Spherical Cell Formation in *Aspergillus oryzae* with Special Reference to Heavy Metal Impurities in Culture Solution.

By

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In the writer's previous paper (1933), a joint work with Prof. SAKAMURA, the following facts were reported on the formation of the spherical cell in *Aspergillus oryzae* and *Asp. niger*: The spherical cell formation is mainly due to the presence of heavy metal compounds which are contained as impurities in the chemicals used for the preparation of the culture solution. If the coal treatment of the culture solution, for the purpose of the removal of impurities, is previously carried out, it does not occur. However, it can be again produced, if Cu-, Zn-, Cd. or Ni-salt is anew added to thus treated solution, while Fe- and Mn-salts act inhibitting its formation.

The present investigation was attempted in order to confirm our conclusion mentioned above, based on more perfect method, especially in relation with the removal of impurities.

Non-alkaline glasswares used for culture were cleaned by rinsing with soap, chrom-sulphuric acid, tap water, distilled water and lastly with redistilled water in order. As culture vessel 100 cc. ERLENMEYER flasks or test tubes were used. The inoculation was carried out with conidial suspension which was secured from 10–15 days koji-agar culture. The cultures were incubated in a thermostat at 30°C.

Influence of dry sterilisation of culture vessels.

In the previous work, the glasswares used for culture were plugged with cotton and previously sterilized in a dry oven at 150°–160°C. That such dry sterilisation at high temperature sometimes causes change of the nature of culture solution was reported in some recent papers. Considering that it might not be impossible that the spherical cell formation is also

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influenced by the dry sterilisation, the author firstly in the present investigation carried out an experiment in this relation. The composition of the culture solution was the same as in the forgoing work, namely as follows:

Stock solution

NH_4NO_3	4 g
KH_2PO_4	2 g
$MgSO_4 \cdot 7H_2O$	1 g

dissolved with redistilled water to 100 cc.

Culture solution

Stock solution	12.5 cc.
Glucose solution (m/2)	25.0 cc.
Redistilled water	12.5 cc.

Fe was not added to the culture medium, because it was present as impurity enough for the growth of fungus. One part of culture vessels, 100 cc. ERLENMEYER flasks, was stopped with cotton plugs and dry sterilized at 150°–160°C, while the other part were not so treated. Fifty cc. portions of the culture solution were placed in each ERLENMEYER flask and sterilized for one hour in a KOCH steam sterilizer. The results of the cultures were shown in Table I. The initial pH-value of the culture solutions were all equally 4.2.

Table I.

	Culture duration (day)	Dry sterilisation	pH	Dry weight of mycelium (g)	Conidium	Spherical cell
A	5	done	3.0	0.316	++	−
	5	not done	3.3	0.161	++	−
	7	done	3.0	0.540	++	−
	7	not done	3.1	0.440	++	−
B	5	done	2.9	0.280	±	−
	5	not done	3.4	0.186	+	−
	8	done	2.9	0.405	±	−
	8	not done	2.8	0.320	+	−
C[1)]	8	done	3.4	0.541	±	−
	8	not done	3.4	0.440	+	−
	11	done	3.5	0.697	±	−
	11	not done	3.8	0.435	+	−

1) The culture media which shown in Table I, C were previously treated with MERCK's medical coal and filtered. See coming pages.

From these results it follows that the mycelial growth occurs better in the dry-sterilized flask than in the non-sterilized. The conidial formation was inhibited a little in the former case. As to the spherical cell formation which was the proper object of this experiment, we could not recognize any difference between the two cases. Therefore, it may practically concluded that the dry-sterilisation exerts no influence upon spherical cell formation.

Heavy metal compounds found as impurities and their reference to the mycelial growth and the formation of the spherical cell.

From the results of the writer's experiments with *Aspergillus oryzae*, which will be reported in detail on other occasion, it will be here briefly summarized, that chemical preparation delivered from the different manufacturing factories give different results in respect to the spherical cell formation, appearance of mycelial mass, formation and colour of conidium and pH-change of culture medium. Such difference was noticed especially between MERCK's and KAHLBAUM's preparations in the case of magnesium sulphate. Using MERCK's preparation of magnesium sulphate, the formation of the spherical cell was remarkable, mycelium grew scattered in many small masses or forming a meshwork on the surface of the culture solution, and no conidium was produced. On the other hand, when KAHLBAUM's preparation was used, the spherical cell formation scarcely occurred, mycelium covered the whole surface of the culture solution in the form of mat, and conidium was formed vigorously. According to the origin of the chemicals the colour of conidium of *Aspergillus oryzae* varies in different degrees from yellow to dark green. Further investigation in this direction is now in progress.

Detection of Fe, Cu and Mn impurities in chemical preparations used for culture.

For the Fe-test the ammonium sulphocyanate method after SHIMODA and NAGAI (1932) was used. The available limit of this reaction was estimated 2×10^{-8} mol. For the detection of Cu the KASTLE-MEYER reaction was employed, of which the available limit was 10^{-6} mol. By addition of glucose to 5% this reaction was interfered with and its limit was displaced to 10^{-4} mol. Phosphate exerted no influence on this reaction even in such high concentration as 0.05 mol. Ammonium nitrate hindered this reaction in relatively high concentration. The Mn-test was practiced applying the potassium persulphate method after REIMAN and MINOT (1920), by means

of wihch reaction Mn was detected up to in 2×10^{-5} mol. The test for Zn, which has been proved to be an important element for the culture of fungus in the meaning either of nutrient or of stimulant, was not conducted, because no suitable method or reagents were within reach.

From the results of the test it will be noticed that the occurrence of heavy metal compounds as impurities in chemical is very common, though their amount is very small. This was the case even in some of KAHLBAUM's or MERCK's preparations with the certificate of warranty. Fe- and Cu-impurities, especially in magnesium sulphate of KAHLBAUM as well as MERCK were very noticeable, and in the former Mn also was detected. It is also a very remarkable fact, that ammonium salts of many kinds, which are often used as the N-source for the culture of mould, contain Cu in tolerably noticeable amounts, while it is found relatively little in nitrates. Fe was detected more or less in nitrates ($NaNO_3$ and KNO_3) and phosphates (NaH_2PO_4, KH_2PO_4 and Na_2HPO_4), but its occurrence in HCl should be announced, regarding which some statements will be made below. Glucose of TAKEDA showed weak Cu-reaction, but not Fe-reaction.

Comparison between KAHLBAUM's and MERCK's preparation of magnesium sulphate in respect to the nutritive effect.

As stated above, KAHLBAUM's preparation of magnesium sulphate differs from that of MERCK in the presence of Mn impurity. Therefore it was not improbable to expect that any difference of cultures between these two origins, if such occurs, may be eliminated by the addition of Mn to the culture solution with MERCK's preparation. Two kinds of culture solution were prepared, in one of which magnesium sulphate of KAHLBAUM and in the other MERCK's preparation were used. The composition of the culture solution was the same as that mentioned above. The coal treatment for the purpose of adsorption of impurities was not here considered.

Table II.

$MgSO_4 \cdot 7H_2O$	Added	Culture duration (day)	pH	Dry weight of mycelium (g)	Conidium	Spherical cell
MERCK	—	7	2.8	0.128	−	++
MERCK	$MnSO_4$	7	2.5	0.328	++	−
KAHLBAUM	—	7	2.6	0.345	++	−
MERCK	$Fe_2(SO_4)_3$	7	2.6	0.270	−	++

($MnSO_4$, 10^{-7}mol) ($Fe_2(SO_4)_3$, 10^{-6}mol).

The results of the experiment met our expectation. As a result of the addition of manganese sulphate ($MnSO_4$) to the MERCK culture it was found that mycelial mass covered the surface of the culture medium, conidium was formed vigorously and the spherical cell formation was hindered, all of which phenomena were recognized in the KAHLBAUM culture. Moreover the dry weight of mycelium attained to the same value as in the KAHLBAUM culture.

Although Fe was detected in MERCK's preparation of magnesium sulphate, a further addition of Fe in 10^{-6} mol showed a similar effect to Mn. This is probably due to the similar nature of Fe and Mn, which appears also in the tendency of the inhibition of the spherical cell formation.

Removal of Fe and Cu in culture solution by means of adsorption with coal.

ROBERG (1931) and LOHMANN (1934) tried to remove impurities by treatment of the culture solution with MERCK's medical coal. To thus treated solution Zn, Fe, Cu-salt etc. separately or combined, were added, in order to ascertain the physiological meaning of these heavy metal salts. According to these authors, the adsorption procedure should be practiced only in weak alkaline solution, because in acid reaction, at pH 6.0, the adsorption power of the medical coal is much diminished. Also in the previous paper the coal treatment was done in alkaline reaction, but it is necessary to determine the suitable pH-region for the removal of Fe and Cu by adsorption. For this purpose salts of these metals in proper concentration were dissolved in 0.005 or 0.05 molar phosphate buffer solution[1] of pH-range 2.0–8.3. The adsorption process went on in the vessel of a WARBURG respiration apparatus at a constant temperature of 18°C, under continuous shaking, where the presence of the manometer had certainly nothing to do with it. After 30 minutes the culture solution was filtered and the Fe- and Cu-tests were tried.

(a) Adsorption of Fe

The following mixture was placed in a WARBURG trough:

$Fe_2(SO_4)_3$ (10^{-4} mol)	1 cc.
Phosphate buffer (0.01 or 0.1 mol)	5 cc.
Redistilled water	4 cc.

1) The buffer solutions were prepared by the combination of NaOH and H_3PO_4, in which some Fe was detected, but Cu was not.

Medical coal MERCK 0.2 g[1]

After 30 minutes the mixture was filtered and the Fe-test was applied to the filtrate. The Fe-reaction after the adsorption is shown in the following table:

Table III.

pH	2.0	2.4	2.6	2.8	3.0	3.5	4.0	4.6	5.1	5.5	5.7	6.0	6.2	6.6	7.0	7.3	7.8	8.3
Fe	++	+	±	±	±	±	−	−	−	−	−	−	±	+	++	++	++	++

In the pH-range 4.0–6.0 the adsorption of Fe was most effective, while in the range 2.0–2.6 and 6.6–8.3 it was incomplete. Similar results were secured with KAHLBAUM's magnesium sulphate in which the presence of Fe impurity is very common. In the pH-region 3.0–7.0 the adsorption was perfect, while in the other regions of higher or lower pH-value it was imperfect.

In high acidity the adsorption of Fe not only went on imperfectly, but its dissoilution out of coal must be considered as possible, as really proved by ROBERG (1931) and others. But it was not always the case in the present experiment, provided the pH-value stands higher than 3.6.

The dissolution of Fe should be taken in consideration also in the case of filter paper, especially if it is not of high quality. Toyo filter paper No. 7 was proved to ful-fill our requirement in this respect, because no dissolution of Fe out of it was recognized.

(b) Adsorption of Cu

The applied method was the same as in the case of Fe, and the concentration of copper sulphate was equal to that of ferric sulphate ($Fe_2(SO_4)_3$). The adsorption was perfect in a less acid reaction than pH 2.6, while in pH 2.0–2.6 the Cu-reaction appeared even after coal treatment. It is a noteworthy fact that a part of Cu impurity could be adsorbed also by filter paper during filtration. But this is limited only to the case of filter paper of lower quality, and No. 7 did not show such remarkable adsorption power in respect to Cu.

Within the available limit of the KASTLE-MEYER reaction it was ascertained that the removal of Cu impurity in KAHLBAUM's preparation of magnesium sulphate by coal treatment was perfect in a 1% solution, but it was difficult in a 2.5% or 5% solution. The same difficulty was

1) This amount of medical coal (%) was adopted according to the ROBERG's procedure (1931).

encountered in attempting to make ammonium nitrate and other ammonium salts absolutely free from Cu by this method.

Relation between the adsorption of impurities and temperature or kind of coal.

According to LOHMANN (1934) the adsorption of impurities in culture solution by treatment with MERCK's medical coal is more effective at low temperatures than at high. In the present experiment the adsorption of Fe impurity in the culture solution, containing MERCK's magnesium sulphate was studied comparatively at 10°, 18°, 30°, 50° and 100°C, and in pH 4.2, 5.5 and 7.3 respectively. The result of the experiment showed that adsorption power was very weak at 50° and 100°C, while at lower temperatures perfect removal of Fe was confirmed.

In respect to the adsorption of Cu at different temperatures no conclusive result was reached, because the Cu-test in the culture solution was rendered difficult by the presence of its other components.

The coal treatment mentioned above was conducted with MERCK's medical coal without exception. The same adsorption procedure was tried using coal of other kinds, KAHLBAUM's blood coal and SCHERING-KAHLBAUM's cane sugar coal.

Table IV.

Coal	Presence of Fe in coal[1]	Adsorption of Fe	Adsorption of Cu	Spherical cell
Medical coal (M)	+	good	good	−
Blood coal (K)	+	good	good	−
Sugar coal (S-K)	−	good in low conc.	bad	++
Medical coal washed with pure HCl solution	±	„	bad	++
Sugar coal washed with pure HCl solution	−	„	bad	++

From these results it will be noticed that, for the purpose of the removal of Fe impurity in the culture solution, coal of any kind used were effective. However, in the case of higher concentration of Fe the adsorption power of the cane sugar coal was insufficient.

1) Coal was rinsed with a pure HCl solution. Fe, which comes out in HCl, was tested.

Medical coal and sugar coal, which were washed repeatedly with pure HCl solution and then washed with redistilled water, until no reaction of Cl appeared, lost the adsorption power against Cu. Therefore it is ont true in this case that the more purified, the higher the adsorption power.

Fe impurity in HCl.

The Fe-test in SCHERING-KAHLBAUM's HCl "for forensic purpose" was tried, and it resulted clearly positively. By means of distillation it was impossible to make HCl free from Fe. Consequently it was necessary to prepare pure HCl, free from Fe, by distillation of mixture distilled H_2SO_4 and KCl. Pure HCl thus prepared was kept in a quartz vessel and used for the regulation of pH-value and for the rinsing of coal.

Preparation of culture solution as free as possible from heavy metal impurities.

From the facts mentioned above we may expect to prepare culture solution for fungus free from heavy metal impurities, though no one could speak of their absolute absence. For this purpose the following procedure was conducted: After glasswares were rinsed as indicated, they were cleaned again with steam and with redistilled metal free water, and then dried. The composition of the culture solution was as follows:

NH_4NO_3(M)	4 g
KH_2PO_4(K: SÖRENSEN)	2 g
$MgSO_4 \cdot 7H_2O$ (K, with certificate)	1 g
Glukose (T)	18.01 g

This mixture was dissolved with redistilled water to 400 cc., and the pH-value of this solution was regulated to about 5.5 by addition of 10.5 cc. NaOH(n/10) and then it was diluted to 800 cc. The salts concentration corresponds to half that of the PFEFFER solution. The reason why such diluted solution should be used is that the more diluted the salts solution is, the more perfect is the removal of impurities. To the culture solution 16 g medical coal was added and after constant shaking it was filtered using Toyo filter paper No. 7. The pH-value of the filtrate was 5.6 or 5.7, which was then regulated with a pure HCl solution. Thus prepared culture solution did not show any Fe, Cu, or Mn reaction.

For the culture 100 cc. ERLENMEYER flasks were used, which were not dry sterilized. The flasks, each containing 50 cc. of culture solution, were sterilized for 10 minutes in a KOCH steam sterilizer.

Formation of the spherical cell.

Using the culture solution free from heavy metals, preparation of which is above described, an experiment on the spherical cell formation was conducted in order to confirm our conclusion in the previous paper. In this experiment, however, 4.44 g of glucose was used. After the coal treatment the pH-value of the culture solution was regulated with HCl to 2.0–5.6. The culture solutions were inoculated with conidial suspension of *Aspergillus oryzae 65* and incubated at 30°C. After two days culture the formation of the spherical cell was examined, but in any culture having high acidity it was not found.

The culture containing KNO_3 (K: with certificate), in which impurities are contained in relatively small amount that can be almost perfectly removed by the coal treatment, showed the same result. By addition of any one of $CuSO_4$(10^{-4} or 10^{-5} mol), $CdCl_2$(10^{-5} or $5\times^{-6}$ mol), $NiCl_2$(10^{-3} or 5×10^{-4} mol) or $ZnSO_4$(10^{-3} or 5×10^{-4} mol) these culture solutions gained the power of spherical cell formation, while $Fe_2(SO_4)_3$ (10^{-5} mol) or $MnZO_4$ (10^{-5} mol) acted antagonistic to it.

The spherical cell formation could be regarded as an indication of incomplete removal of the impurities, especially of Cu, by the adsorption, and it really occurred parallel to the Cu-reaction in the treated culture solutions. When the treatment was made with medical coal no spherical cell was formed, with blood coal a few, and with sugar coal pretty many. From these results it would not be impossible to say that the adsorption went on in this order.

The experimental results and conclusion in the writer's previous paper were confirmed by the more careful experiments in the present paper.

Formation and colour of conidium.

In the course of culture of *Aspergillus oryzae* change of formative tendency and colour of conidium was several times recognized. In the koji-agar culture, which showed the Cu-reaction clearly, conidium was produced vigorously and its colour was yellowish green, while in the PFEFFER solution or in the culture solution indicated above, without coal treatment however, conidium was formed almost in yellow colour. If $NaNO_3$ was used instead of NH_4NO_3 the conidium was yellowish green. In contrast with vigorous conidial formation in the culture solution containing KAHLBAUM's magnesium sulphate, it was scarcely recognized when MERCK's preparation was used. The removal of heavy metal impurities

by the coal treatment caused poor growth of mycelium and conidial formation. By addition of either $Fe_2(SO_4)_3$ in 10^{-6} mol or $CuSO_4$ in 2.5×10^{-7} to such a treated culture solution the mycelial growth was stimulated, but the conidial formation did not occur. Addition of Fe and Cu both together caused remarkable mycelial growth and conidial development.

In the case of *Aspergillus niger* many authors recognized that Cu is necessary for the formation of conidium and the development of its proper colour. A similar relation could be seen also in the culture of *Aspergillus oryzae*. In the culture solution, which was treated with medical coal and to which $Fe_2(SO_4)_3$ and $CuSO_4$ each in 10^{-7} mol were added, yellow conidia were formed. Increased amount of $CuSO_4$ to 10^{-6} mol, however, caused the vigorous formation of conidium in yellowish green colour, with which the addition of Fe in excess interferred.

From these results it will be seen that Fe and Cu both together seem to be indispensable for the conidial formation, and Cu in a suitable concentration for the development of the proper yellowish green colour of *Aspergillus oryzae*.

Summary.

1. Fe, Cu and Mn impurities were chemically tested in the chemical preparations, which were used for fungus culture.

2. It was proved that almost all preparations examined, even those with certificate of warranty, contain Fe or Cu impurity sometimes both of them.

3. For the purpose of removing these impurities, medical coal (not washed) was most powerful, and the adsorption procedure was more effective in weak acid solution (pH 5.5) than in weak alkaline.

4. In thus treated culture solution no spherical cell of *Aspergillus oryzae* was formed in any high acidity. By addition of heavy metal salts it was noticed that Cu, Cd, Ni and Zn act positively causing the spherical cell formation, while Fe and Mn inhibit it. The conclusion in this respect in the writer's previous paper was confirmed by these more carefully conducted experiments.

5. The necessity of Fe and Cu for the mycelial growth and the conidial formation of *Aspergillus oryzae* was ascertained.

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