RESEARCHES ON THE FORMATION OF DIASTASE BY ASPERGILLUS ORYZAE

by

G. L. FUNKE.

Contents.

I. Introduction.

Though the researches which have been done, concerning the amylase of Aspergillus Oryzae, are rather numerous, yet I thought that the one, which I will describe here, might not be superfluous. Many authors studied the taka diastase or taka koji from a technical or purely chemical point of view only, without considering the vital phenomena of the fungus itself; sometimes even without growing it, because they obtained the diastase as a commercial product. Many others only made allusive remarks to the question, as it was their first aim to deal with other qualities of the fungus or with other species. (1-11-17-21-22-25-27).

Only a few treated the matter in a really biological way. One of the first who did so, was Büsgen (4); he investigated what part fungi were acting in the process of making rice wine (sake) in Japan and as the most important, if not the only one of them, he discovered and isolated Aspergillus Oryzae. He gives a rather detailed description of its general habitus, which is very useful as an orientation, but for the rest does not give any physio- logical particulars. He only notes one thing worth mentioning here, viz. that the fungus is not only capable of forming diastase on the amylum of rice, but also on glucose.

A general physiological research has been done by Saïto in 1910 (18); he grew Aspergillus Oryzae on a great number of nutrient solutions, making all sorts of combinations of carbon- and nitrogen sources. Every time when NH_4NO_3 was added, he noticed production of diastase. Only, in my opinion, his investigations could be improved upon in many points: he added carbon compounds in a concentration of 5 %, which seems to me to be too high. Further, he only once stated whether there was diastase present or not, after times of growth which varied between 6 and 37 days, so without considering the age of the fungus. The enzyme was not measured quantitatively, its presence or absence was only indicated by + or −. Neither was the degree of acidity exactly determined; we shall see that especially this last point ought not to have been neglected.

This investigation was somewhat extended by Kita (12); he too noticed the presence of amylase on all sorts of food material, especially when NH_4NO_3 was used as nitrogenous supply. But he did take into consideration the age of the fungus and noted that there is not always the same quantity of enzyme to be found. He mentions e. g. the remarkable fact that often in the first days after

oculation, no amylase appears, but that later on large quantities of it are formed, which arrive simultaneously with the development of the conidia. We shall see that this is indeed often the case, but that there is no causal relation between the two phenomena.

I will say no more here about the results of other authors; under the various subdivisions of my article, I shall find the occasion to refer to the publications, dealing with that part of it.

As the method of this research was essentially the same as in my former investigations about the formation of diastase by Aspergillus niger, I refer to my publications on that subject (7-8-9). I stuck to giving the carbon source in a concentration of only 0,5—1 %; the quantity of salts was only half of that I used formerly, so:

NH_4NO_3	0. 25 %
KH_2PO_4	0. 05 „
$MgSO_4$	0.025 „

In most cases I also investigated the quantity of amylase which the mycelium contained, by rubbing it down with infusorial earth and extracting it with the culture solution. When the mycelium was very scanty or when only small quantities of enzyme were to be expected, I used only 20 c.c.M. of the solution for the purpose. So in the tables two figures will be seen, one for the quantities of amylase in the culture solution and the other for culture solution + mycelium. I might have subtracted the first from the last, in order to know how much enzyme was contained in the mycelium, but I did not do so for several reasons. Firstly, it is of special interest to know the entire quantity of enzyme, produced by the fungus and it matters less how it is divided in- and outside the mycelium; and the more so because, as a rule, in the course of development

all amylase is secreted into the culture solution, as we shall see later. The separation in two different quantities therefore is only imaginary. Further it appeared, that we can never make out exactly how much amylase stays behind in the mycelium, as during the extraction the infusorial earth adsorbs part of it (quite like the acid which is formed in the culture solution). Indeed, when we see that sometimes the mycelium + culture solution seem to contain less amylase than the solution itself, this explanation seems the only satisfactory one for an otherwise absurd fact.

Formerly it was shown, that there exists no close relation between the weight of fungus material and the production of enzyme. I therefore did not always determine the dry weight, but when I did so, it was chiefly in order to compare the conduct of the three strains, with which the research was carried out, on the different sorts of food material.

The pH of the culture solution of every culture was determined in the colorimetrical way and is always indicated in the tables, as in the case of Aspergillus Oryzae it appeared to be of the utmost significance.

Also the formation of glycogen was gone into. It appeared that it was formed on a much smaller scale than by Aspergillus niger. In many cases no trace of it was to be seen, very often it was indistinct or dubious; and then only after some seven or ten days of growth, more rarely even after fourteen days and never at a later period. In the tables its precense or absence is therefore mostly not indicated.

As nearly always only acid phosphate was added to the culture solutions, these remained uncoloured during sterilisation; only when rather large quantities of $K_2 HPO_4$ were added, the sugar and the salt solutions were sterilised separately and afterwards put together.

In order to avoid a possible aftereffect of foregoing culture mediums, I only then considered an experiment as final when the conidia or the mycelium, used for oculation, were taken from cultures, which had been grown at least twice in a medium of the same chemical composition as that, on which I intended to grow them. For the rest I did not pay very much attention this time to this phenomenon.

I worked with three strains, one isolated from dead termites on Java by Dr. Boedijn (named B.), one out of the laboratory of miss Church, Washington (named Ch.) and one out of the botanical laboratory of Utrecht (named U.).

II. A. General phenomena.

In the beginning of my investigation, working with strain U only, I got the following impression of the course of development. The fungus grew rather well on most nutrient solutions but, contrary to Aspergillus niger, the mycelium mostly remained immersed in the liquid. It formed one loose, flocky mass, or often the flocks remained apart and were more sharply defined; it then gave a somewhat granular impression. It made no difference whether oculation was executed with conidia or with bits of mycelium. Sometimes this situation remained as it was and after some weeks the mycelium sunk gradually to the bottom of the flasks. Of course there appeared no conidia. Acid was fairly formed through the pH seldom was lower than 4.- and remained prettily constant.

Amylase could hardly ever be traced; only at the very outset larger or smaller quantities [1]) appeared (vid. tables 1U, 2U, 5U, 11U, etc.). As all authors concur in

[1]) The concentration of amylase was estimated by taking the amount to be = 100, if the time, taken for hydrolysis, was 150 min. (comp. Went, 32). Of course, the word "quantity" is not correct, but I have used it for convenience' sake.

regarding Aspergillus Oryzae as a strong producer or diastase, I was surprised to see so very little of it in my own experiments. Then the outcome of two culture series changed my opinion. One of them was on lactose (table 14 U) and showed no enzyme in the solution and little in the mycelium. On the 34th day however, the fungus had developed on the surface, forming a large quantity of conidia. Amylase at once was abundant; I had to leave this series for a long time and could only look at it again, when it was more than a hundred days old. I thought that it would be exhausted at that time, but the contrary was the case. Dry weight had still increased, conidia had developed very abundantly and large quantities of diastase were present, which had been entirely secreted into the liquid. Hydrogen ion concentration had sunk in the mean time to pH $\pm$ 6.2.

The other series was on amylum and taught me even more (table 16U): after three weeks, no or hardly any diastase had been formed; development of the fungus resembled that, described above. After the 21st day in some flasks a totally different habitus was to be observed: the mycelium had spread on the surface a rather thick, closed pellicle without any conidia. The quantities of enzyme were abundant and the degree of acidity had sunk considerably. The series had thus differentiated into two groups which remained clearly distinct. In the "surface series", pH remained constantly between 6.- and 7.-, conidia never appeared (or if they did, very rare and uncoloured) and amylase was always present in large quantities; in the other flasks the mycelium remained immersed, pH about 4.- and no diastase could be demonstrated (though the amylum in the culture solutions had been entirely hydrolysed!).

It seemed to me that there might be a relation between the production of diastase and the degree of acidity of

the medium, viz. that a high degree of acidity makes formation of diastase impossible and vice versa. Till then I had not thought that the hydrogen ion concentration could have so much influence, because Sherman, Thomas and Baldwin (24) have stated, not only that the amylase of Aspergillus Oryzae works best at pH 4.8 but also, that there is rather a zone of optimal hydrolysing power which extends from pH ± 4.- to ± 6.-, gradually decreasing at either end. The degree of acidity in my liquids therefore remained mostly within the limits of this optimal zone.

Though I could at the moment not yet give an explanation of these facts, I tried however to produce the same result by an artificial keeping down of the degree of acidity. I therefore added 0.5 % K_2HPO_4 to the culture liquid, thus making a buffer solution of it which could directly bind any acid that might be formed. The results are rather striking, as may be seen from table 3: pH remained constantly between 6.— and 7.—; the mycelium, though in the very beginning immersed, formed very soon a thick, closed layer on the surface, which after two or three weeks was covered by brownish green conidia. The quantities of amylase were enormous and it is clear that they were secreted into the liquid, very soon after having been formed. In most cases therefore, I considered it no longer necessary to determine the quantities of enzyme in the mycelium after the first weeks.

The culture solutions were always kept apart and appeared to lose nothing of their enzymatic power, not even after six months.

The photographs may give an idea of two cultures on glucose, on buffered and not buffered solutions, otherwise grown under quite similar conditions and of the same age (28 days).

The phenomena, which I have described here, occurred

on all of the twelve kinds of food material on which I grew Aspergillus Oryzae. Summarizing, on not buffered solutions: mycelium immersed, flocky, degree of acidity high, no or hardly any diastase; on buffered solutions just the opposite.

Often however, on unbuffered solutions the mycelium came on the surface, sooner or later, of its own account and this was always accompanied by formation of diastase and disappearance of the acid, in most cases also with formation of conidia; but this is not necessary (in so far Kita is not quite right); vid. tables 7U, 14U, 18U).

Never, however, were the quantities of enzyme so abundant as on buffered solutions.

An important restriction is to be made, concerning the above described facts. After the first introductory experiments, I continued the research with two other strains, viz. Ch and B. It then appeared how useful it is to do this sort of work with more than one strain, because, if we investigate one strain only and happen to come across a less favourable one, we might get an altogether wrong idea of the physiological qualities of the fungus.

This holds true for strain B: in many respect it was entirely useless, though it was of some value as compared with the others. It differed from U and Ch in so far, that growth was much slower, the average dry weight at the beginning correspondingly less. In unbuffered solutions it came only very exceptionally to the surface; but when it did, development was very strong and especially conidia were formed in much larger quantities, which were besides striking by their clear —, nearly grass green colour. I can confirm Saïto's statement (17) about the colour of the conidia, viz. it differs with the composition of the nutrient solution, but still more with the age of the culture. The conidia are mostly preceeded by a yellow colouring of the purely white mycelium; at first they are either pale —

or grass green, later they fade into a brownish green, where ultimately the brown prevails. Especially on inuline, colour keeps somewhat longer pure green, on lichenine it is at first yellowish brown, but this is of little importance. Only in strain B the clear green colour was, on the whole, preserved during a longer period.

The main difference, however, between B and the others was that B formed extremely little diastase, even on buffered solutions, in any case much less than Ch and U. One glance at any table will suffice to make this clear. This phenomenon constantly appeared on every sort of food material and throughout the ten months during which this research was made. Later on, I shall again refer to this point.

Strain Ch did not differ very much from U. The last one was perhaps a little stronger as producer of enzyme, but this was of no great consequence. What is said generally about the formation of amylase, bears chiefly on strains U and Ch.

B. Production of diastase.

I will now proceed to the discussion of the formation of amylase on the different nutrient solutions. I need not say very much about it, the tables will speak for themselves. Yet one observation should be made, concerning the figures, which indicate the quantities of enzyme. These are calculated in the same way as has been done first by Went (32), viz. the amount of diastase is taken = 100, if the time for hydrolysis is 150 min.

This methods presents some difficulties, when times of hydrolysis are very short, especially as a little inaccuracy of observation will then cause great aberrations. So it occurred repeatedly that the amylum was hydrolysed in 30 or 35 seconds; this gives for the quantities of enzyme 30000, resp. 25710; in the last case I rounded off to

25000 or 26000. I can not warrant that the real time of hydrolysis may not have been one second shorter or longer. Besides, it is of only little importance for this type of work.

Table 1.

Amylase production of A.O. on glucose 1%, KH_2PO_4

Days.	Glycogen.	Diastase in myc. extract.	pH myc. extr.	Diastase in cult. sol.	pH cult. sol.	Dry weight in m.Gr.	Remarks.
				Strain U.			
14	+?	0	4.5	0	4.0	170	immersed.
21	—	0	4.0	0	3.9	126	„
7	(+)	30	5.5	0	4.8	—	„
14	(+)	trace	5.4	0	4.8	100	„
21	—	0	4.5	0	4.1	145	„
27	—	0	4.0	0	4.5	117	„
35	—	0	4.0	0	3.4	194	„
49	—	0	4.0	0	3.6	126	„
63	—	0	3.8	0	3.5	134	„
				Strain Ch.			
14	—			0	4.6	—	immersed.
21	—	12	4.8	0	4.0	—	„
28	—	75	5.6	4	4.7	—	on the surface; con. —
35	—	200	5.8	0	4.2	—	„ „ „ „ —
42	—	10	5.9	0	4.0	—	„ „ „ „ —
				Strain B.			
21	—	0	5.9	0	3.5	—	immersed.
35	—	10	5.2	0	4.2	—	„
42	—	0	5.5	0	4.9	—	„
49	—	0	5.5	0	4.9	—	„
63	—	0	6.1	0	5.8	—	„

As may be seen, no or hardly any diastase, except a little in the mycelium of Ch.

Due to the KH_2PO_4, the culture solution is at the beginning faintly acid, pH ± 5.8. Though I did not think it probable, it might however be possible that this degree of acidity prevents the very first development of amylase. Therefore I grew the three strains also on strictly neutral solutions by adding K_2HPO_4 i. st. of KH_2PO_4. The results are to be seen in table 2.

Table 2.

Amylase production of A.O. on glucose 1 %, K_2HPO_4.

Days.	Glycogen.	Dyastase in myc. extract.	pH myc. extr.	Diatase in cult. sol.	pH cult. sol.	Dry weight in m.Gr.	Remarks.
				Strain U.			
28	—			0	4.5	73	immersed.
7	—	1875	6.0	120	6.0		„
14	—	0	4.9	0	4.4		„
21	—	0	3.5	0	3.5		„
35	—	0	4.0	0	3.5		„
				Strain Ch.			
28	—			0	4.0	172	little flocks on the surface.
7	+?	100	6.2	20	5.9		„ „ „ „ „
14	—	500	5.9	0	4.8		strong development
21	—	185	5.5	0	4.6		„ „ ; con.?
35	—	15	5.5	0	4.4		„ „ ; con.
				Strain B.			
28	—			0	4.1	98	immersed.
7	—	trace	6.0	trace	5.7		„
14	—	0	4.5	0	3.9		„
21	—	0	4.9	0	4.6		„
35	—	0	4.9	0	4.8		„

As was to be expected, things remain more or less the same.

Buffered glucose solution shows a very different result; vid. table 3. Though these cultures make the impression of having developed much stronger than those on unbuffered solutions, it appears from the figures for the dry weight, that in reality this is hardly the case. We shall see something similar on nearly every kind of food material; only on amylum and glycerine there is a notable increase of dry weight on the buffered solutions, as compared with the unbuffered ones.

Table 3.

Am. prod. of A. O. on glucose 1 %, buffered.

Days.	Glycogen.	Diastase in myc. extract.	pH myc. extr.	Diastase in cult. sol.	pH cult. sol.	Dry weight in m.Gr.	Remarks.
				Strain U.			
35	—			26000	6.3	153	
7	—	3000	6.5	350	6.0	77	immersed.
14	—	15000	6.5	7500	5.9	119	on the surface
21	—			20000	6.1	194	clos. pell.; con. +
28	—			18000	6.3	162	„ „ „ + +
35	—			18000	6.5	186	„ „ „ + +
42	—			30000	6.5	140	„ „ „ + +
56	—			30000	6.5	131	„ „ „ + +
77	—			36000	6.5	108	„ „ „ + +
				Strain Ch.			
14	—						immersed.
21	—	7500	6.5	5000	6.2		closed pellicle; con. +
35	—	15000	6.6	15000	6.2		„ „ „ +
				Strain B.			
14	—	20	6.5	8	6.3		immersed.
21	—	300	6.6	10	6,3		on the surface;
28	—	2500	6.6	1000	6.4		„ „ „
42	—	65	6.6	65	6.3		„ „ „ ; con. +

It was shown formerly that for Aspergillus niger the concentration of the foodmaterial should not be higher than 1 %, if we want to get the full production of diastase. Here we see that Aspergillus Oryzae produces it nearly as well on a 5 % solution as on the 0.5 % or 1 % solutions, on which I used to grow it.

Table 4.

Am. prod. of A.O. on glucose 5 %, buffered.

Days.	Glycogen.	Diastase in myc. extract.	pH myc. extr.	Diastase in cult. sol.	pH cult. sol.	Dry weight in m.Gr.	Remarks.
				Strain U.			
12	—			3750	5.2		
15	—	30000	5.9	16500	5.1	367	closed pellicle, no con.
22	—			30000	6.2	570	„ „ „ „
				Strain Ch.			
12	(+)			2500	6.1		
15	(+)	12000	6.2	7500	5.6	425	no con.
22	—			5000	5.5	490	„ „
				Strain B.			
12	—			250	6.2		
15	—	2500	6.2	600	6.3	583	closed pellicle; no con.
22	—			350	6.3	496	„ „ „ „

Table 5.

Am. prod. of A.O. on galactose 1 %.

				Strain U.			
14	—	0	4.2	0	3.5	168	partly on the surface; no con.
7	—	85	6.0	0	4.2	—	„ „ „ „ „ „
14	—	0	4.0	0	3.4	174	„ „ „ „ „ „
21	—	0	4.2	0	3.8	168	„ „ „ „ „ „
28	—	0	4.8	0	3.8	120	„ „ „ „ „ „
34	—	0	4.8	0	3.8	—	„ „ „ „ „ „
49	—	0	4.2	0	3.5	138	„ „ „ „ „ „
63	—	0	4.5	0	3.5	112	„ „ „ „ „ „

Days.	Diastase in myc. extract.	pH myc. extr.	Diastase in cult. sol.	pH cult. sol.	Dry weight in m.Gr.	Remarks.
			Strain Ch.			
28	3750	6.1	7	5.9		on the surface; no con.
14	1250	6.2	0	5.5		„ „ „ „ „
			Strain B.			
28	20	5.9	0	4.7		immersed.
14	0	5.5	0	4.7		on the surface; no con.

Table 6.

Am. prod. of A.O. on galactose 1 %, buffered.

			Strain U.			
21			3000	6.4	190	clos. pell.; con. + +; yell. br.
7	1500	6.4	750	6.1		partly immersed.
14			3750	6.2	231	closed pellicle.
21			6000	6.4	178	„ „
35			9000	6.4	150	„ „ ; con. + +.
			Strain Ch.			
21			1500	6.3	199	
7	1500	6.2	700	6.2		partly immersed.
14			1250	6.3	199	closed pellicle.
21			6000	6.3	187	„ „
35			15000	6.5	111	„ „ ; con. + +.
			Strain B.			
21			0	6.2	190	con. + +, green.
7	6	6.1	0	6.3		closed, thin pellicle.
14			5	6.5	149	„ ; con. + + grass green.
21			0	6.5	115	„ „ + + „ „
35			8	6.5	84	„ „ + + „ „

Table 7.

Am. prod. of A.O. on mannose 0.5 %.

Days.	Diastase in myc. extract.	pH myc. extr.	Diastase in cult. sol.	pH cult. sol.	Dry weight in m.Gr.	Remarks.
			Strain U.			
21			0	4.8	102	strong.
7	30	4.9	0	4.5		partly immersed.
14	185	5.2	0	4.6		„ „
21	0	4.6	0	4.5		„ „
35	0	4.9	0	4.6		„ „
			Strain Ch.			
21			150	6.0	96	strong.
7	180	5.9	0	4.9		on the surface; con. —
14	600	5.8	0	4.6		„ „ „ „ —
28	125	6.1	10	6.0		„ „ „ „ —
			Strain B.			
21			0	4.6	72	immersed.
7	0	6.1	0	4.9		„
14	0	5.9	0	5.2		„
28	0	5.9	10	5.2		„

Table 8.

Am. prod. of A.O. on mannose 0.5 %, buffered.

			Strain U.			
21			30000	6.3		closed pellicle; con. + +
28			25000	6.4	93	„ „ „ + +
			Strain Ch.			
20			12000	6.2	—	closed pellicle; con. —
28			18000	6.5	154	„ „ „ + +
			Strain B.			
20			250	6.2		nearly clos. pellicle; con. +
28			100	6.4	128	„ „ „ „ + +

Table 9.

Am. prod. of A.O. on fructose 1 %.

Days.	Diastase in myc. extract.	pH myc. extr.	Diastase in cult. sol.	pH cult. sol.	Dry weight in m.Gr.	Remarks.
				Strain U.		
7	350	5.5	30	4.9		immersed.
14	0	4.9	0	4.2	160	partly on the surface; no con.
21	30	4.8	0	3.5	162	„ „ „ „ „ „
28	0	4.9	0	4.0	134	„ „ „ „ „ „
42	30	5.6	0	4.8	106	„ „ „ „ „ „
59			350	6.0	115	„ „ „ „ „ „
63			600	6.2	103	„ „ „ „ „ „
				Strain Ch.		
6						immersed.
13	0	4.9	0	3.5	206	on the surface, strong, no con.
20	0	3.8	0	3.5	182	„ „ „ „ „ „
28	0	4.6	0	3.6		„ „ „ „ „ „
14	0	4.6	0	4.1	234	on the surface; no con.
28	0	3.9	0	3.6	187	„ „ „ „ „
7	275	6.1	210	5.9		immersed.
21	0	4.9	0	3.5		partly on the surface.
28	125	5.9	12	4.6		„ „ „ „
35	2500	6.2	600	6.1		„ „ „ „
				Strain B.		
28	0	4.9	0	3.2		on the surface; no con.
21	0	5.8	0	3.7		„ „ „ „ „

Table 10.

Am. prod. of A.O. on fructose 1 %, buffered.

Days.	Diastase in myc. extract.	pH myc. extr.	Diastase in cult. sol.	pH cult. sol.	Dry weight in m.Gr.	Remarks.
			Strain U.			
13			7500			closed; no con.
21			20000	6.3	182	„ „ „
14			15000	6.4	146	„ „ „
			Strain Ch.			
13			15000	6.2	106	no con.
21			20000	6.5	77	„ „
			Strain B.			
7	0	6.1	0	6.2	—	partly on the surface; con. +
14	0	6.2	0	6.3	200	con. + +, grass green.
21	12	6.0	0	6.0	163	„ + +, „ „
28	0	6.2	0	6.1	106	„ + +, „ „
42	6	5.9	trace	5.8	144	„ + +, „ „

Strain Ch shows us on fructose an example of what I described on page 590; one of the series on unbuffered solution was very different from the others, for its degree of acidity had sunk with all the accompanying phenomena.

We have had already many opportunities to see that strain B can not be considered a standard of amylase production of Aspergillus Oryzae.

The dry weights of the three strains do not differ very much from one another; now one of them has developed stronger, then again the others; there seems to be no hard and fast rule in this respect.

With the bioses we get similar phenomena as with the monoses.

Table 11,
Am. prod. of A.O. on saccharose 1 %.

Days.	Diastase in myc. extract.	pH myc. extr.	Diastase in cult. sol.	pH cult. sol.	Dry weight in m.Gr.	Remarks.
			Strain U.			
28			0	3.4	85	immersed.
28			0	4.0	122	„
7	95	6.1	30	5.5		„
14	25	5.8	0	4.2	108	„
21	0	4.8	0	4.0	119	„
28	0	4.5	0	3.3	106	„
35	0	4.6	0	3.6	104	„
49	0	5.0	0	3.8	131	„
63	0	4.8	0	3.5	124	„

Table 12.
Am. prod. of A.O on saccharose 1 %, buffered.

			Strain U.			
28			7500	6.2	187	con. +
7	3500	6.2	350	6.2		partly immersed.
14	13000	6.4	1500	6.3		on the surface; con. —
21	21000	6.5	21000	6.4		„ „ „ „ —
35	20000	6.5	18000	6.4		„ „ „ „ + +
			Strain Ch.			
28			9000	6.2	186	con. +
7	880	6.1	85	6.3		partly immersed.
14	15000	6.3	375	6.5		on the surface; con. —
21	5000	6.4	1000	6.5		„ „ „ „ —
35	9000	6.4	9000	6.4		„ „ „ „ —
			Strain B.			
28			75	6.1	195	con. + +
7	165	6.2	0	6.0		immersed.
14	10	6.5	0	6.1		„
21	30	6.6	0	6.5		partly on the surf.; con. —
35	180	6.5	150	6.5		„ „ „ „ con. + +

Table 13.

Am. prod. of A.O. on maltose 1 %, buffered.

Days.	Diastase in myc. extract.	pH myc. extr.	Diastase in cult. sol.	pH cult. sol.	Dry weight in m.Gr.	Remarks.
			Strain U.			
12			5000	5.8		closed; con. + +
15	30000	6,2	6000	6.1		„ „ + +
			Strain Ch.			
12			5000	5.5		closed; con. —
15	30000	5.9	3000	5.2		„ „ —
10			30000	6.1	161	„ „ —
			Strain B.			
12			150	6.1		closed; con. —
15	600	6.2	500	6.1		„ „ —
10			750	6.2	176	„ „ + +

Table 14.

Am. prod. of A.O. on lactose 1 %.

			Strain U.			
14	20	5.0	0	4.5	81	immersed.
21	125	5.0	10	4.5	—	on the surface; con. +
28	125	5.7	0	5.0	87	„ „ „ „ +
34	5000	5.9	125	5.2	93	„ „ „ „ +
105	2500	6.4	3750	6.2	128	„ „ „ „ + +
7	45	6.1	25	5.8		immersed.
28	40	6.0	0	4.9		„
49	250	6.1	0	4.9		„
98			0	4.8		„
105			0	5.1		on the surf.; con. + pale green
112			0	4.9		„ „ „ „ + „ „
133	180	6.5	0	4.9		„ „ „ „ + „ „

Days.	Diastase in myc. extract.	pH myc. extr.	Diastase in cult. sol.	pH cult. sol.	Dry weight in m.Gr.	Remarks.
			Strain Ch.			
7	0	6.1	0	5.8		immersed.
21	0	6.2	0	5.2		„
28	45	6.1	0	5.0		„
35	30	6.2	35	5.0		on the surface; con. —
56	500	5.9	25	5.1	137	„ „ „ „ —
77	165	6.2	0	4.9		„ „ „ „ —
98			1875	6.2		„ „ „ „ —
119			1500	6.3	115	„ „ „ „ —
			Strain B.			
28	0	6.2	0	5.0		immersed, granular
35	0	6.1	0	5.1		„ „
56	0	6.1	0	4.9	125	„ „
98			25	5.5		„ „
119			0	4.9		„ „
133	5	6.2	0	6.1		„ „ ; con.?

Table 15.

Am. prod. of A.O. on lactose 1 %, buffered.

			Strain U.			
19	500	6.6	300	6.5		poor; con. —
42	12000	6.7	10000	6.6		covers surface; con. +
			Strain Ch.			
19	200	6.5	150	6.4		poor; con. —
42	6000	6.8	3500	6.5		on the surface; con. rare.
			Strain B.			
19	0	6.5	0	6.4		very poor; con. —
42	60	6.7	25	6.5		on the surface; con. rare.

Contrary to Aspergillus niger, Aspergillus Oryzae is very well able to feed on lactose. Kellner, Mori and Nagaoka (II) are wrong when they state that it can not assimilate this sugar (and the same holds for glycerine, as we shall see further on). Only, when we compare its

behaviour on other carbohydrates, we see that Aspergillus Oryzae has much difficulty with hydrolysing the milk sugar; lactose seems to be formed only scantily. Also on buffered solution, growth was slow and the great quantites of amylase appeared only after a long time.

On polyoses the fungus, on the whole, needed some more time to assimilate the food material, but essentially it behaves in the same way. I once more draw the attention to the fact that on unbuffered amylum solutions, strains Ch and U form hardly any diastase. though the iodine reaction indicated, that after ten days at the most, no more amylum was left in the nutrient liquid. I will refer to this question farther on.

Especially on amylum, strain B proves once more to give a quite false impression of the physiological qualities of Aspergillus Oryzae; in the unbuffered series it had not yet hydrolysed the starch after eight weeks; the liquid then still gave a red-violet colour with iodine. If the mycelium was extracted with water, no trace of enzyme appeared either.

Table 16.

Am. prod. of A.O. on amylum 0.5 %.

Days.	Diastase in myc. extract.		pH myc. extr.		Diastase in cult. sol.		pH cult. sol.		Dry weight in m.Gr.	Remarks.	
					Strain U.						
7	0		4.9		0		4.5		110	immersed.	
14	0		5.5		0		4.4		102	„	
7	18		6.2		20		5.5			immersed.	
13	trace		5.5		0		5.0		106	„	
21	0		5.9		0		4.6		72	„	
28	7500	0	6,3	5.1	2500	0	5,8	4.5	88	on the surf.; con. —	immersed.
35	5000	0	6.3	5.5	3750	0	6.1	4.5	101	„ „ „ „ —	„
42						0		4.5	77	„ „ „ „ —	„
56						0		4.7		„ „ „ „ —	„
63	3000		6.6		1500	0	6.3	4.8	84	„ „ „ „ —	„

Days.	Diastase in myc. extract.	pH myc. extr.	Diastase in cult. sol.	pH cult. sol.	Dry weight in m.Gr.	Remarks.
			Strain Ch.			
21	135	5.8	0	4.9	76	immersed.
7	55	6.1	30	5.9	29	„
14			0	5.0	51	„
21			0	4.6	49	„
28			0	4.8	58	partly on surface.
35			0	4.8	71	„ „ „
42			0	5.1		„ „ „
49			10	6.2		covers surface; con. —
56			75	6.2	104	„ „ „ —
			Strain B.			
14	0	5.5	0	4.4	68	immersed.
21			0	5.2	105	„
28			0	5.9	130	„
35			0	4.9	127	„
42			25 0	6.2 4.8	119	„
49			— 0	5.1	131	„
56			12 0	6,3 5.0	113	„
70			0	4,9	125	„

Series B gives a slight indication of a similar phenomenon as with U, viz. the differentiation into two groups with varying degree of acidity.

Table 17.

Am. prod. of A.O. on amylum 0.5 %, buffered.

Days.	Diastase in myc. extract.	pH myc. extr.	Diastase in cult. sol.	pH. cult. sol.	Dry weight in m.Gr.	Remarks.
			Strain Ch.			
35			22000	6.2	134	covers surface; con. —
14	15000	6.2	9000	6.1	108	on the surface.
21			30000	6.3	134	covers surface; no con.
28			18000	6.3	133	„ „ „ „
35			18000	6.6	103	„ „ „ „
42			18000	6.5		„ „ „ „
56			26000	6.5	119	„ „ „ „
70			30000	6.6	113	„ „ „ „
			Strain B.			
35	150	6.1	85	6.2	157	covers surface; con. + +
7	0	6.2	35	6.3		immersed.
14	1000	6.1	8	6.3	98	covers surface.
21	1250	6.4	30	6.5	164	„ „
28			135	6.6	150	„ „ ; con. + +
35			375	6.5	114	„ „ „ + +

Inuline is easily assimilated. New points of view are not to be noted. Only strain Ch makes an exception: on the buffered solution it forms relatively very little enzyme. Development too was extremely poor. When we compare however the behaviour of strain U, I rather believe that we need not attach too much importance to this fact which, though for the moment unexplained, is certainly a mere freak.

Table 18.

Am. prod. of A.O. on inuline 1 %

Days.	Diastase in myc. extract.	pH myc. extr.	Diastase in cult. sol.	pH cult. sol.	Dry weight in m.Gr.	Remarks.
			Strain U.			
7	30	5.5	0	4.2	118	covers surface; con. +
14	185	6.2	12	4.8	190	„ „ „ + +
7	550	5.4	25	4.8	84	strong; con. +
14	150	6.1	10	5.1	144	„ „ +
21	125	5.9	20	5.2	146	covers surface; con. + +
28	165	6.0	10	4.6	103	„ „ „ + +
34	110	5.8	15	4.9	119	„ „ „ + +
42	75	5.8	20	5.0	151	„ „ „ + +
49	60	5.8	20	5.0	140	„ „ „ + +
56	50	5.8	25	5.4	158	„ „ „ + +
			Strain Ch.			
7						very poor.
14	600	6.1	500	6.1		thin pellicle; con. +
21	125	6.1	185	4.9		„ „ „ +
28	100	6.0	25	5.5	95	„ „ „ +
42	100	6.1	35	5.8		„ „ „ +
			Strain B.			
7						immersed, very poor.
14	25	5.9	0	4.9		partly on the surface, con. —
21	20	5.9	6	5.1		strong, con. +
28	35	6.1	0	5.0	95	„ „ +
42	35	5.9	35	5.0		„ „ +

Table 19.

Am. prod. of A.O. on inuline 1 %, buffered.

Days.	Diastase in myc. extract.	pH myc. extr.	Diastase in cult. sol.	pH cult. sol.	Dry weight in m.Gr.	Remarks.
				Strain U.		
13			1250	6.1		covers surface; con. +
21			6000	6.1	191	„ „ „ + +
20			15000	6.2		„ „ „ + +
32			30000	6.5		„ „ „ + +
35			30000	6.5	185	„ „ „ + +
				Strain Ch.		
13			250	6.0		very poor.
21			250	6.2	102	no con.; partly immersed.
35			250	6.5	100	poor.
				Strain B.		
13			250	6.1		covers surface; con. + +
21			20	6.2	185	„ „ „ + +
32			100	6.4		„ „ „ + +
33			80	6.4	248	„ „ „ + +

The following table bears upon cultures on lichenine, a polyose prepared out of Cetraria islandica in the laboratory for technical botany, Delft. This material seems to be in its chemical composition very similar to cellulose; the main product of hydrolysis is glucose. It is not quite soluble in water, but forms a bronze coloured colloidal solution with it. After refrigeration, part of it coagulates into thin skins at the surface and against the walls of the flasks. Aspergillus Oryzae grew very slowly on it; afther three weeks only very small and thin pellicles were formed, which carried yellowish conidia. Yet the lichenine was hydrolysed;

this coud be observed from the culture solution, which passed very easily through the filters, whereas it took some hours when in its original state. The bronze colour remained unchanged. The part of the material which had coagulated, was not attacked by the fungus. So it was left behind in the filters, together with the mycelium; thus the dry weight could not be exactly determined. I approximated it by subtracting from the figures the weight of substance which was left behind in the filter by a sterile culture solution.

As far as amylase production is concerned, lichenine seems to be as good a food material as the others.

Table 20.

Am. prod. of A.O. on lichenine 1 %, buffered.

Days.	Diastase in myc. extract.	pH myc. extr.	Diastase in cult. sol.	pH cult. sol.	Dry weight in m.G.	Remarks.
			Strain U.			
21			6000	6.2	±34	thin pell.; con +, yellowish.
15	1500	6.3	800	6.1		„ „ „ + „
22	9000	6.4	5000	6.4		„ „ „ + „
29	9000	6.4	6000	6.4		covers surf.; con. greenish.
			Strain Ch.			
21			5000	6.2	±31	
15	600	6.1	500	6.3		poor.
22	3750	6.4	3000	6.4		rather strong; con. + +.
29	5000	6.5	3750	6.4		covers surf.; con greenish.
			Strain B.			
21			40	6.2	±33	
15	30	6.1	20	6.1		thin pellicles.
22	125	6.2	80	6.5		„ „ ; con, + +
29	180	6.4	100	6.5		covers surf.; con. greenish.

I grew Aspergillus Oryzae also on some substances, different from: carbohydrates. On unbuffered glycerine, growth is very scanty; the myceluim, which consists of some meagre flocks, remains entirely immersed. On buffered solutions, strain U gives results, similar to those on carbohydrates. Strain Ch again behaves differently, but this time too I am of opinion that this should not be regarded as a phenomenon, essentially differring from the main line of conduct of Aspergillus Oryzae towards its food material.

Table 21.

Am. prod. of A.O. on glycerine 1 %.

Days.	Diastase in myc. extract.	pH myc. extr.	Diastase in cult. sol.	pH. cult. sol.	Dry weight in m.Gr.	Remarks.
			Strain U.			
21			0	5.0	73	immersed.
7	75	6.0	20	5.2		very poor, immersed.
14						„ „ „
21	165	6.0	0	5.8		„ „ „
35	500	6.1	0	5.9		stronger.
			Strain Ch.			
21			0	4.5	82	partly on the surf.; no con.
7	100	6.1	trace	5.1		immersed, poor.
14	750	6.0	150	5.9		„ „
21	30	6.0	10	5.9		„ „
35	100	5.9	0	4.9		„ stronger
			Strain B.			
21			0	4.6	73	immersed.
7	0	6.0	0	4.9		poor; immersed.
21	0	5.9	0	4.9		„ „
35	0	6.2	0	5.1		„ „

Table 22.

Am. prod. of A.O. on glycerine 1 %, buffered.

Days.	Diastase in myc. extract.	pH myc. extr.	Diastase in cult. sol.	pH cult. sol.	Dry weight in m.Gr.	Remarks.
				Strain U.		
21			12000	5.6		covers surface; con. +
28			20000	6.3	160	„ „ „ +
				Strain Ch.		
21			75	6.1		poor, immersed.
28			375	6.2	70	„ „
				Strain B.		
21			50	6.2		on the surface; no. con.
28			15	6.5	188	„ „ „ „ „

Table 23.

Am. prod. of A.O. on calcium lactate 1 %.

				Strain U.		
14	600	5.9	220	5.8		immersed.
7	375	5.9	170	5.9		„
14	500	5.9	170	5.8	32	on the surface; con. +
21	375	6.1	250	5.9		„ „ „ „ +
35	500	6.4	500	6.2	83	„ „ „ „ +
				Strain Ch.		
14				—		very poor; immersed.
21	50	5.8	70	5.8		on the surface; con. —
28			200	6.0	58	„ „ „ „ —
35			45	5.6	52	„ „ „ „ +
42			85	5.9	54	„ „ „ „ +
56			50	5.9	50	„ „ „ „ +
63			85	5.9	63	„ „ „ „ +
				Strain B.		
14	0	6.2	0	6.1		on the surface; con. rare.
21	0	6.0	0	6.0		„ „ „ „ „
35	0	6.1	0	6.0		„ „ „ „ „
42	8	6.1	0	6.0		„ „ „ „ „
56	0	6.5	0	6.2	76	„ „ „ „ „

On calcium lactate Aspergillus Oryzae develops only scantily, though much better than Aspergillus niger. Numerous tiny flocks of mycelium are formed on the surface. The degree of acidity is much lower than on unbuffered carbohydrates, very probably, because only a little part of the foodmaterial is consumed.

The quantities of diastase, considering the very scanty dry weight, are rather considerable.

The behaviour on buffered calcium lactate has not been investigated.

III. Discussion of the results and supplemental investigations.

When briefly recapitulating the results, as they are recorded in the tables, we state the following facts: when Aspergillus Oryzae is grown on a nutrient solution, which has been buffered by adding 0.5 % K_2HPO_4, it develops well, forms a mycelium, which covers the surface of the liquid after two weeks at the utmost and which is mostly, but not always, coloured brownish green by the very numerous conidia. It produces large quantities of diastase, which are soon entirely secreted into the culture solution and which keep their enzymatic concentration for an indefinite time. These facts were observed with every one of the twelve kinds of food material used. The chemical composition of the nutrient solution appeared to have no special influence. All this applies to two of the three strains, with which the research was done.

When, however, the nutrient solutions were not buffered, the phenomena were quite different, the mycelium came occasionally to the surface (e.g. on inuline), but remained mostly immersed. Diastase was not formed, or if so, in scanty quantities and irregularly. The degree of acidity rose to an average pH of about 4.—.

This hydrogen ion concentration is in itself not high

enough to prohibit the diastase action, on the contrary, it is nearly optimal. It is clear, however, that there must be some relation between the production of diastase and of acid, which, so to say, exclude one another. The enzyme is produced before the acid, but as soon as the latter appears, the former disappears. Yet we may not conclude that the acid destroys the amylase, because there are too many instances of series where at length the hydrogen ion concentration goes down and where the diastase then reappears (e.g. T. 9Ch, 14U, 16U etc.). I can not believe that in those cases the enzyme should be newly formed for the second time. All my researches have hitherto confirmed my impression that the fungus only once produces diastase, viz. in the beginning of its lifetime and that this enzyme is kept, when circumstances are favourable for it.

As the hydrogen ion concentration itself can not be the cause of the inactivity of the enzyme, it must be admitted that the chemical composition of the acid plays a role, viz. that there may exist a temporary chemical binding between acid and diastase (leaving the hydrogen ions free). The following data plead in my opinion for this hypothesis: when in a solution of pH 3.— à 5.— there appeared no diastase, I raised the pH to $\pm$ 6.— by carefully adding bases (mostly $Na_2 CO_3$); though I have done this with more than a hundred different liquids, I could never state any enzymatic action in this way.

When an acid liquid contains some material, which could prevent an eventually existing diastase to come to activity, it might be that, when we add some active enzyme to that liquid, this would also lose its activity. A great number of acid liquids were thus mixed with strong enzymic concentrations. Neither immediately, nor after one or two weeks, could the least decrease of activity of the enzyme be shown. (On the contrary, as the active liquids

had a pH between 6.— and 7.—, the mixing with an acid liquid brought their pH tot ± 5.— and the enzyme worked the more intensely for it). So, if such binding exists, it must take place directly after the formation of the amylase.

I have no clear idea myself about the nature of that binding. It is very possible of course, that the enzyme is not bound directly to some acid, but that the protoplasma in some way has a part in it. Indeed, it is only natural it has. Now it would be very complicated to analyse all the chemical processes, which are going on here; the interesting theories and considerations of Kluyver and Donker (13, 14) could perhaps throw more light on this problem, but I myself did not yet venture to apply them. For the moment we must satisfy ourselves with the vague notion that there exists some chemical relation between acid and enzyme but, in any case, we can imagine that the process proceeds somehwat as follows: the diastase is temporarily bound after having been formed (without, however. losing its activity altogether, witness the hydrolysis of the amylum in the amylum cultures); later on, when the foodmaterial has been assimilated and dissimilation gets the upper hand, the protoplasm consumes part of itself, resp. the acids in the first place, the degree of acidity is thus lowered and the enzyme comes free once more (or: by the dissimilation of the protoplasm, proteolytic substances could come free which, having a stronger affinity to the acids, would bind them, thus superseding the enzyme and causing it to reappear).

But if so, this process could perhaps be accelerated by temperature, higher than the room temperature at which I did this research. I therefore placed some cultures on different nutrient liquids in a room where temperature was constantly 35°; I never saw that in those cases the diastase reappeared earlier than in the cultures at ± 20°. So this

seems to plead against the above described cours of things. But I must add, that this outcome may not be considered as final, because I did not repeat the investigation often enough; besides, it might have been arranged in another way, in order to let the high temperature influence the protoplasm more intensely. I have not had enough time to do this.

Moreover, I observed something else; as I saw that the strongest enzyme action was always to be found in liquids of pH between 6.— and 7.—, I wondered whether Sherman, Thomas and Baldwin (24) were right in stating that the optimal hydrogen ion concentration for the amylase of Aspergillus Oryzae was at pH 4.8. So by adding acids to those liquids, I lowered the pH to 4.5 — 5.— and saw that the enzyme action became really stronger, corresponding to the data of Sherman c. s. This was the case when I added phosphates (which were used also by Sherman c. s.) or citric acid. With oxalic acid or HNO_3 however, things turned out somewhat differently. It seems that oxalic acid in higher concentrations acts upon the enzyme, not only by its hydrogen ions, but also in another way, viz. by its chemical properties. At pH 3.— à 3.5 the action is strongly weakened, though still very distinct; when oxalic acid was added, however, not the least diastatic action was to be shown any more at this $(H^{\cdot})$.HNO_3 does the same in a still stronger degree. As the pH of the culture solution, in which no amylase appeared, does not differ very much from that, at which the very strong enzyme showed no more activity in this investigation, it is acceptable that the fungus really forms oxalic acid, thus inhibiting its own enzyme action (with pH 3.— à 4.— correspond concentrations of oxalic acid, which can very well exist in the culture solution). The following facts make this supposition still more probable.

After having inactivated the diastase by oxalic acid, I

added a solution of Na_2CO_3 till the pH was ± 6.2, in order to see if the process was revertible. It appeared that the enzyme regained its former activity for the greater part, when the alkali was added immediately after the acid; the longer, however, the acid was allowed to work upon the diastase, the less the Na_2CO_3 was capable of reactivating the enzyme, as will be seen from the following data:

original concentration of diastase (pH 6.2)	: 9500
Na_2CO_3 added immediately after the oxalic acid	: 7500
„ „ „ 5 min. „ „ „ „	: 7000
„ „ „ 60 „ „ „ „ „	: 375
„ „ „ 24 hours „ „ „ „	: trace?

I think that it is clear, that there exists some kind of chemical binding between acid and enzyme. Now we can imagine that the acid is capable of binding only a limited quantity of the amylase; once it is saturated, we could add more diastase, which then would not lose its activity. This proved to be true: a c.c.M. of oxalic acid inactivates entirely a c.c.M. of the diastase; when once more a c.c.M of diastase are added, one tenth of the last quantity seems not to get bound; all of the diastase, which is further added, is in no way influenced by the acid. All this tallies with what is going on in reality in the cultures and it also explains how an acid inactive culture solution is not capable of weakening the diastase concentration in other liquids.

It it of interest now to know, whether Aspergillus Oryzae really forms oxalic acid (or HNO_3; I did not, however, extend my investigations farther on this substance; nor did I investigate the influence of gluconic acid, though Butkewitsch (5) showed that this too is produced often by fungi). I could not find any exact indication in literature whether oxalic acid occurs in this fungus; Aspergillus niger appears to form it always, except when inorganic nitrates are given as nitrogen source; in the last case, HNO_3 is

produced, which stops the formation of oxalic acid (2-3-10-19-30-31). Yet there is some contradiction on this matter; Wehmer e.g. stated the forming of oxalic acid, though he gave NH_4NO_3 as nitrogen supply. I did not succeed in clearing up this point. When I added $CaCO_3$ to buffer the solution, the formation of amylase took place as usual; also when I used asparagine as nitrogenous supply. On unbuffered solutions with asparagine, the enzyme production was lowered, but not stopped altogether and the degree of acidity was not so high as in solutions with NH_4NO_3 (see table 24); so far, things tally with which was to be expected. But I thought that by adding $CaCO_3$, crystals of calcium oxalate might be formed. Now I could not find them, neither between the hyphae of the mycelium, nor in the sediment at the bottom of the flasks.

From the figures for the dry weight, we see that asparagine seems to be a much better food material for Aspergillus Oryzae, than NH_4NO_3.

Table 24.

Am. prod. of A. O. on glucose 2 %; strain U.

Days.	Diastase in myc. extract.	pH myc. extr.	Diastase in cult. sol.	pH cult. sol.	Dry weight in m.Gr.	Remarks.
buffered by $CaCO_3$; nitrogen source: NH_4NO_3						
10	12000	6.3	10000	6.2	109	thin pellicles; no con.
17	12000	6.5	7500	6.3	132	„ „ „ „
unbuffered; nitrogen source: asparagine						
10	800	6.0	750	5.2	273	covers surface; no. con.
17	6000	6.2	3000	6.1	—	„ „ con. +
buffered by $CaCO_3$; nitrogen source: asparagine						
10	12000	6.4	9000	6.2	266	covers surface; no con.
17	15000	6.5	6000	6.3	—	„ „ con. +

There was still another possibility, which had to be considered. It is striking that the absence of diastase is not only accompanied by a high degree of acidity, but that the fungus in that case is also nearly always immersed. Although the layer of liquid above the mycelium is only thin, we may yet admit that an immersed mycelium has less free oxygenium at its disposal than one, which has grown on the surface and is thus in immediate contact with the air. Though we need not think of an entirely anaerobic respiration, yet it might be that an immersed mycelium gets at least part of its energy by digesting intramolecularly the supplied carbon compounds; oxalic acid might be formed in this way, which could explain the other phenomena. There are, however, strong evidences against this supposition. Though the Aspergilli on the whole, are able to live partly anaerobically, yet they mostly produce their acids when developing strongly, that is, when they dispose fully of free oxygenium. Besides, Tollenaar (29) stated, that taka diastase may be active even in absence of O_2 (which need not prove that it can be formed under that condition).

Yet I would not neglect this side of the question. I therefore grew the fungus in flasks, which could be closed by glass stoppers, part of them were nearly entirely filled with the nutrient liquid, so that there remained a very little space of air, only juist enough for germination; an other part of them were only filled half way up, $\pm$ 50 c.c.M. of air being left. Thus the rate of available oxygenium was lowered considerably. By way of control, a parallel series was oculated on similar flasks, which were closed with wads of cotton wool.

Table 25.

Am. prod. of A.O. on glucose 1 %. buffered; strain Ch.

Days.	Diastase in myc. extract.	pH myc. extr.	Diastase in cult. sol.	pH cult. sol.	Dry weight in m.Gr.	Remarks.
			Flasks entirely filled.			
14	250	6.3	50	6.2		poor, little flocks on the surf.
21	1500	6.3	140	6.1		„ „ „ „ „ „
28	1250	6.5	45	6.3		„ „ „ „ „ „
42	750	6.6	150	6.5		„ „ „ „ „ „
			Flasks halfway filled.			
14	1800	6.2	375			little flocks on the surface.
21			1800	6.2	126	dito, stronger.
28			1500	6.4	154	„ „
42			30000	6.5	115	covers surface; no con.
			Flasks closed with cotton wool.			
14			15000			covers surface, con. —
21			22000	6.5	183	„ „ „ +
28			30000	6.5	123	„ „ „ +

Am. prod. of A.O. on amylum 0.5 %; strain U.

			Flasks halfway filled.			
15	900	4.5	0	4.5	51	on the surface; no con.
22	0	4.3	0	3.8	83	„ „ „ „ „
			Flasks closed with cotton wool.			
15	0	4.9	0	4.8	73	on the surface; no con.
22	0	4.1	0	3.5	103	„ „ „ „ „

The figures of the glucose series seem at first sight to confirm my hypothesis; yet there are a few observations to be made: in the entirely filled flasks, growth was very

poor; only a few little flocks of mycelium were formed; the small quantities of diastase are without doubt due to this. In the halfway filled flasks, growth was almost normal; the mycelium formed a thick, closed pellicle but without any conidia (comp. Thom and Church, 28). Though it lasted somewhat longer, yet the normal quantities of diastase appeared in the end. Moreover, hardly any acid was formed, due to the buffering of the liquid. I therefore repeated the investigation on unbuffered amylum. I expected to force a similar result as with the amylum series of strain U, only with still more telling phenomena. Results turned out negative. In any case it is not doubtful that more or less anaerobic respiration is not vital for the formation of enzyme.

The fact that on unbuffered solutions a mycelium sometimes comes to the surface, and sometimes does not, with all the accompanying phenomena, must, for the moment, remain unexplained.

The results, obtained with Aspergillus Oryzae, arouse my doubts wether the conclusions, drawn from the researches on Aspergillus niger, were quite correct. I thought I was justified in saying that there are kinds of sugars on which Aspergillus niger does not form any diastase, as no trace of it appeared in my experiments, This is especially the case with mannose and fructose. It had not struck me that the degree of acidity could have been of any influence, because the hydrogen ion concentration mostly remains within the limits of optimal diastase action; moreover, Aspergillus niger never forms two sorts of mycelia like Aspergillus Oryzae does, but always develops on the surface of the liquid, notwithstanding the acid. Now it might be possible that yet this acid prevents the diastase from appearing; if this were so, we might perhaps get the diastase free, if Aspergillus niger were grown on

buffered solutions. I have done this with two strains from the laboratories of Schiemann and professor Westerdijk (called Sch and West). (I did not use strains U and G, with which my former tests were done, because the after-effect of their seven passings on milk sugar had not yet ceased, though after the last cultures, which I have recorded in tables 34 and 35 (9), I grew them several times subsequently on amylum).

I grew the strains Sch and West by preference on mannose and fructose, as formerly I had not found any amylase on these substances. The results appear in tables 26, 27, 28 and 29.

Table 26.

Am. prod. of Asp. niger on mannose 0.5 %.

Days.	Diastase in myc. extract.	pH myc. extr.	Diastase in cult. sol.	pH cult. sol.	Dry weight in m.Gr.	Remarks.
			Strain West.			
14			50	4.0	149	numerous flocks; con. +
7	0	5.9	0	4.5		„ „ „ +
14	trace	4.9	25	4.2		„ „ „ + +
21	8	5.1	75	4.8		„ „ „ + +
35	50	6.0	75	5.1		„ „ „ + +
			Strain Sch.			
14			120	4.0	130	numerous flocks; con. +
7	18	4.5	0	4.2		no con.
14	300	4.2	125	4.0		con. + +
21	150	5.0	150	4.6		„ + +
35	75	5.4	75	4.7		„ + +

Table 27.

Am. prod. of A. n. on mannose 0.5 %, buffered.

Days.	Diastase in myc. extract.	pH myc. extr.	Diastase in cult. sol.	pH cult. sol.	Dry weight in m.Gr.	Remarks.
				Strain West.		
14			0	6.0	159	covers surface; con. + +
7	75	5.4	0	5.0		con. +
14	300	6.0	10	5.5		„ + +
21	200	6.2	trace	5.9		„ + +
35	200	6.5	20	5.9		„ + +
				Strain Sch.		
14			65	6.2	148	covers surface; con. + +
7	50	6.2	0	6.0		con. +
14	125	6.2	trace	6.1		„ + +
21	100	6.4	25	6.1		„ + +
35	75	6.5	40	6.1		„ + +

Table 28.

Am. prod. of A. n. on fructose 1 %.

				Strain West.		
14			0	3.8	217	some flocks, con. +
7	50	4.8	0	4.2		strong, no con.
14	50	4.5	trace	4.0		con. +
21	20	4.5	trace	4.0		„ +
35	75	4.5	50	4.9		„ +
63	0	6.1	0	5.9		„ +
				Strain Sch.		
14			35	4.0	224	numerons flocks; con. + +
7	25	4.9	0	4.0		strong; no con.
14	150	4.8	75	4.0		con. +
21	180	5.5	150	4.8		„ +
35	150	6.1	150	5.1		„ +
63	50	6.2	50	6.2		„ +

Table 29.

Am. prod. of A. n. on fructose 1 %. buffered.

Days.	Diastase in myc. extract.	pH myc. extr.	Diastase in cult. sol.	pH cult. sol.	Dry weight in m.Gr.	Remarks.
				Strain West.		
14			60	4.5	271	strong; con. + +
7	5	6.1	0	6.0		con. +
14	150	4.9	20	4.5		„ + +
21	75	4.8	100	4.2		„ + +
35	100	5.5	40	4.5		„ + +
				Strain Sch.		
14			35	4.9	262	covers surface; con. + +
7	18	5.9	0	5.9		con. +
14	150	5.8	10	4.9		„ + +
21	125	5.9	75	5.0		„ + +
35	100	6.2	125	5.9		„ + +

From these tables we may conclude firstly, that I was not right as I admitted that Aspergillus niger does not form at all any diastase on fructose and mannose, as may been seen from the figures on the unbuffered series. My former conclusion was only true for the strains, with which I worked at the time.

Secondly, it appears that 0.5 % K_2HPO_4 is not sufficient for Aspergillus niger to bind all the acid, which is formed on a 1 % or 0.5 % solution of sugar. But when we see the figures for strain Sch on buffered mannose 0.5 %, buffering appears to have kept down the hydrogen ion concentration fairly succesfully. Now, there is no question about a considerable increase of diastase, as compared with the unbuffered series, nor is there in the other series.

That at least strain West is quite well able to produce diastase, may appear from table 30, which records the

results of some cultures on amylum and glucose; here we see again the quantities of diastase, we are used to with Aspergillus niger. It should be noted that the hydrogen ion concentration in these cultures is rather high (though they were buffered) and that the enzyme is not at all hindered by it. The amylase of the starch and glucose cultures kept its concentration for an indefinite time, whereas that of the mannose and fructose cultures lost it gradually within some weeks. Apparently formation of enzyme is prevented by the last two sugars or by their metabolic substances In any case, I think that I have been right formerly in concluding from these and similar facts, that for Aspergillus niger, it is the chemical composition of the food material which in the first place influences the production of diastase, and not the degree of acidity.

Table 30.

Amylase production by Asp. niger

Food material.	Days.	Diastase in myc. extract.	pH myc. extr.	Diastase in cult. sol.	pH cult. sol.	Remarks.
			strain West.			
glucose 1 %, buffered.	12			750	4.8	covers surface; con. + +
	15	1000	5.1	1000	4.8	„ „ „ + +
	10	2500	5.7	2500	5.5	„ „ „ + +
amyl. 0.5 %, buffered.	22			1900	4.9	thin pellicle; con. +
	25	2750	5.1	2000	4.4	
	17	1500	5.8	800	5.5	
			Strain Sch.			
glucose 1 %, buffered.	12			0	5.9	strong
	15	500	6.2	trace	6.3	
	10	350	6.1	200	6.1	covers surface; con. + +
amyl. 1.5 %, buffered.	35	1250	6.6	165	6.3	thin pellicle; con. +
	27	900	6.3	150	6.2	

A few words remain to be said about the strains. That they can differ rather much, we have seen again with Aspergillus Oryzae, especially with strain B. The differences are mainly physiological, appearing from the diastase formation, but also from the different way in which the food material is consumed: hence, apparently less growth, immersed mycelium, high rate of acidity, etc. I have said in the beginning that this strain, shortly before I began my experiments with it, had been isolated from dead termites on Java. So we can say that it is rather a „wild" strain; now it is worth while comparing it with the one of Aspergillus niger, which I have isolated at the time from oak galls. This one represented similar phenomena, viz. slow growth on several sorts of sugar, especially on amylum, no or hardly any formation of enzyme. I have already demonstrated that it needs in no way be looked upon as a heriditarily constant race, but only as the result of a long lasting, strong aftereffect of former mediums, i. c. oak galls. The same can be argued of strain B of Aspergillus Oryzae; not only has it been found on quite different food material (and how long had it grown there?), but besides, it comes from an other climate, a fact which may have influenced it in many ways. It would have been worth while cultivating it at a temperature, higher than in the room where I made my investigations, but unfortunately time failed me.

In the meanwhile, I dare not say, for the same reasons as for strain G of Aspergillus niger, that strain B is a heriditary variety, not even though we see the results of the following supplemental work. Table 31 records the result of a series of subsequent cultures on amylum, after B had been grown for ± 8 months on several kinds of food material. The intention was to make B used to assimilating starch and thus induce it to produce diastase. Now, the quantities of enzyme prove to be hardly or not

greater than in the first series on amylum (table 16) and moreover, they do not increase in the subsequent cultures. I believe that, if any result were to be got, this research ought to be continued for some years at least.

Table 31.

Am prod. of strain B on amylum 0.5 %, buffered.

Foregoing culture medium.	Number of the culture.	Days	Diastase in myc. extract.	pH myc. extr.	Diastase in cult. sol.	pH cult. sol.	Remarks.
Several kinds of carbo hydrates during 8 months	N°. 1	21	1500	6.2	1500	6.1	covers surface; con. (+)
N°. 1	N°. 2	15			250	6,2	covers surface; con. (+)
		26			900	6.6	„ „ „ (+)
		29	750	6.6	750	6,7	„ „ „ (+)
N°. 2	N°. 3	19			200	6.2	„ „ „ (+)
		22	1000	6.5	200	6.2	
N°. 3	N°. 4	22	750	6.4	250	6.1	
N°. 4	N°. 5	10	500	6.2	60	6.1	
N°. 5	N°. 6	14	250	6.1	100	6.0	
N°. 6	N°. 7	20	350	6.3	75	6.2	
N°. 7	N°. 8	21	150	6.3	85	6.4	

Strains Ch and U had been grown for many years in laboratories; we need not wonder, therefore, that this has had its influence on their diastase production and that, on the whole, they are very similar in all respects.

So I can quite agree with the opinion of Thom and Church (28), who are rather sceptical about mutations, hereditary varieties, etc., as they have experienced during twenty years the seeming constancy with which many Aspergilli reacted on change of medium.

Strains Sch and West show little differences, except

for the formation of conidia: with West they were at first spread all over the surface of the mycelium, which in that way was coloured and even grey; with Sch on the contrary, the conidia were formed on some well defined spots only, in large quantities, so that those spots were coloured a deep black, sharply contrasting with the white mycelium. This was especially clear a week after oculating; later on, the mycelia of both strains were equally covered with a thick layer of conidia, so that the difference became indistinct. Yet it could be still recognized after five weeks, especially on the unbuffered solutions.

Those sharply defined spots of black conidia of strain Sch, also its great difficulty in assimilating amylum, its developing only little acid etc., reminded me of similar phenomena in strain G. It appeared, however, that this time these facts were not due to a recent isolation. Both strains had been for fifteen years in the laboratory of professor Westerdijk; West had been isolated out of the air, Sch was got by Schiemann as a mutation (20).

IIII. Summary.

1) On buffered nutrient solutions Aspergillus Oryzae produces diastase in very large quantities; it is secreted into the liquid soon after having been formed and keeps its concentration for an indefinite time.
2) The chemical composition of the food material, twelve kinds of which have been used in the experiments, has no influence whatever on the quantity of diastase.
3) The fungus, on the whole, needs somewhat more time for hydrolysing bioses and polyoses; the above described diastase production occurs all the same, only some weeks later. Especially lactose is hydrolysed with much difficulty.
4) The concentration of the carbonic food material was mostly 0.5 or 1 %; on 5 % solutions, the quantities of diastase produced, were quite the same.

5) On unbuffered solutions Aspergillus Oryzae does not grow so well, produces rather much acid and hardly any diastase. Anaerobic respiration occurs, but seems to play no part in this fact.
6) The acid produced is probably oxalic acid; it is possible that it inactivates the enzyme by binding it chemically; when later on the fungus consumes the acid, the diastase becomes free once more.
7) Investigations in vitro seem to confirm the hypothesis, described in 6).
8) The research was carried out with three strains, two of which were very similar; the third behaved very differently all through the experiment. If is argued that this is probably due to the aftereffect of a quite different former medium and that we are not allowed to think of a hereditary variety.
9) Investigations with Aspergillus niger confirmed in the main my former conclusions, viz. that for this species the formation of diastase corresponds chiefly with the chemical composition of the food material and not with the degree of acidity.

I have once more the great pleasure in thanking professor Dr. G. van Iterson for his kindly allowing me to make this research in his laboratory and for his valuable help in all respects.

Also to professor Dr. Joh. Westerdijk I wish to express my heartfelt thanks for her extreme kindness in providing me with the strains, with which I carried on this investigation.

Finally, I have to thank very cordially my colleague, mr. C. Deelder, Schiedam, for his friendly readiness to help me once more to correct the translation of this article.

Delft, Laboratory for technical botany.
May 1927.

V. Literature.

1) Atkinson, R. W., Proc. Roy. Soc. Vol, 32, 1881, p. 299. On the diastase of Koji.
2) Boas, F. und Leberle, H., Bioch. Z. schr. Bd. 90, 1918, p. 78. Unters. über Säurebildung bei Pilzen und Hefen I.
3) ———. Bd. 92, 1919, p. 170, id. II.
4) Büsgen, M., Ber. D. Bot. Ges. Bd. 3, 1885. Aspergillus Oryzae.
5) Butkewitsch, Wl., Pr. Jahrb. f. wiss. Bot. Bd. 64, 1925, p. 637. Die Säuren als Zwischenglied der oxydativen Umwandlung des Zuckers durch die Pilze.
6) Euler, H. von, Chemie der Enzyme I-II. 1920—1922.
7) Funke, G. L., Proc. Roy. Ac. Sc. Vol. 25 1922. The influence of hydrogen ion concentration on the action of amylase of Aspergillus niger.
8) ———. Rec. des trav. bot. néerl. T. 19. 1922. Researches on the formation of diastase by Aspergillus niger van Tiegh.
9) ———. T, 23. 1926, id. II.
10) Gotoh Kazuo, Bot. Mag. Tokyo Vol. 39, 1925. Ueber den Zusammenhang zwischen der Pufferwirkung der Kulturlösung und der Oxalsäurebildung von Aspergilles niger.
11) Kellner, O., Mori, Y. und Nagaoka, M., Z. f. phys. Ch. Bd. 14, 1889, p. 297. Beiträge zur Kenntniss der invertierenden Fermente.
12) Kita, G., Wochschr. f. Brauerei, Bd. 29, 1912, p. 460. Ueber die Enzyme des Aspergillus Oryzae.

13) Kluyver, A. J. en Donker, H. J. L., Versl. K. Ak. v. Wet. Amst. Vol. 33, 1924, p. 895. De eenheid in het chemisme van de fermentatieve suiker-dissimilatie-processen der microben.

14) ———. Vol. 34, 1925, pag. 237. De katalytische overdracht van waterstof als kern van het chemisme der dissimilatieprocessen.

15) Nikitinsky, J., Pr. Jahrb. f. wiss. Bot. Bd. 40, 1904. Ueber die Beeinflussung der Entwicklung einiger Schimmelpilze durch ihre Stoffwechselprodukte.

16) Oppenheimer, C., Die Fermente, 1925.

17) Saïto, K., Zentralb. f. Bakt. II Bd. 18, 1907. p. 30. Mikrobiologische Studien über die Zubereitung des Batatenbranntweines auf der Insel Hachijo (Japan).

18) ———. Woch.schr. f. Brauerei. Bd. 27, 1910, pag. 181. Der Einfluss der Nährung auf die Diastasebildung durch Schimmelpilze.

19) Sakamura, T., Journ. of the coll. of Agric. Hokk. Imp. Un. Vol. 14, 1924. Ueber die Kultur von Aspergillus niger, mit besonderer Rücksicht auf das Puffervermögen der Nährlösung. Anorganische N.-Quellen und Aciditätsveränderungen der Kulturlösing.

20) Schiemann, E., Zeitschr. f. ind. Abst. und Vererb. Lehre, Bd. 8, 1912, p. 8. Mutationen bei Aspergillus niger van Tieghem.

21) Sherman, H. C. and Baker, J. C., Journ. Am. chem. Soc. Vol. 38, 1916, p. 1638. Experiments upon the amylase of Aspergillus Oryzae.

22) ———. p. 1885. Experiments upon starch as substrate for enzyme action.

23) Sherman, H. C. and Punnett, P. W., ibid. p. 1877. On the products of the action of certain amylases upon soluble starch, with special reference to the formation of glucose.

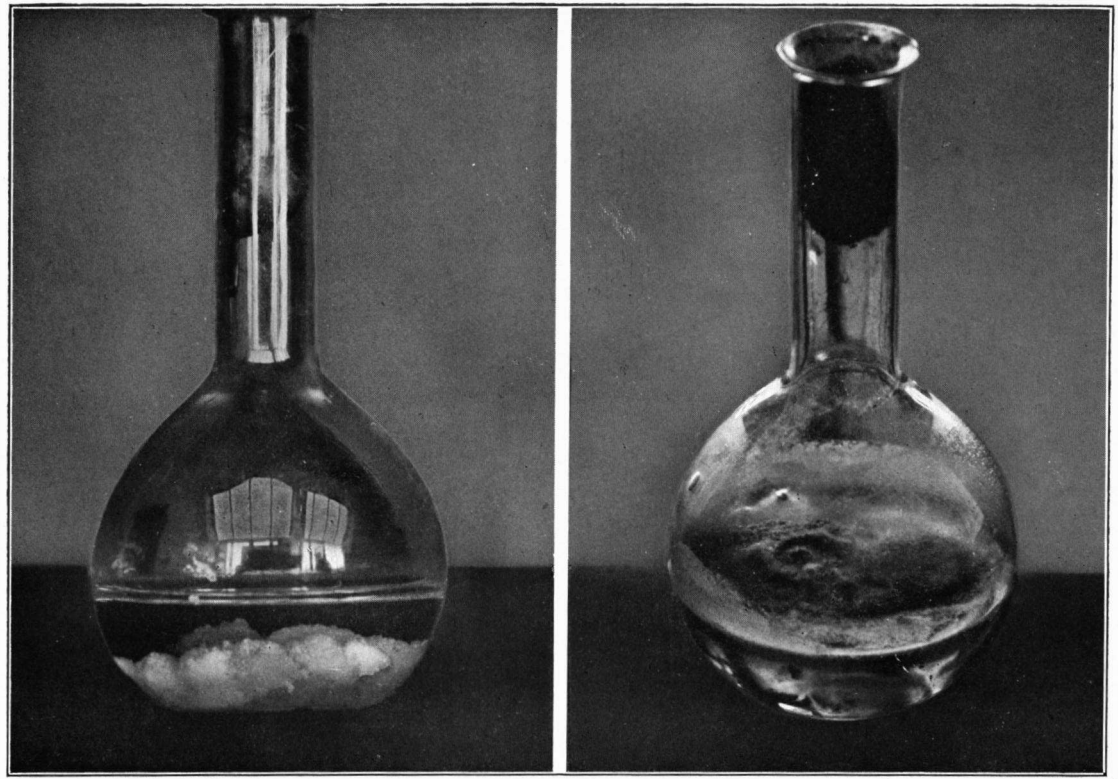
24) Sherman, H. C., Thomas, A. W. and Baldwin, M. F. ibid. Vol. 41, 1919, p. 231. Influence of hydrogen ion concentration upon enzymic activity of three typical amylases.
25) Takamine, J., Journ. Soc. Chem. Ind. Vol. 17, 1898, p. 118. Diastatic substances from fungus growth.
26) ———, p. 437. Simple quantitative determination of diastatic power.
27) ——— and Oshima, K. ibid. Vol. 42, 1920, p 1261. The properties of a specially prepared enzymic extract, polyzime, comparing its starch liquefying power with malt diastase.
28) Thom, Ch. and Church, M. B., The Aspergilli.
29). Tollenaar, D., Lab. landb. scheik., Lab. pl. phys. ond. Wag. 1925. Omzetting van koolhydraten in het blad van Nicotiana tabacum L.
30) Wehmer, C., Ber. D. Bot. Ges. Bd. 24, 1906, p. 381. Die Bildung freier Oxalsäure durch Aspergillus niger.
31) ———. Bioch. Zeitschr. Bd. 59, 1914, p. 63. Der Gang der Acidität in Kulturen von Aspergillus niger bei wechselnder Stickstoffquelle.
32) Went, F. A. F. C., Versl. Kon. Ak. v. Wet. Amst. Vol. 27, 1918. De loop van de vorming van diastase bij Aspergillus niger.
33) Wohlgemuth, J., Bioch. Zeitschr. Bd. 39, 1912, p. 324. Zur Kenntniss der Takadiastase.

VI. Plate.

Two cultures of Aspergillus Oryzae, strain U, on glucose 1 %, old 28 days.

A on unbuffered solution.

B on buffered solution.



A. On unbuffered solution.

B. On buffered solution.