Botany. — *Nitrate Assimilation of Aspergillus niger* VAN TIEGHEM. By S. DE BOER. (Communicated by Prof. J. C. SCHOUTE.)

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Introduction.

The object of this investigation was the study of nitrate metabolism of Aspergillus niger, for the purpose of ascertaining:

a. whether nitrate, after being taken up, is accumulated by the fungus or is directly used in metabolism, and

b. the influence of the pH on these processes.

Further, the influence of glucose was investigated, as it might be expected that glucose will have a similar effect in the case of Aspergillus as of Avena roots where the nitrate uptake und nitrate reduction is increased by the addition of glucose, as POSTMA (7) has proved.

For this investigation the nitrate had to be quantitatively determined in the fungus as well as in the solution. According to the literature the determination of nitrate by the diphenylamine and phenoldisulphonic acid methods has not yielded satisfactory results. ITZEROTT (5) and BÜNNING (4) used the brucine method in determining the nitrate in a culture solution, whereas in ascertaining the mycelium only qualitative observations were made by the diphenylaminesulphuric acid method. Respecting the brucine method, according to ALTEN and WEILAND (1), account must be taken of great variations (as much as 25 %), and although ITZEROTT and BÜNNING state that they have obtained more favourable results, it will be advisable to employ a better method. The xylenol method, first applied by BLOM and TRESCHOW (3) to soil and plants, is a more exact nitrate determination which has been employed in different researches of recent years. This method, which I followed in determining the nitrate in the culture solution and, in a modified form, also for fungi, yielded satisfactory results.

In physiological experiments concerning nitrate assimilation by fungi, the usual procedure is as follows. The fungi are reared on a medium containing the substance to be examined, in this case nitrate. After some days the quantity still remaining of the nitrate first added to the medium is then ascertained. The difference is caused by the nitrate accumulated in the fungus and the nitrate taken up in metabolism partly for building up new protoplasm. In these circumstances it is not possible to separate these two processes. For this reason I employed fungus mats which, moreover, were starved on water. This method, which has been employed

by VAN WAESBERGHE (8) among others, and which suits the fungus, as his experiments show, has the advantage that the growth-phase is separated from the investigation-phase. Moreover, by starving, the reserve substances which might affect the nitrate metabolism, are used up.

§ 1. *Experimental Methods.*

a. *Culture method.*

In a 100 ml Erlemeyer-flask containing 37 ml nitrate-free Raulin-solution, a spore suspension of Aspergillus niger is sown. The fungus is reared for 2 days, after which the mats are starved on water. After one day the water is replaced by a known nitrate solutioa in which, after some time, the nitrate which has not been taken up by the fungus, is determined.

b. *Nitrate determination.*

The nitrate determination in the solution was carried out, with slight modifications, after the directions given by ALTEN et al. (2) and WERR (9):

To 5 ml solution (which must not contain more than 4 mg NO_3) 25 ml 75 vol. % nitrate-free H_2SO_4 is added and, after cooling to about 25°, 3 drops 2 : 4-xylen-1-ol. The flask is closed and well shaken, after which the solution must stand half an hour in the dark, in this time the xylenol is nitrated to nitroxylenol. The solution is then cooled in ice-water and diluted with 100 ml water. The diluted nitroxylenol solution may be kept in the dark till it is distilled with steam. Some nitroxylenol always crystallizes in the condensor; this is removed by shutting off the water of the condensor for some time in order that the crystals may be carried away with the steam. About 70 ml of the distillate is collected in 5 ml 1 % NaOH, in which it forms a yellowish-brown product. Water is added to 100 ml and the extinction of this liquid is determined in a Zeiss Stufen-photometer with S 47 filter.

A standard-curve is made by determining from different nitrate concentrations the respective extinction-values. Between 0.2 and 4.0 mg NO_3 a practically straight line can be obtained. In this way nitrate quantities of 0.2 to 4.0 mg per 5 ml can be determined with 1—2 % of accuracy.

For a nitrate determination in the fungus, it is necessary, according to MCVEY's (6) experiments with meat, to dissolve the nitrate first in water and to remove the protein. This is done in the following way: 2 fungus mats were ground with quartz-sand in a mortar, transferred to a beaker with 20 ml of water, made slightly alkaline with NaOH (indicator litmus) and then heated on a water-bath for an hour. Then this suspension is brought to a volume of 25 ml, and centrifuged. 15 ml of the liquid is acidified with 10 % H_2SO_4 to pH 3.6 (yellow to bromo-cresol green), after which 1 ml 20 % phosphotungstic acid solution is added to precipitate

the protein [1]). In a measuring flask this mass was filled up to 25 ml, centrifuged and 5 ml of the liquid was used for the determination of the nitrate. To determine whether the nitrate-quantum has not increased or diminished by these manipulations, a known quantity of nitrate was added to nitrate-free fungus at different stages of the process, and at the end the quantity of nitrate is determined. These values proved to differ from the original by not more than 2 %.

It further appeared that when equal quantities of nitrate are added to fungi all cultivated and starved in the same way, about equal quantities are recovered some days later in the several series (see Table I).

TABLE I. Aspergillus niger, reared for 2 days on a nitrate-free RAULIN-solution, starved for 1 day, thereafter 1 or 2 days on 40 ml phosphate buffer pH 6 with KNO_3. Figures are mg per 1 fungus mat.

Added mg NO_3	25.2	25.2	25.2	25.2
Found in solution after 1 day	23.0	23.8	23.1	23.1
„ „ fungus „ „ „	1.4	1.4	1.4	1.4
„ „ solution „ 2 days	22.8	22.5	22.7	22.7
„ „ fungus „ „ „	0.8	0.6	0.5	0.4

The differences in these four series of 2 fungus mats are slight. This method is therefore well suited for determining nitrate in fungi and their solutions.

§ 2. *Influence of the pH upon the nitrate assimilation.*

a. *With starved fungus mats.*

The fungi, after being starved, are transferred to sterile phosphate buffers, to which nitrate has been added in the form of KNO_3. The pH changes but little and is restored to the original value again by the addition of phosphoric acid or NaOH. The results of the various experiments have been collected in Table II.

The table shows that nitrate has been taken up by the fungus from the solutions. In the fungus there is always a very small quantity of nitrate and thus no question of nitrate accumulation. No distinct connection can be observed between the pH and the quantum NO_3 taken up.

ITZEROTT (5) and BÜNNING (4) concluded from their experiments that the nitrate assimilation is dependent upon the pH, and observed a nitrate accumulation in the cells if the pH of the solution is lower than 3. But, as

[1]) In this case the protein was not precipitated with trichloro-acetic acid, as indicated by GORTER (10), because Cl has a disturbing effect in the xylenol method for nitrate determination.

TABLE II. Aspergillus niger, reared for 2 days on nitrate-free RAULIN-solution, 1 day starved, thereafter 1 or 2 days on 40 ml phosphate buffer of different pH, with nitrate, the original quantity of which is given in mg. The quantities recovered given in percentages of the quantum added. Figures per 1 fungus mat.

Exp. Nr	Duration of experiment after starvation	Original nitrate quantity mg	Nitrate recovered in % of original quantity	pH 2	pH 4	pH 6	pH 8
26	1 day	45.2	in solution	98.0	91.8	96.5	96.0
			in fungus	0.4	1.8	1.8	1.3
27	1 day	48.3	in solution	98.1	95.8	93.2	
			in fungus	1.8	2.9	5.3	
	2 days		in solution	93.8	97.5	93.8	
			in fungus	2.5	0.2	0.4	
28	2 days	58.5	in solution	84.5	93.2	92.2	
			in fungus	0.8	0.8	0.6	
31	2 days	55.2	in solution	80.0	82.2	86.6	86.1

was remarked above, these authors made no quantitative determinations in the fungus mats, but merely determined nitrate in the buffer solution in experiments with non-starved fungi.

b. *With growing fungi.*

In the case of non-starved, or still growing, fungus mats, nitrate is much more used in metabolism than in the case of starved fungi. Here the pH may have some influence; some experiments were therefore carried out respecting the nitrate assimilation with growing fungi. For this purpose the fungi were reared at least 3 days in Raulin-solutions of different pH to which 5 g KNO_3 per l, had been added. During the growth it was observed that the fungi at high pH formed but very thin mats. After a few days the nitrate quantum in the solutions and fungi, as also the dry-weights, were determined. (See Table III.)

TABLE III. (Exp. nr. 30). Aspergillus niger, reared on 37 ml nitrate containing RAULIN-solution of different pH. Original quantity of nitrate 108,9 mg. Recovered quantities of nitrate in percentages of the original. Figures per 1 fungus mat.

Duration of experiment	Recovered nitrate in %	pH 2	pH 4	pH 6	pH 8
4 days	in solution	54.0	12.0	26.6	75.8
	in fungus	0.0	0.0	0.0	0.0
5 days	in solution	0.0	0.0	0.0	10.5
	in fungus	0.1	0.0	0.0	0.0
4 days	dry weight in mg	184	267	273	117
5 days	dry weight in mg	387	517	457	344

It follows from this experiment that nitrate is quickest taken up by the fungi at pH 4. Nowhere has nitrate been found in the fungus, it is therefore not accumulated. The nitrate when taken up is at once metabolised, and this also takes place most rapidly at pH 4. Likewise the dry-weight is greatest at pH 4. We may thus state that the growth of the fungi is dependent upon the pH and that parallel to this is the nitrate assimilation (the uptake, accumulation and further transformation of nitrate together).

§ 3. *Influence of glucose.*

In contrast with § 2 where either a certain quantum of nitrate was used with starved fungi, or where non-starved fungi were reared on solutions containing nitrate and glucose, in the following experiments both nitrate and glucose were added to the starved fungus mats. By this method it could be ascertained whether carbohydrates are necessary as a source of energy in nitrate assimilation. To bring out the effect of glucose in the two series of experiments given below, different quantities of glucose were used. (See Table IV.)

TABLE IV. Aspergillus niger, reared for 2 days on nitrate-free RAULIN-solution, 1 day starved; thereafter 1 or 2 days on 40 ml phosphate buffer of different pH with nitrate and glucose. The nitrate quantum originally added in mg. The quantities recovered in percentages of the quantum added. Figures per 1 fungus mat.

Exp. Nr	Duration of experiment after starvation	Glucose added mg	Original quantity of nitrate mg	Nitrate recovered in % of original quantity	pH 2	pH 4	pH 6	pH 8
28	2 days	300	58.5	in solution	47.5	54.9	55.0	
				in fungus	0.6	0.2	0.2	
31	1 day	30	55.2	in solution	83.0	85.7	88.4	86.6
	2 days			in solution	71.5	82.4	82.2	79.1

Compare with these the corresponding values without glucose from Table II, experiment Nrs. 28 and 31.

It appears from these experiments that the addition of glucose promotes the assimilation of nitrate, for in this case more nitrate is taken up and metabolised. There is no accumulation here either, as can be seen from the first experiment. At pH 2 the influence of glucose in these cases is slightly greater.

The dry-weights of the fungi, with or without glucose show at different pH practically no differences. In contrast to the non-starved (§ 2 *b*), these fungi have been reared on a solution of the same pH, and the substances added after starvation have apparently had little influence on their dry-weight.

The influence of glucose is particularly noticeable when a larger quantity is added, for it appears that more glucose has a greater influence in promoting the nitrate metabolism. A similar influence of glucose was seen in the experiments of POSTMA (7) with Avena roots. A determination of the respiration in the case of Aspergillus will have to decide whether here, where the addition of glucose promotes the nitrate assimilation, there also will be an increased respiration.

In conclusion I wish to express my thanks to Dr. A. W. H. VAN HERK, and Mr. A. GORTER for their interest in this research.

Summary.

1. The xylenol method for determining the nitrate in fungi and their culture solutions yields satisfactory results.

2. Nitrate is not accumulated in Aspergillus niger, but is metabolised after uptake.

3. In the case of starved fungus mats, the pH is not seen to have any effect upon the nitrate assimilation.

4. In growing fungi the nitrate assimilation is greatest at pH 4 in connection with their growth.

5. Nitrate assimilation is increased by addition of glucose.

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