Botany. — *"Conditions influencing the production of colouring matter of Monascus purpureus* WENT". By Prof. SHIN-ICHI HIBINO. (Communiated by Prof. F. A. F. C. WENT).

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Up to the present time, a large number of bacteria and fungi which produce special colouring matter have been described. Among these organisms, however, those which have been investigated as to the physiology of the production of colouring matter are very limited in number.

Monascus purpureus WENT, which was first described and investigated in detail by WENT (20), is known as producing a characteristic red colouring matter, the chemical nature of which was examined by PRINSEN GEERLIGS (14), who considered it to be an anthraquinon derivative and by BOORSMA (2), who isolated the α- and β-oryzaerubin components. However, no investigation on the conditions influencing the production of colouring matter has been previously done, and its chemical construction is quite unknown, also.

In former research, special attention was paid to the action of magnesium in the culture medium, for Mg must be considered an essential element for the production of colouring matter in bacteria (THUMM (18), KUNZE (8), BENECKE (1), and SAMKOW (15)), and in fungi (KOSSOWICZ (7), MEDISCH (10) and NAUMANN (13)). In addition to Mg, the importance of phosphorus and sulphur in this respect, was also noticed by some of these investigators, especially by GESSARD (4) and JORDAN (6), who maintained that the essential elements are P and S and that Mg was not essential for the pigment bacteria.

The effect of the sources of nitrogen and carbon and other physical conditions relating to the production of the colouring matter of these organisms were also observed by the above mentioned investigators, as well as by MILBURN (11), SCHKORBATOW (16), SELIBER (17), and others.

So far in the investigations on the production of colouring matter by fungi or bacteria, nothing has been done to compare the quantitative relation between growth and the production of colouring matter although this has great importance in the metabolic physiology of the organisms because of the close connection of the two functions.

In this preliminary note a report is given of the present research, in which this relation was observed, as well as a remarkable effect of Mg, which acts as a stimulant, the effect of carbohydrates as a source of carbon, and other conditions dealing with the production of colouring matter of *Monascus purpureus*; the investigation having been done on material cultured in the Botanical Laboratory in Utrecht.

General description of methods.

As to the methods generally used, special attention is drawn to the following:

Glassware. For all the cultures, Jena-glass ERLENMEYER flasks were used. These were previously boiled with 15 % hydrochloric acid for half an hour, then washed carefully with distilled water, prepared by the following method:

Water. Water which was used in the preparation of the culture media and for washing glass-ware was distilled in a Jena-glass still.

Chemicals. "Chemically pure" chemicals were used. Inorganic chemicals were always recrystallized twice or thrice and in each case even the slightest trace of Mg, SO_4, and PO_4, which should be absent in the present experiment, was shown microchemically by the reaction of Mg mixture, $BaCl_2$, and Ammonium molybdate mixture.

LINTNER's soluble starch (which I myself made with great care), saccharose, maltose, dextrose, galactose, and lactose showed almost no trace of these three minerals. Fructose, glycogen, inulin, and dextrine, which are naturally very difficult to purify, showed a slight degree of impurity and were used without recrystallization.

Cotton stoppers. Special attention was paid to the cotton stoppers, as I realized that during sterilization they give some mineral impurity to the media, therefore the cotton was boiled in 2 % HCl, washed with distilled water, and dried.

Sterilization. Media were sterilized in the autoclave, under two atmospheres, for half an hour, the subsequent loss of water being corrected by the addition of sterilized water.

Inoculation. As *Monascus* produces its spores in the culture medium itself and the mycelium mass is very compact, it is not easy to get spores. Therefore the fungus mass on the surface of a new and vigorous normal culture was cut into fine pieces of as nearly similar size as possible and brought into a flask containing a certain amount of distilled water at 30° C., where they were soaked for one hour to purify them of any substance present. Each piece was then used for inoculation.

Temperature. All experiments were carried out in a room of constant temperature of 27° C., the temperature most suitable for this fungus.

Determination of dry weight of fungus body. When all other observations had been made, each mass of fungus was isolated from the medium, was carefully washed in water of 45° C., dried at 105° C., and the constant weight determined.

Determination of colour intensity. AUTENREATH's colorimeter was used. It is, however, difficult to make the determination accurately, as the purification of the colouring matter of this fungus is not yet possible, for it is really a mixture of various colouring matter, as I shall explain later. Therefore I have taken as standard, in a scale 0—1000, the solution from the maltose culture in which this fungus constantly produced the most intense colour. By this means we can make a relative comparison of the intensity of colour, being supplemented afterwards by the qualitative description. In the present investigation, only the water soluble colouring

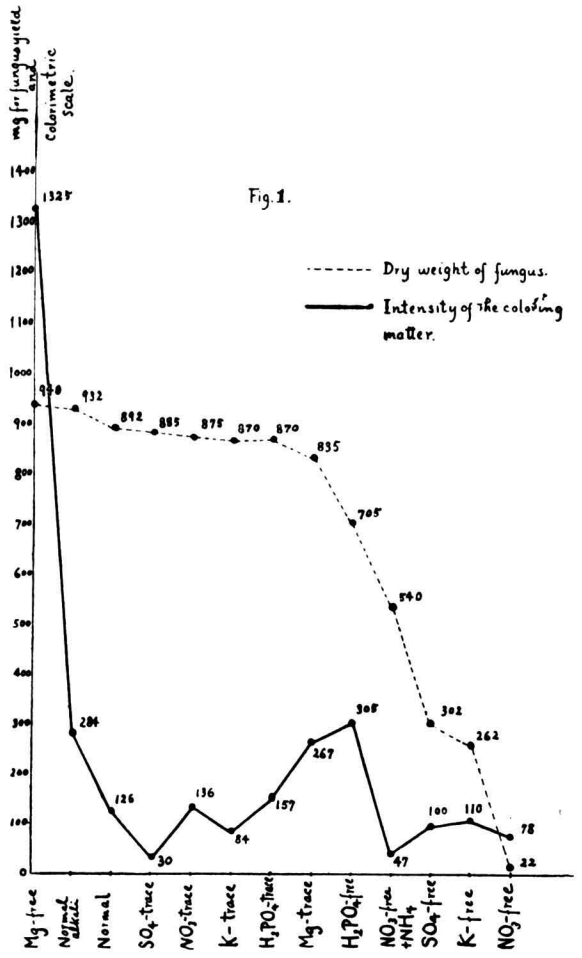
matter was quantitatively observed, the insoluble part being described only qualitatively.

Effects of the essential anions and cations in the culture media upon the production of colouring matter.

The normal culture medium consisted of 3 parts of mineral substances in molecular concentration, $N/20$ KNO_3, $N/100$ KH_2PO_4, and $N/200$ $MgSO_4$ with 3% of soluble starch, and was found to be very suitable for *Monascus*.

In order to investigate the special action of these two anions, Mg and K, and three cations, $-H_2PO_4$, $=SO_4$, and $-NO_3$, thirteen groups of culture media were prepared. Each contained the essential anions and cations in the molecular concentration, with the exception of one anion or cation, which, being omitted, was replaced by other salts ("free culture"), or, a certain small amount was added ("trace culture"). In all cases the total concentration or the media was always isomolecular.

Each ERLENMEYER flask (300 cc.) contained 150 cc. of the medium and 0.5 cc. of $N/5$ citric acid, with one exception ("normal alkaline"). After ten weeks of cultivation, the following results were observed:



The relation between the production of colouring matter and the essential elements was naturally not similar to that between fungus growth and the essential elements.

Fungus growth was almost normal in the case of the following media: "normal alkaline", "SO_4 trace", "NO_3 trace", „H_2PO_4 trace", and „Mg trace". It was hindered in "H_2PO_4 free", and "NH_4 free", still further hindered in "SO_4 free", and "K free", and almost checked in "NO_3 free", as N is needed by the fungus in rather large amounts for plastic substance.

As to the production of colouring matter in the "NO_3 trace", "K trace", and "H_2PO_4 trace" cultures, and in the "SO_4 free", "K free", and "NO_3 free" cultures, the intensity produced was almost similar to the normal culture. It was a little stronger in the "normal alkaline", "Mg trace", and PO_4 trace" cultures.

As the source of nitrogen for the growth of the fungus and the production of colouring matter, NH_4 is less favourable than NO_3.

In the "SO_4 free" culture, the colour of the medium became yellowish.

In general, the mineral constituents of the culture media influence the tone of the colour produced.

It is remarkable that the fungus very intensively produced colouring matter in the "Mg free" culture, while the fungus yield was just the same as in the normal and "Mg trace" cultures. This curious fact causes one to consider that there was still present some small amount of Mg due to the impurity of the chemicals, which were purified, however, with considerable care. This possibility was ascertained by the following experiment.

Influence of the various concentrations of Mg.

Culture media containing 0.5 % KNO_3, 0.1 % KH_2PO_4, and 3 % soluble starch or maltose, to which various amounts of $MgSO_4$ in molecular concentration were added, were inoculated and the results summarized. See fig. 2.

When the Mg concentration was decreased from $\frac{1}{2}N$ to $\frac{1}{100,000}N$, the fungus yield showed almost no difference; the production of colouring matter, however, gradually increased in the descending series, suddenly becoming remarkably intensive in $\frac{1}{100,000}N$, but when the Mg concentration is decreased to an infinitesimal degree, neither fungus yield nor production of colouring matter occurred.

In order to leave Mg out of the media absolutely, more careful purification of the chemicals and glass-ware was undertaken, and in addition to the Jena-glass flasks, quartz flasks were used. Under such conditions, a light yield of fungus and a slight production of colouring matter still occurred, while the almost colourless medium showed a light brown tinge. This result assures one that Mg acts as a stimulant in the production of colouring matter of this fungus; one may say it acts almost oligodynamically. It also seems probable that Mg is not a component of this colouring matter and that, although Mg is of course an essential element

(MOLISCH (12), BENECKE (1), LOEW (9), GÜNTHER (5), and others), a certain small quantity is quite sufficient for the growth of the fungus.

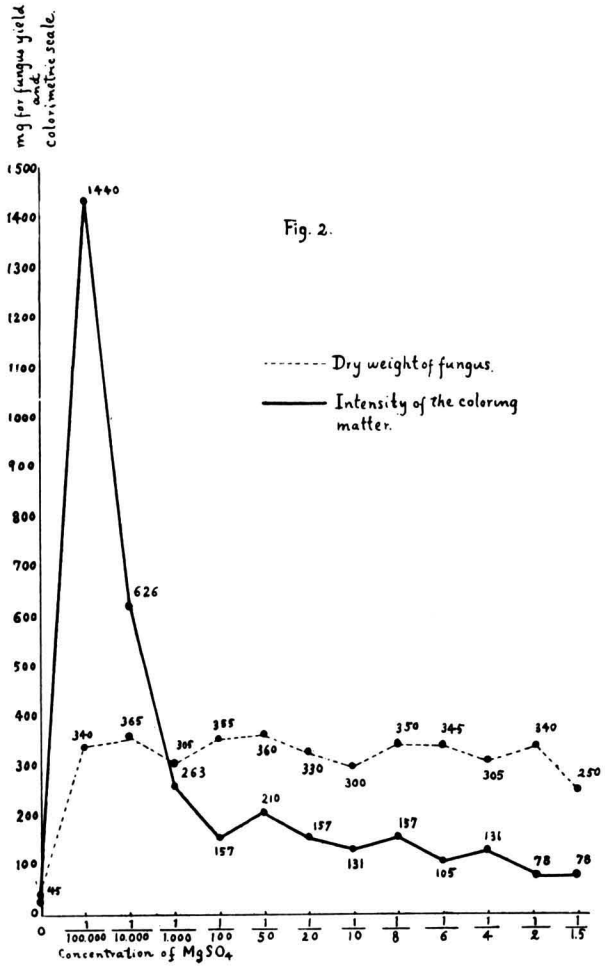


Fig. 2.

Influence of the carbohydrates.

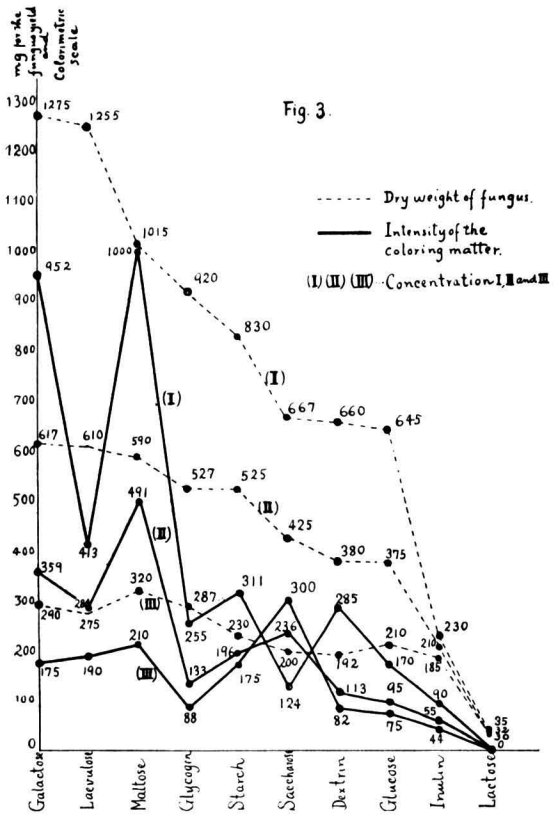
The influence of the carbohydrates, which provide the source of carbon in assimilation and for the production of colouring matter, was investigated. Thirteen sets of culture media were prepared, in which the mineral parts consisted of 0.5% KNO_3, 0.1 % KH_2PO_4, and 0.01 % $MgSO_4$ and to which various carbohydrates were added in three different molecular concentrations, as follows:

Concentration I. $N/2$ for hexoses and polysaccharides and $N/4$ for disaccharides, the concentration of which is isomolecular to $N/2$ hexose, when they are completely hydrolized by the fungus enzyme.

Concentraion II. $N/4$ was substituted in the above procedure for $N/2$ and $N/8$ for $N/4$.

Concentration III. $N/8$ was substituted for $N/2$ and $N/16$ for $N/4$.

For convenience, in the case of polysaccharides, $n = 1$, was taken in the formula $(C_6H_{10}O_5)_n$, under the assumption that these substances are absorbed by the fungus only after they have been hydrolized into hexoses. Figure 3 shows the results.



Lactose had absolutely no value and inulin was only slightly effective in both assimilation and the production of colouring matter. Their hydrolization products, galactose and levulose, were very effective. These results were due to the absence of lactase and inulase in this fungus.

Glucose, dextrose, saccharose, starch, glycogen, maltose, levulose, and galactose were good nutrients for the fungus, but they showed different properties in the production of colouring matter. With maltose and galactose the fungus produced its colouring matter very intensively, while with levulose the intensity was lower, although this hexose gave a large yield of fungus. Saccharose was slowly effective and had a peculiar action for the three different concentrations used, the less concentrated gave the greatest intensity of colour, while the influence of al the other carbohydrates upon the assimilation and the production of colouring matter was rather proportional to their concentrations.

Any of these carbohydrates can be material for the formation of colouring matter, if it is possible for the fungus to assimilate them, though their efficiency in such formation is variable.

In general, contrary to the nature of the mineral constituents in the culture media, carbohydrates do not effect the tone of the colour produced but do influence its intensity.

Influence of oxygen.

In the first stage of the growth, when the spores or the mycelium were sunk into the culture medium, either liquid or solid, the colouring matter was not produced. Later, when some mycelium had reached above the surface of the medium, colouring matter was produced just below the surface. This has been observed previously by WENT (20). At a more advanced stage, the colouring matter was produced either in the submerged or aerial parts of the fungus body, if the medium was favourable for growth. Afterwards the medium became stained by the diffusion of colouring matter, while especially the under side of the floating fungus mass was very intensively stained by the colouring matter within the cells.

In the anaerobic culture, the formation of the colouring matter was greatly hindered, thereby differing considerably from the control, while the growth of fungus occurred to some extent. However, I did not succeed in obtaining a perfectly colourless culture. It is, nevertheless, obvious that oxygen is essential for the production of colouring matter.

Influence of light.

The production of colouring matter was quite indifferent to the intensity of light. Cultures in the dark and those exposed to the sunlight, both direct and diffused, for the same length of time, showed colour formation and growth of fungus.

Influence of temperature.

Temperature had a marked influence upon the production of colouring matter as on the growth of the fungus. The optimum temperature was 27° C.—30° C. Above 35° C. and below 20° C., the production of colouring matter was largely checked.

Influence of water content of medium.

The influence of the water content of solid media was observed and some interesting results were found. For instance, a sterilized and coagulated agar-medium, consisting of 0.5 % KNO_3, 0.1 % KH_2PO_4, and 0.01 % $MgSO_4$, 3 % maltose, and 2 % agar, which is very favourable for the production of colouring matter, was cut into several square blocks of different sizes and placed in a sterilized Petri dish. Every block was inoculated at the same time. After a few days, the colouring matter was produced more quickly in the small blocks than in the large ones, according to the differences in the rate of evaporation of the water in the blocks.

Relation between spore formation and the production of colouring matter.

As seen in many cultures under various conditions, the production of colouring matter seemed to follow the formation of spores. It is to be noticed that the colouring matter developed first in the mycelium which produce spores and in the spores, then later in certain mycelium which were rich in reserve material. When colouring matter was intensively produced, the spore formation was also very vigorous. In the culture with lactose medium, in which the fungus remained colourless, spore formation was very slight or lacking altogether.

Characteristics of the colouring matter.

According to PRINSEN GEERLIGS (14), the chemical nature of the colouring matter of *Monascus purpureus* was considered to be an anthraquinon derivative, and was described as hardly soluble in water and soluble in ethyl alcohol, chloroform, etc., but this investigator used dried material taken from rough cultures on rice medium, in which 0.1 % arsenic acid was used to avoid the propagation of other organisms. BOORSMA (2), in dealing with its solubility in Na hydrate solution, considered that the colouring matter is made up of two components, α- and β-Oryzaerubin.

Although the present research is not concerned with the chemical nature of the colouring matter, it seems from its physical nature that this colouring matter consists of at least four components, which are produced in both liquid and solid media:

a. Water soluble part.

1. Yellow coloured component.
2. Carmine coloured component.

b. Water insoluble part, soluble in ethyl alcohol, chloroform, etc.

3. Yellow coloured component.
4. Carmine coloured component.

These four components of the colouring matter seem to be substances closely related chemically, giving fluorescence in their respective solvents.

The proportion of constituents in colouring matter differs according to the environmental conditions; for this reason the tone of the colouring matter varies from yellow to blood red, or carmine, showing various intermediate colours.

It is to be noticed that the colouring matter decreases in solubility when the coloured fungus mass is heated to a temperature above 100° C., or is long dried, so the water soluble part is not obtained. In extracting the Ang-khak mass, some part of the colouring matter which is insoluble in the various solvents always remains. It may be assumed that this was the case in the research of PRINSEN GEERLIGS (14).

For more detailed results, further investigation is necessary.

This research was carried on at the Botanical Laboratory in Utrecht during my visit to Europe.

It is with great appreciation that I thank Professor F. A. F. C. WENT,

who suggested this problem to me and who always helped me with his kind encouragement, as well as putting at my disposal all the facilities of his laboratory.

My thanks are also due to Dr. ANNIE M. HARTSEMA, Mr. S. R. DE BOER, Mr. F. W. WENT, and Miss WILLY M. COELINGH, who often assisted me, and to Mr. P. A. DE BOUTER, who aided me with his mechanical skill.

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