ENZYMES OF *ASPERGILLUS ORYZAE*

I. THE DEVELOPMENT OF A CULTURE MEDIUM YIELDING HIGH PROTEASE ACTIVITY

By MARGARET E. MAXWELL*

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Summary

When grown on modified Raulin's medium, containing additional sucrose, ammonium tartrate, and phosphate, *Aspergillus oryzae* yields more protease than when cultivated on unmodified Raulin's or Czapek-Dox media. Replacement of sucrose in the medium by fructose, invert sugar, glucose, maltose, starch, or lactose decreases the yield of enzyme in that order. Ammonium tartrate may be replaced by equivalent concentrations of the more readily available salts, sodium potassium tartrate (Rochelle salt) and ammonium chloride, without loss in activity.

Varying the concentration of each of the constituents has shown that the following medium is the one most favourable for protease formation: 4.0 per cent. sucrose, 3.0 per cent. sodium potassium tartrate, 1.1 per cent. NH_4Cl, 0.3 per cent. K_2HPO_4, 0.05 per cent. $MgSO_4.7H_2O$, 0.002 per cent. $FeSO_4.7H_2O$, and 0.1 p.p.m. Zn. The pH is adjusted to 6.2 with HCl. With this medium, maximum yield of enzyme is obtained in 10 days at 22°C. with a ratio of volume to surface area of 0.9.

I. INTRODUCTION

It has been shown that a strain of the mould *Aspergillus flavus-oryzae*† produces high yields of proteolytic enzymes when grown on steamed wheat bran (Maxwell 1950). To purify these enzymes it was considered advisable to develop a protein-free liquid culture medium on which the mould would grow vigorously and produce high concentrations of extra-cellular enzymes.

Twenty-five strains of *A. oryzae* were tested for growth and proteolytic activity on various liquid media. The Czapek-Dox and Raulin media were found to support growth of the organism with the production of small amounts of protease. Strain 292.4795, from the collection of Dr. C. Thom, which had previously been found to produce the highest yields of protease when grown on steamed wheat bran, also proved most suitable for surface culture on liquid media.

This paper describes changes in the production of protease by this mould with variations in the nature and concentration of components of the medium. From these data a medium has been selected for further studies on the enzymes of *A. oryzae*.

* Biochemistry Unit, Wool Textile Research Laboratory, C.S.I.R.O., Melbourne.

† Referred to in this series of papers as *A. oryzae*.

II. Methods

Cultures of *A. oryzae* were prepared by inoculating sterile medium with 0.5 per cent. by volume of a heavy spore suspension of the organism, and incubating at the prescribed temperature. The resulting cultures were filtered, followed by estimation of protease activity of the medium, using the gelatin viscosity reduction method of Lennox and Ellis (1945) and the gravimetric method of W. G. Crewther (in press). The latter method expressed protease activity in terms of the weight of gelatin rendered soluble in 80 per cent. ethanol as a result of protease action. The activity is expressed as mg. of gelatin and may be converted to enzyme units by reference to a standard curve.

Sucrose was estimated by inversion with acid and application of Bertrand's method (1906).

Ammonia nitrogen was determined by direct distillation from alkaline solution and titration, and total nitrogen by digestion with sulphuric acid and Nesslerization.

III. Selection of Medium

In order to determine the composition of a basal medium which could be modified to improve the yield of protease produced by *A. oryzae*, this organism was grown on a number of modifications of Czapek-Dox and Raulin media, one of the latter being that developed by F. G. Lennox (personal communication). The protease activities developed in 14 days at 25-27°C. were compared with the activity of an aqueous extract of a steamed-bran culture of the same organism. Both viscometric and gravimetric methods were used (Table 1). The tartrate medium of Lennox is seen to be considerably better than the other modifications tested, and it has accordingly been used as the basal medium for further investigation. The concentration of each of the ingredients, shown in Table 1, has been changed and combined with that of other ingredients in different ways in order to obtain the best possible yield of protease at 25-27°C. The effects of varying the carbon source and the organic anion, and of the importance of trace metals in the medium were also investigated.

(*a*) *Variation of the Carbon Source*

Table 2 provides figures for the protease activity of cultures of *A. oryzae* grown on the basal medium in which the sucrose was substituted with other sugars or starch. Both the viscometric and gravimetric methods showed sucrose to give the highest protease activities. Experiment has shown this superiority of sucrose to be maintained when the basal medium is changed in other ways.

(*b*) *Concentration of Ammonium and Tartrate Ions*

The ammonium tartrate in the basal medium was replaced by varying amounts of ammonium chloride and tartaric acid, the pH being adjusted to

TABLE 1

PROTEASE ACTIVITIES ON LIQUID MEDIA

Medium	Composition of Medium (g./100 ml.)													Time of Incubation at 25-27°C. for Maximum Yields of Protease (days)	Maximum Protease	
	Sucrose	Molasses	K Acetate	NH_4 Tartrate	NH_4NO_3	Urea	Gelatin	K_2HPO_4	KCl	NaCl	$MgSO_4.7H_2O$	$FeSO_4.7H_2O$	$ZnSO_4$	$MnSO_4$		Viscometric Method (units/ml.)	Gravimetric Method (units/ml.)
Czapek-Dox	1.0	—	—	—	0.3	—	—	0.1	0.05	—	0.05	0.001	—	—	10	0.28 0.31	56 68
Modified Czapek-Dox	1.0	—	—	—	—	—	1.0	0.1	0.05	—	0.05	0.001	—	—	10	0.63 0.56	108 122
Raulin's medium	1.0	—	—	0.5	—	—	—	0.03	0.05	0.2	0.025	0.0002	—	—	8	0.61 0.61	224 240
Modified Raulin's medium	1.0	—	—	0.5	—	—	—	0.05	0.05	0.2	0.025	0.0002	—	—	8	0.56 0.53	394 230
Modified Raulin's medium	1.0	—	—	0.5	—	—	—	0.1	0.05	0.2	0.025	0.0002	—	—	8	0.50 0.48	232 158
Modified Raulin's medium	1.0	—	—	0.5	—	—	—	0.03	0.05	0.2	0.05	0.0002	—	—	8	0.90 0.63	274 280
Modified Raulin's medium	1.0	—	—	0.5	—	—	—	0.03	0.05	0.5	0.025	0.0002	—	—	8	0.48 0.44	200 146
Modified Raulin's medium	1.0	—	—	0.5	—	—	—	0.03	0.05	1.0	0.025	0.0002	—	—	8	0.40 0.30	218 140

TABLE 1 (*Continued*)

PROTEASE ACTIVITIES ON LIQUID MEDIA

Medium	Composition of Medium (g /100 ml.)														Time of Incubation at 25-27°C. for Maximum Yields of Protease (days)	Maximum Protease	
	Sucrose	Molasses	K Acetate	NH_4 Tartrate	NH_4NO_3	Urea	Gelatin	K_2HPO_4	KCl	NaCl	$MgSO_4.7H_2O$	$FeSO_4.7H_2O$	$ZnSO_4$	$MnSO_4$		Viscometric Method (units/ml.)	Gravimetric Method (units/ml.)
Modified Raulin's medium	1.0	—	—	1.0	—	—	—	0.03	0.05	0.2	0.025	0.0002	—	—	8	0.31 0.29	148 124
Modified Raulin's medium	1.0	—	—	0.5	—	0.5	—	0.03	0.05	0.2	0.025	0.0002	—	—	8	0.11	36 38
Modified Raulin's medium	1.0	—	—	0.5	—	—	—	0.03	0.05	0.2	0.025	0.0002	0.0005	0.0002	8	0.28	174
Modified Raulin's medium	1.0	—	0.05	0.5	—	—	—	0.03	0.05	0.2	0.025	0.0002	—	—	8	0.26	152
Modified Raulin's medium	—	2.0	—	0.5	—	—	—	0.03	0.05	0.2	0.025	0.0002	—	—	8	0.36	234
Tartrate medium (Lennox)	1.0	—	—	1.0	—	—	—	0.1	0.05	—	0.05	0.002	—	—	8	2.90 2.88	262 252
Bran digest	—	—	—	—	—	—	—	—	—	—	—	—	—	—	11	3.88 2.78 5.00	380 480 440

6.2 with potassium hydroxide. When grown on such media, *A. oryzae* was found to produce maximum protease activity when the ratio of ammonium chloride to tartaric acid was between 1.4 and 1.7 (Fig. 1). The use of these two salts in the ratio of 1.4 approximates to the addition of ammonium tartrate, as such, to the medium.

TABLE 2

EFFECTS OF CARBOHYDRATES IN THE MEDIUM

Media contain the following percentages of nutrients: carbohydrate, 1; ammonium tartrate, 1; K_2HPO_4, 0.1; $MgSO_4.7H_2O$, 0.05; KCl, 0.05; $FeSO_4.7H_2O$, 0.02. pH 6.2

Carbohydrate	Time for Maximum Protease Production (days)	Protease Activity (units/ml.)		Growth of Mould
		Viscometric Method	Gravimetric Method	
Starch	13	1.48	350	Slow during the first 5 days, then very heavy
Sucrose	8	2.65	370	Heavy mycelial mat developed by third day
Fructose	8	2.43	358	Heavy mycelial mat developed by third day
Invert sugar	8	2.14	358	Heavy mycelial mat developed by third day
Glucose	8	2.06	328	Heavy mycelial mat developed by third day
Maltose	8	1.75	262	Heavy mycelial mat developed by third day
Lactose	12	0.88	268	Heavy mycelial mat developed by third day

(*c*) *Concentration of Ammonium Tartrate and Sucrose*

The optimum ratio of sucrose to ammonium tartrate was determined by growing the mould on media containing varying concentrations of sucrose. For each sucrose concentration, media containing 0.1, 0.5, 1.5, 1.0, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, and 5.0 per cent. ammonium tartrate were tested for protease production. The optimum tartrate concentration, the maximum protease activity, and the weight of mycelium corresponding with each sucrose concentration are shown in Table 3. With the exception of media containing less than 1.0 per cent. sucrose, highest yields of protease were obtained when the ratio

of sucrose to ammonium tartrate was of the order of 2 : 1 by weight. Maximum protease activity was obtained with a concentration of 4 per cent. sucrose, greater concentrations giving rise to higher mycelium weights but rather slower production of the protease.

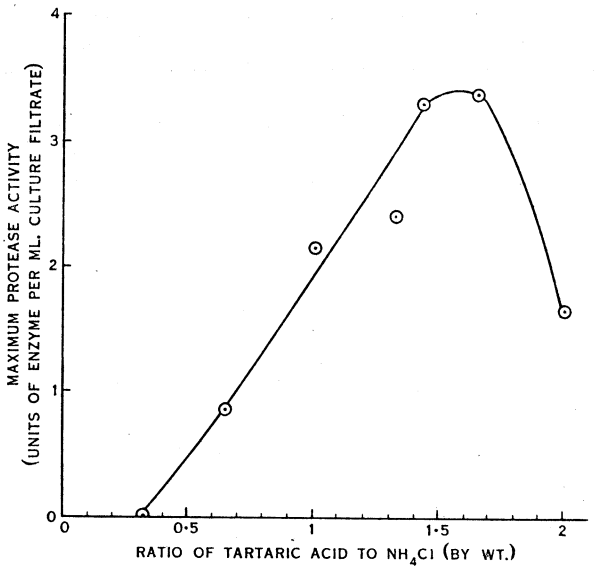

Fig. 1.—Effect of ratio of tartaric acid to ammonia on protease formation (viscometric method).

(*d*) *Comparison of Various Organic Radicals with Tartrate*

It has been found that a number of organic salts can replace tartrate in the medium, with varying degrees of effectiveness for protease production. The optimal concentrations of sucrose and ammonium salts have been determined for media containing salts of malonate, lactate, malate, pyruvate, acetate, formate, fumarate, succinate, citrate, maleate, and oxalate, and it was found that protease production on these media was less than that on the optimal tartrate medium. The activities of the culture filtrates decreased in the order given above.

A more detailed study was made of the changes taking place in media containing tartrate, malonate, lactate, and malate. Table 4 sets out the optimal concentrations of the various components of the media containing these anions as far as protease production is concerned. Sucrose, phosphate, magnesium, and iron were required in the same concentrations as in the tartrate medium.

The changes taking place in these media during growth of the mould are summarized in Table 5. In all media the pH falls initially to values approaching 4.0, then rises above 7.5. With tartrate the minimum pH value is reached

in four days and the pH is restored to 6.0 in six days. However, with the other media tested, although the fall in pH was as rapid, a considerably longer

TABLE 3

EFFECT OF CHANGING SUCROSE AND AMMONIUM TARTRATE CONCENTRATIONS ON THE PRODUCTION OF PROTEASE BY *A. ORYZAE*

Data are provided only for those ammonium tartrate concentrations giving maximum protease activity for each of the sucrose concentrations tested. Incubation temperature 25-27°C.

Sucrose (g./100 ml.)	Ammonium Tartrate (g./100 ml.)	Incubation Time (days)	Viscometric Activity (units/ml.)	Dry Weight Mycelium (g./100 ml. filtrate)
0.1	0.1	7	0.8	0.15
0.5	0.5	6	2.1	0.23
1.0	0.5	6	4.8	0.31
2.0	1.0	6	4.3	0.69
3.0	1.5	6	6.5	1.04
4.0	2.0	6	8.5	1.40
5.0	2.5	7	8.5	1.48
6.0	3.0	8	8.5	1.69
7.0	3.5	9	8.5	1.55
8.0	4.0	10	7.8	1.85
9.0	4.5	10	8.1	2.15
10.0	5.0	12	8.1	2.43

period was required for the pH to rise to 6.0. On the other hand, omission of tartrate or other organic radical resulted in a steady fall of pH to less than

TABLE 4

CONCENTRATIONS OF COMPONENTS OF MEDIA GIVING MAXIMUM YIELDS OF PROTEASE IN THE PRESENCE OF VARIOUS ANIONS

Medium	Molar Concentrations Giving Maximum Protease Activity					
	Organic Anion	NH_4Cl	Sucrose	K_2HPO_4	$MgSO_4 \cdot 7H_2O$	$FeSO_4 \cdot 7H_2O$
Tartrate	0.11	0.21	0.11	0.02	0.002	0.00007
Malonate	0.20	0.09	0.11	0.02	0.002	0.00007
Lactate	0.22	0.05	0.11	0.02	0.002	0.00007
Malate	0.15	0.05	0.11	0.02	0.002	0.00007
No anion	—	0.21	0.11	0.02	0.002	0.00007
No anion but 2% $CaCO_3$* incorporated	—	0.21	0.11	0.02	0.002	0.00007

* $CaCO_3$ was oven-sterilized and added to medium after autoclaving, giving a pH value of 7. The other media were adjusted to pH 6.2.

2.0, and incorporation of calcium carbonate in a medium lacking tartrate resulted in an almost constant pH with little protease production (Table 5).

With all media, the production of mycelium progressed at the same rate, which lends support to the view that the sucrose content of the medium is the chief factor limiting mycelium production.

TABLE 5

EFFECT OF VARIOUS ANIONS ON THE CHARACTERISTICS OF THE CULTURE DURING GROWTH OF *A. ORYZAE** AT 22°C.

Anion in Growth Medium	Maximum Protease (viscometric units)	Weight of Mycelium (g./100 ml. medium)		Maximum Non-Ammonia N (mg./100 ml.)	Minimum Ammonia N (mg./100 ml.)	pH		Final Sucrose Concentration (g./100 ml.)
		Maximum	Final			Minimum	Time for Return to 7.0 (days)	
Tartrate	23 (10 days)	3.0 (6-7 days)	2.0 (12 days)	62 (11 days)	120 (6-7 days)	4.5 (4 days)	8–9	Nil (8 days)
Malonate	18 (11 days)	2.0 (5-7 days)	2.0 (11 days)	54 (11 days)	22 (7-8 days)	4.5 (4 days)	9	Nil (8 days)
Lactate	10 (11 days)	3.0 (7 days)	2.0 (10 days)	23 (11 days)	1 (9 days)	4.5 (5-7 days)	11	0.5 (from 10 days)
Malate	10 (11 days)	3.0 (6-7 days)	2.0 (14 days)	24 (11 days)	1 (10 days)	4.5 (4 days)	11	0.5 (from 9 days)
No anion	0.3 (8 days)	2.0 (6-7 days)	1.0 (10 days)	—	260 (7 days)	1.9 (5 days)	pH constant at 1.9	2.2 (14 days)
No anion; 2% $CaCO_3$	2.0 (14 days)	3.0	—	—	190 (11 days)	5.8 (10 days)	12	Nil (11 days)

* The results in Tables 5 and 6 were obtained with a strain of *A. oryzae*, selected from the original strain, that yielded culture filtrates having higher proteolytic activity than those from the strain used in the earlier experiments.

(*e*) *Inorganic Constituents of the Medium*

For experiments on the inorganic constituents of the medium, the basal medium was modified to contain 2 per cent. ammonium tartrate and 4 per cent. sucrose. The optimal concentration of phosphate in the form of KH_2PO_4 was found to be 0.3 per cent. A seven-day incubation period at 25-27°C. was found to give highest activities for all phosphate concentrations over the range 0.1-0.5 per cent. Figure 2 illustrates the effect of phosphate concentration on the protease activity of the culture.

Media containing varying amounts of magnesium sulphate were prepared for each of a range of phosphate concentrations from 0.1 to 0.5 per cent. The optimum concentration of $MgSO_4.7H_2O$ was found to be independent of the phosphate concentration and was approximately 0.05 per cent. Increasing the magnesium sulphate content above this value led to slower production of the enzyme.

Media that contain potassium in the form of Rochelle salt and potassium phosphate, and chloride as the ammonium salt, do not require the addition of further amounts of potassium chloride.

Of the trace metals, Mn, Co, Ni, Cu, and Mo were slightly inhibitory at a concentration of 0.1 p.p.m., Zn did not inhibit protease production at concentrations up to 1.0 p.p.m., and Fe was found to influence the enzyme production in the manner shown in Figure 3, with optimal production at a concentration of 0.0025 per cent. $FeSO_4.7H_2O$ (5 p.p.m. Fe).

It is almost certain that trace elements are supplied to the mould in the other constituents of the medium. For instance, in large-scale experiments using flat-sided, 2-l. bottles for growing the mould, occasional batches failed, owing to Zn deficiency. It is therefore advisable to add 0.1 p.p.m. of Zn to the medium.

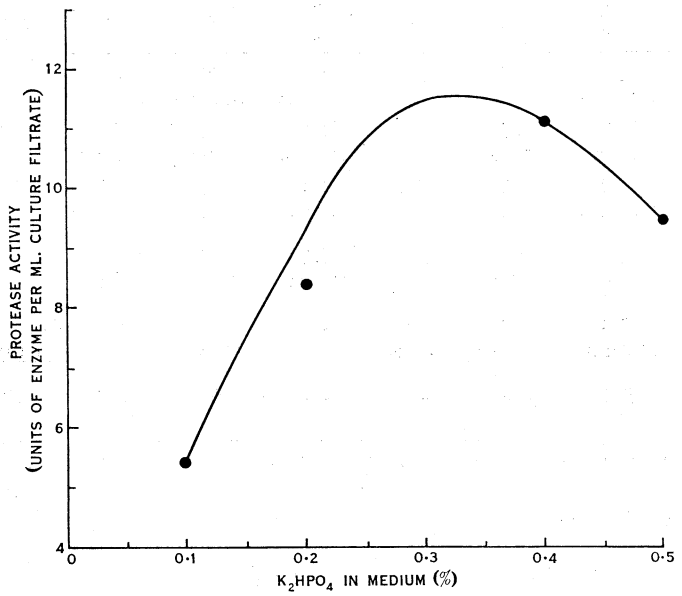

Fig. 2.—Effect of concentration of K_2HPO_4 in the medium on protease formation (viscometric method).

IV. Optimum Conditions for Incubation

Having obtained information regarding the optimum concentration of the medium for production of protease by *A. oryzae* on liquid medium, experiments were continued using flat-sided, 2-l. bottles in place of the 500-ml. conical flasks. The optimum temperature and ratio of volume to surface area were determined for cultures grown in these bottles on the medium described above.

(*a*) *Temperature*

Protease activities were determined by the viscometric and gravimetric methods during incubation at various temperatures. At the higher temperatures the protease developed rapidly but the maximum activity reached was considerably less than in cultures incubated at 18-22°C. (Fig. 4). Thus, at

35°C., the maximum activity of 4.5 viscometric units was produced within four days, whereas at 22°C. the maximum of 27 units required 11 days for its development. Incubation at 18°C. did not provide a higher maximum activity than was obtained at 22°C., and the time for reaching this condition was 21 days. Results obtained by the gravimetric method were similar to the above (Fig. 5).

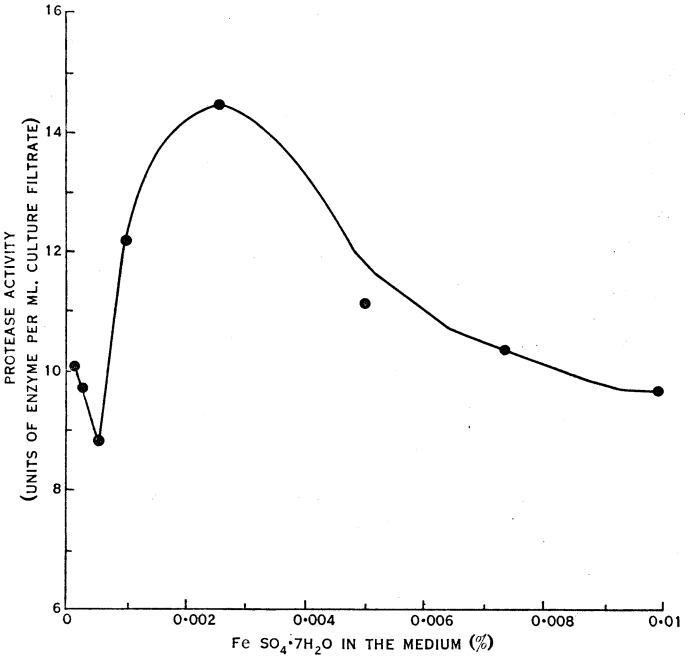

Fig. 3.—Effect of concentration of $FeSO_4.7H_2O$ in the medium on protease formation (viscometric method).

(*b*) *Ratio of Volume to Surface Area*

It has been found that as the ratio of volume to surface area decreased, the activity of the protease produced approached a maximum value. Further decrease of the ratio caused a decrease in the maximum activity reached. Tables 6 and 7 indicate that a volume of 125-225 ml. per bottle, providing a ratio of volume to surface area of 0.52-0.93, gave rise to highest protease activities. As would be expected, increasing the volume of medium per bottle caused a corresponding increase in the time required for the attainment of maximum activity.

During growth of the mould, the volume of medium decreased daily by about 4 ml. per bottle initially, the rate of loss decreasing after about six days to between 1 and 2 ml. per day. At this stage the surface was completely covered with the mycelial mat, which had attained its maximum weight. From Tables 4 and 5 it will be seen that the total recovery of protease per unit

volume of the initial medium was greatest where a volume to surface ratio of 0.93 was used. Using a volume to surface ratio of 0.93, and cultivating the

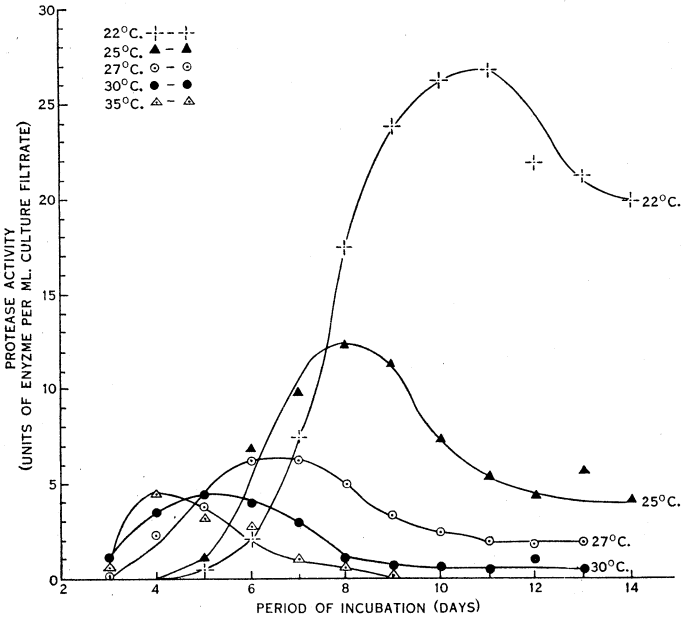

Fig. 4.—Effect of temperature of incubation on protease formation (viscometric method).

TABLE 6

EFFECT OF RATIO OF VOLUME TO SURFACE AREA ON PROTEASE ACTIVITY (VISCOMETRIC) OF CULTURE FILTRATES*

Volume per 2-l. Bottle (ml.)	Volume (ml.) / Area (cm.²)	Maximum Activity (units/ml.)	Time of Incubation at 22°C. (days)	Filtrate Recovered (ml.)	Enzyme Recovered (units/ml. of original medium)
75	0.310	29.0	7	52	20.5
100	0.413	27.5	7	76	20.9
125	0.516	35.0	8	97	27.2
150	0.620	32.7	8	115	25.0
175	0.723	33.7	9	135	26.0
200	0.826	31.5	9	165	26.0
225	0.929	32.5	10	190	27.5
250	1.032	29.0	10	215	25.0
275	1.140	26.0	10	240	22.7
300	1.240	26.2	11	260	22.7
325	1.340	26.0	11	280	22.4
350	1.440	21.0	11	293	17.6
375	1.550	20.4	12	320	17.4

* See footnote to Table 5.

mould at 22°C. for 10 days in 120 bottles, approximately 20 l. of culture filtrate was obtained, with a protease content of 540,000 viscometric units.

V. Final Choice of Medium and Conditions

In the experimental work described earlier, some of the constituents of the medium were tested for optimal concentrations, using a basal medium that differed in some way from the medium found later to give higher protease activities. Further tests were therefore done in which two constituents of the medium were varied and combined in different ways. However, no improvement in the yield of protease could be obtained, and the medium adopted contained 4 g. sucrose, 2.0 g. ammonium tartrate, 0.3 g. K_2HPO_4, 0.05 g. $MgSO_4.7H_2O$, 0.002 g. $FeSO_4.7H_2O$ per 100 ml. of tap water and 0.1 p.p.m. Zn, added as $ZnCl_2$. The optimal initial pH is 6.0-6.5.

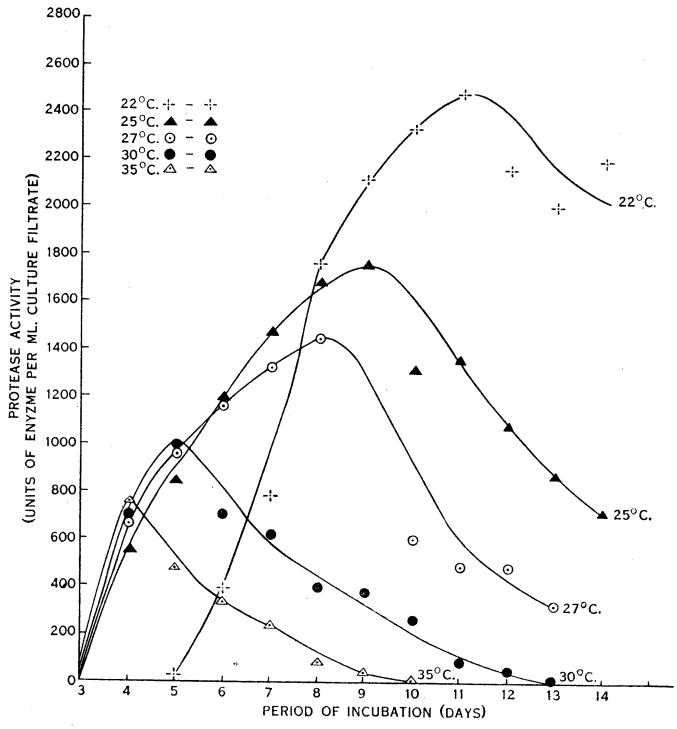

Fig. 5.—Effect of temperature of incubation on protease formation (gravimetric method).

For large-scale enzyme production the ammonium tartrate was replaced by a mixture of sodium potassium tartrate and ammonium chloride. The 2.0 g. of ammonium tartrate in the above medium could be replaced by 3.0 g. of sodium potassium tartrate (Rochelle salt) and 1.1 g. ammonium chloride, and the potassium chloride could then be omitted.

VI. Discussion

During the experimental work described above it has become apparent that there are two requirements for the production of a culture filtrate rich

in proteolytic activity. Firstly, the medium must be capable of supporting an active growth of the mycelial mat responsible for enzyme elaboration, and secondly, its composition and conditions of growth must be such that the required enzyme is produced in substantial amount in the medium. That these are separate considerations is shown, for instance, by the fact that the final protease activity of a culture of *A. oryzae* is not increased by increasing the sucrose content of the medium above 5 per cent., whereas the mycelial weight continues to increase even at double this sucrose concentration.

TABLE 7

EFFECT OF RATIO OF VOLUME TO SURFACE AREA ON PROTEASE ACTIVITY (GRAVIMETRIC) OF CULTURE FILTRATES

Volume per 2-l. Bottle (ml.)	Volume (ml.) / Area ($cm.^2$)	Maximum Activity (units/ml.)	Time of Incubation at 22°C. (days)	Filtrate Recovered (ml.)	Enzyme Recovered (units/ml. of original medium)
150	0.620	3120	8	105	2180
200	0.826	3160	9	155	2450
225	0.929	3120	10	180	2500
250	1.032	3000	11	200	2400
300	1.240	2560	12	250	2130
350	1.440	2520	14	283	2040

It has become generally recognized that moulds are sensitive to low concentrations of trace metals, and the need for magnesium, iron, and zinc in the medium is therefore to be expected. Similarly, the role of phosphate in the metabolism can be conjectured, though not with any degree of certainty. However, the variation of protease production with variation in the concentration of tartrate and with changes in the ratio of tartrate to ammonium salt concentration does not suggest a ready explanation. The fact that omission of the organic radical from the medium leads to a continued fall in the pH of the medium to a very low value, and the observation that no protease is produced in any of the media until the pH has risen from the minimum of 4.0 to approximately the original pH of 6.0 suggests that one role of tartrate is to prevent major changes in the pH of the medium due to assimilation of ammonium ions to form protein. For this to be effective it would be necessary for the tartrate to be metabolized and removed from the medium simultaneously with the ammonium ions. The more rapid return of the pH to the original value in tartrate medium, as compared with media containing other organic radicals, would also support this hypothesis.

A different explanation of the role of tartrate is suggested by the fact that substitution of calcium carbonate for tartrate results in culture filtrate having low protease activities although the pH is buffered at about 6.0. In this medium, growth of the mycelium was excellent. It is apparent, therefore, that tartrate and other organic anions have an important role in the formation or stabilization of the protease complex. Another possible explanation is that

the permeability of the mycelium to enzymes is dependent on the presence of such anions. In the present investigation it has not been possible to establish which explanation is correct.

VII. Acknowledgments

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