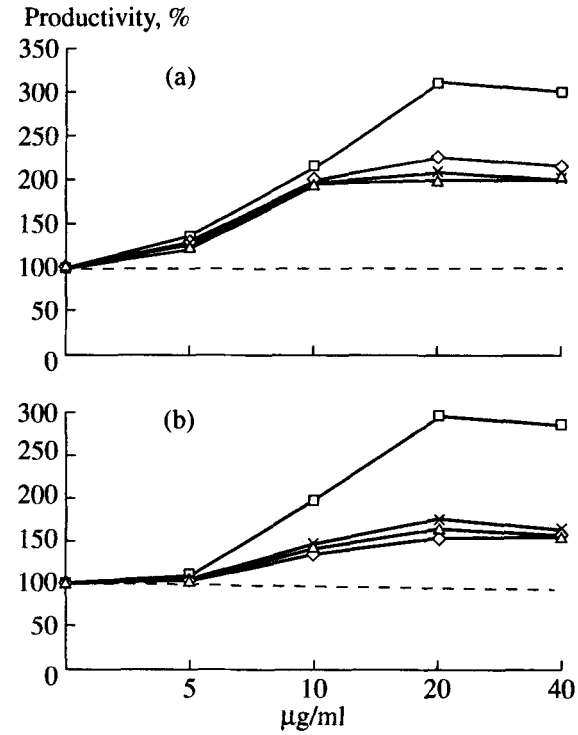

Fig. 1. Influence of various concentrations of 5'-nucleoside monophosphates (μg/ml) on the productivity of extracellular alkaline phosphatase synthesis by *B. intermedius* (a) S3-19 and (b) S7: (□) 5'-AMP, (◇) 5'-UMP, (×) 5'-CMP, (△) 5'-GMP. (– – –) is control medium without the addition of 5'-nucleoside monophosphates (100%).

RESULTS AND DISCUSSION

The effect of different concentrations of 5'-nucleoside monophosphates (from 5 to 40 μg/ml) on the activity of extracellular alkaline phosphatase was studied in two streptomycin-resistant strains—*B. intermedius* S3-19 and S7—differing in the level of extracellular enzyme activity [7]. The results are shown in Fig. 1. It is established that, upon the addition of mononucleotides to the medium at a concentration of 5 μg/ml, the activity of extracellular phosphatase increased in both strains. The maximal effect was attained at 20 μg/ml for all 5'-nucleoside monophosphates, the activity level of extracellular enzyme being higher in *B. intermedius* S3-19 than in *B. intermedius* S7. With the use of 5'-AMP, the specific activity of extracellular alkaline phosphatase of *B. intermedius* S3-19 and *B. intermedius* S7 increased threefold. 5'-GMP, 5'-CMP, and 5'-UMP exerted a stimulating effect to a lesser degree than 5'-AMP and caused the enzyme specific activity to increase, on average, 1.5–2 times in both strains. An increase in the concentration of 5'-nucleoside monophosphates to 40 μg/ml did not enhance the effect.

The dynamics of bacterial growth and of the level of the extracellular alkaline phosphatase activity in *B. intermedius* S3-119 and S7 were studied in the presence of 5'-nucleoside monophosphates added to the medium at an optimal concentration (Fig. 2). In both

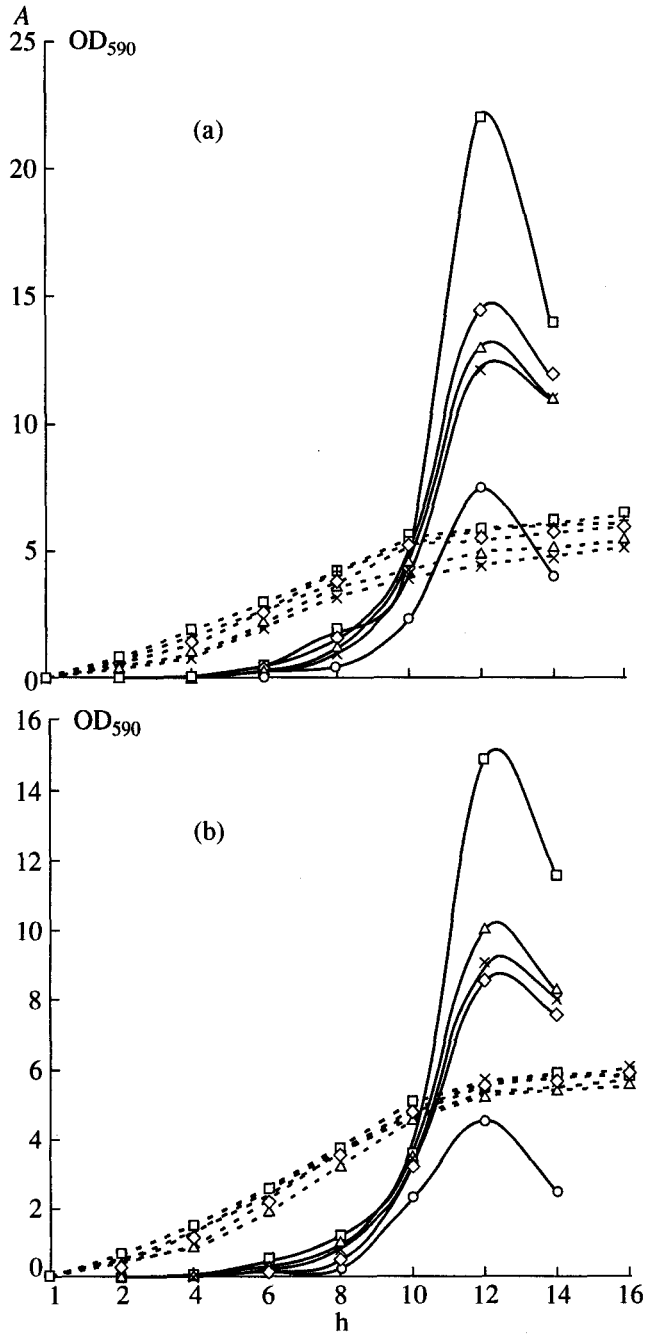

Fig. 2. Dynamics of growth and extracellular alkaline phosphatase activity of *B. intermedius* (a) S3-19 and (b) S7 on media containing (□) 5'-AMP, (◇) 5'-UMP, (△) 5'-CMP, and (×) 5'-GMP. o is control medium without the addition of 5'-nucleoside monophosphates. (—) Phosphatase activity, *A*, μmol/(ml min); (– – –) biomass, OD_{590}.

strains, the mononucleotides cause the greatest increase in the alkaline phosphatase activity in the phase of growth retardation (after 12 h of growth); the effect was maximum in the presence of 5'-AMP, which caused a three- to five-fold increase in the phosphatase activity, whereas 5'-GMP, 5'-CMP, and 5'-UMP increased the activity, on average, by a factor of two.

Thus, the maximal level of extracellular alkaline phosphatase activity in the two *B. intermedius* strains was observed upon the addition of 5'-AMP at a concentration of 20 μg/ml. When studying the catalytic characteristics of this enzyme purified to the homogeneous state, we showed that, being a nonspecific phosphomonoesterase, the enzyme preferentially hydrolyzed 5'-AMP; it could also hydrolyze mono-, di-, and trinucleotides and phosphorylated hexoses [8].

It is known from the literature available that a changeover of the carbon source in nutrient medium results in a change in the level of alkaline phosphatase activity in *B. subtilis* and *B. licheniformis* [1, 2]. We studied the activity of extracellular alkaline phosphatase in the two *B. intermedius* strains, S3-19 and S7, under conditions of active enzyme synthesis and secretion (peptone with a low orthophosphate content); glucose, sodium pyruvate, sodium lactate, or glycerin were added to the medium as the sources of carbon at different concentrations in different experiments.

It was shown that, among media with different glucose contents (from 0.1 to 6%), the maximal specific activity of extracellular alkaline phosphatase was observed when the glucose concentration was 1% (Figs. 3a and 3b). An increase in the glucose concentration to 4–6% decreased the enzyme specific activity. When glucose was replaced with sodium pyruvate, whose content varied from 0.1 to 2%, the maximal specific enzyme activity in the culture fluid was observed at a pyruvate concentration of 0.5% (Figs. 3a and 3b). When glycerol was used as the source of carbon (at a concentration of 0.1 to 2%), the maximal specific phosphatase activity was observed when the glycerol content was 0.5% (Fig, 3a); a further increase in its concentration decreased the specific activity of the enzyme. When sodium lactate at a concentration of 0.5 to 4% was added to the medium, the maximal specific phosphatase activity was observed at a concentration of 0.5% for both strains; an increase in the lactate concentration resulted in a decrease in the enzyme specific activity (Figs. 3a and 3b).

We also compared the activities of the extracellular phosphatase of *B. intermedius* S3-19 grown on glucose, sodium pyruvate, sodium lactate, or glycerol at concentrations causing the maximal stimulation of the extracellular enzyme and the maximal decrease in its activity (Fig. 4). The control medium contained peptone and no additional carbon sources. The maximal phosphatase activity in the culture fluid was observed in medium with 0.5% pyruvate. The enzyme activity level under these conditions exceeded that of phosphatase in the control medium 4 times; that of the enzyme in medium with glucose, 1.6 times; and that of the enzyme in media with lactate and glycerol, 2.5–3 times; the maximal phosphatase activity in medium with sodium pyruvate was recorded earlier (10–11 h of growth) than in media with glucose and glycerol (12 h of growth). Lactate and pyruvate at a concentration of 2% caused

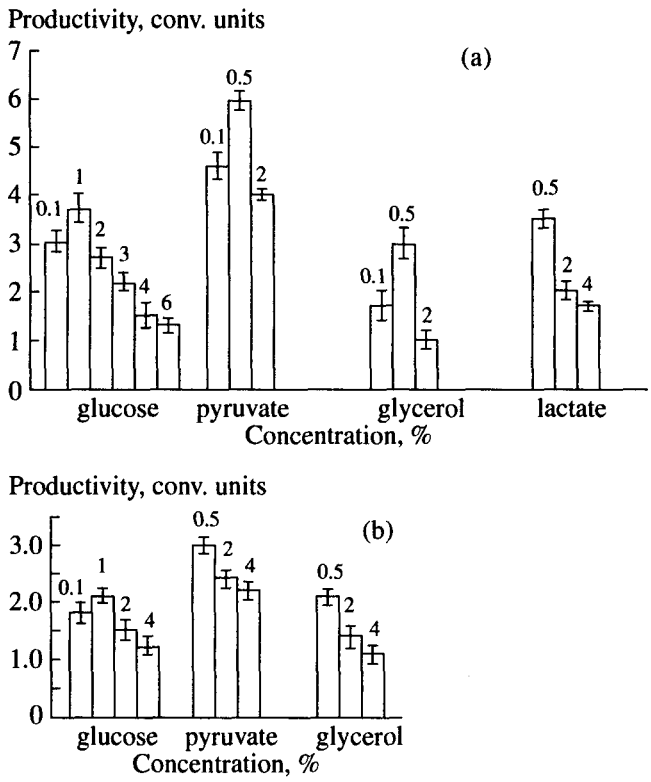

Fig. 3. Influence of various concentrations of carbon sources on the productivity of extracellular alkaline phosphatase synthesis by *B. intermedius* (a) S3-19 and (b) S7. Figures above the columns show the concentrations (%) of the carbon sources.

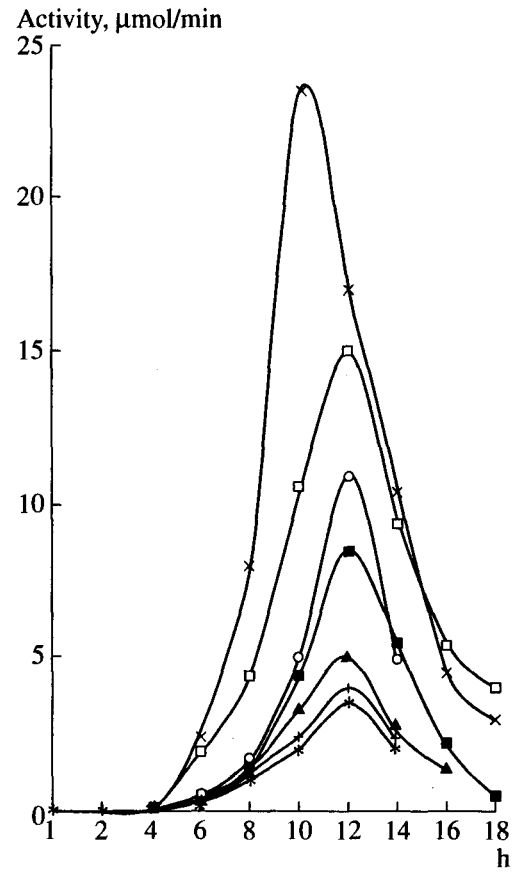

Fig. 4. Production of extracellular alkaline phosphatase by *B. intermedius* S3-19 grown in media with various carbon sources: (×) 0.5% sodium pyruvate, (□) 1% glucose, (○) 0.5% sodium lactate; (■) 0.5% glycerol, (+) 2% sodium lactate; (*) 2% sodium pyruvate (▲) control medium without the addition of carbon-containing components.

the phosphatase activity to decrease by 20–40% in comparison with the control medium (Fig. 4).

Thus, it was established for both strains that sodium pyruvate at a concentration of 0.5% caused the maximal increase in the extracellular phosphatase activity, and sodium lactate and sodium pyruvate at a concentration of 2% caused a decrease in the activity of this enzyme.

Dynamics of growth and production of extracellular enzyme were studied in *B. intermedius* S3-19 cells grown under conditions of maximum stimulation of the extracellular phosphatase activity, i.e., on media with 1% glucose and 0.5% sodium pyruvate, as well as under conditions determining decreased enzyme activity, i.e., on medium with 2% sodium lactate (Fig. 5). The curves of the specific growth rate of the bacteria, specific rate of enzyme accumulation, and specific phosphatase activity are given as well. As seen from Fig. 5, the specific growth rate and enzyme activity curves do not coincide, either on medium with sodium pyruvate or on medium with glucose. The maximal specific rate of enzyme accumulation was observed in the phase of growth retardation, when enzymatic systems became involved in catabolic processes. The maximal value of the specific rate of enzyme accumulation was observed in the 11th h of growth on sodium pyruvate medium and in the 12th h of growth on glucose medium. Figure 5 shows that, in medium with sodium pyruvate, the formation of extracellular alkaline phosphatase in the phase of active growth is characterized by shifts of the curves of the specific rate of enzyme accumulation and of the enzyme specific activity relative to the corresponding curves recorded for medium with glucose.

In the presence of 2% sodium lactate, causing a decrease in the extracellular phosphatase content, a shift in the maximum of the culture specific growth rate occurred (it was observed later, in the 6th h of growth) and prolongation of the lag phase was observed; the maximum enzyme accumulation in the medium was also observed later, in the 13th h of culture growth, nearer to the beginning of the stationary phase.

The data obtained in this work demonstrate that the level of extracellular alkaline phosphatase activity changes depending on the nature of the carbon sources in the medium. Both the formation rate and the amount of extracellular alkaline phosphatase synthesized vary depending on the source of carbon. In the usual low-

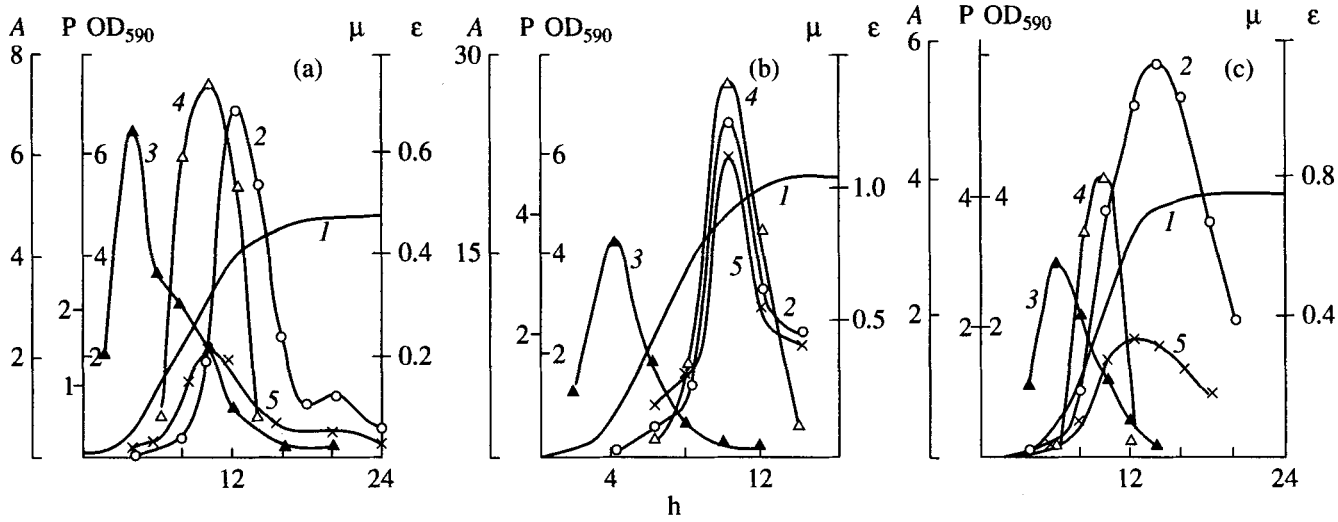

Fig. 5. Dynamics of growth and of the level of extracellular alkaline phosphatase of *B. intermedius* S3-19 in media with (a) 1% glucose, (b) 0.5% sodium pyruvate, and (c) 2% sodium lactate. (*1*) biomass, OD_{590}; (*2*) extracellular alkaline phosphatase activity, *A*, μmol/(ml min); (*3*) specific growth rate, μ; (*4*) the enzyme specific accumulation rate, ε; (*5*) phosphatase specific activity, conv. units.

phosphate medium, glucose and sodium pyruvate at low concentrations (up to 0.5%) increased and, at 2%, decreased the level of extracellular alkaline phosphatase activity in comparison with the control medium. Hydrean *et al.* believe that the mechanism regulating the biosynthesis of alkaline phosphatase may be linked to carbon metabolism [2].

Thus, the results of this work, as well as the data obtained earlier by the other authors [1, 2], suggest the existence of several regulatory mechanisms of the biosynthesis of extracellular alkaline phosphatase; one of these mechanisms is related to carbohydrate metabolism.

REFERENCES

1. Ghosh, A. and Ghosh, B.K., Alkaline Phosphatase Derepression in Vegetative Cells of *Bacillus subtilis* by Glucose and Its Reversal by Lactate, *Biochem. Biophys. Res. Commun.*, 1972, vol. 46, no. 1, pp. 296–304.
2. Hydrean, C., Ghosh, A., Nallin, M., and Ghosh, B.K., Interrelationship of Carbohydrate Metabolism and Alkaline Phosphatase Synthesis in *Bacillus licheniformis* 749/C, *J. Biol. Chem.*, 1977, vol. 252, no. 9, pp. 6806–6812.
3. Ghosh, R., Ghosh, A., and Ghosh, B.K., Properties of the Membrane-bound Alkaline Phosphatase from Glucose- and Lactate-grown Cells of *Bacillus subtilis, J. Biol. Chem.*, 1977, vol. 252, no. 19, pp. 6813–6822.
4. Pirt, S.J., *Principles of Microbe and Cell Cultivation*, Oxford: Blackwell, 1975. Translated under the title *Osnovy kul'tivirovaniya mikroorganizmov i kletok*, Moscow: Mir, 1978.
5. Leshchinskaya, I.B., Balaban, N.P., Kapranova, M.N., and Golubenko, I.A., *Sovremennye metody izucheniya nukleinovykh kislot i nukleaz mikroorganizmov* (Modern Methods for Studying Microbial Nucleic Acids and Nucleases), Kazan: Kazan Gos. Univ., 1980.
6. Plokhinskii, N.A., *Matematicheskie metody v biologii* (Mathematical Methods in Biology), Moscow: Mosk. Gos. Univ., 1978.
7. Sharipova, M.R., Balaban, N.P., and Leshchinskaya, I.B., Production of Extracellular Alkaline Phosphatase by Antibiotic-Resistant Strains of *Bacillus intermedius, Mikrobiologiya*, 1994, vol. 63, no. 1, pp. 52–58.
8. Sharipova, M.R., Balaban, N.P., Nekhotyaeva, N.V., Mardanova, A.M., Dementiev, A.A., and Leshchinskaya, I.B., A Novel *Bacillus intermedius* Extracellular Alkaline Phosphatase: Isolation, Physico-Chemical, and Catalytic Characteristics, *Biochem. Mol. Biol. Int.*, 1996, vol. 38, no. 4, pp. 753–761.
9. Znamenskaya, L.V., Romakhina, E.R., Filatova, S.V., Balaban, N.P., and Leshchinskaya, I.B., Biosynthesis of Extracellular Enzymes by *Bacillus intermedius, Mikrobiologiya*, 1986, vol. 55, no. 3, pp. 449–454.