Diastase Formation by Aspergillus

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CONTENTS

I. Introduction

There are many investigations on the effects of internal and external environments upon the production of diastase by molds, owing to their importance in industries. Some remarkable studies were recently carried out on the components of diastase-system; γ-amylase by Kitahara and Kurushima,[1] gluc amylase by Phillips and Caldwell,[2] and amylo-glucosidase by Kerr *et al.*,[3] and on the multiplicity of the physico-chemical characteristics of α-, β-amylases and of maltase of various mold strains.[4),5]

For studies on diastase formation of mold, it is desirable to select such a strain of mold as to be distinctly verified compositions and characteristics of its diastase, and moreover to produce powerful diastase of a simpler composition, since both compositions and characteristics of diastase would differ greatly according to kinds of mold.

From these standpoints mentioned above, *Asp. kawachii* Kitahara was selected as a suitable strain for the present experiments in which the formation of α-amylase and of maltase was investigated.

II. Materials and Methods

As was mentioned above, *Asp. kawachii* Kitahara was employed for these studies. Kitahara *et al.* studied on diastase of this mold; they pointed out[6),7] that the compositions of diastase of this mold were so simple that no special amylase producing glucose directly from starch

existed, and then they[8] found that this mold produced powerful α-, β-amylases and maltase, and also that most part of α-amylase was sensitive to acid. The homogeneity in physico-chemical properties of β-amylase and of maltase would be expected from the conclusion proposed by Okazaki.[5]

The three components of diastase, α-, β-amylases and maltase, are generally concluded to be physico-chemically homogeneous respectively.

Determination of α-amylase: Activity of α-amylase was determined by the procedure proposed by Sandstedt, Kneen and Blish.[9] One unit of α-amylase was calculated by the amount of enzyme by which one gram of starch treated with β-amylase was dextrinized in one hour at 40°C.

Determination of maltase: 5 ml. of diastase preparation were added to 15 ml. of buffered (by adding 5 ml. of 0.1 N acetate of pH 4.7) maltose (1.5 per cent solution as monohydrate) at 40°C. in a test tube, and then the temperature was kept in water thermostat. After a definite time, 2 ml. of the mixture were transferred into 5 ml. of 0.1 N sulfuric acid in order to stop the reaction. After the acid inactivation, 5 ml. of 0.1 N NaOH were added. 2 ml. aliquots were taken for determination of its reducing value by the method of Hanes.[10] Maltase activity was indicated by ml. of 0.01 N $Na_2S_2O_3$ corresponding to the increase of the reducing value after a definite time.

K, the constant of saccharifying activity in a monomolecular reaction, was calculated by the method of Willstätter and Schudel[11] after measuring the amount of glucose produced. The reaction mixture consisting of 20 ml. of 2 per cent soluble starch solution, 10 ml. of 0.1 N acetate buffer solution (pH 4.7), 7.5 ml. of H_2O and 2.5 ml. of enzyme solution was kept at 40°C., and the reducing value was measured by the same procedure as was mentioned in maltase determination. Sometimes saccharifying activity was noted by the amount (ml.) of 0.01 N $Na_2S_2O_3$ corresponding to the increase of the reducing value after a definite time.

In order to determine the activities of exoenzymes of the mold, the filtrate of the cultured solution was used, and for endoenzymes the extract of the mycelium mat was prepared in the following manners: The harvested cells were crushed with sand, extracted 3 hours at 30°C. with one per cent NaCl solution of the same volume corresponding to the cultural medium, and then filtered.

III. Experiments and Discussion

A. Effects of the concentration of carbon- and nitrogen-sources in the media and the age of cultivation on diastase formation in growing culture.

It was already shown that enzymic constitution of an organism changed remarkably owing to its cultural environments. Thus the actual enzymic constitution of a cell will be a certain portion of its potential enzymic constitution being limited by the conditions under which it was grown. Among the factors revealing the selection of enzymic constitution, Gale[12] summarized that the following articles were predominating: (a) the chemical constitution of cultural medium, (b) the physico-chemical conditions during the growth, and (c) the age of cultivation.

Effect of the concentration of carbon source upon amylase production by mold was studied by Saito,[13] and he reported that as higher the concentration, the amylase activity became poorer.

As regards nitrogen source, Murota and Saruno[14] concluded that the amylase production by mold was proportional to the nitrogen concentration of the media. Tanabe[15] stated that no correlation was found between the amount of assimilated nitrogen and the amylase activity.

The importance of protein content of the cells on proteolytic enzyme system of *Escherichia coli* was found by Virtanen and Winkler.[16] Spiegelman and Dunn[17] showed that the presence of exogenous nitrogen was necessary to produce adaptively galactozymase of yeast.

In order to test effects of concentration of carbon- and nitrogen-sources in the growing culture, six kinds of media shown in Tables 1 and 2 were used.

Table 1.

Composition of cultural medium. (gms. per 1 liter)

Glucose	20, 40 and 60
$(NH_4)_2HPO_4$	1.18 and 5.91
KH_2PO_4	3
Na_2SO_4	0.5
$MgSO_47H_2O$	0.25
$CaCl_22H_2O$	0.05

Table 2.

Concentration of carbon- and nitrogen-sources. (gms. per 1 liter)

Medium	Glucose	$(NH_4)_2HPO_4$
A	20	1.18
B	40	1.18
C	60	1.18
D	20	5.91
E	40	5.91
F	60	5.91

The organism was cultured at 30°C. on 60 ml. of the medium shown in Tables 1 and 2 in 300 ml. Erlenmeyer flask. After two days' incubation, as much $CaCO_3$ was added as one quarter of the quantity of the sugar employed. The activity of α-amylase and of maltase, and the constant of saccharifying activity (K) were determined after four, seven, eleven and fifteen days' incubations respectively (see Figs. 1, 2, 3, 4, 5 and 6).

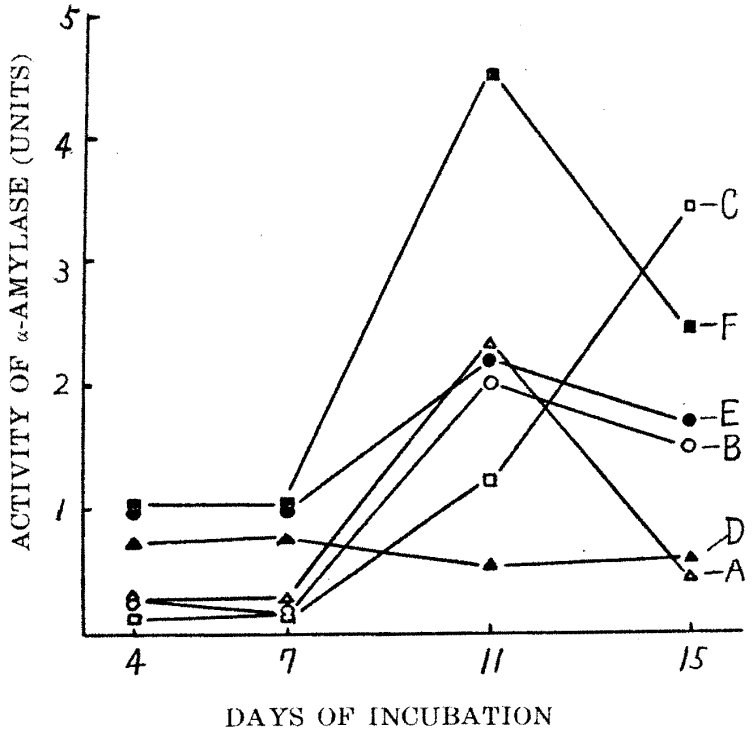

Fig. 1. Production of α-amylase: Activity of endoenzyme.

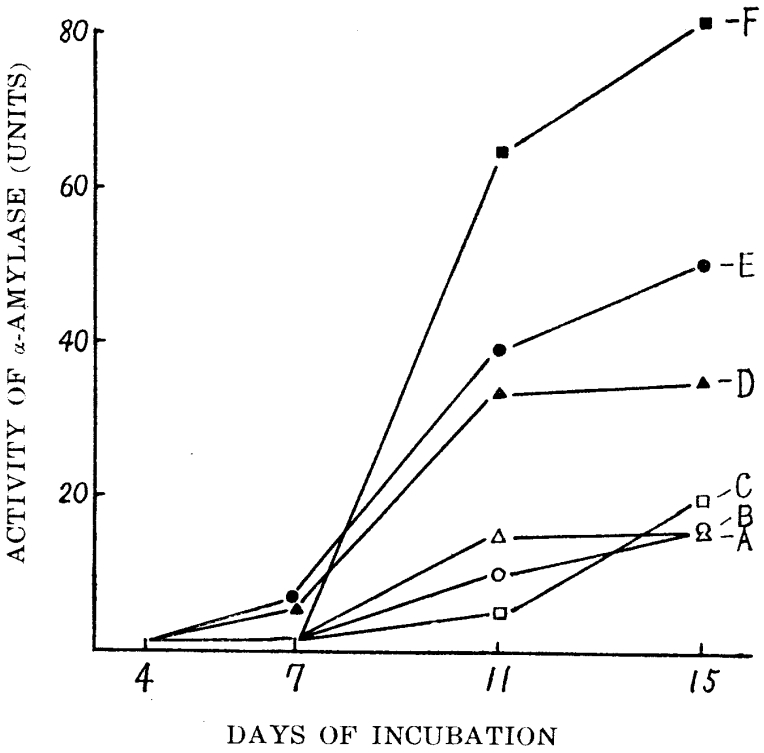

Fig. 2. Production of α-amylase: Activity of total enzyme.

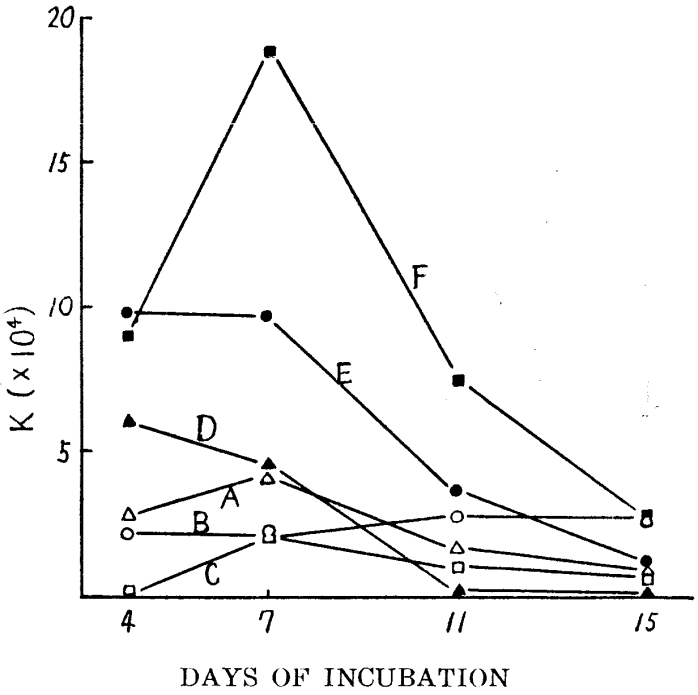

Fig. 3. Saccharifying activity ($K \times 10^4$): Activity of total enzyme.

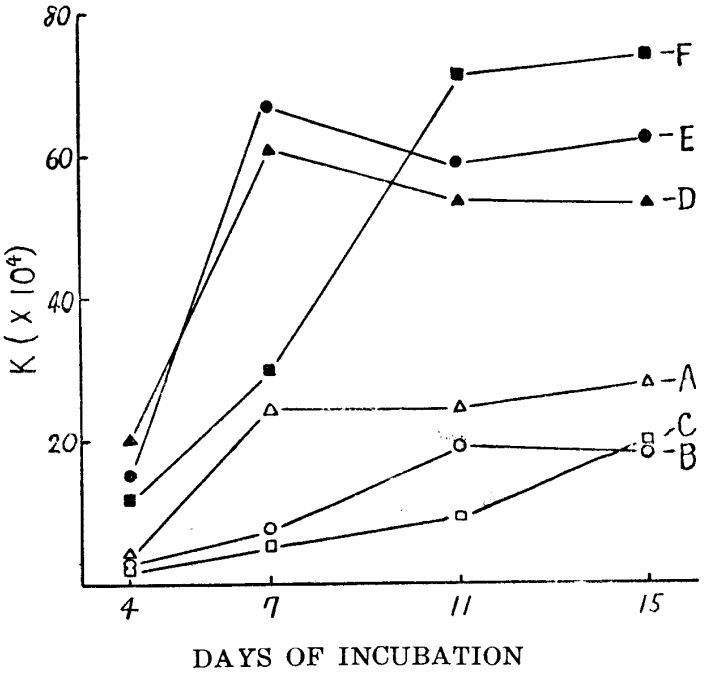

Fig. 4. Saccharifying activity ($K \times 10^4$): Activity of total enzyme.

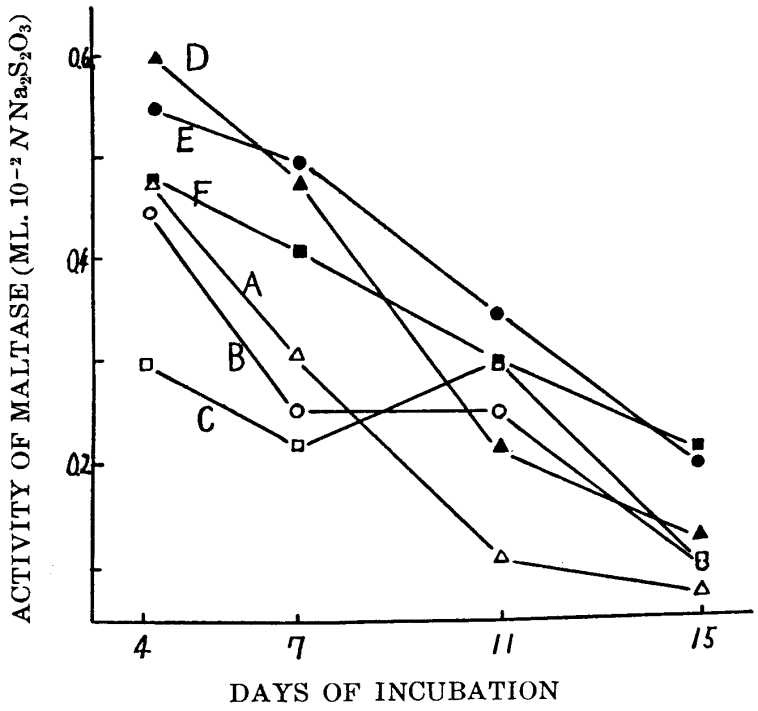

Fig. 5. Production of maltase (reducing value after 1 hour): Activity of endoenzyme.

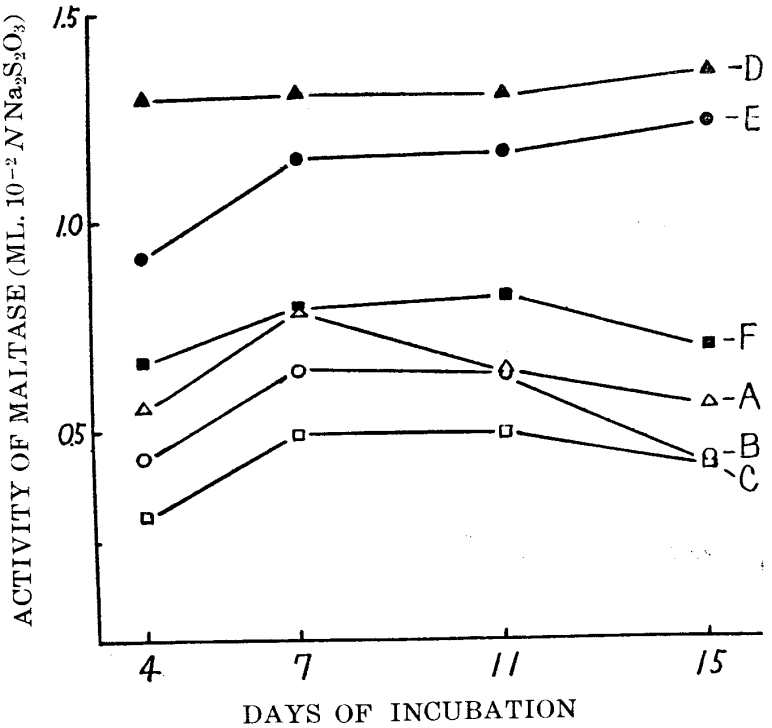

Fig. 6. Production of maltase (reducing value after 1 hour): Activity of total enzyme.

The weight on dry basis of the mold mat obtained by these cultures was shown in Fig. 7.

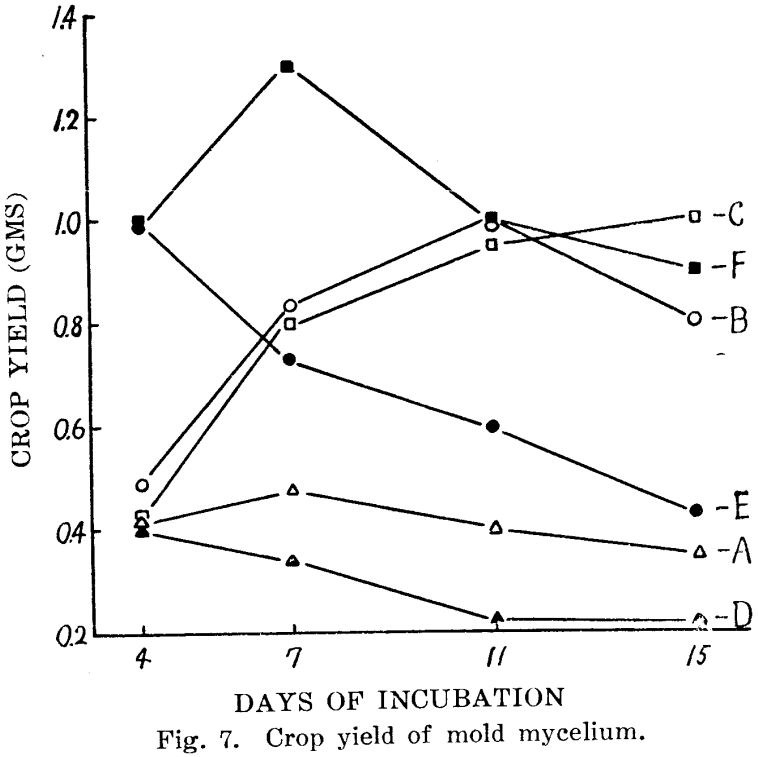

Fig. 7. Crop yield of mold mycelium.

From the results of experiments shown in the above figures, following articles are summarized: (1) concentration of glucose reveals remarkably different effect on α-amylase- and on maltase-formation, (2) concentration of nitrogen source reveals very much the same effect on the two enzymes mentioned above, and (3) effect of the age of cultivation is not the same among the two enzymes.

At earlier stages of incubation, the endoenzyme plays a greater part of the total (endoenzyme+exoenzyme) enzymic activity, but its part falls off with time until the maximum amount of endoenzyme is much inferior to exoenzyme. At the latter stage of incubation, therefore, the total amount of enzyme is very much the same as the amount of exoenzyme. The studies on the formation of enzyme should be discussed on both sides of endo- and total (or exo)-enzymes.

At low concentration of nitrogen source, the effect of the concentration of glucose on the formation of α-amylase was not so distinct, but at high concentration of nitrogen source the formation of α-amylase was observed to be parallel to sugar concentration of the cultural media.

On the other hand, the formation of maltase decreased against the increasing concentration of glucose despite the concentration of nitrogen, and very much the same results were again obtained with maltose media.

It is interesting to note that the relation between the formation of enzyme and the concentration of carbon sources is not the same between the two enzymes (α-amylase and maltase), since the presence of glucose in the medium frequently inhibits more or less the formation of many other enzymes.[12] According to Gale,[18] Monod suggests that the effect of glucose is due to a definite "enzyme suppression". It may be possible that many enzymes arise from a limited supply of a common protein precursor. There are many metabolic pathways at the same time in the cell in which individual enzyme is concerned. In other words, the active enzyme may be produced by some reaction between precursor and substrate revealing a higher affinity to the precursor.

The maltase formation reveals during such an early stage, as is shown in Figs. 5 and 6, when the active synthesis of cell-materials is followed through rapid consumption of the nutrients.

Among the nutrients, Fig. 8 shows that the consumption of sugar takes place very rapidly at early stage of incubation despite the initial concentration of sugar.

Consequently, maltase formation is supposed to be suppressed in the presence of increasing amount of sugar even when the supply of nitrogen is abundant.

In the media of high concentration of nitrogen source, both the

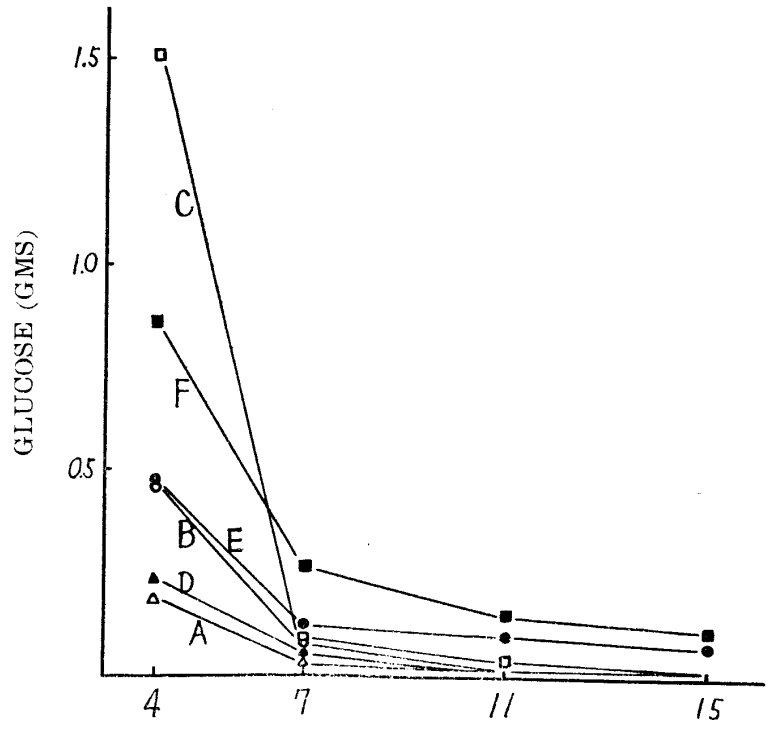

Fig. 8. Remaining sugar in media.

enzyme formation and nitrogen content of the cells harvested are larger than in the other case (see Fig. 9), and nitrogen rich cells appear to contain more enzymic precursor.

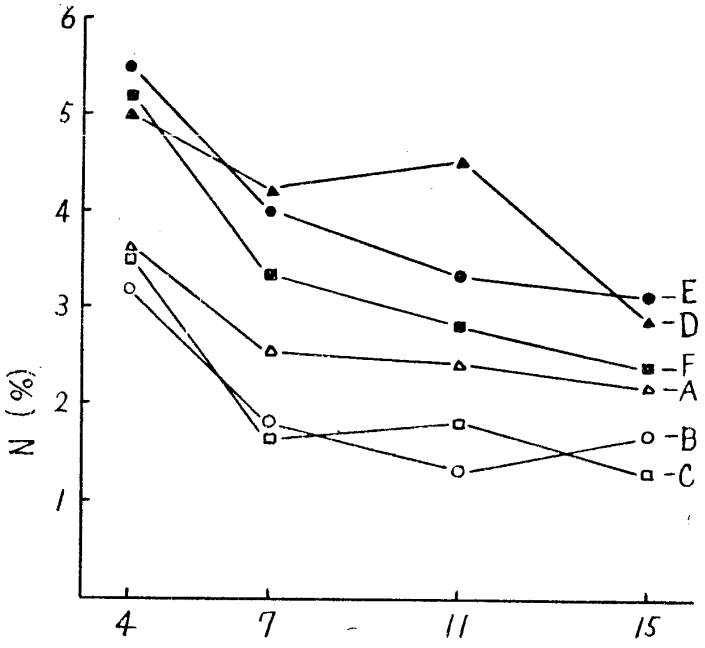

Fig. 9. Nitrogen content of the cells.

The behavior of carbon source, in the presence of available precursor, is thought to be important to the problem on α-amylase formation as will be discussed later on. In the case of low concentration of nitrogen source, available precursor will be produced little, so that the effect of the concentration of sugar appears at nearly the end of cultivation for α-amylase formation.

In the case of maltase, high activities are already observed in the vigorous growth period (early stage of cultivation), while only a little or no activity is proved during the rapid growth period in the case of α-amylase which appears to be formed at the period near the cessation of growth. From these results it is suggested that maltase formation is concerned with the breakdown of substrate into glucose to be metabolized, and formation of α-amylase is accompanied with catabolic mechanism as Gale[18] suggests, although the effect of pH of the media (see Table 3) is not negligible as will be shown later on.

Table 3.

pH of the cultured media.

	Incubation period (days)			
	4	7	11	15
A	3.8	6.5	7.2	7.2
B	3.7	5.0	7.2	7.2
C	3.5	5.0	6.8	6.8
D	4.5	6.5	6.8	6.8
E	4.0	6.5	7.0	7.0
F	3.8	5.8	7.0	7.4

From Figs. of 1 to 7 it is also pointed out that the crop yield of mold mat and production of enzymes are not parallel, and that the maximum growth is carried out at shorter period of incubation with high concentration of nitrogen when compared with low concentration of nitrogen.

As diastase components which control the saccharifying activity are α-, β-amylases and maltase, it will be comprehensible that the values of K change in such tendency as to represent nearly a mean value of activities of α-amylase and maltase according to the initial concentration of glucose employed and the age of cultivation.

B. Diastase formation by replaced culture.

Euler[19] and Willstätter[20] reported that yeast saccharase was produced by suspending yeast cells in sugar solution at 30°C. or autolyzing

them with ammonium phosphate. Analogous result was obtained with yeast maltase.[21] The formation of enzymes by active cell suspensions has been carried out recently by many investigators.

For diastase formation by this mold, the replaced culture using mycelium mat was tried. The mold was cultivated for four days on 30 ml. of the medium containing 2 per cent glucose, 0.16 per cent ammonium phosphate and the other inorganic salts as were shown in Table 1. After washing with distilled water, mycelium mat was placed on 25 ml. of the following media (see Tables 4 and 5) buffered by M/3

Table 4.

Maltase activity of replaced culture.

Media for replaced culture		Incubation time (hours)						pH values after incubation for	
		6		24		48			
		Maltase activity after		Maltase activity after		Maltase activity after			
No.	Composition	1 hr.	3 hrs.	1 hr.	3 hrs.	1 hr.	3 hrs.	24 hrs.	48 hrs.
1	Phosphate alone (pH 3.0)	0.17	0.36	0.32	0.61	0.45	0.96	3.2	3.2
2	Maltose (50 mg.)	0.08	0.10	0.56	1.06	1.00	1.65	3.0	3.2
3	Asparagine (50 mg.)	0.12	0.24	0.40	0.87	0.50	1.16	3.4	3.8
4	No. 2+No. 3	0.17	0.33	0.74	1.25	1.02	1.73	3.3	3.6
5	No. 1+toluene*	0.28	0.50	0.36	0.63	0.43	0.78	3.2	3.2
6	No. 2+toluene*	0.08	0.26	0.27	0.46	0.40	0.69	3.1	3.1
7	No. 3+toluene*	0.25	0.44	0.25	0.50	0.38	0.68	3.1	3.2
8	H_2O (pH 3.0 by HCl)	0.16	0.30	0.19	0.44	0.38	0.65	3.0	3.5

Note: * 2 ml. of toluene were added so as to cover the mold mat.

Table 5.

α-Amylase activity of replaced culture.

Media for replaced culture		α-Amylase activity after incubation for			pH values after incubation for	
No.	Composition	6 hrs.	24 hrs.	48 hrs.	24 hrs.	48 hrs.
1′	Phosphate alone (pH 6.0)	15.48	40.00	45.89	5.9	6.0
2′	Maltose (50 mg.)	11.03	30.97	49.66	5.9	6.0
3′	Asparagine (50 mg.)	16.27	34.29	52.60	6.5	6.5
4′	No. 2+No. 3	15.40	44.32	69.41	6.4	6.6
5′	No. 1+toluene (2 ml.)	16.09	20.28	22.07	6.2	6.5
6′	No. 2+toluene (2 ml.)	17.45	19.46	18.82	5.5	5.5
7′	No. 3+toluene (2 ml.)	19.00	23.13	23.90	5.9	5.9
8′	H_2O (pH 6.0 by HCl)	14.84	24.62	34.29	6.4	6.6

phosphate solution of pH 3.0 and M/15 phosphate solution of pH 6.0 respectively, since maltase and α-amylase are stable in the above pH values as will be shown later.

Modifications of concentration of maltose and of asparagine were tried (see Tables 6 and 7).

Table 6.

Effects of maltose and asparagine.

Media for replaced culture			pH values of No. 9-No. 12 media after incubation for		pH values of No. 9'-No. 12' media after incubation for	
No.	Composition					
	Maltose (mg.)	Asparagine (mg.)	24 hrs.	48 hrs.	24 hrs.	48 hrs.
9 & 9'	500	no	3.2	3.2	4.7	5.7
10 & 10'	no	500	5.0	5.8	7.8	7.8
11 & 11'	500	50	3.1	3.3	5.9	6.4
12 & 12'	50	500	5.0	5.8	7.2	7.8

Note: No. 9-No. 12 media were buffered by *M*/3 phosphate solution of pH 3.0 and No. 9'-No. 12' media by *M*/15 phosphate solution of pH 6.0.

Table 7.

Activity of maltase and of α-amylase.

Media No.	Incubation time (hours)						Media No.	α-Amylase activity after incubation for		
	6		24		48					
	Maltase activity after		Maltase activity after		Maltase activity after					
	1 hr.	3 hrs.	1 hr.	3 hrs.	1 hr.	3 hrs.		6hrs.	24hrs.	48hrs.
9	0.10	0.24	0.38	0.81	0.48	1.02	9'	12.40	34.28	37.16
10	0.25	0.48	0.33	0.60	0.33	0.69	10'	17.04	44.32	69.82
11	0.08	0.18	0.75	1.20	1.72	2.20	11'	15.80	47.33	88.46
12	0.25	0.46	0.44	0.85	0.45	0.94	12'	15.65	51.74	98.48

Mycelium mat before the replaced culture was crushed with one gram of sand and extracted with 25 ml. of one per cent NaCl at 30°C. for 3 hours. Activities of α-amylase and maltase of the extract were determined (see Table 8).

Table 8.

Endoenzyme before replaced culture.

Maltase activity after		α-Amylase activity
1 hr.	3 hrs.	
0.35	0.70	15.00

From these results it is ascertained that α-amylase and maltase are remarkably formed during one to two days in phosphate buffer, and that for maltase formation the addition of maltose is very effective, while asparagine is effective for α-amylase formation. The formation of each individual enzyme is suppressed by the addition of toluene.

Tanabe *et al.*[24] and Bindal *et al.*[23] reported that the addition of asparagine stimulates amylase formation in growing culture. From the present experiments asparagine is very effective for α-amylase formation, in particular, in the presence of maltose. Asparagine is supposed to take a part in enzyme synthesis, since the effect is apparent after a prolonged incubation period. Glutamic acid is observed to reveal almost analogous effect. For α-amylase formation, maltose alone has no effect. Accordingly, in the presence of asparagine, maltose seems to serve as only an energy source.[24] Nevertheless, from the experiments shown in Figs. 1 and 2, it was observed that high concentration of carbon source enhanced α-amylase production in growing culture. The apparent difference between the behavior of carbon source in growing culture and that in replaced culture is thought to have an important significance to discuss α-amylase formation, as will be done later on.

Effect of maltose on maltase formation will be discussed later on, too.

Bacterial amylase-formation by cell-suspensions was reported by Fukumoto *et al.*[25] and phosphate or adenosine triphosphate was pointed out to play an important rôle as was observed by Reiner[26] in the case of galactose adaptation of yeast. From the above experiments (see Tables 4 and 5), it is supposed that phosphate plays a rôle for maltase formation but it does not for α-amylase formation.

C. Effect of pH of the culture on diastase formation.

Gale[12] showed that alteration of pH of the medium during the growth had a marked effect on enzyme production. Diastase formation in growing culture will be supposed to be affected by pH of the medium, as was revealed by the age of cultivation.

Effect of pH was tested with the replaced culture containing glucose (50 mg.) and peptone (50 mg.) buffered by M/5 phosphate solution. The stability of α-amylase was tested by keeping the enzyme preparation at 30°C. for 38 hours in M/5 phosphate buffer solution (see Fig. 10).

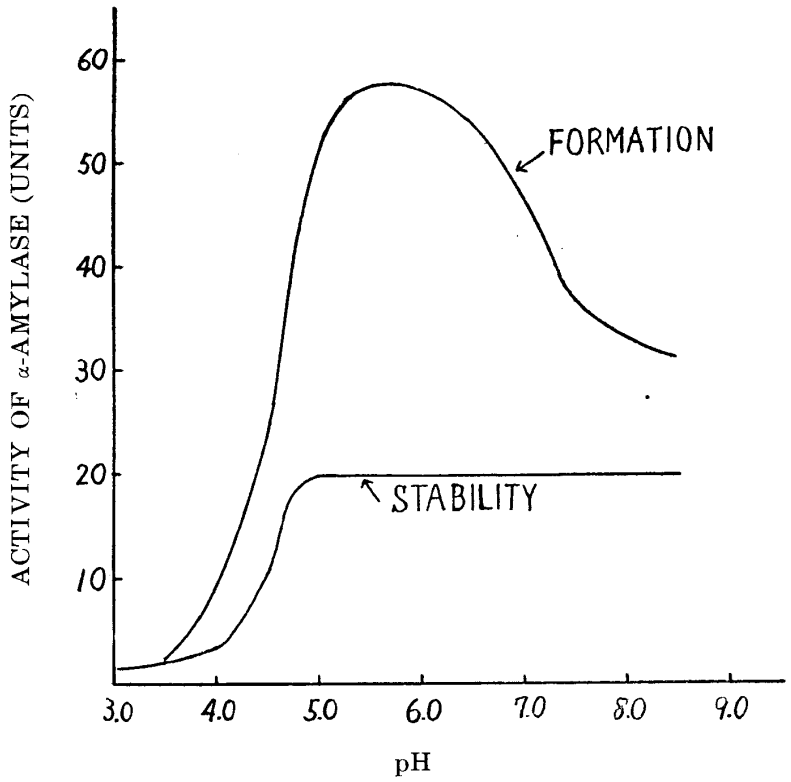

Fig. 10. Effect of pH on formation and stability of α-amylase.

Fig. 10 shows that the optimum pH for α-amylase formation is 5.7–6.3. These pH values coincide with the value on which α-amylase was produced most vigorously in growing culture as was shown in Figs. 1, 2 and Table 3.

As the effect of pH on α-amylase formation was cleared, it is necessary to re-examine the effect of the age of cultivation mentioned previously. This was done by replaced culture with cells at different stages of precultivation (see Table 9).

From the Table 9 it is known that the cells at too late stage of cultivation and also those at too early stage of growth when maltase was produced most vigorously as was shown in Figs. 5 and 6 are not fitting for α-amylase formation.

Also effect of pH on maltase formation and on stability of maltase was studied and the results were shown in Tables 10 and 11.

Table 9.

Effect of the age of cells upon α-amylase formation by replaced culture.

Preculture*(1)		Replaced culture*(2)	
Incubation time (hrs.)	pH of the cultured media	Weight of mycelium mat used (gms.)	α-Amylase activity
66	2.4	0.16	28.23
92	2.1	0.17	33.87
120	2.2	0.21	19.72

Notes: *(1) Composition of the preculture:
Glucose.....2%
$(NH_4)_2HPO_4$.....0.16%
Other inorganic salts are as shown in Table 1 in the amounts.
*(2) Replaced culture was done on phosphate buffer solution of pH 5.8 containing asparagine (50 mg.).

Table 10.

Effect of pH on maltase formation by replaced culture*.

pH of replaced culture	Maltase activity after	
	1 hr.	3 hrs.
2.5	0.80	1.70
3.0	1.03	1.94
3.5	1.01	1.88
4.5	0.88	1.80
5.5	0.53	1.12
6.5	0.47	1.06
7.4	0.20	0.40
8.3	0.17	0.35

Note: * Replaced culture was done on phosphate buffer solution containing glucose (50 mg.) and peptone (50 mg.).

Table 11.

Stability of maltase on various pH*.

pH	Maltase activity after	
	1 hr.	3 hrs.
3.0	0.58	1.31
4.0	0.56	1.28
5.0	0.55	1.21
6.0	0.35	0.70
7.0	0.22	0.46
Before treatment	0.62	1.35

Note: * Maltase activity was determined after keeping the enzyme solution of various pH at 30°C for 38 hours.

The optimum pH for maltase formation was proved to be 3.0–3.5 and the pH on which maltase is formed vigorously shows the value (see Figs. 5, 6 and Table 3) near the optimum pH mentioned above.

It is summarized that not only the optimum pH for α-amylase formation is different from that for maltase formation, but also the age of cultivation favorable for α-amylase formation differs from that for maltase formation. With any of these enzymes, it is found that the optimum pH for enzyme formation coincides with the optimum pH for stability of the enzyme.

D. Effect of various carbon sources on diastase formation.

It is reported that substances having closely related chemical structure with the substrate which is easily decomposed by the enzyme affect more efficient stimulation to the formation of the enzyme. Thus, in the case of *Aspergillus* species, maltose reveals as the strongest stimulant for the production of maltase,[27] and for amylase formation, starch is the strongest stimulant, followed by dextrin and glucose.[28]

For an explanation to the fact that the substrate added to the cultural media during the growth of microorganism results a marked stimulation on the enzyme formation, theory of natural selection was first given by Massini[29] and then proved by Levis.[30]

Although a variant strain of this mold which will be described later, produces a least amount of maltase, the stimulating effect of maltose on maltase formation is proved with both the parent and the variant strains (see Table 12), and that it is ascertained that both strains remain the morphological characteristics in these cultures.

Maltase activity in Table 12 was measured with the shaking cultures incubated for 48 hours on 50 ml. of the media containing one per cent

Table 12.

Maltase formation in maltose and glucose solutions.

Strain	Sugar	Maltase activity after		
		1 hr.	2 hrs.	4 hrs.
Parent	Glucose	1.08	1.54	1.80
	Maltose	1.46	1.86	2.18
Variant	Glucose	0.54	0.96	1.28
	Maltose	1.06	1.44	1.84

sugar and 0.66 per cent peptone and the others in Table 1, and analogous result was obtained on surface culture.

From these results, it is suggested that the stimulation on maltase formation will be due to chemical mechanisms in the cells, but not to the natural selection mentioned above.

Effect of various carbon sources on maltase formation was tested, with the results as shown in Table 13 which illustrated the maltase activities of the filtrates of the four days' cultures on the solutions containing 2 per cent various carbon sources and 0.16 per cent $(NH_4)_2HPO_4$ and the others in Table 1.

Table 13.

Effect of various carbon sources on maltase formation.

Carbon sources	Growth	pH of the cultured media	Maltase activity after	
			1 hr.	3 hrs.
Arabinose	++	2.0	0.02	0.05
Inositol	++	2.3	0.10	0.22
Xylose	++	2.0	0.05	0.08
Mannitol*	+ (++)*	2.2*	0.15*	0.28*
Dulcitol	++	1.9	0.45	0.81
Fructose	++	2.0	0.42	0.76
Mannose	++	2.2	0.25	0.68
Sorbitol	++	2.0	0.55	1.25
Sucrose	++	2.1	0.75	1.29
Trehalose	++	2.2	0.66	1.15
Glucose	++	2.1	1.51	2.14
Raffinose	++	2.1	1.79	2.27
Maltose	++	2.1	1.98	2.57
Starch	++	2.3	2.09	2.65

Notes: (1) * Measured with five days' culture.
(2) Sugars and the other constituents of the cultural media were pasteurized separately.

As will be seen in the above table, the carbon sources are divided into four groups with regard to their ability for stimulating maltase production. The reason of the stimulation by starch is uncertain, since it is difficult to verify whether it is due to the effect of starch itself or hydrolyzed products by diastase, and moreover maltase and β-amylase are at present not successfully separated.

Although such a distinct relation between the chemical structure

and enzyme formation as in the case of the formation of adaptive enzymes[31] was not found, maltase formation was proved to be suppressed in the presence of stranger carbon sources such as pentoses and some sugar alcohols. On the contrary, the marked effect of maltose and starch (or hydrolysis product) is suggested to be due to that these carbon sources will have a specific affinity to a precursor of enzyme in the course of enzyme synthesis in the cells. Effect of raffinose is difficult to understand.

Although maltose alone was already shown to reveal little effect on α-amylase formation in replaced culture, effect of various carbon sources in growing culture on α-amylase formation was examined as was shown in Table 14 in which the α-amylase activity and the crop yield of mold cells were observed by keeping the mats for two days on the cultures added $CaCO_3$ to one per cent, after the determination of maltase activity.

Table 14.

Effect of various carbon sources on α-amylase formation.

Carbon sources	α-Amylase activity	Crop yield*
Arabinose	44.65	0.13
Inositol	31.66	0.14
Xylose	41.73	0.12
Mannitol	—	—
Dulcitol	50.27	0.16
Fructose	37.64	0.17
Mannose	41.13	0.17
Sorbitol	46.46	0.16
Sucrose	45.02	0.09
Trehalose	48.00	0.10
Glucose	38.40	0.12
Maltose	37.25	0.10
Starch	39.50	0.12

Note: * Crop yield was weighed on dry basis.

No remarkable difference was observed on the effect on α-amylase formation among various kinds of carbon sources.

It is interesting to note that starch reveals no remarkable effect on α-amylase formation. Cultured on the modified Czapek solution containing 2 per cent glucose or starch which showed no low pH values during cultivation, effect of starch was compared with that of glucose. Table 15 shows the α-amylase activity of the culture.

Table 15.

Effect of glucose and of starch on α-amylase formation.

	Incubation period (days)			
	5		9	
	pH	α-Amylase activity	pH	α-Amylase activity
Glucose	6.0	23.38	7.8	79.42
Starch	6.5	27.15	7.9	81.88

Also from the above results no marked difference between starch and glucose was found contrary to the result by Goodman.[32] An analogous result was observed by replaced culture, too. It is difficult to suppose that exogenous starch will affect the formation of α-amylase which is synthesized in the cells, since the cell membrane is impermeable to starch. Knapp[33] also could not find the special effect of starch on the formation of amylase by *Aspergillus*.

Although exogenous starch was found not to affect the formation of α-amylase, endogenous starch was not concerned at all.

It is known that high concentration of carbon source in growing culture results a transient accumulation of polysaccharide within the cell.[31),35] This was again proved in the following experiment (see Table 16) by measuring the polysaccharide by the method described by Ergle[36] with the cells grown on media containing 5 per cent or 2 per cent glucose (besides 0.5 per cent peptone and the others in Table 1) for five days.

Table 16.

Analysis of polysaccharide in mold cells.

Cultural media	Polysaccharide on dry basis (%)		
	Free	Bound	Total
5% glucose	0.62	0.92	1.54
2% glucose	0.20	0.25	0.45

The polysaccharide isolated from the mold mat was found to be blue by iodine solution. This polysaccharide in mold cells may be possible to affect α-amylase formation by its special affinity in the course of the enzyme synthesis. The effect of the concentration of glucose in growing culture (Figs. 1 and 2) confirms the above suggestion.

E. Variation and diastase.*

Kitahara *et al.*[37] reported that a mutant of *Aspergillus* is not different from its parent in their diastase constitution. Takeda[38] also pointed out an analogous conclusion in *Rh. javanicus* Takeda.

Sakaguchi and Ishitani[39] reported that *Asp.* species varied naturally.

In the course of repeated subculture on malt extract agar, a variant strain of *Asp. kawachii* was isolated, and its diastatic constitution and other characteristics were compared with the parent strain.

1. Morphological characteristics[40] (see Table 17).

Table 17.

Morphological characteristics.

	Parent	Variant
On malt extract agar	ochre color	greyish-white color
Mycelium	colorless, septate 4-6 μ in width	do, 5-6 μ in width
Conidiophore	400-750 μ in length, 7.5-10 μ in width, surface smooth, aseptate	350-550 μ in length, 5-7 in width, do, septate (one to some)
Blase	almost spherical, (25-35 μ) no definite line of demacation from the stem	oval (20 × 35 μ), do,
Sterigmata	simple phase, clevate, radial, no sterigma on the lower one quarter part of blase, 7-12 μ in length, 2.5-4 μ in width, numbers in optical section; 24	do, do, sterigma only on the upper one quarter part of blase, 10-12 μ in length, 3.5 μ in width, 6-10 (ordinarily 8)
Conidia	spherical (3-4.5 μ), rough, ochre color	do, do, greyish-white color
Perithecium	not yet certified	do

2. Acid formation.

50 ml. of medium containing 5 grams of glucose, 0.1 gram of $(NH_4)_2SO_4$, 0.05 gram of KH_2PO_4, 0.005 gram of $MgSO_4 7H_2O$ and 0.005 gram of $CaCl_2 2H_2O$ were placed in 300 ml. Erlenmeyer flask and incubated at 30°C. (see Table 18).

* The writer thanks Mr. Kazuyuki Morihara for assistance in the experiments of this part.

Table 18.

Acid formation.

	Incubation period (days)					
	7		14		21	
	Acidity	Cells weighed	Acidity	Cells weighed	Acidity	Cells weighed
Parent	9.27	0.86	18.05	1.03	5.15	1.08
Variant	5.15	0.74	4.55	0.99	4.15	0.98

Notes: (1) Acidity: Ml. of 0.1 *N* NaOH to neuralize 10 ml. of cultured solution.
(2) Cells weighed: Weight (gms.) on dry basis.

3. Diastase activity.

5 grams of the wheat bran-koji were crushed and extracted at 37°C. for one hour with 50 ml. of one per cent NaCl. Diastase activity was measured with the filtered extract (see Figs. 11 and 12). Reaction mixture for determination of diastase activity is shown in each experiment. Determination of reducing value is as was described in II. Materials and Methods.

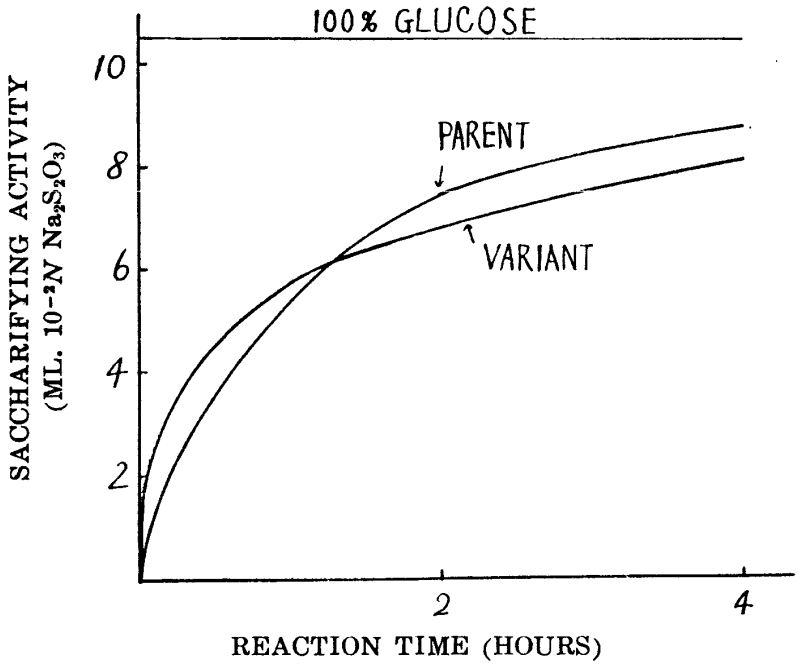

Fig. 11. Saccharifying activity. Reaction mixture consists of 20 ml. of 2 per cent soluble starch solution, 10 ml. of 0.1 *N* acetate buffer solution of pH 4.7, 4 ml. of H_2O and 1 ml. of enzyme solution.

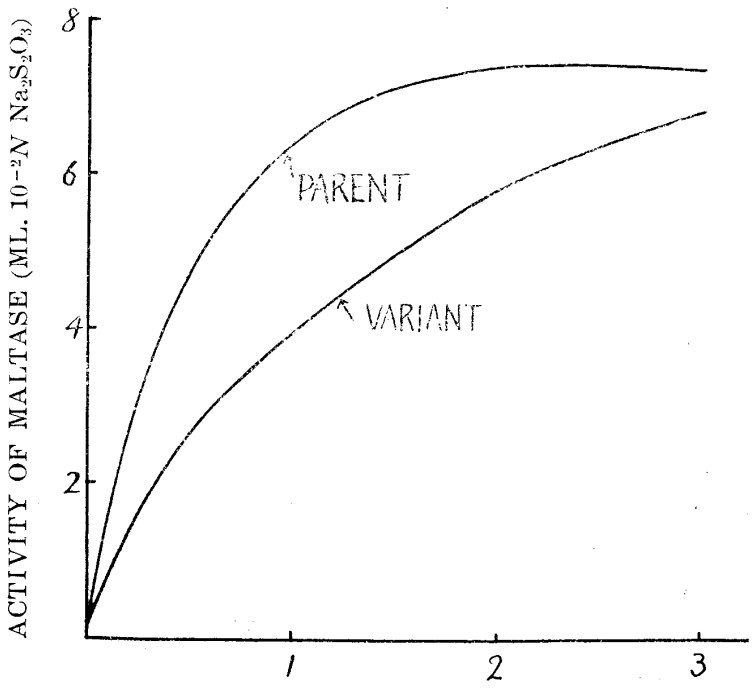

Fig. 12. Maltase activity. Reaction mixture consists of 20 ml. of 2 per cent maltose solution, 5 ml. of 0.1 *N* acetate solution of pH 4.7 and 5 ml. of enzyme solution.

From the above figures it is supposed that activities of β-amylase and maltase of parent strain are stronger, while variant strain produces powerful α-amylase. This conclusion was again ascertained by the methods described by Kitahara and Kurushima,[7] by which the activity of α-amylase or of β-amylase and of maltase was traced as was shown in Figs. 13, 14 and 15.

It will be worth to note that the variant strain kept its characters after several transfers.

From these results, it is concluded that, when any given gene would naturally be altered, *i.e.*, a natural variant comes out, diastatic constitution of the variant strain often alters from that of the parent strain as was already pointed out with the other biochemical activities.[11),12),13)] Matsuyama[11)] has recently reported analogous results on diastase of Akakoji fungus (a strain of *Asp. kawachii*).

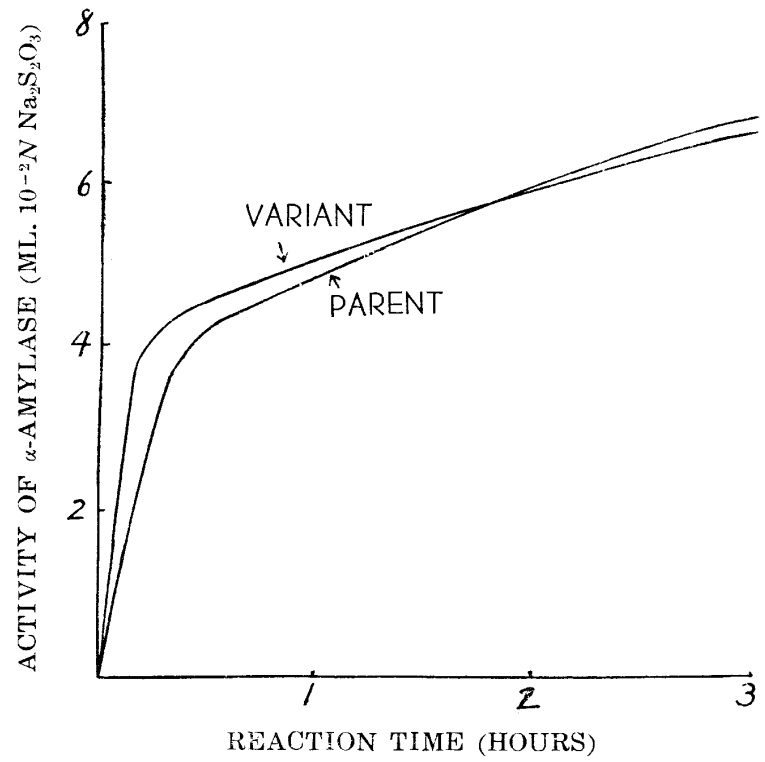

Fig. 13. Saccharifying activity of the enzyme treated at pH 7.0 and 55°C. for 15 min.: Activity of α-amylase. Reaction mixture consists of 20 ml. of 2 per cent soluble starch solution, 10 ml. of 0.1 *N* acetate buffer solution of pH 4.7 and 10 ml. of enzyme solution treated.

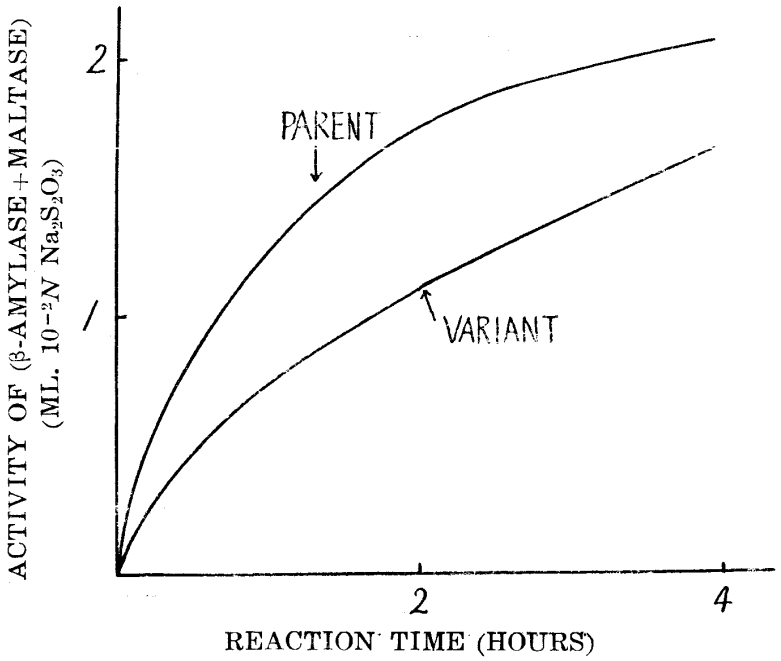

Fig. 14. Saccharifying activity of the enzyme treated at pH 3.5 and 50°C. for 15 min.: Activity of (β-amylase+maltase). Reaction mixture is as shown in Fig. 13.

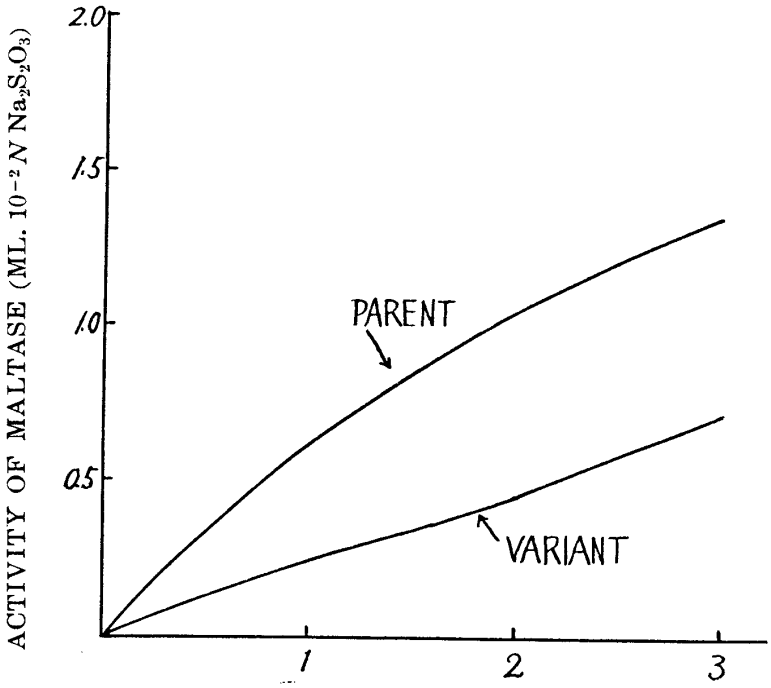

Fig. 15. Maltase activity of the enzyme treated as described in Fig. 14. Reaction mixture consists of 20 ml. of 2 per cent maltose solution, 10 ml. of 0.1 *N* acetate solution of pH 4.7 and 10 ml. of enzyme solution treated.

IV. Acknowledgement

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V. Conclusion

For the investigation of diastase formation by mold, *Asp. kawachii* Kitahara was selected as a suitable strain, and the formation of α-amylase and of maltase was studied.

Effects of the concentration of carbon- and nitrogen-sources in growing cultures upon the formation of each individual enzyme were compared, and initial concentration of glucose was found to have a different effect on the formation of respective enzyme.

That high concentration of glucose in growing culture suppresses

maltase formation will be due to the effect of the age of cultivation when maltase is vigorously formed. In other words, active enzymes necessary for vigorous consumption of glucose will be suggested to suppress simultaneous maltase formation at high concentration of glucose.

For α-amylase formation, high concentration of glucose was found to be favorable. Since α-amylase is formed at the period near the cessation of growth, enzyme suppression owing to glucose consumption will not take place, and moreover at high concentration of glucose a transient accumulation of polysaccharide within the cell was shown to be brought about. The writer suggests that accumlated polysaccharide within the cell will take part in α-amylase formation as substrate. No marked effect of exogenous starch on α-amylase formation both in growing culture and in replaced culture confirms this suggestion.

A sufficient supply of nitrogen substance is favorable for the formation of the two enzymes. At low concentration of nitrogen source, a precursor of enzyme will be formed in less quantities.

Even by replaced culture, formation of diastase was clearly proved. It was observed that maltose revealed favorable effect on maltase formation and that asparagine was favorable for α-amylase formation. It is supposed that maltose combines with enzyme precursor and then the compound thus produced takes part in maltase formation, and that asparagine will serve as a substance for precursor synthesis and perhaps the precursor produced combines with endogenous polysaccharide and results in α-amylase formation.

The optimum pH values for the formation of both enzymes are different: 3.0–3.5 for maltase and 5.7–6.3 for α-amylase in replaced culture. These pH values coincide not only with the pH of the ordinary growing culture on which these enzymes are formed most vigorously, but also with the optimum pH for stability of these enzymes, respectively.

Effects of the various carbon sources on α-amylase formation are not so evident as on maltase formation. No remarkable difference among various carbon sources upon α-amylase formation will be due to that the formation of α-amylase is accompained with catabolic pathway. On the contrary, remarkable difference among various carbon sources upon maltase formation will be due to that the formation of maltase is concerned with the break-down of substrate. Thus in the presence of pentoses and of some sugar alcohols little maltase was formed.

A natural variant strain of the mold was separated, and morphological and physiological characteristics were compared with those of the parent strain. The diastatic constitution of the variant strain was proved to be different from that of the parent strain.

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