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EXPERIMENTAL ARTICLES

Optimization of Cultivation Medium for the Production of *Bacillus intermedius* 3-19 Glutamyl Endopeptidase

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Abstract—The effect of some components of cultivation medium on the growth of the streptomycin-resistant *Bacillus intermedius* strain 3-19 and on the production of glutamyl endopeptidase was investigated using factorial experimental design, which allowed the concentrations of peptone and inorganic phosphate to be optimized for the maximum production of the enzyme. Experiments with different peptones and casamino acids showed that the enzyme production is maximum with peptone 3 of plant origin. The addition of casamino acids or amino acids to the peptone-containing cultivation medium inhibited the production of glutamyl endopeptidase.

Key words: glutamyl endopeptidase, spore-forming bacteria, amino acids, biosynthesis, factorial experimental design.

Proteolytic enzymes are vitally important to the living cell. They are involved in catabolic processes, the post-translational processing of proteins, and cell differentiation. In prokaryotes, they take part in spore formation and cell dormancy, implementing the selective proteolysis of certain proteins. Proteolytic enzymes with a narrow substrate specificity are widely used in molecular biology to study the primary structure of proteins and peptides.

Bacillus intermedius synthesizes several serine proteinases, one of which is glutamyl endopeptidase (EC 3.4.21.19), an enzyme cleaving peptide bonds formed by the α-carboxyl groups of glutamic and aspartic amino acid residues [3, 6].

Glutamyl endopeptidases, which can be considered as a separate evolutionary branch of proteolytic enzymes, are widely spread among bacteria, including *Staphylococcus aureus, Bacillus licheniformis, B. subtilis, B. intermedius, Streptomyces griseus, Str. fradiae, Str. thermovulgaris, Actinomyces* sp., and *Thermoactinomyces* sp. [1]. In spite of the fact that the structural, physicochemical, and catalytic properties of glutamyl endopeptidases have been extensively studied [1–3], the function of these enzymes in bacteria remains obscure. In *S. aureus*, glutamyl endopeptidase serves as an epidermolytic toxin [2].

The regulation of the synthesis of glutamyl endopeptidases is also poorly studied, while the understanding of the regulation mechanism may provide insight into the functional role of these enzymes in bacterial cells, including their role in cell differentiation and adaptation.

Our recent study [4] dealt with the effect of cultivation conditions on the biosynthesis and localization of *B. intermedius* 3-19 glutamyl endopeptidase. The aim of the present work was to optimize the cultivation medium of *B. intermedius* 3-19 for the maximum production of the enzyme.

MATERIALS AND METHODS

The streptomycin-resistant strain *Bacillus intermedius* 3-19 was derived from the wild-type *B. intermedius* strain 7P (the collection of microorganisms of the Department of Microbiology, Kazan State University) through selection for resistance to 500 μg/ml streptomycin and maximum proteolytic activity. The strain was deposited in the All-Russia Collection of Industrial Microorganisms as strain B-3833.

Basal medium for the cultivation of this strain contained (%) peptone, 2; $CaCl_2 \cdot 2H_2O$, 0.06; $MgSO_4 \cdot 7H_2O$, 0.05; NH_4Cl, 0.02; NaCl, 0.3; and $MnSO_4$, 0.01 (pH 8.5). The medium was sterilized at 1 atm. Some ingredients of the cultivation medium were sterilized separately and were added to the medium immediately before inoculation. These were solutions of inorganic phosphate (Na_2HPO_4) and yeast extract (Serva), which were sterilized at 1 and 0.5 atm, respectively. Yeast extract was added to the medium at concentrations of 0.5, 1, or 2%. Peptone was obtained from three manufacturers, meat processing plants in Semipalatinsk (peptone 1) and Vinnytsya (peptone 2) and a factory in

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Table 1. The composition of peptones used in the work

Component	Content, %		
	Peptone 1	Peptone 2	Peptone 3
Oligopeptides	95	89	71
Total nitrogen	14	14.5	13.5
Amines	3.8	3.4	3
Sulfate ashes	3	4.4	5.8
Chlorides	0.85	0.7	0.8
Total phosphorus	0.17	0.18	0.29
Inorganic phosphorus	0.005	0.01	0.005

Tbilisi (peptone 3 of plant origin). Solutions of amino and casamino acids were also sterilized separately. Amino acids in L- and D,L-form were added to the cultivation medium at concentrations of 0.05 and 0.1 mg/ml, respectively. Casamino acids were added at concentrations of 0.5, 1, and 2%.

The strain was cultivated at 30°C in flasks 20% full of the growth medium on a shaker (200 rpm). The medium was inoculated with 1 vol % of a 12-h-old culture grown in the presence of 500 μg/ml streptomycin. Total phosphorus content in peptone was determined by the commonly accepted method [5]. Inorganic phosphorus was determined as described earlier [6]. Bacterial growth was monitored by measuring culture turbidity at 590 nm using a KFK-2 spectrophotometer and a 1-cm pathlength cuvette.

Proteolytic activity was determined as described in [3], with *N*-carbobenzoxy-L-glutamic acid *p*-nitroanilide (*N*-CBZ-Glu *p*-nitroanilide). One unit of proteolytic activity was defined as the amount of enzyme that hydrolyzed 1 nmol of the substrate per 1 min. The efficiency of enzyme production was defined as the ratio of the proteolytic activity of the culture liquid to the biomass and was expressed in arbitrary units.

Data were statistically processed as described in the handbook [7] using BIOPT software [8].

RESULTS AND DISCUSSION

The accumulation of extracellular enzymes in the cultivation medium considerably depends on its composition. In particular, a high content of complex organic substrates, such as peptone, casein hydrolysate, and yeast extract, enhances bacterial growth and the biosynthesis of extracellular proteinases [6, 9]. This can be explained by a good assimilation of physiologically active compounds, microelements, and other nutrients present in such substrates and their beneficial effect on biosynthetic processes in bacterial cells.

Peptones prepared from different raw materials by different manufacturers considerably differ in the content of peptides and mineral salts, which may influence the growth of bacteria and enzyme production. According to manufacturer's information, peptones 1 and 2 differ insignificantly. At the same time, peptone 3 contains 20–26% less oligopeptides and 1.6 times more total phosphorus than peptones 1 and 2 (Table 1).

Bearing this in mind, we attempted to determine the type of peptone, its concentration, and the concentration of inorganic phosphate that provided for the most efficient production of *B. intermedius* 3-19 glutamyl endopeptidase using the factorial experimental design B2. Factors were tested at three levels. The experimental design characteristics, tested variables, and the values of biomass (expressed in optical density units), proteinase activity, and the efficiency of enzyme production averaged over triplicate measurements for each peptone type are summarized in Tables 2–4. Data processing using BIOPT software allowed the following regression equations with a confidence level of 95% to be obtained:

Peptone 1: $Y = 15 - 5X_1^2 - 2.7X_2^2$;

Peptone 2: $Y = 12.5 - 3.6X_1^2 - 0.4X_2^2 + 1.5X_1X_2$;

Peptone 3: $Y = 23.95 - 11.6X_1^2 - X_2^2$.

The absence of linear coefficients with respect to X_1 and X_2 in these equations indicates that the concentrations of peptone (X_1) and inorganic phosphate (X_2) that are optimal for the synthesis of glutamyl endopeptidase are within the tested ranges of the variables. The optimal concentrations of peptone 1 and inorganic phosphate (X_1 = 20 g/l and X_2 = 0.2 g/l, respectively) correspond to a theoretical maximum of glutamyl endopeptidase activity, Y = 14.6 U/ml. The optimal concentrations of peptones 2 and 3 are the same as that of peptone 1; however, they correspond to different theoretical maxima of enzymatic activity, Y = 12.5 and 24 U/ml, respectively. The results of these bifactorial experiments are presented graphically in Figs. 1a–1c as Y isolines on the inorganic phosphate–peptone response surface. As is evident from these figures, the graphical concentration optima of inorganic phosphate and peptone correspond to the theoretical maxima of the glutamyl endopeptidase activity. Peptone 3 is the best source of carbon and nitrogen for enzyme production, since it provides for about two times higher glutamyl endopeptidase activity in the cultivation medium than peptones 1 and 2 (Tables 2–4).

In the case of peptone 3, the glutamyl endopeptidase activity (as well as the efficiency of enzyme production) is close to maximal at peptone concentrations of 19 to 21 g/l and at inorganic phosphate concentrations of 0.17 to 0.23 g/l. Peptone 3 favorably differs from peptones 1 and 2 in that it contains a greater amount of inorganic phosphate than the two other peptones. However, for the efficient production of glutamyl endopeptidase, the cultivation medium of *B. intermedius* 3-19 should be additionally supplemented with 0.2 g/l inorganic phosphate. As was shown earlier, if the cultivation medium of *B. intermedius* does not contain inor-

Table 2. The optimization of peptone 1–containing nutrient medium for the biosynthesis of glutamyl endopeptidase by *B. intermedius* 3-19 using the factorial experimental design B2

Factor level				Biomass, OD units	Glutamyl endopeptidase, U/ml	Productivity
peptone		inorganic phosphate				
X_1	g/l	X_2	g/l			
+	30	+	0.3	12.5	6.8	0.54
–	10	+	0.3	5.9	8.0	1.33
+	30	–	0.1	11.4	7.6	0.67
–	10	–	0.1	5.3	6.1	1.16
+	30	0	0.2	11.9	8.6	0.72
–	10	0	0.2	5.3	11.0	2.11
0	20	+	0.3	9.9	12.8	1.28
0	20	–	0.1	8.9	11.0	1.25

Table 3. The optimization of peptone 2–containing nutrient medium for the biosynthesis of glutamyl endopeptidase by *B. intermedius* 3-19 using the factorial experimental design B2

Factor level				Biomass, OD units	Glutamyl endopeptidase, U/ml	Productivity
peptone		inorganic phosphate				
X_1	g/l	X_2	g/l			
+	30	+	0.3	12.2	7.7	0.63
–	10	+	0.3	6.6	6.02	0.91
+	30	–	0.1	11.2	8.17	0.73
–	10	–	0.1	5.3	12.04	2.27
+	30	0	0.2	11.2	8.17	0.73
–	10	0	0.2	5.3	9.63	1.83
0	20	+	0.3	8.6	12.10	1.41
0	20	–	0.1	8.6	10.58	1.23

Table 4. The optimization of peptone 3–containing nutrient medium for the biosynthesis of glutamyl endopeptidase by *B. intermedius* 3-19 using the factorial experimental design B2

Factor level				Biomass, OD units	Glutamyl endopeptidase, U/ml	Productivity
peptone		inorganic phosphate				
X_1	g/l	X_2	g/l			
+	30	+	0.3	14.0	10.7	0.76
–	10	+	0.3	5.5	9.0	1.64
+	30	–	0.1	12.5	11.5	0.92
–	10	–	0.1	5.9	14.6	2.47
+	30	0	0.2	13.9	12.0	0.86
–	10	0	0.2	5.3	12.8	2.42
0	20	+	0.3	12.0	23.8	1.98
0	20	–	0.1	10.9	22.2	2.04

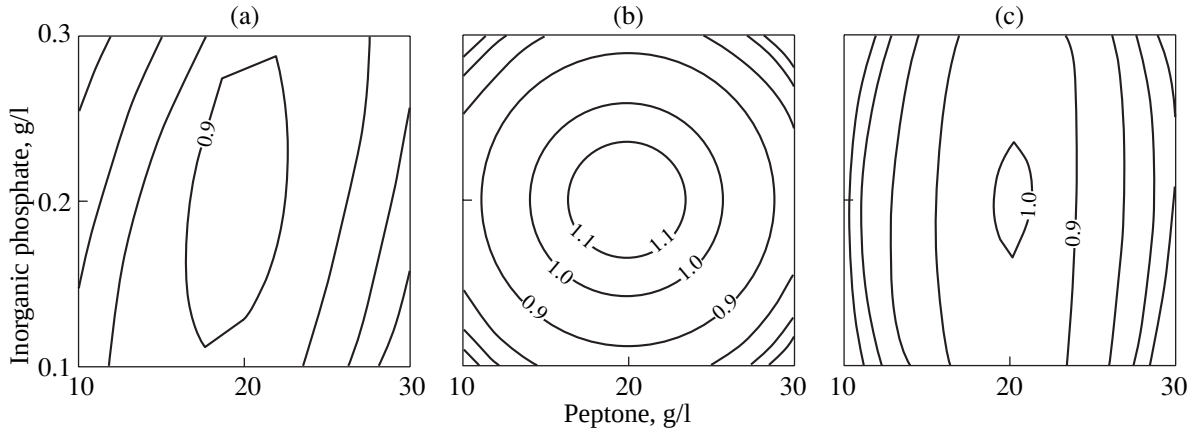

Fig. 1. The glutamyl endopeptidase isolines in bifactorial experiments with (a) peptone 1, (b) peptone 2, and (c) peptone 3. The maximal activity of glutamyl endopeptidase in each experiment was taken to be unity.

ganic phosphate, the content of proteinase in the cell walls increases, while its content in the culture liquid decreases [10]. Presumably, inorganic phosphate influences the secretion of hydrolases from bacterial cells.

Taking into account the observation that the presence of corn or yeast extract in the cultivation media of bacteria promotes the biosynthesis of proteinases [9, 11], we investigated the effect of yeast extract, as a supplementary source of vitamins, amino acids, peptides, and microelements, on the synthesis of glutamyl endopeptidase by *B. intermedius* 3-19. Experiments showed that yeast extract at concentrations of 0.5, 1, and 2% enhanced bacterial growth and the total glutamyl endopeptidase activity in the culture liquid. However, the productivity of enzyme synthesis per unit biomass did not change (Fig. 2).

Proteolytic enzymes present in the culture liquid may produce amino acids from proteinaceous substrates. These amino acids or those originally present in the cultivation medium can inhibit the biosynthesis of

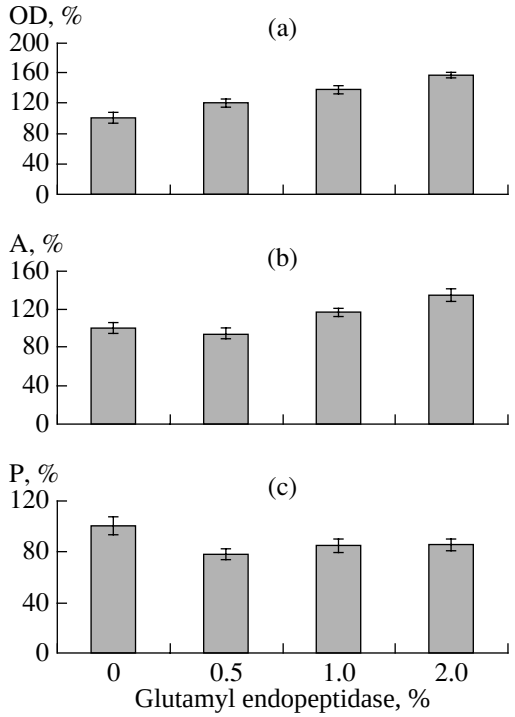

Fig. 2. The effect of yeast extract on the (a) biomass, (b) glutamyl endopeptidase activity, and (c) productivity of enzyme synthesis by *B. intermedius* 3-19. The biomass, enzymatic activity, and productivity of strain 3-19 in the absence of yeast extract in the cultivation medium were taken to be 100%.

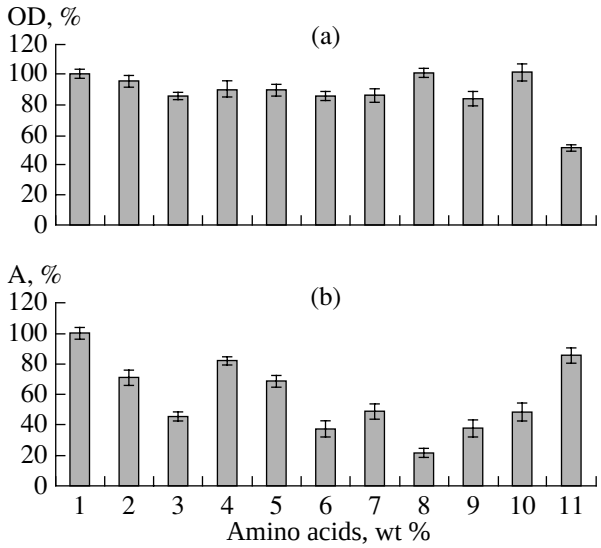

Fig. 3. The effect of (2) alanine, (3) glycine, (4) cysteine, (5) asparagine, (6) glutamine, (7) aspartic acid, (8) glutamic acid, (9) tryptophan, (10) histidine, and (11) casamino acids on the (a) biomass and (b) glutamyl endopeptidase activity of *B. intermedius* 3-19. The biomass and the glutamyl endopeptidase activity of strain 3-19 without the addition of amino and casamino acids to the cultivation medium (control 1) are taken to be 100%.

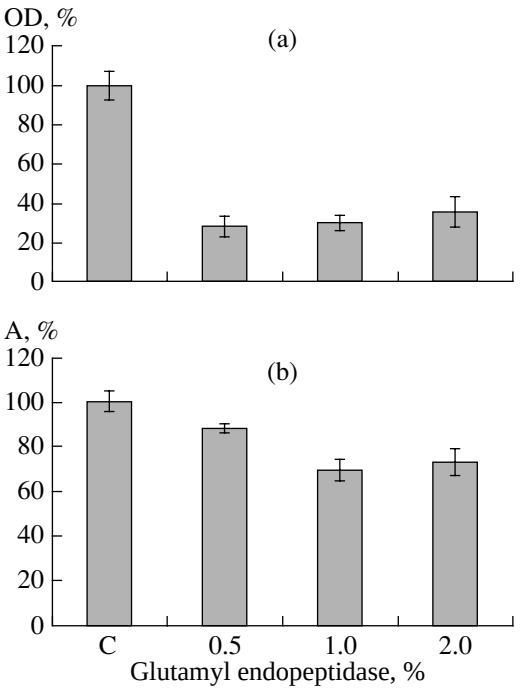

Fig. 4. (a) Biomass and (b) glutamyl endopeptidase activity of *B. intermedius* 3-19 in the cultivation medium with casamino acids as the sole source of carbon and nitrogen. The bars marked by the letter C show the biomass and glutamyl endopeptidase activity in the control cultivation medium containing 2% peptone as the source of carbon and nitrogen.

the enzymes by a feedback mechanism, as was shown for the exoproteases of *Aspergillus candidus* and the intra- and extracellular proteinases of *Bacillus megaterium* [12, 13].

Bearing this in mind, we investigated the effect of individual amino acids (alanine, glycine, cysteine, tyrosine, histidine, asparagine, glutamine, aspartic acid, and glutamic acid) and casamino acids (a mixture of amino acids present in the complete casein acid hydrolysate) on the synthesis of glutamyl endopeptidase by *B. intermedius* 3-19. As can be seen from Fig. 3, the addition of casamino acids to the cultivation medium of strain 3-19 inhibited its growth by about two times and decreased the glutamyl endopeptidase activity of the culture liquid by 15%. Individual amino acids (particularly glutamic acid), as well as their mixtures, also inhibited the biosynthesis of the enzyme, which is in agreement with the data available in the literature [14].

Experiments in which casamino acids served as the sole source of carbon and nitrogen showed that they provided for a 3.5 times poorer growth of *B. intermedius* 3-19 and a 15–20% slower synthesis of glutamyl endopeptidase than the peptones (Fig. 4). Although the productivity of enzyme synthesis in this case increased (due to a low bacterial biomass), the yield of glutamyl endopeptidase was considerably lower than in the peptone-containing medium. A similar effect was observed earlier for the serine proteinase of *B. intermedius* and for the acid proteinase of *Candida albicans* [6, 15].

To conclude, the optimal concentrations of peptone 3 and inorganic phosphate for the production of glutamyl endopeptidase by *B. intermedius* 3-19 are 20 and 0.2 g/l, respectively. The addition of amino acids to the peptone-containing cultivation medium was found to inhibit the biosynthesis of the enzyme. The substitution of peptone for casamino acids as the sole source of carbon and nitrogen is inexpedient, since such a substitution slows down the synthesis of glutamyl endopeptidase.

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