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Studies on the Adaptation of Yeast to Copper

X. Effect of Nutrients and Inhibitors on the Growth and Pigmentation in the Presence of Copper

By

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When streaked on certain agar media which are rich in nutrients and contain copper in the order of 1 mM/l, the strain of *Saccharomyces ellipsoideus* used in this laboratory usually forms only a slight film of background which, after a matter of a few days, becomes spotted with colonies, mostly brown-coloured and containing copper resistant cells. Cells of these colonies give brown confluent growths when restreaked on a fresh medium which contains the same order of concentration of copper. The copper resistant substrain obtained by successive subcultures on a copper medium forms white colonies which are apparently similar to the normal strain when transferred on to the unpoisoned medium. The strain, however, does not lose the copper resistance even after several passages through the unpoisoned medium, but will grow rapidly, confluently and brown coloured, if inoculated again on the copper medium. The growth on the copper medium is coloured within the first 2–3 days, but with ageing it gradually becomes tinged with green, accompanied by the diffusion of greenish matter into the agar. And the growth becomes covered by whitish layer of cells after 2–3 weeks. By extracting the cells of the brown colonies, brown and green pigments are obtained, of which the precise nature is not yet determined.

Observations similar to the above have been reported with respect to the copper cultures of bacteria, namely for marine bacteria by Waksman *et al.* (1) and for *Azotobacter* by Lewis (2). So the brown pigmentation on copper-media seems to be a rather general phenomenon common to ceratin microorganisms.

Not only the nature of the pigmented substances, but also the roles played by them are not known clearly. There is even a possibility that they are insoluble products of cell metabolism carried on in the presence of copper. The

vigorous growth on the copper medium, however, is always accompanied by the brown colouration, at least for the resistant substarain used. Hence the colouration seems to present an accessible approach to the mechanism by which resistant cells can grow vigorously in the presence of copper.

The present paper deals with the effect of culture conditions, including the amino acid and organic acid compositions of medium, and the presence of some enzyme inhibitors on the pigmentation and the growth vigour of colonies on the copper-containing media. It will be inferred that particular amino acids play important roles in the pigment formation, and possibly help the cells to grow in the presence of copper.

Materials and Methods

The strain of *Saccharomyces ellipsoideus* used was the same as in the previos reports (3, 4, 5, 6). The copper resistant substrain, which has been derived from it by successive subcultures on MH medium poisoned with 1 mM/l copper, is called R_{lb}.

Used were the three kinds of culture medium, MH, H and h, of which the compositions are given in Table I. For solid medium, agar was added to be 2 per cent.

Table I

Composition of H- and MH-media

H-medium		MH-medium	
Peptone	5g	H-medium	1000ml
KH_2PO_4	5g	Malt extract, Bé 8°	360ml
$MgSO_4 \cdot 7H_2O$	2g		
Cane sugar	100g		
H_2O	1000ml		

Composition of h-medium

KH_2PO_4	3g	Thiamine	200γ
$MgSO_4 \cdot 7H_2O$	1g	Pyridoxine	200γ
$(NH_4)_2SO_4$	1g	Nicotinic acid	200γ
Glycine	1g	Pantothenic acid	200γ
Cane sugar	50g	Biotin	2γ
H_2O	1000ml	Inositol	10,000γ
		Riboflavin	200γ
		PABA	200γ

Copper medium was prepared by adding a calculated amount of sterilized $CuSO_4$ solution to the sterilized culture medium which had been cooled to room temperature, or, in the case of agar medium, to 45° C. Inhibitors were also mixed with the culture medium after cooling. Concentrations of $CuSO_4$ and

inhibitors are given in mM (millimoles/l.). The incubation temperature was 30° C.

As an orienting study, the colouration was recorded subjectively. The growth vigour is not particularly depicted. However, unless otherwise specified, the description, "pigmentation," implies also that the growth occurred in one step, instead of being two-stepped as when the sensitive strain is inoculated on the copper medium.

Results

1. Copper concentration. Cells were transfered successively to MH media of higher and higher copper contents. Up to 8 mM, the more concentrated the copper, the darker was the colour of growth.

With the h-medium, in which cells were more susceptible to copper than in MH, lower concentrations of copper were used. In this case, the colouration of growths became more and more intense from 0.01 to 0.5 mM, being darker as a whole than in the case of MH.

2. Concentration of KH_2PO_4, $MgSO_4$, and sucrose. In order to see the effect on pigmentation of concentrations of KH_2PO_4 and sucrose, the h-medium was modified for these two ingredients.

Each of the two modifications in the concentration of KH_2PO_4, namely 0.5 and 5.0 g/l, contained 10 and 100 g/l of sucrose. When R_{lb} was inoculated, the colour of growth was the lightest on the medium that was poor in both ingredients, and was the deepest on the one that was rich in both.

Similarly, when the $MgSO_4$ content was made 0.3 and 5.0 g/l, with 10 and 100 g/l sugar in each, the colour of colonies was the lightest on the medium containing lower amounts of the two nutrients, and the deepest on the one higher in the amounts of both.

3. Enzyme inhibitors. To the MH-medium enriched with 1 mM $CuSO_4$, enzyme inhibitors were added to give the final concentrations as indicated in Table II. The prior concentration of each inhibitor in the table was such that permitted 75 % or more of viable cells of the sensitive parent strain grow in the MH plate unpoisoned with copper. On the other hand, the higher limits of concentration were such that, when added to the copper-medium, to permit only scattered growth of the streaked parent strain.

The R_{lb} substrain was streaked on the slant of 1 mM Cu-MH medium supplemented with the inhibitors in concentrations within the ranges given in Table II. The "complete" inhibition of pigmentation, as described in the table, signifies that the strain grew to a considerable extent without appreciable colouration, and the "no" inhibition represents the brown colouration of growth, which was confluent with lower concentrations of the inhibitors and was only scattered with the highest concentration.

Table II. Inhibition by enzyme inhibitors of the pigmentation of copper-resistant strain growing on 1 mM Cu-MH slant

Inhibitors	Concentation (mM)	Inhibition of pigmentation	Remark
Na-fluoride	7.5 – 10.0	Complete	
Na-nitrite	2.0 – 3.0	Complete	
J-acetate	0.3 – 0.35	Complete	
Na-bisulfite	6.0 – 20.0	No	
Na-azide	0.03 – 0.1	No	No growth *in the highest concentration*
2,4-dinitrophenol	0.2 – 0.5	No	
Ethyl urethane	5.0 – 14.0	No	Growth inhibited almost completly
Hydroxylamine	3.5 – 5.0	No	
Malonate	300 – 1000	Considerable	

The three fermentation inhibitors, monoiodoacetate, fluoride, and nitrite, inhibited the colouration, while bisulfite did not. The CO_2 liberation is known to be hindered by the former three, and not by bisulfite. So it is tentatively suggested that the CO_2 production has some bearing on the Cu-pigmentation.

The two inhibitors of oxidizing enzymes, azide and hydroxylamine, did not inhibit the pigmentation. Malonate inhibited it strongly, but not completely. Hence it seems that the oxidation itself is not essential to the pigment production, and the dehydrogenation of succinate might have a limited connection to this. The lack of inhibition by 2,4-dinitrophenol and azide suggests that the pigment formation is not dependent on the formation of energy rich bonds (7, 8).

4. Anaerobic condition and shaking. The R_{1b} strain was inoculated on the 1 mM Cu-MH slant in a vial, which was put in a larger vial containing a sufficient amount of alkali-pyrogallol solution. Under the anaerobic condition thus furnished, the pigmentation was not inhibited, conforming to the fact that it was not inhibited by respiration inhibitors.

While the cells were coloured brown when cultured unshaken in liquid MH containing 1 mM $CuSO_4$, the pigmentation was not observed when the culture was shaken.* When an aliquot of the standing culture using the liquid 1 mM Cu-MH medium was shaken after the nutrient had been consumed considerably, the population which had been brown turned greenish in 20 hours. And when another aliquot of the same culture was harvested by centrifugation, resuspended

* A 2 l conical flask containing 50 ml of culture medium were shaken at sixty 6 cm strokes per minute.

in an equivolume of fresh 1 mM Cu-MH medium and shaken, the cells lost the brown colour in a day, though the cell proliferated only a small fraction.

Hence the shaking seemed not only to be unfavourable for the pigmentation but rather to destruct the pigment already formed in the cell.

In order to see the relation between the anaerobic condition and the shaking, the shaking culture was made under nitrogen stream, and it was found that the pigmentation of cells was inhibited in this case. This may suggest that the shaking hampers the pigmentation by preventing the culture from over-saturation of CO_2.

5. Vitamins. The synthetic h-medium contained eight vitamins, as shown in Table I. Eight media which lacked each vitamin respectively, and the one lacking all of the vitamins were prepared. After adding $CuSO_4$ to each of the media to give the concentration of 0.5 mM, they were inoculated with R_{Ib} which had been kept culturing on a 1 mM Cu-MH slant. Essentially the Cu-pigmentation was not affected by the vitamin deficiencies in the media, though the amount of growth varied from each of the others to some extent.

6. Nitrogen source. The colour of growth was deeper with the Cu-MH medium than with the Cu-H medium; and the higher the corcentration of malt extract in MH, the darker was the colour, the Cu-concentration being 1 mM in each case. The Cu-pigmentation became lighter when a part of peptone in the H-medium was replaced by $(NH_4)_2SO_4$. Yeast extract, on the other hand, made the pigmentation lighter when it is added to the H-medium. The more the yeast extract, the lighter the colour. Thus, the Cu-pigmentation seemed to be conditioned by the quality and quantity of nitrogen source in the nutrient medium.

In order to see the role of amino acids, glycine of the synthetic h-medium was substituted by $(NH_4)_2SO_4$ and each one of the seven other amino acids as named below. The concentration of each amino acid was made such that the amount of nitrogen was the same in each medium, except tyrosine and histidine. Copper was added to give the concentration of 0.3 and 0.5 mM. The parent strain and R_{Ib} were streaked on separate slants, and the growth behaviour and the pigmentation were recorded.

The results as presented in Table III show that the parent strain inoculated on media of which the nitrogen source was glycine, tyrosine, arginine β-phenylalanine or α-alanine, grew as thin, white film at first, followed by the secondary growth of discrete brown colonies in a few days. On the other hand, the Cu-medium which was provided with glutamate or aspartate, permitted the sensitive parent strain to grow in one step, resulting in the confluent and brown-coloured growth, as if the strain were R_{Ib}.

The copper-trained strain, R_{Ib}, could grow confluently on any of the media represented in Table III, except the one provided with L-histidine. The brown pigmentation, however, differed according to the nitrogen source, α-alanine, aspartate and glutamate being favourable for the pigmentation. When fluoride, which

Table III. Pigmentation at 5 days after the inoculation of the parent and Rlb strains on Cu-h-slant of which glycine was replaced by the various amino acids. The amont of each amino acid added is shown in brackets in grams per 1 nutrient. Concentrations of copper and of supplemented inhibitors are also given.

+++, ++ and + represent, respectively, deep, moderate and slight pigmetation of the confluent growth; (+) represents that the primary growth was white and the secondary colonies were pigmented.

Strain	Parent	Rlb			
	1	2	3	4	5
Inhibitors added (conc. in mM)	None	None	NaF (7.5)	NaF (7.5) Malonate (300)	$NaNO_2$ (2.0)
$CuSO_4$ conc. in mM.	0.5	0.3	0.3	0.3	0.3
$(NH_4)_2SO_4$ (1.0)	−	+	−	−	−
Glycine* (1.0)	(+)	+	−	−	−
DL-α-Alanine (1.4)	(+)	++	−	−	−
Arginine (0.66)	(+)	+	−	−	−
L-Phenylalanine (2.3)	(+)	+	−	−	−
Tyrosine (Satur.)	(+)	+	−	−	−
L-Histidine (1.2)	no growth	no growth			
Aspartic acid** (2.0)	++	++	++	++	−
Glutamic acid** (2.2)	+++	+++	+++	+++	−

* The original h-medium.
** Added after neutralization by NaOH.

had been shown to inhibit the pigmentation, was added, intensive pigmentation occurred only on the medium supplied with glutamate or aspartate. Even when both malonate and fluoride were present, the pigmentation occurred, if glutamate or aspartate was furnished.

Fluoride may reduce the production of CO_2, of which a sufficient supply was inferred in a preceding section to be necessary for the pigmentation. But glutamate and aspartate caused the pigmentation even in the presence of this inhibitor. The inhibition of pigmentation due to malonate, which may inhibit succinic dehydrogenase, was overcome also by the two amino acids. The effect on the pigmentation of the concentration of KH_2PO_4 and sucrose, as reported above, was also masked by the two amino acids.

It might be suspected that the dicarboxylic amino acids combine copper and reduce the concentration of copper ion in the medium. But the brown pigmentation which is considered to be conditioned by the entrance of copper into cells

is a good indication that cells are well under the influence of copper. Polarographic measurements correctly reproduced the amount of copper added to the medium containing glutamate or aspartate.

7. *Organic acids.* In connection with the facts that the pigmentation is strongly reduced by fermentation inhibitors but not by respiration inhibitors, and that glutamate and aspartate prominently favour the pigmentation, need was felt for studies into the relation of organic acids to the pigmentation.

As the growth was significantly suppressed on the media in which organic acids were the sole source of carbon, each of seven organic acids was added to the 0.3 mM Cu-h-medium to give the concentration of 0.2 M. In order to inhibit the pigmentation due to sucrose contained in the medium, nitrite or fluoride was added in the concention as shown in Table II.

Table IV. Pigmentation of the parent and R_{1b} strains observed 5 days after the inoculation on the 0.3 mM Cu-h-slants supplemented with organic acids and enzyme inhibitors.

Strain	Parent	R_{1b}			
	1	2	3	4	5
Inhibitors added (conc. in mM)	NaF (7.5)	NaF (7.5)	$NaNO_2$ (2.0)	NaF(7.5) Malonate (300)	$NaNO_2$(2.0) Malonate (300)
Sucrose only	−	−	−	−	−
Lactate	−	−			
Pyruvate	±	±	−		
Acetate	+++	+++	+	++	−
Citrate	+++	+++	++	+++	+
Succinate	+++	+++	+	++	−
Fumarate	±	±	−		
Malate	++	++	+	++	+

Notations as in table III. ± : Insignificant pigmentation.

Results are presented in Table IV. On the Cu-h-medium supplemented with acetate, citrate, succinate or malate, the sensitive parent strain grew in one step, giving rise to the heavily pigmented confluent growth, just as R_{1b} does. As the pigmentation occurred in the presence of fluoride, it must be owing to the supplemental acids as in the case of glutamic and aspartic acids. On the other hand, the media containing a second group of acids, lactate, pyruvate and fumarate, and also the one with no added acid permitted only thin, white film of growth.

Even the resistant strain coloured well only on the medium enriched with

one of the first group of acids. The differential effect between the two groups of acids was apparent also when nitrite was added, although the colour was lighter in this case. The inhibition by malonate was not significant when this was given in combination with fluoride, but was considerable in association with nitrite, as seen in columns 4 and 5. The malonate inhibition is apparent with acetate and succinate, but not with citrate and malate. It seems that the succinic dehydrogenase system is not dispensable when either of the latter two acids are presented. It is interesting to consider this fact in relation to the particular effect of glutamate and aspartate among amino acids.

Discussion and conclusion

When the composition of copper-containing medium was changed, and also when some enzyme inhibitors were added to it, the vigorous growth was usually accompanied by the brown pigmentation of cells. It has been determined by Naiki *et al.* (6) that cells of the same strain come to contain very much copper when they grow in the copper-containing medium. If, however, copper is added to killed cells, they are coloured with a rather bluish tint. Hence the brown coloured matter seems to be produced through the active cell metabolism taking place in the presence of copper.

When the wild strain is spread on copper media white colonies grow, as a matter of order of thousandth, among brown ones. So there certainly is a variant which can grow in copper media whithout producing brown substances. But the variant, the most stable and predominating in the medium containing moderate concentration of copper, is the one which grows brown coloured in copper-media (3, 5). And the present investigation is an approach to the copper-resitant metabolism of this brown pigment-forming variant.

Among enzyme inhibitors, only those which reduce the production of CO_2 inhibited the pigmentation. The shaking of liquid culture was unfavourable for the pigmentation even in the nitrogen atmosphere. These facts may suggest that a high partial pressure of CO_2 is necessary for the pigment formation in the routine copper medium. In the medium of lower sugar and KH_2PO_4 contents, which was found less favourable for the pigmentation than that of higher contents, a less CO_2 evolution was observed manometrically than in the latter.

Among the amino acids tested, glutamic and aspartic acids have special activity for the pigmentation. Among several organic acids, on the other hand, four were found to be so.

It has been shown in the yeast cell that the tricarboxylic acid cycle is present, at least as a component reaction if not the main path of oxidation (9), and also that amino acids can be formed from pyruvate and acetate (10, 11). As to whether the organic acids contribute to the pigmentation as the source of amino acids, or whether the reverse relation exists between the two groups of

substances, the former alternative seems to be more probable, because: 1) The amino acids are effective in the concentration of 1/60–1/70 M, while the organic acids must be provided in 1/5 M; 2) The unfavourable effect of reducing the contents of both sugar and KH_2PO_4 is overcome only by the the addition of amino acids; 3) Alanine is not effective, whereas it might be if organic acids be what are needed.

In the coexistence of nitrite, malonate inhibits the pigmentation due to acetic and succinic acids but not that due to malate and citrate. This may be explained as the difficulty in producing glutamic or aspartic acids from acetic and succinic acids under that condition, namely when both of the succinic dehydrogenase activity and the CO_2 evolution by fermentation are inhibited.

If the above series of reasoning is admitted, it will be stated that the high partial pressure of CO_2 is necessary for the pigmentation in the routine medium as a factor leading to the production of glutamic and aspartic acids. And the conclusion is also possible that the brown copper-resistant strain can synthesize those amino acids in the presence of copper, while the original sensitive strain cannot, and that, in the presence of copper, the sensitive strain cannot form organic acids in an amount sufficient for producing essential amino acids.

Although many observed facts point to the conclusion as above, some remain to be explained: 1) The pigmentation by glutamic and aspartic acids is inhibited by nitrite. The decomposition of amino acids is conceivable, but no evolution of gas could be detected by Warburg's manometer. 2) Fumarate is not effective. A possible explanation might be that it is impermeable or that it is the reducing system of succinic dehydrogenase that is necessary for the pigmentatin.

Summary

The luxuriant growth of *Saccharomyces ellipsoideus* in copper-containing media is accompanied by brown colouration of cells. This pigmentation in copper media is favoured by (1) high partial pressure of CO_2, (2) certain organic acids, and (3) glutamic and aspartic acids. (1) and (2) seem to be effective by contributing to the production of (3). It is inferred that the original strain cannot, and the copper-trained substrain can synthesize a sufficient amount of the two amino acids in the presence of copper, in the usual media which do not contain much of them.

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