The Influence of Metal-complexing Agents on Citric Acid Production by *Aspergillus niger*

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(Received 17 September 1965)

SUMMARY

Addition of any of the chelating agents ethylenediaminetetra-acetic acid, diamino-cyclohexane-*N,N*-tetra-acetic acid or diethylenetriamine-penta-acetic acid at about 1.0 mM to a sugar + NH_4NO_3 + salts medium stimulated citric acid production by *Aspergillus niger*. The greatest stimulation (about tenfold) was obtained with EDTA (1.0 mM). In contrast, potassium ferrocyanide at 84 μM stimulated citric acid production about 20-fold. Ferrocyanide was effective only when present at the beginning of the mycelial growth period, whereas the chelating agents were effective at any time during the growth period. Stimulation of citric acid production by ferrocyanide could be repressed by transferring the mycelium to fresh medium without ferrocyanide, or by removal of the ferrocyanide precipitate in the medium. Analyses of the iron, copper and zinc contents of the mould mycelium are given. Ferrocyanide did not affect the copper and zinc contents of the mycelium: EDTA did not affect the iron, copper and zinc contents of the mycelium.

INTRODUCTION

Aspergillus niger produces large amounts of citric acid from sugars under certain conditions and this organism is used for the industrial production of citric acid. Crucial conditions in the fermentation are: the strain of the organism, the metal ion nutrition, and the physical environment of the mould. Perlman & Sih (1960) reviewed work on this process. The cultivation process is empirical and its mechanism not understood. One of the unexplained effects is the increase in citric acid yield caused by addition of a small amount of ferrocyanide to the medium. We chose the citric acid fermentation for a study of the effects of metal-chelating agents (as metal buffers) in culture media and have observed that certain chelating agents, like ferrocyanide, increase the yield of citric acid. The present paper reports these effects and discusses their significance. In a previous paper (Choudhary & Pirt, 1965*a*) the effects of metal buffers on the growth of *Aspergillus niger* were discussed.

METHODS

General. Details of media and cultural methods were described previously (Choudhary & Pirt, 1965*a*). The general procedure for the production of citric acid with *Aspergillus niger* was as follows: 25 ml. of medium M2 (carbon source, glucose at 150 g./l.) was placed in a 250 ml. conical flask, inoculated with conidia and incubated at 25° for 7 days on a rotary shaker. Potassium ferrocyanide, when

required, was added 24 hr after inoculation unless otherwise stated; other chelating agents were added before inoculation unless otherwise stated. In addition to the two strains of *A. niger*, Wis 72-4 and IMI-31821 (i), previously described (Choudhary & Pirt, 1965*a*) we also used the strain N-548 of Noguchi & Johnson (1961), supplied by Professor M. J. Johnson (Biochemistry Dept., University of Wisconsin). Media were sterilized by autoclaving; potassium ferrocyanide solution was sterilized by membrane filtration. The medium was initially at pH 3.5 and finally at about pH 2.

Wet ashing for trace metal analysis. Trace metals in mycelium and culture filtrate were determined after wet ashing. Cultures were filtered through filter paper in Buchner funnels. After thorough washing with de-ionized water the mycelium was transferred to a watch-glass using a glass rod, and dried overnight at 105°. Mycelium (0.8–1.0 g.) was placed in a 300 ml. Kjeldahl flask, 5 ml. HNO₃ and 1 ml. H₂SO₄ added and the flask heated gently so that the solution just started to boil. A further addition of 5 ml. HNO₃ was made as the solution began to char and when the oxidation had practically ceased, 1 ml. perchloric acid was added and the flask heated slightly more vigorously until white fumes began to form. Heating was continued until the solution was colourless. Samples of culture medium (10–15 ml.) were transferred to 300 ml. Kjeldahl flasks and evaporated to dryness. Wet ashing was then done by the procedure described above.

All the vessels and apparatus brought in contact with trace metal solutions for analysis were cleaned by standing for 2–3 hr in a boiling sulphuric acid + sodium nitrate mixture. The glass apparatus was then washed three or four times with glass-distilled water.

Estimations of Fe³⁺, Zn²⁺, Cu²⁺ and Mn²⁺. Iron was determined by means of the α, α' -dipyridyl method of Jackson (1938). The digest was diluted with water to about 10 ml. and neutralized by dropwise addition of approximately 8 ml. concn. ammonium hydroxide (bromophenol blue as internal indicator). The solution was then diluted to 100 ml. and a sample taken for the analysis.

To determine copper the Zincon method of McCall, Davis & Stearns (1958) was first tried but the reported colour was not developed. Instead, the diethyldithiocarbonate method of Eden & Green (1940) as modified by Arman (1957) was used. A sample of the neutralized digest was used for the analysis.

Zinc was determined by the dithizone 'mixed colour' method described by Sandell (1944). A sample of the neutralized digest was used for the analysis.

Manganese was estimated by the permanganate method of Sandell (1944), the un-neutralized digest of the mycelium or culture filtrate being used for analysis.

Other analyses. The CO₂ and O₂ in the effluent gas from the continuous-flow culture were analysed by the methods described by Pirt & Callow (1958). Samples of the culture filtrate were used for assaying citric acid and reducing sugar content. Citric acid was estimated colorimetrically by the method of Marier & Boulet (1958). The sample range was 20–200 μ g. citric acid/ml.; the estimation was unaffected by glucose up to 2.0 mg./ml.

Reducing sugars (glucose and hydrolysed sucrose) were estimated by a modification of the ferricyanide method of Fujita & Iwatake (1931). The estimation was unaffected by the citric acid present in the sample. Sucrose was hydrolysed by the method of Pirt & Callow (1958).

Yield of citric acid (%) is expressed as: (g. citric acid produced/g. sugar utilized) $\times$ 100.

Chelating agents were obtained from Hopkin and Williams Ltd., Chadwell Heath Essex, England.

RESULTS

Action of chelating agents on citric acid production

Effect of concentration of chelating agent. The effect of chelating agents on citric acid production by *Aspergillus niger* was examined by growing the mould in medium M2 in shake-flasks with the addition of one of the following chelating agents: ethylenediaminetetra-acetic acid (EDTA), 1,2-diaminocyclohexane-*N,N*-tetra-acetic acid (CTDA), diethylenetriaminepenta-acetic acid (DTPA), nitrilotriacetic

Fig. 1

Fig. 2

Fig. 1. The effect of EDTA (0–9.4 mm; added at the time of inoculation) on citric acid production from glucose after 7 days by strain 72-4. Citric acid concn. ○; % citric acid yield, ●; mycelial dry-wt. concn., △.

Fig. 2. The effect of CDTA (0–3.13 mm; added at the time of inoculation) on citric acid production by strain 72-4. Citric acid concn., ○; % citric acid yield, ●; mycelial dry-wt. concn., △.

acid (NTA) and dihydroxyethylglycine (DHEG). The effects of EDTA, CDTA and DTPA at different concentrations are shown in Figs. 1, 2 and 3. Each of these chelating agents stimulated citric acid production, the maximum stimulation being reached with the chelating agent in the range 1–3 mm. Similar results were obtained with strain N-548. The results with strain IM-31821(i) were qualitatively similar but the concentration and percentage yield of citric acid were only about one-third

of those obtained with the other two strains. The agents NTA and DHEG at concentrations up to 9.4 mM had no effect on citric acid production.

Effect of time of addition of chelating agent. The effect of adding EDTA or DTPA to shake-flask cultures at different times after inoculation with conidia was investigated. The effect of time of addition of 1.04 mM-EDTA with strain 72-4 is illustrated in Fig. 4. The results show that the chelating agents stimulated the production of citric acid when added at any time up to 72 hr after inoculation but maximum stimulation occurred with the addition between 0 and 24 hr. In a control medium (without added chelating agent) strain 72-4 produced 3.0 ± 0.5 g./l. of citric acid after 7 days with a 3% yield and the mycelial dry wt. was 22.0 ± 1.5 g./l.

Fig. 3

Fig. 4

Fig. 3. The effect of DTPA (0–9.4 mM; added at the time of inoculation) on citric acid production by strain 72-4. Citric acid concn., ○; % citric acid yield, ●; mycelial dry-wt. concn., △.

Fig. 4. The effect of EDTA (1.04 mM), added at various times after inoculation, on citric acid production by strain 72-4. Citric acid concn., ○; % citric acid yield, ●; mycelial dry-wt. concn., △.

Effect of increasing both ferric ion and EDTA concentrations. An experiment was made to see whether the stimulation of citric acid production was controlled by the Fe^{3+} :EDTA ratio. In a preliminary experiment it was found that maximum citric acid production occurred when the Fe^{3+} :EDTA molar ratio was about 1:30. In a second experiment the Fe^{3+} content of the medium was increased and the EDTA concn. was also increased to keep the Fe^{3+} :EDTA ratio constant at either of 3 values; 1:12.5, 1:25 or 1:37.5. The results are shown in Table 1. The iron present as a contaminant in the medium constituents was determined colorimetrically and allowed for in calculating the Fe^{3+} :EDTA ratio. The results show that the mycelial dry wt. was unaffected. At each Fe^{3+} :EDTA ratio, an increase in the Fe^{3+} concn. decreased the citric acid production. Therefore the Fe^{3+} :EDTA ratio alone did not control the citric acid production; some other factor must also have been involved. However, with the lowest Fe^{3+} :EDTA ratio there was some suppression of the inhibition caused by increased Fe^{3+} concn.

Table 1. Effect of varying Fe^{3+} concentration and Fe^{3+} :EDTA ratio on citric acid production by strain 72-4

Fe ³⁺ concn. (μ M)*	Fe ³⁺ :EDTA molar ratio								
	1:12.5			1:25			1:37.5		
	Mycelial dry wt. (g./l.)	Citric acid (g./l.)	% citric acid yield	Mycelial dry wt. (g./l.)	Citric acid (g./l.)	% citric acid yield	Mycelial dry wt. (g./l.)	Citric acid (g./l.)	% citric acid yield
42	17.2	15.2	15.2	17.5	25.0	26.0	16.5	23.0	23.0
96	18.0	5.5	5.5	15.0	7.0	8.1	18.3	9.0	11.3
266	17.0	1.1	2.3	17.0	1.0	1.1	18.0	4.5	5.8
518	16.5	3.5	4.0	18.3	2.5	2.5	18.6	6.5	11.5

* Added iron + iron contamination (14 μ M) in medium constituents.

Fig. 5

Fig. 6

Fig. 5. The effect of ferrocyanide concn. on the synthesis of citric acid from glucose by strain 72-4. The ferrocyanide was added 24 hr after inoculation with conidia and the culture duration was 7 days. Citric acid concn., $\circ$; % citric acid yield, $\bullet$; mycelial dry-wt. concn., Δ .

Fig. 6. The effect of time of addition of ferrocyanide (84 μ M) on the production of citric acid from glucose by strain 72-4. Citric acid concn., $\circ$; % citric acid yield, $\bullet$; mycelial dry-wt. concn., Δ .

Action of ferrocyanide on citric acid synthesis

Effect of ferrocyanide concentration. Potassium ferrocyanide was added to the media in shake-flask experiments to characterize the ferrocyanide action and to compare it with that of the chelating agents. The influence of ferrocyanide concn. is shown in Fig. 5. The maximum concentration and percentage yield of citric acid were reached with 84 μ M ferrocyanide. The decrease in citric acid production at higher ferrocyanide concentration may be attributed to the reduced amount of mycelium. Strain N-548 showed a similar response to ferrocyanide but produced less mycelium and consequently gave a lower citric acid yield (44 g./l.). When sucrose was used as the carbon source, even larger amounts of citric acid (80 g./l.) were produced by strain 72-4 in 7 days with a 65% yield. Apparently sucrose was converted more quickly than glucose into citric acid.

Effect of time of addition of ferrocyanide. The effect of adding the optimum amount

of ferrocyanide ($84\ \mu\text{M}$) to the culture at different times is shown in Fig. 6. At $84\ \mu\text{M}$ and pH 8.5, ferrocyanide inhibited the germination of conidia and hence could not be added initially. The most notable point is that the ferrocyanide unlike the chelating agents was without effect when added at 48 hr or later. Moreover, the mould grew in the small pellet form (Choudhary & Pirt, 1965*a*) only when the ferrocyanide was added at 24 hr, i.e. at the beginning of the mycelial growth period. It may be postulated that growth of the mould in the presence of ferrocyanide

Table 2. *Citric acid production by strain 72-4 in nitrogen-free medium M2 with and without ferrocyanide*

Nitrogen source	Ferro-cyanide concn. (μM)	Inoculum	Time of sample (days)	Mean mycelial dry wt. (g./l.)	Mean citric acid amount		Replicates
					(g./l.)	% yield	
Absent	0	48-hr mycelium	3	9.5	3.3	7.0	2
			5	12.5	3.8	11.5	
			7	10.0	4.0	12.0	
Absent	84	48-hr mycelium	3	9.5	5.0	16.0	3
			5	9.2	8.0	21.0	
			7	9.6	8.5	20.0	
Absent	0	72-hr mycelium	2	9.5	5.0	16.0	3
			4	13.5	7.5	15.0	
			6	13.6	8.0	16.0	
Absent	84	72-hr mycelium	2	10.0	10.0	30.0	3
			4	12.0	14.0	30.0	
			6	14.0	15.0	29.0	
Present	0	Conidia	5	17.0	2.5	4.8	2
			7	20.8	3.0	3.3	
Present	84	Conidia	5	—	27.5	32.0	2
			7	20.0	60.0	50.0	

induces a mechanism for acid accumulation which is unaffected by subsequent removal of ferrocyanide. This hypothesis was tested in experiments with washed mycelium grown in the presence of ferrocyanide. Mould mycelium was grown in the presence of $84\ \mu\text{M}$ -ferrocyanide for 48 and 72 hr; the mycelium was then filtered off in a chamber of sterile air (Harris-Smith, Pirt & Firman, 1963) and thoroughly washed with sterile distilled water. The washed mycelium was then aseptically transferred to 30 ml. of medium M2, with or without ferrocyanide ($84\ \mu\text{M}$), and without NH_4NO_3 to prevent further growth. The flasks were then incubated on the shaker. As controls, unfiltered cultures were run in parallel, with and without ferrocyanide ($84\ \mu\text{M}$). At intervals 4 ml. samples of culture were taken by pipette and analysed for citric acid, glucose and mycelial dry wt. The results (Table 2) show that for maximum citric acid production the nitrogen-free replacement medium needed to have ferrocyanide added. The omission of ferrocyanide from the replacement medium decreased the production of citric acid by about 50 %. Some of the citric acid producing ability induced by the preliminary growth in ferrocyanide was retained. This result is interpreted to mean that the medium untreated with ferrocyanide contains some material which can reverse the change which ferrocyanide induces in the mycelium.

Influence of ferrocyanide precipitate on citric acid production. One hypothesis to

account for the role of ferrocyanide is that it makes the medium suitable for citric acid production by precipitating iron or other cations, thus causing the mycelium to be deficient in iron. It may therefore be expected that if ferrocyanide is added to the medium and the precipitate removed, this treated medium should give maximum citric acid production when inoculated. This hypothesis was tested in the following way. Conidia were germinated in medium M2 and the resultant mycelium after incubation for 26 hr was thoroughly washed with de-ionized water in sintered-glass crucibles placed in the cabinet with a sterile atmosphere. Part of the washed mycelium was used to inoculate medium M2 to which 84 μM -ferrocyanide had been added 24 hr previously. Another part of the washed mycelium was used to inoculate medium M2 after removing the ferrocyanide precipitate by membrane filtration 24 hr after the addition of ferrocyanide at 84 μM . The mould in the replacement

Table 3. *Effect of removal of ferrocyanide precipitate from medium M2 on the production of citric acid by strain 72-4*

Ferro- cyanide concn. (μM)	Inoculum	Medium treatment	Time of sample (days)	Mean dry wt. (g./l.)	Citric acid		Repli- cates
					Mean concn. (g./l.)	% yield	
0	Conidia	None	7	22.5	3.0	3.0	2
84	Conidia	None	7	17.5	58.0	50.0	2
0	Washed myce- lium 26 hr	None	6	23.0	2.0	2.0	2
84	Washed myce- lium 26 hr	Ferrocyanide added 24 hr before inocu- lation	6	20.0	23.0	22.0	4
84	Washed myce- lium 26 hr	Ferrocyanide added, ppt. removed after 24 hr and then inocu- lated	6	20.0	2.2	2.0	4

medium with ferrocyanide precipitate developed in the form of large filamentous but separate pellets. In the same medium with ferrocyanide precipitate removed the mould developed as a viscous conglomerate of large filamentous pellets. The effects on citric acid production and mycelial dry weight are shown in Table 3. The removal of the ferrocyanide precipitate from the replacement medium prevented stimulation of citric acid production. Some decrease of stimulation of citric acid formation resulted just from the transfer of the mould to the replacement medium. The important point established is that the presence of the ferrocyanide precipitate in the medium was essential for the stimulation of citric acid production. The function of the ferrocyanide precipitate may be to maintain a low concentration of Fe^{3+} or other metal ions in the medium.

Effects of metal-complexing agents on the trace-metal uptake by the mould

Experiments were made to determine whether or not there was a relation between the uptake of trace metals by the mycelium and citric acid production. Iron, copper and zinc in the mycelium and medium were estimated. The concentration of man-

ganese in the mycelium (1–2 g.) and in the filtrate (10–15 ml.) was below the amount (1 μ g.) which could be estimated. Iron was not estimated in the cultures containing ferrocyanide.

Trace-metal analyses were done on mycelium and culture filtrates from cultures grown with and without EDTA, and with and without ferrocyanide. Citric acid production and the mycelial dry weights were determined, to correlate them with the trace-metal analyses. The results are given in Table 4. The excess of trace metal over that added represents the amount of metal contamination in the medium. The results show that EDTA did not significantly affect the uptake of Fe^{3+} , Cu^{2+} and Zn^{2+} by the mycelium. Also the ferrocyanide did not affect the Cu^{2+} and Zn^{2+} uptakes by the mycelium. Thus no relation was found between the trace-metal uptake by the mycelium and the citric acid production.

Table 4. *Trace-metal analyses of mycelium and medium from 7-day cultures of strain 72-4 in medium M2 with and without metal-complexing agents**

Chelating agent (concn.)	Mycelial dry wt. (g./l.)	Citric acid		Replicates	Trace metals present†					
		Concn. (g./l.)	% yield		Mycelium (μ g./g. dry wt.)			Culture filtrate (μ g./l.)		
					Fe ³⁺	Cu ²⁺	Zn ²⁺	Fe ³⁺	Cu ²⁺	Zn ²⁺
None	22.4	3.0	3.0	5	46	5	19	1100	161	297
EDTA (1.04 mm)	20.8	25.0	26.0	6	48	6	21	1084	139	320
None	22.5	3.0	3.0	5	46	5	22	1100	161	350
Ferrocyanide (84 μ M)	20.0	59.0	51.0	4	NA‡	5	21	NA‡	171	280

* When present, EDTA and ferrocyanide were added before or 24 hr after inoculation respectively.

† In each case the initial concentration of trace metals in the medium plus inoculum of conidia was (μ g./l.): Fe^{3+} , 2120; Cu^{2+} , 242; Zn^{2+} , 676.

‡ No analysis.

The effect on citric acid production of omitting single trace metals from the medium, both with and without ferrocyanide, was examined (Table 5). Omission of iron did not affect the mycelial dry wt. but it caused the iron content of the mycelium to be markedly decreased. In the absence of added iron, ferrocyanide only slightly stimulated citric acid production. Also in the absence of added iron, ferrocyanide did not modify the mould morphology to the small discrete pellet form; the growth remained as in the control. It may be concluded that the minimum iron supply for maximum citric acid production is greater than the minimum iron supply for mycelium production. Copper was taken up by the mycelium to a much smaller extent than iron or zinc. The copper present as contaminant in the medium (182 μ g./l.) was well above the maximum amount taken up by the mould, hence it is not surprising that omission of copper had no effect. Omission of zinc caused limitation of the mould growth by the zinc supply and greatly decreased the zinc content of the mycelium. Ferrocyanide did not stimulate citric acid production in zinc-starved mycelium. The morphology of the mycelium was in the form of large fimbriated pellets without zinc and ferrocyanide added. The presence of ferro-

Table 5. Effect of omitting single trace metals from medium M2 on citric acid production by 7-day cultures of strain 72-4 both with and without ferrocyanide

Ferro- cyanide concn. (μ M)	Metal omitted	Mean mycelial dry wt. (g./l.)	Citric acid		Trace metals present							
					Medium with conidia inoculum (μ g./l.)				Mycelium (μ g./g. dry wt.)			
			Mean concn. (g./l.)	% yield	Fe ³⁺	Cu ²⁺	Zn ²⁺		Fe ³⁺	Cu ²⁺	Zn ²⁺	Culture filtrate (μ g./l.)
0	None	20.0	3.0	3.0	1810	280	610		42	5	25	1000
84	None	19.0	60.0	56.0	NA	NA	NA		NA	7	23	NA
0	Fe ³⁺	21.6	2.5	3.0	428	229	610		14	8	27	196
84	Fe ³⁺	21.0	6.0	7.0	NA	NA	NA		NA	6	28	NA
0	Cu ²⁺	22.5	2.0	2.0	1920	182	608		47	4	20	1100
84	Cu ²⁺	23.0	64.0	50.0	NA	NA	NA		NA	5	26	NA
0	Zn ²⁺	15.0	2.0	2.1	1971	250	270		51	4	14	1000
84	Zn ²⁺	10.0	2.0	4.0	NA	NA	NA		NA	3	12	NA

NA = no analysis.

cyanide without added zinc produced the typical small discrete pellets usually obtained with added ferrocyanide. This result shows that growth in the small pellet form was not by itself a sufficient condition for maximum citric acid production.

DISCUSSION

The mechanism whereby *Aspergillus niger* produces citric acid in large amount under certain conditions is a problem of great interest. Citric acid is presumably a normal intermediate in the terminal respiratory metabolism of the mould and its massive excretion is apparently caused by a derangement of this metabolism. The conditions which favour the production of citric acid are: (1) special strains of the mould, (2) high oxygen supply, (3) a low pH value, about 2, (4) a special trace-metal balance. The latter condition is deduced largely by a consensus of opinion rather than from precise knowledge of the trace-metal balance. The stimulation of citric acid production by ferrocyanide may be interpreted as due to a favourable modification of the trace-metal balance. The stimulation by metal-chelating agents such as EDTA first reported by Choudhary & Pirt (1965*b*) similarly seems to be due to modification of the trace-metal balance. The observation that the $\text{Fe}^{3+}:\text{EDTA}$ ratio should be kept low and that ferrocyanide does not stimulate unless iron is present in excess of that required for growth suggests that there should be an unlimited supply of iron but supplied at a critical concentration which is controlled by ferrocyanide or chelating agent. The stimulations by ferrocyanide and chelating agents differ markedly in the effect of the time of addition. Chelating agents can stimulate when added to the culture at any time up to 72 hr from time of inoculation or possibly later; on the other hand ferrocyanide stimulated only when added before 48 hr, that is, early in the growth period. This limitation of the ferrocyanide effect is evidence against the suggestion put forward by Martin (1955) that ferrocyanide has a direct action on the mould in stimulating citric acid production. The effect of the chelating agent at a later period, when most of the mycelium is formed, may be attributed to permeation of the lipid cell membrane by the undissociated acid and the binding of trace metals which have been taken up by the cell. It is assumed that ferrocyanide cannot do this. The lack of stimulation of citric acid production by the chelating agents NTA and DHEG may be attributed to the same properties which, as Choudhary & Pirt (1965*a*) suggested, prevent their having any action on mould morphology and on conidial germination.

Under the optimum conditions for citric acid production the mould always grew in the form of small discrete pellets like a suspension of sand particles. Perhaps this type of pellet formation should be included as the fifth special condition for citric acid production; it is, however, difficult to believe that this is anything other than a correlated effect caused by the same conditions which stimulate citric acid production.

The trace-metal analyses of mycelia indicate that the agents which stimulate citric acid production do not affect the total uptake of Fe^{3+} , Cu^{2+} and Zn^{2+} by the mould. The analytical method for Mn^{2+} was not sensitive enough to detect this element in the medium or mycelium. It would be interesting to do so in view of the observation of Noguchi & Johnson (1961) that Mn^{2+} may strongly inhibit citric acid production by *Aspergillus niger*; also the observation of Clark, Ito & Tymchuk

(1965) that ferrocyanide is particularly effective in removing Fe^{3+} and Mn^{2+} from the medium.

We also grew the mould in a stirred fermenter and in continuous-flow culture, but in these forms of culture citric acid was not produced in large amount and its production was not stimulated by ferrocyanide or chelating agents. In the stirred fermenter the mould grew in the viscous filamentous form. It thus appears that some other controlling factor remains to be discovered, or the agitation has to be of a type which permits formation of the right type of pellet morphology.

An equipment grant from the Science Research Council (D.S.I.R.) is gratefully acknowledged.

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