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EXPERIMENTAL ARTICLES

Biosynthesis of the *Bacillus intermedius* Subtilisin-Like Serine Proteinase by the Recombinant *Bacillus subtilis* Strain

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Abstract—The effect of certain nutrients on the growth and production of the *Bacillus intermedius* subtilisin-like serine proteinase by the recombinant strain *Bacillus subtilis* AJ73(pCS9) was studied. Glucose was found to inhibit the synthesis of proteinase in the early (28 h of growth) but not in the late stationary phase (48 h of growth). The inhibitory effect of the other mono- and disaccharides studied was less pronounced. Casamino acids added to the medium at concentrations of 0.1–1% as an additional carbon and nitrogen source stimulated enzyme biosynthesis. Individual amino acids (cysteine, asparagine, glutamine, tryptophan, histidine, and glutamate) also stimulated enzyme biosynthesis in the early stationary phase by 25–30%, whereas other amino acids (valine, leucine, alanine, and aspartate) were ineffective or even slightly inhibitory to enzyme production. The stimulatory effect of the first group of amino acids on the synthesis of proteinase in the late stationary phase was negligible. In contrast, the bivalent ions Ca^{2+}, Mg^{2+}, and Mn^{2+} stimulated biosynthesis of proteinase in the late stationary phase (by 20–60%) and not in the early stationary phase. The data indicate that there are differences in the biosyntheses of proteinase by the recombinant *B. subtilis* strain during the early and late periods of the stationary phases.

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Key words: subtilisin-like serine proteinase, biosynthesis, growth conditions, recombinant strain.

Bacilli produce a wide range of extracellular and intracellular proteinases, including subtilases [1]. The latter enzymes belong to an abundant (more than 200 members) superfamily of subtilisin-like serine proteinases. Analysis of the amino acid sequences of subtilases (known are more than 170 complete sequences) showed that this superfamily is characterized by a high level of homology of the conservative core region of the polypeptide chain [1]. The common catalytically active amino acid residues (Asp-32, His-64, and Ser-221) are surrounded by the conservative amino acid sequences [2]. Subtilases, subtilisin in particular, are of commercial importance [3, 4]. Bacilli produce proteinases both during the period of active growth and during sporulation. The expression of bacillar genes is controlled by a complex regulatory system, which includes specific regulatory proteins (Spo0, AbrB, CcpA, DegU, SinR, Hpr, and others) according to the current physiological status of bacillar cells [5].

Earlier studies showed that the bacterium *Bacillus intermedius* secretes into the culture liquid a subtilisin-like proteinase with two maxima of activity, one in the early (24 h of growth) and the other in the late (44–46 h of growth) stationary phase [6]. The first peak of activity corresponds to the initial stage of sporulation, whereas the second peak corresponds to the stage of release of mature endospores. A comparative analysis of the physicochemical and enzymatic properties of proteinases isolated from the culture liquid of the early and late stationary phases showed that they are similar in the main biochemical characteristics (such as substrate specificity, inhibition characteristics, and *N*-terminal amino acid sequences) but considerably differ in the catalytic parameters K_m and k_{cat} [7, 8]. Recently, using the multicopy plasmid pCS9, we cloned the whole gene of the *B. intermedius* subtilisin-like proteinase in the recipient *Bacillus subtilis* AJ73 cells, which are deficient in their own extracellular proteinases [9].

The aim of this work was to comprehensively study the biosynthesis of the *B. intermedius* subtilisin-like serine proteinase by the recombinant strain *B. subtilis* AJ73 in the early and late periods of its stationary phase.

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MATERIALS AND METHODS

The recipient strain *Bacillus subtilis* AJ73, which is deficient in its own extracellular proteinases, was kindly provided by Y. Yomantas from the State Scientific Research Institute of Genetics and Selection of Industrial Microorganisms in Moscow, Russia. This strain was transformed with plasmid DNA as described by Anagnostopolous and Spizizen [10]. The multicopy plasmid pCS9 was constructed on the basis of vector pCB22 carrying the entire gene of the *B. intermedius* 3-19 subtilisin-like serine proteinase [9].

The recombinant strain *B. subtilis* AJ73(pCS9) was grown at 30°C on a shaker (200 rpm) in 100-ml flasks with 20 ml of a cultivation medium. The medium was inoculated with 18-h cells from the linear growth phase. The basal cultivation medium contained (%) peptone, 2; $CaCl_2 \cdot 2H_2O$, 0.06, $MgSO_4 \cdot 7H_2O$, 0.05; NaCl, 0.3; NH_4Cl, 0.02, $MnSO_4$, 0.01, Na_2HPO_4, 0.035 (pH 8.5). The medium was supplemented with 20 μg/ml erythromycin, since plasmid pCS9 bears the erythromycin resistance gene. The medium was sterilized at 1 atm. Solutions of inorganic phosphate, NH_4Cl, $(NH_4)_2SO_4$, ammonium citrate, and inorganic metal salts were sterilized separately at 1 atm. Solutions of carbon sources were sterilized at 0.5 atm and added to the medium immediately before inoculation. Solutions of casamino acids and individual amino acids were also prepared and sterilized separately. L- and D,L-amino acids were added to the medium at concentrations of 0.05 and 0.1 mg/ml, respectively. Casamino acids were added to concentrations of 0.025, 0.1, 0.25, 0.5, 1.0, and 2.0 mg/ml. Bacterial biomass was measured spectrophotometrically at 590 nm.

The activity of subtilisin-like serine proteinase was measured with the chromogenic substrate Z- Ala-Ala-Leu-*p*NA as described by Lyublinskaya et al. [11]. One unit of activity was defined as the amount of enzyme that hydrolyzed 1 nmol of substrate per min.

The efficiency of production of subtilisin-like serine proteinase was defined as the ratio of proteolytic activity in the culture liquid to the biomass and expressed in arbitrary units. The data obtained were statistically processed using Microsoft Excel software. They were considered to be significant at the standard deviation $\sigma \le 15\%$. The statistical significance of these data was evaluated using Student's *t*-test for significance level $P \le 0.05$.

RESULTS AND DISCUSSION

It is known that bacillar serine proteinases, including the subtilisin-like serine proteinase of *B. intermedius*, are subject to repression by glucose and other carbohydrates [12, 13]. In our experiments, we studied the effect of mono- (glucose, mannose, and galactose) and disaccharides (sucrose, maltose, and lactose) on the synthesis of the *B. intermedius* subtilisin-like serine proteinase by the recombinant strain *B. subtilis* AJ73

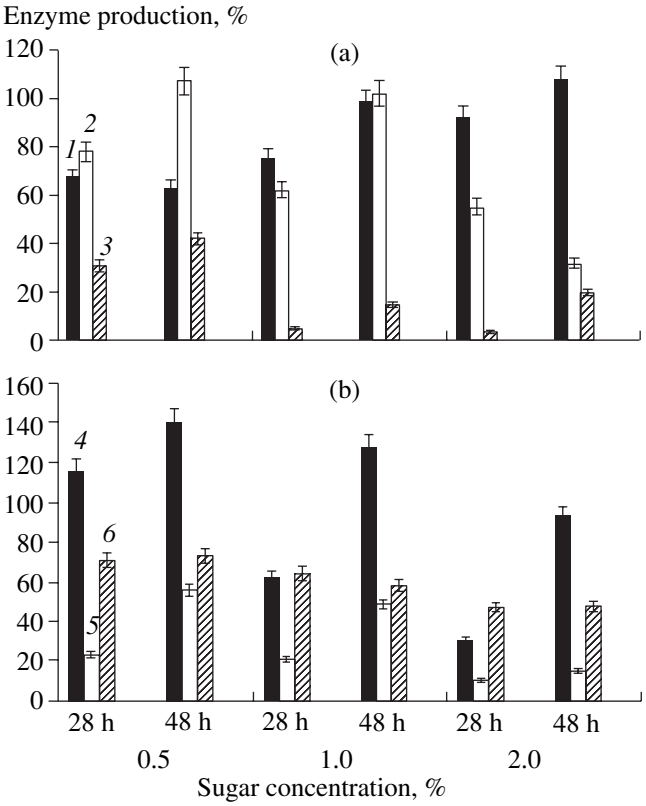

Fig. 1. The effect of (a) mono- and (b) disaccharides on the production of subtilisin-like proteinase by the recombinant strain *B. subtilis* AJ73(pCS9) in the early (28 h of growth) and late (48 h of growth) periods of the stationary phase: (*1*) galactose, (*2*) mannose, (*3*) glucose, (*4*) sucrose, (*5*) maltose, and (*6*) lactose. The enzyme production without added saccharides was taken to be 100%.

(Fig. 1). As can be seen from this figure, all of the above saccharides enhanced the growth of the recombinant strain and diminished the specific activity of subtilisin-like proteinase. Glucose added to the medium considerably decreased (by 70 and 90% at concentrations of 0.5 and 1%, respectively) the first peak of extracellular proteinase in the culture liquid of the recombinant strain (28 h of growth). However, the inhibitory effect of glucose on the second peak of proteinase activity in the late stationary phase was considerably lower (10–40%). Mannose and 0.5% galactose added to the cultivation medium diminished both peaks of proteinase activity by 10–50%. At the same time, galactose at a concentration of 2% slightly enhanced proteinase synthesis (by 10%).

Among the disaccharides studied, maltose showed the maximum inhibitory activity (90% decrease in both peaks of proteinase activity). Lactose added to the cultivation medium inhibited proteinase production by 40%. Sucrose was found to be the least inhibitory disaccharide. Moreover, its addition to the cultivation medium at concentrations of 0.5 and 1% stimulated proteinase production in the late stationary phase by 40 and 20%, respectively. Only at a concentration as

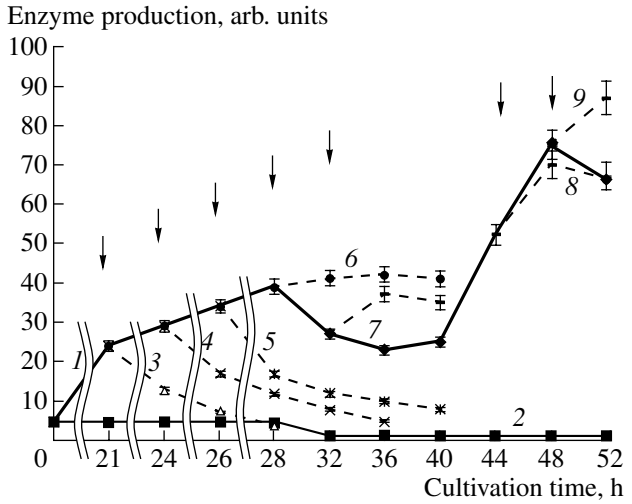

Fig. 2. The production of subtilisin-like proteinase by the recombinant strain *B. subtilis* AJ73(pCS9) in the medium containing (*1*) no glucose, (*2*) 1% glucose, and (*3–9*) 1% glucose added at different times indicated by the vertical arrows.

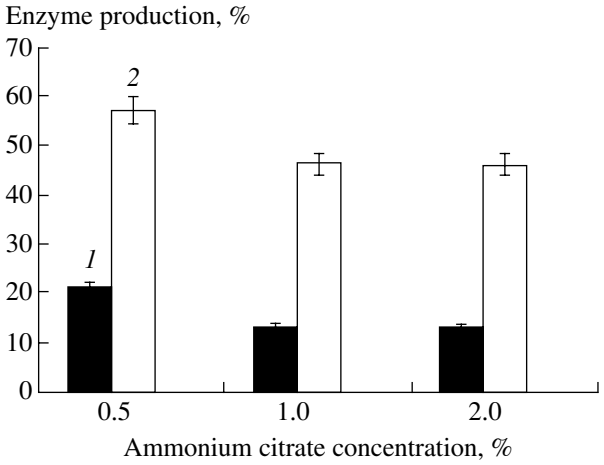

Fig. 3. The effect of ammonium citrate on the production of subtilisin-like proteinase by the recombinant strain *B. subtilis* AJ73(pCS9) in the early (28 h of growth) and late (48 h of growth) periods of the stationary phase. The enzyme production without added ammonium citrate was taken to be 100%.

high as 2% did sucrose slightly inhibit (by 10%) the enzyme production in the late stationary phase. To study the role of catabolite repression in the regulation of the expression of the subtilisin-like serine proteinase gene, glucose (1%) was added to the culture liquid in different growth phases (Fig. 2). When it was added to the medium at a time with the inoculum, the synthesis of proteinase was completely inhibited. When 1% glucose was added to the culture liquid at the 21st, 24th, and 26th h of growth (i.e., in the linear and retardation growth phases), the specific activity of subtilisin-like proteinase in the later growth stages considerably decreased. In contrast, no decrease in proteinase activity occurred when glucose was added to the culture liquid at the 28th h of cultivation and later. These data suggest that the synthesis of subtilisin-like serine proteinase in the early stationary phase (28 h of growth) is subject to catabolite repression by glucose. However, glucose does not repress the synthesis of this enzyme in the late stationary phase (52 h of growth).

Our earlier studies showed that the addition of an inorganic nitrogen source (for example, ammonium sulfate) to the medium already containing an organic nitrogen source (for example, peptone) does not influence the synthesis of subtilisin-like serine proteinase by the parent strain *B. intermedius* 3-19 [12]. In the present study, similar experiments were carried out with the recombinant strain *B. subtilis* AJ73(pCS9). As is evident from Fig. 3, ammonium citrate as an additional nitrogen source induced the growth of this strain but inhibited the production of subtilisin-like serine proteinase. Other inorganic ammonium salts, NH_4Cl and $(NH_4)_2SO_4$, at all the concentrations tested also inhibited the production of subtilisin-like serine proteinase, especially in the early stationary phase (28 h of growth) (Fig. 4). The

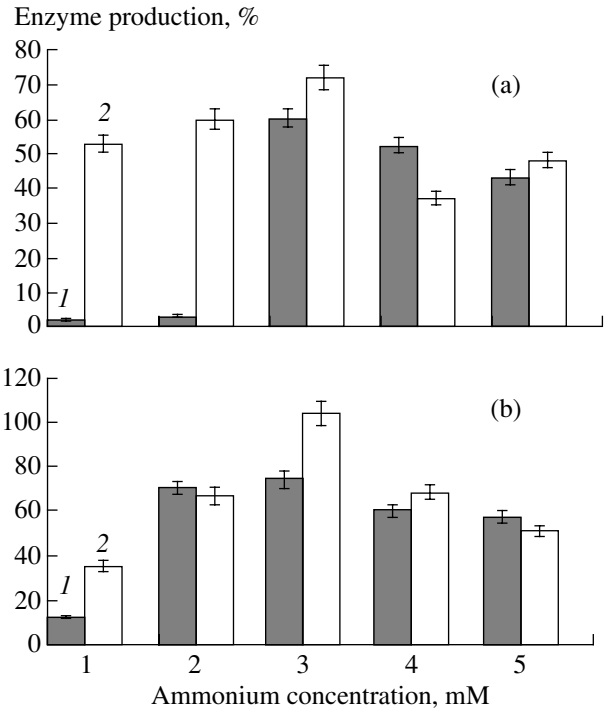

Fig. 4. The effect of (*1*) ammonium chloride and (*2*) ammonium sulfate on the production of subtilisin-like proteinase by the recombinant strain *B. subtilis* AJ73(pCS9) (a) in the early (28 h of growth) and (b) late (48 h of growth) periods of the stationary phase. The enzyme production without added ammonium ions was taken to be 100%.

dependence of the inhibitory effect of ammonium ions on their concentration was not linear. For example, ammonium sulfate at a concentration of 3 mM almost did not affect the enzyme production, although lower concentrations of this salt were inhibitory.

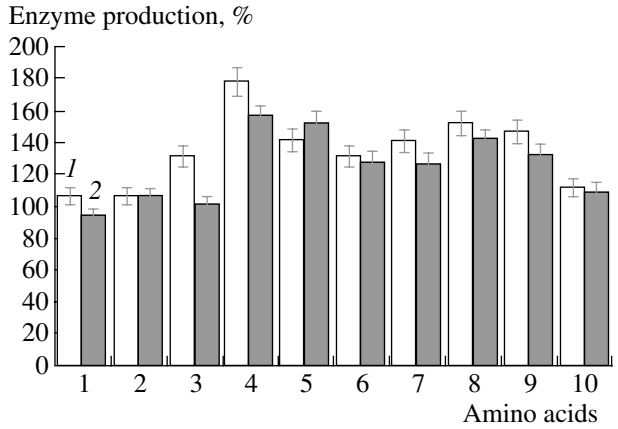

Fig. 5. The effect of individual amino acids on the production of subtilisin-like proteinase by the recombinant strain *B. subtilis* AJ73(pCS9) (*1*) in the early (28 h of growth) and (*2*) late (48 h of growth) periods of the stationary phase: (1) L-alanine, (2) L-valine, (3) D,L-leucine, (4) D,L-cysteine, (5) D,L-asparagine, (6) D,L-glutamine, (7) D,L-tryptophan, (8) D,L-histidine, (9) D,L-glutamic acid, and (10) L-aspartic acid. D,L- and L-amino acids were added at concentrations of 0.1 and 0.05 mg/ml, respectively. The enzyme production in the medium to which amino acids were not added was taken to be 100%.

It is known that the biosynthesis of extracellular proteinases can be regulated by their end products, including amino acids. As a rule, the addition of amino acids to the medium exerts an inhibitory action on the synthesis of exoproteases [14], although some amino acids at certain concentrations can exert a stimulatory effect. In particular, the production of the extracellular serine proteinase of *Bacillus thuringiensis* was found to be stimulated by five amino acids (aspartic and glutamic acids, glutamine, asparagine, and glycine) [13].

In this study, we investigated the effect of various groups of amino acids on the synthesis of the *B. intermedius* 3-19 subtilisin-like serine proteinase in the recombinant strain *B. subtilis* AJ73(pCS9). These groups included nonpolar hydrophobic amino acids (alanine, valine, leucine, and tryptophan), polar hydrophilic amino acids (glycine, cysteine, asparagine, and glutamine), negatively charged acidic amino acids (aspartate and glutamate), positively charged alkaline amino acids (arginine and histidine), and complex amino acids (casamino acids). The addition of individual amino acids (cysteine, asparagine, glutamine, tryptophan, histidine, glutamate, and aspartate) to the medium that contained peptone stimulated the enzyme synthesis in the early stationary phase by 80, 40, 30, 40, 50, 30, and 10%, respectively (Fig. 5). The effect of these amino acids on the enzyme biosynthesis in the late stationary phase was, on the average, 20% slighter. It is evident that the amino acids that stimulate the production of subtilisin-like proteinase are hydrophilic. Presumably, they serve as an additional source of nitrogen. The effect of hydrophobic amino acids on enzyme synthesis was negligible, except that leucine slightly stimulated it in the early stationary phase.

It was of interest to compare the effects of amino acids on the synthesis of extracellular proteinase in different *Bacillus* strains. According to available data, the dicarboxylic amino acids aspartate and glutamate induce two to five times the synthesis of exoproteases in *B. alvei* [13]; lysine, methionine, asparagine, proline, and alanine exert no effect; and threonine, valine, leucine, phenylalanine, and norvaline inhibited two to five times the enzyme biosynthesis. The addition of casamino acids (0.025 and 0.5%) to the peptone-containing cultivation medium of *B. subtilis* AJ73(pCS9) elicited the production of subtilisin-like proteinase by 20–50% (Fig. 6),

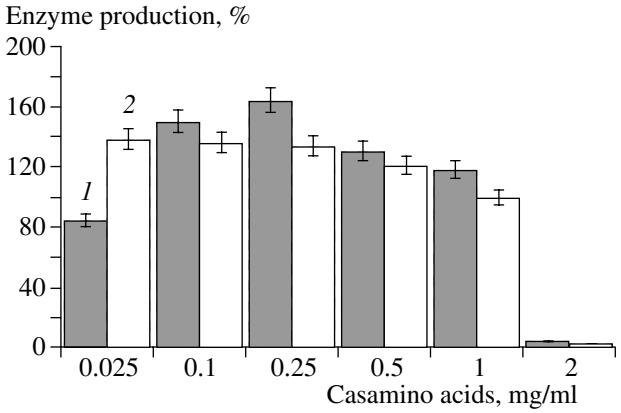

Fig. 6. The effect of casamino acids as an additional carbon and nitrogen source on the production of subtilisin-like proteinase by the recombinant strain *B. subtilis* AJ73(pCS9) (*1*) in the early (28 h of growth) and (*2*) late (48 h of growth) periods of the stationary phase. The enzyme production in the peptone-containing medium to which casamino acids were not added was taken to be 100%.

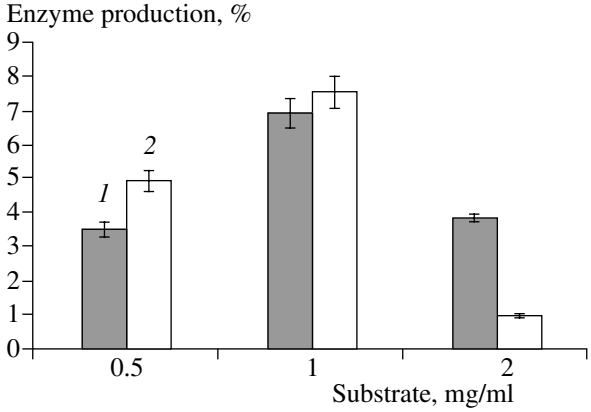

Fig. 7. The effect of casamino acids as the sole carbon and nitrogen source on the production of subtilisin-like proteinase by the recombinant strain *B. subtilis* AJ73(pCS9) (*1*) in the early (28 h of growth) and (*2*) late (48 h of growth) periods of the stationary phase. The enzyme production in the medium containing 20% peptone as the carbon and nitrogen source was taken to be 100%.

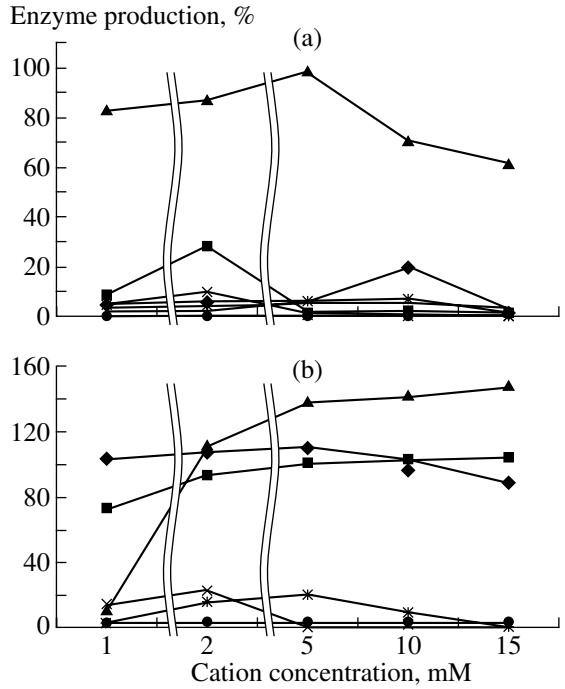

Fig. 8. The effect of bivalent cations on the production of subtilisin-like proteinase by the recombinant strain *B. subtilis* AJ73(pCS9) (a) in the early (28 h of growth) and (b) late (48 h of growth) periods of the stationary phase: Mn^{2+} (◆), Mg^{2+} (■), Ca^{2+} (▲), Zn^{2+} (×), Fe^{2+} (∗), Cu^{2+} (●).

whereas the same acids added at a higher concentration (2%) somewhat inhibited enzyme synthesis.

The addition of casamino acids to the medium containing no peptone stimulated the enzyme production at all the concentrations tested (Fig. 7); however, the activity of proteinase in the medium with 2% casamino acids was lower than in the peptone-containing medium. Therefore, as with the proteinase of *Candida albicans* [15], the replacement of peptone with casamino acids as the carbon and nitrogen source does not favor the synthesis of proteinase by the recombinant strain *B. subtilis* AJ73(pCS9).

Typically, the serine proteinases of bacilli do not require bivalent metal ions for activity. The X-ray analysis of the active centers of bacillar subtilisins indicated their structural identity, which explains a similarity of the substrate specificities of these enzymes [1]. The tertiary structure of subtilisins is characterized by the presence of three surface Ca-binding sites [16], due to which Ca^{2+} ions stabilize the tertiary structure of these enzymes, enhance their thermostability, and prevent the unfolding of the protein globule. The blocking of the Ca-binding sites results in a loss of the specific activity of subtilisins because of an incorrect protein folding [16].

The study of the effect of bivalent metal cations showed that, at concentrations ranging from 1 to 15 mM, all but Ca^{2+} ions inhibited the production of proteinase by the recombinant *B. subtilis* strain in the early stationary phase (Fig. 8). In contrast, the production of proteinase in the late stationary phase was stimulated by Ca^{2+} ions by 40%. The stimulating effect of Mg^{2+} and Mn^{2+} ions was less profound (10–20%), whereas the other metal ions (Zn^{2+}, Fe^{2+}, and Cu^{2+}) inhibited the enzyme biosynthesis by 80%. These data suggest that Ca^{2+} ions probably stabilize the structure of the late-stationary-phase subtilisin-like proteinase.

Thus, the data provide evidence that glucose represses the synthesis of extracellular subtilisin-like proteinase by the recombinant *B. subtilis* strain in the early stationary phase (the period of transition from vegetative growth to sporogenesis) but does not influence the enzyme production in the late stationary phase. This finding suggests that the synthesis of subtilisin-like serine proteinase is regulated differently in different growth stages. Some individual amino acids at concentrations of 0.05 and 0.1 mg/ml, as well as casamino acids added to the medium at concentrations up to 2 mg/ml, stimulate the production of proteinase by the recombinant strain. The bivalent cations Ca^{2+}, Mg^{2+}, and Mn^{2+} beneficially influence enzyme biosynthesis in the late stationary phase. These data may be useful for developing an optimal medium for the production of subtilisin- like serine proteinase. The results indicate that there are certain differences in the syntheses of proteinase by the recombinant *B. subtilis* strain in the early and late stationary phases. In other respects, the production of extracellular subtilisin-like proteinase in the recombinant *B. subtilis* strain resembles that in the parent *B. intermedius* strain.

ACKNOWLEDGMENTS

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