Microbiology, Vol. 73, No. 3, 2004, pp. 279–285. Translated from Mikrobiologiya, Vol. 73, No. 3, 2004, pp. 335–342.

EXPERIMENTAL ARTICLES

The Regulation of *Bacillus intermedius* Glutamyl Endopeptidase Biosynthesis in the Recombinant *Bacillus subtilis* Strain AJ73 during Sporulation

I. B. Chastukhina*, M. R. Sharipova*,[1], L. A. Gabdrakhmanova*, N. P. Balaban*, D. R. Safina*, S. V. Kostrov***, G. N. Rudenskaya**, 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. Kurchatova 46, Moscow, 123182 Russia*

Received March 4, 2003; in final form, July 3, 2003

Abstract—The growth of the recombinant *Bacillus subtilis* strain AJ73 carrying the *Bacillus intermedius* 3-19 glutamyl endopeptidase gene on a multicopy plasmid and the effect of some nutrients on the efficiency of extracellular glutamyl endopeptidase production in the stationary growth phase were studied. In this phase, the concentration of glutamyl endopeptidase in the culture liquid peaked at the 48th and 78th hours of cultivation and depended on the composition of the cultivation medium. Unlike the synthesis of glutamyl endopeptidase in the trophophase (i.e., during vegetative growth), which was suppressed by glucose, the synthesis of this enzyme during sporulation was resistant to glucose present in the cultivation medium. A multifactorial experimental design allowed optimal proportions between the concentrations of major nutrients (peptone and inorganic phosphate) to be determined. Inorganic phosphate and ammonium ions augmented the production of glutamyl endopeptidase by 30–150%, and complex organic substrates, such as casein and gelatin, enhanced the production of glutamyl endopeptidase by 50–100%. During sporulation, the production of glutamyl endopeptidase was stimulated by some bivalent cations (Ca^{2+}, Mg^{2+}, and Co^{2+}) and inhibited by others (Zn^{2+}, Fe^{2+}, and Cu^{2+}). The inference is drawn that the regulatory mechanisms of glutamyl endopeptidase synthesis during vegetative growth and sporulation are different.

Key words: glutamyl endopeptidase, regulation of biosynthesis, recombinant strain, growth conditions, sporulation.

Glutamyl endopeptidases (EC 3.4.21.19) form a subfamily of serine proteinases in the chymotrypsin family. These proteinases have a narrow substrate specificity and cleave only peptide bonds between the α-carboxylic groups of glutamic and aspartic acids [1]. In previous works, we described the properties of the glutamyl endopeptidase that is secreted by *Bacillus intermedius* 3-19 during the phase of vegetative growth [2], the regulatory mechanisms that control glutamyl endopeptidase synthesis in *B. intermedius*, the cloning of the complete enzyme gene in *Bacillus subtilis*, and the conditions that promote the accumulation of extracellular glutamyl endopeptidase in the culture liquid of the recombinant strain in the growth retardation phase [3–5]. The synthesis of the glutamyl endopeptidase that appears in the culture liquid of *B. subtilis* in the growth retardation phase was shown to be completely suppressed by glucose and activated by inorganic phosphate, casein, and gelatin. It was also found that the bacterium *B. intermedius* secretes glutamyl endopeptidase in the late stationary growth phase, during sporulation stages V and VI [6]. Both the active accumulation of glutamyl endopeptidase in the culture liquid of *B. intermedius* 3-19 and the initiation of sporulation of this bacterium are subject to catabolite repression by glucose [6], which is in agreement with the supposition that sporulation and alkaline proteinase synthesis are regulated coordinately [7].

This work was aimed at studying the synthesis of the *B. intermedius* 3-19 glutamyl endopeptidase in the recombinant *B. subtilis* strain AJ73 in the late growth stages.

MATERIALS AND METHODS

Bacillus subtilis strain AJ73 with the chromosome from which the genes of its own extracellular proteinases were deleted was kindly provided by Yu. Jomantas

[1] Corresponding author; e-mail: Margarita.Sharipova@ksu.ru

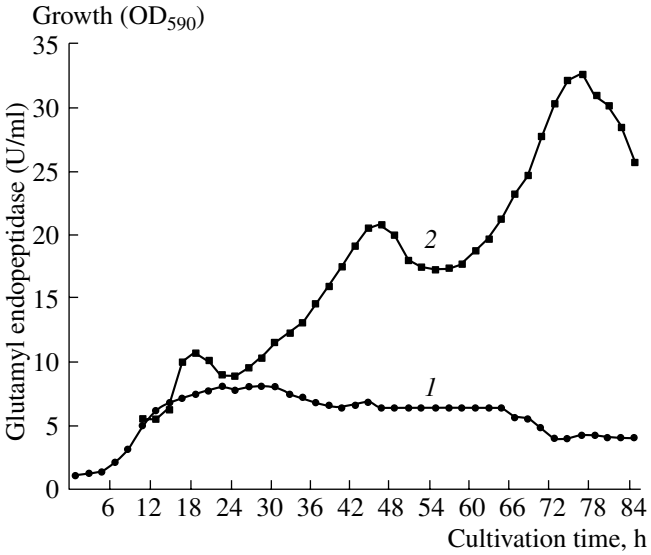

Fig. 1. Dynamics of (*1*) culture growth and (*2*) glutamyl endopeptidase activity in the culture liquid of the recombinant *B. subtilis* strain AJ73(pV).

from the State Research Institute of Genetics and Selection of Industrial Microorganisms (Moscow, Russia).

Competent *B. subtilis* cells were transformed with plasmid DNA as described by Glover [8]. The multicopy plasmid pV was constructed on the basis of vector pCB22 carrying the complete gene of *B. intermedius* glutamyl endopeptidase [3].

B. subtilis was cultivated at 30°C on a shaker (200 rpm) in 100-ml flasks containing 20 ml of a nutrient medium. The basal medium for the cultivation of *B. subtilis* contained (%) peptone, 1.7; yeast extract, 0.5; gelatin, 1; $CaCl_2 \cdot 2H_2O$, 0.01; $MgSO_4 \cdot 7H_2O$, 0.05; NaCl, 0.3; $MnSO_4$, 0.01; NH_4Cl, 0.01; and Na_2HPO_4, 0.036 (pH 8.5). The medium was supplemented with 20 μg/ml erythromycin since plasmid pV bears the erythromycin resistance gene. The medium was sterilized at 1 atm. Solutions of inorganic phosphate (Na_2HPO_4), NH_4Cl, $C_6H_6O_7(NH_4)_2$, and salts of bivalent metals were sterilized separately at 1 atm and added to the medium immediately before inoculation. Casein and gelatin solutions were sterilized at 0.5 atm. Yeast extract, gelatin, and Hammarsten casein were purchased from Difco Laboratories, Sigma, and Serva, respectively. Peptone was obtained from a meat-processing plant in Tbilisi.

The material for inoculation was an 18-h culture in an amount of 1 vol %. Bacterial biomass was evaluated nephelometrically at 590 nm.

Proteolytic activity was measured as described previously [2] with *N*-carbobenzoxy-L-glutamic acid *p*-nitroanilide (BZ-Glu-*p*-NA). One unit of proteolytic activity was defined as the amount of enzyme that hydrolyzes 1 nmol of substrate per minute.

The efficiency of glutamyl endopeptidase production was defined as the ratio of proteolytic activity in the culture liquid to the biomass and was expressed in arbitrary units.

Spores were detected by the microscopic examination of specimens that were stained with Gram or Peshkov stains [10]. Viable cells and spores were enumerated by counting colonies grown on agar plates that were inoculated with bacterial suspensions.

The data obtained were statistically processed as described by Plokhinskii [11]. The results of multifactorial experiments were processed using the STATGRAPHICS program.

RESULTS AND DISCUSSION

In the first set of experiments, we investigated the dynamics of culture growth and the accumulation of *B. intermedius* 3-19 glutamyl endopeptidase in the culture liquid of the recombinant *B. subtilis* AJ73(pV) strain.

As is evident from the results presented in Fig. 1, glutamyl endopeptidase activity in the culture liquid of *B. subtilis* AJ73 showed three peaks, one peak being in the growth retardation phase (at the 18th hour of cultivation) [9] and two peaks being in the late stationary growth phase (at the 48th and 78th hours of cultivation). Proteolytic activity in the second and third peaks exceeded proteolytic activity in the first peak by 2 and 3 times, respectively. The glutamyl endopeptidase that accumulated in the culture liquid in the growth retardation phase was arbitrarily named early glutamyl endopeptidase, whereas the enzyme that accumulated in the late stationary growth phase was named late glutamyl endopeptidase.

After 48 and 78 h of cultivation, the amount of sporulating cells and spores in the *B. subtilis* AJ73 culture reached 25 and 50%, respectively. At later cultivation periods, the percent of spores in the culture remained at a level of (50 ± 3)%. These data are of interest from the standpoint of the possible existence of a relationship between the processes of sporulation and glutamyl endopeptidase synthesis.

It is known that the transition of *B. subtilis* cells from vegetative growth to sporulation is accompanied by a change in the cell response to glucose. During the stage of vegetative growth, the presence of glucose in the medium suppresses (completely or partially) sporulation by the mechanism of catabolite repression. After the initiation of sporulation, however, glucose does not influence this process [12].

Bearing this in mind, we investigated the effect of glucose on the production of late glutamyl endopeptidase by the *B. subtilis* AJ73 culture occurring at different developmental stages. Glucose was added, to a final concentration of 1%, in the trophophase (0, 6, and 10 h of cultivation) and following the growth retardation phase (20, 32, 44, 56, and 70 h of cultivation). The addition of glucose to actively growing *B. subtilis* cells (i.e.,

Table 1. Optimization of nutrient medium for the biosynthesis of glutamyl endopeptidase by the recombinant *B. subtilis* strain AJ73(pV) at the 48th hour of cultivation

Factor value				Biomass, OD units	Endopeptidase, U/ml	Productivity
Peptone		Inorganic phosphate				
X_1	g/l	X_2	g/l			
0	20	–	0.1	20.4	39.7	1.9
+	30	+	0.3	20.8	29.0	1.4
–	10	0	0.2	16.3	47.4	2.9
+	30	0	0.2	18.7	23.1	1.2
0	20	+	0.3	10.0	15.9	1.6
+	30	–	0.1	14.3	16.6	1.1
–	10	+	0.3	11.6	17.2	1.5
0	20	0	0.2	19.6	47.4	2.4
–	10	–	0.1	15.5	31.4	2.0

Table 2. Optimization of nutrient medium for the biosynthesis of glutamyl endopeptidase by the recombinant *B. subtilis* strain AJ73(pV) at the 78th hour of cultivation

Factor value				Biomass, OD units	Endopeptidase, U/ml	Productivity
Peptone		Inorganic phosphate				
X_1	g/l	X_2	g/l			
–	10	+	0.3	9.5	24.4	2.6
+	30	–	0.1	20.4	44.4	2.2
0	20	0	0.2	15.0	62.9	4.2
–	10	–	0.1	12.8	28.1	2.2
+	30	0	0.2	17.2	48.1	2.8
–	10	0	0.2	12.0	42.2	3.5
0	20	–	0.1	15.5	44.4	2.9
+	30	+	0.3	17.4	40.7	2.3
0	20	+	0.3	13.6	49.6	3.6

in the trophophase) drastically suppressed the synthesis of late glutamyl endopeptidase. However, beginning from the 20th hour of cultivation (after the initiation of sporulation), the addition of glucose did not affect the synthesis of late glutamyl endopeptidase. Consequently, unlike the synthesis of early glutamyl endopeptidase, the synthesis of late glutamyl endopeptidase by *B. subtilis* AJ73(pV) cells is resistant to catabolite repression. This suggests that the regulatory mechanisms that control the expression of the glutamyl endopeptidase gene during vegetative growth and during sporulation are different.

It is known that recombinant strains usually require elevated concentrations of nitrogen and phosphorus sources in the cultivation medium. For this reason, we investigated the effect of different concentrations of major nutrients (peptone and inorganic phosphate) in the cultivation medium on the production efficiency of late glutamyl endopeptidase. Experiments were designed according to a bifactorial scheme. The concentrations of peptone and inorganic phosphate were varied at three different levels (Tables 1, 2). The tabulated values of biomass (estimated as culture turbidity), glutamyl endopeptidase activity, and productivity of enzyme synthesis are the means of triplicate experiments.

The results of these bifactorial experiments are graphically presented in Fig. 2 as isolines of glutamyl endopeptidase activity, which form zones of peptone and inorganic phosphate concentrations that are optimal for glutamyl endopeptidase activity in the culture liquid. Similarly, Fig. 3 illustrates zones of peptone and inorganic phosphate concentrations that are optimal for the efficiency of glutamyl endopeptidase production.

The analysis of these figures showed that the production of glutamyl endopeptidase at the 48th hour of

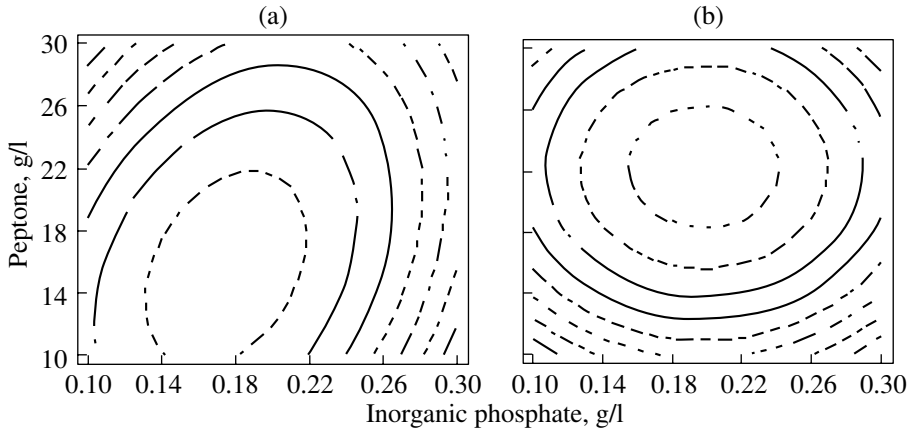

Fig. 2. Isolines of glutamyl endopeptidase activity constructed from the results of bifactorial experiments with the recombinant *B. subtilis* strain AJ73(pV): (a) at the 48th hour of cultivation; (b) at the 78th hour of cultivation.

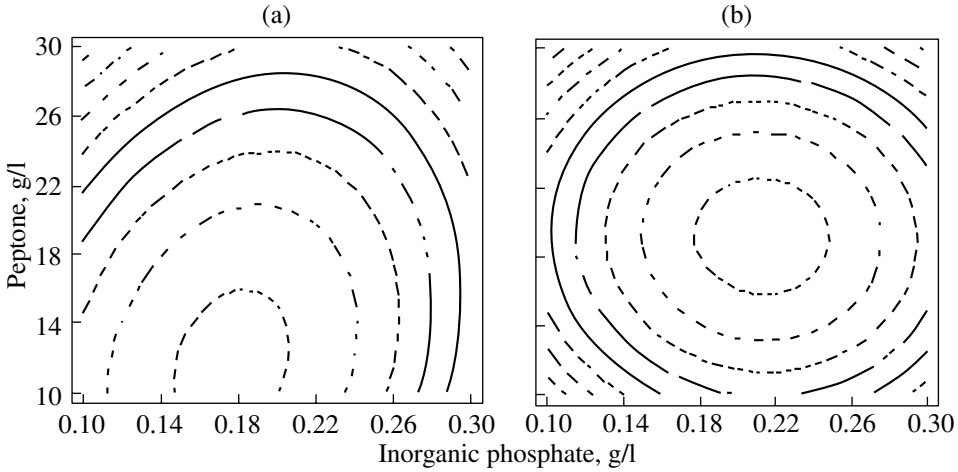

Fig. 3. Isolines of glutamyl endopeptidase production constructed from the results of bifactorial experiments with the recombinant *B. subtilis* strain AJ73(pV): (a) at the 48th hour of cultivation; (b) at the 78th hour of cultivation.

cultivation must be the most efficient in the medium containing 16 g/l peptone and 0.18 g/l inorganic phosphate, whereas the production of glutamyl endopeptidase at the 78th hour of cultivation must be the most efficient in the medium containing 22 g/l peptone and 0.2 g/l inorganic phosphate. This concentration of inorganic phosphate is two times higher than that necessary for the efficient production of glutamyl endopeptidase in the growth retardation phase. This fact can be explained by the transition of a certain fraction of stationary-phase cells to a resting state.

It is known that the presence of an enzymatic substrate or its derivative in a cultivation medium can stimulate the synthesis of the corresponding enzyme [12]. Our previous experiments showed that the addition of casein or gelatin (proteinase substrates) to the cultivation medium of *B. subtilis* AJ73(pV) favorably influences the synthesis of glutamyl endopeptidase in the stage of vegetative growth [9]. Similar experiments in this work showed that the addition of 0.5% casein to the cultivation medium of *B. subtilis* AJ73(pV) enhanced proteinase synthesis by 40% at the 48th hour of cultivation and by 100% at the 78th hour of cultivation (Fig. 4). At still higher casein concentrations, the efficiency of glutamyl endopeptidase production decreased. The addition of 1% gelatin to the medium augmented proteinase synthesis by 50% at the 48th hour of cultivation and by 110% at the 78th hour of cultivation.

Thus, the biosynthesis of both early and late glutamyl endopeptidases by the recombinant *B. subtilis* strain is activated in the presence of complex organic substrates.

Taking into account our earlier observations that the production of glutamyl endopeptidase by *B. intermedius* 3-19 [4] and *B. subtilis* AJ73(pV) [9] cells in the growth retardation phase is higher when the cultivation

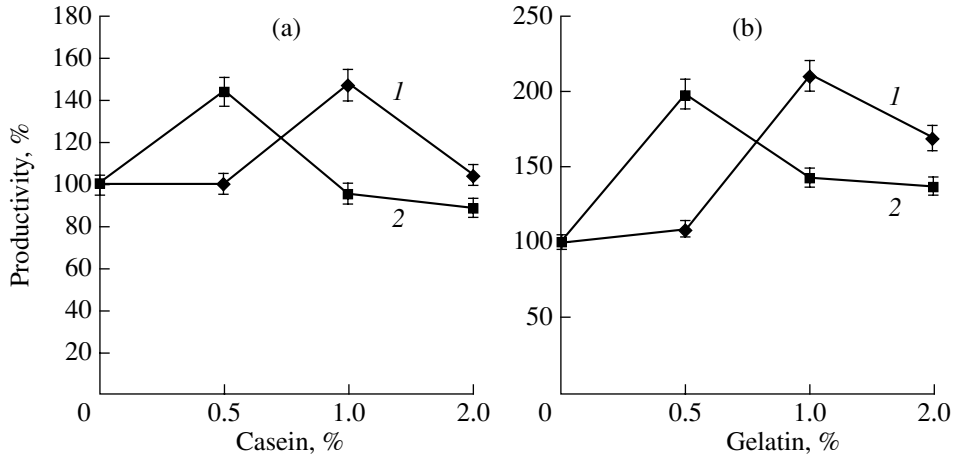

Fig. 4. The effect of (*1*) casein and (*2*) gelatin on the productivity of glutamyl endopeptidase synthesis by the recombinant *B. subtilis* strain AJ73(pV): (a) at the 48th hour of cultivation; (b) at the 78th hour of cultivation. The productivity of enzyme synthesis in the medium without casein and gelatin was taken to be 100%.

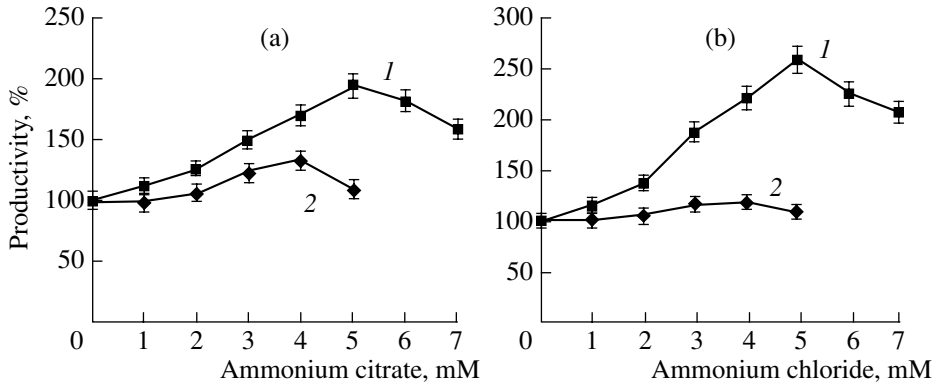

Fig. 5. The effects of (*1*) ammonium citrate and (*2*) ammonium chloride on the productivity of glutamyl endopeptidase synthesis by the recombinant *B. subtilis* strain AJ73(pV): (a) at the 48th hour of cultivation; (b) at the 78th hour of cultivation. The productivity of enzyme synthesis in the medium without ammonium ions was taken to be 100%.

medium contains both organic (peptone) and inorganic (ammonium ions) nitrogen sources [4], we investigated the effect of ammonium ions on the production of late glutamyl endopeptidase. As can be seen from Fig. 5, the addition of ammonium chloride to the medium at a concentration of 4 mM stimulated glutamyl endopeptidase production by 30% at the 48th hour of cultivation and by 15% at the 78th hour of cultivation. Ammonium citrate was found to be a better source of inorganic nitrogen than was ammonium chloride (Fig. 5, curves *1*). The optimal concentration of ammonium citrate for the production of glutamyl endopeptidase was found to be 5 mM.

Bivalent metal cations play an important part in maintaining a catalytically active conformation of proteinases [13, 14]. In particular, glutamyl endopeptidases are Ca^{2+}-dependent enzymes [1, 2]. Previous experiments showed that Ca^{2+} ions present in the reaction mixture stabilize the *B. intermedius* glutamyl endopeptidase and increase its activity [14]. Co^{2+} ions also stabilize the enzyme molecule [4]. Ca^{2+}, Mg^{2+}, and Co^{2+} ions favorably influence the biosynthesis of glutamyl endopeptidase by the parent *B. intermedius* 3-19 [4] and the recombinant *B. subtilis* AJ73(pV) strains [9].

Bearing this in mind, we investigated the effect of some bivalent cations on the synthesis of late glutamyl endopeptidase by *B. subtilis* AJ73(pV) (Fig. 6). Experiments showed that Zn^{2+} ions at concentrations of 1–2 mM did not influence the production of late glutamyl endopeptidase. Higher Zn^{2+} concentrations diminished the efficiency of glutamyl endopeptidase production by 40% at the 48th hour of cultivation and by 70% at the 78th hour of cultivation. Fe^{2+} and Cu^{2+} cations at concentrations of 1–10 mM inhibited the synthesis of late glutamyl endopeptidase by *B. subtilis* AJ73(pV), Fe^{2+} ions being the most inhibitory at the 48th hour of cultivation and Cu^{2+} ions being the most inhibitory at the 78th hour of cultivation. Mn^{2+} ions did not influence the production of late glutamyl endopeptidase. Mg^{2+} ions

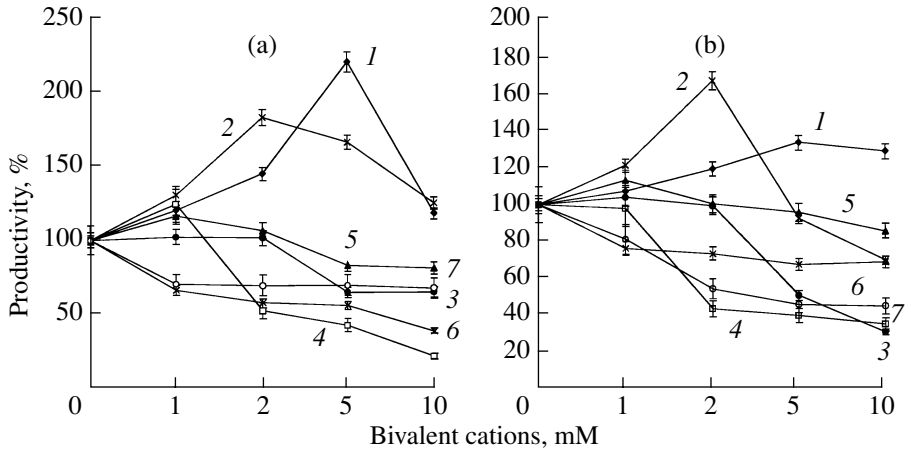

Fig. 6. The effects of (*1*) Ca^{2+}, (*2*) Co^{2+}, (*3*) Zn^{2+}, (*4*) Mg^{2+}, (*5*) Mn^{2+}, (*6*) Fe^{2+}, and (*7*) Cu^{2+} cations on the productivity of glutamyl endopeptidase synthesis by the recombinant *B. subtilis* strain AJ73(pV): (a) at the 48th hour of cultivation; (b) at the 78th hour of cultivation. The productivity of enzyme synthesis in the medium without bivalent cations was taken to be 100%.

at a concentration of 1 mM stimulated the synthesis of late glutamyl endopeptidase by 20%, but higher Mg^{2+} concentrations inhibited the synthesis by 70–80%.

Ca^{2+} ions at a concentration of 5 mM stimulated the synthesis of late glutamyl endopeptidase by 2.2 times at the 48th hour of cultivation and by 1.3 times at the 78th hour of cultivation. Such a difference in the stimulatory effects of Ca^{2+} ions can be explained by the different conformations of the enzyme synthesized at the 48th and 78th hours of cultivation [15].

Co^{2+} cations at concentrations higher than 1 mM exerted a beneficial effect on the biosynthesis of late glutamyl endopeptidase. At a concentration of 2 mM, Co^{2+} ions stimulated the production of late glutamyl endopeptidase by 80% at the 48th hour of cultivation and by 70% at the 78th hour of cultivation. On the other hand, Co^{2+} ions inhibited the growth of bacilli.

The data obtained allow the inference to be drawn that the presence of easily metabolizable glucose in the cultivation medium does not influence the biosynthesis of late glutamyl endopeptidase by the recombinant *B. subtilis* strain AJ73(pV), which can be due to different regulatory mechanisms that control this biosynthesis in the early and late growth stages. Glucose inhibits the initiation of sporulation but does not influence the late stages of this process. It is tempting to suggest that the synthesis of late glutamyl endopeptidase and endospore formation are regulated coordinately.

In most aspects, the synthesis of late glutamyl endopeptidase by the recombinant *B. subtilis* strain AJ73(pV) is similar to the synthesis of other extracellular serine proteinases. The nutrient medium that is used for the growth and the efficient synthesis of glutamyl endopeptidase by the recombinant strain must be enriched in organic and inorganic nitrogen sources and contain an enzymatic substrate (casein or gelatin). The bivalent Ca^{2+}, Mg^{2+}, and Co^{2+} ions favorably influence the production of glutamyl endopeptidase by the recombinant strain. The biosynthesis of glutamyl endopeptidase in the late growth stage (during sporulation) requires less inorganic phosphate than in the phase of vegetative growth.

ACKNOWLEDGMENTS

This work was supported by grant no. 01-04-48037 from the Russian Foundation for Basic Research and by grant no. REC 007 from the US Civilian Research & Development Foundation.

REFERENCES

1. Rudenskaya, G.N., Microbial Glutamyl Endopeptidases, a New Subfamily of Chymotrypsin Proteinases, *Bioorg. Khim.*, 1998, vol. 24, no. 4, pp. 256–261.
2. 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., Glutamylendopeptidase of *Bacillus intermedius* Strain 3-19, *FEBS Lett.*, 1997, vol. 404, pp. 241–244.
3. 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* Glutamylendopeptidase Gene, *J. Prot. Chem.*, 1999, vol. 18, pp. 21–26.
4. Gabdrakhmanova, L.A., Shakirov, E.V., Balaban, N.P., Sharipova, M.R., Leshchinskaya, I.B., and Rudenskaya, G.N., Biosynthesis and Localization of Glutamylendopeptidase of *Bacillus intermedius* 3-19, *Microbios*, 1999, vol. 100, pp. 97–108.
5. Gabdrakhmanova, L.A., Balaban, N.P., Sharipova, M.R., Kostrov, S.V., Akimkina, T.V., Rudenskaya, G.N., and Leshchinskaya, I.B., Optimization of *Bacillus intermedius* Glutamylendopeptidase Production by Recombinant Strain of *Bacillus subtilis* and Localization of

Glutamylendopeptidase in *Bacillus subtilis* Cells, *Enzyme Microb. Technol.*, 2002, vol. 31, pp. 256–263.

6. Sharipova, M.R., Balaban, N.P., Gabdrakhmanova, L.A., Shilova, M.A., Kadyrova, Yu.M., Rudenskaya, G.N., and Leshchinskaya, I.B., Hydrolytic Enzymes and Sporulation in *Bacillus intermedius, Mikrobiologiya*, 2002, vol. 71, no. 4, pp. 494–499.
7. Klier, A., Msadek, T., and Rapoport, G., Positive Regulation in the Gram-Positive Bacterium *Bacillus subtilis, Annu. Rev. Microbiol.*, 1992, vol. 46, pp. 429–459.
8. *DNA Cloning: A Practical Approach*, Glover, D.M., Ed., Oxford: IRL, 1985. Translated under the title *Klonirovanie DNK. Metody*, Moscow: Mir, 1988.
9. Gabdrakhmanova, L.A., Shakirov, E.V., Balaban, N.P., Sharipova, M.R., Rudenskaya, G.N., Kostrov, S.V., Akimkina, T.V., and Leshchinskaya, I.B., Optimized Medium for the Efficient Production of *Bacillus intermedius* Glutamyl Endopeptidase by the Recombinant *Bacillus subtilis* Strain AJ73, *Mikrobiologiya*, 2000, vol. 69, no. 5, pp. 653–659.
10. *Rukovodstvo k Prakticheskim Zanyatiyam po Mikrobiologii* (Workbook for Laboratory Classes in Microbiology), Egorov, N.S., Ed., Moscow: Mosk. Gos. Univ., 1983.
11. Plokhinskii, N.A., *Matematicheskie Metody v Biologii* (Mathematical Methods in Biology), Moscow: Mosk. Gos. Univ., 1978.
12. *Biosintez mikroorganizmami nukleaz i proteaz* (Biosynthesis of Nucleases and Proteases by Microorganisms), Imshenetskii, A.A., Ed., Moscow: Nauka, 1979.
13. Khaidarova, N.V., Rudenskaya, G.N., Revina, L.P., Stepanov, V.M., and Egorov, N.S., Glu,Asp-Specific Proteinase from *Streptomyces thermovulgaris, Biokhimiya*, 1989, vol. 54, no. 1, pp. 46–52.
14. Leshchinskaya, I.B., Shakirov, E.V., Itskovich, E.L., Balaban, N.P., Mardanova, A.M., Sharipova, M.R., Blagova, E.V., Levdikov, V.M., Kuranova, I.P., Rudenskaya, G.N., and Stepanov, V.M., Glutamyl Endopeptidase from *Bacillus intermedius* 3-19: Isolation, Properties, and Crystallization, *Biokhimiya*, 1997, vol. 62, no. 62, pp. 1052–1059.
15. Balaban, N.P., Mardanova, A.M., Sharipova, M.R., Gabdrakhmanova, L.A., Sokolova, E.A., Garusov, A.V., Mil'gotina, E.I., Rudenskaya, G.N., and Leshchinskaya, I.B., Isolation and Characterization of Glutamyl Endopeptidase 2 from *Bacillus intermedius* 3-19, *Biokhimiya*, 2003, vol. 68, no. 11, pp. 1514–1521.