ENZYMES OF *ASPERGILLUS ORYZAE*

II. THE YIELD OF ENZYMES FROM MUTANTS PRODUCED BY ULTRAVIOLET IRRADIATION

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[*Manuscript received July* 2, 1951]

Summary

Conidia of a strain of *A. oryzae* were irradiated at five different wavelengths, 2480, 2650, 2804, 3132, and 3650 Å. Those germinating after irradiation were isolated by the single spore technique and examined for changes in morphology and for enzyme production on liquid medium. Mutants of the normal strain were produced at all wavelengths, including "lethal mutants."

The 60 stable mutants recovered could be broadly classified into five types. Type *A* comprised strains in which the protease responsible for the reduction in viscosity of gelatin was produced at an earlier stage in growth than either the esterase or the protease acting on the lower molecular weight components of gelatin. Type *B* gave a slightly higher yield, and type *C* a slightly lower yield of proteases than the original strain. Types *D* and *E* developed a much greater mycelial weight and much less enzyme than the normal but the two types were distinguishable on the basis of their appearance.

I. INTRODUCTION

Changes in the production of enzymes by sexually reproducing fungi as a result of the induction of mutation by ultraviolet irradiation of the spores of *Neurospora crassa* (Beadle and Tatum 1941) and of *Chaetomium globosum* (McAuley and Ford 1947*a*, 1947*b*) have been demonstrated, and increased production of penicillin has been obtained by selecting mutants of *Penicillium notatum* produced by neutron bombardment (Hanson *et al.* 1946). These results suggested that an increase in the yield of protease obtained from cultures of *A. oryzae* grown on an artificial medium (Maxwell 1951) might be achieved by induced mutation of the spores of this organism.

This paper describes changes in the appearance and enzyme production of *A. oryzae* on irradiation with ultraviolet light at five different wavelengths.

II. METHODS

The apparatus used was that described by McAulay and Ford (1947*a*). Since the conidia of *A. oryzae* were found to be much more susceptible to drying than the ascospores of *C. globosum*, no air was passed over the spores

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during irradiation, and moist filter paper strips were held between the quartz glass slides to maintain the humidity close to saturation.

Spores of the normal strain were spread on a thin marked line on a quartz glass slide, using a dissecting microscope. The dosage necessary to give at least a 50 per cent. mortality was determined for wavelengths of 2480, 2650, 2804, 3132, and 3650 Å. After irradiation, groups of 50 to 100 spores were transferred to a petri dish containing beer wort agar, and spread over the surface in 0.5 ml. normal saline with a sterile wire. As each spore germinated it was transferred to a separate agar plate, to be later compared with the original strain in morphology and biochemical reactions. A control was included in each experiment, and for this the spores were spread on the quartz glass slide but were shielded during irradiation. Afterwards they were plated out as described above, and a number of germinating spores, at least equal to those spores taken from the irradiated samples, were transferred to separate plates to check for spontaneous mutation or variation. Cultivation was carried out at 22°C. on the modified Raulin's medium described by Maxwell (1952).

The protease activities were estimated viscometrically by the method of Lennox and Ellis (1945) and gravimetrically by the method of W. G. Crewther (in press). Esterase was estimated by a procedure based on the method of Huggins and Lapides (1947).

Table 1

EFFECT OF WAVELENGTH ON MORTALITY AND MUTATION

Wavelength (Å)	Dosage to Give 10 per cent. Mutants (joule/cm.^{2})	Optimal Dosage for Mutant Production (joule/cm.^{2})	Kill at Optimal Dosage (%)	Mutants at Optimal Dosage (per cent. of experimental colonies)
2480	0.045	0.720	75	22
2650	0.017	0.033	80	50
2804	0.021	0.053	77	36
3132	7.5	7.5	40	10
3650	60	60	20	9

III. Experimental and Results

(*a*) *Effect of Wavelength on Mutation*

The results of three or more irradiation experiments at each of the five wavelengths are shown in Table 1. The wavelength 2650 Å was the most effective in producing mutation, closely followed by 2804 Å and 2480 Å. Even at 3132 and 3650 Å, mutants were produced after prolonged irradiation. At 3650 Å, 10 per cent. kill and 10 per cent. of mutants resulted from 4-5 days' continuous irradiation. It was not feasible to increase the dosage at 3132 and 3650 Å to obtain a kill comparable with that obtained at shorter wavelengths, as long exposure resulted in poor germination of the controls.

As beer wort agar is rich in nutrients, it was expected to serve as a satisfactory medium for all mutants. However, a number of "lethal mutants" of the type described by Ford (1948) were observed under the microscope. Conidia of these mutants germinated, but after one or two cell divisions the cell walls ruptured and the cytoplasm was extruded.

It was not practicable to test every colony isolated for the production of enzymes and accordingly the following were selected for study:

(i) Colonies showing differences from the normal strain in rate of growth or in the appearance of the spores or mycelium.

(ii) Colonies developing from slowly germinating conidia or showing irregular development of the mycelium.

(iii) Some apparently normal colonies exceeding in number those included in (i) and (ii).

Before testing the production of enzymes on liquid media, the mutant colonies were subcultured through at least five generations by single spore isolation.

TABLE 2

DISTRIBUTION OF MUTANT TYPES, EXPRESSED AS A PERCENTAGE OF THE TOTAL ISOLATIONS FOR EACH WAVELENGTH

Wavelength (Å)	Total Isolations	Appearance on Wort Agar— Appearance on Liquid Medium— Production of Enzymes on Liquid Medium—	Normal			Abnormal	
			Normal		Abnormal	Normal	Abnormal
			Normal (%)	Abnormal (%)	Abnormal (%)	Normal (%)	Abnormal (%)
2480	44		52	23	2	23	—
2650	54		41	4	9	42	4
2804	80		34	10	16	30	10
3132	8		25	25	0	50	0
3650	17		35	47	0	12	6

The results of testing the 203 cultures from irradiated conidia for growth and protease production on the liquid medium are summarized in Table 2. It will be seen that, after repeated subculturing, a high proportion of form mutants reverted to the original strain. Of those irradiated at wavelengths 2480, 2650, and 3132 Å, only 18-25 per cent. appeared to be stable mutants showing some variation from the normal in enzyme activity on the liquid medium. At the wavelengths 2804 and 3650 Å, the proportions of stable mutants were 36 and 53 per cent. respectively. As will be shown later in this paper, the mutants obtained at 3650 Å were almost all of the same type and were characterized by a slight reduction in yield of proteases. All strains showing abnormal growth on liquid medium produced abnormal yields of enzyme.

Comparison of the 60 mutants with the normal strain in respect of protease and esterase activity and mycelial weight can be drawn from the data given in Table 3.

The strains have been divided into five types (*A* to *E*) as follows:

Type *A* strains include those that produce maximum gelatin viscosity-reducing enzyme earlier than the normal strain. The production of protease acting on the lower molecular weight components of gelatin and esterase production are normal. The maximum viscometric activity is slightly higher than normal but the organism appears normal on both wort agar and on liquid medium. The three mutants of this type were obtained at a wavelength of 2804 Å, but in view of the small number isolated, the figures are not significant.

TABLE 3

ENZYME PRODUCTION BY MUTANTS

Mutant Group	No. of Mutants Tested	Maximum Enzyme Activity: Viscometric Protease (% of normal activity) Mean	Viscometric Protease Standard Deviation	Gravimetric Protease (% of normal activity) Mean	Gravimetric Protease Standard Deviation	Esterase (% of normal activity) Mean	Esterase Standard Deviation	Mean Optimum Incubation Period (days): Viscometric Protease	Gravimetric Protease	Esterase	Dry Wt. of Mycelium After 12 days at 22°C. (% of normal wt.) Mean	Standard Deviation
A	3	121	4	111	9	120	12	7	10	10	89	4
B	19	124	13	122	8	105	16	10	12	10	86	7
C	27	78	9	82	13	84	11	10	11	10	119	14
D	5	10	8	16	7	25	16	11	12	10	199	60
E	6	25	22	37	25	60	25	11	12	10	147	58

Type *B* comprises strains that appear normal on wort agar but give greatly reduced sporing on liquid medium. The dry weight of mycelium is low, but production of proteolytic enzymes is greater than normal. Esterase production is not changed. With one exception, mutants of this type originated at wavelengths of 2650 or 2804 Å.

Type *C* strains give decreased yields of protease and esterase, the dry weights of mycelium being greater than the normal strain. The majority of mutants obtained at 3650 Å are of this type but they also appeared at all wavelengths.

Types *D* and *E* show the greatest divergence from normal (Table 5). These strains yield less than 50 per cent. of the normal protease production. Type *D* develops up to twice the normal mycelium weight and spores profusely, type *E* produces few or no spores, the mycelium weight being usually higher than the normal. These types predominate at a wavelength of 2804 Å.

The occurrence of specific mutations of *A. oryzae*, similar to that described by Ford (1948) with *Chaetomium*, is not excluded by the above results; but the 203 colonies examined, of which all but 60 reverted to the normal strain on repeated subculture, are too few to give more than an approximate indication of the effect of wavelength on mutation. The wavelengths 2650 and 2804 Å were by far the most lethal for conidia of *A. oryzae* (Table 1). If change in metabolism and enzyme production are accepted as criteria of cell

damage, the results in Table 4 indicate that 2804 Å is the most damaging wavelength of those tested in these experiments. Mutants of types *D* and *E*, and to a lesser extent type *B*, show the greatest differences from the original strain, and these occur from two to four times more frequently after irradiation at 2804 Å than at the other wavelengths used.

TABLE 4

EFFECT OF WAVELENGTH ON TYPE OF MUTANT*

Mutant	Total Number Isolated	Number Isolated at Following Wavelengths				
		2480 Å	2650 Å	2804 Å	3132 Å	3650 Å
Normal strain *A. oryzae*	122	23	22	27	28	22
Type *A* mutant	3	—	—	3	—	—
Type *B* mutant	19	1	5	13	—	—
Type *C* mutant	27	10	2	5	2	8
Type *D* mutant	5	—	1	4	—	—
Type *E* mutant	6	—	1	4	—	1

* These results refer to strains found to be stable after several sub-cultivations.

(*b*) *Metabolism of Mutants in Relation to Enzyme Production*

Mutants of the same type showed a gradation in enzyme production rather than a clear-cut divergence from the original. For example, gelatin viscosity reduction activity in type *D* mutants ranged from 2 to 21 per cent. of normal. One mutant of each type was grown on the liquid medium for 14 days at 22°C. Changes in the composition of the medium and the production of proteases and esterase were followed daily from the third day onwards. At this stage, no attempt was made to determine the optimum concentrations of the components of the liquid medium for each mutant.

Table 5 summarizes the appearance of the mycelium, utilization of sugar, and variation in pH of the medium during growth. Except for type *A* mutants, there is considerable variation in growth and in the colour of the liquid medium. A bright yellow culture filtrate is normally associated with high enzyme activity, and a very pale or orange filtrate with low activity. The bright orange liquid of type *D* was very slow to filter and contained large crystals of the potassium salt of an unidentified aliphatic organic acid. Sugar utilization by types *A* and *B* was the same as for the normal strain. The more rapid protease formation of type *A* was not correlated with more rapid removal of sugar from the medium. Types *D* and *E* required longer than normal to complete sugar utilization. The estimated 0.5 per cent. reducing sugar present after seven days with type *C* may be a reducing compound synthesized by the mould.

In general, mutants that induced the usual changes in pH of the medium during growth, that is, caused a decrease of pH to approximately 4.5 by the fourth day and restored the pH to the original value by the seventh day, produced enzyme yields that compared favourably with that of the original culture. Types *D* and *E* required longer periods for restoration of the pH and gave low yields of enzyme.

The dry weights of mycelium from cultures on 225 ml. medium are shown in Table 3. Types *A* and *B* both attained maximum mycelial weight two days earlier than the normal strain, and types *C* and *E* one day earlier, while the dry weight of the type *D* mycelium increased to nearly double that of the others. All mutants lost weight when proteases appeared in the medium, and this decrease continued till approximately the twelfth day, after which there was little further change. Autolysis of the mycelium of type *D* mutants, which produced very small amounts of extra-cellular enzymes in the medium, did not liberate more enzymes, nor did disintegration of the mycelium in the Waring Blendor.

TABLE 5

GROWTH AND METABOLISM OF MUTANTS

Mutant	Appearance of Growth on Liquid Medium	Utilization of Sucrose in Liquid Medium During Growth	pH of Liquid Medium During Growth
Normal strain	Even, cinnamon-brown sporing; liquid golden yellow	All sugar disappeared from medium by eighth day	Decreased to 4.5 by fourth day; increased to 6 by seventh day then slowly to 7.5 on thirteenth day
Type *A*	As above	As above	As above
Type *B*	Poor and irregular sporing; liquid bright citron yellow	As above	As above
Type *C*	Even, cinnamon-brown sporing; liquid yellow orange	Fell to 0.5 per cent. on eighth day; then constant	As above
Type *D*	Profuse sporing, bottle green; liquid orange and slightly viscous	All sugar disappeared from medium by tenth day	Decreased to 4.5 by fourth day; remained constant at 6.5-6.8 from eighth day onwards
Type *E*	Few spores; dense white mycelium tending to sink below surface; liquid pale yellow	As above	Decreased to 4.5 by fourth day; increased to 6 by tenth day then slowly to above 7 by fourteenth day

Nitrogen utilization is recorded in Table 6. The nitrogen content of the mycelium varied considerably during growth, decreasing with the age of the mycelium. Types *A*, *B*, and *C* differ little from the normal in the percentage of available nitrogen built into the mycelium, but type *D* used almost twice

TABLE 6

NITROGEN UTILIZATION BY MUTANTS

Mutant	Mycelium Nitrogen		N of Medium During Growth		Maximum Protease Production		
	Uptake from Medium (% of N available)	Maximum Loss to Medium (mg.)	Change in Ammonia N (mg./ml.)	Change in Soluble Non-Ammonia N (mg./ml.)	Visco-metric Activity (units/ml.)	Gravi-metric Activity (units/ml.)	Maximum Esterase Activity (units/ml.)
Normal strain	25	40-50 at tenth day	Minimum at eighth day; rises to 300 at tenth day, then constant	Maximum 100-120 at ninth to eleventh day, then decreases with further incubation	37-38 at tenth day	4600 at eleventh day	428-466 at tenth day
Type *A*	26	40 at seventh day	Minimum 270 at seventh day; rises to 280 at ninth day, then constant	Maximum 130 at seventh day, then constant	41 at seventh day	4600 at tenth day	494 at tenth day
Type *B*	28	80 at tenth day	Minimum 270 at eighth day; rises to 390 at tenth day, then constant	Maximum 130-140 at ninth to tenth day, decrease with further incubation	56 at tenth day	6800 at twelfth day	532 at tenth day
Type *C*	26	40 at tenth day	Minimum 270 at eighth day; rises to 340 at thirteenth day	Maximum 60 at eighth day, then constant	34 at tenth day	4200 at tenth day	361 at tenth day
Type *D*	43	30 at thirteenth day	Falls steadily to 186 at fourteenth day	Maximum 37 at thirteenth to fourteenth day	2 at twelfth day	3400 at thirteenth day	93 at twelfth day
Type *E*	15	30-40 at thirteenth day	Minimum of 325 at ninth day; rises to 391 at fourteenth day	Maximum 50 at eleventh day, then constant	4 at eleventh day	100 at twelfth day	150 at twelfth day

as much and type *E* very much less than the normal strain. Consequently the residual ammonia in the medium was much lower for type *D* and higher than normal for type *E*. The maximum loss of nitrogen from the mycelium to the medium from the time of first appearance of the enzymes corresponded both in time and quantity with the appearance of the proteases and esterase in the solution. It seems probable that this nitrogen loss from the mycelium is due to liberation of enzyme protein, there being simultaneous appearance of soluble non-ammonia nitrogen in the medium.

Type *C* mutants resembled the normal strain when grown on a zinc-deficient medium. Protease and esterase activities were all low and the culture filtrate was more orange than yellow. However, the addition of higher concentrations of zinc to the medium did not improve the yield of enzyme with type *C* mutants.

IV. Discussion

In experiments involving purification of the proteases produced by *A. oryzae* it is of considerable importance to prevent autodigestion of the enzymes during production and handling. The production of highly active protease solution in a relatively short incubation time by type *A* mutants suggests that these strains may find application in the production of protease preparations for purification.

Although no conclusions can be drawn from the limited amount of information concerning specific effects of certain wavelengths, the data presented here indicate that major changes in the production of enzymes by asexual fungi, some of a desirable character, may be induced by ultraviolet irradiation.

V. Acknowledgments

The author wishes to express her gratitude to Professor A. L. McAulay for hospitality and facilities offered for the irradiation experiments described, and for valuable criticism; also to Dr. J. M. Ford for helpful collaboration and comment. The metabolism studies were greatly facilitated by the technical assistance of Miss B. Lyons and Miss M. C. Robinson, to whom grateful thanks are recorded.

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