Metal-Complexing Agents as Metal Buffers in Media for the Growth of *Aspergillus niger*

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SUMMARY

The influence of metal-complexing agents on the mycelial growth rate, conidial germination and morphology of *Aspergillus niger* in shake-flask cultures was studied. The agents tested were: ethylenediaminetetraacetic acid (EDTA), diaminocyclohexane-*N,N*-tetra-acetic acid (CDTA), diethylenetriaminepenta-acetic acid (DTPA), and nitrilotriacetic acid (NTA), which form soluble complexes, and ferrocyanide, which forms insoluble complexes. The agents were added singly to culture media in concentrations up to 9.4 mM. The sum of the concentrations of the complex-forming metals (Fe, Cu, Zn and Mg) added to the medium was 1.04 mM. At pH 6.5 mycelial growth rate was almost unaffected by any of the agents when they were added after the germination of conidia. At pH 3.5 ferrocyanide at 84 μ M increased the mycelial doubling time by 30 %, but the other agents had little effect on the mycelial growth rate at pH 3.5. EDTA, CDTA and DTPA at 1.04 mM inhibited germination of conidia at pH 6.5; lowering the pH partially relieved this inhibition. Ferrocyanide at 9.4 mM was without effect on germination at pH 6.5, but at pH 3.5 it strongly inhibited germination at 84 μ M. The morphology of the organism was markedly affected by EDTA, CDTA, DTPA and ferrocyanide but not by NTA. In cultures without complexing agents or with NTA the organism grew in the form of large pellets with a filamentous periphery; such pellets formed a gelatinous conglomerate in the culture. With either EDTA, CDTA or DTPA at 1.04 mM or ferrocyanide at 84 μ M, the organism grew in the form of small pellets with a smooth exterior; such pellets remained discrete and dispersed in the medium. These physiological effects are attributed to changes in the metal ion concentrations in the medium brought about by the complexing agents.

INTRODUCTION

The trace-metal concentrations in culture media may be controlled in either of two ways. The commoner method is to purify the medium so as to remove certain metal ions and then add known amounts of the corresponding metal salts. This method is exacting, laborious and subject to failure by recontamination of the medium from dust and the walls of vessels. A newer and as yet little used method is to add metal-chelating agents to the medium so as to decrease the concentration of free metal ions to the desired values. This method has the advantage that the metal complex acts as a 'metal buffer' which reversibly dissociates so as to replace free metal ions as they are consumed by a growing organism, or to combine with metal ions added to the system. For wider aspects of the subject the symposium in

Federation Proceedings (1961) should be consulted. Many metal-complexing agents are available but their physiological effects have been little investigated and need to be better known if metal buffers are to be used more widely. The object of the present work was to study the effect of some metal-complexing or chelating agents on the growth of *Aspergillus niger*. The accumulation of citric acid in the medium when this mould is grown is a process known to be sensitive to the concentrations of trace metals. The theory of metal-complexing agents, which is analogous to that of hydrogen-ion buffers, has been treated, for example, by Chaberek, Bersworth & Martell (1955). The dissociation of a simple metal complex, MX , is represented by

where M^{+2} is the metal ion and X^{-n} is the anion of the chelating agent of valency n . The 'stability constant' K is given by

$$K = \frac{[MX^{2-n}]}{[M^{+2}][X^{-n}]},$$

where the brackets indicate molar concentrations. The stability constant is used as a measure of the degree of chelation. Values of the stability constants for the metals and chelating agents used in this work are given in Table 1.

Table 1. *Log K values (stability constants) of metal chelates**

Metal	DHEG	NTA	EDTA	CDTA†	DTPA
Fe ³⁺	?	15.87	25.1	31.0	28.6
Cu ²⁺	8.15	12.68	18.8	21.3	21.1
Zn ²⁺	5.36	10.45	16.5	18.67	18.3
Fe ²⁺	4.31	8.84	14.33	?	16.5
Mn ²⁺	3.15	7.44	14.04	16.78	?
Mg ²⁺	1.15	7.00	8.69	10.32	?

* All values from Bjerrum, Sillén & Schwarzenbach (1957), except † from Simons, Swidler & Benedict (1963).

? = Not known.

The removal of iron and other biologically important metals from molasses by the formation of complexes with ferrocyanide ions was suggested by Mezzadrolì (1938) in order to make this cheap source of carbohydrate suitable for citric acid production by *Aspergillus niger*. Since then, potassium ferrocyanide has been extensively used for the treatment of molasses to be used as an ingredient in media for citric acid production. Ferrocyanide ions form highly insoluble precipitates with the ions of many heavy metals. The order of increasing solubility of metallic ferrocyanides of importance in this study is as follows: Fe³⁺, Cu²⁺, Zn²⁺, Mn²⁺, Fe²⁺, Mg²⁺ (*American Cyanamid*, 1953). It has proved useful to compare the biological actions of the soluble chelate-metal complexes with those of the insoluble ferrocyanides. In this paper the effects on the growth of *Aspergillus niger* are considered.

METHODS

Strains of organism. The strain of *Aspergillus niger*, Wis 72-4, kindly supplied by Professor M. J. Johnson (Biochemistry Department, University of Wisconsin), was used. Some work was done with the Kew strain IMI-81821(2) derived from Wis 72-4, and supplied by the Mycological Institute, Ferry Lane, Kew, Surrey, U.K.

Media. The compositions of the media used are given in Table 2. All reagents were of analytical grade. Glass-distilled water was used for the preparation of solutions unless otherwise stated. All media, unless otherwise stated, were autoclaved at 121° for 15 min. Media were divided into several parts, in graduated bottles, for sterilization: the parts were: for medium M1: (1) glucose 50 %, w/v; (2) KH_2PO_4 ; (3) NH_4NO_3 ; (4) FeCl_3 ; (5) $\text{MgSO}_4 + \text{CuSO}_4 + \text{ZnSO}_4$. The salt solutions were ten times concentrated. Medium M3 was sterilized in three parts: (1) glucose 50 %, w/v; (2) $(\text{NH}_4)_2\text{SO}_4$ ten times concentrated; (3) inorganic salt solutions containing citrate buffer, double strength. Decrease of volume, because of evaporation in the autoclave, was made up in the graduated bottles before mixing all the solutions aseptically. The pH value of a medium was adjusted with sterile N-NaOH or N-HCl after mixing all solutions.

Table 2. *Composition of media used for growth of Aspergillus niger*

	M1	M2	M3	M4*
	g./l.			
Constituents				
Glucose	150	150	150	91
Agar	20	0	0	30
NH_4NO_3	2.5	2.5	0	0.45
$(\text{NH}_4)_2\text{SO}_4$	0	0	4.7	0
KH_2PO_4	1.0	2.5	2.5	0
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.25	0.25	0.25	0.06
Na citrate buffer (pH 6.4)†	0	0	Present	0
	mg./l.			
Trace metals‡				
Fe^{3+}	2.20	1.3	1.3	0
Cu^{2+}	0.48	0.06	0.06	0
Zn^{2+}	3.80	0.25	0.25	0
	pH value			
	4.5	3.5	6.5	4.5

* Also contained beer (English bitter), 60 ml./l.

† 0.054 mM citrate, pH adjusted with NaOH.

‡ Fe as FeCl_3 ; Cu as CuSO_4 ; Zn as ZnSO_4 .

Inoculum preparation. Stock cultures were maintained as freeze-dried conidial suspensions. The mould was transferred from the stock culture to surface cultures in medical flat bottles plugged with cotton-wool. The bottles were of 8 oz. capacity and contained 15 ml. agar medium. Medium M1 was used for strain 72-4 and medium M4 for the Kew strain. The bottles were incubated at 30° for 5–7 days. To wash off the spores the cultures were wetted with 5 ml. of 0.05 % aqueous solution of 'Monoxal O.T.' (di-octyl ester of sodium sulphosuccinic acid). The agar was washed twice with sterile distilled water and the combined washings were made to 50 ml. and shaken with glass beads to break up clumps of conidia. The number of conidia was determined with a Thoma counting chamber. The final stock conidial suspension was adjusted to contain 50 million conidia/ml.; 1 ml. of this suspension was used to inoculate 25 ml. medium.

Shake-flask culture. For shake-flask culture 25 ml. medium including 1 ml. conidial suspension was held in a 250 ml. conical flask plugged with cotton-wool. The flasks were shaken on a rotary shaker (throw 25 mm., 180 cye./min.) and incubated at 25°. In the kinetic experiments on growth a series of shake-flasks were set up and duplicate flasks removed at intervals for analysis.

Stirred-fermenter culture. *Aspergillus niger*, strain 72-4, was also grown in a 2 l. fermenter of the type described by Elsworth, Meakin, Pirt & Capell (1956). The vortex aeration system (Callow & Pirt, 1961) was used. The impeller was a vaned disc of 63 mm. diameter and vane depth 10 mm.; the stirrer speed was 1120 rev./min. Polyglycol P2000 (R. W. Greef and Co., London) was added at a concentration of 0.2 ml./l. to prevent foaming.

Metal-complexing agents. The chelating agents were dissolved and brought to pH 7-8 by adding NaOH. Potassium ferrocyanide was used as source of ferrocyanide ions; the solution was sterilized by membrane filtration.

Mycelial dry weight was determined by filtering off mycelium in a tared sintered glass crucible (porosity 2), washing the mycelial mat thoroughly with water and drying to constant weight at 100-105°.

RESULTS

Effects of chelating agents and ferrocyanide on mycelial growth rates of Aspergillus niger

To determine the mycelial growth rate, mycelial dry weights in shake-flask cultures were determined at intervals after the conidia had germinated, that is about 24 hr after inoculation. On plotting the logarithms of dry weights against time, a straight line was obtained. An example of such a plot is shown in Fig. 1. This straight line relation shows that growth of the mycelium followed the exponential law, $dx/dt = \mu x$, where x is the mycelial dry weight, t is time and μ is a constant called the 'specific growth rate'. The doubling time of the mycelium (t_d) is given by the formula $t_d = (\log_e 2)/\mu$. The growth rates of the mycelium under different conditions are expressed in terms of the specific growth rate or the doubling time. To examine the effects of chelating agents on mycelial growth rates, these

Table 3. *Effects of chelating agents added after germination on the mycelial doubling times of Aspergillus niger strain 72-4*

Concn. (mm)	Chelating agent			
	CDTA	DTPA	EDTA	NTA
Initial pH 6.5; medium M3, doubling time (hr)				
0.00	6.9	6.9	6.9	6.9
1.04	7.5	7.4	7.4	7.2
3.13	7.5	7.5	7.5	7.5
9.40	8.1	8.0	7.3	7.2
Initial pH 3.5; medium M2, doubling time (hr)				
0.00	7.7	7.7	7.7	7.7
1.04	8.0	7.7	7.7	7.7
3.13	8.0	8.1	8.0	7.9
9.40	8.3	8.3	8.0	7.9

agents were added to shake-flask cultures after the conidia had germinated, and the doubling times determined (Table 3). The coefficients of variation of the doubling times were 6% in medium M3 and 7% in medium M2. The pH value decreased from 6.5 to 3.5 in medium M3 and from pH 3.5 to 1.8 in medium M2 over 48 hr. The exponential growth phase in both media generally came to an end about 55 hr after inoculation.

The growth rates were only slightly decreased by increasing the molarity of the chelating agent up to nine times the summed molarity of Mg + Fe + Cu + Zn. The addition of the chelating agents did not affect the maximum mycelial dry weight attained.

Addition of ferrocyanide to the medium gave rise to a blue colour which may be attributed to formation of Prussian blue, a ferric ferrocyanide complex. The mycelial growth rates obtained when potassium ferrocyanide was added after the conidia had germinated are given in Table 4. The pH value of the medium had a marked effect on the action of ferrocyanide: at pH 6.5 the growth rate was hardly affected by ferrocyanide to 9.4 mM, whereas, at pH 3.5, 84 μ M ferrocyanide appreciably decreased the growth rate.

Table 4. *Effect of ferrocyanide on the mycelial doubling time of Aspergillus niger strain 72-4*

Ferrocyanide (mM)	Doubling time (hr)
Initial pH 6.5; medium M3	
0.00	6.9*
1.04	7.2
3.13	7.6
9.40	7.2
Initial pH 3.5; medium M2	
0.00	7.7†
84 μ M	10.0

* Standard deviation 0.052 hr.

† Standard deviation 0.071 hr.

Effect of chelating agents and ferrocyanide on germination of conidia

Some of the chelating agents tested markedly inhibited the germination of conidia. The effects of the agents on germination were determined by adding them to the medium before inoculation with conidia. Inhibition of germination showed as an increase in the lag before the exponential increase of mycelial weight occurred (Fig. 2). The lag increased progressively with increase in concentration of EDTA. When EDTA at 9.4 mM was present before the conidia germinated it had a more marked inhibitory effect on mycelial growth than when added after germination. At the lower concentrations, however, the only effect of EDTA was to inhibit germination and the subsequent mycelial growth rate was unaffected. Table 5 shows the effects of CDTA, DTPA, EDTA and NTA on the germination period and subsequent growth rate of *Aspergillus niger* strain 72-4; similar results were obtained with the Kew strain. The most powerful inhibitor of germination was CDTA; at

3.13 mM and pH 6.5 it completely inhibited germination. In contrast, NTA was without effect on germination up to 9.4 mM at pH 8.5 and 6.5. The effect of decreasing the pH value of the medium from 6.5 to 3.5 was to alleviate the inhibition (except with NTA). Since decreasing the pH value would decrease the degree of metal chelation the pH effect indicates that the inhibition was due to metal chelation.

Fig. 1. Mycelial growth curve for *Aspergillus niger* strain 72-4 in medium 3 in shake-flasks.

Fig. 2. Increase in the lag (germination period) of *Aspergillus niger* strain 72-4 brought about by EDTA in medium M3 in shake-flask cultures. A, No EDTA; B, 1.04 mM-EDTA; C, 3.13 mM-EDTA; D, 9.4 mM-EDTA.

Table 5. *Effect of chelating agents on germination period and subsequent mycelial doubling time of Aspergillus niger strain 72-4*

Chelating agent concn. (mM)	CDTA		DTPA		EDTA		NTA	
	Increase in germination period (hr)	Doubling time (hr)	Increase in germination period (hr)	Doubling time (hr)	Increase in germination period (hr)	Doubling time (hr)	Increase in germination period (hr)	Doubling time (hr)
Initial pH 6.5; medium M3								
0.00	0	6.9	0	6.9	0	6.9	0	6.9
1.04	35	8.5	18	7.5	18	7.2	0	7.5
3.13	No germination	{ —	21	7.2	27	7.2	0	7.2
9.40			40	9.3	38	11.4	0	7.5
Initial pH 3.5; medium M2								
0.00	0	7.7	0	7.7	0	7.7	0	7.7
1.04	24	9.2	5	8.4	7.5	9.2	0	7.6
3.13	31	13.0	12	8.5	21.0	9.2	0	8.0
9.40	No germination	—	23	8.7	33.0	16.2	0	8.4

Standard deviations of doubling time in control media M3 and M2 were 0.052 and 0.071 hr, respectively.

Ferrocyanide up to 9.4 mM was without effect on germination in medium M3 at pH 6.5; but in medium M2 at pH 3.5 it was a powerful inhibitor of germination; concentrations above 28 μ M prevented germination.

Effect of increasing EDTA and metal concentrations in parallel

An experiment was done to see whether inhibition of germination and of the subsequent mycelial growth by EDTA could be antagonized by adding more metal ions. The EDTA concentration was increased up to 9.4 mM and extra amounts of the Mg, Fe, Cu and Zn sources were added to keep the EDTA:metal ratio constant. The lag period brought about by inhibition of germination, and the mycelial growth rates in the medium after inoculation with conidia are given in Table 6. These results show that the inhibition of germination and of mycelial growth was not antagonized by adding extra Mg, Fe, Cu and Zn salts. The failure to prevent the inhibition may be attributed to chelation of some trace metal other than one of those added, or to some effect of the chelating agent other than to deprive the organism of metal ions.

Table 6. *Effect of increasing concentrations of EDTA and of metal ions (Mg + Fe + Cu + Zn) on the lag (germination) period and growth rate of strains of Aspergillus niger*

EDTA concn. (mM)	Metal concentration relative to concentration in medium M3	Strain 72-4		Kew strain	
		Increase in lag period (hr)	Doubling time (hr)	Increase in lag period (hr)	Doubling time (hr)
0.00	1	0	6.9	0	6.3
1.04	1	19	7.3	21.0	8.2
3.13	3	26	8.0	24.0	8.6
9.40	9	37	12.1	39.0	17.3

Effects on mould morphology. Ferrocyanide and EDTA, CDTA and DTPA affected the morphology of the mould growth in shake-flask cultures. In medium M2 without complexing agents the mould grew as a viscous conglomerate of pellets (average diameter 2 mm.) with a filamentous periphery. The filamentous exteriors of the pellets interlocked to form a gelatinous conglomerate. In the presence of 1.04 mM-EDTA, CDTA, DTPA or 84 μ M-ferrocyanide, the mould formed smaller pellets (average diameter, 1.3 mm. with EDTA; 0.7 mm. with ferrocyanide) with smooth exteriors. Such pellets remained dispersed in the medium as granules. In contrast, addition of NTA did not affect the mould morphology.

The highly viscous conglomerate was obviously much less readily agitated and aerated than the smooth pellet suspension. Considerable significance is attached to this morphological alteration in the citric acid fermentation, the smaller smooth pellets being considered desirable (Clark, 1962). It was also observed that citrate added as a pH buffer in medium M3 caused the formation of smooth separate pellets, like those obtained with EDTA, except that the citrate pellets were slightly larger. Addition of EDTA, CDTA, DTPA or ferrocyanide to medium M3 decreased the pellet size to that usually obtained with the complexing agent.

The effect of EDTA, CDTA and DTPA on morphology was modified when a

conidial rather than a mycelial inoculum was used. When a conidial inoculum was used, smooth pellets were obtained only when the concentration of chelating agent was 1.04 mM, not at 3.13 or 9.40 mM.

Stirred fermenter culture. The mould was grown in medium M2 in the stirred fermenter with excess of available oxygen and with growth limited by the oxygen supply. Under both these conditions the organism grew in the form of a uniformly filamentous and highly viscous mass; it did not form pellets, as was the case in shake-flasks.

The growth rate was decreased in the stirred fermenter, the doubling time in medium M2 being 13 hr as compared with 7.7 hr in shake-flasks with the same medium. Addition of ferrocyanide (126 μ M) slightly decreased the growth rate but did not affect the morphology of the mould growth. It is concluded that vigorous stirring can counteract the tendency of the organism to grow in the pellet form.

DISCUSSION

Exponential growth of filamentous moulds in mass culture is now becoming generally recognized; it has been previously reported for *Neurospora* (Zalokar, 1959), *Penicillium* (Pirt & Callow, 1960) and *Gibberella* (Borrow *et al.* 1964). *Aspergillus niger* is now shown to be another mould which will grow exponentially under mass-culture conditions. The achievement of exponential growth greatly facilitates growth studies in that the mycelium doubling time can be used for the quantitative comparison of growth rates.

The effects of the metal-complexing agents on growth of two *Aspergillus niger* strains have been analysed in terms of growth rate, germination period and morphological changes. The nature of the effects on growth produced by the complexing agents were qualitatively similar but differed quantitatively, and were also considerably dependent on the pH value of the medium. EDTA, CDTA and DTPA at concentrations up to nine times the summed molarity of the complexed metals (Mg, Fe, Cu, Zn) had little effect on growth rate when added after germination, either at pH 6.5 or pH 3.5. Ferrocyanide up to 9.4 mM had no effect on the growth rate at pH 6.5, but at pH 3.5 it strongly inhibited growth. The conidia were much more sensitive than was the mycelium to the complexing agents. EDTA, CDTA and DTPA at low concentrations inhibited germination at pH 6.5 and 3.5; ferrocyanide at comparable concentrations did not affect the germination rate at pH 6.5, but, at pH 3.5, strongly inhibited germination at a concentration about equal to that of the Fe ions. Inhibition of the germination of conidia by *Aspergillus niger* by ferrocyanide at low pH has been previously observed by Martin (1955).

The form of the mass of growth in shake-flask cultures was also highly sensitive to the metal-complexing agents: EDTA, CDTA, DTPA and ferrocyanide induced the formation of small smooth granular easily dispersed pellets of mycelium, in contrast to the viscous conglomerate of larger pellets with filamentous peripheries formed in the absence of metal-complexing agents. On a molecular concentration basis, ferrocyanide was most effective in producing this change of form of growth.

The effects of the agents may be attributed to their metal-chelating power. This conclusion is supported by the following evidence. (1) The only known action of physiological importance which the agents have in common is to complex with

metal ions. (2) The antagonism of the effect of EDTA, CDTA or DTPA on conidial germination caused by decreasing the pH value of the medium can be accounted for by the decreased degree of metal chelation at the lower pH values. The lack of physiological action by NTA may be attributed to two possible mechanisms. One is that it has the lowest complexing power of the agents tested. The other is that it is trivalent and therefore will form a neutral complex with the Fe^{3+} ion. Such a neutral complex would be expected to be lipid soluble and therefore able to diffuse through the plasma membrane into the cell and thus make the iron available. This point suggests that the iron concentration may be largely responsible for the effects observed with the agents. Other evidence in favour of the iron concentration being critical is that the chelating agents and ferrocyanide complex iron before Cu, Zn or Mg, and that the effective concentration of ferrocyanide was about the same as the concentration of added Fe ($23.3 \mu\text{M}$).

The anomalous results in vigorously stirred cultures remain unexplained. Here the high shearing effect, and the presence of stainless steel parts in the vessel which possibly contributed traces of metal, especially at the low pH value, are new factors to be taken into account.

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