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EXPERIMENTAL ARTICLES

Conditions of the Biosynthesis of an Extracellular Subtilisin-Like Proteinase by *Bacillus pumilus* KMM 62

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Abstract—The influence of the cultivation conditions on *Bacillus pumilus* KMM 62 growth and effectiveness of the production of a subtilisin-like serine proteinase were investigated. Enzyme accumulation in the culture fluid reached the maximum value after 32 and 46–48 h of growth; it depends on the composition of the nutrient medium. The ratio of the concentrations of two main components of the medium, peptone and inorganic phosphate, which was optimal for enzyme biosynthesis was determined by multifactor experiments. Ammonium salts, when introduced as an additional nitrogen source, had different effects on the proteinase biosynthesis at different growth stages: they suppress enzyme production at the early stationary growth phase and stimulate the biosynthesis of the enzyme after 46–48 h of growth. Complex organic substrates (albumin, casein, hemoglobin, and gelatin) have a repressive effect on the biosynthesis of the enzyme. The effect of amino acids on culture growth and enzyme biosynthesis during the early and late stationary growth phase is different. Hydrophilic amino acids, glutamine, and glutamic acid exhibit the most pronounced repressive action on biosynthesis. The involvement of different regulatory mechanisms of the synthesis of this proteinase is assumed in the early and late stationary phases of growth.

Key words: Bacillus pumilus, subtilisin-like proteinase, biosynthesis, conditions of growth and biosynthesis.

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Bacilli produce diverse extracellular enzymes; the numerous group of serine proteinases (subtilases, or subtilisin-like proteinases) is one of the best studied [1]. The genes of many bacillary subtilases have been cloned and sequenced, and the three-dimensional structures have been determined for some proteins [1–3]. However, the question of the functional role of extracellular subtilisin-like proteinases and the mechanisms of the regulation of their biosynthesis still remains open.

The culture *Bacillus pumilus* KMM 62 is the producer of an extracellular serine proteinase. The gene of *B. pumilus* serine subtilisin-like proteinase has been cloned and its sequence has been determined [4]. However, published data concerning the patterns of the biosynthesis of this enzyme and the effect of exogenous factors on the expression of the gene of this protein are still scarce.

Global changes in cellular physiology and metabolism are known to occur on transition to the stationary phase, in particular, those caused by the changes in the apparatus of transcription. Thus, expression of a large number of genes occurs, which ensure resistance to unfavorable conditions [5]. Upon transition of the cells to the stationary phase, the synthesis of different secondary metabolites, particularly hydrolytic enzymes, is activated [6]. The synthesis of extracellular proteinases by bacilli during the stationary growth phase is apparently among the multitude of reactions of adaptation to unfavorable conditions.

Earlier, it has been shown that bacteria *B. intermedius* excrete serine proteinases into the medium with the maximum activity after 24 and 44–46 h growth [7, 8]. With the aid of recombinant strains, the patterns governing the biosynthesis of the subtilisin-like enzyme have been investigated; the differences in its synthesis in the early and late stationary growth phase have been revealed [9]. It was interesting to investigate the patterns governing the biosynthesis of *B. pumilus* KMM 62 subtilisin-like proteinase during different growth phases of the culture. The selection of the optimum cultivation conditions for the maximum yield of *B. pumilus* subtilisin-like proteinase is the initial stage of the work on the elucidation of the mechanisms of biosynthesis and on the subsequent preparative production of this enzyme.

The goal of the present work was to study the patterns of growth, spore formation, and biosynthesis of

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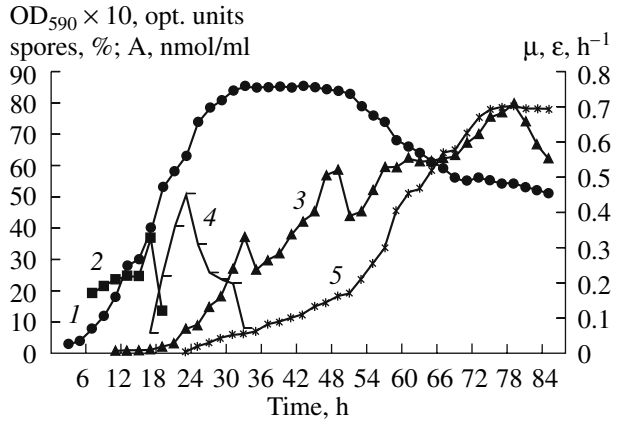

Fig. 1. Dynamics of culture growth (*1*), specific growth rate μ (*2*), proteolytic activity (*3*), specific rate of proteinase accumulation ε (*4*), and spore formation (*5*) by *B. pumilus* KMM 62.

subtilisin-like proteinase in *B. pumilus* KMM 62, as well as the effect of various exogenous factors on the level of enzyme accumulation in the culture liquid at different growth stages of the bacterial culture.

MATERIALS AND METHODS

Strain *Bacillus pumilus* KMM 62, isolated from seawater, was used in the work (from the collection of the Department of Microbiology, Kazan State University).

The initial nutrient medium for the cultivation contained (g/l): peptone, 20; $CaCl_2 \cdot 2H_2O$, 0.1; $MgSO_4 \cdot 7H_2O$, 0.3; NaCl, 3.0; $MnSO_4$, 0.1; $Na_2HPO_4 \cdot 12H_2O$, 0.3; pH 8.5 [7]. The medium was sterilized at 1 atm. The final concentration of peptone in the multifactor experiments was 15–40 g/l. The solutions of inorganic phosphate ($Na_2HPO_4 \cdot 12H_2O$), ammonium citrate ($C_5H_7O_5COONH_4$), and ammonium chloride (NH_4Cl) were sterilized separately at 1 atm and added into the medium before inoculation; the final concentration of inorganic phosphate was 0.1–0.4 g/l, and of ammonium salts, 1–5 mM.

The solutions of Hammersten casein (Serva), albumin, gelatin, and hemoglobin (Sigma) were sterilized at 0.5 atm and introduced into the medium at final concentrations of 0.1, 0.5, and 1.0%. Solutions of amino acids (leucine, tryptophan, asparagine, glutamine, and glutamic acid) were prepared and sterilized separately. Amino acids were introduced into the medium at the final concentration of 0.1, 0.5, and 1.0%.

The ratio between the volume of flask and the volume of the medium was 1 : 5. An aliquot (1 vol %) of 12–15 h culture was used as inoculum. The cultivation was performed at 37°C on a vibrostand (200 rpm). The biomass increase was determined nephelometrically using a FEK-56M colorimeter (λ = 590 nm). The quantity of biomass was expressed in units of light absorption in the cuvette of 1 cm thickness.

A synchronous culture was used to study the spore formation. In order to obtain it, a 50-h inoculum was subjected to short-term heating at 80°C for 30 s (5–6 times). To enumerate free spores, the preparation was stained according to Peshkov [10]. The number of free spores was expressed as the percentage with respect to the total number of vegetative and sporulating cells (100%); the cells were counted at 1600× magnification under a phase contrast microscope (Carl Zeiss, Jena, Germany) in 5–10 microscope fields.

The activity of subtilisin-like proteinase was determined by hydrolysis of the chromogenic substrate Z-Ala-Ala-Leu-*p*NA according to the method described previously [11]. The amount of the enzyme which hydrolyzed 1.0 nmol of the substrate in 1 min under experimental conditions was used as the unit of activity. The productivity of the culture with respect to the synthesis of subtilisin-like proteinase was defined as the ratio of the proteolytic activity of the culture fluid to the amount of biomass; it was expressed in arbitrary units or as percentages of the productivity of culture on the control medium. The specific growth rate $\mu = \frac{d}{dt}\ln D$ and the specific rate of increase of proteinase activity $\varepsilon = \frac{d}{dt}\ln A$ were calculated.

The mathematical processing of the data obtained was performed using the Microsoft Excel software package by calculating the standard deviation (σ). The results were considered reliable when the standard deviation σ did not exceed 15%. The results of multifactor experiments were processed with the aid of the BIOPT software package [12].

RESULTS AND DISCUSSION

We identified a proteinase in *B. pumilus* culture fluid, which hydrolyzed the synthetic substrate Z-Ala-Ala-Leu-*p*Na, specific for subtilisins. The dynamics of growth and accumulation of the enzyme in the course of cultivation was investigated (Fig. 1). The maximum speed of growth (0.28 h^{-1}) was observed during the period from the 14th to the 16th hour of cultivation. Trace amounts of the enzyme were detected in the culture fluid after 20 h. Afterwards, the level of its activity increased, reaching two maximums, after 32 and 46–48 h of cultivation (Fig. 1). The highest rate of accumulation of the subtilisin-like proteinase (ε) was observed after 20–24 h of growth. These results are close to the previously published data concerning the biosynthesis of a subtilisin-like proteinase by *B. intermedius*. In the process of cultivation of these bacteria, two maximums of accumulation of the specific enzymatic activity in the culture fluid were also revealed; they corresponded to 24–26 and 44–46 h of growth [7, 8]. However, in the case of *B. intermedius*, the first maximum of activity accumulation occurred earlier (24–26 h) compared to *B. pumilus*. Thus, the first maximum of accumulation of

the proteolytic activity in *B. pumilus* (32 h) coincided with the beginning of the stationary phase of growth, while the intensive synthesis of the enzyme occurred when the growth decreased; this is characteristic of other species of bacilli as well.

The second maximum of accumulation of the extracellular proteinase occurred during the late stationary growth phase. The question arises about the nature of accumulation of this protein in the culture fluid and the correlation of this process with spore formation. The results of the study of the dynamics of spore formation (Fig. 1) demonstrated that only 5% of the spores were detected 32 h after inoculation; after 46–48 h, the number of free spores was still low and did not exceed 15%. Such a substantial increase in the extracellular proteolytic activity (the activity of the second peak approximately two times higher than that of the first one) cannot be explained by cell lysis alone. These data indicate that the subtilisin-like proteinase is an excreted enzyme, which is not accumulated in the medium as a result of lysis. However, the increase of the extracellular activity in the phase of cell extinction (68–78 h) apparently coincides with the beginning of the massive cell lysis and the release of endospores. It has been previously demonstrated in the study of the biosynthesis of a subtilisin-like proteinase by *B. intermedius* with the aid of the β-galactosidase reporter protein that the level of this enzyme remained constantly low up to 44 h of growth. Afterwards, the activity of both β-galactosidase and of *B. intermedius* subtilisin-like proteinase increased [8]. The participation of this proteinase in splitting of the proteins of the envelope of the mother cell was assumed, which contributes to the spore release into the medium [9].

Since the synthesis of extracellular enzymes depends on the medium composition to a considerable degree, we investigated the effect of the ratio of two main components of the nutrient medium, peptone and inorganic phosphate, on the biosynthesis of the subtilisin-like proteinase. The optimum concentrations of peptone and inorganic phosphate (P_i) for the synthesis of *B. pumilus* subtilisin-like proteinase during the early and late stationary phases were determined in two-factor experiments. The concentrations of the factors indicated varied at three levels. The diagram of the experiment and the concentration of the components of the medium, as well as the values of biomass (optical density), proteolytic activity, and productivity (averages of three repeats) are presented in Tables 1 and 2.

The results of two-factor experiments are most clearly represented in Fig. 2 in the form of the lines of activity level, where the zone can be seen, which is optimal for the two factors in question. Processing of the results on the productivity of the culture with respect to proteinase synthesis revealed optima analogous to those obtained for activity. Thus, it was shown that active production of the subtilisin-like proteinase after 32 h of cultivation occurred when the initial peptone concentration in the medium was 35 g/l and that of inorganic phosphate was 0.35 g/l. Lower concentrations of peptone (26 g/l) and slightly lower concentrations of inorganic phosphate (0.32 g/l) are required for active production of the subtilisin-like proteinase after 48 h of growth. Thus, the optimal peptone concentrations for the synthesis of subtilisin-like proteinases in

Table 1. Optimization of the nutrient medium composition for the biosynthesis of *B. pumilus* subtilisin-like proteinase, secreted after 32 h of growth

Levels of the factors				Biomass, optical units	Activity, nmol/ml	Productivity, arbitrary units
Peptone		P_i				
X1	g/l	X2	g/l			
+	40	+	0.4	15.6	380.57	23.95
–	20	+	0.4	5.6	52.86	9.44
+	40	–	0.2	16.4	290.7	17.7
–	20	–	0.2	7.6	84.6	11.1
+	40	0	0.3	14	327.7	23.41
–	20	0	0.3	6	68.43	10.57
0	30	+	0.4	16.8	359.4	21.4
0	30	–	0.2	14.4	338.3	23.5
Initial medium				8.6	37.1	4.31

Table 2. Optimization of the nutrient medium composition for the biosynthesis of *B. pumilus* subtilisin-like proteinase, secreted after 48 h of growth

Levels of the factors				Biomass, optical units	Activity, nmol/ml	Productivity, arbitrary units
Peptone		P_i				
X1	g/l	X2	g/l			
+	35	+	0.35	18.4	338	18.4
–	15	+	0.35	3.2	42.2	13.2
+	35	–	0.15	21.2	465.2	22
–	15	–	0.15	6	105.8	17.6
+	35	0	0.25	18	422.8	23.4
–	15	0	0.25	4.4	80.2	18.4
0	25	+	0.35	5.6	232.4	41.4
0	25	–	0.15	17.6	380.6	21.6
Initial medium				8.4	58.5	6.96

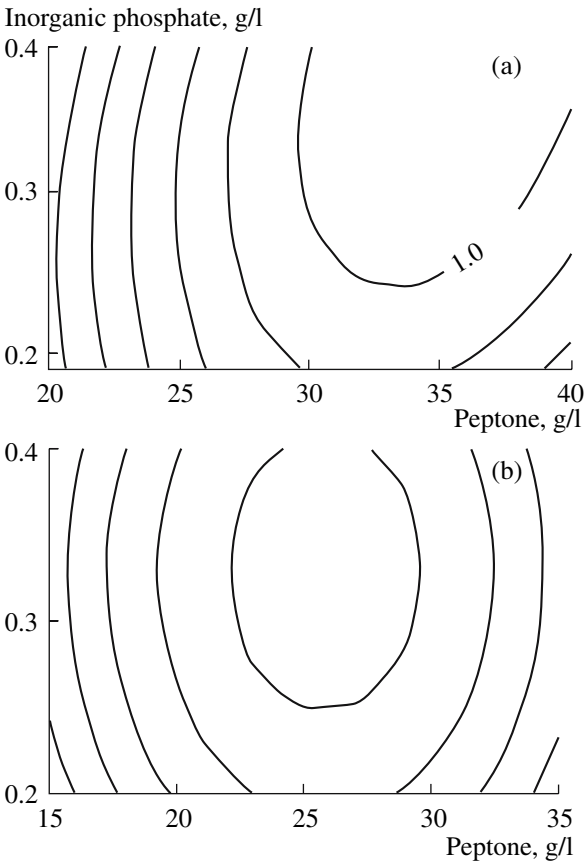

Fig 2. Lines of the activity level of subtilisin-like proteinase in two-factor experiments for *B. pumilus* after 32 (a) and 48 (b) h of growth.

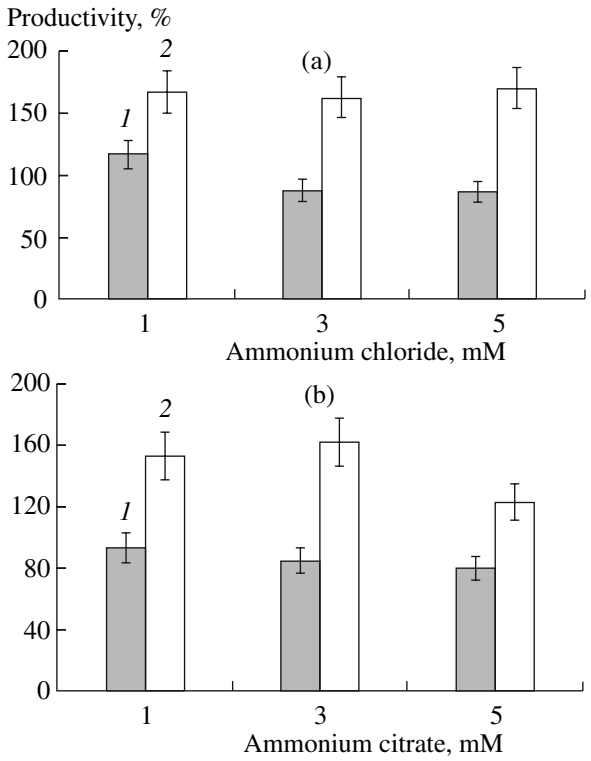

Fig. 3. Influence of different concentrations of ammonium chloride (a) and ammonium citrate (b) on the production of *B. pumilus* subtilisin-like proteinase after 32 (*1*) and 48 (*2*) h of cultivation. The productivity of the culture on the medium with no ammonium salts added was 100%.

the early and late stationary growth phases are different. The obtained results showed that active synthesis of the enzyme by *B. pumilus* cells required sufficiently high levels of the main nutrient sources in the medium. For example, the optimal peptone concentrations for *B. intermedius* were 20 and 22 g/l [7, 8].

The addition of nitrogen compounds in combination with peptone is known to have a substantial effect on enzyme synthesis and secretion [13]. The influence of ammonium ions on the synthesis of *B. pumilus* subtilisin-like proteinase was therefore investigated. Ammonium ions introduced into the medium in the form of organic ($C_5H_7O_5COONH_4$) or inorganic salts (NH_4Cl) had different effects on proteinase biosynthesis at different phases of growth (Fig. 3). Ammonium chloride (1 mM) somewhat increased the productivity of the culture after 32 h of growth. However, ammonium chloride (3 and 5 mM) and ammonium citrate at all the concentrations tested decreased the enzyme production in the early stationary growth phase by 10–20%, but stimulated by 30–70% the synthesis of subtilisin-like proteinase in the late stationary phase.

It is interesting that the addition of ammonium ions to the nutrient medium stimulated the secretion of all exoenzymes in *Aspergillus oryzae*. Repression of the synthesis and secretion of an extracellular protease in the presence of mineral sources of nitrogen has been reported for *Asp. nidulans* [14]. The repressive effect of sulfate of ammonium and other ammonium salts on the secretion of the acid proteinase by *Candida albicans* [15] and on the proteolytic activity of the micromycete *Alternaria alternata* [16] has been revealed.

Since proteolytic enzymes are degradative in nature, they must be induced by the substrate. Thus, some extracellular proteinases of the microscopic fungus *Paecelomyces variotii* were secreted only in the presence of protein substrates in the medium [17]. Addition of bovine serum albumin to the medium resulted in a 1000-fold increase in the activity of *Candida albicans*. Addition of some other substrates (hemoglobin, ovalbumin, histones) also resulted in increased activity [15].

We investigated the influence of complex protein substrates introduced as additional sources of nitrogen (albumin, casein, hemoglobin, and gelatin) on the production of *B. pumilus* subtilisin-like proteinase (Fig. 4). The introduction of albumin into the medium repressed the productivity of the culture with respect to the synthesis of proteinase both in the early and in the late stationary growth phase. Addition of casein (0.1–1.0%)

led to a 30–50% increase in biomass but inhibited the synthesis of proteinases by 30–90%. A similar pattern was revealed upon the addition of gelatin to the medium; its inhibitory action on proteinase biosynthesis was found to be less expressed in comparison with casein. Only hemoglobin (0.5%) caused an increase (by 70%) in the production of the subtilisin-like proteinase after 32 h of growth; a 0.1% concentration of hemoglobin increased the enzyme production after 48 h of growth by 20–25%.

Similar results on the effect of casein and gelatin were obtained in the study of the biosynthesis of serine proteinases by two marine bacteria, *Bacillus firmus* 44b and *B. oligonitrophilus* 21p [18]. It has been shown that the introduction 1% casein and gelatin into the growth medium significantly decreased the synthesis of the investigated proteinases by *B. firmus* 44b and to a lesser degree by *B. oligonitrophilus* 21p. It has previously been shown that hemoglobin has no substantial effect on the biosynthesis of serine proteinase by *B. inermedius* grown on the medium with peptone, while casein and gelatin increased the enzyme production twofold [8]. Thus, complex protein substrates have different effects on the biosynthesis of serine proteinases.

The significant decrease in the productivity of the synthesis of *B. pumilus* subtilisin-like proteinase caused by protein substrates may be the result of repression by the end products of nitrogen metabolism. The creation of reserves of amino acids for different biosynthetic purposes may be one of the possible functions of extracellular proteolytic enzymes. Repression by the end products (amino acids accumulating as a result of the hydrolysis of protein substrates) may therefore be one of the most efficient control mechanisms for the synthesis of these enzymes.

It is known that individual amino acids introduced into the medium as additional sources of nitrogen have different effects on the synthesis of the proteolytic enzymes [19]. Induction of the synthesis of proteinases occurs in some cases and repression in others. We investigated the influence of individual amino acids belonging to different groups on the biosynthesis of *B. pumilus* subtilisin-like proteinase (Fig. 5). Nonpolar hydrophobic (leucine, tryptophan) and polar hydrophilic (asparagine, glutamine, glutamic acid) amino acids were introduced into the medium.

The investigated amino acids had different effects on the culture growth and enzyme production in the early and late stationary growth phases. The most efficient growth suppression was achieved with glutamine (by 40–60%) and glutamate (by 70%). Asparagine, tryptophan, and leucine had practically no effect on biomass accumulation after 32 and 48 h of growth. The accumulation of proteolytic activity at the different stages of growth strongly depended on the type and concentration of amino acids. Hydrophobic amino acids leucine and tryptophan had different effects on the accumulation of proteinase during the early and late

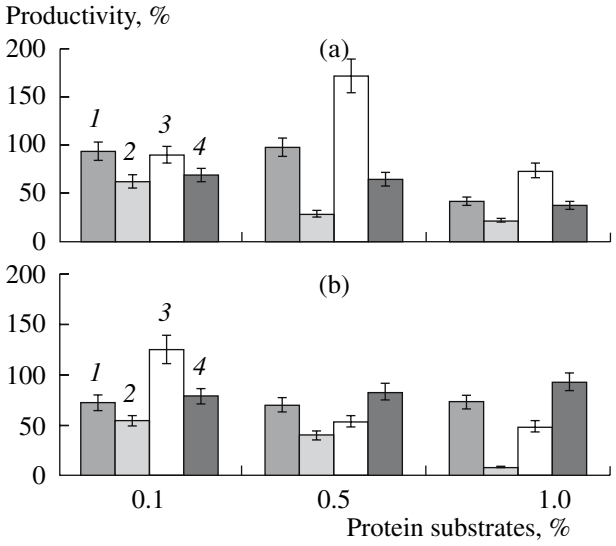

Fig. 4. Effect of different concentrations of albumin (*1*), casein (*2*), hemoglobin (*3*), and gelatin (*4*) on the production of *B. pumilus* subtilisin-like proteinase after 32 h (a) and 48 h (b) of growth. The productivity of the culture on the medium with no protein substrates added, 100%.

stationary growth phases. In the presence of 0.1% leucine, the level of proteinase biosynthesis after 32 h of growth decreased; it increased, however, after 48 h. The introduction of tryptophan into the medium led to a decrease in the production of the subtilisin-like proteinase after 32 h and an increase after 48 h of growth. Asparagine (0.1–0.5%) had no effect or insignificantly increased the productivity of proteinase by the culture. However, in the presence of 1% asparagine, the biosynthesis of the enzyme after 32 h of growth was inhibited by 45%. Glutamic acid (0.1–0.5%) practically did not influence the accumulation of the subtilisin-like proteinase. An increase in the concentration of this amino acid to 1% decreased the level of proteinase biosynthesis after 32 h of growth by 40%, and after 48 h by 70%.

Thus, hydrophilic amino acids, glutamine and glutamic acid, had a more pronounced effect on the biosynthesis of *B. pumilus* subtilisin-like proteinase; this is apparently the result of the regulation of the enzyme synthesis according to the mechanism of repression by end product. However, from the data obtained it is not possible to unambiguously conclude that the repressive effect is connected with the hydrophilic or hydrophobic nature of amino acids.

It is known that diverse amino acids and their combinations differently influence the synthesis of proteinases in different species of bacilli. For example, it has been shown that the mixture of isoleucine and threonine caused the maximum repression of the exoproteinase synthesis by *B. megaterium* KM. In the case of *B. cereus*, such repression occurs in the presence of threonine and histidine or of a mixture of nine amino acids, which also suppresses sporulation. It has been

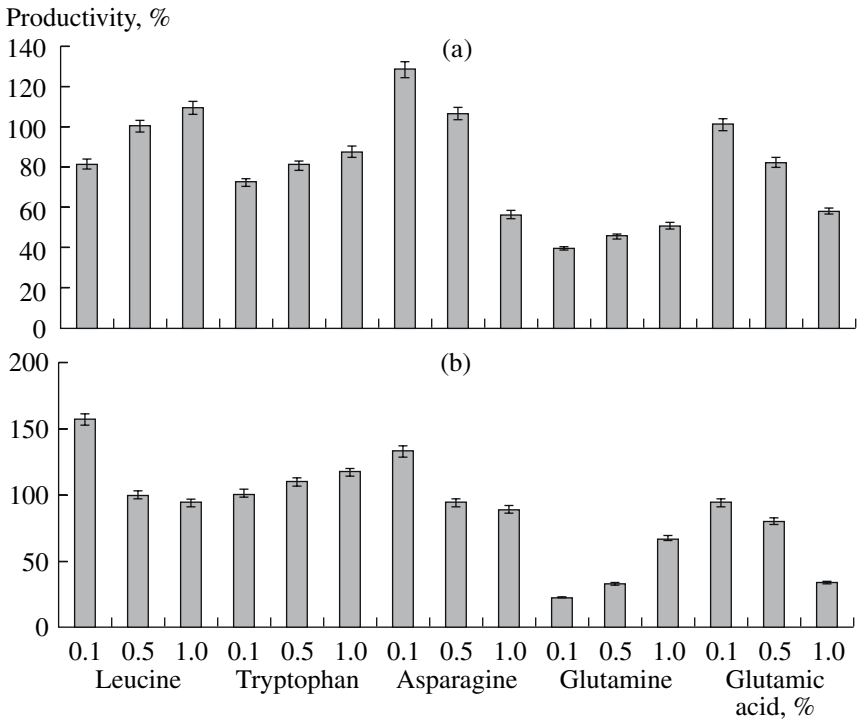

Fig. 5. Effect of amino acids on the production of *B. pumilus* subtilisin-like proteinase after 32 (a) and 48 (b) h of growth. The productivity of the culture on the medium with no amino acids added was 100%.

shown that the amino acid mixture containing arginine, histidine, methionine, and lysine stimulates proteinase synthesis in *B. subtilis* A-50 [20].

The present investigation has demonstrated that the *B. pumilus* extracellular subtilisin-like proteinase is one of the enzymes of the second phase of growth, the synthesis of which occurs during the phase of decreased growth rate and the transition of the culture to the stationary growth phase. Two maximums of the protease activity were revealed in the culture fluid, corresponding to the early and late stationary phases. The character of the enzyme biosynthesis is analogous to that of *B. intermedius* 3-19; it is therefore possible to assume a general regularity in the biosynthesis of subtilisin-like proteinases in different *Bacillus* species. Active proteinase synthesis correlates with the spore formation, which is also characteristic of a number of other bacilli [8, 9, 13]. Formation of the proteinases secreted at different stages of growth depends on the composition of the nutrient medium; sufficiently high concentrations of peptone and inorganic phosphate are required. Ammonium ions were demonstrated to affect differently the biosynthesis of *B. pumilus* subtilisin-like proteinase at different growth stages. Repressive action of a number of individual amino acids, and also of protein substrates was revealed; this finding makes it possible to assume that the mechanism of repression of enzyme synthesis by the end product is involved in the regulation of the synthesis of extracellular serine proteinases. The addition of proteins and amino acids to peptone-containing nutrient media was shown to be inexpedient for the purpose of an increase in productivity. On the other hand, the productivity of the synthesis of the subtilisin-like proteinase increased after 48 h of growth in the presence of ammonium salts in the concentration range 1 to 5 mM. The differences in the influence of exogenous factors on the effectiveness of enzyme production during the early and the late stationary growth phase revealed in this work may indicate changes in the expression of the gene of subtilisin-like proteinase upon transition of the cells to the stationary growth phase.

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