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Enzymatic Breakdown of Polymetaphosphate

VI. Influence of Nutritional Factors on the Polymetaphosphatase Production of *Aspergillus niger*

G. LINDEBERG and H. MALMGREN

Institute of Microbiology, the Royal Agricultural College, and Institute of Biochemistry, University of Uppsala, Sweden

During earlier investigations on polymetaphosphatase in *Aspergillus niger* one of the authors [1] (M.) found it desirable to obtain the enzyme in as large quantities as possible. Therefore, an investigation was begun in order to elucidate the optimum conditions of polymetaphosphatase production. Special emphasis was placed on a study of the following factors: carbon and nitrogen sources, trace elements and pH.

METHODS

Aspergillus niger van Tieghem (no. 594 from the National Collection of Type Cultures maintained in Britain by the Medical Research Council) was used as a test organism. The stock culture was grown on malt extract agar (2.5 % malt extract; 2 % agar-agar). The chemicals used in the nutrient solution were of the following qualities: crude and refined commercial sucrose; saccharose, purum; the other chemicals of reagent grade. The trace element content of the chemicals used was determined colorimetrically *. The following amounts of Fe, Zn, Mn and Cu were found (μg/g).

	Fe	Zn	Mn	Cu
Glucose	0.0	0.1	0.2	0.4
K_2HPO_4	8.0	0.0	0.0	1.1
$MgSO_4 \cdot 7$ aq.	0.7	0.0	0.1	0.2
$(NH_4)_2SO_4$	1.0	0.0	0.2	0.3
NH_4-tartrate	2.0	2.2	0.9	0.6
$NaNO_3$	3.4	0.1	0.0	0.3
Asparagine	0.0	1.5	0.0	0.4
Glycine	0.3	8.8	0.0	8.2
Urea	0.0	1.1	0.0	1.0

Thus, in most experiments the trace element content of the chemicals used is small in comparison with the addition of the same elements.

* The determinations were carried out according to the official methods of Kungl. Lantbruksstyrelsens Kungörelser, Nr 9, 1950 and Sandell: Colorimetric determination of traces of metals. New York (1950).

The water was distilled first in a stainless steel apparatus and then in a pyrex glass apparatus. 300 ml Erlenmeyer flasks (Pyrex) were used as culture vessels. They were carefully cleaned with dichromate-sulphuric acid, hot water, and finally streaming vapor before use. Each flask contained 40 ml of sterile nutrient solution. Suspensions of conidia in sterile water were prepared for the inoculation. These suspensions were filtered through thin layers of sterile cotton before use. Each flask was inoculated with 1 ml suspension. The flasks were incubated at 30° C. Eight parallels were harvested from each series each time. Four of them were used for the determination of the mycelium dry weight and — in some cases — analysed for nitrogen and phosphorus. These mycelia were rinsed for about half an hour in running tap water and finally with distilled water, care being taken that the conidia-bearing surface was not wetted. The remaining four flasks were used in determining the polymetaphosphatase activity of the mycelia and the nutrient solution.

The polymetaphosphatase activity was determined viscosimetrically according to a method described by Ingelman and Malmgren [2]. As a relative measure of the enzyme activity (using the same substrate and the same substrate concentration) a quantity z is used. This quantity is defined by:

$$z = (\eta_{sp})_{t=0} \times \frac{d\left(\frac{1}{\eta_{sp}}\right)}{dt}$$

where η_{sp} = the specific viscosity, t = time after incubation with enzyme of the substrate solution. As substrate was used a potassium metaphosphate $(KPO_3)_n$, n being of the order 10 000. z is expressed in 10^{-4} min.$^{-1}$.

The relative amount of polymetaphosphatase secreted into the nutrient solution, A_s, was calculated as the product of the z-value and the volume (ml) of the solution. The corresponding value for the quantity of enzyme in the mycelium, A_m, was calculated analogously after extracting the mycelia in the following way: After addition of acetate buffer (pH = 5.4) to a total volume of 25 ml per mycelium, the mycelia were ground up in a Waring blendor. After about one hour the suspension was centrifuged and the activity of the supernatant was determined. The total polymetaphosphatase production, *i. e.* the sum of A_s and A_m, is designated ΣA. All these quantities are given in units of $z \cdot 10^{-4}$.

Control experiments were made in order to check that too much enzyme was not lost by extracting the mycelia with only one portion of buffer. The sediment was washed repeatedly until the washings did not show any activity. It was found that the losses of polymetaphosphatase depending on incomplete extraction were small, less than 5 %. In only one case, where asparagine was used as a nitrogen source, did the loss of enzyme amount to 10 %.

The determinations of the nitrogen content of the mycelia were made according to Kjeldahl, the phosphorus determinations following the method of Lowry and Lopez [3].

EXPERIMENTS AND RESULTS

Influence of the nature of the carbon source

In some earlier experiments a mixture of crude and refined sucrose was used as carbon source (2.5 % and 7.5 % resp.). In experiment no. 1 the growth

Table 1. Influence of the carbon source on enzyme production. Time of incubation: 6 days.

Carbohydrate	Mycelium dry weight (ω) in g	pH	ΣA	$\frac{\Sigma A}{\omega}$
Crude sucrose + refined sucrose	0.93	1.7	6	6
Refined sucrose	0.94	1.6	6	6
Saccharose	0.95	1.6	7	7
Glucose	0.95	1.7	5	5

and the enzyme production of the mould was compared when using pure glucose and different qualities of saccharose as carbon source.

Experiment no. 1. Basal medium: 5 g $(NH_4)_2SO_4$, 2 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7$ aq., 100 ml tap water, distilled water to 1 000 ml (including the sugars). Four series according to Table 1.

The growth and enzyme production of the mould was almost the same in all four cases. Owing to the necessity of using as well-defined chemicals as possible glucose was preferred as carbon source during the continued work.

Influence of trace elements

Preliminary experiments showed that an addition of tap water to the nutrient solution had a considerable influence on the enzyme production. Hence tap water must contain some metal ions of importance in this connection.

In the next experiment the influence of different metals on the enzyme production of the mould was investigated.

The following metals, which have earlier been shown to be essential for *A. niger* (for references see Steinberg [4]), were tested: iron, zinc, manganese and copper.

Experiment no. 2. Basal medium: 100 g glucose, 5 g $(NH_4)_2SO_4$, 2 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7$ aq., redistilled water to 1 000 ml (including the trace element additions). The following salts of the trace elements were used: Fe-citrate (ferric), $MnSO_4 \cdot 4$ aq., $CuSO_4 \cdot 5$ aq. and $ZnSO_4 \cdot 7$ aq. The final series contained the trace elements in the following concentrations: Fe, Cu and Zn 1 p.p.m., Mn 3 p.p.m. Twelve series according to Table 2.

Enzyme production in the control series was found to be rather low. Fe and Cu had a stimulating effect both on growth and enzyme production.

Table 2. Influence of different trace elements on the enzyme production. Time of incubation: 6 days.

Metals added	Mycelium dry weight (ω) in g	pH	ΣA	$\frac{\Sigma A}{w}$
Control	0.25	2.2	11	44
Fe	0.44	1.8	39	89
Cu	0.34	1.9	31	91
Mn	0.45	2.1	21	47
Zn	0.65	1.8	1	2
Zn, Fe	0.72	1.7	2	3
Zn, Cu	0.46	1.7	1	2
Zn, Mn	0.46	1.8	1	2
Fe, Mn	0.39	2.2	25	64
Fe, Cu	0.30	2.0	32	107
Cu, Mn	0.25	2.0	17	68
Fe, Mn, Cu	0.48	1.8	36	75

Mn alone stimulated growth but caused no increase in the relative enzyme production, while in combination with Fe or Cu, it seemed to limit slightly the stimulating effect of these metals. This result is difficult to explain without further experiments. An addition of 1 mg Zn per liter almost completely inhibited enzyme production, and also caused a marked inhibition of conidial formation (cf Roberg [6]). Since high enzyme production and the most uniform growth of the mould occurred on addition of Fe, Mn and Cu, these metals were added in most of the following experiments.

In an additional experiment, the influence of calcium, which under certain conditions affects growth of several fungi (cf Lindeberg [5]), was also tested. However, at a concentration of 20 p.p.m. this metal had no action either on growth or on polymetaphosphatase production.

Although zinc at a concentration of 1 p.p.m. inhibits the enzyme production, it cannot be presumed that this metal should also have an inhibitory effect at lower concentrations. The influence of different concentrations of zinc on the enzyme production was therefore studied.

Experiment no. 3. Basal medium: 100 g glucose, 5 g $(NH_4)_2SO_4$, 2 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7$ aq, Fe and Cu 1 p.p.m. Mn 3 p.p.m., redistilled water to 1 000 ml. The results of 5 series are given in Table 3.

The mycelial weight increased with increasing Zn-concentration until a maximum was reached at 100 μg Zn per liter. At 1 000 μg per liter the mycelial weight was somewhat lower. At concentrations of 1—100 μg Zn per liter no

Table 3. Influence of different concentrations of zinc on enzyme production. Time of incubation: 7 days.

µg Zn added per liter	Mycelium dry weight (ω) in g	pH	ΣA	$\frac{\Sigma A}{w}$
0	0.25	1.8	54	220
10	0.57	1.7	53	93
100	1.04	1.6	59	57
1 000	0.81	1.6	3	4

pronounced effect on the total enzyme production was observed, whereas at 1 000 µg Zn per liter it was strongly inhibited. The enzyme production per gram mycelium $\frac{\Sigma A}{w}$ decreased on increasing additions of Zn.

Influence of the nature of the nitrogen source and of hydrogen ion concentration

At an early stage of the investigation it was found that the nature of the nitrogen source was of great importance for the polymetaphosphatase production. One experiment which also demonstrates the role of the incubation time will be described here.

Experiment no. 4. Basal medium: 25 g crude and 75 g refined sucrose, 2 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7$ aq, 100 ml tap water; distilled water to 1000 ml. Two series containing 5 g $(NH_4)_2SO_4$ resp. 7.7 g KNO_3 per liter. Fig. 1.

In the ammonium sulphate series the mycelial dry weight reached a (maximum) value of about 0.85 g.; simultaneously the pH decreased from 6.7 to 1.7. Subsequently both mycelial weight and pH remained practically unchanged. Autolysis of the mycelium was not observed. The total amount of enzyme ΣA reached a value of about 3 after 6 days, and remained virtually the same. The activity of the mycelial extracts was low, only $\sim 1/6$ of the total amount of enzyme being found in the mycelium. Thus, in this case most of the polymetaphosphatase was secreted into the medium.

In the nitrate series the mycelial dry weight passed a maximum of $\sim$ 1.20 g. after 6 days after which a rather pronounced autolysis began. After 2 days the pH had decreased to 3.8 but when the autolysis set in the pH increased again, reaching the value of 8.5 after 10 days and the value of 9.0 after 13 days. The total enzyme production was somewhat higher than in the former series,

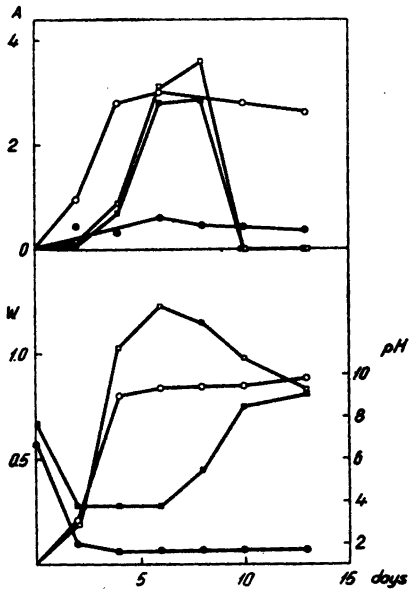

Fig. 1. Abscissa: time of growth in days. Ordinata to the left, lower part of the figure: mycelium dry weight in g □ = KNO_3; ○ = $(NH_4)_2SO_4$. *Ordinata to the right, lower part of the figure: pH of the medium;* ■ = KNO_3; ● = $(NH_4)_2SO_4$. *Ordinata, upper part of the figure: relative amount of enzyme;* □ = Σ A KNO_3; ■ = A_m KNO_3; ○ = ΣA $(NH_4)_2SO_4$; ● = A_m $(NH_4)_2SO_4$.

but most of the enzyme was found in the mycelium, only a slight amount being secreted into the nutrient solution.

The large secretion of enzyme in the ammonium sulphate series is due probably to the increased concentration of hydrogen ions in this series. Hence, one might expect an adjustment of the pH to a lower value than the "normal" one of, for instance, the nitrate medium, to result in an increased secretion. Furthermore, if a certain minimum amount of enzyme is needed inside the mycelial cells, an increase of the secretion might result in an increased total enzyme production.

The effect of the hydrogen ion concentration of the medium on the production of certain enzymes of microorganisms has earlier been shown. In different bacteria Gale [7,8] observed an increased production of catalase, urease, fumarase, and certain dehydrogenases when the pH of the medium was lowered. Lindeberg and Fåhraeus [9] (unpublished) found an increased production of laccase in the hymenomycetes *Polyporus zonatus* and *P. versicolor* when the hydrogen ion concentration was increased.

As demonstrated above the polymetaphosphatase production is dependent on the nitrogen source, and it was suggested that this fact might possibly be related to a variation of the amount of assimilated nitrogen in the mycelium. The question also arises if there is any relation between the polymetaphosphatase production and the phosphorus content of the mycelium. It could

be assumed that a high assimilation of phosphorus would facilitate the enzyme production. On the other hand it might be expected that the presence of more enzyme in the cells would cause a more intense synthesis of polymetaphosphate, the occurrence of which in the mycelium has been demonstrated by Ingelman and Malmgren [10]. In the following experiments the factors discussed above have been taken into consideration. The nitrogen sources used were: equivalent amounts of $(NH_4)_2SO_4$, NH_4-tartrate, $NaNO_3$, asparagine, glycine and urea, i.e. ammonium salts of one strong and one weak acid, anionic inorganic nitrogen, amido-amino-, pure amino- and pure amido-nitrogen.

Experiment no. 5. Basal medium: 100 g glucose, 2 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7$ aq, Fe and Cu 1 p.p.m., Mn 3 p.p.m., Zn 0.1 p.p.m., redistilled water to 1000 ml (including the nitrogen compounds). In different series were added: 5 g $(NH_4)_2SO_4$, 7 g NH_4-tartrate, 6.4 g $NaNO_3$, 5 g asparagine, 5.7 g glycine and 2.3 g urea resp.

Each nitrogen source (except $(NH_4)_2SO_4$) was added to two series, one of which was used for pH-adjustment. In this series the pH was adjusted to $\sim$ 2 by sterile addition of 1 *M* H_2SO_4 after 2 days' incubation when a continuous mat had been formed by the fungus. The mycelia were analysed for nitrogen and total phosphorus.

The results are presented in Tables 4 and 5.

As seen in Table 4 there was a distinct difference between enzyme production on different nitrogen sources. Regarding the „normal" conditions of growth (no pH-adjustment), it is found that ammonium sulphate and sodium nitrate gave the highest total enzyme production, followed by ammonium tartrate, glycine and urea. With asparagine as nitrogen source the polymetaphosphatase production was very low. The secretion of enzyme into the nutrient solution also varied. In the ammonium sulphate series (the lowest pH-value) only 25 per cent of the total activity remained in the mycelium, in the asparagine series 80—90 per cent.

The effect of pH-adjustment was as expected. The most pronounced effect was observed in the asparagine series in which the total enzyme production was increased 4—5 times. The pH-adjustment also resulted in a decreased formation of conidia and a mycelium dry weight lower than in the corresponding reference series.

The $\frac{A_m}{w}$-values were highest for the sodium nitrate series and lowest for the asparagine series. It may be mentioned here that in several other experiments — not described in this paper — the lowest $\frac{A_m}{w}$-value, 3, was also found in the asparagine series.

The analytical data of Table 5 show no correlation between the assimilation of nitrogen and phosphorus on the one hand and the enzyme production on the other. As shown by Behr [11], the percentage of nitrogen in the mycelium

Table 4. Influence of the nitrogen source on growth and enzyme production.

Nitrogen source	Time of incubation in days	Mycelium dry weight (ω) g	pH	ΣA	$\frac{A_m}{\Sigma A}$	$\frac{A_m}{w}$
$(NH_4)_2SO_4$	4	0.76	1.5	44	0.24	14
	7	0.95	1.9	43	0.25	11
NH_4-tartrate	4	0.95	2.2	16	0.45	8
	7	1.07	3.0	28	0.25	7
pH adjusted	4	0.85	1.5	56	0.36	24
	7	1.03	1.9	45	0.19	8
$NaNO_3$	4	0.81	3.6	47	0.79	46
	7	0.97	4.5	43	0.55	24
pH adjusted	4	0.78	2.5	57	0.78	57
	7	0.97	2.2	96	0.46	46
Asparagine	4	0.90	2.3	4	0.84	4
	7	1.13	3.0	4	0.91	3
pH adjusted	4	0.69	1.7	21	0.40	12
	7	0.92	2.0	15	0.17	3
Glycine	4	0.77	2.3	21	0.40	11
	7	1.03	2.7	13	0.58	7
pH adjusted	4	0.62	1.5	30	0.20	10
	7	0.85	1.7	36	0.12	5
Urea	4	0.53	2.5	13	0.30	7
	7	0.73	2.7	12	0.28	5
pH adjusted	4	0.48	1.9	16	0.31	10
	7	0.66	2.0	30	0.13	6

decreases with time. The total nitrogen uptake from the medium varied between about 28 and 38 mg per flask, corresponding to 70—90 % of the available nitrogen. In all cases maximum nitrogen uptake seems to have been reached after four days. On the contrary, the phosphorus uptake in most series increased from the fourth to the seventh day, causing in several series an

Table 5. Analytical data of mycelia grown on media with different nitrogen sources.

Nitrogen source	Time of incubation in days	% N	% P	mg N per mycelium *	mg P per mycelium *
$(NH_4)_2SO_4$	4	4.4	0.74	33	5.6
	7	2.7	0.47	26	4.5
NH_4-tartrate	4	3.5	0.71	33	6.7
	7	3.1	1.07	33	11
pH adjusted	4	3.7	0.87	32	7.4
	7	3.2	0.79	33	8.1
$NaNO_3$	4	4.7	0.79	38	6.4
	7	3.5	1.06	34	10
pH adjusted	4	4.9	0.79	38	6.2
	7	3.9	1.07	38	10
Asparagine	4	3.4	0.64	31	5.8
	7	2.8	1.25	31	14
pH adjusted	4	4.1	0.74	29	5.1
	7	3.0	0.95	28	8.8
Glycine	4	4.2	0.70	32	5.4
	7	3.4	1.21	35	12
pH adjusted	4	5.3	0.76	33	4.7
	7	3.5	0.67	30	5.7
Urea	4	5.2	0.82	28	4.3
	7	3.7	0.81	27	5.9
pH adjusted	4	5.9	0.86	28	4.1
	7	2.3	0.89	15	5.8

* In all series the medium contained 42 mg N and 14 mg P per flask (40 ml)

increase in the relative phosphorus content of the mycelium also. Of course, it would be very desirable to know also the polymetaphosphate content of the mycelium. However, owing to the great difficulties implied in the quantitative determination of polymetaphosphate, the question of the amount of this compound present in mycelia from different media must be left open in this work.

Influence of an addition of polymetaphosphate

The polymetaphosphatase of *A. niger* is a constitutive enzyme in the sense that it is synthesized by the organism even if the nutrient medium does not contain the specific substrate of the enzyme (Karström [12]). The difference between constitutive and adaptive enzymes is, however, not sharp as shown by Karström and others. An organism may exhibit a certain degree of adaptation even with regard to constitutive enzymes. Among the nitrogen compounds tested asparagine gives the lowest polymetaphosphatase production and an adaptive feature in the character of the enzyme might be expected to be more easily revealed with asparagine as a nitrogen source than with the other compounds. In order to investigate whether an addition of polymetaphosphate to the medium stimulates the formation of polymetaphosphatase the following experiment was carried out.

Experiment no. 6. Basal medium 100 g glucose, 2 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7$ aq, 5 g asparagine, Fe and Cu 1 p.p.m., Mn 3 p.p.m., Zn 0.1 p.p.m., redistilled water to 875 ml. To this medium 125 ml 0.4 % polymetaphosphate in 0.1 *M* NaCl, resp. 125 ml 0.1 *M* NaCl were added in different series.

The results are presented in Table 6.

Table 6. Influence of polymetaphosphate on enzyme production.

	Time of incubation in days	Mycelium dry weight (ω) in g	pH	ΣA	$\frac{A_m}{\Sigma A}$
Control	4	1.05	2.8	27	0.88
	7	1.06	6.7	14	0.73
$(KPO_3)_n$ added	4	1.02	2.8	21	0.74
	7	1.13	5.5	15	0.85

As seen from the Table the addition of polymetaphosphate had no influence on the enzyme production.

SUMMARY

1. The influence of certain factors on the polymetaphosphatase production of *A. niger* has been studied.

2. The enzyme production was about the same on media containing glucose or saccharose as carbon sources.

3. An increased enzyme production was achieved upon addition of Fe, Cu (1 p.p.m.) and Mn (3 p.p.m.), Fe being the most effective of these metals. Ca had no effect.

4. The enzyme production per gram mycelium decreased on addition of zinc (10—1 000 μg/liter). The total enzyme production was strongly inhibited in the presence of 1 mg Zn per liter.

5. The nature of the nitrogen source greatly affected the enzyme production. Highest production was found with nitrates and ammonium salts, definitely lower production with organic nitrogen sources, *e. g.* glycine, urea and, especially, asparagine.

6. With increasing concentration of hydrogen ions the secretion of enzyme into the medium was increased. By lowering the pH of the nutrient solution to about 1.5—2 the enzyme production was greatly stimulated.

7. No relation could be observed between the total nitrogen and phosphorus content of the mycelium and the enzyme production.

8. Addition of polymetaphosphate to the medium did not increase the enzyme production.

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