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EXPERIMENTAL ARTICLES

# Growth Conditions and Production of the *Bacillus intermedius* Subtilisin-Like Serine Proteinase by the Recombinant *Bacillus subtilis* Strain

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**Abstract**—The effect of the components of the nutrient medium on growth and production of the *Bacillus intermedius* subtilisin-like serine proteinase by the recombinant strain *Bacillus subtilis* AJ73(pCS9) was studied. The production of proteinase was found to be dependent on the composition of the nutrient medium and showed two peaks, at the 28th and 48th h of growth. The concentrations of the main components of the nutrient medium (peptone and inorganic phosphate) optimal for the biosynthesis of subtilisin-like serine proteinase at the 28th and 48th h of growth were determined in factorial experiments. Complex organic substances, casein at concentrations of 0.5–1%, gelatin at concentrations of 0.5–1%, and yeast extract at a concentration of 0.5%, stimulated the production of subtilisin-like serine proteinase by the recombinant strain. The study of the sporulation dynamics in this strain showed that the proteinase peaks at the 28th and 48th h of growth correspond, respectively, to the initial stage of sporulation and to the terminal stages of endospore formation (V–VII stages of sporulation).

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*Key words*: *Bacillus intermedius*, extracellular subtilisin-like serine proteinase, biosynthesis, sporulation, recombinant strain.

In recent years, there has been increasing interest among researchers in processes associated with the stationary growth phase of bacteria. This phase is distinguished by activation of various signaling systems, which initiate the transcription of specific genes [1]. Under growth-limiting conditions, the general response of a cell population and individual cells is to survive stress and to continue proliferation. For this purpose, bacilli secrete into the medium various proteins, including proteolytic enzymes. Many of these extracellular enzymes, such as subtilisins, are of commercial importance [2]. Subtilisin-like serine proteinases with a broad substrate specificity cleave the bonds between hydrophobic amino acids [3, 4]. It is known that the biosynthesis of subtilisin-like serine proteinases is associated with sporulation in bacilli [5]; however, the functional role of these proteinases in sporulation is far from being well understood. In particular, it is unknown whether subtilisin-like serine proteinases are directly involved in endospore formation or if the processes of proteinase synthesis and sporogenesis are merely regulated by a common mechanism.

Earlier studies showed that the bacterium *Bacillus intermedius* secretes into the culture liquid a subtilisin-like proteinase with maxima of activity at the 24th h of growth (early stationary phase) and 44–46th h of growth (late stationary phase) [6, 7]. The first peak of activity corresponds to the initial stage of sporulation, whereas the second peak corresponds to the stage of endospore maturation and cell autolysis. Experiments with the reporter protein β-galactosidase showed that the occurrence of subtilisin-like proteinase in the medium is due to its secretion from bacterial cells in the late stationary growth phase, rather than to lysis of the cell wall [8]. A comparative analysis of the physicochemical and enzymatic properties of proteinases isolated from the culture liquid in the early and late stationary phases [9, 10] showed that they differ in certain kinetic characteristics and physicochemical properties. Theoretically, these two proteinases may be encoded either by one or by two different genes. Recently, on a multicopy plasmid we cloned the whole gene of subtilisin-like proteinase

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in the *Bacillus subtilis* AJ73 cells, which are deficient in their own extracellular proteinases [11].

The aim of this work was to study the growth, sporogenesis, and biosynthesis of the *B. intermedius* subtilisin-like serine proteinase in the recombinant strain *B. subtilis* AJ73.

## MATERIALS AND METHODS

The recipient strain *Bacillus subtilis* AJ73, which is deficient in its own extracellular proteinases, was kindly provided by Yu. Yomantas from the State Scientific Research Institute of Genetics and Selection of Industrial Microorganisms, Moscow, Russia. This strain was transformed with plasmid pCS9 as described by Anagnostopolous and Spizizen [12]. The multicopy plasmid pCS9 was constructed on the basis of vector pCB22 and carried the entire gene of the *B. intermedius* subtilisin-like serine proteinase [11].

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. 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 carries the erythromycin-resistance gene. The medium was sterilized at 1 atm. Supplementary ingredients were sterilized separately and added to the medium immediately before inoculation. The solution of inorganic phosphate was sterilized at 1 atm, and those of casein, gelatin, and yeast extract were sterilized at 0.5 atm. Yeast extract was purchased from Difco Laboratories; gelatin, from Sigma; casein Hammarsten, from Serva; and peptone, from a plant in Tbilisi.

Bacterial biomass was measured spectrophotometrically at 590 nm.

The activity of subtilisin-like serine proteinase was determined with the chromogenic substrate Z- Ala-Ala-Leu-*p*NA as described by Lyublinskaya et al [13]. One unit of activity was defined as the amount of enzyme that hydrolyzed 1 nmol of substrate per min.

The production efficiency of subtilisin-like serine proteinase was defined as the ratio of proteolytic activity in the culture liquid to the cell mass and expressed in arbitrary units.

The activity of extracellular β-galactosidase was measured according to Miller [14]. The specific β-galactosidase activity was calculated per 1 g of dry biomass.

Endospores were visualized by Gram staining of bacterial cells [15]. Spore-containing cells were counted in five microscopic fields at a magnification of 1600× under a phase-contrast microscope (Carl Zeiss, Jena, Germany). The number of spore-containing cells was expressed as a percentage of the total number of vegetative and sporulating cells. Alternatively, spores were assayed by the method based on their resistance to treatment with chloroform. An aliquot of the *B. subtilis* AJ73(pCS9) culture grown for 28 to 48 h was treated with chloroform and plated onto nutrient agar. The number of colony-forming units (CFUs) was considered to be equal to the total number of spore-containing cells and free spores. The particular stages of sporulation were distinguished as described in [16].

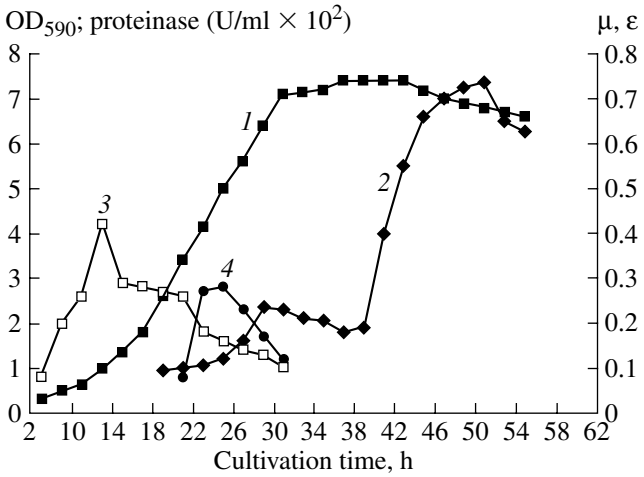


**Fig. 1.** Dynamics of (*1*) growth, (*2*) proteolytic activity, (*3*) specific growth rate (μ), and (*4*) the specific rate of enzyme synthesis (ε) by the recombinant strain *B. subtilis* AJ73(pCS9).

The data obtained were statistically processed using Microsoft Excel. The data were considered to be significant when the standard deviation σ was σ ≤ 15%. The statistical significance of the data was evaluated by using Student's *t*-test for significance level $P \le 0.05$.

The data of factorial experiments were processed using the Statgraphics program, which makes it possible to derive regression equations, to evaluate the significance of tested models, and to obtain graphic images of the response surface.

## RESULTS AND DISCUSSION

The first set of experiments was performed to investigate the dynamics of growth, enzyme accumulation, and spore formation in the recombinant strain *B. subtilis* AJ73(pCS9). The dynamics of enzyme accumulation in the culture liquid of this strain was found to be almost the same as in the parent strain *B. intermedius* 3-19 [7]: there were two peaks of proteolytic activity, which corresponded to the early and late periods of the stationary growth phase. The second peak was three times higher than the first peak (Fig. 1). The specific accumulation rate of subtilisin-like serine proteinase expressed in U/h (symbolized ε) increased as the specific growth rate decreased (Fig. 1). Unlike the parent strain *B. subtilis* 3-19, which shows the first peak of proteolytic activity at the 24th h of cultivation [6], the recombinant strain *B. subtilis* AJ73(pCS9) showed the

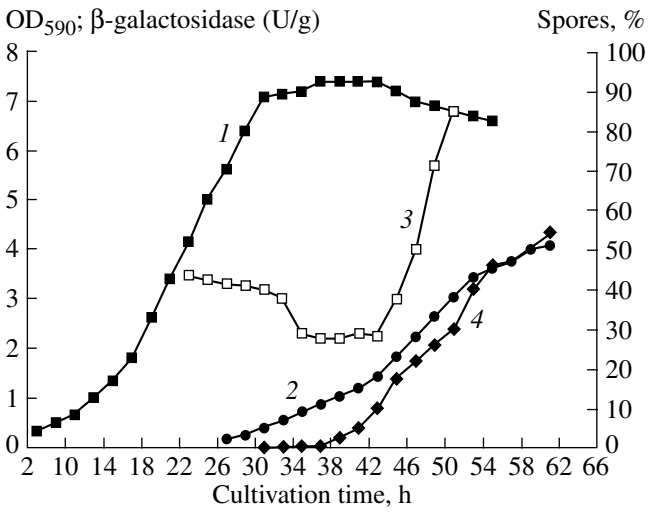


**Fig. 2.** Dynamics of (*1*) growth, (*2*) spore formation, (*3*) β-galactosidase activity, and (*4*) the number of chloroform-resistant spores in the recombinant strain *B. subtilis* AJ73(pCS9).

first peak of subtilisin-like serine proteinase at the 28th h of cultivation. The second peak of enzyme activity (subtilisin-like proteinase 2) is observed on the 48th h of cultivation. We believe that the proteinases of both peaks are encoded by the same gene, whose expression is regulated differently in different growth stages.

The study of spore formation in the recombinant strain (Fig. 2) showed the presence of only a few mature spores in the 30-h-old culture. After 40 h of growth, the number of spores substantially increased to reach a relative value of ~50% by the 50th h of cultivation. This time corresponded to V–VII stages of endospore formation, which are characterized by the synthesis of spore envelopes, endospore maturation, the autolysis of sporangium, and the subsequent release of mature spores into the medium. The assay of the reporter enzyme β-galactosidase, which is an intracellular protein and appears in the culture liquid due to cell lysis, showed that the activity of this enzyme in the culture liquid remained at a low level (2–4 U/g) until the onset of cell lysis and the release of endospores into the medium (44 h of growth). This observation was also confirmed by the culture microscopy and the enumeration of chloroform-resistant cell forms (Fig. 2). After 44 h of cultivation, the activity of β-galactosidase in the culture liquid gradually increased to 6 U/g. These data suggest that bacterial cells in the culture liquid remained intact for as long as 44 h of cultivation and that the first peak of extracellular proteolytic activity corresponds to the initial stage of spore formation, whereas the second peak corresponds to the stage of endospore maturation and sporangium autolysis. At the stage of release of mature endospores, subtilisin-like proteinases may participate in the cleavage of cell wall proteins, favoring the release of spores into the medium.

It is known that recombinant strains typically require more nitrogen and phosphorus for growth than do wild strains. The effect of the main sources of these elements in the nutrient medium (peptone and inorganic phosphate) on the biosynthesis of the subtilisin-like serine proteinase in the early and late periods of the stationary phase was studied in two-factor experiments. The concentrations of peptone and inorganic phosphate that are optimal for the biosynthesis of subtilisin-like serine proteinase were determined in factorial experiments designed according to the two-factor scheme B2. The results of these experiments were processed using the Statgraphics program. Two variables (the concentrations of peptone and inorganic phosphate) took three levels. The data obtained for the biomass (estimated as the culture turbidity), proteolytic activity, and the productivity of enzyme synthesis are presented in Table 1 (data for the 28th h of cultivation) and Table 2 (data for the 48th h of cultivation). The data presented in these tables are the means of triplicate experiments. Regression equations 1 and 2 for the activity of subtilisin-like

**Table 1.** Optimization of nutrient medium for the production of the *B. intermedius* subtilisin- like serine proteinase by the recombinant strain *B. subtilis* AJ73(pCS9) in the early stationary phase (28 h of growth)

| Factor levels | | | | Biomass (culture turbidity) | Proteinase activity, U/ml | Productivity, U/g dry cells |
|---|---|---|---|---|---|---|
| Peptone | | Inorganic phosphate | | | | |
| $X_1$ | g/l | $X_2$ | g/l | | | |
| 0 | 30 | 0 | 0.2 | 17 | 0.98 | 0.058 |
| – | 20 | 0 | 0.2 | 8 | 0.7 | 0.087 |
| 0 | 30 | + | 0.3 | 11.3 | 0.99 | 0.088 |
| – | 20 | – | 0.1 | 9.4 | 0.22 | 0.023 |
| – | 20 | + | 0.3 | 10 | 0.59 | 0.059 |
| + | 40 | – | 0.1 | 13.4 | 0.22 | 0.016 |
| + | 40 | + | 0.3 | 17 | 0.69 | 0.04 |
| 0 | 30 | – | 0.1 | 15 | 0.42 | 0.028 |
| + | 40 | 0 | 0.2 | 18 | 0.55 | 0.03 |

**Table 2.** Optimization of nutrient medium for the production of the *B. intermedius* subtilisin- like serine proteinase by the recombinant strain *B. subtilis* AJ73(pCS9) in the late stationary phase (48 h of growth)

| Factor levels | | | | Biomass (culture turbidity) | Proteinase activity, U/ml | Productivity, U/g dry cells |
|---|---|---|---|---|---|---|
| Peptone | | Inorganic phosphate | | | | |
| $X_1$ | g/l | $X_2$ | g/l | | | |
| 0 | 30 | 0 | 0.2 | 17 | 1.3 | 0.07 |
| – | 20 | 0 | 0.2 | 7.7 | 1.01 | 0.13 |
| 0 | 30 | + | 0.3 | 8.8 | 1.32 | 0.15 |
| – | 20 | – | 0.1 | 5.3 | 0.56 | 0.1 |
| – | 20 | + | 0.3 | 10.4 | 1.09 | 0.1 |
| + | 40 | – | 0.1 | 17.6 | 0.67 | 0.03 |
| + | 40 | + | 0.3 | 16 | 0.99 | 0.06 |
| 0 | 30 | – | 0.1 | 12.4 | 0.77 | 0.062 |
| + | 40 | 0 | 0.2 | 15.5 | 0.95 | 0.06 |

serine proteinase in the 28- and 48-h-old cultures, which were derived from the data presented in Tables 1 and 2, have the form

$$Y = -2.89 + 0.169X_1 + 10.8X_2 - 0.0029X_1^2 + 0.025X_1X_2 - 23.166X_2^2, \quad (1)$$

$$Y = -2.416 + 0.152X_1 + 11.575X_2 - 0.00238X_1^2 + 0.0525X_1X_2 - 19.33X_2^2. \quad (2)$$

The presence of negative square and positive linear terms in these equations indicates favorable effects of both factors tested.

The results of these experiments are presented in Fig. 3 as the isolines of subtilisin-like proteinase activity. The optimal concentrations of peptone and inorganic phosphate for the total enzyme activity and the enzyme biosynthesis after 28 and 48 h of cultivation were found to be, respectively, 30 and 29 g/l peptone and 0.24 and 0.26 g/l inorganic phosphate. For comparison, the optimal concentrations of peptone for the production of subtilisin-like proteinases 1 (24 h of growth) and 2 (48 h of growth) in the parent strain *B. intermedius* 3-19 are, respectively, 20 and 22 g/l peptone [6].

It is known that the addition of complex organic substances to nutrient media favorably influences enzyme biosynthesis [17]. Our earlier experiments also showed that the addition of casein at a concentration of 1% and gelatin at a concentration of 0.5% to the nutrient medium of *B. intermedius* 3-19 favorably affects the biosynthesis of subtilisin-like proteinase by this strain, although these substances at a higher concentration (2%) diminished both the absolute and specific proteinase activities [6, 7]).

Similar experiments with *B. subtilis* AJ73(pCS9) showed that a gelatin concentration of 1% is optimal for the production of subtilisin-like proteinase in the 28-h-old culture (an 80% increase in production), whereas a gelatin concentration of 0.5% is optimal for the production of subtilisin-like proteinase in the late stationary phase (a 50% increase in production) (Fig. 4). At higher gelatin concentrations, the specific activity of subtilisin-like proteinase decreased. Casein at concentrations of 0.5 and 1% augmented the enzyme production in the

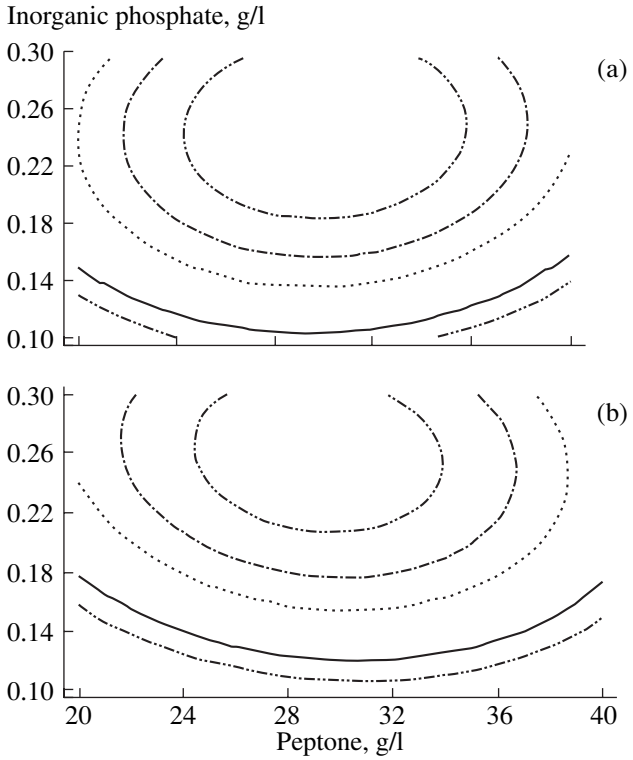


**Fig. 3.** The isolines of activity of subtilisin-like serine protei nase in the culture liquid of the recombinant strain *B. subtilis* AJ73(pCS9) after (a) 28 h and (b) 48 h of growth. The isolines were plotted on the basis of two-factor experiments.

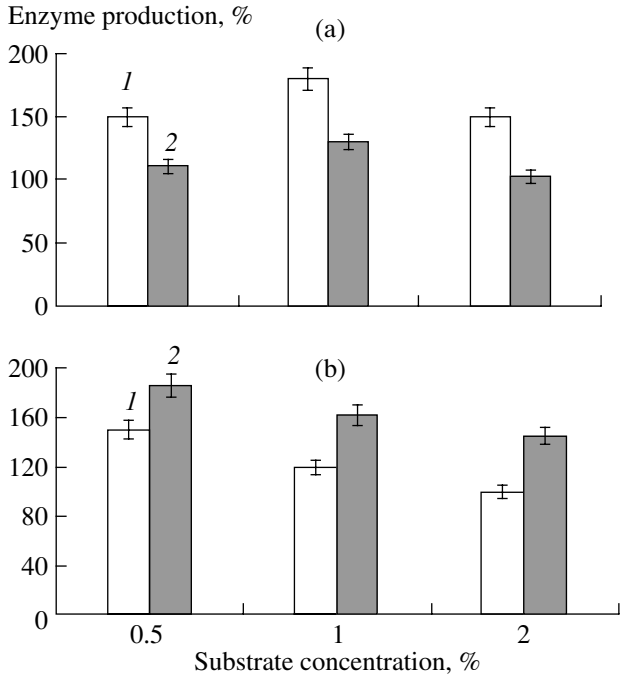


**Fig. 4.** The effect of (*1*) gelatin and (*2*) casein on the production of subtilisin-like serine proteinase by the recombinant strain *B. subtilis* AJ73(pCS9) in (a) the early (28 h) and (b) late (48 h) stationary phase. The production of proteinase in the medium without these substances was taken as 100%.

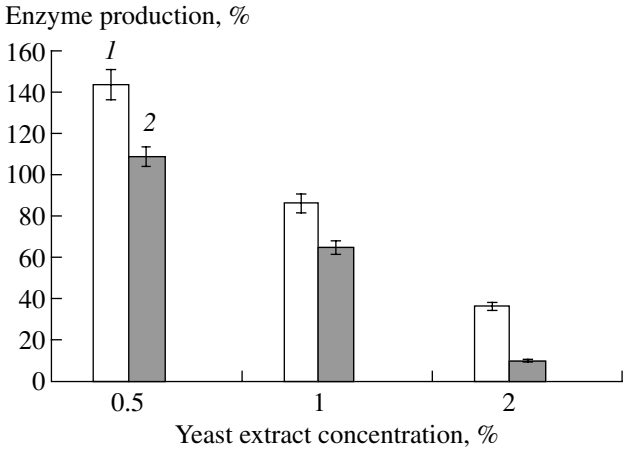


**Fig. 5.** The effect of yeast extract on the production of subtilisin-like serine proteinase by the recombinant strain *B. subtilis* AJ73(pCS9) in (*1*) the early (28 h) and (*2*) late (48 h) stationary phase. The production of proteinase in the medium without yeast extract was taken as 100%.

early stationary phase only by 10 and 30%, respectively. At the same time, 0.5% casein enhanced the production of subtilisin-like proteinase in the late stationary phase by 80% (Fig. 4).

Thus, the complex organic substances gelatin and casein added to the medium at moderate concentrations increase the production of subtilisin-like proteinase by the recombinant strain *B. subtilis* AJ73(pCS9). The increase in the enzyme production is higher in the late (48 h of growth) than in the early (28 h of growth) periods of the stationary phase, which can be accounted for by different regulation of the biosynthesis of subtilisin-like proteinase during these periods of growth.

In order to improve the production of proteinases, the nutrient media of producing strains are often supplemented with corn or yeast extracts, which are valuable sources of growth factors, such as vitamins, amino acids, peptides, and microelements [17]. The addition of yeast extract at a concentration of 0.5% to the cultivation medium of *B. subtilis* AJ73(pCS9) increased the production of subtilisin-like proteinase by approximately 50% in the early stationary phase and only slightly in the late period of this stage. It should be noted that this concentration of yeast extract is inhibitory (approximately by 2 times) to the biosynthesis of subtilisin-like proteinase by the parent strain *B. intermedius* 3-19 [18]. Yeast extract at a concentration of 2% unfavorably influenced the production of proteinase by both the recombinant and parent strains (Fig. 5).

Thus, the accumulation dynamics of subtilisin-like serine proteinase in the culture liquid of the recombinant strain *B. subtilis* AJ73(pCS9) is similar to that in the parent strain *B. intermedius* 3-19. The two peaks of proteinase activity in the culture liquid correspond to the early and late periods of the stationary phase. The enzyme production during these periods depends on the same factors that determine the biosynthesis of other serine proteinases, including the subtilisin-like serine proteinase of the parent strain. In the case of the recombinant strain, the optimal concentrations of nutrient substances are different for the biosynthesis of subtilisin-like serine proteinase in different growth phases. The enrichment of the cultivation medium of the recombinant strain in phosphorus, nitrogen, and carbon sources, as well as in yeast extract, as the source of vitamins and other biologically active substances favorably influences strain growth and enzyme production. The maximal production of subtilisin-like proteinase by the recombinant strain is observed at a higher peptone concentration than in the case of the parent strain. The addition of casein and gelatin to the cultivation medium of the recombinant strain also stimulates proteinase. These data can be used for optimizing the cultivation medium for the recombinant strain *B. subtilis* AJ73(pCS9) in order to enhance the synthesis of subtilisin-like serine proteinase and thereby facilitate its isolation from the culture liquid in a pure form.

## ACKNOWLEDGMENTS

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