ON THE YEAST *CANDIDA PULCHERRIMA* AND ITS PIGMENT

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE TECHNISCHE WETENSCHAP AAN DE TECHNISCHE HOGESCHOOL TE DELFT, KRACHTENS ARTIKEL 2 VAN HET KONINKLIJK BESLUIT VAN 16 SEPTEMBER 1927, STAATSBLAD No. 310 EN OP GEZAG VAN DE RECTOR MAGNIFICUS Dr O. BOTTEMA, HOOGLERAAR IN DE AFDELING DER ALGEMENE WETENSCHAPPEN, VOOR EEN COMMISSIE UIT DE SENAAT TE VERDEDIGEN OP WOENSDAG 18 JUNI 1952, DES NAMIDDAGS TE 2 UUR

DOOR

JOHANNES PETRUS VAN DER WALT

GEBOREN TE PRETORIA

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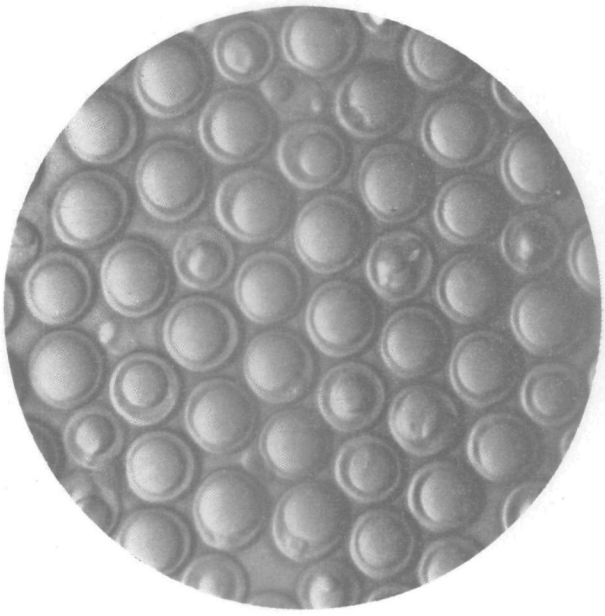
PROF. DR IR A.J.KLUYVER



Candida pulcherrima (Lindner) Windisch
BEIJERINCK's fat yeast

AAN MY MOEDER

AAN DIE NAGEDAGTENIS VAN MY VADER

Met genoeë wil ek my erkentlikheid betuig teenoor die Studiefonds voor Zuid-Afrikaansche Studenten vir die Studiebeurs wat ek gedurende my verblyf in Nederland mog ontvang het.

Ook is dit my aangenaam om die Suid-Afrikaanse Wetenskaplike en Nywerheidsnavorsingsraad vir 'n Studiebeurs te bedank.

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E R R A T A

Page 10 line 42 read: anascosporogenous

Page 20 line 39 read: SABOURAUD-agar

Page 25 line 36 read: repeatedly

Page 31 line 10 read: influence

Page 33 line 18 read: as far as

Page 35 line 44 read: incapable

Page 34 line 9 omit: on

Page 44 line 8 read: addition

Page 54 line 29 read: 0.5 square metres

Page 105 line 19 read: hybriden

INTRODUCTION

In 1901 LINDNER described a hitherto unknown yeast species to which he gave the name of *Torula pulcherrima*. The specific name chosen for this yeast was directly connected with the fact that on examination of microscopical preparations of the organism sometimes very beautiful pictures were encountered. Under certain conditions the whole field of the microscope was filled with large strictly spherical cells of quite uniform size, each containing a highly refractive globule. LINDNER characterizes these globules correctly as consisting of "ein Amalgam von Fett und Plasma".

The first investigator after LINDNER to study this yeast was BEIJERINCK (1912) who describes that he could isolate the organism – to which he gives the name *Saccharomyces pulcherrimus* – quite regularly from the stomach of bees and bumble-bees. He made some observations on the instability of the globular cell forms, and is inclined to consider the change of these forms into a generation of budding cells of much smaller dimensions as a case of mutation. BEIJERINCK is much impressed by the large size of the oil drops occurring in the globular generation, and introduces the indication 'fat yeast' for the organism. In 1916 BEIJERINCK emphasizes this point of view, and discusses the possibility of applying the organism for industrial fat production. He finally rejects this idea on ground of the pronounced mutability of the organism leading to the formation of the smaller cell type which does not produce fat and for which he introduces the name of *Saccharomyces pulcherrimus secundarius*.

In 1918 BEIJERINCK draws the attention to quite another property of his 'fat yeast', *viz.* its ability to produce a dark red pigment on media containing some soluble iron salt. BEIJERINCK gives convincing evidence that this pigment is quite different from that of the common red yeasts – the "Rosahefen" of the German bacteriological literature – where the pigments are known to be typical lipochromes. The nature of the pulcherrimus-pigment is, however, not elucidated, although BEIJERINCK suggests a relationship with the anthocyanins. Finally it is worth stressing that according to BEIJERINCK also the property of pigment production is not uniformly distributed over all cells. Many of the cells form perfectly colourless colonies also on iron-containing media, and BEIJERINCK concludes that the pigmented colonies are derived from a mutant form which, however, is not at all identical with the *Saccharomyces pulcherrimus secundarius* referred to above. A further complication is that the pigmented mutant is far from stable: it easily reverts to the colourless main form. Finally

somewhat older cultures of the mutant in question have a strong tendency of dying off.

The preceding may suffice to establish the conviction that LINDNER's *Torula pulcherrima* – which in accordance with the modern views on yeast taxonomy will henceforth be named *Candida pulcherrima* – is a yeast which is fully entitled to be the subject of further study. In the first place there is the ability to form under certain conditions large quantities of some lipid: according to BEIJERINCK not seldom from 60-80% of the cell volume is occupied by this substance. Secondly there is the pronounced mutability of the organism which may lead either to BEIJERINCK's *secundarius* mutant, or to a quite different pigment-producing mutant. Finally there is the dependency of the pigment formation on the presence of iron, at first sight suggesting that the pigment itself contains iron. At the time of BEIJERINCK's investigations the important rôle which haemin compounds play also in vegetable cells had not yet been discovered, and, therefore, no attention had been given to the possibility that the red 'iron-yeasts' might owe their colour to the presence of some 'vegetable blood pigment'.

All this seems to justify a renewed investigation of the yeast in question, and of its remarkable pigment.

It is true, that since BEIJERINCK *Candida pulcherrima* has been studied by various other investigators. As such the names of PUNKARI and HENRICI (1933, 1935), WINDISCH (1938, 1940), PORCHET (1938), CASTELLI (1940) and ROBERTS (1946a, 1946b) should be mentioned here. However, with due acknowledgement to the valuable contributions made by several of these authors, it cannot be said that they have succeeded in giving a clear picture of *Candida pulcherrima* in its manifold manifestations. To the contrary, as will be set forth at various stages in this thesis, several of their conclusions have in various respects enhanced the already existing confusion.

Since, moreover, in these later studies the nature of the red pigment has practically been left out of consideration, there seemed to be all reason to make another attack on the various mysteries which still enshroud *Candida pulcherrima*.

Chapter I

EARLIER OBSERVATIONS ON THE MORPHOLOGY OF CANDIDA PULCHERRIMA

In this chapter a brief survey will be given of the work done by earlier investigators regarding the main morphological and cultural properties of the yeast presently known as *Candida pulcherrima*.

It should, however, be remarked that all observations dealing with the question of pigment production will be practically left out of consideration here. This particular point will be more amply discussed in chapter IV.

LINDNER's original description of the yeast in question – which appeared in 1901 – is very brief. He first states that he has encountered the yeast both in overripe plums, and in the excrements of larvae found in apples. He then reports that the yeast formed in beer wort normal ellipsoidal, budding cells, but that after the fermentation was over, and under conditions of sufficient air supply, the cells increased in size, rounded off, whilst after some time large highly refractive globules were formed in the interior of the cells. If these cells were allowed to germinate in fresh wort, they were seen to throw off their outer membrane, and a new generation of small cells was formed. No ascospore formation was observed by LINDNER.

As has already incidentally been mentioned in the Introduction BEIJERINCK's views on the yeast in question differ rather markedly from those of its discoverer. Here a summary will be given of his observations, as reported in the three papers in which he refers to the organism (BEIJERINCK, 1912, 1916, 1918). Apparently BEIJERINCK is more or less fascinated by those forms in which the fat globules give a characteristic aspect to the yeast, hence his indication of the organism as 'the fat yeast'. BEIJERINCK reports that he regularly found the organism in the stomach of bees and bumble-bees, but he also isolated it from polishings of barley, from grapes and from the nectar of flowers, especially from those of *Lamium* species. There can scarcely be any doubt that the early recognition of the yeast in these materials was mainly based on the observation of the typical 'fat cells', usually indicated by him as '*pulcherrima*-cells'. BEIJERINCK also observed that inoculation of these cells in suitable fermentation media led to the formation of a yeast generation consisting of much smaller, ellipsoidal cells which did not contain any fat. BEIJERINCK is

inclined to consider this change as a typical mutation, and he introduces the name *secundarius* mutant for this new, in his eyes rejectable, form.

This at first sight rather surprising conclusion must primarily be explained by the fact that BEIJERLINCK's first observations on the yeast were made at a time when he had centered his attention on the wide occurence of the mutation phenomenon in the microbe world. Apparently BEIJERINCK ignored the possibility, already clearly indicated by the drawings in LINDNER's publication, that the *pulcherrima*-cells are merely latent forms ("Dauerzellen") which on being transferred into fresh media just lead to the 'normal' cells by germination. However, BEIJERINCK made a second observation which at least gives some further clue to his vision. He found, namely, that on directly plating the organism on solid media, like wort gelatin or agar – with probably very high sugar content – not all colonies consisted of the small ellipsoidal cells, but that sometimes some of the colonies appeared to consist of large fat-containing cells. Apparently these had been formed out of the *pulcherrima*-cells in the inoculant by budding, immediately followed by fat formation in the buds.

Seen in this light – the occurrence of two types of colonies after streaking a pure culture on one and the same medium – the idea of mutation, or at least of 'dissociation', as this phenomenon has been called by later investigators, seems, indeed more or less justified. However, the use of the name *'secundarius'* for the form by far prevailing in all normal fermentation media does not seem fortunate, and only finds its explanation in the fact that BEIJERINCK was chiefly interested in the 'fat cells'.

Finally it should be remarked that BEIJERINCK states clearly that his *secundarius*-form under certain conditions not seldom reverts to the *pulcherrima*-cells, and also that it is not at all easy to maintain the latter form.

The next authors who studied *Candida pulcherrima* were PUNKARI and HENRICI (1933) whose paper throws a clear light on the great variability of the organism, as manifested by sector formation in giant colonies. Since in this study – and also in a second paper (1935) – the question of pigment formation plays a preponderant rôle, these publications will not be discussed here in any detail. However, it should be mentioned that they also found a dissociation into a smooth and a rough form, the latter characterized by the formation of a primitive mycelium.

In her monograph on the anascorporogenous yeasts LODDER (1934) also gives a clear description of the organism – classified by her as *Torulopsis pulcherrima* – in which, however, no personal observations on the occurrence of the typical *pulcherrima*-cells

are given. Some of the strains investigated showed a marked variability; these were brought to a new variety: *T.pulcherrima* var. *variabilis*.

The next study is that of PORCHET (1938). The author isolated ten strains from various fruits grown in Switzerland. She reports that six of these strains conformed very well to LODDER's description. The four other strains showed, however, a markedly different behaviour. In the first place a well differentiated pseudomycelium was formed. Secondly PORCHET describes – and gives photomicrographs of – round cells formed at the end of elongated cells. These terminal cells she calls conidia, and even casually compares them to basidiospores. Finally these four strains were characterized by a typical dissociation phenomenon leading to pigmented colonies with large round cells containing oil drops. Some other correlating differences in the properties of the two forms are mentioned. Each of these forms may revert to the other.

In 1938 WINDISCH published a note which tended to revolutionize the then existing views on the morphology and the taxonomic position of the organism. A yeast strain isolated by him had been identified by the Yeast Division of the "Centraalbureau voor Schimmelcultures" at Delft as *Torulopsis pulcherrima*. WINDISCH then showed in the first place that the organism could produce a profuse and typical pseudomycelium which banned it from the genus *Torulopsis*. Much more startling, however, was WINDISCH's claim of having observed ascospore formation. His description of this phenomenon can be summarized as follows.

In aged cultures, in and on various media, the occurrence of large round cells, constantly containing large oil globules, often occupying up to four-fifths of the cell volume, were noted. To many of these "Riesenzellen", the remnants of what he considered to be ruptured asci, were still attached. Often these 'asci' still contained a body which WINDISCH regarded as ascospore. Accompanying these giant cells were numerous representatives of a smaller type, differing from the former by their ability of rapid multiplication by bud formation. On a slide culture made of the small-celled generation which incidentally was contaminated with a *Penicillium*-species he claimed to have witnessed copulation which was preceded by the formation of conjugation tubes. Through these tubes, migration of the nucleus of one of the cells of a copulating pair might have taken place, leading to a nuclear fusion in the second cell. This cell functions as "Askogon" and by rounding off and increase in size becomes a "Karpogon". In this transformation also the included oil globule increases in size, whilst a stout membrane is formed. After eight days, the thick-walled "Karpogon" formed a thin-walled attachment, or fragile

ascus in which four ascospores developed. The thin-walled ascus was clearly separated from the "Karpogon" by the thick membrane of the latter.

The ascus ruptured at the seat of attachment and liberated the ripe spores which, however, were reported to be irregular in shape. The spores increased in size and then sprouted. The author could not repeat these observations in slide cultures in the absence of the *Penicillium* contaminant. On SABOURAUD-agar, however, similar structures were noted.

CASTELLI (1940) published a study on twenty three strains of *Candida pulcherrima* freshly isolated by him from musts, especially of the Chianti type. Of these strains twenty one answered quite satisfactorily to the standard description given by LODDER in 1934. The two remaining strains differed amongst other by the more elongated cell form in malt extract, a more rapid gelatin liquefaction, and a stronger pigment production. CASTELLI was unable to obtain ascospore formation by any of his strains, and expresses doubt regarding WINDISCH's results in this respect.

Soon after this WINDISCH (1940) reported extensively on a continuation of his studies.

The two types primarily distinguished by PORCHET were considered by WINDISCH to be successive phases in a life-cycle.

He found one type of colony to give weak concentric rings on the agar, at the periphery of which pseudomycelium occurred in abundance. The cells in these cases were elongate ovals and corresponded to the description given by LODDER (1934). The second type distinguished itself in that the cells were predominantly spheroid. The colonies of this type, granular in appearance, were slow in development, and measured only a fraction of a millimetre. In aged colonies, giant cells with oil drops occurred. Attached to these cells remnants of membranes were often found. The occurrence of these structures was always correlated with the presence of small cells. Subcultures of these small cells on SABOURAUD-agar displayed a tendency towards lipid formation, usually one globule per cell. The oil globule assumed an elongated form, and after some time, exerted a pressure on the cell wall. Under this strain the wall produced a protrusion which ensnared the one half of the oil drop. The protrusion was then partitioned from the mother cell. Due to the highly refractive oil globule, WINDISCH found it impossible to witness the actual 'spore formation', until the 'ascus' ruptured at the seat of attachment, and liberated the 'spores' and protoplasmic debris. Intact 'asci' were only rarely observed. The author sought to explain all this by the supposition that the 'ascus' membrane was exceedingly fragile and sensitive. The "Epiplasma" surrounding the 'spores' was supposed to have a

tendency to swell, and the attraction of water would then lead to a rupture of the fragile membrane. Apart from this, the so-called 'asci' ripened with an astounding rapidity, and consequently remained intact only for a short while. The ruptured 'asci' remnants remained attached to the 'ascus mother cells'. It should be remarked by the way that WINDISCH makes the startling statement that these 'ascus mother cells' at the same time bear the character of "Chlamydosporen" or "Gemmen".

The 'ascus mother cell' was also capable of bud formation, even whilst the 'ascus' was still attached. The daughter cells detached themselves and normally multiplied by budding. Remarkably they could also develop into 'asci', the author reports to have noted single cells with visible spore tetrades.

A fusion of representatives of haploid cells derived from the ascospores, without the formation of conjugation tubes, was also claimed to have been seen. After fusion the larger of the two copulants sprouted, the diploid bud detaching itself from the husks of the copulants.

WINDISCH finally proposed the following life cycle: The 'ascospores' give rise to a small-celled generation. Copulation then follows and the vegetative form is obtained, characterized by cells of normal dimensions and for which the pseudomycelium is also representative. The vegetative form gives rise to 'asci' which again produce the small-celled haplophase.

WINDISCH attempts to support his scheme by claiming that nuclear staining according to the WINGE-FEULGEN technique revealed a binucleate condition of the vegetative form.

The German author extended his observations to *Candida tropicalis* Berkhout, and states to have established the same phenomena for this organism.

As for the taxonomy of *Candida pulcherrima*, WINDISCH concludes in the first place that already the formation of abundant pseudomycelium under certain conditions makes it impossible to retain the yeast in the genus *Torulopsis*, and he transfers it to the genus *Candida* Berkhout. Since this generic name has also been accepted by later authors the correct scientific name of the organism is at present *Candida pulcherrima* (Lindner) Windisch.

However, it should be realized that at the same time WINDISCH gives a new generic definition of *Candida*, quite different from that of *Candida* Berkhout as redefined by DIDDENS and LODDER (1939).

On ground of his revolutionary observations the genus *Candida* amend. Windisch is an ascosporogenous genus. In this respect WINDISCH also refers to the early observations on *Candida albicans* made by VUILLEMIN and by OKABE who – in contrast to numerous other investigators – report to have observed ascospore formation for

this organism. WINDISCH goes still further by denying that the family of the *Candidaceae*, to which the genus *Candida* is brought, should belong to the *Saccharomycetes*. According to WINDISCH the "*Candidae*" differ from the latter amongst other by their mode of ascus formation, the occurrence of special 'ascus mother cells', the presence of "Epiplasma" in the asci, and the budding of the ascus-mother cells. He is inclined to accept a relationship between the "*Candideae*" and the *Exoascaceae*, amongst which he especially mentions the genus *Taphrina*.

In 1942 DIDDENS and LODDER published the second part of the book "Die anaskosporogenen Hefen". In this book they maintained the genus *Candida* in the sense as proposed by them in 1939. On the other hand they agreed with WINDISCH's view that, indeed, LODDER's acceptance in 1934 of the name *Torulopsis pulcherrima* (Berlese) Saccardo could not be maintained. With a view to the pseudomycelium formation a transfer of the yeast to their genus *Candida* was clearly indicated.

ROBERTS in 1946 reported on a study made to check a possible relationship between the *Taphrinales* and the true yeasts. She pointed out that the haploid saprophytic phase of *Taphrina deformans* could be classified as belonging to the genus *Torulopsis*, if the classification of LODDER (1934) was employed. She then refers to WINDISCH's claims that *Torulopsis pulcherrima* was capable of sporulation and heterogamous copulation, and that it could occur in a binucleate state.

For her comparative study she chose *Taphrina deformans* as representative of the *Taphrinales*, and *Torulopsis pulcherrima* as representative of the true yeasts.

ROBERTS found similar structures in her cultures, as were described by WINDISCH, but she was unable to observe the actual discharge of the ascospores from the 'asci', and the germination of the so-called 'spores'. Although only slight indications for copulation could be found, the occurrence of conjugation tubes is claimed to have been noted. Thick-walled cells, with papillate protrusions, were also encountered in cultures of *Taphrina deformans*, and were regarded by the author also to be ascogenous structures. Microscopical examination revealed that 90% of the pigmented cultures of *Torulopsis pulcherrima* contained the typical large thick-walled cells; in 65% of the cultures daughter cells were found to be attached to them. Of the non-pigmented cultures only 7% contained these *pulcherrima*-cells, whilst only 2% had daughter cells attached. No such correlations, however, could be found for *Taphrina deformans*. Contrary to WINDISCH, ROBERTS could not demonstrate the binucleate condition in *Torulopsis pulcherrima*.

ROBERTS granted a superficial relationship between *Torulopsis*

pulcherrima and the *Taphrinales* based on cultural similarities, and on the phenomena regarded by her and WINDISCH as ascospore formation, but not on grounds of nuclear data.

She thus concluded that WINDISCH's assumption of a natural relationship between the mentioned organisms was incorrect. However, she subscribed to WINDISCH's claims regarding the ascospore formation for *Torulopsis pulcherrima*, thus justifying its removal from the fungi imperfecti. Correctly ROBERTS maintained that already the presence of pseudomycelium necessitated its removal from the *Torulopsidoideae* as formulated in 1934 by LODDER. At that time ROBERTS was unaware that a transfer of the species into the genus *Candida* had already been accepted in 1942 by DIDDENS and LODDER.

Finally it should be added that LANGERON and LUTERAAN (1947) in their study on the application of staining techniques in yeast investigations incidentally subject WINDISCH's concepts to a severe criticism. For these authors the so-called asci are merely cell wall-remnants of *pulcherrima*-cells which have died off.

The above review will suffice to give the impression that the later studies on the life-cycle of *Candida pulcherrima* have led to a rather confused picture which contains several elements conflicting with the observations of the earlier investigators.

Chapter II

PERSONAL INVESTIGATIONS ON THE MORPHOLOGY OF CANDIDA PULCHERRIMA

§ 1 Introductory remarks

The main object of the present study has been an investigation of the remarkable pigment produced by *Candida pulcherrima*, and of the conditions determining its production. In consequence hereof the first step made has been a comparative test of all strains of *Candida pulcherrima* present in the Delft collection of the "Centraalbureau voor Schimmelcultures" on their pigment producing ability.

For details regarding the way in which this investigation has been carried out the reader is referred to Chapter IV § 2. Here it may suffice to state that of the 12 strains tested strain No. 35.2.8 was characterized by its exceptionally strong production of pigment. Hence this strain has been selected for further study, and all observations reported in this thesis refer to this particular strain, unless otherwise stated.

The strain had been received by the "Centraalbureau" in 1929 from Dr K. BENEDEK of Leipzig, labeled as *Cryptococcus interdigitalis* Pollacci et Nannizzi. In 1926 the last-mentioned authors described this yeast species which they isolated from an interdigital affection of the hand of an Italian workman engaged in the wine industry. LODDER (1934) on studying the organism has identified it with *Torulopsis pulcherrima*, whilst DIDDENS and LODDER (1942) confirmed this identification, and brought the organism to the species *Candida pulcherrima* (Lindner) Windisch.

Whilst the problems connected with the pigment production will be discussed in the following chapters, a perusal of the earlier work on the morphological and cultural characteristics of the species – as reported in chapter I – made it inevitable also to collect some personal experience regarding this aspect of the organism.

In the following sections the observations made, and the conclusions which can be derived from these, will be reported. Since the observations have been mainly restricted to one particular strain, it is not claimed that they have led to a final description of the life-cycle of the species in its fullest extent.

§ 2 Cultural and morphological observations

It was indicated to submit the organism in question in the first place to the various tests adopted by LODDER, and by DIDDENS and LODDER, for their standard description.

Thus, the culture was first inoculated in malt extract. Good growth, accompanied by a weak fermentation, occurred. After 36 hours by far the majority of the cells were ovoid, this being in agreement with LODDER's description to which we refer for all further details. Some cells were united in a chain-like formation, suggesting a primitive pseudomycelium. In older cultures formation of a thin pellicle had occurred, whilst along the surface of the medium a ring had developed. Microscopical examination revealed, besides the normal cells, a few giant cells.

In plating the culture on malt agar a uniform type of colonies developed; white to cream-coloured, smooth, moist and glistening. Cells were more elongated ovals; in the vacuoles not seldom "Tanzkörperchen" could be observed.

Some of the cells had attained considerable larger dimensions, however, no round *pulcherrima*-cells of the type first described by LINDNER were encountered. In malt agar cultures 4 to 5 days old there was unmistakable evidence of the presence of a rudimentary pseudomycelium. Noteworthy too were structures showing a striking similarity with those described by PORCHET as "formes conidiennes" (See Plate I Fig. 1). As has already been set forth by DIDDENS and LODDER (1942) there is, however, no reason to consider these oval cells at the top of one or more strongly elongated cells to be conidia; it seems much more logical to accept these structures as a primitive beginning of a pseudomycelium.

In order to test the ability to form pseudomycelium under more favourable conditions the current technique of slide-cultures on potato agar was applied. Although after 7 days pseudomycelium formation was only scanty, a rather characteristic growth had developed. As is shown in Plate I Fig. 2 several large ovoid to round cells were present which all contained one or two strongly refractive bodies which proved to consist of fat, or at least of some material rich in lipids. Here for the first time cells were encountered reminiscent of LINDNER's *pulcherrima*-cells, and of BEIJERINCK's 'fat yeast'. Yet it must be acknowledged that they differed from these by the fact that the cells were not perfectly spherical, whilst the 'lipid globules' were much smaller than those in the cells pictured by these authors.

In view of the reports by WINDISCH (1938, 1940) and by ROBERTS (1946a) on the occurrence of asci in *Candida pulcherrima* it seemed most desirable to test the strain under investigation also on those media which are recommended for ascospore formation in yeasts.

In the first place use was made of the gypsum block technique; after 14 days asci could not be found. Cells approximating the *pulcherrima*-type containing large oil drops were abundant.

A preparation made from a 14 days old culture on a carrot wedge offered a similar picture, with the exception that several pseudomycelium units were encountered. Asci again were absent.

A 14 days old slant culture on V8-agar similarly contained a fair number of cells reminiscent of the *pulcherrima*-type, whilst pseudomycelium was rather well developed.

On GORODKOWA-agar the normal cell type dominated, again no asci were encountered.

On summarizing the foregoing it strikes that the four media most currently in use for inducing ascospore formation failed to reveal any indications of the occurrence of structures which could be considered as asci.

Yet another attempt was made to bring about ascospore formation by testing the acetate-medium as recently recommended for this purpose by ADAMS (1949) in the case of bakers' yeast. This medium consists of 0.04% glucose, 0.14% anhydrous sodium acetate and 2% agar. The organism is first cultivated in tomato juice for 24 hours at 30°C., and the cells collected by centrifugation. After two washings with sterile water the sediment is evenly distributed over a slant of the acetate medium and maintained at 25°C. for at least 7 days. On examining the culture after this period it appeared that only very moderate development had taken place; no asci could be found in the microscopical preparation.

Re-examination some two weeks later, however, revealed the presence of numerous large thick-walled cells containing well-developed oil globules. Most striking was the fact that many cases were to be seen in which the large cells appeared in various stages of the process of discarding their outer membranes. Not seldom cells were found where this membrane only remained attached to the cells at one spot. Also present were typical stalagmoid cell forms which contained oil globules.

Since the demonstration of the occurrence of the true *pulcherrima*-cells had not been successful – often large cells with quite noticeable fat contents were encountered, but they missed the regular shape so characteristic for LINDNER's picture – it seemed tempting to bring the organism also on a medium especially suitable for fat formation. As such the medium which STARKEY (1946) had used for this purpose in his study on the yeast, at present known as *Lipomyces starkeyi*, was chosen. This medium contains 3% glucose, 0.01% yeast extract and 2% agar (pH=7), and therefore is very poor in nitrogenous nutrients.

In these experiments, as before, a washed pre-culture in tomato

juice was used; the cells again being evenly distributed over the surface of the slant. The cultures were incubated at 25°C. After 14 days growth was rather moderate. Microscopical examination, however, revealed a most pleasing picture, which is reproduced as frontispiece. Lipid formation had occurred in great abundance, and large, round, thick-walled cells of rather uniform shape, almost completely filled with oil, were present in profusion.

Most striking was that many of these thick-walled cells again showed the phenomenon of casting off the outer membrane. All stages, from those in which the latter had just commenced its detachment to the final stage in which the enveloping membrane remained attached only at one point to the liberated cells, were to be seen.

Appearing next to the thick-walled cells containing the large lipid bodies were smaller cells of normal and even subnormal dimensions. These cells were more or less ovoid in shape. Interesting was that it not infrequently appeared that these smaller cells were ensnared by the empty husks which had been shed by the giant cells. Cases could regularly be found where the discarded sacs contained one to two small cells, especially, if on making the microscopical preparation the cell mass was thoroughly suspended in the water drop. Less frequent were the cases where three to four - and even five - cells were trapped in these membranous pouches. These structures often showed a most striking similarity to those described by WINDISCH, and also by ROBERTS, as asci. This is illustrated by Plate I fig. 3 and 4, in which respectively two and three small cells are shown to be entrapped.

Several arguments can now be forwarded against the interpretation of the said structures as asci. In the first place it was notable that nowhere in the preparation structures could be found which could be interpreted as asci in an early stage of formation. Nowhere giant cells were found which showed a budding, possibly leading to the formation of an ascus. To the contrary all structures resembling asci had more or less the same dimensions as those of the cells described by WINDISCH as 'ascus-mother cell' ("Karpogon"). Moreover, in none of these cases the cell-wall of the alleged asci was intact. It is clear that this situation is in perfect harmony with the view that this 'cell' is nothing but a discarded outer membrane of the giant cell, and this view was fully corroborated by the fact that in the preparations all grades of loosening of the outer membrane were encountered.

All these observations favour the assumption that we have to regard the thick-walled giant cells as resting stages in the development of the yeast, resting stages as have been found to occur in very diverse types of fungi, and which are generally indicated

as chlamydospores. For this type of cells the process of casting off the outher membrane has often been described. Plate I fig. 5 gives for instance the reproduction of a wall tableau drawn by BEIJERINCK in 1387 in which the process in question is clearly pictured as occurring in *Mucor racemosus*. Plate I fig. 6 shows a personal observation regarding the great similarity in the behaviour of a chlamydospore of this fungus with the phenomenon observed in *Candida pulcherrima*.

However, in degrading the alleged ascus structures to chlamydospores to which a shedded elastic outer membrane has remained locally attached, the necessity arises to explain the presence of the small cells within the open sacs. It then strikes that the cells in question do not at all resemble ascospores; there is a lack in uniformity, and moreover they strikingly resemble the very numerous oval yeast cells which occur all over the preparation. Under these conditions it seems quite acceptable that the alleged ascospores are just normal vegetative cells incidentally trapped in the open sacs with their elastic membranes.

However, as set forth in chapter I, WINDISCH has not flinched from advancing the theory that the yeast in question is characterized by two different types of 'ascus'-formation. According to this author the large cells may also give rise to fat-containing daughter cells which may be directly transformed into an ascus. It is true that WINDISCH must have recourse to the remarkable hypothesis that next to the ascospores the cells remain filled with a "Epiplasma" and the large oil drop. This drop interferes with the observation of the ascospores, but at a certain moment these cells burst open, and WINDISCH describes how he observed ascospores in the discharging mixture of lipid and "Epiplasma".

It has been carefully tried to repeat these observations, but without success. It was found that sometimes a slight pressure on the cover glass led to the rupture of the cell wall of a chlamydospore, allowing the protoplasmic debris and oil globules to stream out of the rupture, as pictured by WINDISCH for his asci of the second type. In such cases the liberated oil globules were stainable with Sudan III, but no spores were ever observed.

WINDISCH reports that most of his observations on which he based his theory of ascus formation were made in cultures on SABOURAND-agar. It seemed, therefore, desirable to study the organism also on this medium. Moreover, it was decided to include also the particular strain used by WINDISCH in the investigation.

It was, indeed, found that both strains formed also on SABOURAUD-agar chlamydospores in great abundance, although always the majority of the cells in the preparations was of the normal yeast type. Also in WINDISCH's strain it could easily be established that the

chlamydospores often shed their outer membranes. In Plate II fig. 1 and 2 one can observe some early stages in this process.

It seems probable that the shedding of the outer membrane should be considered as the first step in the resumption of active life by the chlamydospore. The liberated cell will under favourable conditions show bud formation, herewith giving rise to another generation of normal vegetative cells.

However, this is not the only way in which a chlamydospore returns to active life; in other cases a process may occur which can be described as germination.

Remarkably it was found that in a later stage of the culture of the main strain on SABOURAUD-agar several chlamydospores had spontaneously ended their period of dormancy by germination. This is clearly to be seen in Fig. 3 and 4 of Plate II.

In Plate II fig. 3 the outer membrane of the chlamydospore has at one place been pierced by the cell which remains surrounded by the inner part of the membrane. In this particular cell the pierced outer membrane is still clearly visible adhering to the back part of the cell. Here a typical dactylate cell form has resulted which by some of the earlier authors may well have been interpreted as a conjugation tube. However, any indication that conjugation actually occurs was lacking in the preparations.

Plate II fig. 4 gives very much the same picture.

Plate II fig. 5 and 6 show further stages of the germination process. Here the cell has already grown out, and practically left the outer membrane which is still clearly to be seen at the pole opposite to the site where germination started.

It is obvious that the germinated chlamydospore soon starts to proliferate by bud formation. This is amply demonstrated by Fig. 8, 9 and 11 of Plate II which pertain to germinating chlamydospores which were observed in a two months old plate culture on a biotin deficient medium used in connection with the investigation by CUTTS and RAINBOW (1950), for which the reader is referred to Chapter IX.

Plate II fig. 7 is in so far remarkable as it provides documentation for the germination of a chlamydospore of the red mutant of the yeast. Plate II fig. 8 and 9 show that bud formation may already occur in very early phases of the germination process. Plate II fig. 10 and 11 show that the empty outer membrane may remain attached to the germinating chlamydospore even in the stage that the latter has already attained the normal yeast form.

§ 3 Discussion

On summarizing the foregoing observations and considerations, the conclusion seems warranted, that the theory put forward by

WINDISCH (1938, 1940), and supported by ROBERTS (1946a), according to which *Candida pulcherrima* should be considered to be an ascomycete, has to be rejected.

In stead hereof the following description of morphological changes, as pictured in the diagram reproduced in Fig. I, may be tentatively proposed.

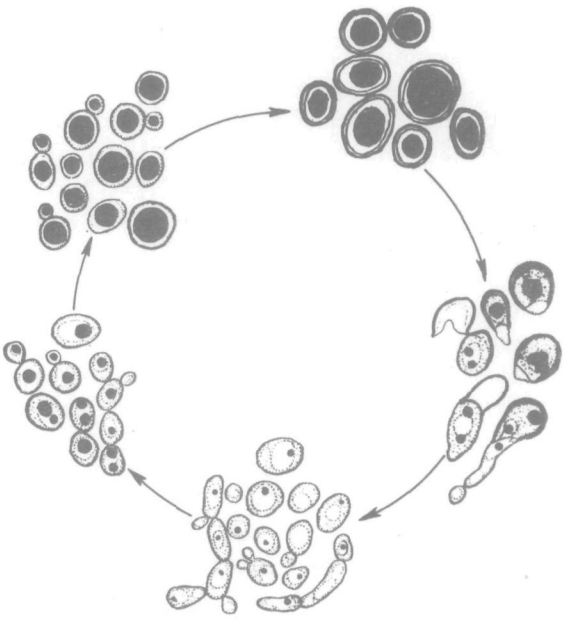


Fig. I. The proposed life cycle for *Candida pulcherrima*

The oval to round vegetative cells as present in young cultures in well-balanced media containing a suitable sugar and the necessary nitrogenous compounds – protein building stones – occur either singly or in pseudomycelial formations. These cells multiply by a normal budding process as long as the nutritional conditions remain favourable. As soon as certain nutrients become deficient, or metabolic products accumulate in the medium, the budding process slackens. Under aerobic conditions, and if carbohydrates are not yet completely exhausted, there is a tendency for the cell population to assume a more spherical shape, whilst lipids are formed in abundance in the cells. Both these cells and the lipid globules increase in size, thus giving rise to the typical *pulcherrima*-cells of LINDNER and BEIJERINCK. Under certain conditions a thickening of the cell wall takes place, ultimately leading to the formation of the typical chlamydospores. Usually

a period of dormancy sets in, during which the chlamydospores apparently may maintain themselves at the expense of the high energy reserve nutrient: the lipid drop. When conditions again become favourable for active growth the ripe chlamydospores either shed their outer membranes, and multiply by budding, or a process of germination sets in, in which the cell pierces the outer membrane, sometimes forming dactylated structures, which ultimately also give rise to the normal vegetative forms by budding. These blastospores may remain united in chain-like structures, thus forming a pseudomycelium.

However, the general picture as given above, is clearly in want of some supplementation. It seems apt to remind here of the observations made by BEIJERINCK, as referred to in chapter I. It will be clear that the form indicated by this author as the *secundarius* mutant is considered in the foregoing as the main normal form of the organism in question. However, it is not permissible to neglect BEIJERINCK's primary observation that on one and the same plate of malt agar or malt gelatin he obtained both colonies of his *secundarius* type and of what he calls *pulcherrima* cells, which in the foregoing have been considered as the precursors of the chlamydospores. Although with the strain under investigation it has never been possible to repeat these observations, one cannot escape the conclusion that in BEIJERINCK's case there must have been some dissociation between colonies in which the normal cells maintained themselves, and others which were characterized by a predominant formation of *pulcherrima*-cells. Moreover, since BEIJERINCK reports that he was able to maintain at least to a certain extent this *pulcherrima*-generation – he admits that reversion to his *secundarius* type frequently occurs – one is more or less compelled to conclude that there must also be conditions under which *pulcherrima*-cells show proliferation by budding, leading to young cells also characterized by the presence of large oil globules which gradually increase in size, thus giving rise to new *pulcherrima*-cells.

Chapter III

SOME REMARKS ON THE OCCURENCE OF CHLAMYDOSPORES IN YEASTS

§ 1 Introductory remarks

As appears from the previous chapter typical chlamydospores are not seldom encountered in *Candida pulcherrima*. In this respect it should be reminded that even WINDISCH does not refrain from occasionally indicating his 'ascus-mother cells' by this name. The occurrence of chlamydospores in the yeast in question has also been clearly recognized by LANGERON and LUTERAAN (1947, 1949).

It should be added at once that more or less casual references to the occurrence of chlamydospores in other yeast species are frequently found in literature.

Already in 1899 DAIREUVA gave a clear description of chlamydospores in *Candida albicans*, and this observation was confirmed in the same year by VUILLEMIN. Since then several other authors have made the same observation, so for instance LANGERON and TALICE in 1932.

For *Candida mortifera*, a yeast later identified as *Candida pseudotropicalis*, REDAELLI (1925) reports the occurrence of chlamydospores. CARNEVALE-RICCI (1926) did the same for *Torulopsis tonsillae (Candida pelliculosa)*. Analogous reports were made by POLLACCI and NANNIZZI (1928) for *Monilia fioccoi (Candida parapsilosis)*, and by REDAELLI and CIFERRI (1934) for *Blastodendrion flareri (Candida intermedia)*.

Outside the genus *Candida*, MOORE (1935) described chlamydospores in the yeast *Hemispora coremiformis (Trichosporon cutaneum)*.

In view of these references the question arises whether the ability to form chlamydospores is a general property of large, more or less well-defined, groups of yeasts, and if so, whether the occurrence of this characteristic cell form has any significance from a taxonomic standpoint.

The first part of the question cannot be answered on the basis of the available data. However, it seems certain that it is impermissible to conclude to the absence of a chlamydospore producing ability in certain species, merely because until now such spores have not yet been encountered.

It should be realized that a systematic investigation in this direction has never been made, simply because the conditions which

determine chlamydospore formation in fungi, and more particularly in yeasts, are not at all, or only very imperfectly known.

This leads to the question whether an attempt to collect these data may be deemed worth-while.

In this respect the statement of DIDDENS and LODDER (1942, p. 241); "Jedenfalls kann der Chlamydosporenbildung kein grosser systematischer Wert beigelegt werden" is certainly not encouraging.

Now it must be acknowledged that this statement is in so far quite justified that the occurrence of chlamydospores may under present conditions not be considered to be specific for the genus *Candida*. For the said authors have incontestably established the occurrence of chlamydospores also in an ascosporeforming yeast, namely *Saccharomyces fragilis*, the perfect stage of *Candida pseudotropicalis*.

On the other hand, however, LANGERON and LUTERAAN have in two rather recent papers (1947, 1949) again drawn attention to the chlamydospores as a characteristic worthy of consideration in yeast description. They point out that the chlamydospore has particular affinities for certain dyes. This is clearly evidenced by the fact that the chlamydospore – in contrast to the normal vegetative yeast cell – shows a marked degree of acid-fastness on staining, a property which remarkably is shared by the ascospores. This even leads the French authors to the statement that ascospores can be considered as functional chlamydospores.

Such a dictum throws, of course, a new light on the possible biological status of the chlamydospores, and it is tempting to conclude that a further investigation of the conditions determining the formation of chlamydospores, together with a more systematic search for these structures in the yeast domain, may well yield interesting results.

§ 2 Personal observations on the occurrence of chlamydospores

In the previous chapter it has been stressed that particular STARKEY's medium for fat production was very favourable for the induction of chlamydospore formation in *Candida pulcherrima*, an observation which was repeadtedly confirmed.

It was then decided to check on these spores LANGERON and LUTERAAN's statement that chlamydospores are characterized by a high degree of acid-fastness.

Flame-fixed preparations were steamed for five minutes with ZIEHL-NEELSEN carbol fuchsin, and then decolorized for three to five minutes with acid alcohol (97% alcohol, 3% conc. HCl). The chlamydospores contrasted beautifully by their red to red-black colour with the colourless vegetative cells (Fig. 12 of Plate II).

The next step was to test the suitability of STARKEY's medium for chlamydospore formation in some other yeast species belonging to divergent genera.

Candida reukaufii, *Torulopsis diffluens* and *Debaryomyces hansenii* were chosen as such. These organisms were cultivated for 24 hours in tomato juice. Growth was collected by centrifugation, washed twice with sterile water and transferred to slants of STARKEY medium. These cultures were maintained at 25°C. for two weeks.

Microscopical examination of the culture of *Torulopsis diffluens* revealed many thick-walled cells. Some of these, as is shown in Plate III fig. 1, had detached their outer membranes to form husks similar to those observed for *Candida pulcherrima* under the same circumstances. Other cases were noted in which germination of a chlamydospore was evident, as is shown in Plate III fig. 2. Flame fixed preparations were stained with carbol fuchsin, and subsequently treated with alcohol containing 3% HCl. The chlamydospores retained the dye quite convincingly.

The culture of *Candida reukaufii* gave a rather different microscopical picture. Typical was the marked tendency to form pseudomycelium with large, terminal or intercalar, thick-walled cells of spherical or stalagmoid form (cf. Plate III figs. 3 and 4). These thick-walled cells readily split their outer membranes allowing the encysted cells to emerge from the remnants. Typical for these large cells again was their marked degree of acid-fastness.

In the case of *Debaryomyces hansenii* microscopical examination revealed a tendency towards fat production in most cells. Further present were numerous larger thick-walled cells (Plate III fig. 5), several of which had again detached their outer membraned (Plate III fig. 6). Acid-fast structures were readily demonstrated according to the ZIEHL-NEELSEN technique (Plate III fig. 7). No true asci were encountered in either vital or stained preparations.

The demonstration of chlamydospore production in the three organisms mentioned – more or less selected at random – offers tangible support for the need of a detailed investigation into the occurrence of, and the conditions most favourable for, the formation of these spores.

The superficial survey of the literature, as given in § 1, indicates that these structures have in the past most frequently been encountered in the genus *Candida*. The fact, however, that chlamydospore formation could be demonstrated in the case of *Torulopsis diffluens* creates the impression that these structures may be encountered in the cases of many other asporogenous yeasts.

The demonstration of chlamydospores in the case of *Debaryomyces hansenii*, and the fact that DIDDENS and LODDER (1942) encountered chlamydospores in a culture of *Saccharomyces fragilis* offers sufficient evidence that – contrary to what LANGERON and LUTERAAN (1949) presumed – chlamydospore formation may also occur in sporogenous yeasts.

A first condition for a thorough investigation into the distribution of chlamydospores in yeasts, is, of course, the elaboration of methods suitable for the induction and demonstration of these structures. Media such as BENHAM's corn meal agar and potato infusion or potato agar have been recommended for this purpose, especially in the case of *Candida albicans*. The medium of STARKEY used in this study has the advantage of having given reproducible results for four divergent yeast species, and therefore recommends itself for further application.

We find here a confirmation of LANGERON and LUTERAAN's conclusion that conditions which favour lipid production also seem to be suitable for the induction of chlamydospore formation. It has to be admitted that at present methods for this induction are mainly empirical, but the same also holds for the methods generally in use for ascospore formation.

The marked degree of acid-fastness and the thick cell walls, the outer part of which under certain conditions may be shed, offer easily recognizable characters for the establishment of the occurrence of chlamydospores.

Altogether the conclusion seems warranted that the chlamydospore is not merely an old vegetative cell characterized by a thickened cell wall, but a well devised resting stage in the life-cycle of the organism.

§ 3 Suggested significance of chlamydospores for the pathogenicity of yeasts

Candida albicans is the first yeast species in which the occurrence of chlamydospores was established with certainty. It is, curiously enough, also the yeast which is most frequently encountered as an infective agent in affections of the mouth, lungs, vagina, skin and nails and also in systemic "moniliasis". As appears amongst other from Chapter II *Candida pulcherrima* can also occur in such infections.

Other members of this genus, however, such as *Candida krusei*, *Candida tropicalis*, *Candida pseudotropicalis*, *Candida guillermondii*, *Candida pelliculosa* and *Candida parapsilosis* have also been isolated from pathological conditions of the lung. The remarkable feature is that for many of these yeasts also the occurrence of chlamydospores has been proved, and for the others such an occur-

rence must be deemed very probable.

It is of topical interest that in recent years as a result of treatment of human patients with the more recently discovered antibiotics, cases of "bronchomoniliasis" and of "moniliasis" of other organs have become much more frequent, and here again as a rule *Candida albicans* is found.

All this suggests a connection between pathogenicity of yeasts and their ability to form chlamydospores. The production of chlamydospores might provide the invading yeasts with a natural defence against the forces of the "milieu intérieur" of the invaded host.

The following considerations seem to have a bearing on the subject. CASTELLANI (1905, 1910) was the first to make a thorough study of "bronchomoniliasis" and other bronchomycoses, and it cannot be denied that in some of his cases, especially the more serious ones, the clinical picture showed a great resemblance to pulmonary tuberculosis. Later on, PIJPER (1916, 1924) in rather extensive studies on "bronchomoniliasis" and *Monilia*-fungi in sputum gave photomicrographs of sputum which *inter alia* show structural elements, now identifiable with chlamydospores.

The question thus arises whether it is merely fortuitous that the two types of micro-organisms which cause similar pulmonary affections, *Mycobacterium tuberculosis* and the various *Candida* species from lungs, have one remarkable and rather rare property in common, namely the formation of acid-fast elements.

It happens frequently that yeasts, either alone, or in combination with acid-fast bacteria, are isolated from patients suffering from or suspected of pulmonary tuberculosis. This had led REENSTIERNA (1912) to his original and bold idea that the acid-fast bacilli of KOCH are "merely fragments or evolutionary forms of lower, probably quite common place, in nature occurring fungi, which at one time far back in the past entered and at times still enter the human organism...." (REENSTIERNA, 1936).

This idea has found supporters in Sweden. HOLLSTRÖM in 1943 presented a report of a detailed study in which he claims to prove the correctness of the earlier views of REENSTIERNA, and also those of GULLBERG of 1938. The main argument of HOLLSTRÖM is the finding of acid-fast cells in pure cultures of *Monilia pinoyi* (probably *Candida albicans*), which yeast he had isolated from patients suffering from or suspected of pulmonary tuberculosis. It must be pointed out that HOLLSTRÖM also observed the presence of double-contoured cells in stained sections of the brains of experimentally infected animals. This finding, in combination with the recognized acid-fast nature of these elements strongly suggests that the cells observed were merely the chlamydospores so often

present in *in vitro* cultures of *Candida albicans*.

There is therefore every reason to reject the views of the Swedish authors about heterogenesis from a yeast to a tubercle bacillus. But it seems worth while to consider whether the higher degree of natural resistance which might well go with acid-fastness has not something to do with the prolonged existence and infectiveness of both yeasts and tubercle bacilli in lungs. It is their one common characteristic and both microbes are found under similar pathological conditions, either separately or together. It must be left to further investigations to provide an answer to this question.

Chapter IV

THE ISOLATION AND MAINTENANCE OF A RED MUTANT

§ 1 Survey of earlier investigations

In Chapter I a survey has been given of the earlier observations on the morphology of *Candida pulcherrima*. In this survey no mention has been made of one of the properties which is responsible for a great part of the interest which this yeast has aroused in later years. Herewith reference is made to the fact that under certain conditions cells can be encountered which are characterized by the production of a red pigment, and which may ultimately lead to a deep red coloration of the yeast colony.

The first author who mentions the faculty of *Candida pulcherrima* to produce a red pigment is LINDNER who in 1915 – fourteen years after his first description of the yeast – casually remarks that "rötlich gefärbte Kolonien" may occur.

In the same year GROSBÜSCH (1915) made a series of observations on the pigment production by a yeast named by him *Torula rubifaciens*, but which has been identified by later authors with *Candida pulcherrima*. GROSBÜSCH noted that his isolates, obtained from apple rinds, produced a red to maroon seemingly water soluble pigment on various liquid and solid media. He used many divergent media, partly of a very complex nature. To a medium consisting of sugar, ammonium phosphate and some tartaric acid he added apple must and malt extract. But also yeast water glucose, wort, milk and grape- and apple musts were used. Solid media were prepared by the addition of gelatin to the above, while potato- and apple wedges were also used. In liquid culture the pigment was either retained in the sediment or in the pellicle, but it could also be dispersed in the medium. On solid media the pigment was similarly either retained in the colonies, or diffused into the gelatin. As far as liquid media were concerned, it was found that pigmentation was more striking in complex semi-synthetic media than in yeast water glucose. Further observations led him to conclude that pigmentation was dependent on four factors. GROSBÜSCH considered the nature of the carbohydrate source to be of primary importance. With arabinose, raffinose, maltose, lactose and galactose better pigmentation was obtained than with saccharose, which on its turn was preferable to glucose or fructose. Secondly, an increase in sugar concentration above 2.5% depressed pigmentation progressively in the case of saccharose. The third factor, according to GROSBÜSCH, was the sterilization time, which was

inversely proportional to the intensity of pigmentation. Lastly he mentions the degree of acidity. He found that the addition of tartaric acid – except in very small quantities – was unfavourable for pigmentation.

GROSBÜSCH offers the following partial explanation of his observations. Sugars which can not be fermented afforded the best pigmentation. Sterilization invariably leads to hydrolysis, and the ensuing formation of fermentable sugar leads to the production of large quantities of carbon dioxide and ethanol which depress pigmentation. Light was without any influende on the production of pigment. Whether or not the presence of oxygen was essential for pigmentation he was unable to give a definite opinion on.

BEIJERINCK's classical study in 1918 of *Candida pulcherrima* revealed a quite new aspect of the problem of pigment production. He established that *Candida pulcherrima* owed its pigment to the presence of iron salts in the medium. Moreover, he found that the dependency of pigment production on iron was shared, although to a much smaller degree, by various other yeasts normally occurring in milk and cheese. BEIJERINCK then developed a medium facilitating the identification of his *Saccharomyces pulcherrimus* by adding a sufficient amount of an iron salt to a 2% glucose malt agar. On this medium not seldom typically pigmented colonies developed. Since the organism was an avid monose fermenter, it could readily be distinguished from the vulgar non-fermentative red yeasts which owe their colour to the presence of lipochrome pigments.

BEIJERINCK still made a second important observation. He cultivated the yeast on an agar medium to which no extra iron addition had been made, and after colourless growth had occurred he made some soluble ferric salt diffuse into the medium. In the diffusion zone not only the colonies turned red, but pigment was also produced in the agar medium at some distance from the colonies. BEIJERINCK concluded from this observation that the yeast secreted some colourless precursor ("chromogène incolore") which reacts with iron to give the pigment. However, BEIJERINCK also noted that, if the plate was partially covered by a cover slip, no pigment was produced under the cover slip. He interprets this result by accepting that besides the precursor and the iron also free oxygen is indispensable for pigment production.

BEIJERINCK supports his theory of pigment formation by the demonstration that on media with a low iron concentration the precursor is formed in excess to the iron available in the immediate vicinity of the cells, so that pigment production only occurs in the medium surrounding the colonies. If more iron is available in the medium, the conversion of the precursor into the pigment already takes place in the cells themselves.

BEIJERINCK stresses the instability of the property of pigment production: upon plating a pigmented colony on a malt medium with a suitable iron content, besides colonies which are more or less strongly pigmented, always also perfectly white colonies will develop. BEIJERINCK considered the pigmented type to be a mutant, since it was by far the less stable of the two, and it could only be maintained by frequent subculturing. Thus when an aged red culture was plated on an agar medium containing sufficient iron, the majority of the colonies proved to be white. BEIJERINCK was inclined to explain this reversion to the stable non-pigmented form, by the assumption that strongly pigmented cells soon became non-viable due to the fact that during pigmentation lethal iron concentrations had accumulated in these cells.

He states explicitly that the mutation into the red type was not at all linked with the earlier described differentiation into the fat-forming and his so-called *secundarius*-type. Both types were able to yield red forms.

Fifteen years later, in 1933 PUNKARI and HENRICI again took up the study of the colour variations occurring in cultures of *Candida pulcherrima*. For their investigations they chose a subculture of GROSBÜSCH's original strain of *Torula rubifaciens* which they found to be identical with *Torula pulcherrima*. They were of the opinion that the variations in *Torula pulcherrima* were of the same nature as those exhibited by many bacteria. Like BEIJERINCK, they considered the organism particularly favourable for the study of microbic variation due to the fact that single cell-cultures were easily obtainable, the colour variations striking and sexual reproduction excluded. Their choice in media fell on a 5% glucose peptone agar, to which they did not add iron, despite the fact that they were acquainted with BEIJERINCK's paper.

Four single-cell isolates were made and these cultivated into four giant colonies. These giant colonies gave rise to the occurrence of red mutants. Partly these appeared at irregular spots or in rings in the colonies, sometimes typical red sectors or lobes were formed. A second generation was cultivated by making fresh single cell cultures from different areas of the four giant colonies of the first generation. This process was repeated until four generations had been obtained, the investigators having studied the individual characteristics of three-score giant colonies. From their observations they conclude that neither sexual reproduction, nor life-cycles were responsible for the observed variations. In short they were unable to state for fundamental cause for the transformations.

The dissociative variations could be split into two types, *i.e.* red and white, next to rough and smooth. These four characterist-

ics could occur independently of one another. Red-smooth, red-rough and white-smooth occurred frequently, but in no case was white-rough encountered. The rough-red forms were always associated with the tendency to form rudimentary mycelium. The authors ascribe their failure to establish the cause for these at random occurring transformations to two facts, *viz.*, to the artificial selection which had been practised during the entire duration of their investigation, and secondly to the fact that the degree of mutability of the giant colonies increased with age.

Two years after their first publication PUNKARI and HENRICI in 1935 gave a second account of the spontaneous variations of *Torula pulcherrima*. They had continued their observations through a further six generations, although the paper dealt only with the progeny of one of the colonies from the fourth generation of their first study. Having abandoned the single cell technique, they now resorted to mass transfers, while for the rest, the technique remained essentially the same. Their study was instructive in so fas as that they discovered what they concluded to be the fourth variant, *i.e.*, white-rough, and further that the transformations of white into red occurred in the young cells at the periphery of the colony, while the reverse took place in aged cells towards the centre of the colony. Like BEIJERINCK they found that the red form was the less stable of the two, and they expressed their disbelief that the red form could be stabilized indefinitely.

PORCHET in 1938 in a comparative study of ten strains of *Torulopsis pulcherrimus*, isolated from Swiss fruit, applied pigmentation as one of the characteristic properties. The media used consisted of fruit juices, fruit juice-gelatin with and without addition of peptone, yeast water with various sugars, and GORODKOWA-agar. On fruit juice-gelatin and GORODKOWA-agar red, rose-white and bi-coloured colonies having mat or glistening surfaces, were noted. In liquid cultures, a pellicle was formed and later a sediment. In some cases the pellicle was fragile and colourless, while in others it assumed a rose-coloured, slimy, smooth or wrinkled appearance. In the case of pigmented pellicles, the cells were elongate and lateral bud formation occurred. Pseudomycelium was thus only encountered in pigmented forms. Pigmentation was capricious. She considered the pigment to be distinctly different from the lipochrome pigments occurring in the *Rhodotorula* and *Sporobolomyces* species, since the pigment of *Candida pulcherrima* could, in contrast to the carotenoid pigments, diffuse into both liquid and solid media. PORCHET, aware of the fact that BEIJERINCK connected pigmentation with the presence of iron salts, made additions of ferrous sulphate or ferric chloride to her media, but

found that pigmentation was not particularly enhanced. From this, she concluded that iron played no rôle, and that pigmentation was to be attributed to some internal and yet unknown factor. She was inclined to ascribe the dissociation noted to changes in the chromosome structure or in their distribution.

It is clear that PORCHET bore no knowledge of the investigations of PUNKARI and HENRICI (1933, 1935).

CASTELLI (1940) opposed the opinion of PORCHET that iron played no rôle in the pigmentation of *Candida pulcherrima*. He isolated twenty-three strains of this organism from musts, especially of the chianti type. He investigated the pigmentation of his strains on grape juice-agar, malt agar and peptone-saccharose agar, with or without additions of iron. His findings led him to subscribe fully to the claims of BEIJERINCK (1918).

As already set forth in Chapter II, ROBERTS in 1946 published a report on an investigation in which she sought to establish relationship between the *Taphrinales* and the yeasts. For her comparative study she chose *Taphrina deformans* as representative of the former and *Candida pulcherrima* of the latter. Her cultures were maintained on a mixed vegetable agar and on a potato glucose agar. On solid media both organisms displayed pigmentation, the pigment of *Candida pulcherrima* differing from that of *Taphrina deformans* in that the former could diffuse into the agar. An analysis was made of no less than approximately three hundred and fifty single cell isolates of *Candida pulcherrima*. It was found that isolates from non-pigmented colonies and from white sectors of bicoloured colonies usually gave rise to white colonies. Isolates from red sectors, as a rule gave rise to bicoloured colonies.

The stability of the white variant and the instability of the pigmented areas were found constantly in 90% of her isolates. In the remaining 10% three different cases could be distinguished. Firstly, single cell isolates from the marginal areas of pigmented sectors gave rise to entirely pigmented colonies which failed to show reversion to white. The second exception was the occurrence of brown sectors in a red portion of a bicoloured colony. Single cell isolates from these brown sectors again gave rise to normal red colonies. Lastly was the comparatively rare variation where pigmented colonies arose from single cell cultures from either white colonies or from white sectors of bicoloured colonies. ROBERTS unable to account for the variations observed in the pigmentation phenomenon tentatively suggested that the predominant unidirectional mutation from white to red may be regarded as a type of dual phenomenon, in which she considered the stable white form as the homo-type. She is inclined to connect the variations with the alleged sporulation as described by WINDISCH (1938, 1940)

and which she thought to have confirmed. She based this conclusion on the fact that in about 65% of her pigmented cultures thick-walled cells with attached daughter cells, *i.e.* ascogenous cells, were encountered, while only 2% of the non-pigmented cultures contained these structures.

In a second paper in the same year ROBERTS gave an account of a study made on the effect of iron and other factors influencing pigmentation of *Torulopsis pulcherrima*. Potato-dextrose agar was chosen as medium for this investigation. In the first instance it was found that pigmentation was closely correlated with the amount of nutritional substance at the disposal of the organism. Maximum production of pigment occurred where isolated colonies grew on sufficient amounts of substrate.

The rôle of oxygen was then investigated and the author proved beyond all dispute that molecular oxygen was a decisive factor, in the absence of which no pigmentation could occur.

Light was without influence. Temperature had no effect on the production of pigment, but temperatures below 19°C. seemed to favour the retention of the pigment in the colonies. The author then set out to verify BEIJERINCK's statement that pigmentation was dependent on the presence of iron. Initially additions of ferric ammonium sulphate and ferrous ammonium sulphate were made, and it was found that an increase of the iron content of the medium up to 0.1% progressively enhanced pigmentation. Higher concentrations were inhibitory to growth. Concentrations of 0.0001% to 0.001% gave an increase of diffusion of the pigment in the agar. At 0.01% diffusion of the pigment was greatly inhibited, and at higher concentrations the pigment was retained in the colonies. Since iron salts are subjected to a high degree of hydrolysis above pH 5, ferric ammonium citrate, as originally proposed by BEIJERINCK, was employed by her to determine at what concentration of this salt diffusion was inhibited. This was found to vary for the different isolates. The hydrogen ion concentration was found to influence pigmentation too. Batches of media, to which no additional iron was added, were made up to different pH values. It was then found that as the pH increased and accordingly the ionic iron concentration fell, pigmentation decreased. From some observations ROBERTS is inclined to conclude that the presence of iron stimulates the synthesis of the chromogen postulated by BEIJERINCK. An equal number of single cell isolates of pigmented and hyaline cells were made from a pigmented colony growing on potato-dextrose, 0.1% ferric ammonium citrate agar. Analysis showed that 70% of the hyaline cells proved to be viable, while all pigmented cells were found to be incapale of budding. This may be deemed to be an experimental proof of BEIJE-

RINCK's postulate that pigmented cells are poisoned by the retention of such high concentrations of iron. She links this with her observation that growth is barely supported by a medium containing 1% ferric ammonium citrate.

§ 2 The isolation of a red mutant of strain 35.2.8

As already stated, the chief aim of the present study has been an investigation of the remarkable pigment produced by *Candida pulcherrima*, and the factors determining its production. For this purpose it was necessary to obtain a strain which under favourable conditions gave maximum pigmentation, and in which this property could be well maintained.

Previous workers who had incidentally studied the pigmentation of *Candida pulcherrima* in the "*Laboratorium voor Microbiologie*" at Delft, had paid some attention to these aspects. Their observations indicated that two strains, present in the collection namely No 35.2.8 *Cryptococcus interdigitalis* (POLLACCI et NANNIZZI) and No 35.2.1. *Torula* spec. No 44 (Melliger), were the most suitable for the production of the pigment. The general experience was that by plating the strains on a peptone, 4% saccharose agar medium, as originally prescribed by BEIJERINCK and to which a trace of ferric chloride, or preferably of ferric ammonium citrate, was added, a mixture of red and white colonies was obtained. By the subsequent plating of a single well-isolated pigmented colony, plates were obtained on which only red colonies had developed. Similarly these earlier workers found that cultivation at 30°C. gave most abundant growth and most profuse pigmentation.

The twelve strains of *Candida pulcherrima* present in the collection of the Yeast Division of the Centraalbureau were all tested on their ability to elaborate the characteristic red pigment in the presence of iron salts under suitable nutritional conditions. By subculturing these strains from the as a rule quite colourless malt agar cultures on agar slants of the following composition: 2% saccharose, 1% cornsteep liquor, 0.05% ferric ammonium citrate, 2% agar, pH 6 in all cases a pronouncedly pigmented growth developed. The property of pigment production may, therefore, be regarded as characteristic for the species.

Since in accordance with the experiences of the earlier workers strain 35.2.8 proved to be the best pigment producer, this strain was selected for the further investigations.

A culture from malt agar was plated on the following agar medium: 4 g saccharose, 2 g Bacto-peptone, 0.2 g KH_2PO_4, 0.05 g $MgSO_4 . 7\ H_2O$, 1 ml yeast autolysate, 2 g agar, 0.05 g ferric ammonium citrate, 100 ml tap water. The plates were incubated at 30°C. This medium is essentially the same as that prescribed by

BEIJERINCK, and will be henceforth be referred to as Modified BEIJERINCK Medium, abbreviated by M.B.M.

After seven days two colony types could be distinguished: large, white and smooth in appearance – some of which had commenced with slight pigmentation at the centre – next to a smaller maroon to bordeaux red type, generally rough in appearance.

By subculturing a well isolated rough maroon colony on a plate of the same composition the satisfactory result was obtained that only pigmented colonies developed.

On examining a young well-pigmented colony microscopically the occurrence of pigmented cells was at once noticed. Two types of coloured cells could be distinguished, *i.e.*, the exopigmented and the endopigmented cells. The former are by far the most striking. In these exopigmented cells, generally ovoid and measuring on the average (5.8×8.0) μ, evidently the pigment is fully localized at the outer surface of the cells wall, where it is deposited as a deep red somewhat wrinkled membrane enclosing the cell. In the case of the endopigmented cell the occurrence of the pigment is limited to a pink to red vacuole.

Associated with the pigmented phase is the occurrence of fat or oil globules in the cells, which globules readily stain with Sudan III. Not uncommon too is the occurrence of pigmented and hyaline filamentous structures which can be regarded as a primitive pseudomycelium.

In the young colony the exo- and endopigmented cells averaged from 10 to 20% of the total. Since, however, on plating a suspension of this colony one may obtain 100% red colonies, it should be concluded that also the cells which do not show any pigmentation when observed under the microscope are nevertheless potential pigment producers.

From one colony of the plate in question a subculture on a slant of M.B.M. was made. This subculture gave rise to an apparently homogeneous red growth.

Experience has shown that it was possible to maintain subcultures from this culture in the same state, and these subcultures have been the starting-point for all further experiments reported in this thesis.

Although this has proved to be quite satisfactory from a practical point of view, the results communicated in the following section should be taken into account.

§ 3 The maintenance of the red mutant and its reversion to the non-pigmented form

As has already been remarked at the end of the preceding section, it has been found possible to maintain the red mutant in an

apparently pure state for almost three years.

However, from time to time some complications have been encountered. These complications proved to be of a twofold nature. Partly they were due to the fact that at first sight negligible changes in the medium proved to injure the pigment production.

But secondly it has been found that, despite the apparent homogenity of the culture, it always contains cells which have reverted to the non-pigmented form and which produce white colonies also under conditions optimal for pigment production.

In the following a survey will be given of the said experiences.

In the first place it was then found that sometimes fresh batches of M.B.M. failed to produce a suitable red culture. It is true that in these cases pigmentation invariably occurred in the agar, but the actual cell mass remained white or only became very poorly pigmented, despite the fact that the same additions of iron had been made as in the earlier experiments. The disturbing factor was traced to be present in the peptone as is clear from the following experiments.

A comparison was made of the following nitrogen sources: Difco Bacto-peptone, Difco casamino acids (technical), G.B. casein hydrolysate (enzymatic) and a batch of Organon peptone which had given the unsatisfactory pigmentation. These preparations were on turn substituted as nitrogen source in M.B.M.; a high grade agar (Difco Bacto agar) was used in all cases. The media were prepared in the usual manner with the addition of 0.05% ferric ammonium citrate. Plates were made and inoculated from a suspension of a young culture of the red mutant and incubated at 30°C. for four days.

Growth on all the plates was satisfactory, perhaps with the exception of the plate in which the Difco Casamino acids were used, where growth was less abundant. Pigmentation, however, showed greater variations.

Where Bacto-peptone served as nitrogen source fine deep maroon colonies were obtained with no pigment formation in the agar. Pigmentation on the plates containing Bacto casamino acids (technical) as nitrogen source was less intense than on the plates containing Bacto-peptone. On the plates where the enzymatic casein hydrolysate served as nitrogen source, pigmentation of the growth was poor, pigment was neither present in the substrate. In the case of plates containing Organon peptone the colonies were rose tinted, with a profuse pigment formation in the agar. It seems probably that this particular batch of peptone is rich in chelating compounds, so that the free iron content in this medium will be extremely low.

On ground of these experiments later Bacto-peptone has always

been applied for the preparation of M.B.M. used for the stock cultures.

A second experience proved to be of a quite different nature. If subculturing was done every seven to ten days results always were quite satisfactory. However, it was found that on subculturing older cultures not seldom white growth zones developed in the young cultures.

In agreement herewith it was observed that also on aged plates pigmented colonies not infrequently reverted to non-pigmented secondary growth, which when plated again gave rise to both red and white colonies. Similarly it was found that colonies which initially were white slowly turned red on aging.

In this respect it is interesting to refer to the statement of PUNKARI and HENRICI (1933) "that the degree of variation observable in a giant colony progresses steadily with the age of the culture".

On the other hand it would not be justified to conclude from the benificiary result of frequent subculturing on the maintenance of uniformly pigmented cultures that the young cultures do not contain cells able to revert to the non-pigmented form.

In this connection it seems of interest to report here some observations which were made incidentally, namely in a series of experiments which were made to answer the question, whether complex organic nitrogen containing compounds were essential for pigment formation.

To this purpose a five days old well-pigmented culture of the red mutant grown on M.B.M. was plated on a synthetic medium in which ammonium sulphate was the sole source of nitrogen.

The medium had the following composition:

10 g saccharose, 5.0 g $(NH_4)_2SO_4$, 1.0 g KH_2PO_4, 0.16 g K_2HPO_4, 0.1 g $MgSO_4.7H_2O$, 0.5 g NaCl and 20 g Bacto-agar, which had been washed fifteen times with destilled water and once with redistilled ethanol, were dissolved in 990 ml distilled water. To this was added 1 ml of a trace element solution containing 400 γ Zn as $ZnSO_4.7H_2O$, 1150 γ Fe as $FeSO_4.(NH_4)_2SO_4.6H_2O$, and 25 γ Cu as $CuSO_4.5H_2O$. The medium was further supplemented with 1 ml of a vitamin solution containing 200 γ thiamine, 200 γ pyridoxine, 200 γ nicotinic acid, 200 γ calcium pantothenate and 2 γ biotin. Batches of 99 ml were autoclaved at 110°C. for 15 minutes.

All chemicals used were of analytical purity.

To each of these batches 1 ml of a sterile 5% solution of ferric ammonium citrate was added.

The plate was incubated at 30°C.

After 48 hours white colonies had appeared on the plate.

After 72 hours the colonies had commenced with pigment form-

ation. The majority was, however, still colourless; pigmentation commenced either at the periphery or more or less in the centre, and then expanded throughout the whole colony. On viewing the plate from the reverse side, it was noted that the cores of the pigmented colonies were still white.

After 96 hours had elapsed, a great number of smooth and glistening colonies characterized by a deep maroon to bordeaux-red colour was present. However, next to these there were several colonies which were devoid of any pigment whatsoever, as is shown on Plate IV fig. 1.

The plate was left for another seven days at room temperature. The colonies were now entirely pigmented with the exception of a few colonies which had remained perfectly colourless. Pigmented colonies were a deep maroon with either smooth glistening or rough mat surfaces (see Plate IV fig. 2).

In the first place these observations leave no doubt that pigment production can occur in the complete absence of an organic nitrogen source in the medium.

The main result, however, is a convincing demonstration that also in a young apparently homogeneous culture of the red mutant cells occur which also under conditions suitable for pigment production give rise to colonies which remain completely colourless.

Finally a series of experiments has been made in order to test in how far the stability of both the white and the red form would be influenced by continued subculturing on media with a low and a high iron concentration respectively. In this respect it should be remembered that ROBERTS (1946b) is inclined to conclude that iron is not only a reactant in the final step of pigment formation, but also acts as a stimulant in the production of the precursor. This might imply that a continued cultivation on an iron-poor medium might favour the stabilisation of the white form, whilst an iron-rich medium might promote the predominance of the red mutant. In order to test this the following experiments were made. 5 g peptone, 10 g saccharose, 0.5 g KH_2PO_4, 0.01 g $MgSO_4 . 7H_2O$ and 2.5 ml yeast autolysate were dissolved in 250 ml water contained in a litre flask and shaken for 30 hours together with 50 ml of a 0.1% solution of 8-hydroxyquinoline in redistilled chloroform. The nutrient solution was shaken with a second portion of the 0.1% 8-hydroxyquinoline for a further twenty hours, and then shaken with several fresh portions of pure chloroform to remove possible traces of the quinoline. Quantitative removal of iron was not aimed at, nor could this be expected, since peptone normally contains chelating agents such as α-amino acids and α-hydroxy acids which will compete with oxine.

To batches of 99 ml of the nutrient solution, containing 0.1 ml

trace elements solution, was added 2 g Bacto agar, and the medium then autoclaved at 110°C. for 15 minutes. To one batch of the sterile agar sol 1 ml 5% ferric ammonium citrate solution was added and slants were prepared. Similarly a set of slants was made to which no additional iron was added.

From a four days old culture on M.B.M. on which both pigmented and non-pigmented colonies were present, two colonies were selected, the one being well pigmented and rough, while the second was smooth and white. Of both colonies subcultures were made on agar slants of high and low iron concentration, and the slants incubated at 30°C.

After three days the cultures were examined. Subcultures of the white colony were luxuriant, smooth and spreading. The growth on the tubes of low iron concentration was entirely white with no tendency to produce any pigment. The growth on the tube with the higher iron concentration was almost white with a very faint pink tint.

On the slants with the subcultured pigmented rough colony strong pigment production had occurred. In the case of the lower iron concentration growth was almost white, but had assumed a warted yet glistening appearance, whilst a red zone in the agar was manifest. In the tube with the higher iron concentration the culture had continued to grow in the typical rough form, while all pigment was retained in the cell mass.

Of each of the four tubes a subculture was made on a second tube of the same iron concentration as the first. The second series was incubated at 30°C. while the first series was placed at 4°C. for eventual comparison. Examination of the second series after three days incubation presented the same pictures as the first series. After subcultures were made, the second series was similarly stored at 4°C.

After elapse of another three days at 30°C. the third series was examined and gave the same impression as the foregoing two. The third series was then also stored at 4°C. together with the first and the second.

Nine days later the three series were then mutually compared.

All the successive transfers of one and the same type were identical, all presented the same picture as the first series. In the three cultures of the progeny of the white colony on the medium with high iron concentrations the pale pink tint originally present had now deepened to a red-maroon. Nevertheless the intensity was not yet quite comparable to that of the progeny of the rough red colony on the same iron concentration. In the subcultures of the white colony grown on the lower iron concentration during the storage at 4°C. weakly coloured zones had developed in

the substrate, these being less intense, however, than the zones produced by the subcultures of the pigmented colony on media with the same iron concentration.

It is clear that these results do not permit to draw far-reaching conclusions regarding the influence of the composition of the medium on the stability of the two forms. The fact that the successive cultures of one and the same type were fully identical seems to contradict the existence of such an influence.

It should be realized that the considerable reduction in colour of the cell mass of the red mutant on the iron-poor medium, as compared with that observed on the iron-rich medium, will only be due to the fact that in the first medium the low iron content has become a limiting factor in the pigment production.

Chapter V

FACTORS DETERMINING THE PIGMENT PRODUCTION BY THE RED MUTANT

§ 1 Introductory remarks

With a view to the ultimate aim of producing the pigment of *Candida pulcherrima* on a somewhat larger scale it seemed indicated to investigate in further detail the factors which determine the production of the pigment by the red mutant described in the preceding chapter.

BEIJERINCK's pioneer study (1918) leaves no doubt that both iron and oxygen are indispensable, but regarding the nutritional conditions of the organism optimal for pigment production on the data present in the literature are only fragmentary and partly contradictory.

It was, therefore, indicated to make some experiments regarding the influence of variations in the carbon source and in hydrogen ion concentration on the pigment production.

As for the nitrogen source sufficient insight had already been obtained through the experiments reported in Chapter IV § 3 which had shown that even with ammonium nitrogen both satisfactory growth and pigment formation occur, but that certain organic nitrogen preparations like Bacto-peptone still were more favourable.

As regards iron there seemed to be reason to make a quantitative investigation of the influence of this metal, since it is clear from the investigations of BEIJERINCK and ROBERTS (1946b) that the amount of iron is decisive for the mode of formation of the pigment, either in the cells, or in the medium.

Because ROBERTS (1946b), as well as GROSBÜSCH (1915), states quite definitely that light does not influence pigment production, no further attention has been given to this point.

§ 2 The influence of the nature of the carbon source

As had been reported in Chapter IV § 1 GROSBÜSCH has concluded from his experiments that the nature of the carbon source offered to the organism in so far effected pigmentation that non-fermentable disaccharides gave a markedly better pigmentation than was the case with the fermentable hexoses. His observations were made in liquid cultures, and no attention was given to the essential rôle of iron in pigmentation.

In order to obtain a better insight into the problem it seemed desirable to investigate first which carbon compounds are assim-

ilated by the yeast under aerobic conditions. This was done in liquid media according to the technique recommended by WICKERHAM (1948). In this method the carbon compound to be investigated is introduced in a liquid synthetic medium containing $(NH_4)_2SO_4$ as nitrogen source next to the necessary nutrient salts and vitamins. After inoculation the tubes were incubated at 25°C. for three weeks, when the density of growth was recorded.

In additions the fermentability of the various compounds was checked in the usual way with the aid of EINHORN tubes, using a 2% solution of the compound in yeast water.

The following results were obtained:

Compound	*Assimilation*	*Fermentation*
Glycerol	+	–
Sodium lactate	–	–
Glucose	+	+
Fructose	+	+
Mannose	+	+
Galactose	+	± or (–)
Sorbose	+	–
m-Inositol	–	–
Saccharose	+	–
Lactose	–	–
Maltose	+	–
Raffinose	–	–
Melizitose	+	–

It was indicated to test the suitability of the assimilable carbon compounds for pigment production under the conditions which had already been found favourable in the case of saccharose. To this purpose 1 g of the compound to be tested was dissolved in 49.5 ml yeast water pH 6.5, together with 1 g Bacto-agar, and sterilized at 110°C. for 15 minutes. After sterilization 0.5 ml of a sterile 5% solution of ferric ammonium citrate was added, and plates made. These were inoculated with a suspension of a young well-pigmented culture. The plates were incubated at 30°C. for three days, and then kept at room temperature for four days.

Examination showed that on all plates good growth had occurred. Moreover, there were no noticeable differences in pigmentation which in all cases was profuse.

All compounds which can serve as carbon source are thus equally suitable for pigment production. It should be particularly stressed that, if other conditions for pigmentation are favourable, fermentable hexoses can serve as well as non-fermentable di- and tri-saccharides.

It seems probable that the differences in suitability of the various carbon sources, as observed by GROSBÜSCH, will have at

least partly been due to different amounts of iron present as impurities in the compounds tested.

§ 3 The influence of the hydrogen ion concentration

ROBERTS (1946 b) has already paid some attention to this factor. She conducted her experiments with a potato glucose agar, to which no further additions of iron had been made.

On varying the pH of this medium from 4.0 to 6.2, she noted that formation of the pigment took place progressively in the agar. At pH 4.0 all the pigment was retained in the colonies, while at pH 6.2 the growth was colourless, and the pigment was formed in the agar. She gave a very acceptable explanation on the basis that iron salts are subject to increasing hydrolysis as the hydrogen ion concentration falls. This lowers the concentration of available iron below the critical value for pigmentation to occur within the cell mass.

The question arose as to what effect pH variations would produce, if the iron concentration was constantly maintained above this level. In order to answer this question it was decided to grow the yeast over a range of pH values between 4.5 and 8.0, with increments of 0.5 pH units. The use of ferric ammonium citrate seemed suitable, since this compound is a relatively tight chelate.

The same medium was used as in the foregoing section the pH being adjusted to the required values. Slants were prepared, inoculated with a 3 days old rough culture of the red mutant, and incubated at 30°C. for 3 days.

Examination revealed that pH had no significant influence. Pigmentation had in all cases occurred within the cell mass, no pigment was present in the agar.

At pH values below 6 the growth had a decisive mat surface, while the colour had a somewhat brown tint. On viewing the growth under low magnification secondary mycelium was visible.

The colour of growth in the tubes with pH values above 6 was bordeaux-red with a metallic sheen. In all the tubes growth had continued in the rough form. The deeper colour of the growth in this case was probably due to a somewhat increased pigment production.

These results may be considered as a confirmation of ROBERTS' assumption that the rather marked effect which the hydrogen ion concentration had in her experiments on the distribution of the pigment in the culture should be ascribed to the influence of this concentration on the hydrolysis of the ferric salt present in her medium. It should be understood that in these experiments ROBERTS did not add any extra iron, and, therefore, pigment production

depended on the traces of iron introduced with the potato decoction.

In the present experiments this secondary action of the pH has been eliminated. Under these conditions the influence of the pH on pigment production is not marked, although there was some indication for a slight favourable effect of pH values above 6.0.

§ 4. The influence of iron concentration

All observations on the rôle of iron in the pigment production of *Candida pulcherrima* prior to those of ROBERTS were of a qualitative nature. Despite the merit of the study of this author her experimental work suffers from some imperfections. The iron content of her basal potato glucose medium was unknown, being based on a theoretical figure for the iron content of potato tubers. The presence of iron in the agar and water used for the media was neglected. Moreover, the source of iron used by her for making additions, ferric ammonium citrate, is a compound liable to decomposition. For this reason on using such a preparation its iron content should be determined analytically, and this was not done by ROBERTS.

For a more accurate investigation it was imperative to work with a medium of known iron content.

OLSON and JOHNSON (1949) devised a method for the quantitative removal of iron from synthetic media for yeast cultivation with the aid of 8-hydroxyquinoline. It seemed possible to profit from their experiences.

In order to obtain media of varying iron concentrations the following procedure was adopted.

10 g of saccharose together with 1 g KH_2PO_4 and 0.25 g $MgSO_4.7H_2O$ were dissolved in 500 ml distilled water contained in a 2 litre flask. The pH of the solution was adjusted to 5.0, and 10 ml of a 0.1% solution of 8-hydroxyquinoline in redistilled chloroform was added. The flask with contents was then placed in a mechanical agitator and shaken for twenty hours. The liquid was than transferred to a separating funnel and the greenish chloroform layer withdrawn, while the aqueous layer was repeatedly extracted with fresh redistilled chloroform, until the last traces of colour had disappeared. After this still five more extractions were conducted. The pH of the solution was then adjusted to 6.0 with the aid of redistilled aqueous ammonia.

Samples of Nobel Bacto-agar and Bacto-peptone were analysed for iron. Weighed samples were incinerated, and the inorganic residue treated with 2N HCl and a trace of bromine. The iron content was then determined colorometrically with potassium thiocyanate. Analysis gave the iron content of the specific batches

of agar and peptone as 0.014% and 0.0048% respectively.

To 450 ml of the purified basal solution contained in a litre flask, 10 g of Nobel Bacto-agar and 5 g Bacto-peptone were added together with 0.5 ml of trace element solution, containing 400 γ zinc as $ZnSO_4.7H_2O$ and 25 γ copper as $CuSO_4.5H_2O$ per ml, as well as 0.5 ml of a vitamin solution containing 200 γ thiamine, 200 γ pyridoxine, 200 γ nicotinic acid, 200 γ calcium pantothenate and 2 γ biotin per ml. The flask with contents was heated au bain-marie until the agar and peptone were dissolved. The contents were then transferred to a measuring cylinder, the flask rinsed with basal medium, the washings added to the rest, and the volume made up to 500 ml with basal medium. Batches of 49 ml of the agar sol were then transferred to 100 ml flasks and autoclaved at 110°C. for 15 minutes. The iron content of this medium was 0.00032% w/v.

Immediately after autoclaving 1 ml of a sterile ferric ammonium citrate solution of the desired concentration was aseptically added to each batch. Thus a series of flasks was obtained in which the iron concentrations ranged from 0.00032% to 0.015% w/v with a fair degree of accuracy. From these media slants were made.

Ferric ammonium citrate was selected as source for supplementing iron, since inorganic iron salt are firstly subject to hydrolysis leading to the precipitation of basic salts and insoluble hydroxides, and are secondly liable to double decomposition with phosphates. Such reactions will lead to the presence of unavailable iron compounds in the medium.

The ferric ammonium citrate used in this case was a preparation of MERCK's *ferri ammonium citricum fusc. in lamellis*. Samples of this preparation were analysed, and found to contain 27.9% Fe.

Slants with the following iron concentrations (w/v)% 0.00032, 0.0005, 0.001, 0.005, 0.01 and 0.015 were incoculated in triplicate from a 3 days old culture of the red mutant. The slants were incubated at 30°C.

The cultures were first examined after 24 hours. In all tubes growth had occurred, that of the first two sets being somewhat less then in the remaining four. At the 0.00032% Fe level, no pigmentation had taken place, while in the series of 0.0005 and 0.001% Fe a definite red zone had established itself, darker in the case of the higher concentration. In the case of 0.005% Fe the zone was much narrower, being restricted to the area immediately under the streak. The colour was markedly deeper than in the preceding cases. Growth itself at 0.00032, 0.0005 and 0.001 as well as at 0.005% Fe was white. At the 0.01 and 0.015% Fe levels no pigment formation had occurred in the substrate, all pigment being retained in the cell masses, giving the streaks a fine maroon appearance.

After a further 24 hours at 30°C., the cultures were again examined. Growth in the first two sets was still less abundant than in the rest. The first set now too displayed a faint coloured zone, while in the second and third set the colour intensities of the zones had increased, the whole showing an increase in the colour intensity as the iron concentrations increased from 0.00032 to 0.001%. The depth of the zones in these cases was approximately the same for all three series, circa 6 mm.

In the case of the 0.005% Fe, at the most 3 mm deep, and then again restricted to the area immediately surrounding the growth, while colour intensity of the zone was much deeper than in the preceding cases. Growth itself had now assumed a pale rose colour, indicating that part of the pigment had been retained in the cell mass.

In the case of 0.01% Fe the zone was completely absent. All pigment was retained in the cell mass giving the growth a fine bordeaux-red appearance.

In the last set, in which the iron amounted to 0.015% Fe, growth had assumed a magnificient dark maroon colour, even deeper than was the case in the 0.01% Fe set.

From the above there remains no doubt that the pigment production of *Candida pulcherrima* is directly bound to the presence of available iron in its environment. Below a definite iron level, pigmentation does not occur within the cell mass, but is formed in the substrate, while the colour strength of the zones is proportional to the iron concentration. At the value 0.005% Fe pigmentation occurs in the cell mass as well as in the substrate. Above this transitional value the pigmentation process occurs solely in the cell mass, and a minimum iron concentration of 0.01% seems recommendable, if pigment production within the cells is aimed at.

It will be superfluous to remark that all these observations are in perfect harmony with BEIJERINCK's idea regarding the production by the yeast of a colourless precursor which only in contact with iron is converted into the pigment.

§ 5 The indispensability of molecular oxygen

GROSBÜSCH was unable to give a decisive opinion whether or not, the presence of oxygen affected pigmentation. That this is, indeed, the case, was first noted by BEIJERINCK. He found that aerobic cultures grown on media of low iron content, failed to develop a red colour on addition of an iron salt, if a coverslip had been placed on the colonies, so that air was excluded. He concluded that "le pigment est formé par un chromogène incolore qui produit le pigment, aussi bien dans la cellule qu'au dehors en

présence de sel de fer et *d'air*".

ROBERTS (1946b) was able to confirm BEIJERINCK's observation regarding the indispensability of oxygen for pigment production. In her experiments she grew plate cultures in an atmosphere which was made anaerobic with the aid of alkaline pyrogallate. After nine days the fully colourless growth was considerably less than in the aerobic controls, where pigmentation had occurred.

At first sight there seem to be two possibilities to account for the absence of colour in the anaerobic cultures. Either under anaerobic conditions BEIJERINCK's chromogen had not been formed, or the chromogen was already there only waiting for oxygen to manifest its presence by pigment formation.

ROBERTS concludes to the correctness of the latter assumption, since after admitting air to the nine days old anaerobic cultures within 18 hours the entire surface of the colonies had become distinctly red. It should be remarked that this demonstration is not yet quite conclusive, because in the period mentioned growth under aerobic conditions had clearly occurred, so that a production of chromogen during the said 18 hours remains possible.

However, in a second series of experiments, in which she used two days old anaerobic cultures on a medium containing 0.1% ferric ammonium citrate, she noted the development of pigment within 5½ hours after the restoration of aerobic conditions. This seems, indeed, to prove her claim.

Nevertheless, it seemed worth-while to collect some personal experience regarding this question.

To this purpose four heavily seeded plate cultures were made of the red mutant on M.B.M., to which, however, no extra iron had been added. After seven days the plates were covered by a rather even white growth. Now circular discs of agar were removed from the plates, and the cavities filled with a 4% solution of ferric chloride. Two plates were left exposed to atmospheric oxygen, while the remaining two were maintained in an atmosphere of carbon dioxide.

In the case of the plates exposed to atmospheric oxygen, pigmentation occurred within 24 hours. Most remarkable was the fact that pigmentation only developed as a concentric pigmented band around the cavity (see Plate V fig. 1). In this band the colonies had become reddish, whilst beneath the colonies a red zone in the agar could be observed. Striking was the fact that this pigmented zone was preceded by a zone in which the colonies had remained colourless.

In contrast hereto the plates which had been maintained under carbon dioxide failed to show any pigment.

After 24 hours one anaerobic plate was removed from the carbon

dioxide atmosphere, and exposed to atmospheric oxygen. Only after a further 24 hours a pigmented zone appeared, quite similar to that noted in the case of the aerobic plates after 24 hours. The plate which had remained under anaerobic conditions still failed to display any pigmentation.

In the case of the aerobic plates the pigmentation bands had increased in both width and intensity.

On leaving the plates exposed to the atmosphere for a further week, pigmentation spread to all colonies except to those which were located in 5 mm wide zone immediately surrounding the cavity in which the ferric chloride solution had been brought.

It remains another question how these results should be interpreted. The fact that pigmentation did not occur instantaneously on admittance of air to the ferric chloride treated plates previously kept in carbon dioxide can only difficulty be reconciled with BEIJERINCK's view that the pigment owes its origin to a direct action of oxygen on some oxidizable chromogen.

The long period which elapses after the admittance of air to the yeast colonies already exposed to ferric ions before pigment formation occurs seems to suggest that this formation is ultimately due to metabolic activities of the yeast cells in which free oxygen plays some rôle. This assumption is, of course, also strongly supported by ROBERT's experiments dealing with the behaviour of anaerobic cultures on an iron-containing medium after admittance of air.

If, however, aerobic metabolism of *Candida pulcherrima* would in all cases lead to a production of a precursor which would only need iron to be converted into pigment one should observe that pigment formation would occur instantaneously if ferric ions were added to yeast colonies grown on an iron-poor medium, and this independent of the presence of *living* yeast cells.

In order to test this hypothesis the following experiments were made.

Another set of four heavily seeded plate cultures on M.B.M. from which iron salts were omitted was made. After 7 days cultivation at 30°C., two plates were exposed to chloroform vapours for 24 hours, while the remainding two were left at room temperature. Hereupon both series of plates were flooded with 0.5% $FeCl_3$ solution for 30 seconds and the fluid decanted. After 17 minutes the viable plate cultures showed the first signs of a red coloration, while the nonviable plate failed to show pigmentation even after seven days. In the first place these experiments show clearly that living cells are indispensable for pigment formation.

The whole of the experimental evidence available indicates, however, that neither aerobic metabolism leads to some precursor which produces pigment on iron addition, nor does anaerobic metabolism in the presence of iron lead to some chromogen which produces pigment by autoxidation.

In view of all this the only possible conclusion seems to be that pigment formation is due to aerobic metabolic activities of the yeast cells, but only in so far as metabolism is under the influence of a suitable concentration of ferric ions.

In other words pigment production in Candida pulcherrima only occurs in the living cells under the condition that both free oxygen and iron act concomitantly.

Chapter VI

THE PREPARATION OF THE PIGMENT OF CANDIDA PULCHERRIMA

§ 1 Introductory remarks

It again was BEIJERINCK who was the first to pay attention to the nature of the pigment of *Candida pulcherrima*. He concluded to an acidic character of the pigment, since it was readily dissolved by caustic alkali, from which solution it could again be precipitated – in apparently unchanged state – by addition of a sufficient amount of acid. The pigment was remarkably stable, and even resisted boiling with dilute sulphuric acid.

BEIJERINCK reports further that the pigment was somewhat soluble in water, however, insoluble in alcohol, ether, carbon disulphide, chloroform, carbon tetrachloride, or benzene.

The solubility of the pigment in alkali together with the fact that pigmentation only took place in the presence of iron presumably induced BEIJERINCK to think of a polyphenolic nature of the pigment, and this made him suggest that it might belong to the group of the widely occurring natural pigments, the anthocyanines. However, BEIJERINCK himself forwards the objection that the latter pigments do not contain iron.

§ 2 Preliminary orientation regarding the properties of the pigment

The first problem to be solved in the preparation of the pigment was apparently the elaboration of a suitable method for the extraction of the pigment from a deeply coloured cell mass. It seemed indicated to try firstly the extraction of the dried cells with organic solvents.

The following were subjected to a test:

Hydrocarbons: Petroleum ether fractions of b.p. 40°-60°C. and of b.p. 60°-80°C., benzene, toluene.

Halogen derivatives: Chloroform, carbon tetrachloride, ethylene dichloride, butyl chloride.

Alcohols: (in the presence and absence of 5% HCl): Methanol, ethanol, propanol, isopropanol, butanol, isobutanol, n-pentanol.

Acids: Formic acid, glacial acetic acid, monochloroacetic acid, trichloroacetic acid, propionic acid, butyric acid, acetic acid anhydride.

Esters: Methyl acetate, methyl benzoate, methyl butyrate, ethyl acetate, ethyl butyrate, amyl formate, amyl acetate, amyl butyrate.

Ethers: diethyl ether, dioxane.

Polyalcohols: Ethylene glycol, butylene glycol, glycerol, trimethylene glycol, dipropylene glycol.
Ketones: Acetone.
Phenols: Phenol, p-cresol.
N-containing compounds: Formamide, acetamide, pyridine, collidine, piperidine, nitrobenzene, concentrated ammonium-hydroxide.

None of these solvents, however, did elute the pigment from the yeast cells, neither at room temperature nor at 100°C. (or at the boiling points of the solvents, if these were below 100°C.).

As for the suitability of water as a solvent, it should be remarked that, indeed, a slightly coloured solution was obtained; the greater part of the pigment, however, was retained by the cells.

Under these conditions there remained very little else, but to resort for the extraction to the use of either sodium, potassium or barium hydroxide solutions. In these liquids the pigment readily dissolved to give a deep yellow solution. After removing the undissolved cell residues by centrifugation, and acidification of the clear supernatant, with acetic or dilute hydrochloric acid, the pigment was precipitated as amorphous red flakes. The pigment thus liberated from the cell mass was again tested for solubility in organic solvents, since the possibility did not seem excluded that in the cells it would have been linked to some protein, and for this reason would have failed to dissolve in organic solvents.

However, the liberated pigment still retained its insolubility in all solvents except alkali.

The dissolved and reprecipitated pigment was then subjected to the action of pancreatin. This was done partly in order to eliminate proteins which might be present as contaminating compounds, partly because it seemed possible that during the preceding operations the pigment had remained attached to a carrier of protein nature.

A washed sample of the pigment was suspended in a phosphate buffer at pH 8 together with an active pancreatin preparation in the presence of toluene at 37°C. After 5 days the pigment was collected, washed and again tested for solubility in a great number of solvents, but also this time results were negative.

Since the pigment seemed only to dissolve at relatively high hydroxyl ion concentrations, the minimum pH for solubility was determined, making use of SØRENSEN's ($\frac{M}{10}$ glycine + $\frac{M}{10}$ NaCl) + $\frac{M}{10}$ NaOH buffers.

To 10 ml of the buffers contained in rubber stoppered tubes 0.05 ml of a well-washed suspension of the crude pigment was added, the tubes were thouroughly shaken and left for several hours. After agitating once more, the pigment suspension was then viewed through the entire length of the column of the liquid.

At pH values below 11.9 the pigment failed to dissolve, so that the liquid remained red. Between pH 11.9 and 12.2 there obviously was a transition stage. At pH 11.9 the colour was still predominantly red, at pH 12.2 it was predominantly yellow. Only at pH 12.5 and higher the pigment dissolved to give a distinct yellow solution. This offers an explanation why concentrated ammonium hydroxide with a pH of approximately 11.6 failed to dissolve the pigment.

Due to the failure to obtain a good dispersion of the pigment in water, and its insolubility in all organic solvents, attempts to purify the pigment by absorption met with no success. The pigment was readily absorbed by shaking an aqueous suspension with MERCK's Ultra carbon, BROCKMAN's aluminium oxide and silica. However, elution with organic solvents failed, as was also the case with concentrated ammonium hydroxide, even in the presence of detergents.

Attempts to obtain soluble acetyl derivatives of the pigment either in pyridine or in alkali were also unsuccessful. The pigment readily dissolved in alkali, but on addition of acetyl chloride the resultant lowering of the pH of the system at once led to a reprecipitation of the pigment as insoluble amorphous flakes. There thus remained for a more or less quantitative extraction of the pigment nothing but the use of alkali.

Since proteins also readily dissolve in aqueous alkali it was deemed favourable to use methyl alcohol as a solvent for the potassium hydroxide. Yet by this treatment only a partial recovery of the pigment on subsequent acidification proved to be possible. This difficulty could, however, be overcome by an addition of some ferric chloride.

§ 3. Finally adopted procedure

The following procedure was eventually adopted for the preparation of the pigment. Large quantities of yeast were grown in KOLLE flasks. These flasks were preferred to PETRI dishes in view of airborne mold infections. The harvested yeast was washed with methyl alcohol, and subsequently extracted with 5% KOH in methyl alcohol. The liquid phase was clarified by centrifugation, and the clear deep yellow to brown supernatant fluid neutralized by addition of 10% HCl, the pH being finally adjusted to 2.5 to 3. A 5% $FeCl_3$ solution was then added dropwise in order to ensure complete precipitation. The liquid was once more centrifuged, and the precipitate washed with 0.001 N HCl. The pigment was than suspended in distilled water and dialysed, first against running tap water and then against distilled water, until the washings gave no reaction for chloride or sulphate. The pigment was

again collected by centrifugation. In order to remove possibly present protein the pigment was repeatedly extracted with 90% phenol, as recommended by PALMER and GERLOUGH (1940) for the preparation of protein-free antigen for the removal of proteins. After extraction the phenol was removed by extractions with ether and the pigment dried *in vacuo*.

A more detailed description of the various steps in the preparation is now given.

For the large scale cultivation of the yeast, the following medium was selected.

Saccharose 40.0 g, peptone 20.0 g, KH_2PO_4 2.0 g, $MgSO_4.7H_2O$ 0.5 g, yeast autolysate 10 ml, agar 20 g, tap water 1000 ml. The ingredients were heated au bain-marie until the agar dissolved.

Portions of 150 ml of the above medium were introduced into 20 plugged, sterile KOLLE flasks of 18.5 cm diameter, and autoclaved at 110°C. To the sterile agar sol 1.5 ml of a sterile 5% ferric ammonium citrate solution was added under aseptic conditions, and the medium allowed to set. Each flask was inoculated with a suspension of a slant culture of the red mutant, obtained by suspending an entire slant culture in approximatily 5 ml sterile water. The inoculation took place by flooding the entire surface of the plate by tilting. The flasks were then placed in a horizontal position at 30°C. for 7 days.

After incubation, the deep maroon to bordeaux-red cells mass, which had a typical esterlike odour, was harvested with the aid of a stout DRIGALSKI-spatula, and the growth was suspended in 1 litre methyl alcohol, contained in a 2 litre beaker. After harvesting the increase in weight of the beaker with contents gave the yield of moist yeast. From 20 flasks with an effective culture surface of approximately 2.5 square meters, 206 g moist yeast were obtained.

The yeast suspension was filtered through a coarse glass-wool mat to remove particles of agar. The filtered suspension was then transferred to four centrifuge flasks, and the yeast mass collected by centrifugation at 3000 r.p.m. The somewhat yellow supernatant liquid was discarded, and 1000 ml of a 5% KOH solution in methyl alcohol distributed over the four flasks. The compact yeast mass was then thoroughly agitated and homogeneously emulsified. A deep yellow-brown suspension was thus formed, while the maroon yeast mass lost its red colour. The alkaline alcoholic suspension was again centrifuged at 3000 r.p.m., and the clear deep yellow-brown supernatant fluid carefully decanted and filtered. The clear liquid was then neutralized with 10% HCl, and the pH adjusted to 2.5 to 3.0. On acidification the colour changed to red brown with partial precipitation of the pigment. A 5% ferric chlo-

ride solution was then carefully added dropwise, under rapid stirring, until no further precipitation of the voluminous maroon red flakes occurred. The precipitate was then allowed to settle overnight at 4°C. The partly decolorized yeast mass was then once more extracted with a further 500 ml methyl alcohol and treated similarly and the final preparation added to the first portion. After sedimentation, as much as possible of the clear supernatant liquid was withdrawn, and the precipitate compacted by centrifugation. The pigment was subsequently washed with two portions of 250 ml 0.001 N HCl. The washed pigment precipitate was then suspended in 250 ml distilled water, transferred to collodion sacks and dialyzed against running tap water for 48 hours. This was followed by dialysis against distilled water, until the washings gave no reaction for sulphate or chloride.

The supernatant liquid was withdrawn from the sacks and the pigment collected by centrifugation. The compact mass was then thoroughly emulsified with 50 ml 90% phenol, and left overnight. The liquid phase was then removed by centrifugation, and the pigment subjected to a further two phenol extractions. The phenol extracts were separated from the undissolved pigment. Since the phenol phase was still somewhat coloured it was left for several days, after which some finely dispersed pigment settled out and could be worked up again.

After the third phenol extraction the pigment was collected on a fine glass filter 3 G 4, and repeatedly washed with re-distilled ether, until free from all traces of phenol. The final product was then dried *in vacuo* over calcium chloride. Yield: 1.04 g. This means approximately 0.5% of the moist yeast, or 2% of the dry yeast.

The properties of the product finally obtained can be summarized as follows.

The caked pigment can readily be disintegrated to an amorphous deep maroon to bordeaux-red powder, which is insoluble in organic solvents, but again readily dissolves in alkali.

It possesses no melting point, but gradually decomposes on heating.

In the course of time several batches of yeast have been worked up according to this procedure: Altogether about 3.7 g of pigment have been isolated.

It is fully acknowledged that the procedure applied suffers from serious shortcomings as regards purification, and unusual application of strong alkali for the separation of a biological substance. In view of the embarrassing properties of the pigment no better method could be devised.

Chapter VII

INVESTIGATIONS ON THE CHEMICAL NATURE OF THE PIGMENT OF CANDIDA PULCHERRIMA

§ 1 Elementary analysis

A sample of the most purified preparation of the pigment was submitted to elementary analysis. By the kind courtesy of Professor Dr P. E. VERKADE this analysis was performed by Mr M. VAN LEEUWEN of the Department of Organic Chemistry of the Technical University, for whose able assistence the author's thanks are due.

In the first place a qualitative analysis showed the presence of carbon, hydrogen, nitrogen and iron, whilst sulphur, phosphorus and halogens were found to be absent.

A quantitative nitrogen determination according to the DUMAS method yielded the following result:

7.000 mg pigment gave 0.521 ml nitrogen gas at 17°C. and 761 mm Hg, hence 8.61% N.

A quantitative nitrogen determination according to the TER MEULEN method yielded the following result:

15.95 mg pigment gave ammonia, equivalent to 5.46 ml 0.0182 N HCl, hence 8.7% N.

On combustion 40.05 mg pigment yielded 23.4 mg H_2O, 71.4 mg CO_2 and 6.2 mg ash (apparently pure Fe_2O_3).

From these figures one arrives at the following percentage composition:

6.5% H; 48.6% C; 8.6% N; 10.8% Fe.

It seemed desirable to check the latter value by a direct determination of iron in the ash.

100 mg pigment were combusted, and the residue taken up in hydrochloric acid. After subsequent treatment with bromine for complete oxidation, the iron was determined colorimetrically with the aid of the thiocyanate method, using a BECKMAN spectrophotometer D.U. The determination was made at 495 m μ, slit width 0.995 mm.

The iron content of the pigment was thus established to be 10.7%, in satisfactory agreement with the earlier obtained value.

Summarizing the foregoing percentage composition of the pigment results:

C = 48.6%	O = 25.6%	Fe = 10.7%
H = 6.5%	N = 8.6%	

Accepting one atom of iron to be present per molecule computation leads to the following elementary composition:

$C_{21.1}\ H_{33.7}\ O_{8.3}\ N_{3.2}\ Fe$.

This result is, of course, unsatisfactory, and one cannot escape the conclusion that, notwithstanding the serious efforts made, a complete purification of the amorphous pigment has not been achieved.

Allowing for the presence of small amounts of impurities in the pigment preparation, it is evidently possible to suggest some compounds which in their elementary composition do not deviate considerably from the data actually found.

This is demonstrated by the following:

	% C	%H	%O	%N	%Fe
Actually found:	48.6	6.5	25.6	8.6	10.7
Calculated for $C_{21}H_{34}O_8N_3Fe$:	49.2	6.7	25.0	8.2	10.9
Calculated for $C_{20}H_{34}O_8N_3Fe$:	48.0	6.8	25.6	8.4	11.2

§ 2 Acid hydrolysis of the pigment

It seemed indicated to subject the pigment to an acid hydrolysis, in the hope that this procedure would lead to the formation of some identifiable compounds.

As has already been remarked, at room temperature the pigment is apparently quite resistant to the action of strong mineral acids. For this reason it was decided to attempt an acid hydrolysis at a higher temperature. To 5 mg of the pigment 2 ml concentrated hydrochloric acid (sp.w. 1.19) was added, and after sealing the mixture in a thickwalled glass tube, the tube was maintained for 24 hours at 100 °C.

At the end of the operation the liquid had assumed a deep yellow colour, while a dark solid mass had remained undissolved. The clear supernatant liquid gave reactions for ferric ions.

The liquid was further analyzed for the presence of amino acids by using the two dimensional paper partition chromatography according to DENT (1948). After application of the hydrolysate on the paper the excess acid was neutralized with ammonia vapour.

The iron then precipitated as brown iron hydroxide. Phenol in the presence of ammonia, and collidine in the presence of diethylamine were chosed as the successive liquid phases.

The application of the ninhydrin reagent yielded only three spots, two of which were very faint, while the third was quite spectacular. One of the spots could not be identified with any of the usual amino acids, and on ground of its location suggested some peptide. The second faint spot could be identified with some certainty as aspartic acid, which then must have only been present in traces.

As for the prominent spot this strongly suggested to be leucine.

Since, however, a paper chromatographic separation of leucine, isoleucine and norleucine is not so easily attained, it seemed important to repeat the analysis with a second portion of hydrolysate, this time giving special attention to the three mentioned amino acids. To this purpose an onedimensional chromatographic analysis was applied on six paper strips. On each of the first three an authenic sample of one of the three amino acids was brought, whilst on the next three the hydrolysate together with one of the three amino acids was applied. This time butanol in the presence of ammonia in the gas phase was used as an elutant. After development of the strips with ninhydrin the presence of norleucine in the hydrolysate could with certainty be excluded. The results were fully in favour of the idea that the amino acid present in the hydrolysate was, indeed, leucine. It must be admitted, however, that the presence of isoleucine too could not be quite certainly excluded.

As most important result of the investigation the absence of other amino acids next to the leucine (resp. isoleucine) should be stressed. For this brings definite proof that the *pulcherrima* pigment is devoid of a protein moiety.

§ 3 Pyrolysis of the pigment

As has already been remarked in Chapter VI the pigment has no melting point; on heating a sample a gradual decomposition takes place, starting at about 150°C.

Nevertheless, a careful examination of this pyrolytic process led to some noteworthy observations. In the first place it was found that at the colder part of the tube in which pyrolysis was carried out a crystalline compound accumulated. Moreover, pungent vapours evolved which gave a red coloration when brought into contact with a paper strip dipped in EHRLICH's reagent for pyrrole derivatives (p - aminodimethylbenzaldehyde in acid solution). A pine splinter dipped in concentrated hydrochloric acid also turned red. Addition of a 5% solution of mercuric chloride to the aqueous leachings of the condensed vapours gave a white precipitate. All this gives a strong indication for the precense of some heterocyclic derivative amongst the products of pyrolysis.

It seemed, of course, important to give full attention to the crystalline product which accumulated during the pyrolysis. In order to obtain a somewhat larger quantity of this product 2.8 g of the pure pigment preparation was brought in a porcelain boat, heated electrically at 300°C. in a hard glass tube of 60 cm length under passage of a slow current of nitrogen. The pigment decomposed with copious evolution of yellow vapours which condensed together with the white crystalline product in the cooler regions

of the tube. As soon as no further separation of degradation products occurred, the tube and contents were allowed to cool. The tube was cut in two and the entire contents quantitatively washed with redistilled ether into the glass container of a SOXHLETT apparatus. The contents were then thoroughly extracted with ether. The crystalline product proved to be insoluble in ether, while the other products were readily extracted to give a deep brown solution. Extraction of the mass was continued until the ether washings were colourless. The dark coloured ether solution was set aside. The crystalline mass together with the charred residue was subsequently extracted with 250 ml redistilled methanol which dissolved the crystals. However, owing to its limited solubility in this solvent, the crystals again separated out in the brown coloured methanol extract in the flask.

After complete extraction of the crystalline mass, the methanol extract was allowed to cool and left overnight at 4°C. The separated solid was quantitatively collected by centrifugation and recrystallized from 75 ml methanol, from which it separated as fine, colourless needles. The product was redissolved in a minimal quantity of boiling methanol, treated with a trace of ultracarbon and recrystallized. A light, feathery, white crystalline mass was obtained. Yield: 41 mg.

The substance proved to be very soluble in pyridine, showed a limited solubility in methanol, less in ethanol and was insoluble in water. The substance showed no definite melting point. However, sublimation occurred which commenced at approximately 220°C. In a sealed capillary the compound melted with decomposition at 285°-287°C. (uncorrected).

*Micro-elementary analysis *):*

4.704 mg substance yielded 3.48 mg H_2O and 11.23 mg CO_2
%H = 8.28 %C = 65.15%
4.128 mg substance yielded 0.465 ml nitrogen at 25°C. and 754 mm Hg.
%N = 12.8.

Found:	65.15%C	8.28%H	12.8%N
Calculated for C_6H_9NO:	64.86%C	8.17%H	12.6%N.

No molecular weight could be determined according to RAST, since the substance proved to be insoluble in camphor. Methoxy groups were found to be absent.

*) The author wishes to express here his most sincere thanks to Professor Dr J.P.WIBAUT, of the University of Amsterdam for the valuable advice and interest shown in connection with the analysis of the compound in question. The micro-elementary analysis was carried out by Mr. P.J.HUBERS of the Department of Organic Chemistry of the University of Amsterdam.

The determination of carbon bound methyl groups according to the procedure of KUHN-ROTH:

5.445 mg substance = 2.78 ml 0.01 N NaOH

% CH_3 = 7.68.

This result seems to indicate the presence of one methyl group on a molecular formule $C_{12}H_{18}N_2O_2$, thus doubling the empirical formula C_6H_9NO.

Chemical properties.

The compound $C_{12}H_{18}N_2O_2$ gave no colour reaction with EHRLICH's reagent, neither at room temperature, nor at 100°C. Alkaline $KMnO_4$ was not decolorized. No colour reaction was obtained with isatin and sulphuric acid, nor with a solution of ferric chloride. Diazotized sulphanilic acid yielded no azo-dye.

Acetylation with either acetyl chloride in pyridine, or in acetic acid anhydride with a trace of concentrated sulphuric acid yielded a tar which could not be purified by crystallization.

The ultraviolet absorption of the compound, dissolved in ethanol, was recorded with a BECKMAN spectrophotometer Model D.U.

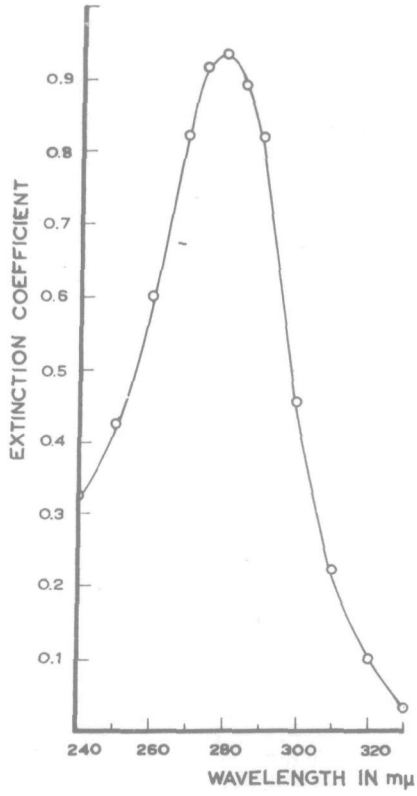
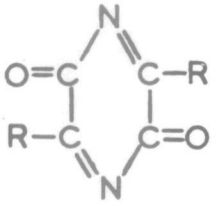


Fig. II – Ultraviolet absorption spectrum of an ethanolic solution of crystalline pyrolisis product of pulcherrimin

As appears from Fig. II the compound gave a marked absorption in the untraviolet region, with a clear maximum at 280 m μ.

The foregoing data are clearly insufficient to give any idea of the nature of the crystalline product of pyrolysis. It happened, however, that attention was drawn to the studies of DUTCHER (1947a, b) on the structure of aspergillic acid. This author obtained from the mother liquors of bromoaspergillic acid, a colourless neutral product of high melting point. It possessed the formula $C_{12}H_{18}N_2O_2$, gave no reaction with $FeCl_3$ and gave a strong absorption in the ultraviolet with a maximum at 285 m μ. DUTCHER established that this product could be catalytically reduced to a diketopiperazine. He proposed a pyrazinedione formula as the most likely structure of the unknown compound:



$R = C_4H_9$

Formula I

The equality of the molecular formulae, the ultraviolet absorption, the sublimation and high melting point, the inability to give a colour reaction with $FeCl_3$, and the fact that no acetyl derivatives could be obtained, together suggest a close relationship between the $C_{12}H_{18}N_2O_2$ product obtained by DUTCHER and the product $C_{12}H_{18}N_2O_2$ obtained by pyrolysis of the pigment produced by *Candida pulcherrima*.

On the ground of these similarities one is inclined to attribute also to the product obtained from the pigment a pyrazinedione nucleus $C_4N_2O_2$. Subtraction of these elements from the molecular formula leaves the aliphatic residue C_8H_{18}.

The fact that leucine was demonstrated to be present in the pigment molecule suggests that the compound of pyrolysis is formed by the degradation of the diketopiperazine of leucine. This would then imply that formula I would also hold for the structure of the product of pyrolysis, although there may be structural differences in the side-chains R.

§ 4 Spectroscopical observations on the pigment and on some other iron complexes

It has to be acknowledged that the data obtained in the foregoing chemical investigations of the pigment are of a too fragmentary character to permit a clear insight into the nature of the pigment. An explanation of this unsatisfactory situation must partially be sought in the limited amount of pigment available, and partially in the embarassing properties of this complex iron compound with greatly impeded chemical manipulation.

It seemed, however, possible that a spectroscopical examination of the pigment might open some new prospect for the establishment of the class of compounds to which the pigment should be reckoned.

A spectroscopical analysis of the purified pigment preparation met, however, with the difficulty of the insolubility of this preparation in all solvents, except alkali, in which case, however, the red colour is discharged. In the meantime the observation had been made that on suspending a well pigmented culture of the red mutant in destilled water, after settling of the yeast cells, the relatively clear supernatant maintained a red colour, apparently because part of the pigment remained in a colloidal solution. It

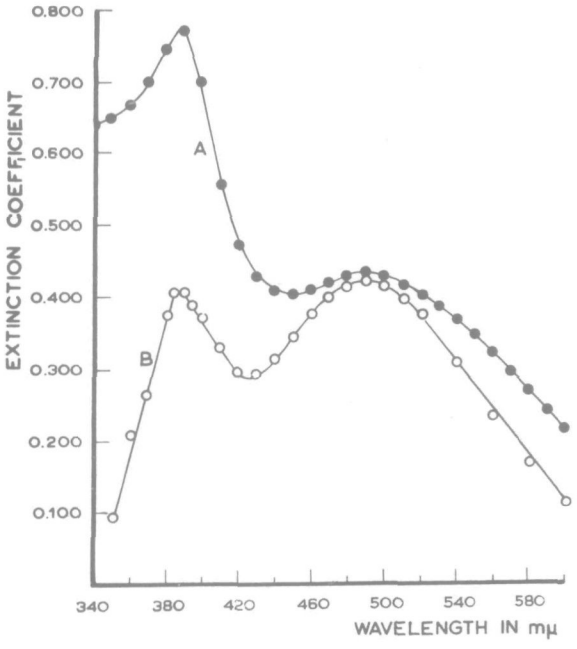


Fig. III. Absorption spectrum of: A, pulcherrimin; B, ferric complex of aspergillic acid

was, therefore, decided to use this solution, after removing the solid particles by centrifugation, for the spectroscopical examination. A preliminary test had shown that on filtration of the aqueous phase through a SEITZ clarifying filter the pigment was retained by the filter, indicating that in the solution the pigment was only present in a dispersed state.

The absorption spectrum of the colloidal solution was determined against distilled water with the aid of a BECKMAN spectrophotometer. Measurements were made in corex glass cells of 1.000 cm and at minimum slit width.

As will be seen from curve A in Fig. III maxima of absorption are present in the vicinity of 390 m μ and 490 m μ.

Even a superficial examination of the spectrum suffices to show that the pigment fundamentally differs from those of the haemin type.

In § 3 it has been reported that the study of the pyrolysis product of the pigment had led to the postulation of its identity with some byproduct isolated by DUTCHER in the preparation of some derivative of aspergillic acid.

On reading DUTCHER's publication it struck the author that in contrast to the said byproduct – which did not give a coloration with iron, no more than the pyrolysis product – aspergillic acid itself gives a beautiful red colour on addition of ferric ions.

This made it especially attractive to study also the absorption spectrum of the iron complex of aspergillic acid. At the request of Professor KLUYVER, Dr JAMES D. DUTCHER of the Squibb Institute for medical Research at New-Brunswick (N.J.) had the great kindness to put a preparation of aspergillic acid at our disposal.

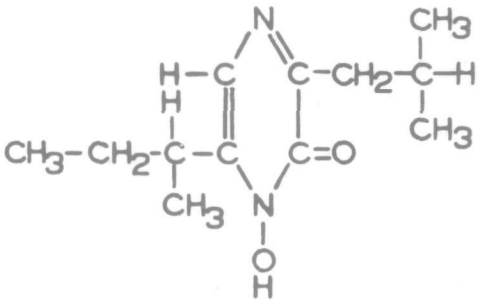
To 5 ml of a solution of aspergillic acid in methanol, containing 2.5 mg per ml, 1 mg ferric chloride and a few drops of concentrated hydrochloric acid were added (pH = 1.7). A beautiful bordeaux-red colour developed quite comparable to that of the pigment. This solution was diluted to give a suitable colour intensity for the determination. Measurements were made against a solution of aspergillic acid in methanol, the solution having the same concentration as in the experiment in which ferric chloride was present. Also some hydrochloric acid was added.

The spectrum of the iron complex thus obtained is recorded in curve B Fig. III.

It will be seen that the agreement between the spectrum of the pigment and that of the iron complex of aspergillic acid is most striking. The two maxima in absorption coincide in an almost perfect way.

In this connection it is important to dwell for a moment on the constitution of aspergillic acid, and on what is known regarding the nature of its red iron complex.

Owing to the investigations of DUTCHER, supplemented by some observations of DUNN *et al.* (1949), it is now practically certain that aspergillic acid has the following constitution:



Formula II

The formation of the red iron complex is now generally accepted to be connected with the hydroxamic grouping occurring in the molecule. Is is a well-known fact that several aliphatic hydronamic acids also yield intensively red iron complexes in which one atom of iron is linked with three identical ligands. From the studies of CHANTRENNE (1948) it has, moreover, appeared that the iron complexes of all aliphatic hydroxamic acids examined by him also gave a maximum of absorption in the vicinity of 500 m μ.

It was deemed desirable to check this observation, and this led to the determination of the absorption spectrum of the iron complex of butyrohydroxamic acid which is reproduced in curve A of Fig. IV. Here again the absorption maximum is, indeed, situated between 490-500 m μ.

At first sight it was, of course, very tempting to conclude from these spectroscopical observations that the pigment would also contain some hydroxamic grouping responsible for the formation of the iron complex.

It seemed, however, prudent to test the specificity of this absorption maximum for the hydroxamic grouping, since so many divergent compounds are known to give a red coloration with ferric ions. For this reason also the spectra of ferric thiocyanate, and of the reaction product of ferric chloride and kojic acid were determined. As is seen from the curves B and C of Fig. IV which represent the said spectra, the result is quite disappointing in as far as here too maxima of absorption at 490-500 m μ occur.

It is, therefore, clear that it would be premature to conclude from the situation of the absorption maxima to the presence of a hydroxamic grouping in the molecule. On the other hand the possibility hereof is neither excluded, and it was, therefore, most desirable to make an attempt at a direct demonstration of the

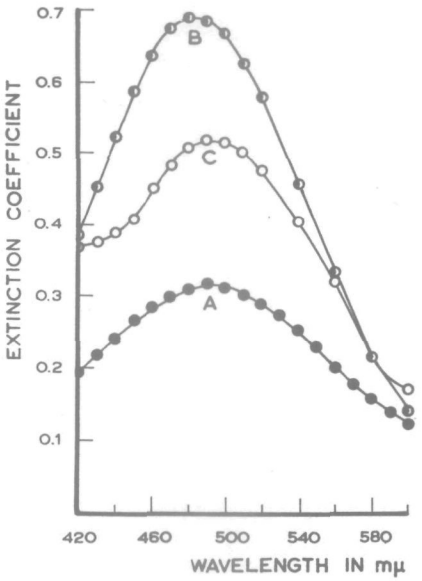


Fig. IV. Absorption spectrum of ferric complexes of: A, butyrohydroxamic acid; B, thiocyanate; C, kojic acid.

occurrence of the said grouping in the molecule. The more so, since such a demonstration would give a direct link with the pyrazinedione ring postulated to occur in the product of pyrolysis.

FEIGL (1947) states that hydroxylamine is generated from hydroxamic acids when these acids are subjected to the action of strong hydrochloric acid. The presence of hydroxylamine is readily demonstrated by either oxidation to nitrous acid, and subsequent diazotation and coupling of sulphanilic acid with α-napthylamine, or with diacetylmonoxime in the presence of nickel ions.

Hence 500 mg pigment was suspended in 5 ml concentrated hydrochloric acid, and evaporated to dryness on a water bath. The mass was leached with 2 ml distilled water, neutralized with ammonium hydroxide, and filtered. The filtrate was once more neutralized with hydrochloric acid and again filtered. In the final filtrate hydroxylamine was readily demonstrated by both procedures mentioned above.

a. Two drops of the test solution were mixed on a spot plate with a few mg sodium acetate, 2 drops of sulphanilic acid in 75% acetic acid and 1 drop of 0.1 N iodine in glacial acetic acid. The mixture is left for 3 minutes. The excess iodine is removed by the addition of 0.1 N sodium thiosulphate solution, and one drop of α-napthylamine is added. A deep cherry red colour developed giving a positive reaction for hydroxylamine.

b. A few drops of the test solution were transferred to a hollow ground object glass and neutralized with ammonia vapour. An equal volume of the diacetylmonoxime-nickelchloride reagent was added. After several minutes the characteristic red crystals of nickel dimethylglyoxime were to be seen under low magnification, indicating the presence of hydroxylamine in the test liquid.

These successful demonstrations of hydroxylamine amongst the products of acid hydrolysis of the pigment, together with the close agreement between the spectrum of the pigment and that of the iron complexes of hydroxamic acids, make the occurrence of a hydroxamic group in the pigment extremely probably.

§ 5 Discussion of results

In this section an attempt shall be made to coordinate the experimental results reported in the preceding sections, and to arrive at a sober picture of our limited knowledge of the chemical nature of the pigment.

On ground of all experiences there is all reason to conclude that the pigment of *Candida pulcherrima* is a chemical entity, and the name *pulcherrimin* seems to be indicated. Then it should be at once stressed that especially amongst the vegetable pigments pulcherrimin takes a special place for two reasons.

In the first instance pigments with iron contents as high as 10.7% are very rare, as follows from the fact that the iron content of the blood pigment haemin and of the haemin moiety of pigments like cytochrome are in the neighbourhood of 8.5%.

In the second place it is a quite unusual feature of a natural pigment to be insoluble in all solvents with the exception of rather strong alkali, and to resist the action of this solvent so that it is recovered apparently unchanged after acidification.

It will be understood that when the high iron content of pulcherrimin had been established at once the suspicion arose that this pigment would be a new representative of the group of haemin compounds. However, both the absorption spectrum of pulcherrimin, and the ratio N/Fe in the pigment leave no doubt that it does not belong to the haemins.

Now it may be remarked that outside the haemin field natural pigments containing iron are rare. An iron content has been claimed for aspergillin, the black pigment isolated by LINOSSIER (1891) from the conidia of *Aspergillus niger*. The investigation made by QUILICO and DI CAPUA (1933) has yielded a qualitative confirmation of this claim, but the pure pigment only contained 0.26% iron, so that it certainly belongs to a class of compounds different from pulcherrimin.

Next mention should be made of the brown pigment occurring in

the spleen, bone marrow and liver and which has first been obtained in a crystalline state by LAUFBERGER (1937). This compound which has been named ferritin has the surprisingly high iron content of about 20%. However, as was first shown by GRANICK and MICHAELIS (1943) the iron can be removed from ferritin leaving a typical, colourless, iron-free protein, the so-called apoferritin, of a molecular weight of 460.000. Both in the hydrolysates of ferritin and apoferritin GABRIO and TISHKOFF (1950) demonstrated with the aid of paper chromatography the presence of at least 14 different amino acids. It will be clear from these data that pulcherrimin does neither show any relationship with ferritin.

With a view to the above the conclusion seems to be warranted that pulcherrimin must be considered to be a representative of a hitherto unknown class of pigments.

The question then arises what can be said regarding the chemical nature of pulcherrimin.

Then it seems appropriate to dwell first for some moments on the fact that hydroxylamine has been shown to occur amongst the products of hydrolysis of pulcherrimin.

Until recently the occurrence of a hydroxamic grouping in natural products had not been proved with certainty. It is true that the occurrence of free hydroxylamine has often been postulated as an intermediate product in the processes of nitritication, denitrification and biological nitrogen fixation. Especially for the latter process VIRTANEN and collaborators (cf. VIRTANEN, 1948) have given experimental proof that under certain conditions small amounts of oxime nitrogen are formed. In this case the hydroxylamine apparently originates from an incomplete oxidation of molecular nitrogen.

In the case of *Candida pulcherrima* we are at first sight more or less obliged to accept that hydroxylamine will arise from an oxidation of the ammonium ion, this being in most experiments the only nitrogen source made available to the organism. This oxidation may occur either directly, or after the ammonia has been converted into some amide group, or in a peptide bond. But in any case we must ascribe to *Candida pulcherrima* the ability to produce a metabolic compound containing a trivalent hydroxylated nitrogen atom.

That a hydroxamic grouping can occur in a natural compound was first definitely shown by DUTCHER for the case of aspergillic acid and was recently demonstrated to be also the case for the new antibiotic nocardamin by Stoll *et al.* (1951).

In addition it seems of importance to point out that in recent years several authors have studied the formation of hydroxamic acids out of hydroxylamine under the influence of enzymes. Thus

WAELSCH *et al.* (1950) and GROSSOWICZ (1950) have demonstrated that cell-free extracts of *Proteus vulgaris* catalyze the following reactions, provided that ATP is added to the system:

Glutamine + NH_2OH → glutamohydroxamic acid + NH_3

Asparagine + NH_2OH → aspartohydroxamic acid + NH_3

VIRTANEN and BERG (1951) similarly demonstrated the synthesis of benzohydroxamic acid from benzoic acid or benzamide and hydroxylamine, with the aid of enzymes obtained either from sheep liver, or from bakers' yeast. ATP distinctly activated this synthesis in the case of benzoic acid. Similarly benzohydroxamic acid could be hydrolyzed by the respective enzymes into benzoic acid and NH_2OH.

The foregoing may suffice to stress that the occurrence of hydroxylamine amongst the hydrolytic products of pulcherrimin is a rather noteworthy fact, and moreover that this phenomenon may well be due to the occurrence of a hydroxamic acid grouping in the pulcherrimin molecule.

Now returning to the fact that the same holds for aspergillic acid, as has been shown by DUTCHER, the striking similarity between the absorption spectrum of pulcherrimin and that of the ferric complex of aspergillic acid gains increased importance. It strongly suggests that also in pulcherrimin the ferric iron binding ligands contain a hydroxamic group present in some ring system.

That this ring system may well be closely related to that occurring is aspergillic acid is probable on ground of the following considerations. In the first instance it is certainly a remarkable coincidence that the crystalline compound obtained in the pyrolysis of pulcherrimin had exactly the same composition: $C_{12}H_{18}O_2N_2$ as a byproduct obtained by DUTCHER in the preparation of bromodesoxyaspergillic acid.

Also in various other respects the similarity between the two compounds is striking. For the said product DUTCHER has proved the presence of a pyrazinedione ring which implies a dehydrogenation of the pyrazine hydroxamic acid structure as present in aspergillic acid.

It seems not at all excluded that also in the pyrolysis of pulcherrimin a similar dehydrogenation of the pigment takes place, which would mean that as in aspergillic acid also in ligands of pulcherrimin a pyrazine hydroxamic acid structure would be present. Such a conclusion is in gratifying harmony with the observation regarding the identity of the absorption spectra of the iron complex of aspergillic acid and of pulcherrimin.

In further support of this postulation the following should be pointed out. DUTCHER has shown that on hydrogenation of bromoaspergillic acid and of bromodesoxyaspergillic acid a compound

is formed which contains a diketopiperazinering. Hydrolysis of this compound yielded according to the investigations of DUNN *et al.* (1948, 1949 a, b) a mixture of leucine and isoleucine.

Now it seems another remarkable coincidence that amongst the products of the hydrolysis of pulcherrimin the only compound which with certainty could be identified was leucine, possibly accompanied by isoleucine *.

In summarizing the author is inclined to conclude that probability is in favour of the view that the iron binding property of the ligands in pulcherrimin will be connected with the occurrence of a pyrazine hydroxamic acid nucleus in these moieties.

Further investigations should bring definite proof for this hypothesis, and should reconcile this view with the data for the empirical composition of the pigment, which data apparently are in want of some correction.

*) These observations were made long before any relationship between the pigment and aspergillic acid had been surmized.

Chapter VIII

ON THE POSSIBILITY OF SUBSTITUTING IRON BY OTHER METALLIC ELEMENTS IN THE PIGMENT OF CANDIDA PULCHERRIMA

§ 1 Some remarks on the biological significance of chelation

After it had been found that iron in the ferric state was present in pulcherrimin as a co-ordinated atom it seemed worthwhile to investigate whether *Candida pulcherrima* would be able to produce analogous chelates with metallic elements other than iron.

In later years it has become increasingly evident that chelated compounds carrying different metals are not at all rare in living nature. An excellent review of our present knowledge in this field has been given by HUNTER, PROVASOLI, SCHATZ and HASKINS (1950).

To this class of chelated compounds belong the all-important prosthetic groups of many enzymes and further several biologically active compounds. Thus iron presents itself in the vitally important haeme complexes of the respiratory enzymes, such as cytochromes, catalases and other hydroperoxidases. A similar haeme-complex, leghaemoglobin, has in recent years been demonstrated by VIRTANEN and associates (1947 a, b) to fulfil a fundamental rôle in the symbiotic nitrogen fixation in the leguminous root nodules.

Similarly copper has been realized to be a vital trace element, although its 'locus of action' is as yet incompletely understood. Copper is not known to occur as an integral part of smaller prosthetic groups, as is the case with iron in the haeme-complexes, but rather appears to be part of large protein molecules with some respiratory function. The respiratory pigment of molluscs and arthropods has been shown to be a copper-protein, haemocyanin, the prototype of the copper proteins. Spectroscopic observations seem to indicate that copper in this compound is present in the tetraco-ordinated condition. Furthermore copper has been shown to be present in the tyrosinase and ascorbic acid oxidases.

The rôle of cobalt, long recognized in nutrition, blazed into the lime light with the discovery of vitamin B_{12}, now known to be required not only by vertebrates, but also by several micro-organisms. It is at present known that the cobalt atom in vitamin B_{12} occurs in a co-ordinate condition. BRINK, KUEHL and FOLKERS (1950) found that the vitamin contains one cyano group which is bound co-ordinatively to the cobalt atom.

For the other metallic elements known to be involved in cellular activities, such as magnesium, zinc, manganese and molyb-

denum, even less is known regarding their mode of action.

Apart from the essential rôle which many chelates play in cellular metabolism there is still another side to the great biological significance of chelation. It is a well-known fact that several metallic ions, if their concentration surpasses a certain lower level, have a distinct toxic effect on various living cells. It is, therefore, more or less selfevident that chelation can be an important factor in counteracting this toxic effect.

So it is a common experience in bacteriology that certain metallic ions exert a harmful influence on the growth of many microorganisms in a synthetic medium, whilst this effect is not observed, if complex substances like peptone are added to the medium. Since peptone contains several chelating compounds, such as α-amino acids and α-hydroxy acids, the metallic ions added will be for a large proportion stored in relatively non-toxic chelates.

It seems very probable that nature will not have neglected this possibility of protecting life against harmful influences, or in other words many cells may well have acquired the ability to produce chelate-forming compounds in order to ward off metallic ions. It is even tempting to see the pigment production by the red mutant of *Candida pulcherrima* in this light.

In this trend of thought one might expect that the chelate-forming activity of the yeast would not be restricted to ferric ions, but would also manifest itself in the presence of other metallic ions, thus leading to the formation of new pigments analogous to, but different from pulcherrimin. This point has, therefore, been submitted to an experimental test.

It seemed appropriate to give special attention to the elements copper, cobalt and nickel, since these metals have not seldom been encountered in biological materials, whilst they are known to form often surprisingly stable organic complexes.

§ 2 Formation of a copper-containing pigment by Candida pulcherrima

In the investigation on the behaviour of *Candida pulcherrima* towards copper ions, as well as in the experiments dealing with the other metals, always M.B.M., in which the iron had been substituted by the metal under investigation, has been used. The medium thus contained:

2.0 g Bacto-peptone, 4 g saccharose, 0.2 g KH_2PO_4, 0.05 g $MgSO_4$, $7H_2O$, 1 ml yeast autolysate, 2.0 g Bacto-agar and 100 ml distilled water. After adjusting the pH to 6.0 and sterilization at 110°C. for 15 minutes, the metal in question was added in such a quantity that the final concentration amounted to approximately 10^{-3}M. The metal was introduced as sulphate in a molar solution. To these

solutions ammonium sulphate, also in molar concentration, had been added in order to counteract hydrolysis during sterilization at 110°C. To 100 ml agar sol, 0.1 ml of the solution was added and plates prepared.

The plates were then inoculated with a suspension of a young vigorous culture of the red mutant of *Candida pulcherrima* and incubated at 30°C. for 4 days.

On a plate to which copper sulphate had been added good growth occurred. The colonies has assumed a rough form – cf. Plate V fig. 2 – and were lemon yellor in colour. No reversion to a 'non-pigmented' growth form had occurred. Microscopical examination revealed, next to normal cells, a larger type of cells which displayed a yellow granular outer layer. These cells were somewhat similar to the typical *pulcherrima*-cells, since lipids were present although not as marked as in the true *pulcherrima*-cells. See Plate V fig. 3. The cultures were quite viable, and subcultures could readily be maintained on the same medium. Nevertheless, the presence of copper was easily demonstrated in the ash of the cells, by fusion with a borax bead. Staining of live cultures with a dilute aqueous solution of methylene blue, however, revealed the occurrence of many non-viable cells, generally of the 'pigmented' type. That copper had been removed from the agar medium was readily demonstrated by flooding a plate from which growth had been rinsed, with ammonium sulphide. While a faint black coloration due to the formation of cupric sulphide developed in the upper layer of the agar, clear zones occurred at those spots where growth had previously manifested itself.

There can be no doubt on ground of the preceding observations that *Candida pulcherrima* has succeeded in incorporating the cupric ion into a greenish yellow pigment complex. Like the pulcherrimin the pigment proved to be dissolved by caustic alkali yielding a bile-green solution, but on acidification of the solution the pigment could not be recovered: only a brown precipitate resulted.

The formation of the copper pigment is more or less in accordance with expectation, in as far as it is well-known that hydroxamic acids easily form insoluble copper complexes.

In order to test the stability of the copper complex the following experiment was made.

In a copper containing plate an annular cavity was cut and filled with 4% ferric chloride solution. After 18 hours the ferric chloride had partly diffused into the agar causing a brown coloration. The yellow colonies, even beyond the brown area of the agar assumed a distinct red colour, typical for *Candida pulcherrima*, when growth on iron containing media.

It must, therefore, be concluded that ferric ion easily expels the copper out of the copper pigment.

§ 3 Formation of a cobalt-containing pigment by Candida pulcherrima

On a similar cobalt-containing agar also good growth occurred. In contrast to what held for the growth on the copper-containing medium, the colonies were entirely smooth and deep yellow in colour. However, a fair degree of dissociation into non-pigmented colonies had occurred; these colonies were cream-white in colour. Microscopical examination of the yellow colonies revealed cells of normal dimensions. Cases were again encountered where 'pigmented' cells were coated with a yellow granular outer layer. Staining of the cells with dilute aqueous methylene blue again showed the 'pigmented' cells to be non-viable. Contrary to exceptations and to findings of NICKERSON and VAN RIJ (1949) filamentous forms were not encountered. Cobalt was readily demonstrated in the ash of the cells by fusion with a borax bead. Treatment of the plates with ammonium sulphide readily demonstrated the removal of cobalt from the medium. Treatment with ferric chloride solution as described for the case of growth on the copper containing medium, gave – as is shown in Plate V fig. 4 – red colonies only where pigmentation had occurred, *i.e.* with the yellow colonies. Here again the affinity of iron for the ligands surpasses that of cobalt, so that the latter metal is expelled.

Subcultures could readily be maintained on the cobalt containing medium, but were always accompanied by reversion to the non-pigmented form.

The recent development of our insight into the rôle of cobalt in nutrition suggested the possibility that *Candida pulcherrima*, under the influence of the cobalt ion, could elaborate a compound or compounds which could show vitamin B_{12}-activity. A preliminary survey of the organisms known to produce this physiologically active compound was not encouraging for this hypothesis, since PEELER *et al.* (1949) only found very slight activity in the yeasts examined by them.

For the purpose of testing *Candida pulcherrima* on its B_{12}-producing ability, the following medium was compounded of reagents of analytical purity. Saccharose 10 g, KH_2PO_4 1.0 g, K_2HPO_4 0.16 g, 12.5 ml vitamin-free casein hydrolysate (=3 g casein), $MgSO_4.7H_2O$ 0.7 g, NaCl 0.5 g, $Ca(NO_3)_2$ 0.4 g, 1 ml trace element solution, 200 γ thiamine hydrochloride, 200 γ pyridoxine hydrochloride, 200 γ nicotinic acid, 200 γ Ca-pantothenate, 2 γ biotin, 1000 ml distilled water. Two portions of 150 ml of the above medium were introduced into KOLLE flasks of 18.5 cm diameter and autoclaved at 110°C. for 15 minutes. After sterilizing 0.15 ml of a sterile solution containing M $CoSO_4$ and M $(NH_4)_2SO_4$ was added to the agar medium, and the medium allowed to set. The plates were inoculated with thick suspensions of a 5 days old culture of

Candida pulcherrima grown on slants of the same composition. The cultures were incubated at 30°C. for 7 days. Growth again exhibited a fine brilliant yellow hue. The growth was harvested in two portions of 10 ml distilled water, the cells were collected by centrifugation, and the supernatant was put aside. From two plates 2.4 g moist yeast was obtained. The supernatant was adjusted to pH 4.5 by addition of suitable amounts of sodium acetate and acetic acid. In this liquid the cells were once more suspended, after which 0.2 ml toluene was added to the suspension. The suspension was transferred to a rubber stoppered flask and the cells autolysed at 50°C. for three days. The mass was autoclaved at 120°C., and the sediment removed by filtration through a sintered glass bacterial filter. The solution was again adjusted to pH = 5, and the clear extract autoclaved at 110°C. for 15 minutes.

The extract was then assayed for vitamin B_{12} activity with *Lactobacillus leichmanni* A.T.C.C. 9830 (313) according to the method of HOFFMAN and STOKSTAD (1949), as well as with *Euglena gracilis* according to the procedure of HUTNER *et al.* (1949). Both assays failed, however, to indicate any B_{12}-activity whatsoever.

§ 4 Formation of a nickel-containing pigment by Candida pulcherrima

Growth on a nickel containing agar was very similar to that on the cobalt containing medium. Thus rough colonies were not noted, while the colour of the pigmented colonies was a rich yellow. Microscopical examination revealed pigmented cells, which as in the cases of copper and cobalt proved generally to be non-viable, since they readily stained with dilute aqueous methylene blue as in the case of cobalt there was a marked tendency to give non-pigmented colonies.

Subcultures could be carried on, but these frequently reverted to the non-pigmented phase. Nickel could be demonstrated in the ash of the pigmented growth, and treatment of the yellow colonies with 4% $FeCl_3$ solution again gave a transformation of colour quite analogous to what happened in the cases of copper and nickel.

§ 5 Formation of a titanium-containing pigment by Candida pulcherrima

It seemed interesting to test also another trivalent metallic ion, and as such titanium was chosen.

To 100 ml sterile agar sol as used in the previous cases 0.12 ml 12% $TiCl_3$ solution was added, so that a Ti concentration of approximately 10^{-3} M was obtained. Titanous chloride being strongly subject to hydrolysis gave a precipitate of basic compounds which consequently decreased the effective Ti^{+++} concentration to a value lower than that calculated.

Growth was somewhat slower in developing, but after a week smooth pigmented colonies had developed which when viewed by transmitted light were deep orange. It was noteworthy that the pigment was partly present in the medium, as happened on media in which the iron concentration was below the value necessary for pigmentation to occur in the cell mass. Microscopical examination revealed pigmented cells, which were orange-brown in colour, and which as a rule were non-viable. Treatment with 4% $FeCl_3$ solution again expelled the titanium out of the pigment, thus leading to bordeaux-red colonies.

§ 6 Discussion

The results reported in the preceding sections throw some new light on the general phenomenon of pigment production by *Candida pulcherrima*. Whilst until now the pigment production seemed to be a specific reaction of the mutant cells on the occurrence of ferric ions in the medium, it has now been shown that several other metallic ions likewise induce these cells to produce some organic compound with marked chelating properties.

All these chelates are characterized by colours which depend on the nature of the metal responsible for the induction of the chelating compound.

The fact that in all cases the action of ferric chloride on the pigments led to the formation of the normal iron-pigment, pulcherrimin, proves in the first instance that all the metals tested are less strongly bound to the ligands then holds for iron. It is even more important that herewith proof is given that under the influence of all metals the same chelating compound has been formed.

Although no attempts have been made to isolate the chelates containing the various metals, it seems quite safe to conclude to their occurrence, firstly because of the different colours displayed by the cells, but secondly because chemical analysis indicates that the metals occur in the living cells in amounts which could scarcely be tolerated, if these metals occurred in the ionic state.

Chapter IX

SIMILAR PIGMENTS PRODUCED BY OTHER MICRO-ORGANISMS

§ 1 The behaviour of other yeast species on the medium optimal for pigmentation in Candida pulcherrima

During his study of *Candida pulcherrima* BEIJERINCK (1918) noted that this yeast had the property to produce a red pigment, when cultivated in the presence of iron salts, in common with "diverses levures du lactose". He came to this conclusion by the fact that many lactose fermenting yeasts, when grown on whey-agar assumed a reddish tint. Since then it has become common knowledge that certain strains of *Saccharomyces lactis* irregularly give rise to pigmented growth on certain solid media. SACCHETTI thus in 1938 described a lactose fermenting yeast, which he designated as *Zygosaccharomyces versicolor*, and which apparently was closely related to *Zygosaccharomyces lactis (Saccharomyces lactis)*. The new isolate differed from his strains of *Saccharomyces lactis* only in the ability to produce a red pigment, when grown on saccharose yeast water agar. SACCHETTI, aware of BEIJERINCK's study (1918) on the pigmentation of *Candida pulcherrima*, considered the presence of lactic acid, rather than iron, as being determinative for pigment production. A subculture of *Zygosaccharomyces versicolor* was forwarded to the "Centraalbureau voor Schimmelcultures", where it was found to be identical with *Saccharomyces lactis*. A specific separation of the new yeast from *Saccharomyces lactis* was not accepted, since a certain amount of pigmentation was long known to occur in genuine strains of *Saccharomyces lactis* maintained in the collection.

Similarly a pigmented yeast was isolated from a rotting sisal plant by ASHBY, and described by CORDRO'CH in 1937 who named it *Zygosaccharomyces ashbyi* for its isolator. Giant colonies produced reddish brown sectors, similar to *Saccharomyces lactis*. CORDRO'CH felt that *Zygosaccharomyces ashbyi* was closely allied to *Zygosaccharomyces marxianus (Saccharomyces marxianus)*. *Zygosaccharomyces ashbyi* was incapable of fermenting lactose, but readily assimilated this disaccharide.

In view of the above it seemed quite possible that the pigment so characteristic for *Candida pulcherrima* could also occur in other yeasts, provided that the requisite conditions were conformed to. Lack of other pertinent data, however, suggested that pigmentation for other yeasts could hardly be as striking as was the case for *Candida pulcherrima*.

A selection of both lactose- and non-lactose fermenting yeasts were consecutively grown on the following media which all received sufficient iron additions.

i) 99 ml whey + 2 g agar, pH 6.0 and autoclaved at 110°C. for 15 minutes. After sterilizing 1 ml of a sterile 5% solution of ferric ammonium citrate was added.

ii) M.B.M. containing 0.05% ferric ammonium citrate pH 6.0.

iii) Malt agar, pH 6.0 with 0.05% ferric ammonium citrate.

iv) 1 g cornsteep liquor + 2 g saccharose + 2 g of agar + 99 ml tapwater pH 6.0 and autoclaved at 110°C. for 15 minutes. After sterilizing 1 ml of a sterile 5% ferric ammonium citrate solution was added.

v) 2 g of molasses + 0.1 g $(NH_4)_2SO_4$ + 2 g agar + 99 ml tapwater pH 6.0 and autoclaved at 110°C. for 15 minutes. After sterilizing 1 ml of a sterile 5% ferric ammonium citrate solution was added.

vi) 99 ml yeast water + 2 g saccharose + 2 g agar pH 6.0 was autoclaved at 110°C. for 15 minutes and 1 ml of a sterile 5% ferric ammonium citrate solution was added.

vii) READER-medium to which was added 2% agar. After sterilizing 0.05% ferric ammonium citrate was introduced.

Cultivation was done on slant cultures, except in the case of vii where giant colonies were grown to save time and material. The cultures were incubated at 30°C. for 3 to 4 days, and then kept at room temperature. The results are given in the table on page 78, followed by some details regarding the cases in which pigmentation occurred.

We may conclude from the table pigmentation that occurs in both lactose fermenting and non-lactose fermenting groups.

However, in no instance was pigmentation found to be as striking as in the case of *Candida pulcherrima*. On the contrary great difficulty was often enough experienced, in deciding definitely whether or not growth truly had a reddish tint especially, when the cultures had to be examined in artificial light. Growth often assumed a brown colour which could entirely mask any suggestions of red.

For *Saccharomyces marxianus* (strain *ashbyi*) the dependency of pigmentation on iron was clearly shown by growing the organism on M.B.M. with varying iron concentrations. For *Candida pulcherrima* pigment formation in the agar was completely inhibited at 0.05 mg/ml, while for *Saccharomyces marxianus* this occurred already at 0.01 mg/ml. *Saccharomyces marxianus* assumed a deep red colour at optimal iron concentrations, but pigmentation was never so intense and so striking as that of *Candida pulcherrima*.

The pigmentation of various lactose and non-lactose fermenting yeasts on media suitable for pigmentation of *Candida pulcherrima*

Organism	Medium						
	i	ii	iii	iv	v	vi	vii
Saccharomyces lactis, strain:							
2.5.1.	-	±	-	-	-	+	+
2.5.2	-	-	-	-	-	D	-
2.5.3	-	-	-	-	-	-	-
2.5.4	-	-	-	-	-	-	-
U L 43	-	-	-	-	-	-	-
U L 72	-	-	-	-	-	-	-
"casei"	-	-	-	-	-	-	-
"versicolor"	-	-	-	-	-	+	+
Saccharomyces fragilis, strain:							
8.12.1	-	-	-	-	-	-	±
8.12.2	-	-	-	-	-	D	-
8.12.3	-	-	-	-	-	D	-
8.12.4	-	-	-	-	-	-	±
8.12.5	-	-	-	-	-	-	±
8.12.6	-	-	-	-	-	-	±
8.12.7	-	-	-	-	-	-	±
8.12.8	-	-	-	-	-	-	-
8.12.9	-	-	-	-	-	-	-
8.12.10	-	-	-	-	-	D	-
8.12.11	-	-	-	-	-	-	-
Saccharomyces marxianus, strain:							
2.7.1	-	-	-	-	-	-	±
2.7.2	-	-	-	-	-	-	-
"macedoniensis" 1	-	-	-	-	-	-	-
" 2	-	-	-	-	-	-	-
" 3	-	-	-	-	-	-	-
" 4	-	-	-	-	-	-	-
" 5	-	-	-	-	-	-	-
" 6	-	-	-	-	-	-	-
" 7	-	-	-	-	-	-	-
" 8	-	-	-	-	-	-	-
"ashbyi"	-	++	±	-	-	+	+
Saccharomyces cerevisiae							
8.1.67	-	-	-	-	-	-	-
Candida pseudotropicalis, strain:							
35.6.1	-	-	-	-	-	D	±
35.6.2	-	-	-	-	-	D	-
35.6.3	-	-	-	-	-	-	-

Organism	Medium						
	i	ii	iii	iv	v	vi	vii
Candida pseudotropicalis, strain:							
35.6.4	-	-	-	-	-	D	-
35.6.5	-	-	-	-	-	-	-
Candida pseudotropicalis var. *lactosa*, strain:							
35.6A.1	-	-	-	-	-	-	-
35.6A.2	-	-	-	-	-	-	-
35.6A.3	-	-	-	-	-	-	-
Candida macedoniensis, strain: 35.12.1	-	-	-	-	-	-	-
35.12.2	-	-	-	-	-	-	-
35.12.3	-	±	±	-	-	-	±
Brettanomyces claussenii	-	-	-	-	-	-	-
Torulopsis sphaerica, strain:	-	-	-	-	-	-	-
Candida pulcherrima, strain:							
35.2.8	++	+++	+++	+++	++	+++	+++

+++ : deep maroon
++ : deep red
+ : pink
± : cream pink
D : red diffusion in substrate

The effect of iron was not so readily demonstrated for *Saccharomyces lactis*. SACCHETTI (1938) reports that additions of iron did not improve pigmentation of *Zygosaccharomyces versicolor*. However, he contends that the addition of 1% lactic acid affords better pigmentation. Nevertheless, it was felt that the pigmentation of *Saccharomyces lactis* was related to that of *Candida pulcherrima*, and the experiments of SACCHETTI were repeated. The pH of a yeast agar, 2% saccharose, 1% lactic acid was found to be 4.7. As control a batch of yeast water, 2% saccharose agar was prepared, the pH being adjusted to 4.7 with concentrated HCl. Analysis showed the yeast water to contain only 0.007% lactic acid. No additional iron was added to either batch. Slant cultures were made of *Saccharomyces lactis* strain 2.5.1 and strain *versicolor* and incubated at 30°C. Within 16 hours pigmentation had commenced on both media in equal intensity. After eight days on both media pigmentation had occurred in the cell mass, no noticeable difference in intensity could be observed. Evidently for the

pigmentation of *Saccharomyces lactis* to occur in the cell mass no relatively high iron concentration was necessary, as has been shown to be the case for *Candida pulcherrima* and *Saccharomyces marxianus*.

A further indication that the pigmentation in the case of *Saccharomyces lactis* and *Saccharomyces marxianus* is very similar to that of *Candida pulcherrima* was obtained by growing these organisms on READER agar which was allowed to solidify in vertical culture tubes. No addition of iron was made. The tubes were incubated for three weeks at 30°C. During incubation red pigmented zones originated in the agar. These zones were comparable in colour as well as in intensity to that of *Candida pulcherrima*, when grown under the same conditions.

§ 2 Pigmentation in yeasts in consequence of biotin deficiency

As has been shown in Chapter IV § 2 *Candida pulcherrima* grew well and showed excellent pigmentation on media devised to satisfy the vitamin requirements of normal yeasts. It was, therefore, somewhat startling when CUTTS and RAINBOW in December 1950 reported that in the course of their studies on the biotin requirements of various fermentative yeasts, they had observed a red pigmentation when these yeasts were grown on sub-optimal biotin levels in the presence of methionine. Most of their observations were conducted with a brewery top yeast. A pink colour developed in the cell mass, when the D-biotin concentration was maintained between 0.003 to 0.04 γ per litre. Pigmentation failed to develop when i) methionine was replaced by another amino acid, ii) the biotin level was raised to 0.08 γ per litre, or when iii) the biotin concentration was maintained at optimal values and calcium pantothenate, p-aminobenzoic acid or meso-inositol were added in sub-optimal quantities. Where p-aminobenzoic acid was replaced by adenine, and methionine by histidine, no pigment was formed. The addition of 25 mg adenine chloride per litre to a medium, otherwise conforming to the requirements for pigment production, greatly suppressed pigmentation. It should be stressed in this connection that the yeast in question was *not* adenine-dependent. The same observations regarding the appearance of a pink colour were also made for strains of *Schizosaccharomyces pombe*, *Saccharomycodes ludwigii*, *Saccharomyces carlsbergensis* and *Saccharomyces cerevisiae*. In the cases of these yeasts pigmentation also occurred *without* the addition of methionine. The authors suggested that a precursor of adenine was possibly responsible for the pigmentation encountered, and they further thought it possible that this type of pigmentation might be related to that encountered by LINDEGREN and LINDEGREN (1947) in their studies of adenine-

dependent haploid mutants of *Saccharomyces cerevisiae*. In this case an interaction of the adenine precursor with methionine was held to be responsible for pigmentation.

Whatever the case may be, it seemed worth-while to repeat and to extend the observations of CUTTS and RAINBOW by testing a large number of yeasts species on their ability to form a red pigment under the conditions as prescribed by these authors.

It was decided to include in this investigation in the first place all forty-four strains which in § 1 of this chapter had been tested – although with generally negative results – on their ability to form a red pigment on the iron-containing media which had been found more or less optimal for pigment production by *Candida pulcherrima*. With a view to the yeast species investigated in the study of CUTTS and RAINBOW it seemed, however, desirable to include also these species in the investigation. For this reason four strains of *Saccharomycodes ludwigii*, ten strains of *Schizosaccharomyces pombe* and ten strains of *Saccharomyces carlsbergensis* were also examined.

For these additional strains the behaviour on the iron-containing M.B.M. was first established. On this medium only four of the strains of *Schizosaccharomyces pombe* gave a faintly pink growth, suggesting that here a pigment had been formed of the type of that of *Candida pulcherrima*.

Next all 68 strains were plated on the medium of CUTTS and RAINBOW to which 2% Nobel Bacto Agar was added.

The medium used by CUTTS and RAINBOW contains:
10 g glucose, 4 g $(NH_4)_2HPO_4$, 2.0 g KH_2PO_4, 1.0 g $MgSO_4.7H_2O$, 0.25 g $CaCl_2$, 3 ml lactic acid, 12 ml potassium lactate, 500 mg L-methionine, 10,000 γ inositol, 1000 γ Ca-D-pantothenate, 1000 γ aneurin, 1000 γ pyridoxine, 1000 γ nicotinic acid, 50 γ riboflavin, 10 γ para-aminobenzoic acid, 100 γ KI, distilled water 1000 ml and 1 ml trace element solution. To this medium 0.01 γ D-biotin and 20 g Nobel Bacto agar were added. The medium was then sterilized at 110°C. for 15 minutes. The plates were incubated at 30°C. for two days, and then kept at room temperature for three days before examination took place.

The results are recorded below.

Saccharomyces lactis. Six of the seven strains gave a great number of pink to red colonies. Strain 2.5.1 was exceptionally fine, in this case all colonies were a deep red. See Plate V fig. 5. Such intense pigmentation had never been observed in the earlier experiments with this strain. Deeply pigmented cells closely resembling those observed in *Candida pulcherrima*, when grown on a iron-containing medium, were noted in profusion (cf. Plate V fig. 6).

Saccharomyces fragiles. Five of the eleven strains gave a fair number of pink colonies. Strains 8.12.5 and 8.12.7 gave the most pronounced pigmentation.

Saccharomyces marxianus. Of the eight strains investigated six gave pink to rose coloured colonies in fair number. Strain 8.12.7 was found to give the strongest pigmentation.

Saccharomyces cerevisiae. Strain 1.8.67 give fine pink to red colonies. Here microscopical examination revealed that the pigment was generally only present in the vacuoles.

Candida pseudotropicalis. Only strain 35.6.3 gave one red colony. Difficulty was experienced in discerning the colour, since growth in general had the tendency to turn brown, thus masking any possible pink.

Candida pseudotropicalis var. *lactosa.* Strain 35.6A.2 gave one definitely pink colony. The same difficulty was encountered as in the case of *Candida pseudotropicalis.*

Torulopsis sphaerica. No coloured colonies were encountered.

Candida macedoniensis. On the low biotin concentration the organism failed to grow.

Brettanomyces claussenii. This organism grew very poor and colonies soon turned brown.

Schizosaccharomyces pombe. All eleven strains investigated gave pigmented colonies. Brown shades were encountered in some cases. Strain 1.1.10 seemed to give the best pigmentation.

Saccharomycodes ludwigii. All four strains only assumed a more brown tint.

Saccharomyces carlsbergensis. No pink or red colonies were noted, since all strains readily turned brown.

It is clear that the foregoing observations give a full confirmation of the claims of CUTTS and RAINBOW that biotin deficiency induces many yeast species to the production of a pink to red pigment.

In how far this pigment is in any way related to the iron-induced pigment of *Candida pulcherrima* remains to be seen. In any case it seemed worth-while to test also whether biotin-deficiency would in any way influence pigment production in *Candida pulcherrima.*

Cultures were made from a young culture of the red mutant on the medium of CUTTS and RAINBOW. The plates were incubated at 30°C.

Growth was slow in developing, and initially colourless. Pigmentation was first noted after the third day. After four days the plates were placed at room temperature. As could be expected, the amount of pigment formed was not large, since the iron concentration was low. Remarkably this time all pigment was retained

in the colonies giving a more or less pink colour. This was in contrast to the earlier observations according to which at such low iron concentrations and in the presence of sufficient biotin for maximum growth, pigmentation, inevitably occurred in the agar. Low biotin concentrations thus seem to favour the retention of the pigment in the cell mass.

Subsequently two batches of the medium containing 2 and 0.01 γ biotin respectively, were prepared and plate cultures made. The plates were incubated at 30°C. and examined after 48 hours. Growth on the medium containing 2 γ biotin was rapid in development, and pigmentation had already commenced. On these plates the pigment was dispersed in the agar, while on the plate containing 0.01 γ biotin, where growth was poor and pigmentation was scant, no pigment in the agar was noted. The colonies on plates with the higher biotin concentrations were generally colourless, while on the plate with the lower concentration several pink colonies had developed. After five days the plates were removed to room temperature.

After a further five days the plates were again examined. The colonies on the plates containing 2 γ biotin per litre, had established intense colour zones, whilst the colonies themselves were generally cream-coloured. Colonies on the plates containing 0.01 γ biotin, had now assumed a red colour, without pigment in the agar.

It seems very likely that the explanation for this absence of the pigment in the agar must be sought in the amount of precursor produced. On 2 γ biotin per litre, the yeast could carry on its 'normal' functions. The chromogen produced readily diffused into the agar, producing the typical bordeaux-red zones. On 0.01 γ biotin, growth was slight, and in consequence the production of chromogen poor. In this case iron diffused into the colonies, giving them their typical colour.

These results bring at least a first indication that there is some close relationship between the iron-induced pigmentation of *Candida pulcherrima* and the pigmentation induced in a great number of yeast species by biotin deficiency.

§ 3 The production of a pink pigment in haploid yeast mutants and their hybrids

As has already been casually remarked in § 2 LINDEGREN and LINDEGREN (1947) in their studies on the mutation of haploid segregants of *Saccharomyces cerevisiae* encountered a red mutant which was characterized by a beautiful pink coloration (cf. LINDEGREN, 1949 fig. 15.1).

The particular mutant had been obtained by TATUM and REAUME by subjecting a haplophase segregant of *Saccharomyces cerevisiae* to mustard gas treatment.

LINDEGREN and LINDEGREN established the interesting fact that the mutant in question differed from the original form in that it was incapable of growing in a medium which was deficient in adenine. TATUM and REAUME had also discovered a methionine-dependent mutant likewise produced by mustard gas treatment. By crossing, LINDEGREN and LINDEGREN then produced hybrids heterozygous for adenine- and methionine-dependence. A genetic analysis of this hybrid then revealed that all cultures which were both adenine- and methione-dependent were white, whilst the adenine-dependent forms which were methionine-independent developed the pink-colour. Apparently methionine is indispensable for the pigment production, and this was further corroborated by the observation that also some white adenine-dependent and mentionine-dependent cultures produced the pink pigment, when they were grown on peptone agar to which an excess of methionine had been added.

EPHRUSSI, HOTTINGUER and TAVLITZKI (1949) report on the isolation of a mutant of a haploid form of *Saccharomyces cerevisiae* ("Yeast Foam") which was characterized by the production of a pink pigment. In this mutant ("grande rouge") several mutants appeared, partly spontaneously, partly by treatment with acriflavine, which although they contained a gene for pigment production formed white colonies. The remarkable point is that the original pink culture was again characterized by its inability to grow in the absence of adenine which, however, could be replaced by hypoxanthine.

Also WINGE and ROBERTS (1950) obtained an adenine-dependant, recessive, red-coloured, haploid mutant which had arisen through the effects of RÖNTGEN irradiation. By crossing this mutant with a normal white type and by subsequent inbreeding, a sporulating homozygotic red yeast was produced. WINGE and ROBERTS considered the adenine-dependant mutant to be identical with that of TATUM and REAUME.

It is indeed remarkable that the pink haploid mutants of *Saccharomyces cerevisiae* obtained in three independent series of investigations have the property of adenine-dependency in common.

For the present study these observations are particularly important, because EPHRUSSI and LEDERER have made an attempt to elucidate the chemical nature of the pigment in question. The pigment proved to be more or less soluble in water, insoluble in organic solvents, but readily soluble in caustic alkali. From this solution it was again precipitated as deep red amorphous flakes by addition of acid. Apparently the pigment had an acid nature. The colour was readily discharged by SO_2 and H_2S at room temperature. Hydrolysis of their most purified preparation with 6 N HCl for 24 hours at 100°C. yielded a complex mixture of compounds. By paper chromatography glumatic acid, glycine, serine,

valine, leucine, aspartic acid and proline were identified. On the evidence at their disposal the authors concluded that the pigment was composed of a polypeptide entity with a quinoid prosthetic group. The authors gave the following elementary analysis: %C 33.1-38.7, %H 6.1-7.6, %N 8.4-9.1, %P 1.4-3.4, %ash 0.39-0.71. The pigment showed an absorption band in the vicinity of 525 m μ.

On considering the above data, it first strikes that the pigment studied by the French authors in one respect shows a great similarity with pulcherrimin, *viz.* in its behaviour towards various solvents. Here again, we meet with a compound which is insoluble in all organic solvents, but which readily dissolves in caustic alkali, and which can be reprecipitated from this solution by acidification.

However, the analytical figures do not show much resemblance to those obtained for pulcherrimin. Although the %H and %N are in fair agreement with those of pulcherrimin, the analytical figures for the carbon are almost 10% lower than those for pulcherrimin. Further pulcherrimin does not contain any P, and its ash content of 15.5% is in strong contrast to the low ash content 0.39-0.71%, reported by EPHRUSSI and LEDERER for their pigment.

The most striking point of difference is, however, that the French authors do not refer at all to any iron content of their pigment.

Then there is the great variety of amino acids which was obtained in the hydrolysis of the pigment which also presents a marked difference with pulcherrimin.

EPHRUSSI and LEDERER, however, point out that the said amino acids may well have their origin in proteins or peptides present as impurities in their pigment preparation. Once accepting this to be the case, it is clear that neither much value can be attached to their analytical figures.

The author's experience with the group of the pink adenine dependent mutants of *Saccharomyces cerevisiae* has been restricted to a superficial study of the homozygous hybrid obtained by WINGE and ROBERTS (1950). These authors forwarded the culture to the Yeast Division of the "Centraalbureau" in Delft in June 1948, where it was included in the collection as No 8.1.72.

By plating the culture on ordinary malt agar or saccharose peptone agar, and on subsequent incubation at 30°C.; two types of colonies could be distinguished after 3 to 4 days. Next to the white colonies a second pale pink type appeared. As these colonies aged the colour deepened to a very pronounced pink which, however, markedly differed from the maroon to bordeaux-red, so typical for the red mutant of *Candida pulcherrima*. Frequently pigmentation was limited to the surface of the colonies, which suggests that

atmospheric oxygen may be involved in the pigmentation phenomenon. The cultivation on media of relatively high iron concentrations such as M.B.M. in no way enhanced pigmentation.

On aging of the plates it was not seldom noted that non-pigmented secondary growth arose, probably as a result of back mutation. In contrast to what holds for *Candida pulcherrima* pigmented colonies eventually fade after several weeks and turn brown, a phenomenon never noticed in cultures of the red mutant of *Candida pulcherrima* even after 6 to 7 months.

It was found that the pigment of the red adenine-dependent mutant of *Saccharomyces cerevisiae* No 8.1.72, similarly to what was shown to hold for the pigment of the red adenine-dependent mutant of *Saccharomyces cerevisiae* studied by EPHRUSSI and LEDERER, could be extracted by alkali, and reprecipitated amorphously by subsequent acidification. This opened the possibility for a spectroscopical examination of the pigment, as reported in § 5.

On summarizing the foregoing it can be said that on the one hand the properties of the adenine-dependent pink yeasts show some resemblance to those of pulcherrimin, but that on the other hand there are several strong arguments which speak against the identity of the two types of pigments.

§ 4 On the formation of a red pigment by aerobic sporeforming bacteria

Incidentally the author came across a culture of some aerobic sporeforming bacterium which was characterized by the formation of a red zone in a medium which consisted of plain peptone agar. It drew the attention that the general picture of the culture showed a great similarity to that obtained by cultivating *Candida pulcherrima* on media with low iron concentration.

This stimulated a closer investigation of this particular type of bacterial pigmentation.

With the aid of the monographic study by SMITH *et al.* (1946) on the genus *Bacillus* it was not difficult to identify the organism as some variant of *Bacillus subtilis*.

Now it is well-known that 'red' representatives of the group of aerobic sporeforming bacteria are frequently encountered. As long ago as 1888, GLOBIG isolated and described such an organism, to which he gave the name of *Bacillus mesentericus ruber*. Since then several, although mostly casual, references to the occurrence of such bacteria have appeared in the literature.

In his handbook of systematic bacteriology MIGULA (1897) accepted GLOBIG's *Bacillus* as a new species for which he introduced the name *Bacillus globigii*. In more recent systematic sur-

veys the organism is no longer recognized as a separate species, and is usually included into the species *Bacillus subtilis*.

There is, therefore, all reason to conclude that the culture of the author was identical with or at least closely related to GLOBIG's bacterium.

The author's curiosity once being aroused, it was tempting to test whether also in this case pigment production would be stimulated by an addition of iron to the medium, as holds for *Candida pulcherrima*.

In order to check this the bacterium was streaked on two slants: one consisting of common peptone agar, the second of the same medium to which 0.05% ferric ammonium citrate had been added. It was now most satisfactory that, indeed, on the second medium a striking increase in pigment production could be observed. However, in this case a coloured zone was almost absent; the pigment was practically completely retained by the bacterial growth.

This demonstration also seemed to offer an explanation for the more or less capricious character of pigment production by strains of this type, to which often reference is made in literature. This might well be connected with changes in 'free' iron content of the media employed.

Taking this into account it seemed worthwhile to ascertain whether on media with a special addition of iron the capacity to form pigment might not be quite widely distributed within the genus *Bacillus*.

To this purpose 60 strains were selected, representing the 28 species of the genus *Bacillus* present in the collection of the "Laboratorium voor Microbiologie" at Delft.

All strains were cultivated directly on peptone agar with 0.05% ferric ammonium citrate and incubated at 30°C. for 3 days. Only the thermophilic strain *Bacillus kaustophilus* was incubated at 45°C.

Results:

Bacillus strain	*Production of red pigment*
Bacillus subtilis	
1.3.1	+
1.3.2	-
1.3.3	-
1.3.4	-
1.3.5	-
1.3.6	+++
1.3.7	-
1.3.8	±
1.3.9	±

Bacillus strain	*Production of red pigment*
Bacillus subtilis	
1.3.10	++
1.3.11	-
1.3.12	-
1.3.13	+++
1.3.14	black pigment
1.3.15	-
1.3.16	+++
1.3.17	-
1.3.18	+++
1.3.19	++
Bacillus mesentericus	
1.2.1	-
1.2.2	-
1.2.3	-
Bacillus licheniformis	
1.55.1	-
1.55.2	+
Bacillus mycoides	
1.4.3	-
1.4.6	-
1.4.7	-
Bacillus cereus	
1.11.4	-
Bacillus undulatus	
1.11.1	-
1.11.3	-
Bacillus megaterium	
1.5.2	-
1.5.10	-
1.5.11	-
Bacillus pasteurii	
1.9.1	-
1.9.2	-
1.9.3	-
Bacillus nitroxus	
1.21.1	-
Bacillus leubii	
1.10.1	-

Bacillus strain	*Production of red pigment*
Bacillus firmus	
1.65.1	-
Bacillus brevis	
1.58.1	-
Bacillus funicularis	
1.17.1	-
Bacillus sphaericus	
1.61.1	-
1.61.2	-
1.61.3	-
Bacillus multivorans	
1.15.1	-
1.15.2	-
Bacillus agri	
1.12.1	-
Bacillus kaustophilus	
1.19.1	-
Bacillus amarus	
1.13.1	-
Bacillus palustris	
1.23.1	-
Bacillus pimulus	
1.62.1	-
Bacillus guttulatus	
1.20.1	-
1.20.2	black pigment
Bacillus paradoxus	
1.7.1	-
Bacillus fastidiosus	
1.18.1	-
Bacillus circulans	
1.8.2	-
Bacillus laterosporus	
1.64.1	-
Bacillus aminovorans	
1.16.1	-
Bacillus alvei	
1.63.2	-
Bacillus lentus	
1.66.1	

The number of + in the above list indicates the intensity of pigmentation of the bacterial growth. It was again striking that the iron addition to the medium had almost suppressed the formation of pigmented zones in the agar.

As is evident from the above, the typical iron dependent pigmentation is restricted strictly to the strains belonging to the species *Bacillus subtilis* and *Bacillus licheniformis*. Furthermore it appears that this property is rather capricious, in so far that there are both qualitative and quantitative differences between strains belonging to one and the same species.

It was later found that a particularly fine demonstration of pigment formation in the agar could be obtained by cultivating a suitable strain on a high layer of an agar medium containing 1% peptone, 2% saccharose, 0.05% K_2HPO_4, 0.01% $MgSO_4.7H_2O$.

Observations were made after nine days. Due to the low iron concentration of this medium the pigmented zones were very pronounced, and quite comparable to those obtained with *Candida pulcherrima* under comparable conditions.

The foregoing experiments strongly suggest that there will be a close relationship between the bacterial pigment and that of *Candida pulcherrima*.

§ 5. Spectroscopical analysis of the various pigments referred to in the preceding sections

Lack of time preventing a thorough investigation of the various red pigments produced by the micro-organisms discussed in the preceding sections, it seemed nevertheless most desirable to check a possible identity of the various pigments with the aid of a superficial spectroscopical analysis.

It was indicated to give first attention to the pigments produced by the selected strains of *Saccharomyces lactis* and of *Saccharomyces marxianus*, since in these cases the iron-dependency of pigment formation had been duly demonstrated.

The yeast crop obtained from one plate culture of *Saccharomyces lactis* on the biotin deficient medium as specified by CUTTS and RAINBOW was suspended in 5 ml N KOH. The pigment was readily extracted from the yeast mass to give a yellow solution, which was separated from the yeast by centrifugation. On acidification and subsequent addition of a trace of $FeCl_3$ the red pigment was again precipitated. After settling the supernatant still had a distinct red colour, apparently due to the fact that a small part of the pigment remained dissolved in the colloidal state. The solution was diluted to a suitable concentration with distilled water, and the absorption spectrum of the red liquid recorded with the BECKMAN spectrophotometer. Measurements were conducted with minimum

slit width, using a blank sample of neutralized alkali containing the same quantity of iron.

The results of the measurements have been plotted in Fig. V as curve B. For the sake of comparison also the earlier determined spectrum of pulcherrimin has been included in Fig. V as Curve A. It strikes at once that both curves show an almost coinciding maximum in the vicinity of 390 m μ. The maximum of pulcherrimin in the vicinity of 490 m μ is in the spectrum of *Saccharomyces lactis* less pronounced, but nevertheless clearly indicated.

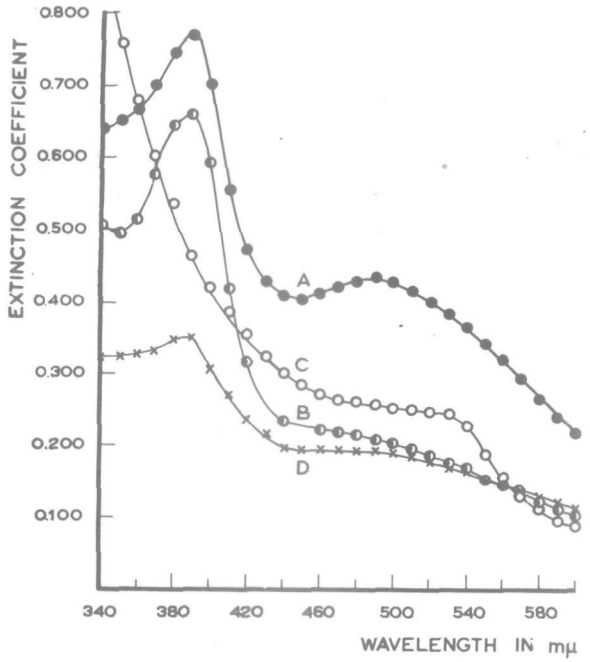


Fig. V. Absorption spectrum of: A, pigment of *Candida pulcherrima*; B, *Saccharomyces lactis*; C, *Saccharomyces cerevisiae*; red hybrid and D, *Bacillus subtilis*

Altogether there is sufficient reason to conclude, if not to the identity of, then at least to a very close relationship between the two pigments.

Difficulty was encountered in the case of *Saccharomyces marxianus* strain *ashbyi* as the pigment immediately precipitated as coarse flakes making spectroscopic measurements impossible.

Here the conclusion regarding the identity of this pigment and pulcherrimin – or at least their close relationship – must rest for the time being on the identity of the factors determining the mode of pigment formation in the two cases.

As has already been remarked in § 3 the only representative of

the red adenine-dependent mutants accessible to the author was the homozygous strain of *Saccharomyces cerevisiae* described by WINGE and ROBERTS (1950).

Since with this type of pink yeasts pigment production is neither dependent on the presence of excess iron, nor on the use of a medium with a sub-optimal biotin level, the organism was cultivated on plain malt agar.

The growth from one plate was suspended in 5 ml N KOH. This again led to an extraction of the pigment. The yeast mass was separated from the liquid by centrifugation. The clear supernatant liquid was withdrawn. On acidification of this liquid the red colour was restored with the concomitant precipitation of deep red amorphous flakes. The residual liquid remained somewhat coloured and after a suitable dilution the absorption spectrum was determined. Measurements were again carried out with minimum slit width, using N KOH neutralized with acetic acid as blank.

The results are recorded as curve C in Fig. V. It strikes at once that the general appearance of this curve is strongly divergent from that of the curves A and B. There is not the slightest indication for a maximum in the vicinity of 390 m μ. Nor is a maximum near 490 m μ indicated. At most it is worthy of notice that the extinction coefficient between 525 and 520 m μ remains constant. In this connection it should be mentioned that EPHRUSSI and LEDERER report for the pigment of their red adenine-dependent mutant an absorption band in the vicinity of 525 m μ.

The spectroscopical investigation, therefore, leads to the conclusion that the pigment of the adenine-dependent mutant is dissimilar to that of *Candida pulcherrima* and *Saccharomyces lactis*. This result is in harmony with the fact that the conditions determining the pigmentation of the adenine-dependent mutants are so different from those which determine the formation of pulcherrimin. Nevertheless, it cannot be denied that some of the chemical properties of both yeast pigments, to wit the solubility in and resistance against strong alkali, and the possibility of recovery by acidification of the said solution, are so strikingly similar, that one may expect that further investigation will reveal a certain degree of relationship.

Finally attention has been given to the absorption spectrum produced by some strains of *Bacillus subtilis*. For this investigation strain Nr 1.3.18 was selected, and the organism was grown as a surface culture on peptone water containing 0.05% ferric ammonium citrate. The organism formed a rose coloured pellicle on the medium. A seven days old culture was as usually extracted with alkali. The bacterial cells were removed by centrifugation, and the residual yellow supernatant again neutralized with simultane-

ous addition of a trace of iron. Again a red precipitate was formed; some colour remained in the solution which was used for the determination of the absorption spectrum. The result is recorded as curve D in Fig. V. Here again the two absorption maxima of pulcherrimin are at least clearly indicated, which strongly suggests the remarkable identity of the yeast and the bacterial pigments.

It is clear that all observations reported in this section only bear a provisional character.

A more detailed study of the four pigments investigated is evidently required for complete verification. Before concluding the author wishes to state explicitly that he is not at all convinced that further investigation will show that the pigments of *Candida pulcherrima*, *Saccharomyces lactis* and *Bacillus subtilis* are identical. But, most probably, they will belong to the same group of compounds.

It should be realized that there are numerous examples showing that a number of structurally related natural pigments are found in various organisms, sometimes closely related, sometimes widely dispersed in the kingdom of life.

Chapter X

SOME REMARKS ON THE MECHANISM OF PULCHERRIMIN PRODUCTION

In this final chapter some remarks will be made regarding the – it is true very restricted – insight obtained into the mechanism of pulcherrimin production.

This survey should start with a tribute to BEIJERINCK who was the first investigator to indicate clearly the factors which determine the production of the said pigment by *Candida pulcherrima*.

BEIJERINCK stresses in his paper that the pigment production is not a common property of all cells belonging to this species. He clearly showed that many cells developed fully colourless colonies under conditions which were optimal for pigment production by other cells. This implies that the first condition for pigment production is a genetic change in part of the individual cells. Cells showing this change have been referred to in this publication as 'the red mutant' BEIJERINCK secondly discovered that the presence of iron in the medium was an essential condition for pigment formation, and he convincingly demonstrated that an increase of the iron concentration of the medium led to an enhanced pigment production.

The fact that it was possible to obtain pigment formation by adding iron after the development of the yeast had already practically come to an end, made BEIJERINCK rightly conclude that in the presence of a suboptimal iron level some compound had been formed which only after addition of the iron was converted into the pigment.

BEIJERINCK proved, however, that this pigment formation was not merely a chemical reaction between his precursor ("chromogène incolore") and the ferric ion, since he established that pigment was only formed, if free oxygen had access to the system.

Notwithstanding the fact that several authors have denied the essential rôle of iron in the formation of the pigment, the correctness of BEIJERINCK's views have been fully corroborated by the investigation of ROBERTS (1946b), whilst also the author has arrived at the same result (cf. Chapter V).

Moreover, BEIJERINCK has pointed out that his concept also yields a full explanation of the fact that on media with a high iron level the occurrence of the pigment is restricted to the colonies, whilst in case of a relatively low iron content of the medium the colourless precursor diffuses into the surrounding

ous addition of a trace of iron. Again a red precipitate was formed; some colour remained in the solution which was used for the determination of the absorption spectrum. The result is recorded as curve D in Fig. V. Here again the two absorption maxima of pulcherrimin are at least clearly indicated, which strongly suggests the remarkable identity of the yeast and the bacterial pigments.

It is clear that all observations reported in this section only bear a provisional character.

A more detailed study of the four pigments investigated is evidently required for complete verification. Before concluding the author wishes to state explicitly that he is not at all convinced that further investigation will show that the pigments of *Candida pulcherrima*, *Saccharomyces lactis* and *Bacillus subtilis* are identical. But, most probably, they will belong to the same group of compounds.

It should be realized that there are numerous examples showing that a number of structurally related natural pigments are found in various organisms, sometimes closely related, sometimes widely dispersed in the kingdom of life.

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solid medium, and in doing so leads to a diffuse pigment precipitation in a wide zone around the colony.

Now it should be remarked that these fundamentally important observations on the essential rôle of iron in the formation of the red pigment of *Candida pulcherrima* do not bring a definite proof that this pigment should be iron-containing. In the first place there is the example of etiolation of green leaves in consequence of iron-deficiency, showing the indispensability of iron for the production of the iron-free pigment chlorophyll. In addition BEIJERINCK's observation regarding the indispendability of oxygen for pigment production opens the possibility that the rôle of iron might be restricted to a catalytical action on the oxidation of the precursor.

The investigations reported in this thesis regarding the chemical nature of pulcherrimin are the first to prove definitely that this pigment is, indeed, an organic compound containing iron.

Another aspect of the process underlying pigment production has come to the fore by the spectroscopical demonstration of the identity of pulcherrimin and the pigment produced by several yeast species when grown on a suboptimal biotin level, as discovered by CUTTS and RAINBOW. This tends to show that in these yeast species biotin-deficiency leads to a derailment of some normally occurring metabolic process in such a way that the pulcherrimin-precursor is formed.

This justifies the conclusion that in some yeasts, like the red mutant of *Candida pulcherrima*, such a derailment will for some reason happen independently of the presence of biotin, whilst in other yeasts biotin-deficiency is indispensable for this course of affairs.

In this respect it is now worthy to stress that on considering various yeast species there seems to be a gradual transition in the degree to which biotin-deficiency is essential for pigment production.

Starting with the red mutant of *Candida pulcherrima* it is certain that this organism can elaborate pigment on media with biotin levels optimal for growth, and even surpassing this value. This holds not only for the strain 35.2.8 used throughout this investigation, but as well for 11 other strains tested for this property.

The same has been shown to be the case for strain *ashbyi* of *Saccharomyces marxianus*, but seven other strains of this species did not respond to iron addition to the media by pigment production. However, on cultivating these strains on a medium relatively deficient in biotin, six of the seven strains gave convincing proof that they too were able to produce the pigment.

An analogous situation was encountered in *Saccharomyces lactis*.

Here the reaction on iron addition was still less marked than in *Saccharomyces marxianus*. Only strain 2.5.1 and strain *versicolor* gave a noticeable pigmentation on some of the media suitable for pigment production by *Candida pulcherrima*. On five other media which were equally suitable for the latter organism, pigmentation failed to develop. Yet on media containing a sub-optimal biotin level six of the seven strains of the species all gave splendid pigmentation.

Next comes a considerable number of yeast species for which no strains have been encountered which ever give any pigment production on media containing normal biotin levels. But in agreement with the original observations made by CUTTS and RAINBOW it has been found to be possible to induce pigment production by a relative biotin-deficiency in certain strains of the species *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Moreover, the same behaviour was observed for *Saccharomyces fragilis*, *Candida pseudotropicalis* and its variety *lactosa*.

It is exactly this gradual transition in the degree of necessity of relative biotin-deficiency for the phenomenon of pigment production which makes it so highly probable that the pigment observed in the cases discussed above will indeed be nothing but pulcherrimin.

This leads to the interesting conclusion that pigment production by *Candida pulcherrima* is not the more or less strictly specific prerogative of this yeast, but has to be considered as an excessive manifestation of some metabolic derailment which under certain conditions may occur as well in other yeast species, and – as the case of *Bacillus subtilis* shows – even in so far distanced micro-organisms as bacteria.

Taking into consideration all this, the following tentative scheme of the reactions ultimately leading to the production of pulcherrimin may be given.

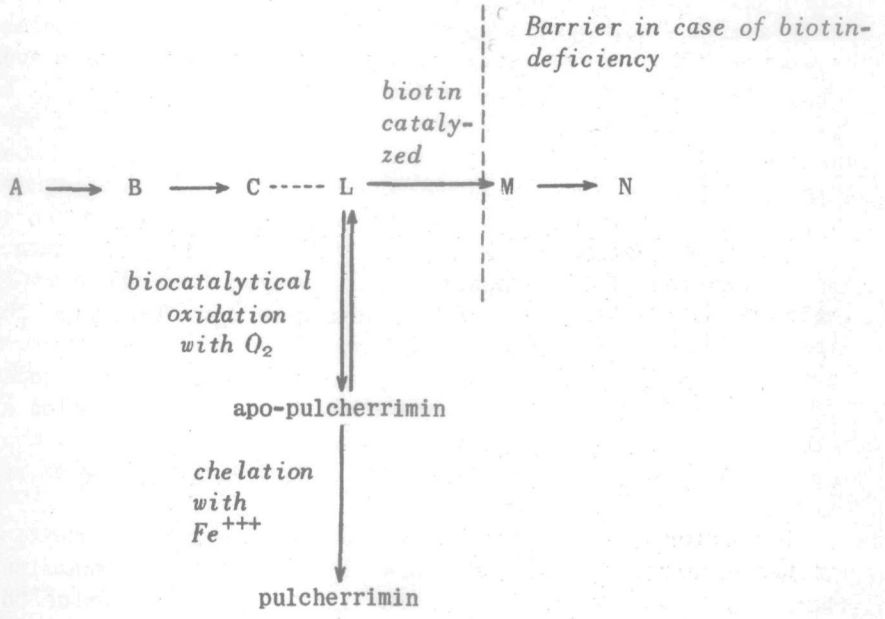
Under aerobic conditions a nutrilite A is converted into some normal cell component N. In the chain of reactions leading from A to N biotin acts as the prosthetic group of some enzyme system catalyzing one of the elementary reactions. It is difficult to say which type of reaction this will be, since biotin has successively been made responsible for a catalytic action on deamination (LICHSTEIN and UMBREIT, 1947), on carbon dioxide fixation (LICHSTEIN 1950), and on succinate oxidation (AJL *et al.*, 1950) etc.

How this may be, it seems logical to accept that biotin-deficiency prevents the normal reaction chain A → N to proceed, with the result that some intermediate metabolite L accumulates.

In order to take account of the indispensability of living cells and oxygen for pigment production one might assume that the

cell oxidizes the accumulated L to some precursor – apo-pulcherrimin – which in the presence of a suitable concentration of ferric ions is at once converted into pulcherrimin, but which otherwise diffuses into the medium and then is only gradually precipitated as pigment.

In the case of the red mutant of *Candida pulcherrima* in which biotin deficiency plays no rôle, it must then be accepted that L accumulates for some other still unknown reason, inherent in the mutation. Moreover, the presence of ferric ions leading to the conversion of the apo-pulcherrimin into the insoluble pigment should also promote the conversion of L into apo-pulcherrimin.



As for the pink pigment of the adenine dependent mutants of LINDEGREN, of EPHRUSSI and of WINGE, in view of the similarity between the general chemical properties of this pigment and pulcherrimin, it seems probable that also in this case we are dealing with some metabolic derailment possibly starting from some other spot in the reaction chain and thus leading to some analogous, though different pigment.

Evidently all these considerations are in want of further investigations, before they can be accepted as a representation of what really goes on in the pigmentation of the yeasts in question.

SUMMARY

In Chapter I a survey is given of the earlier observations on the morphological and cultural characteristics of the yeast *Candida pulcherrima* (Lindner) Windisch. Special attention was paid to the studies of LINDNER, BEIJERINCK, WINDISCH and ROBERTS. It was stressed that many points regarding the morphology and the taxonomic position of the organism are still controversial, particularly because the two last-mentioned authors claim to have witnessed ascospore formation, whilst all earlier workers have concluded to the asporogenous character of the yeast.

In Chapter II an account is given of the author's personal investigations on the life-cycle of *Candida pulcherrima*. It was shown that the structures interpreted by WINDISCH and by ROBERTS to be asci are nothing but open sacs which are formed when chlamydospores in the process of germination shed their outer membrane. These sacs may partly remain attached to the chlamydospores. In these elastic membranes not seldom small yeast cells become ensnared, and these cells have then been wrongly interpreted as ascospores by WINDISCH and by ROBERTS. The claims of these authors regarding the ascosporogenous nature of the yeast are, therefore, rejected. On testing various media on their suitability to induce the formation of chlamydospores, it appeared that those media which promote lipid production in yeasts were also favourable for the development of chlamydospores. Remarkably in a later stage of these cultures the chlamydospores sometimes spontaneously ended their period of dormancy, and gave rise to another generation of budding yeast cells. In this germination process the outer membrane is not always shed, but it may also be pierced by the emerging cell.

In Chapter III some attention is given to the general question of the occurrence of chlamydospores in yeasts. It is emphasized that a better insight into the conditions which determine the formation of chlamydospores is most desirable, and a method is given which yielded satisfactory results in the case of four rather divergent yeast species. It is suggested that more attention should be given to the fact that chlamydospores are well-devised resting stages in many organisms, as is manifested amongst other by the acid-fastness of these structural elements. The question is raised, in how far the formation of chlamydospores by various *Candida* species may be connected with their pathogenic properties in cases of broncho-moniliasis.

Chapter IV deals with the isolation and maintenance of the red mutant of *Candida pulcherrima*. In the first place the earlier

publications on the appearance of red-pigmented forms in the yeast are reviewed. Hereupon the twelve strains of *Candida pulcherrima* present in the collection of the Yeast Division of the "Centraalbureau voor Schimmelcultures" were screened on their pigment producing ability, the strain once described by POLLACCI and NANNIZZI as *Cryptococcus interdigitalis* being finally selected as the most profuse pigment producer. On a medium closely resembling the medium recommended by BEIJERINCK for pigment production both smooth white and red-pigmented colonies developed, the latter being usually rough in appearance. In agreement with the observations of earlier investigators it was found that either of these forms will under certain conditions revert to the other. However, on cultivation of the red form under conditions favourable for pigment production – *i.e.* on media with a relatively high iron content – and by regular transference within short intervals, it proved possible to maintain the red mutant in a state which for all practical purposes could be considered to be pure.

In Chapter V an inquiry is made into the factors which determine the pigment production by the red mutant. In contrast to the claims of GROSBÜSCH the nature of the carbon source used proved to be immaterial, provided that the compound in question did sustain normal growth. Also the influence of pH was found to be only of secondary importance, at least in as far as an iron chelate, like ferric ammonium citrate, was used as source of iron.

In agreement with the original observations of BEIJERINCK iron was found to be indispensable for pigment formation. It was clearly established that the concentration of the iron decided whether the pigment was formed and deposited in the cells, or originated in the agar zones in the immediate vicinity of the colonies. BEIJERINCK's conclusion that free oxygen is essential for the production of pigment could fully be corroborated.

In Chapter VI a report is given about manifold attempts to extract the pigment from cell masses of the red mutant. The only feasible procedure appeared to be the extraction of the cells with caustic alkali followed by reprecipitation of the pigment by acidification of the alkaline solution. A detailed description is given of the procedure adopted for the mass cultivation of well-pigmented yeast, and of the methods applied for the isolation and purification of the pigment.

Chapter VII reports on an attempt made to obtain some insight into the chemical nature of the pigment, for which the name pulcherrimin is proposed. Analyses showed the pigment to contain carbon, hydrogen, oxygen, nitrogen and iron, whilst sulphur, phosphorus and halogens were found to be absent. The most remarkable outcome is the high iron content (10.7%) of the pigment.

Whilst at room temperature the pigment is remarkably resistant against the action of strong acids, a prolonged acid hydrolysis at 100°C. led to a decomposition. Amongst the hydrolytic products initially only leucine could be identified with certainty; moreover, indications for the simultaneous presence of isoleucine were obtained. On pyrolysis of the pigment small quantities of a white crystalline product, having the molecular formula $C_{12}H_{18}N_2O_2$, were obtained. The properties of this compound closely resemble those of a compound of exactly the same molecular constitution isolated by DUTCHER as a derivative of aspergillic acid. This suggests the presence of a pyrazinedione nucleus in the said pyrolytic product. A second indication for some relationship between pulcherrimin and aspergillic acid was obtained, when it was found that the absorption spectrum of the pigment practically coincided with that of the iron complex of aspergillic acid. Since the latter complex is generally accepted to be connected with the presence of a hydroxamic grouping in the molecule, the establishment of hydroxylamine amongst the hydrolytic products of pulcherrimin may be considered as a further support for the postulated relationship between the two compounds in question. Also the identification of leucine (and isoleucine?) amongst the hydrolytic products of pulcherrimin is in favour of this supposition.

In Chapter VIII it is shown that *Candida pulcherrima* forms various other pigments, if the iron in the medium is substituted by other metallic compounds, such as copper, cobalt, nickel and titanium. Under these conditions apparently chelates are formed analogous to pulcherrimin, but in which the iron is substituted by the metal in question. Investigation showed that the yeast grown on a cobalt-containing medium, and which obviously contained a deep yellow cobalt-chelate, did not have any B_{12}-activity.

Chapter IX reports on investigations regarding the production of red, non-carotenoid pigments by yeast species other than *Candida pulcherrima*. By testing a large number of yeasts under the conditions of optimal pulcherrimin production, only some strains of *Saccharomyces lactis* and of *Saccharomyces marxianus* were found to be capable of a pigment production somewhat comparable to that characteristic for *Candida pulcherrima*.

In the meantime a short publication by CUTTS and RAINBOW had appeared in which it was shown that several common yeast species produce a red pigment, if cultivated on a medium deficient in biotin. This observation was checked for 68 yeast strains belonging to various species, and for several of these the said observation could be confirmed. A preliminary spectroscopical analysis tends to show the identity of this pigment with pulcherrimin, and this is supported by the fact that also pulcherrimin production

by *Candida pulcherrima* is influenced by biotin-deficiency.

In contrast hereto the pink pigment produced by adenine-dependent haploid mutants of *Saccharomyces cerevisiae* and their hybrids – as studied by LINDEGREN, by EPHRUSSI and by WINGE – seems to differ from pulcherrimin as judged by the spectroscopical data. Nevertheless, some relation between the chemical natures of these two types of pigment seems likely.

Lastly it has appeared very probably that under certain conditions pulcherrimin can also be formed outside the yeast domain, namely by certain strains of *Bacillus subtilis* and *Bacillus licheniformis*.

In the final Chapter X a brief discussion is given of our very restricted insight into the metabolic processes leading to the formation of pulcherrimin. In this connection it is stressed that the ability to form pulcherrimin is apparently a property common to a great number of yeast species, and that there seems to be amongst these species a gradual transition in the degree of necessity of relative biotin-deficiency for the production of the pigment.

S A M E N V A T T I N G

In Hoofdstuk I wordt een overzicht gegeven van de eerdere onderzoekingen betreffende de morphologische eigenschappen van de gist *Candida pulcherrima* (Lindner) Windisch. In het bijzonder werd aandacht geschonken aan de onderzoekingen van LINDNER, BEIJERINCK, WINDISCH en ROBERTS. Er wordt de nadruk op gelegd, dat aangaande vele punten van de morphologie en taxonomie van het organisme geen overeenstemming is bereikt. In het bijzonder treft het, dat de beide laatstgenoemde schrijvers aangeven ascosporeforming te hebben waargenomen, terwijl alle voorgaande onderzoekers tot een asporogeen karakter van de gist hadden besloten.

In Hoofdstuk II wordt een verslag gegeven van de eigen onderzoekingen over de levenscyclus van *Candida pulcherrima*. Deze leiden tot de conclusie, dat de structuren welke door WINDISCH en ROBERTS als asci worden opgevat, niets anders zijn dan open zakvormige elementen, welke ontstaan doordat chlamydosporen tijdens het kiemingsproces somtijds de buitenste celwand afwerpen. Deze membranen kunnen gedeeltelijk aan de chlamydosporen gehecht blijven. Niet zelden geraken nu kleine gistcellen in deze elastische membranen verstrikt. Het zijn nu deze cellen, welke door WINDISCH en door ROBERTS ten onrechte als ascosporen zijn geïnterpreteerd. De beweringen van deze schrijvers betreffende de ascosporogene natuur van de gist worden daarom verworpen. Bij het onderzoek van verschillende media op hun geschiktheid om chlamydosporen te doen ontstaan, bleek het, dat media die vetvorming in gisten bevorderen, ook gunstig zijn voor de ontwikkeling van chlamydosporen. Het was opmerkelijk, dat in een later stadium van deze cultures de chlamydosporen soms spontaan hun rustperiode beeindigen en een nieuwe generatie van knopvormende gistcellen deden ontstaan. In dit kiemproces werpen de sporen niet altijd de buitenste celwand af, somtijds wordt deze plaatselijk doorboord door de zich ontwikkelende cel.

In Hoofdstuk III wordt enige aandacht gegeven aan de algemene vraag betreffende het voorkomen van chlamydosporen in gisten. De wenselijkheid wordt bepleit van een nader onderzoek naar de voorwaarden, welke de vorming van chlamydosporen bepalen. Een methode wordt beschreven, waarmede in bedoeld opzicht bevredigende resultaten werden verkregen bij vier zeer uiteenlopende gistsoorten. Tenslotte wordt betoogd, dat er aanleiding bestaat om meer aandacht te schenken aan het feit, dat chlamydosporen voor vele micro-organismen natuurlijke rust-stadia zijn, zoals o.a. in de opmerkelijke zuurvastheid van deze structuren tot uiting komt. De vraag wordt voorts opgeworpen in hoeverre de vorming van chlamy-

dosporen door verschillende *Candida*-soorten mogelijk samenhangt met hun pathogene eigenschappen in gevallen van broncho-moniliasis.

In Hoofdstuk IV wordt de isolatie en het aanhouden van de rode mutant van *Candida pulcherrima* besproken. In de eerste plaats wordt een overzicht gegeven van de eerdere publicaties betreffende het optreden van een rood pigment vormende stammen van deze gist. De twaalf stammen van *Candida pulcherrima*, welke aanwezig zijn in de verzameling van de Gist-Afdeling van het „Centraalbureau voor Schimmelcultures", werden aan een vergelijkend onderzoek onderworpen wat betreft hun vermogen tot pigmentvorming; de eens door POLLACCI en NANNIZZI als *Cryptococcus interdigitalis* beschreven stam werd tenslotte uitgekozen als zijnde de beste pigmentvormer. Op een medium, dat t.o.v. het door BEIJERINCK aanbevolen medium voor pigmentproductie slechts op een enkel punt was gewijzigd, ontwikkelden zich zowel glädde witte als geplooide rode koloniën. In overeenstemming met de waarnemingen van oudere onderzoekers werd vastgesteld, dat beide vormen onder bepaalde voorwaarden in elkaar over kunnen gaan. Indien evenwel de rode vorm werd gekweekt onder voorwaarden, welke optimaal waren voor pigmentvorming, dus op media met een relatief hoog ijzergehalte, en tevens voor regelmatig overenten binnen korte tijdsperioden werd gezorgd, kon de rode mutant in een vorm worden aangehouden, welke voor alle practische doeleinden als rein kon worden beschouwd.

In Hoofdstuk V wordt verslag uitgebracht over een onderzoek aangaande de factoren, welke de pigmentvorming door de rode mutant bepalen. In tegenstelling tot de opinie van GROSBÜSCH bleek de geaardheid van de koolstofbron van geen belang te zijn, althans in zoverre als de betreffende verbinding een normale groei van het organisme mogelijk maakt. Ook bleek het, dat de invloed van de pH slechts van een secundaire betekenis is, mits als bron voor het ijzer een ijzercomplex, zoals ferri ammonium citraat, werd gebruikt. In overeenstemming met de oorspronkelijke waarnemingen van BEIJERINCK werd gevonden, dat ijzer onmisbaar is voor pigmentvorming. Er werd voorts vastgesteld, dat de ijzerconcentratie bepaalde of het pigment in de cellen werd afgescheiden, dan wel in de agar zone in de onmiddellijke omgeving van de koloniën optrad. De gevolgtrekking van BEIJERINCK, dat aanwezigheid van vrije zuurstof noodzakelijk is voor de productie van pigment kon volledig worden bevestigd.

In Hoofdstuk VI wordt een overzicht gegeven van talrijke pogingen om het pigment uit de cellen van de rode mutant te extraheren. De enige bruikbare werkwijze bleek te zijn een extractie van de cellen met loog, gevolgd door een herprecipitatie van het pigment door aanzuren van de alkalische oplossing. De gevolgde werkwijze voor het cultiveren van een sterk gekleurde gist op wat

groter schaal, alsmede de methoden gebruikt voor de isolatie en zuivering van het pigment worden beschreven.

Hoofdstuk VII vermeldt de pogingen, welke zijn verricht om tot enig inzicht te komen in de chemische aard van het pigment, waarvoor de naam pulcherrimine wordt voorgesteld. De analysen leerden, dat het pigment koolstof, waterstof, zuurstof, stikstof en ijzer bevatte, doch dat zwavel, phosphor en halogenen afwezig waren. Het meest opmerkelijke resultaat is het hoge ijzergehalte (10.7%) van het pigment. Terwijl het pigment bij kamertemperatuur merkwaardig resistent is tegen de inwerking van sterke zuren, treedt bij langdurige hydrolyse met zuur bij 100°C. ontleding op. Onder de producten der hydrolyse kon aanvankelijk alleen leucine met zekerheid worden geidentificeerd; bovendien werden aanwijzingen verkregen voor de gelijktijdige aanwezigheid van isoleucine. Door pyrolyse van het pigment werden kleine hoeveelheden van een wit kristallijn product, met de moleculaire formule $C_{12}H_{18}N_2O_2$ verkregen. De eigenschappen van deze stof bleken zeer veel te gelijken op die van een verbinding met geheel dezelfde moleculaire samenstelling, welke door DUTCHER als een derivaat van aspergilluszuur was verkregen. Dit doet de aanwezigheid van een pyrazinedion-kern in het bewuste pyrolyseproduct vermoeden. Een tweede aanwijzing voor een verwantschap tussen pulcherrimine en aspergilluszuur werd verkregen, toen werd gevonden, dat het absorptiespectrum van het pigment practisch overeenkomt met dat van het ijzercomplex van aspergilluszuur. Aangezien algemeen wordt aangenomen, dat dit laatstgenoemde complex zijn ontstaan dankt aan de aanwezigheid van een hydroxaamgroepering in het molecuul, mag het aantonen van hydroxylamine onder de hydrolytische producten van pulcherrimine als een verdere steun voor de aangenomen verwantschap tussen de twee besproken stoffen worden beschouwd. Ook spreekt de identificatie van leucine (en isoleucine?) onder de producten van de hydrolyse van pulcherrimine ten gunste van deze veronderstelling.

In Hoofdstuk VIII wordt aangetoond, dat *Candida pulcherrima* verschillende andere pigmenten vormt, als in het medium het ijzer wordt vervangen door andere metalen, zoals koper, cobalt, nikkel en titaan. Onder deze voorwaarden worden blijkbaar verbindingen gevormd overeenkomend met pulcherrimine, maar waarin het ijzer is vervangen door één van deze metalen. Indien de gist groeit op een cobalt-houdend medium, wordt een helder geel cobaltcomplex gevormd; een onderzoek leerde, dat de gist geen vitamine B_{12}-activiteit bezat.

Hoofdstuk IX heeft betrekking op onderzoekingen aangaande de productie van rode-, niet-lipochrome, pigmenten door andere gistsoorten dan *Candida pulcherrima*. Door een groot aantal gisten on-

der voorwaarden voor optimale productie van pulcherrimine te cultiveren, werden voor enkele stammen van *Saccharomyces lactis* en *Saccharomyces marxianus* vastgesteld, dat zij in staat waren een pigment te vormen, dat grote overeenkomst vertoonde met dat van *Candida pulcherrima*. Inmiddels was een korte publicatie van CUTTS en RAINBOW verschenen, waarin werd aangetoond, dat verschillende gewone gistsoorten een rood pigment vormen, als zij worden gekweekt op een medium, waarin biotine in sub-optimale hoeveelheid aanwezig is. Deze waarneming werd gecontroleerd voor 68 giststammen, welke tot zeer verschillende soorten behoorden. Voor verschillende van de onderzochte stammen kon het resultaat der Engelse onderzoekers worden bevestigd. Een voorlopig spectroscopisch onderzoek gaf aanwijzingen, dat er overeenkomst bestaat tussen het onder deze omstandigheden gevormde pigment en pulcherrimine. Deze zienswijze werd gesteund door het feit, dat ook de pulcherrimine-productie door *Candida pulcherrima* door een tekort aan biotine wordt beinvloed. In tegenstelling hiermede schijnt het rose pigment, dat wordt gevormd door adenine-behoeftige haploide mutanten van *Saccharomyces cerevisiae* en hun hydriden, welke door LINDEGREN, EPHRUSSI en WINGE zijn bestudeerd, in spectroscopisch opzicht van pulcherrimine te verschillen. Desniettemin lijkt enige verwantschap in de chemische aard van de beide soorten pigment aannemelijk. Voorts bleek met grote waarschijnlijkheid, dat pulcherrimine onder bepaalde omstandigheden ook buiten het rijk der gisten kan worden gevormd, n.l. door sommige stammen van *Bacillus subtilis* en *Bacillus licheniformis*.

Tenslotte is in Hoofdstuk X een korte bespreking gewijd aan het zeer beperkte inzicht, dat is verkregen in de stofwisselingsprocessen, welke tot de vorming van pulcherrimine leiden. In dit verband is naar voren gebracht, dat het vermogen om pulcherrimine te vormen blijkbaar aan een groot aantal gistsoorten eigen is en dat er blijkbaar bij deze soorten een geleidelijke overgang bestaat, wat betreft de noodzakelijkheid van een tekort aan biotine voor de productie van het pigment.

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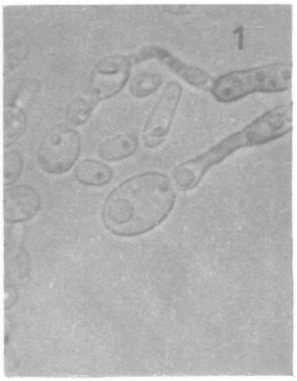
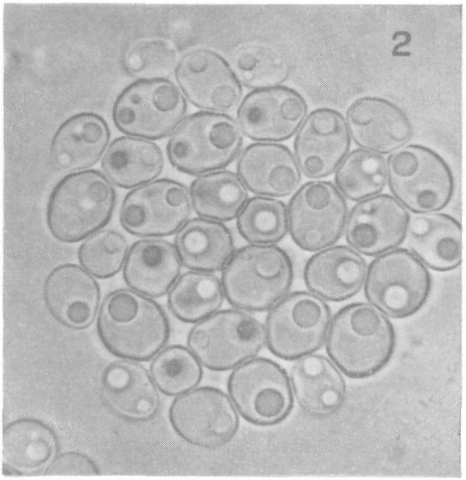
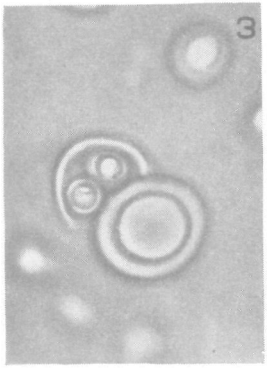
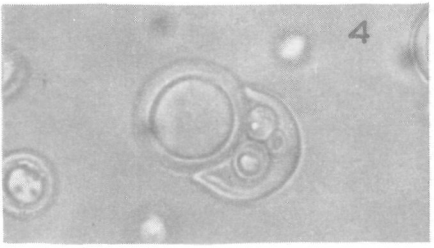
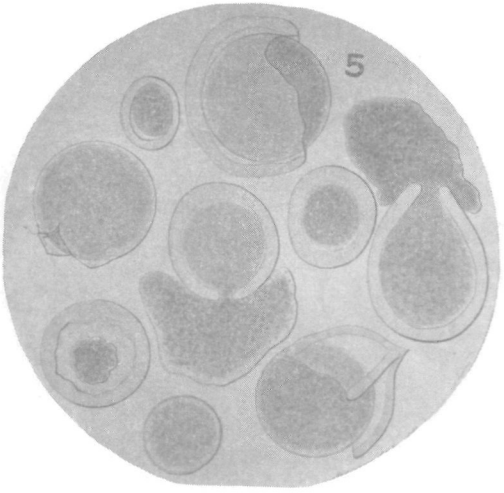
PLATE I.











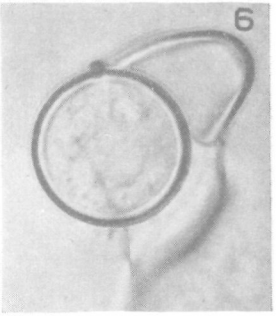


Plate I*

Fig.1 *Candida pulcherrima* on malt agar. "Forms conidiennes". Mag. 1000x

Fig.2 *Candida pulcherrima* from a slide culture. Note lipid globules. Mag. 1000x

Fig.3 and 4 *Candida pulcherrima* from a 14 days old culture on STARKEY's medium. Note the small cells trapped within the husks of the germinating chlamydospore. Mag. 1150x.

Fig.5 A wall-tableau drawn by BEIJERINCK in 1887, illustrating the desquamation of cells in an anaerobic culture of *Mucor racemosus* (Reproduced by the courtesy of Prof. Dr. A. J. Kluyver).

Fig.6 An anaerobic culture of *Mucor racemosus*, showing a cell shedding its outer membrane. Mag. 1000x

* The author's thanks are due to Mr: J.A. Schuur and to Mr. H.R. Veenhoff for taking the photomicrographs and photographs, respectively.

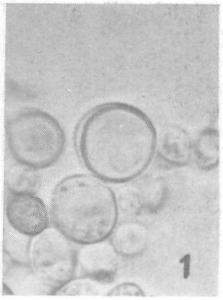
Plate II

Figs.1 and 2 Chlamydospore of *Candida pulcherrima* strain No 35.2.6 shedding their outer membranes. Culture on SABOURAUD-agar, 14 days old. Mag. 1000x

Figs.3,4,5 and 6 Chlamydospores of *Candida pulcherrima* strain No 35.2.8 shedding their outer membranes. Culture on SABOURAUD-agar, 14 days old. Mag. 1000x

Figs.7,8,9,10 and 11 Germinating chlamydospores of *Candida pulcherrima* on a biotin-deficient medium, 2 months old. Mag. 1000x

Fig.12 Chlamydospores of *Candida pulcherrima* stained according to the ZIEHL-NEELSEN technique. Weakly counter-stained with aqueous methyleneblue for contrast. Mag. 1000x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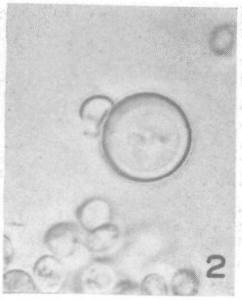
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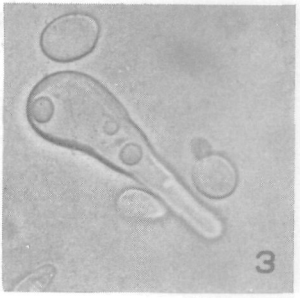
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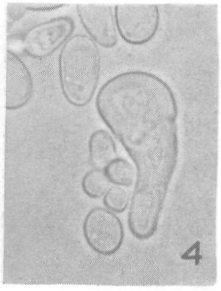
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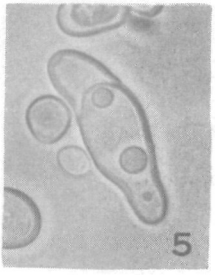
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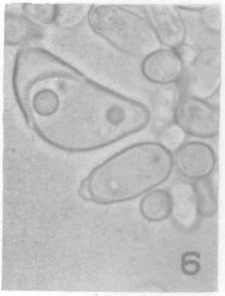
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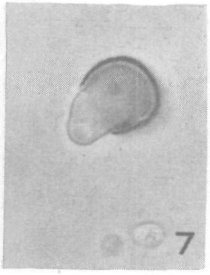
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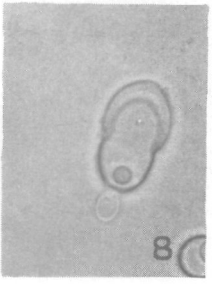
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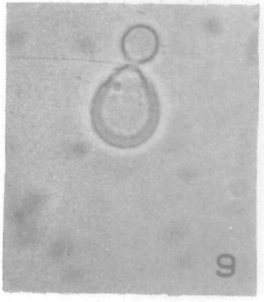
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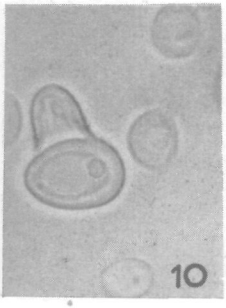
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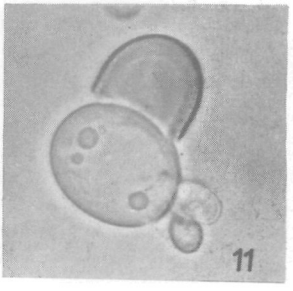
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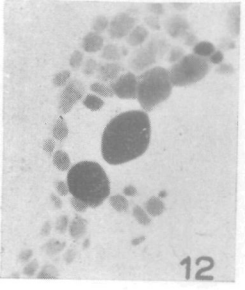
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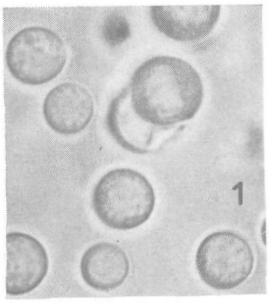
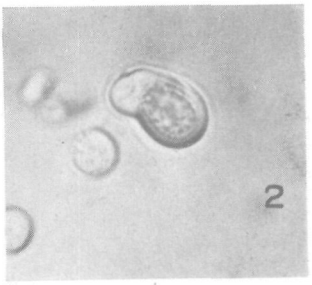
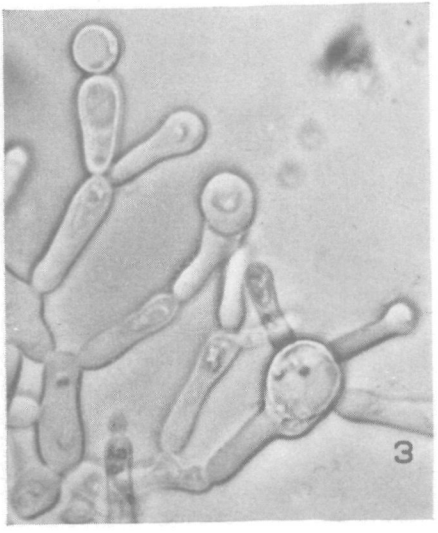
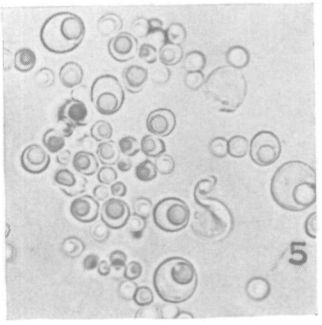
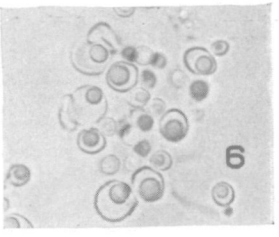
PLATE III

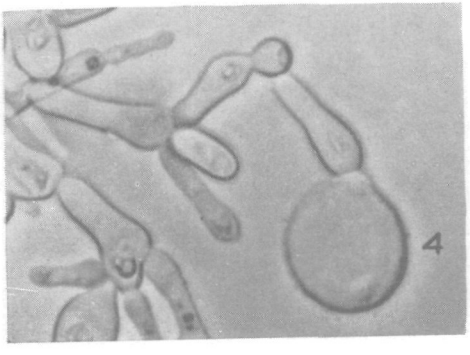












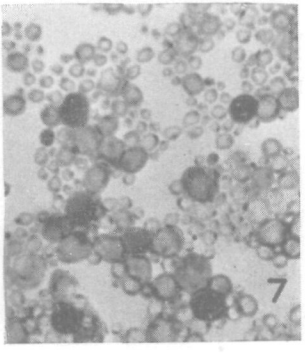


Plate III

Figs.1 and 2 Chlamydospores of *Torulopsis diff-luens*. Culture on STARKEY's medium, 14 days old. Mag. 1150x

Figs.3 and 4 Chlamydospores of *Candida reukaufii*. Culture on STARKEY's medium, 14 days old. Mag. 1150x.

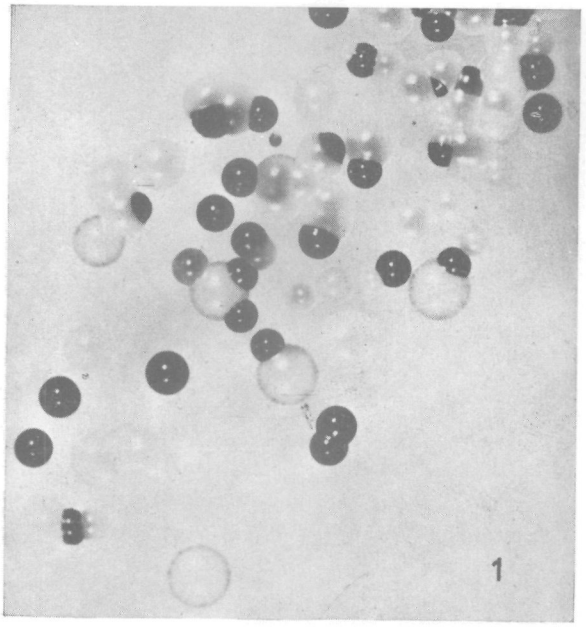
Figs.5 and 6 Chlamydospores of *Debaryomyces hansenii*. Culture on STARKEY's medium, 14 days old. Mag. 1000x

Fig.7 Chlamydospores of *Debaryomyces hansenii* on STARKEY's medium, 14 days old. Stained according to the ZIEHL-NEELSEN technique. Slightly counter-stained with aqueous methylene-blue for contrast. Mag. 1000x

Plate IV

Fig.1 Plate culture of *Candida pulcherrima* on synthetic medium containing 0.05% ferric ammonium citrate. 4 days old. Mag. 6x

Fig.2 Same as Fig.1. Culture 11 days old. Mag. 6x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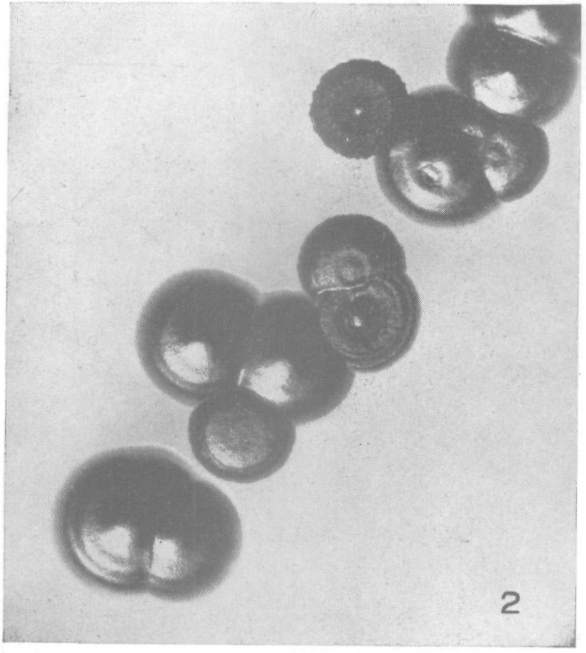
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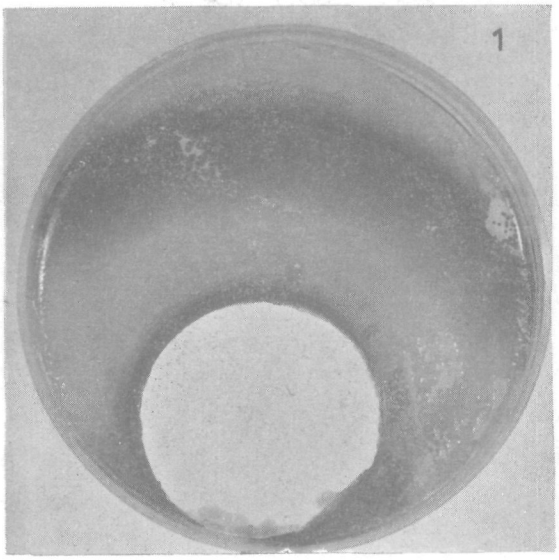
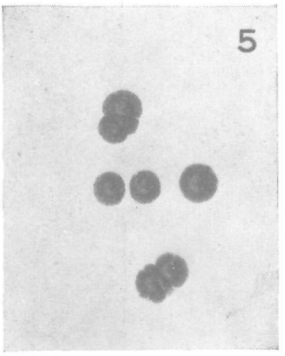
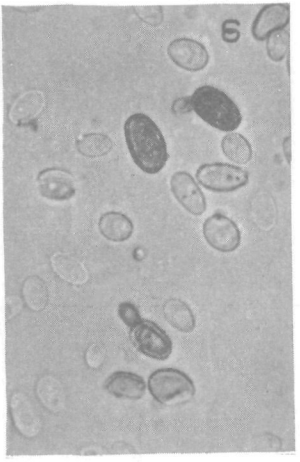
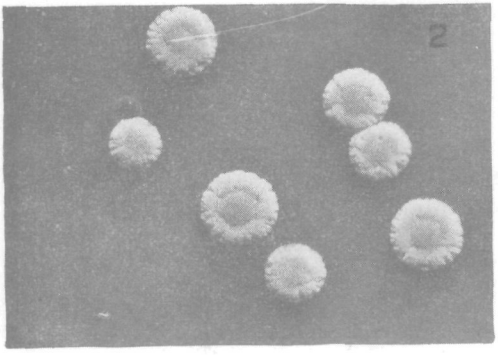
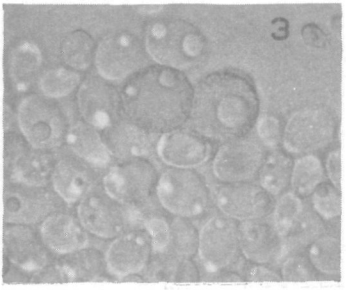
PLATE V











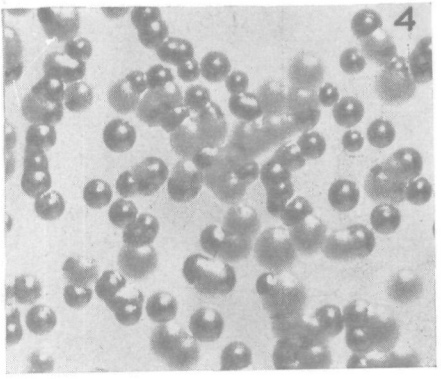


Plate V

Fig.1 Plate culture of *Candida pulcherrima*, demonstrating the pigmented zone after treatment with ferric chloride solution. Mag. 7/9x

Fig.2 Plate culture of *Candida pulcherrima* on copper-containing medium. Mag. 6x

Fig.3 *Candida pulcherrima* on copper-containing medium. Mag. 1000x

Fig.4 Plate culture of *Candida pulcherrima* on cobalt-containing agar after treatment with 4% $FeCl_3$ solution. Mag. 4x

Fig.5 Plate culture of *Saccharomyces lactis* (strain 2.5.1) on biotin-deficient medium. Mag. 6x

Fig.6 *Saccharomyces lactis* on biotin-deficient medium. Mag. 1000x

STELLINGEN

I

De productie van een rode kleurstof door *Candida pulcherrima* is niet een specifieke eigenschap van deze gistsoort, doch moet worden beschouwd als een excessieve uiting van een stofwisselingsanomaliteit, welke onder bepaalde omstandigheden ook in andere gistsoorten optreedt.

II

Torulopsis minor (Poll. et Nann.) Lodder mag worden beschouwd als een mutant van *Rhodotorula rubra* (Demme)Lodder, welke het vermogen tot vorming van carotenoide kleurstoffen heeft verloren.

III

Ten onrechte wordt ook nog in vele recente studieboeken de indruk gewekt, dat *Penicillium glaucum* niet in staat is het l(–) wijnsteenzuur aan te tasten.

IV

In tegenstelling tot de zienswijze van KNIGHT en PROOM moet het door SMITH, GORDON en CLARK als diagnostisch hulpmiddel gebruikte kenmerk van de groei van aerobe sporevormende bacteriën op glucose-nitraat agar als belangrijk worden beschouwd.

B.C.J.G.KNIGHT and H.PROOM, J.gen.Microbiol. **4**, 508, 1950.

V

Een uitbreiding van de fraaie „donkerveld"-studiën van PIJPER over de verschillende agglutinatie-typen van *Salmonella typhosa* tot vertegenwoordigers van andere bacteriën-geslachten kan er in belangrijke mate toe bijdragen om de bestaande onzekerheid inzake de betekenis van de bacteriën-flagellen op te heffen.

A.PIJPER, J.Path.Bact. **47**, 1, 1938.
A.PIJPER, J.Bact. **57**, 111, 1949.

VI

De uitspraak van MANIL, dat er een correlatie bestaat tussen het voorkomen van de antigenen H en O en het uiterlijk der koloniën ("rough" of "smooth") is onjuist.

P.MANIL, Microbes et actions microbiennes. Liège, 1945.

VII

De veranderingen in de polyphenolen, welke optreden gedurende de fermentering van thee en cacao en welke resp. leiden tot de vorming van thee-aroma en de "precursor" voor het cacao-aroma, zijn van essentieel verschillende aard.

W.G.C.FORSYTH and J.E.ROMBOUTS, Report Cocoa Conference. London, 1951.

VIII

Van de verschillende soorten algen voorkomende langs de kusten van Zuid-Afrika biedt *Ecklonia maxima* de grootste mogelijkheid voor industriële verwerking.

R.A.SHUTTLEWORTH, S.African Ind.Chemist. V, 115, 1951.

IX

De conclusie van SERFONTEIN inzake de groei van een tyrosinebehoeftige mutant van *Bacterium coli*, dat „wanneer 'n substituent aan die aminogroep in tirosien is, kan die verbindings blykbaar glad nie of baie sleg deur die organisme benut word", is niet in overeenstemming met de door deze auteur zelf verkregen resultaten.

W.J.SERFONTEIN, 'n Bydrae tot die kennis van enkele aspekte van die eiwitchemie. Proefschrift, Leiden 1952.

X

De mening van RAMART-LUCAS, dat naast p-nitrosophenol en benzochinonmonoxim nog een derde zgn. β-structuur voorkomt, welke niet een ion der eerstgenoemde verbindingen is, is onjuist.

P.RAMART-LUCAS, Bull.soc.chim. **12**, 477, 1945;
Ibid. **15**, 571, 1948; **16**, 53, 1949.
E.HAVINGA and A.SCHORS, Rec.trav.chim. **69**, 457, 1950.

XI

De door KOLTHOFF aangegeven modificatie van de volumetrische bepaling van ferri-ijzer met titanochloride en kaliumrhodanide levert onbetrouwbare uitkomsten.

I.H.KOLTHOFF und H.MENZEL, Die Massanalyse. Berlin, 1931.
F.P.DWYER and N.J.GIBSON, Analyst **76**, 548, 1951.

XII

Het is niet waarschijnlijk, dat bij de polymerisatie van lijnolie intramoleculaire reacties in de eerste phase van het proces een essentiële rol spelen.

H.E.ADAMS and P.O.POWERS, Ind.Eng.Chem. **36**, 1124, 1944.
E.SCHAUENSTEIN, Oesterr.Chem.Ztg. **50**, 2, 1949.

XIII

Ten onrechte menen PICON en FLAHAUT, dat koolstof in tegenwoordigheid van manganosulfide aan een destillatieproces onderhevig is.

M.PICON et J.FLAHAUT, Bull.soc.chim. **17**, 1070, 1950.

XIV

De sleutel tot een gezonde, economische en politieke ontwikkeling van de Unie van Zuid-Afrika is gelegen in een doelbewuste bevordering van de landbouw in dit land.