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Influence of Nitrogen Nutrition on the Excretion of Protease by Gelatin-Liquefying Bacteria

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Virtanen and Tarnanen [1] and Virtanen and Suolahti [2] have shown that *Pseudomonas fluorescens* excretes into the nutrient solution a protein-hydrolyzing enzyme. This gave an explanation to the liquefaction of gelatin effected by the bacteria group called gelatin-liquefying bacteria. Peptidases are not excreted by these bacteria, but remain in the cells.

In connection with the studies made in this laboratory on the mutual relation of the nitrogen content and the enzymatic activity of the cells, we have also examined to what extent the formation of the protease excreted by *Ps. fluorescens* depends on the amount of nitrogen nutrition and, hence, on the N-content of the cells. At the same time we have also studied the dependence of the growth velocity of cells on the amount of N-nutrition and the percentage of the N-fraction insoluble in trichloracetic acid in cells with widely varying N-content. The results are reported in the present paper.

EXPERIMENTAL

The strain of *Pseudomonas fluorescens* used in the experiments was isolated from sewage. The selected strain dissolved gelatin with the greatest rapidity. Pepton broth was used for the stock culture in the laboratory. In the experiments proper, a mineral nutrient solution was used in which glycerin served as the only carbon source. The composition of the nutrient solution was: 50 g glycerin, 25 g K_2HPO_4, 15 g NaCl, and 0.2 g $MgSO_2 \cdot 7H_2O$ per 5 l tap water. Ammonium sulphate was used as the nitrogen source, its amount ranging from 0.05 to 25 g per 5 l. As it was important to induce as vigorous a growth of bacteria as possible, experiments were at first conducted in order to find out the conditions for the best growth. The growth was therefore compared in thin layers in Roux-flasks, and in thick layers in Kluyver-flasks where a strong current of air was passed through the solution by means of a compressor. The latter method, however, gave only about half of the bacterial yield obtained from the former, and therefore cultivation in the unmoving solution in the Roux-flasks was adopted.

Each Roux-flask contained 100 ml nutrient solution. An inoculation of 1 ml liquid culture of *Ps. fluorescens* was made into each flask, and ammonium sulphate was simultaneously added. Each experiment comprised seven different concentrations of ammonium sulphate, and for each concentration 10 Roux-flasks were employed (= 1 litre nutrient solution). The ammonium sulphate concentrations used were: 0.05 g, 0.1 g, 0.5 g, 5 g, 10 g, 15 g, and 25 g per 5 l nutrient solution.

It was attempted to maintain the pH of the nutrient solution at 6.8—7 throughout the growth period. It was controlled by means of indicator paper, and when the pH decreased NaOH solution was added.

The growth temperature was about 30° C, and the period of growth varied from 2 to 3 days.

When the growth was interrupted, the bacteria were separated from the solution by centrifugation, suspended in water, and separated again. All of the clear growth solutions with the same $(NH_4)_2SO_4$-concentration were combined as well as the bacterial masses, which were suspended in 30 ml of water. The growth of bacteria was determined both by the turpidity of the culture solution, using a Klett-Summerson photometer, and by determining the dry matter of the bacterial suspension obtained. In the lower bacterial concentrations, both methods gave a similar growth curve; but in higher concentrations, the photometric determinations gave too low values.

The nitrogen soluble in trichloracetic acid was determined by allowing the wet bacterial mass to stand for 24 hours in 20 % trichloracetic acid. After that time, it was centrifuged and the solution filtered through bacterial filter (Jena G 5 auf 3) for complete removal of bacteria. Nitrogen was determined in the clear solution.

The activity of the protease present in the growth solution was determined by examining its ability to decompose both casein and gelatin. Decomposition was followed by determining amino-N by the Cu-method of Pope and Stevens. Total N was determined by the Kjeldahl method introduced by Miller [4]. Ten per cent of casein was dissolved in water by adding NaOH to raise the pH to 9. This basal solution was added to the growth solution, to make it 2 per cent with regard to casein. In the experiments with gelatin, 2 per cent solutions were also used. The pH of the growth solutions was adjusted to 7. In all experiments, infections were prevented by means of toluene. A control, with distilled water instead of growth solution, was included in each experiment. The insignificant amount of amino-N formed therein was always subtracted from the results in the experiment proper.

The clear growth solution was used in the same experiment series in amounts that always corresponded to equal amounts of bacteria. If, for instance, the yield of bacteria was 762 mg dry substance in one litre nutrient solution with 10 g ammonium sulphate, and 132 mg bacteria in one litre with 0.1 g ammonium sulphate, the protease activity of the growth solution was determined by using, in the former case, 45.6 ml and, in the latter, 263.6 ml growth solution.

The increase of amino-N in casein and gelatin solutions is expressed in the results as mg per g total N of bacteria.

The peptidase activity of the bacterial mass was determined by following the increase of amino groups in a Witte-pepton solution. A known amount of bacterial mass was suspended in 2 % pepton solution, and the pH adjusted to 7.

The peptidase activity of bacteria grown in different ammonium sulphate concentrations was compared, in the same experiment series, by using the same amount of dry bacteria in each experiment. As the N-content of the bacteria was known, decomposition

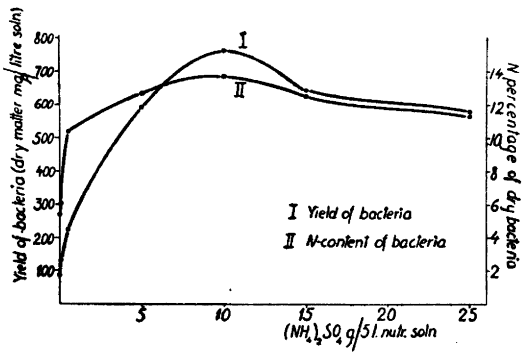


Fig. 1. Dependence of the growth and N-content of Ps. fluorescens on the ammonium sulphate concentration of the nutrient solution.

could be calculated per nitrogen in them. The increase of amino-N is expressed in the results as mg per g total N of bacteria.

RESULTS

In the preliminary experiments different problems were examined, especially the effect of ammonium sulphate concentration on the growth of bacteria, on the N-content of bacteria, and on the protease activity of the clear growth solution. After that, four complete experiments were made in which all of these questions were simultaneously examined. The dependence of growth and N-content of the bacteria on the ammonium sulphate concentration was similar in all experiments, as can be seen from Table 1 and Fig. 1.

The results show that 10 g $(NH_4)_2SO_4$ is optimum for the growth. The N-content of the cells also shows a maximum at this concentration. The decrease of N-content accompanying the lowering of $(NH_4)_2SO_4$ is very distinct. Table 2 shows the uptake of ammonium nitrogen by bacteria during the experiment.

Table 1. Dependence of the NH_4^+-concentration of the nutrient solution on the growth and N-content of Ps. fluorescens.

$(NH_4)_2SO_4$ g/5 l	Expt. 5 (growth 72 h)		Expt. 6 (growth 48 h)		Expt. 7 (growth 72 h)		Expt. 8 (growth 72 h)	
	Dry bact. mg/l	N % of dry bact.	Dry bact. mg/l	N % of dry bact.	Dry bact. mg/l	N % of dry bact.	Dry bact. mg/l	N % of dry bact.
25	504	12.9	259	10.5	577	11.3	516	11.5
15	—	—	—	—	646	12.5	556	11.8
10	—	—	—	—	762	13.7	824	12.4
5	550	11.1	391	11.2	595	12.7	544	11.9
1	294	10.2	—	—	—	—	—	—
0.5	141	10.0	209	10.7	224	10.4	237	10.1
0.1	—	—	112	6.1	132	6.1	137	6.1
0.05	57	5.4	76	5.6	87	5.4	94	5.4

Table 2. The amount of ammonium nitrogen present in the nutrient solution at the end of the experiment. Inoculation contained 4 mg N.

$(NH_4)_2SO_4$ g/5 l	N in bacteria mg/l	N remaining in solution mg/l
25	59	995
15	66	569
10	102	322
5	65	149
0.5	24	0
0.1	8	0
0.05	5	0

In an ammonium sulphate concentration of 0.5 g/5 l, the bacteria had consumed the entire nitrogen nutrition. The very distinct decrease in the nitrogen content of cells from this concentration on is evidently due to the fact that the cells are still able to form cell material to some extent although nitrogen nutrition from the outside is no longer available. Thus the decrease in the N-content of cells is natural. However, it is surprising that the nitrogen content of the cells is somewhat lower in the superoptimum ammonium sulphate concentration than in the optimum one.

In order to get a rough idea about to what extent the nitrogen compounds are similar in high-nitrogen and low-nitrogen cells, we determined, in experiments 6 and 8 the N-fractions soluble and insoluble in trichloracetic acid. The results appear in Table 3.

Table 3. N-fraction insoluble in trichloracetic acid in Ps. fluorescens with normal and low-nitrogen content.

$(NH_4)_2SO_4$ g/5 l	N insoluble in trichloracetic acid, % of total N	
	Expt. 6	Expt. 8
25	75 (10.5)	76 (11.5)
15	—	81.8 (11.8)
10	—	85.9 (12.4)
5	84.5 (11.2)	84.3 (11.9)
0.5	—	77.2 (10.1)
0.1	—	76.4 (6.1)
0.05	—	75.0 (5.4)

The parenthetical figures indicate the N-percentage of bacteria per dry matter.

It can be seen from the table that when the N-content of the cells is highest, the proportion of the so-called protein-N (insoluble in trichloracetic acid) to total N is also highest. In low-nitrogen cells this N-fraction is considerably lower. It is of special interest that the same applies also to cells grown in nutrient solutions with excess ammonium nitrogen, although their N-content is not much lower than that of the cells grown in optimum NH_4^+-concentration. The bacteria separated from cultures grown for 48 h and 72 h gave similar results. The age of the cells is, accordingly, not a decisive factor in the variations in the N-content of the cells or in the changes in the N-fraction insoluble in trichloracetic acid. A more detailed analysis of the N-compounds of the cells grown in nutrient solutions containing different amounts of N-nutrition was recently made by Virtanen and Miettinen [5]. They found that the lack of nitrogen nutrition had the greatest effect on the decrease of nucleic acid and protein fractions. Soluble nitrogen compounds other than nucleic acid did not at all decrease. The N-fraction from *Torulopsis* yeast soluble in trichloracetic acid contains, according to Roine [6], chiefly free amino acids and their amides (glutamic and aspartic acids, their amides, and alanine) and, in addition, nucleotides. Peptides are found, at most, in very small amounts. The composition of the soluble N-fraction was not examined quantitatively with *Ps. fluorescens*. If it is similar to that in *Torulopsis*, it is evident that in the low-nitrogen bacteria protein synthesis has not proceeded as far as in the normal-nitrogen cells.

The dependence of the activity of protease excreted into the nutrient solution was, in all four experiments, very similar. The results are therefore presented only from experiment 8.

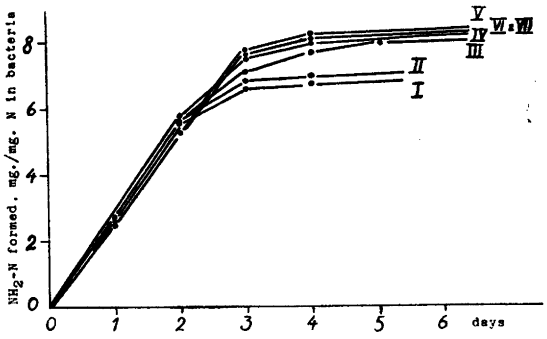
In each experiment, both with casein and gelatin, the quantity of the growth solution corresponded to 37.6 mg bacterial mass. Thus the experimental solutions had the following compositions (Table 4).

Table 4. Composition of the solution in experiments on the decomposition of casein by the extracellular protease of P s. f l u o r e s c e n s.

$(NH_4)_2SO_4$ g/5 l	Clear growth solution ml	Water ml	10 % casein solution ml
0.05	400	—	100
0.1	275	125	100
0.5	158.6	241.4	100
5	69.2	331.8	100
10	45.5	354.5	100
15	67.7	332.3	100
25	70.9	329.1	100
Control	—	400	100

Fig. 2. Influence of N-nutrition on the formation of amino-N from casein by excreted protease of Ps. fluorescens.

I.	$(NH_4)_2SO_4$	*0.05*	*g/5l*
II.	»	*0.1*	»
III.	»	*0.5*	»
IV.	»	*5.0*	»
V.	»	*10.0*	»
VI.	»	*15.0*	»
VII.	»	*25.0*	»



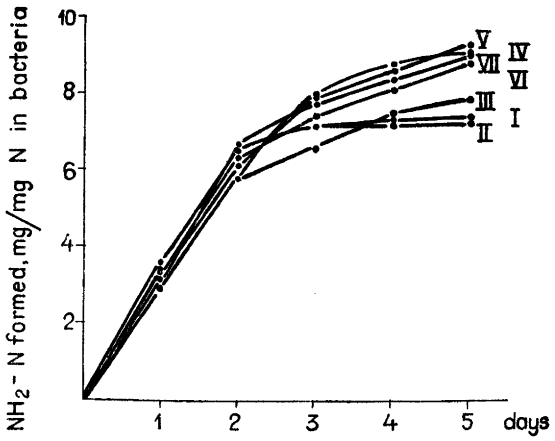
When gelatin was used as substrate, the experimental arrangement was the same. The results appear from the curves in Figs. 2 and 3.

The curves illustrate that the formation of amino-N, from both casein and gelatin, by the action of protease excreted into the culture solution, is practically independent of the ammonium sulphate concentration of the solution. Only in the lowest NH_4^+-concentrations is the hydrolysis of proteins by the growth solution retarded more rapidly than in higher concentrations. The formation of the extracellular protease in the cells is, on the whole, largely independent of the nitrogen content of the cells.

Fig. 4 shows the hydrolysis of pepton caused by the bacterial mass. The N-content of the bacteria has no noticeable effect even on this reaction. Virtanen and Winkler[7] have earlier noted that the proteolytic ability of *Escherichia coli* does not change as the N-content of the cells decreases from 13 to 9.5 %.

Fig. 3. Influence of N-nutrition on the formation of amino-N from gelatin by excreted protease of Ps. fluorescens.

I.	$(NH_4)_2SO_4$	*0.05*	*g/5l*
II.	»	*0.1*	»
III.	»	*0.5*	»
IV.	»	*5*	»
V.	»	*10*	»
VI.	»	*15*	»
VII.	»	*25*	»



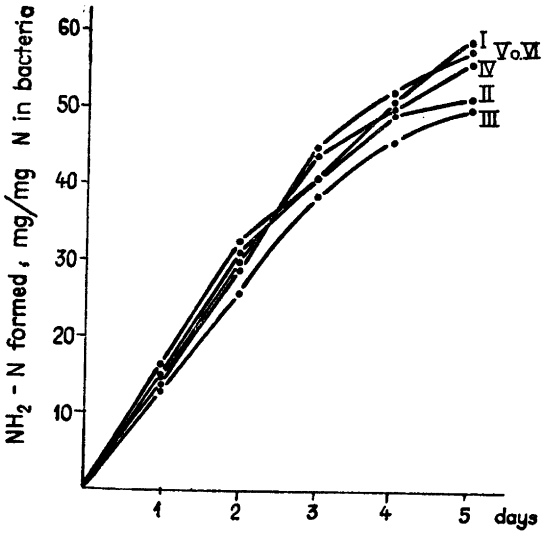


Fig. 4. Decomposition of pepton caused by Ps. fluorescens masses grown in different ammonium sulphate concentrations.

I.	$(NH_4)_2SO_4$	*0.05 g/5l*
II.	»	*0.1* »
III.	»	*0.5* »
IV.	»	*5* »
V.	»	*10* »
VI.	»	*15* »
VII.	»	*25* »

As the enzymes belong to the fraction insoluble in trichloracetic acid, it would be most natural to calculate the activity of the enzymes per nitrogen of this fraction and not per total nitrogen of the cells. The results, however, would not change in principle by this change of the calculation basis. We have therefore again used the total nitrogen basis since insoluble nitrogen was not determined in the earlier investigations with *E. coli*.

SUMMARY

The N-content of the cells of *Ps. fluorescens* was highest in the ammonium sulphate concentrations optimum to growth. The nitrogen content of the cells decreased as the N-nutrition was lowered and the growth weakened. When the ammonium nitrogen content of the nutrient solution was so low that it was rapidly consumed by the cells, the N-content of the cells decreased to less than half of the normal.

The percentage of the N-fraction insoluble in trichloracetic acid (so-called protein N) was highest in high-nitrogen cells in the ammonium concentration optimum to growth. As the N-content of the cells lowered, the percentage of this N-fraction decreased. The same occurred when the ammonium sulphate concentration of the nutrient solution was superoptimum. The activity of the protease excreted by *Ps. fluorescens* into the culture solution was largely independent of the ammonium sulphate concentration of the culture solution

and of the N-content of the cells. Both in the decomposition of gelatin and casein this fact could be noted. The enzymes which cause the hydrolysis of pepton and which are not excreted by the cells are also independent of the N-content of the cells.

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