Microbiology, Vol. 69, No. 5, 2000, pp. 546–551. Translated from Mikrobiologiya, Vol. 69, No. 5, 2000, pp. 653–659.

EXPERIMENTAL ARTICLES

Optimized Medium for the Efficient Production of *Bacillus intermedius* Glutamyl Endopeptidase by the Recombinant *Bacillus subtilis* Strain AJ73

L. A. Gabdrakhmanova*, E. V. Shakirov*, N. P. Balaban*, M. R. Sharipova*, G. N. Rudenskaya, S. V. Kostrov***, T. V. Akimkina***, and I. B. Leshchinskaya***

**Kazan State University, ul. Kremlevskaya 18, Kazan, 420008 Russia*
***Moscow State University, Vorob'evy gory, Moscow, 119899 Russia*
****Institute of Molecular Genetics, Russian Academy of Sciences, pl. Akademika Kurchatova 46, 0-182, Moscow, 123182 Russia*

Received December 3, 1999

Abstract—A nutrient medium was elaborated for the efficient production of glutamyl endopeptidase by the recombinant *Bacillus subtilis* strain AJ73 bearing the *Bacillus intermedius* 3-19 glutamyl endopeptidase gene within a multicopy plasmid. Optimal concentrations of the main nutrients, peptone and inorganic phosphate, were found using a multifactor approach. To provide for active growth and efficient glutamyl endopeptidase production, the cultivation medium of the recombinant strain should be enriched in phosphorus, organic and inorganic nitrogen sources, and yeast extract. Complex protein substrates, such as casein and gelatin, enhanced the biosynthesis of glutamyl endopeptidase. At the same time, easily metabolizable carbon sources suppressed it. The production of glutamyl endopeptidase was stimulated by the bivalent cations Ca^{2+}, Mg^{2+}, and Co^{2+}.

Key words: glutamyl endopeptidase, Glu,Asp-specific proteinase, biosynthesis, recombinant strain, growth conditions

Microbial proteolytic enzymes with a narrow substrate specificity, which hydrolyze only peptide bonds formed by certain amino acids, attract the attention of researchers as possible tools for studying the primary structure of proteins and peptides and as model objects for the investigation of the evolution of microbial enzymatic systems.

Glutamyl endopeptidases (Glu,Asp-specific proteinases), whose substrate specificity is determined by the presence of the negatively charged side chains of glutamic and aspartic acids in the substrate molecule, refer to serine protease. Glutamyl endopeptidases were first found in staphylococci (1972) [1] and then in streptomycetes and bacilli [2]. The glutamyl endopeptidases known to date are secretory proteins with a molecular mass of 18–29 kDa and widely varying isoelectric point. The physicochemical and catalytic properties of glutamyl endopeptidases have been studied in great detail [2].

Analysis of the nucleotide sequences of the respective cloned genes allowed the amino acid sequences of glutamyl endopeptidases from *Staphylococcus aureus*, *Bacillus licheniformis*, *B. subtilis*, *B. intermedius*, *Streptomyces griseus*, and *Str. fradiae* to be established [3]. Based on these data, the evolutionary tree of glutamyl endopeptidases from different sources was constructed. The tree shows that glutamyl endopeptidases fall into three distinct groups, one of which includes the glutamyl endopeptidases of sporogenous bacteria [3].

The enzyme from the streptomycin-resistant *B. intermedius* strain 3-19 is the most studied glutamyl endopeptidase, for which physicochemical and catalytic properties, biosynthetic pathways, and cellular location have been described [4, 5]. At the same time, little is known about the regulation of the biosynthesis of these proteinases.

Genetic engineering provides an efficient approach to relevant investigations. After the complete gene of *B. intermedius* 3-19 glutamyl endopeptidase had been cloned in *B. subtilis* AJ73 within a multicopy plasmid [6], it was necessary to elaborate a cultivation medium providing for the efficient production of endopeptidase by the recombinant strain.

This work was aimed at studying the effect of the main ingredients of the cultivation medium on the synthesis of *B. intermedius* glutamyl endopeptidase in the recombinant *B. subtilis* strain.

MATERIALS AND METHODS

Bacillus subtilis strain AJ73 with a chromosome from which the genes of its own extracellular proteinases were deleted was donated by Y. Yomantas from the

0026-2617/00/6905-0546$25.00

State Scientific Research Institute of Genetics and Selection of Industrial Microorganisms, Moscow, Russia.

Competent *B. subtilis* cells were transformed with plasmid DNA as described in the handbook [7]. The multicopy plasmid pV was constructed on the basis of vector pCB22 carrying the complete gene of *B. intermedius* glutamyl endopeptidase [6].

B. subtilis was cultivated in 100-ml flasks with 20 ml of medium on a shaker (200 rpm) at 30°C. The medium was supplemented with 20 μg/ml erythromycin, since plasmid pV bears the erythromycin resistance gene. Material for inoculation was an 18-h culture in an amount of 1 vol %. Basal medium for the glutamyl endopeptidase production by the recombinant *B. subtilis* strain (this medium is usually used for the cultivation of *B. intermedius*) contained (%) peptone, 2; $CaCl_2 \cdot 2H_2O$, 0.01; $MgSO_4 \cdot 7H_2O$, 0.03; NaCl, 0.3; and $MnSO_4$, 0.01 (pH 8.5). The medium was sterilized at 1 atm. Supplementary ingredients were sterilized separately and added to the medium immediately before inoculation. The solutions of inorganic phosphate (Na_2HPO_4), NH_4Cl, and salts of bivalent metals were sterilized at 1 atm, and those of carbohydrates, casein, and gelatin were sterilized at 0.5 atm. Hemoglobin solution was sterilized by boiling it for 15 min. Yeast extract was purchased from Difco Laboratories; hemoglobin and gelatin, from Sigma; casein Hammarsten, from Serva; and peptone, from a Vinnitsa meat-processing plant.

Bacterial biomass was evaluated nephelometrically.

Proteolytic activity was measured as described earlier [4] with *N*-carbobenzoxy-L-glutamic acid *p*-nitroanilide (*N*-CBZ-Glu *p*-NA). One unit of proteolytic activity was defined as the amount of enzyme that hydrolyzed 1 nmol of substrate per min.

The efficiency of glutamyl endopeptidase production was defined as the ratio of the proteolytic activity of the culture liquid to the biomass and was expressed in arbitrary units. The specific growth rate was calculated by the formula $\mu = d(\ln D)/dt$. The specific rate of the accumulation of glutamyl endopeptidase was calculated by the formula $\varepsilon = d(\ln A)/dt$.

Data were statistically processed as described in the handbook [8]. The results of multifactor experiments were processed using the BIOPT software package [9].

RESULTS AND DISCUSSION

Recombinant strains are commonly cultivated in nutritionally rich media, since plasmid-bearing cells spend some amounts of nutrients for the replication of heterologous plasmids and the expression of foreign genes [10]. The presence of recombinant plasmids in cells may change the level of certain cellular metabolites and thus cause global perturbations in cellular metabolism [11]. This may increase the requirements of cells for nutrients and oxygen, which should be taken into account when optimizing the production of foreign proteins by recombinant bacterial strains.

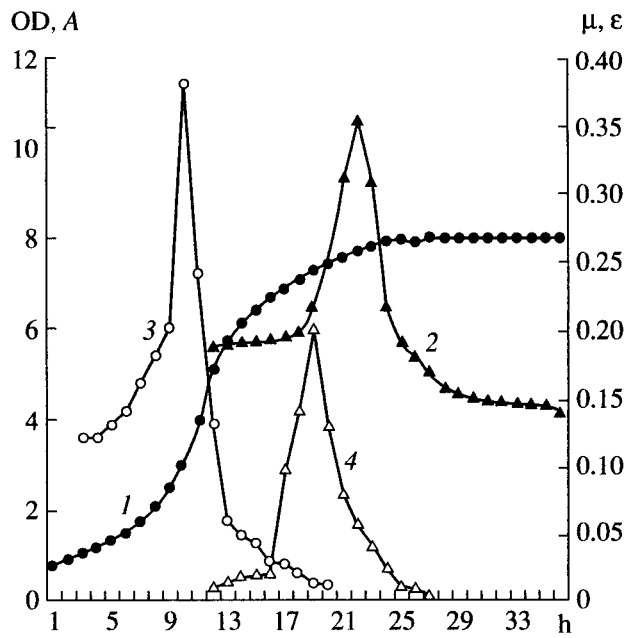

Fig. 1. Dynamics of culture growth and the biosynthesis of glutamyl endopeptidase by the recombinant *B. subtilis* strain AJ73: (*1*) OD_{590}, units; (*2*) glutamyl endopeptidase activity (*A*), E/ml; (*3*) specific growth rate (μ), h^{-1}; (*4*) specific rate of enzyme accumulation (ε), h^{-1}.

In the first set of experiments, we investigated the dynamics of culture growth and the accumulation of glutamyl endopeptidase in the culture liquid of the recombinant *B. subtilis* strain.

As is evident from the results presented in Fig. 1, glutamyl endopeptidase activity appeared in the culture liquid of *B. subtilis* AJ73 in the growth retardation phase and reached a maximum in the late stationary phase. The specific rate of enzyme accumulation increased after the specific growth rate of culture had reached its maximum. It should be noted that all stages of enzyme production by the recombinant *B. subtilis* strain were shifted to later growth stages as compared with the parent *B. intermedius* strain [5]. Furthermore, the recombinant strain showed poor growth and inefficient enzyme production in the medium that was developed for *B. intermedius*.

In the next set of experiments, attempts were made to optimize this medium in order to attain an efficient production of glutamyl endopeptidase by the recombinant strain. Taking into account that recombinant strains typically require increased concentrations of nitrogen and phosphorus sources, we investigated the effect of different concentrations of peptone and inorganic phosphate in the cultivation medium on the efficiency of production of recombinant glutamyl endopeptidase. Experiments were designed according to the double-factor scheme B2. In experiment 1, each of the two variables (concentrations of peptone and inorganic

Table 1. Optimization of nutrient medium for the biosynthesis of glutamyl endopeptidase by *B. subtilis* AJ73 in experiment 1 designed according to the double-factor scheme B2

Factor value				*Bacillus subtilis* pV		
Peptone		Inorganic phosphate		Biomass, OD units	Endopeptidase, U/(ml min)	Productivity
*X*1	g/l	*X*2	g/l			
+	30	+	0.3	5.3	6.8	1.3
−	10	+	0.3	0.8	8.0	7.9
+	30	−	0.1	7.0	6.2	0.9
−	10	−	0.1	1.0	3.0	3.0
+	30	0	0.2	6.6	6.5	1.0
−	10	0	0.2	1.3	5.9	4.4
0	20	+	0.3	2.3	7.5	3.3
0	20	−	0.1	4.6	6.0	1.3

Table 2. Optimization of nutrient medium for the biosynthesis of glutamyl endopeptidase by *B. subtilis* AJ73 in experiment 2 designed according to the double-factor scheme B2

Factor value				*Bacillus subtilis* pV		
Peptone		Inorganic phosphate		Biomass, OD units	Endopeptidase, U/(ml min)	Productivity
*X*1	g/l	*X*2	g/l			
+	20	+	0.4	7.6	7.9	1.0
−	10	+	0.4	4.4	7.9	1.8
+	20	−	0.2	8.8	7.9	1.0
−	10	−	0.2	6.8	4.9	0.7
+	20	0	0.3	10.9	9.9	0.9
−	10	0	0.3	6.8	6.9	1.0
0	15	+	0.4	10.0	10.9	1.1
0	15	−	0.2	10.4	5.6	0.5

phosphate) took three values (Table 1). Data on the biomass (estimated as culture turbidity), glutamyl endopeptidase activity, and productivity of enzyme synthesis (Table 1) are the means of triplicate experiments. The regression equation for glutamyl endopeptidase activity derived from the data presented in Table 1 has the form (its confidence level is 95%):

$$Y = 6.9 + 12X2 - 0.7X1^2 - 0.2X2^2 - X1 \cdot X2.$$

As is evident from this equation, optimum peptone concentration for the biosynthesis of glutamyl

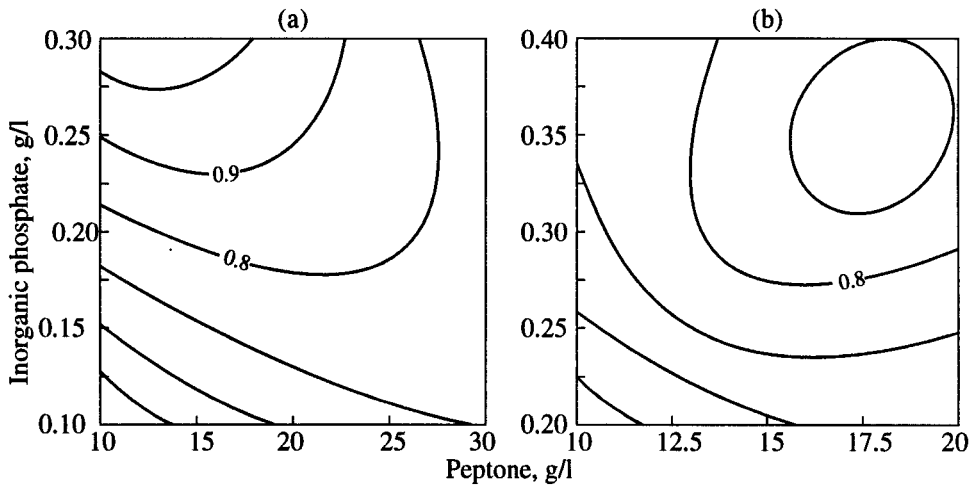

Fig. 2. Isolines of glutamyl endopeptidase activity in the double-factor experiments with the recombinant *B. subtilis* strain AJ73: (a) experiment 1 and (b) experiment 2. The maximum activity of glutamyl endopeptidase in particular experiments was taken as 1.

endopeptidase ($X1$) is within the range of the peptone concentrations used in experiment 1, whereas the optimum concentration of inorganic phosphate ($X2$) turned out to be lower than the minimum concentration of inorganic phosphate used in this experiment (Fig. 2a).

Taking into account these results, we increased the concentrations of inorganic phosphate used in experiment 2 (Table 2). The regression equation derived from the data presented in this table is as follows (its confidence level is also 95%):

$$Y = X1 + 1.4X2 - 1.1X1^2 - 1.2X2^2.$$

The results of the double-factor experiment 2 are presented in Fig. 2b as the isolines of glutamyl endopeptidase activity. The theoretical maximum of glutamyl endopeptidase activity with $Y = 10.9$ E/ml is attained at the point with coordinates $X1 = 17.5$ g/l (peptone) and $X2 = 0.36$ g/l (inorganic phosphate). The maximum productivity of enzyme synthesis coincided with the maximum of enzyme activity.

Thus, the recombinant strain efficiently synthesized glutamyl endopeptidase in the medium with 17.5 g/l peptone and 0.36 g/l inorganic phosphate. This concentration of inorganic phosphate is considerably higher than that necessary for the efficient production of glutamyl endopeptidase by the parent strain *B. intermedius* 3-19. This confirms the assumption that recombinant (plasmid-bearing) bacterial strains have increased requirements for nutrients.

The addition of yeast extract at a concentration of 0.5% to the growth medium enhanced the culture growth and enzyme activity accumulated in the medium about twofold (consequently, the productivity of enzyme synthesis by *B. subtilis* AJ73 did not change). Raising the concentration of yeast extract up to 3% not only failed to increase the productivity of enzyme synthesis but even caused it to slightly decrease. In view of this, all subsequent experiments were carried out using media with 0.5% peptone.

The degree of aeration did not significantly affect the productivity of enzyme synthesis.

Taking into account our earlier observations that glutamyl endopeptidase is efficiently synthesized by the parent *B. intermedius* strain in a medium containing both organic (peptone) and inorganic (NH_4Cl) nitrogen sources [5], we investigated the effect of ammonium ions on the production of glutamyl endopeptidase by the recombinant *B. subtilis* strain. As can be seen from Fig. 3, the addition of ammonium ions to the medium at a concentration of 2 mM stimulated the glutamyl endopeptidase production by 20%.

According to data available in the literature (see, for instance, [12]), the synthesis of extracellular enzymes may be enhanced by the addition of their substrates to the medium. Earlier, we found that casein, gelatin, and hemoglobin failed to stimulate the biosynthesis of glutamyl endopeptidase by *B. intermedius* 3-19 [5]. Similarly, hemoglobin at concentrations of 0.5–2% did not promote the biosynthesis of glutamyl endopeptidase by *B. subtilis* AJ73, although casein and gelatin at these concentrations exerted a considerable stimulating effect. As can be seen from Fig. 4, 0.5% casein in the medium enhanced the synthesis of glutamyl endopeptidase by the recombinant *B. subtilis* strain by 60%. Still greater stimulating effect (by 100%) was observed in

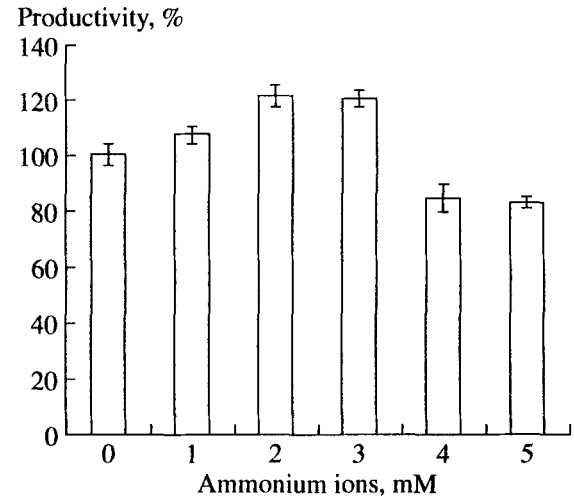

Fig. 3. Effect of ammonium ions on the glutamyl endopeptidase production by the recombinant *B. subtilis* strain AJ73. The productivity of enzyme synthesis in the ammonium-free cultivation medium was taken as 100%.

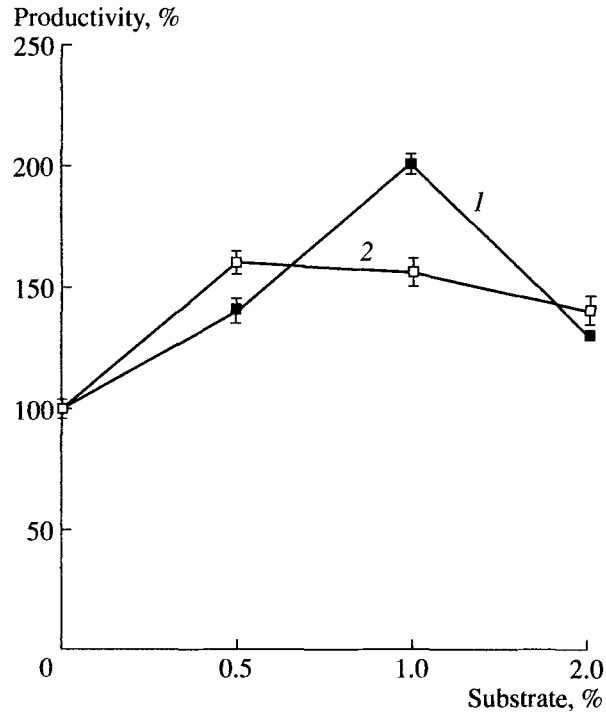

Fig. 4. Effect of casein and gelatin on the glutamyl endopeptidase production by the recombinant *B. subtilis* strain AJ73: (*1*) gelatin and (*2*) casein. The productivity of enzyme synthesis in the medium lacking enzyme substrates was taken as 100%.

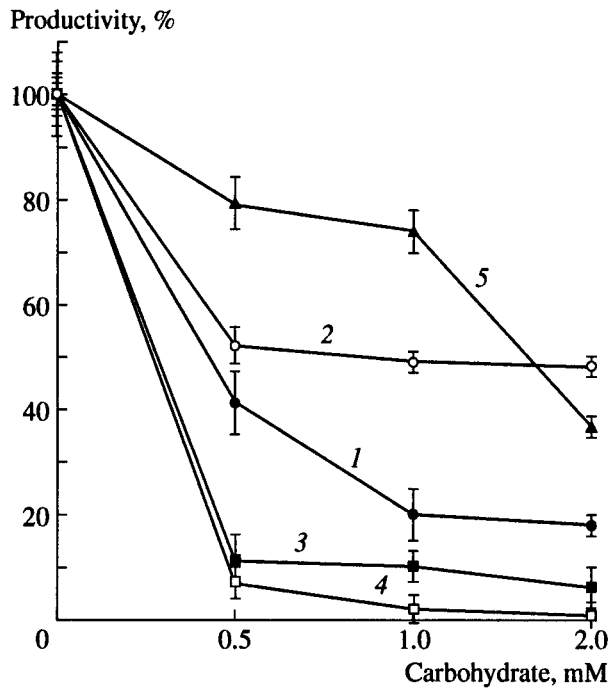

Fig. 5. Effect of carbohydrates on the glutamyl endopeptidase production by the recombinant *B. subtilis* strain AJ73: (*1*) maltose, (*2*) galactose, (*3*) glucose, (*4*) sucrose, and (*5*) lactose. The productivity of enzyme synthesis in the medium lacking carbohydrates was taken as 100%.

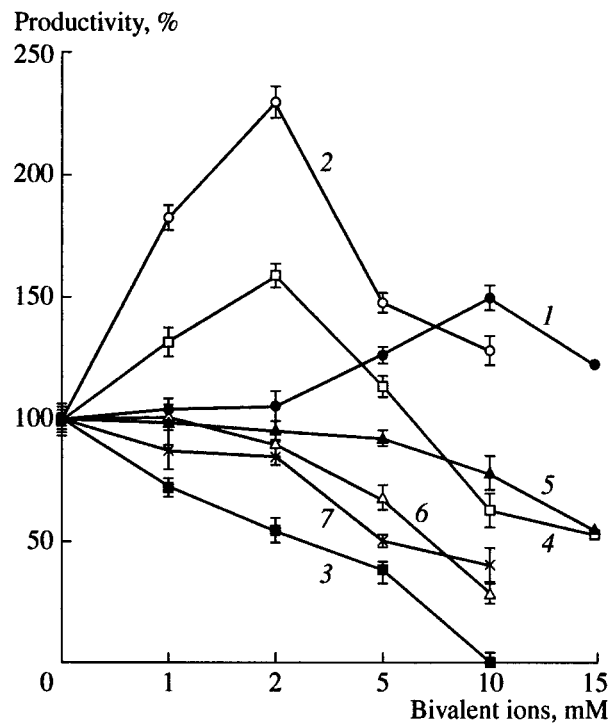

Fig. 6. Effect of bivalent cations on the glutamyl endopeptidase production by the recombinant *B. subtilis* strain AJ73: (*1*) Ca^{2+}, (*2*) Co^{2+}, (*3*) Zn^{2+}, (*4*) Mg^{2+}, (*5*) Mn^{2+}, (*6*) Fe^{2+}, and (*7*) Cu^{2+}. The productivity of enzyme synthesis in the medium lacking bivalent ions was taken as 100%.

the presence of 1% gelatin in the medium (Fig. 4, curve *1*). In this case, culture growth did not change. Based on these results, 1% gelatin was included into the formulation of the cultivation medium optimal for the production of glutamyl endopeptidase by *B. subtilis* AJ73.

Our data and those of other authors [5, 12] show that the synthesis of serine proteases, including *B. intermedius* glutamyl endopeptidase, is suppressed by glucose and other carbohydrates. However, the requirements of recombinant producers of proteases for carbohydrates may be different. In view of this, we investigated the effect of monosaccharides (glucose and galactose) and disaccharides (sucrose, maltose, and lactose) on the growth of the recombinant *B. subtilis* strain and production of glutamyl endopeptidase. As can be seen from Fig. 5, carbohydrates stimulated the growth of *B. subtilis* AJ73 but suppressed the synthesis of glutamyl endopeptidase by this strain. The strongest inhibitory effect on the enzyme production (90%) was observed in the presence of 0.5% sucrose in the medium. The suppressing effect of glucose, galactose, and maltose was less pronounced, and that of lactose was the least: 1% lactose present in the medium decreased the productivity of enzyme synthesis by only 25%.

Generally, alkaline proteinases do not require bivalent cations for their activity and stability, although glutamyl endopeptidases are known to be Ca^{2+}-dependent enzymes [2, 4]. Furthermore, the glutamyl endopeptidase of *B. intermedius* 3-19 was shown to be favorably influenced not only by Ca^{2+} but also by Mg^{2+} and Co^{2+} ions [5].

The data presented in Fig. 6 show that the bivalent ions Zn^{2+}, Mn^{2+}, Fe^{2+}, and Cu^{2+} at concentrations of 1–10 mM inhibited the synthesis of glutamyl endopeptidase by *B. subtilis* AJ73. The strongest inhibitory effect was exerted by Zn^{2+} ions; at a concentration of 10 mM, they completely suppressed glutamyl endopeptidase activity. Conversely, Ca^{2+} and Mg^{2+} cations exerted a favorable effect; 10 mM Ca^{2+} and 2 mM Mg^{2+} stimulated the biosynthesis of glutamyl endopeptidase by 50 and 60%, respectively. All bivalent ions tested did not influence the growth of the recombinant strain except for Co^{2+} ions, which suppressed it considerably. Bearing this in mind, we did not include cobalt sources into the formulation of the optimal medium despite of the fact that 2 mM Co^{2+} increased the production of this enzyme by the recombinant strain by more than two times (Fig. 6, curve *2*).

As was shown for the parent *B. intermedius* strain, cobalt ions can influence the process of enzyme detachment from the cell membrane and promote the release of membrane-bound proteins to the medium, thus giving rise to the extracellular forms of enzymes. This implies that the increase in the glutamyl endopeptidase activity of the culture liquid of *B. subtilis* AJ73 under the action of Co^{2+} ions may not be related to the activation of biosynthetic processes.

Thus, the accumulation of glutamyl endopeptidase in the culture liquid of the recombinant *B. subtilis* strain AJ73 bearing plasmid pV with the gene of this protease depends on the same factors which affect the biosynthesis of other serine proteases, including the glutamyl endopeptidase of *B. intermedius* 3-19. The growth of the recombinant producer of glutamyl endopeptidase and the enzyme production rate are stimulated by the enrichment of the cultivation medium with phosphorus, organic and inorganic nitrogen, yeast extract (as the source of vitamins and other bioactive substances), as well as by the addition of casein and gelatin (as the substrates of endopeptidase) and the bivalent ions Ca^{2+}, Co^{2+}, and Mg^{2+} to the medium.

Based on the results presented above, the medium optimal for the glutamyl endopeptidase production by the recombinant *B. subtilis* strain AJ73 should contain (%) peptone, 1.7; yeast extract, 0.5; gelatin, 1; $CaCl_2 \cdot 2H_2O$, 0.11; $MgSO_4 \cdot 7H_2O$, 0.05; NaCl, 0.3; $MnSO_4$, 0.01; NH_4Cl, 0.01, and Na_2HPO_4, 0.036 (pH 8.5).

REFERENCES

1. Drapeau, G.R., Boily, Y., and Houmard, J.J., Purification and Properties of an Extracellular Protease of *Staphylococcus aureus*, *J. Biol. Chem.*, 1972, vol. 247, no. 20, pp. 6720–6726.
2. Rudenskaya, G.N., Microbial Glutamyl Endopeptidases: A New Subfamily of Chymotrypsin Proteinases, *Bioorg. Khim.*, 1998, vol. 24, no. 4, pp. 256–261.
3. Demidyuk, I.V. and Kostrov, S.V., Some Peculiarities of the Functional Organization of Glutamyl Endopeptidases, *Mol. Biol.*, 1999, vol. 33, no. 1, pp. 100–105.
4. Leshchinskaya, I.B., Shakirov, E.V., Itskovitch, E.L., Balaban, N.P., Mardanova, A.M., Sharipova, M.R., Viryasov, M.B., Rudenskaya, G.N., and Stepanov, V.M., Glutamyl Endopeptidase of *Bacillus intermedius*, Strain 3-19, *FEBS Lett.*, 1997, vol. 404, pp. 241–244.
5. Gabdrakhmanova, L.A., Shakirov, E.V., Balaban, N.P., Sharipova, M.R., Leshchinskaya, I.B., and Rudenskaya, G.N., Biosynthesis and Localization of Glutamyl Endopeptidase of *Bacillus intermedius* 3-19, *Microbios.*, 1999, vol. 100, pp. 97–108.
6. Rebrikov, D.V., Akimkina, T.V., Shevelev, A.B., Demiduyk, I.V., Bushueva, A.M., Kostrov, S.V., Chestukhina, G.G., and Stepanov, V.M., Molecular Cloning and Nucleotide Sequence of *Bacillus intermedius* Glutamyl Endopeptidase Gene, *J. Protein Chem.*, 1999, vol. 18, pp. 21–26.
7. *DNA Cloning: A Practical Approach*, Glover, D.M., Ed., Oxford: IRL, 1985. Translated under the title *Klonirovanie DNK. Metody*, Moscow: Mir, 1988.
8. Plokhinskii, N.A., *Matematicheskie metody v biologii* (Mathematical Methods in Biology), Moscow: Mosk. Gos. Univ., 1978.
9. Krasnov, S.I. and Znamenskaya, L.V., BIOPT Software Package for Optimizing Biological Research, *Biol. Nauki*, 1992, vol. 2, p. 15.
10. Lenski, R.E. and Nguen, T.T., Stability of Recombinant DNA and Its Effects on Fitness, *Trends Biotechnol. Ecol. Evol.*, 1988, vol. 6, pp. 51–53.
11. Georgiou, G., Optimizing the Production of Recombinant Proteins in Microorganisms, *AICHE J.*, 1988, vol. 34, pp. 1233–1248.
12. Itskovich, E.L., Znamenskaya, L.V., Balaban, N.P., Ershova, T.A., and Leshchinskaya, I.B., Synthesis of Extracellular Alkaline Proteinase by *Bacillus intermedius*, *Mikrobiologiya*, 1995, vol. 64, no. 5, pp. 623–629.