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FORMATION OF EXTRA- AND INTRACELLULAR AMINO ACIDS BY MICROMYCETES

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A comparative study has been made of extra- and intracellular amino acids in the dynamics of the growth and development of the cellulolytically active fungus Trichoderma harzianum *and of a protoplast culture and a hybrid culture of the fungus obtained by the methods of cell engineering.*

Among biochemical processes in a natural medium the synthesis of amino acids by micromycetes is of great importance. A capacity of various microorganisms for synthesizing and accumulating amino acids intracellularly and also of producing them during their growth and development in a nutrient medium has been found [1-3]. In view of this, it appeared of interest to investigate the compositions of the extra- and intracellular amino acids in the cellulolytically active fungus *Trichoderma harzianum* and also in a protoplast culture and a hybrid culture of the fungus obtained by the methods of cell engineering.

We have carried out a comparative study of the dynamics of the accumulation of amino acids in the culture liquid and the mycelium of the above-mentioned culture. The results of the investigation are presented in Tables 1-6.

It was established that *Trichoderma harzianum* produced the following amino acids: lysine, arginine, aspartic acid, serine, leucine, alanine, and methionine. The maximum amount of amino acids was observed on the eighth day of growth and amounted in total to 117.0 μg/ml. The protoplast culture of *Trichoderma harzianum* secreted into the culture liquid lysine, arginine, aspartic acid, serine, glycine, alanine, methionine, leucine, and tyrosine, and the hybrid culture secreted lysine, arginine, aspartic acid, serine, glycine, alanine, methionine, leucine, and tyrosine.

With respect to their qualitative composition, the amino acids of the culture liquid taken for investigating the culture had similarities and differences. The common properties must be taken to include the fact that the micromycetes studied produced seven identical amino acids: lysine, arginine, aspartic acid, serine, leucine, alanine, and methionine. The maximum amounts of these amino acids were observed, in the main, on the sixth-eighth day of growth of the micromycetes, although for each strain the percentage of the combined sum of amino acids changed with the age of the culture. For example, on the sixth day of growth the hybrid *Trichoderma harzianum* culture formed the maximum combined sum of amino acids — 267.3 μg/liter, while the greatest formation of glycine and tyrosine, at 27.4 and 11.4 μg/liter, respectively, was observed on the fourth day.

In the case of the hybrid culture of *Trichoderma harzianum,* the pattern of accumulation of lysine and methionine was as follows. The culture began to produce these amino acids on the second day of growth, the amounts of methionine and lysine in the culture liquid then amounting to 2.3 and 3.7%, respectively, of the combined sum of the amino acids. As follows from Tables 1-3, their maximum accumulation in the culture liquid took place on the sixth to eighth days of growth. The percentages of these amino acids in the combined sum of amino acids on the sixth day of growth amounted to 11.4 and 9.6%.

It was noted that, together with a capacity for producing extracellular amino acids, the cultures investigated could accumulate amino acids in the cells of the mycelium, and these in considerably greater number than in the culture liquid. Thus, the protoplast culture of *Trichoderma harzianum* synthesized 16 amino acids in the mycelium, and secreted only 9 into the culture liquid (Tables 2 and 5).

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TABLE 1. Dynamics of the Formation of Amino Acids in a Culture Liquid of the Initial Fungus *Trichoderma harzianum*

Amino acid	Days											
	second		fourth		sixth		eighth		tenth		twelfth	
	μg/liter	%	μg/liter	%	μg/liter	%	μg/liter	%	μg/liter	%	μg/liter	%
1. Lys	0.3	1.12	1.4	2.2	3.2	2.90	2.4	2.05	1.6	1.70	1.0	1.3
2. Arg	4.5	16.7	15.0	22.8	26.0	23.80	31.0	26.40	22.0	23.80	18.0	24.3
3. Asp	6.2	23.1	10.4	15.7	18.0	16.50	16.2	13.844	9.4	10.20	8.4	11.3
4. Ser	–	–	11.0	16.7	16.0	14.60	22.0	18.80	19.0	20.39	13.0	17.6
5. Leu	–	–	3.1	4.7	8.4	7.80	8.4	7.50	11.2	11.72	9.2	12.3
6. Ala	8.4	31.38	13.8	20.9	16.0	14.60	21.0	17.90	17.0	18.20	15.0	20.3
7. Met	7.4	27.6	11.2	16.9	21.3	19.51	16.0	13.60	13.2	14.10	9.6	12.9
Sum of the amino acids, μg/liter	26.8	100.0	65.9	100.0	108.9	100.00	117.0	100.00	93.4	100.00	74.2	100.0

TABLE 2. Dynamics of the Formation of Amino Acids in the Culture Liquid of a Protoplast Culture of the Fungus *Trichoderma harzianum*

Amino acid	Days											
	second		fourth		sixth		eighth		tenth		twelfth	
	μg/liter	%	μg/liter	%	μg/liter	%	μg/liter	%	μg/liter	%	μg/liter	%
1. Lys	1.2	2.9	1.6	2.1	2.4	1.7	1.7	1.2	1.0	0.8	0.6	0.6
2. Arg	–	–	–	–	7.4	5.2	14.2	9.8	10.4	8.9	8.6	9.2
3. Asp	16.4	40.0	21.2	29.0	42.44	30.0	40.0	27.4	32.4	27.8	26.0	27.6
4. Ser	9.6	23.5	14.0	19.2	22.0	15.6	21.4	14.6	17.4	14.9	13.2	14.0
5. Gly	–	–	12.0	16.4	24.4	17.2	28.0	19.2	21.2	18.4	17.4	18.5
6. Ala	3.6	8.8	5.7	7.79	6.3	4.5	9.2	6.3	10.4	8.9	8.4	8.9
7. Met	8.6	21.0	14.0	19.2	21.4	15.1	16.0	10.9	11.0	9.4	8.7	9.3
8. Leu	1.5	3.8	3.2	4.4	7.4	5.5	6.0	4.2	4.8	4.1	4.2	4.4
9. Tyr	–	–	1.5	2.0	7.4	5.2	9.3	6.4	8.0	6.8	7.2	7.6
Sum, μg/liter	40.9	100.0	73.2	100.0	141.2	100.00	145.8	100.00	116.6	100.00	94.3	100.0

TABLE 3. Dynamics of the Formation of Amino Acids in the Culture Liquid of a Hybrid Culture of the Fungus *Trichoderma harzianum*

Amino acid	Days											
	second		fourth		sixth		eighth		tenth		twelfth	
	mg/liter	%	mg/liter	%	mg/liter	%	mg/liter	%	mg/liter	%	mg/liter	%
1. Lys	1.4	2.2	6.8	4.4	10.2	3.8	8.6	3.8	8.6	3.8	6.4	4.0
2. Arg	–	–	15.4	9.9	22.5	8.4	20.4	7.6	17.4	7.7	7.3	4.5
3. Asp	26.2	41.2	44.0	28.4	82.4	30.8	74.0	27.8	62.0	27.5	42.0	27.5
4. Ser	17.0	26.7	32.4	20.8	62.0	23.1	60.4	22.8	48.2	21.4	36.2	22.6
5. Gly	–	–	22.4	14.4	27.4	10.2	33.0	12.4	30.4	13.5	21.2	13.2
6. Ala	5.4	8.5	7.6	4.9	9.4	3.5	14.0	5.2	18.7	8.2	18.2	11.3
7. Met	2.4	3.8	4.6	2.9	9.6	3.6	8.2	3.2	6.4	2.8	4.1	2.6
8. Leu	11.2	17.6	19.4	12.5	31.2	11.6	28.0	10.6	21.0	9.3	16.2	10.1
9. Tyr	–	–	2.7	1.8	11.4	4.6	17.4	6.6	13.0	5.8	8.6	5.2
Sum of the amino acids, mg/liter	63.6	100.0	155.3	100.0	267.3	100.00	265.6	100.00	225.2	100.00	160.2	100.0

TABLE 4. Dynamics of the Accumulation of Amino Acids in the Mycelium of the Initial *Trichoderma harzianum* Culture

Amino acid	Days											
	second		fourth		sixth		eighth		tenth		twelfth	
	μg/liter	%	μg/liter	%	μg/liter	%	μg/liter	%	μg/liter	%	μg/liter	%
1. Lys	1.42	6.90	1.96	8.00	2.18	7.52	3.04	9.10	2.34	8.2	2.08	8.4
2. His	2.12	10.40	2.44	9.90	2.86	9.80	3.22	9.62	3.02	10.5	2.74	11.30
3. Arg	1.34	6.50	1.78	7.27	2.16	7.50	2.84	8.41	2.12	7.31	1.96	7.92
4. Asp	1.28	6.20	1.54	6.29	1.76	6.12	2.04	6.12	1.88	6.5	1.42	5.75
5. Thr	2.04	9.90	2.28	9.32	2.46	8.50	2.88	8.54	2.34	8.2	2.12	8.59
6. Ser	1.14	5.67	1.36	5.57	1.84	6.31	2.28	6.67	2.16	7.4	1.96	7.95
7. Glu	2.12	10.40	2.44	9.90	2.88	10.00	3.18	9.45	3.04	10.52	1.76	7.20
8. Ala	0.88	4.30	1.14	4.70	1.28	4.40	1.56	4.63	1.4	4.81	1.16	4.70
9. Val	0.94	4.59	1.24	5.23	1.52	5.30	1.7	5.1	1.42	4.9	1.24	5.22
10. Cys+Met	2.12	10.40	2.46	10.00	2.84	9.87	3.04	9.0	2.64	9.1	2.24	8.48
11. Ile	1.86	9.10	2.24	9.21	2.92	10.10	3.24	9.63	2.76	9.51	2.38	9.63
12. Leu	1.16	5.56	1.30	5.30	1.74	6.00	1.88	5.59	1.42	4.90	1.30	5.36
13. Tyr+Phe	2.04	9.90	2.28	9.30	2.46	8.58	2.74	8.2	2.34	8.12	2.32	9.51
Sum of the amino acids, μg/liter	20.46	100.0	24.46	100.0	28.90	100.00	33.63	100.00	28.88	100.00	24.68	100.0

TABLE 5. Dynamics of the Accumulation of Amino Acids in the Mycelium of a Protoplast Culture of *Trichoderma harzianum*

Amino acid	Days											
	second		fourth		sixth		eighth		tenth		twelfth	
	mg/liter	%	mg/liter	%	mg/liter	%	mg/liter	%	mg/liter	%	mg/liter	%
1. Lys	3.88	11.78	4.16	11.44	3.32	8.63	4.48	10.91	4.02	10.96	3.6	10.77
2. His	3.18	9.62	3.46	9.51	3.32	8.63	4.52	11.02	3.94	10.74	3.04	9.10
3. Arg	2.14	6.05	2.42	6.65	3.02	7.80	3.36	8.19	3.02	8.02	2.64	7.90
4. Asp	2.24	6.80	2.32	6.38	2.54	6.60	2.60	6.33	2.42	6.60	2.04	6.10
5. Thr	2.70	8.20	2.96	8.14	3.24	8.42	2.72	6.62	3.04	8.03	2.76	8.26
6. Ser	1.64	4.97	1.88	5.17	2.04	5.30	2.36	5.70	2.12	5.85	1.70	5.08
7. Glu	3.04	9.20	3.22	8.50	3.48	9.14	2.60	6.33	3.21	8.74	2.88	8.63
8. Gly	1.96	5.95	2.24	6.90	2.38	6.18	3.21	7.82	2.36	6.43	2.04	6.12
9. Ala	1.44	4.40	1.86	5.10	2.24	5.82	1.52	3.70	1.32	3.60	2.02	6.10
10. Val	1.58	4.80	1.76	4.84	1.94	5.04	2.24	5.46	2.04	5.55	1.68	5.02
11. Cys+Met	3.02	9.10	3.16	8.70	3.36	8.73	3.58	8.72	3.22	8.78	2.64	7.90
12. Ile	2.36	7.20	2.64	7.32	2.80	7.28	2.96	7.21	1.38	3.75	2.22	6.54
13. Leu	1.48	44.50	1.64	4.50	4.50	1.82	2.04	4.97	1.70	4.63	1.62	4.85
14. Tyr+Phe	2.32	7.04	2.64	7.32	7.32	2.96	3.04	7.40	2.88	7.84	2.52	7.54
Sum of the amino acids, mg/liter	32.92	100.00	36.36	100.00	38.46	100.00	41.03	100.00	36.67	100.00	33.48	100.0

TABLE 6. Dynamics of the Accumulation of Amino Acids in the Mycelium of a Hybrid Culture of *Trichoderma harzianum*

Amino acid	Days											
	second		fourth		sixth		eighth		tenth		twelfth	
	mg/liter	%	mg/liter	%	mg/liter	%	mg/liter	%	mg/liter	%	mg/liter	%
1. Lys	3.74	12.53	3.96	11.15	4.11	9.22	5.08	9.97	3.40	8.52	3.24	9.20
2. His	3.11	10.42	3.42	9.58	3.61	8.17	3.80	7.46	3.02	7.57	2.80	7.94
3. Arg	2.04	6.83	2.70	7.57	3.04	6.87	3.42	6.72	3.24	8.12	2.60	7.38
4. Asp	1.87	6.26	2.42	6.78	2.70	6.19	3.05	5.99	2.64	6.61	2.31	6.56
5. Thr	2.46	8.24	2.92	8.19	3.44	7.49	4.70	9.23	3.04	7.62	2.60	7.38
6. Ser	1.42	4.75	1.84	5.24	3.62	8.12	3.80	7.67	3.34	8.28	3.21	9.10
7. Glu	2.48	8.30	3.32	9.30	5.56	12.48	4.40	8.64	4.34	10.88	3.86	10.95
8. Gly	1.84	6.16	2.04	5.71	2.64	5.92	3.02	5.93	2.34	5.86	2.20	6.24
9. Ala	1.18	3.95	1.34	3.75	2.04	4.57	2.46	4.83	2.12	5.31	1.76	4.99
10. Val	1.24	4.16	1.92	5.38	2.42	5.41	2.80	5.50	2.32	5.81	2.02	5.73
11. Cys+Met	2.94	9.85	3.30	9.25	3.80	8.53	4.02	7.89	3.42	8.57	2.94	8.35
12. Ile	2.07	6.93	2.24	6.28	2.60	5.83	2.94	5.77	2.30	5.76	2.02	5.73
13. Leu	1.34	4.49	1.80	5.04	2.20	4.34	3.40	6.67	1.70	4.26	1.36	3.87
14. Tyr+Phe	2.16	7.23	2.42	6.67	2.84	6.36	3.04	5.97	2.68	6.71	2.32	6.58
Sum of the amino acids, mg/liter	29.84	100.00	35.67	100.00	44.65	100.00	49.93	100.00	39.90	100.00	35.24	100.0

The cultures investigated differed in their percentage contents of one and the same amino acid out of the series mentioned as a function of the age of the mycelium. Thus, for the protoplast culture of *Trichoderma harzianum* the level of lysine in the mycelium in the first two days was 3.88 mg/g and in the following days the amount of lysine varied between 3.88 and 11.44 mg/g. The proportion of lysine in the combined sum of amino acids accumulated after two days of growth amounted to 11.78%.

The initial *Trichoderma harzianum* culture produced lysine intracellularly in the first two days in an amount of 1.43 mg/g, while its maximum amount was observed on the eighth day of growth — 3.04 mg/g — with a slight fall on the twelfth day of growth — to 2.08 mg/g. The proportion of lysine in the combined sum of amino acids rose from 6.9% on the second day to 9.1% on the eighth day of growth of the culture.

Thus, the micromycetes that we have studied produce the maximum amounts of extra- and intracellular amino acids on the sixth to eighth days of growth.

EXPERIMENTAL

A protein-rich cellulolytically active fungus, *Trichoderma harzianum*, isolated from the soil of the Tashkent oblast, a protoplast culture of *Trichoderma harzianum* obtained by the protoplasting and regeneration of protoplasts of this species of fungus [4], and a hybrid *Trichoderma harzianum* culture obtained by fusing morphological mutants of *Trichoderma harzianum* were investigated [5].

The seed material was prepared in Czapek–Dox liquid nutrient medium. Fermentation was carried out by the deep cultivation method in Erlenmeyer flasks containing 100 ml of fermentation medium at a growth temperature of 28°C on a shaking machine at 220 rpm. The time of cultivation was from 48 to 288 h [6]. The amount of seed material was 5% on the volume of the fermentation medium.

The fermentation medium used was a modified Czapek–Dox medium containing as the source of carbon 2% of ground maize cobs on the volume of the fermentation medium. After the end of the time of cultivation, the micromycetes of the biomass were collected by filtration and dried at 50°C to constant weight. Amino acids were determined in samples. In the determination of the amount of amino acids in the fungal mycelium [7], the biomass was hydrolyzed in sealed glass tubes in 6 N HCl (2 ml per 100 mg of mycelium) at 105-120°C for 24 h.

After the completion of hydrolysis, the humins were filtered off, and the hydrochloric acid was eliminated by repeated evaporation under vacuum. The hydrolysate was desalted by evaporation on the water bath to form a syrup. The residue was dissolved in acidified 96% ethanol (0.5% of concentrated HCl per 100 ml of ethanol) and was kept in the refrigerator for 16 h. This operation was repeated three times.

In order to eliminate lipids, the hydrolysate was shaken several times in a separatory funnel with diethyl ether and was then evaporated on the water bath. The dry residue was dissolved in a standard buffer with pH 2.2. The amino acids were determined quantitatively on an AAA-881 automatic amino acid analyzer.

REFERENCES

1. T. Asai, K. Aida, and K. Oishi, Bull. Agric., Chem. Soc., **2**, 21 (1957).
2. S. Kinoshita, Adv. Appl. Microbiol., **1**, 201 (1959).
3. C. Grebins, J. Delretin, and A. Cremieux, Bull. Soc. Chim. Biol., **47**, No. 6, 1281 (1965).
4. T. N. Kozhina, N. V. Mironenko, and O. V. Chepurnaya, Mikol. Fitopatol., **17**, No. 3, 248 (1983).
5. I. A. Zakharov, S. A. Kozhina, T. N. Kozhina, and I. V. Fedorova, A Collection of Methods on the Genetics of Yeast — Saccharomycetes [in Russian], Nauka, Moscow (1984), p. 144.
6. V. N. Nazarenko, V. N. Sokolov, A. I. Ginak, and B. S. Oster, Prikl. Biokhim. Mikrobiol., **3**, 437 (1993)
7. Methods of Experimental Mycology, A Handbook [in Russian], Naukova Dumka, Kiev (1982), p. 225.